INFECTION AND IMMUNITY, June 1970, p. 526-531
Vol. 1, No. 6
Copyright © 1970 American Society for Microbiology
Printed in U.S.A.

Cryptococcus neoformans

IV. The Not-So-Encapsulated Yeast

FERESHTEH FARHI, G. S. BULMER, AND J. R. TACKER

Department of Microbiology and Immunology, University of Oklahoma School of Medicine, Oklahoma City, Oklahoma 73104

Received for publication 14 February 1970

Nonencapsulated cells of *Cryptococcus neoformans* which may have a diameter of less than 4 μm are capable of producing experimental cryptococcosis in mice. It has been established that this relatively small, nonencapsulated yeast can exist in soil. In this form, the organism could be more readily disseminated by air currents, and it is more likely to be inhaled into the lungs than the larger encapsulated yeast. Nonencapsulated cells produce sufficient capsular material to inhibit phagocytosis by 50% when incubated for 5 to 10 hr with human lung tissue in vitro. The general assumption that the encapsulated cells are the etiological agent of naturally acquired cryptococcosis may have to be revised.

In a recent communication (6), we presented a theory on the pathogenesis of cryptococcosis. This proposal rested on two, as yet unproven concepts: (i) that the infectious particle of *Cryptococcus neoformans* is a nonencapsulated yeast, and (ii) that this organism is usually killed by human leukocytes after ingestion. This report is directed to the nature of the infectious particle. Information on the fate of the ingested particle will be published later (Tacker, Bulmer, and Farhi, *unpublished data*).

C. neoformans is regularly described as a heavily encapsulated yeast, which, with its capsule, has a diameter of 10 to 20 μm. The first indication that this might not always be true was presented by Emmons in 1962 (7). He noted that cells of *C. neoformans* in pigeon excreta usually did not possess a visible capsule. In 1968, Ishaq, Bulmer, and Felton (11) reported on environmental factors affecting the viability of *C. neoformans*. During these investigations, the size of the cell and capsule was measured after incubation in soil for various time intervals. Their data indicated that the longer the organism was incubated in soil the smaller the capsule became. This report is an extension of these observations.

MATERIALS AND METHODS

Soil. Soil, collected from Kingfisher, Okla., was obtained from F. Felton, Veterans Administration Hospital, Oklahoma City, Okla.

Cryptococcus neoformans strains. Eight strains, designated as CIA (serotype A), A, B, CDC, CS, CS10, UH1, and UH3, were obtained from F. Felton. All were human isolates, except strains CS and CS10 which were isolated from soil.

Media. Contents of the essential salt solution (ESS) were described previously (6).

Synthetic medium with a low *p*H (LpH) level, used with the addition of 2% agar, inhibited capsule production. This medium was prepared by adding 0.02 g of glucose per ml and 5.0 μg of thiamine monochloride per ml to a 1:10 dilution of ESS. HCl (0.1 N) was used to adjust the *p*H level to 5.0.

Synthetic medium with a high *p*H (HpH) level, used to promote capsule production, was prepared by adding 0.01 to 0.03 g of glucose per ml, 0.1 g of sodium glutamate per ml, and 5.0 μg of thiamine monochloride per ml to a 1:10 dilution of ESS. The *p*H level of the medium was adjusted to 7.0 with 0.1 N NaOH. When solid medium was desired, 2% agar was added.

Soil inoculation. Suspensions of *C. neoformans*, used to inoculate soil, were prepared in the following manner. Water (150 ml) was added to 25 g of soil and stirred for 30 min on a magnetic stirrer; the supernatant fluid was decanted and autoclaved for 15 min at 15 psi of pressure. Cells of *C. neoformans*, cultured for 48 hr on HpH agar, were added to 15 ml of the sterile-soil washings to give the following final concentrations per ml: strain CIA, 0.42×10^9; strain A, 1.2×10^9; strain B, 0.35×10^9; strain CDC, 0.3×10^9; strain CS, 0.25×10^9; strain CS10, 0.53×10^9; strain UH1, 1.6×10^9; strain UH3, 1.5×10^9. To 5.0 g of sterile soil in screw-cap bottles (3 by 5 cm; autoclaved on two successive days for 20 min at 15 psi) was added 0.2 ml of soil washing-yeast cell suspension. Bottles were incubated in the dark, at 25 C and 85% humidity (11). At various time intervals, two bottles were removed and treated in the following manner. Sterile distilled water (10 ml) was added to each bottle; the bottles were shaken manually for 2 min and allowed to stand at 26 C for 5

