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GRADUATE COLLEGE

AN IMMUNOLOGICAL AND CHEMICAL CHARACTERIZATION OF *CRYPTOCOCCUS NEOFORMANS*

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ROBERT F. DEVLIN

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AN IMMUNOLOGICAL AND CHEMICAL CHARACTERIZATION

OF CRYPTOCOCCUS NEOFORMANS

APPROVED BY

DISSERTATION COMMITTEE

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AN IMMUNOLOGICAL AND CHEMICAL CHARACTERIZATION OF *CRYPTOCOCCUS NEOFORMANS*

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

History

Cryptococcosis, the disease caused by *Cryptococcus neoformans* (*Torula histolytica*, *Cryptococcus hominis*), has been referred to as "European blastomycosis" in European literature and as "torulosis" in the older American literature. The geographically defining term "European blastomycosis" is misleading, since the disease has occurred sporadically throughout the world.

The organism was first isolated from peach juice by Sanfelice (1894). He was able to demonstrate its pathogenicity in laboratory animals and, because of its "yeast-like nature" and its "tumor-forming ability", he suggested the name *Saccharomyces neoformans*. In 1895 Busse and Buschke reported isolation of a yeast-like organism from a lesion in the tibia of a woman. Curtis (1896) described a similar organism which had been isolated from the hip of a patient with a myxomatous tumor and proposed the name *Saccharomyces subcutaneous tumefaciens*. Klein (1901), recovered a yeast which was pathogenic for guinea pigs from the sediment of a sample of milk. Weis (1902) compared

the isolates of Sanfelice and Klein and found them both to be pathogenic for mice and to be morphologically and physiologically similar. Vuillemin (1901) placed these organisms in the genus Cryptococcus.

In 1905, von Hanseman described a case of meningitis in which gelatinous "cysts" were observed. Verse (1914) reported a case of human cryptococcosis with central nervous system involvement. However, it remained for Stoddard and Cutler (1916) to delineate the clinical features of this disease, to isolate the etiologic agent by cultural methods, and to demonstrate its pathogenicity by animal inoculation, and histologic sections. They differentiated the disease from other mycotic infections of the central nervous system and referred to the organism as Torula histolytica. All of these fungi are now considered to be identical and are named Cryptococcus neoformans (Benham, 1935; Lodder and Kreger - Van Rij, 1952).

The Nature of C. neoformans

Cryptococcus neoformans can be cultivated at room temperature or at 37 C on most common laboratory media. On Sabouraud's dextrose agar at room temperature, the colonies are glistening, mucoid, and cream to tan in color. Organisms are best prepared for microscopic examination by emulsifying growth from an agar culture in a drop of India ink (Weidman and Freeman, 1924). Such preparations reveal thick-walled, ovoid to spherical, budding yeast cells, 5-15 microns in diameter which are surrounded by a large gelatinous capsule frequently large enough to give the organisms a total diameter of 20-30 microns. No endospores or mycelium are produced.

Cryptococci are nonfermenting yeasts which produce extracellular starch when grown on a defined medium containing dextrose and thiamine at a pH below 5 (Mager and Aschner, 1946; Lodder and Kreger - Van Rij, 1952). Also, all cryptococci are urease positive (Seeliger, 1956). Carbon and nitrogen assimilation tests are used to distinguish the single pathogenic species (C. neoformans) from other encapsulated, closely related, nonpathogenic species. C. neoformans does not assimilate nitrate (KNO_3) or lactose, but does assimilate glucose, maltose, sucrose, and galactose (Benham, 1955). Its ability to grow at 37 C also distinguishes this species from nonpathogenic cryptococci. C. neoformans is pathogenic for the mouse when injected intraperitoneally; all other species of cryptococci are nonpathogenic.

In summary, C. neoformans can be identified by the following criteria: (1) positive urease test; (2) growth at 37 C; (3) assimilation of KNO_3, lactose, glucose, maltose, sucrose, and galactose; (4) pathogenicity for the mouse. Benham (1935), used agglutination and precipitin-absorbtion techniques, as well as morphological characteristics, to divide the genus Cryptococcus into 4 groups, with strains of C. neoformans recovered from human disease placed in group III. Evans (1950 and 1951) subdivided C. neoformans into 3 serological types, A,B, and C, based on agglutination, precipitin, and capsular reactions. Serotyping of C. neoformans into serotypes probably depends on differences in the capsular pentosan, which confers capsule type specificity (Evans, 1951).

Fine details of the internal and external morphology of C. neoformans, as seen in ultrathin sections, were shown by Edwards, et al.

(1967). The capsule contained microfibrils (30-40 Å in diameter) that appeared to radiate from the cell wall and to coil and intertwine in various directions. These thin, uniformly structured, electron-dense filaments were believed to represent complex polysaccharide molecules. The internal morphology of C. neoformans was in many ways similar to that of other yeasts. The cell was uninucleate with a single nucleolus. The nuclear envelope, a pair of unit membranes interrupted by pores, was typical of that found in eucaryotic organisms. Smooth endoplasmic reticulum, mitochondria, vacuoles, storage granules, and ribosomes were consistent features of the cytoplasm. In addition, C. neoformans cells demonstrated organelles, derived from the plasma membrane which were comparable to bacterial mesosomes and mitochondria of an annulate type.

According to Benham (1935), Littman and Zimmerman (1956), and Littman (1958), C. neoformans is the only pathogenic fungus which produces a capsule. The gelatinous capsular material is composed, essentially, of 2 polysaccharides. Aschner, Mager, and Leibowitz (1945) and Mager and Aschner (1946) described another extracellular component, a starch which was produced in both liquid and solid media. A crystalline amylose and a pentose-containing polysaccharide were isolated by Hehre et al. (1949). A polysaccharide giving a positive Molisch test, a positive test for pentose and hexuronic acid, and a negative test for protein and amino acids were obtained in 1951 by Evans. In the same year, Evans and Mehl, using filter paper chromatography, reported a polysaccharide substance to contain xylose, mannose, galactose, and uronic acid. However, the findings of Drouhet, Segretain, and Aubert (1950) were slightly different in that they found

no galactose. They also reported that hyaluronidase would destroy the capsule, suggesting that it was composed of hyaluronic acid. Foley and Uzman (1952), using hot alkali extraction, reported the isolation of a highly polymerized polysaccharide which was not affected by hyaluronidase. Their observation that the capsular polysaccharide was not digested by hyaluronidase led to the conclusion that glucosamine or hexosaminidic linkages were not involved in the capsule structure.

Few reports exist on tne structure of the capsular polysaccharide and most of these deal primarily with the nature of the monosaccharide units. Miyazaki (1961) proposed a branched mannose backbone for the capsule of an unidentified Cryptococcus species. Rebers, et al. (1958), through the use of specific precipitation with type XIV pneumococcal antiserum, obtained evidence for side chains terminating in galactose and glucuronic acid in the capsular polysaccharide of Type A C. neoformans.

Blandamer and Danishefsky (1966) studied the composition and structure of the capsular polysaccharide of C. neoformans, Type. B. Acid hydrolysis of the polysaccharide, followed by paper chromatography, showed components corresponding to mannose, xylose, glucuronic acid, and galactose. On the basis of carbazole and cysteine-sulfuric acid reactions, the molar ratio for these components was calculated to be 6:4:2:1, respectively. They found that mannose was libereated slowly from the polysaccharide, while xylose was rapidly liberated, suggesting that the xylose was located on external side chains of a mannan backbone. Additional evidence for a mannan structure was provided by the identification of mannitol in hydrolysates of the reduced oligo-

saccharides prepared by partial acid hydrolysis of the capsular polysaccharide. These findings on the composition of the oligosaccharides and on the rate of release of the specific monosaccharides suggest that the polysaccharide consists of a mannan backbone with branches of xylose and glucuronic acid. At the present time, no information is available on the types of linkages or the sequence of monosaccharide repeating units involved in the composition of the capsular polysaccharide derived from C. neoformans.

The extracellular polysaccharides isolated from a non-pathogenic encapsulated strain of Cryptococcus laurentii have been studied by Abercrombie, et al. (1960). They isolated an acidic polysaccharide containing D-mannose, D-xylose, and D-glucuronic acid, and a neutral polysaccharide containing D-glucose only. The results of preliminary structural studies on the acidic polysaccharide suggested that it consists of a mannose-containing backbone with xylose and glucuronic acid as end groups, while the glucan contains 1-3, 1-4, 1-2, and/or 1-6 linked residues.

