

STUDIES ON THE PATHOGENESIS OF
EXPERIMENTAL CANDIDIASIS

by

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A thesis submitted to the faculty of the University
of Utah in partial fulfillment of the requirements for
the degree of

Doctor of Philosophy

Department of Bacteriology

University of Utah

August 1959

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The writer wishes to express his appreciation to Dr. Louis P. Gebhardt for his assistance throughout the preparation of this thesis, and to Drs. Stanley Marcus, Paul S. Nicholes and John Bachtold for their helpful suggestions and criticisms. Acknowledgement is made to the members of the departments of Bacteriology, Anatomy and Pharmacology for their aid in carrying out the experiments to be described.

Portions of this research were supported by the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Department of Health, Education and Welfare.

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STUDIES ON THE PATHOGENESIS
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Introduction

Five years before Pasteur completed his researches for his doctoral thesis in chemistry, Gruby (1842) described a microorganism, Sporotrichum sp. (Candida albicans), as the etiological agent of a human disease now referred to as thrush or oral candidiasis. In the past 117 years considerable work has been reported in the literature on various human infections caused by this organism. Information available at the present time firmly establishes the fact that the yeast-like organism, C. albicans, can and does act as the primary etiological agent of a multitude of different human and animal infections. Equally well established is its secondary role in as many different human infirmities, both infectious and noninfectious.

There is, nevertheless, a decided lack of information concerning the exact means by which this organism is able to manifest itself as a parasite in tissues of susceptible hosts either under natural or experimental conditions of infection. Also, data relating to immune mechanisms of hosts which enable them to resist the limited parasitic attributes of this organism are meager.

The organism of main medical importance in the genus Candida is C. albicans. Rarely, however, other organisms of the genus have been

isolated from diseased humans under conditions which would strongly incriminate them as disease-producing agents. In the 100 odd years since its original isolation no less than 172 different names have been used to describe this organism (Conant et al., 1944). A significant portion of the apparent confusion existing in the older literature is explicable on this basis. In modern times standardization and use of the name of Candida albicans seems almost complete. Rather than moniliasis, etc., the general term candidiasis is enjoying current favor to denote disease caused by this organism. This nomenclature for the organism and for the disease caused by it will be used in this study.

With the advent of the "antibiotic and steroid" era, a significant increase in the incidence of human candidiasis has occurred. This increase in the number of cases is manifest mainly in patients undergoing extensive and prolonged therapy with antibiotics of the tetracycline structure class and/or antiphlogistic steroid hormones. The magnitude of this problem is sufficient in itself to justify re-evaluation and study of the parasitic role of C. albicans in primary and secondary infectious disease.

The purpose of the work presented in this thesis has been to study under laboratory conditions certain host-parasite relationships in experimental candidiasis.

REVIEW OF THE LITERATURE

I. The Role of Candida Species in Natural Human Infections.

Candida albicans has never been isolated from a nonparasitic habitat (Skinner, 1947). The majority of strains of this organism has been found in association with humans. The exact pathological significance of the isolation of C. albicans from certain human sources is questionable. Many authors have reported the presence of C. albicans and other yeast-like organisms in cultures of specimens from apparently normal mouths, vaginas and upper respiratory and gastrointestinal tracts.

Tanner et al. (1927) isolated yeasts, including Candida, from the mouths of 10 per cent of a series of healthy young adults. Epstein (1924) found the "Soorpilz" in the mouths of 54 per cent of a series of infants 2 to 6 weeks of age, in 46 per cent of those up to one year and in 39 per cent of those of the 1 to 6 year age group. No adequate distinction was made between C. albicans and other species. Ten per cent of the mouths of 1,002 healthy college students were found to harbor C. albicans (Todd, 1937). Other authors have reported data indicating the relatively high incidence of the occurrence of C. albicans and other yeast-like organisms in the oral cavity. Of 48 children, the throats of 12 and the tongues of 6 yielded C. albicans, and 30 of 122 samples of saliva yielded this species (Fisher, 1936). Knighton (1939) isolated yeasts from 32 per cent of 123 mouths without dentures and from 61 per cent of 23 cases with full or partial plates. This author reported that the percentage incidence of C. albicans in each group, dentate and edentate, was approximately the same.

The flora of the normal female vagina has been reported to include yeast-like organisms. Weinstein and Wickerham (1938), Carter and Jones (1937) and Flass et al. (1931) have found normal carrier rates in females varying from 7 to 15 per cent. The incidence of C. albicans alone has been reported to be approximately 7 per cent (Dawkins et al., 1953). Others (Castellani and Taylor, 1925), however, have reported the inability to find Candida in the absence of clinical disease. The report of Jones and Martin (1938) on the occurrence of species of Candida in the vagina is of considerable interest. They isolated yeast-like organisms from the vaginas of 32 per cent of a series of pregnant women and 14 per cent of non-pregnant women. C. albicans was isolated only from those individuals exhibiting symptoms. In the normal vagina, the closely related fungus they designated as Candida albicans var. stellatoidea was sometimes found. This organism was considered non-pathogenic. These findings have not been substantiated by others, however. Rauramo (1950) and Dawkins et al. (1953) have reported an inability to isolate yeast-like organisms from female genitalia with the supposed characteristics of C. albicans var. stellatoidea. The latter authors isolated 102 strains of yeast-like organisms from vaginas and vulvas of normal females and not one of them qualified as C. albicans var. stellatoidea.

C. albicans has been reported to be a member of the normal flora of the gastrointestinal tract. The results of studies cited by Skinner (1947) indicate that about 15 per cent of humans harbor this yeast-like organism in their intestinal contents.

The possibility exists that C. albicans may be a member of the normal flora of the skin surfaces of man. Conant et al. (1944) states

without reservation or documentation that C. albicans is a normal inhabitant of the skin. That this may not be so is strongly suggested by the data of various workers. Among them are Benham and Hopkins (1933), Croft and Black (1938), Downing et al. (1937), Drake (1945) and Winter and Foley (1956a) and MacKinnon (1946) who have all failed to find C. albicans on the normal skin.

The ecological relationships to man of other *Candida* species have not been studied as extensively as has C. albicans. Skinner (1947) has cited extensive references to the isolation of Candida krusei, Candida tropicalis, Candida pseudotropicalis, Candida guilliermondi and others from non-human or non-animal origin. These and other organisms, however, are frequently isolated from human clinical specimens. Winter and Foley (1956) have reported on the frequency and species distribution of yeast-like organisms from the throats and mouths of children seen at a tumor therapy clinic. Of a total of 2,032 positive cultures, 110 proved to be C. tropicalis; 15, C. pseudotropicalis; 10, C. krusei; and 7, C. parapsilosis (C. parakrusei). Of 500 patients visiting a contraceptive clinic, Dawkins et al. (1953) found 3 strains of C. krusei, 3 of C. tropicalis and one strain each of C. parakrusei and C. mycoderma. These organisms were isolated from vulval and vaginal swabs.

From a consideration of the investigations cited above, it has been concluded that C. albicans infections in man are of both endogenous and exogenous origin (Skinner, 1947 and Dobias, 1957).

The many clinical forms that candidiasis can assume in the human have been the subject of numerous publications. Over 100 years of human clinical literature have established the fact that C. albicans

is principally a parasite of skin and mucous membranes of the oral, vaginal, respiratory and gastrointestinal tracts (Dobias, 1957). As early as 1838, Valleix (1838) stated that the succession of symptoms of thrush or oral candidiasis had not been studied as thoroughly as it should have been. He suggested that those persons who believe that oral candidiasis means nothing more than a local pseudomembraneous infection of the mouth have not looked for signs of more extensive infection. The disease entities in man caused by C. albicans range from the most benign form, lasting but a few days, to acute dissemination proving fatal in from days to weeks, or to protracted, chronic illness lasting many years. In this review no attempts will be made to present a comprehensive survey of the clinical literature relating to symptoms and variations of symptoms of human candidiasis. Emphasis will be placed on literature pertaining to a more thorough understanding of the pathogenesis of human candidiasis.

Candidiasis may affect humans at any age with infection occurring more frequently in early neonatal life or early infancy than at any other time. The majority of cases in younger age groups are those with involvement of the oral cavity (Dobias, 1957). The portal of entry is most likely the mouth, and the symptoms become evident on an average of about 8 or 9 days after birth (Ludlam and Henderson, 1942, Anderson et al., 1944). The most probable source of infection in the newborn has been suggested to be the C. albicans organisms in the birth canal of the mother (Woodruff and Hesseltine, 1938). These authors suggested that the child has 35 times the chance of getting oral candidiasis if the mother is carrying the causitive fungus in her vagina.

Oral infections caused by C. albicans in older age groups have been reported frequently in the literature. Fisher (1936), Knighton (1939) and others have reviewed this subject. However, on the basis of data previously discussed, it is more difficult to make a definitive diagnosis on the basis of positive cultures.

Evidence has been presented that some denture-sore mouths are caused by C. albicans (Bartels, 1937 and Cahn, 1936).

Perleche, caused by C. albicans, is an infection of the commissures of the mouth. Finnerud (1929) reported clinical observations on 100 human cases. He reported that the experimental infection could be produced in human volunteers by use of pure cultures of C. albicans. Skinner (1947) discussed the findings of numerous authors who assume that perleche is in many cases due to C. albicans, although similar pathological conditions are due to vitamin deficiencies and that the disease is often an infection towards which such a deficiency predisposes. He suggested that unless the clinical symptoms are clear and the yeast-like organisms found in abundance, the presence of C. albicans in the oral cavity should have no more significance than the finding of Staphylococci in an open lesion.

In the gastrointestinal tract the esophagus is one of the most common sites of Candida infections (Dobias, 1957, Ludlam and Henderson, 1942, and Lederer and Todd, 1949, etc.). These infections occur more frequently in young infants. Lederer and Todd (1949), in a series of 204 necropsies on infants under 12 months of age, encountered 26 cases in which candidiasis was directly or indirectly responsible for the fatal outcome. Involvement of the esophagus was a common finding.

De Gavaller (1953) reported lesions caused by C. albicans in 72 of 288 autopsied newborn infants. In one-half of these 72 children the esophagus was infected.

Lesions of other portions of the gastrointestinal tract occur much less frequently (Dobias, 1957). This author reviewed the works of many investigators and concluded that infections of the stomach wall and ulceration of the small and large intestine, which may lead to perforation and subsequent peritonitis, can be caused by C. albicans.

Skinner (1947) has reviewed the literature in connection with the possibility of the existence of intestinal candidiasis with symptoms of diarrhea. He concluded that there is little if any evidence that yeast-like organisms are related to infection of this tract. However, data cited by Dobias (1957) tends to strongly suggest that C. albicans can infect the lower gastrointestinal tract causing symptoms of diarrhea. He reported that of 70 cases of cutaneous moniliasis, 19 per cent had intestinal symptoms. Eighty-seven per cent of 60 cases in which stools were examined had Candida in their feces. Stool cultures in the majority were negative and symptoms lacking after a course of specific therapy.

Differing opinions have been expressed relative to the frequency of occurrence of pulmonary candidiasis. Robertson (1948) and others doubt the existence of primary lung disease and suggest that it occurs only as a secondary invasion of an underlying tuberculosis, neoplastic or bronchiectatic lesion. The American literature on "primary pulmonary" candidiasis has been reviewed by Cohen (1953) and he, as well as others, concluded that primary disease of humans is possible. He warned, however, that recovery of the organism and proof of its virulence does not

constitute positive proof of the infection. He suggested that the diagnosis of primary pulmonary disease is justified only if C. albicans is recovered regularly from fresh sputum specimens, and if all other possible etiological agents are excluded. He reported a case which terminated fatally and postmortem examination showed miliary granulomata of lungs and kidneys with aneurysms of the abdominal aorta, the left femoral and right common iliac arteries. The granulomatous lesions were not tuberculous and postmortem blood cultures were negative for bacteria and fungi. Examination of tissues from the wall of the aneurysm failed to show C. albicans. Prior to death repeated sputum cultures revealed the presence of C. albicans and a consistent absence of any other etiological agent. Tuberculin skin tests were positive but histoplasmin and coccidioidin tests were negative.

Lesions of the respiratory tract reportedly caused by C. albicans have been found in infants (Dobias, 1957). As in adults, however, bronchial and pulmonary candidiasis in infants and younger children is difficult to diagnose. Pathological changes in the bronchi and lungs have been reported to accompany other symptoms of candidiasis in individuals of this age group (Smith and Sano, 1933, de Gavaller, 1953 and others). Schürmann (1953) has found *Candida* in pure culture in lung tissue and in blood of a premature infant who died at the age of 2 days. The histological evidence suggested possible hematogenous spread to the lung.

On the basis of numerous reports in the literature, few investigators doubt the role of C. albicans as a secondary invader of pulmonary tissue (Skinner, 1947, Dobias, 1957, Winter and Foley, 1956a and Gonzalez-

Mendoza and Bojahl, 1958). The tendency of many workers, however, has been to consider C. albicans as "only a secondary invader" (Robertson, 1948).

Cutaneous candidiasis has been the subject of an enormous number of clinical papers. These infections have been reported to occur more commonly in infants and young children (Dobias, 1957). In infants, more frequently, lesions start around the anus, or on the prominences of the buttocks and spread to the genital region, upper thighs and lower abdomen. Similar lesions are frequently found on the face. Rarely the entire body may be covered. Dobias (1957) has suggested that the skin of infants becomes infected from fecal material containing the fungus as a result of oral and successive gastrointestinal candidiasis.

Other means of transmission of C. albicans to the skin of the infant have been proposed. Direct contamination of the skin during passage through the birth canal has been suggested (Dobias, 1957). Other means may be by contamination of the skin with C. albicans from a nurse's or mother's hands (Holzel, 1953), from insufficiently sterilized diapers (Mayer et al., 1951) and as a result of the child with an oral infection sucking his fingers and transferring organisms to skin surfaces (Ludlam and Henderson, 1942).

Candidiasis of the skin of the newborn has been reported to be common, especially in lower economic groups of the population. Bound (1956) found an incidence of almost 3 per cent in the first 6 months of age. A high incidence of 35 per cent in hospitalized infants of less than one month of age has been indicated (Plenert, 1955).

Cutaneous candidiasis in older individuals has been mentioned frequently (Skinner, 1947). Intertrigo or infection of moist skin areas occurs more frequently in diabetic and obese patients (Greenwood and Rockwood, 1930) supposedly due to increased blood glucose and moist surfaces. It was reported by Cornbleet (1935) that the sweat of persons with cutaneous candidiasis contained more than normal amounts of reducing sugars. Humans whose hands are habitually wet are more likely to become infected with C. albicans. This was found to be true by Hopkins and Benham (1929), who were able to experimentally infect humans by inoculation of pure cultures of C. albicans under simulated conditions of natural infection.

A rather unusual clinical form of cutaneous candidiasis which has been reported exhibited chronic deep granulomatous lesions (Hauser and Rotman, 1950). Horn-like structures were often superimposed over the primary skin lesions. Large numbers of filaments of C. albicans were observed in the granulomatous tissues.

Vaginal and vulvo-vaginal candidiasis is one of the more common infections caused by C. albicans in the female. These infections have been the subject of numerous clinical reports (reviewed by Plass et al., 1931). Pre-adolescent and post-menopausal females are less commonly infected (Skinner, 1947). The incidence of yeast in the vagina of pregnant women has been reported to be high. Twenty-eight per cent of 300 women of all economic classes were found to harbor yeast-like organisms (Woodruff and Hesselstine, 1938). Nearly half of these patients had symptoms of vaginitis. These data were collected on the basis of vaginal smears and included all yeasts and yeast-like fungi. Others

(Carter et al., 1940) have reported even higher figures for the incidence of vaginal yeasts and yeast-like organisms in the gravid female. The above cited workers did not distinguish C. albicans from other yeast-like organisms. Negroni (1935) found that 8 per cent of 100 non-pregnant, as opposed to 33 per cent of pregnant women, harbored C. albicans in their vaginas. The paper of Jones and Martin (1938) has been considered of importance. They reported yeast-like fungi in the vaginas of 32 per cent of a series of pregnant and 14 per cent of non-pregnant women. C. albicans was only isolated from those patients exhibiting symptoms. In the normal vagina the closely related organism they described as C. albicans var. stellatoidea was found. This yeast-like organism was considered non-pathogenic. Skinner (1947) has suggested that the work of Jones and Martin (1938) may cast a considerably different light on the many reported findings of C. albicans in the genital tract of normal females. The ordinary methods of identification of C. albicans do not enable one to distinguish the so-called variety from the species. Skinner (1947) further suggested the possibility that C. albicans as distinct from the variety may not be a part of the normal vaginal flora. He proposed that the source of the pathogen for vaginal candidiasis could best be explained on the basis of contamination with fecal material from the gastrointestinal tract, which in many persons definitely contains C. albicans. As has been noted previously, however, others have been unable to isolate yeast-like organisms from female genitalia with the supposed characteristics of C. albicans var. stellatoidea (Rauramo, 1950, and Dawkins et al., 1953).

The reason for the greater incidence of genital infections in the pregnant female is not definitely known. The increase in glycogen in the vaginal mucosa during the third trimester has been offered as an explanation (Skinner, 1947). Definitive data indicating that this is the true explanation have never been collected.

Experimental infections have been produced in both pregnant and non-pregnant females (Hesseltine et al., 1934, and Bland et al., 1937). Cultured C. albicans cells were instilled into the vagina while using other Candida species as controls. Subjective symptoms and increased discharge were produced using cultures of C. albicans only. Bland et al. (1937) indicated that pruritus and increased discharge began 24 to 96 hours after inoculation into the vagina.

Systemic candidiasis, with involvement of many different organs, occurs relatively rarely. On the basis of diagnostic difficulties during life and at autopsy, Lederer and Todd (1949), Ludlam and Henderson (1942) and others have suggested that C. albicans may be responsible for a more significant number of fatal infections. According to these authors, systemic candidiasis may pass unrecognized at autopsy unless specific cultural and histological examinations are carried out, since, generally, there is a lack of characteristic gross pathological changes. Plenert (1955) has suggested that diagnosis of systemic infection caused by C. albicans should only be made when the organisms are demonstrated in the liver, brain or parenchyma of the kidney. To this list of affected organs or tissues the following should be added: spleen, subcutaneous tissues, myocardium, diaphragm and other muscles, peritoneum, pleura, endocardium, pericardium, urinary bladder, ureters, lung, bone, thyroid,

and others (Dobias, 1957, Ludlam and Henderson, 1942, Harrell and Thompson, 1958, Schaberg et al., 1955, etc.). Dissemination by means of the blood stream and lymphatics has been the suggested means of infection of these organs and tissues under natural conditions (Dobias, 1957).

Certain groups of humans have been observed to be more susceptible to infections caused by C. albicans. Among these individuals are those suffering from metabolic and endocrine disturbances, neoplasia, and other states of debilitation and pathological changes in pulmonary tissue. The increased incidence of C. albicans vaginitis in pregnant females has already been considered.

One group of females showing the highest incidence of vaginal candidiasis is that suffering from diabetes melitus (Hesseltine, 1933). The common term for the disease has been diabetic vaginitis. Hesseltine and Campbell (1938) have suggested that these individuals are more susceptible because of the recurrent washing of the genital skin and mucous membranes with glucose-containing urine. They stated that this encourages the growth of the yeast-like organism and with diet or insulin therapy the condition is usually cured or ameliorated immediately after the disappearance of the glucosuria. The disease was reported to be less common in the pre-adolescent and post-menopausal years. They were able to produce an experimental disease in women by applying glucose in powder or solution to normal vaginal surfaces which harbored C. albicans. If yeast-like organisms were absent, however, no symptoms were produced. Hesseltine and Campbell emphasized that it is not the glucose, acetone bodies or other compounds in diabetic urine which cause symptoms, but rather that the glucose favors the growth of the C. albicans which is

already in the vagina. This hypothesis apparently has not been examined further. As has been discussed previously, an increased incidence of cutaneous candidiasis in diabetics has also been reported (Greenwood and Rockwood, 1930).

Clinical associations between alteration in adrenocortical and parathyroid function and superficial candidiasis have been observed (Whitaker et al., 1956). In all the patients in whom it was present, cutaneous and oral candidiasis preceded the hypoparathyroidism or Addison's disease by various periods of time, from 3 months to as long as 13 years. The authors suggested that the absence of evidence of visceral candidiasis in any of the 6 autopsy cases precluded the possibility that C. albicans infection of the parathyroid or adrenal glands led to the malfunction. Rather, they speculated that the hypoparathyroidism and hypoadrenocorticism resulted in a depression of normal host resistance which allowed manifestation of the superficial infections.

The increasing incidence of several different clinical forms of candidiasis in patients suffering from leukemia or various forms of lymphomatous disease has been reported by Winter and Foley (1956a) and Keye and Magee (1956). Both groups of investigators suggested that the increase is not due to greater awareness in diagnosis of candidiasis, but to the different therapeutic agents used in attempts to control the neoplastic growth and to the extensive use of antibiotics in these patients.