Downloaded from http://iai.asm.org/ on September 19, 2020 by guest

min. Viable plate counts and India ink preparations were made from the supernatant fluid to determine the number of viable cells, the percentage of encapsulated cells, and the size of cells and capsules. All microscopic measurements were made with a Porton Reticle ocular micrometer. In all instances, at least 100 cells were examined. The size of the capsules was determined microscopically in India ink preparations by subtracting the diameter of the cell from the diameter of the cell plus capsule. Virulence was determined by inoculating 1.0 ml of soil washings containing *C. neoformans* intraperitoneally (IP) into five 8-week-old mice obtained from the University of Oklahoma Medical Center mouse colony.

Capacity for capsule production after incubation in soil. Cells, incubated in soil for 1 year, were divided into three groups. The first group was killed by autoclaving, the second group was killed with chloroform, and the third group was untreated. Cells were separated from soil as described above. Autoclaved and chloroform-treated cells were inoculated into 2.0 ml of water and 2.0 ml of HpH broth; untreated cells were inoculated into 2.0 ml of HpH broth, 2.0 ml of water, and also into 2.0 ml of 0.1 M glucose solution containing 10^{-4} M dinitrophenol. The cultures were aerated during incubation at 25 C for 18 hr; the percentage of encapsulation was then measured in India ink preparations.

Generation time. Cells of strain CIA grown on Sabouraud agar for 48 hr 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. Cell suspension (1 ml) was added to the broth, giving a final concentration of 7×10^4 cells/ml. Cells of CIA, incubated in soil for 1 year, were harvested as soil washings. A 1.0-ml amount of the soil washing-cell suspension was added to 50 ml of HpH broth containing 2% glucose to give a final concentration of 7×10^4 cells/ml.

Both cultures were incubated, under aeration, at 26 C. After intervals of 2, 4, 6, 8, 20, 28, 36, 44, 52, and 64 hr, samples were removed, and the number of viable cells was determined by plate counts on Sabouraud agar. Generation time was calculated as $G = 0.3010\, t/(\log_{10} b - \log_{10} a)$, where G is generation time, t is time elapsed, 0.3010 is $\log_{10}$ of 2, b is the number of yeast cells at the end of t, and a is the number of yeast cells at the beginning of t (13).

Effect of water on capsule production. Various amounts of water (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ml) were added to *C. neoformans*-containing soil samples which had been incubated for 1 year. Control soil samples were autoclaved for 30 min before the addition of water. After 72 hr incubation at 25 C, the percentage of encapsulated cells was determined.

Effect of salts on capsule production. Highly encapsulated cells, which had been grown for 48 to 72 hr at 26 C on HpH agar, were inoculated into HpH broth supplemented with 5% of each of the following salts: NaCl, $NaNO_3$, Na_2SO_4, KCl, KH_2PO_4, and K_2HPO_4. After 3 subculturings at 48-hr intervals, the number of encapsulated yeast cells was determined.

Effect of concentration of lactose on capsule production. Cells which were highly encapsulated as a result of growth on HpH agar for 48 hr at 26 C were transferred to HpH broth containing 5, 10, 20, 30, or 40% lactose. At various time intervals after incubation at 26 C, samples were removed and the percentage of encapsulation was determined. Cell growth was determined by observing optical density changes with a colorimeter (Spectronic-20; Bausch & Lomb, Inc., Rochester, N.Y.) at 690 nm.

