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AN EVALUATION OF VARIOUS ENVIRONMENTAL FACTORS AFFECTING THE
PROPAGATION OF CRYPTOCOCCUS NEOFORMANS

A DISSERTATION

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in partial fulfillment of the requirements for the

degree of

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Oklahoma City, Oklahoma

1965

AN EVALUATION OF VARIOUS ENVIRONMENTAL FACTORS AFFECTING THE
PROPAGATION OF CRYPTOCOCCUS NEOFORMANS

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AN EVALUATION OF VARIOUS ENVIRONMENTAL FACTORS AFFECTING THE
PROPAGATION OF CRYPTOCOCCUS NEOFORMANS

CHAPTER I

INTRODUCTION

The genus Cryptococcus is composed of relatively simple fungi, consisting of yeast cells which reproduce by budding. These fungi produce neither true nor pseudomycelium. The latter characteristic differentiates them from the monilia group while the absence of ascospore formation distinguishes them from the true yeasts (Saccharomyces group). Cryptococcus spp. are known to be widespread in nature. They are found frequently in animals and vegetable material (Benham, 1935) and have been isolated from such sources as soil, skin, milk, and milk products, fruit and fruit juices.

Cryptococcus neoformans is the only species known to be pathogenic to man and animals. The disease it causes is known as cryptococcosis (torulosis, European blastomycosis, Busse-Buschke's disease). C. neoformans exists both as the etiologic agent of human disease and as a saprophyte in nature. Busse and Buschke, in 1894, isolated an encapsulated yeast from the ulcerous tibia of a woman and referred to it as Saccharomyces spp. At about the same time, Sanfelice recovered a yeast-like organism from fermenting peach juice. He was able to demonstrate

its pathogenicity for dogs, guinea pigs, and chickens. Because of its tumor forming properties in experimental animals, Sanfelice named this fungus Saccharomyces neoformans.

In 1901, Vuillemin transferred the fungus to the genus Cryptococcus because of lack of ascospore formation which is typical of organisms in the Saccharomyces group and called Busse's organism C. hominis. Stoddard and Cutler, in 1916, misinterpreted the mucoid capsule of the fungus as evidence of histolysis of host tissue and named the fungus Torula histolytica. Skinner, 1950, established that the valid name of this fungus was C. neoformans. The use of European blastomycosis as a synonym for cryptococcosis caused further confusion. This argument was resolved by Benham who supported the use of the term blastomycosis solely for the disease produced by Blastomyces dermatitidis (North American blastomycosis).

Since the time of Busse's description of the first case of cryptococcosis, approximately 500 other instances of human infection have been recorded from most parts of the world. The severity of the disease and high fatality rate by the fungus increased interest among mycologists and other workers.

C. neoformans is the most frequent cause of mycotic meningitis in man and is often fatal. Although the involvement of the central nervous system with subacute and chronic meningitis is the most familiar form of this mycosis, other systems are also involved. Generalized infections with lesions in bones and viscera have been observed. In addition, infections have been reported in a variety of domestic and captive animals such as horse, cattle, dog, cat, marmoset, pig, cheetah, guinea pig, and ferret (McGrath, 1954; Barron, 1955). Trautwein and Nielson (1962)

reported cryptococcosis in two cats, a dog and a mink. A severe outbreak of bovine mastitis was reported to be caused by C. neoformans (Pouden, 1952). In a Holstein dairy herd, 106 cows out of 235 were positive. Emmons (1952) also isolated this fungus from the teats and milk of a cow. They reported that this infection was apparently caused by the inoculation of the fungus into the teats, most probably by contaminated milking machines. The report was confirmed when thirty-seven days after feeding a 10-week old calf with 200 ml of broth culture of C. neoformans in milk, Emmons could not demonstrate the organism in the tissues. In this outbreak of bovine mastitis, there were no reports of infection in herdmen. This strengthens the belief that the infection is exogenous, soil borne, and there is no hazard of animal-to-man transmission. The lesions, especially in the facial region of other infected animals, also suggest that this fungus exists free in nature. Isolation of C. neoformans from peaches by Sanfelice (1894), from milk by Klein (1901), and later, re-isolation from milk in the absence of infection in cows by Carter and Young (1950), also testified to its saprophytic occurrence in nature. Emmons (1951-1954) isolated it repeatedly again from a saprophytic source. Many of the specimens he collected were from various environments, such as barnyards, granneries, in and around the vicinity of chicken houses and other miscellaneous sources. The results revealed no specific pattern of distribution. The fungus has also been isolated by others in soil samples from various parts of the United States, Hawaii, Brazil and Japan (Yamamoto, Ishida, and Soto, 1957; Ajello, 1958; Silva and Paula, 1963; Hasenclever and Emmons, 1963; Muchmore, Rhoades, Nix, Felton, and Carpenter, 1963).

All of the previous and present reports indicate that C. neoformans is present in man's environment. It is highly probable that the disease begins as a respiratory involvement with circumscribed pulmonary lesions. This was speculated first by Cox and Tolhurst (1946) and later by Wager and Calhoun (1954) after observing focal lesions in the lungs and pleura of patients with cryptococcal meningitis. Ritter and Larsh (1963) were able to infect mice following an intranasal installation and supported the theory that the respiratory tract probably is a portal of entry of the organism into the host. Smith, Ritter, Larsh, and Furcolow (1964) infected mice by two methods: first, by exposing mice to loam soil seeded with the organism, and second, by exposing mice in a Henderson apparatus to an aerosol of C. neoformans. These two methods of exposure of mice to airborne C. neoformans showed that the mouse is susceptible to infection when the fungus is inhaled under simulated natural conditions.

There are some instances in which other tissues are involved in the primary inoculation of the organisms (Freeman, 1930; Freeman and Weidman, 1923; Wade and Stevenson, 1941). Skin or subcutaneous inoculation produces only a dermal type of lesion (Levine, Zimmerman, and Scorza, 1957). However, the majority of clinical evidence supports the concept that the respiratory tract is a primary portal of entry for C. neoformans.

Cryptococcus neoformans in Association with the Pigeon and Canary

C. neoformans is reported to be found frequently in close association with pigeon and canary excreta. Emmons (1955) in a study conducted in Loudoun County, Virginia, isolated C. neoformans from 8 of 11 specimens which were essentially old, weathered pigeon nests and droppings taken from the attic of a partially demolished house. Later (1960) Emmons

again showed the relationship of pigeon manure to the presence of C. neoformans in specimens collected in or near Washington, D. C. Kao and Schwarz (1957), utilizing an animal inoculation technique, recovered the fungus from 41 out of 107 specimens collected from pigeon nests. From 9 premises in the locality of Cincinnati, Ohio, Ajello (1958) recovered the fungus from 14 of 1,127 soil samples. Littman and Schneirson (1959) isolated the fungus from 72 out of 201 specimens collected from indoor and outdoor locations in the 5 boroughs of the city of New York. They also indicated that excreta collected from indoor pigeon coops were more heavily and more frequently infected with C. neoformans than those obtained from coops in outdoor sites. Hasenclever and Emmons (1963) isolated C. neoformans from pigeon droppings and cereal waste from Baltimore and Hagerstown, Maryland, and from Charlottesville and Norfolk, Virginia. The comparison between the C. neoformans isolated from patient and soil isolates showed that isolates from cases of cryptococcosis were more virulent than the soil strains. Muchmore, Rhoades, Nix, Felton, and Carpenter (1963) reported 3 cases of cryptococcal meningitis in Kingfisher, Oklahoma. They also were able to isolate C. neoformans from bird droppings and other organic materials in the immediate environments of these three patients.

Cases of cryptococcosis and isolation of C. neoformans from pigeon excreta also have been reported from world areas other than the United States. Yamamoto, Ishida, and Soto (1957) isolated the fungus from a cat and pigeon droppings in Japan. Silva and Paula (1963) recovered it from droppings and nestings of pigeons at Salvador, Brazil. Despite the frequent association of C. neoformans with pigeon droppings, Emmons failed

to isolate the organism from spleen, liver, kidney or digestive tract of any of twenty young pigeons. Staib (1961, 1962, 1963) isolated C. neoformans from canary and other bird manure specimens collected from zoological gardens and pet shops. After feeding C. neoformans to a canary, he recovered the fungus from the feces but found no indication of infection to the intestinal tract of the bird.

The failure to isolate C. neoformans from the digestive tract of pigeons and canaries suggests that the birds' manure merely provides a medium in which the fungus grows freely. This view was supported by Staib (1962) in assimilation tests. He also found that a powdered sterile suspension of canary and pigeon manure were superior to Sabouraud's dextrose broth.

Effect of Temperature and pH on Growth of Cryptococcus neoformans

There are no reports available at present which show the optimum temperatures and pH for the growth of C. neoformans in soil. Kuhn (1939, 1949) studied the growth of C. hominis at mouse and rabbit body temperature. He suggested that the higher body temperature of rabbits 39°-40°C (102°-104°F) was unfavorable for the multiplication of the organisms, whereas the lower body temperature of the mouse 37.2°C (99.1°F) was favorable for its growth.

In support of this hypothesis, Kuhn reported that when cultured in vitro at 39.4°C (104°F), C. neoformans were killed slowly, but at 40.6°C (105°F) no viable cells remained after 6 days incubation. Later, Kligman, Crane, and Norris (1951) confirmed the previous reports that at 39°C multiplication is inhibited; 40°-42°C is lethal both in vivo and in vitro; and the organisms were completely destroyed when incubated at 40°C

for 8 days. Staib (1963) studied the resistance of C. neoformans to desiccation and high temperature. He reported that C. neoformans isolated from canary droppings remained viable for 480 days in dry river sand and garden soil; for 420 days in dry canary droppings; and 240 days in dry bird manure other than canary. When damp river sand seeded with C. neoformans and stored for one and one-half years was heated to 50°C for 24 hours, not one cell remained viable. Organisms kept in dry sand survived and growth occurred even when subjected to a temperature of 80°C for 24 hours.