Jeanes, et al. (1964) demonstrated that the acidic extracellular polysaccharide of Cryptococcus contained mannose, xylose, and glucuronic acid in the molar ratio of 5:2:1. Structural studies indicated that this polysaccharide has a backbone of mannose residues with xylose and glucuronic acid end groups. As with the polysaccharide from C. neoformans, no information is available on the types of linkages or the sequence of monosaccharide repeating units of the cellular polysaccharide derived from C. laurentii.

Investigations by many workers (Northcote and Horne, 1952;

Nickerson, et al., 1961; Peat, et al., 1961; Sakaguchi, et al., 1967; Suzuki, et al., 1967) into the structure of other species of yeasts have demonstrated that the major polysaccharide components found in the cell walls are polymers of glucose (glucan) and mannose (mannan).

Yeast mannan, one of the main soluble polysaccharides of yeast cell walls, can be obtained easily by autoclaving whole yeast cells or by treating cell walls with dilute alkali or neutral buffers (Nickerson, et al., 1961; Peat, et al., 1961). Chemical and structural analyses of these mannans have shown that they are composed exclusively of mannose, however they differ in the types and sequence of the linkages involved in the backbone and side chain groupings. Through the use of methylation analysis, mannans isolated from yeasts have been found to contain alpha (1-6), alpha (1-3), and alpha (1-2) linkages and it is thought that the backbone structure consists of alpha (1-6) linkages, while the other linkages constitute the side chain groups. Mannans have been shown to be the serologically reactive polysaccharides in yeast cell walls (Summers, et al., 1964; Sakaguchi, et al., 1967; Suzuki, et al., 1967).

The cell wall constituent, glucan, is the component responsible for the shape and rigidity of the yeast cell (Northcote and Horne, 1952; Nickerson, 1963). The glucan is insoluble in water, yields only glucose on hydrolysis, and is composed mainly of beta (1-6) and beta (1-3) linkages (Tanaka, 1963). These components, mannan and glucan, have not been studied in C. neoformans.

Cryptococcosis

The disease cryptococcosis is a subacute or chronic infection caused by C. neoformans, an organism which has a marked predilection for the central nervous system. It may infect the skin, lungs, or other tissues of the body, but usually disseminates to the meninges. The cutaneous form may be primary or may appear as a manifestation of an already established systemic infection. Lesions may appear as acneform pustules, punched out granulomatous ulcers, subcutaneous tumors, or deep abscesses. Many cutaneous cases progress to generalized infections with involvement of the lungs, other visceral organs, and the central nervous system. The incidence of pulmonary cryptococcosis is secondary to the central nervous system disease caused by this organism. Primary pulmonary infections resemble neoplasms or tuberculosis. Brain infections may resemble an encephalitis, acute or chronic meningitis of bacterial orgin (especially tuberculosis), brain tumor, or brain abscess.

There is frequent association (10 to 30%) of cryptococcosis and the leukemia - lymphoma group of diseases (Hutter and Collins, 1962). Concurrent fungus infections may be due to the debilitated state of the patient, who acquires exogenous infection, or to activation of an existing latent infection. The latter hypothesis is borne out by the finding at autopsy of small subpleural nodules containing C. neoformans in patients whose death was due to a number of unrelated causes (Baker and Haugen, 1955). Therapy with steroids, urethane, mustard gas, and folic acid antagonists may activate latent infections by C. neoformans and other fungi (Zimmerman and Rappaport, 1954; Zimmerman, 1955; Keye

and Magee, 1956; Baker, 1962; Goldstein and Rambo, 1962).

The histology of brain lesions varies greatly from a minimal inflamatory reaction to pseudotubercles with giant cells, epithelioid cells, and lymphocytes. The typical lesion in other tissues, particularly in the skin, is a granuloma (Baker and Haugen, 1955).

Despite its classical association with meningitis or meningoencephalitis, there is increasing clinical evidence that cryptococcosis is primarily a pulmonary infection. Supportive evidence was provided by the demonstration that mice can be infected with airborne C. neoformans (Littman, 1959; Ritter and Larsh, 1963). It is commonly believed that the organisms are disseminated from the lungs hematogenously (Littman, 1959). However, Takos (1956) has shown penetration of the intestinal wall by cryptococci which had been either swallowed directly or after being swept from the tracheobronchial tree.

Epidemiology of C. neoformans

Sources of infection in human disease have not been precisely determined. At present, however, evidence indicates that the source is exogenous. Although C. neoformans has been isolated from the intestinal tract of man (Benham, 1935) and horses, sheep, goats, and swine (Van Uden, 1958), there is no evidence for transmission from animal to animal, from animal to man, or from man to man.

Benham (1935) reported the isolation of C. neoformans from normal skin and mucous membranes. The organism has been isolated from soil in various parts of the United States, Hawaii, and Brazil (Emmons, 1951, 1954; Ajello, 1958; Hasenclever and Emmons, 1963; Muchmore, et al.

1963). C. neoformans has been closely associated with pigeon excreta (Emmons, 1954, 1955, 1960; Kao and Schwartz, 1957; Littman and Schneirson, 1959). Since the pigeon is ubiquitous in nature it is probable that Cryptococcus species has world wide distribution.

Everson and Lamb (1964) recovered C. neoformans from 9 of 20 samples of slime flux exuded by mesquite trees (Prosopsis jaliflora) growing in the Tucson area of Arizona. The organisms have been isolated from wood (McDonough, et al. 1961) as well as soil (Ajello, 1958; Frey and Durie, 1964; Kolukanov and Vojtsekhovskii, 1965). These findings indicate that C. neoformans may exist in small numbers in soil which is free of avian droppings (Ajello, 1967). It has been postulated that cells from such areas are carried by wind currents and pigeons to areas having a high concentration of bird excrement where they find more favorable growth conditions. Hence, avian habitats, especially those of the pigeon, have become the prime source of human and animal infection.

It should also be emphasized that the relationship of birds to C. neoformans is probably indirect. Natural infections of birds have not been recorded. Attempts to produce experimental disease via the intravenous route in pigeons have failed (Kao and Schwarz, 1957; Staib, 1963), although infection has been achieved by the intracerebral and intraocular routes (Littman, et al., 1965; Sethi and Schwarz, 1966). It has been suggested that the high body temperature of birds (Kligman, et al., 1951) and rabbits (Kugh, 1939; Kuhn, 1949) is unfavorable for the multiplication of C. neoformans.

The results of epidemiological and immunological studies lend

credence to the hypothesis that pigeon habitats serve as reservoirs for human infections, and also that subclinical cryptococcosis may be more prevalent than is realized. A complement-fixation fluorescent antibody test for C. neoformans was applied to the sera of 134 pigeon breeders (Walter, et al., 1966). About 22 per cent were positive as compared to 3 per cent of a control group composed of 36 non-pigeon breeders. Positive reactions were observed only with C. neoformans Type A and B cells. The authors concluded that the pigeon breeders had been infected previously with C. neoformans Type A and B cells. Moreover, 48 out of 49 isolates of C. neoformans cultured from the pigeon habitats of 72 pigeon breeders studied belonged to serotype A.

The use of a slide agglutination test for the serotyping of 472 isolates of C. neoformans obtained from clinical and natural sources revealed 5 serologic groups (Walter, 1968). These included Evans types A, B, and C (Evans, 1950; 1951) and, in addition, a fourth serotype which did not react with type-specific antisera for either A, B, or C. The fifth group was designated Type B (variant) since strains of Type B fell into 2 distinct serologic groups. Serotype A accounted for 100 per cent (189 cultures) of the isolates from avian habitats, 95.4 per cent of nonavian saprophytic isolates, and 89.1 per cent from human infections. Vogel (1966) also demonstrated a new serotype of C. neoformans, designated Type R.

Hypersensitivity, of either the immediate or delayed type, to C. neoformans in man or experimental animals has been reported only rarely. Essentially, all reported cases of cutaneous hypersensitivity have been of the delayed type and the majority of these have been cases

of human cryptococcosis (Berghausen, 1927; Kessel and Holtzward, 1935; Dienst, 1938; Carton, 1953; Leopold, 1955). In 1961, Salvin and Smith injected a soluble cell wall antigen intradermally into guinea pigs sensitized with living cells of C. neoformans, and elicited a delayed type skin response. Bennett, Hasenclever and Baum (1965) confirmed the work of Salvin and Smith by the use of a similarly prepared antigen. They were able to demonstrate skin reaction areas of erythema and induration exceeding 5 mm diameter within 24 hrs, in 14 of 16 patients with treated, inactive cryptococcosis and in 4 of 9 patients with active cryptococcosis. They observed delayed-type reactions in 15 of 22 normal volunteers and in 8 of 17 patients with a diagnosis of other types of mycotic disease. Later, 5 positive reactors to the C. neoformans antigens were found among 107 adults and children tested at the Government Hospital in Israel.