In vitro production of capsules in human lung tissue. A piece of "normal" human lung was obtained from the Oklahoma State medical examiner. The tissue came from a 46-year-old white male who had died 5 hr previously as a result of a gunshot wound in the stomach. Material was handled aseptically and kept refrigerated until use. A cube (0.5 cm^3) of tissue was placed in each of a number of sterile tubes. Each tube was inoculated with 1.5 ml of saline suspensions containing 10^7 to 2×10^7 cells/ml nonencapsulated cells which had been harvested from a 48-hr, LpH agar culture. Tubes were placed on a rotator and after 0, 1, 3, 6, 8, and 24 hr of incubation at 37 C, 0.2 ml of fluid was removed and added to 1.0 ml of human serum and 1.0 ml of autologous peripheral leukocyte suspension. After 90 min of incubation at 37 C, the percentage of phagocytosis was determined (5). Capsule production was detected in India ink preparations.

Isolation of soluble cryptococcal polysaccharide (SCIA). Soluble polysaccharide was obtained from a 4- to 5-day-old culture of strain CIA grown at 25 C with aeration in HpH broth supplemented with 3% glucose. Cells, killed by the addition of chloroform (3%, final concentration), were removed by centrifugation at $300 \times g$. Sodium acetate (10%, final concentration), acetic acid (1%, final concentration), and 2 volumes of ethanol were added to the supernatant; the mixture was stored at 4 C for 48 hr. The precipitate was collected after centrifugation at $300 \times g$. One volume of absolute ethanol was added to the supernatant fluid, and the mixture was stored at 4 C for 48 hr. The precipitate was then collected after centrifugation at $300 \times g$. The two precipitates were pooled, washed with 200 ml of absolute ethanol, centrifuged at $300 \times g$, and resuspended in distilled water to give a 1% solution. The material was then centrifuged at $22{,}000 \times g$ for 60 min at 4 C to remove insoluble impurities. The supernatant fraction was deproteinized by eight extractions with chloroform and *n*-butanol (14). To the deproteinized supernatant fluid were added sodium acetate (10%), acetic acid (1%), and 3 volumes of absolute ethanol. After 48 hr of incubation at 4 C, the precipitate was collected after centrifugation at $300 \times g$ and dissolved in water. It was then dialyzed against four changes of tap water and then against four changes of distilled water at 4 C. After lyophilization, the material (hereafter referred to as SCIA) was stored in a screw-cap vial at room temperature.

Isolation of adhered capsular polysaccharide (ACIA). Acetone-killed cells of strain CIA (cultured on HpH agar supplemented with 3% glucose) were suspended in distilled water (10%, final concentration). The capsule was removed by sonic oscillation (6). The supernatant fluid of the sonically treated

Downloaded from http://iai.asm.org/ on September 19, 2020 by guest

cells was treated according to the above-mentioned protocol for SCIA. This material is hereafter referred to as ACIA.

Characterization of capsule polysaccharide. Total nitrogen was determined by the micro-Kjeldahl method (12), by using 4-mg samples. Total carbohydrate was determined by the anthrone test. A standard curve was obtained with mannose. A Spinco model E ultracentrifuge was used for ultracentrifugal studies. Each polysaccharide sample was dissolved in Veronal buffer (*p*H 8.6; ionic strength, 0.15) to a final concentration of 13.3 mg/ml. The samples were centrifuged at 52,640 rev/min for 90 min. Qualitative identification of monosaccharides present in each polysaccharide was made by descending paper chromatography on SS (Schleicher and Schuell Co., Keene, N.H.) filter paper (22.5 by 50 cm) with the following solvent systems: *n*-butanol–pyridine–0.1 N HCl (5:3:2, v/v, 20 hr) and ethyl acetate–acetic acid–formic acid–water (18:3:1:4, v/v, 18 hr). 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-cap tube and hydrolyzed in boiling water for 3 hr. The sample was then dried in vacuo over NaOH and resuspended in 0.05 to 0.1 ml of distilled water. The entire sample was applied to the paper. Standards used with each run included galactose, glucuronic acid, glucuronolactone, glucose, mannose, ribose, and xylose. Monomers were detected with 2-amino-biphenol reagent (9) or anilin-phthalic acid reagent (17). Polysaccharide monomers were determined quantitatively in the following manner. Differential hydrolysis of polysaccharides was accomplished by dissolving 0.5-mg samples in 0.5 ml of distilled water and in 0.5 ml of 3 N HCl. Samples were hydrolyzed in closed tubes in a boiling-water bath for 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 24 hr, dried in vacuo over NaOH, resuspended in distilled water, dried, and then resuspended in 0.05 to 0.1 ml of distilled water. Samples were applied as spots on the chromatographic paper. Chromatograms were run in *n*-butanol–pyridine–0.1 N HCl solvent for 20 hr. Anilin-phthalic acid reagent was used for staining. The amount of each monomer was determined by the method described by Wilson (16).