Mosberg and Choudens (1951) reported the effect of changes in pH and temperature on the growth of C. neoformans. This study was done on culture medium (Sabouraud's dextrose agar) ranging in pH from 5.4 to 8.7. They found that no appreciable growth was obtained when the pH of this medium was alkaline (pH 7.3 to 8.7) but when the medium was slightly acid (pH 6.5) good growth occurred. However, their study did not include the effects on C. neoformans of changes in the pH of soils.

Relationship of Encapsulation of Cryptococcus neoformans to Virulence

The literature contains many references pertaining to the effect of encapsulation on virulence of bacterial species; however, studies of this nature on the only encapsulated yeast (C. neoformans) are limited. Drouhet, Segretin, and Aubert (1950) noted that an encapsulated strain of C. neoformans, isolated from a patient, was more virulent for mice than the thinly encapsulated parent strain. In contrast, Kao and Schwarz (1957) failed to observe a correlation between capsule thickness of C. neoformans and strains recovered from pigeon nests and virulence in white mice. Gadebusch (1958) observed that a variant strain of C. neoformans

which possessed a large capsule resisted phagocytosis by mouse polymorphonuclear leucocytes while thinly encapsulated yeast variants were readily ingested.

Evaluation of *Cryptococcus neoformans* and other Pathogenic Fungi

C. neoformans and most other potential pathogenic fungi have been isolated from the soil. The most common method of propagation of fungi is by the production and dispersal of highly resistant asexual spores. These reproductive structures are capable of withstanding relatively extreme variations in temperature, humidity and weather conditions. Spores can remain viable for a long time even in dried state. Another remarkable capacity of most pathogenic fungi is being dimorphic (mold-like when cultured at room temperature; yeast-like in tissues or when cultured on special media at 37°C) with the exception of C. neoformans, Candida albicans¹, and Geotricum spp.¹. Once a vegetative spore of pathogenic fungus enters a host, it germinates in the yeast phase and adapts itself to the new environment.

C. neoformans is a monomorphic (same morphology in cultures and tissues) encapsulated yeast which reproduces by budding. There is only one report concerning the ability of this fungus to withstand high temperature. Staib (1963) reported that C. neoformans isolated from bird manure remained viable in sun-dried sand for a period of one and one-half years.

The natural environmental factors and nutritional substances in soil play an important role in the geographical distribution of fungi. Aspergillus fumigatus (etiological agent aspergillosis) is abundant in

¹Candida and Geotricum spp., in certain environments, may form a rudimentary or pseudomycelium.

soil. Thom and Raper (1945) demonstrated its occurrence in soil or in decaying vegetation. Sporotrichum schenckii has been isolated from soil, wood, and plants by Beurmann and Gougerot (1908). This fungus is found in the north central part of the United States and Johannesburg, Africa. Nocardia asteroides was isolated by Gordon and Hagen (1936) and by Emmons (1951). This fungus is most prevalent in the tropical and subtropical areas. Emmons (1951) and Ajello and Zeidberg (1951) isolated Allescheria boydii from soil enriched with decaying organic substances. It has been frequently isolated in soil in eastern, central and northern United States and Canada. Histoplasma capsulatum was isolated from the soil by Emmons (1949) and Ajello and Zeidberg (1951). This fungus is more frequently recovered from river valleys than from desert areas. Isolation has been reported from three major river valleys of South America and Africa. Burma, India, Thailand and Indonesia are also included in its geographic distribution. Anderson (1958) reported its existence in the soil collected from urban areas and under trees where birds had roosted. Edwards, Ajello, Moore, Jacobs and Aronson (1960) isolated H. capsulatum from soils in and around Dalton, Georgia. The areas of this town from which they most frequently isolated this organism were shaded. Thousands of starlings frequented the trees in these sections of town. Emmons (1958, 1961) recovered H. capsulatum from soil around the foundation walls of a dwelling which sheltered a large colony of the common house bat, Eptesicus fuscus. The fungus was isolated with great regularity from samples of soil taken adjacent to the foundation wall. Beyond 9 feet from the wall the fungus could not be isolated from the soil. The studies on natural and experimental epidemiology of histoplasmosis by Larsh (1960) indicated that

H. capsulatum was more frequently isolated from soil samples near shady and humid areas in which the pH of the soil was 6.0 and 6.5. When the higher pH prevailed in similar areas, no organism could be isolated. This suggests that the growth of H. capsulatum is associated very closely with shady and humid soil (pH 6.0 - 6.5), enriched with bird droppings, particularly where there are significant aggregations of urban birds (pigeons, starlings, chickens) and bats.

Another highly infectious fungus, Coccidioides immitis was isolated from arid and semiarid climates, desert areas of Southern California (Smith and Baker, 1941), Arizona, New Mexico (Emmons, 1942), Northern Argentina and Paraguay (Nino and Urzua, 1950). Maddy (1957) studied the ecological factors relating to its geographical distribution and established its association with the so-called Lower Sonoran Life Zone. The Lower Sonoran Life Zone is characterized by low rainfall and hot summers; by characteristic plant association in which Palo Verde, certain cacti and creosote bush are prominent members; and by the occurrence of characteristic rodents. The saprophytism of the fungus enables it to flourish over vast areas of the southwestern United States. Friedman, Smith, Pappagianis, and Berman (1956) reported the effect of temperature and humidity on survival of C. immitis. They found that dry arthrospores survived well except when persistent low humidity was associated with persistent high temperature. Diurnal variations of both temperatures and humidity and the protective effect of upper layers of soil ensures the survival of the fungus from season to season. Smith et al. (1946) suggested that rainfall and winter were factors beneficial for survival and multiplication of C. immitis. There was a high proportion of

isolations from topsoil at the end of the spring rains as compared with no isolation at the end of the preceding hot summer. These qualities of the fungus show its adaptation for survival in its arid habitat and give some clues as to why the organism is so rare in nature other than southwest America (Smith et al., 1961). Other two pathogenic fungi with limited geographical distribution are Blastomyces brasiliensis (South American blastomyces) and Blastomyces dermatitides (North American blastomyces). B. brasiliensis is distributed predominantly in and around Brazil. Batista et al. (1962) isolated it from the soil and reported that the fungus occurs as a saprophyte on vegetation and in the soil. B. dermatitides distribution extends southward from Canada, Mexico, Central America, northern South America and in the Mississippi Valley. It is assumed that the fungus grows as a saprophyte in soil. All attempts (except one) to isolate it from soil have failed. For the first time, Denton (1961) isolated it from a tobacco-stripping barn in Lexington, Kentucky. The barn had been contaminated with exudates and secretions of a dog who died with blastomycosis. The pH value of the positive soil sample was 5.8. McDonough, Prooien and Lewis (1965) reported on the lysis of B. dermatitidis yeast-phase cells. This explains why this fungus has not, in general, been isolated from soil. Most probably this is due to brief survival of yeast-phase of the fungus in nature.

Preventive measures for all the pathogenic fungi are successful only when the ecological factors pertaining to their survival and growth have been established. Emmons and Piggott (1963) speculated that the eradication of Histoplasma capsulatum is possible by covering the infested area with 6 - 8 inches of clay and shale oil. Previously, the application

of pentachlorophenol in fuel oil proved to be successful in Maryland. This was discontinued due to its destructive properties to all vegetation. Complete dust control was shown to be very effective against Coccidioides immitis by Smith et al. (1946-1961). Grassing the potentially infected areas (lawns, parks, and play grounds, etc.) greatly reduced the incidence of disease.

Reports of Emmons and others regarding the association of C. neoformans with pigeons has recently stirred the public and many health officials in New York. It has been suggested that control measures might be achieved by exterminating all the pigeons. However, Schneidu (1946) opposed this idea because C. neoformans has never been found in the pigeons per se.

In view of the cited references, extensive information about the natural history of the fungi and detection of their habitats are necessary before one can understand the epidemiology of the diseases that they produce.

Although at present C. neoformans has been isolated from soil and certain birds' manure almost throughout the world, the effects of different soil, temperature, humidity, direct sunlight, darkness and animal or bird manure on the growth, survival, pathogenicity and size of capsule have yet to be elicited. The present study is an attempt to assess the effects of these factors on the preservation and proliferation of C. neoformans.

CHAPTER II

MATERIALS AND METHODS

Materials

Collection of Soil

Soil samples were collected in and around Kingfisher, Oklahoma, from: (a) a private dwelling at 322-1/2 West Broadway, designated hereafter as sample (No. 1); (b) the east-side entrance of the city park (No. 2); and (c) a freshly plowed field two miles east of Kingfisher (No. 3). Layers of soil 0.5 to 2 inches in depth were scraped by hand and placed into clean plastic bags of 300 gram capacity. All samples were stored in the laboratory at room temperature.

Cryptococcus neoformans Strain

The Emmons strain of C. neoformans, American type culture collection No. 11239 dated 2/25/64, originally isolated by Emmons from an environmental sample, was supplied by Dr. Frances G. Felton, University of Oklahoma Medical Center, Oklahoma City, Oklahoma. The C. neoformans culture was stored at 6°C on mycophil slants (Baltimore Biological, Inc.).