Muchmore, et al., (1964), using an antigen prepared by Salvin, reported delayed type skin reactions in 26 out of 86 long-time residents of Kingfisher, Oklahoma. There appeared to be a correlation between isolation of C. neoformans from soil or pigeon droppings with 3 cases of active cryptococcosis (1963).

A delayed-type hypersensitivity was described by Lomanitz and Hale (1963) in both rabbits and guinea pigs sensitized with heat-killed whole cells and with cell walls of C. neoformans prepared by sonic vibration. Whole cells and cell "soma" were used as skin test antigens. Passive transfer of sensitivity to decapsulated cells and to cell wall antigens was accomplished by using a mixture of peritoneal exudate cells and antigen. An immediate-type hypersensitivity was not observed in

this study.

Perceval (1965) demonstrated delayed type hypersensitivity in mice vaccinated with viable cryptococci. Hypersensitivity reactions were elicited by injecting whole cells into the hind foot pad and examining for accelerated swelling which reached a maximum in 24 hours.

The most recent report concerning delayed hypersensitivity to C. neoformans is that of Atkinson and Bennett (1968). Skin test material (cryptococcin) prepared from a urea extract of C. neoformans elicited dermal reactions of the delayed type. The authors were able to obtain reactions greater than 5 mm in diameter in 42 per cent of patients with active cryptococcosis and in 50 per cent of patients with inactive disease. Only 1 reactor was detected in a group of 21 normal control subjects. However, among patients with blastomycosis, histoplasmosis, and coccidioidomycosis the reactor rates were 50, 22, and 25 per cent, respectively. The authors postulated that these patients may have been exposed to C. neoformans in addition to the fungus responsible for their clinical disease, however their experimental results in guinea pigs also suggested that cross-reactivity might be another possible explanation.

The recent investigations of Salvin and Smith (1961), Bennett, et al., (1965), and Atkinson and Bennett (1968) provided skin test antigens which may prove useful in epidemiological surveys. It is possible, however, that not all patients with active cryptococcosis will respond to skin test antigens. Atkinson and Bennett (1968) reported that over 50% of patients with active or inactive cryptococcosis were anergic to cryptococcin.

Immunology of C. neoformans

Despite the mounting evidence which points to the ubiquity of C. neoformans, cryptococcosis develops in only a small percentage of persons who are exposed to the fungus. The reason for this is not understood, however, it is thought that patients with clinical disease represent only a small portion of those who are probably infected. Little is understood about the immunology of cryptococcosis or of the host-parasite relationship. In active advanced cases of cryptococcosis the host fails to mobilize a completely effective, defensive cellular reaction or to combat the infection by any observable immunologic mechanism. However, the host apparently is able to resist initial invasion in some manner, except under certain unusual circumstances. Among experimental animals, it appears that the mouse is uniformly susceptible to infection with C. neoformans, whereas the rabbit is resistant. Thus, these 2 animal species have been used extensively to study natural host resistance and antibody response to the organisms.

Anticryptococcal factors in human serum which resembled beta lysin were first described by Allen (1955) and later confirmed by Howard (1961), Baum and Artis (1961), and Gadebusch (1961). Gadebusch (1961) also reported that the anticryptococcal factor was not related to complement or properdin. In 1963, Baum and Artis confirmed the finding that the human anticryptococcal serum factor was not related to complement or properdin and that it was heat stable at temperatures up to 65 C. Summers and Hasenclever (1964) demonstrated a cryptococcal growth inhibiting factor in experimentally produced mouse ascites fluid and in serum from the same mice.

Igel and Bolande (1966) presented evidence for an anti-cryptococcal factor in normal human salivary secretions and serum. Normal pooled human cerebrospinal fluid contained no such material. Using chromatographic separation of human serum they demonstrated a relationship between the inhibitor and the beta globulin fraction. In contrast, Szilagyi, et al., (1966) found a growth inhibiting factor for C. neoformans in the alpha 2 and gamma 2 serum globulins.

Gadebusch and Johnson (1966) have recently shown that a number of cationic proteins from mammalian tissues possess potent in vitro and in vivo anticryptococcal activity. Microscopic examination of C. neoformans cells treated with these agents revealed cytological alterations ranging from overt lysis of the cells (polymorphonuclear leukocyte lysosomal cationic protein) to change in plasticity (muramidase) and/or vacuolation (plakin) or granulation of the cytoplasm (histones). Gadebusch (1966) later showed that cryptococcal cells were especially sensitive to muramidase during the budding process and that such treatment resulted in cells with osmotic instability, impaired capsular synthesis, and inability of reproductive units to separate. Lysosomal cationic protein (LCP) and plakin were found to be capable of releasing nucleic acids from the cells. The same effect, but to a lesser extent, was caused by lysine-rich histone, spermine, and RNase. In addition, LCP and plakin rapidly inhibited oxygen uptake of C. neoformans.

As mentioned earlier, the cellular response to infection with C. neoformans is minimal and is characterized by giant cell formation. Drouhet and Segretain (1951) were the first to report that cryptococcal polysaccharide inhibited migration of guinea pig leukocytes in vitro.

Gadebusch (1959) reported that leukocytes from anemic mice would phagocytize small-capsule variant cells more readily than large-capsule strains. The formation of histocyte rings in response to C. neoformans infection in mice and also in tissue culture was reported by Schnearson-Porat (1965). Rings were observed only when the yeast had a large capsule and when the cells were previously exposed to immune serum. Gadebusch (1966) also reported that rabbit peritoneal and alveolar macrophages were able to phagocytize C. neoformans. He reported that peritoneal macrophages exhibited 1 per cent and 76 per cent phagocytosis for a large capsule type and a small capsule type, respectively. Similar results were seen with alveolar macrophages.

Bulmer and Sans (1967) reported that only 24 per cent of human leukocytes phagocytized an encapsulated strain of C. neoformans, whereas phagocytosis of non-encapsulated mutants ranged from 74 to 84 per cent. They also found that isolated cryptococcal polysaccharide was a specific and potent inhibitor of phagocytosis. These results with human leukocytes correlate well with those of Gadebusch (1966) who used rabbit macrophages.

C. neoformans causes a varied and minimal immunologic response in infected humans and animals as well as in immunized animals. Failure to demonstrate antibodies in serum from clinical cases has been attributed to poor antigenicity of the organism (Salvin, 1959; Seeliger, 1962), immunologic paralysis (Seeliger, 1962), neutralization of antibodies by an abundance of circulating fungus antigens in the body fluids (Salvin, 1959; Neill, et al., 1951), and also to the inadequacy of serologic tests (Walter and Jones, 1968). The presence of crypto-

coccal polysaccharide in the body fluids of patients with cryptococcosis has been demonstrated by Neill, et al., (1951), Seeliger and Christ (1958), Anderson and Beech (1958), Bloomfield, et al., (1963), and Bennett, et al., (1964). These findings would appear to support the theory that excess antigen might neutralize antibody as rapidly as it is formed.

Following a report by Rappaport, et al., (1926), cryptococcal antibodies in humans were not detected again until 1960 when Seeliger demonstrated agglutinins. Since then, with the development of improved serologic techniques, antibody to C. neoformans has been reported several times. Pollock and Ward (1962) reported cryptococcal antibodies in patients with the disease by use of a hemagglutination test. Using fluorescent antibody techniques, Vogel, et al., (1961), Marshall, et al., (1961), Vogel, (1966), and Pidcoe and Kaufman (1968) reported antibodies to C. neoformans in patients with active disease. Walter and Atchison (1966) obtained similar results using a complement-fixation fluorescent antibody test. Gordon and Vedder (1966) showed that antigen and antibodies could be detected by latex-fixation and tube agglutination tests. Walter and Jones (1968) confirmed these results with complement-fixation, slide agglutination, and latex-fixation tests.