That the widespread use of antibiotics has resulted in increased numbers of cases of candidiasis has been reported many times (Harris, 1950, Woods et al., 1951, Lipnik et al., 1952, etc.). Deaths due to

disseminated *Candida* infections have been increasing in frequency (Pappenfort and Schnall, 1951). The antibiotics most commonly incriminated are members of the tetracycline structure class and chloramphenicol. A number of theories have been proposed to explain the increased incidence of candidiasis as a complication of antibiotic therapy.

One of the commonly proposed theories is the "suppression with substitution" concept suggested by Miller (1951). This theory holds that the administration of the antibiotics upsets the normal flora of an area resulting in an overgrowth of resistant species of microorganisms which can overwhelm the host resistance. McVay and Strunt (1951) in cultural studies of oral, vaginal and rectal specimens found that when chlortetracycline was given, 63 per cent of a total of 186 patients had *C. albicans* in their stools. None of these individuals harbored the organisms before therapy. Winter and Foley (1956a) have found a definite correlation between antibiotic therapy and increased oral carrier rates for *C. albicans*. Among those patients receiving no antibiotic therapy, 7 per cent had positive cultures, while cultures were positive in 31 per cent of those receiving antibiotics. McGovern et al. (1953) have also observed an increased carrier rate in children following administration of antibiotics. However, others have been unable to find an increase in the number of antibiotic-treated patients with *C. albicans*. Robinson (1954) studied the occurrence of *C. albicans* in the stools, vagina or oropharynx of patients who had received penicillin and/or one of the "broad-spectrum" antibiotics in contrast to those who had not been treated with antibiotics for at least 3 months. His results indicated that there were no significant differences in *C. albicans* carrier rates

in antibiotic treated and non-treated individuals. In all patients the carrier rates ranged between 13 and 17 per cent. An experimental study has indicated the administration of chlortetracycline or oxytetracycline at therapeutic levels did not materially alter the numbers of C. albicans in the fecal material of turkey poults given organisms via the oral route (Sieburth and Roth, 1954).

A second theory proposed is that members of the normal flora of the gastrointestinal tract supply certain essential nutrients to the host (Harris, 1950). This author has suggested that the inhibition of growth of organisms in the gastrointestinal tract by the antibiotics results in nutritional disturbances which affect the integrity of the mucous membranes, opening a portal of entry for microorganisms which normally are unable to penetrate the intact healthy mucosa. Harris stated that the administration of vitamin preparations will alleviate symptoms in some patients which develop infections of the intestinal tract following extensive tetracycline therapy.

A third proposed explanation suggested that some of the antibiotics directly stimulate the growth and/or virulence of C. albicans. Many workers have presented data to support the validity of this theory.

Moore (1951) reported that crystalline chlortetracycline hydrochloride at a concentration of 0.2 mg per ml in Sabouraud's glucose broth stimulated the growth of C. albicans. This finding was corroborated by Pappenfort and Schnall (1951). Six different lots of chlortetracycline as prepared for oral administration showed stimulatory effects, while no apparent increase in growth of C. albicans resulted when exposed to either of two lots of the drug prepared for parenteral injection.

Huppert et al. (1953) found that when chlortetracycline hydrochloride was present in a broth culture medium in concentrations greater than 0.1 mg per ml significant stimulation of growth of C. albicans took place. In their studies, growth was determined by increase in total cell nitrogen. Under the same experimental conditions, penicillin, chloramphenicol, streptomycin and oxytetracycline did not show a similar effect. In a later publication (Huppert and Cazin, 1955), a stimulatory effect on the growth of C. albicans was found with neomycin, bacitracin and chlortetracycline. No effect on growth of this fungus was noted with magnamycin, erythromycin or a preparation of chlortetracycline which contained parabens. The authors reported no correlation between in vitro growth stimulation and the association of the antibiotic with the development of candidiasis.

An approach to the study of the in vivo effects of antibiotics on *Candida* has been made by Huppert et al. (1955). They indicated that an intestinal carrier state in mice could be established by oral inoculation of suspensions of cells of C. albicans and different antibiotics. Mice inoculated with cell suspensions and water did not excrete C. albicans in their feces beyond 24 hours. The carrier establishing effects were observed with all antibiotics studied, which included chlortetracycline, chloromycetin, oxytetracycline, dihydrostreptomycin, magnamycin, neomycin, erythromycin, penicillin and tetracycline. The dose of each antibiotic used except penicillin was calculated on the basis of the standard dose for a 70 kg man. A statistically significant difference was noted with two different strains of mice. The RAP strain of Rockland Farms tended to be more susceptible to oral exposure to a suspension of C. albicans

administered during a course of antibiotic treatment and to be more uniform in response than the CF-1 strain of Carworth Farms. The authors concluded that direct stimulation of C. albicans growth cannot be the fundamental mechanism for the increased incidence of candidiasis in antibiotic-treated patients, since stimulation of growth was only evident with chlortetracycline, neomycin and bacitracin. However, all of the antibiotics tested were capable of inducing the carrier state in mice.

Another approach to the study of in vivo effects of antibiotics on candidiasis has been made by Seligmann (1952). Using a strain of C. albicans which was non-pathogenic via the intraperitoneal route, the addition of 2 mg of chlortetracycline to the inoculum resulted in fatalities in the majority of mice. A similar effect was noted when the drug inoculation preceded the intraperitoneal injection of Candida cells by as long as 24 hours. Given 8 and 24 hours after infection, chlortetracycline was ineffective. The "virulence enhancing activity" of the antibiotic was destroyed by standing at room temperature or by boiling, and no indication of toxin production or biological alteration by C. albicans was found in vitro. The author suggested that the mechanism of action of chlortetracycline was one of lowering the animal's resistance. In a later publication (Seligmann, 1953), oxytetracycline was found to have similar effects on experimental candidiasis induced by intraperitoneal injection. Penicillin, bacitracin and chloromycetin did not exhibit "virulence enhancement." It was further found that only the intraperitoneal injection of chlortetracycline or oxytetracycline and C. albicans cells resulted in increased mortality rates. Studies of all

other routes of administration and combinations failed to demonstrate the effect. The author indicated that the intravenous inoculation of both drug and fungus resulted in decreased mortality in mice as compared to groups of animals receiving only the organisms intravenously.

A problem of considerable interest, with clinical as well as theoretical significance, has become evident in the treatment of neoplastic diseases since the advent of use of steroid hormones, adrenocorticotrophic hormone, nitrogen mustards, tri-ethylene melamine, antagonists of folic acid, 6-mercaptopurine, and other anti-leukemic drugs, as well as multiple antibiotics. A significant increase in candidiasis as well as other mycotic infections has been observed in patients suffering from leukemia, lymphomas, Hodgkin's disease and other neoplastic diseases (Keye and Magee, 1956). In an extensive study of *Candida* infections, Winter and Foley (1956a) have reported a significantly increased *C. albicans* carrier rate in children with malignant disease as compared to those suffering from other illnesses. Comparisons were made between 165 patients of a tumor therapy clinic and 1,004 general pediatric patients. The carrier rate was also increased in those groups of children with diabetes mellitus, cystic fibrosis of the pancreas, nephrotic syndrome and rheumatic disease. Treatment with antibiotics and/or cortisone, general condition of the patient and primary diagnosis were all factors affecting the carrier rate.

Keye and Magee (1956) have studied the results of 15,845 consecutive autopsies performed from 1919 to June, 1955. Material for this survey consisted of clinical records, autopsy protocols, gross and histological material and data from cultural studies of all patients with fungus

infections. These cases totaled 88. The authors were able to come to the following conclusions. (a) A significant increase in the number of fungus infections has occurred in recent years. This increase was mainly due to secondary fungal infections. (b) Histoplasmosis, cryptococcosis, mucormycosis, aspergillosis and candidiasis accounted for the increased incidence. No change was observed in the incidence of primary mycotic infections. (c) There was a tendency for more extensive dissemination of the fungal infections in recent years, as compared with that observed before the use of antibiotics. (d) A large number of recently observed patients with leukemia or lymphoma had secondary fungal infections. None of the patients with these malignant diseases prior to 1948 had complicating fungus infections.

Keye and Magee interpreted their findings as indicating a definite relationship between the observed increase of secondary fungal infections and the increased use of antibiotics, cortisone and chemotherapeutic agents used in the treatment of leukemia and lymphomas. One possible explanation in all cases of superinfections caused by mycotic agents is that normal host defense reactions are depressed as a result of attempted therapy of the primary disease, infectious or otherwise. The data may support a statement that such superinfections as candidiasis in patients being treated with drugs which depress normal host defense mechanisms are, indeed, "diseases of therapy."

On the basis of published information, *Candida* species other than *C. albicans* apparently are of little importance as causative agents of human candidiasis. However, these "non-pathogenic" *Candidas* have been reported to cause human disease (Dobias, 1957). Among the incriminated

organisms were C. tropicalis, C. pseudotropicalis, C. krusei, C. parapsilosis (C. parakrusei) and C. guilliermondi. Five cases of endocarditis were cited by Skinner (1947) in which the only organisms isolated from blood cultures were C. parakrusei or C. guilliermondi. C. tropicalis has been associated with broncho-pulmonary disease (Castellani, 1910).

II. The Role of Candida Species in Natural Animal Infections.

Few studies have been reported in the literature on the ecological relationships of Candida to animals other than man. Skinner (1947) has cited references which have reported the isolation of C. albicans from laboratory animals, rats, pigeons and the European hedgehog. Other investigators reported the isolation of the yeast-like organism from rabbits, guinea pigs and rats (Coutelen and Cocket, 1942), and from fowl, a lamb, a cat, a dog, a turkey and donkeys (Tallice, 1932). Jordan (1953) recovered C. albicans from the crop of 21 per cent of 378 fowls. Huppert et al. (1955) have observed the apparent absence of Candida from the intestinal contents of laboratory mice. C. albicans has been found not to be a member of the normal flora of the bovine intestinal tract (van Uden and Sousa, 1957). These authors founded their conclusions after culturing caeca contents of 252 adult cows. One hundred and thirty-one strains of yeasts were isolated and not one exhibited the morphological or biochemical characteristics of C. albicans. The suggestion was made that C. tropicalis is substituted in the bovine intestinal flora for C. albicans and that this might explain the apparent

rareness of candidiasis in cows. Forty-five strains of the former organism were cultured.

Candida albicans has been reported to produce disease in chickens and turkeys and can cause considerable losses in a bird population (Skinner, 1947 and van Uden and Sousa, 1957). The infections were reported to be thrush-like and involved the mouth, crop, proventriculus and gizzard. In young birds, candidiasis was often fatal while older animals usually recovered.

From the published information available in the literature, natural candidiasis in domestic mammals must be rare. Hagan and Bruner (1956) have stated that "...authors refer to monilia infections of the oral mucosa in calves and colts. No additional information about them is available. Presumably they are of little consequence."

III. Studies on Experimental Candidiasis.

One of the first attempts to study experimental candidiasis was made by Klemperer in 1885. He apparently injected the yeast-like organisms intravenously into laboratory animals. In the intervening years since that time, few studies have been made and recorded in the literature on experimental disease caused by C. albicans. Until quite recently most investigators were mainly interested only in whether the yeast-like organism isolated from a clinical specimen was "pathogenic or non-pathogenic." The procedures used for these determinations of pathogenicity allowed many false conclusions to be drawn (Dodge, 1935). Associated with this confusion were the problems referable to the

almost extreme degree of entanglement in identification and nomenclature of members of the genus now known as *Candida*. Reference has already been made to the large number of synonyms given to the single organism, *C. albicans* (Conant et al., 1944).

Within the past 25 to 30 years gradual increased interest in the host-parasite relationships in experimental mycotic diseases has become evident with candidiasis being included. Since about 1950, with the observed significant increase in incidence of many forms of *C. albicans* infections in humans treated for bacterial infections, investigative publications on experimental candidiasis have become more numerous. Perhaps, with a more thorough understanding of the host-parasite interactions under conditions of induced infections in laboratory animals, a more logical approach to the control of natural infections in the human could be made.

Most common laboratory animals have been reported to have been infected experimentally with *C. albicans*. Ashford (1916) was able to induce fatal infections in rabbits, rats and guinea pigs. He injected a broth culture of "Monilia X" into the tongue of a rabbit. This strain of organism had been isolated from gastrointestinal contents of a patient with severe symptoms of sprue. During the process of injection of the broth culture, "...the animal received also about 2 c.c. per oram." After 48 hours violent symptoms of diarrhea developed and the rabbit died 75 hours after injection. Extensive pathological changes were noted in the internal viscera and pure cultures of "Monilia X" were isolated from the spleen, liver, kidneys, lungs and heart's blood. The organism was also recovered from tissues at the original site of injection and

from the mucous membrane of the stomach and small intestine. The author reported that the organism lost its virulence upon continued cultivation but it could be recovered by passage through susceptible animals, "...and even reach such a point as to sicken or kill animals by continued feeding." Subcutaneous injection of this strain into rabbits resulted in the development of a "blastomycotic ulcer." Smith (1924) observed similar effects in guinea pigs and rabbits. He reported that infection of the gastrointestinal tract could be produced in guinea pigs by intraperitoneal injection or by introduction of organisms directly into the stomach. These animals had been maintained on a vitamin C deficient diet. Using guinea pigs fed a normal diet, Nye et al. (1929) observed no gastrointestinal symptoms or lesions in animals fed for a month with fresh cultures of "Parasaccharomyces A" obtained from stools and gastric contents of cases of pernicious anemia. Fatal infections in rabbits could be produced, however, with these strains by either intravenous or intraperitoneal inoculations. White mice have been reported to have been infected with yeast-like organisms. Van der Linden (1926) produced fatal infections by intravenous injection of cells of several different "Monilia albicans" strains, and others (Tanner et al., 1927) have found that yeast-like organisms isolated from throats of normal individuals were pathogenic for mice inoculated via the intraperitoneal route.

As has been stated previously, much of the early literature has contributed little to an understanding of the problems concerning differences in virulence of strains of C. albicans and the factors referable to the host and/or parasite which can modify this quantitative expression of pathogenicity.

Van der Linden (1926) has been reported to have been able to detect differences in virulence of 3 strains of "Monilia albicans" (Dowling, 1930). He produced fatal infections in mice in from 1 to 12 days by intraperitoneal inoculation of these organisms. The strains were reported to exhibit "marked" differences in virulence when injected intraperitoneally or intravenously. Others (Stovall and Pessin, 1933) measured differences in rabbit virulence of "Monilia" species. Strains of "Monilia parapsilosis" (C. parapsilosis or C. parakrusei) were found to be avirulent for rabbits when injected intravenously in numbers as high as 200 million per 100 gm body weight. "Monilia candida" (C. tropicalis) strains were of intermediate virulence, with approximately 30 million cells per 100 gm body weight necessary to produce lethal effects. These authors reported that the lethal dose of 5 strains of "Monilia albicans" (C. albicans) varied between 1.5 and 6.0 million cells injected intravenously into rabbits. MacKinnon (1936) determined by intravenous inoculation the "dosis minima mortal" for rabbits of several strains of "Micotorula albicans" (C. albicans). His results indicated that the minimum lethal dose of separate isolates varied between 2.5 and 20 million cells. In none of the three preceding works were the numbers of animals used to make these virulence determinations noted.

Salvin et al. (1952a) have reported quantitative studies on differences in virulence of separate isolates of C. albicans. Mice were inoculated intraperitoneally with counted numbers of cells of different strains suspended in gastric mucin solutions. The addition of hog gastric mucin to suspensions of cells of pathogenic fungi was shown by Strauss and Kligman (1951) to increase the virulence of these organisms for mice

when injected intraperitoneally. Salvin's data indicated significant differences in virulence of C. albicans for white mice. Other members of the genus *Candida* were found to be lethal for 21-day-old female mice when these animals were challenged by the use of the same techniques (Salvin, 1952b). Data were collected from groups of animals which received relatively large numbers of cells of strains of C. albicans and other species of *Candida* via the intraperitoneal route. Mean lethal doses (LD50) for the organisms employed were calculated and compared on the basis of a 48-hour observation period. Unfortunately no attempts were made to arrive at an estimate of the errors of these determinations. The necessary data for these calculations were not presented. The author concluded that *Candida* species other than C. albicans may be of importance in infection of man. Assuming no errors in the LD50 determinations, one could conclude from the data presented that one isolate of C. krusei was more virulent than 3, equal in virulence to one and less virulent than 2 of the strains of C. albicans studied.

In the study of a host-parasite relationship, factors referable to the parasite which result in variation in the extent and the outcome of the interaction are of extreme importance. Among the more important of these is the stability of virulence of the parasite under in vivo and in vitro conditions.

Early workers on experimental candidiasis reported that continued growth of C. albicans on artificial media resulted in reduced virulence for laboratory animals (Ashford, 1916 and Kurotchkin and Lim, 1930). The former author indicated that an original culture of "Monilia X" which was virulent for rabbits, "...after 5 months, during which the

original culture had become old and full of mycelium, and had also been several times replanted," was unable to produce symptoms in rabbits when injected intraperitoneally. The inoculum for these animals consisted of 5 ml of a 30-day broth culture. Kurotchkin and Kim described an experiment using a strain of "Monilia tropicalis" that was isolated from a case of "bronchomoniliasis." The "virulence" of this organism for hamsters was determined by intraperitoneal injection. Very soon after the original isolation, 18 million cells were found to be necessary to result in death of animals within 48 hours. After three months of cultivation on Sabouraud's medium, 270 million cells were necessary to produce the same effect. MacKinnon (1936) reported that the strains of "Micotorula albicans" that he studied maintained their virulence for rabbits, guinea pigs, mice and rats for long periods of time. Over shorter periods of cultivation on artificial media, Hill (1958) found that the LD50 for mice of one strain of C. albicans did not significantly vary.

Ashford (1916) and Kurotchkin and Lim (1930) also reported that strains of presumably C. albicans and perhaps C. tropicalis regained or increased their virulence when passed through animals. Contrary to these suggested findings, MacKinnon (1936) observed that animal passage did not increase the virulence of "Micotorula albicans." In the recent literature no accounts of controlled studies on variation in virulence of C. albicans strains have been found.

C. albicans has been reported to produce "variants" which have been called membranous or lethal variants (MacKinnon, 1940). These dissociated forms are characterized by differences in colonial morphology, microscopic morphology, agglutinability in saline and tryptaflavin

solutions, etc. In the case of the membranous variants, partial reversion to the normal yeast-like form was noted. However, the reverted forms were unstable and tended to return to the membranous form. The lethal variants were characterized by a greatly reduced growth rate and by increasing difficulty to produce mycelial growth. The author reported that membranous variants were of reduced virulence for laboratory animals as compared to the normal yeast-like organisms. Lethal variants were characterized by greatly diminished or complete loss of virulence. Similar results have been reported by others (di Menna, 1952 and Eisman et al., 1953). These investigators reported rough colonial variants exhibiting reduced virulence. In the cases of the experiments of the latter authors, differences in virulence of strains were determined on the basis of average survival time of groups of mice after intraperitoneal injection of cells suspended in 4 per cent gastric mucin. Winsten and Murray (1956) have reported that the addition of 0.25 per cent cysteine would suppress filamentous growth of rough variants of C. albicans and allow the growth of normal yeast-like cells. This effect of reducing substances on the growth of filamentous variants of C. albicans was reported earlier by Nickerson and Chung (1954). Winsten and Murray found that the rough, filamentous strain grown in a broth medium with added cysteine exhibited increased virulence for mice as compared to the same organism grown in a broth without added cysteine.

Of equal importance to the understanding of host-parasite interactions, are the many sources of variation in the relationship which are contributed by the host. Attempts to evaluate some of these factors have been made.

Few accounts are found in the literature of experiments designed to study the effects of diet, age, sex or environmental conditions on experimental candidiasis. Ashford (1916) and Smith (1924) reported that guinea pigs fed on a diet low in vitamin C were more easily infected with what were probably strains of C. albicans.

Age as a host factor has been reported to be of importance in susceptibility to experimental candidiasis. Mice 3 weeks of age were found to exhibit decreased survival time as compared to older animals when challenged intraperitoneally with cells of C. albicans suspended in 2.5 per cent hog gastric mucin (Salvin, 1952b). Sex, however, had no marked influence on the susceptibility of 60-day-old mice to infection.

One of the few studies on the effects of environmental factors on experimental candidiasis has been reported by Scherr (1953a). Groups of mice received a uniform inoculum of cells of C. albicans intraperitoneally and were then maintained in incubators whose temperature ranges were 5°-12°C., 15°-20°C., 28°-32°C. and 35°-37°C. Mortality data were collected and at the end of 30 days all surviving animals were autopsied. Mice infected and kept at temperatures of 5°-12°C. and 35°-37°C. showed a higher death rate and more severe dissemination than those maintained at 15°-20°C. and 28°-32°C. This effect of temperature on experimental candidiasis in mice was suggested to contribute to difficulties encountered with infected mice during the summer months.

