STUDIES ON THE CELL WALLS AND EXTRACELLULAR MATERIAL OF VIRULENT AND AVIRULENT *CRYPTOCOCCUS* SPECIES

by

ADRIANNE ROSS

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ABSTRACT

Relationships between cell envelope biochemistry, pathogenicity and taxonomy of virulent and avirulent Cryptococcus species have been studied.

Variation in growth medium allowed controlled production of cells with or without extensive capsule synthesis. Thiamine (10 µg/ml) and a pH of 7.0 were necessary for optimal growth. Heat treatment was used to kill cells. There was no detectable damage to cell envelope materials.

Preparations of purified cell wall and extracellular material were analyzed for amino acids, amino sugars and neutral sugars after serial hydrolysis. Extracellular material was also analyzed for phosphorus and uronic acids by colorimetric methods and for O-acetyl groups by gas-liquid chromatography.

The protein portions of cell walls and extracellular material contained the same amino acids in different proportions, specific for each strain. Extracellular material from two strains of Cr. neoformans, 365-16 and 365-26, was very similar in amino acid composition. Ethanolamine and glucosamine occurred in all preparations, cysteine/cystine only in Cr. albidus and Cr. laurentii, and galactosamine only in Cr. neoformans walls. Glucose was the predominant cell wall monomer; xylose, mannose and galactose occurred in smaller quantities. Extracellular material contained large amounts of mannose, a trace of glucose and, in addition, glucuronic acid. Only the Cr. neoformans strains contained O-acetyl groups. Extracellular material and cell wall composition were sufficiently different to suggest

independent synthesis. The amount of capsule synthesis and polysaccharide composition were easily affected by growth conditions.

Extracellular polysaccharide produced by the most virulent strain of Cr. neoformans (365-11), contained more mannose, glucuronic acid and O-acetyl groups than the less virulent strains. Cell walls of Cr. neoformans 365-26 had more glucose and less glucosamine, mannose and xylose than did the walls of the nonpathogenic Cr. laurentii.

Both cell wall and extracellular polymers were similar in composition to those produced by Tremella mesenterica and other basidiomycetes. Hyphal Cr. neoformans (Coward strain) produced septate hyphae and clamp connections. A close taxonomic relationship to the heterobasidiomycetes is proposed.

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ABBREVIATIONS

Symbols used for Monomers

Ala	alanine
Arg	arginine
Asp	aspartate
Asx	aspartate or asparagine (undefined)
Cys	cysteine
Gal	galactose
GalN	galactosamine (2-amino-2-deoxy-galactose)
Glc	glucose
GlcN	glucosamine (2-amino-2-deoxy-glucose)
GlcNx	glucosamine or N-acetyl-glucosamine (undefined)
Glu	glutamate
Glx	glutamate or glutamine (undefined)
Gly	glycine
His	histidine
Hyp	4-hydroxyproline
Ile	isoleucine
Leu	leucine
Lys	lysine
Man	mannose
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Tyr	tyrosine
Val	valine
Xyl	xylose

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CHAPTER I

INTRODUCTION

Incidence and Distribution

Cryptococcus neoformans (Sanfelice) Vuillemin is the sole causative agent of cryptococcosis, an infectious pulmonary disease of man and animals, which frequently spreads to the central nervous system in susceptible patients. The disease is world wide in occurrence but its actual incidence is unknown. Since 1952 the number of deaths reported due to *Cr. neoformans* in the United States has averaged 66 per year (Ajello 1967). Littman and Schneierson (1959) estimated that 5,000 - 15,000 cases of subclinical or clinical pulmonary cryptococcosis occur annually in New York City alone. Bird droppings are thought to constitute the main reservoirs of infection (Ajello 1958, Emmons 1954, Littman and Schneierson 1959) and *Cr. neoformans* is found infrequently in natural soils and plant sources (Staib and Altmann 1973, Felton *et al.* 1974, Sneller and Swatek 1974). The presence of this fungus in the atmosphere and certain soils has been explained by prior exposure to human and animal, particularly bird, excreta and secretions (Ajello 1967, Gentles and La Touche 1969). Staib (1962, 1963) suggested that the particular close association of *Cr. neoformans* with bird droppings was related to creatinine, a constituent of bird urine, which the yeast can utilize as a sole source of nitrogen. Staib *et al.* (1972) also found that *Cr. neoformans* was able to

colonize dead plants and proposed that decaying plant material might provide an additional reservoir for Cr. neoformans in nature.

Other members of the genus Cryptococcus are non-pathogenic and occur universally in soils, the phyllosphere, including mosses, and the atmosphere (Carmo-Sousa 1969): they have also been isolated from 117 different species of plants (Staib and Altmann 1973). Recent studies by Sneller and Swatek (1974) have shown a relationship between the average daily temperatures and soil pH and the species of Cryptococcus isolated from Southern California soils. Cr. albidus strains preferred a temperature range of 15-22C and a pH of 5.4-6.6; Cr. diffluens was found in regions from 21-26C with pH 6.9-7.9 and Cr. laurentii occurred at 27-29C and pH 8.1-8.3. Ishaq et al. (1967) found that sterile soils seeded with Cr. neoformans supported growth best under alkaline conditions (pH 7.7 as compared with 7.4 and 6.8) and when humidity was high and temperatures moderate to low.

Although Cr. neoformans grows in mammals at 37C it does not grow well at 39.4C, and the optimum temperature for growth appears to be 29C (Kuhn 1949). This sensitivity to temperatures above 39C may explain why birds, which have a higher body temperature than mammals, are merely carriers of the disease. They are seldom infected with the fungus, although Littman et al. (1965) found that intracerebral inoculations of pigeons caused a fatal systemic infection. Of the other species of Cryptococcus, only some strains of Cr. laurentii have the

ability to grow at 37C. There have been occasional reports of Cr. laurentii being pathogenic to man (Blair et al. 1970), and more recently Cr. albidus has been implicated as the causal agent of cryptococcosis (Da Cunha 1973, Wieser 1973), although this species has a lower optimum growth temperature (Phaff and Fell 1970).

Systematics

Phaff and Fell (1970) based classification of the genus on (a) the ability to assimilate inositol as a carbon source for growth, (b) the production of a heteropolysaccharide capsule, (c) the absence of a well developed pseudomycelium, and (d) the lack of ability to produce sexual stages (ascospores, teliospores or ballistospores) or to ferment sugars. However, Bab'yeva and Golubev (1970, cited by Golubev et al. 1971) described cultures of Cryptococcus which do not assimilate inositol but are typical in other respects. An increasing number of reports have suggested the existence of sexually active species and have proposed a relationship to the lower basidiomycetes. Van der Walt (1967) observed a type of endospore formation which resulted from internal budding after conjugation of haploid Cr. albidus cells. However, he did not conclusively demonstrate that the zygote was diploid. Kurtzman (1973) isolated mating strains of Cr.laurentii. These formed conjugation tubes which gave rise to hyphae, 5% of which were binucleate: the hyphae also produced chlamydospores. The conjugation tubes were similar to those found in Tremella spp. (Bandoni 1963) and in Sporobolomyces odorus Derx (Bandoni et

al. 1971). Kurtzman (1973) was unable to obtain any mating response between haploid forms of either Tremella aurantia or Tremella encephala and Cr. laurentii var laurentii although these strains had similar carbon assimilation patterns. He related the presence of haustoria-like structures in Cr. laurentii to a possible parasitic existence in nature.

No clamp connections were observed on Cr. laurentii mycelia (Kurtzman 1973) although Shadomy (1970) isolated two strains of Cr. neoformans which produced hyphae and formed clamp connections. Pseudomycelium has been reported several times in the literature (Cox and Tolhurst 1946, Shadomy and Utz 1966) as well as occasional reports of sexual reproduction (Todd and Hermann 1936, Benham 1955). Lurie and Shadomy (1971) have demonstrated hyphae in eleven strains of Cr. neoformans and consequently regard hyphal forms as other than chance mutations. However, sexual stages have yet to be confirmed in Cr. neoformans.

The major difference between Cr. neoformans, the type species of the genus (Phaff and Fell 1970), and the other species, is its pathogenicity in man and mammals.

Pathology and Immunology

Cr. neoformans is thought to enter the human lungs as a nonencapsulated yeast less than 5 μm in diameter (Farhi et al. 1970). In a normal individual the yeast will be engulfed and killed by polymorphonuclear leukocytes (Tacker et al. 1972) without production of disease (Atkinson and Bennett 1968). Killing of microorganisms by leukocytes normally requires

opsonization (Mayer 1973): that is, recognition of the invader by an antibody, activation of the complement system by that antibody and fixation of the complement on the invading cell's surface. This is the classical complement pathway. The alternate or properdin pathway does not require antibody to initiate it, but instead involves assembly of properdin enzymes in response to microbial polysaccharides (such as yeast zymosan), which then activate the later complement components. Either way the complement serves to lyse the invading cell or render it susceptible to phagocytosis (Mayer 1973).

Most studies on cryptococcosis have suggested that host cellular responses, including phagocytosis, play the major role in conferring resistance against this infection (Abrahams et al. 1970), Gadebusch 1972), and it was thought that humoral anticryptococcal polysaccharide did not have a protective role (Goren and Middlebrook 1967). However, potent anticryptococcal factors have been found in normal human serum (Baum and Artis 1963, Szilagyi et al. 1966) and Bulmer and Tacker (1975) believed that these, along with cationic proteins released from host cells, enhance phagocytosis. Baum and Artis (1963) concluded that the anticryptococcal serum factor was not related to complement or properdin since it was heat stable at temperatures up to 65C. However, Diamond et al. (1972) and Cline et al. (1968) found a requirement for a heat labile factor in phagocytosis of cryptococci. Diamond et al. (1974) recently suggested that complement does play a role in the phagocytosis of cryptococci by human peripheral blood leukocytes. They were

able to demonstrate antibody to cryptococci in normal serum by a sensitive absorption technique, although it could not be identified with the usual indirect fluorescent antibody technique. The classical pathway appeared to be functioning primarily to activate the alternate pathway, which in turn was responsible for opsonization. Spinal fluid did not opsonize cyptococci and no complement was detected on the surface of cryptococci obtained from the spinal fluid of patients with cryptococcal meningitis. However, the fact that spinal fluid from patients with the disease could mediate opsonization of cryptococci in absorbed normal serum suggested that anti-cryptococcal antibody and possibly properdin were present in the spinal fluid. Igel and Bolande (1966) were also unable to detect an anticryptococcal factor in spinal fluid. According to Gadebusch and Johnson (1966) cationic proteins stimulate leukocyte emigration and adhesion and increase capillary permeability. Chemotaxis, immune adherence, and release of histamine (which increases permeability of blood capillaries from leukocytes, mast cells and platelets) are all also activities of the late complement components (Mayer 1963).

Individuals who develop cryptococcosis are thought to have deficient immune systems. The disease is encountered more frequently in patients with Hodgkin's disease, lymphosarcoma, leukemia, and diabetes mellitus and in those receiving prolonged steroid therapy (Littman and Walter 1968). Farhi et al. (1970) proposed that in these people, phagocytosis of the unencapsulated yeast is delayed sufficiently to allow large amounts of

capsular material to be produced which then inhibits phagocytosis. Borowski et al. (1974) found that subinhibitory concentrations of amphotericin B inhibited capsule formation in cryptococcal cells which were then more readily phagocytized. Mitchell and Friedman (1972) also found that the degree of phagocytosis was proportional to capsular thickness. However, the ability of the macrophages to kill the yeast cells was unrelated to capsule thickness and seemed largely strain dependent. Bulmer and Tacker (1975) believed that the capsule itself was the virulence factor and they also proposed that myeloperoxidase in normal human saliva may provide an additional anti-cryptococcal factor. However, it seems to me that the initial failure to activate opsonization of the cryptococci is the crucial factor in the development of the disease and its ultimate spread to the central nervous system. This may be dependent upon failure of the individual to form antibodies to the unencapsulated yeast or failure of the complement system to adhere to activated yeast cells. The latter now seems more likely in view of Diamond et al.'s work (1974).

Farmer and Komorowski (1973) recently reported a case of a patient infected with a capsule-deficient strain of Cr. neoformans. The capsule-deficient yeast invoked an intense inflammatory response in mice which was characterized by early suppuration and phagocytosis followed by marked histiocytic and fibroblastic reaction, limiting the infection. These features were also described by Shadomy and Lurie (1971) working with infections caused by the unencapsulated hyphal Coward

strain of Cr. neoformans. Thus the cryptococcal antigens may involve cell wall as well as capsular components, and normal antiserum may contain antibodies to both (Vogel 1966). Most investigations have demonstrated that the capsular polysaccharide is not a complete antigen (Cozad et al. 1963, Goren 1966, 1967) and Gadebusch (1960) reported that the antigenic material resides in the capsular polysaccharide that is closely bound to the wall. Devlin (1969) found that zeolite ghost preparations of whole cells were the most efficient immunizing agents and that the cell wall also had antigenic properties. Farmer and Kamorowski (1973) and Devlin (1969) have implied that the antigenic factor beneath the capsule may be proteinaceous. The cell walls of another pathogenic yeast, Histoplasma capsulatum, appeared the most antigenically active fraction in complement fixation tests (Odds et al. 1971). Pine and Boone (1968) found larger quantities of both chitin and amino acids in the cell walls of antigenic as compared with nonantigenic strains of Histoplasma capsulatum.

Higher levels of cell wall chitin, protein and phospholipid have also been related to virulence (for mice) in strains of Blastomyces dermatitidis (Cox and Best 1972 and Di Salvo and Denton 1963).

Cell Wall and Capsule Biochemistry

Only recently has the cell wall of Cr. neoformans received any attention, perhaps because the cells are so difficult to disrupt (Devlin 1969, Erke and Schneidau 1973). Cook et

al. (1970) compared cell walls of Cr. neoformans isolated from patients and from soil and, in essentially qualitative work, noted differences in lipid, carbohydrate and hexosamine content. No amino acid studies have been performed except by Uzman et al. (1956) on somatic proteins extracted from a strain of Cr. neoformans by treatment of the cells with hot alkali. The cell walls of nonpathogenic species Cr. albidus and Cr. terreus consisted mainly of α- and β-linked glucans, whose proportions were affected by the composition of the growth medium (Jones et al. 1969, Bacon et al. 1968). Glucan represented 75% of the Cr. albidus cell walls (Bacon et al. 1968). The main nitrogenous constituent was glucosamine, present as chitin. Jones et al. (1969) found that the chitinous residue, after acid and alkaline extraction, was composed of microfibrils rather than being granular as in Saccharomyces species.

The capsular polysaccharides of Cr. neoformans have antigenic activity and have been classified into four serological types; A, B, C and R, differentiated on the basis of precipitin, agglutination and "quelling" reactions (Evans and Kessel 1951). From structural and molecular studies the capsule appears to consist of a mannose backbone with branches of xylose and glucuronic acid as shown in Fig. 1 (Miyazaki 1961, Blandamer and Danishefsky 1966). Mannose also occurred as side units attached by (1→4) linkages.

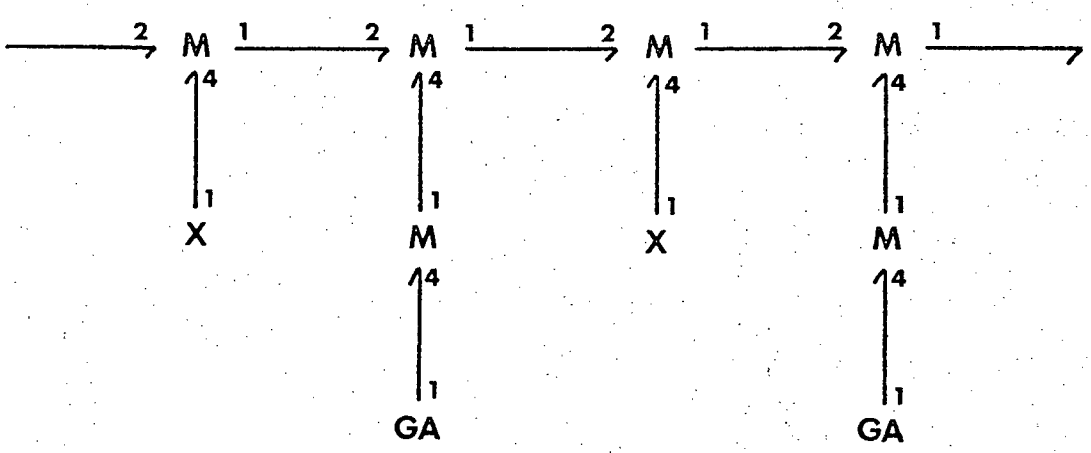


Fig. 1 Proposed structure of capsular polysaccharide of Cr. neoformans according to Miyazaki (1961). M = α-D-mannopyranosyl residue; X = α-D-xylopyranosyl residue; GA = α-D-glucuronopyranosyl residue.

The structure and composition of both acidic and neutral polymers produced by Cryptococcus species have been extensively reviewed (Phaff 1971; Gorin and Spencer 1968). Some of the studies demonstrate the close relationship between Cryptococcus species and the heterobasidiomycetous genus, Tremella (Slodki et al. 1966, Helms et al. 1969). However, values for the proportions of xylose to mannose recovered from the heteropolysaccharides differed among the various reports (Phaff 1971) and not all workers tested for, and found, galactose and O-acetyl groups. Both Evans and Theriault (1953) and Rebers et al. (1958) believed that Cr. neoformans produced two polysaccharides, both types contained glucuronic acid but only one contained galactose.

Evans and Mehl (1951) were unable to demonstrate any qualitative differences in acid hydrolysates of the three cryptococcal polysaccharide antigens (A, B and C). However,

infrared spectroscopy (Levine et al. 1959) revealed that differences in the serological types may depend in part on the degree of acetylation of the polysaccharide side chains. Kozel and Cazin (1971), compared the chemistry of soluble polysaccharides from virulent and weakly virulent strains and found the virulent strain had a larger uronic acid content and a larger molecular size. The terminal glucuronic acid units have also been implicated as antigenic agents in cross-reactions between type II antipneumococcal serum and Cr. laurentii and Cr. neoformans (Helms et al. 1969). The polysaccharide from Tremella mesenterica did not cross-react, and Fraser et al. (1973b) have recently shown this to be due to steric interactions. Xylose units located in the vicinity of the glucuronic acid residues may be responsible for this steric hindrance. In addition, Cr. laurentii had xylose present only as nonreducing end groups (Miyazaki 1961), whereas Tremella mesenterica was shown to have 2-O-β linked xylopyranose side chains, thus forming a much more highly branched structure (Fraser et al. 1973a).

Although the protein content of cryptococcal capsular and extracellular material has not previously been investigated, Masler et al. (1966) reported the presence of immunologically active extracellular polysaccharide-protein complexes in cell-free culture fluid of Candida albicans. Phaff (1971) suggested that this protein may have been lost from cells during shaking or harvest rather than being a true exocellular product. However, Müller and Sethi (1972) described a proteo-

lytic activity of Cr. neoformans against human plasma proteins, and yeasts are known to produce external invertase and acid phosphatases (Lampen 1968).

The factors responsible for the variations in composition of cryptococcal acidic heteropolysaccharides observed by Phaff (1971) may involve differences in growth media, origin and purity of the polysaccharides, and different methods of chemical analysis. Growth conditions are known to affect both composition and production of extracellular and capsular polysaccharide (Farhi et al. 1970, Bulmer and Sans 1968, Littman 1958, Slodki et al. 1970, Foda et al. 1973). Foda et al. (1973) found that whereas an acidic polysaccharide was synthesized by Cr. laurentii between pH 3 and 7, a neutral glucan was produced only when the medium pH dropped to below 3.0 and growth was slowed. They did not consider this amylose to be a true extracellular polysaccharide. The α-D-glucan isolated from Cr. laurentii in casamino acids - salts medium at pH 5 by Abercrombie et al. (1960) was not an amylose, and Phaff (1971) proposed that it may have originated from the cell wall. Bulmer and Sans (1968) found that a low pH inhibited the heteropolysaccharide capsule production of Cr. neoformans whereas high pH stimulated capsule synthesis: different carbohydrate carbon sources also affected capsule production. Farhi et al. (1970) showed that addition of salts and water to soil samples containing Cr. neoformans inhibited capsule production. Littman (1958) studied the nutritional growth factors affecting capsule production in Cr. neoformans and demonstrated a thiamine

and sodium glutamate requirement. However, Farhi (1969) was unable to establish a growth stimulatory effect with thiamine within 24 hr. Slodki et al. (1970) reported that under phosphate limiting conditions Hansenula holstii and H. capsulata no longer produced exocellular phosphomannans, but instead released non-phosphorylated mannans. The mannans were not only lacking in phosphate, but their structure was changed: H. capsulata replaced the normal mixture of α- and β-linkages with α-linked mannan units and the H. holstii mannan was branched rather than linear. A galactomannan from Sporobolomyces sp. which under normal growth conditions had both phosphate and O-acetyl substituents was found to have many of the phosphate groups replaced by O-acetyl groups when grown under phosphate-limiting conditions. This might explain the variability in recovery of O-acetyl groups from polysaccharides of Cr. laurentii and Tremella spp. reported by Slodki et al. (1966).

Extracellular polysaccharides have been either precipitated (with ethanol) from the cell free growth medium or extracted directly from the cells, as adhered material, by a variety of methods (Gorin and Spencer 1968). In many cases the growth media used were not chemically defined and although the neopeptone was dialyzed by Evans and Theriault (1953), there may still have been amino contaminants from the medium which would have precipitated with ethanol. Most workers dealing with the pathogen Cr. neoformans have used a chemical method (for example 1% phenol, Evans and Kessel 1951) to kill the cells before harvesting. Treatment with strong acids or

bases, alcohol, or heat may cause denaturation of protein components or hydrolysis or oxidation of polysaccharides. Similarly, analytical procedures which do not account for losses due to degradation or insufficient release of certain components, would give underestimations of the sugar constituents of the polysaccharides. None of the studies so far reported have employed critical quantitative analytical methods; sugars were measured solely by paper chromatography or colorimetric methods. Since xylose appears to be an easily liberated side chain moiety in cryptococcal and Tremella polysaccharides (Miyazaki 1961, Blandamer and Danishefsky 1966, Cameron 1973) it is likely that the discrepancies in xylose: mannose ratios were either a result of xylose degradation or an incomplete release of mannose.

An investigation of the cryptococcal cell envelope, comprising cell wall, capsule and extracellular material, would have significance from both a taxonomic and pathogenic viewpoint. The research described in this thesis had the following aims:

(1) To determine the optimum conditions of temperature, pH and nutrients for growth and capsule synthesis of each of three strains of Cr. neoformans, two of Cr. albidus, and one of Cr. laurentii.

(2) To develop a method of killing pathogenic cells that does not alter wall or capsule chemistry.

(3) To isolate and purify cell walls and extracellular material from each of these virulent and avirulent Cryptococcus species including a pathogenic strain of Cr. albidus (H1354)

and a hyphal strain of Cr. neoformans.

(4) To analyze the cell walls and extracellular material for carbohydrates, protein and minor constituents such as phosphorus.

(5) To determine the virulence (LD50) of all strains against mice.

(6) To compare and contrast the analyses from different strains and relate these to virulence and the taxonomic position of the species.

(7) To investigate the pathogenicity of the Cr. albidus H1354 strain.

(8) To find a medium that will support and establish mating strains of the Cryptococcus species and to demonstrate sexual stages.

CHAPTER II

EXPERIMENTAL PROCEDURE

1. MATERIALS

A. Organisms

Cryptococcus neoformans strains 365-11, 365-16 and 365-26 were isolated from sputum and were pathogenic to mice. *Cr. laurentii* 371-1 was isolated from a tracheal aspiration, and *Cr. albidus* 367 from air. These five cultures were obtained from Dr. L. Kapica, Department of Microbiology, McGill University, Montreal. A pathogenic strain of *Cr. albidus*, H1354, isolated from human cerebrospinal fluid, was obtained from Dr. H.G. Wieser, Aarau, Switzerland. All the cultures were maintained at 4C on Sabouraud dextrose (4%) agar slants and subcultured monthly. A hyphal strain of *Cr. neoformans* (Coward strain), obtained from Dr. H.J. Shadomy, Virginia Commonwealth University, Richmond, Virginia, was pathogenic to mice and was maintained in the hyphal state on V8 juice agar (Phaff and Mrak 1949). *Cr. laurentii* NRRL Y1 4920 (U.B.C. #81114), *Cr. albidus* 72539 (U.B.C. #900) and *Cr. terreus* U.B.C. #8157 were obtained from Dr. R.J. Bandoni, Department of Botany, University of British Columbia.

B. Chemicals

Reagents were obtained from the suppliers as indicated:-

2-hydroxypyridine (Aldrich Chemical Company, Inc., Milwaukee); Beckman Amino Acid Calibration Mixture Type 1 (Beckman Instruments, Inc., Spinco Division, Palo Alto, California); thiodiglycol (Bio-Rad Laboratories, Richmond, California); D-galactose, pyridine AnalaR ACS (British Drug Houses, Ltd., Poole, England); all amino acids (Calbiochem, Los Angeles, California); all culture media (Difco Laboratories, Detroit, Michigan); trifluoroacetic acid, metaphenyl phenol, ascorbic acid (Eastman Kodak Company, Rochester, New York); Alcian Blue 8G-S, benzene, Bromophenol Blue, Coomassie Brilliant Blue, D-mannose, cyclohexane certified ACS spectranalyzed, mannitol recrystallized Fisher certified, N, N-dimethylformamide 99% mole pure (Fisher Scientific Company, Fair Lawn, New Jersey); dimethylsulfoxide (Koch Light Laboratories Ltd., Colnbrook, England); merthiolate (E. Lilly and Company, Canada Ltd., Box 4037, Terminal A, Toronto); D-arabinose, D-glucose, D-xylose (Nutritional Biochemicals Corporation, Cleveland, Ohio); p-dimethylaminobenzaldehyde (MCB, Norwood, Ohio); methyl cellosolve, ninhydrin, hexamethyldisilazane, trimethylchlorosilane (Pierce Chemical Company, Rockford, Illinois); D-glucosamine hydrochloride, myo-inositol, thiamine hydrochloride (Sigma Chemical Company, St. Louis, Missouri). All other chemicals were obtained locally. "Baker Analyzed" grade (J.T. Baker Chemical Company, Phillipsburg, New Jersey) or equivalent was used when available.