RESULTS

Viability, encapsulation, and virulence during incubation in soil. Figure 1 presents the mean data obtained with eight strains of *C. neoformans* which had been incubated in soil for 18 months. During the first 2 months of incubation, there was approximately a twofold increase in the number of viable cells. The number of viable cells remained constant for the next 7 to 8 months and then slowly decreased.

After 5 days of incubation in soil, the number of nonencapsulated cells increased from the original 5 to 45% of the total population. (The only parameter used to judge nonencapsulation was microscopic observations of cells in India ink preparations.) After 60 days of incubation

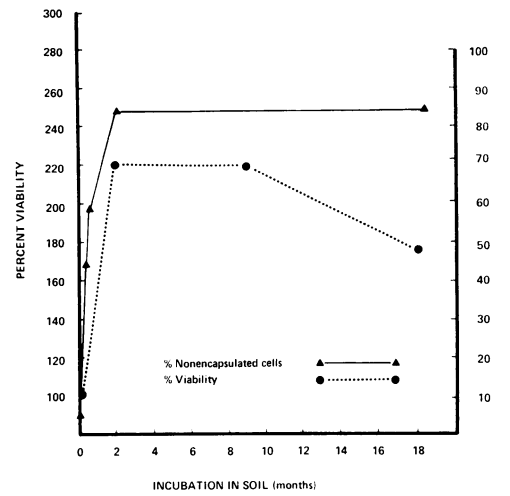

FIG. 1. *Viability and capsular production of eight strains of Cryptococcus neoformans incubated in soil.*

in soil, 85% of the cells were nonencapsulated. The per cent of nonencapsulated cells remained constant throughout the remainder of the experiment. It is possible that the remaining 15% encapsulated cells were never viable from the beginning of the experiment. The average diameter of the cells, exclusive of capsule, decreased from 4.2 to 3.2 μm during the 18 months of incubation in soil.

After 0, 1, 6, 12, and 18 months of incubation in soil, 1.0 ml of soil washings containing 0.79×10^6 to 4.3×10^6 viable cells were inoculated IP into each of five mice. All mice died within 4 weeks after inoculation and had symptoms of cryptococcal meningitis. Large encapsulated yeast cells were observed in brain tissues at autopsy, and *C. neoformans* was isolated in pure culture from brain tissues. At the dosages used, there was no apparent change in the virulence of the organisms during 18 months of incubation in soil.

Generation time. When cells of CIA grown on Sabouraud agar were transferred into HpH broth, there was a lag period of at least 2 hr before the cells entered the logarithmic phase. The culture reached stationary phase 18 hr after inoculation, and the generation time of cells was 2.5 hr. Cells maintained in soil for 1 year had a 4-hr lag period when subsequently inoculated into HpH broth; during the logarithmic phase which lasted approximately 18 hr, generation time was 2.5 hr.

Effect of water, salts, and lactose on capsule production. To determine whether the disappearance of capsule after incubation in soil was due to dehydration, cells of strain CIA which

Downloaded from http://iai.asm.org/ on September 19, 2020 by guest

had been incubated in soil for 1 year were suspended and incubated in distilled water, HpH broth, Sabouraud broth, or a 0.1 M glucose solution containing 10^{-4} M dinitrophenol (DNP). Cells were then examined after no further treatment, after chloroform killing, or after autoclaving. Initially 13% of the cells were encapsulated. After 18 hr of incubation at 26 C, 63, 91, and 93% of untreated cells taken from the water, HpH broth, and Sabouraud broth, respectively, were encapsulated. After similar incubation, 30% of the cells in the DNP-glucose solution had capsules. Chloroform-killed or autoclaved cells had no apparent increase in the number of encapsulated cells.

