69-17,650

FARHI, Fereshteh, 1941-

THE INFECTIOUS PARTICLE OF CRYPTOCOCCUS NEOFORMANS.

The University of Oklahoma, Ph.D., 1969
Bacteriology

University Microfilms, Inc., Ann Arbor, Michigan

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE INFECTIOUS PARTICLE OF *CRYPTOCOCCUS NEOFORMANS*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1969

THE INFECTIOUS PARTICLE OF *CRYPTOCOCCUS NEOFORMANS*

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

The author is most grateful to Dr. Glenn S. Bulmer for his encouragement, constructive support and patience during the course of this investigation. Sincere appreciation is expressed to the other members of the Department of Microbiology and Dissertation Committee for their criticisms and support.

The author is grateful to Dr. Gilbert A. Castro for his help in preparation of sugar derivatives and the use of a gas-liquid chromatography apparatus, and to Dr. Johan A. Wulff for the use of an ultracentrifuge.

Acknowledgment is made to Public Health Service grant AI 05022 and National Iranian Oil Company for financial support.

TABLE OF CONTENTS

Page

LIST OF TABLES..... v

LIST OF ILLUSTRATIONS..... vii

Chapter

I. INTRODUCTION AND LITERATURE REVIEW..... 1

II. MATERIALS AND METHODS..... 12

III. RESULTS..... 43

IV. DISCUSSION..... 79

V. SUMMARY..... 92

LIST OF REFERENCES..... 95

LIST OF TABLES

Table		Page
1.	Protocol Used to Study the Effect of Various Substrates on Capsular Production	19
2.	Protocol Used to Study the Effect of Various Concentrations of Thiamine on Capsular Production	20
3.	Protocol Used to Study the Effect of Various Metabolic Inhibitors on Capsular Production	21
4.	Procedure Used to Study Capsular Production by C. neoformans, CIA, in Mouse Tissues in vitro	26
5.	Effect of Soil Incubation on the Size of Cells, Capsule, and Viability of C. neoformans, CIA	44
6.	Effect of Various Carbohydrates on Capsule Production by Resting Cells of C. neoformans, CIA	53
7.	Effect of Inorganic Salts on Capsular Production by Six Strains of C. neoformans	57
8.	Capsular Production by Eight Strains of C. neoformans on Various Laboratory Media	60
9.	Capsular Production by C. neoformans, CIA, in Mouse Tissue in vitro	62
10.	Characteristics of C. neoformans, CIA, and Mutants S_1, S_2, S_3, and S_4	63
11.	Qualitative Analysis of Polysaccharides Isolated from Parental Strain CIA and Mutants S_1 and S_2	66
12.	Quantitative Studies of ACIA, SCIA and S_1 Polysaccharides	69
13.	Immunological and Chemical Evidence for Cryptococcal Polysaccharide in Human Serum	74

14. Phagocytosis of C. neoformans, Strains CIA and S_1 by Human Leukocytes..... 77

15. Effect of Various Treatments of C. neoformans, CIA, on Phagocytosis by Human Leukocytes, in vitro..... 78

LIST OF ILLUSTRATIONS

Figure		Page
1.	Assay Procedure for Determining Location of Factor Responsible for Inhibition of Phagocytosis of C. neoformans by Human Leukocytes in vitro	36
2.	Procedure for Removal of Factor From Human Serum Responsible for Inhibition of Phagocytosis	40
3.	Effect of Soil Incubation on Viability of Eight Strains of C. neoformans	46
4.	Effect of Soil Incubation on Capsule of Eight Strains of C. neoformans	47
5.	Effect of H_2O on Capsular Production by C. neoformans, CIA, Incubated in Soil for One Year	49
6.	Capsular Production by Environmentally Induced Nonencapsulated Cells of C. neoformans, CIA	51
7.	Effect of pH on Capsular Production by C. neoformans, CIA	55
8.	Effect of Various Concentrations of Lactose on Capsular Production by C. neoformans, CIA	59
9.	Gas Chromatographic Analysis of TMS Derivatives of Hydrolyzed S_1, SCIA, ACIA, and Known Carbohydrates	67
10.	Percent Phagocytosis of Nonencapsulated C. neoformans, CIA, Incubated in Excised Human Lung Tissue for Varying Time Periods	71
11.	Percent Phagocytosis of Nonencapsulated C. neoformans, CIA, Incubated in Human Sera for Varying Time Periods	72

THE INFECTIOUS PARTICLE OF *CRYPTOCOCCUS NEOFORMANS*

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Cryptococcus neoformans is an encapsulated yeast which is widely distributed in nature and causes a human infection called cryptococcosis, torulosis, or European blastomycosis. The organism has a pronounced predilection for the central nervous system, although it may cause a subacute or chronic infection involving skin, lungs, or other parts of the body (Littman and Zimmerman, 1956). It seems likely that minor, subclinical infections of the lungs are common and that only a small number of these infections progress into the disseminated state (Walter and Atchison, 1966; Davis, *et al.*, 1967; and Lewis, *et al.*, 1958). The increasing incidence of the latter form was noted by Aikat, Chatterjee and Banerjee (1967).

C. neoformans was discovered simultaneously by several investigators. Sanfelice (1894) isolated a yeast-like organism from fermenting peach juice and demonstrated the pathogenicity of this organism for laboratory animals. Owing to the "tumor-forming" ability of the organism, he named it *Saccharomyces neoformans*. Busse described the first human case, a localized infection, which later became generalized (1894, 1895). Curtis (1896) isolated a similar yeast-like organism from a myxomatous

tumor of the hip. He named the organism S. tumefaciens. Vuillemin (1901) transferred these organisms into the genus Cryptococcus since they did not form ascospores. Weis (1902) compared the so-called "tumor-forming" yeasts with those isolated from milk and found that both types were pathogenic for experimental animals and quite similar in other aspects. Ironically, the first case of meningitis due to C. neoformans was not reported until 1905, at which time von Hansemann recovered yeast cells from a patient with tubercular meningitis (von Hansemann, 1905).

Currently, it is believed that cryptococcosis is caused only by C. neoformans. The clinical forms are classified into five types; pulmonary, central nervous system, dermal, osseous, and visceral (Emmons, Binford, and Utz, 1963).

According to Benham (1955, 1956), the genus Cryptococcus is characterized by; budding yeast cells, presence of a capsule, mucous culture which is yellow to yellow-brown in color; starch formation, absence of pseudo or true mycelium, absence of sugar fermentation (Kreger-van Rij, 1964), and urease production (Seeliger, 1956). Division of the genus into species is based upon the shape of the cells, assimilation reactions with various sugars and nitrate, growth at 37C, and pathogenicity for mice. C. neoformans has the following characteristics:

1. Thick walled, ovoid to spherical, 5-20μ in diameter, budding yeast cells surrounded by a gelatinous capsule.
2. Assimilation of galactose, glucose, maltose, and sucrose. Unable to assimilate lactose as sole source of carbon or nitrate as sole source of nitrogen.
3. Growth at 37C.

4. Pathogenicity for mice when injected intracerebrally or intraperitoneally.

Cryptococcosis is a sporadic disease found throughout the world. Although spontaneous infections in animals have been reported, there are no known instances of animal-to-man or man-to-man transmission of the disease. Benham (1935) reported the isolation of Cryptococcus sp. from skin and feces of healthy individuals. This finding suggested an endogenous origin of cryptococcosis. However, Emmons in 1951 was the first to isolate this organism from the soil. Since then, this organism has been cultured from soils throughout the world, mainly from those containing pigeon excreta (Ajello, 1958; Kao and Schwarz, 1957; Littman and Schneierson, 1959; Emmons, 1960; Bergman, 1963; Muchmore, et al., 1963, Keall, Wilkie and Binns, 1968). Few workers currently hold to the endogenous origin concept.

Emmons (1962), while studying sections of pigeon excreta containing C. neoformans, noted that the cells appeared nonencapsulated and produced very thin capsules when first isolated on laboratory media. Ishaq, Bulmer, and Felton (1968), reported that the capsule of a heavily encapsulated strain of C. neoformans almost completely disappeared during soil incubation. Their data indicated that, in conditions such as high humidity, lack of sunlight, and room temperature, C. neoformans remains viable in soil for periods in excess of one year. Their studies led them to speculate that the infectious particle of C. neoformans may be nonencapsulated.

In man, the route of infection of the organism is believed to be respiratory. Freeman and Weidman (1923) isolated C. neoformans from

various tissues of guinea pigs after intranasal instillation of the cells. Wade and Stevenson (1941) inoculated mice intranasally with this organism and reported that several of the mice died of systemic cryptococcosis. Cox and Tolhurst (1946) infected one out of four mice by intranasal instillation. Ritter and Larsh (1963) demonstrated a 50 percent mortality of white mice after intranasal instillation of about 10,000 viable cells of C. neoformans. Subsequently, Smith and associates (1964) were able to infect white mice with an aerosol of the organism. Basu Mallik, et al. (1966) showed that, after white mice were exposed to a massive spray of C. neoformans, a persistent lung infection developed. Invasion of blood occurred within 18 hours and the brain became involved after three days.

The role of pigeons and other birds in dissemination of cryptococcosis has been a highly debatable subject. Emmons (1955) reported that he was not able to isolate C. neoformans from various organs and the digestive tract of birds. Staib (1962) isolated the organism from canary and other bird excreta. Littman, Borok, and Dalton (1965) were able to establish an infection in pigeons following intracranial inoculation of C. neoformans. Littman and Borok (1968) fed C. neoformans to pigeons and were able to isolate viable cells from the gullet and excreta within the first one or two hours. Organisms were also recovered from the beaks, feathers, and feet. All of these reports seem to indicate that birds may be incidental carriers of C. neoformans, i.e. they do not contract cryptococcosis but may carry and disseminate viable yeast cells on external portions of their body or pass it in excreta after ingestion of contaminated materials.

Perhaps one of the most outstanding characteristic of organisms of the genus Cryptococcus is the ability to produce a capsule. The capsule may be five times the diameter of the cell, especially in tissues. While many pathogenic bacteria possess capsules, C. neoformans is the only encapsulated pathogenic yeast. The relationship of capsules to bacterial pathogenicity has been known for many years and it is generally accepted that the capsules of pathogenic bacteria fall into three categories (MacLeod and Bernheimer, 1965):

1. Nontoxic, antigenic capsules that act by inhibiting phagocytosis, and antibodies produced against the capsule are protective. The best example of an organism possessing this type of capsule is Diplococcus pneumoniae, in which isolated, type specific, pneumonoccal capsule inhibits phagocytosis in vitro, and when injected into animals it induces the production of specific antibody which subsequently protects the animal against that specific type of D. pneumoniae (MacLeod and Krauss, 1950).
2. Nontoxic, antigenic capsules that act by inhibiting phagocytosis, but antibodies against the capsule are not protective. One of the best examples of an organism in this category is Bacillus anthracis. In this case, the capsule inhibits phagocytosis in vitro, but the antibodies produced against it are not protective in vivo (Smith and Keppie, 1955).
3. Nontoxic, nonantigenic capsules which inhibit phagocytosis. The hyaluronic acid capsule of streptococci of Group A is an example of this category. While hyaluronic acid is antiphagocytic in vitro, it is recognized as "self" in vivo, and thus

no antibody is produced against. (Kass, and Seastone, 1944).

Serologic differences between strains of C. neoformans were first reported by Benham in 1935. After studying several strains of the organism by means of serological tests and virulence assays, she divided them into two types. Later, Evans (1949), on the basis of agglutinin titers, divided 12 strains of C. neoformans into three serotypes, A, B, and C. He showed that type specificity was due to the capsular polysaccharide. More recently, Walter and Atchison (1966), Vogel (1966), and Wilson, Bennett, and Bailey (1968) presented evidence which suggests that at least four different serotypes exist in C. neoformans.

C. neoformans produces a minimal and variable immunological response in diseased humans. Several investigators (Shapiro and Neal, 1925; Kimball, Hasenclever, and Wolff, 1967) failed to demonstrate any type of antibody production in diseased humans, while others (Rappaport and Kaplan, 1926; Vogel, Sellers, and Woodward, 1961) demonstrated antibody in patients' sera using agglutination and indirect fluorescent antibody techniques. Bloomfield, Gordon, and Elmendorf (1963) and Gordon and Veddar (1966), using antibody-sensitized latex particles in the slide agglutination test and yeast cell agglutination, detected antigen and/or antibody in the sera of patients suffering from cryptococcosis. The antigen detected in patients with cryptococcosis may be composed largely of cryptococcal capsular polysaccharide. Neill, Sugg, and McCauley (1951), using anticryptococcal antibody in precipitin and complement fixation tests, detected serologically active "soluble antigens" in the spinal fluid, blood, and urine of a patient with cryptococcal meningitis. Absorption of antiserum with cryptococcal polysaccharide removed the

capacity of the serum to react with the fluids of the patient.

In experimental animals, the immune response varies considerably among different species and with the type of antigen used. Neill, Abrahams and Kapros (1950) showed that killed, weakly encapsulated cells of C. neoformans were a better immunogen than killed, heavily encapsulated cells. Comparisons of the antibody responses included not only agglutination of the cells, but also precipitation of cryptococcal polysaccharide and "quellung" reaction on the heavily encapsulated cells. However, this phenomenon differs from that seen with the pneumococci in that it appears to be due to a change in the capsule which makes it more readily seen, rather than to an increase in the size of the capsule (Neill, et al., 1949). Evans, et al. (1956) suggested that visualization of the Cryptococcus capsule may be due to the formation of a thin line of precipitation around the capsule. Kase and Metzger (1962), using a dry variant of C. neoformans, reported the production of high titers of antibody (1:640 to 1:1280) to different antigenic preparations of C. neoformans which contrasts with the minimal antibody responses obtained by most investigators. Devlin and Patnode (1968) showed that cryptococcal antigens prepared by zeolite treatment of formalized yeast cells were quite antigenic and produced a strong humoral antibody response in rabbits. Gadebusch (1963) reported that the injection of purified cryptococcal capsular polysaccharide into rabbits resulted in the production of specific anticapsular antibody. Other investigators (Cozad, Richey, and Larsh, 1963; Devlin, 1969), however, presented strong evidence to the contrary. Goren and Middlebrook (1967) demonstrated that cryptococcal polysaccharide

conjugated to bovine gamma-globulin is a potent antigen for mice. However, Goren (1967) found that such antibodies were not protective. Bennett and Hasenclever (1965) demonstrated the persistence of capsular material in the blood of rabbit and mice for several weeks. The animals were susceptible to subsequent inoculation with viable yeast cells. Thus, it seems that cryptococcal capsular material, while not immunogenic, is haptenic and that antibodies produced against it are not protective.

Whereas the importance of bacterial capsules as virulence factors has been amply documented, the importance of the cryptococcal capsule remained rather obscure until recently, when Bulmer, Sans, and Gunn (1967), using encapsulated parental cells and nonencapsulated mutants of C. neoformans, showed a correlation between the presence of capsule and the virulence of the organism for mice.

One of the first reports on the effect of cryptococcal capsule on leukocytes was that presented by Drouhet and Segretain in 1951. They reported that the cryptococcal capsule inhibited the migration of guinea pig leukocytes in vitro. Gadebusch (1958, 1959) demonstrated that a small-capsule variant of C. neoformans was phagocytized more readily than a heavily encapsulated variant by leukocytes from anemic mice. Louria and Kaminski (1965), as a result of studies with small- and large-capsule strains, suggested that the immunity to cryptococcal infection in mice was "tissue bound". Abrahams (1966) reached similar conclusions. Bulmer and Sans (1967, 1968) demonstrated that isolated cryptococcal polysaccharide inhibited the phagocytosis of the organism by human peripheral leukocytes in vitro. Later, Bulmer, Farhi, and Tacker (in

preparation) demonstrated intracellular killing of C. neoformans by peripheral blood leukocytes.

The cryptococcal capsule appears to have the following effects on the host:

1. Isolated cryptococcal capsule inhibits the phagocytosis of C. neoformans. The antiphagocytic nature of the material is very specific and results from the adherence of the material to the yeast cells. Isolated capsule does not effect leukocytes, since it does not inhibit the phagocytosis of other microorganisms (Bulmer and Sans, 1968).
2. Inoculation of the material along with unrelated immunogens does not inhibit antibody production (Devlin, 1969).
3. Although it has been shown that a soluble fraction released from cryptococcal cells, as well as whole cells, produces a fever response in rabbits (Briggs and Atkins, 1966; Haley, Myer, and Atkins, 1966), it was shown by the same investigators that small amounts of purified polysaccharide are nonpyrogenic for rabbits. The minimal pyrogenic dose of the yeast polysaccharide appeared to be 10-20 mg, whereas 0.0003-0.03 mg of purified lipipolysaccharide from gram-negative bacteria produced the same response in rabbits (Landy, et al., 1955; Snell and Atkins, 1965).

Thus, it would appear that the cryptococcal capsule is nontoxic and antiphagocytic, and that the antibody produced against it is not protective.

The cryptococcal capsule is a polysaccharide. Evans and Kessel

(1951) showed that the polysaccharide contains hexose, pentose, and hexuronic acid. Evans and Mehl (1951), using paper chromatography, identified galactose, mannose, xylose, and hexuronic acid. These investigators suggested that the difference between serotypes was due to quantitative rather than qualitative changes in the amount of different monomers present in capsular polysaccharide. Drouhet, Segretain, and Auber (1950) reported the presence of only mannose, xylose, and glucuronic acid in cryptococcal polysaccharide, although they suggested that the capsule may contain a hyaluronic acid-like substance (Drouhet and Segretain, 1949). Foley and Uzman (1952) isolated a highly polymerized capsular polysaccharide from C. neoformans by hot alkali and were unable to detect hyaluronic acid-like substances. Einbinder, Benham, and Nelson (1954) reported that the capsular polysaccharide of C. neoformans contained 31 percent pentose (arabinose), 18.1 percent hexose (glucose), and 6.1 percent hexuronic acid (glucuronic acid). Rebers, et al. (1958), using antibody absorption techniques concluded that the capsular side chains terminate in galactose and glucuronic acid in Type A C. neoformans. They also suggested that the cryptococcal polysaccharide may contain at least two types of polymers. Miyazaki (1961) proposed a branched mannose backbone for an untyped C. neoformans. Blandamer and Danishefsky (1966) reported molar ratios of mannose to xylose and glucuronic acid to galactose to be 6:4 and 2:1, respectively for Type B C. neoformans. Their findings on the composition of oligosaccharide, obtained by partial hydrolysis of native polysaccharide, and the rate of release of the specific monosaccharides suggested that cryptococcal polysaccharide consists of a mannan backbone with branches of xylose and glucuronic acid.

The objectives of this investigation were to study:

1. The effect of soil incubation on cells and capsules of C. neoformans.
2. The conditions under which capsular production by environmentally or genetically induced nonencapsulated cells of C. neoformans is initiated.
3. The chemical nature of capsular and soluble polysaccharides produced by C. neoformans.
4. The time required for the cells of C. neoformans to produce enough capsular material in human tissues to impair phagocytosis by human leukocytes.