2. METHODS

A. Kill Procedure

Several methods were investigated to kill the cells without altering wall or capsule chemistry. The need for this study arose from the health hazards caused by the production of aerosols during harvesting and breakage procedures and from the fact that cell fragmentation (assumed to be cell death) was incomplete.

(1) Chemical Treatments

Shake cultures of each strain were prepared in 125 ml Erlenmeyer flasks containing 50 ml Sabouraud dextrose (2%) broth. The flasks were shaken at 125 rev/min in a R77 Metabolyte shaker water bath (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 25C. After 36 hr, formalin (final concentration 0.5%), phenol (final concentration 0.5%) or KCN (final concentrations between 10^{-6} and 10^{-2} M) was added to a fask of each strain and incubation continued. Samples (0.1 ml) were withdrawn at intervals up to 12 hr from the formalin and phenol treatments and up to 48 hr from the KCN treatments and spread on Sabouraud dextrose agar plates which were incubated at 25C for 7 days. Plates were checked daily for growth.

(2) γ-Radiation

Dembitzer et al. (1972) reported that Cr. neoformans cells subjected to 1×10^6 rads lost their ability to reproduce.

A 36 hr Cr. laurentii culture was exposed for 90 min to a Co^{60} source giving 73.44 x 10^4 rads/hr. Samples were withdrawn and plated on Sabouraud dextrose agar every 25 min.

(3) Heat Treatment

Batches (100 ml) of each pathogenic strain were shaken for 36 hr at 25C. The flasks were then swirled in a water bath at 50, 60, 75 and 85C for 1 hr. Samples were taken at intervals, plated as before, and observed for 1-2 weeks.

Heat effects were also assessed for all the Cryptococcus strains when grown in Littman's Capsule medium (LCM). Duplicate flasks (100 ml medium) for each strain were treated at 55C for up to 2 hr. Samples were withdrawn and inoculated as drop colonies on Sabouraud agar plates (two drops per duplicate flask).

B. Decapsulation

To obtain pure cell walls, all capsular material should be removed and conversely, capsular material must be free from cytoplasmic or wall contaminants. Both walls and capsules should be unaltered chemically by the treatment. According to Bulmer and Sans (1968) sonic oscillation removed 80% of the capsular material without disrupting cells. Heat killed cells of Cr. neoformans 365/11 (20g) and 20g glass beads (0.45 mm Braun, Canadian Laboratory Supplies, 14823, 118 Ave., Edmonton) were mixed to a thick slurry and were then cooled in an ice bath. The suspension was sonic oscillated at 200 watts for four 5 min intervals using a Blackstone Ultrasonic Probe.

The cells were kept chilled in an ethanol-ice bath throughout and the probe was immersed in ice between treatments. Cells were observed by phase contrast microscopy after each treatment. The methods of Goren and Middlebrook (1967) and Vogel (1966) using dimethylsulfoxide and 0.5 N HCl respectively, were also tried as were treatments of cells with 8 M urea, 5 M sucrose and 5 M NaCl.

C. Growth Conditions

Several authors (Littman 1958, Bulmer and Sans 1968, Foda et al. 1973) showed that extent of capsulation in Cryptococcus species depended on the growth medium. Therefore the optimum conditions for capsule production and for secretion of extracellular material were determined for all of the strains. Liquid media used were Sabouraud dextrose broth (SAB; 1% neopeptone, 2% dextrose) and Littman's modified capsule medium (LCM; Littman 1958) with 1.5 mg litre^{-1} $NaNO_3$ replacing 1.5 mg litre^{-1} $Na_2MoO_4.2H_2O$ (to avoid precipitation of the medium) and varying concentrations of thiamine. Exponential growth was established for each strain in both media at 25 and 37C and pH optima for growth and capsule synthesis were determined.

(1) Measurement of growth and thiamine requirements

Samples (50 ml) of LCM were dispensed in 125 ml flasks and thiamine HCl (to give final concentrations between 0 and 50 µg/ml) added to half the flasks. Filter sterilized thiamine (the same range of concentrations) was added to the

remaining flasks after autoclaving. Sterile dextrose (1% final concentration) was also added to all the flasks at this time. A suspension of each strain was made in sterile water from a loopful of a 3 day old slant culture and agitated thoroughly with a Vortex mixer. Cell counts were obtained with a haemocytometer and 1 ml, containing approximately 3×10^7 cells, was added to each flask. A sterile-water cell suspension was left 24 hr at room temperature and these vitamin depleted cells were used to inoculate medium containing no thiamine. All the flasks were incubated at 25C in a psychrotherm controlled environment reciprocal shaker incubator at 100 rev/min. Samples were removed periodically from each flask and growth determined spectrophotometrically at 650 mm against a medium blank. Medium was also used to make dilutions as cultures became too dense to read. When cells had reached stationary phase the experiment was terminated, and pH measurements taken for each flask. Ocular micrometer measurements were made of capsule and cell diameters with fungicidal India ink mounts (Littman 1958). The mean diameter (microns) of 50 cells excluding capsule was subtracted from the mean diameter of cells including capsule: this figure, divided by two, denoted capsule thickness (Ishaq, Bulmer and Felton 1968). Photographs were taken under phase contrast microscopy to show differences in cell shape and capsule size.

Special precautions were taken during growth experiments with the pathogenic strains. All inoculation and transfer procedures were performed in a negative pressure microbio-

logical hood R 3.5 MP, fitted with ultraviolet lights (Germ Free Laboratories, Miami, Florida). Labelled spectronic 20 tubes were fitted with foam stoppers and autoclaved immediately readings had been taken. pH measurements were made with the electrode within the hood.

D. Hyphal Growth Forms

Hyphal Cr. neoformans, Coward strain, was grown in V8 juice agar at 25C and 30C. Sabouraud agar and corn meal agar did not give good filamentous growth. After 10 days, agar containing the submerged filaments was transferred to 10 ml sterile distilled water and macerated with a glass rod. This suspension was used to inoculate V8 juice broth, Sabouraud broth and Littman's capsule medium. Flasks were incubated at 25C; either shaken very slowly or left as still cultures. Most of the growth was yeast-like and insufficient filamentous growth was obtained for cell wall or capsule preparations even after two weeks.

The other yeast strains, including Cr. laurentii #8114, Cr. terreus #8157 and Cr. albidus #900 were also tested for hyphal growth and formation of conjugation tubes. Media used were corn meal (Difco), Sabouraud (2% dextrose), malt extract and V8 agars. Cultures were streaked on the agar plates and incubated at 15, 25, 30 and 37C. The two strains of Cr. laurentii, 371 and 8114, and of Cr. albidus 367 and 900 were tested for mating responses by Kurtzman's method (1973) and incubated on malt extract agar and V8 agar at 15C.

E. Extraction and Purification of Extracellular Material

Inoculum cultures of each strain were prepared in 50 ml Littman's capsule medium (Littman 1958): Dextrose (1% final concentration) was added after autoclaving. With Cr. albidus #367, thiamine (10 µg/ml final concentration) was also added after autoclaving. The cultures were shaken at 100 rev/min in the psychrotherm reciprocal shaker incubator at 25C until each strain reached exponential phase. Samples (5 ml) of the exponential phase cultures were transferred to 350 ml of the same medium in 1 litre Erlenmeyer flasks and shaken at 90 rev/min at 25C.

When the cultures reached stationary phase, as judged by pH and absorbance measurements, the cells were harvested by centrifugation at 16,000 xg. Cells of pathogenic strains were killed before harvesting by heating the flasks in a water bath at 55C for 90 min with occasional swirling. To prevent damage to polysaccharide constituents by heating in dilute acid (final pH of medium was ca. 3.0) the medium was adjusted to ca. pH 6.0 with 0.1N NaOH before the heat treatment. The cells were washed twice with water, by resuspension and centrifugation, to dislodge as much adhering capsular material as possible. Washings were combined with the culture fluid and passed through a millipore filter (0.45 µ pore size) to remove whole cell and cell fragment contaminants.

Soluble polysaccharide complexes were then extracted according to the following modifications of the procedure of Farhi, Bulmer and Tacker (1970). The combined culture fluids

were concentrated to 1/10 volume by rotary evaporation (Flash-Evaporator, Buchler Instruments, Fort Lee, New Jersey) at 50c in vacuo and two volumes of absolute ethanol were added to the concentrate. After 48 hr at 4C, the precipitate was collected by centrifugation at 300 x g. One volume of absolute ethanol was added to the supernatant and the solution was left for a further 48 hr. The precipitate was collected by centrifugation at 300 x g. The two precipitates were pooled, washed twice with absolute ethanol, centrifuged at 300 x g and resuspended in deionized water. The suspension was dialyzed against three changes of deionized water in a 20 litre Multiple Dialyzer (Oxford Laboratories) at 4C. The non-diffusible material was centrifuged at 27,000 x g for 60 min at 2C to remove insoluble material, dispensed into pre-weighed plastic beakers and lyophilized. The beakers were then reweighed and the white fluffy material stored in screwcap vials at 4C.

At least two preparations were made from each strain, grown under identical conditions.

F. Cell Wall Extraction and Purification

Seed cultures of Cr. laurentii and Cr. neoformans 365-26 were prepared from agar slants by inoculating 100 ml of Sabouraud dextrose (2%) broth in 250 ml flasks and shaking at 25C at 100 rev/min. After 36 hr (during the exponential growth phase; capsules less than 0.2 μ diameter), 10 ml samples of each seed culture were transferred to 2800 ml Fernbach flasks containing 1 litre of broth and were shaken at 25C for 36 hr. Prior to

harvesting, Cr. neoformans cells were killed by heating, with constant swirling, in a 65C water bath for 25 min. The pH of the medium after 36 hr incubation was about 6.0, therefore no adjustment was necessary to avoid acid conditions. The cells were harvested by centrifugation at 9000 x g for 10 min, washed twice with water to remove adhering extracellular material, and cooled for 15 min in an ice bath. Portions (10 ng) of washed cells were mixed into a paste with 2 ml ice-cold water and 50 g glass beads (0.45 - 0.50 mm diameter, Braun, Canadian Laboratory Supplies, Edmonton) and disrupted in a Braun MSK homogenizer for four to six x 2 min periods. The process of breakage was assessed using phase contrast microscopy. Four x 2 min periods gave approximately 90% breakage of Cr. laurentii cells but six x 2 min periods were required to break approximately 70% of the Cr. neoformans cells.

The bead paste was washed 5 x with cold deionized water, by resuspension and centrifugation, and the supernatants combined. Whole cells of Cr. laurentii were removed by centrifugation at 950 x g for 5 min at 2C. The supernatant was then centrifuged at 27,000 x g for 10 min to recover cell wall fragments. With Cr. neoformans, however, the combined supernatants from the bead paste had to be shaken with a Vortex mixer to disperse aggregates of whole cells and wall fragments. The resulting suspension was centrifuged at 120 x g for 5 min and the pellet of whole cells and fragments washed 5 times to extract wall fragments into the supernatant. The supernatants were combined and centrifuged once more to remove remaining whole

cells. The cell wall fragments were then collected by centrifugation at 27,000 x g for 10 min.

The washing procedure was based on that of Mitchell and Taylor (1969). The cell wall fragments were washed 5 times with ice-cold deionized water (centrifugation at 27,000 x g for 10 min for Cr. laurentii, 12,000 x g for Cr. neoformans), 5 times with ice-cold 1.0M NaCl solution (centrifugation at 12,000 x g for both preparations), 5 times with cold water and twice with 8.0 M urea solution (centrifugation at 27,000 x g for 10 min for Cr. laurentii, 12,000 x g for Cr. neoformans). The fragments were suspended overnight in 8.0 M urea solution at 4C, then centrifuged and washed a further 3 times with 8M urea solution, 5 times with water, 5 times with 1.0N NH_4OH solution (centrifugation at 1200 x g for 10 min for both preparations) and 5 times with water, or until free from cytoplasmic contamination (as judged by phase contrast microscopy). The final wash was extended for 20 min and the cell walls were lyophilized and stored at -20C. The walls also appeared free from any adhering capsular material (as judged by India ink staining and phase contrast microscopy).

G. Analytical Procedures

Stock solutions containing known amounts (approximately 2 mg/ml) of each dry lyophilized extracellular preparation were made in water. Samples from these stocks were used in the analytical procedures to minimize weighing errors. Where necessary, the samples were freeze dried in 13 x 100 mm test tubes before use.

(1) Elemental and Ash Analyses

Analyses for nitrogen were performed by Organic Microanalysis, Montreal, Quebec. Phosphorus was determined spectrophotometrically by the method of Ames (1966) with KH_2PO_4 as standard.

Ash was determined gravimetrically. Coors crucibles (15 x 9 mm, Canadian Laboratory Supplies Ltd., Edmonton), were weighed on a 6-place balance and approximately 20 mg extracellular material weighed into each. They were left in a desiccator over P_2O_5 overnight, reweighed, heated to constant weight (12 hr) in a muffle furnace at 800C, cooled in a desiccator and weighed again.

(2) Amino Acids

Cell walls and extracellular material (4-5 mg) were hydrolyzed in vacuo with 0.5 ml of 6N HCl containing 1 mg/ml oxalic acid (James 1972), at 110C for 24 and 48 hr (Tristram and Smith 1963, Cameron 1973). The hydrolysates were dried in vacuo over concentrated H_2SO_4 and KOH pellets, then redissolved in 1.0 ml pH 2.2 sodium citrate buffer (0.02N). Aliquots of each solution together with 20 nm of the internal standard solution (α-amino-β-guanido-propionic acid for basic amino acids and norleucine for neutral and acidic amino acids) in pH 2.2 buffer were applied to the appropriate column of a Beckman Amino Acid Analyzer Model 120C. The analysis was based on the methods of Spackman, Stein and Moore (1958) and Cameron (1973). Basic amino acids were separated on a 16.5 x 0.9 cm

column and eluted with pH 5.25 sodium citrate buffer. After application of the sample, the space above the resin bed was filled with pH 3.25 sodium citrate buffer and the buffer line containing pH 5.25 buffer was connected. This procedure allowed separation of lysine from ethanolamine which occurred in most of the preparations, and also resolved phenylalanine from tyrosine and glucosamine from galactosamine. Acidic and neutral amino acids were separated using a 58 x 0.9 cm column. Glucosamine was adequately separated from phenylalanine as long as column length was greater than 58 cm. Hydroxyproline was determined after hydrolysis in 0.5 ml 6N HCl in vacuo for 14 hr at 110C, by the spectrophotometric method of Bergman and Loxley (1970), using *p*-dimethylaminobenzaldehyde.

(3) Amino Sugars

Cell walls and extracellular material (2-4 mg) were hydrolyzed in vacuo with 0.5 ml of 2N HCl (Oates and Schrager 1967) containing 1 mg/ml oxalic acid at 105C for 8, 16, 24, 72 and 96 hr. Hydrolysates were dried in vacuo over concentrated H_2SO_4 and KOH pellets, dissolved in 1 ml of 0.2N sodium citrate buffer, pH 2.2, and analyzed using the 16.5 x 0.9 cm column of the amino acid analyzer.

(4) Neutral Sugars

Neutral sugars were determined by two separate procedures. In both cases the free sugars were estimated by gas-liquid chromatography as trimethylsilyl (TMS) derivatives.

The first procedure was a modification of Cameron's

method (1973) based on the procedures of Albersheim et al. (1967) and Sweeley et al. (1963). Cell walls and extracellular material (approximately 2 mg) were hydrolyzed in sealed tubes at 105C with 0.5 ml 2N CF_3COOH for 30, 60, 120 and 240 min to release the neutral sugars. Hydrolysates were dried in vacuo over KOH pellets and 0.5 ml of the internal standard, myo-inositol (1 mg/ml in deionized water) added. The solution was dried, then dissolved in 1 ml pyridine and treated, successively, with 0.1 ml hexamethyldisilazane and 0.05 ml trimethylchlorosilane. The mixture was shaken for a few seconds, and after 30 min was freeze dried to remove the pyridine. The TMS derivatives were redissolved in 1 ml cyclohexane and aliquots of this solution injected into a Varian Aerograph dual column gas chromatograph Model 1740, equipped with flame ionization detectors. The flow rates of N_2 and H_2 were 25 ml/min and air 250 ml/min. Columns (4.9m x 3mm) containing 10% silicone fluid SF 96 on acid washed DMCS treated 60/80 mesh flux-calcined diatomite (Chromosorb W, Chromatographic Specialities, Brockville, Ontario) were used. They were linearly temperature programmed from 130C (at injection) to 230C at 2 degrees/min. Peak areas were measured with a Varian Aerograph 477 integrator. Only one internal standard, myo-inositol, was employed since the integrator gave accurate readings when the gas-liquid chromatograph peaks were off-scale.

The second procedure involved freeing the neutral sugars by resin hydrolysis according to the method of Lehnhardt and Winzler (1968). Approximately 1 mg of extracellular

material (0.5 ml stock solution) was hydrolyzed with an equal volume of 40% w/v AG 50 x 2 (H+) 200/400 mesh resin (Bio-Rad Laboratories, Richmond, California) in 0.02N HCl in sealed test tubes at 100C for 24, 48 and 72 hr (Niedermeier 1971). Samples were cooled, the seal was broken, 0.4 mg of the internal standard (mannitol) was added and the solution was mixed immediately with a Vortex mixer. The mixture was transferred to a Pasteur pipette column plugged with glass wool and the hydrolysates collected in Erlenmeyer flasks. The hydrolysis tubes were washed several times with deionized water and the washings poured through the pipette column. A total of 30 ml deionized water was siphoned through the resin and glass wool and collected in the Erlenmeyer flasks. The hydrolysates plus washings were passed through a second Pasteur pipette column containing 1 ml of a 20% W/V suspension of AG1-X8 (HCO_3-) 200/400 mesh resin (Bio-Rad) in water. The Erlenmeyer flasks were rinsed several times with a 50% v/v solution of methanol-water and the washings siphoned through the columns. The eluates were collected in round bottom flasks and concentrated to a small volume with a rotary evaporator. The concentrates were then transferred to 10 ml round bottom flasks and dried with the rotary evaporator. The samples were then treated with 0.5 ml N,N-dimethylformamide containing 0.1 M 2-hydroxypyridine and allowed to mutarotate overnight at 40C (Reid et al. 1970). The sugars were now at mutarotation equilibrium and were silylated by shaking for 30 min with 0.5 ml hexamethyldisilazane and 0.25 ml trimethylchlorosilane.

Samples (2 µl) of the mixtures were injected into a Hewlett Packard 7610A dual column gas chromatograph fitted with flame ionization detectors and direct on-column injection. The flow rate of He (carrier gas) was 60 ml/min, H_2 35 ml/min, and air 500 ml/min. Dual copper columns (2.4 x 6 mm) containing 10% (w/w) SE-52 on 80/100 mesh diaport S were used, and the chromatograph was operated isothermally at 190C with a flash heater temperature of 260C and a detector temperature of 270C. Peak areas were measured with an electronic integrator. Molar response factors for each sugar relative to mannitol (Dutton et al. 1968), and the percentage composition of each of the sugars at mutorotation equilibrium, had been previously determined.

(5) Uronic Acids

Uronic acids were estimated colorimetrically with metaphenylphenol according to the method of Blumenkrantz and Asboe-Hansen (1973). The authors claimed this method to be both more sensitive and more specific for uronic acids than the earlier methods described by Dische (1947) and Brown (1946). Glucuronolactone was used as the standard.

(6) O-Acetyl

The method of O-acetyl determination involved transesterification with sodium methoxide (0.1 M in absolute methanol) at 0C for 30 min (Whistler and Jeanes 1943). The resulting methyl acetate was estimated by gas chromatography according to the method of Reid et al. (personal communication). 30 µl

benzene standard (89.9 mg/ml) were added to 10 mg extracellular material before treatment with 0.1M sodium methoxide and 2 µl was injected into a Hewlett Packard F and M 402 gas chromatograph fitted with flame ionization detectors. The flow rate of He was 25 ml/min, H_2 40 ml/min and air 400 ml/min. Dual copper columns (1.8m x 3mm) of Chromosorb 101 were linearly temperature programmed from 100 (at injection) to 150C at 4 degrees/min.

H. Electrophoresis and Staining

(1) Polyacrylamide gels

Approximately 25 mg of Cryptococcus albidus extracellular material was dissolved in 1.5 ml deionized water by degassing in a small bell jar. Sample (0.05 - 0.2 ml) was applied to 0.5 x 6.0 cm polyacrylamide gel columns (pH 9.0) prepared by the method of Ornstein and Davis (1962) as modified by Fox, Thurman and Boulter (1964) except that the large pore gel was omitted. Gels were run at 3 ma per tube until the marker dye reached the end of each tube. Protein was detected in the gels by staining with 1% w/v amido black in 7% w/v acetic acid for one hour. Destaining was conducted for one to three days in 7% acetic acid. Carbohydrate was detected by a modified periodic acid Schiff stain (Page and Stock 1974). Band mobility (Rp) was expressed as a fraction of the mobility of the bromophenol blue front-tracking dye. Distance was measured from the middle of each band.

(2) Cellulose acetate strips

Samples (2 mg) of extracellular material were dissolved in 500 µl tris-barbital-sodium-barbital buffer, pH 8.8, 0.05 M (Gelman High Resolution Barbital buffer, Gelman Instrument Company, Ann Arbor, Michigan). Samples (5 µl) were applied to Gelman Sepraphore 111 cellulose acetate strips (2.5 cm x 15 cm). Electrophoresis was carried out in a Gelman electrophoresis unit at 3 ma per strip for 40 min. To differentiate and demonstrate both glycoprotein and acidic polysaccharide, the strips were cut in half with a razor blade and either stained for glycoprotein by the Alcian blue method of Wardi and Allen (1972) or for carboxyl groups with Alcian blue (1% in 2.5% acetic acid) alone. The latter strips were destained in two changes of 1:1 (v/v) 0.1 M citrate buffer (pH 3.0): absolute ethanol, and cleared with two changes of absolute methanol (60 sec each) and one rinse 10% acetic acid v/v in absolute methanol (60 sec). Separate strips were stained for protein with 0.25% w/v Coomassie brilliant blue in 7% w/v acetic acid for 15 min (Dulaney and Touser1970). These strips were destained in four changes 5% aqueous acetic acid and cleared as described above. The cleared strips were placed on a grease free glass plate and dried in an oven at 60C for 15 min. Band mobility was expressed as a fraction of the mobility of a standard applied to separate strips: heparin for the Alcian blue stain and hyland control serum for Coomassie blue.

I. Gel Chromatography

Approximately 15 mg of Cr. neoformans #365-26 extracellular polysaccharide were suspended in 10.0 ml of 0.02 M pyridine-HCl buffer (pH 5.5) and degassed to dissolve. The solution was dialyzed at 4C against three changes of the same buffer and chromatographed on a column of DEAE Bio Gel-A (100/200 mesh, Bio-Rad) which had been equilibrated with pyridine-HCl buffer pH 5.5. The flow rate was 10 ml per hr and the void volume was 250 ml. After elution of the column with 400 ml of the buffer, a convex gradient from 0-3.0 M NaCl (700 ml) in 0.02 M pyridine-HCl buffer (700 ml) was applied. Fractions of 10 ml were collected and portions (2 ml) assayed for carbohydrate by the phenolsulphuric acid method (Dubois et al. 1956), and for protein by ultraviolet absorption at 280 nm with a Beckman DU spectrophotometer. Conductivity was measured using a conductivity bridge model RC 216B2 and was proportional to the molarity of NaCl.

J. Infrared Spectra

Approximately 1.5 mg dry, lyophilized cell wall or extracellular material were ground with 200 mg dry KBr in an agate mortar. The dry, finely ground powder was scraped into an evacuable die and pressed into a clear pellet with a hydraulic press at 18 tons. The 13 mm discs were scanned using a Unicam SP 200G spectrophotometer.

K. Virulence Testing with Mice

The virulence of each strain used was tested by inoculating 12 Swiss white mice per strain with 0.02 - 0.04 ml of a 10^6 cell suspension in sterile saline. The suspensions were prepared from three-day-old cultures of each strain and injected intracerebrally with a 26 gauge needle. Cell counts were made with a haemocytometer and serial dilutions plated out on Sabouraud dextrose agar to check the viable count. Deaths began to occur after four days and the experiments were terminated after four weeks. Mortality rate was expressed as the time taken to kill 50% of the mice. Autopsies were performed, agar slants inoculated with brain tissue and smears examined with fungicidal India ink by phase contrast microscopy.

CHAPTER III

RESULTS

1. Kill Procedures

Potassium cyanide (10^{-6} - 10^{-2} M) failed to kill any of the Cryptococcus strains tested even after 48 hrs incubation. Capsule synthesis, as judged by average capsule size, was not affected.

Exposure to 10^{6} rads from a Co^{60} source slowed the growth but failed to kill Cr. laurentii 371-1. There was no detectable loss of capsule. Little morphological change was observed after exposure to lower doses of radiation, but after 10^{6} rads some cells appeared distorted and were budding abnormally.