Pigeon Manure

A sack of pigeon manure weighing 700 grams was obtained from Dr. Frances G. Felton, University of Oklahoma Medical Center, Oklahoma

City, Oklahoma. The manure had been collected from the Burris-Mills, Kingfisher, Oklahoma, on September 7, 1962.

Culture Dishes

For all experiments sterile, disposable, plastic petri dishes 15 x 100 mm were used.

Phosphate-Buffered Saline M/15¹

Stock solutions of sodium phosphate and potassium phosphate were prepared by dissolving the following quantities in 0.87% of saline solution and making each solution up to one liter.

Na_2HPO_4 anhydrous	9.47 g
KH_2PO_4	9.08 g

Sixty-one and one-tenth ml of Na_2HPO_4 and 38.9 ml KH_2PO_4 were mixed together. The pH value was checked and found to be pH 7.0.

Stock solutions and mixtures were kept in the refrigerator in Pyrex glass bottles.

Humidity Chambers

The humidity chambers were prepared, with some modification, as described by Ritter, Larsh, and Furcolow (1964). The humidity chambers consisted of one-quart, wide mouth Kraft mayonnaise jars with screw-cap lids.

Mice

Male white mice were obtained from Dr. A. J. Stanley, University of Oklahoma Medical Center. Male and female white, Webster and brown C₃H strain mice were obtained from Roscoe B. Jackson Memorial Lab, Bar

¹Hawk, Oser and Summerson. Practical Physiological Chemistry (New York: The Blakiston Co., Inc.) 13th ed. pg. 702, 1954.

Harbon, Maine. Mice weighed 15-20 grams each. The animals were housed in plastic cages and were given Rockland Mouse/Rat diet and water ad libitum.

Methods

Determination of the Presence of C. neoformans in Pigeon Manure

Determination of the presence or absence of Cryptococcus neoformans from the pigeon manure was made by a procedure described by Hasenclever and Emmons (1963). Aqueous sample suspensions of the pigeon manure were placed on plates of Sabouraud's dextrose agar containing 50 micrograms of chloromycetin per ml. Plates were incubated for 48-72 hours at 37°C. Any colonies having a creamy morphology were recultured. Subsequently, cells from the suspected colonies were examined microscopically in India ink mounts. In no instance could the presence of C. neoformans be demonstrated by the methods used.

Determination of the Presence of C. neoformans in Soil

One gram of each soil specimen was placed in an 18 x 150 mm test tube; 10 ml of sterile physiological saline was added and a sterile rubber stopper was inserted. The tubes were shaken vigorously by hand for about 5 minutes. The suspensions were allowed to settle briefly and 5 ml of each supernatant was pipetted into sterile tubes. Two plates of Sabouraud's dextrose agar and two plates of Sabouraud's dextrose agar containing 50 micrograms of chloromycetin were inoculated as described under Plating Method. One plate of each type of medium was incubated at room temperature (20°-24°C) and the other plates at 37°C for 24-36 hours.

Soil suspensions of 0.5 ml (containing 2 mg of streptomycin and

5 mg of penicillin per ml) were injected intraperitoneally into 5 adult laboratory white mice.

Preparation of Pigeon Manure Extract

Approximately 600 grams of manure was placed in a polyethylene carboy containing 15 liters of distilled water and agitated overnight by means of an electric stirrer. This suspension was then filtered twice through six-layers of cheese cloth. Three-quarters of the filtrate was collected in milk cartons and stored at -20°C for future use. The remaining quarter was centrifuged at $1,150 \times G$ for 30 minutes. The supernatant was sterilized by being passed through a Seitz filter apparatus. This sterilized filtrate, containing 4.6 milligrams of manure per ml, was designated as 100% pure extract. One per cent, 5%, 20%, 35%, and 50% extracts were prepared by diluting the stock extract with sterile distilled water. The sterility of the various extracts was checked by inoculating samples onto Difco Sabouraud's dextrose agar; corn meal agar; nutrient agar and Littman oxgal agar slants. No growth resulted on any of the media. The extracts were stored at refrigerator temperature (6°C) in screw-capped bottles.

Preparation of C. neoformans for Seeding

The inocula was prepared from a 24 hour culture grown at 37°C on Sabouraud's dextrose agar. A loopful of this culture was transferred to an Erlenmeyer flask containing 50 ml of sterile mycophil broth. The flask was incubated with continuous aeration at 37°C in a water bath for 18 hours. Subsequently, 10% of this suspension (5 ml) was inoculated into 200 ml sterilized mycophil broth and incubated for 24 hours, as

previously. This broth culture was centrifuged for 10 minutes at 1,290 x G. The packed cells were washed twice with sterile physiological saline and then resuspended in 200 ml of the salt solution. All cultures were tested for contamination with a gram stain and India ink preparations.

Counting Method

Total cell count. Total cell counts were made with a Spencer, "Bright-Line" hemacytometer, by counting the cells of C. neoformans in 5 central squares (0.04 sq mm each) and multiplying by 50,000 or by counting the yeast cells in all 25 squares and multiplying by 10,000. These figures then represented the number of cells per ml.

Viable cell count. Six serial 10-fold dilutions of the suspension were prepared in sterile physiological saline and plated on Sabouraud's dextrose agar by the spreader (described earlier). Colonies were counted with the aid of a Spencer, Quebec, darkfield model colony counter. The actual viable cells per ml were obtained from the dilution plate containing from 30-100 colonies.

Plating Method

Petri dishes containing Sabouraud's dextrose agar were inoculated with 0.1 ml quantities of diluted suspension of the soil specimens which were delivered slowly from a 1 ml serological pipette graduated in one-hundredth of a ml. The inocula were evenly distributed over the surface of the plates by means of a bent glass rod. Prior to each distribution the rods were immersed in 95% ethyl alcohol, flamed and cooled.

Soil Seeding with C. neoformans for Pilot Study

Four-gram sterile samples of each sterile soil specimen collected

from the three different sites were placed in twelve 16 x 100 mm test tubes. Morton stainless steel culture tube closures were placed on each tube. The tubes were autoclaved for an hour at a pressure of 15 psi for three consecutive days. After cooling, suspensions of a few of the soil samples were made and checked for sterility by culturing on nutrient and Sabouraud's dextrose agar. Gram stain and India ink preparations were also made. One-half ml of C. neoformans cells (approximately 10 million cells) was pipetted into each tube. Subsequently, 0.5 ml of sterilized M/15 phosphate-buffered saline was added. All tubes were incubated at 24°C for 14 days.

Soil Seeding with C. neoformans for Full Scale Study

The 600 tubes, each containing 4 grams of sterile soil sample (No. 1) were divided into five groups (120 each). To each tube in the first four groups, 0.5 ml pigeon manure extract (1%, 5%, 20%, and 35%, respectively) was added. In the fifth group (controls) sterile phosphate-buffered saline was substituted for the extract. The five groups of tubes were then further subdivided into sets of 12 each (Table 1). The tubes were seeded with 0.5 ml C. neoformans (11×10^6 organisms) with the aid of Kimax glass automatic burets, 25 ml capacity and graduated in one-tenths of ml (the use of burets was found to be more rapid and accurate). The tubes were incubated at different temperatures (6°C, 24°C, and 37°C), and under various atmospheric conditions such as sunlight, darkness, 100% relative humidity (artificially provided) and atmospheric humidity varying from 10-50%. Hundred percent relative humidity was provided by placing the sterile soil tubes into the humidity chambers containing about 150 ml distilled water previously sterilized for 15 minutes at pressures of 15 psi (Figures 1 and 2).

TABLE 1

EXPERIMENTAL DESIGN FOR FULL SCALE STUDIES ON SURVIVAL AND GROWTH OF CRYPTOCOCCUS NEOFORMANS

Temperature	Number of Tubes With and Without Manure Extract									
	Controls		1% Extract		5% Extract		20% Extract		35% Extract	
	Rel. Humidity 100%	Atm. Humidity	Rel. Humidity 100%	Atm. Humidity	Rel. Humidity 100%	Atm. Humidity	Rel. Humidity 100%	Atm. Humidity	Rel. Humidity 100%	Atm. Humidity
4 ^o -6 ^o C	12	12	12	12	12	12	12	12	12	12
20 ^o -24 ^o C	12	12	12	12	12	12	12	12	12	12
37 ^o C	12	12	12	12	12	12	12	12	12	12
Sunlight ¹	12	12	12	12	12	12	12	12	12	12
Darkness ²	12	12	12	12	12	12	12	12	12	12

100% relative humidity was provided by placing the tubes in the humidity jars.

¹Tubes were placed on the roof of our laboratory building in full sunlight (July-February).

²Darkness was maintained by keeping the tubes (on the roof) covered with a black bucket (July-February).

Figure 1.--Seeded soil tubes enclosed with "Morton stainless steel culture tube closures", and incubated in atmospheric humidity.

Figure 2.--Seeded soil tubes enclosed with "Morton stainless steel culture tube closures", and incubated in 100% humidity.

Adjustment of pH of Soils

Two hundred-gram samples of soil specimen No. 1 (pH 7.7) were placed in a Waring blender. About 200 ml of sterile distilled water was added to each and the mixture was blended thoroughly. One N HCl (approximately 20-30 ml) was added to the contents of each blender. At various intervals the pH of each was checked with a Beckman expanded scale pH meter. When the pH of one mixture reached approximately 4.0 and the other roughly 6.0, the suspensions were transferred to aluminum foil dishes and dried under a hood. The final pH values were found to be 6.0 and 7.2, respectively.

In studies on the effect of pH on survival and growth of C. neoformans, four grams of sterile soil of each pH were placed in tubes and seeded with 54×10^6 viable cells.