CHAPTER II

MATERIALS AND METHODS

The eight encapsulated strains of Cryptococcus neoformans (CIA, A, B, CDC, CS, CS10, UH1, UH3) used in these studies were obtained from Dr. Glenn S. Bulmer, Department of Microbiology, University of Oklahoma School of Medicine. All of these organisms were human isolates except for CS and CS10 which were cultured from soil. Strain CIA was classified in serotype A by J. Walter (personal communication). Methods for obtaining mutants and culturing C. neoformans in a nonencapsulated state are described below. Unless otherwise stated, strain CIA was used for these investigations. Stock cultures of all strains were transferred weekly on Sabouraud-Dextrose broth plus 2% agar (Difco).

Animals

White mice, 4-8 weeks of age, obtained from the University of Oklahoma Medical Center, were used in virulence, transformation and capsular production studies.

Soil Sample

Soil, used for incubation of C. neoformans was collected from Kingfisher, Oklahoma and obtained from the laboratory of Dr. F. Felton, Veterans Administration Hospital, Oklahoma City, Oklahoma.

Media

Bacto-Yeast Nitrogen Base Without Amino Acids (Difco)

This medium was supplemented with 5.0µg/ml thiamine monochloride and 1.0 mg/ml sodium glutamate. One percent (final concentration) of the following was added; D-galactose, D-glucose, D-sodium glucuronate, D-lactose, D-maltose, D-mannose, sucrose, and D-xylose.

Bacto-Yeast Carbon Base (Difco)

This medium was supplemented with 5.0 µg/ml of thiamine monochloride and 5.0 mg/ml of sodium nitrate.

Essential Salt Solution (ESS)

ESS was prepared by dissolving the following in 100 ml of distilled water: KH_2PO_4, 2.0 gm; $(NH_4)_2SO_4$, 2.0 gm; $MgCl.6H_2O$, 0.2 gm; $CaCl_2.2H_2O$, 0.002 gm; $FeCl_2.4H_2O$, 0.04 gm; $MnCl_2.4H_2O$, 0.0015 gm; and NaN03, 0.0015 gm.

Low pH Synthetic Medium (LpH)

LpH was prepared by adding 0.02 gm/ml glucose and 5.0 µg/ml thiamine monochloride to a 1:10 dilution of ESS. Hydrochloric acid (0.1N) was used to adjust the pH to 5.0. This medium allows the growth of C. neoformans without the production of capsule.

High pH Synthetic Medium (HpH)

This medium was prepared by adding 0.01-0.03 gm/ml glucose, 0.1 mg/ml sodium glutamate, and 5.0 µg/ml thiamine to a 1:10 dilution of ESS. The pH of the medium was adjusted to 7.0 with 0.1N NaOH. In this medium C. neoformans produces a large capsule.

Other Media

Sabouraud dextrose broth (hereafter referred to as Sabouraud medium), trypticase soy agar (Difco), potato-dextrose agar (Difco), and Raper's complete agar (Raper, 1966) were also used. In all instances, 2% agar was added when solid medium was desired.

India Ink Measurement of the Capsule

Unless otherwise stated, the size of the capsules of C. neoformans was measured by mixing a drop of yeast cell suspension on a microscope slide with a drop of Higgins American India Ink diluted 1:2 with distilled water. After the addition of a cover slip, 100 cells were viewed microscopically and the number of encapsulated and nonencapsulated cells was counted and converted into percentages. A Porton Ritcle Ocular micrometer was used for microscopic measurements. The diameter of the capsule of encapsulated cells was determined by subtracting the diameter of the cell from the diameter of the cell plus capsule.

Incubation of C. neoformans in Soil

The suspensions of C. neoformans used to inoculate the soil were prepared in the following manner: one hundred and fifty ml of water were added to 25 gm soil and stirred for 30 minutes on a magnetic stirrer (Fisher Scientific Co. Model 14-511-1). After centrifugation at 350 x g for 15 minutes, the supernatant fluid was removed and autoclaved. Cells of C. neoformans, cultured for 48 hours on HpH agar, were added to 15 ml of the sterile soil washings to give the following concentrations: Strain CIA, 1.2×10^8 (preliminary study) and 4.2×10^8; strain A, 1.2×10^9; strain B, 3.5×10^8; strain CDC, 3.0×10^8; strain CS, 2.5×10^8; strain

CS10, 5.3 x 10^8; strain UH1, 1.6 x 10^9; and strain UH3, 1.5 x 10^9. Control bottles received 0.2 ml of autoclaved encapsulated yeast cells.

Five gm of soil was added to each of 500 screw capped bottles (3 x 3 x 5 cm.) which were divided into 8 groups. Bottles in each group received 0.2 ml of a suspension of one of the eight strains of C. neoformans. All bottles were shaken manually to disperse the yeast cells throughout the soil and were then incubated at 25C in a dark incubator having a humidity of 85%.

At various time intervals, two bottles from each set were removed from the incubator. Ten ml of sterile water were added to each bottle. The bottles were shaken manually for 2 minutes and allowed to stand at 25C for 5 minutes; viable plate counts and India ink preparations were made with samples removed from the supernatants to determine the number of viable cells in each bottle, the percent encapsulated and nonencapsulated cells, and the diameter of the capsule and cells.

Capsular Production of C. neoformans After One Year Incubation in Soil

The following experiments were carried out using 4 bottles of soil containing cells of CIA which had been incubated in soil for one year. To each bottle 10 ml of distilled water was added and yeast cell suspensions were prepared as described previously. The suspensions were pooled and 0.5 ml was added to 24 test tubes (13 x 100 mm) in the following experimental systems: The first system contained six tubes; 2 tubes contained 2.0 ml of HpH broth, 2 tubes contained 2.0 ml of Sabouraud broth, and 2 tubes containing 2.0 ml of distilled water. All tubes received 0.5 ml of water. The second system contained 6 tubes and

was a replica of the first system with the exception that all tubes received 0.5 ml of chloroform instead of 0.5 ml of water. The third system consisted of six tubes each containing 2 ml of 0.1 M glucose and the following concentrations of dinitrophenol (DNP, Fisher Scientific Company, Fair Lawn, N.J.), 10^{-4}M, 10^{-5}M and 10^{-6}M. Four additional bottles containing cells of CIA which had been incubated in soil for one year were autoclaved for 30 minutes. A cell suspension was prepared by adding 10 ml of sterile water to each of the autoclaved bottles and manually shaking the bottles for two minutes. After 5 minutes without shaking, the supernatant fluids from the bottles were pooled and used as inocula (0.5 ml/tube) for two tubes containing 2.0 ml of HpH broth, two tubes containing 2.0 ml of Sabouraud broth, and two tubes containing 2.0 ml of sterile distilled water.

All 24 tubes were incubated on a rotator (Multi-Purpose Rotator, Model 150V, Scientific Industries, Inc., Springfield, Mass.) at a speed of 15 rpm for 24 hours at 25C, at which time India ink preparations of each sample were made and the percent encapsulation and diameter of the cells and capsules were determined.

Virulence of C. neoformans Incubated in Soil

After 0, 1, 6, 12, and 18 months incubation in soil, cells of CIA were collected as described previously and 1.0 ml samples were injected intraperitoneally into each of the five mice. The number of cells inoculated into each animal varied from 0.79×10^6 to 4.3×10^6. Four weeks post-inoculation, the brains of infected mice were examined by direct microscopy for the presence of encapsulated yeast cells, using India ink preparations, and by culturing the tissues on Sabouraud agar

plates. After 3-5 days incubation at 25C, the plates were examined for the presence of encapsulated yeast colonies.

Effect of Water on Capsular Production by C. neoformans Incubated in Soil for One Year

Various amounts of distilled water, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ml, were added to 5 gm soil samples containing cells of CIA incubated for one year. Control samples were autoclaved for 30 minutes before the addition of water. After 72 hours incubation at 25C, the yeast cells were examined in India ink preparations for the percent encapsulation and size of the capsule.

Generation Time of C. neoformans Grown on Laboratory Medium and Incubated in Soil

Cells of CIA grown on Sabouraud agar for 48 hours were harvested and suspended in 10 ml of sterile distilled water. To a 250 ml Erlenmeyer flask was added 50 ml of HpH broth containing 2% glucose. One ml of the cell suspension was added to the medium to give a final concentration of 5×10^5 cells/ml.

Cells of CIA, incubated in soil for one year, were harvested as soil washings, as described previously. To 50 ml of HpH broth containing 2% glucose was added 1.0 ml of the soil washing cell suspension to give a final concentration of 7×10^4 cells/ml.

Both cultures were incubated at 25C under aeration. After 2, 4, 6, 8, 20, 28, 36, 44, 52, and 64 hours, samples were removed and the number of viable cells was determined by dilution plate counts on Sabouraud agar. Generation time was calculated from the following formula:

$$G = \frac{0.3010t}{\log_{10} b - \log_{10} a}$$

Where G is generation time, t is time elapsed, 0.3010 is $\log_{10}$ of 2, b is the number of bacteria at the end of t, and a is the number of bacteria at the beginning of t (Oginsky and Umbreit, 1959).

Environmentally Induced Nonencapsulated Cells of C. neoformans

Cells of CIA were cultured on LpH agar at 26C to obtain environmentally induced nonencapsulated cells. After 2-3 subculturings at 3-5 day intervals, 95% of the cells produced very small, or no, capsules as observed in India ink preparations.

Capsular Production by Environmentally Induced Nonencapsulated Cells of C. neoformans

Forty-eight hour cultures of nonencapsulated cells, grown on LpH agar, were harvested, suspended in 50 ml of 0.3 M phosphate buffer (pH 5.0), and shaken overnight on a horizontal shaker (Burrell Wrist Shaker). The suspension was then centrifuged at 300 x g for 30 minutes. The supernatant fluid was discarded and the cells were resuspended in sterile distilled water to a concentration of 1-2 x 10^9 cells/ml. One tenth ml of this suspension was added to each of the systems shown in Table 1, Table 2, and Table 3, in attempts to study the time and substrates required for capsular production, the effect of different concentrations of thiamine on initial capsular production, and the effect of the following metabolic inhibitors on capsular production; sodium fluoride (NaF, J. T. Baker Chemical Co., Phillipsburg, N.J.), potassium iodoacetate (KIAc, Eastman Organic Chemicals, Rochester, N.Y.), and DNP. All systems were incubated on a horizontal shaker at 25C. After 0, 1, 3, 5, 7, 10, 18, and 24 hours, samples were removed for assay of cell

TABLE 1

PROTOCOL USED TO STUDY THE EFFECT OF VARIOUS SUBSTRATES ON CAPSULAR PRODUCTION

Flask No.	Constituents of Each Flask[a]					
	ESS[b] (ml)	Glucose (0.1 gm/ml) (ml)	Glutamate (0.02 gm/ml) (ml)	Thiamine (50 µg/ml) (ml)	$(NH_4)_2SO_4$ (0.02 gm/ml) (ml)	H_2O (ml)
1	2.0	2.0	2.0	2.0	2.0	10.0
2	2.0	-	2.0	2.0	2.0	12.0
3	2.0	-	-	2.0	-	16.0
4	2.0	-	-	2.0	2.0	14.0
5	2.0	2.0	-	-	2.0	14.0
6	2.0	2.0	-	-	-	16.0

[a]All solutions were prepared in distilled H_2O. The pH was adjusted to 7.0 with 0.1 N NaOH prior to sterilization.

[b]Essential Salt Solution

TABLE 2

PROTOCOL USED TO STUDY THE EFFECT OF VARIOUS CONCENTRATIONS OF THIAMINE ON CAPSULAR PRODUCTION

Flask No.	Constituents of Each Flask[a]					
	ESS[b] (ml)	Glucose (0.1 gm/ml) (ml)	Glutamate (0.02 gm/ml) (ml)	Thiamine (100 µg/ml) (ml)	$(NH_4)_2SO_4$ (0.02 gm/ml) (ml)	H_2O (ml)
1	2.0	2.0	2.0	0.0	2.0	12.0
2	2.0	2.0	2.0	0.2	2.0	11.8
3	2.0	2.0	2.0	0.4	2.0	11.6
4	2.0	2.0	2.0	0.5	2.0	11.5
5	2.0	2.0	2.0	1.0	2.0	11.0

[a]All stock solutions were prepared in distilled H_2O. The pH was adjusted to 7.0 with 0.1N NaOH prior to sterilization.

[b]Essential Salt Solution

TABLE 3

PROTOCOL USED TO STUDY THE EFFECT OF VARIOUS METABOLIC INHIBITORS ON CAPSULAR PRODUCTION

Flask No.	Constituents of Each Flask[a]						
	ESS[b] (ml)	Glucose (0.1 gm/ml) (ml)	Glutamate (0.02 gm/ml) (ml)	Thiamine (50.0 µg/ml) (ml)	$(NH_4)_2SO_4$ (0.02 gm/ml) (ml)	Inhibitor	H_2O (ml)
1	2.0	2.0	2.0	2.0	2.0	--	10.0
2	2.0	-	2.0	2.0	2.0	--	12.0
3	2.0	2.0	2.0	2.0	2.0	KIAc	8.0
4	2.0	2.0	2.0	2.0	-	KIAc	10.0
5	2.0	2.0	2.0	2.0	2.0	NaF	8.0
6	2.0	2.0	2.0	2.0	-	NaF	10.0
7	2.0	2.0	2.0	2.0	2.0	DNP	8.0
8	2.0	2.0	2.0	2.0	-	DNP	10.0

[a]All stock solutions were prepared in distilled H_2O. Final concentration of the inhibitors was: KIAc, 1×10^{-3}M; NaF, 1×10^{-2}M, and DNP, 1×10^{-4}M.[2] The pH was adjusted to 7.0 with 0.1N NaOH prior to sterilization.

[b]Essential Salt Solution

viability, as measured by the dilution plate count method and the trypan blue method (McLimans, et al., 1957). Percent encapsulation as observed microscopically in India ink preparations.

Effect of Various Carbohydrates on Capsular Production by Nonencapsulated Resting Cells of C. neoformans

Cells of CIA were cultured for 48-72 hours on LpH agar and harvested by suspending the cells in sterile distilled water. After two washings with sterile distilled water, the cells were suspended in sterile distilled water to a concentration of 1-2 x 10^8 cells/ml. Aliquots (0.2 ml) of this suspension were added to test tubes containing 2.0 ml 0.85% NaCl (saline), 0.1M KH_2PO4 and 1.0% (final concentration) of either galactose, glucose, glucuronic acid, mannose or xylose. Control tubes contained no carbohydrate. The tubes were incubated at 25C on a rotator. After 18 hours incubation the size of the capsules and the percent encapsulation was measured in India ink preparations. The percent viability of the cells was determined with trypan blue.

Optimal pH for Capsular Production by Nonencapsulated Resting Cells of C. neoformans

Three different buffers, Walpole's acetate buffer (pH range of 3.6-5.6), Sorenson's phosphate buffer (pH range of 5.0-8.2), and McIlvaine's citrate phosphate buffer (pH range of 2.2-8.0) were used to assay for the optimal pH range of capsular production by resting cells of C. neoformans (Diem, 1968). The inoculum was prepared from a 24-48 hour culture of cells of CIA grown on LpH. The cells were incubated in 0.3M phosphate buffer (pH 5.0) at 25C for 4-5 hours, removed by centrifugation at 300 x g for 30 minutes, and resuspended in sterile distilled

water to concentration of 1-2 x 10^8 cells/ml. Each tube contained 3.0 ml of a given buffer (pH range of 3.0 to 8.2, in 0.4 increments), 0.1 ml of a 33% glucose solution, and 0.1 ml of cell inoculum. At various time intervals, samples were removed and the percent encapsulation, capsular diameter, and viability were determined. The India ink used for detecting capsular production at pH values under 5.0 was buffered with Sorenson's phosphate buffer at pH of 8.0 to prevent aggregation of India ink.

Incubation of Nonencapsulated Cells of C. neoformans with Cryptococcal Polysaccharide

Cells of CIA, grown on LpH agar for 3 days at 25C, was used as inoculum. Medium was prepared consisting of Bacto-Yeast Nitrogen Base supplemented with 1.0 mg/ml of capsular polysaccharide (ACIA) isolated from encapsulated CIA cells (See below, Isolation of Adhered Capsular Polysaccharide of C. neoformans, CIA) as a sole source of carbon. To 50 ml of medium in each of two 125 ml Erlenmeyer flasks was added cryptococcal cells to a final concentration of 1-5 x 10^4 cells/ml. The flasks were incubated at 25C, one flask without aeration and the other under aeration. After 0, 1, 3, 5, 7, 14, 21, and 28 days, 2.5 ml samples were removed. A 0.5 ml portion was dilution-plated and the remaining 2.0 ml was passed through a millipore filter (HAWP 025 00 HA 0.45μ). The filtrate was dialyzed at 4C against 4 changes of tap water and 3 changes of distilled water and then the total carbohydrates were determined. For this purpose, the nondialyzable solution was diluted 1:5 with distilled water and 1.0 ml of this material was used with 2.0 ml of Anthrone reagent (Seifter, et al., 1950). The quantity of

carbohydrate present in each sample was determined by reading in a Beckman DU spectrophotometer at 620 mμ. A standard curve was prepared using known concentrations of ACIA polysaccharide.

Effect of Inorganic Salts on Capsular Production by Growing Cells of C. neoformans

Sabouraud broth and HpH broth were used to study the effect of NaCl on capsular production. The media were supplemented with 0, 1, 3, 5, 7, and 10% NaCl. The inocula consisted of either encapsulated or environmentally induced nonencapsulated cells of CIA. To 5.0 ml of each of the media was added 0.1 ml of distilled water containing 1-2 x 10^5 cells. The tubes were incubated on the rotator at 25C. After 1, 3, 5, and 7 days, samples were removed and observed in India ink preparations for the presence of capsule and tested for viability with trypan blue dye and by recording turbidity at 690 mμ in a Bausch and Lomb Spectronic 20.

In further experiments, encapsulated cells of six strains of C. neoformans were cultured in HpH broth containing 5% (final concentration) of either NaCl, $NaNO_3$, Na_2SO_4, K_2SO_4, KCl, KH_2PO_4, or K_2HPO_4. Percent encapsulation was determined in India ink preparations after the organisms had been subcultured twice at 48-72 hour intervals.

Effect of Lactose on Capsular Production by C. neoformans

To study the effect of various concentrations of lactose on capsular production of CIA, HpH broth was supplemented with 0, 5, 10, 20, 30, and 40% lactose. To 5.0 ml of each of the concentrations of lactose was added 0.1 ml (1-3 x 10^6 cells) of an aqueous suspension of

CIA cultured on LpH. The tubes were incubated at 25C on a rotator. After 0, 1, 2, 4, and 7 days, samples were removed and the percent encapsulation and growth were determined by India ink preparations and turbidimetric techniques, respectively.

Capsular Production by C. neoformans Grown on Various Media

Capsule production by 8 strains of C. neoformans cultured on nutrient agar, trypticase soy agar, HpH agar, Sabouraud agar, potato-dextrose agar and Raper's complete agar was determined. Each strain was inoculated onto each of the media and incubated at 25C for 72-96 hours. After 3 subculturings the size of the capsule was measured in India ink preparations.