Incubation with formalin or phenol at 25C for 2 and 6 hr, respectively, killed all the Cr. neoformans strains (Table 1). However, pretreatment with formalin led to extensive flocculation of wall fragments with whole cells during the breakage procedure. Treatment of Cr. laurentii cells with phenol for 10 hr prior to harvest was also an unsuitable method of killing the cells since the subsequent recovery of amino acids from the cell walls was substantially reduced (Table II).

The effect of high temperatures for up to 1 hr on the three Cr. neoformans strains is shown in Table III. All the strains were sensitive to high temperatures. Heat treatment

TABLE I

EFFECTS OF INCUBATION, FOR DIFFERENT TIMES, WITH FORMALIN (FINAL CONCENTRATION 0.5% HCHO) AND PHENOL (FINAL CONCENTRATION 0.5%) ON GROWTH OF CR. NEOFORMANS

Incubation time in hr	Growth of Strains at 25C[a]					
	Phenol			Formalin		
	365-11	365-16	365-26	365-11	365-16	365-26
0	+++	+++	+++	+++	+++	+++
1	+++	+++	+++	+	++	+
2	++	++	+	-	-	-
3	+	++	-	-	-	-
6	-	-	-	-	-	-
8	-	-	-	-	-	-
10	-	-	-	-	-	-
12	-	-	-	-	-	-

[a]Scale of growth from - = no growth to +++ = maximum growth of colonies on Sabouraud dextrose agar plates inoculated with 0.1 ml treated cells and incubated at 25C for 7 days. Results are averages of growth recorded on two plates/treatment/time.

TABLE II

AMINO ACID CONTENT OF CELL WALLS OF CR. LAURENTII 371-1 AFTER TREATING THE CELLS WITH PHENOL (0.5% FINAL CONCENTRATION) FOR 10 HR PRIOR TO HARVEST

(μg anhydro amino acid recovered/mg cell wall[a] preparation)

24 hr hydrolysis with 6N HCl

Amino acid	Untreated cells	Phenol treated cells	% decrease
Lys[b]	1.3	1.3	0.0
His	0.6	0.5	16.7
Arg	0.6	0.4	33.3
Asx	1.9	1.3	31.6
Thr	1.1	1.0	9.1
Ser	1.7	1.5	11.8
Glx	2.4	1.3	45.8
Pro	0.9	0.5	44.4
Gly	1.0	0.9	10.0
Ala	1.3	0.9	30.8
Cys	0.0	0.0	0.0
Val	0.9	0.6	33.3
Met	0.2	0.1	50.0
Ile	0.8	0.4	50.0
Leu	1.3	0.8	38.5
Tyr	0.7	0.6	14.3
Phe	0.8	0.6	25.0
Total Recovery	17.2	12.7	

[a] partially purified wall preparations were used

[b] Lysine poorly resolved

TABLE III

EFFECT OF TEMPERATURE ON GROWTH OF THREE STRAINS OF CR. NEOFORMANS

Incubation time in min.	Growth of Strains at 25C[a]											
	365-11				365-16				365-26			
	Treatment Temperatures °C											
	50	60	75	85	50	60	75	85	50	60	75	85
0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5	+++	++	+	-	+++	++	+	-	+++	+++	++	-
10	+++	-	-	-	+++	+	-	-	+++	+	-	-
15	+++	-	-	-	+++	-	-	-	+++	-	-	-
30	+++	-	-	-	+++	-	-	-	+++	-	-	-
45	+++	-	-	-	+++	-	-	-	+++	-	-	-
60	+	-	-	-	++	-	-	-	+++	-	-	-

[a]Scale of growth from - = no growth to +++ = maximum growth on Sabouraud dextrose agar plates inoculated with 0.1 ml treated cells and incubated at 25C for 7 days. Results are averages of growth recorded on two plates/treatment/time.

did not appear to affect protein or polysaccharide composition of extracellular material (Table IV). In all the following experiments cells were killed by heat treatment at 65C for 25 min. (prior to harvest) for cell wall extraction and at 55C for 90 min (prior to harvest) for extracellular material preparation.

Table V shows differences in resistance to heat treatment at 55C among the six Cryptococcus strains growing in LCM. The two Cr. albidus strains were the least resistant, and Cr. laurentii 371-1 the most resistant to heat. There was little difference in resistance among the Cr. neoformans strains: strain 365-16 seemed the most resistant to heat.

2. Decapsulation

None of the methods tested gave complete decapsulation. Short periods of sonic oscillation (1-5 min) removed little or no capsular material and, in contrast to Bulmer and Sans' work (1968), periods of above 5 min ruptured some of the cells. Extended treatment (overnight) with dimethylsulfoxide (Goren and Middlebrook, 1967) removed some of the capsules but even large volumes of solvent (500 ml/40 ml cells) failed to completely decapsulate all the cells. Consequently cells for wall preparations were grown in SAB for 36 hr (minimum capsule production) and for extracellular material LCM was used (5 days growth gave maximum capsule production). Adhering capsular material was not extracted and analyzed in this study because of these difficulties.

TABLE IV

AMINO ACID AND NEUTRAL SUGAR CONTENT OF EXTRACELLULAR MATERIAL OF CR. NEOFORMANS 365-26 AFTER HEAT TREATMENT AT 55C FOR 90 MIN PRIOR TO HARVEST

µg anhydro amino acid recovered/mg extracellular material. 24 hr hydrolysis with 6N HCl[a]			µg anhydro neutral sugar recovered/mg extracellular material. 1 hr hydrolysis with 2N TFA[b]		
	Untreated cells	Heat treated cells		Untreated cells	Heat treated cells
Lys	0.7	0.8	Xyl	175.64	174.74
His	0.2	0.2	Man	239.50	241.99
Arg	0.3	0.3	Gal	54.21	51.43
Asx	4.8	4.8	Glc	10.82	11.55
Thr	2.9	2.8	Total Recovery	480.17	479.71
Ser	4.5	4.6			
Glx	2.4	2.5			
Pro	4.3	4.2			
Gly	0.8	0.8			
Ala	3.9	3.8			
Cys	0.0	0.0			
Val	1.9	1.9			
Met	0.0	0.0			
Ile	2.1	2.0			
Leu	0.9	0.9			
Tyr	0.1	0.1			
Phe	2.5	2.4			
Total Recovery	32.3	32.1			

[a] Mean of duplicate hydrolysis

[b] Mean of duplicate hydrolyses. Sugars estimated as TMS derivatives by gas chromatography (Cameron 1973). For details of procedure see Chapter II, Section 2 G(4).

TABLE V

EFFECT OF HEAT TREATMENT OF 55C ON SIX CRYPTOCOCCUS STRAINS GROWING IN LCM[a]

	Cr. neoformans 365-11				Cr. neoformans 365-16				Cr. neoformans 365-26				Cr. laurentii 371-1				Cr. albidus 367				Cr. albidus H1354			
Time Min	Drop no.[b] 1	2	3	4	Drop no. 1	2	3	4	Drop no. 1	2	3	4	Drop no. 1	2	3	4	Drop no. 1	2	3	4	Drop no. 1	2	3	4
0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
15	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+++	+++	+++	+++	++	++	++	++	++	+++	++	+++
20	+++	++	++	+++	+++	+++	+++	+++	++	++	++	-	+++	+++	+++	+++	-	-	-	-	++	+	-	-
30	++	+	-	-	+	++	++	+++	+	+	++	+	++	++	++	+++	-	-	-	-	++	-	-	-
40	+	-	-	-	+	+	++	+++	+	-	-	-	+	+	+++	+	-	-	-	-	-	-	-	-
60	+	-	-	-	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	+[c]	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
110	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
120	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a: Littman's Capsule Medium containing 10 ug/ml thiamine HCl
b: 4 drops/strain (2 from each of duplicate flasks), were placed on Sabouraud agar plates (1 strain per plate) and incubated at 25C for 1 week. - = no growth of drop +++ = maximum growth of drop
c: plate contaminated, colony atypical of Cr. neoformans

3. Growth Conditions

A. Temperature

All the strains grew well at 25C in SAB and LCM. Exponential phase was established as 36 hr in both media for five of the strains. Cr. albidus 367 was the exception: logarithmic phase was reached after 72 hr in LCM and after 65 hr in SAB. Cr. laurentii 371-1 and all the Cr. neoformans strains grew well at 37C but Cr. albidus 367 grew poorly at 33C and not at all at 37C. Cr. albidus H1354 grew well at 33C but also showed no growth at 37C.

B. pH

The optimum pH for capsule production of all six strains was between pH 6.5 and pH 7.0. This confirmed the work of Littman (1958) and Bulmer and Sans (1968). Above pH 7.0 both growth and capsule synthesis were much reduced: Cr. albidus strains 367 and H1354 exhibited an extended lag phase of 48 hr followed by scanty growth. In contrast Bulmer and Sans (1968) reported maximum capsule size at pH 7.5 for a strain of Cr. neoformans, CIA. All the strains produced much smaller capsules in SAB (0.2 - 1μ capsule thickness) than in LCM (1.5 - 4.0μ). The percentage of encapsulated cells was also lower in SAB than LCM and logarithmic phase cells in SAB produced few capsules, mostly less than 0.5μ in width.

C. Thiamine

Littman (1958) reported a stimulatory effect of thiamine on capsule synthesis and growth over a range of concentrations

from 0.0001 to 0.1 µg/ml. Figures 2 to 4 compare the effects of autoclaved and unautoclaved thiamine on cell growth (measured as increased absorbance at 650 nm) and capsule thickness. Only Cr. albidus 367 gave poor growth with autoclaved thiamine: the other strains could use heat separated thiamine (into thiazole and pyrimidine, during autoclaving, Fries 1965) as easily as they could the intact molecule. Although Littman reported a reduction in capsule width at thiamine concentrations above 0.1 µg/ml, all of the strains here showed increases up to 10 µg/ml; Cr. albidus 367 and Cr. neoformans 365-26 gave largest values at 50 µg/ml. Figure 5 shows the differences in capsule size between cells grown in 0.1 and 10.0 µg/ml thiamine for each strain. Cr. laurentii 371-1 showed little difference in capsule size between the two concentrations of thiamine. Cells of Cr. neoformans strains 365-26 and 365-16 were larger when grown in 10 µg/ml thiamine. The histograms in Figures 2-4 indicate no substantial differences in capsule thickness between cells grown in autoclaved and those in unautoclaved thiamine.

As a result of these experiments, 10 µg/ml thiamine was added to large scale cultures grown for capsule extraction before autoclaving except for Cr. albidus 367 to which it was added aseptically afterwards.

4. Cell and Capsule Morphology

The photographs (Figure 5) show the differences in cell size and shape among the strains. Cr. albidus 367 has oval cells which exhibit polar budding. This strain was pink in liquid

FIGURE 2

Effect of thiamine on growth and capsule synthesis of Cr. albidus 367 and Cr. albidus H1354 in BSM measured after 120 hr (Cr. albidus 367) and 72 hr (Cr. albidus H1354) incubation at 25C, shake culture

▲--▲ , represent unautoclaved thiamine

△--△ , represent autoclaved thiamine

a: first reading for vitamin-depleted cell control

capsule thickness expressed as:

$$\frac{\text{diameter of cell + capsule - cell diameter}}{2}$$

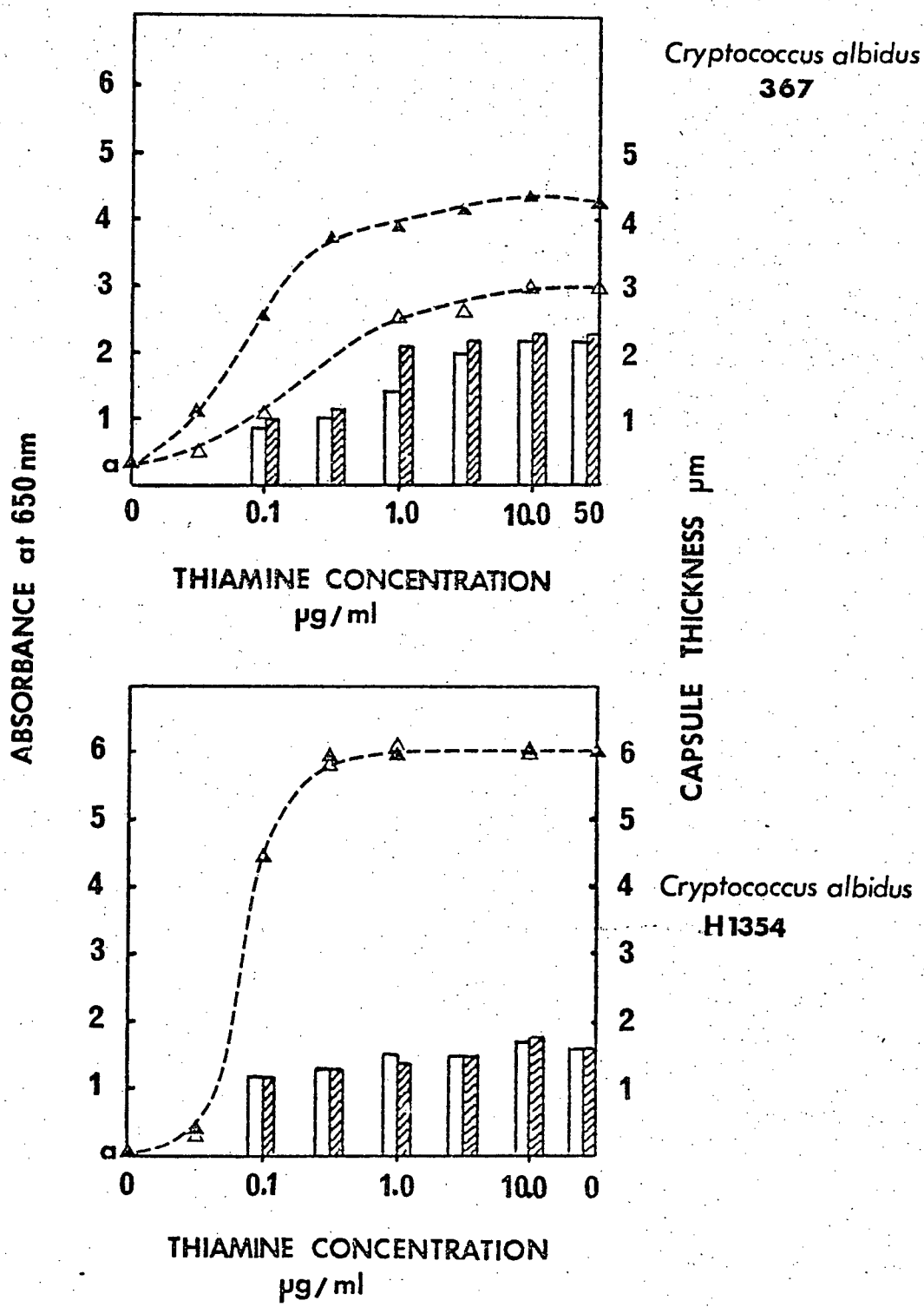

Cryptococcus albidus
367
ABSORBANCE at 650 nm
CAPSULE THICKNESS µm
THIAMINE CONCENTRATION
µg/ml
0
0.1
1.0
10.0
50
Cryptococcus albidus
H1354
THIAMINE CONCENTRATION
µg/ml
0
0.1
1.0
10.0
0

FIG. 2

FIGURE 3

Effect of thiamine on growth and capsule synthesis of Cr. laurentii 371-1 and Cr. neoformans 365-16 in BSM measured after 72 hr incubation at 25C, shake culture.

▲--▲ represent unautoclaved thiamine

△--△ represent autoclaved thiamine

Capsule thickness expressed as:

$$\frac{\text{diameter of cell + capsule - cell diameter}}{2}$$

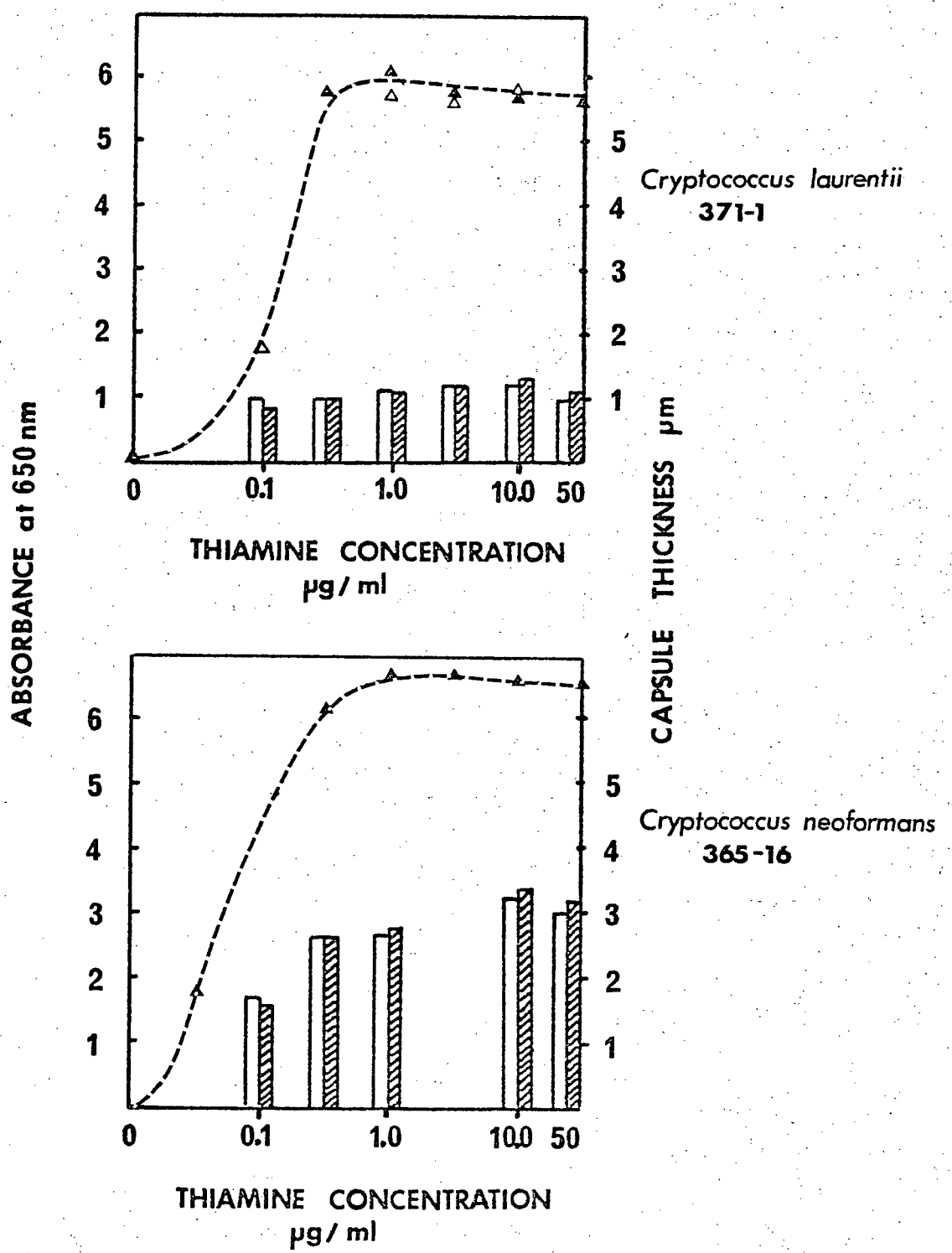

ABSORBANCE at 650 nm
CAPSULE THICKNESS µm
6
5
4
3
2
1
0
0.1
1.0
10.0
50
THIAMINE CONCENTRATION
µg / ml
Cryptococcus laurentii
371-1
Cryptococcus neoformans
365-16

FIG. 3

FIGURE 4

Effect of thiamine on growth and capsule synthesis of Cr. neoformans strains 365-11 and 365-26 in BSM measured after 72 hr incubation at 25C, shake culture.

▲--▲ , represent unautoclaved thiamine

△--△ , represent autoclaved thiamine

Capsule thickness expressed as:

$$\frac{\text{diameter of cell + capsule - cell diameter}}{2}$$

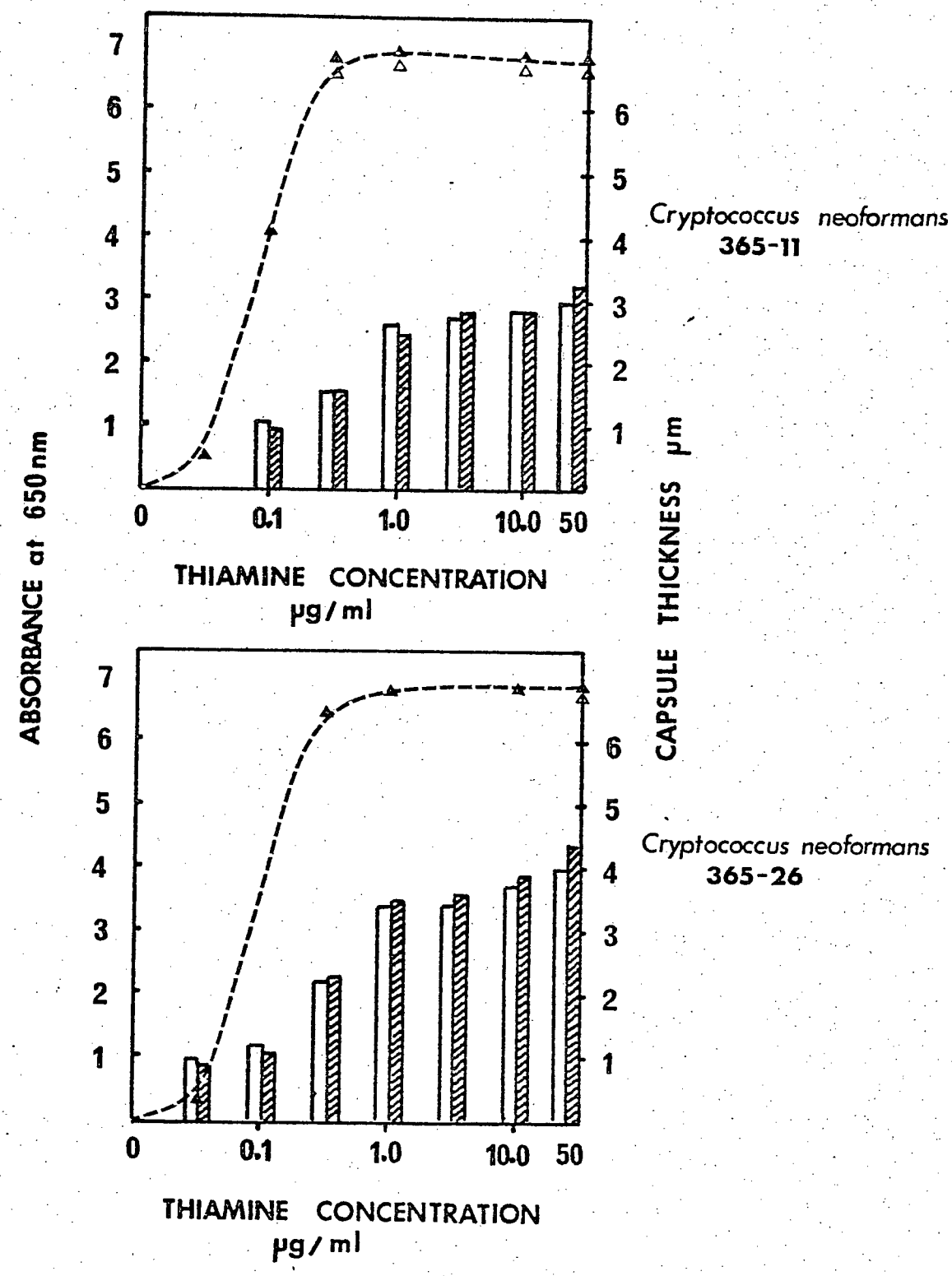

ABSORBANCE at 650 nm
CAPSULE THICKNESS μm
Cryptococcus neoformans
365-11
THIAMINE CONCENTRATION
μg/ml
Cryptococcus neoformans
365-26
THIAMINE CONCENTRATION
μg/ml
0
0.1
1.0
10.0
50

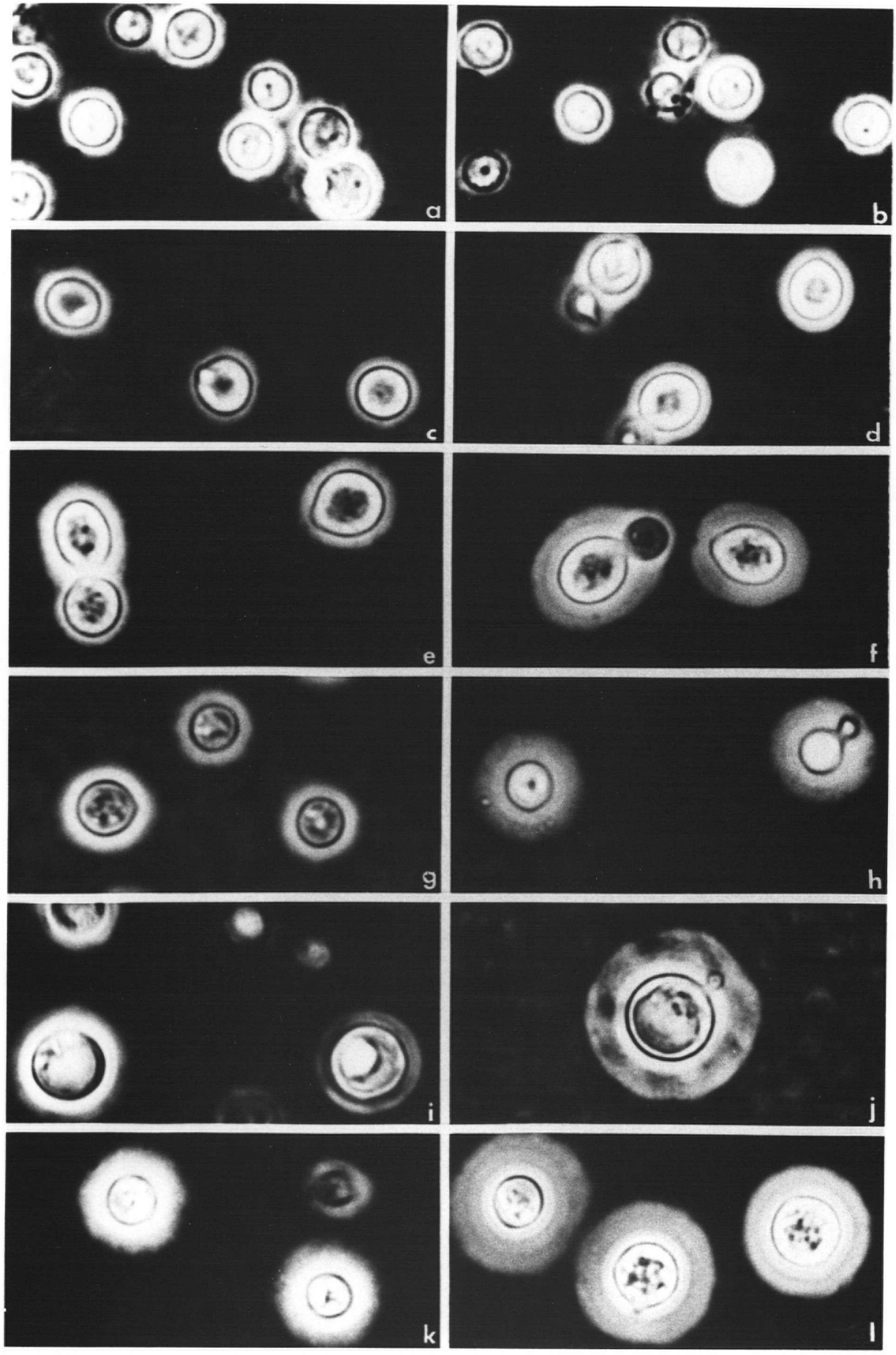
FIG. 4

FIGURE 5

DIFFERENCES IN CAPSULE SIZE BETWEEN CELLS GROWN IN 0.1 AND 10.0 µg/ml THIAMINE FOR EACH CRYPTOCOCCUS STRAIN

(India ink preparations, ⊢——⊣ = 15.3 µm)

With 0.1 µg/ml thiamine	With 10 µg/ml thiamine
a. Cr. laurentii 371-1	b. Cr. laurentii 371-1
c. Cr. albidus H1354	d. Cr. albidus H1354
e. Cr. albidus 367	f. Cr. albidus 367
g. Cr. neoformans 365-11	h. Cr. neoformans 365-11
i. Cr. neoformans 365-16	j. Cr. neoformans 365-16
k. Cr. neoformans 365-26	l. Cr. neoformans 365-26


a
b
c
d
e
f
g
h
i
j
k
l

culture and very mucilaginous in appearance on slants. Cr. albidus H1354 was paler pink and also mucilaginous. Cr. laurentii 371-1 had smaller, spherical cells and cultures were yellowish, colonies being drier and somewhat wrinkled. All three strains of Cr. neoformans had spherical cells, were white in gross appearance and growth was slimy and mucilaginous. Cells of Cr. neoformans 365-11 appeared smaller than the other two strains.