When, after 1 year of incubation, various amounts of water were added to the bottles containing soil and cells of strain CIA, there was an increase in the number of encapsulated cells, proportional to the amount of water added (Fig. 2). There was no increase in the number of encapsulated cells when the bottles were autoclaved before the addition of water.

Six strains of *C. neoformans* cultured on HpH agar were inoculated into HpH broth containing 5% concentration of various salts. All six strains exhibited decreased ability to produce capsules in the salt solutions. In general, after two transfers into the salt solutions, the number of encapsulated cells in the population dropped to a 10% average (Table 1).

Increasing concentrations of lactose also caused the number of encapsulated cells to decrease. After 24 hr of incubation, 65% of the cells possessed capsules in the presence of 5% lactose, whereas 20% of the cells possessed capsules in the presence of 40% lactose. After 7 days of incubation in the presence of 5 and 40% lactose, less than 30 and 10% of the cells, respectively, possessed capsules. Cells were viable and multiplying in the concentrations of lactose used, as determined spectrophotometrically.

Effect of incubation in lung tissue. If the cells of *C. neoformans* lose their capsule in soil, and since the capsule is antiphagocytic (6), it becomes important to determine whether *C. neoformans* can produce capsules in lung tissue. Figure 3

TABLE 1. *Effect of inorganic salts on capsule production by six strains of Cryptococcus neoformans*

Salts[b]	Per cent encapsulation in strain[a]					
	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

[a] Measured after two transfers.
[b] Five per cent concentration in broth with a high *p*H level.

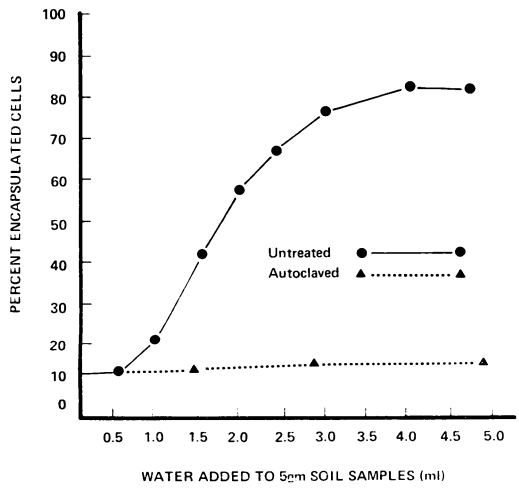

FIG. 2. *Effect of water on capsular production by Cryptococcus neoformans strain CIA incubated in soil for one year.*

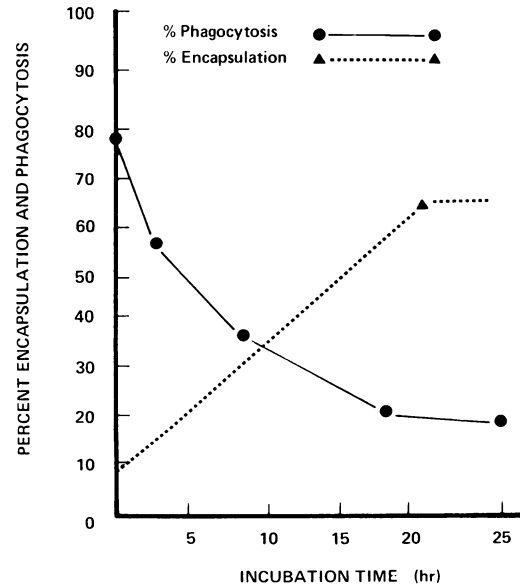

FIG. 3. *Capsular production and phagocytosis of Cryptococcus neoformans in human lung tissue in vitro.*