Walter and Jones (1968) observed that antibodies were difficult to detect in patients with active or recent disease, but that circulating antigen could be detected readily. Antibodies to C. neoformans were observed only after therapy, when the patients became culturally negative and when antigen titers had subsided. It was postulated that

the presence of antibodies in man reflects a state of recovery or a low grade infection. These investigators also suggested that their findings tend to disprove the hypothesis of non-antigenicity of C. neoformans and the concept of immunologic paralysis. They favor, instead, Salvin's (1959) hypothesis that excess antigen in the body neutralizes antibody.

It would appear, therefore, that C. neoformans is highly antigenic and that cryptococcal antibody might be protective. However, attempts to produce potent antisera and vaccines have met with minimal and variable results. Also, attempts to actively or passively immunize experimental animals have met with varying degrees of success, and have lead to some controversy.

Early investigators (Hoff, 1942; Kligman, 1947; Stanley, 1949) failed to demonstrate significant amounts of humoral antibodies in rabbits. Evans (1950) reported the production of "high titered" antisera (1:320) when 3 of 9 strains of C. neoformans were used to immunize rabbits. Neill, Abrahams, and Kapros (1950) showed that high titered antisera could be obtained in rabbits if closely spaced, large doses of antigen from weakly encapsulated strains of C. neoformans were used. This observation suggested that there is an inverse relationship between the size of the capsule and the resulting antibody titer. Gadebusch (1958b) observed that variant strains of C. neoformans which possessed large capsules resisted phagocytosis by mouse polymorphonuclear leukocytes, whereas thinly encapsulated variants were readily ingested. Similar findings have been observed with rabbit peritoneal and alveolar macrophages (Gadebusch, 1966) and with human polymorphonuclear leukocytes (Bulmer and Sans, 1967).

In regard to virulence of C. neoformans and its relationship to morphological characteristics, there appears to be some discrepancy in the literature. The reports of Drouhet, Segretain, and Aubert (1950), Kao and Schwarz (1957), Gadebusch (1958b), and Littman and Schneirson (1959) disagree with regard to a correlation between thickness of the capsule and virulence of the organism for mice. Ishaq (1965) reported that, when grown in soil for 1 month, the size of the capsule decreased by 55 per cent, and that after 6 months it decreased by 77 per cent. He also reported that in mice injected intraperitoneally with organisms grown in soil for 1 month 100 per cent of the mice died. The mortality rate decreased to 40 per cent when cells grown for 6 months were used. Conversely, Vanbreuseghem (1967) reported that although the virulence of various strains may differ, virulence is not modified by maintenance of the organisms for years in the laboratory or by inoculation into animals, nor is it related to the size of the capsule.

Gadebusch (1960), employed a highly specific enzyme isolated from a soil microorganism (Alcaligenes species), to remove the capsular polysaccharide from intact cells of C. neoformans. Using these decapsulated cells as antigen, he was able to produce, in rabbits, agglutinating antibody titers of 1:640, as compared with titers of 1:160 when formalin-killed or Waring Blender-ground, encapsulated cells were used. By using a fluorescent antibody technique, he showed that the cells possessed the capsular antigen, even though no capsular material was visible with India ink. Gadebusch suggested that the antigenic material resides in a thin layer of capsular material that is closely bound to the cell wall. When these cells are subjected to repeated enzymatic

action or to mild acid treatment they lose their ability to stimulate the formation of either protective or agglutinating antibodies.

Kase and Metzger (1962) treated a highly encapsulated, mucoid strain of C. neoformans with Wescodyne G to isolate a dry, thinly encapsulated variant. They reported the production of "high titered" antisera (1:640 to 1:2560) with the dry variant, as compared to titers of 1:2 with the parent strain. Kaufmen and Blumer (1965) obtained agglutinating antibody titers of up to 1:640 in rabbits immunized with 2 weakly encapsulated strains of C. neoformans. Using agar double diffusion techniques with this system they observed 1 to 4 precipitin bands.

Attempts to develop potent vaccines from various fungi have been hampered by the inherent characteristics of the fungi themselves. Induced immunity to the fungi is strongly influenced by the morphological character of the fungi from which the vaccine is made. Another very important consideration is the dosage used. This was emphasized for C. neoformans by Abrahams and Gilleran (1960) when they observed that, with killed vaccines, dosages either above or below a certain range were suboptimal for protection. Kong and Levine (1967) postulated that this is the most probable reason for the failure of other workers (Gadebusch, 1958a; Levine, 1962) to demonstrate strong immunity to C. neoformans in mice vaccinated with killed organisms. With killed vaccines, immunity to experimental cryptococcosis generally declines over a relatively short period of time. Mice vaccinated with multiple doses of killed C. neoformans cells survive longer when challenged 7 to 14 days later than they do if challenged 21 days later

(Abrahams and Gilleran, 1960). To date, the effect of the route of vaccination has not been investigated.

Gadebusch (1958a) reported that "crude" polysaccharide prepared from capsular material of C. neoformans failed to induce the formation of protective antibodies in mice. On the other hand, a "purified" polysaccharide preparation, especially when combined with a resin, was capable of protecting mice. An attempt to immunize mice passively with rabbit anticryptococcal sera failed (Gadebusch, 1958b). In 1963, Gadebusch reported similar findings, emphasizing that protection could be greatly enhanced if the "pure" polysaccharide material was coupled to an ion exchange resin prior to injection. In the same year, Louria (1963) noted an increased survival rate in mice immunized with live C. neoformans cells and challenged 1 to 4 months after immunization. Pretreatment of mice with bacterial endotoxin prior to immunization with live C. neoformans cells also resulted in protection against cryptococcal challenge. Endotoxin-protected animals showed no ability to limit the infection until circulating antibody could be detected. Also, control of multiplication of cryptococcal cells could be correlated with the presence of antibody, but could not be correlated with an enhanced cellular response. Louria postulated that "although protection against cryptococcal challenge appears to be antibody dependent, it is unclear whether protective antibody is to be found in plasma or if it is tissue bound."

Cozad, et al., (1963) reported that capsular polysaccharide from C. neoformans did not stimulate antibody production in rabbits, but that it apparently reacted with circulating antibody in the serum

of animals hyperimmunized with whole cells. In contrast, Gadebusch et al. (1964) reported that capsular polysaccharide per se was "highly antigenic" in mice, rabbits, and rats and resulted in hemagglutinating antibody titers of 1:10, 1:20, and 1:40 respectively. In these studies, a single injection of 600 ug polysaccharide was used in mice and rats, and a total of 36 mg, given in 2 injections 21 days apart, was used in rabbits. Gadebusch stated that the failure of Cozad, et al., (1963) to demonstrate antibody to polysaccharide was probably due to the presence of an excess of circulating polysaccharide which combined with antibody as soon as it was formed.

Gordon and Lapa (1964) reported that immune globulin afforded no protection against experimental cryptococcosis in mice. However, combined globulin and amphotericin B therapy resulted in either prolonged survival time of the animals or reduction in the amount of amphotericin b needed to provide a given level of protection. Following therapy of human cryptococcosis with rabbit antiserum, Gordon and Vedder (1966) detected both rabbit and human antibody in patient's sera. This was taken to indicate that human antibody, not previously detected due to the presence of excess antigen, was now detectable by virtue of the neutralization of antigen by rabbit antibody. In contrast to the hypothesis of Louria (1963), these authors proposed that, while circulating protective antibody is not measured by agglutination tests, serum titers of agglutinating antibody may parallel those of the former and thereby, may serve as an index of host resistance. Goren (1966, 1967) reported that, when conjugated with bovine gamma globulin, purified polysaccharide from C. neoformans is highly antigenic in mice and may

induce the production of agglutinating antibody titers of the order of 1:2000. However, despite this high level of humoral antibody, animals challenged with 20-50 doses of homologous organisms exhibited no increase in survival time over controls. Contrary to the findings of Gadebusch (1958a, 1963, 1964), Goren found that his polysaccharide was non-antigenic for the mouse by itself or when conjugated to mouse gamma globulin. In 1968, Goren and Warren reported that a pseudo-immune reaction can occur at the surface of cryptococcal cells with complement from fresh mouse, rabbit, guinea pig, and human sera.

Goren (1967), studied the progressive changes in the fungal population of the tissues of infected, nonimmune mice, and obtained evidence for a "dual natural immune response", i.e. production of non-protective, humoral, anti-polysaccharide antibody and stimulation of an, at least partially, effective defense mechanism that remains uncharacterized, but which may reside at the cellular level. He suggested that "the 'immunologic paralysis' postulated as a consequence of cryptococcal infection is probably relevant only to polysaccharide antigen (soluble or capsular) and as such has but little influence on active immunity."