The capsules appear (see Figure 5) to be divided into a bright viscous inner zone and a less viscous outer mucous halo. All the Cr. neoformans strains examined here and Cr. albidus 367 exhibited an outer mucous halo. Cr. laurentii 371-1 showed only a slight halo and it was not visible in Cr. albidus H1354. It seemed that increase in capsule width corresponded to increase in the outer zones in the Cr. neoformans strains and Cr. albidus 367.

5. Growth of Hyphal Forms

Cr. neoformans, Coward strain, grew well on V8 juice agar at 25 and 30C, producing branching septate filaments as submerged growth. The surface colonies were largely yeast-like and mucoid. Figure 6 shows clamp connections but it was not possible to see if the filaments were dikaryotic. The yeast-like cells had small capsules. It was not possible to obtain sufficient filamentous growth for cell wall or capsule extraction. None of the other strains showed hyphal growth or conjugation tubes on the sporulation media tested. However, Cr. albidus 367 and Cr. terreus 8157 developed many cigar-shaped cells and

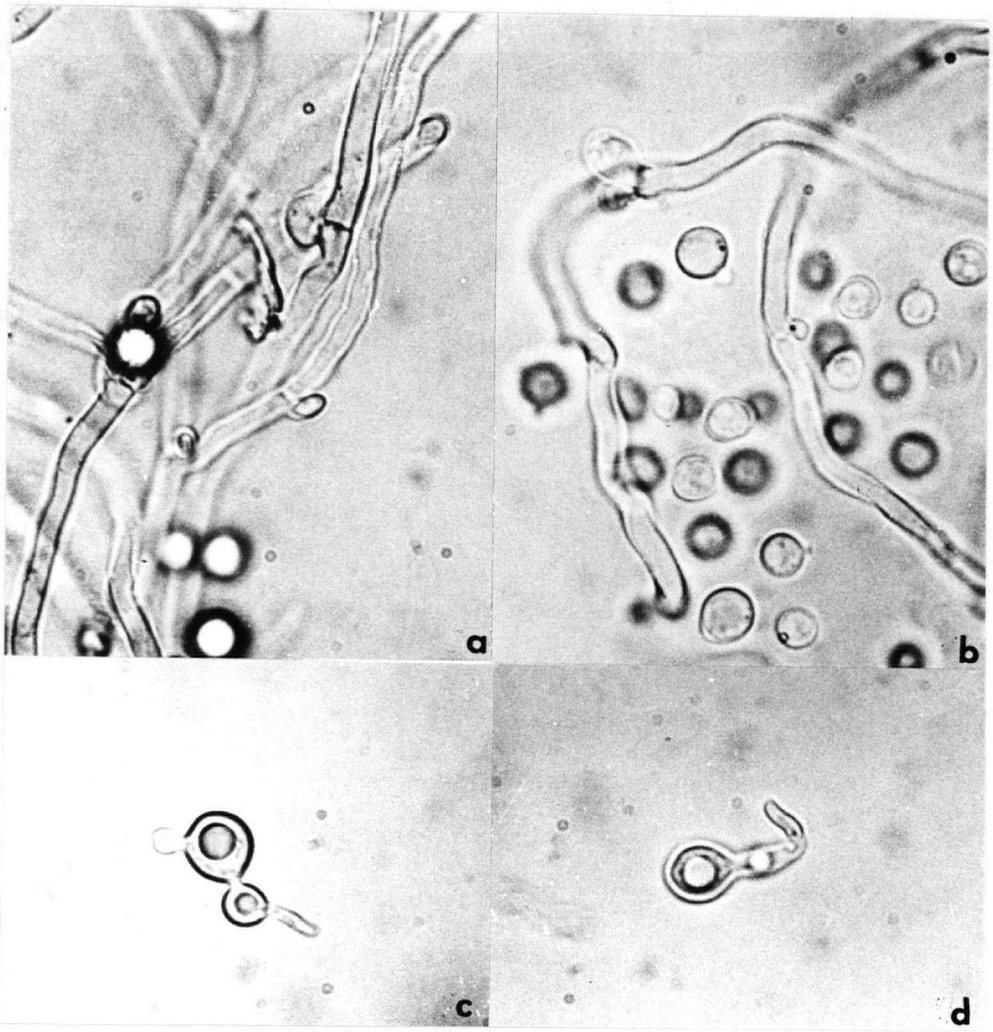


FIGURE 6. HYPHAL GROWTH FORMS IN CR. NEOFORMANS

(Unstained preparations examined under phase contrast microscopy all photographs x 200)

1a Cr. neoformans, Coward strain, clamp connections

1b Cr. neoformans, Coward strain, septate hyphae

2a Cr. neoformans, 365-26 abnormally budding cell after heat treatment.

2b Hyphal protrusion from Cr. neoformans 365-26

hyphal bodies when growing in LCM or SAB after incubation periods of seven days or more. One of the Cr. neoformans strains, 365-26, produced hyphal outgrowths (Figure 6) and possible conjugation tubes after heat treatment (10 min at 60C). All the strains which I examined, except the Coward strain, appear to produce hyphal forms only in response to adverse conditions such as heat treatment or nutrient deficiency.

6. Extracellular Material - Extraction

Initial preparations were brown or yellowish presumably because the dextrose in the medium charred during autoclaving. This was resolved by autoclaving dextrose and medium separately and then adding the dextrose aspectically. The physical appearance of the ethanol precipitate from each strain and the yield of pure extracellular material are given in Table VI. Precipitates from the Cr. neoformans strains were extremely viscous and appeared highly polymerized, yielding large amounts of extracellular material. Apparently the older the culture, the lower the pH became and the higher was the yield of polysaccharide. Fode et al. (1973) showed that a strain of Cr. laurentii produced heteropolysaccharide capsule in the pH range from 3 to 7 and synthesized amylose when pH dropped below 3. The ethanol precipitate of Cr. laurentii 371-1 and both Cr. albidus strains contained a water insoluble fraction which may have been starch-like although produced at a pH of above 3, and showing no reaction with iodine. Very small amounts of insoluble material were recovered from the Cr. neoformans preparations.

TABLE VI

PHYSICAL APPEARANCE AND YIELD OF EXTRACELLULAR MATERIAL FROM *CRYPTOCOCCUS* SPECIES

	Strain and Batch No.	Age of Culture Hr.	Final pH of culture fluid	Appearance of Ethanol precipitate in concentrated culture fluid	Yield of Pure Material mg/ 100 ml culture fluid	Ash content % dry wt.
Cr. alb.	367[1]	120	4.63	[a]yellow suspension	45.00	ND
Cr. alb.	367[2]	144	4.01	non-fibrous,white	16.43	4.64
Cr. alb.	367[3]	120	3.09	slightly fibrous	43.00	6.88
Cr. alb.	H1354[1]	144	6.08[b]	slightly fibrous	12.00	ND
Cr. alb.	H1354[2]	120	3.33	fibrous	14.50	8.52
Cr. alb.	H1354[3]	120	ND	fibrous	29.60	3.61
Cr. laur.	371-1[1]	120	3.15	non-fibrous suspension	43.04	43.00
Cr. laur.	371-1[2]	120	3.19	non-fibrous	14.87	11.63
Cr. laur.	371-1[3]	120	ND	non-fibrous	30.26	5.71
Cr. neof.	365-11[1]	108	3.75	fibrous	22.00	9.55
Cr. neof.	365-11[2]	120	3.10	viscous, fibrous	70.30	4.54
Cr. neof.	365-16[1]	120	ND	very fibrous	58.67	2.27
Cr. neof.	365-16[2]	110	3.40	viscous, fibrous	50.15	2.12
Cr. neof.	365-26[1]	110	3.32	viscous, fibrous	56.00	2.19
Cr. neof.	365-26[2]	112	3.18	viscous, fibrous	59.00	2.25

[a]dextrose autoclaved with medium-charring occurred-precipitate took on colour of medium

[b]high initial pH may have inhibited growth

7. Cell Walls - Extraction

Numerous methods were tried to break the cells of Cryptococcus species. Each species (even each strain) needed different conditions to break the wall. Even though cells were grown in medium for minimal capsule production, some strains had more capsule adhering and were therefore more resilient. Cr. laurentii cells proved the most easily broken and the cells in 6l medium yielded 80 mg cell walls after breakage with a Braun homogenizer. Only 60 mg/6 l were obtained from Cr. neoformans 365-26 after laborious separation procedures. As found by many other workers, Cr. neoformans walls, whatever the method of breakage, have a tendency to aggregate with remaining whole cells and cytoplasmic debris. Attempts to separate these constituents by sucrose gradients, differential centrifugation and sonication, yielded insufficient amounts of pure walls for complete analysis. The cell wall preparations (three from Cr. laurentii 371-1, including one from phenol treated cells, and one from Cr. neoformans 365-26) showed no evidence of cytoplasmic contamination by phase contrast light microscopy. No capsular material was detected with the India ink test.

8. Analytical Studies

A. Cell Wall Analysis

(1) Amino Acids

Duplicates of each wall preparation were hydrolyzed in vacuo in 6N HCl at 110C for 24 hr. Serial hydrolyses were not performed because of the limited amounts of cell walls available.

Table VII shows the amounts of amino acids recovered/mg pure, dry cell wall preparation from Cr. laurentii and Cr. neoformans and Tremella mesenterica (Cameron 1973). There were large differences in total recovery but, as can be seen from Table VIII, there were differences between the proteins when the percentages of total amino acids were compared. In view of the proposed taxonomic relationship between Cryptococcus and Tremella (Slodki, Wickerham and Bandoni 1966), it is interesting to compare the amino acid compositions of the two species. Histidine and serine are lower in Tremella than in Cryptococcus species whereas glutamic acid and methionine are higher. Neither Cryptococcus species possessed hydroxyproline and cysteine/cystine was detected in Cr. laurentii only. The two Cryptococcus species were very similar in amino acid composition except that Cr. neoformans had substantially more lysine.

(2) Amino Sugars

Glucosamine was found in the cell walls of both species and a trace of galactosamine was detected in Cr. neoformans 365-26. The recoveries of glucosamine after hydrolysis of the cell wall preparations with 2N and 6N HCl are presented in Table IX. Cameron (1973) reported massive degradation of glucosamine with 6N HCl and obtained the best estimate for Tremella (23.9 µg/mg cell wall) after 72 hr in 2N HCl. All the hydrolyses here were performed in the presence of 1 mg/ml oxalic acid (James 1972); this appeared to reduce degradation of glucosamine in 6N HCl. The maximum value for Cr. neoformans was obtained after 9 hr in 6N HCl and for Cr. laurentii, after

TABLE VII

AMINO ACIDS IN THE CELL WALLS OF CR. LAURENTII 371-1, CR. NEOFORMANS 365-26 AND TR. MESENTERICA

μg anhydro amino acid recovered/mg cell wall preparation[a]

	Cr. neoformans[b]	Cr. laurentii[b]	Tr. Mesenterica[c]
Lys	1.32	1.79	3.3
His	0.47	1.36	1.2
Arg	0.85	1.70	3.5
Asx	1.07	3.20	5.0
Thr	0.63	1.66	2.5
Ser	0.79	1.75	1.8
Glx	1.40	3.91	7.8
Pro	0.64	1.55	2.6
Gly	0.66	1.30	2.3
Ala	0.81	2.16	3.3
Cys	0.00	0.20	0.0
Val	0.65	1.71	2.8
Met	0.21	0.35	1.3
Ile	0.43	1.27	2.5
Leu	0.76	2.23	4.3
Tyr	0.50	1.32	2.0
Phe	0.49	1.59	2.2
Hyp.[d]	0.00	0.00	0.4
Total Recovery	11.68	29.05	48.9

a: hydrolyzed with 6N HCl for 24 hr
b: average of triplicate analyses (Cr. laurentii)and duplicates (Cr. neoformans)
c: results expressed as best estimate (maximum value) of serial hydrolyses with 6N HCl performed by D.S.Cameron (1973)
d: determined spectrophotometrically (Bergman and Loxley 1970)

TABLE VIII

AMINO ACIDS IN THE CELL WALLS OF CR. LAURENTII, CR. NEOFORMANS AND TREMELLA MESENTERICA

(Amino Acids as percent of total µg anhydro amino acids recovered)

Amino Acid	Cr. neoformans	Cr. laurentii	Tremella Mesenterica
Lys	11.3	6.2	6.8
His	4.0	4.7	2.5
Arg	7.3	5.9	7.2
Asx	9.2	11.0	10.3
Thr	5.4	5.7	5.1
Ser	6.8	6.0	3.7
Glx	12.0	13.5	16.0
Pro	5.5	5.3	5.3
Gly	5.7	4.5	4.7
Ala	6.9	7.4	6.8
Cys	0.0	0.7	0.0
Val	5.6	5.9	5.7
Met	1.8	1.2	2.7
Ile	3.7	4.4	5.1
Leu	6.5	7.7	8.8
Tyr	4.3	4.5	4.1
Phe	4.2	5.5	4.5
Hyp.	0.0	0.0	0.8

TABLE IX

AMINO SUGARS IN THE CELL WALLS OF CR. LAURENTII 371-1 AND CR. NEOFORMANS 365-26

(μg anhydro GlcNx/mg cell wall preparation)

	Duration of Hydrolysis with 2N HCl				
	8 hr	16 hr	32 hr	72 hr	96 hr
Cr. laur.	6.2	9.1	14.3	28.4	36.1
Cr. neof.	ND[a]				22.4 0.2[b]

	Duration of Hydrolysis with 6N HCl				
	7 hr	8 hr	9 hr	24 hr	Best Estimate[c]
Cr. laur.	ND	39.71	ND	40.0	40.0
Cr. neof.	26.0	ND	30.6	20.7	30.6

a: ND = not determined

b: 0.2 μg/mg anhydro galactosamine detected in Cr. neoformans

c: maximum value

24 hr in 6N HCl. The results show that release and degradation of glucosamine from each species' cell wall preparation may be different and requires serial hydrolyses, with both 2N and 6N HCl, in the presence of oxalic acid.

(3) Neutral Sugars

Xylose, mannose, galactose and glucose were identified in the cell walls of both Cryptococcus species. The results are presented in Tables X and XI. The cell walls of Cr. neoformans contained a much larger percentage of glucose with small amounts of the other sugars. Tremella cell walls contained larger amounts of xylose and smaller amounts of glucose: they also contained small quantities of arabinose, fucose and rhamnose.

B. Extracellular Material Analysis

(1) Amino Acids

The results presented in Tables XII and XIII show that all the usual protein amino acids were present in the preparations from all the strains tested. Cysteine/cystine was detected in both strains of Cr. albidus, in Cr. laurentii, a small amount occurred in Cr. neoformans 365-11 and trace amounts only were present in Cr. neoformans 365-16 and 365-26. The protein hydrolysates of all strains contained large quantities of serine, threonine, aspartic and glutamic acids. Cr. neoformans 365-16 and 365-26 had large amounts of proline and showed substantial differences in the ratios of Gly/Ala, Ile/Leu and Tyr/Phe from the other strains (Table XV). Cr.

TABLE X

NEUTRAL SUGARS IN THE CELL WALL OF CR. LAURENTII 371-1

(µg anhydro sugar/mg cell wall preparation)

	Duration of Hydrolysis with 2N CF_3COOH[a]				
	1 hr	2 hr	3 hr	best estimate[b]	% total µg sugar
Xyl	57.5	56.9	49.9	57.5	11.1
Man	47.6	62.5	61.9	62.5	12.0
Gal	13.6	15.4	11.3	15.4	3.0
Glc	321.1	384.1	382.0	384.1	74.0
Total Recovery				519.5	

a: sugars detected as TMS derivates by method of Cameron (1973)
b: maximum value

TABLE XI

NEUTRAL SUGARS IN THE CELL WALL OF CR. NEOFORMANS 365-26

(µg anhydro sugar/mg cell wall preparation)

	Duration of Hydrolysis with 2N CF_3COOH				
	1 hr	2 hr	3 hr	best estimate	% total µg sugar
Xyl	11.3	10.5	9.0	11.3	2.4
Man	15.1	18.2	18.3	18.3	3.9
Gal	2.6	2.8	2.8	2.8	0.6
Glc	360.0	432.0	435.0	435.0	93.1
Total Recovery				467.4	

TABLE XII

AMINO ACIDS IN THE EXTRACELLULAR MATERIAL OF *CR. NEOFORMANS* (365-11, 365-16 AND 365-26), *CR. ALBIDUS* (367 AND H1354) AND *CR. LAURENTII* (371-1)

Duplicate samples (4 - 5 mg/ml) were hydrolyzed with 6N HCl containing 1 mg/ml oxalic acid for 24, 48 and 72 hr

(µg anhydro amino acid recovered/mg extracellular material)[a]

	Cr.Alb. H1354 #2	*Cr.Alb.* 367	*Cr.Laur.* 371-1	*Cr.Neof.* 365-11	*Cr.Neof.* 365-16	*Cr. Neof.* 365-26
Lys	0.84	0.35	1.03	0.73	0.34	0.80
His	0.78	0.29	2.26	0.51	0.21	0.20
Arg	1.28	0.65	1.30	0.64	0.28	0.35
Asx	4.12	2.53	5.77	3.96	2.86	4.88
Thr	4.72	3.24	4.42	3.16	1.85	2.95
Ser	5.12	3.52	5.10	4.07	3.19	4.57
Glx	7.44	3.25	6.78	3.79	1.85	2.49
Pro	2.60	1.33	3.75	1.40	2.06	4.51
Gly	3.55	1.48	2.82	1.38	0.79	0.90
Ala	4.81	2.88	5.29	2.51	2.51	3.94
Cys	2.11	0.34	0.91	0.23	0.00	0.00
Val	2.86	1.54	2.07	1.40	1.22	1.86
Met	0.20	0.08	0.31	0.28	0.04	0.04
Ile	1.42	0.74	1.58	0.86	1.40	2.12
Leu	1.77	0.78	2.33	1.26	0.65	0.91
Tyr	1.23	0.63	1.64	1.08	0.27	0.14
Phe	1.12	0.53	1.67	0.71	1.33	2.48
Hyp[b]	0.00	0.00	0.00	0.00	0.00	0.00
Total Recovery	45.97	24.16	49.03	27.97	20.85	33.14

a: Values represent the mean of the best estimates (maximum value) from two preparations of each strain

b: determined colorimetrically (Bergman and Loxley 1970)

TABLE XIII

AMINO ACIDS IN THE EXTRACELLULAR MATERIAL OF *CR. NEOFORMANS* (365-11, 365-16 AND 365-26), *CR. ALBIDUS* (367 AND H1354) AND *CR. LAURENTII* (371-1)

Duplicate samples (4-5 mg/ml)were hyrolyzed with 6N HCl containing 1 mg/ml oxalic acid for 24, 48 and 72 hr

(Amino acids as percent of total µg anhydro amino acids recovered)[a]

	Cr.Alb. H1354 #2	*Cr.Alb.* 367	*Cr.Laur.* 371-1	*Cr.Neof.* 365-11	*Cr.Neof.* 365-16	*Cr.Neof.* 365-26
Lys	1.8	1.5	2.1	2.6	1.6	2.4
His	1.7	1.2	4.6	1.8	1.0	0.6
Arg	2.8	2.7	2.7	2.3	1.3	1.1
Asx	9.0	10.5	11.8	14.2	13.7	14.7
Thr	10.3	13.4	9.0	11.3	8.9	8.9
Ser	11.1	14.6	10.4	14.6	15.3	13.8
Glx	16.2	13.5	13.8	13.6	8.9	7.5
Pro	5.7	5.5	7.7	5.0	9.9	13.6
Gly	7.7	6.1	5.8	4.9	3.8	2.7
Ala	10.5	11.9	10.8	9.0	12.0	11.9
Cys	4.6	1.4	1.9	0.8	0.0	0.0
Val	6.2	6.4	4.2	5.0	5.9	5.6
Met	0.4	0.3	0.6	1.0	0.2	0.1
Ile	3.1	3.1	3.2	3.1	6.7	6.4
Leu	3.9	3.2	4.8	4.5	3.1	2.7
Tyr	2.7	2.6	3.3	3.9	1.3	0.4
Phe	2.4	2.2	3.4	2.5	6.4	7.5

a: Percentages derived from figures and totals in Table XII

neoformans 365-11 bore more resemblance to the other, nonpathogenic, species although, like Cr. neoformans 365-16 and 365-26, it had a large amount of aspartic acid. A comparison of amino acids in extracellular material and cell walls is shown in Tables XIV and XV. The extracellular material differed from cell wall preparations in having less basic amino acids and more serine and threonine. The amino acid composition of somatic proteins extracted from Cr. neoformans C-94 by Uzman, Rosen and Foley (1956) are included in Tables XIV and XV and compared with the values from cell walls and extracellular material obtained in this study.

Ethanolamine was detected in all the preparations but it was not possible to quantify since three peaks were observed: two before lysine and one before ammonia. All the strains showed ethanolamine as a shoulder on the lysine peak. Cr. albidus 367 and H1354 gave an extra, separate peak well before lysine and Cr. laurentii 371-1 a third peak before ammonia. Both liquid ethanolamine (BDH) and the solid form (Calbiochem) gave four distinct peaks (one just before lysine and three around ammonia) but the proportions of these peaks were different in each case.