Study of Growth of C. neoformans under Various Conditions

At various intervals, the entire soil content of the tubes were poured into 50 ml Erlenmeyer flasks, containing 40 ml sterile physiological saline. The flasks were shaken vigorously until the soil was evenly suspended and were then allowed to stand for 5 minutes. Four 10-fold serial dilutions were made from each flask and 0.1 ml of each dilution was plated on Sabouraud's dextrose agar in duplicate. All suspensions were distributed on the medium with a glass rod. Colonies were counted on each plate and total viable cell counts were calculated according to the dilution.

Virulence Tests

The pathogenicity of C. neoformans was established by injecting

intraperitoneally 0.5 ml of seeded soil suspensions into laboratory male and female white mice weighing 15-20 grams each. When the animals died, small amounts of organs were smeared on microscopic slides and India ink mounts were made. Portions of liver, kidney, spleen, lung and brain of each of the animals were cultured on Sabouraud's dextrose agar and incubated at 37°C. Comparative studies on mouse resistance to cryptococcosis were done with brown C₃H strain and white Webster mice, 8 weeks old and weighing approximately 15-18 grams.

Production of Capsules of C. neoformans

In vitro. Using India ink mounts, the capsule thickness of C. neoformans grown in mycophil broth was measured and recorded as the initial thickness. Subsequently, soil samples were seeded with the organism. At 3 month intervals, suspensions of the seeded soil were prepared and plated on Sabouraud's dextrose agar. Capsule thickness was measured both of the organism in the soil suspension directly (before plating) and those growing on Sabouraud's dextrose agar plates for 48 hours.

In vivo. The seeded soil suspensions with C. neoformans, after one month, 3 months and 6 months intervals (without pigeon manure extract and incubated at 20°C-24°C in 25-30% relative humidity), were injected intraperitoneally into 10 adult, male, white mice, using a dosage of 125,000 viable cells for each mouse. Mice that were sick or died of cryptococcosis were necropsied and the capsule thickness of yeast in various organs was measured.

Capsule measurement technique. Slide preparations of C. neoformans grown in soil, on Sabouraud's dextrose agar and in vivo (mice) were made

with India ink. A small portion of the colony grown on Sabouraud's dextrose agar was lifted from the center with a sterilized needle. In the case of the organisms grown in soil for a period of 0-6 months, suspensions were made and a loopful of suspension was picked up with a sterile wire loop. In vivo samples of different organs of the autopsied mice were smeared onto slides, using sterile forceps. India ink was added and cover slips were applied and semipermanent mounts were made with the aid of paraffin and vaseline.

Under the high dry lens (43 x) of the microscope the diameter of cell (including capsule) was measured. Subsequently, the cell diameter (without capsule) was measured. The thickness of the capsule was determined by subtracting the cell diameter (without capsule) from the cell plus capsule and dividing by 2, as shown in formula:

$$t_c = \frac{D_1 - D_2}{2}$$

Encapsulated yeast cell

D_1 = Diameter of cell + capsule

D_2 = Diameter of the cell w/o capsule

t_c = Thickness of the capsule

Using a Porton Reticle ocular micrometer, measurements were made of each yeast cell as it came into view. Budding cells were avoided in these measurements to limit the discrepancies in measurement. Careful data were taken as to the size of the cell and thickness of the capsule.

Histopathological Studies

Mice showing symptoms of cryptococcosis, such as intracranial pressure resulting in the bulging of the skull at the occipital region, were sacrificed by ether anesthetic and the livers, spleens, kidneys,

and brains were immediately removed and fixed in 10% formalin. Tissue sections were prepared and stained with hematoxylin-eosin and periodic acid-Schiff stain.

CHAPTER III

RESULTS

Determination of the Presence of *C. neoformans* in Different Soil Specimens

Of the nine specimens of soil representing three different locations (in front of a dwelling at 322-1/2 West Broadway; from the east-side entrance of the city park; and from a freshly plowed field 2 miles east of town) one sample from each location was used to investigate the presence of *C. neoformans* naturally occurring in those soils. Cultures of three soil samples were made as described (in Materials and Methods). India ink wet-mount preparations of the colonies appearing on the media indicated that none of the organisms were *C. neoformans*. Colonies which appeared to be mucoid and yeast-like were subcultured and incubated at room temperature. In a few instances in wet-mounts of yeast-like colonies, true mycelium which fragmented into arthrospores was observed. These colonies were presumed to be *Geotrichum* spp. However, in India ink wet-mount preparations no encapsulated yeast was seen. Since the soil for the study undertaken was to be sterilized, no attempt was made to identify other organisms.

Determination of the Growth and Survival of *C. neoformans* in Different Soil Specimens

Each of the three seeded soil samples (described under Pilot Study in Materials and Methods) containing 10×10^6 organisms and incubated at

24°C for 14 days, were examined for growth and survival of C. neoformans. At the end of 14 days, one tube of each soil was transferred to a 50 ml Erlenmeyer flask containing 40 ml sterile saline solution. Four 10-fold dilutions were prepared and plated, as described under Plating Method (in Materials and Methods). All plates were incubated at 37°C for 36-48 hours. On gross examination at the end of 36 hours, the resulting colonies were yeast-like and varied from moist to mucoid to waxlike; the color of these colonies was light and cream-like initially but changed to yellow as the culture aged. Following an incubation period of 36 hours, the colonies could be counted in certain of the dilution plates (Figures 3-6); these data were converted to the number of viable cells per tube. The results (Table 2) after 14 days incubation show that in soil No. 1 (in front of a dwelling) the number of viable cells increased from an initial 10×10^6 cells to 10.8×10^6 . In soil No. 2 (City Park) the viability dropped to 9.4×10^6 and in soil No. 3 (from a field) to 8.4×10^6 cells. Since the number of cells was higher in the case of soil No. 1 than in either No. 2 or No. 3, No. 1 was selected for further studies. The pH values of these soils was found to be pH 7.7 for soil No. 1, 7.4 for soil No. 2, and 6.8 for soil No. 3. Wet-mount preparations in India ink from colonies grown on Sabouraud's dextrose agar revealed C. neoformans cells that were approximately 6 to 9 μ in diameter, with distinct cell walls but relatively narrow capsules 2 to 3 μ in thickness. The soil suspension preparation showed yeast cells 6 to 8 μ in diameter which had a capsule of about 2.0 μ in thickness. Representative of each soil suspension containing 125,000 viable cells were injected intraperitoneally into 5 adult mice. At the end of 4 - 6 weeks all animals died. On autopsy, cells of C. neoformans were present in lungs, kidneys, livers, spleens and brains of the dead animals.

Figure 3.--Growth of C. neoformans on Sabouraud's dextrose agar. The method for isolating the colonies involved the use of a bent glass rod. Colonies are too numerous to count.

Figure 4.--Growth of C. neoformans on Sabouraud's dextrose agar. The method for isolating the colonies involved the use of a bent glass rod. Serial dilution 10^2 giving rise to 120-130 colonies.

Figure 5.--Growth of C. neoformans on Sabouraud's dextrose agar. The method for isolating the colonies involved the use of a bent glass rod. Note the isolation of colonies. Serial dilution 10^3 giving rise to 12 colonies.

Figure 6.--Growth of C. neoformans on Sabouraud's dextrose agar. The method for isolating the colonies involved the use of a bent glass rod. Serial dilution 10^4 giving rise to 1 colony.

TABLE 2

COMPARATIVE GROWTH AND SURVIVAL OF CRYPTOCOCCUS NEOFORMANS IN SOIL SPECIMENS FROM THREE DIFFERENT LOCATIONS (PILOT STUDY)

Soil Specimens	Incubation Period in Days	Plate Count Per 0.5 ml of Culture		Average	Total Viable Count
		Duplicate Count			40,000 ¹
	0				10.0 x 10 ⁶
1	14	290	250	270	10.8 x 10 ⁶
2	14	255	215	235	9.4 x 10 ⁶
3	14	227	193	210	8.4 x 10 ⁶

Soil specimens were collected in and around Kingfisher, Oklahoma. Soil No. 1 was obtained in front of a dwelling at 322 1/2 West Broadway; Soil No. 2 from the east side entrance of the City Park; and No. 3 from freshly ploughed field 2 miles east of town.

¹Dilution factor 1:40,000

Initial inoculum in soil 10 x 10⁶ cells/0.5 ml/tube.

Determination of the Effects of Various Conditions on Growth
and Survival of *C. neoformans* in a Full Scale Study

Effect of Different Temperatures

Every 30 days the number of viable cells of *C. neoformans* was determined in representative tubes that had been incubated at 4^o-6^oC, 20^o-24^oC, and 37^oC. These data (Table 3) indicate that when *C. neoformans* was incubated at 4^o-6^oC in 35-40% relative humidity, there was an increase in the number of cells from an initial 11×10^6 to 35.6×10^6 by the end of the first month. The number of cells at the end of 2nd, 3rd, and 4th months, though still well above the initial number, had dropped to 32.0, 31.6, and 28.0×10^6 , respectively. At the 5th month there was a rapid decrease in the number of cells with a further decrease to 4.8×10^6 by the end of the 6th month. However, at room temperature (20^o-24^oC) in 25-30% relative humidity there was an increase in number of cells only during the first two months which was followed by a marked decrease during the next four months. At 37^oC in 20-25% relative humidity, the number of cells dropped from an initial 11×10^6 to 4.1×10^5 at the end of the first month. By the end of six months the number of cells was only 1.8×10^3 , a decrease of approximately 99.6%. These results indicate that the cells of *C. neoformans* survive longer when stored in soil at 4^o-6^oC as opposed to 20^o-24^oC or 37^oC incubation. Of the latter two incubation temperatures, 37^oC is by far the most detrimental to the survival of this organism (Figure 7).