Downloaded from http://iai.asm.org/ on September 19, 2020 by guest

TABLE 2. *Quantitative studies of ACIA[a] and SCIA[b] polysaccharides*

Type of polysaccharide	Molar ratio of			
	Mannose	Xylose	Glucuronic acid	Galactose
ACIA	9.0	4.0	3.5	2.0
SCIA	9.0	4.0	3.0	2.0

[a] Adhered capsule from strain CIA.
[b] Soluble capsule from strain CIA.

presents the data obtained by incubating *C. neoformans* with excised human lung tissue. As the incubation time with lung tissue increased, the number of encapsulated cells increased. Concurrently, there was a corresponding decrease in the ability of human leukocytes to phagocytize the yeast cells.

Nature of capsular polysaccharide. Both soluble and adhered polysaccharides demonstrated one sharp peak in the ultracentrifuge with calculated molecular weights of 10,000 to 15,000. Total protein was less than 1.2%. Trace amounts of nucleic acid were detected by spectrophotometric readings at 260 and 280 nm. The molar ratios of the monomers are shown in Table 2.

DISCUSSION

Salts, especially NaCl, affect the production of different macromolecules in bacterial cells (3, 15), although the mechanism of action is unknown. Thus, it was not surprising to find in this study that all of the salts tested inhibited capsular production of *C. neoformans* (Table 1). Since addition of water to soil samples containing only viable cells of *C. neoformans* initiated capsule production (Fig. 2), it would appear that the presence of salts in soils may reduce the amount of active water or decrease its chemical activity and thus inhibit capsule production. Disappearance and reappearance of the capsule may be controlled by the amount of active water, or its relative activity, present in the system. To test this hypothesis, another method (1) was used to reduce the amount of available water in the system, i.e., increasing carbohydrate concentrations. It was found that *C. neoformans* grew in the presence of 40% lactose in a synthetic medium with a high *p*H level, and, although growth was slower than in the presence of 10% or less lactose, a decrease in the number of encapsulated cells was associated with high lactose concentrations. Decrease in available water may be an important factor relative to the disappearance of capsule of *C. neoformans* in soil. However, the disappearance and reappearance of capsules appears to be more than a mere hydration phenomena, as evidenced by the findings that viable cells are necessary for capsule production and this process, which is inhibited by dinitrophenol, is energy-dependent.

About the same time the number of nonencapsulated cells increased dramatically, the number of viable cells more than doubled. This elevated mitotic activity may also be related to the disappearance of the capsule.

During soil incubation, the size of the average cell decreased to approximately 3 μm. These cells were viable and infectious. Thus, contrary to current concepts, the infectious particle of *C. neoformans* appears to exist as a relatively small and nonencapsulated organism. The observation that cells remain viable in soil over extended periods suggests that the infectious particle may exist in a state of reduced or shifted metabolic activity, perhaps analagous to the spores of other fungi or bacteria. In theory, this concept of the infectious particle of *C. neoformans* supports the hypotheses of the lung as the primary portal of entry. Brown et al. (4), reported that nasal efficiency is practically 100% for removal of particles larger than 5 μm, and that there is little alveolar retention for particles larger than 4 to 5 μm. The calculations and data of Hatch (10) substantiate these findings. Thus, it is difficult to envision the deposition to any significant depth of an encapsulated yeast 10 to 20 μm in diameter.

If *C. neoformans* resides in soil in a nonencapsulated state, and since the importance of the capsule as a virulence factor has been demonstrated (6), the time and conditions required for capsular production takes on a new importance. Our findings indicate that capsule production is affected by the amount of available water and that cells must be viable for this energy-dependent process.

Since *C. neoformans* appears to reside in soil in a nonencapsulated state and since the capsule is antiphagocytic (6), it then becomes important to determine whether this organism can produce capsules in lung tissue. Our in vitro findings indicate that, not only are capsules produced when nonencapsulated cells are incubated with human lung tissue, but enough are present in 5 to 10 hr to inhibit phagocytosis by 50%.