(2) Amino Sugars

The results in Table XVI show that glucosamine was recovered from all of the extracellular material preparations. No galactosamine was detected in the strains studied here although I found it in extracellular material from Cr. terreus 8157. The amounts of glucosamine recovered were much smaller

TABLE XIV

AMINO ACIDS IN THE EXTRACELLULAR MATERIAL AND CELL WALLS OF *CR. NEOFORMANS* 365-26 AND *CR. LAURENTII* 371-1

(Amino acids as percent of total µg anhydro amino acids recovered)

Hydrolysis in 6N HCl

	Cr. laur. 371-1 Cell wall[a]	*Cr. laur.* 371-1 Ex. mat.[b]	*Cr. neof.* 365-26 26 Cell wall	*Cr. neof.* 365-26 26 Ext. mat.	*Cr. neof.* C-94
Lys	6.2	2.1	11.3	2.4	7.3
His	4.7	4.6	4.0	0.6	2.2
Arg	5.9	2.7	7.3	1.1	3.9
Asx	11.0	11.8	9.2	14.7	9.4
Thr	5.7	9.0	5.4	8.9	6.0
Ser	6.0	10.4	6.8	13.8	6.6
Glx	13.5	13.8	12.0	7.5	9.9
Pro	5.3	7.7	5.5	13.6	4.2
Gly	4.5	5.8	5.7	2.7	7.5
Ala	7.4	10.8	6.9	11.9	9.6
Cys	0.7	1.9	0.0	0.0	0.4
Val	5.9	4.2	5.6	5.6	7.5
Met	1.2	0.6	1.8	0.1	0.0
Ile	4.4	3.2	3.7	6.4	8.1
Leu	7.7	4.8	6.5	2.7	11.5
Tyr	4.5	3.3	4.3	0.4	1.8
Phe	5.5	3.4	4.2	7.5	4.6
	100.1	99.7	100.2	99.9	100.5

a: Values from Table VIII
b: Extracellular material - values from Table XIII
c: Amino acid composition of somatic proteins extracted from *Cr. neoformans* C-94 by treatment with hot NaOH. Proteins (20 mg) hydrolyzed with 1 ml 6N HCl and 0.5 ml glacial acetic acid for 24 hr at 110C (Uzman, Rosen and Foley 1956)

TABLE XV

AMINO ACID RATIOS IN THE EXTRACELLULAR MATERIAL OF *CR. NEOFORMANS* (365-11, 365-16 AND 365-26), *CR. ALBIDUS* (367 AND H1354) AND *CR. LAURENTII* (371-1) AND THE CELL WALLS OF *CR. NEOFORMANS* (365-26) AND *CR. LAURENTII* (371-1)

	Ratios of percent anhydro amino acids[a]		
Strains	Gly/Ala	Ile/Leu	Tyr/Phe
Cr. alb. H1354 #2	0.73	0.80	1.13
Cr. alb. 367	0.51	0.97	1.18
Cr. laur. ex. mat.[b]	0.54	0.67	0.97
Cr. laur. wall	0.61	0.57	0.83
Cr. neof. 365-11	0.54	0.69	1.56
Cr. neof. 365-16	0.32	2.16	0.20
Cr. neof. 365-26 ex. mat.	0.23	2.37	0.05
Cr. neof. 365-26 wall	0.83	0.57	1.02
Cr. neof. C-94[c]	0.78	0.70	0.39

a: ratios calculated from percentage values in Table XIV
b: extracellular material
c: amino acids of somatic proteins extracted from *Cr. neoformans* C-94 by treatment with hot NaOH (Uzman, Rosen and Foley 1956)

TABLE XVI

AMINO SUGARS IN THE EXTRACELLULAR MATERIAL OF *CR. NEOFORMANS* (365-11, 365-16 AND 365-26), *CR. ALBIDUS* (367 AND H1354) AND *CR. LAURENTII* (371-1)

(μg anhydro GlcNx/mg extracellular material)

	Hydrolyzing agent				
	6N HCl + 1 mg/ml oxalic acid		2 HCl + 1 mg/ml oxalic acid		
	Duration of Hydrolysis				Best Estimate[a]
	24 hr	48 hr	72 hr	96 hr	
Cr. alb. H1354 #2	1.50	0.57	0.90	1.26	1.50
Cr. alb. 367	0.66	0.41	0.65	0.68	0.68
Cr. laur. 371-1	1.63	1.41	1.89	2.11	2.11
Cr. neof. 365-11	1.51	1.26	1.04	1.35	1.51
Cr. neof. 365-16	0.41	0.37	0.37	0.39	0.41
Cr. neof. 365-26	0.49	0.33	0.34	0.50	0.50

a: maximum value

than those from the cell walls (Table IX).

(3) Neutral Sugars

The same four sugars (xylose, mannose, galactose and glucose) were detected in the extracellular material as were found in Cr. laurentii and Cr. neoformans cell walls, although the proportions differed. The results of analysis by two different procedures are presented in Tables XVII and XVIII. Total recoveries were much higher by the resin hydrolysis method of Lehnhardt and Winzler (1968) than after hydrolysis with 2N TFA (Cameron 1973). Since the individual percentages of total sugar were the same for both methods, the error may lie in the mannitol standard used in the first procedure, or the myoinositol used in the second. Maximum values for mannose were obtained after 72 hr hydrolysis with 0.02N HCl and 4 hr with 2N TFA. Xylose was released and degraded more rapidly, maximum values being 24 hr and 1 hr respectively. Galactose and glucose gave best recoveries between 48 and 72 hr and 2 - 4 hr.

(4) Uronic Acids

The results of uronic acid analyses are presented in Table XIX. The pathogenic Cr. neoformans strains contained much more uronic acid than the nonpathogenic species. The relative proportions of uronic acid and neutral sugars are shown in Table XXI.

(5) O-Acetyl

O-acetyl groups were detected only in the Cr. neoformans strains as shown in Table XX. Strain 365-11 had substantially

TABLE XVII

NEUTRAL SUGARS IN THE EXTRACELLULAR MATERIAL OF *CR. NEOFORMANS* (365-11, 365-16 AND 365-26), *CR. ALBIDUS* (367 AND H1354) AND *CR. LAURENTII* (371-1)

(μg anhydro sugar/mg extracellular material preparation)

	Cr.Alb. 367 #3	*Cr.Alb.* H1354 #3	*Cr.Laur.* 371-1 #3	*Cr.Neof.* 365-11 #2	*Cr.Neof.* 365-16 av.	*Cr.Neof.* 365-26 av.
A						
Xyl	349.0	165.1	260.1	158.3	205.4	297.3
Man	591.1	392.9	404.0	707.7	414.6	538.0
Gal	90.8	359.2	195.9	119.3	270.2	92.4
Glc	56.2	231.3	36.0	29.6	99.8	26.4
Total Recovery	1087.1	1148.5	896.0	1014.9	990.0	954.1
B						
Xyl	215.3	89.7	200.4	103.1	138.7	187.8[a]
Man	355.7	214.2	287.5	368.5	251.4	319.8
Gal	46.7	177.4	138.5	67.0	159.5	59.2
Glc	35.1	117.7	26.8	15.8	58.3	17.0
Total Recovery	652.8	597.0	653.2	554.4	607.9	583.8

A - neutral sugars determined by procedure [see Chapter II, G (5)], according to a modification of Lehnhardt and Winzler's method (1968). Results are best estimates (maximum value) of duplicate 24, 48 and 72 hr resin hydrolyses with 0.02N HCl.

B - neutral sugars determined by procedure [see Chapter II G(5)] according to modification of Cameron's method (1973). Results are best estimates of duplicate 1, 2 and 4 hr hydrolyses with 2N TFA.

a: mean values of two preparations taken for *Cr. neoformans* 365-16 and 365-26.

TABLE XVIII

NEUTRAL SUGARS IN THE EXTRACELLULAR MATERIAL OF CR. NEOFORMANS (365-11, 365-16 AND 365-26), CR. ALBIDUS (367 AND H1354) AND CR. LAURENTII (371-1)

(neutral sugars as percent total µg anhydro neutral sugar recovered)[a]

A	Cr.Alb. 367 #3	Cr.Alb. H1354 #3	Cr.Laur. 371-1 #3	Cr. Neof. 365-11 #2	Cr.Neof. 365-16 av.	Cr.Neof. 365-26 av.
Xyl	32.1	14.4	29.0	15.6	20.8	31.2
Man	54.4	34.2	45.1	69.7	41.9	56.4
Gal	8.4	31.3	21.9	11.8	27.3	9.7
Glc	5.2	20.1	4.0	2.9	10.1	2.8
Total Recovery	100.1	100.0	100.0	100.0	100.1	100.1
B						
Xyl	32.9	15.0	30.7	18.6	22.8	32.2
Man	54.5	35.8	44.0	66.5	41.4	54.8
Gal	7.2	29.6	21.2	12.1	26.2	10.2
Glc	5.4	19.7	4.1	2.9	9.6	2.9
Total Recovery	100.0	100.1	100.0	100.1	100.0	100.1

A - neutral sugars determined by procedure [Chapter II G(5)] according to a modification of Lehnhardt and Winzler's method (1968)

B - neutral sugars determined by procedure [Chapter II G(5)] according to a modification of Cameron's method (1973)

a: percentages calculated from values in Table XVII

TABLE XIX

URONIC ACIDS IN THE EXTRACELLULAR MATERIAL OF *CR. NEOFORMANS* (365-11, 365-16 AND 365-26), *CR. ALBIDUS* (367 AND H1354) AND *CR. LAURENTII* (371-1)

(μg uronic acid/mg extracellular material)[a]

	Cr.Alb. 367	Cr.Alb. H1354	Cr.Laur. 371-1	Cr.Neof. 365-11	Cr. Neof. 365-16	Cr.Neof. 365-26
b	73.1	56.0	32.2	158.0	112.2	151.2

a: Determined spectrophotometrically (Blumenkrantz and Asboe-Hansen 1973)

b: Mean of duplicate of two preparations of each strain

TABLE XX

O-ACETYL GROUPS IN THE EXTRACELLULAR MATERIAL OF *CR. NEOFORMANS* (365-11, 365-16 AND 365-26), *CR. ALBIDUS* (367 AND H1354) AND *CR. LAURENTII* (371-1)

(μg O-acetyl/mg extracellular material)[a]

Cr.Alb. 367	Cr.Alb. H1354	Cr.Laur. 371-1	Cr.Neof. 365-11	Cr. Neof. 365-16	Cr.Neof. 365-26
0.0	0.0	0.0	67.1	14.2	18.1

a: Detected as methyl acetates by gas chromatography. For details of procedure see Chapter II, G (b).

TABLE XXI

NEUTRAL SUGARS AND URONIC ACIDS OF CRYPTOCOCCAL EXTRACELLULAR MATERIAL EXPRESSED AS MOLE PERCENT[a] OF TOTAL NM SUGAR

	Cr.Alb. 367	Cr.Alb. H1354	Cr.Laur. 371-1	Cr.Neof. 365-11	Cr.Neof. 365-16	Cr.Neof. 365-26
Xyl	34.4	16.4	33.8	17.5	22.9	30.1
Man	46.3	31.9	39.5	51.0	33.9	41.8
Gal	6.1	26.4	19.0	9.3	21.5	7.7
Glc	4.6	17.5	3.7	2.2	7.9	2.2
Uronic Acid	8.8	7.7	4.1	20.2	13.9	18.2
	100.2	99.9	100.1	100.2	100.1	100.0

a: Mole percentages were calculated from the figures in Tables XVIII and XIX divided by the anhydro molecular weight for each sugar.

TABLE XXII

NEUTRAL SUGARS, URONIC ACIDS AND O-ACETYL GROUPS OF EXTRACELLULAR MATERIAL FROM CR. NEOFORMANS (365-11, 365-16 AND 365-26) EXPRESSED AS MOLE PERCENT[a] OF TOTAL NM SUGAR

	Cr. Neof. 365-11	Cr. Neof. 365-16	Cr. Neof. 365-26
Xyl	13.0	21.4	27.7
Man	37.7	31.6	38.4
Gal	6.9	20.0	7.1
Glc	1.6	7.3	2.0
Uronic acid	14.9	13.0	16.7
O-acetyl	25.9	6.7	8.1
	100.0	100.0	100.0

a: Mole percentages were calculated from the figures in Tables XVIII, XIX and XX divided by the anhydro molecular weight for each sugar.

larger amounts than either 365-16 or 365-26. Table XXII shows the relative proportions of uronic acid, neutral sugar and O-acetyl in these three strains. From these results I estimated that 35% of the total polysaccharide was O-acetylated in the extracellular material from Cr. neoformans 365-11. The values for 365-16 and 365-26 were 7% and 9% respectively. It was not possible to determine from these figures where the O-acetyl groups were substituted or in which sugars they occurred.

(6) Elemental and ash analysis

Two different preparations of extracellular material from each strain were analyzed; the results are shown in Table XXIII. In most of the preparations there was a substantial difference between the nitrogen value determined by Organic Microanalysis and the total nitrogen calculated from amino acid nitrogen plus ammonia nitrogen as detected on the amino acid analyzer. The nitrogen values balanced in only one of the preparations, Cr. albidus H1354 #3. This preparation gave a much higher recovery of total amino acids and amino sugars than any of the other preparations. Table XXIV compares the recoveries of amino acids and amino sugars in Cr. albidus H1354 preparations #2 and #3.

(7) Complete analysis of extracellular material

The total recovery of extracellular material components for the six Cryptococcus strains is summarized in Table XXV. Approximately 80% of the weight of each extracellular material preparation was recovered, leaving 20% unaccounted.

TABLE XXIII

ASH, PHOSPHORUS, AND NITROGEN ANALYSIS OF EXTRACELLULAR MATERIAL FROM CR. NEOFORMANS (365-11, 365-16 AND 365-26), CR. ALBIDUS (367 AND H1354) AND CR. LAURENTII (371-1)

(µg/mg extracellular material preparation)

	Ash[a]	P[b]	N[c]	Total N[e]
Cr. alb. 371 #2	4.6	1.1	ND[d]	ND
Cr. alb. 371 #3	6.9	1.3	1.4	0.5
Cr. alb. H1354 #2	8.5	1.5	ND	1.0
Cr. alb. H1354 #3	3.6	1.6	1.8	1.8
Cr. laur. 371-1 #1	43.0	0.1	1.2	1.0
Cr. laur. 371-1 #3	5.7	1.3	1.4	1.0
Cr. neof. 365-11 #1	9.6	1.9	ND	ND
Cr. neof. 365-11 #2	4.5	0.4	1.8	0.6
Cr. neof. 365-16 av.[f]	2.2	0.2	1.1	0.5
Cr. neof. 365-26 av.	2.2	0.2	1.1	0.5

a: determined gravimetrically. See Chapter II G(1) for details of procedure.
b: determined spectrophotometrically (Ames 1966)
c: analysis performed by Organic Microanalysis, Montreal, Quebec.
d: ND = not determined.
e: nitrogen calculated from amino nitrogen plus ammonia nitrogen as detected on the amino acid analyzer.
f: little difference between duplicate preparations of Cr. neoformans 365-16 and 365-26, therefore average value presented.

TABLE XXIV

DIFFERENCES IN RECOVERIES OF AMINO ACIDS AND AMINO SUGARS IN TWO SEPARATE PREPARATIONS OF EXTRACELLULAR MATERIAL FROM *CR. ALBIDUS* H1354

	μg anhydro amino acid recovered/mg extracellular material[a]		Amino acids as percent of total μg anhydro amino acids recovered	
	Cr. albidus H1354 #2	*Cr. albidus* H1354 #3	*Cr. albidus* H1354 #2	*Cr. albidus* H1354 #3
Lys	0.84	2.04	1.8	1.9
His	0.78	1.14	1.6	1.1
Arg	1.28	2.53	2.7	2.4
Asx	4.12	9.08	8.7	8.5
Thr	4.72	10.63	9.9	9.9
Ser	5.12	13.11	10.8	12.2
Glx	7.44	15.41	15.7	14.4
Pro	2.60	7.34	5.5	6.8
Gly	3.55	8.30	7.5	7.7
Ala	4.81	9.93	10.1	9.3
Cys	2.11	4.16	4.5	3.9
Val	2.86	5.41	6.0	5.0
Met	0.20	0.39	0.4	0.4
Ile	1.42	3.18	3.0	3.0
Leu	1.77	5.19	3.7	4.8
Tyr	1.23	3.08	2.6	2.9
Phe	1.12	2.52	2.4	2.4
GlcNx[b]	1.50	3.84	3.2	3.6
Total Recovery	47.47	107.28	100.1	100.2

a: hydrolyzed in 6N HCl with 1 mg/ml oxalic acid for 24, 48 and 72 hr. Values represent best estimates (maximum value).

b: hydrolyzed in 6N HCl with 1 mg/ml oxalic acid for 24 and 48 hr and in 2N HCl for 72 and 96 hr. Values represent best estimates (maximum value).

TABLE XXV

COMPLETE ANALYSIS OF EXTRACELLULAR MATERIAL OF *CR. NEOFORMANS* (365-11, 365-16, 365-26), *CR. ALBIDUS* (367, H1354) AND *CR. LAURENTII* (371-1)

(µg/mg extracellular material preparation)

	Cr.alb. 367 #3	*Cr.alb.* H1354 #3	*Cr.laur.* 371-1 #2	*Cr.neof.* 365-11 #2	*Cr.neof.* 365-16 av.	[a] *Cr.neof.* 365-26 av. [a]
Polysaccharide						
anhydro neutral sugar	652.8	599.0	653.2	554.4	607.9	583.8[b]
anhydro amino sugar	0.7	3.8	2.1	1.5	0.4	0.5
anhydro uronic acid	73.1	56.0	32.2	158.0	112.2	151.2
O-acetyl	0.0	0.0	0.0	67.1	14.2	18.1
Protein						
anhydro amino acid	24.2	103.4	49.0	28.0	20.9	33.1
Ash	6.9	3.6	11.6	4.5	2.2	2.2
Phosphorus	1.3	1.6	1.3	0.4	0.2	0.2
Total Recovery	759.0	767.4	749.4	813.9	757.7	788.7[c]

a: average of complete analysis of two preparations of 365-16 and 365-26

b: total neutral sugar values taken from Table XVII (Cameron's method 1973)

c: totals of *Cr. neoformans* 365-11, 365-16 and 365-26 corrected for those H atoms on the sugar molecule which were substituted by O-acetyl groups (calculated from number of moles of O-acetyl).

Lipid determinations were not included since gravimetric methods (Bartnicki-Garcia and Nickerson 1962; Weete, personal communication) were found to be unreliable on the small quantities available. However, the values obtained were never less than 3% for the three strains tested (Cr. neoformans 365-26, Cr. albidus 367 and Cr. laurentii 371-1).

9. Gel electrophoresis and Staining

A. Polyacrylamide gels

The electrophoretic patterns of Cr. albidus extracellular material, as detected by amido black (for protein) and periodic acid-Schiff stain (for carbohydrate) are shown in Figure 7. Initial runs with the PAS stain indicated that far less material was needed per gel to give good resolution of carbohydrate than was needed to detect a thin protein band with amido black. All the Cr. neoformans strains were too viscous in solution for sufficient quantities to be applied to detect protein. Congruence of the PAS and amido black bands suggest the presence of one or more glycoprotein moieties,or the presence of two species with the same mobility. Protein band C was very faint, present on only two out of five gels.

B. Cellulose acetate strips

The electrophoretic patterns of Cryptococcal extracellular material from four different strains are shown in Figures 8 and 9. Strips were stained for protein, carbohydrate and carboxyl moieties after electrophoresis. The Coomassie blue stain did not detect protein presumably because it proved impossible to apply sufficient amounts to the strips to visualize protein because the material was too viscous and of limited

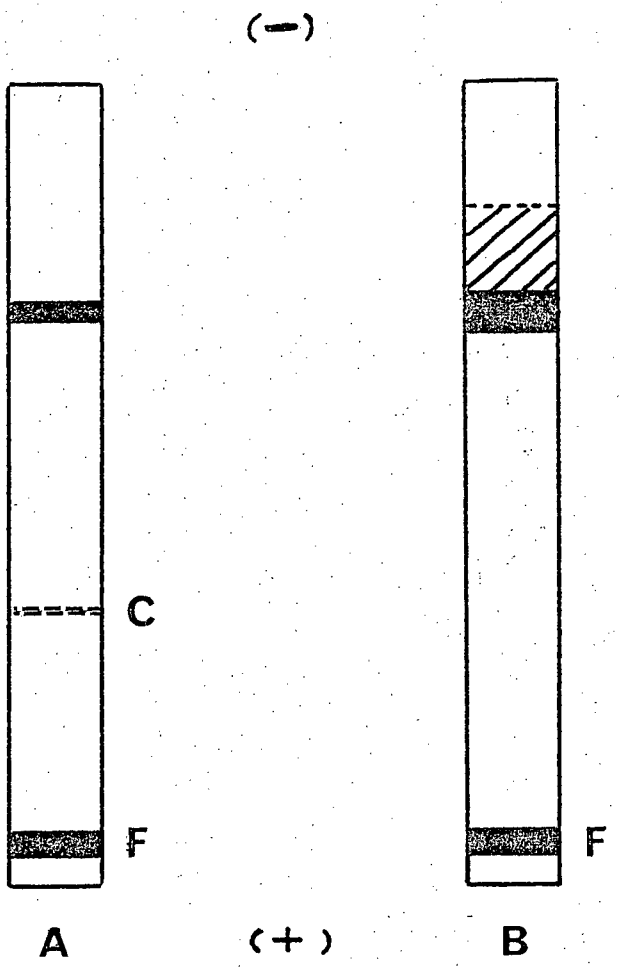


FIGURE 7. Electrophoretic pattern of Cr. albidus 367 #1 extracellular material applied to polyacrylamide gels. A: 3 mg material (in 0.2 ml) applied to gel, and protein detected with 1% w/v amido black. B: 1 mg material (in 0.2 ml) applied to gel, and carbohydrate detected by modified PAS stain (Page and Stock 1974). F: electrophoretic front. C: present on two gels only. Band positions drawn to scale (1 cm represents 0.5 cm length) and taken from mean of 5 gels per stain. See Chapter II H(1) for conditions of electrophoresis.

FIGURE 8

Electrophoretic pattern of cryptococcal extracellular material on cellulose acetate strips.

A: stained with Alcian blue alone to detect carboxyl groups

B: stained for glycoprotein by Alcian blue method of Wardi and Allan (1972).

Band positions taken as mean of duplicate samples and drawn to scale.

P: point of application of sample (5 µl)

See Chapter II H(2) for conditions of electrophoresis.

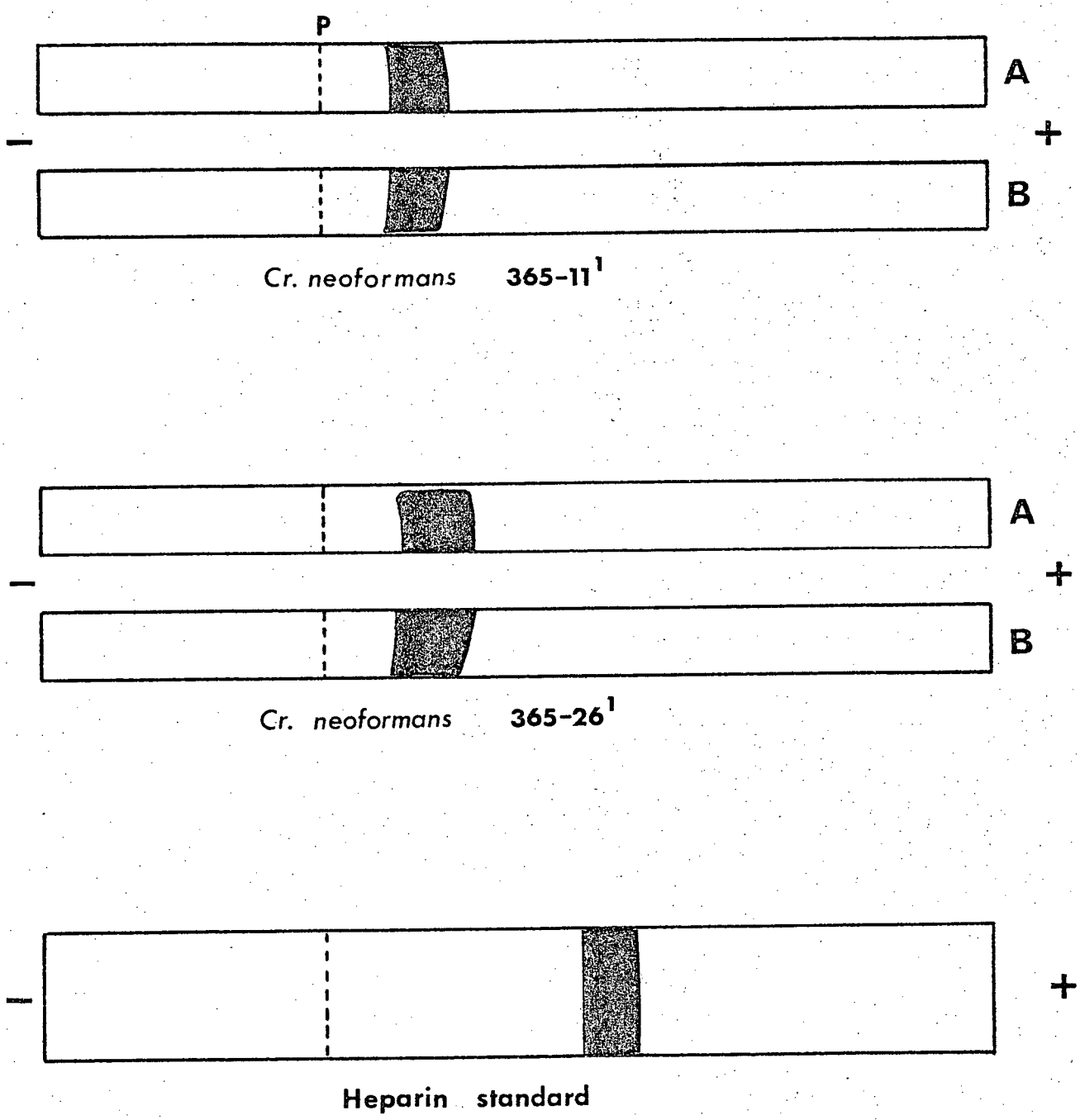

P
A
B
–
+
Cr. neoformans 365-11[1]
A
B
–
+
Cr. neoformans 365-26[1]
–
+
Heparin standard

FIG. 8

FIGURE 9

Electrophoretic pattern of cryptococcal extracellular material applied to cellulose acetate strips.