Studies have shown that the administration of hormones to infected animals can modify the course of experimental candidiasis. Cortisone in the dose range of 1 to 4 mg per day increased the susceptibility of mice to disseminated infection (Seligmann, 1953, Friedman et al., 1954, Roth

et al., 1957, etc.). Scherr (1955, 1956) has reported results of extensive studies of the effects of hormones and other drugs on experimental candidiasis in mice. Suggestions were made that the enhancing effect on infected mice was, among other things, a function of the severity of the infection. He reported that cortisone can, under certain conditions, have an efficacious action on infected animals. That these enhancing effects of cortisone may be dependent on the amount of hormone given to infected animals has been established by Hill et al. (1954). Adrenalectomized mice infected intravenously with C. albicans and treated with varying amounts of cortisone exhibited definite differences in mortality rates. Cortisone, at a dose of 0.01 mg per day, provided a significant degree of protection to these mice. Doses above and below this optimal amount resulted in increased mortality rates. Adrenalectomized mice given 0.01 mg of cortisone acetate per day were found to be almost the equivalent of intact mice with respect to resistance to experimental candidiasis.

Mankowski (1955) studied the influence of other steroid hormones and alterations in the hormonal balance of mice experimentally infected with C. albicans and other pathogenic fungi. Under the described conditions, estradiol administration decreased the survival of mice infected with C. albicans, Histoplasma capsulatum or Cryptococcus neoformans. This effect was reported to be more conspicuous in females than males. Progesterone was reported to inhibit the deleterious effects of estradiol on fungus-infected female mice. Ovariectomized mice infected with C. albicans exhibited an appreciably longer survival time.

Very few attempts to modify experimental candidiasis by immunization have been recorded in the literature. Van der Linden (1926) was reported to have been unable to protect animals against infection with "Monilia albicans" by repeated subcutaneous injections of living organisms (Dowling, 1930). Marcus and Rambo (1952) injected formalin-killed cells of C. albicans and subsequently challenged mice intravenously with living organisms. No significant differences between mortality rates of immunized and control animals were noted. However, data on cultural studies of surviving animals indicated that there were fewer numbers of immunized animals with positive kidney cultures than those from the control groups. Studies on other animals (Winner, 1956) have indicated that attempted immunization against C. albicans infections afforded little if any protection against death from experimental candidiasis. Rabbits were found to possess a considerable degree of natural immunity to intravenously injected C. albicans cells. The presence of natural or of artificially-induced agglutinins did not protect the animals from the lethal effects of the organisms. Prior repeated injection of formalin-killed cells did not result in a difference in the pathological lesions produced after inoculation of large doses of living organisms.

A number of different studies on the pathogenesis of experimental candidiasis have been made. Radaelli (1924) used several strains of what must have been C. albicans and injected various animals by different routes of inoculation. When introduced intraperitoneally, intravenously or intraarterially into rabbits, rats and guinea pigs, these organisms were pathogenic and produced extensive kidney damage. Rarely were the heart muscle, spleen, mesenteric lymph nodes or liver found to exhibit

macroscopic lesions. The investigator suggested that the organism acts as an embolus with blockage of small vessels. The organisms grew and penetrated from the primary site of the embolus into surrounding tissues. They did this, he suggested, by radiating filaments from a central point. Nye et al. (1929) found essentially the same pathological effects in rabbits inoculated with "Parasaccharomyces A" strains. Stovall and Pessin (1933) maintained that simple mechanical plugging of vessels could not explain the nature of pathogenicity of C. albicans, but that the ability to grow in the animal body and invade tissue were qualities essential for virulence. Using cells of 3 different species, "Monilia albicans," "M. parapsilosis" and "M. candida," only "M. albicans" (C. albicans) produced filaments in vivo.

MacKinnon (1936) in an extensive study on experimental infections induced by the injection of cells of "Micotorula" strains reported that two distinct types of kidney lesions were produced depending on the virulence of the strain and the number of organisms injected. In rabbits challenged intravenously, "lesiones de siembra" or microabscesses developed if the animals survived only a short time. Animals surviving at least a week exhibited large triangular shaped kidney lesions, named by him, "lesiones de eliminacion."

Within the last 5 years increased interest in the pathological effects of C. albicans in experimental animals has become evident. Evans and Winner (1954) reported their studies on the histogenesis of lesions in experimental candidiasis in the rabbit. Animals were injected intravenously with constant numbers of cells of C. albicans and tissues were removed from sacrificed rabbits at 6, 24, 48, 72 hours and

after death from infection. In initial lesions only a few yeast-like cells were seen. Tissue reactions to these organisms consisted of slight necrosis which was sometimes only limited to pyknosis of a few cells. Little inflammatory response occurred as evidenced by cellular reactions. By 24 hours after injection, proliferation of organisms in tissues was apparent with budding and mycelial formation. Polymorphonuclear reactions of variable intensity were evident and were associated with more extensive tissue necrosis. In some of the lesions histiocytes were present, but neither destruction nor phagocytosis of the organisms were apparent. The authors suggested that spread of the lesion appeared to be by local invasion of the tissues, the developing organism passing directly through cells and membranes, thus increasing the area of tissue damage and inflammation. By 48 hours after intravenous inoculation, extensive formation of filamentous organisms was seen. From the blood stream, strands of pseudomycelia were observed to enter adjacent tissues by direct penetration of the capillaries. All organs and tissues examined contained proliferating organisms and an even distribution of lesions was described. Abscesses were seen more commonly in the renal cortex and ventricular myocardium with no localizations evident in the spleen or bone marrow. New foci of infections with the previously described characteristics were apparent.

Two distinctly different types of tissue changes were observed in the kidneys of rabbits injected intravenously. The first consisted of local necrosis of tissues where organisms were found to lie in the glomerular and intertubular capillaries. With increasing time after injection more abundant pseudomycelial formation was evident. The other

type of tissue change became evident by examination of specimens removed 48 hours after injection. This consisted of hyaline thickening of the basement membrane of a few of the capillary loops of some glomeruli. Not all glomeruli were affected and no direct correlation between presence of organisms and development of the change was shown. The hyaline material took a positive stain with the periodic-acid Schiff technique and was noted to increase in amount with time until there was complete obliteration of the capillary loops. No evidence was found to suggest that capillary embolism by the fungus was responsible for this lesion. No explanations were offered as to the nature or origin of this hyaline material.

The study of the pathogenic effects of C. albicans in mice injected intravenously has indicated that these animals react very similarly to rabbits (Adriano and Schwarz, 1955). In these experiments, lesions were most prominent in the myocardium, kidney, brain and spleen. Depending on the number of organisms injected, myocarditis or kidney damage were suggested to be the immediate cause of death. Central nervous system involvement was believed to be a late manifestation of the experimental disease.

The intraperitoneal route of inoculation for inducing experimental Candida infections in mice has been used by many different investigators (MacKinnon, 1936, Straus and Kligman, 1952, Salvin, 1952 and others). Studies on the pathogenesis of disease initiated by this means have indicated that the hematogenous spread may be the usual course of events in establishment of widely disseminated candidiasis (Seligman, 1952 and Winter and Foley, 1956a). The major pathological changes were

confined to the renal tissues and consisted of miliary abscesses deep within the substance of these organs. Suspension of the inoculum in a solution of hog gastric mucin was observed to have no significant effect on the mortality rates or character of renal lesions in infected mice. Mice inoculated with organisms via the intravenous route were reported to develop the kidney lesions sooner. The administration of chlortetracycline, oxytetracycline, tetracycline or cortisone by intraperitoneal injection to mice challenged intraperitoneally, resulted not only in increased mortality, but resulted in massive invasion of the renal cortex from the peritonealized surfaces of the kidney. This type of lesion was not seen in untreated animals. The authors suggested that the action of the antibiotics on enhancement of this experimental disease appeared to be due to decreased hematogenous dissemination with more significant damage to the kidney resulting from cortical invasion. Young (1958) found that 2 hours after injection of heavy suspensions of C. albicans intraperitoneally into mice, tissue sections of abdominal wall, mesenteric lymph nodes, spleen, liver, kidney and intestine were negative for organisms. However, in the pancreas, the mycelial organisms were invariably present in the connective tissue capsule and septa, and in some of the blood vessels. He suggested that the route of invasion from the peritoneal cavity was by way of the pancreas and its blood vessels.

Some of the pathological changes in tissues of animals suffering from experimental candidiasis might be explicable on the basis of a toxic substance associated with the organism, C. albicans (Winner, 1956). Twenty-one-day-old female mice were found to die as the result of intra-

peritoneal injection of large numbers of dead cells of this organism (Salvin, 1952b). This lethal effect of dead cells of C. albicans, Candida species and other pathogenic and non-pathogenic fungi was more evident when 1 to 2 mg of dried tubercle bacilli were added to the inoculum. There appeared to be few, if any, correlations between toxicity of pathogens and their virulence for mice. Others (Roth and Murphy, 1957) have reported a substance released from living cells of C. albicans by supersonic vibration. This water-soluble substance was lethal to mice treated intraperitoneally with chlortetracycline and of decreased lethality to mice treated with oxytetracycline. Evidence they presented may indicate that this toxic substance is a surface component of cells of C. albicans.

From the foregoing discussion of literature concerning the role of C. albicans in natural human infections, one of the more definite conclusions that can be drawn is that this organism is a relatively common parasite of man and does cause significant clinical disease.

Because of the common association of C. albicans with man, as a member of his normal flora, many if not most human infections have an endogenous origin. Under conditions of close association, especially infants in hospitals, exogenous infections also occur.

Although some of the factors or states which predispose to development of active infection are known, more definitive information on normal host defenses against this organism would seem to be essential. Studies on the pathogenesis of both natural and experimental candidiasis may yield information as to specific or non-specific means to prevent

the development of these infections in humans who are more prone to become infected. Considering the indisputable evidence indicating an increased incidence of primary and secondary human infections caused by C. albicans, especially patients given prolonged treatment with antibiotics, steroid hormones, and certain chemotherapeutic drugs, it would appear to be more than of academic interest to obtain more knowledge of the exact nature of the host-parasite interactions in this infectious disease.

MATERIALS AND METHODS

I. Organisms.

The strains of Candida albicans and other members of the same genus used in this study were obtained from either Dr. S. Marcus, Dr. S. B. Salvin, local hospitals or human clinical material. The following isolates of Candida were those commonly used:

Candida albicans

Strain 520 was obtained from Dr. S. Marcus. It was isolated from a case of vaginal candidiasis and originated from the laboratory of Dr. M. Littman, Tulane University.

Strain 3148 was kindly furnished by Dr. S. B. Salvin, Rocky Mountain Laboratories, P.H.S. It was originally from the collection at the National Institutes of Health and was reported to have been isolated from a case of endocarditis.

Strain 3160 was kindly supplied by Dr. S. B. Salvin. This culture was originally obtained by him from the American Type Culture Collection.

Strain 3161 was isolated from a patient with oral candidiasis and kindly furnished by Dr. S. B. Salvin.

Strain U #308 was obtained from the Department of Bacteriology Culture Collection, University of Utah.

Strain U #309, obtained from the above source, labeled C. albicans, Vets Hospital, mouse virulent.

Strain U #312, designated Candida (Monilia) albicans #4551, was obtained from the Department of Bacteriology Culture Collection, University of Utah.

Strain U #313 Giles was originally isolated from lesions in an adult patient with oral candidiasis. This strain was obtained from the same source as above.

Strain H. X. was isolated from the blood of a patient who died of systemic candidiasis following antibiotic therapy for bacterial endocarditis.

Strain Nick was isolated from the oral cavity of an infant with clinical symptoms of infection.

Strain Larsen was isolated from an oral lesion in the mouth of a 5-year-old child.

Strain Vet #2 was obtained from a local hospital and was of unknown origin.

Strain Vet #4 was isolated at a local hospital from a sputum specimen submitted for routine culture.

Strain Vet #5 was isolated from the sputum of a patient in a local hospital.

Strain Anderson was obtained from a local clinic and was of unknown origin.

Strain Finlayson (oral) was isolated from a female patient with a single oral lesion.

Strain Finlayson (vaginal) was isolated from the above woman who had complained of symptoms of vaginal candidiasis of several years duration.

Strain Lewis #2 was obtained from the sputum of a patient with Hodgkin's disease. The specimen also contained a strain of Candida tropicalis, Lewis #1.

Strain Goring was isolated from fluid of a K B cell tissue culture. This tissue culture had been inoculated with washings from a swab specimen from a chronic oral lesion.

Strain Sekino was isolated from the vagina of an 18-year-old student of nursing who had described symptoms of "burning and itching" for several years.

The following *Candida* species were all obtained by Dr. S. Marcus from the Northern Regional Research Laboratories: *Candida pseudo-tropicalis*, Y-83; *Candida krusei*, Y-301; *Candida tropicalis*, Y-619; *Candida parapsilosis* (*Candida parakrusei*), Y-316; and *Candida guilliermondi*, Y-304, and kindly made the above culture available to the author. An additional strain of *C. tropicalis*, Lewis #1, was isolated from clinical material as described above.

In the early parts of this study all organisms were maintained on both 15 per cent human blood agar and Sabouraud-maltose agar slant cultures incubated at 37°C. and transferred at weekly intervals. Later, because of apparently decreased growth on the blood agar medium, weekly transfers were made only on the Sabouraud-maltose agar slants.

In the case of all strains of organisms used in this work, original cultures as received were streaked for isolation on Sabouraud-maltose agar plates and inocula for pure stock cultures were taken from single isolated colonies with the aid of a dissecting microscope.

Procedures used for identification of individual strains were: chlamydospore formation on the polysaccharide medium of Nickerson and Mankowski (1953), carbohydrate fermentation characteristics using the technique of Widra (1957) and other methods based upon morphological

alterations of C. albicans and C. stellatoidea to be described later. Another method used for identification of some of the strains of *Candida* was that of Pagano et al. (1957). It was based upon colonial characteristics of *Candida* species on a medium containing 2, 3, 5-triphenyl tetrazolium chloride. The differentiating quality depends on the varying capacity of different yeast-like organisms to reduce a given concentration of the tetrazolium compound and impart color to the colonies.

For purposes of animal inoculation and certain other uses, cell suspensions were adjusted to known concentrations by direct enumeration using a clinical hemocytometer. Organisms were washed from the surface of agar slant cultures, filtered through sterile Pyrex glass wool, diluted, counted, and on the basis of the count, further diluted to the desired number of cells per desired volume. Duplicate determinations were made using the two chambers of the hemocytometer and at least a total of 600 organisms were counted.

II. Animals.

The mice used in these experiments were of three different strains, CBA, CFW and the strain of Mus musculus maintained at the Rocky Mountain Laboratories (R.M.L.). Mice of the CBA strain were obtained from the colonies of either Dr. T. F. Dougherty or Dr. S. Dickman, CFW from the Department of Bacteriology breeding colony and the R.M.L. strain from Rocky Mountain Laboratories, Hamilton, Montana. In most experiments animals of both sexes were used and fed on either Purina Laboratory Chow or Rockland Mouse Diet. In most cases tap water was given ad libitum.

Adrenalectomy of mice was accomplished using ethyl ether as an anesthetic. A midline incision was made down the back of the animal and the adrenals removed by making a small incision into the peritoneal cavity on either side directly over the kidneys. The skin wound was closed with several skin clips and unless both adrenal glands were successfully removed without excessive bleeding the mice were discarded. All adrenalectomized animals were given a solution of 1 per cent sodium chloride to drink rather than tap water. A period of at least 48 hours lapsed between the time of the operational procedures and the use of the animals in different experiments.

III. Media.

The two solid media used in these studies to maintain stock cultures of the *Candida* species had the following compositions:

Modified Sabouraud Agar

Sabouraud-maltose agar (Difco).	65.0 gm
Agar (Difco).	5.0 gm
Yeast extract (Difco)	0.1 gm
Beef extract (Difco).	0.5 gm
Distilled water	1,000 ml

After dissolving the ingredients, the medium was sterilized at 15 pounds pressure for 15 minutes.

Blood Agar

Tryptose-phosphate broth (Difco).	29.5 gm
Agar (Difco).	20.0 gm
Distilled water	1,000 ml

After dissolving the ingredients and sterilization in the autoclave, the medium was cooled and 15 per cent citrated human blood aseptically added.

The liquid medium used in certain experiments had the following ingredients:

Glucose	20.0 gm
Peptone (Difco)	5.0 gm
Yeast extract (Difco)	0.1 gm
Malt extract (Difco).	5.0 gm
Asparagine.	0.2 gm
Monopotassium phosphate	0.1 gm
Distilled water	1,000 ml

The pH of the liquid medium was adjusted to approximately 5.5 and sterilized by autoclaving at 15 pounds for 15 minutes.

The medium used for isolation of certain strains of the yeast-like organisms was the modified Sabouraud agar described previously.

IV. Challenge Experiments.

The experiments on the lethal effects of injection of cells of different strains of C. albicans were accomplished using the intravenous route of inoculation. Prior to seeding the agar slants from which the cells were taken for injection purposes, the stock cultures of the organisms were transferred at 24 hour intervals for at least two passages. This probably resulted in a maximum number of viable cells in the inocula used for injection.

Suspensions of cells of individual species of Candida albicans and other Candida species were prepared from 15 per cent human blood agar slant cultures or in most cases from modified Sabouraud-maltose agar cultures which had been incubated at 37°C. for from 24 to 48 hours. These suspensions were diluted to desired concentrations as previously described.

Mice were inoculated intravenously in the tail vein with 0.25 ml of inoculum. In the case of injection of mice of the CBA strain it was necessary to use an illuminating device to visualize the veins.

V. Techniques Used to Study Local Tissue Reactions to Injected Organisms.

The methods used to study local tissue reactions to injected Candida cells were modifications of those of Schneebeli and Dougherty (1951) and of Selye (1953). This latter technique was employed by Higginbotham et al. (1956) with modification.

The first method consisted of the injection of suspensions of cells of members of the genus Candida into the subcutaneous tissues of mice, and the inflamed areas were studied at various times after injection. One-tenth ml of the cell suspensions was injected into the subcutis of the animal's abdomen. At different time intervals the mice were sacrificed and thin sheets of loose connective tissue were excised from within the area of injection. These sheets of areolar tissue were quickly spread on microscope slides, air-dried and stained by the May-Grünwald-Giemsa method. The cells of the Candida stained a light blue by this procedure, which made them easily distinguishable from host cells.

A second method similar to the first was later used because of better localization of the injected organisms and because of greater ease in preparation of the connective tissue spreads. It was a modification of the "granuloma pouch" technique. One ml of air was injected subcutaneously into the nape of the neck of mice. Subsequently, the organisms contained in 0.2 ml of diluent were injected into the formed air pouch. At different times after inoculation, animals were sacrificed and the intact air pouch partially dissected free. Sheets of connective tissue comprising the wall of the base of the pouch were removed and treated as previously described.

VI. Reducing Compound Determinations.

Oxidation-reduction potential measurements were made using the Beckman Model G pH meter with a platinum indicator electrode and a saturated calomel reference electrode. The platinum electrode was cleaned in concentrated nitric acid after each series of determinations and rinsed with distilled water and dried between each sample. Because of needle drift, readings were taken one minute after immersing the electrodes in the solution under test.

Three different methods for the determination of sulfhydryl compounds were used. They were the iodometric procedure of Woodward and Fry (1932), the nitroprusside method of Grunert and Phillips (1952) and the amperometric titration technique of Benesch and Benesch (1948).