Effect of Different Relative Humidities

The combined effects of temperatures and relative humidities are illustrated by the data in Table 4. It may be seen that at the end of 6 months incubation the *C. neoformans* cells seeded in soil and held at 4^o-

Figure 7.--The survival and growth of *C. neoformans* seeded in soil incubated at constant but different temperatures.

TABLE 3

THE SURVIVAL AND GROWTH OF CRYPTOCOCCUS NEOFORMANS SEEDED IN SOIL
INCUBATED AT CONSTANT BUT DIFFERENT TEMPERATURES

Temperature	Viable Count Per 0.5 ml Cells/Tube After Incubation						
	0	One Month	Two Months	Three Months	Four Months	Five Months	Six Months
4°-6° C	11 x 10 ⁶	35.6 x 10 ⁶	32.0 x 10 ⁶	31.6 x 10 ⁶	28.0 x 10 ⁶	10.0 x 10 ⁶	4.8 x 10 ⁶
20°-24° C ¹	11 x 10 ⁶	34.5 x 10 ⁶	22.8 x 10 ⁶	3.4 x 10 ⁶	1.2 x 10 ⁶	11.8 x 10 ⁵	8.8 x 10 ⁵
37° C	11 x 10 ⁶	4.1 x 10 ⁵	3.5 x 10 ⁴	2.1 x 10 ⁴	1.3 x 10 ⁴	2.9 x 10 ³	1.8 x 10 ³

Only atmospheric relative humidities were provided (described in Table 4).

¹20°-24° C was room temperature.

TABLE 4

THE EFFECT OF DIFFERENT RELATIVE HUMIDITIES AND TEMPERATURE ON SURVIVAL AND GROWTH OF
CRYPTOCOCCUS NEOFORMANS

Incubation Period in Months	<u>Temperature 4°-6° C</u>		<u>Temperature 20°-24° C</u>		<u>Temperature 37° C</u>	
	Atmospheric Humidity 35-40%	Artificially Added Humidity 100%	Relative Humidity 25-30%	100% Humidity	Relative Humidity 25-30%	100% Humidity
	<u>x10⁶</u>	<u>x10⁶</u>	<u>x10⁶</u>	<u>x10⁶</u>		
0	11	11	11	11	11.0 x 10 ⁶	11.0 x 10 ⁶
1	35	38	34	42	4.1 x 10 ⁵	8.2 x 10 ⁵
2	32	33	22	52	3.5 x 10 ⁴	2.0 x 10 ⁵
3	31	33	3	39	2.1 x 10 ⁴	6.8 x 10 ⁴
4	28	30	2	36	1.3 x 10 ⁴	4.5 x 10 ⁴
5	10	21	1	32	2.9 x 10 ³	7.8 x 10 ³
6	4	18	0.8	30	1.8 x 10 ³	4.5 x 10 ³

100% relative humidity was provided by setting tubes in the humidity jars. Relative humidity at 4°-6° C was 35-40%; at room temperature (20°-24° C) was 25-30%, and at 37° C was 20-25%. There was a great decrease in number of cells at 37° C regardless of the 100% relative humidity. Initial inoculum was 11 x 10⁶ per 0.5 ml/tube.

6°C in 100% relative humidity demonstrated an increase in the number of cells from an initial 11.0×10^6 to 18.0×10^6 cells. Whereas, those incubated at 20°C-24°C in 100% relative humidity increased to 30.0×10^6 . These values represent approximately increases of 65% and 270%, respectively. However, there was roughly a 60% decrease in the number of cells at 4°C-6°C in 35-40% relative humidity by the end of 6 months. There was an even greater decrease (approximately 92%) when incubation was carried out at room temperature (20°C-24°C) and held in 25-30% relative humidity. At 37°C there was a rapid decrease in the number of cells incubated in both 20-25% and 100% relative humidities. The number of cells decreased from an initial 11.0×10^8 to 1.8×10^3 and 4.5×10^3 , respectively, by the end of 6 months. It will be seen that at lower temperatures (4°C-6°C) the humidity has less effect on the growth and survival of C. neoformans. At room temperature (20°C-24°C), the growth and survival of the fungus requires 100% relative humidity. At 37°C, there was a relative rapid loss of viability by the end of 6 months regardless of the relative humidity.

Effect of Freezing

When the suspension of C. neoformans was prepared for seeding into soil, about 10 ml of this suspension previously cultured in mycophil broth was stored at -15°C. Viable cell counts made 24 hours after freezing and thawing showed a drop in number of cells from 290×10^7 to 220×10^7 per ml (about a 24% decrease). In the case of a sample which was frozen for 8 months, the number of cells dropped to 116×10^7 (roughly 63% decrease). In a separate experiment, approximately 10.0×10^6 cells per ml of C. neoformans were suspended in phosphate-buffered saline (M/15, pH 7.0). This suspension was frozen at -15°C and after 6 months storage it was

Figure 8.--Effect of pigeon manure extract on growth of C. neoformans seeded in soil incubated at 20°-24°C in 100% relative humidity.

TABLE 5

INFLUENCE OF PIGEON MANURE EXTRACT ON SURVIVAL AND GROWTH
OF CRYPTOCOCCUS NEOFORMANS¹

Incubation Time in Months	<u>Pigeon Manure Extract</u>				
	<u>No Extract</u>	<u>1%</u>	<u>5%</u>	<u>20%</u>	<u>35%</u>
	Viable Cell Count Per 0.5 ml/Tube (X10 ⁶)				
0	11	11	11	11	11
1	42	46	51	55	58
2	52	58	63	65	67
3	39	44	46	52	58
4	36	39	42	47	55
5	32	38	39	40	50
6	30	33	34	37	40

¹Initial inoculum was 11×10^6 cells per 0.5 ml/tube and incubated at room temperature (20° - 24° C) in 100% relative humidity.

thawed out and viable counts were made. No decrease in viability was noted. Apparently, the phosphate-buffered saline greatly contributed to the maintenance of viability of cells of C. neoformans when stored under the described conditions.

Effect of Pigeon Manure Extract

Emmons (1955, 1960), Staib (1962), and others, have established the association of C. neoformans with manure of pigeons and other birds. Data obtained from experiments with varying concentrations of pigeon manure are shown in Figure 8 (data from Table 5). The number of viable cells of control soil (0.5 ml of C. neoformans suspension and 0.5 ml saline solution) incubated at room temperature for 6 months in 100% relative humidity increased from an initial 11×10^6 cells to 42.0×10^6 after one month incubation and by the end of six months the viability had dropped to 30×10^6 (2.7-fold increase over initial inoculum). In seeded soil samples containing 0.5 ml of varying concentrations of pigeon manure extract (1%, 5%, 20%, and 35%) the number of cells increased exponentially with increasing concentrations of the extract. By the end of 6 months, there was a gradual decrease for all concentrations. These data support previous reports that birds' manure provides an enrichment medium in nature for the growth of C. neoformans.

Effect of Variation in pH of Soil

The results of growth and survival of C. neoformans in three soils (pH 6.0, pH 7.2, and pH 7.7) are shown in Table 6 and Figure 9. pH values of the soil samples were adjusted as described in Materials and Methods and inoculated with 54×10^6 (5.4×10^7) C. neoformans cells. The tubes

TABLE 6

COMPARISON OF THE SURVIVAL AND GROWTH OF CRYPTOCOCCUS NEOFORMANS
IN SOIL SAMPLES OF DIFFERENT pH VALUES

Incubation Time in Months	Relative Humidity %	<u>pH Values of Soil Samples</u>		
		pH 6.0	pH 7.2	pH 7.7
		Viable Cells Per 0.5 ml/tube ($\times 10^6$)		
0		54	54	54
1	100	32	90	114
	25-30	2	33	38
2	100	4	94	116
	25-30	None	1	14
3	100	3	120	136
	25-30	None	1	29

Initial inoculum was 54×10^6 viable cells, incubated at room temperature and 25-30% and 100% relative humidity.

Figure 9.--Comparison of the survival and growth of *C. neoformans* in soil samples of different pH incubated at 20°-24°C in 100% relative humidity.

were maintained at room temperature (20° - 24° C) in 25-30% and 100% relative humidity. Viable cell counts were made monthly for 3 months. By the end of the first month, when the organisms were maintained in soil at pH 6.0 in 100% relative humidity, the number of cells dropped from an initial 54×10^6 to 32×10^6 and by the end of the third month to 3×10^6 . However, when only 25-30% relative humidity was provided, the number of cells dropped much more rapidly; from 54×10^6 to 2.0×10^6 by the end of the first month; and by the end of the second month no viable organisms remained. On the other hand, at pH 7.2 in 100% relative humidity, the number of cells increased at monthly intervals from an initial 54×10^6 to 90×10^6 , 94×10^6 and 120×10^6 , respectively. At pH 7.7 in 100% relative humidity the number of viable cells increased at monthly intervals from an initial of 54×10^6 to 114×10^6 , 116×10^6 and 136×10^6 , respectively. After three months incubation the number of cells decreased (approximately 50%) at pH 7.7 in 25-30% relative humidity, while at pH 7.2 only a very limited number of cells survived (1×10^6 cells).

It is clear that the growth and survival of C. neoformans varied with the pH of the soil; in more acid soil fewer organisms survived and conversely, more organisms survived in soil with a higher pH.