Capsular Production by C. neoformans in Mouse Tissue In Vitro

Cardiac blood was collected from 8 week old mice and pooled in a heparinized tube. Brains and lungs of the animals were removed and cut into approximately 0.5 cm^3 pieces. Various combinations (Table 4) of lung, brain, blood, saline, and HpH broth were inoculated with 1-2 x 10^6 nonencapsulated cells of CIA which had been cultured on LpH agar. The tubes were incubated on a rotator at 37C. At various time intervals, capsular production was measured in India ink preparations. For this study, India ink was prepared in phosphate buffer (pH 8.0) to reduce aggregation of ink particles. A similar protocol was used to study the ability of nonencapsulated mutants, S_1 and S_2, to produce capsules in mouse tissue in vitro.

TABLE 4

PROCEDURE USED TO STUDY CAPSULAR PRODUCTION BY C. NEOFORMANS, CIA, IN MOUSE TISSUES IN VITRO*

Tube No.	Blood (ml)	Brain (mm^3)	Lung (mm^3)	0.85% NaCl (ml)	HpH Broth (ml)
1	0.5	0.5	--	--	1.0
2	0.5	--	0.5	0.5	--
3	--	--	0.5	0.5	--
4	0.5	0.5	--	0.5	--
5	--	0.5	--	0.5	--
6	--	--	--	--	1.0

*All tubes were inoculated with 1-2 x 10^6 nonencapsulated cells of C. neoformans, CIA, which had been cultured on low pH synthetic agar (LpH).

Isolation of Nonencapsulated Mutants of C. neoformans

Spontaneous, nonencapsulated mutants of strain CIA were isolated by a method similar to that described by Goodlaw, Mika, and Braun (1950) for the isolation of nonencapsulated mutants of Brucella abortus. Sabouraud broth (100 ml) was inoculated with a heavy suspension of a 48 hour culture of encapsulated cells grown on HpH and incubated at 25-27C in a stationary state. Samples were taken at three day intervals, for a period of 4 weeks and plated onto Sabouraud agar. Cells from dry, rough appearing colonies were isolated and examined in India ink preparations for the presence of a capsule. Nonencapsulated isolates were characterized auxanographically and by the tube assimilation techniques as to their ability to utilize galactose, glucose, lactose, maltose and sucrose and nitrate (Ajello, et al., 1966). The ability to produce urease was determined by using urea disks (Difco). Further characterization of these mutants as to their ability to utilize various monomers of cryptococcal capsule, i.e., galactose, glucuronic acid (potassium salt), mannose, and xylose, was done by culturing the cells on Bacto-Yeast Nitrogen Base supplemented with 2% agar and 1% of each of the above sugars. Cells were transferred at least three times on each medium.

The virulence of the mutants and the parental strains was determined by inoculating eight weeks old mice intraperitoneally (I.P.) with 1.0 ml of a saline suspension containing 1-3 x 10^6 cells or by inoculating 4-6 week old mice intracerebrally (I.C.) with 0.05 ml of a saline suspension containing 1-3 x 10^8 cells/ml. Ten mice were inoculated with each strain and observed for 9 months. Surviving animals were sacrificed

and brain, lung, spleen and liver of all were homogenized using mortar and pestle and examined by direct microscopy for the presence of yeast cells using India ink preparations, and by culturing the tissues on Sabouraud agar. Animals which expired were examined in a similar manner.

Transformation Studies

A method similar to that of Griffith (1928) was used. Eighteen mice were injected I.P. with 1.0 ml of a mixture containing 1 x 10^6 cells of heat-killed (autoclaved for 10 minutes) encapsulated cells of CIA and 1-2 x 10^6 cells of one of the nonencapsulated mutants, S_1, S_2, or S_3 (See Chapter III for characteristics of these mutants). Two mice were sacrificed at 1, 3, 5, 7, 9, 11, 13, 15 and 20 days. Lung, brain, kidney, and blood were removed, homogenized, and plated onto Sabouraud agar. After 3-10 days incubation at 25C, the plates were examined for the presence of colonies of encapsulated yeast.

Isolation of Soluble Polysaccharide From C. neoformans

Three liters of HpH broth, supplemented with 3% glucose, were inoculated with 50 ml of a 48 hour culture of either CIA or the mutants, S_1 and S_2. After 96 hour incubation at 25C, under aeration, the cells were killed by the addition of 3% chloroform (final concentration), and removed by centrifugation at 300 x g for 30 minutes. Sodium acetate (10%), acetic acid (1%), and two volumes of absolute ethanol were added to the supernatant fluid. The mixture was stored at 4C for 48 hours. The precipitate was collected following centrifugation at 300 x g for 15 minutes. One volume of absolute ethanol was added to the supernatant fluid and the mixture was stored at 4C for 48 hours. The precipitate was then

collected following centrifugation at 300 x g for 15 minutes. No additional precipitate could be obtained by the further addition of ethanol to the supernatant fluid. The two precipitates were pooled, washed in 200 ml of absolute ethanol, centrifuged at 300 x g for 15 minutes, and resuspended in distilled H_2O to give a 1% solution. The material was then centrifuged at 22,000 x g for 60 minutes at 4C to remove insoluble impurities. The supernatant was deproteinized by 8 extractions with chloroform and n-butanol (4:1 v/v, Sevag, Lackman, and Smolens, 1938). To the deproteinized supernatant fluid were added sodium acetate (10%), acetic acid (1%), and 3 volumes of absolute ethanol. After 48 hours incubation at 4C, the precipitate was collected following centrifugation at 300 x g for 15 minutes, and dissolved in water, dialyzed against 4 changes of tap water and 4 changes of distilled water at 4C. After lyophilization, the material was stored at room temperature. The isolated polysaccharides are hereafter referred to as: S_1, soluble polysaccharide isolated from S_1 cells; S_2, soluble polysaccharide isolated from S_2 cells; and SCIA, soluble polysaccharide isolated from cells of CIA.

Isolation of Adhered Capsular Polysaccharide of C. neoformans, CIA

After 72-96 hour incubation on Sabouraud agar at 25C, cells of strain CIA were collected and suspended in 250 ml acetone. The suspension was incubated at 25C under constant mixing using a magnetic stirrer. After 6 changes of acetone at 8 hour intervals, the cells were collected by centrifugation at 300 x g for 10 minutes and dried *in vacuo*. Ten gm of dried cells were suspended in distilled water (10% final concentration).

To each 20 ml of this suspension was added 20 gm of glass beads (Type 110, Minnesota Mining and Manufacturing Co., St. Paul, Minnesota). The mixture was placed in a Raytheon Model DF-101 sonic oscillator (Raytheon Manufacturing Co., Walton, Massachusetts) and treated for 20 minutes. The supernatant fluid was collected following centrifugation at 12,000 x g for 30 minutes. Enough distilled water was added to the sediment to bring the total volume up to 30-35 ml and this mixture was sonic oscillated again for 15 minutes and centrifuged as before. India ink preparations of the sediment revealed that about 70-80% of the capsule was removed from the cells without apparent damage to the cells. The two supernatant fluids were pooled and treated in a manner identical to that used for the isolation of soluble polysaccharides. The isolated polysaccharide is hereafter referred to as adhered capsule from cells of CIA (ACIA).

Characterization of Polysaccharides

Total nitrogen was determined by the micro-Kjeldahl method (Lang, 1958) using 4 mg samples of each polysaccharide.

Total carbohydrate was determined by the Anthrone test. A standard curve was obtained by using various concentrations of mannose.

For ultra centrifugal studies a Spinco Model E ultracentrifuge was used. Each polysaccharide was dissolved in veronal buffer (pH, 8.6; ionic strength, 0.15) to a final concentration of 13.3 mg/ml. The samples were centrifuged at 52,640 rpm for 90 minutes.

For serological studies an immunodiffusion technique similar to that of Ouchterlony (1949) was used. Diffusion plates were prepared as follows: A one percent solution of Ionagar #2 (Colab Laboratories,

Inc., Chicago, Ill.) containing a 0.01% final concentration of merthiolate (Eli Lilly, Indianapolis, Indiana) was prepared in barbital buffer (pH 7.4). Into 10 x 10 cm plastic petri plates 15 ml of this mixture was poured and allowed to solidify. The plates were stored at 4C. The wells were punched 1.0 cm apart. Each well received approximately 0.1 ml of either anticryptococcal antibody (courtesy of R. F. Devlin, Department of Microbiology, University of Oklahoma Medical Center) or antigens (1-2 mg/ml of purified polysaccharide, S_1, SCIA, or ACIA).

Qualitative identification of monosaccharides present in each polysaccharide was made by descending paper chromatography on S and S (Schleicher and Schuell) filter paper (22.5 x 50 cm) with the following solvent systems: n-butanol - pyridine - 0.1N HCl (5:3:2, V/V, 20 hours) and ethyl acetate - acetic acid - formic acid - water (18:3:1:4, V/V, 18 hours). The latter was used only to elicit a better separation of xylose and ribose. Samples were prepared by dissolving 0.5 mg of polysaccharide in 0.5 ml of distilled water and 0.5 ml of 3 N HCl. The mixture was placed in a sealed screw capped test tube and hydrolyzed in boiling water for 3 hours. The sample was then dried in vacuo over NaOH, resuspended in 1.0 ml of distilled water, dried again, and resuspended 0.05-0.1 ml of distilled water. The entire sample was applied to the paper. The standards used with each run consisted of galactose, glucuronic acid, glucuronolactone, glucose, mannose, ribose, and xylose. Monomers were detected with 2 aminobiphenol reagent (Gordon, Thornburg, and Werum, 1956) or anilin-phthalic acid reagent (Wilson, 1959).

Further qualitative characterization of ACIA, SCIA, and S_1 polysaccharides was carried out using gas liquid chromatography. For this

purpose, trimethylsilyl (TMS) derivatives of the hydrolized polysaccharides were prepared. Ten mg of each polysaccharide, as well as 10.0 mg of standard containing 2.5 mg galactose, glucuronic acid, mannose and xylose were hydrolyzed in 1.5N HCl for 6 hours. The hydrolysates were dried overnight in vacuo over NaOH, resuspended in distilled water, and dried again. The TMS derivatives were prepared according to the method of Sweeley, et al. (1963). A Varian Aerography gas chromatograph (Model 1740-1) with a six foot x 1/8 inch column packed with 3% SE-30 mesh Varaport 30 (Varian Aerograph, Houston, Texas) was used. It was operated at 175C with a nitrogen flow of 30 ml/min. A hydrogen flame detector was used to detect the compounds coming off the column.

Quantitative determination of each polysaccharide monomer was done in the following manner: Differential hydrolysis of polysaccharides was accomplished by dissolving 0.5 mg or 1.5 mg samples in 0.5 ml or 1.5 ml of distilled water and 0.5 ml or 1.5 ml of 3N HCl, as described previously. Samples were hydrolyzed for 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 24 hours, dried in vacuo, resuspended in distilled water, dried, and resuspended in 0.05-0.2 ml of distilled water. Samples containing 0.5 mg of polysaccharide were applied as spots on the chromatographic paper, while those containing 1.5 mg were applied as a band 5 cm in length. Chromatograms were run in n-butanol - pyridine - 0.1N HCl solvent for 20 hours and dried. Those containing 0.5 mg samples were stained with anilin-phthalic acid reagent. Stained carbohydrate spots (3 x 3 cm) were cut into smaller pieces and eluted for one hour in 2.0 ml of 0.7N HCl in 80% ethanol. The supernatant fluid was removed and read in a Beckman DU at 360 mμ (for hexoses) and 420 mμ (for uronic acids)

(Wilson, 1959). Chromatograms containing 1.5 mg of carbohydrates were not stained, but were cut into 5 x 3 cm strips which were then further cut into smaller pieces, added to 5 ml of distilled water, placed at 4C for 48 hours (with occasional shaking), centrifuged at 350 x g for 30 minutes, and then 2.0 ml samples were removed for the phenol-sulfuric acid test (Dubois, et al., 1956) for hexoses and pentoses and for the carbazole test (Dische, 1950) for hexuronic acid. Standard curves galactose, glucose, glucuronic acid, mannose, and xylose were prepared by using 10-200 µg of each sugar.

Galactose oxidase was also used for quantitative measurements of galactose in each sample. In this case, polysaccharides were hydrolyzed for six hours, dried, resuspended in water (as described previously) and used directly with galactostat reagents (Worthington Biochemical Corp., Freehold, N.J.).

Collection of Human Serum and Leukocytes

Normal human blood was collected by venipuncture, aspirated into sterile tubes, and allowed to clot. The resulting serum was used to prepare yeast suspensions and growth medium. Serum collected in this manner was used within 5-24 hours after collection.

Autologous peripheral blood leukocytes were collected by the method of Braunsteiner, Pakesch, and Vetter (1952). Ten to 15 ml of venous blood was drawn into a 20 ml plastic syringe containing 100-150 units of heparin (Liquaemin Sodium "10", Organon, Inc., W. Orange, N.J.). Immediately, 4-6 ml of a 3.5% solution of polyvinylpyrrolidine (Type NP-K30) in saline was added to the blood and gently mixed. The syringe was then placed at a 45° angle at 4C in a beaker and the supernatant

fluid in the syringe was aspirated very carefully into a 13 x 100 mm siliconized tube containing 50 units of heparin. The number of leukocytes in the suspension was determined by standard hemocytometer counts. The viability of the leukocytes was determined with trypan blue. Generally, 95% of the leukocytes were viable and the concentration of leukocytes varied from 3.8×10^6 to 4.5×10^6 cells/ml.

Phagocytosis System

One ml of human serum containing $5\text{-}6 \times 10^6$ yeast cells and a 1.0 ml suspension of autologous human leukocytes were placed in each 13 x 100 mm siliconized tube. The tubes were placed at approximately a 30° angle on the rotator in a 37C incubator. After 90 minutes incubation a drop of the mixture from each tube was placed on a microscope slide, spread with another slide to an area of one square inch, dried, and stained with Wright-Giemsa stain. With each slide, 100 leukocytes containing yeast cells was determined. The number of leukocytes containing yeast cells per one hundred leukocytes counted is hereafter referred to as the percent phagocytosis.

Phagocytosis of Cells of *C. neoformans* Incubated in "Normal" Human Lung *In Vitro*

"Normal" human lung was obtained from the office of the Oklahoma State Medical Examiner. The lung tissue was obtained from a 65 year old man who had died as the result of a gunshot wound in the stomach 5 hours previously.

To 0.5 cm^3 portions of tissue in 13 x 100 mm siliconized test tubes was added 1.5 ml of saline containing $1\text{-}2 \times 10^7$ cells/ml of a 48 to 72 hour culture of nonencapsulated cells of CIA. The tubes were

incubated on a rotator at 37C. After 0, 3, 7, 11, 18, and 24 hours, 0.2 ml of fluid was transferred to a 13 x 100 mm siliconized tube containing 1.0 ml of serum and 1.0 ml of the autologous leukocyte suspension. After incubation on the rotator at 37C for 90 minutes the percent phagocytosis was determined as described previously. Controls for this experiment consisted of tubes containing nonencapsulated cells of CIA, 1.0 ml of serum, 1.0 ml of autologous leukocytes, and 0.85 mg/ml of isolated ACIA capsular polysaccharide.

Capsule production was detected in buffered India ink preparations.

Phagocytosis of Cells of C. neoformans Incubated in Human Serum In Vitro

One tenth ml of a saline suspension containing 6-7 x 10^6 nonencapsulated cells of C. neoformans, CIA, was added to siliconized tubes (13 x 100 mm) containing 0.9 ml of human serum. After 0, 2, 6, 11, and 24 hours, 1.0 ml of autologous leukocyte suspension was added to each tube. Phagocytosis experiments were initiated and the percent phagocytosis were determined as described previously. Control tubes contained 1.0 ml of serum containing 6-7 x 10^6 nonencapsulated cells of C. neoformans, 1.0 ml of autologous leukocytes, and 0.85 mg/ml of isolated ACIA.

Location and Nature of Factor Responsible for the Inhibition of Phagocytosis of Cells of C. neoformans Incubated in Serum for 24 Hours

To determine whether the inhibition of phagocytosis of cells of C. neoformans, CIA, incubated in serum was due to soluble or adhered polysaccharide, the following experiments were carried out (Figure 1). For each experiment, the leukocytes and serum were derived from the

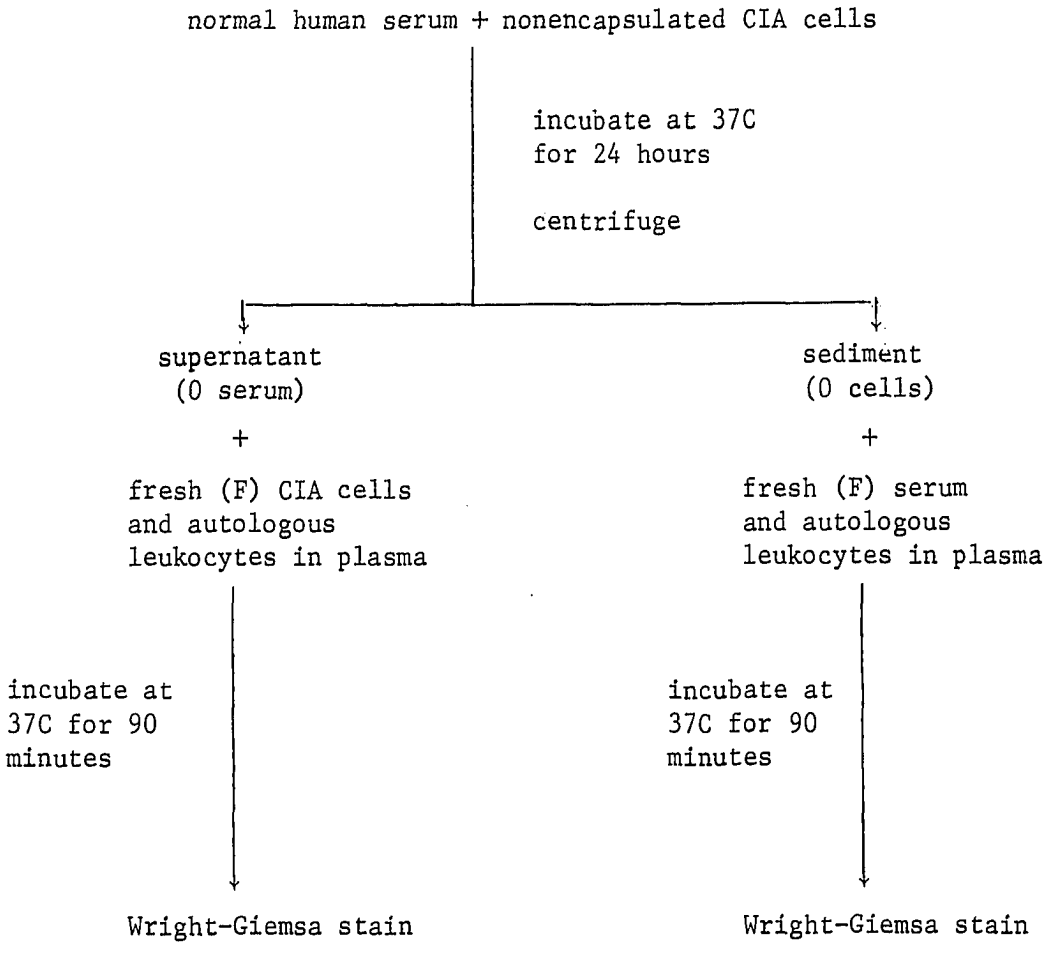

Figure 1--Assay procedure for determining location of factor responsible for the inhibition of phagocytosis of C. neoformans by human leukocytes in vitro.

blood of single donor. Four tubes, each containing 2.0 ml of human serum, were inoculated with 7.5 x 10^7 cells/ml of nonencapsulated cells of C. neoformans. These tubes, plus a tube containing 8.0 ml of uninoculated serum, hereafter referred to as fresh (F) serum, were incubated on the rotator at 37C for 24 hours. The contents of the tubes containing cells of C. neoformans were pooled and centrifuged at 200 x g for 30 minutes. The sediment is hereafter referred to as old (O) cells and the supernatant as old (O) serum. Four ml of O serum was passed through a 0.45μ millipore filter and was used for chemical and immunological studies (see below). The remaining 4.0 ml were used for phagocytosis studies. To each of five siliconized tubes (13 x 100 mm) was added 1.0 ml of either F or O serum. To the O serum was added 0.1 ml of saline containing 6.0-7.0 x 10^6 cells of fresh (F) nonencapsulated yeast cells (cultured for 48-72 hours on LpH agar). To the F serum was added 0.1 ml of saline containing 6.0-7.0 x 10^6 O cells. Controls consisted of 1.0 ml of O serum and 6.0-7.0 x 10^6 O cells; 1.0 ml of F serum and 6.0-7.0 x 10^6 F cells; and 1.0 ml of F serum, 6.0-7.0 x 10^6 F cells, and 0.85 mg/ml of ACIA. To each tube was added 1.0 ml of autologous leukocytes. Phagocytosis experiments were initiated and the percent phagocytosis was determined as described previously.