The iodometric method involves titration of a 2 per cent sulfosalicylic acid centrifugate with 0.001 N potassium iodate at 20°C. in the presence of excess potassium iodide, with starch as the internal

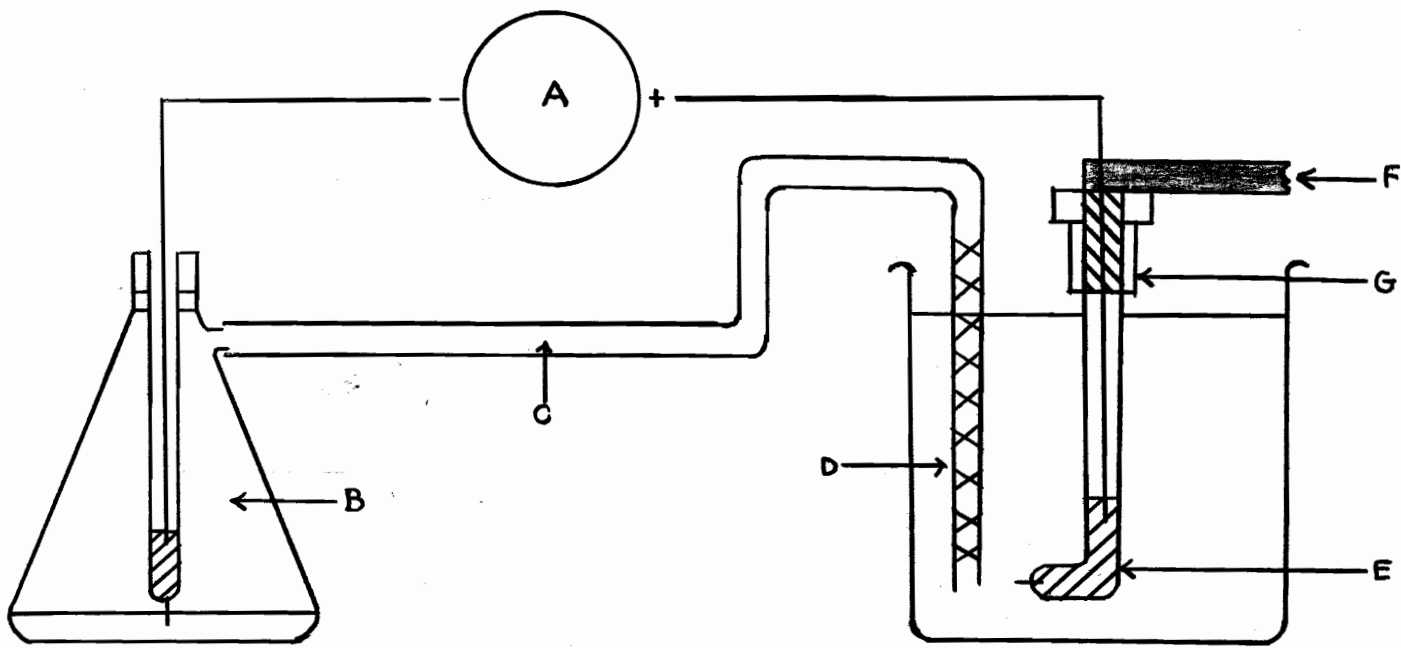
indicator. The volume of iodate solution is proportional to the amounts of sulfhydryl compounds. The technique of Woodward and Fry was modified for use of more convenient volumes.

The nitroprusside method of Grunert and Phillips depends on the formation of a rose-colored complex of glutathione in the presence of sodium nitroprusside. The intensity of the color is temperature dependent and all reagents and the reaction mixture were maintained in a water bath held at 20°C. The intensity of the developed color was determined using the Coleman Junior Spectrophotometer and light of 520 m μ . Because of the slow fading of the developed color, readings were taken 45 seconds after the addition of the final reagents. Metaphosphoric acid at a concentration of 3 per cent was used to precipitate protein, and in the case of liquid cultures, to aid in the removal of organisms when centrifuged. This method has been reported to measure compounds with a mercapto group such as glutathione and cysteine. Grunert and Phillips (1952) reported that the cysteine-nitroprusside complex faded rapidly even in the presence of cyanide ion which stabilizes the glutathione complex.

The method of amperometric titration of sulfhydryl groups with 0.001 N silver nitrate solution utilizes a rotating platinum electrode and a mercury/mercuric iodide reference electrode. A suitable current-measuring device measures the flow of current between the two half cells. When a sulfhydryl compound in an aqueous alcoholic solution is titrated with aqueous silver nitrate, the insoluble silver mercaptide is precipitated. A negligible current flows until there is an excess of silver ions. At this point the diffusion current of silver ions to the

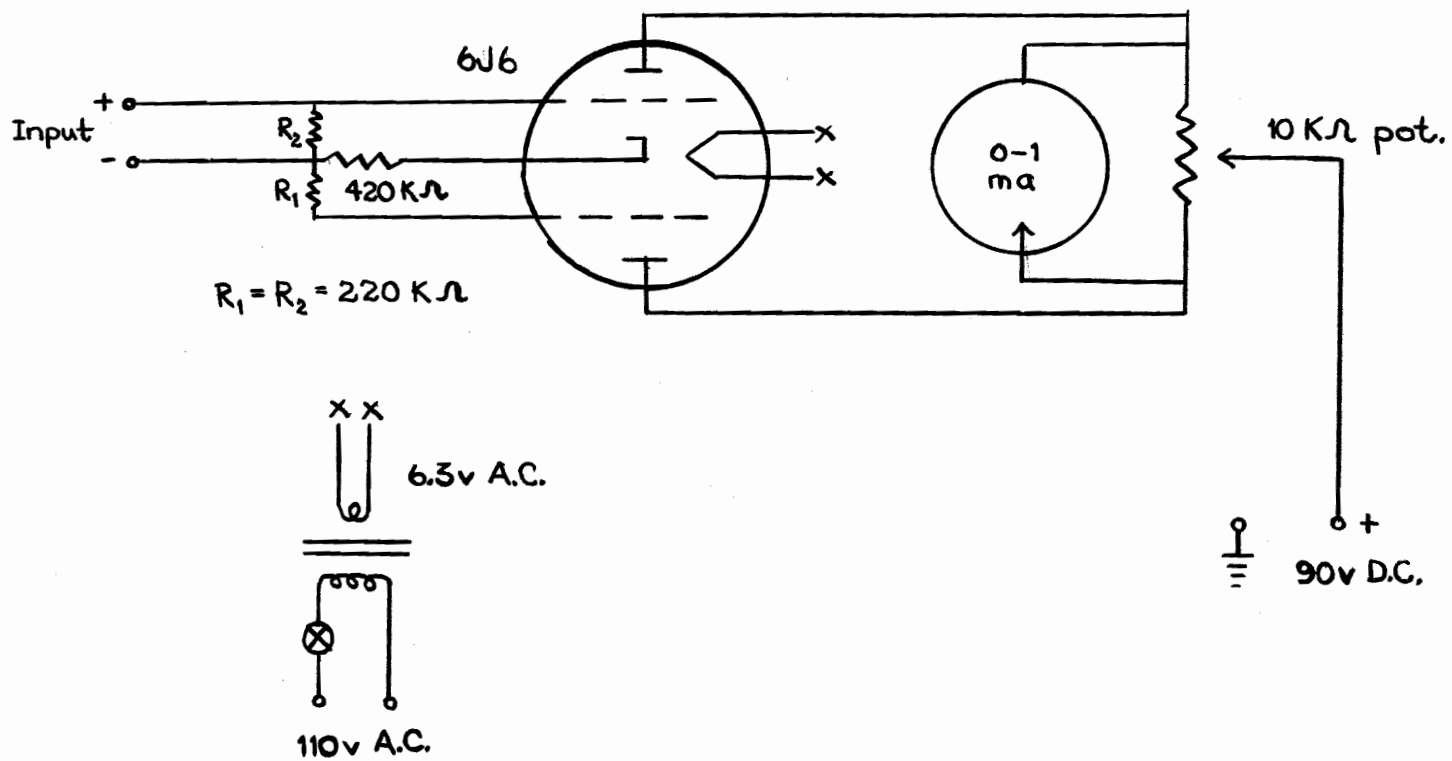
platinum electrode rises sharply and in proportion to the concentration of these ions in solution. The end-point of the titration was obtained graphically by plotting current readings against the volume of added silver nitrate and noting the point of intersection of the two straight lines. A diagram of the apparatus for amperometric titration is given in Figure 1.

The current measuring device was a vacuum tube micrometer whose circuit is shown in Figure 2. The values of R_1 and R_2 were chosen by trial and error such that full-scale deflection of the 0-1 millimeter was equal to approximately 10 microamperes. This was determined using a mercury cell rated at 1.34 volts and a variable resistance decade box. The current response of the instrument is given in Figure 3. The response was not linear. However, it was linear enough over small current changes to make the instrument usable as a diffusion current indicator.



- A. Microammeter
- B. Mercury/mercuric iodide reference cell
- C. Plastic tubing, containing saturated KCL
- D. Salt bridge with sat. KCL in 3 per cent agar
- E. Rotating platinum electrode
- F. Rubber belt from the shaft of the stirring motor
- G. "Plexi glas" bearing

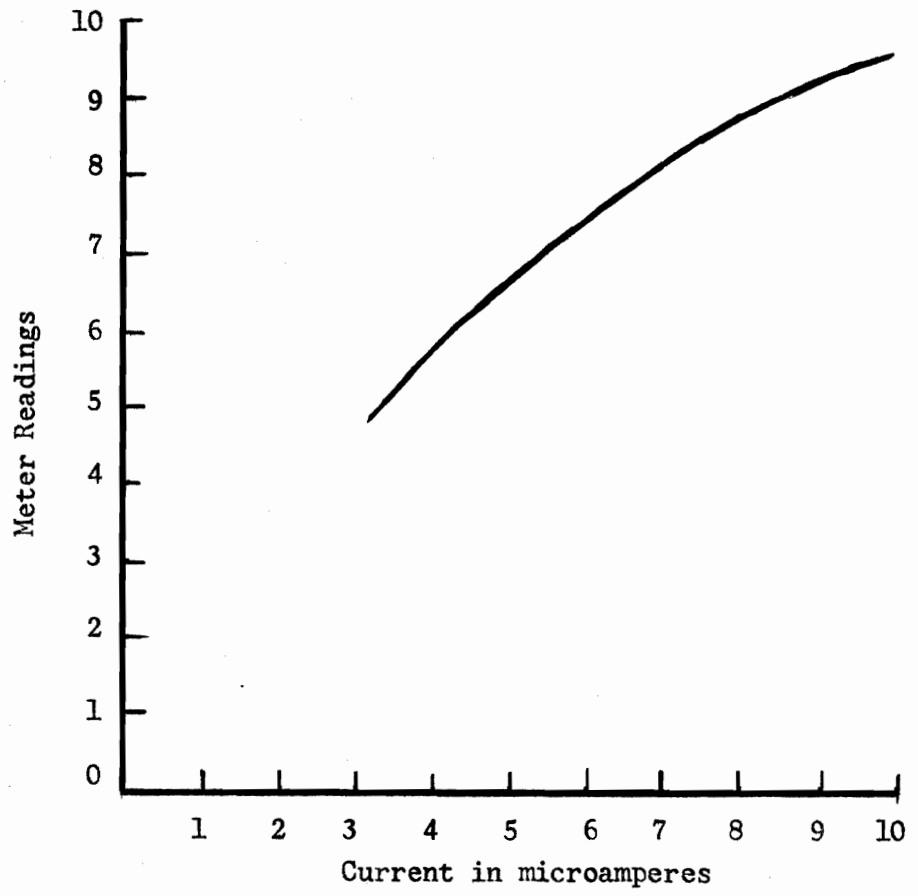
Figure 1. Apparatus for Amerpometric Titration



(50)

Figure 2. Vacuum Tube Microammeter

Figure 3. Current Response of the Microammeter



EXPERIMENTAL RESULTS

I. Studies on the Virulence for Mice of Strains of Candida.

The ability of an organism to produce death as a result of infection is only one of many factors which has been associated with the virulence of parasites for defined hosts. Other effects such as ability to infect, to invade or to produce toxic substances may serve, under certain conditions, to define the quantitative expression of pathogenicity. However, in most cases these parameters are much more difficult to measure quantitatively. One of the logical prerequisites to the study of the nature of a host-parasite relationship is the detection of significant differences in virulence of strains of the parasitic organism. With the establishment of differences, various comparisons can then be made between virulent strains and those of reduced virulence. In this study, virulence of *Candida* for mice will be equated with the ability to infect and produce death when injected into laboratory mice via the intravenous route.

As suggested by the work of Marcus and Rambo (1952), early experiments designed to detect differences in virulence of strains of *C. albicans* employed the technique of intravenous injection of mice with cells suspended in brain-heart-infusion broth (Difco). For reasons to be discussed later, however, the use of this suspending medium was abandoned in favor of 0.85 per cent sodium chloride solution. Table 1 presents the results of a study on the lethality of strains of *C. albicans* for adult CBA mice. Groups of animals of both sexes were used in these experiments. In the case of *C. albicans* #520, the organisms for challenge were suspended in brain-heart-infusion broth, while cells of *C. albicans* #3160 and #3161 were suspended in saline solution. Mice were injected

TABLE 1

LD50 OF STRAINS OF CANDIDA ALBICANS

FOR ADULT CBA MICE

Strain	No. of organisms infected I.V.	Mortality Ratio at 18 days	LD50
<u>C. albicans</u> #520	10 ⁴ 10 ⁵ 10 ⁶ 10 ⁷	1/25 12/25 23/25 25/25	114,800 ±46,000
<u>C. albicans</u> #3160	10 ⁴ 10 ⁵ 10 ⁶ 10 ⁷	1/10 6/10 8/10 10/10	125,900 ±95,000
<u>C. albicans</u> #3161	10 ⁴ 10 ⁵ 10 ⁶ 10 ⁷	2/9 7/10 9/10 10/10	41,690 ±44,600

with the inocula contained in 0.25 ml and observed for an 18-day period. Any animals found dead earlier than 48 hours after challenge with the infecting organisms were not included in the data. Estimates of the LD50 dose of the organisms were made using the method of Miller and Tainter (1944). The data indicated that there were no significant differences in the virulence of these three stock strains of C. albicans for mice (CBA) under the described experimental conditions.

All mice found dead during the experimental observation period were autopsied and gross findings noted. Moribund animals which would not have survived to the next day were sacrificed and tissues removed for histological studies. These tissues were from the lungs, liver, spleen and kidneys. After sectioning, the tissues were stained using the routine hematoxylin-eosin technique and the Hotchkiss-McManus procedure described by Kligman et al. (1952). The only organ studied in which gross or microscopic lesions were observed was the kidney. Multiple sections of tissues from the lungs, liver and spleen failed to exhibit any apparent lesions. Duplicate sections of these tissues stained by the Hotchkiss-McManus technique failed to show any Schiff-positive bodies which could be interpreted as being C. albicans cells. Massive involvement of the kidneys was observed with numerous organisms in the necrotic centers of the abscesses. Many of the cells of C. albicans found in the kidneys of infected mice demonstrated filamentous morphology. Figure 4 is a photomicrograph of a kidney section from an infected animal stained by the Hotchkiss-McManus procedure.

It was proposed that if a more susceptible host were used, differences in virulence of strains of C. albicans would be measurable. Adult

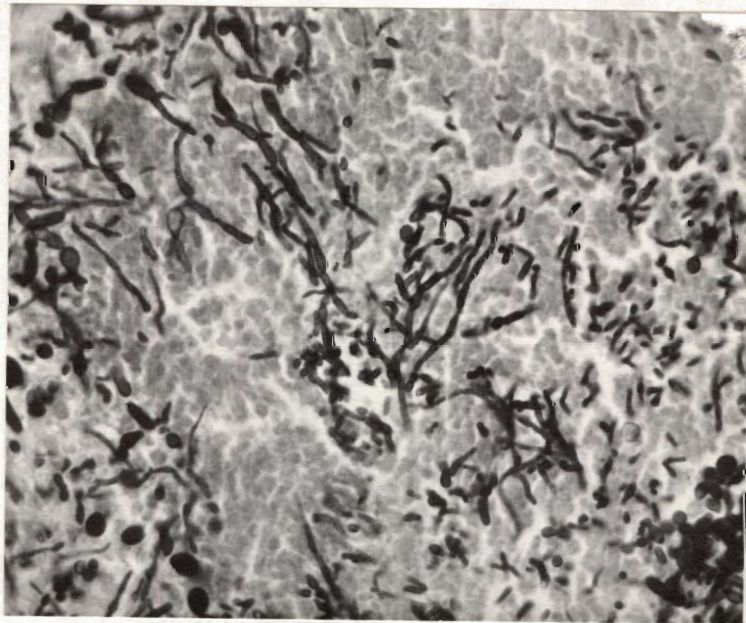


Figure 4. Hotchkiss-McManus stained tissue section of kidney from a mouse infected with Candida albicans #520.

CBA mice of both sexes were adrenalectomized and inoculated intravenously approximately 48 hours later with varying numbers of cells of C. albicans #520. Intact animals of the same strain were similarly challenged and served as controls. In this preliminary experiment the cell suspensions for inoculation were made up in sterile brain-heart-infusion broth. Less than 24 hours after intravenous injection of the cell suspension into adrenalectomized mice, 19 out of 40 animals were found dead. None of the intact, challenged mice died in the same period. A group of 10 adrenalectomized mice were injected intravenously with the same volume of the sterile suspending medium and within 24 hours, 7 of them had died. On the basis of the apparent toxicity of brain-heart-infusion broth for adrenalectomized mice, all subsequent challenge experiments were made using sterile 0.85 per cent sodium chloride solution as the cell-suspending medium.

A large group of adult CBA mice were obtained and randomly divided into two groups. Animals of one group were adrenalectomized as previously described while the other animals served as controls. Mice of both groups were challenged intravenously with cells of C. albicans #520 suspended in saline solution, observed for 18 days and mortality data collected. Animals found dead were autopsied and observed for gross pathological changes. Table 2 presents a summary of the collected data. The results indicated that adrenalectomized CBA mice were significantly more susceptible to the lethal effects of intravenously injected cells of C. albicans #520 than were intact animals of the same strain. Under the described conditions the data further indicated that adrenalectomized

TABLE 2

LETHALITY OF CELLS OF CANDIDA ALBICANS #520
FOR ADRENALECTOMIZED AND INTACT CBA MICE

Animals	No. of Organisms Injected I.V.	Mortality Ratio at 18 days	LD50 ¹
Adrenal- ectomized	10 ⁴	14/36	21,000 (42,000- 10,500)
	10 ⁵	25/33	
	10 ⁶	35/36	
Intact	10 ⁴	2/35	98,000 (171,500- 56,000)
	10 ⁵	18/35	
	10 ⁶	32/35	
	10 ⁷	35/35	

¹Estimates of the LD50's and their 95 per cent confidence limits were made by the method of Wilcoxon and Litchfield (1949).

Potency ratio $\frac{98,000}{21,000} = 4.6$ (11.17 - 1.89).

CBA mice were somewhere in the range of from about 2 to 11 times more susceptible to death from intravenous injection of this strain of organism than intact animals.

Salvin (1952b) has reported that C. albicans #3160 and #3161 differed significantly in virulence for mice when injected intraperitoneally. These two isolates were obtained from Dr. Salvin and their lethality for adrenalectomized CBA mice determined. Suspensions of these two different strains were prepared in saline such that the final inocula contained approximately 50,000 cells per 0.25 ml. Forty-eight hours after adrenalectomy, groups of mice were challenged intravenously with these organisms and observed over an 18-day observation period. Table 3 presents the results of this experiment which indicated that, under the described conditions, no significant differences exist between the lethality of these two isolates of C. albicans for adrenalectomized mice. These data substantiated the conclusions drawn with regards to the virulence of the same strains of organisms for intact mice.

Because of the previously described lack of apparent differences in mouse virulence of stock cultures of C. albicans, strains were isolated from various clinical sources and tested for their virulence for intact mice. The hypothesis was made that the three stock cultures of C. albicans had been originally selected on the basis of a high virulence for experimental animals and that this might explain the absence of measurable differences in pathogenicity for mice. Tables 4 and 5 summarize the distinguishing characteristics of the strains of C. albicans selected for further study. For comparative purposes the results of differentiating studies on a known strain of C. albicans and other *Candida*

TABLE 3

INTRAVENOUS CHALLENGE OF ADRENALECTOMIZED CBA MICE
WITH STRAINS OF CANDIDA ALBICANS

Strains of <u>C. albicans</u>	No. of Organisms Injected I.V.	Mortality Ratio	P Value ¹
#3160	50,000	19/45	---
#3161	50,000	27/45	0.2- 0.1

¹The probability value was estimated by calculation of χ^2 (Fisher, 1950) with Yates correction.

species are also presented. The method of Hill and Gebhardt (1956) and the morphological alteration of C. albicans in 50 per cent horse serum will be described later. These two techniques, however, enable the differentiation of C. albicans and C. stellatoidea from other members of the genus *Candida*.

Measurements of mouse virulence of the selected C. albicans strains were made by intravenous injection of standard numbers of cells as determined by hemocytometer counts. All possible care was exercised in standardization of the inocula for injection. Approximately 300 adult mice of the RML strain were pooled and randomly selected for intravenous injection. For each strain of organism studied, approximately 30 animals were injected. The mice were maintained under standard laboratory conditions and observations for mortality made daily. Any animals found dead up to 48 hours after inoculation were discounted from consideration. The results of these experiments are given in Table 6 at 7, 14 and 18 days after injection with cells of different strains.