The relationship of polysaccharide antigens to immunity in cryptococcosis still remains to be completely elucidated. In general, only minimal antibody responses occur in animals immunized with antigens (whether particulate or soluble) containing a relatively high content of capsular polysaccharide. There are several possible explanations for this phenomenon: first, the capsule has been shown to inhibit phagocytosis of cryptococcal cells; second, the capsular polysaccharide

may combine with antibody *in vivo* as rapidly as it is formed; third, the capsule may have a toxic effect on the reticuloendothelial system; finally, the host may be in a specific state of immune tolerance.

In general, the immunity induced with other cryptococcal fractions is relatively weak and virtually no characterization of the antigenic components has been accomplished. Isolated cryptococcal cell walls are, as yet, unstudied immunologically and the nature and potency of this antigen in *C. neoformans* is relatively unknown.

The objectives of the present research were to: (1) isolate cell fractions, both particulate and soluble from *C. neoformans*, (2) characterize these fractions as to immunogenicity and antigenic components, (3) study the biological effects of certain of the fractions, and (4) determine the chemical nature of these fractions.

CHAPTER II

MATERIALS AND METHODS

Animals

Rabbits

Male albino, New Zealand rabbits (Oryctolagus cuniculus), weighing approximately 7-8 pounds, were used for the preparation of antisera. The animals were obtained from local commercial sources and were fed a diet of commercial pellets. All animals were caged individually and maintained at least 2 weeks in the animal quarters before use in order to acclimate them to the laboratory environment.

Guinea Pigs

Male albino, guinea pigs (Cavia porcellus), weighing approximately 250-300 grams, were used in the passive cutaneous anaphylaxis tests. The animals were obtained from local commercial sources and maintained on a diet of commercial pellets which was supplemented twice weekly with fresh cabbage leaves. These animals were housed 5 per cage and held at least 1 week in the animal quarters before use.

Organism, Maintenance and Cultural Techniques

Organism

A virulent, highly encapsulated human isolate of C. neoformans Type A[1] (C1A) was used throughout these studies. The organisms were maintained by weekly transfer on Sabouraud's Dextrose Agar[2] and incubation at room temperature.

Culture Techniques

The encapsulated C. neoformans cells used for preparation of antigens were grown for 4 days at 25 C on Sabouraud's Dextrose Agar plates containing 2.0 per cent agar[2]. The use of 2.0 per cent agar resulted in a firm agar surface which facilitated removal of the organisms from the culture surface.

The same strain (C1A) was decapsulated by growth on a synthetic, low pH (4.5), culture medium containing 1.0 per cent dextrose and 2.0 per cent agar (Bulmer and Sans, 1968). A description of the synthetic culture medium is presented in Table 1.

Preparation of Antigens

Formalinized Whole Cell Antigens

Cryptococcal cells, either encapsulated or non-encapsulated, were removed from the culture medium with a rubber spatula under

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[1]Courtesy of Dr. Glenn S. Bulmer, University of Oklahoma Medical Center, Oklahoma City, Oklahoma.

[2]Difco Laboratories, Detroit, Michigan.

TABLE 1

COMPOSITION OF MODIFIED SYNTHETIC CULTURE MEDIUM
USED TO CULTURE CRYPTOCOCCUS NEOFORMANS
IN THE NON-ENCAPSULATED STATE

KH_2PO_4	2.0	gm
$(NH_4)_2SO_4$	2.0	gm
$MgCl_2 \cdot 6H_2O$	0.2	gm
$CaCl_2 \cdot 2H_2O$	0.02	gm
$FeCl_2 \cdot 4H_2O$	0.04	gm
$MnCl_2 \cdot 4H_2O$	0.0015	gm
$NaNO_3$	0.0015	gm
Sucrose	10.0	gm
Thiamine	0.001	gm
Agar	20.00	gm
Distilled Water	1.0	liter

Adjust final pH to 4.5 before autoclaving

aseptic conditions and treated with 0.6 per cent formalin. The cell suspension was stirred continously for 48 hours using a magnetic stirrer[3]. It was then washed three times with sterile 0.85 per cent NaCl and tested for sterility using Thioglycollate Medium[2] with indicator and Sabouraud's Liquid Medium[2]. The formalinized cells were taken up in 0.85 per cent NaCl containing 0.3 per cent formalin and the cell suspension was adjusted turbidimetrically to equal a No. 10 McFarland nephelometer standard (approximately 11 x 10^6 cells/ml by hemocytometer count). The whole cell antigen suspensions were then stored at -20 C until used for immunization.

Sonicated Antigens

A 50 per cent suspension of washed C. neoformans cells in 0.85 per cent NaCl and a volume of glass beads[4] (size 110) equal to the volume of packed whole cells were placed in a Raytheon Sonic Oscillator[5] (Model DF 101) for 45 minutes. This treatment resulted in approximately 60 to 80 per cent breakage of the cells as determined by direct microscopic examination. The sonically treated material was centrifuged in an International refrigerated centrifuge[6] (Model HR-1) for 10 minutes at 1,100 x g to remove cellular debris as well as intact cells and glass beads. The supernatant fluid constituted the soluble sonicated

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[3]Fisher Scientific Company, Fairlawn, New Jersey.

[4]Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.

[5]Raytheon Manufacturing Company, Waltham, Massachusetts.

[6]International Equipment Company, Boston, Massachusetts.

antigen (SON).

For the purpose of isolating cell walls and cytoplasmic material, a 50 per cent whole cell suspension was treated as described above, however, the soluble sonicated antigen was centrifuged at 10,000 x g for 30 minutes in a Sorvall superspeed refrigerated centrifuge (Model RC2-B)[7]. The sediment (crude cell wall material) was then washed 10 times with 0.85 per cent NaCl; this material constituted the cell wall material (C.W.).

The supernatant material from the first 10,000 x g centrifugation was used as crude cytoplasmic material (CYT). The antigens used for production of antisera were adjusted as to protein and carbohydrate content by the Lowry (1951) and anthrone (1950) methods, respectively. The antigens were stored at -20 C until used for immunization.

The preparation of all antigens was carried out aseptically. The sterility tests used were the same as those described under the preparation of formalinized whole cell antigens. A schematic representation of the sonication procedures is shown in Figure 1.

Preparation of Crude Capsular Polysaccharide

Crude capsular polysaccharide material was obtained by subjecting a 50 per cent suspension of highly encapsulated cryptococcal cells to the Raytheon Sonic Oscillator[5] treatment for 20 min. The material was then centrifuged for 30 min at 10,000 x g in a Sorvall

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[7] Ivan Sorvall, Inc., Norwalk, Connecticut.