Immunological Detection of Capsular Polysaccharide in Old Serum

Hyperimmune anticryptococcal rabbit sera were obtained from R. F. Devlin, Department of Microbiology, University of Oklahoma Medical Center. The rabbits had been immunized with zeolite treated antigens of CIA. The agglutination titer of pooled sera was greater than 1:640

when turbidity of the antigen used was 2 McFarland units (Devlin, 1969). To 5.0 ml of the rabbit anticryptococcal serum was added 4.0 mg of ACIA. The mixture was incubated at 37C for one hour, refrigerated at 4C overnight, and the precipitate was removed by centrifugation at 350 x g for 10 minutes. Since, when it was used in a ring test (Campbell, et al., 1964), additional antibody was detected in the supernatant fluid, the absorption was carried out 2 more times using 2.5 mg and 0.5 mg of antigen, respectively. At this time, the ring test indicated a very slight antigen excess. This absorbed and the nonabsorbed anticryptococcal sera were used to examine the 0 serum for the presence of capsular polysaccharide. For this purpose, 0.1 ml of 0 serum was layered over 0.1 ml of either absorbed or nonabsorbed sera in small test tubes. The tubes were incubated at 37C for 60 minutes and for the presence of a precipitin band was recorded arbitrarily as +++, ++, +, ±, - (strong to no band, respectively).

Chemical Detection of Capsular Polysaccharide in Old Serum

Two ml of old serum was diluted with 18 ml of distilled water. To this solution were added 4.0 ml of 2% $ZnSO_4 \cdot 7H_2O$ and 4.0 ml of 1.8% $Ba(OH)_2 \cdot 8H_2O$. The solution was mixed well and then centrifuged at 350 x g for 10 minutes at 4C. The supernatant fluid was dialyzed at 4C against 3 changes of tap water and 3 changes of distilled water and then lyophilized. The lyophilized material was resuspended in 1.0 ml of distilled water. The presence of hexuronic acid was measured by the carbazole test (Dische, 1950). The standard curve for the carbazole test was prepared by adding the following amounts of purified capsular

material (ACIA) to 1.0 ml of serum and treating each sample as described previously; 0.00 (blank), 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 mg. All samples were read in the Beckman DU Spectrophotometer at 535 mμ.

Removal of Factor from Human Serum Responsible for Inhibition of Phagocytosis

To determine whether fresh, nonencapsulated cells of C. neoformans could remove all or any of the inhibitory factor produced by cells of C. neoformans incubated in serum, the protocol in Figure 2 was used. Nonencapsulated cells of C. neoformans, strain CIA, were incubated in 4.0 ml of human serum as described previously for phagocytosis experiments. After 24 hours incubation at 37C, the mixture was centrifuged at 200 x g for 30 minutes and the supernatant fluid was collected. To 3.0 ml of this fluid was added 1-2 x 10^9 fresh (F) (48-72 hour culture) nonencapsulated cells of C. neoformans. The mixture was incubated for 10 minutes at 37C on a rotator, after which it was centrifuged at 350 x g for 10 minutes. The sediment (treated cells) was washed 3 times with saline. Treated cells were added to 1.0 ml of F serum to a concentration of 5.0-6.0 x 10^6 cells/ml. To the 1.0 ml of treated serum were added 6.0-7.0 x 10^6 fresh yeast cells. Controls consisted of: 0 cells and 0 serum; 0 cells and F serum; F cells and 0 serum; F cells and F serum; and F cells, F serum, and 0.85 mg/ml ACIA. Each tube received 1.0 ml of autologous leukocytes. Phagocytosis experiments were initiated and the percent phagocytosis was determined as described previously.

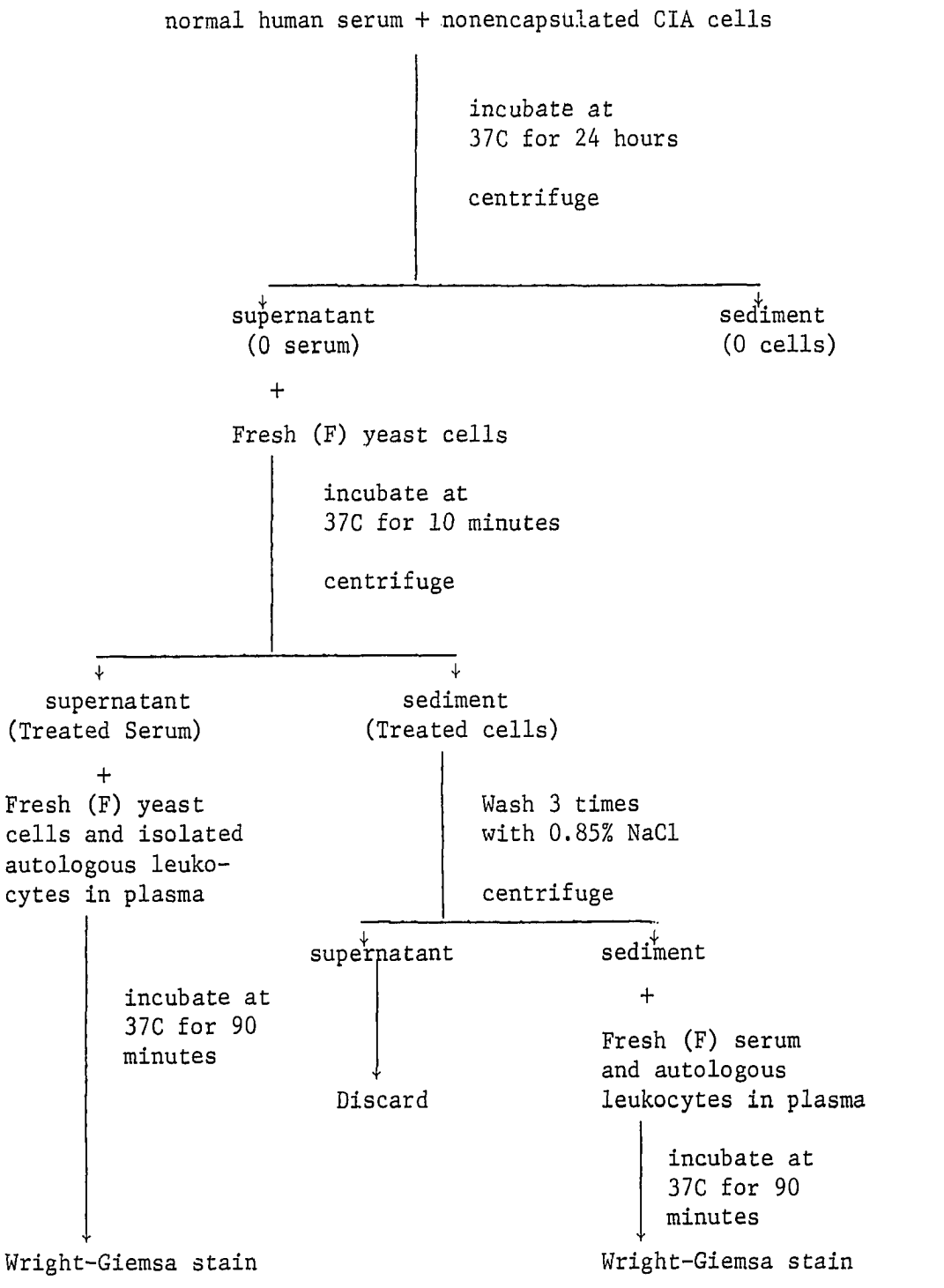

Figure 2--Procedure for removal of factor from human serum responsible for inhibition of phagocytosis.

Comparison of C. neoformans, Strains CIA and S_1 Mutant, by Phagocytosis

Cells of strains CIA and S_1, cultured on Sabouraud agar at 25C for 3-5 days, were diluted in normal human serum to a final concentration of 6.0-7.0 x 10^6 cells/ml. Tubes containing 1.0 ml of serum with either S_1 or CIA cells received either 0.5 ml of saline, 0.5 ml saline containing 4.0 mg/ml of ACIA, 0.5 ml saline containing 4.0 mg/ml of soluble CIA polysaccharide (SCIA), 0.5 ml saline containing 4.0 mg/ml soluble S_1 polysaccharide, or 0.5 ml of saline containing 4.0 mg/ml of capsular polysaccharide of C. laurentii (courtesy of Dr. D. S. Feingold, University of Pennsylvania, Philadelphia, Pa.). Each tube received 1.0 ml of autologous leukocyte suspension. Phagocytosis experiments were initiated and the percent phagocytosis was determined as described previously.

Effect of Various Treatments of C. neoformans on Phagocytosis by Human Leukocytes

Forty-eight to 72 hour cultures of C. neoformans, CIA, grown on LpH agar at 26C were harvested and treated in the following manner: (1) a heavy suspension of cells was prepared in 5.0 ml of saline and autoclaved for 15 minutes at 121C under 15 lb. pressure; (2) a heavy suspension of cells was prepared in 5.0 ml of 0.25% sodium desoxycholate (DOC) and incubated at 37C for 20 minutes. The suspension was centrifuged at 300 x g for 10 minutes and the sediment was washed three times with saline. These cells were used immediately in phagocytosis experiments; (3) a heavy suspension of yeast cells was prepared in 50 ml of acetone, incubated for eight hours at 25C with constant mixing (using a magnetic stirrer). Cells were collected by centrifugation and

resuspended in 50.0 ml of fresh acetone. This procedure was repeated at least 6 times and the cells were then dried in vacuo; (4) a heavy suspension of cells was prepared in saline containing 0.6% formalin and stirred on a magnetic stirrer for 48 hours. Cells were collected by centrifugation at 300 x g for 10 minutes, washed in 0.85% NaCl, and stored at 4C in 0.85% NaCl containing 0.3% formalin. At the time of use cells were washed four times with saline. The viability of cells from all four treatments was determined by culturing a sample on Sabouraud agar. Only the DOC-treated cells could be cultured. Trypan blue preparation of DOC-treated cells showed that 40-50% of the cells were viable. To each of 5 siliconized test tubes was added 1.0 ml of serum containing 5.0-6.0 x 10^6 untreated, autoclaved, DOC-treated, acetone-treated, or formalin-treated cells. Each tube received 0.5 ml of saline. A second set of 5 tubes was prepared in a similar manner except each tube received 0.5 ml of saline containing 2.0 mg of ACIA. To all tubes was added 1.0 ml of autologous leukocyte suspension. Phagocytosis experiments were initiated and the percent phagocytosis was determined as described previously.

CHAPTER III

RESULTS

Soil Studies

Table 5 contains the data obtained from a preliminary study on the effect of soil incubation on cells of strain CIA. During the first 30 days of incubation in soil, there was an increase in the number of viable cells from 0.79×10^7 to 4.2×10^7. The number of cells remained rather constant (approximately 4×10^7) for the next 270 days and then decreased to 2.8×10^7 cells after 500 days incubation in soil.

At the time of inoculation into soil, 100% of the cells possessed capsules with an average capsular diameter of 4.1μ. After 30 days incubation in soil the percent of nonencapsulated cells increased from zero to 66. The average capsular diameter of the remaining cells decreased from 4.1μ to 2.5μ. The percent of nonencapsulated cells reached a maximum of 93% after 100 days incubation in soil. The average capsular diameter of encapsulated cells decreased to 1.7μ during this time period. The percent nonencapsulated cells and the average capsular diameter of encapsulated cells remained essentially constant for the remainder of the experiment (500 days).

During the 500 days of incubation in soil, there was a reduction in the diameter of the cells, as well as in the diameter of the capsules. Initially, the average diameter of cells (excluding the

TABLE 5

EFFECT OF SOIL INCUBATION ON THE SIZE OF CELLS, CAPSULE, AND VIABILITY OF C. NEOFORMANS, CIA

Incubation Time (Days)	Viable Cell Count Per 5.0 gm Soil x 10^7	Percent Nonencapsulated Cells	Average Diameter of Cells (μ)	Average Capsule Diameter of Encapsulated Cells (μ)
0	0.79	0.0	5.2	4.1
30	4.20	66.0	4.2	2.5
70	4.20	88.0	3.7	2.2
100	5.40	93.0	3.7	1.7
160	4.30	88.0	3.6	1.9
300	3.70	90.0	3.3	1.6
500	2.80	91.0	3.2	1.8

capsule) was 5.2μ. After 70 days of incubation, the diameter of the cells decreased to 3.7μ. After 500 days incubation, the average diameter of the cells was 3.2μ.

From the results of this preliminary study it appeared that during soil incubation cells of C. neoformans remain viable, however there is a reduction in the diameter of the cells and capsules and a marked increase in the number of nonencapsulated cells.

In further studies, 8 different strains of C. neoformans were incubated in soil for 18 months. The results of this experiment are shown in Figure 3. During the first 2 months of incubation, there was approximately a two fold increase in the number of viable cells. The number of viable cells remained constant for the next 7 months and then slowly decreased. The average diameter of the cells decreased from 4.2μ to 3.2μ during the 18 months of incubation in soil.

After 5 days incubation in soil (Figure 4), the percent of nonencapsulated cells increased from 5 to 45. After 60 days of incubation in soil 85% of the cells were nonencapsulated. The percentage of nonencapsulated cells remained constant throughout the remainder of the experiment (540 days).

To determine whether the disappearance of capsule during soil incubation was due to a dehydration phenomenon, cells of CIA which had been incubated in soil for one year were suspended in distilled water and then used without further treatment, with chloroform treatment, or after autoclaving, as previously described. Initially, the percent encapsulated cells was 13. After 17 hours of incubation in high pH synthetic broth (HpH), Sabouraud broth, distilled water, or 0.1M glucose

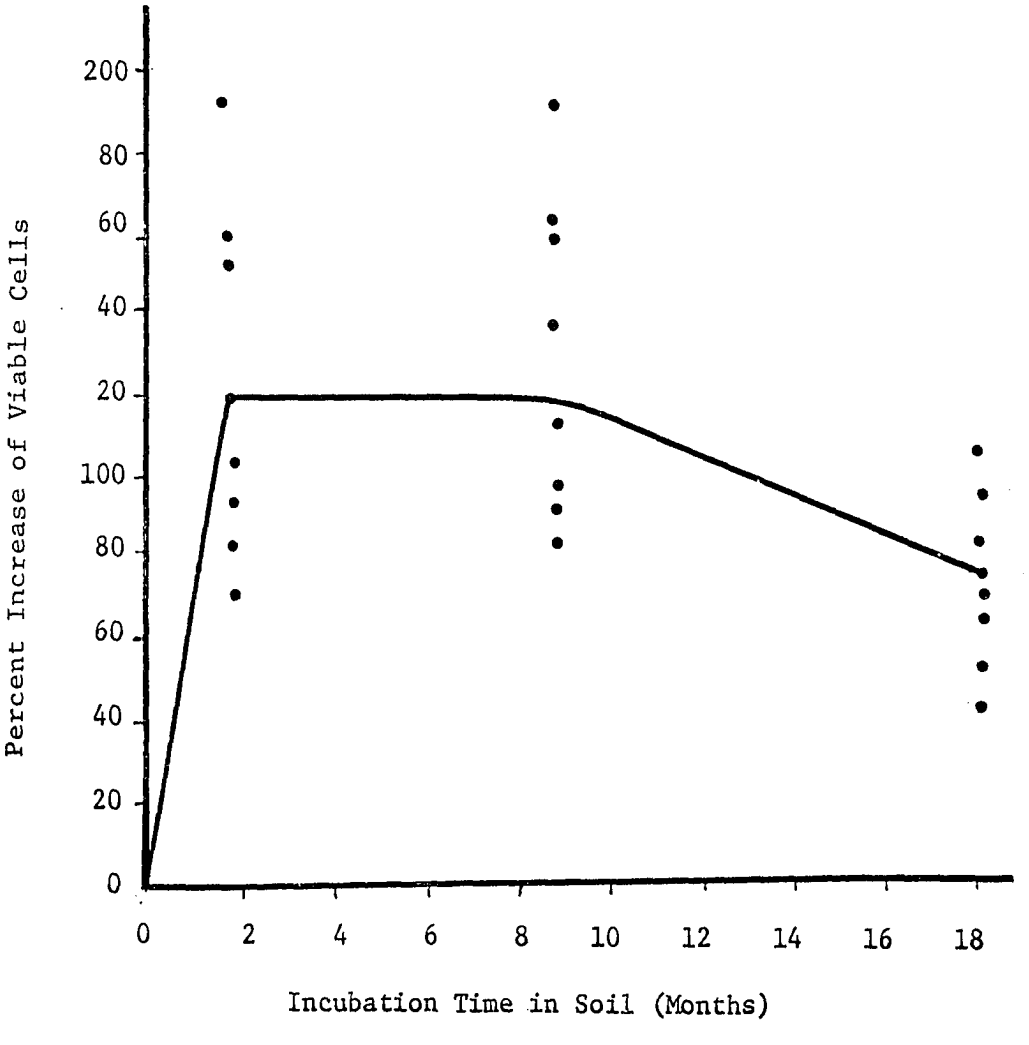

Figure 3--Effect of Soil Incubation on Viability of Eight Strains of C. neoformans.