A: stained with Alcian blue alone to detect carboxyl groups

B: stained for glycoprotein by Alcian blue method of Wardi and Allan (1972)

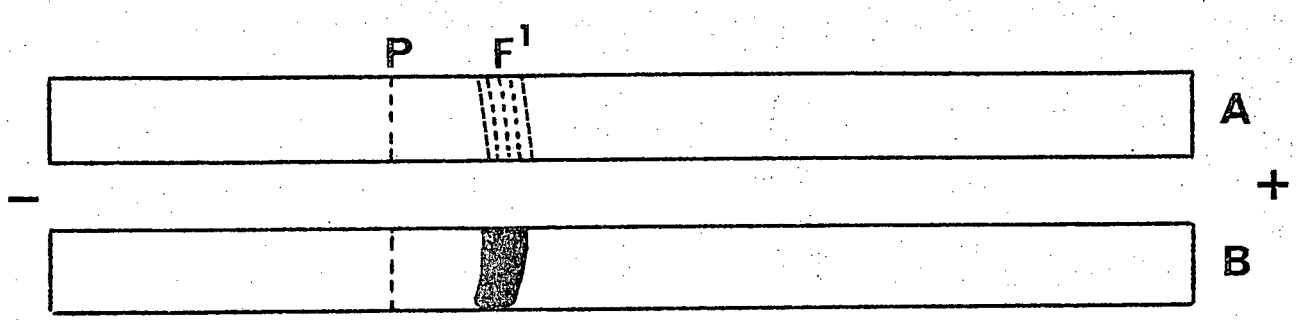
Band positions taken as mean of duplicate samples and drawn to scale.

P: point of application of sample (5 µl)

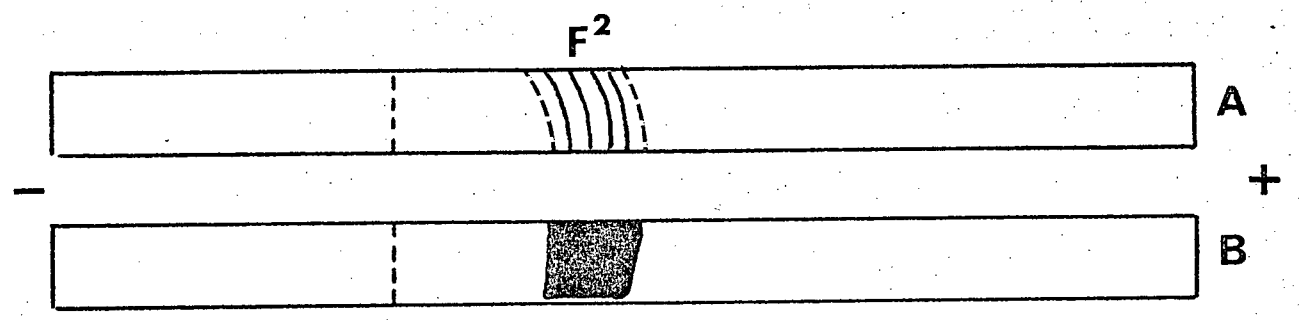
F^1: very faint band

F^2: faint band

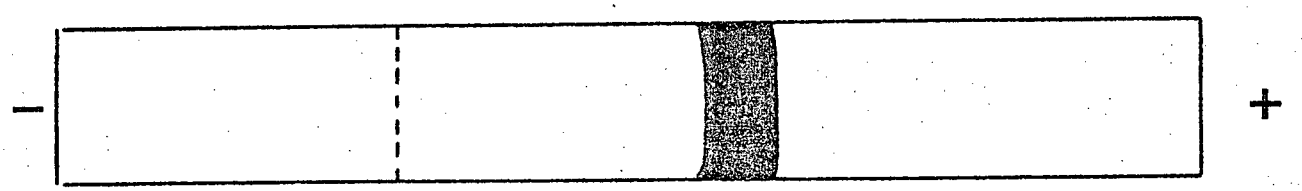
See Chapter II H(2) for conditions of electrophoresis



Cr. albidus **367**[3]



Cr. laurentii **371–1**[1]



Heparin standard

FIG.9

solubility in water or tris-barbital-sodium-barbital buffer. Preparations from the different species stained equally with the PAS technique but both Cr. neoformans preparations stained much more intensely with Alcian blue alone than did Cr. laurentii or Cr. albidus. A comparison of Rp values shows the congruence of protein and carbohydrate bands (Table XXVI) and of possible glycoprotein and acidic polysaccharide (Table XXVII) in preparations of extracellular material from Cryptococcus species. This again infers the possibility of the presence of a glycoprotein complex containing acidic groupings.

10. Gel Chromatography

The fractionation patterns of purified Cr. neoformans 365-26 extracellular material on a DEAE BioGel-A column are shown in Figure 10. The material eluted as a single sharply defined band just at the start of the NaCl gradient. Protein and carbohydrate moieties were coincident within this band suggesting that the small protein fraction may be bound to the large carbohydrate moiety. The material did not appear to be contaminated with any other molecular species.

11. Infrared Spectroscopy

Cryptococcal extracellular material from the six different strains and Cr. laurentii cell walls showed identical spectral patterns for wavenumbers between 2000 and 4000 cm^{-1} (Figure 11). Authentic mannan showed the same absorption maxima at 2900 and 3250 - 3550 cm^{-1} (Iida and Finnerty 1973). The preparations also had similar patterns for wavenumbers between

TABLE XXVI

MEAN Rp VALUES OF GLYCOPROTEIN AND PROTEIN BANDS OF EXTRACELLULAR MATERIAL FROM CRYPTOCOCCUS ALBIDUS 367 #1 ON POLYACRYLAMIDE GELS

	Periodic Acid[a] Schiff Stain	Amido Black[b] Stain
Band No. 1	0.18	ND[c]
2	0.28[d]	0.27[d]
3	ND	0.72[e]

a: PAS stains for carbohydrate b: amido black stains for protein c: not detected d: darkest band e: faint band
Rp = band mobility

TABLE XXVII

MEAN Rp VALUES OF GLYCOPROTEIN AND CARBOXYLIC ACID BANDS OF EXTRACELLULAR MATERIAL FROM CRYPTOCOCCUS SPECIES ON CELLULOSE ACETATE STRIPS

	Alcian Blue[a] Stain	Alcian Blue[b] Stain	Uronic Acid ug/mg
Cr. alb. 367 #1	0.28[c]	0.27	41.3
Cr. laur. 371-1 #1	0.48	0.46	21.5
Cr. neof. 365-11 #1	0.31	0.31	100.1
Cr. neof. 365-26 #1	0.34	0.34	151.2

a: stains for carboxyl groups
b: stains for carbohydrate (Wardi and Allan 1972)
c: faint bands

FIGURE 10

Chromatography of extracellular polysaccharide from Cr. neoformans 365-26 on a DEAE BioGel-A column (37 x 2.5 cm). A convex gradient from 0 - 3M NaCl in 0.02 M pyridine-HCl buffer (pH 5.5) was applied after elution with 400 ml pyridine-HCl buffer. Fractions of 10 ml were collected.

● = carbohydrate

O = protein

Δ = NaCl gradient

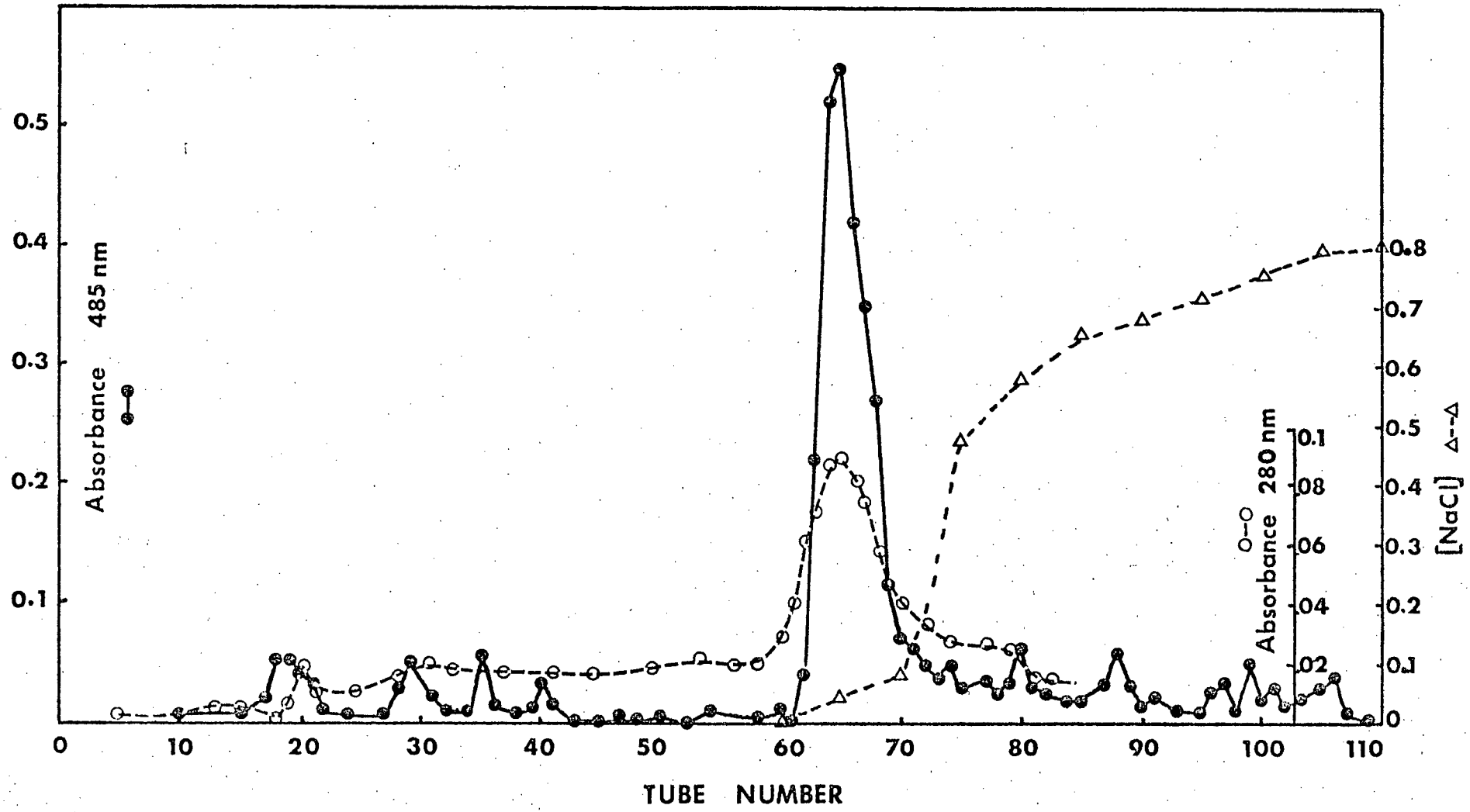

Absorbance 485 nm
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
90
100
110
TUBE NUMBER
O–O Absorbance 280 nm
0.1
08
06
04
02
0
Δ–Δ [NaCl]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

FIG. 10

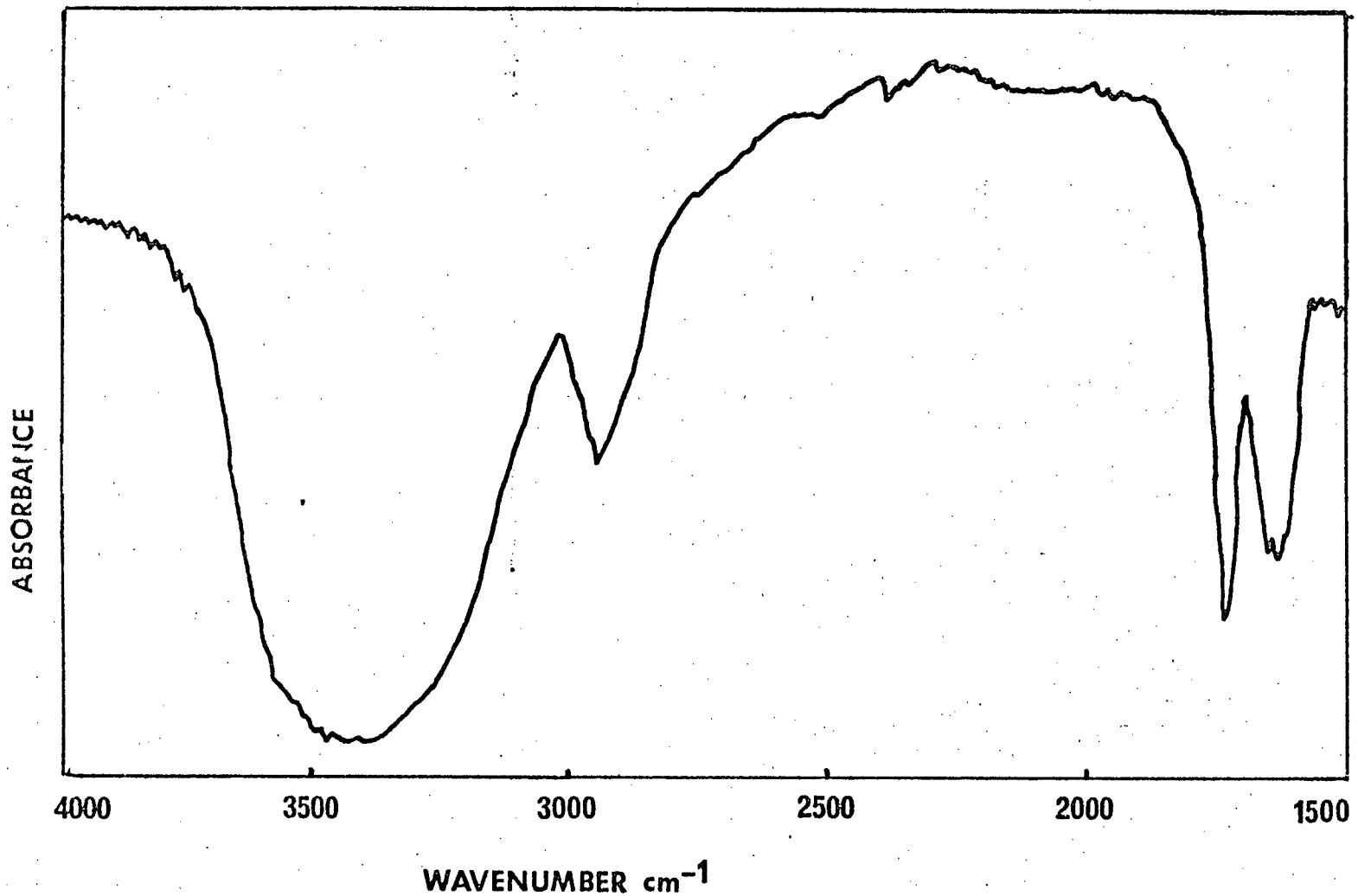


Figure 11. Infra red absorption spectrum of extracellular material from Cr. neoformans 365-26

650 and 1800 cm^{-1} (Figure 12) but there were some differences in intensity particularly at 1730, 1650, 1550, 1375, 1250 and 800 cm^{-1}. Figure 13 shows the spectra of Cr. laurentii cell wall, Cr. laurentii extracellular material and standard for GlcN NHl. These spectra all lacked the intense peak at 1730 cm^{-1} which is due to carbonyl stretching vibrations. This was characteristic of the Cr. neoformans strains. The vibrations at 1550 and 1620 - 35 cm^{-1} were attributed to C = O and NH groups (Beran et al. 1972). Only Cr. laurentii (cell wall and extracellular material) and Cr. albidus H1354 had peaks at 1550 cm^{-1} which correlated with the larger amounts of amino acid and glucosamine recovered from these strains compared with Cr. neoformans strains and Cr. albidus 367. The peaks at 800 and 900 cm^{-1} are indicative of equatorial α-H-C_1 and also occur in yeast mannan. Cr. laurentii cell wall, which was largely glucan and glucosamine had very weak vibrations at these wavenumbers but a slight peak at 890 cm^{-1} indicative of β-linkages and also characteristic of yeast glucan (Beran et al.1972). The large peak at 1620 - 1650 cm^{-1}, present in all the strains, was a characteristic peak of carbohydrates such as mannan and glucan (Beran et al. 1972). The increasing intensity of vibrations at 1250 cm^{-1} (CO of acetyl) and 1730 cm^{-1} (C = O) from spectrum b - f correlated with the increase in both acetylation and uronic acid content in the six strains (Table XXV). A similar slight increase in methyl H at 1375 cm^{-1} and in carboxylate anion at 1425 cm^{-1} also confirmed the increase in O-acetyl and carboxylic acid content. Cr. neoformans 365-11 had the most intense bands at all these wavenumbers.

FIGURE 12

Infrared absorption spectra of extracellular material from Cryptococcus species.

a: Cr. albidus 367

b: Cr. laurentii 371-1

c: Cr. albidus 371-1

d: Cr. neoformans 365-16

e: Cr. neoformans 365-26

f: Cr. neoformans 365-11

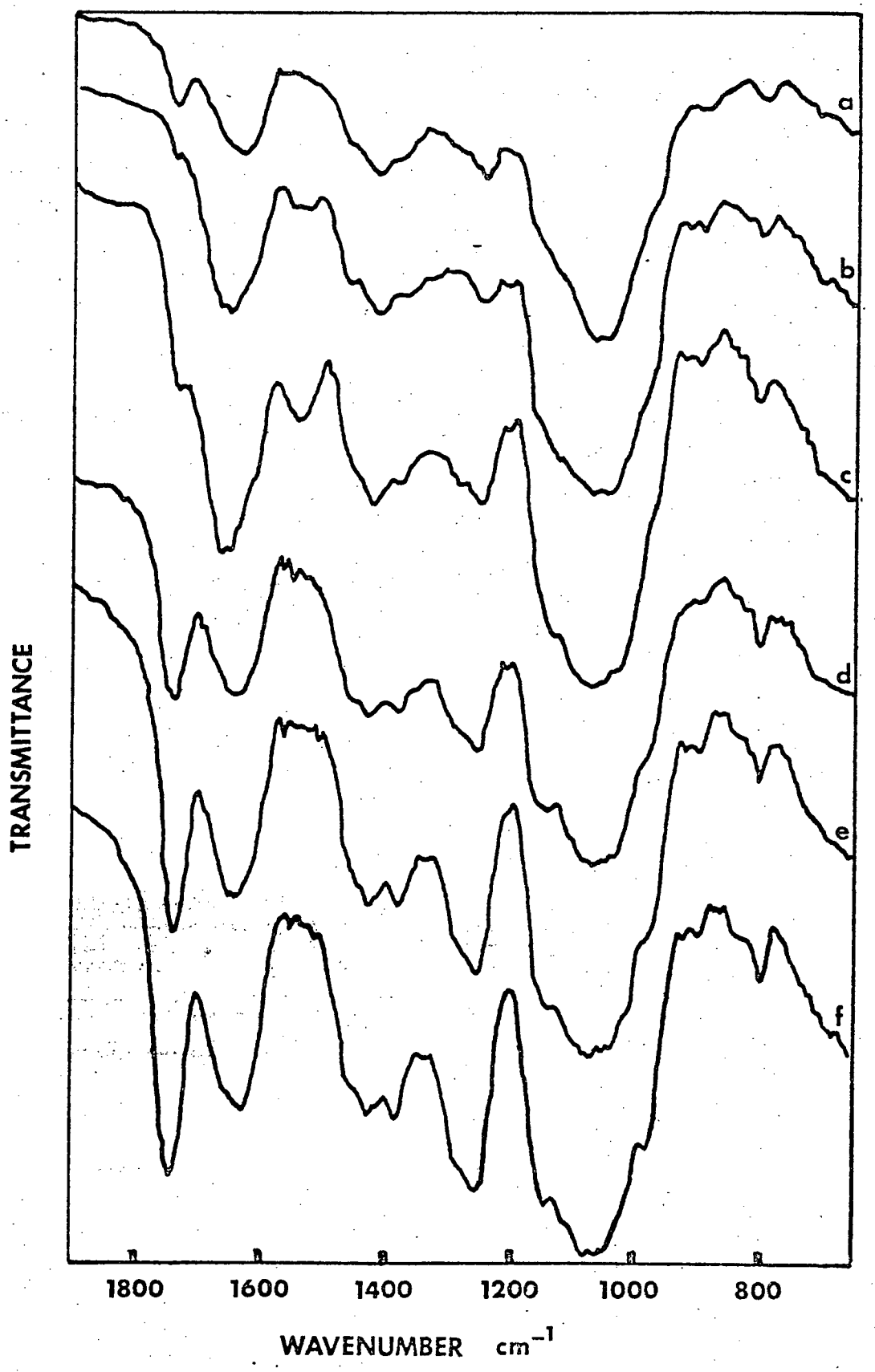

a
b
c
d
e
f
TRANSMITTANCE
1800
1600
1400
1200
1000
800
WAVENUMBER cm⁻¹

FIG. 12

FIGURE 13

Infrared absorption spectra of cell walls and extracellular material from Cr. laurentii 371-1 compared with spectrum from glucosamine HCl

a: Cr. laurentii cell wall

b: Cr. laurentii extracellular material

c: Glucosamine HCl

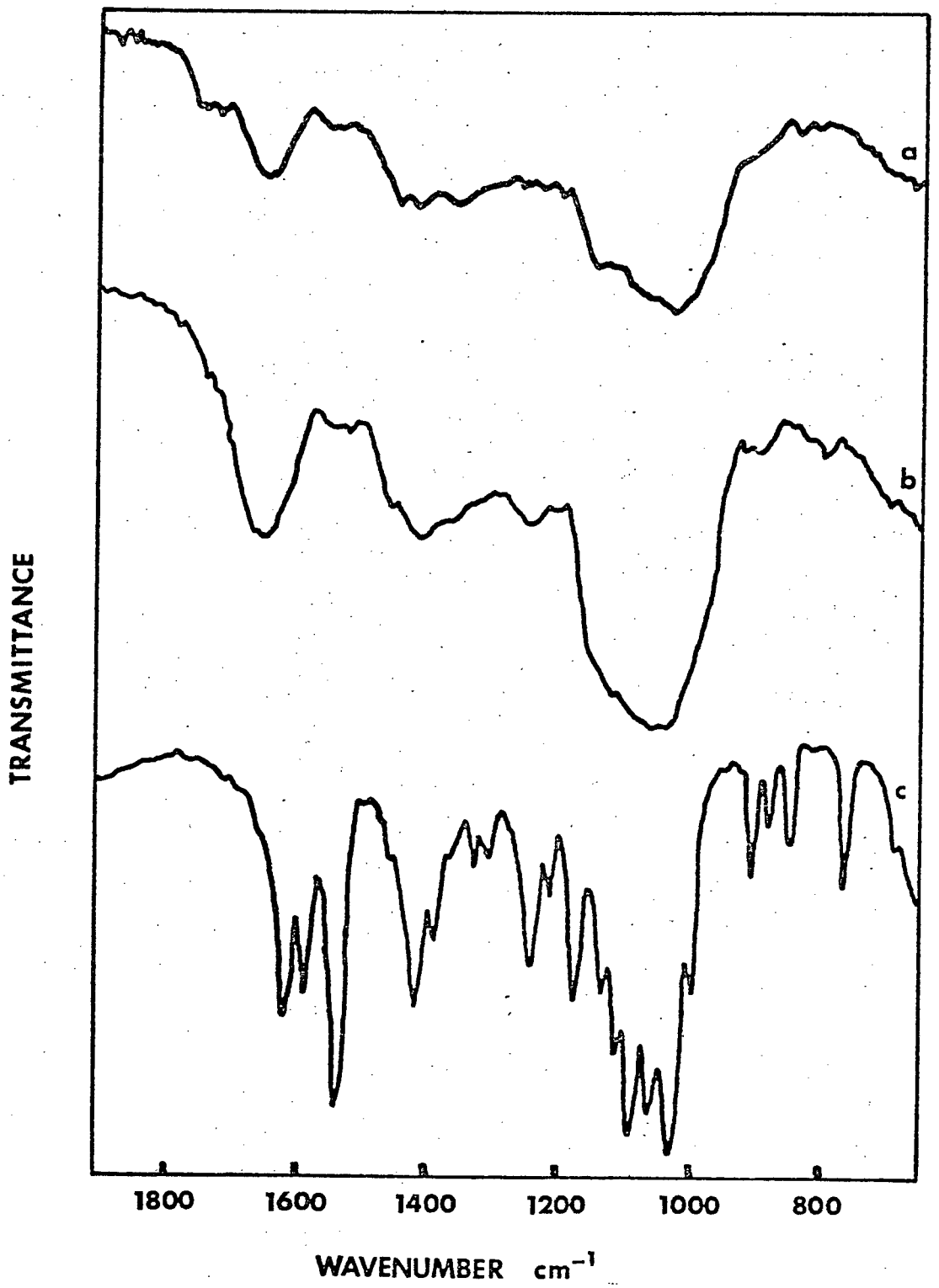

a
b
c
TRANSMITTANCE
1800
1600
1400
1200
1000
800
WAVENUMBER cm⁻¹

FIG.13

12. Virulence Testing with Mice

The results of virulence testing with mice are expressed in Table XXVIII. Cr. albidus strains 367 and H1354 and Cr. laurentii 371-1 all failed to kill any of the mice. Cr. neoformans 365-11 killed 50% of the mice after five days, while the mortality rate for 365-16 was 50% on day 18. The mice showed characteristic symptoms of domed skull, staggering, twitching, dull fur and no increase in size. 80% of the mice were still alive 28 days after inoculation with 365-26. Mice were killed and smears and cultures made from the brain tissue. Cells from brain tissue of a mouse killed by this strain were subcultured and reinoculated into a further 12 mice. After this passaging, 50% mortality occurred after 19 days. Fig. 14 shows cells from mouse brain before and after passaging, demonstrating the increase in cell size in brains of mice killed by the yeast. The majority of cells also had larger capsules in the more virulent form.