The generation time of *C. neoformans* (strain CIA) was calculated to be 2.5 hr, regardless of whether the cells were originally cultured on laboratory medium or removed from soil after 1 year of incubation. However, the latter cells did exhibit a 2-hr longer lag phase. This indicates

Downloaded from http://iai.asm.org/ on September 19, 2020 by guest

that the 5- to 10-hr incubation period required for significant in vitro capsule production with human lung tissue may be a slightly conservative figure when applied to the inhalation of the infectious particles in nature.

The chemical characterization of adhered and soluble polysaccharides showed them to be quite similar, if not identical. Adhered capsular material contained slightly more glucuronic acid than did the soluble polysaccharide, whereas the same monomers, galactose, glucuronic acid, mannose, and xylose, were found as in type B polysaccharide (2). Molar ratio of the monomers from the two polysaccharides varied somewhat. These findings tend to support the hypothesis of Evans and Mehl (8), which supposes that type specificity of cryptococcal polysaccharide is due to quantitative rather than qualitative difference.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Research Career Program Award K03-AI 13188 (to G. S. B.) and by grant AI 05022, both from the National Institute of Allergy and Infectious Diseases. F. F. was the recipient of a National Iranian Oil Company scholarship.

LITERATURE CITED

1. Anand, J. C., and A. D. Brown. 1968. Growth rate patterns of the so-called osmophilic and non-osmosphilic yeasts in solutions of polyethylene glycol. J. Gen. Microbiol. **52**:205–212.
2. Blandamer, A., and I. Danishefsky. 1966. Investigations on the structure of the capsular polysaccharide from *Cryptococcus neoformans* type B. Biochim. Biophys. Acta **117**: 305–313.
3. Brown, A. D. 1964. Aspects of bacterial response to the ionic environment. Bacteriol. Rev. **28**:296–329.
4. Brown, J. H., K. M. Cook, F. G. Ney, and T. Hatch. 1950. Influence of particle size upon the retention of particulate matter in the human lung. Amer. J. Pub. Health Nat. Health **40**:450–458.
5. Bulmer, G. S., and M. D. Sans. 1967. *Cryptococcus neoformans*. II. Phagocytosis by human leukocytes. J. Bacteriol. **94**:1480–1483.
6. Bulmer, G. S., and M. D. Sans. 1968. *Cryptococcus neoformans*. III. Inhibition of phagocytosis. J. Bacteriol. **95**:5–8.
7. Emmons, C. W. 1962. Natural occurrence of opportunistic fungi. Lab. Invest. **11**:1026–1032.
8. Evans, E. E., and J. W. Mehl. 1951. A qualitative analysis of capsular polysaccharides from *Cryptococcus neoformans* by filter paper chromatography. Science **114**:10–11.
9. Gordon, H. T., W. Thornburg, and L. N. Werum. 1956. Rapid paper chromatography of carbohydrates and related compounds. Anal. Chem. **28**:849–855.
10. Hatch, T. F. 1961. Distribution and desposition of inhaled particles in respiratory tract. Bacteriol. Rev. **25**:237–240.
11. Ishaq, C. M., G. S. Bulmer, and F. G. Felton. 1968. An evaluation of various environmental factors affecting the propagation of *Cryptococcus neoformans*. Mycopathol. Mycol. Appl. **35**:81–90.
12. Lang, C. A. 1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. Anal. Chem. **30**:1692–1694.
13. Oginsky, E. L., and W. W. Umbreit. 1959. An Introduction to bacterial physiology, 2nd ed., p. 55. W. H. Freeman & Co., San Francisco.
14. Sevag, M. G., D. B. Lackman, and J. Smolens. 1938. The isolation of the components of streptococcal nucleoproteins in serologically active form. J. Biol. Chem. **124**:425–436.
15. Smithies, W. R., N. E. Gibbons, and S. T. Bayley. 1955. The chemical composition of the cell and cell wall of some halophilic bacteria. Can. J. Microbiol. **1**:605–613.
16. Wilson, C. M. 1959. Quantitative determination of sugars on paper chromatograms. Anal. Chem. **31**:1199–1201.

Downloaded from http://iai.asm.org/ on September 19, 2020 by guest