These data suggested that rather broad variations were evident in the mouse virulence of cultures of C. albicans identified on the basis of the described techniques. Comparisons of the 18-day mortality in groups of mice injected with a standard number of cells of the isolates were made by the method of Mainland and Murray (1952) and are presented in Table 7. The results of the studies with C. albicans Vet. #4 are not included because of the previously noted fact (Table 6) that all animals inoculated with cells of this strain had died by the 16th day of observation. Apparent differences were even more evident in the response of groups of challenged mice when the data were considered on the basis of

TABLE 4

FERMENTATION CHARACTERISTICS OF CANDIDA STRAINS																		
Strain	dextrose			galactose			lactose			maltose			raffinose			sucrose		
	day			day			day			day			day			day		
	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5
<u>C. albicans</u>																		
Vet. #4	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	-	-	-
Vet. #5	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	a	a	a
Anderson	g	g	g	a	a	g	-	-	-	g	g	g	-	-	-	a	a	a
Vet. #2	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	s	a	a
Giles	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	s	a	a
Finlayson (oral)	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	a	a	a
Goring	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	s	a	a
Sekino	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	a	a	a
Finlayson (vaginal)	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	a	a	a
#U308	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	a	a	a
<u>C. stellatoidea</u>	g	g	g	a	a	a	-	-	-	g	g	g	-	-	-	-	-	-
<u>C. krusei</u>	g	g	g	a	a	a	-	-	-	-	-	-	-	-	-	-	-	-
<u>C. guillermondi</u>	a	a	a	a	a	a	-	-	-	-	-	-	a	a	a	s	a	a
<u>C. tropicalis</u>	g	g	g	a	a	g	-	-	-	g	g	g	-	-	-	g	g	g
	g = gas			a = acid			- = no reaction											

TABLE 5
OTHER IDENTIFYING CHARACTERISTICS OF
CANDIDA STRAINS

Strain	Chlamydo. ¹	<u>In vivo</u> morph. ²	<u>In vitro</u> morph. ³	Colonies ⁴
<u>C. albicans</u>				
Vet. #4	pos.	pos.	pos.	pos.
Vet. #5	pos.	pos.	pos.	pos.
Anderson	pos.	pos.	pos.	pos.
Vet. #2	pos.	pos.	pos.	pos.
Giles	pos.	pos.	pos.	pos.
Finlayson (oral)	pos.	pos.	pos.	pos.
Goring	neg.	pos.	pos.	pos.
Sekino	pos.	pos.	pos.	pos.
Finlayson (vaginal)	pos.	pos.	pos.	pos.
U. #308	pos.	pos.	pos.	pos.
<u>C. stellatoidea</u>	?	pos.	pos.	neg.
<u>C. krusei</u>	neg.	neg.	neg.	neg.
<u>C. guilliermondi</u>	neg.	neg.	neg.	neg.
<u>C. tropicalis</u>	neg.	neg.	neg.	neg.

¹Chlamydospore production.

²Morphological alteration 2 hours after injection subcutaneously in mice.

³Morphological alteration in 50 per cent horse serum at 37°C for 8 hours.

⁴Colonial characteristics of C. albicans on tetrazolium agar medium.

TABLE 6

MORTALITY RATIOS OF GROUPS OF MICE INOCULATED WITH
500,000 CELLS OF STRAINS OF CANDIDA ALBICANS

Strain	No. of Animals Dead/Total No. Injected		
	7 days	14 days	18 days
<u>C. albicans</u> Vet. #4	16/28	26/28	28/28 ¹
<u>C. albicans</u> Vet. #5	5/30	22/30	27/30
<u>C. albicans</u> Anderson	11/29	24/29	27/29
<u>C. albicans</u> Vet. #2	4/29	19/29	21/29
<u>C. albicans</u> U. #313 Giles	0/29	12/29	19/29
<u>C. albicans</u> Finlayson (oral)	2/26	15/26	18/26
<u>C. albicans</u> Goring	1/30	5/30	12/30
<u>C. albicans</u> Sekino	2/29	9/29	12/29
<u>C. albicans</u> Finlayson (vaginal)	0/30	5/30	14/30

¹All animals in the group had died by the 16th daily observation period.

mortality as a function of time after inoculation. Figure 5 represents a plot of this relationship and compares groups of animals injected intravenously with cells of the different strains which were studied.

Animals which survived for approximately 35 days after challenge were sacrificed and autopsied. Gross pathological changes of the kidneys were noted and these organs cultured by streaking a cut surface on Sabouraud-maltose agar. The results of these studies are summarized in Table 8. The majority of mice surviving for 35 days after challenge were found to be chronically infected on the basis of cultural recovery of organisms. The unexpected observation was made that, of those animals showing visible kidney lesions, 17 out of a total of 23 had involvement of the right kidney only. In only one instance, a mouse which survived inoculation of cells of C. albicans Vet. #5, was an animal observed with macroscopic lesions of the left kidney and not of the right. In this case, however, organisms were cultured from both organs.

As has been considered previously, certain authors have suggested that *Candida* species other than C. albicans were virulent for experimental animals. A limited number of experiments were designed to study the virulence of some of these organisms for mice. Suspensions of cells of C. pseudotropicalis, C. krusei, C. tropicalis, C. parapsilosis (C. parakrusei), C. guilliermondi and C. stellatoidea were prepared in saline to contain 1×10^6 organisms per 0.25 ml and injected intravenously into adult mice (CBA). Groups of 10 animals each were inoculated and observed for a 21-day period. Any animals which died during this time period were autopsied and touch-impression smears prepared from the kidneys. These smears were stained using the May-Grünwald-Giemsa

TABLE 7

COMPARISONS OF THE VIRULENCE OF CANDIDAALBICANS STRAINS FOR MICE¹

Strain	Mortality Ratio	P Value
<u>C. albicans</u> Vet. #5	27/30	---
<u>C. albicans</u> Anderson	27/29	>.05
<u>C. albicans</u> Vet. #2	21/29	>.05
<u>C. albicans</u> U. #313 Giles	19/28	>.05
<u>C. albicans</u> Finlayson (oral)	18/26	>.05
<u>C. albicans</u> Goring	12/30	.01
<u>C. albicans</u> Sekino	12/29	.01
<u>C. albicans</u> Finlayson (vaginal)	14/30	.01

¹Comparisons were made between C. albicans Vet. #5 and all others using mortality data collected on the 18th day after challenge.

Figure 5.

Mortality of Groups of Mice Infected with Various Strains of Candida albicans.

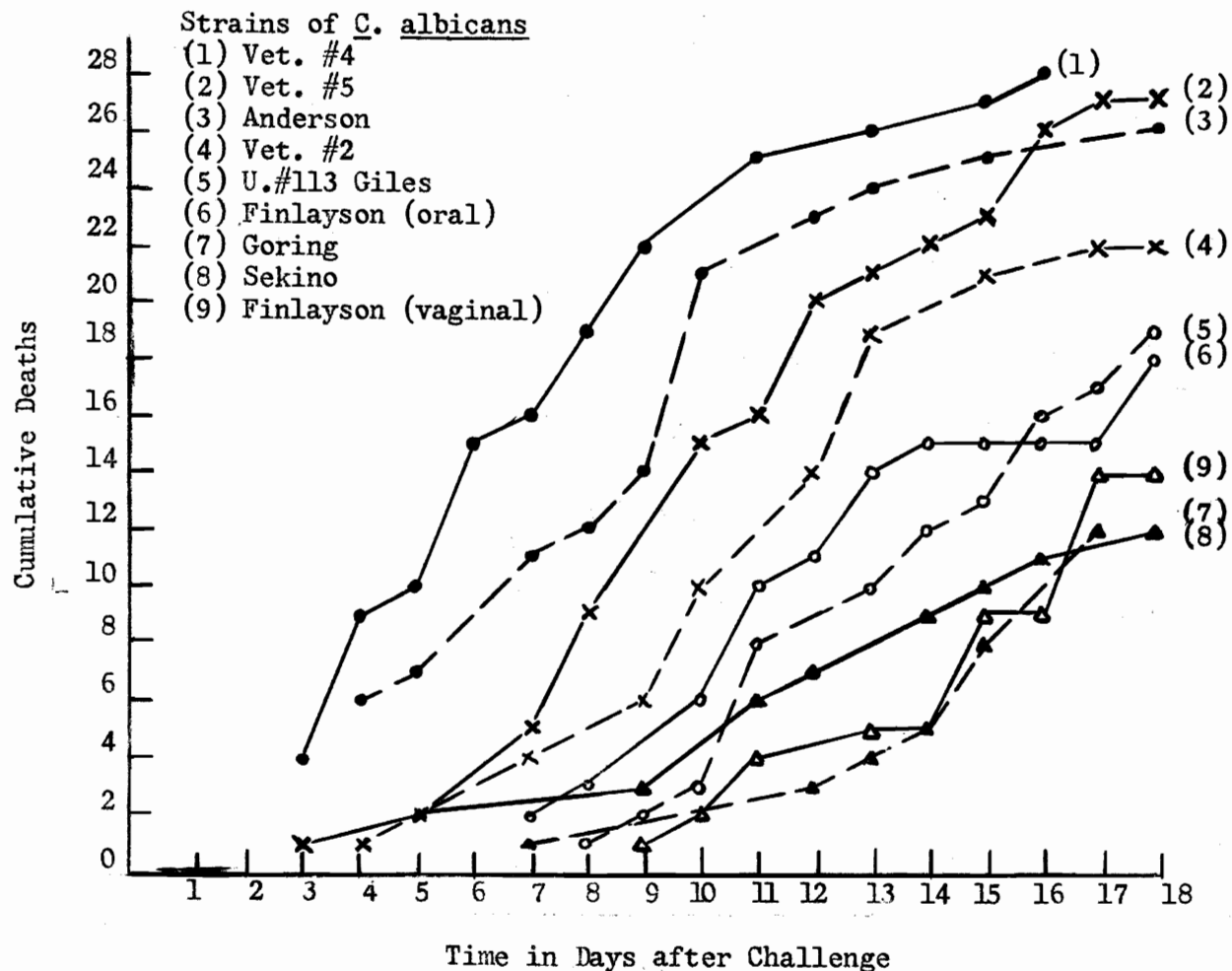


TABLE 8

RESULTS OF AUTOPSY AND CULTURAL STUDIES ON MICE SURVIVING
 INTRAVENOUS INJECTION OF 500,000 CELLS OF
 VARIOUS CANDIDA ALBICANS STRAINS

Strain	Kidney Cultures No. pos./total Surviving	Gross Lesions Observed		
		Right pos. Left neg.	Both pos.	Both neg.
<u>C. albicans</u> Finlayson (vaginal)	6/7	5	1	1
<u>C. albicans</u> Sekino	6/9	4	1	4
<u>C. albicans</u> Goring	10/10	7	3	0
<u>C. albicans</u> Giles	1/2	0	1	1
<u>C. albicans</u> Vet. #5	2/2	1	0	0

technique. No deaths which could be attributed to infection occurred in groups of animals injected with cells of the strains of nonpathogenic *Candida* which were studied. Survivors of the 21-day period of observation were sacrificed and examined grossly. No visible kidney lesions which could be attributed to infection were apparent. It was concluded that these organisms possessed little if any virulence for adult mice of the CBA strain when injected in numbers which would have resulted in high mortalities in similar groups of animals challenged intravenously with cells of virulent *C. albicans* strains.

II. Effects of Cortisone Acetate on Experimental Candidiasis.

Since the introduction of antiphlogistic steroid hormone and adrenocorticotrophic hormone, it has been shown that these compounds have a profound effect on the host-parasite relationship in various infectious diseases. Thomas (1952) has suggested that these substances may provide investigative tools to study basic problems of infectious disease such as "... the meaning of natural resistance and susceptibility, mechanisms involved in tissue damage and infection, and the relationship between tissue responses to bacteria, virus, and bacterial toxins."

Experimental studies on the effects of cortisone on candidiasis in mice have indicated that, in the dose range of 1.0 to 4.0 mg per day, a significantly increased mortality rate resulted (Seligmann, 1953, Friedman et al., 1954, Roth et al., 1957, etc.). Scherr (1953b) reported that the effect of cortisone on the course of systemic candidiasis in mice was influenced by the severity of infection. He found that this

hormone, suspended in an aqueous vehicle, "... enhanced the severity of a mild monilial infection, did not alter the severity of a moderate infection, and significantly reduced the severity of a severe infection."

The reports in the literature of the varying effects of administered cortisone on experimental infections caused by pathogenic fungi were made on the basis of use of intact animals. These animals received different amounts of the hormone plus the contributions made by their own intact adrenals. The questions arise as to what effects to the host-parasite interaction are due to exogenous hormone and what are related to the endogenous hormones secreted by the animal's adrenal glands? The administration of graded amounts of cortisone to adrenalectomized mice infected by intravenous inoculation of cells of C. albicans was attempted to partially answer these questions.

Male mice (CBA), 14 to 16 weeks of age, were used in these experiments. They were adrenalectomized as previously described and used for challenge 48 hours later. The organism used in this study was C. albicans #520 and cells were grown on 10 per cent blood agar. The inoculum for intravenous injection was prepared from 48 hour slant cultures and contained 50,000 organisms per 0.25 ml. The animals were challenged intravenously with 0.25 ml of the suspension and randomly separated into 5 groups of approximately 40 mice each. The hormone used was cortisone acetate* and was diluted to the desired concentrations with sterile pyrogen-free saline solution. These concentrations were such that the animals in the individual groups received 1.0 mg, 0.1 mg, 0.01 mg or 0.001 mg of hormone per day given in two divided doses of 0.1 ml each. These injections were given into the subcutaneous tissues of the nape of

*"Cortone," Merck and Co., Rahway, New Jersey.

the neck at approximately 12-hour intervals. The control group of mice received 0.1 ml of sterile saline by the same route and at the same time intervals.

The groups of animals were observed over a period of 14 days and mortalities noted. Individuals found dead were autopsied and touch-impression smears prepared from the kidneys. Moribund animals which would not have lived to the next observation period were sacrificed and tissues from the lungs, liver, spleen and kidneys removed for sectioning. These tissues were fixed in Zenker-acetic acid solution and prepared sections of these tissues were stained by the routine hematoxylin-eosin procedure and the Hotchkiss-McManus technique as described by Kligman et al. (1952).

The results of experiments on groups of adrenalectomized CBA mice challenged with 50,000 cells of C. albicans and treated with various amounts of cortisone acetate are presented in Table 9. The doses of hormone, number of animals per group, per cent mortality, the "chi²" values obtained by comparing each treated group with the control group of mice receiving only saline solution are given. These same data converted to per cent survival are given graphically in Figure 6. The survival of these groups of animals as a function of time is presented in Figure 7.

The results of these experiments indicated that there was a dose of cortisone which, when given to an adrenalectomized mouse infected with C. albicans, offered a significant degree of protection. Doses above and below this resulted in increased mortality. The optimal

amount of cortisone for protection was found to be between 0.01 and 0.1 mg administered subcutaneously in two divided doses per day.

All of the mice which died during the observation period were infected with C. albicans as determined by the finding of characteristic kidney lesions and the presence of organisms in stained smears prepared from the kidneys. It is interesting to note that the only organs examined in which macroscopic or microscopic lesions could be demonstrated were the kidneys. Multiple sections made from the lungs, liver and spleen of moribund animals and stained by the Hotchkiss-McManus technique revealed no Schiff-positive bodies which could be construed to be fungus cells.

No apparent gross or microscopic differences could be noted in the size, number or distribution of lesions between mice of the control or treated groups. The kidneys of mice in the treated and control groups were severely infected and multiple abscesses were evident. The necrotic centers of these lesions contained large numbers of mycelial-like and yeast-like bodies. Figure 8 is a photomicrograph of a kidney section from an infected animal stained by the Hotchkiss-McManus procedure. No observable differences in the degree of cellular response to the fungus cells could be noted in the treated and control groups of mice.

TABLE 9

MORTALITIES IN GROUPS OF MICE INFECTED WITH
CANDIDA ALBICANS # 520 AND TREATED
 WITH CORTISONE

Group No.	Dose of Cortisone in mg/day ¹	No. of Animals per Group	Per Cent Mortality ²	"Chi ² " Values ³	P Value
1	Saline control	42	45.2	---	---
2	0.001	40	42.5	0.38	0.5
3	0.01	39	7.7	16.39	0.001
4	0.1	40	12.5	10.58	0.01
5	1.0	39	92.3	20.56	0.001

¹in two divided doses per day.

²for a 14-day observation period.

³as calculated by the technique of Fisher (1950).

Figure 6. Effects of Graded Doses of Cortisone on Survival of Adrenalectomized Mice Infected with Candida albicans #520.

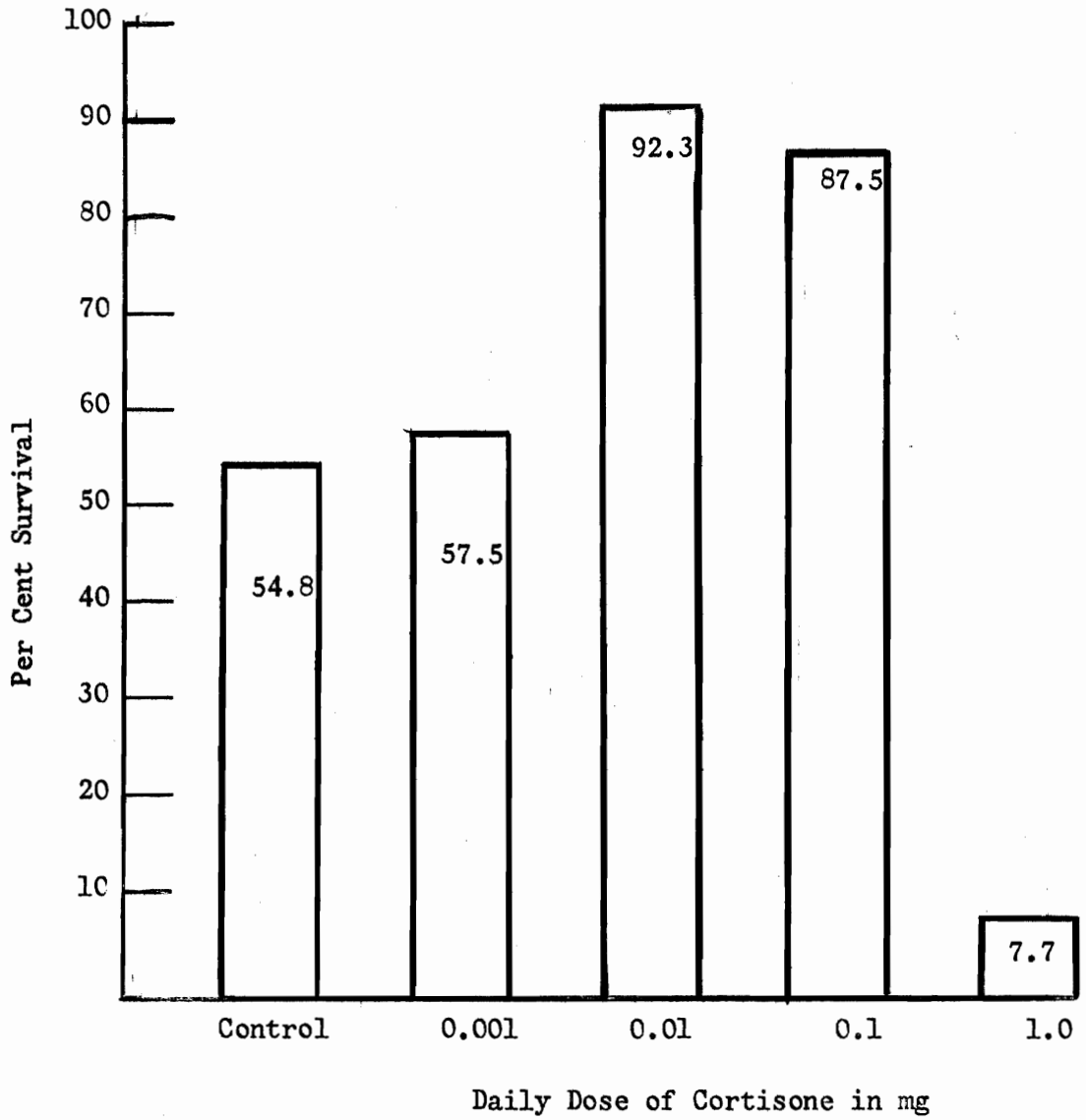
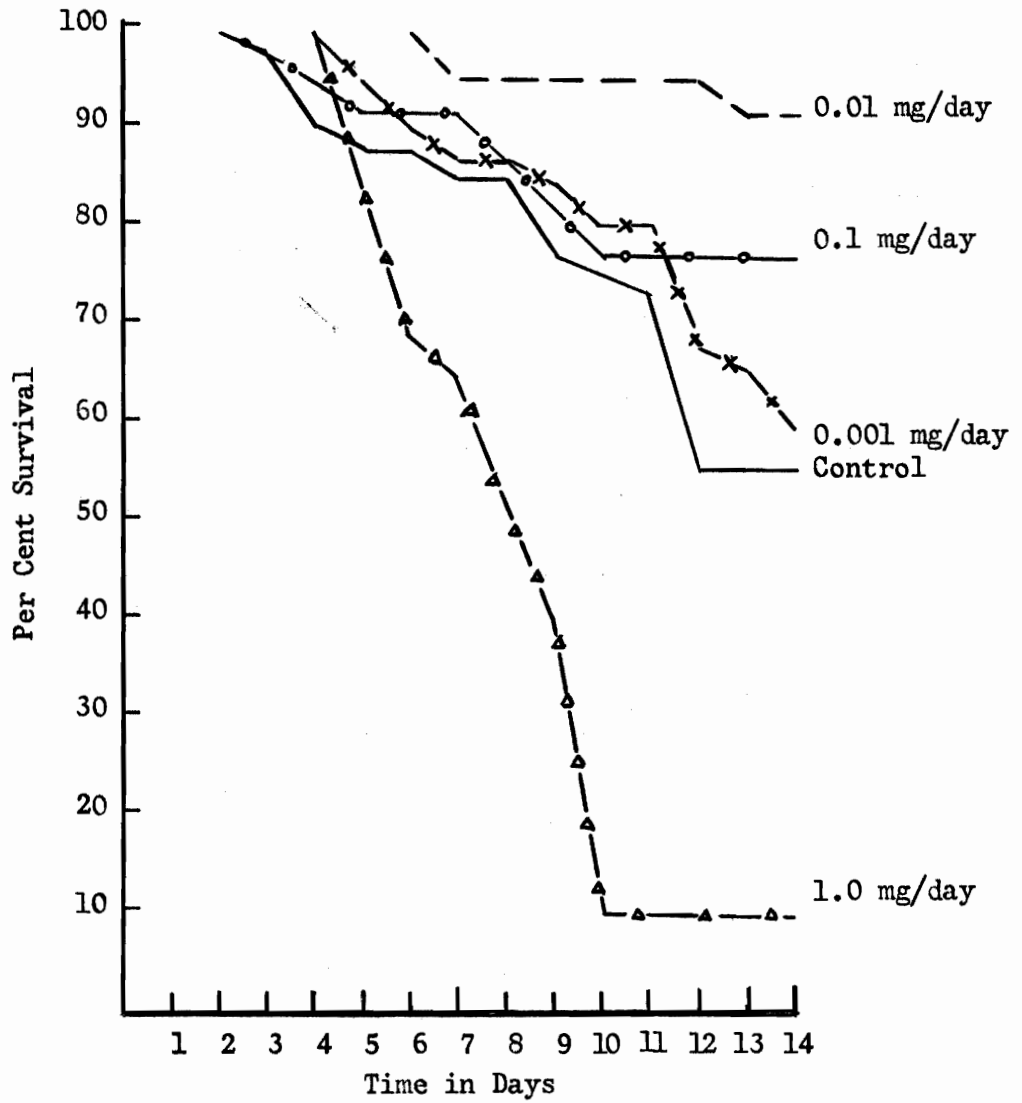


Figure 7. Survival of Adrenalectomized Mice Infected with Candida albicans #520 and Treated with Graded Doses of Cortisone.