Summer and Winter Conditions in Oklahoma on Growth and Survival of C. neoformans

Sunlight and Darkness During Summer

This investigation was started in July 1964 and continued until February 1965. The tubes of soil were seeded with 11×10^6 C. neoformans cells and allowed to set out on the roof of the laboratory building. One batch of tubes was incubated under direct sunlight in 100% relative humidity

and the second batch of tubes was incubated also in direct sunlight but in atmospheric humidity approximately 10-20% during the summer and 40-50% during the winter. A third batch of tubes was incubated under the same conditions covered with a black bucket. The number of viable cells per tube was ascertained after an initial incubation of 15 days, and thereafter at monthly intervals for 6 months. Results for the first 30 days are shown in Figure 10. By the end of 15 days, the organisms which were subjected to 100% relative humidity in sunlight showed a rapid drop in number of cells from an initial 11×10^6 to 6×10^3 , and by the end of 30 days no viable cells remained. However, in the case of the organisms incubated only in atmospheric humidity (10-20% in sunlight) the number of viable cells dropped from an initial 11×10^6 to 3×10^6 (73% decrease) and by the end of 6 months decreased to 6.9×10^3 (Table 7). It will be seen (Figure 10) that during the period from July 24 to August 24 a high temperature $102-106^{\circ}\text{F}$ ¹ ($40^{\circ}-41.5^{\circ}\text{C}$) prevailed for 12 days.

The number of viable cells incubated in the dark in 100% relative humidity dropped from an initial 11×10^6 to 2.5×10^6 by the end of 15 days. However, in atmospheric humidity (10-20%) the number of cells dropped only to 3.7×10^6 . These figures represent a decrease of roughly 75% and 65%, respectively. By the end of 6 months in 100% relative humidity, the viable count dropped to 2.6×10^5 , and in case of 10-20% humidity the number of cells dropped to 1.4×10^5 . These results suggest that during the summer in Oklahoma C. neoformans cells kept in darkness survived longer than those incubated in the direct sunlight.

¹Figures of temperatures (highs and lows) were obtained from the Weather Bureau, Oklahoma City, Oklahoma.

Figure 10.--Effect of sunlight and humidities during summer on growth and survival of C. neoformans. (July - August, 1964)

TABLE 7
EFFECT OF SUNLIGHT AND DARKNESS ON SURVIVAL AND GROWTH
OF CRYPTOCOCCUS NEOFORMANS IN SUMMER CONDITIONS
(JULY-NOVEMBER)

Incubation Time	Relative Humidity % ¹	Viable Cell Count/0.5 ml/Tube	
		Direct Sunlight ²	In Dark ³
0 Days		11.0×10^6	11.0×10^6
15 Days	100	6.0×10^3	2.5×10^6
	10-20	3.0×10^6	3.7×10^6
1 Month	100	None	1.9×10^6
	10-20	1.4×10^5	2.3×10^6
2 Months	100	None	1.6×10^6
	10-20	4.4×10^4	4.8×10^5
3 Months	100	None	1.5×10^6
	10-20	6.5×10^3	3.5×10^3
4 Months	100	None	1.4×10^6
	10-20	7.8×10^3	3.3×10^5
5 Months	100	None	12.0×10^5
	40-50	5.3×10^2	2.2×10^5
6 Months	100	None	2.6×10^5
	40-50	6.9×10^3	1.4×10^5

Initial inoculum was 11×10^6 viable cells.

¹Atmospheric humidity was not controlled (average 10-20 during summer and 40-50 during winter).

²Direct sunlight was provided by setting the tubes on the roof of the laboratory building.

³Darkness was maintained by keeping the tubes covered with a black bucket.

Sunlight and Darkness During Winter

The tubes containing soil were seeded with 5.4×10^7 (54×10^6) C. neoformans cells and kept on the roof of the laboratory building in direct sunlight during the months of November 1964 to February 1965. The results are shown in Table 8. The organisms which were incubated in 100% relative humidity showed an increase in the number of viable cells from an initial 5.4×10^7 to 10.0×10^7 by the end of the first month, and 14.0×10^7 a month later. At the end of 3 months, the viability had decreased slightly to 12.0×10^7 (still higher than the initial). However, in 40-50% relative humidity the number of viable cells increased from an initial 5.4×10^7 to 7×10^7 at the end of 30 days, and decreased to 2.0×10^7 at the end of 90 days. It is shown in Figure 11 that during the month of November the low temperatures varied from 35° - 40° F while the highs were roughly 56° - 58° F. In December, the low ranged 30° - 35° and high 42° - 46° F. During the month of January, the low 25° - 30° F and high 37° - 42° F. Table 8 shows that during the month of November the number of viable cells in 100% relative humidity doubled that of the initial; there was a 3-fold increase in December that had decreased to 2.5-fold by the end of January (still well above the initial). These data indicate that the growth and survival of the fungus was better under the winter conditions than of the summer.

Cryptococcus neoformans Capsule in Relation to Virulence

The results of the measurement of 1,500 cells of C. neoformans grown in soil, on Sabouraud's dextrose agar and in vivo (mice) are shown (Tables 9, 10, and 11). It is seen (Table 9) that as the incubation period of seeded soil is prolonged, the size of the cell (cell diameter + capsule) and thickness of capsule (cell diameter + capsule - cell diameter) is

Figure 11.--Effect of winter on growth and survival of C. neoformans. (November 1964 - February 1965)

TABLE 8
EFFECT OF SUNLIGHT AND DARKNESS ON SURVIVAL AND GROWTH
OF CRYPTOCOCCUS NEOFORMANS IN WINTER CONDITIONS
(NOVEMBER-FEBRUARY)

Incubation Period in Months	Relative Humidity %	Viable Cells ¹ Per 0.5 ml (X10 ⁷)
0		5.4
1	100 40-50	10.0 7.0
2	100 40-50	14.0 5.0
3	100 40-50	12.0 2.0

Initial inoculum was 5.4×10^7 cells/0.5 ml/tube.

Experiment was carried out from the month of November 1964 to February 1965.

Atmospheric relative humidity and temperature data were obtained from the weather bureau, Oklahoma City, Oklahoma.

¹Approximately the same results were obtained when incubated in the dark.

TABLE 9

MEASUREMENT OF SIZE OF CELLS AND THICKNESS OF CAPSULES OF CRYPTOCOCCUS NEOFORMANS IN SOIL SUSPENSIONS. SEEDED SOIL WAS INCUBATED AT ROOM TEMPERATURE IN 25-30% RELATIVE HUMIDITY.

Incubation Period in Months	Number of Viable Cells Measured	Size of Cells in Microns (μ)		Thickness of Capsules in Microns (μ) $t_c = \frac{D_1 - D_2}{2}$
		Diameter + Capsule (D_1)	Diameter w/o Capsule (D_2)	
0		8.4	3.0	2.7
1	139	8.4	5.9	1.2
	6	5.9	4.2	0.9
	5	4.2	3.0	0.6
Total $\bar{x}$	150 (Mean)	<u>8.1</u>	<u>5.7</u>	<u>1.1</u>
3	128	5.9	4.2	0.9
	12	4.2	3.0	0.6
	10	8.4	5.9	1.2
Total $\bar{x}$	150	<u>5.9</u>	<u>4.2</u>	<u>0.9</u>
6	135	4.2	3.0	0.6
	13	5.9	4.2	0.9
	2	8.4	5.9	1.2
Total $\bar{x}$	150	<u>4.4</u>	<u>3.1</u>	<u>0.63</u>

Measurements were made with Porton Reticle Ocular Micrometer.

reduced. By the end of the first month, the thickness of capsule reduced from an initial 2.7 microns to 1.1 microns in thickness (approximately 55% reduction) and by the end of 3 months it decreased to 0.9 microns and after 6 months incubation reduced to 0.63 microns in thickness (77% reduction). The same effect is observed in size of the cell. The size is reduced from an initial 8.4 microns to 8.1, 5.9 and 4.4 microns, respectively. However, when the soil suspensions were plated directly on the Sabouraud's dextrose agar and incubated at 37°C for 36 hours, the measurement of the size of the cell and thickness of capsule did not vary appreciably from the initial size and thickness (Table 10). When the suspensions of the soil after one month, 3 months and 6 months intervals were injected into mice, the narrowly encapsulated cells (capsules varying from 0.9 to 0.63 microns in thickness) developed into thick ones (Table 11). Variations in capsule thickness in vitro and in vivo are shown in Figures 12-17.

It was interesting to observe that when mice were inoculated with seeded soil suspensions at 3 month intervals, the virulence of C. neoformans decreased as the seeded soil incubation increased. Suspensions of soil seeded with C. neoformans that had been incubated at room temperature (20°-24°C) for 1-6 months period were prepared at monthly intervals (1 month, 3 months and 6 months). The suspensions were diluted to contain approximately 125,000 cells and then injected intraperitoneally into adult white mice in groups of ten. The control mice were injected with sterile soil suspension. All mice were observed for 10 weeks. It is shown (Table 12) that the mice injected with soil suspension at the end of 1 month incubation died in 4-6 weeks (100% total mortality). Seventy percent of mice died that were injected with samples incubated for 3 months. Whereas only

TABLE 10

MEASUREMENT OF SIZE OF CELLS AND THICKNESS OF CAPSULES OF CRYPTOCOCCUS NEOFORMANS GROWN
ON SABOURAUD'S DEXTROSE AGAR

Incubation Period in Months	Number of Viable Cells Measured	Size of Cells in Microns (μ)		Thickness of Capsules in Microns (μ) $t_c = \frac{D_1 - D_2}{2}$
		Diameter + Capsule (D_1)	Diameter w/o Capsule (D_2)	
0	145	18.5	3.0	2.8
(before seeding the soil)	5	8.4	4.2	2.1
	Total 150			
	$\bar{x}$ (Mean)	<u>8.5</u>	<u>3.0</u>	<u>2.70</u>
	140	8.4	3.0	2.7
1	5	8.4	4.2	2.1
	5	5.9	2.1	1.9
	Total 150			
	$\bar{x}$	<u>8.3</u>	<u>3.0</u>	<u>2.65</u>
	127	8.4	3.0	2.70
3	4	8.4	4.2	2.1
	19	5.9	2.1	1.9
	Total 150			
	$\bar{x}$	<u>8.1</u>	<u>2.9</u>	<u>2.58</u>
	116	8.4	3.0	2.7
6	11	8.4	4.2	2.1
	23	5.9	2.1	1.9
	Total 150			
	$\bar{x}$	<u>8.1</u>	<u>3.5</u>	<u>2.53</u>

Measurements were made with Porton Reticle Ocular Micrometer.