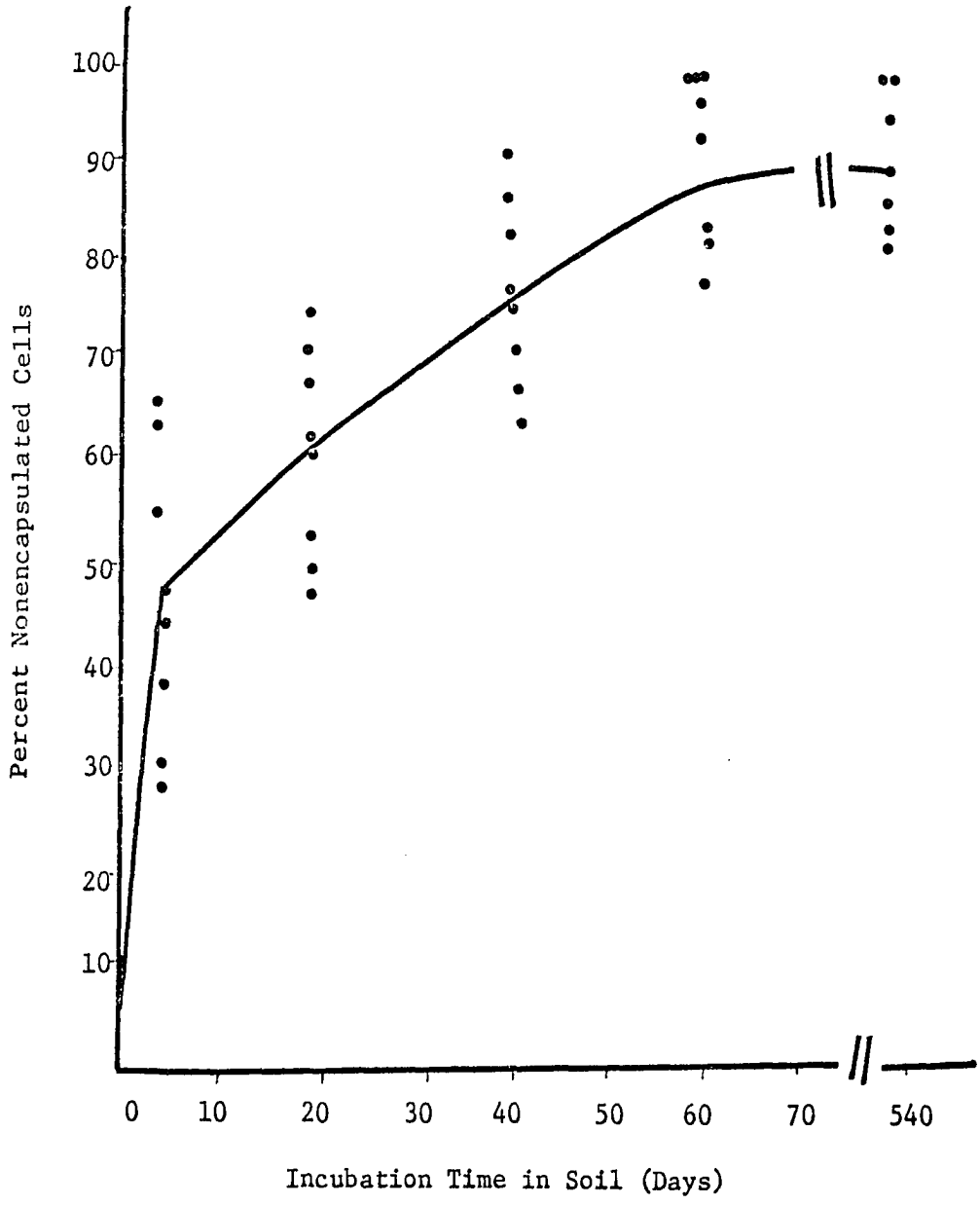

Figure 4--Effect of Soil Incubation on Capsule of Eight Strains of C. neoformans.

containing various concentrations of dinitrophenol (DNP), the percent of encapsulated cells was determined. Ninety-three, 91 and 63 percent of untreated cells were encapsulated in HpH, Sabouraud broth, and distilled water, respectively. At the lowest concentration of DNP (1 x 10^{-6}M), the percent encapsulation of untreated cells was about 70. In the highest concentration of DNP (1 x 10^{-4}M) only 30 percent of the cells were encapsulated. The chloroform treated or autoclaved cells shows no increase in the percent encapsulated cells in any of the media used.

Effect of Soil Incubation on Virulence of C. neoformans

After 0, 1, 6, 12, and 18 months incubation in soil, 1.0 ml of soil washings containing 0.79-4.3 x 10^6 cells were inoculated intraperitoneally (I.P.) into each of 5 mice. All mice died within four weeks after inoculation and had symptoms of cryptococcal meningitis. Large, encapsulated yeast cells were observed in brain tissues at autopsy. C. neoformans was isolated in pure culture from brain tissues of the infected mice. At the dosage used, there was no apparent reduction in virulence of the organisms during 18 months of incubation in soil.

Effect of Water on Capsular Production by C. neoformans Incubated in Soil

The effect of water on capsular production by cells of C. neoformans incubated in soil for one year was determined by adding various amounts of water to 5 gm soil samples containing the cells of strain CIA. The results are presented in Figure 5. There was no apparent increase in percent of encapsulated cells in the soil sample treated with

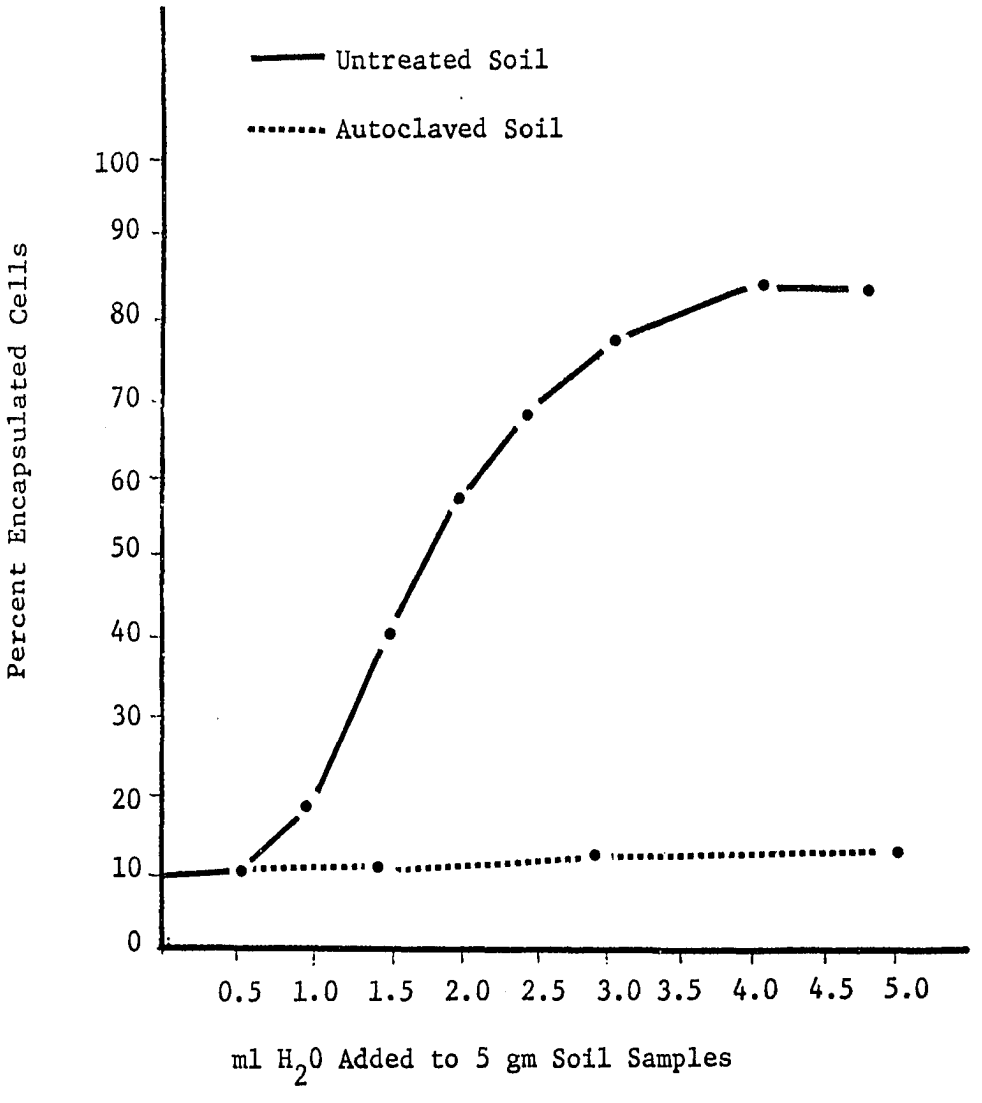

Figure 5--Effect of H_2O on Capsular Production by C. neoformans, CIA, Incubated in Soil for One Year.

0.5 ml of water. However, there was a direct relationship between the amount of water added and percent encapsulation in the range of 1.0 - 4.0 ml of water. The control samples, i.e., those autoclaved before the addition of water, demonstrated no increase in the percent of encapsulated cells.

Generation Time Studies of C. neoformans

When cells of CIA grown on Sabouraud agar were transferred into HpH broth, there was a lag period of at least 2 hours before the cells entered the logarithmic phase. The culture reached the stationary phase approximately 18 hours after inoculation. The generation time of the cells was 2.5 hours. Whereas, cells maintained in soil for one year had a 4 hour lag period when subsequently inoculated into HpH broth. During logarithmic phase which lasted approximately 18 hours, the generation time was 2.5 hours. Thus, the only difference in the growth cycles of the two types of cells was the longer lag period for cells incubated in soil.

Capsule Production by Nonencapsulated Cells of C. neoformans

Following cultivation on low pH synthetic agar (LpH) at 26C for 3-5 days, 90-95% of the cells of strain CIA are nonencapsulated. These cells were incubated in phosphate buffer (pH 5.0) overnight to reduce the endogenous metabolites and used to evaluate the effects of various compounds on capsule production (Figure 6). In the medium lacking carbohydrates no capsule was detected after 22 hours incubation. When glucose and ammonium sulfate were added, capsule production was observed during the first hour of incubation. After 8 hours incubation

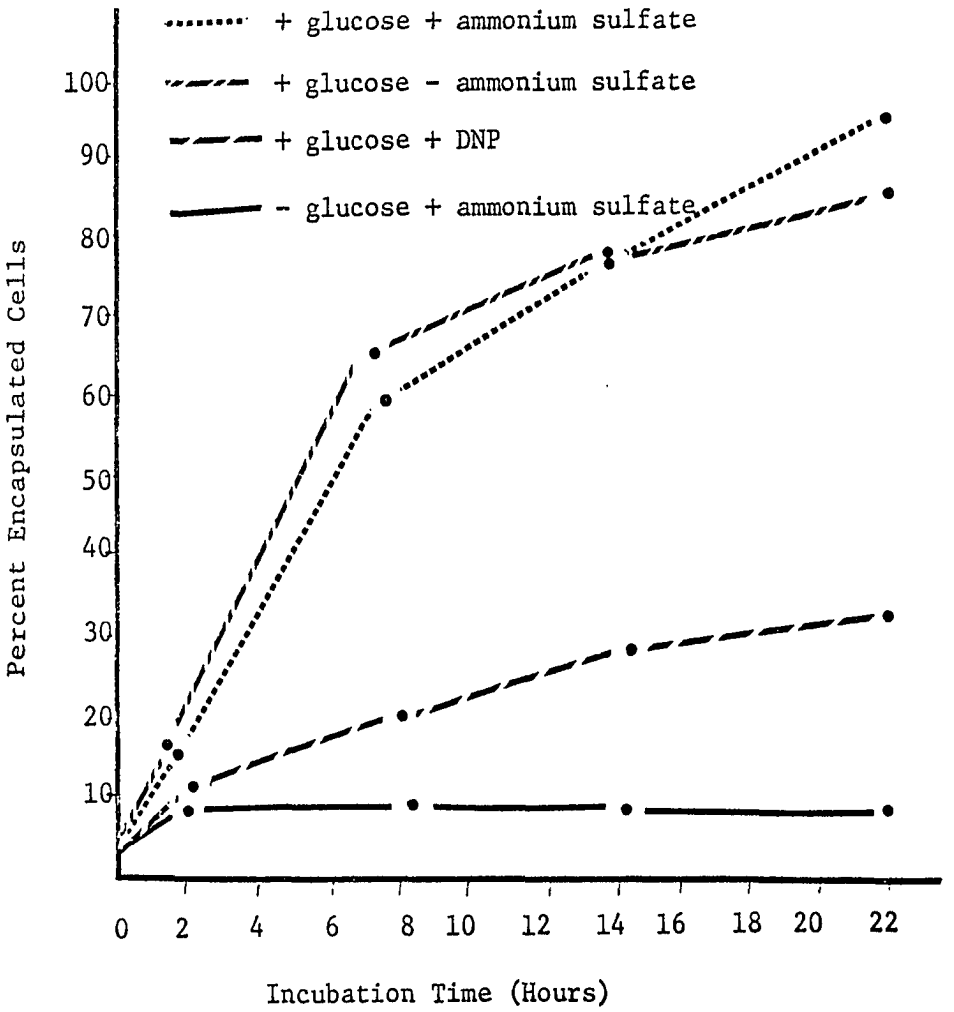

Figure 6--Capsular Production by Environmentally Induced Non-encapsulated Cells of C. neoformans, CIA.

approximately 65% of the cells were encapsulated and after 22 hours over 90% of the cells possessed capsules. During the initial 16 hours of incubation capsule production was almost as rapid with resting cells (i.e., cells in medium containing glucose but lacking ammonium sulfate) as with actively proliferating cells (i.e., cells in medium containing glucose and ammonium sulfate). After 22 hours incubation, 80% of the resting cells were encapsulated and 93% of the actively proliferating cells were encapsulated.

The addition of various concentrations of thiamine (0.0 - 10.0 µg/ml) to the media had no effect on the production of capsule during 22 hours incubation.

When DNP was added to medium containing glucose but no ammonium sulfate, the percent encapsulation was reduced from approximately 80 to less than 30. Sodium fluoride or potassium iodoacetate failed to inhibit capsule production.

Effect of Different Carbohydrates on Capsule Production of Resting Cells by C. neoformans

The ability of nonencapsulated resting cells of CIA to utilize carbohydrates for capsular production was studied. The carbohydrates chosen were those found as monomers in the capsular polysaccharide of C. neoformans, i.e. galactose, glucuronic acid (sodium salt), mannose, and xylose. Glucose was used as a control. After 18 hours incubation 70-76% of the cells were viable regardless of the media (Table 6). Capsule production occurred in the presence of all the carbohydrates but not in the medium lacking a source of carbon. Cells having the largest capsule, as well as largest percent encapsulation, were those

TABLE 6

EFFECT OF VARIOUS CARBOHYDRATES ON CAPSULE PRODUCTION BY RESTING CELLS OF C. NEOFORMANS, CIA*

Carbohydrate	Percent Encapsulation	Diameter of Capsule (μ)	Percent Viability
Galactose	65	2.1	75
Sodium Glucuronate	48	1.6	70
Mannose	70	3.8	71
Xylose	43	1.2	76
None	9	1.0	75
Glucose (control)	76	2.5	75

*Measurements were made after 18 hours of incubation at 25C. Initially only 7% of the cells were encapsulated.

incubated in mannose. Cells having the smallest capsule, as well as the smallest percent encapsulation, were those incubated in xylose and glucuronic acid.

pH Optimum for Capsular Production by Resting Cells of *C. neoformans*

Different buffers were used to study the pH optimum for capsular production. Using Walpole's acetate buffer (pH 3.6-5.6) inhibited capsule production by cells of CIA. Thus, McIlvaine's phosphate buffer (pH 2.2-8.0) was used for this study. After two hours incubation at 25C, maximum number of encapsulated cells was observed between pH 4.2 and 5.4 (Figure 7). About 40-43% of the cells had capsules. The average capsule diameter of the encapsulated cells was 1.5μ between pH 3.4 and 7.4.

After 24 hours incubation at 25C, the optimal pH range shifted to slightly higher values. Maximum capsular production was observed in the pH range 6.2-7.0. In this pH range, 77% of the cells possessed capsules. The average capsular diameter of encapsulated cells was approximately 3.0μ between pH 3.0 and 5.2, 4.0μ between pH 5.6 and 6.8, 3.0μ between pH 7.2 and 7.6, and 1.0μ at the higher pH levels. The trypan blue dye test revealed that 75% of the cells were viable in all tubes (pH 3.0-8.2) after 24 hours incubation.

Ability of *C. neoformans* to Degrade Cryptococcal Capsule

The ability of *C. neoformans* to utilize its own capsule was tested by inoculating nonencapsulated CIA cells into 50 ml of Bacto-Yeast Nitrogen Base supplemented with 1.0 mg/ml of isolated capsular

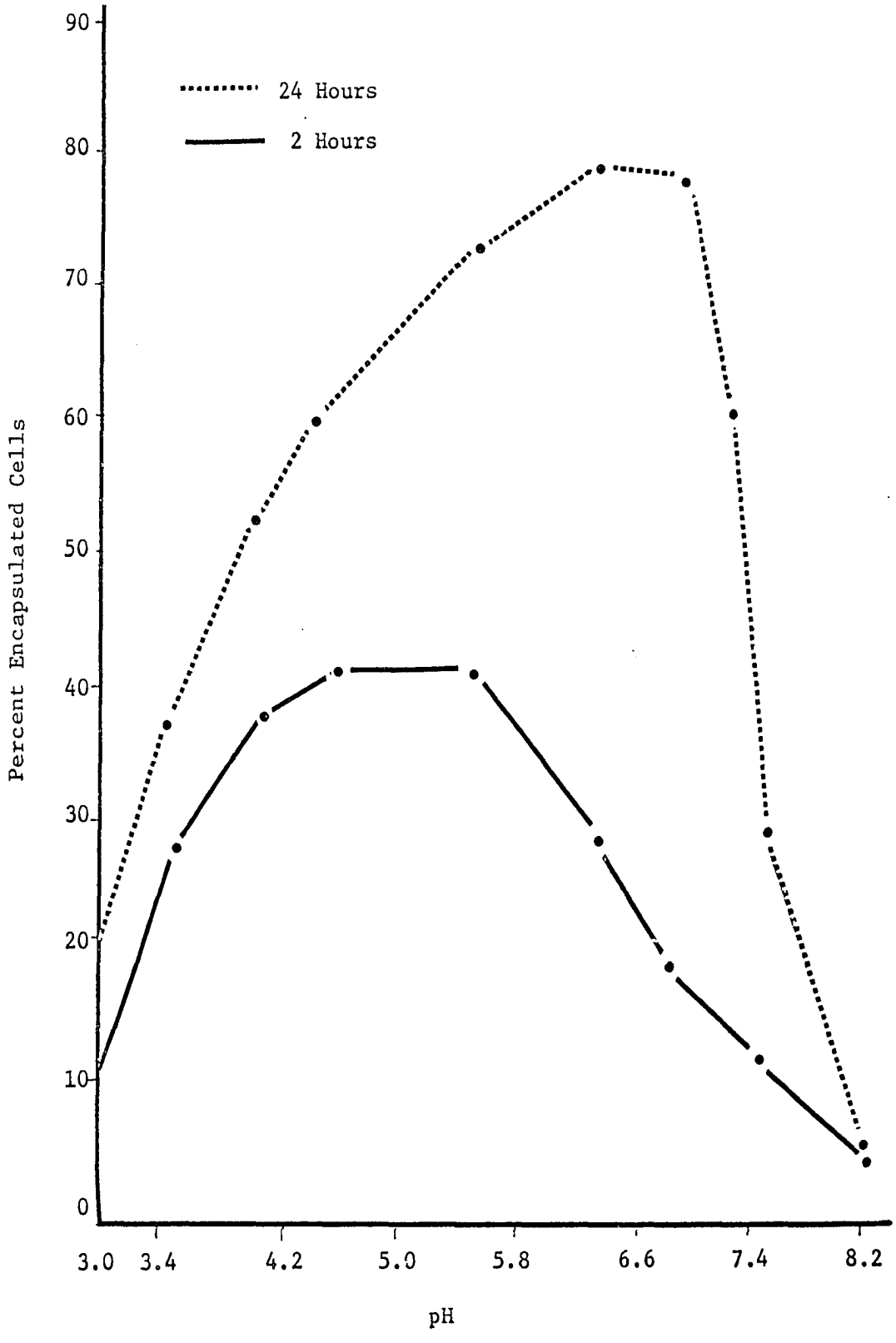

Figure 7--Effect of pH on Capsular Production of C. neoformans, CIA.

material (ACIA) as a sole source of carbon. At various times, the viability of the cells and the amount of nondialyzable polysaccharide present in the culture filtrate were determined. After 2 days incubation at 25C under aeration, cell numbers increased from 0.30×10^6 to 1.2×10^6 per ml. The number of cells increased to 2.0×10^6/ml during the remainder of the study (28 days). The amount of polysaccharide present in the culture filtrate decreased from 1.0 mg to 0.9 mg/ml within the first 2 days of incubation and remained almost constant for the duration of study.