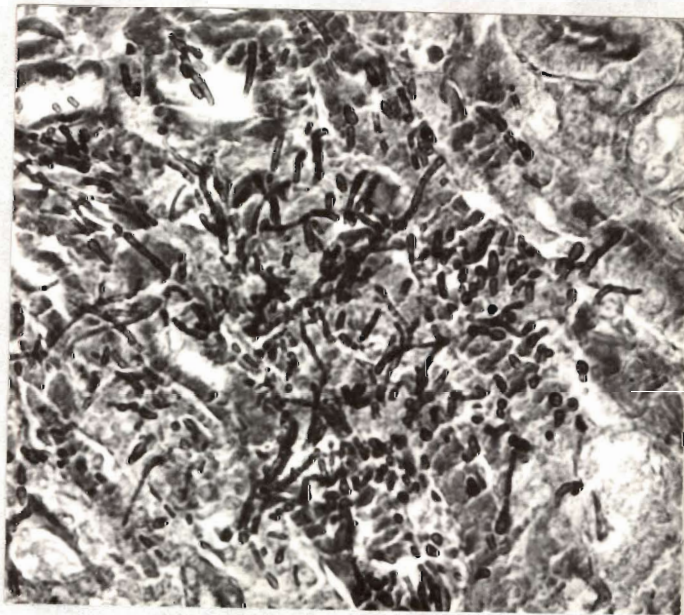


Figure 8. A photomicrograph of a kidney section from an animal infected with Candida albicans #520.

III. In Vivo Morphological Alterations of Candida Species.

Cells of C. albicans with filamentous morphology have been observed frequently in specimens from human cases of candidiasis. As has been discussed previously, morphologically altered organisms have been observed in tissues of most human organs. In rabbits experimentally infected by intravenous injection of C. albicans, large numbers of filamentous cells have been observed in kidney tissues (Stovall and Pessin, 1933 and Winner, 1956).

While studying the host tissue reactions to washed yeast-phase cells of pathogenic fungi, it was observed that cells of C. albicans rapidly underwent morphological alterations after injection into the subcutaneous tissues of mice. With the exception of C. stellatoidea, the other members of the genus *Candida* which were studied did not exhibit a similar change. The morphological alteration of cells of C. albicans was so rapid as to suggest a possible role for this phenomenon in the pathogenesis of experimental candidiasis in the mouse.

Adult mice of the RML strain and of the CFW strain were used in the majority of these studies. Washed cells of members of the genus *Candida* were injected into the subcutaneous tissues of animals and the inflamed areas studied at various times after injection. A striking morphological alteration occurred in the cells of all the isolates of C. albicans in this study. Within 60 minutes after injection into the subcutaneous tissues of mice almost all of the yeast-like cells of C. albicans had formed a short rudimentary pseudomycelium (Figure 9). Cells suspended in the saline diluent retained their typical yeast-like morphology when incubated at 37°C. Various stages in development of the altered organisms

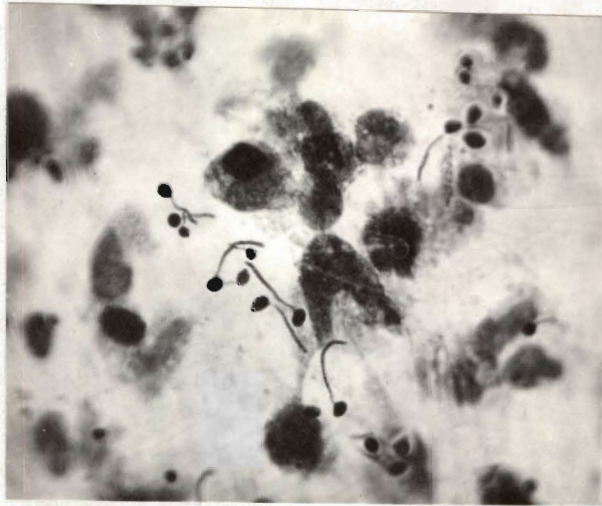


Figure 9. Appearance of cells of C. albicans one hour after injection into the subcutaneous tissues of mice.

were observed. Slides prepared 2 hours after injection revealed an increase in length of the pseudomycelium with beginning septa formation (Figure 10). Four hours after injection, definite septa were present (Figure 11). With the exception of C. stellatoidea, these in vivo alterations in morphology were not observed when other species of *Candida* were injected. Cells of these organisms retained their typical yeast-like morphology. Four hours after injection there appeared to be a tendency for a few cells of C. tropicalis and C. krusei to exhibit elongation (Figure 12 and 13). The single strain of C. stellatoidea used in this study exhibited in vivo pseudomycelium formation similar to that shown by C. albicans strains.

At 6, 12 and 24 hours after injection of yeast-like cells of C. albicans typical pseudomycelia were observed in the spreads prepared from the loose connective tissues of mice. Some were observed which were an oil-immersion field in length. The formation of lateral blastospores was observed in several instances. As judged by their staining characteristics, the majority of the organisms seen 24 hours after injection appeared to be degenerating. In the case of other species of *Candida* which were studied it was increasingly difficult to demonstrate organisms in the subcutaneous connective tissues of mice after 12 hours.

Phagocytosis of yeast-like cells was observed in subcutaneous tissues taken from mice injected with all species of *Candida* studied. The ingested cells exhibited various stages of digestion as evidenced by loss of the ability to take the basophilic stain. With preparations from animals injected with cells of C. albicans rarely was a phagocyte observed which had ingested an organism showing any degree of pseudomycelial

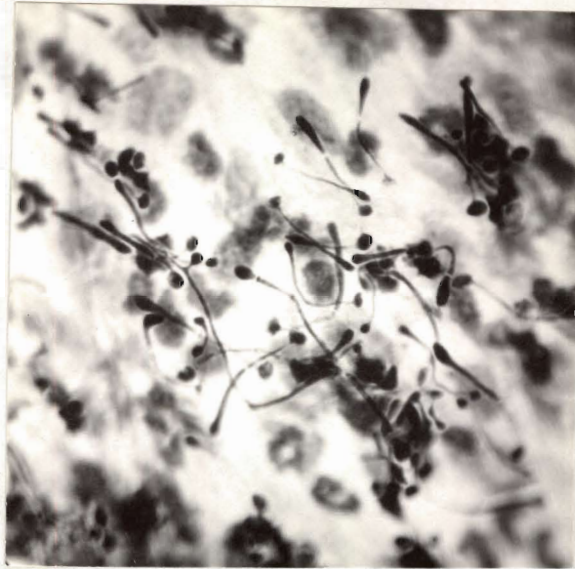


Figure 10. Morphology of C. albicans cells 2 hours after inoculation into mice.

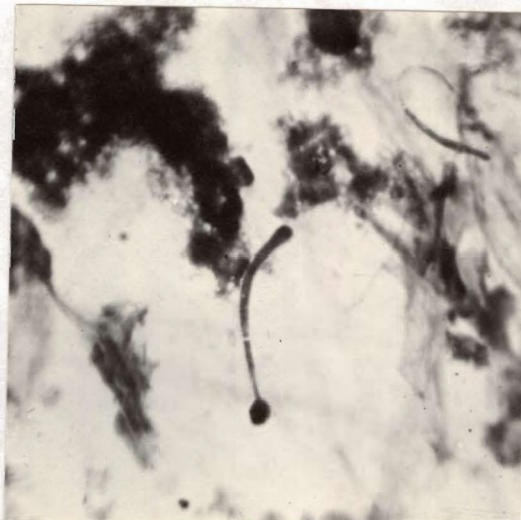


Figure 11. Appearance of cells of C. albicans after 4 hours in the subcutaneous tissues of mice.

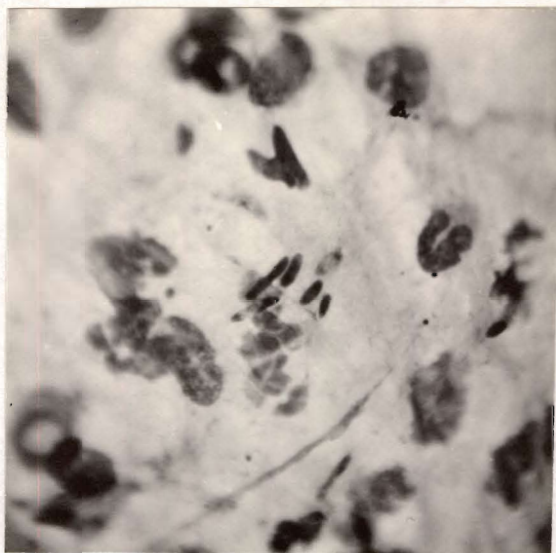


Figure 12. C. krusei cells 4 hours after subcutaneous injection into mice.

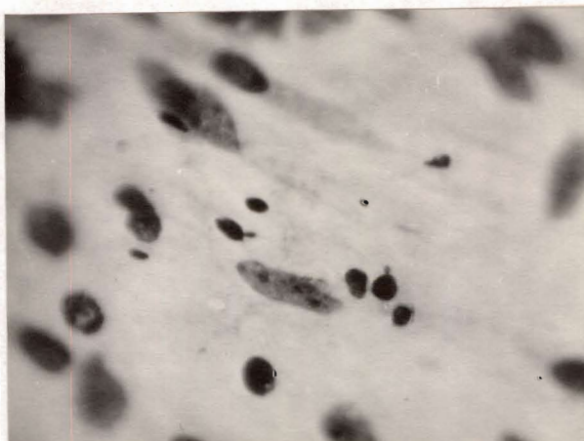


Figure 13. C. tropicalis cells 4 hours after inoculation into mice.

formation (Figure 14). Also, rarely was a polymorphonuclear neutrophil observed which had ingested cells of C. albicans. Phagocytic cells were observed to line up along the pseudomycelia in preparations taken 4 hours after injection of yeast-like cells (Figure 15). It would appear reasonable to suggest that this morphological alteration of cells of C. albicans favored survival of the fungus in the host tissues by a mechanical interference of ingestion by phagocytes.

On the basis of the speculation that the yeast-like form of C. albicans is best adapted to development in tissues, Scherr (1951) postulated that any agent which would transform C. albicans from the yeast-like phase to the mycelial phase in vivo might arrest the multiplication of the pathogen. To examine this hypothesis, he tried a number of compounds which in vitro resulted in yeast-like to mycelial transformation. These substances were given to mice systemically infected with C. albicans. The results of these studies indicated no salutary effects of these compounds on experimental candidiasis. The fact that large numbers of filamentous C. albicans cells are found in tissues of infected mice and rabbits may indicate that exactly the opposite relationship may exist, that the filamentous form of C. albicans is best adapted for persistence in tissues of an infected host. Experiments to study this possibility were designed on the basis of the report of Nickerson and van Rij (1949) that glutathione and cysteine would suppress filamentous formation of C. albicans in vitro.

Injections of suspensions of C. albicans Vet. #4 in solutions of cysteine hydrochloride and glutathione were made into previously formed air pouches in mice. Two different concentrations of these compounds



Figure 14. Phagocytosis of cells of C. albicans in connective tissue of mice 2 hours after injection.

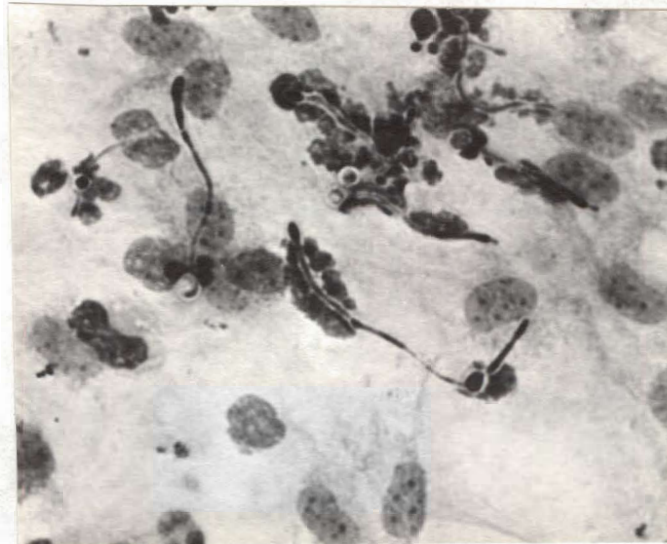


Figure 15. Inability of phagocytic cells to engulf cells of C. albicans 4 hours after injection into subcutaneous tissues of mice.

were used, 10 and 100 mg per ml. These suspensions were adjusted to approximately pH 7 with 0.1N sodium hydroxide solution and injected in volumes of 0.2 ml which contained approximately 1×10^6 organisms. A fifth group of mice were inoculated with organisms suspended in saline and these animals were immediately sacrificed and their bodies incubated at 37°C. Four hours after injection, sheets of connective tissue forming the base of the air pouch were spread on microscopic slides, stained and examined. At least 5 animals were used per group and animals inoculated with organisms suspended in saline solution served as controls. No apparent inhibition was evident of in vivo morphological alteration of cells of C. albicans Vet. #4 which were suspended in solutions of cysteine or glutathione. However, observation of preparations from animals which had been killed and incubated after injection of yeast-like organisms failed to reveal cells exhibiting pseudomycelial formation (Figure 16). Preparations from control animals 4 hours after inoculation indicated the presence of typically altered organisms (Figure 17).

Attempts to protect experimentally infected mice by administration of reducing compounds were made using glutathione and 2,3-dimercaptopropanol (BAL)*. The working hypothesis was proposed that repeated administration of these substances might inhibit in vivo morphological transformation of cells of C. albicans and protect experimentally infected mice against candidiasis. Doses of BAL were selected on the basis of toxicological studies on animals reported by Durlacker et al. (1946) and of glutathione from studies made by Cater et al. (1957). Groups of adult mice (RML) were infected by intravenous challenge with 500,000 cells

*Hynson, Westcott and Dunning, Inc., Baltimore 1, Maryland.

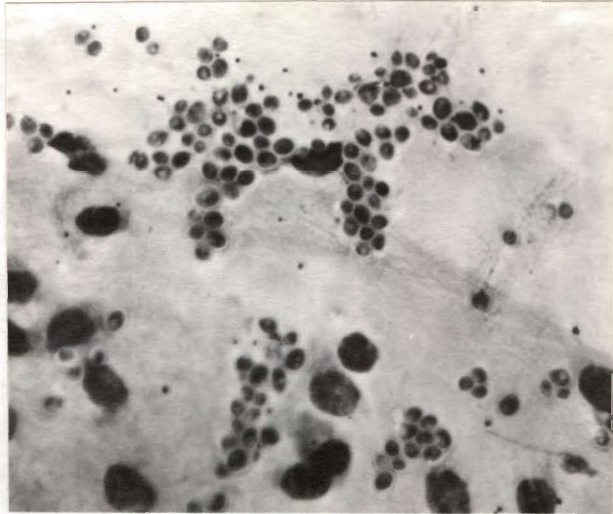


Figure 16. Appearance of cells of *C. albicans* in tissues of a dead mouse incubated at 37°C for 4 hours.

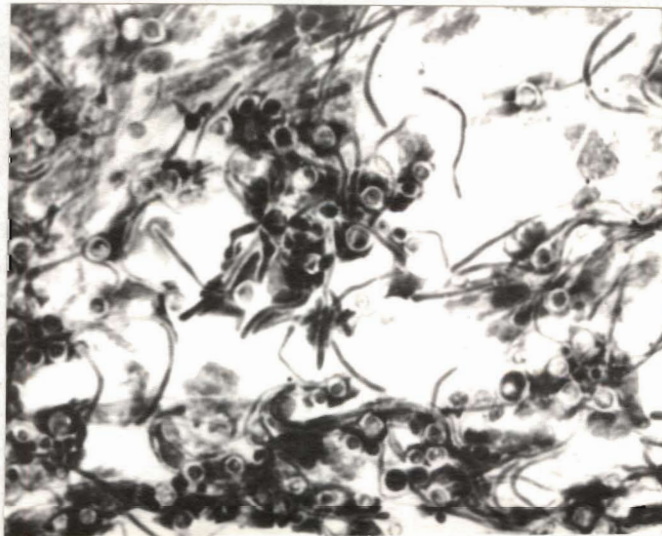


Figure 17. Appearance of cells of *C. albicans* in connective tissue of a mouse 4 hours after injection.

of C. albicans Vet. #4 and the first subcutaneous injection of drug or control material given immediately afterwards and then at 2, 4 and 6 hours. Control groups of animals received the same volume of either saline solution or peanut oil containing 20 per cent benzyl benzoate which was the diluent used in the original BAL preparation. Groups of 10 noninfected animals received the same amounts of BAL, glutathione or the two diluents at the same time periods. The results of these experiments are presented in Table 10. No significant differences in mortality of treated or control groups of animals were apparent when data were compared after a 14-day observation period. The selected doses of BAL and glutathione were not toxic for noninfected control animals.

IV. In Vitro Morphological Alterations of Candida Species.

The dimorphic phenomenon exhibited by yeast-like organisms, especially C. albicans, has been studied extensively. Scherr and Weaver (1953), McClary (1952) and Skinner (1947) have reviewed the many purported factors which affect a yeast-like to filamentous morphological transformation. The experiments reported in this study were designed to result in information of value in the elucidation of conditions or factors which control the morphology of C. albicans in vitro. This information could prove useful in understanding the in vivo environment which affects the changes in morphology of this pathogenic fungus.

As a first approximation to the in vivo environment of C. albicans in tissues of experimental animals, washed cells of this organism and other Candida were added to an equal volume of sterile horse serum. The

TABLE 10

EFFECTS OF 2,3-DIMERCAPTOPROPANOL (BAL) AND GLUTATHIONE
ON MICE WITH EXPERIMENTAL CANDIDIASIS

Challenge dose of <u>C. albicans</u> Vet. #4 ¹	Treatment given at 0, 2, 4 and 6 hours. ²	Mortality Ratio at 14 days.
500,000 organisms	BAL, 1 mg/0.1 ml	15/19
500,000 organisms	BAL control diluent, 0.1 ml	18/20
500,000 organisms	glutathione, 10 mg/0.1 ml	19/20
500,000 organisms	saline solution, 0.1 ml	20/20
-----	BAL, 1 mg/0.1 ml	0/10
-----	glutathione, 10 mg/0.1 ml	1/10
-----	BAL control diluent, 0.1 ml	0/10

¹injected intravenously.