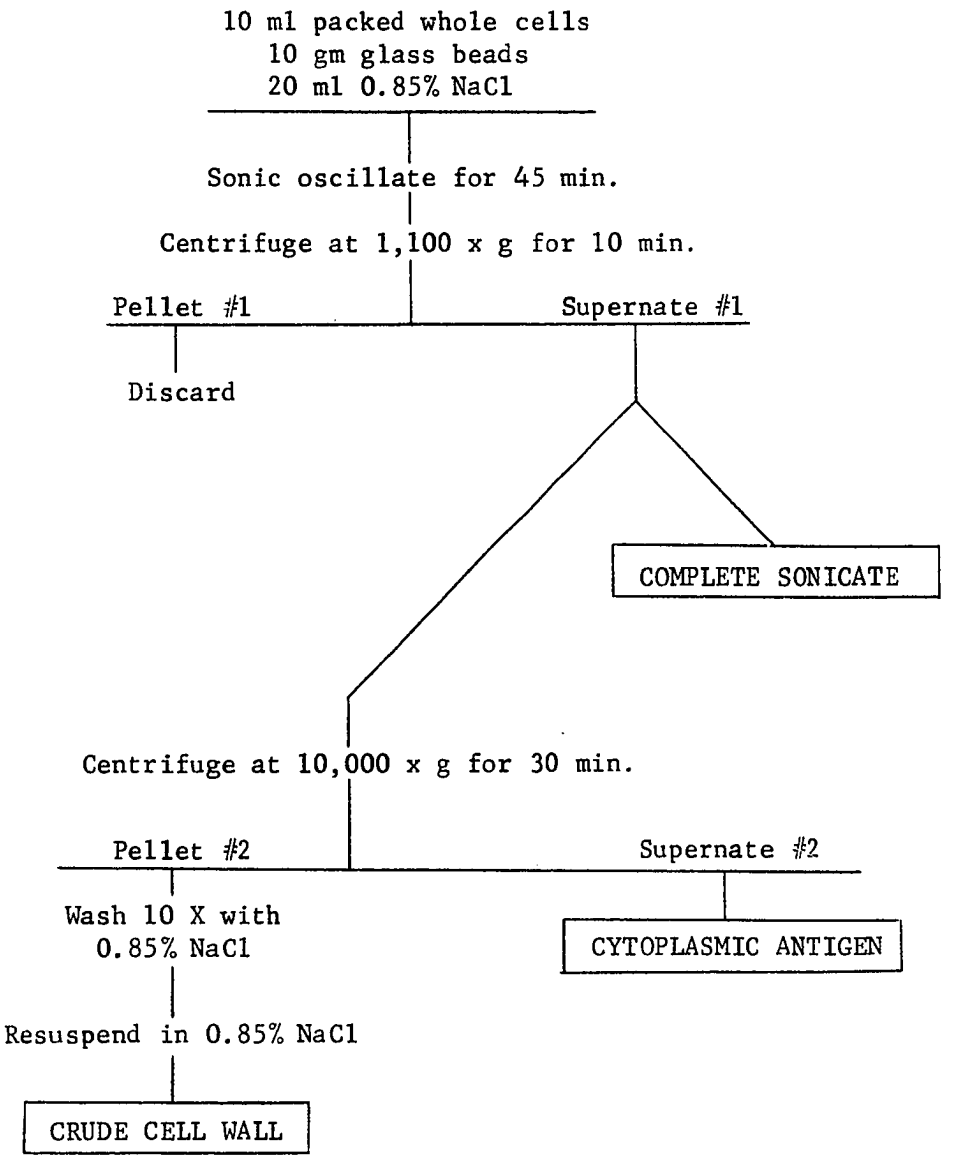

Figure 1. Preparation of Antigens from Cryptococcus neoformans by Sonication.

refrigerated centrifuge (Model RC2-B)[7]. The supernate was mixed with 5 volumes of cold (4 C) 95 per cent ethyl alcohol and allowed to stand overnight at 4 C. The precipitate was sedimented at 3,300 x g for 10 min, redissolved in distilled water, and reprecipitated 2 more times with 5 volumes of 95 per cent ethanol. The crude capsular polysaccharide (CAP) was then dried in a vacuum desiccator over $CaCl_2$ and stored at 4 C until ready for use (Figure 2).

Phenol Extraction Method

The phenol extraction procedure is shown in outline form in Figure 3. Forty grams (wet weight) of washed cryptococcal cells were suspended in distilled water to make a final volume of 200 ml. An equal volume of 90 per cent phenol was added to the cell suspension, making a final concentration of 45 per cent phenol. The extraction procedure was performed at 25 C for 30 min with continous mixing by a magnetic stirrer[3]. The mixture was then centrifuged at 10,000 x g for 30 min in a Sorvall superspeed refrigerated centrifuge (Model RC2-B)[7]. The residue was resuspended in 200 ml of distilled water and extracted with phenol exactly as described for the original cell suspension. The phenol phases were separated from the water phases by centrifugation for 30 min at 5,000 x g and the equivalent phases were combined.

The pooled phenol phase was extracted with 100 ml volumes of anhydrous ethyl ether until the extract was negative to a 10 per cent ferric chloride test. The pooled water phase was washed twice with 200 ml of 90 per cent phenol. The pooled phenol and water phases were both

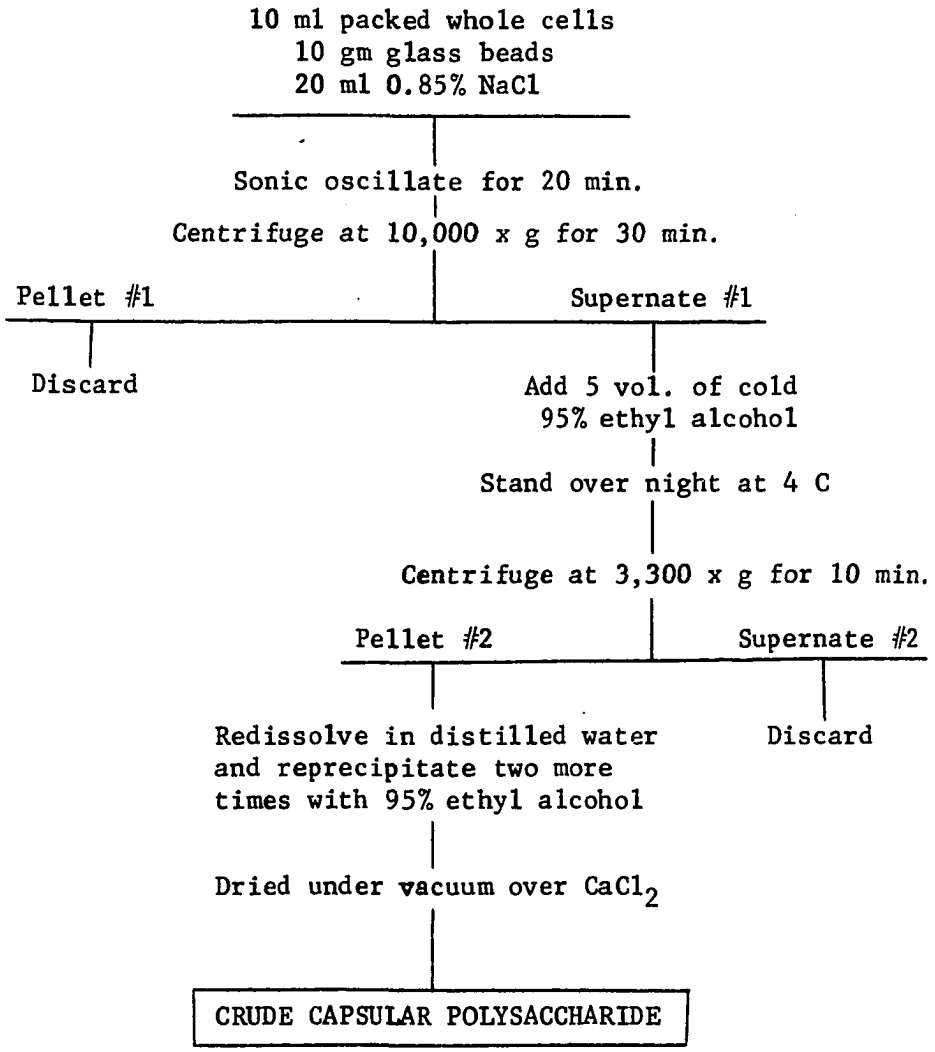

Figure 2. Preparation of Crude Capsular Polysaccharide from Cryptococcus neoformans.