Effect of Various Inorganic Salts on Capsular Production of C. neoformans

In preliminary experiments using cells of CIA grown on either LpH (nonencapsulated cells) or Sabouraud agar (encapsulated cells) and various concentrations of NaCl it was found that with increasing concentration of NaCl, the percent encapsulation decreased. Cells grew well in 5% NaCl but not in 10% NaCl. The data obtained from a more extensive study are presented in Table 7. Six encapsulated strains of C. neoformans cultured on HpH agar were inoculated into HpH or Sabouraud broths containing 5% concentrations of various salts. All six strains demonstrated a decreased ability to produce capsules in the salt solutions. In general, after two transfers into the salt solutions the percent encapsulation dropped to an average of 10. In the presence of NaCl, KH_2PO_4, and K_2HPO_4, all strains studied demonstrated less than 7% encapsulation. With $NaNO_3$ and KCl, all strains tested, with the exception of CS, demonstrated 10% encapsulation or less. In the presence of Na_2SO_4 and K_2SO_4, all strains tested demonstrated 16% encapsulation

TABLE 7

EFFECT OF INORGANIC SALTS ON CAPSULAR PRODUCTION
BY SIX STRAINS OF C. NEOFORMANS

Salts	Strain Percent Encapsulation CIA	A	B	CDC	CS	UH3
None	100	95	82	98	84	99
NaCl	1	0	5	3	4	0
$NaNO_3$	0	10	3	1	28	8
Na_2SO_4	1	4	16	6	51	11
K_2SO_4	1	5	10	1	54	32
KCl	3	1	10	1	14	0
KH_2PO_4	0	5	6	0	1	0
K_2HPO_4	2	0	4	1	1	0

or less, with the exception of CS (51% and 54%, respectively) and UH_3 (32% encapsulation in the presence of K_2SO_4).

Effect of Lactose on Capsular Production of C. neoformans

The results obtained during 7 days incubation of cells of CIA in HpH broth with various concentrations of lactose are shown in Figure 8. Capsular production decreased as lactose concentration increased. After several days, cells in all concentrations of lactose demonstrated less than 30% encapsulation. There was a slower increase in growth (turbidity) in cultures containing 30 and 40% lactose than in the others.

Capsular Production by C. neoformans on Various Laboratory Media

The ability of C. neoformans to produce a capsule on various laboratory media was studied using nutrient agar (NA), trypticase soy agar (TSA), Sabouraud agar, potato-dextrose agar (PDA), HpH, and Raper's complete agar. Table 8 contains the results of this study. With exception of CDC, none of the strains produced any visible capsules on NA and TSA. All of the strains produced varying amounts of capsule on all of the mycological media used. In general, the cells produced the largest capsules on PDA medium.

Capsular Production by C. neoformans in Mouse Tissue In Vitro

The ability of nonencapsulated cells of CIA to produce capsules when incubated in mouse blood, brain, and lung tissues was studied. After 24 hours incubation, the capsule diameter and percent encapsulated cells was less in the tubes containing lung or brain tissue in saline

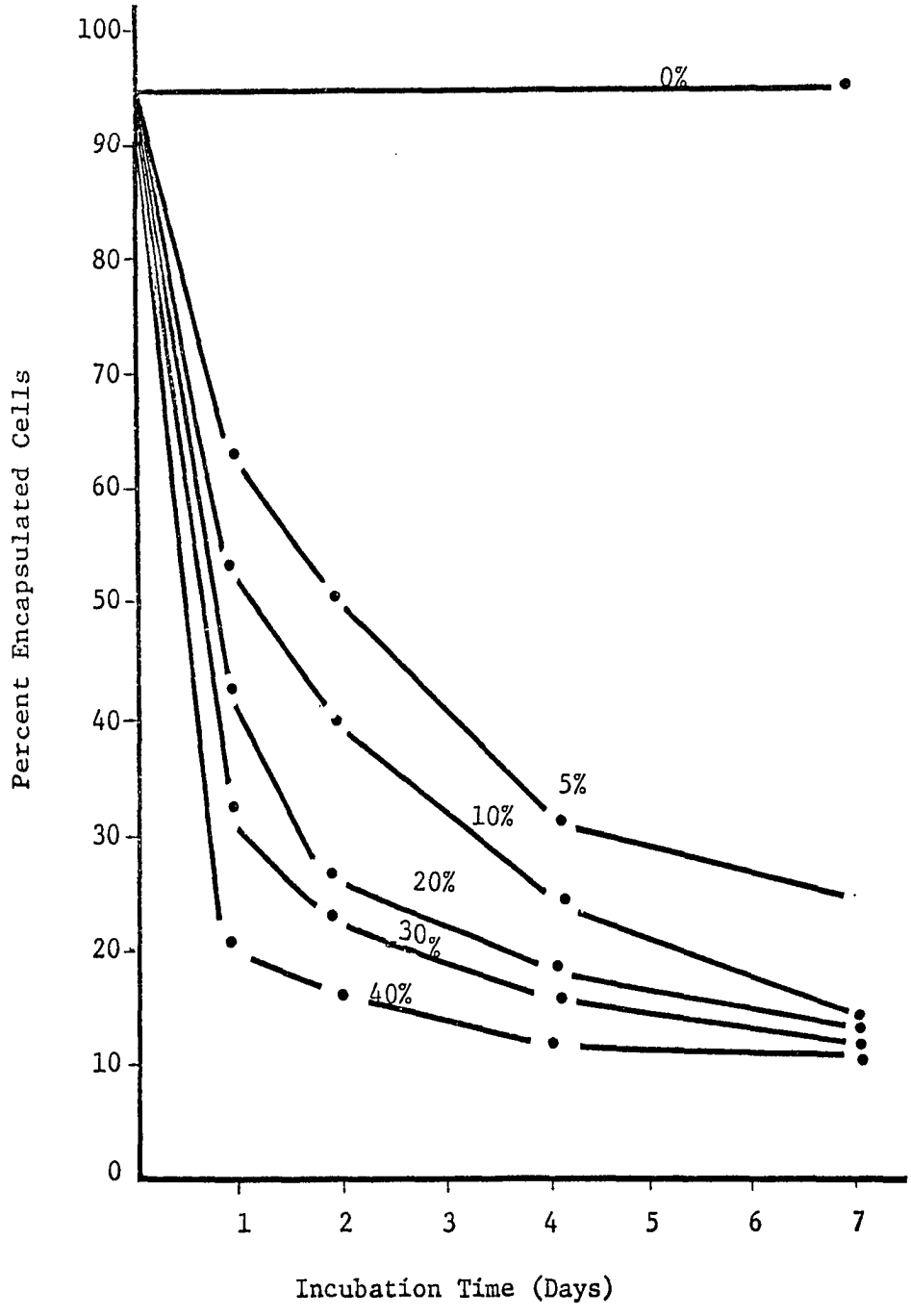

Figure 8--Effect of Various Concentrations of Lactose on Capsular Production by C. neoformans, CIA.

TABLE 8

CAPSULAR PRODUCTION BY EIGHT STRAINS OF C. NEOFORMANS ON VARIOUS LABORATORY MEDIA*

Strain	Medium					
	Nutrient Agar	Trypticase Soy Agar	HpH Agar	Sabouraud Agar	Potato-Dextrose Agar	Raper's Complete Agar
A	-	-	±	±	++	+
B	-	-	±	±	++	+++
CDC	-	±	++	++	+++	+++
CIA	-	-	+	+	++	+
CS	-	-	++	++	+++	+++
CS10	-	-	++	++	++	++
UH_1	-	-	+	±	++	+
UH_3	-	-	±	±	++	+

*-, no capsular production; ±, not all cells possess capsules and the average capsular diameter of encapsulated cells was less than 2μ; +, most cells were encapsulated with average capsular diameter of 4.0-4.5μ; ++, most cells were encapsulated with average capsular diameter of 4.5-5.0μ; +++, most cells were encapsulated with average capsular diameter of more than 5μ.

than in all other combinations (Table 9). The addition of blood to these tubes resulted in an increase in capsule diameter and percent encapsulation. After 66 hours incubation, only the cells suspended in brain and saline had a capsule diameter less than 4.0μ.

Isolation and Characterization of Nonencapsulated Mutants of C. neoformans, CIA

Cells of CIA were inoculated into Sabouraud broth and incubated at 25C without aeration. At various times, samples were removed and examined for the presence of stable nonencapsulated mutants. Nonencapsulated colonies were not observed until after the first 10 days of incubation. Thereafter, approximately 20 nonencapsulated colonies were isolated. Four of these organisms were chosen for further study and hereafter are referred to as S_1, S_2, S_3, and S_4. All four strains had the following characteristics in common:

1. Ability to utilize galactose, glucose, maltose, and sucrose, but not lactose, as a sole source of carbon.
2. Inability to utilize nitrate as a sole nitrogen source.
3. Ability to produce urease.
4. Ability to grow at 37C.

All four isolates varied in their ability to produce capsules and in virulence for mice (Table 10). Whereas S_1 and S_3 did not produce capsules under any circumstances, and were avirulent for mice, S_4 produced capsules most of the time and was virulent for mice. S_2 was intermediate in these characteristics. It did not produce capsules on laboratory media and when it was inoculated intraperitoneally, it was avirulent for mice. However, when it was inoculated intracerebrally it

TABLE 9

CAPSULAR PRODUCTION BY C. NEOFORMANS, CIA, IN MOUSE TISSUE IN VITRO

Constituents of Each Tube	Incubation Time (Hours)									
	0		18		24		48		66	
	CD[a]	PE[b]	CD	PE	CD	PE	CD	PE	CD	PE
Blood + Brain + Minimal Broth	1.0	5	4.2	95	4.2	95	4.2	95	4.2	95
Blood + Lung + 0.85% NaCl	1.0	5	2.8	92	3.5	95	4.0	95	4.0	95
Lung + 0.85% NaCl	1.0	5	1.25	65	1.0	70	2.8	95	4.0	95
Blood + Brain + 0.85% NaCl	1.0	5	2.8	92	3.0	92	4.0	95	4.0	95
Brain + 0.85% NaCl	1.0	5	2.3	60	2.5	63	2.5	95	3.0	95
Minimal Broth	1.0	5	4.0	95	4.0	95	4.0	95	4.0	95

[a]Average capsular diameter of encapsulated cells in microns.

[b]Percent encapsulated yeast cells.

TABLE 10

CHARACTERISTICS OF *C. NEOFORMANS*, CIA, AND MUTANTS S_1, S_2, S_3 and S_4

Characteristic	Organism				
	CIA	S_1	S_2	S_3	S_4
Capsule	+	-	±[a]	-	±[b]
Growth at 37C	+	+	+	+	+
Virulence I.P.[c]	+	-	-	-	+
Virulence I.C.	+	-	+	-	+

[a]Produced capsule *in vivo* (I.C. inoculation) but not *in vitro*.

[b]Segregated into encapsulated and nonencapsulated colonies, but eventually reverted to the encapsulated state.

[c]I.P. = intraperitoneal
I.C. = intracerebral

killed mice. The mice died after a longer incubation period (2-4 weeks) than those inoculated with the parental strain (1 week). Examination of brain tissue revealed the presence of encapsulated yeast cells. However, upon inoculation of brain tissue on Sabouraud agar, the cell isolates were nonencapsulated. Using mouse tissues suspended in saline or in broth (the same type of experiment as with CIA reported previously) and the S_1, S_2, and S_3 mutants, it was found that in the presence of brain, blood, or lung tissues, or combinations of these tissues, none of the mutants was able to produce visible capsules after 96 hours incubation.

In further studies of the mutants, their ability to grow in the presence of the monomers of cryptococcal capsule, i.e. galactose, glucuronic acid, mannose, and xylose, was examined. All of the mutants grew in the presence of any one, or combination of these carbohydrates, but under no circumstances did they produce visible capsules.

Transformation studies using dead encapsulated cells of the CIA strain and viable nonencapsulated mutants (S_1, S_2, and S_3) were negative. Two days post inoculation, a few (less than 10) nonencapsulated colonies were isolated from tissue homogenates of inoculated mice. No organisms were isolated from the infected animals thereafter. None of the animals showed any symptoms of cryptococcosis, i.e. bulging of cranium, or tumor-like masses in the liver and kidney, during the course of the experiment (20 days).

Characterization of Isolated Polysaccharides

Soluble polysaccharides of S_1, S_2, and CIA (SCIA) cells and adhered capsular polysaccharide of CIA (ACIA) were isolated by alcoholic

treatment of culture broths. The characteristics of the materials are shown in Table 11. The amount of protein, as determined by the micro-Kjeldahl method, was 1.2% or less. Nucleic acids, as determined by spectophotometric readings at 260 and 280 mμ and by paper chromatography, were present in trace amounts. At no time, were ribose or deoxyribose detected by chromatography of acid hydrolysates of these polysaccharides. The amount of carbohydrate present was determined by the Anthrone test, using mannose as a standard. S_1 contained 55.0%, S_2 contained 57.0%, SCIA contained 56.0%, and ACIA contained 66.5% carbohydrate assuming the capsule was made of pure mannan. The purity of S_1, SCIA, and ACIA polysaccharides was determined by ultra centrifugation technique. All three polysaccharides exhibited a single peak with calculated sedimentation coefficients of 1.265, 1.365, and 1.385, respectively. The molecular weight of these polysaccharides was estimated to be between 10,000 and 15,000.

On double diffusion plates, ACIA produced one very strong precipitin band, with possibly a weak second band when it was reacted with anticryptococcal antibody. SCIA and S_1 polysaccharides produced three precipitin bands. All three bands demonstrated partial identity with the strong band of ACIA.

Qualitative paper chromatography of an acid hydrolysate of each of the three polysaccharides indicated the presence of galactose, glucuronic acid, glucuronolactone, mannose, and xylose. Gas-liquid chromatography revealed the presence of the same monomers (Figure 9) except that it was difficult to determine whether or not galactose was present in ACIA, although it was detected in S_1 and SCIA. Galactose was detected

TABLE 11

QUALITATIVE ANALYSIS OF POLYSACCHARIDES ISOLATED FROM PARENTAL STRAIN CIA AND MUTANTS S_1 AND S_2

Type of Polysaccharide*	% Total Protein	% Total Nucleic Acid	% Total Carbohydrates
S_1	<1.0	Trace	55.0
S_2	<1.0	Trace	57.0
SCIA	1.2	Trace	56.0
ACIA	<1.0	Trace	66.5

*S_1 and S_2 soluble polysaccharides isolated from HpH culture broths of S_1 and S_2 mutants.

SCIA - Soluble polysaccharide isolated from HpH culture broths of CIA strain.

ACIA - Adhered capsular polysaccharide isolated by sonic oscillation from CIA cells cultured on Sabouraud agar.

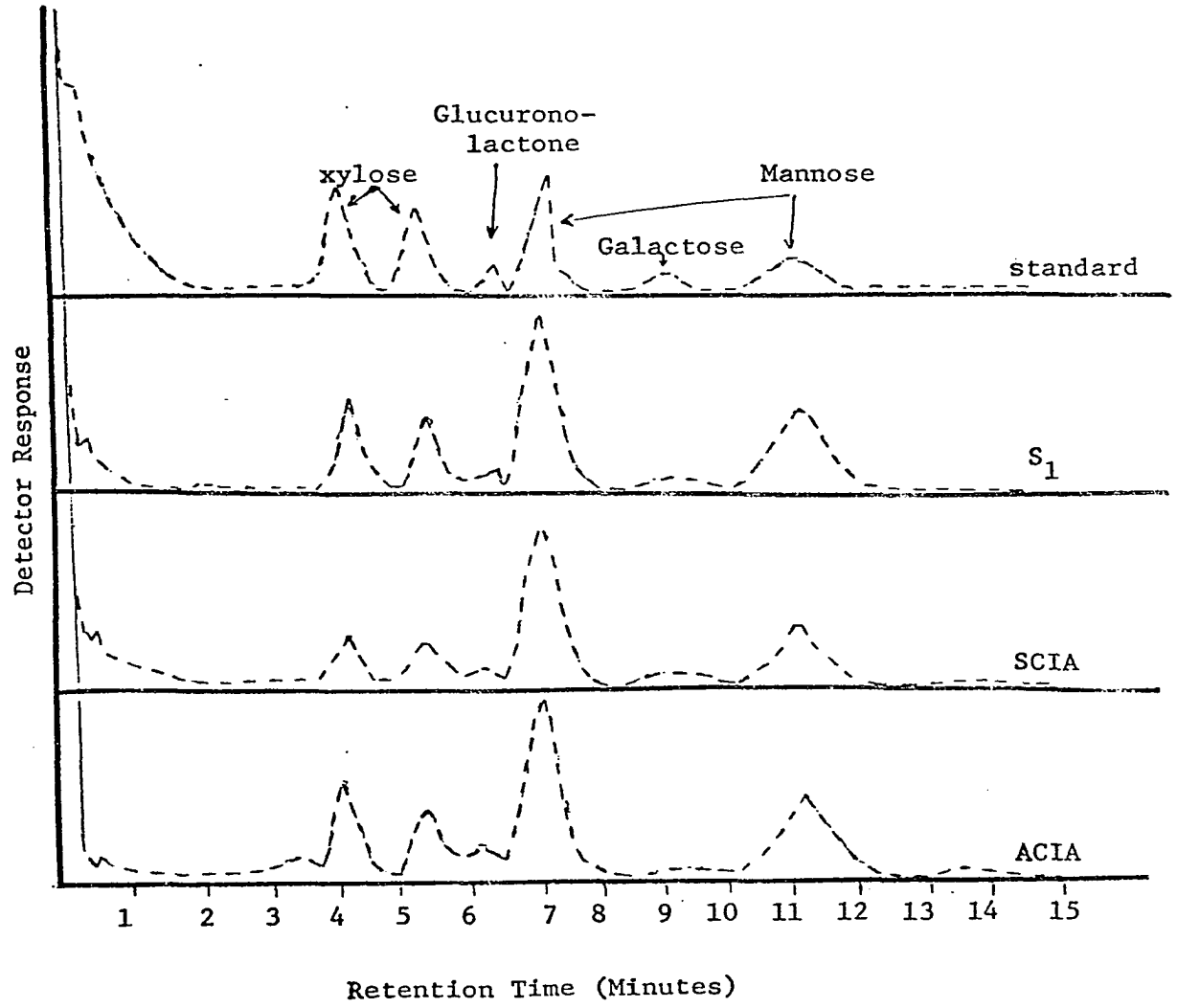

Figure 9--Gas Chromatographic Analysis of TMS Derivitives of Hydrolyzed S_1, SCIA, ACIA, and Known Carbohydrates.

with galactose oxidase in all three polysaccharides.