TABLE 11

MEASUREMENT OF SIZE OF CELLS AND THICKNESS OF CAPSULES OF CRYPTOCOCCUS NEOFORMANS IN VIVO (MICE)¹.

Incubation Period in Months	Number of Viable Cells Measured	Size in Cells of Microns (μ)		Thickness of Capsules in Microns (μ) $t_c = \frac{D_1 - D_2}{2}$
		Diameter + Capsule (D_1)	Diameter w/o Capsule (D_2)	
0	on SAB ²	8.4	3.0	2.7
1	99	23.7	11.8	5.9
	45	16.8	8.4	4.2
	6	11.8	4.2	3.8
	Total 150 $\bar{x}$ (Mean)	<u>21.1</u>	<u>10.5</u>	<u>5.3</u>
3	95	23.7	11.8	5.9
	48	16.8	8.4	4.2
	7	11.7	4.2	3.8
	Total 150 $\bar{x}$	<u>20.9</u>	<u>10.3</u>	<u>5.2</u>
6	91	23.7	11.8	5.9
	43	16.8	8.4	4.2
	16	11.7	4.2	3.8
	Total 150 $\bar{x}$	<u>20.4</u>	<u>10.1</u>	<u>5.2</u>

¹The mice were periodically injected intraperitoneally with 0.5 ml (125,000 cells) of seeded soil suspensions of Cryptococcus neoformans. Measurements were made with Porton Reticle Ocular Micrometer.

²Sabouraud's dextrose agar.

Figure 12.--Wet-mount preparation in India ink of C. neoformans grown on Sabouraud's dextrose agar. The cells show a variation in size and thickness of the capsules. X 450.

Figure 13.--Wet-mount preparation in India ink of C. neoformans in soil at the end of two months incubation. The capsules are relatively narrow. X 450.

Figure 14.--Section of a formalin-fixed mouse spleen experimentally infected with C. neoformans. In this section there are numerous cryptococci with maximal cellular response. Great variation in capsule size is seen. PAS stain, X 205.

Figure 15.--Section of a formalin-fixed mouse kidney experimentally infected with C. neoformans. The fungus cells are entrapped in glomerular capillaries. Relatively scant inflammatory response is evoked. PAS stain, X 480.

Figure 16.--Section of a formalin-fixed mouse lung experimentally infected with C. neoformans. The alveoli are filled with the encapsulated yeast cells. Capsules are quite distinct. H & E stain, X 640.

Figure 17.--Section of a formalin-fixed mouse brain experimentally infected with C. neoformans. Cryptococcus cells and their capsules are demonstrated very well. H & E, X 205.

TABLE 12

CAPSULE THICKNESS OF CRYPTOCOCCUS NEOFORMANS CELLS IN RELATION TO VIRULENCE

Period of Incubation of Cells in Months	Average Capsule Thickness in Microns (μ)			No. of Mice	Percentage Mortality	Average No. of Weeks Required to Cause Death
	In Soil	In SAB	In Vivo			
0	-	2.7	-	-	-	-
1	1.10	2.6	5.3	10	100	4-6
3	0.90	2.1	5.2	10	70	10
6	0.63	2.1	5.2	10	40	10

The mice were injected intraperitoneally with 0.5 ml soil suspensions containing 125,000 Cryptococcus neoformans cells. The seeded soil was incubated at room temperature (20°-24° C) in 25-30% relative humidity.

40% mortality was observed in the group of mice injected with soil suspension incubated for 6 months. All control mice survived. Drouhet and Segretin (1950), Kao and Schwarz (1957), Gadebush (1958), Littman and Schneirson (1959), and Littman and Tsubura (1959) did not agree with each other with regard to correlation between thickness of the C. neoformans capsule and virulence of the organism for mice.

However, the results of the experiment conducted in our laboratory indicate that there might be correlation between capsule thickness and virulence. When the organisms' capsule was 2.6 microns in thickness, it produced 100% of total mortality in mice by 4-6 weeks in comparison with 40% total mortality after 10 weeks when on prolonged incubation capsule thickness had reduced to 0.63 micron.

Sex and Strain Resistance in Experimental Mice

A study was conducted to see whether host defenses vary between sex and strain of mice. Twenty white adult Webster strain and 20 brown C₃H strain, 10 male and 10 female of each strain, 8 weeks old and weighing 15-18 grams, were used during the experiment. Five hundred thousand viable C. neoformans cells of strain No. 11239 were injected intraperitoneally into each mouse. Results (Table 13) show that 100% of the white male Webster strain were dead by the end of 10 weeks, whereas only 70% of the white female were dead by the end of 14 weeks. However, in the case of brown male C₃H strain, only 10% died by the end of 14 weeks. All of the female brown mice survived. The results suggest that females of both strains appear to be more resistant than the males. All dead mice were examined and found to have died of cryptococcosis.

TABLE 13

STUDIES ON SEX AND STRAIN RESISTANCE TO EXPERIMENTAL CRYPTOCOCCOSIS IN MICE

Strain	Sex	# of Mice	Intraperitoneally Dose of Cells	% Mortality	# of Weeks Required To Cause Death
White Webster	Male	10	500,000	100	10
White Webster	Female	10	500,000	70	14
Brown C ₃ H	Male	10	500,000	10	14
Brown C ₃ H	Female	10	500,000	0	-

The mice were injected intraperitoneally with 0.5 ml (500,000 cells) seeded soil suspensions. The soil had been incubated at 20°-24° C in 25-30% relative humidity for a month.

Histopathological Studies

Tissue sections of lung, kidney, spleen and brain were stained with hemotoxylin and eosin and periodic acid-Schiff stain. The organisms in the tissues appeared to be rounded and encapsulated and measured approximately 16 to 20 μ in diameter, with distinct capsules of about 3 - 5 μ in thickness (Figures 14-17). Both Sabouraud's dextrose agar grown and soil grown (without pigeon manure extract and incubated at 20^o-24^oC in 25-30% relative humidity) suspension of cells produced equally thick capsules in vivo. Pathological changes in tissue were observed. Microscopic studies of spleen showed reactive hyperplasia of the lymphoid follicles. In lungs and kidneys, C. neoformans cells were scattered abundantly throughout the organs and elicited a focal inflammatory reaction consisting of a mixture of polymorphonuclear leukocytes, lymphocytes and histiocytes. Involvement of central nervous system was characterized by numerous focal lesions scattered diffusely throughout the brain (Figures 14-17).

CHAPTER IV

DISCUSSION

The experiments conducted serve to show the effect of various factors, such as temperature, humidity, freezing, pigeon manure extract, different pH of the soils, and seasonal changes (summer and winter weather), on the survival and growth of C. neoformans seeded in sterilized soil. The relation of capsule thickness to virulence was also investigated. In addition, studies were made to investigate the host resistance of strain and sex to cryptococcosis using white Webster and brown C₃H mice.

Three cases of cryptococcal meningitis were reported in Kingfisher, Oklahoma, by Muchmore, Rhoades, Nix, Felton, and Carpenter (1963), suggesting the prevalence of C. neoformans in that community. In view of this report, the soil samples for use in these studies, as well as pigeon manure, were collected from in and around that area. Most of the soil samples supported the growth of C. neoformans in the laboratory. However, soil with pH 7.7 maintained better growth than soils with pH 7.2 and 6.8. These results show that the fungus grows better in a slightly alkaline soil. Similar results were obtained in a separate experiment by adjusting the pH of soils to 6.0 and 7.2. It was interesting to note that previous moistening of the soil (simulating natural conditions) was required to support growth. The soil samples of pH 6.0 and 7.2 prepared from the soil by adding HCl and drying completely prior to inoculation supported

no growth in the presence of 25-30% or even in 100% relative humidity. In contrast, the parent soil incubated under the same conditions supported the growth of C. neoformans. This is in conformity with the observation of Menges, Furcolow, Larsh and Hinton (1952) with regard to the growth of Histoplasma capsulatum. These authors reported that if the soil was thoroughly dried, growth was usually meager and rather slow.

It should be noticed that the growth and survival of C. neoformans varied with the pH of the soil. When the soil was alkaline (pH 7.7) the growth was greater than in an acid soil. By contrast, in the laboratory the growth is optimum in an acid medium (Sabouraud's dextrose agar) of pH 5.5 - 5.6 (Mosberg and Choudens, 1951). This is indicative of an extraordinary ability of the fungus to metabolize alkaline medium (soil, manure and urine) in nature and slightly acid medium in the laboratory. This view can be supported by the observation of Staib (1963) on the assimilation of creatinine from bird urine and manure. The fresh manure and urine, being strongly alkaline, does support the growth of C. neoformans in nature. Other fungi have also been isolated from an alkaline soil in nature, particularly Allescheria boydii and H. capsulatum. Emmons and Piggott (1963) isolated H. capsulatum from a soil sample with pH 8.0 and from 6 samples with pH 7.0 or higher.