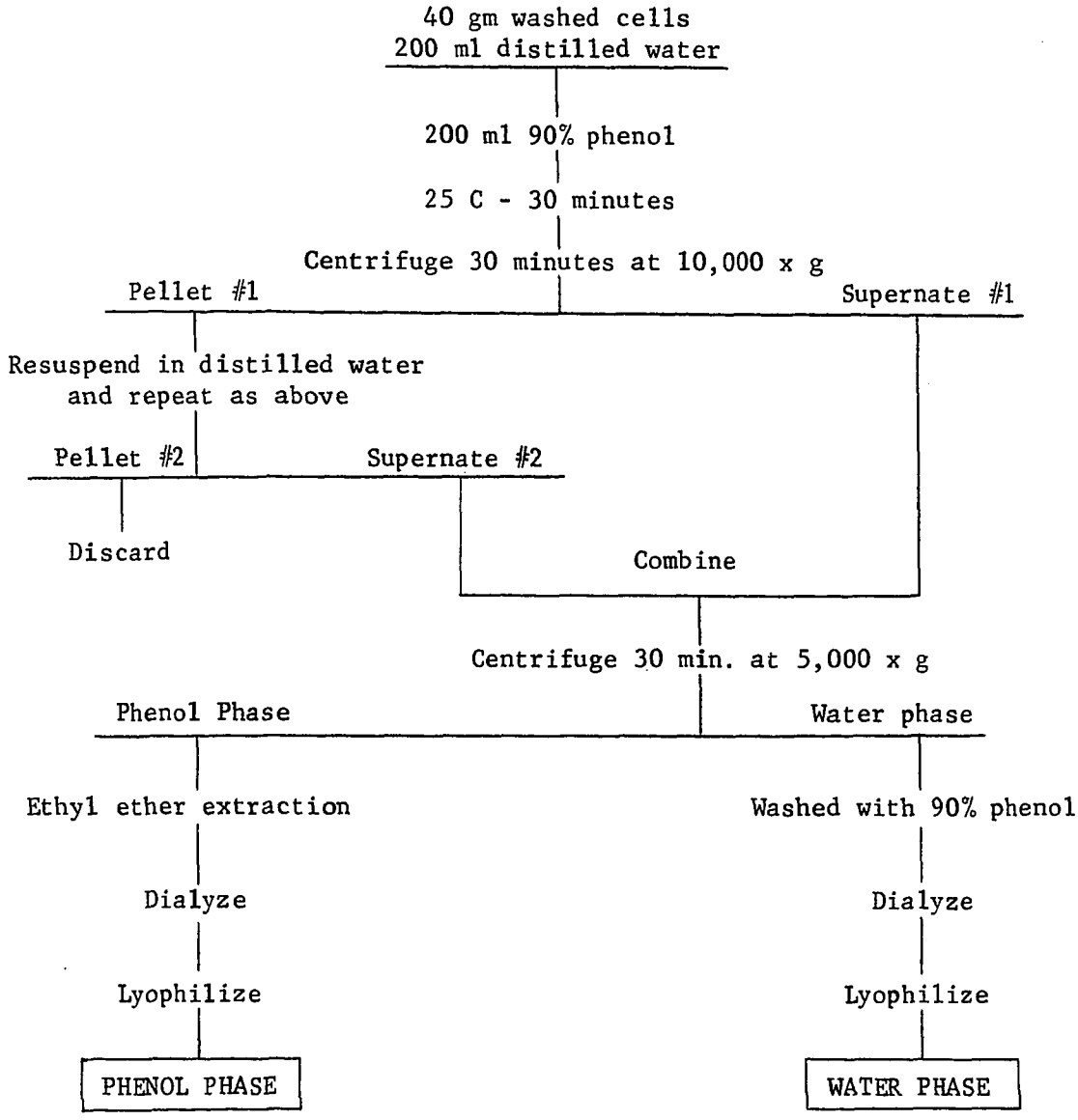

Figure 3. Extraction of Cryptococcus neoformans with 45 Percent Phenol.

dialyzed extensively for 5 days against running tap water, lyophilized[8] and stored at 4 C. These fractions will be referred to as phenol phase (P.P.) and water phase (W.P.).

Mannan Extraction Method

The mannan extraction procedure (Peat, 1961) is shown in outline form in Figure 4. The 16.35 gram sample of acetone-dried cryptococcal cells or zeolite ghosts were stirred into 400 ml of 19 mM citrate buffer (pH 7.2) until a homogenous suspension was obtained. The mixture was autoclaved at 140 C for 2 hr and then centrifuged at 40,000 x g in a Sorvall superspeed refrigerated centrifuge (Model RC2-B)[7] for 30 min. Next, the sediment was resuspended in 400 ml of citrate buffer and treated exactly as described for the original cell suspension. The supernatant fluids (mannan extracts) were combined, adjusted to 1N with acetic acid, centrifuged at 10,000 x g for 20 min to remove a brown gelatinous precipitate, and then neutralized with 6 N NaOH. The polysaccharide was precipitated from the neutralized solution by addition of absolute ethyl alcohol to a final concentration of 60 per cent. The precipitate was washed 3 times with 100 ml volumes of 60 per cent ethanol, dissolved in 500 ml distilled water, and made alkaline with 1 N NaOH. Fehling's solution, made by mixing equal volumes of a $CuSO_4$ solution (34·65 grams/500 ml) and a sodium potassium tartrate solution (125.0 grams KOH and 173.0 grams sodium tartrate/500 ml), was then added until the supernate above the precip-

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[8] Virtis Unitrap, Virtis Research Equipment, Gardiner, New York.

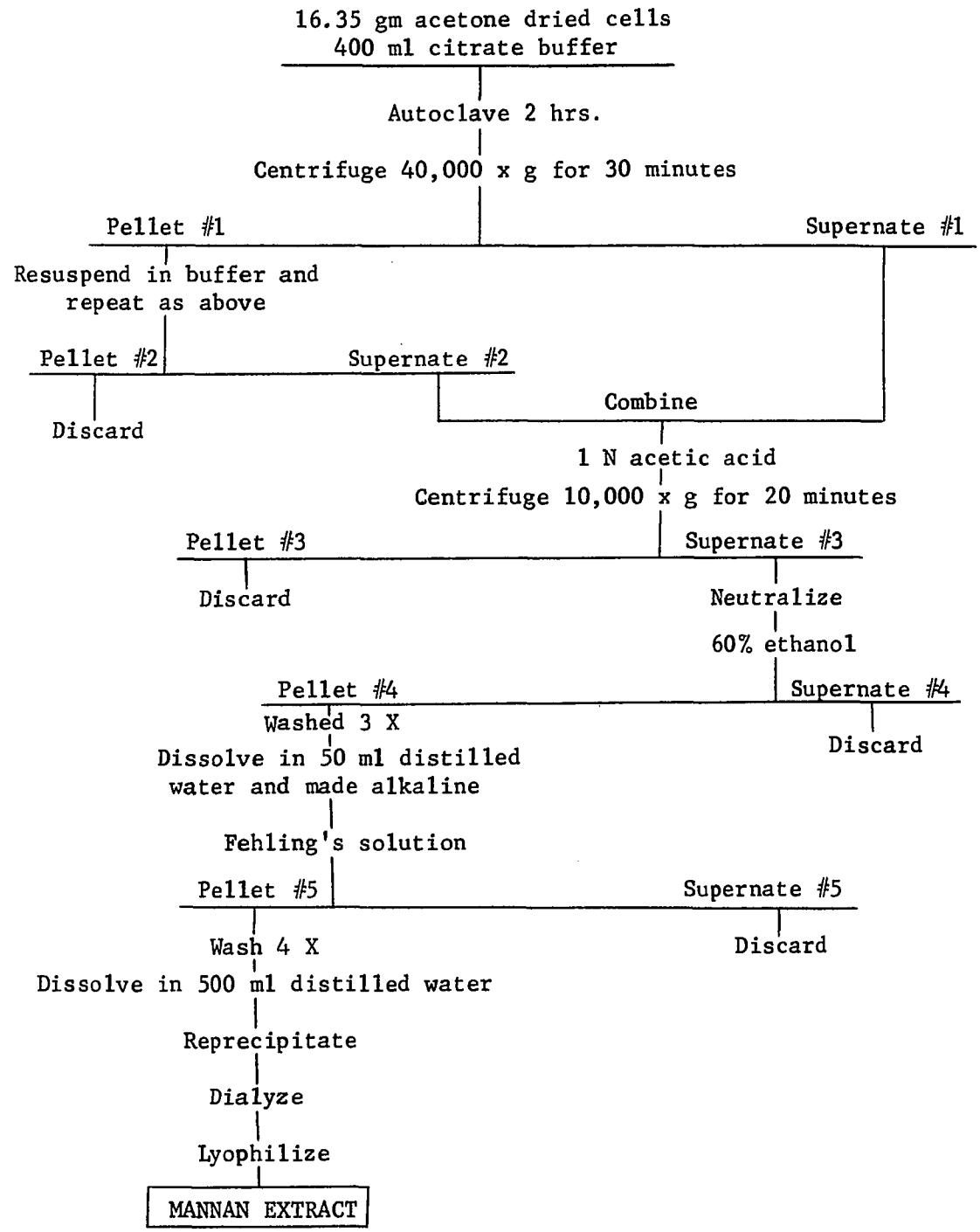

Figure 4. Extraction of Cryptococcus neoformans by the Mannan Extraction Method of Peat.

itated, grey-blue polysaccharide-copper complex was deep blue (approximately 50 ml) and no further precipitin occurred. The polysaccharide-copper complex was collected by centrifugation at 5,000 x g for 10 min and washed 4 times with warm (40 C) distilled water. The complex was dissolved in 500 ml distilled water by adding concentrated HCl slowly with continous stirring until the resultant solution was slightly acid (pH 6.5). The polysaccharide was precipitated by 60 per cent ethanol, washed 3 times with 60 per cent ethanol, dialyzed against distilled water for 72 hr at 4 C, lyophilized[8], and stored at 4 C. By this method 2.56 grams (dry weight) of mannan (MAN) was recovered, with a percentage yield of 15.67.