²injected subcutaneously.

screw-cap tubes containing this mixture were rotated in a roller-tube, tissue culture drum at 37°C. Suspensions of the organisms in 0.85 per cent sodium chloride solution served as controls. At various times aliquots were removed, smeared, stained with methylene blue and examined microscopically. The results indicated that after 6 to 8 hours incubation in 50 per cent horse serum only cells of C. albicans and C. stellatoidea formed rudimentary pseudomycelia similar to those described after inoculation of yeast-like cells into subcutaneous tissues of mice (Figure 18). None of the other members of the genus *Candida* exhibited a similar change in morphology. Two of the nonpathogenic *Candida* (Figures 19 and 20) were found to produce elongated budding forms which could be distinguished easily from morphologically transformed cells of C. albicans and C. stellatoidea. The organisms suspended in saline and incubated at 37°C for the same length of time retained their characteristic yeast-like forms. Similar changes in morphology of C. albicans and C. stellatoidea occurred in the presence of sterile rabbit serum, sterile ascitic fluid and 2.5 per cent gelatin.

Cultures of 7 different strains of C. albicans and 6 other species of *Candida* were given to an impartial person who was asked to erase the original labels and give each culture a code number. The organisms from the separate cultures were suspended in saline solution, added to sterile horse serum and treated as previously described. After observation of microscopic morphology, comparisons of the results with the code key were made. A complete correlation was found between the ability of an organism to undergo the described alteration in morphology and whether or not the culture was C. albicans or C. stellatoidea.



Figure 18. In vitro morphological alteration of cells of C. albicans in 50 per cent horse serum after 8 hours at 37°C.

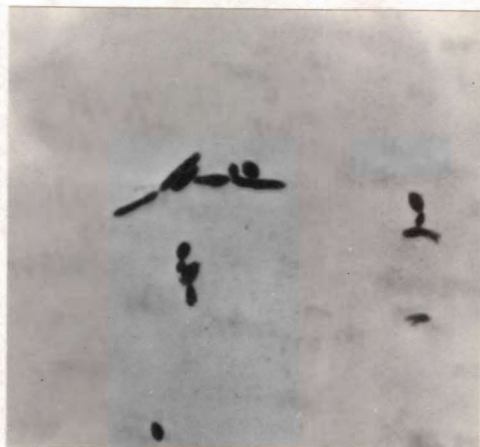


Figure 19. Morphology of C. tropicalis in 50 per cent horse serum after 8 hours incubation at 37°C.



Figure 20. Morphology of C. krusei in 50 per cent horse serum after 8 hours incubation at 37°C.

In the course of studies on the respiratory metabolism of pathogenic fungi, Carter (1955) described a granular type of growth of C. albicans in aerated liquid medium. The same culture medium was used in this study and the observations were made that after 6 to 8 hours of incubation at 35°C on a mechanical shaker, strains of C. albicans and C. stellatoidea produced cells with short pseudomycelia. Examination of stained smears prepared from cultures of other members of the genus indicated that cells of these organisms retained their typical yeast-like morphology. At 12 hours after inoculation, cells of C. albicans strains and those of one strain of C. stellatoidea which was studied had produced definite pseudomycelia. Smears from liquid cultures of C. albicans and C. stellatoidea taken 18 to 24 hours after inoculation showed increasing numbers of cells with yeast-like morphology.

Nickerson and van Rij (1949) reported that the addition of glutathione or cysteine at concentrations of M/100 to a medium which normally supported the filamentous growth of C. albicans resulted in production of yeast-like forms. Nickerson and Chung (1954) indicated that cysteine is produced by growing cells of C. albicans and in sufficient concentrations may allow growth of the organism in the yeast-like phase. In this study, the working hypothesis was made that the 12 to 18 hour period of incubation of C. albicans was necessary for establishment of reducing conditions in the aerated liquid medium conducive to growth of the cells in the yeast-like phase.

Culture flasks containing 500 ml of the liquid medium, described in the section Materials and Methods, were inoculated with 5 ml of a washed cell suspension of the various strains of organisms used.

Triplicate samples for assay of sulfhydryl compounds were taken at 2, 6, 8, 18 and 24 hours of incubation on the mechanical shaker. Smears of the liquid cultures were made at the same time intervals, stained and examined to determine the morphological characteristics of the organisms. Oxidation-reduction potential determinations on the liquid cultures were also made, and the results are presented in Table 11. The values given are the average of three determinations and are not corrected in reference to the standard electrode. A small decrease in measured potential was noted in aerated liquid cultures of all organisms studied. No consistent differences were observed between cultures of the various *Candida* investigated.

Under the conditions of the reported experiments no detectable amounts of sulfhydryl compounds were evident in aerated broth cultures of the different *Candida* used in this study. In all instances the concentrations of cysteine and glutathione were apparently below the levels of sensitivity of the three different assay methods used. With known amounts of glutathione in 0.001 M ethylene diamine tetraacetate solution, the method of Benesch and Benesch (1948) was found to be sensitive to at least 10 ug per ml. Using a 5 ml burette the approximate sensitivities of the iodometric and amperometric titration of glutathione and cysteine were at least 0.1 uM per ml.

As in other studies using this liquid medium, filamentous forms of *C. albicans* and *C. stellatoidea* were observed in smears prepared from cultures of these organisms. The cells of other members of the same genus retained their typical yeast-like morphology.

TABLE 11

OXIDATION-REDUCTION POTENTIAL MEASUREMENTS
OF LIQUID CULTURES OF CANDIDA SPECIES¹

Organism	Time in hours after inoculation				
	2	6	8	18	24
<u>C. albicans</u> #520	+.234	.220	.178	.188	.190
<u>C. albicans</u> H. X.	+.205	.225	.225	.220	.250
<u>C. stellatoidea</u>	+.225	.200	.180	.165	.205
<u>C. guilliermondi</u>	+.217	.185	.145	.135	.170
<u>C. krusei</u>	+.208	.175	.120	.150	.150
<u>C. tropicalis</u> Lewis #1	+.255	.250	.165	.170	.175

¹Values given are in volts.

V. Studies on the Endotoxin of Candida albicans.

The suggestion has been made that certain of the pathological changes in natural or experimental candidiasis could be explained on the basis of a toxic substance associated with cells of C. albicans (Henrici, 1940 and Winner, 1956). The latter author was apparently unaware of the published work of Salvin (1952b) who found that the intra-peritoneal inoculation of large numbers of killed cells of C. albicans and other pathogenic and nonpathogenic fungi resulted in rapid death of mice. The addition of killed tubercle bacilli to the inocula augmented this lethal effect. Twenty-one-day-old female mice were reported to be more susceptible to what Salvin considered an endotoxin.

Because of previously collected data which indicated that adrenalectomy increased the susceptibility of adult mice to experimental candidiasis, studies were initiated to determine the toxicity of killed C. albicans for adrenalectomized mice.

The animals used in these studies were of three types. They were adult mice of the RML strain, adrenalectomized mice of the same strain and 21-day-old female mice of the CFW strain. Adrenalectomy was carried out as previously described and a period of 48 hours lapsed between the time of removal of the adrenals and use of the animals in an experiment.

The toxic preparation was similar to that described by Salvin (1952b). Cells of C. albicans #3148 were grown in a liquid medium at 37°C with constant shaking and recovered by centrifugation. After washing with 0.85 per cent sodium chloride solution, the organisms were killed by exposure to 0.5 per cent formalin for 3 days. The resulting cells were again washed, dried by several washings with cold acetone and used to

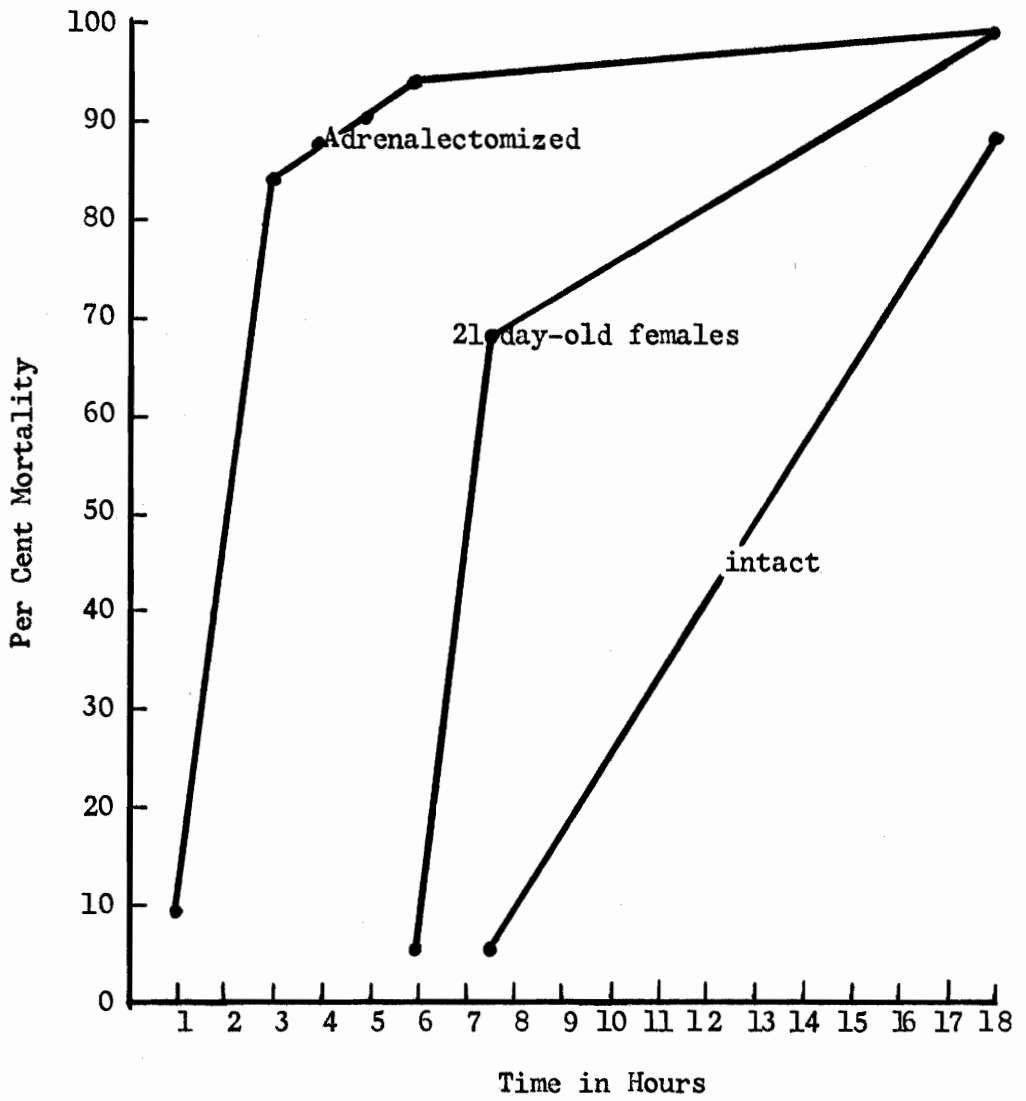
prepare the inocula. The adjuvant employed was cells of Mycobacterium tuberculosis, Jamaica strain, which had been heat-killed and acetone dried. This latter preparation was kindly supplied by Dr. Salvin.

Figure 21 represents the cumulated mortality data collected from three different groups of mice injected intraperitoneally with 20 mg of killed, acetone-dried C. albicans cells plus 2 mg of tubercle bacilli contained in 0.5 ml of saline solution. At least 34 animals were included in each group. Ten adrenalectomized control mice were injected with 0.5 ml of the above diluent and observed over the 18-hour period. These animals survived with no apparent symptoms.

These data indicated that the adrenalectomized mouse injected intraperitoneally with killed, acetone-dried cells of C. albicans and adjuvant was much more susceptible to the lethal effects than were either intact adult or intact 21-day-old mice. Thirty-one out of 34 adrenalectomized mice were dead before there were any deaths in the other groups of animals.

As soon after death as possible all animals were autopsied and gross changes observed. In some instances this was done immediately after cessation of breathing. The gross pathological findings were essentially negative with the exception of increased congestion of the large veins entering the heart. The pulmonary arteries and branches also appeared to be engorged with blood. The pleural cavity of most animals contained a small amount of clear fluid and the lung surface of some showed small areas of hemorrhage. The mice which were autopsied immediately after cessation of breathing were found to have contracting hearts. These heart beats continued for some time after breathing had stopped.

Figure 21. Lethal Effects of Killed, Acetone-Dried Cells of Candida albicans on Mice.



A study of the effect on mortality of adrenalectomized mice injected with killed C. albicans cells alone, the killed tubercle alone and the two preparations in combination was made. Three groups of adrenalectomized mice (RML), 10 animals per group, were injected with 20 mg of killed, acetone-dried yeast-like cells, 2 mg of killed, acetone-dried tubercle bacilli or 20 mg of yeast-like cells plus 2 mg of tubercle bacilli. The cell preparations were suspended in 0.5 ml of saline solution and injected intraperitoneally. A fourth group of 10 adrenalectomized mice were injected with the diluent and served as controls. The results are presented in Table 12.

These data indicated that heat-killed, acetone-dried tubercle bacilli of the Jamaica strain possess a toxic activity for adrenalectomized mice. At a concentration of 20 mg per 0.5 ml, killed, acetone-dried cells of C. albicans were also lethal when injected intraperitoneally into operated animals.

Similar gross pathological changes were observed in adrenalectomized mice injected with killed C. albicans cells alone and in combination with killed tubercle bacilli. No differences between these animals and intact animals injected with both cell preparations were evident. Adrenalectomized mice injected intraperitoneally with 2 mg of killed tubercle bacilli did not appear to exhibit congestion of the right side of the heart or engorgement of the pulmonary arteries.

One of the described actions of cortisone and certain other adrenal steroid hormones has been their ability to protect experimental animals against the lethal effects of bacterial toxins (Higginbotham and Dougherty, 1954, Spink and Hall, 1952 and others). An experiment was designed to

TABLE 12

LETHAL EFFECTS OF KILLED, ACETONE-DRIED CANDIDA ALBICANS CELLS,
KILLED, ACETONE-DRIED TUBERCLE BACILLI OR A
COMBINATION OF BOTH IN ADRENALECTOMIZED RML MICE.

Amounts and Organisms Injected Intraperitoneally.	Mortality Ratio at 13 hours.
20 mg of <i>C. albicans</i> cells	9/10
2 mg of tubercle bacilli	10/10
20 mg of <u><i>C. albicans</i></u> cells and 2 mg of tubercle bacilli	10/10
Saline controls	0/10

study the possible protective effect of cortisone in mice injected intraperitoneally with the toxic preparation of C. albicans cells.

A group of 24 female mice (CFW) approximately 21 days old was treated with 2.5 mg of cortisone acetate. This amount of hormone was contained in 0.1 ml of 0.85 per cent sodium chloride solution and was injected subcutaneously one hour before challenge with the toxin preparation. Control animals were inoculated with the same volume of diluent by the same route. One hour after receiving the hormone or saline injection, mice of both groups were injected with 20 mg of killed, acetone-dried C. albicans #3148 cells and 2 mg of killed, acetone-dried tubercle bacilli. This amount of the toxin preparation plus adjuvant was contained in 0.5 ml of saline solution and was given intraperitoneally. Animals in both groups were observed at hourly intervals for 13 hours and mortalities noted. Shortly after death, mice from both groups were autopsied, examined and tissues from lungs, liver, spleen, kidneys and brain taken for histological examination.

Figure 22 summarizes the results of mortality of mice in the cortisone-treated and the saline-treated control groups. Data collected from observations made on the two groups of animals at the thirteenth hour after inoculation of the toxin preparation plus adjuvant are compared in Table 13. It was apparent that cortisone acetate offered a significant degree of protection against the combined toxic effects of killed cells of C. albicans and tubercle bacilli.

Gross examination of animals which died in both groups of mice indicated the presence of less fluid in the pleural cavity of animals

pretreated with cortisone as compared to those which received saline solution.

No apparent differences were noted in the distribution of Schiff-positive material in tissues of hormone-treated or control mice. Large numbers of Schiff-positive bodies were observed in tissue sections from the lungs of animals in both groups. Microscopic examination of stained kidney tissue revealed large amounts of Schiff-positive material localized in the glomerular tufts (Figure 23).

Figure 22. Effect of Cortisone Acetate on 21-Day-Old Female Mice (CFW) Inoculated with Candida albicans Toxin Plus Adjuvant.

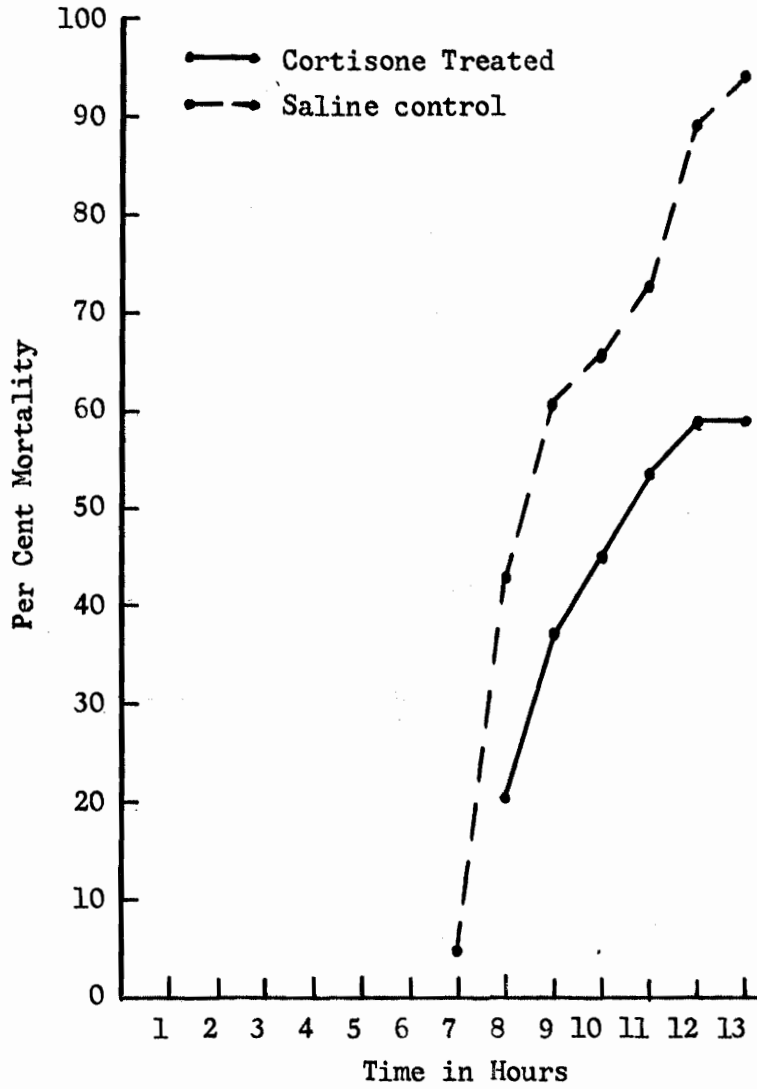


TABLE 13

EFFECT OF CORTISONE ADMINISTRATION ON THE LETHALITY
OF CANDIDA ALBICANS TOXIN PLUS ADJUVANT
IN YOUNG FEMALE CFW MICE

Treatment 1 hour prior to challenge ¹	Mortality Ratio	Per Cent Mortality	P Value ²
Saline	20/21	95.2	---
2.5 mg Cortisone Acetate	14/24	58	.02- .01

¹The challenge dose consisted of the intraperitoneal inoculation of 20 mg of killed, acetone-dried cells of C. albicans plus 2 mg of killed, acetone-dried tubercle bacilli per animal.

²The P value was obtained by calculation of χ^2 with application of Yates correction.

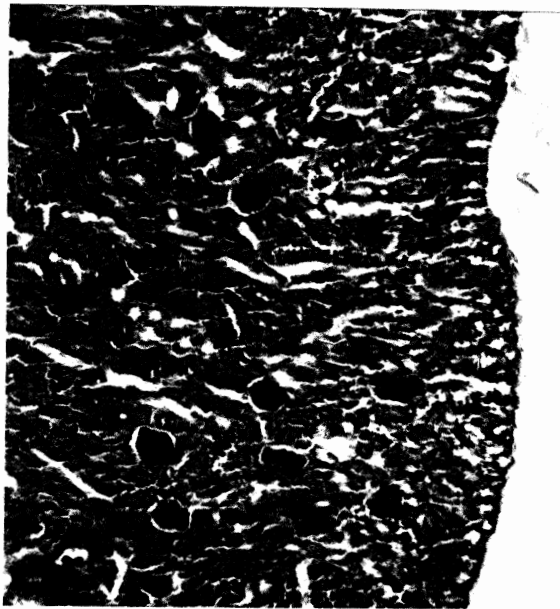


Figure 23. A photomicrograph of a Hotchkiss-McManus stained section of kidney tissue from a toxin-treated mouse.

DISCUSSION

Various investigators (van der Linden, 1926, Stovall and Pessin, 1933, MacKinnon, 1936 and others) have reported the isolation of strains of C. albicans which differed in virulence for experimental animals as measured by the lethal effects produced after infection. None of these authors, however, have presented data which would allow assessment of the errors involved in their determinations. In few of these studies were the numbers of animals used noted.