The rate of release of monomers during acid hydrolysis was similar in all polysaccharides. Maximum amounts of xylose were released within the first 3 hours, and the release of galactose reached a peak after 8 hours of hydrolysis. With ACIA, maximum amounts of glucuronic acid and glucuronolactone were detected after 6 hours of hydrolysis. The release of glucuronic acid and glucuronolactone from S_1 and SCIA was detected after 8 hours of hydrolysis.

The data from quantitative paper chromatography, using either the phenol sulfuric acid test or the aniline-phthalic acid stain, are shown in Table 12. The molar ratio of mannose to xylose to glucuronic acid to galactose was (9:4:3:2) found to be identical in the soluble polysaccharides (S_1 and SCIA). ACIA appeared to contain slightly more glucuronic acid.

Phagocytosis Studies

When cells of CIA grown on Sabouraud agar (large capsules) were used for phagocytosis studies *in vitro*, about 25% of the peripheral blood leukocytes phagocytized these cells. When cells of CIA grown on LpH medium (very small capsules) were used, about 79% of the leukocytes phagocytized them. This indicated, as was shown by Bulmer and Sans (1968), that capsular polysaccharide inhibits phagocytosis of cells of CIA.

Phagocytosis of Nonencapsulated *C. neoformans* Incubated with Human Lung Tissue *In Vitro*

Nonencapsulated cells of CIA grown on LpH agar were transferred into tubes containing small pieces of excised human lung tissue and saline. With increasing incubation time in human lung tissue from zero

TABLE 12

QUANTITATIVE STUDIES OF ACIA, SCIA, AND S_1 POLYSACCHARIDES

Type of Polysaccharide	Molar Ratio			
	Mannose	Xylose	Glucuronic Acid	Galactose
ACIA	9.0	4.0	3.5	2.0
SCIA	9.0	4.0	3.0	2.0
S_1	9.0	4.0	3.0	2.0

to 24 hours, the percent encapsulation increased from 5% to 80%. At the same time, the percent phagocytosis decreased (Figure 10). After 5-10 hours incubation in human lung tissue the percent phagocytosis was inhibited by 50%. The bar on the right (control) represents the percent phagocytosis of nonencapsulated cells of CIA when 0.85 mg/ml of isolated capsular material (ACIA) was added to the system. Approximately 20 hours incubation in exised human lung were required for the nonencapsulated cells of CIA to produce enough capsule to reduce phagocytosis from 75% to 24%.

Phagocytosis of Nonencapsulated *C. neoformans*, Incubated in Human Serum *In Vitro*

Nonencapsulated cells of CIA (grown on LpH agar) were incubated in human serum. As the incubation time in serum increased (Figure 11) the percent phagocytosis decreased. It required 8-10 hours incubation to reduce phagocytosis by 50%. After 24 hours, the percent phagocytosis was reduced to 31. The bar on the right (control) represents the percent phagocytosis of nonencapsulated cells of CIA when 0.85 mg/ml of isolated capsular material (ACIA) was added to the system. The India ink preparations of the cells after 24 hours incubation showed that less than 18% of the cells were encapsulated.

To locate the phagocytic inhibitory factor, CIA cells (0 cells) and serum (0 serum) were separated by centrifugation after 24 hours incubation. To these were added fresh serum (F serum) and fresh cells (F cells), respectively. The percents phagocytosis found in these systems were as follows:

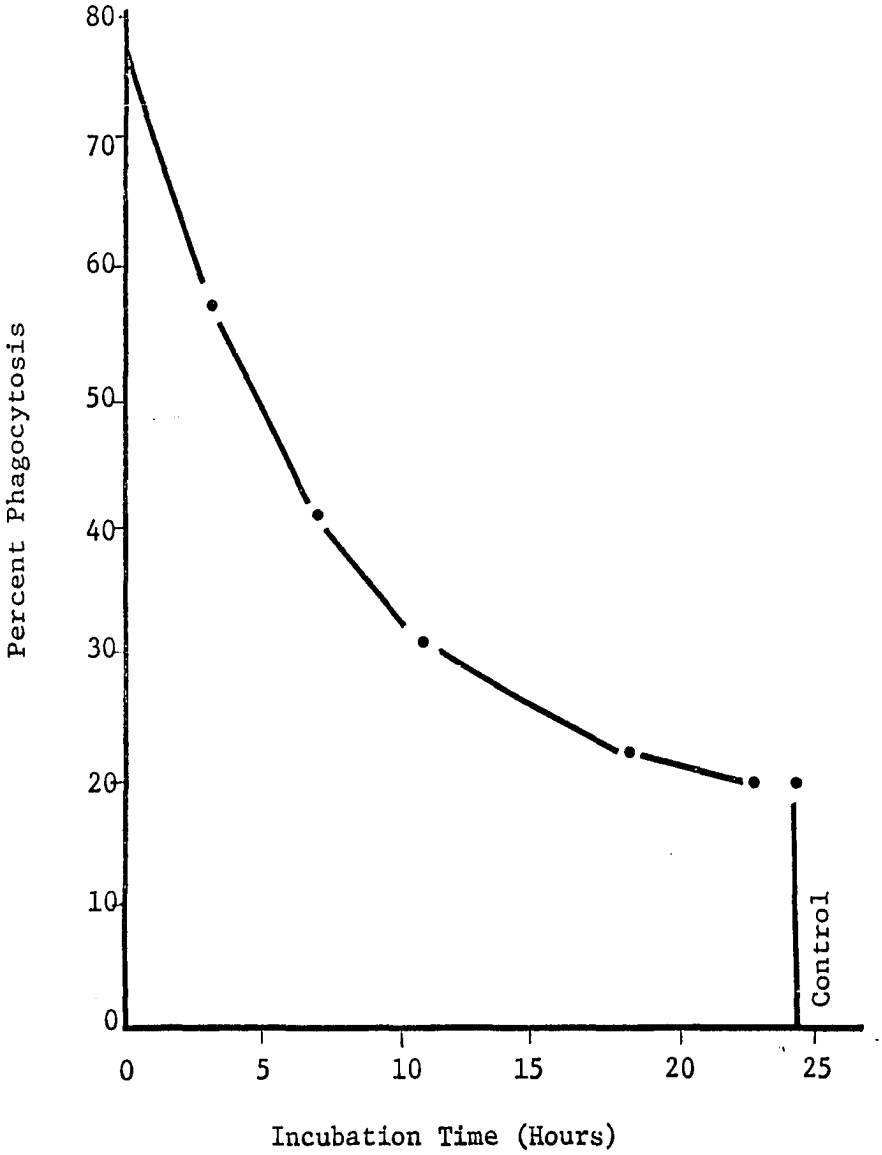

Figure 10--Percent Phagocytosis of Nonencapsulated C. neoformans, CIA, Incubated in Excised Human Lung Tissue for Varying Time Periods.

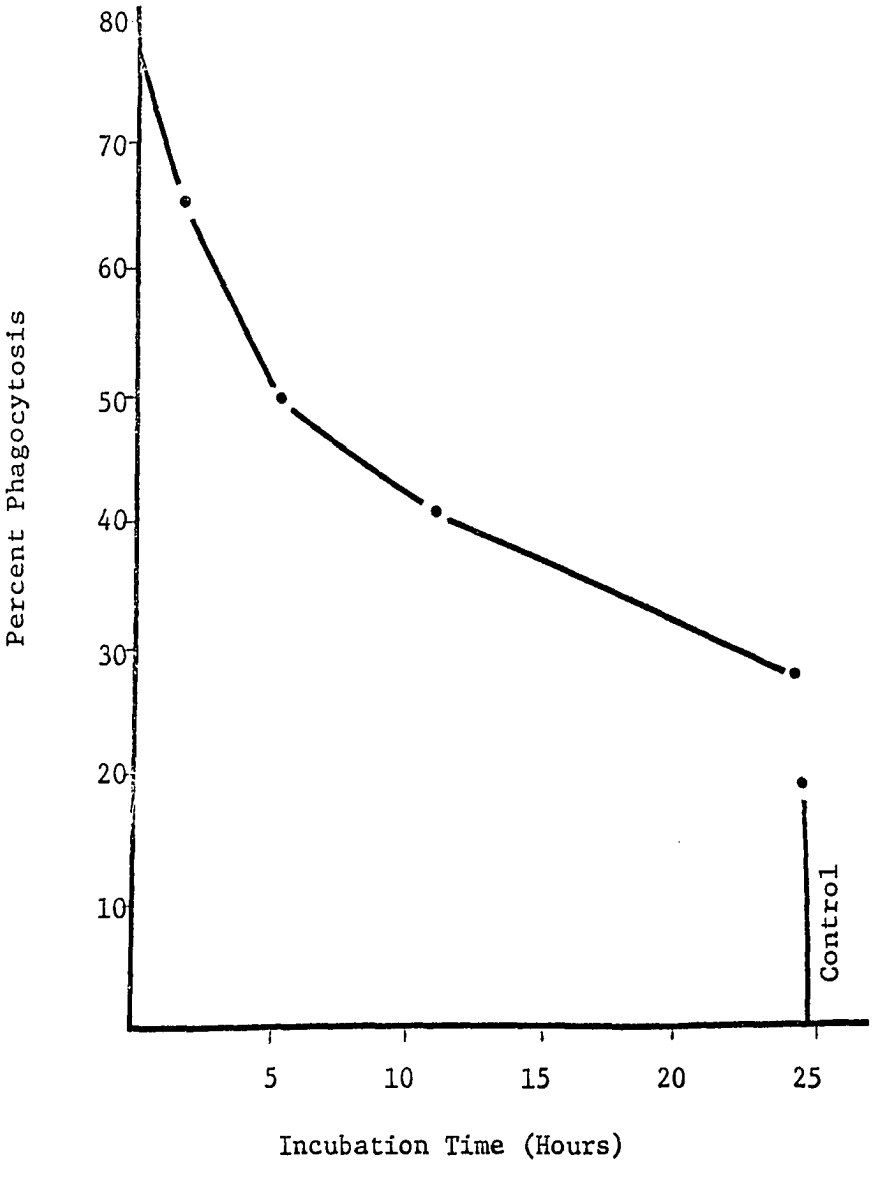

Figure 11--Percent Phagocytosis of Nonencapsulated C. neoformans, CIA, Incubated in Human Serum for Varying Time Periods.

0 cells + 0 serum, 31%

0 cells + F serum, 78%

F cells + 0 serum, 30%

F cells + F serum, 79%

F cells + F serum + 0.85 mg/ml ACIA, 28%

These results indicated that the inhibitory factor was located in the serum rather than on the cells. Since capsular polysaccharide is antiphagocytic, attempts were made to detect it in the serum. Cells of CIA were incubated in serum and after 24 hours were removed by centrifugation. The serum was examined immunologically and chemically for the presence of capsule. Immunologically, the presence of capsule was measured with nonabsorbed and absorbed (purified ACIA) rabbit anticryptococcal antibody. As shown in Table 13, the sera from 4 individuals reacted very strongly with nonabsorbed rabbit serum, while there was very little, if any, reaction with absorbed serum. This indicated that capsular material was present in the sera. Chemically, the presence of capsular polysaccharide was detected by the carbazole test in which the presence of hexuronic acid, and to some extent xylose, was measured. As it can be seen in Table 13, 0.58-0.66 mg of capsular material could be detected per ml of serum. The control represents serum which was not inoculated with cells of CIA.

Bulmer and Sans (1968) reported that isolated capsular polysaccharide is adsorbed by fresh nonencapsulated cells of CIA. Phagocytosis of the latter cells is then inhibited. To determine whether the inhibitory factor present in serum could be adsorbed, serum which had been incubated with cells of CIA for 24 hours (0 serum) was treated with

TABLE 13

IMMUNOLOGICAL AND CHEMICAL EVIDENCE FOR CRYPTOCOCCAL POLYSACCHARIDE IN HUMAN SERA

Experimental Subject	Precipitation Ring Test		Polysaccharide in Serum (mg/ml)
	Nonabsorbed Anticrypto-coccal Serum	Absorbed Anti-cryptococcal Serum	
1	+++	±	0.60
2	+++	±	0.66
3	++	-	0.58
4	+++	+	0.63
Control	-	-	0.00

fresh cells (F cells) for ten minutes and then the fresh nonencapsulated treated cells (FT cells) and the serum (OT serum) were used in phagocytosis studies (Figure 2). The percent phagocytosis found in these experiments was as follows:

0 cells + 0 serum, 30%

F cells + F serum, 75%

F cells + 0 serum, 30%

FT cells + F serum, 44%

F cells + OT serum, 41%

It thus appears that fresh nonencapsulated cells of CIA can remove a substance, probably polysaccharide, from serum, since serum treated cells were not as well phagocytized as untreated cells.

Comparative Phagocytosis Studies of *C. neoformans*, Strains CIA and S_1

Cells of the nonencapsulated mutant S_1 were tested in phagocytosis experiment and the percent phagocytosis of these cells was 80. This mutant produces a soluble polysaccharide which is identical with that produced by CIA. However, when this organism was incubated in serum for varying periods of time, up to 24 hours, there was very little reduction in the percent phagocytosis (from 80 to 66). Ring tests, using both absorbed and nonabsorbed rabbit serum, indicated the presence of capsule in the serum. When nonencapsulated cells of CIA were incubated in the serum, phagocytosis decreased from 78% to 22%. Similarly, serum in which cells of CIA had been incubated for 24 hours reduced the phagocytosis of nonencapsulated cells of CIA from 78 to 31%, whereas it reduced the phagocytosis of cells of S_1 from 80% to only 70%. A study was therefore designed to determine whether isolated cryptococcal

polysaccharides have the same effect on phagocytosis of cells of S_1 and CIA. The results obtained in this type of study are presented in Table 14. It will be seen that phagocytosis of nonencapsulated cells of CIA decreased to 26% with ACIA, to 39% with SCIA, and to 42% with S_1 polysaccharide. Phagocytosis of S_1 cells was 65% with ACIA, 70% with SCIA, and 71% with S_1 polysaccharide. The capsule from C. laurentii was used as a control to test for specificity of the C. neoformans capsule in inhibiting phagocytosis of CIA cells. This polysaccharide reduced the phagocytosis of nonencapsulated cells of CIA to 63%. Thus, it appeared that although phagocytosis of nonencapsulated cells of CIA was inhibited by C. neoformans capsule, the latter material had little effect on phagocytosis of the nonencapsulated mutant S_1.

Effect of Various Treatments of C. neoformans, CIA, on Inhibition of Phagocytosis by Human Leukocytes In Vitro

Nonencapsulated cells of CIA were treated with heat (autoclaved for 15 minutes), sodium desoxycholate, acetone, or formalin. Cells treated in each manner were used in phagocytosis studies. All of the cells were killed except those treated with DOC, in which case about 50% of the cells were viable. The results are presented in Table 15. With nontreated cells, the percent phagocytosis was 78% in the absence of cryptococcal polysaccharide and 26% in its presence. When heat, acetone, or formalin treated cells were used there was very little, if any, reduction in the percent phagocytosis in the presence of capsular material. When DOC treated cells were used, there was approximately a 40% inhibition of phagocytosis in the presence of capsule. It thus appears that killing or injuring the cells interferes with the inhibition of phagocytosis.

TABLE 14

PHAGOCYTOSIS OF C. NEOFORMANS, STRAINS CIA AND S_1, BY HUMAN LEUKOCYTES IN VITRO

Strain	Type of Capsule*	Percent Phagocytosis
CIA	--	79
S_1	--	80
CIA	ACIA	26
S_1	ACIA	65
CIA	SCIA	39
S_1	SCIA	70
CIA	S_1	42
S_1	S_1	71
CIA	CL	63

*ACIA: Adhered CIA polysaccharide
SCIA: Soluble CIA polysaccharide
S_1: Soluble S_1 polysaccharide
CL: C. laurentii polysaccharide

TABLE 15

EFFECT OF VARIOUS TREATMENTS OF C. NEOFORMANS, CIA, ON INHIBITION OF PHAGOCYTOSIS BY HUMAN LEUKOCYTES IN VITRO

Cells of CIA Pretreated With	ACIA in NaCl	NaCl	Percent Phagocytosis
—	-	+	78
	+	-	26
Heat (Autoclaved)	-	+	75
	+	-	63
Sodium Desoxycholate	-	+	72
	+	-	44
Acetone	-	+	87
	+	-	84
Formalin	-	+	91
	+	-	90

CHAPTER IV

DISCUSSION

Until recently, when Ishaq, et al., (1968) speculated that Cryptococcus neoformans may reside in soil in a nonencapsulated state, it was generally accepted that C. neoformans was found only in the encapsulated state and that the infectious particle consisted of an encapsulated yeast 10-20 microns in diameter.

In the present study, information concerning the state of C. neoformans in soil was obtained using eight different strains of the organism. It was found that the cells remained viable in soil for at least 18 months. A more interesting aspect of the findings was that an increase in the number of nonencapsulated cells occurred during incubation in soil. The number of nonencapsulated cells increased rapidly during the first two months of incubation and thereafter remained fairly constant at 85%. It is possible that the remaining 15% encapsulated cells were not viable at the time of inoculation into soil, or died soon thereafter before losing their capsules. The loss of capsule appears to correlate directly with an increase in number of viable cells (Figures 3 and 4). Evans (1960) was able to demonstrate a reduction of cryptococcal capsule during short term exposure to dehydrating substances. This phenomena did not seem to apply during soil incubation because: (1) encapsulated dead cells did not lose capsule during 18 months of soil

incubation; (2) nonencapsulated cells, which had been incubated in soil for one year, did not produce capsules when they were killed before removal from the soil; (3) nonencapsulated cells produced capsules only when actively metabolizing; and (4) capsular production is energy dependent. It thus appears that both the appearance and disappearance of capsule require metabolizing cells, and a dehydration-rehydration phenomenon is not responsible.

If the disappearance of capsule is not based on a dehydration phenomenon, then, it seemed possible that the cells are actually able to utilize their own capsule as an energy and carbon source, i.e., the capsule may act as an extracellular storage compartment. When isolated cryptococcal polysaccharide was used as the sole source of carbon for nonencapsulated cells of C. neoformans, the polysaccharide decreased from 1.0 mg to 0.9 mg/ml in the culture filtrate within the first two days of incubation. During the same period there was a four-fold increase in the number of viable cells. The concentration of polysaccharide did not decrease further after the first two days of incubation. Under the experimental conditions used, little evidence was obtained to substantiate this proposal, however, this phenomenon has been reported in other organisms. Several investigators (Davis, Rhodes, and Shulke, 1965; Buck, et al., 1968; and Szaniszlo, Wirsen, and Mitchell, 1969) demonstrated degradation and utilization of polysaccharides by the organisms which produced them. Szaniszlo, et al., (1969) reported that Leptosphaeria albopunctata, a filamentous fungus which produces a polysaccharide containing a large amount of glucose and a small amount of mannose, apparently utilized its own capsular polysaccharide.