Other striking results were the humidity requirements of the organism. The growth was greater and survival time longer in 100% humidity at all of the temperatures tested below 37°C.

In addition to humidity, temperature affected the growth of C. neoformans. Prolonged incubation at 37°C was deleterious to the fungus. At room temperature (20°C-24°C) the growth of the fungus was increased in

the presence of 100% relative humidity, whereas in atmospheric humidity (25-30%) only 7% viable cells remained at the end of 6 months. However, at low temperatures (4° - 6° C) the growth occurred regardless of the humidity. This is most probably due to the fact that in a refrigerator the humidity is high enough to provide moisture for the survival of C. neoformans. A similar observation was recorded when the fungus was stored at -15° C. The encapsulated yeast was still viable at the end of 8 months.

It was seen that higher temperatures coupled with high humidity (summer heat) was lethal to the organism. However, heat alone had little effect on the survival of the organism. The organism incubated in atmospheric humidity in 10-20% survived at the end of 6 months. These results are consistent with the findings of Staib (1963). He observed that if C. neoformans seeded in dry sand (stored at room temperature for $1\frac{1}{2}$ years) was heated to 60° , 72° , and 80° C for 24 hours, the fungus was still viable. However, when the seeded sand was moistened and then heated to 50° C, no viable cells could be cultured after 24 hours.

Another interesting observation was that organisms which were kept in the dark survived quite well regardless of the high temperature and humidity. Since there was no way of checking the temperature of the seeded soil incubated in the dark, it is difficult to determine whether the organism subjected to direct sunlight were killed by the high temperature, ultraviolet rays or both.

Furthermore, C. neoformans demonstrated a far greater capacity for survival during winter. In view of the results obtained, it is possible to speculate upon its existence and survival in nature. Those fungus

cells which remain in the shade have a better chance to survive and therefore to germinate subsequently, than the ones exposed to sunlight. This speculation is strengthened by the previous reports of isolation of C. neoformans from nature in shaded areas. Ajello (1956) isolated the organism from 8 samples of soil which came from the edge of a pond, a barn, and a tractor shed. Littman and Schneirson (1959) isolated it from an old barn, as well as pet and bird shops. They stated that indoor pigeon coops were more heavily contaminated with C. neoformans than outdoor coops. Emmons (1960) isolated the fungus from warehouses, former barns, and old office buildings; many others have isolated C. neoformans from areas where there was no direct sunlight.

The enhancing effects of pigeon manure extract on the growth of C. neoformans supports the observation of Emmons and others that the fungus is associated with the manure of pigeons. As in the case of H. capsulatum the ecological relationship of this fungus with bird manure demands further clarification. Staib (1963) reported that C. neoformans accumulates purines from the bird urine, particularly uric acid, xanthine and guanine. However, he was unable to explain why C. neoformans is found in bird manure. He assumed that in the bird manure the compounds such as purines and creatinine have a great affinity for water and it is therefore reasonable to believe that the water of hydration of these compounds may protect C. neoformans from dessication. Bird manure may thus serve as a reservoir for the organisms.

It is known that strains of C. neoformans isolated from soil, as well as cultivated on laboratory media, vary considerably not only in thickness of their capsules but also in their virulence for mice. Drouhet

et al. (1950) found that a very small encapsulated variant had less virulence than the large encapsulated parent yeast. Kao and Schwarz (1957), studying strains of C. neoformans isolated from pigeon nests, reported no correlation between capsule size and virulence. Gadebush (1959) found that yeast cells of a small encapsulated variant were more readily phagocytized by polymorphonuclear leukocytes from anemic mice than yeast cells of the large encapsulated parent strain. The studies of Littman and Tsubura (1959) showed that enlargement of the capsule as a result of cultivating C. neoformans on capsule agar (CCA) did not increase the virulence as compared with that of the same strain grown on Sabouraud's dextrose agar (SAB). Hasenclever and Mitchell (1960) reported that three strains of C. neoformans revealed a considerable variation in virulence for the mice, and mentioned that there was no correlation between virulence and the thickness of the capsule.

The experiments conducted in our laboratory indicate that mice inoculated with soil suspensions containing C. neoformans (without pigeon manure extract and incubated at 20°-24°C in 25-30% relative humidity) a month previously showed a total mortality of 100% by 4-6 weeks, and while those inoculated following 3 months incubation showed only 70% mortality in 10 weeks. Other groups of mice inoculated at the end of 6 months incubation had 40% mortality by the 10th week. When autopsied, some of the remaining animals revealed the presence of C. neoformans while others were sterile. The narrowing of C. neoformans capsules from an initial 2.7 to 0.9 and 0.63 microns in thickness resulting from long storage presumably reduced the virulence of the organism.

The studies of environmental factors influencing the growth of

C. neoformans, as well as findings in mouse inoculation studies, suggest that C. neoformans is probably prevalent in the state of Oklahoma and people in urban and rural areas may be exposed. It is probable that mild and sub-clinical pulmonary infections of cryptococcosis do occur as they do in the case of histoplasmosis but these lesions heal spontaneously because of innate resistance or because of immunity acquired during the slow evolution of the pulmonary lesion; thus in only a small percentage of cases the fungus reaches the central nervous system. The immunity in cryptococcosis has been demonstrated in experimental mice by many investigators. Louria (1960) reported that infection with a small number of highly virulent C. neoformans or large numbers of less virulent strains of C. neoformans when injected intraperitoneally, protected animals against intravenous challenge two weeks later. Subsequently, Louria, Kaminski and Finkel (1963) showed that death was delayed and survival markedly increased when mice were challenged 1 to 4 months following the initial inoculation with 10^3 cells of a virulent strain intravenously. In addition to antibody production in cryptococcosis, Baum and Artis (1963) suggested that a growth inhibiting factor is present in normal human serum.

Studies in our laboratory show that innate resistance to C. neoformans varies with the sex and strain in experimental mice. The female white mice were more resistant than the males and the brown C₃H strain was found to be less susceptible to cryptococcosis than the white Webster strain.

These quantitative studies in the evaluation of various environmental factors support the view of Muchmore, Rhoades, Nix, Felton, and

Carpenter that there may be a high rate of exposure to C. neoformans in the Kingfisher community. Since the weather conditions (summer and winter) in the state of Oklahoma are favorable for the growth of this organism, there may be additional unrecognized cases of cryptococcal infection in other parts of the state.

CHAPTER V

SUMMARY

Several experiments were performed to determine the effect of different temperatures (-15° , 4° - 6° , 20° - 24° , and 37°C) and relative humidities (10-100%) on the growth and survival of C. neoformans seeded into previously sterilized soil. The soil and pigeon manure were collected from in and around Kingfisher, Oklahoma.

There was no decrease in the number of viable cells when the temperature was below 37°C in 100% relative humidity. However, at 4° - 6°C in 35-40% relative humidity there was only a slight decrease in the number of viable cells during the first five months, but by the end of the sixth month the viability had dropped approximately 60% of the initial inoculum. At room temperature (20° - 24°C) in 25-30% relative humidity, there was a noticeable decrease in the number of viable cells by the end of the second month. Approximately 7% of the cells remained viable at the end of six months. It was noted that humidity plays an important role in the growth of C. neoformans. The growth was better at 20° - 24°C in 100% relative humidity. When the temperature was maintained at 4° - 6°C , relative humidity had little effect. There was a rapid decrease in the number of viable cells at 37°C , regardless of the humidity. A similar effect of humidity was noted in soil samples of different pH values (pH 6.0, 7.2, and 7.7). Growth was slight when the pH of the soil was 6.0, well marked at pH 7.2, and better at pH 7.7.

During the summer (July-August), high temperatures of 104°-106°F with 100% relative humidity was lethal for C. neoformans. After 15 days there was an 185-fold drop in the number of cells and by the end of 30 days all cells had died. Although there was some decrease in the number of cells in atmospheric relative humidity (10-20%), approximately 12% of the cells were still viable at the end of six months. In contrast, the survival of C. neoformans was more satisfactory when the organisms were incubated under the same conditions except that sunlight was excluded by covering the seeded soil with a black bucket. On the other hand, the growth and survival of the fungus was better under the winter conditions than the summer. There was a 2-fold increase in November, 3-fold increase in December, and 2.5-fold increase by the end of January.

Freezing at -15°C in mycophil broth resulted in 40% reduction in number of cells by the end of eight months incubation. There was no change in viability of the cells when frozen in a phosphate-buffered saline solution for the same length of time.

In the presence of pigeon manure extract (1%, 5%, 20%, and 35%), the number of viable cells increased exponentially with increasing concentration of the extracts.

After prolonged incubation in soil, C. neoformans cells diminished in size, and thickness of capsules. When mice were inoculated intraperitoneally with soil suspensions incubated for six months, the total mortality was only 40% by the 10th week as compared with 100% total mortality within 4-6 weeks when animals were injected with soil suspensions of C. neoformans that had been incubated for one month. A longer time was required for the mice to develop symptoms and death was delayed. It is believed that the virulence of C. neoformans is decreased upon prolonged incubation in soil.

Sex and strain resistance in experimental mice showed that white females were more resistant to infection than males, and brown strain C₃H was found to be less susceptible than the white Webster strain.

The survival of C. neoformans for at least eight months in various experimental environments, from -15°C to 37°C, in different humidities, approximately 10-100%, under summer and winter weather, and in various concentrations of pigeon manure, is evidence of the successful adaptation of this fungus to a wide range of climatic conditions. This may explain why this fungus persists widely in nature.

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