TABLE XXVIII

INOCULATION[a] OF MICE INTRACEREBRALLY WITH VIABLE CELLS OF CR. NEOFORMANS (365-11, 365-26 AND 365-16), CR. ALBIDUS (367 AND H1354) AND CR. LAURENTII (371-1)

	No. of deaths/day									From 12 mice
	4	5	6	8	14	18	21	25	28[b]	% survival
Cr. albidus 367	0	0	0	0	0	0	0	0	0	100
Cr. albidus H1354	0	0	0	0	0	0	0	0	0	100
Cr. laurentii 371-1	0	0	0	0	0	0	0	0	0	100
Cr. neoformans 365-11	2	4	4	2	-	-	-	-	-	0
Cr. neoformans 365-16	0	0	0	0	1	6	5	-	-	0
Cr. neoformans 365-26	0	0	0	0	0	0	0	1	1	83
Cr. neoformans[c] 365-26	0	0	0	0	0	1	1	0	2	66
Cr. neoformans[d] 365-26	0	0	0	0	1	4	7	-	-	0

a: mice inoculated intracerebrally with 0.02 - 0.04 ml of a 10^6 suspension of cells of each strain in sterile saline.
b: experiment terminated on day 28.
c: inoculations repeated after subculturing strain twice on Sabouraud agar at 36C.
d: inoculations repeated with cells removed from brain tissue of mouse killed by this strain and subcultured at 36C.

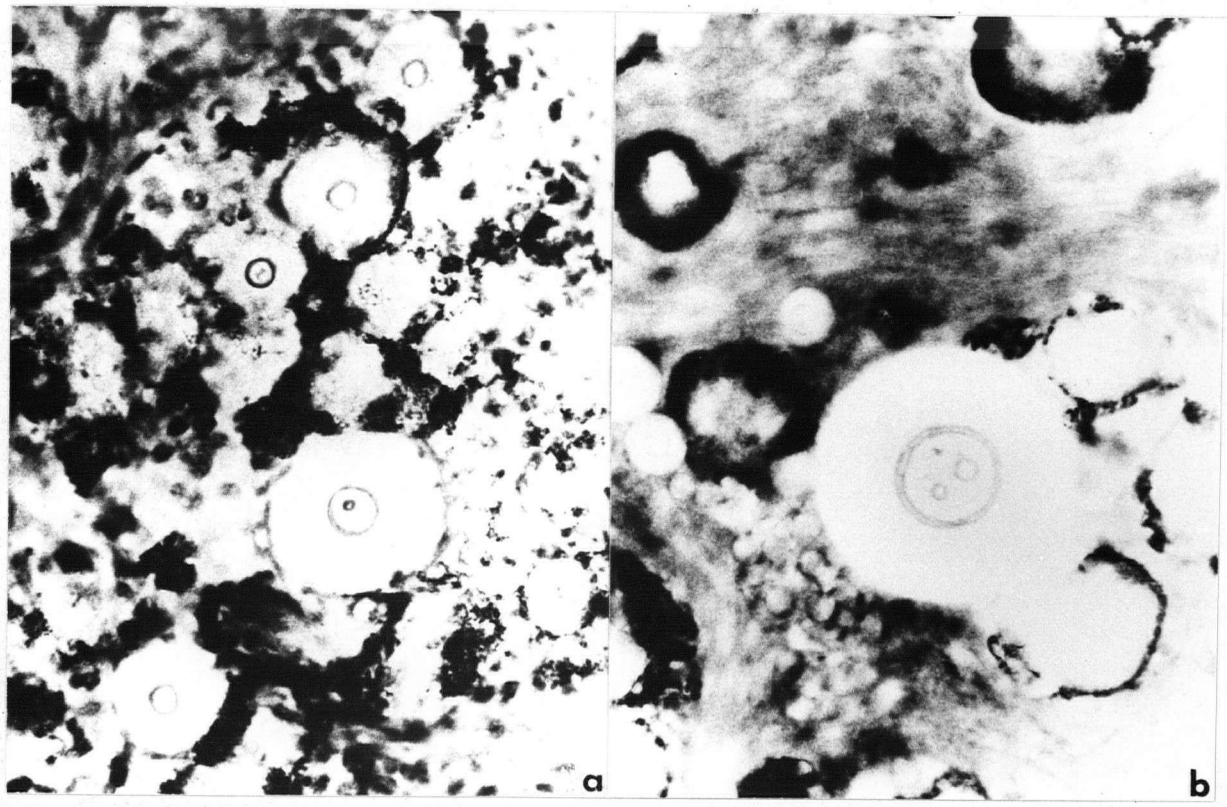


FIGURE 14. CR. NEOFORMANS 365-26 IN BRAIN SMEARS OF SWISS WHITE MICE.

Smears were stained with fungicidal India ink to show the capsule. Photographs x 200.

a. Cr. neoformans 365-26 in brain tissue of mouse not killed by the organism.

b. Cr. neoformans 365-26 in brain tissue of mouse killed by the organism after passaging through mice and subculturing.

CHAPTER IV

DISCUSSION

Preliminary Studies and Growth Conditions of Cryptococcus Species

Preliminary studies were undertaken to find a suitable method for killing Cr. neoformans cells to avoid health hazards during harvest and extraction. Previous workers used 1% phenol (Evans and Kessel 1951), 0.5% formalin (Gadebusch and Johnson 1968, Kobayashi et al. 1974), 3% chloroform (Farhi, Bulmer and Tacker 1970) or autoclaving at 82C for 35 min (Goren and Middlebrook 1967). Phenol was shown to reduce amino acid content of Cr. laurentii cell walls (Table III) and formalin caused flocculation of Cr. neoformans cell walls with whole cells during wall extraction. Autoclaving under the acid conditions of the medium (Goren and Middlebrook 1967) probably degraded readily released sugars such as xylose. I found that metabolic poisons such as potassium cyanide did not kill the cells. Farhi (1969) reported that neither sodium fluoride nor potassium iodoacetate inhibited glucose utilization by a strain of Cr. neoformans. Perhaps the capsule prevented these chemicals from entering the cell. As mild heat treatment did not affect chemical composition of walls or capsules (Table IV), this method was chosen to kill cells prior to harvest, for all preparations made in this study. Slight differences in temperature and holding time were needed for cells grown in different media for different incubation

periods. In addition, for extracellular preparations, the pH of the medium was adjusted to about 6.0 before heat treatment to avoid heating under degradative acid conditions.

Bulmer and Sans (1968) and Golubev et al. (1971) extracted adhered cryptococcal capsular material and reported qualitative analysis. I was unable to obtain such preparations by any of the published methods (Bulmer and Sans 1968, Goren and Middlebrook 1967, Vogel 1966) without cell wall or cytoplasmic contaminants.

Standard conditions of temperature, pH, incubation time and vitamin requirements for maximum capsule production in LCM were determined for each strain (Figures 2 - 5). A temperature of 25C was chosen for all large scale preparations since neither of the Cr. albidus strains grew at 37C but both showed optimum growth at 25C. The lack of growth of Cr. albidus Hl354 above 33C is of interest in view of the supposed pathogenicity of this strain, which was isolated from human cerebrospinal fluid (Wieser 1973). Castellani (1963, cited by Phaff and Fell 1970), isolated two Cryptococcus species from lesions on male genital organs. These capsulated isolates showed similar assimilation patterns to Cr. albidus but did not assimilate nitrate or grow at 37C. Tang and Howard (1973) showed that whereas Cr. neoformans could utilize glutamate at 37C, at this temperature uptake of L glutamic acid by Cr. albidus was severely inhibited. It may be that although Cr. albidus cells could not grow in vitro at 37C, in vivo some Cr. albidus strains could incorporate and utilize L glutamic acid. There are also several examples of fungi being

unable to grow above certain temperatures unless supplied with essential vitamins or amino acids (Deverall 1965). Perhaps conditions *in vivo* provide these substances which *Cr. albidus* H1354, for example, is able to incorporate. However, Tang and Howard (1973) reported that both adenine incorporation into DNA and nuclear migration prior to nuclear division by *Cr. albidus* were also temperature sensitive. If this were true of all *Cr. albidus* strains, it is still unclear why *Cr. albidus* H1354 was able to grow *in vivo*, but not *in vitro*, at 37C. It is also possible that the organism may have mutated to a lower maximum temperature as Wieser (1973)suggested.

All the strains grew poorly above pH 7.0, consequently media for extractions were adjusted to the optimum pH, 6.9 - 7.0, before autoclaving. This contrasts with Bulmer and Sans' work (1968) where maximum capsule size was obtained on solid LCM at pH 7.5. The cells must also be able to grow at pH 7.34 (spinal fluid) and 7.39 (blood plasma) *in vivo*, thus it is unclear why the strains grew poorly above pH 7.0 here. Although the optimum pH for cell growth and subsequent capsule production was 6.5 - 7.0 at inoculation, capsular material and visible capsules did not appear in the medium or on the cells, until the pH had dropped to 5.0 or lower.

All the strains required thiamine for growth, and all except *Cr. albidus* 367 could utilize it in the heat separated form (Figures 2 - 5). *Cr. albidus* 367 could not utilize thiazole and pyrimidine as well as it could use the intact thiamine molecule although capsule size was little reduced in autoclaved

thiamine. Farhi (1969) reported no effect of thiamine within 24 hr incubation either on growing or resting cells, but with the cells tested here (including vitamin depleted cell controls) growth enhancement was observed after 16 hr. As 10 µg/ml thiamine gave maximum capsule production in these studies, this was added to LCM instead of 1 µg/ml as recommended by Littman (1958). The results from these growth studies, which showed differences among the strains, emphasized the need to determine and standardize the optimum growth conditions for each strain before any further studies or extractions were carried out. This should also be considered in future work.

Cell Morphology of Yeast and Hyphal Forms of Cryptococcus

There were differences in both cell shape and capsule size among the strains (Figure 5). Cr. albidus cells were typically oval with polar budding whereas Cr. neoformans and Cr. laurentii cells were always spherical. Cr. neoformans 365-26 had large capsules of up to 4.5 µ in width whereas 365-11 and 365-16 capsules were slightly smaller. The cells of Cr. neoformans 365-11 were also smaller than the other two strains. Both Cr. albidus H1354 and Cr. laurentii 371-1 had small capsules. It is difficult to compare the capsule sizes observed here with those of other workers since many authors do not state how capsule width was calculated. Littman (1958) for example, referred merely to capsule diameter, whereas Bulmer and Sans (1968) defined capsule width as 1/2 (diameter of cell and capsule - diameter of cell).

The photographs (Figure 5) show two distinct zones in the capsule. Stoetzner and Kemmer (1971) and Al-Doory (1971) have also described two capsule regions in a few strains of Cr. neoformans, although many other strains have no indication of a mucous halo. A number of workers have published electron microscopic evidence (Lurie et al. 1971) and fluorescence studies (Vogel 1966) to show that the outer wall and inner capsule are similar in structure. Takeo et al. (1973) described a two layered wall with dense, 20 nm diameter particles most prevalent in the outer layer and also present in the inner capsule. Because capsular microfibrils were recognized by freeze etching but not freeze fracture, the authors considered the capsule to be highly hydrated. Capsule width increased considerably when the Cr. neoformans strains were transferred into mouse brains (cf. Figures 5 and 14) where virulence appeared to be related to capsule size. When Cr. neoformans 365-26 produced predominantly small capsules it did not kill the mice. The cells of virulent strains were slightly larger in vivo than in vitro. It was possible that once the yeast reached the brain, cell division slowed down and metabolic activity was directed towards synthesis of wall, capsule and cytoplasmic material. The freeze etching studies of Takeo et al. (1973) support this view since the large vacuoles and accumulation of storage organelles observed in the cytoplasm of in vivo cells suggested a lower rate of multiplication, and the large number of vesicles suggested a high secretion activity.

The production of branching, septate hyphae with clamp

connections (Figure 6 a, b) in the Coward strain of Cr. neoformans supports the view of several authors (Lurie and Shadomy 1971, Kurtzman 1973, Gordon and Devine 1970) that some species of Cryptococcus may be the imperfect forms of basidiomycetes. I was not able to confirm a perfect basidiomycete state since dikaryotic hyphae were not detected and no sexually reactive cultures were found in any of the strains on the sporulation media tested. Conjugation tubes and hyphal outgrowths were observed in Cr. neoformans 365-26 after heat treatment (Figure 6 c, d). These resembled the structures described by Gordon and Devine from sodium deoxycholate induced mutants. Unlike these authors, I did not observe endogenous sporulation. Cr. neoformans 365-11 and 365-26 are probably type A strains (as judged by O-acetyl peak in infrared spectra, Figure 12), and since Gordon and Devine detected sporulation in type C only, this may be one reason why endogenous sporulation was not observed in Cr. neoformans 365-11 and 365-26. Old cultures of both Cr. albidus strains and Cr. terreus 8157 developed many hyphal bodies and cigar-shaped cells. These were similar in appearance to cells of Cr. albidus which had been incubated at 37C for 18 hr (Tang and Howard 1973). Therefore it appeared that in these cases, hyphal production was in response to the adverse conditions of high temperature and nutrient deficiency as was suggested by Evans (1969). The Coward strain, however, did produce true mycelia under normal cultural conditions, and on the basis of the high degree of DNA homology between this strain and other non hyphal Cr. neoformans strains (Erke and Schneidau 1973), can be classified as Cr. neoformans.

This strain did not produce hyphae in the liquid media SAB and LCM, and therefore I was unable to obtain hyphal cell walls.

Isolation of the Cryptococcal Cell Envelope

Extracellular material was isolated from three strains of Cr. neoformans, two of Cr. albidus and one of Cr. laurentii. In agreement with work by Abercrombie et al. (1960) and Foda et al. (1973) I found that all the strains here produced large amounts of heteropolysaccharide in LCM at an initial pH of 7.0 (Table VI). Recoveries of extracellular material were poor when cells were harvested above pH 4.5. No amylose was detected in any of the strains although this polymere has been reported for other Cryptococcus strains (Foda and Phaff 1969), Kooiman 1963, Gorin et al. 1966). This may have been because the pH did not drop below 3.0 in any instance. I did isolate a water-insoluble fraction (after ethanol precipitation) from the culture medium of the nonpathogenic species. This, like the neutral polysaccharide isolated from Cr. laurentii by Abercrombie et al. (1960), did not stain blue with iodine. After five days incubation in LCM the Cr. neoformans cultures were much more mucilaginous and slimy than the non pathogens and their ethanol precipitates in culture fluid were more fibrous (Table VI). The increase in viscosity of these precipitates seemed to be accompanied by an increase in O-acetyl and glucuronic acid content (Tables VI, XIX and XX).

Cell walls were extracted from Cr. neoformans 365-26 and Cr. laurentii 371-1. I have already mentioned the difficulties involved in obtaining wall preparations from Cr. neoformans

strains. Erke and Schneidau (1973), in their DNA extractions from Cryptococcus species, also found Cr. neoformans cells much more difficult to disrupt than those of Cr. laurentii due to the presence of capsular material. They were unable to recover spoolable DNA from the Cr. neoformans strains even after freeze-thaw procedures and disruption in a French Pressure cell. They considered the French pressure cell more effective than the Braun homogenizer whereas Phaff (personal communication) found the latter to be more effective with Cr. laurentii. Devlin (1969) reported only 20% disruption of Cr. neoformans with the Braun homogenizer but obtained 80% by sonic oscillation and an even higher value by the zeolite extraction procedure (Zipper and Person 1966). I did not obtain more than 60% breakage with zeolite, sonic oscillation or with an Edebo press (AB Biotec Sweden X25 491). I found these methods unsuitable since there must be at least 90% breakage to avoid aggregation of the walls with remaining whole cells and cytoplasmic debris. This aggregation may be due to binding capacities of the highly charged capsular material (acid groups) with sites on the walls or remaining whole cells. A successful procedure for breakage and subsequent isolation of pure cell walls remains to be developed for Cr. neoformans strains.

Chemical Composition of Cell Walls

The cell walls isolated from Cr. laurentii and Cr. neoformans showed some striking similarities in chemical composition to the walls of other basidiomycetes. Previous studies on

basidiomycete cell walls (Crook and Johnston 1962, O'Brien and Ralph 1966, Angyal et al. 1974) showed glucose and glucosamine to be the major monosaccharides with mannose and xylose in lesser amounts and traces of galactose or fucose. Galactose found in both Cryptococcus species (Tables X and XI), was present only in Ustilago maydis (Crook and Johnston 1962), Coniophora cerebella (O'Brien and Ralph 1966) and Tremella mesenterica (Cameron 1973). The Cryptococcus species contained no fucose and much smaller amounts of glucosamine than most basidiomycetous yeasts, except Tremella. Kobayashi et al. (1974) claimed to have detected ribose or rhamnose in an endotoxic substance (hot phenol-water extract) from Cr. neoformans. The peak, which they observed by gas-liquid chromatography as a TMS derivative, was also present in small amounts in my wall and extracellular material samples. However, I did not consider it had the same retention time as either ribose, rhamnose or arabinose. Arabinose has been claimed to be a minor component of Cr. laurentii cell walls (Ankel et al. 1969). However, this peak seemed most likely to be one of the first minor xylose peaks. Infrared spectroscopy (Figure 13) showed that the cell wall of Cr. laurentii had both α- and β-linked glycans (840 and 890 cm^{-1} respectively). The spectrum was very similar to that of a Cr. albidus cell wall preparation examined by Jones et al. (1969). These authors found that the α-glucan composition was considerably reduced by growing the yeast under unfavourable conditions. Kanetsuna and Carbonell (1970, 1971) also found that α-glucan decreased and β-glucan synthesis increased, during the yeast to

mycelial conversion which was associated with the lowering of temperature in Paracoccidioides brasiliensis and Blastomyces dermatitidis. Polysaccharides from the cell wall of Polyporus tumulosus include β-1, 3-glucan, α-glucan and chitin as well as a xylomannan which has similar molar proportions of mannose and xylose (1.2:1) to those in Cr. laurentii cell wall (Angyal et al. 1974). Other basidiomycete cell walls (O'Brien and Ralph 1966) have similar mannose:xylose proportions except Tremella which has much larger amounts of xylose than mannose. This again infers a close relationship between Cryptococcus and the basidiomycetes. The main difference in sugar content between the walls of the two Cryptococcus species was the much larger amount of glucan in Cr. neoformans; 93%, in contrast to 74% in Cr. laurentii (Tables X and XI). The latter figure was similar to the 75% glucose recovered from Cr. albidus walls (Bacon et al. 1968). The other sugars, including glucosamine, were present in smaller amounts in Cr. neoformans (Tables IX, X and XI), although the proportion of glucosamine was still higher than in Tremella cell walls. Cr. neoformans 365-26 also contained a small amount of galactosamine which has not previously been detected in yeast cell walls.

In the only published study of the cell walls of Cr. neoformans Cook et al. (1970) reported a very low recovery of hexosamine (0.3%) in walls of both patient and soil isolates. However, they did not use critical serial hydrolyses and therefore probably underestimated the glucosamine. Devlin (1969) also examined cryptococcal cell walls and "zeolite ghosts" by

qualitative chemical methods. He failed to detect glucosamine, but the mild hydrolytic conditions employed (1.5N HCl for 3 hr at 97C) were probably insufficient to release glucosamine. He was also unable to release N-acetyl glucosamine by hydrolysis with purified chitinase and concluded that the walls contained no chitin. In view of his poor chemical techniques, it is likely that he did not detect the relatively small amount of N-acetyl glucosamine that I consider to be present. Vibrations at 1380, 1550, 1640 and 2940 cm^{-1} in the infrared spectra of Cr. laurentii cell walls (Figure 13), are typical of chitin, which suggests that the glucosamine present is N-acetylated and may be occurring as chitin. The shoulder at 1725 cm^{-1} is indicative of carbonyl vibrations and may be due to N-acetylation (perhaps of glucosamine) rather than to carboxylation, in Cr.laurentii cell walls. However, chitin does not have a peak at this wavenumber. Bowden and Hodges (1970) postulated that the small amounts of glucosamine present in the yeast wall as N-acetyl glucosamine, need not necessarily occur as a chitin-like polymer but rather act as attachment points for peptide moieties to mannan and glucan components. Nakajima and Ballou (1974) have recently used enzymic techniques to isolate an oligosaccharide corresponding to the linkage region between polysaccharide and protein parts of Saccharomyces cerevisiae mannan. A single N-acetyl glucosamine was attached to the reducing end of 12 mannose units by a β-1, 4 linkage. If the glucan layer was combined with or covering the cell wall chitin (Domanski and Miller 1968) then chitinase (as used by Devlin

1969) would not release N-acetyl glucosamine without prior application of a β1-3 glucanase.

Very few fungal cell walls have been examined quantitatively for amino acids and only one study employs corrective serial acid hydrolyses (Cameron 1973). The amino acid patterns of Cr. laurentii and Cr. neoformans cell walls were very similar to those of Tremella mesenterica (Table VIII). All were high in Asx and Glx, the amino acids associated with Alkali stable glycopeptide bond formation. Nakajima and Ballou (1974) proposed that yeast mannan is linked to protein through a di-N-acetylchitobiose to asparagine: this may be the case in Cryptococcus cell walls. Cr. laurentii cell walls, in fact, not only had more glucosamine but also had a slightly higher percentage of Asx than Cr. neoformans (Tables VI, IX) suggesting more of these glycoprotein linkages. Most workers have failed to detect cysteine/cystine in the fungi examined (Roy and Landau 1972) but Pine (1972) detected very small amounts in Histoplasma capsulatum and H. dubiosii cell walls. I detected cysteine/cystine only in Cr. laurentii. Hydroxyproline has not been previously reported in fungi with chitinous cell walls (Bartnicki-Garcia 1968) and it was not detected in the Cryptococcus species tested here. It is possible that the presence of hydroxyproline in Tremella mesenterica cell walls (Cameron 1973) was related to the absence of chitin. These cell walls have yet to be tested for the presence of N-acetylated glucosamine.

Cryptococcal cell walls were also similar ultrastructurally

to those of other basidiomycetous yeasts (Kreger-van Rij and Veenhuis 1971) in having a lamellar wall structure and a similar method of bud formation and septation. These features differed from those recorded for ascomycetous yeasts. Cryptococcus and Tremella differed mainly from other basidiomycetous yeasts in having less glucosamine. This seemed to be replaced by larger amounts of glucan but it is unclear at present exactly how the polymer is arranged to form the two layered lamellar wall observed by electron microscopy.

Relation of Cell Wall Chemistry to Pathogenicity

It is difficult to relate differences in cell wall chemistry to pathogenicity. Cox and Best (1972) related higher wall phospholipid, chitin and protein content to virulence in Blastomyces dermatitidis: less virulent strains had more glucan. On the other hand, Cook et al. (1970) found more hexose and less lipid and hexosamine in the cell wall of the patient isolate of Cr. neoformans than in the soil isolate. However, they did not determine the degree of virulence of the two strains for mice. Although I did not perform lipid and phosphorus analyses, my results agree with Cook et al's in that the pathogen, Cr. neoformans 365-26, had much larger amounts of glucan (equivalent to hexose, Cook et al.) and a smaller amount of glucosamine and probably protein in the cell wall than the nonpathogenic Cr. laurentii. Cr. neoformans 365-26 cell wall also had a substantially larger percentage of lysine than Cr. laurentii.

It is premature to conclude that larger amounts of glucose and smaller amounts of glucosamine and possibly glycoprotein in

the cell walls of Cryptococcus species, are related to pathogenicity. Cell walls from more species and strains must be examined both chemically and structurally, as well as tested for antigenicity.

Chemical Composition of Extracellular Material

The extracellular material isolated from the six Cryptococcus strains contained the same monosaccharide constituents: mannose, xylose, galactose, small amounts of glucose and glucuronic acid as well as O-acetyl substitutions (Tables XVII to XX). The mannose, xylose and glucuronic acid occurred in approximately the same molar proportions as those determined by other workers (Farhi et al.1970, Blandamer and Danishefsky 1966, Slodki et al. 1966, Helms et al. 1969). The presence of galactose is a point of dispute since some workers consider the capsular material to be a mixture of two polysaccharides, one containing galactose and the other not (Evans and Theriault 1953, Rebers et al. 1958). Cr. neoformans 365-16 and Cr. albidus H1354 preparations contained considerably more galactose than the other strains (Tables XVII, XVIII). Judging from the diffuse carbohydrate band in gel electrophoresis of Cr. albidus extracellular material (Figure 7) it is quite possible that the polysaccharide portion is heterogeneous. However, uronic acid and carbohydrate moieties did migrate together in the cellulose acetate strips (Figures 8 and 9) suggesting that the molecule may be homogeneous. Golubev et al. (1971) considered the capsule and extracellular material to be different on the basis of lack of galactose in the former. However, Farhi et al. (1970) found no difference in percentage galactose between

adhered and soluble polysaccharides. Perhaps Golubev *et al.* lost the galactose during the autoclaving procedure they used to remove adhered capsule. Glucose was not detected in any previous studies.

The major differences between the strains were in uronic acid content and the degree of O-acetyl substitution. (O-acetyl has not been estimated quantitatively previously and some authors (Farhi *et al.* 1970, Blandamer and Danishefsky 1966) did not test for it at all).