Since it was not apparent that cells of C. neoformans were able to utilize capsular material, the effect of various salts on capsular production was studied. Salts, especially NaCl, affect the production of different macromolecules in bacterial cells, although the mechanism of their action is not known (Smithies, Gibbons, and Bayley, 1955; Brown, 1961; Brown, 1964). Thus, it was not surprising to find that all of the salts tested in the present study inhibited capsular production of C. neoformans (Table 7). Since addition of water to soil samples initiated capsular production (Figure 5), it would appear that the presence of salts in soils may reduce the activity of water, thus inhibiting capsular production. In other words, disappearance and reappearance of capsule may be controlled by the amount of active water present in the system. To test this hypothesis, another means was used to reduce the water activity of the system. It has been reported that several species of yeasts and filamentous fungi are able to grow in environments of low water activity, that is in the presence of high sugar concentrations (Anand and Brown, 1968). Sucrose and fructose were used as carbohydrates of choice by these investigators. However, since C. neoformans utilizes these carbohydrates, but not lactose, the latter was used in the present investigation. It was found that C. neoformans is able to grow in the presence of 40% lactose in a high pH synthetic medium (HpH). Although the growth of the cells was slower in this concentration of lactose than in concentrations of 10% or less, there was a decrease in the number of encapsulated cells. Since salts and lactose diminish the water activity in a given system, it seems possible that the decrease in water activity may be partially responsible

for the disappearance of capsule of C. neoformans in soil.

Another interesting finding was the effect of soil incubation on the size of the cell. Concurrently, with an increase in the number of nonencapsulated cells, the average diameter of the cells decreased from 4.2μ to 3.2μ. The fact that the average number of nonencapsulated cells increased to 85% during the course of the experiment and the average size of the cells decreased to 3.2μ indicates that the overall average diameter of the particles of C. neoformans in soil is approximately 3.2μ for the eight strains used in this investigation. In other words, the infectious particle of the organism probably resides in soil in a relatively small, nonencapsulated state. It has been shown (Hatch, 1961) that the highest probability for deposition of inhaled particles in the respiratory spaces of the lung occurs within the size range of 1-2μ in diameter. As the particle size increases, penetration and deposition in the lungs decreases simply because fewer particles escape trapping in the upper respiratory. With particles larger than 10μ in diameter penetration into the lung is practically zero. In light of this, it is difficult to imagine how C. neoformans could lodge in human lungs if the infectious particles are 10-20 microns in diameter, as generally accepted. Results of these investigations indicate that the dissemination and infectious particles are less than 4 microns in diameter. If this measurement is a true reflection of all strains of C. neoformans, it becomes much easier to accept the respiratory tract in man as the principal portal of entry.

The loss of a capsule, and the small size of cells of C. neoformans in soil, would be of little significance if there were a

simultaneous and parallel decrease in the virulence of the organism. At the dosage used (0.79 - 4.3 x 10^6 cells) there was no apparent decrease in the virulence of C. neoformans isolated from soil. Ishaq (1965) reported a slight decrease in the virulence of one strain of C. neoformans ATCC 11239. This discrepancy may be due to strain differences or to the size of the inocula used for infecting animals.

It can be postulated that the presence of salts and lack of water activity in soil inhibits capsular production. In addition, when the cells divide, the newly formed daughter cells do not possess capsules. Due to as yet unknown environmental factors these cells are smaller in size. When the endogenous supply of carbohydrate is depleted, as can be demonstrated by a long (8 hour) lag phase once the organisms are transferred into suitable medium, the cells remain viable and virulent in soil, as do the spores of other fungi and bacteria. It is in this state that they are probably disseminated.

If C. neoformans resides in soil in a nonencapsulated state, and since the importance of capsule as a virulence factor has been demonstrated (Bulmer, et al., 1967), the time and conditions required for capsular production take on a new importance. Since the isolation of nonencapsulated cells from the soil was not mechanically feasible, cells were grown on low pH synthetic medium (LpH) to inhibit capsular production. Such nonencapsulated cells of strain CIA were able to produce visible capsules in the presence of glucose and essential salts. Capsular production by these cells, as with those incubated in soil, was energy dependent, since the addition of dinitrophenol (DNP), an uncoupling agent of oxidative phosphorylation (Mahler and Cordes, 1966),

decreased the production of capsule. Thus, it appears that glucose acts as both a carbon and an energy source. Similar findings have been reported with Diplococcus pneumoniae, which can synthesize capsular polysaccharide in the resting state in the presence of glucose and salts (Bernheimer, 1953).

Attempts to inhibit glucose utilization by sodium fluoride (NaF) and potassium iodoacetate (KIAc) were unsuccessful. At the concentration levels used, these inhibitors had no effect on either the growth of the cells or on capsular production. It is possible that these inhibitors were not able to enter the cells.

Thiamine and glutamic acid had no effect on capsular production by C. neoformans within the first 24 hours incubation with either resting or growing cells. Although Reid (1949) reported that C. neoformans requires thiamine for growth and Littman and Zimmerman (1956) showed that the largest amount of capsule is produced in synthetic media containing glutamic acid and thiamine, it should be noted that their investigations were long term growth studies and it is very difficult to completely deplete the intracellular levels of these compounds in short term (i.e., 24 hours) studies.

The pH optimum for capsular production after 2 hours of incubation was in the acidic range (4.6 - 5.8), and after 24 hours of incubation maximal capsular production occurred at a more neutral pH, approximately 7.0.

Spontaneous nonencapsulated mutants of C. neoformans were isolated from the parental encapsulated strain, CIA. At least 20 different nonencapsulated colonies were detected but only four were chosen

for further studies. Although mutant strains showed the same biochemical pattern as the parental strain, they varied in their ability to produce capsules (Table 10). Mutants S_1 and S_3 did not produce capsules under any of the experimental conditions used, either *in vivo* or *in vitro*. These two mutants were avirulent for mice. Mutant S_4, on the other hand, produced capsules both *in vivo* and *in vitro*. However, the encapsulated cells segregated into encapsulated and nonencapsulated cells and eventually all of the cells had capsules, at which time this mutant behaved in a manner similar to that of the encapsulated parental strain.

Mutant S_2 produced capsules and killed mice when it was inoculated intracerebrally, but not when it was inoculated intraperitoneally. Since the peritoneum contains a large number of highly active phagocytes, especially after provocation, the organisms probably were phagocytized soon after intraperitoneal inoculation, whereas in the brain, with its lower phagocytic activity, the cells had sufficient time, and the required nutrients, to produce capsules and thereby inhibit phagocytosis even further. It should be mentioned that, whereas the parental strain, CIA, produced capsules in mouse tissue both *in vitro* and *in vivo*, S_2 cells did not produce capsules in the presence of mouse tissues *in vitro*. Capsular production by S_2 cells in mouse brain *in vivo* was not due to the back mutation of these nonencapsulated cells to their original encapsulated state since the cells of S_2 were encapsulated *in vivo*, they were nonencapsulated when the infected tissue was cultured onto laboratory media.

Several mycological and bacteriological media were used to culture various encapsulated strains, as well as nonencapsulated mutants

of C. neoformans. With one exception, none of the strains produced capsules on bacteriological media. Thus, such media as nutrient agar and trypticase soy agar cannot be used to detect the presence of encapsulated yeasts. Whereas all of the encapsulated cells produced capsules on mycological media, S_2, a virulent mutant which produces capsules in vivo, did not produce capsules on any of the bacteriological or mycological media. These findings indicate that upon culturing clinical specimens, C. neoformans may be encountered in either the encapsulated or the nonencapsulated state.

Although mutants S_1 and S_2 did not produce capsules in vitro, they produced a soluble polysaccharide when they were cultured in a liquid medium. The parental strain (CIA) produced capsules, as well as a soluble polysaccharide, when it was cultured in liquid media. Soluble polysaccharides isolated from the broth cultures by ethanol precipitation contained galactose, glucuronic acid, mannose, and xylose. The fact that the polysaccharide from the mutants contained the same monomers as those identified in the parental strain, plus the fact that the mutants were able to utilize monomers of the capsule as a carbon source, suggest that the mutation occurred at a site which controls the attachment of the polysaccharide to the cell wall rather than at one of the sites which controls the interconversion of the sugars.

Further chemical and serological studies of S_1 and soluble CIA (SCIA) polysaccharides showed them to be identical. There was a slight difference between soluble polysaccharides (S_1 and SCIA) and capsular polysaccharide (ACIA) of the parental strain. This difference, was detected both serologically and chemically. Serologically, the soluble

polysaccharides showed three precipitation bands on double diffusion plates, while ACIA demonstrated one very strong precipitation band, and possibly a second band. It should be noted that the three precipitation bands of the soluble polysaccharides were partially identical with that of ACIA. Chemically, ACIA contained a larger amount of glucuronic acid. This suggests that glucuronic acid may play a role in attachment of polysaccharide to the yeast cells. S_1, SCIA, and ACIA polysaccharides had similar sedimentation coefficients in the ultracentrifuge. Under the conditions used, the molecular weights of these polysaccharides were estimated to be between 10,000 and 15,000.

Differential hydrolysis of Type A cryptococcal capsular polysaccharide (ACIA), as well as the soluble (SCIA) and S_1 polysaccharides, indicated that mannose was liberated slowly from the polysaccharide, while xylose was liberated rapidly, suggesting that xylose is located on the external side chains of the polysaccharide and that mannan forms the backbone.

While the same monomers, galactose, glucuronic acid, xylose, and mannose, were found in Type B cryptococcal capsular polysaccharide (Blandamer and Danishefsky, 1966), the molar ratios of these monomers varied with the different serologic types of cryptococcal polysaccharide.

The molar ratio of mannose to xylose to glucuronate to galactose was 6:4:2:1 in Type B polysaccharide (Blandamer and Danishefsky, 1966), whereas it was 9:4:3:2 in Type A soluble polysaccharide and 9:4:3.5:2 in Type A adhered polysaccharide. These findings support the hypothesis proposed by Evans and Mehl (1951) that the type specificity of cryptococcal polysaccharide is due to quantitative rather than

qualitative differences in the monomers.

The results of *in vitro* phagocytosis studies of *C. neoformans* in its encapsulated and nonencapsulated states, using human peripheral leukocytes, once more emphasized the importance of the capsule as an antiphagocytic substance. The results of phagocytosis studies in which nonencapsulated cells of *C. neoformans* were incubated with human lung tissue indicated that, after 5-10 hours incubation, enough capsular polysaccharide is produced to inhibit phagocytosis by 50%. As the incubation time in lung tissue increased, the percent encapsulation of the yeast cells increased and the percent phagocytosis decreased. Within 24 hours, enough capsule was present to inhibit phagocytosis by 64% (Figure 10). During incubation in serum, the same pattern of inhibition of phagocytosis was detected. Although phagocytosis was not inhibited as much as in lung tissue, the percent phagocytosis was reduced by about 60% after 24 hours incubation in serum (Figure 11). However, few encapsulated cells were observed in India ink preparation. It then became of interest to determine whether phagocytosis was inhibited by a substance(s) that was produced by the cells and adsorbed to them, but which could not be visualized microscopically, or a substance(s) produced by the cells and excreted into the serum. Further studies indicated that the material was produced by the cells and excreted into the serum. Chemical and immunological studies of the serum revealed the presence of cryptococcal polysaccharide (Table 13). Thus, it appears that whereas visible capsular polysaccharide seemed to be responsible for inhibition of phagocytosis in lung tissue, soluble polysaccharide was the responsible factor in serum.

The soluble polysaccharide responsible for the inhibition of phagocytosis apparently must attach itself to the yeast cells to be effective (see below). If this is so, then one wonders why the cells incubated in serum for 24 hours, centrifuged, washed, and suspended in fresh serum did not inhibit phagocytosis. One explanation for such a finding may be that during the repeated washing the polysaccharide was eluted from the cells and therefore was not present during the actual phagocytosis experiments.

Although soluble polysaccharide can inhibit phagocytosis, and the S_1 mutant can produce a soluble polysaccharide quite similar to that of SCIA, the former organism was not virulent for mice. One possibility is that this organism is able to produce soluble polysaccharide in vitro only on suitable media. However, whereas incubation of these cells in human serum for 24 hours did not inhibit phagocytosis of S_1 cells, immunological studies (precipitin ring test) with the serum revealed the presence of capsular material. Although the serum had no inhibitory effect on phagocytosis of S_1 cells, it inhibited the phagocytosis of CIA cells. Further, serum which had been incubated with CIA cells inhibited the phagocytosis of this strain of C. neoformans, but it had no inhibitory effect on phagocytosis of the S_1 strain. The reason for this might have been that (1) cells of the S_1 strain adhered together more so than the cells of the CIA strain, thus allowing for better surface phagocytosis or (2) a "receptor site", i.e., point of attachment of polysaccharide to the yeast cell, was missing or altered on the surface of the S_1 cells. It was shown by Bulmer and Sans (1968) that isolated polysaccharide adheres to cryptococcal cells and thus inhibits phagocytosis. In the present study, it was shown that fresh, nonencapsulated cells of CIA are

able to remove portions of the inhibitory factor from the serum which had been incubated with CIA for 24 hours. These findings support the hypothesis that the S_1 mutation occurred at a locus which is involved in the formation of the receptor sites. If the cells contained receptor sites which are the point of attachment for the capsule, it seems possible that they could be destroyed. The results presented in Table 15 indicate that killing the cells under certain conditions alters the ability of the capsule to inhibit phagocytosis. Thus, the receptor site hypothesis seems to be the most feasible and satisfactory explanation for the high degree of capsular specificity.

It now seems possible to correlate some of the information already known about C. neoformans with that obtained from these investigations and suggest a mechanism for the pathogenesis of cryptococcosis. The infectious particle of C. neoformans is probably a nonencapsulated yeast less than 4 microns in diameter. Without its capsule, dissemination of the organism by air currents would be greatly facilitated. This concept also makes it easier to accept the respiratory tract as the portal of entry, since few particles over five microns in diameter are capable of entering the human lung (Hatch, 1961).

Once in the lungs, the organism is probably phagocytized and killed (Bulmer and Sans, 1967; Bulmer and Sans, 1968; Bulmer, et al., in preparation) or destroyed by serum factors (Baum and Artis, 1961; Baum and Artis, 1963; Bulmer, et al., in preparation; Igel and Bolande, 1966; Szilagyl, Reiss and Smith, 1966; Zimmerman and Rappaport, 1954). It seems possible that certain individuals develop cryptococcosis because of an underlying deficiency of their immunological system. Such

a deficiency, induced either naturally or artifically (Bennington, Haber, and Morgenstern, 1964; Brunning, Foley and Fortuny, 1963), could afford adequate time for capsular production, which in turn would inhibit phagocytosis. In other instances, individuals may inhale a large enough dose of yeast cells to create a locus in the lung. The time required to remove all of these cells would allow some of them to produce enough capsular material to inhibit phagocytosis. From in vitro studies, it appears that 5-10 hours are required for nonencapsulated cells to produce enough capsule to inhibit phagocytosis by 50%. Thus, the size of the inoculum, and more importantly, the speed at which the cells are able to produce capsules in vivo appear to be of primary importance to the pathogenesis of cryptococcosis.

CHAPTER V

SUMMARY

Encapsulated cells of eight strains of C. neoformans remained viable in soil during 18 months of incubation. Eighty-five percent of the cells lost their capsules within the first two months of incubation and remained nonencapsulated thereafter. The disappearance of the capsule was not due to dehydration. Actively metabolizing cells were required for disappearance and reappearance of capsule. Capsular synthesis seemed to be controlled by the amount of available water present. Due to as yet unknown factors, the size of the cells, as well as the size of the capsule, decreased during 18 months of incubation in soil.

Under suitable conditions, capsular synthesis by environmentally induced nonencapsulated cells was initiated in one hour. Capsular production was demonstrated in both growing and resting cells of C. necformans. It was not observed in the absence of a carbon and/or energy source. Salts in excess of 5% inhibited capsular production. The optimum pH for initial capsular production (after 2 hours incubation) by resting cells of C. neoformans was 4.0 - 5.8. After 24 hours incubation, the largest amount of capsule was produced at pH 6.2 - 7.0. No capsular production was observed below pH 3.0 or above pH 8.0.

None of the genetically induced mutants produced capsules in

vitro. One of them, S_2, produced capsules in vivo when it was inoculated intracerebrally into mice, and it was virulent for mice when injected I.C. This mutant, however, was not virulent when it was inoculated intraperitoneally. The other mutants were avirulent for mice regardless of the route of inoculation.

Although the nonencapsulated mutants did not produce adhered polysaccharide (capsule) in vitro, they produced a soluble polysaccharide when cultured in synthetic broth (pH 7.0). Adhered and soluble polysaccharides were isolated by ethanol precipitation of either supernatant fluid of the sonic oscillated encapsulated cells or broth cultures of the encapsulated parental strain or nonencapsulated mutant strains. Qualitatively, both adhered and soluble polysaccharides contained galactose, glucuronic acid, mannose, and xylose. Soluble polysaccharides isolated from mutant S_1 and parental CIA cells were serologically and chemically identical. With both materials, molar ratio for mannose to xylose to glucuronic acid to galactose were 9:4:3:2. Adhered polysaccharide from parental CIA cells contained slightly more glucuronic acid than did the soluble polysaccharides. The percent molar ratio of the adhered polysaccharide was mannose, 9; xylose, 4; glucuronic acid, 3.5; and galactose, 2. The results of differential hydrolysis of soluble and adhered polysaccharides suggested that these polysaccharides contained a mannan backbone with xylose at the external ends of the branches.

Phagocytosis studies confirmed the fact that cryptococcal polysaccharides inhibit the phagocytosis of the organism by human leukocytes. The inhibitory action of polysaccharide seems to depend upon the direct

attachment of the macromolecule onto the yeast cells rather than a direct effect on the leukocytes. Experimental data are presented to suggest the presence of specific attachment sites for the polysaccharide on the yeast cells. In human lung tissue or in serum, five to eight hours were required for environmentally induced nonencapsulated cells of C. neoformans to produce enough capsule to inhibit phagocytosis by 50%.

In summary, C. neoformans cells remain viable in soil without apparent loss of virulence for more than 18 months. The cells lose capsules within the first two months of incubation in soil. The average diameter of the cells decreases to less than five microns. It is postulated that dissemination and inhalation of the organism by individuals are thereby facilitated and that, once in the tissue of a susceptible host, capsule production is initiated. The capsule is a polysaccharide composed of mannose, xylose, glucuronic acid, and galactose and it inhibits the phagocytosis of the organism by human leukocytes. The antiphagocytic nature of the polysaccharide is very specific and is directed towards yeast cells that have compatible receptor sites. Whereas all virulent strains of C. neoformans produce capsules in vivo, they may not do so in vitro.

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