As discussed previously Salvin et al. (1952a) found that several strains of C. albicans differed in virulence for mice. These animals were injected intraperitoneally with relatively large numbers of cells of individual isolates suspended in hog gastric mucin. Data were collected over a 48-hour observation period and mean lethal doses of the organisms calculated on this basis. The apparent discrepancies between his results and those reported herein with C. albicans #3160 and #3161 could be explicable on the basis of different routes of inoculation, but particularly upon the differing degrees of infection induced by the challenge doses. C. albicans endotoxin may well have contributed greatly to the rapid death of animals injected intraperitoneally with the numbers of cells that Salvin et al. used. However, the results presented in this thesis on the lethality of various isolates of C. albicans for mice may well indicate that differences in virulence of these organisms are more evident within a shorter observation period.

Although it was shown that adrenalectomy resulted in a considerable increase in susceptibility to fatal infections with C. albicans #520, the use of operated animals to detect differences in virulence of C. albicans #3160 and #3161 was not successful. The lethality of cells of these two

strains for adrenalectomized mice was not significantly different. Use of a much more susceptible host did not allow the expression of the variation of virulence in mice reported for these two isolates (Salvin et al., 1952a).

No conclusive statements of significance can be made concerning a possible relationship between the source of isolation of an organism and its virulence for mice as defined in these experiments. However, two typical strains of C. albicans which were originally obtained from the vaginas of females with clinical symptoms were of reduced virulence for mice as compared to some isolates from other human sources. Two strains, C. albicans Finlayson (oral) and C. albicans Finlayson (vaginal), which had been grown on artificial media for only two passages, appeared to be of reduced virulence for mice. Data collected and compared after an 18-day observation period indicated no statistically significant differences between the lethality of these organisms. However, comparisons made on the fourteenth day after challenge tended to indicate a variation in virulence of these two isolates obtained from the same infected patient.

One of the supposed characteristics which allows distinctions to be made between C. albicans and C. stellatoidea is sucrose fermentation (Conant et al., 1944, Widra, 1957, etc.). An interesting observation made in this study was that C. albicans Vet. #4, which was unable to produce acid in the sucrose fermentation medium, appeared to be of high virulence for mice. By the criteria used in many clinical laboratories, this organism would be classified as C. stellatoidea and nonvirulent characteristics would be ascribed to it. The truth may be that there is

no constant relationship between sucrose-fermenting ability and the virulence of an isolate for experimental animals or for humans. The results of these studies on C. albicans Vet. #4 suggested the fallaciousness of a dogmatic association between the supposed degree of pathogenicity of an organism and other more easily measured biological characteristics. Winter and Foley (1955) have concluded that C. albicans and C. stellatoidea are extremes in a broad spectrum of biological variation.

From some of the earliest studies on experimental candidiasis (Radaelli, 1924, Nye et al., 1929, Stovall and Pessin, 1933), it is well established that the kidney is the main organ involved in this infection. When organisms are injected either intravenously or intraperitoneally, this phenomenon was noted. It has been suggested that the organism, C. albicans, acts as an embolus and blocks small blood vessels (Radaelli, 1924). The observations of others, however, contradict this hypothesis (Stovall and Pessin, 1933 and Evans and Winner, 1954). In relation to the apparent predilection of intravenously injected staphylococci for kidney tissue of experimental animals, Gray et al. (1957) have speculated that the absence of a system of fixed reticuloendothelial phagocytes may render the kidneys potentially vulnerable to damage. This would appear to be a logical explanation for the results observed in this study on experimental candidiasis.

The establishment of chronic infections as a result of injection of cells of C. albicans into mice has been reported previously (Young, 1958). This study indicated that cells of this yeast-like organism can persist in kidney tissues for long periods of time. The present finding that suggested a differential distribution of lesions of this organ caused

by C. albicans may in part explain the ability of some mice to survive an infection which proved fatal to the majority of injected animals. If it is true that death of mice, infected with C. albicans, results mainly from loss of functional kidney tissue (Adriano and Schwarz, 1955), the ability of an individual mouse to limit extensive damage to one kidney would explain its survival and ability to become chronically infected.

Among the factors which have been associated with increased host susceptibility to natural or experimental infections are those referable to depression of normal function of the host defense mechanisms. That adrenalectomy results in the decreased resistance of animals to many noxious agents, infectious or otherwise, hardly requires documentation. Equally well known are the virulence enhancing activities of certain adrenocortical hormones. However, it is apparent that no simple relationship exists between normal adrenocortical function and resistance to infectious disease. A demonstration of the complexity of aspects of this relationship was noted when the effects of graded doses of cortisone on experimental candidiasis were investigated in adrenalectomized mice. These studies indicated that the mortality enhancing activity of exogenous cortisone was dosage dependent. Daily administration of cortisone acetate in two divided doses resulted in either increased mortality of infected adrenalectomized animals. It was shown that the optimal total dose of this hormone for protection was in the range of from 0.01 to 0.1 mg per day. It should be pointed out that the administration of doses in this range resulted in mortality ratios in groups of infected adrenalectomized mice which would be expected in intact animals infected by the injection of similar numbers of cells of

C. albicans. Beneficial effects of injection of cortisone into animals infected with other pathogenic organisms have been described (Robinson et al., 1953 and Jawetz et al., 1955). A proposed explanation for these observations and for the results herein described could be that infection and adrenalectomy result in an adrenocortical insufficiency which acts to depress the function of many host defense mechanisms. The administration of a "physiological" amount of cortisone to infected animals could restore these functions to normal. One of the normal functions which may require certain adrenal steroids is the ability to detoxify substances of both endogenous and exogenous origin (Higginbotham and Dougherty, 1955). More than optimal amounts of cortisone probably acts by suppression of inflammation (Schneebeli and Dougherty, 1951, Kass and Finland, 1953 and others).

Subsequent to completion of the observations on the in vivo morphological alterations exhibited by cells of C. albicans, the work of Stovall and Pessin (1933) was found in the literature. They studied the behavior of injected cells of "Monilia albicans" (C. albicans), "Monilia candida" (C. tropicalis) and "Monilia parapsilosis" (C. parapsilosis) in rabbits. It was reported that only cells of "M. albicans" would form pseudomycelia in vivo. The authors suggested that this organism produced lesions by a "purely mechanical plugging of capillaries and arterioles." However, on the basis of measurements of cells grown for 48 hours on malt agar, they were unable to find a correlation between cell size and pathogenicity of the three organisms studied. The principal quality which accounted for the virulence of "M. albicans" was suggested to be the ability to grow in the animal body. It is apparent that the size of yeast-like cells

of C. albicans grown on malt agar has little relationship to the size of filamentous cells in vivo. The findings reported in this thesis partially substantiated this early work of Stovall and Pessin. Of all strains of organisms studied, only C. albicans and C. stellatoidea exhibited filamentous morphology in the tissues of the mouse. All species of *Candida* usually considered nonpathogenic failed to develop pseudomycelia in vivo. The fact that the strain of C. stellatoidea behaved similarly to the strains of C. albicans would appear to indicate their close relationship as suggested by others (Winter and Foley, 1955). The rapidity of the alterations in morphology of C. albicans would seem to indicate a significant role for this alteration in the pathogenesis of experimental candidiasis. It would appear reasonable to suggest that this transformation favors survival of the fungus in the host by a mechanical interference of ingestion by phagocytes. Credence to this suggestion was shown by the work of Young (1958), who observed a similar transformation of cells of C. albicans after injection into the peritoneal cavity of mice.

Various authors including Scherr and Weaver (1953) have speculated that the yeast-like form of C. albicans is best adapted to development in tissues. The fact that large numbers of filamentous organisms are found in the tissues of experimentally infected animals may indicate that exactly the opposite relationship may exist, i.e., that the filamentous form of C. albicans is best adapted to growth in animal tissues.

Under the conditions of the described experiments attempts to inhibit the in vivo morphological alteration of C. albicans were unsuccessful. Cysteine and glutathione at concentrations of 10 and 100 mg per ml failed

to alter the described phenomenon. Under cultural conditions described by Nickerson and van Rij (1949) these compounds were shown to allow growth of C. albicans with yeast-like morphology. The inability of these reducing substances to alter in vivo morphological transformation of C. albicans may be best explained because of the probable rapid oxidation of them under these conditions. Attempts to protect mice infected with C. albicans by repeated inoculations of glutathione or BAL indicated that these substances afforded no apparent protection. The concentrations of reducing substances found necessary to prevent yeast-like to pseudomycelial transformation in vitro (Nickerson and van Rij, 1949) may not be attained by the parenteral administration of glutathione or BAL.

An interesting observation made was that conditions in the subcutaneous tissues of dead mice incubated at 37°C would not allow the morphological alteration of cells of C. albicans to occur. In tissues of live animals this transformation readily takes place. It is suggested that under in vivo conditions at least a resemblance to normal physiological conditions is necessary.

From an examination of suggested factors governing in vitro morphological alteration of cells of C. albicans (Skinner, 1947, Scherr and Weaver, 1953, etc.), little understanding can be gained as to the factor or factors responsible for the formation of pseudomycelia under the described conditions of these researches. Anaerobiasis has been implicated in certain studies (McClary, 1952), however, under conditions of aeration in a liquid medium, cells of C. albicans developed rudimentary pseudomycelia. Attempts to correlate the possible accumulation of cysteine, glutathione or other measurable reducing substances with yeast-like to

pseudomycelial interconversions were unsuccessful. During the time after inoculation when large numbers of cells of this organism reverted back to the yeast-like morphology, no detectable amounts of cysteine or glutathione were present in samples taken from aerated liquid cultures. These experiments indicated that if these substances are responsible for control of the morphology of C. albicans, changes in their concentrations must occur intracellularly.

As has been discussed previously, with rare exceptions, *Candida* species are the only yeast-like organisms isolated from human clinical materials. It is suggested that the described in vitro variations in morphology of cells of C. albicans and C. stellatoidea may serve as a basis for routine techniques for partial identification of these organisms. Under the described conditions cells of no other *Candida* species exhibited a similar change in morphology when incubated at 37°C. These techniques would have the distinct advantages of rapidity and ease of accomplishment.

The relative importance of the reported endotoxin (Salvin, 1952b) in the pathogenesis of either experimental or natural candidiasis is unknown. Data collected during this study indicate that adrenalectomized mice may serve as well as young intact mice for demonstration and assay of this toxic substance. It was shown that in operated animals the addition of killed tubercle bacilli as an adjuvant to the inoculum was not necessary. The results of studies suggested that the enhancing effect of the adjuvant in mice inoculated intraperitoneally with killed cells of C. albicans could be explained on the basis of additive toxicity. The injection of 2.5 mg of killed tubercle bacilli intraperitoneally into adrenalectomized mice resulted in rapid death.

Several observations suggested possible explanations of the pathogenic effects of C. albicans endotoxin. The presence in injected animals of engorged vessels of the right side of the heart and of numerous Schiff-positive bodies in the lung tissues may indicate that blockage of pulmonary circulation is a significant aspect of the lethal effects of this toxic substance. The accumulation of large amounts of Schiff-positive material in the kidneys of animals injected intraperitoneally with killed cells of C. albicans plus adjuvant could indicate, however, that function of these organs may be affected. It should be recalled that Evans and Winner (1954) described a Schiff-positive hyaline material as being present in glomerular capillaries of rabbits experimentally infected with cells of C. albicans. This material was noted to increase in amount until complete obliteration of these structures was evident.

As might have been suspected from studies of Higginbotham and Dougherty (1954 and 1955) the pretreatment of mice with 2.5 mg of cortisone acetate afforded a degree of protection to these animals injected with a lethal amount of C. albicans endotoxin. The suggestion seems logical that further studies on the antiendotoxic activity of cortisone might result in information of value in an understanding of the mode of action of the toxic substance of this organism.

SUMMARY

1. Under the described conditions of this experimental study, 3 stock strains of C. albicans, #520, #3160 and #3161, were found not to differ significantly in virulence for CBA mice. Statistical treatment of data collected at 18 days after intravenous challenge indicated that the observed differences in the mean lethal doses could have been due to chance.

2. Adrenalectomy was shown to significantly increase the susceptibility of CBA mice to experimental candidiasis induced by intravenous injection of cells of C. albicans #520. The use of operated animals to detect differences in virulence of C. albicans #3160 and #3161 was not successful.

3. The study of 9 different cultures of C. albicans, which were isolated and identified by morphological, biochemical and cultural means, indicated wide variations in lethality for laboratory mice. Their virulence for mice was compared by the intravenous inoculation of a standard number of cells of each individual organism. A tendency was noted that the longer the experimental observation period the less apparent were differences in mortality of the groups of animals.

4. No conclusive statements could be made concerning a possible relationship between the source of isolation of the organisms and their virulence for mice. An interesting observation was that C. albicans Vet. #4, which was unable to produce acid in the sucrose fermentation medium, appeared to be of high virulence as determined by the designed experimental procedures.

5. In mice which were infected by intravenous challenge, the kidneys were the only organs studied which showed significant involvement. Observations made on animals which had been infected for at least 35 days indicated that strains of C. albicans could establish chronic infections of the kidneys. In a large percentage of animals the right kidney was found to be involved grossly while the left organ appeared normal. No explanation was apparent for the differential distribution of lesions in the kidneys of chronically infected mice.

6. When compared by techniques similar to those used to study strains of C. albicans, other species of *Candida* were found to be avirulent for adult CBA mice. No significant lethal effects or evidence of the presence of progressive infection were apparent in animals injected with large numbers of cells of these organisms.

7. Experiments, designed to elucidate the role of adrenal steroid hormones in resistance to experimental candidiasis, demonstrated that cortisone offered a significant degree of protection to adrenalectomized mice infected with C. albicans. Doses in the range of 0.01 to 0.1 mg per day protected animals to a significant extent when compared to adrenalectomized mice receiving no hormone. Amounts of cortisone both above and below the apparent optimal dose resulted in increased mortality over a 14-day observation period. Under the described experimental conditions, the steroid hormone caused no apparent gross or microscopic differences in the size, number or distribution of lesions caused by C. albicans in adrenalectomized CBA mice.

8. A morphological alteration of yeast-like cells of C. albicans and C. stellatoidea was shown to occur within 1 hour after injection

into the subcutaneous tissues of mice. Yeast-like cells of other *Candida* failed to exhibit these alterations under the same conditions. The cells of *C. albicans* formed elongated pseudomycelia as soon as 1 hour after injection and the postulation was made that the filamentation of this organism in vivo served as a mechanical hindrance to ingestion by mouse phagocytes. Attempts to inhibit this in vivo morphological transformation with cysteine and glutathione and to protect experimentally infected animals by repeated injections of glutathione or 2,3-dimercaptopropanol (BAL) were without success. However, examination of tissue spreads from animals injected with yeast-like cells of *C. albicans* and sacrificed immediately afterwards showed few cells which exhibited any degree of pseudomycelial formation.

9. Studies on the in vitro morphology of *Candida* species proved that a similar alteration occurred when cells of *C. albicans* and *C. stellatoidea* were incubated at 37°C in various sera or other protein solutions. A liquid medium was described which allowed the yeast-like to pseudomycelial transformation to occur. None of the other members of the same genus were found to show a similar change in morphology. No correlations were observed between this in vitro morphological alteration and the accumulation of measurable concentrations of glutathione or cysteine in aerated liquid cultures. A small drop in oxidation-reduction potential was observed to occur in cultures of all *Candida* species studied under the same conditions.

10. Investigations concerning the endotoxin of *C. albicans* suggested that the adrenalectomized mouse was more susceptible than either intact adult or 21-day-old female animals of the CFW strain.

Further experiments demonstrated that the addition of killed tubercle bacilli to the toxin preparation was not necessary to result in death of adrenalectomized RML mice. Pretreatment of 21-day-old female animals (CFW) with 2.5 mg of cortisone acetate resulted in a minimal degree of protection against the lethal effects of this toxic preparation of cells of C. albicans.

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STUDIES ON THE PATHOGENESIS OF
EXPERIMENTAL CANDIDIASIS

by

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An abstract of a thesis submitted to
the faculty of the University of Utah
in partial fulfillment of the require-
ments for the degree of

Doctor of Philosophy

Approved by the faculty committee in

August 1959

Dr. Louis P. Gebhardt, Chairman, Supervisory Committee

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1959

As defined in this study, wide variability in the virulence of strains of Candida albicans for mice was observed. These variations were apparent on the basis of significantly different mortality ratios in groups of animals injected intravenously with standard numbers of cells of isolates of different strains of this organism. Three stock strains of C. albicans exhibited similar mean lethal doses for CBA mice. Although it was shown that adrenalectomy resulted in an increase in susceptibility to fatal infections with C. albicans #520, the use of operated animals to detect differences in virulence of C. albicans #3160 and #3161 was not successful.

No conclusive statements could be made concerning possible relationships between the source of isolation of the organisms and their virulence for mice. An interesting observation made was that C. albicans Vet. #4, which was unable to produce acid in the sucrose fermentation medium, appeared to be of high virulence for mice.

In animals which were infected by intravenous challenge, the kidneys were the only organs studied which exhibited significant involvement. Observations on mice which had been infected for at least 35 days showed that strains of C. albicans could establish chronic infections of the kidneys. In a large percentage of animals the right kidneys were found to be infected grossly while the left organ appeared normal. No explanation was apparent for the differential distribution of lesions in the kidneys of chronically infected mice.

When compared by techniques similar to those used to study strains of C. albicans, other species of *Candida* were found to be avirulent for adult CBA mice. No evidence of progressive infection was noted in

animals injected intravenously with large numbers of cells of these organisms.

It was demonstrated that a dose of cortisone, in the range of 0.01 to 0.1 mg per day, offered a significant degree of protection for adrenalectomized mice infected with C. albicans. Doses of the hormone above and below this optimal amount resulted in increased mortality over a 14-day period. In the amounts used, cortisone administration caused no obvious gross or microscopic differences in the size, number or distribution of lesions in infected adrenalectomized CBA mice.

A morphological alteration of yeast-like cells of C. albicans and Candida stellatoidea occurred within 1 hour after injection into subcutaneous tissues of mice. Cells of other Candida species failed to exhibit these alterations. The cells of C. albicans had formed elongated pseudomycelia by 1 hour. It was postulated that filamentation by this organism in vivo served as a mechanical hindrance to ingestion by mouse phagocytes. Attempts to inhibit this in vivo morphological alteration with cysteine and glutathione and to protect infected animals by repeated injection of glutathione or 2,3-dimercaptopropanol (BAL) were without success. However, injection of yeast-like cells into mice which were sacrificed and their carcasses incubated at 37°C for 4 hours resulted in almost complete lack of this morphological transformation.

Studies on the in vitro morphology of Candida species indicated that similar alterations occurred when cells of C. albicans or C. stellatoidea were incubated at 37°C in sera, other protein solutions or an aerated liquid medium. None of the other members of Candida studied showed a similar change in morphology. No correlations were observed

between this in vitro morphological alteration and measurable concentrations of reducing substances in aerated liquid cultures. Under these same conditions, a small drop in oxidation-reduction potential was observed in cultures of all *Candida* studied.

Investigations concerning the endotoxin of C. albicans suggested that adrenalectomized mice were more susceptible than intact adult or 21-day-old female animals. Further experiments indicated that an adjuvant was not necessary to demonstrate the toxic nature of killed cells of C. albicans. Pretreatment of animals with 2.5 mg of cortisone resulted in a minimal amount of protection against this toxic preparation of cells of C. albicans.

RESEARCH PROPOSALS

1. The predilection of Candida albicans for the kidneys in systemic candidiasis of experimental animals is well known. The hypothesis is enunciated that the kidney tissues of these animals have a reduced capacity to eliminate certain parasitic organisms by phagocytic means.

2. Attempts will be made to correlate the virulence of selected strains of Candida albicans with in vitro and in vivo generation times, their ability to be ingested by phagocytes and other biological characteristics.

3. Most human cases of local candidiasis are infections of the mucous membranes and moist skin surfaces. The proposal is made to investigate the hypothesis that pseudomycelial formation by C. albicans allows persistence of this organism on these body surfaces.

4. It has been reported that C. albicans possesses a toxic surface component which is distinct from the described endotoxin. The question of the pathological significance of these substances in experimental candidiasis will be considered.

5. The proposal is made to study the apparent difference between the incidence of involvement of the right and left kidneys of mice chronically infected with Candida albicans.

6. Little is known concerning the importance of specific acquired resistance in either natural or experimental candidiasis. What is the significance of humoral and cellular defense mechanisms in these infections?

7. The proposal is made to study local tissue reactions to pathogenic fungi using techniques of time-lapse microcinematography.

8. A fruity odor is commonly associated with cultures of Candida albicans. Do strains of this organism produce esters, what are they and what are the metabolic pathways for their synthesis?

9. It is suggested that the yeast-like to pseudomycelial transformation of Candida albicans may serve as a model to study growth and differentiation of mammalian cells. It is proposed that the investigation of factors which affect the morphology of this yeast-like organism may indicate avenues of approach for the study of animal cellular differentiation.

10. What is the natural history of western equine encephalomyelitis virus? Present information suggests the presence of an animal reservoir(s) which maintains the virus over the winter and in the spring serves as a source of infection for the important insect vector.