These differences were clearly demonstrated in the infrared spectra (Figure 12). Although no methyl acetates were detected in transesterified samples of *Cr. albidus* (H1354 and 367) and *Cr. laurentii* 371-1, *Cr. albidus* 367 does have a peak at 1725 cm^{-1} (indicative of C = O) and *Cr. albidus* H1354 a slight shoulder. Difficulties in interpretation of cell wall and polysaccharide spectra often arise because of the additive effects and influences of the absorptions of the different chemical groups present. Thus the more heterogeneous the compound, the more complicated becomes the interpretation. For example, the peaks at 1730 and 1250 cm^{-1} are regarded by some authors (Blandamer and Danishefsky 1966, Maschessault 1962) to be indicative of carboxyl groups whereas Levine *et al.* 1959, Goren and Middlebrook 1967 and Kobayashi *et al.* 1974 consider these vibrations to be due to acetyl functions. The peak at 1250 cm^{-1} is undoubtedly due to C-O stretching vibrations, but the line between ester function at 1725 - 1750 cm^{-1} and carboxyl at 1650 - 1725 is hard to distinguish especially if both functions are

known to be present. Ukai *et al.* (1974) working with *Tremella* polysaccharide and Goren and Middlebrook with *Cr. neoformans*, found that the ester function, presumed to be O-acetyl, was hydrolytically cleaved under mild alkaline conditions with resulting loss of absorption at 1725 and 1250 cm^{-1}. Levine *et al.* (1959) considered the peaks at 1650 and 1425 cm^{-1} to represent carboxylate but both glucan and mannan have large peaks at 1650 as does glucosamine. Because none of the nonpathogenic strains contained O-acetyl functions, but all had uronic acid, it is possible that the vibrations at 1730 cm^{-1} were due to both acetyl and carboxyl functions. This would certainly correlate with the increase in peak size at this wavenumber from the trace in *Cr. laurentii* to the large peak in *Cr. neoformans* 365-11 (Figure 12). If the effect were additive it would explain why the difference in peak size between *Cr. neoformans* 365-11 and the other two *Cr. neoformans* strains is not as marked as would be expected from the differences in their O-acetyl content. None of the strains showed a carboxyl salt peak at 1600 cm^{-1}.

The higher glucosamine content of *Cr. albidus* H1354 #3 and *Cr. laurentii* 371-1 (Table XVI) can also be observed in the spectra from the vibration at 1550 cm^{-1} (Figure 12). Thus the slight shoulder at 1730 cm^{-1} on the H1354 spectrum may also be due to N-acetylation. Both glucose and glucosamine have not previously been detected in cryptococcal extracellular polysaccharides or capsules although other yeasts and both yeast and mycelial phases of *Sporothrix schenckii* produced extracellular

polysaccharides which contained glucosamine and N-acetyl glucosamine (Jeanes et al. 1971, Toriello and Mariat 1974). All the strains showed peaks at 800 and 900 cm^{-1} representative of α-linked glycan. Beran et al. (1972) observed that these vibrations, as well as vibrations at 980 cm^{-1}, were present in the spectrum of yeast mannan but absent from that of yeast glucan. Since Cr.neoformans 365-11, which contained more mannose than the other strains, also had the most intense peak at 800 cm^{-1}, it is possible that the mannose present is α-linked.

The polysaccharides were similar in composition to those isolated from Tremella species (Slodki et al. 1966, Fraser et al. 1973a), in that they contained mannose, xylose, glucuronic acid and O-acetyl groups. Slodki et al. (1966) and Helms et al. (1969) also found three Cr. laurentii strains which contained O-acetyl groups although many do not. However, Slodki et al. (1970) showed that the phosphorus content of mannans may be replaced by O-acetyl groups in phosphate limiting medium. The medium he used contained 0.01% phosphorus whereas the LCM used here contained 0.2%. Cadmus et al. (1962) found that increase in concentrations of phosphorus inhibited capsule production in a Cr. laurentii strain. This may explain why I detected less capsule and capsular material from Cr. laurentii than from the other strains, and why no O-acetyl groups were detected. Ukai et al. (1974) isolated a homogeneous polysaccharide from hot water extracts of Tremella fuciformis and obtained similar molar ratios of sugars to those obtained from other Tremella species (Slodki et al. 1966). The main difference between

Tremella and Cryptococcus is that Tremella has more xylose than mannose in the extracellular polysaccharides and in the cell walls. The Cr. neoformans strains I examined, also had more uronic acid and O-acetyl groups, than Tremella species. Fraser et al. (1973b) demonstrated that the O-acetyl groups occurred on the C-3 positions of the glucuronic acid residues.

This study is the first report of amino acid analyses from Cryptococcal polysaccharide. The only other amino acid analysis relates to somatic proteins extracted from Cr. neoformans with hot alkali (Uzman 1956). From 20 to 100 µg amino acids were recovered per mg extracellular material for all the strains and all contained at least 1% nitrogen (Tables XII and XXIII). Most authors have regarded the nitrogen component as a contaminant and have used the deproteinization procedures of Kabat and Mayer (1967) during purification (Kozel and Cazin 1970, Farhi et al. 1970, Murphy and Cozad 1972). These procedures, involving chloroform-butanol extractions, would not have removed covalently bound protein. After deproteinization, about 1.7% protein was detected in the cryptococcal polysaccharide by the Folin Lowry procedure. However, the Folin Lowry method is often inaccurate since certain amino acid sequences are far more chromogenic than others and hence the value obtained depends on the standard used and on the composition of the protein (Chou and Goldstein 1960). Thus there was probably more protein present in the original cryptococcal extracellular material than these authors detected. Also the cells had been killed with 3% chloroform or 1% phenol (which reduces amino

acid recoveries in cell walls, Table II) before polysaccharide extraction and this may have caused additional losses in protein recovery.

Both gel electrophoresis (Figure 7, Tables XXVI and XXVII) and column chromatography (Figure 10) showed that the extracellular material (at least of Cr. neoformans 365-26 and Cr. albidus 367) migrated as a single species, thus I consider the protein portion to be a true component rather than contaminating material. The use of chemically defined media [with $(NH_4)_2SO_4$ as the sole nitrogen source] and extensive dialysis and reprecipitation during purification, rule out the possibility of this protein being either a medium contaminant or from free amino acids produced by the cells' metabolism or breakdown of the cell walls.

There were some interesting similarities and distinct differences in the amino acid patterns of extracellular material from the six Cryptococcus strains. Threonine, serine and glutamate were the predominant amino acids in both Cr. albidus strains whereas Cr. neoformans 365-11 had large amounts of aspartate, serine and glutamate (Tables XII and XIII). In the other two Cr. neoformans strains aspartate and serine were the predominant amino acids. Cr. laurentii 371-1, like the cell wall preparations, had large amounts of aspartate and glutamate. The fact that the percentages of threonine and serine were generally higher in the extracellular material than in the cell wall preparations, and that there was less glucosamine, might suggest the presence of a different type of glycopeptide linkage. It is interesting that both Cr. laurentii and Cr. neoformans

365-11 extracellular material, which were high in aspartate, also had larger amounts of glucosamine than the other two Cr. neoformans strains. However, this was not the case for the Cr. albidus strains.

Both Cr. neoformans 365-16 and 365-26 were unusually high in proline and phenylalanine compared to the other strains. As the percentage of glutamate is also noticeably lower in these two strains, it may be that they are deficient in proline oxidase which converts proline to Δ'-pyrroline-5-carboxylic acid and then to glutamic acid (Meister 1965). Anderson et al. (1971) found a very high percentage of proline in the amino acid content of a culture filtrate from the pathogen Coccidioides immitis. They suggested that proline along with O-methyl mannose and mannose constituted the major components of skin test activity. Bradley et al. (1974) also found a high proline level in H and M reactive components of histoplasmin, a diagnostic agent for histoplasmosis. Perhaps the high percentage of proline in these two Cryptococcus strains is connected with antigenic activity.

I found that comparison of the ratios of Gly:Ala, Ile:Leu and Tyr:Phe (Table XV) revealed some consistent patterns in the proteins. The ratios were very similar in the cell wall preparations. That of Cr. laurentii was almost identical to Tremella mesenterica. However, Cr. neoformans 365-16 and 365-26 showed some striking differences. The Gly:Ala and Tyr:Phe ratios were much lower than the other strains and the Ile:Leu ratio was much higher. The Tyr:Phe ratio for Cr. neoformans 365-11 was the highest of all the strains. The somatic protein extract of Cr. neoformans (Uzman et al. 1956) had a similar Gly:Ala ratio to Cr. neoformans

365-26, the Ile:Leu value was slightly higher but the Tyr:Phe value resembled the extracellular material. It is possible that Cr. neoformans 365-16 and 365-26 have developed a deficiency in phenylalanine hydrolase and hence are unable to convert phenylalanine to tyrosine (Chandra and Vining 1968). These two strains also lacked cysteine which was present in trace amounts in 365-11 and Uzman's preparation and was 2 - 5% of the total amino acids in the other strains. The close relationship of 365-16 and 365-26 is also borne out by their similar glucosamine, uronic acid and O-acetyl content (Tables XVI, XIX and XX). Their infrared spectra were very alike (Figure 12) although Cr. neoformans 365-26 had larger peaks at 1730 and 1250 cm^{-1} indicative of slightly higher O-acetyl and uronic acid content. Cr. neoformans 365-16 and 365-26 appeared more closely related to each other than to 365-11 which seemed more similar in Gly:Ala, Tyr:Phe and Ile:Leu ratios to the nonpathogens and had slightly more basic amino acids.

Ethanolamine, detected in all the strains, has only recently been reported in fungal exocellular glycopeptides (Rick et al. 1974). Although I did not quantify the ethanolamine it is significant that it occurred in large amounts in those strains that also contained more phosphorus (Table XXIII). Cr. neoformans 365-16 and 365-26 had small ethanolamine peaks and only 0.2% phosphorus. Perhaps the ethanolamine was attached to the glycoprotein through a phosphodiester linkage. However, it is difficult to make any conclusions since although

I established the presence of lipids in the extracellular material, I obtained no quantitative data.

Phosphorus values were larger for the nonpathogenic strains (Table XXIII), which seems to rule out the possibility that a high phospholipid content is related to virulence in Cr. neoformans as was postulated by Cox and Best (1972) for Blastomyces dermatitides. Ash content ranged from 2 - 11% except for one preparation of Cr. laurentii which had 43% ash (Tables VI and XXIII). This preparation was not used for total analyses. Although I did not analyze any of the material for sulphate, it has recently been detected in the capsule by autoradiographic and histochemical techniques (Mahvi et al. 1974). Perhaps sulphate comprises a small percentage of the material that was unaccounted for in my total recoveries. Cr. neoformans 365-16 and 365-26 were consistent in all respects in the duplicate batches, whereas other strains showed slight variations especially in ash content.

Cr. albidus H1354 preparations #2 and #3 showed large differences in protein content. Although I suspected that Cr. albidus H1354 #3 represented the true amino acid and amino sugar content and that the other strains were underestimated for protein, the infrared spectra seem to indicate that this strain did have more amino groups. However, it is obvious from the discrepancies in nitrogen recoveries (Table XXIII) that losses in amino acids and amino sugars occurred. The extracellular material contained large amounts of polysaccharide which caused humin production during acid hydrolysis (James 1972).

This may have been responsible for degradative losses in amino acid, amino sugar and neutral sugar components. Robel (1973) and Altosaar (1974) showed that losses of certain amino acids were due to adsorption to the glass tubes during desiccation. Robel's procedure for correcting such losses was tried in this study, but was found impractical since protein content of the extracellular material was so small.

The total neutral sugar values included in Table XXV are from Cameron's method (1973). Values from Lehnhardt and Winzler's method (Table XVII) appear to be overestimations, and it may be that the true sugar value lies between the two sets of data presented. These discrepancies in total neutral sugar emphasize the need for an accurate and reliable internal standard. More studies must be also done on release and subsequent degradation of glucosamine, especially, from complex polysaccharides such as these. It is well known that the extent of degradation varies for individual monomers, depending on the type of linkage between them and on the conditions of hydrolysis.

Although protein, hexose and acid functions migrated together in electrophoresis and gel chromatography, suggesting protein and polysaccharide are tightly bound, it is quite likely that chemical fractionation would reveal several components. Is the protein structural, a by-product of cell metabolism, or is it an enzyme closely bound to the polysaccharide? Yeasts have been shown to produce invertase and acid phosphatases which are located at the cell surface (Odds and Trujillo-Gonzales

1974, Odds and Hierholzer 1973). These authors showed that Cr. neoformans had a high level of acid phosphatase activity at pH 3.9 and that a similar enzyme from Cr. albidus was mannoprotein in nature with a hexose:protein ratio of 7:1. They also suggested that the hexose protein was antigenic.

Mahvi et al. (1974) recently demonstrated acid phosphatase activity in the capsule and cell wall of Cr. neoformans. They reported greater activity at 25 than at 37C. Perhaps genetic repression of enzyme synthesis at the higher temperature causes slow growth of some strains at 37C. They proposed that the viscous polysaccharide together with acid phosphatase within the capsule serve in holding and degrading nutrients at the cell surface. However, Liu (1959) found alkaline phosphatase in the capsule under in vivo conditions only. He concluded that the enzyme was induced and secreted in the parasitic state. Proteolytic activity has also been demonstrated in Cr. enoformans (Müller and Sethi 1972). The fungus was able to degrade human α_2HS-glycoprotein and to digest human fibrinogen in vitro. As there are differences in amino acid composition among the strains it is interesting to speculate whether these may have any relation to enzymic function and perhaps virulence.

In view of the differences in wall and extracellular material chemistry, it seems unlikely to me that extracellular material is entirely a result of over synthesis or autolysis of material as suggested by Kikuchi et al. (1973) and Carmo-Sousa and Barroso-Lopes (1970) working with Candida species. Phaff (1971) has suggested that some components of the capsule

and extracellular slime may be results of over synthesis, but that other components are retained in the wall. However, the Cryptococcus cell wall is largely glucose (with smaller amounts of xylose, mannose, glucosamine and galactose) and the capsule is predominantly mannose, uronic acid and xylose. The amino acids of cell walls and extracellular material also showed differences: the cell walls contained more basic amino acids (Table XIV). The Tyr:Phe and Gly:Ala ratios were much larger in the cell wall of Cr. neoformans 365-26, than in the extracellular material, whereas the Ile:Leu ratio was much smaller (Table XV). Therefore, I consider the cell wall and extracellular polymers to be synthesized independently, to a large extent. Ankel et al. (1970) isolated an enzyme from Cr. laurentii which catalyzed the incorporation of ^{14}C mannose from GDP-^{14}C mannose into an endogenous polysaccharide acceptor. Their results indicated that ^{14}C mannose was transferred only to the neutral wall heteropolysaccharide suggesting that the acidic heteropolymer was assembled by a different mannosyl transfer system. Also, xylosyl transfer to acidic extracellular polysaccharide, unlike mannosyl transfer, was dependent on the presence of added primer obtained from the acidic polysaccharide. The actual mode of capsule production has yet to be elucidated. Takeo et al. (1973) proposed that capsule material precursors were synthesized in vesicles or paramural bodies and secreted to the cell wall. At the outer layer of the wall, polymerization occurs from the microfibrils of the capsule. The particles they observed in the outer wall and inner capsule appeared to be involved in this polymerization.

Relation of Strain Virulence to Chemistry of the Cryptococcal Cell Envelope

According to the virulence tests that I made with Swiss white mice, Cr. neoformans 365-11 was the most virulent, Cr. neoformans 365-16 and 365-26 (after passaging) were less virulent, and the other three strains were avirulent. This does not agree with preliminary tests made by Dr. L. Kapica with the same Cr. neoformans strains five years ago. All the strains killed 100% of Ajax mice when injected intraperitoneally. 365-16 killed 80% of C-57 black mice, 365-26, 30% and 365-11 killed none. Townley-Price and Bulmer (1974) working with Cr. neoformans, and Chick and Roberts (1974) using Histoplasma capsulatum, also found variations in virulence of the pathogens when injected intraperitoneally into different strains of mice. However, Goren and Middlebrook (1967) reported that virulence declined over a period of two years subculturing, even though morphology and capsular appearance were the same. Therefor I assume the results I obtained to be valid for the three Cr. neoformans strains at the time they were tested, and will attempt to relate these differences in virulence to the chemistry of cell walls and extracellular material also extracted at this time.

There are several interesting possibilities. The infrared spectra, uronic acid and O-acetyl data, and cellulose acetate strip electrophoresis all indicate the higher carboxyl and ester content of the Cr. neoformans strains: 365-11 (the most pathogenic) being the highest and 365-26 and 365-16

somewhat less. 365-11, the most virulent, also has more mannose and glucosamine (and phosphate) than the other two pathogens. However, it is difficult to relate these factors alone to virulence since nonpathogenic species do have some uronic acid and O-acetyl components, but perhaps it is the polymeric structure which is important. It is similarly hard to conclude that the differences in amount of wall glucan between Cr. neoformans and Cr. laurentii might affect pathogenicity, without doing further studies on more species. Bulmer et al.(1967) related actual presence of capsule or capsular material to virulence in unencapsulated Cr. neoformans mutants which were avirulent for mice. They based their evidence for lack of capsule on acid hydrolysis (3N HCl at 100C for 4 hr) of whole cells: only glucose was recovered from unencapsulated mutants but xylose, mannose, galactose and glucuronic acid were recovered from encapsulated cells. Either the mutants' cell walls were deficient in xylose, mannose and galactose or the strong conditions of hydrolysis employed had destroyed the small amounts of these sugars that I have demonstrated in Cr. neoformans cell walls. Thus cell wall deficiencies as well as deficiencies in capsule synthesis may have affected pathogenicity in these mutants.

I have already stated that the reactivity of the different serotypes of cryptococcal polysaccharides depends to a certain extent on the degree of acetylation (Levine et al. 1959, Goren and Middlebrook 1966). These serotypes have not been assessed for differences in virulence which might be attributable to O-acetyl. Vogel (1966) suggested that B and C

antigens (having less O-acetyl) were built on basic A structure but with B or C antigenic components having a peripheral orientation on the capsule. Removing the capsule by acid treatment (0.5N HCl at 60C for 2 hr) transformed B antigenic cells into A antigens. Assuming that extracellular material and capsule are of similar composition, I propose that A antigen types, such as 365-11, have more mannose, O-acetyl and uronic acid but less xylose (13% see Table XXII) side chains. If the B types had more xylose (as do 365-16 and 365-26 - 27% Table XXII) then acid treatment and heating would remove the labile xylose chains revealing the stable mannose and acidic groupings, which, in now altered proportions, would give A reactions. According to BeMiller (1967) any restriction of the flexibility of a polysaccharide chain decreases the rate of hydrolysis. Consequently the larger amounts of glucosamine and protein in Cr. neoformans 365-11 might reduce flexibility of the polysaccharide and provide resistance to hydrolysis. In this way the particular structure of the capsular material might also confer resistance to phagocytic enzymes since it is known (Mitchell and Friedman 1972) that the ability of macrophages to kill engulfed cells is largely strain dependent and unrelated to capsule thickness.

If the capsule is a soluble extension of the antigenic component found in the cell wall (Devlin 1969) then it is possible that the antigen may be mannan-protein. I did not test the Cr. neoformans cell wall fraction for uronic acids or O-acetyl: these groupings, if present, might also have antigenic properties. Kozel and Cazin (1971) and Bulmer (personal

communication) found that injection of capsular polysaccharide from a virulent strain of Cr. neoformans did not inhibit phagocytosis, in vitro, of a non encapsulated strain or of Cr. laurentii. Farhi (1969) proposed that for inhibition of phagocytosis to occur, capsular material must be able to adhere to specific sites on the cell wall. If this is true, these sites again may involve protein or highly charged acetyl and acidic groupings.

Vogel (1966) also mentioned that cultural conditions affected the antigenic properties of the cells: B types might give A reactivity with certain media, and if they were poorly encapsulated. In this study I found that extracellular material preparations from batches of cells that grew poorly or were harvested before the pH had dropped to about 3.5 were less viscous and had a lower uronic acid content. Different growth conditions stimulate the production of different polymers. Perhaps different mouse strains provide different environments for any one strain which might induce differences in the extracellular or wall polymer produced and hence lead to differences in virulence.

The possibility that Cr. neoformans secretes extracellular enzymes, perhaps proteolytic, may be important to pathogenicity. I do not know whether the protein isolated here is enzymic, but at this stage although amino acid composition may affect antigenicity, it does not seem related to virulence. I believe that the amino acid composition of cryptococcal extracellular material and cell walls is under tighter genetic control

than the heteroglycan portion of the polymer. The latter is easily affected by cultural conditions. Amino acid patterns seem constant and distinctive for each strain and show the relationships between strains and species. Uzman et al. (1956) also found that the somatic proteins of two strains which differed in degree of encapsulation, had similar amino acid compositions.

Cr. albidus H1354, the cerebrospinal fluid isolate, was avirulent for mice, had a lower uronic acid content than Cr. albidus 367, no O-acetylation and did not grow at 37C. However, like Cr. neoformans 365-16, Cr. albidus H1354 had a large amount of galactose and like Cr. neoformans 365-11, a relatively large percentage of glucosamine and the same nitrogen content. If the protein portion was enzymic (or antigenic) then these similarities to Cr. neoformans 365-11 may be the explanation for H1354's pathogenicity. It would seem that other enzymic systems have become heat sensitive on the transfer from in vivo to in vitro, or that the spinal fluid was providing unknown factors to account for deficiencies at 37C. Otherwise this strain was very similar to the other Cr. albidus strain except in neutral sugar proportions.

CONCLUSIONS

Heat treatment was found to be a suitable method for killing Cr. neoformans cells without altering wall or capsule chemistry, providing the pH of the medium was adjusted previously to about 6.0. Killing with both phenol and formalin altered cell wall chemistry.

Thiamine was shown to be growth stimulatory and to cause increase in capsule thickness in concentrations of up to 10 µg/ml. Cr. albidus 367 alone required unautoclaved thiamine. None of the strains grew well in vitro above pH 7.0 although the pathogen is known to grow well in vivo in spinal fluid, pH = 7.4, and in blood plasma pH=7.39. Neither of the Cr. albidus strains grew at 37C. These results emphasized the need for carefully controlled and standardized growth conditions in future work: optimum conditions must be determined for each strain.

I observed septate hyphae and clamp connections with the Coward strain of Cr. neoformans and conclude that this is a member of the heterobasidiomycetes. I was not able to demonstrate dikaryotic hyphae or establish mating strains.

Cell walls were isolated only from two species of Cryptococcus: Cr. neoformans and Cr. laurentii. Previous methods in the literature were of limited success in breaking the cell walls. A successful method must be developed for future studies.

The pathogenic Cryptococcus strain had more glucose and less glucosamine, glycoprotein, mannose and xylose in the cell walls than did the nonpathogen, Cr. laurentii. Cr. neoformans also had a trace of galactosamine. Only the cell walls of Cr. laurentii contained cysteine/cystine. More strains must be examined before these differences can be related to pathogenicity.

Infrared spectra demonstrated the presence of α- and β-linked glycans in the walls of Cr. laurentii. High quantities of Asx and Glx in amino acid composition of both species suggested the presence of alkali stable glycopeptide bonds. Perhaps mannan is linked to protein through di-N-acetylchitobiose to asparagine.

The amino acid composition of Cryptococcus cell walls was very similar to that of Tremella cell walls. The mannose:xylose ratio was similar to those of other basidiomycete walls although different from Tremella. Again a relationship to Tremella and the lower basidiomycetes is suggested.

All the strains produced an extracellular polysaccharide containing α-linked mannan, xylose, galactose, glucuronic acid and small amounts of glucose. Only the three Cr. neoformans strains had O-acetyl substituents. These polysaccharides were similar in composition to those produced by Tremella mesenterica and Candida humicola except that Tremella had more xylose than mannose while Cr. neoformans strains had more O-acetyl and glucuronic acid. This further supports the relationship to lower basidiomycetes.

On the basis of congruent bands in electrophoresis and gel chromatography, I considered the protein portion of the extracellular material might be linked to the polysaccharide moiety and thus the material may contain a glycoprotein component. This "glycoprotein" contained the same amino acids as the cell wall glycoprotein although proportions were different. Percentages of serine and threonine were higher than in the wall preparations: they may also have been involved in glycopeptide linkage. Ethanolamine was present in all the preparations.

Comparison of the Gly/Ala, Tyr/Phe and Ile/Leu ratios showed striking similarities between Cr. neoformans strains 365-16 and 365-26. These were different from the other 4 strains. These two strains had large amounts of proline and phenylalanine (possibly due to deficiencies in proline oxidase and phenylalanine hydrolase) and both lacked cysteine/cystine. Their infrared spectra were much alike and they had similar glucosamine, O-acetyl and uronic acid content. 365-16 had more galactose than 365-26. On the basis of this work these two strains seem to be closely related. Amino acid composition was less affected by growth conditions than were carbohydrate constituents.

According to mouse virulence tests, Cr. neoformans 365-11 was the most pathogenic. The polysaccharide produced by this strain in vitro contained more mannose, glucuronic acid, O-acetyl and protein than the other Cr. neoformans strains. The combination of these factors and their spatial arrangement may be related to virulence, or function in the antigenicity

of the organism.

Extracellular material and cell wall composition were sufficiently different to suggest independent synthesis. The degree of capsule synthesis and the composition of the polymers were easily affected by growth conditions such as pH, temperature and nutrient supply. The yeast produced larger capsules in susceptible mice than it did either in resistant mice or *in vitro*. Perhaps both the composition and rate of synthesis of the capsule are affected by variations in the environments provided by susceptible hosts as opposed to normal hosts. This in turn could affect production of antibody to the particular antigen synthesized by the yeast and ability of complement to attach to the antibody-antigen complex or cause opsonization.

Once inside the phagocytic leukocytes death of the yeast cell may be related to resistance of different capsule structures to phagocytic enzymes. Strains with less xylose and more mannose and protein may be more resistant.

Cr. albidus H1354, the pathogenic isolate, showed few similarities to the *Cr. neoformans* strains 365-16 and 365-26. It did not grow at 37C and was very similar to the other *Cr. albidus* strain, suggesting a close relationship. However, *Cr. albidus* H1354 had the same nitrogen content as the virulent strain 365-11. If the protein portion were enzymic or antigenic this might explain the pathogenicity *in vivo* of this temperature sensitive strain.

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