Braun Homogenate

The Braun cell homogenate was prepared in a Braun cell homogenizer (Model MSK 2876)[9] by superspeed oscillation, for 5 min, of a vial containing 5 gm (wet weight) of cryptococcal cells in 20 ml barbital buffer, pH 8.4, I.S. 0.15, and 50 grams of glass beads (size 0.45-0.50 mm, kat. nr. 54170)[9]. Cooling was accomplished with liquid carbon dioxide. The cell homogenate was then centrifuged in a Sorvall superspeed refrigerated centrifuge (Model RC2-B)[7] for 30 min at 10,000 x g. The supernate was decanted and stored at -20 C until ready for use. This material constituted the Braun cell homogenate (B.H.).

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[9]Bronwill Scientific, Rochester, New York.

Zeolite Extraction Method

Zipper and Person (1966) described a method for the rapid disruption of intact cells of Candida albicans and Saccharomyces cerevisiae using synthetic zeolite, a hydrated alkali-aluminum silicate ($Na_2O \cdot Al_2O_3 \cdot (SiO_2)_x \cdot H_2O_x$) capable of exchanging sodium for calcium and magnesium. They obtained 90 per cent or greater breakage of yeast cells, with a recovery of 20 to 25 per cent of the total solubilized cell protein (biuret).

A schematic representation of the modified zeolite procedure used in the present studies for the isolation of soluble and "cell ghost" fractions from C. neoformans is shown in Figure 5. To 5 gm (wet weight) of freshly harvested cells (in a chilled mortar set in crushed ice) was added a total of 10 gm of dry zeolite[10]. The zeolite was added in small portions and incorporated by hand-pestle grinding which was continued for an additional 10 min. The mixture was then added, with rapid stirring, to 30 ml of ice cold distilled water and allowed to stand for 5 min. This treatment resulted in a supernatant suspension, presumably of broken cells and a pellet of zeolite particles. On microscopic examination, the supernatant suspension was found to contain a mixture of small zeolite particles and empty yeast cells which had the appearance of red cell ghosts.

The technique for separating the cell ghosts from zeolite particles consisted of suspending the mixture in 1.0 M sucrose, shaking

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[10] Courtesy of Culligan Water Conditioning Company, Oklahoma City, Oklahoma.

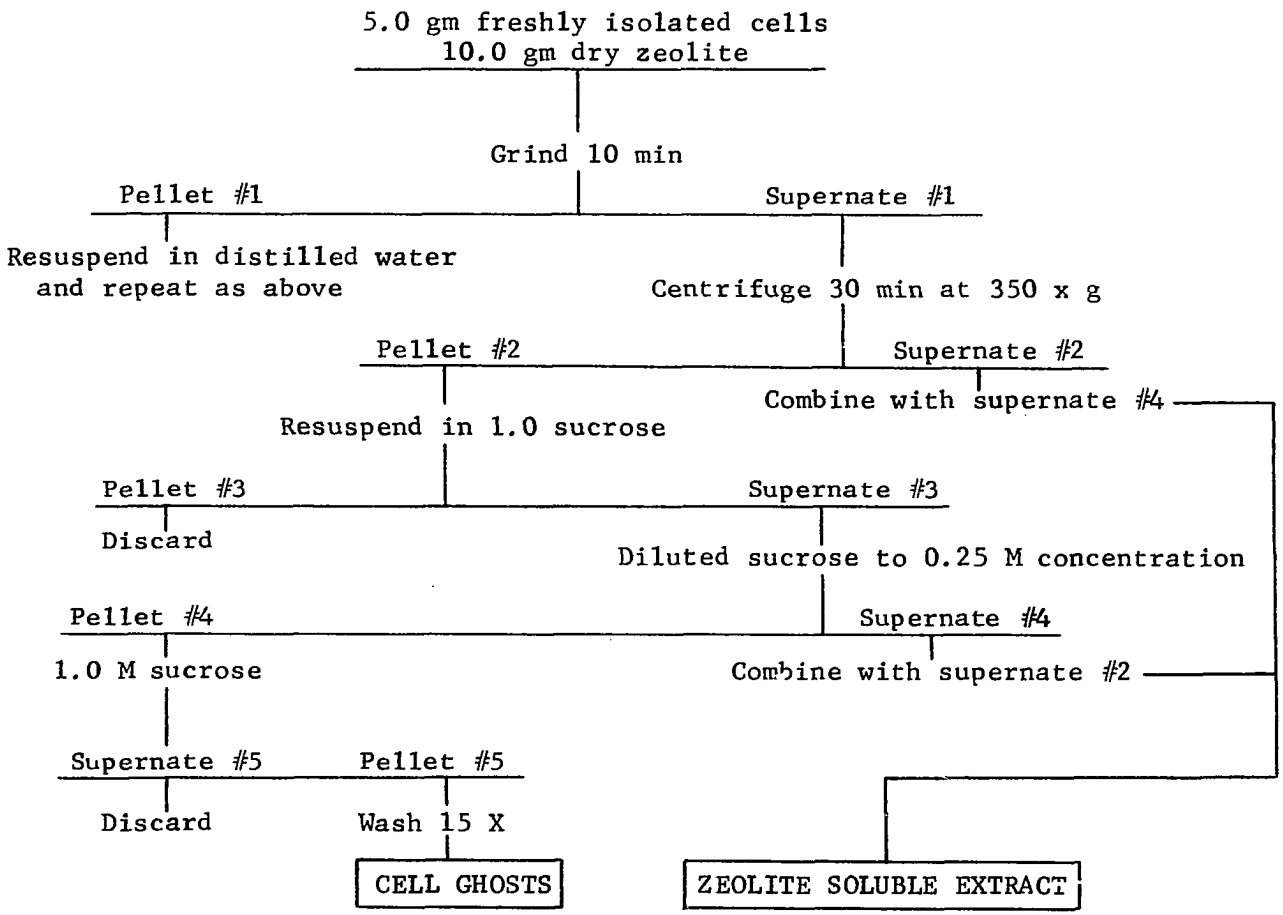

Figure 5. Extraction of Cryptococcus neoformans by the use of synthetic zeolite.

the suspension with an Eberbach shaker[11] for 15 min at 4 C, and centrifuging it at 150 x g for 15 min in an International refrigerated centrifuge (Model PR-1)[6]. Supernate number 3 was diluted with distilled water to a final sucrose concentration of 0.25 M, shaken for 15 min, and centrifuged[6] for 25 min at 150 x g. Supernates number 2 and 4 were combined and dialyzed for 72 hr at 4 C against 3 changes of tap water. The dialysate was then concentrated against Carbowax 4000[12] diluted to 40 X concentration with barbital buffer, pH 8.4, I.S. 0.15, divided into 5.0 ml portions, and stored at -20 C. This constituted the zeolite soluble extract (ZS).

The isolated cell ghosts in pellet number 5 were washed 15 times, alternating between 1 liter volumes of distilled water and 0.25 M sucrose. They were then washed 3 times with 1 liter volumes of distilled water and stored at -20 C. This material constituted the zeolite "cell ghost" fraction (ZG).

Culture Filtrate

A culture filtrate was prepared by growing C. neoformans (C1A) in a basic salts medium (BSM), pH 7.0, described in Table 2. The basic salts medium was made up in 500 ml volumes in 1 liter erlenmeyer flasks which were inoculated with 5.0 ml of a 48 hr BSM stock culture. The cultures were incubated at 25 C for 10 weeks with daily agitation.

The filtrates were harvested by centrifuging the cultures in

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[11]Eberbach Corporation, Ann Arbor, Michigan.

[12]Union Carbide Chemicals Company, 270 Park Ave. N.Y., N.Y.

TABLE 2

COMPOSITION OF THE BASIC SALTS LIQUID MEDIUM USED FOR THE PREPARATION OF CULTURE FILTRATES

KH_2PO_4	0.8	gm
K_2HPO_4	2.2	gm
$(NH_4)_2SO_4$	2.0	gm
$MgSO_4 \cdot 7H_2O$	0.125	gm
NaCl	0.01	gm
$FeSO_4 \cdot 7H_2O$	0.01	gm
$MnCl \cdot 4H_2O$	0.025	gm
Thiamine	0.003	gm
Glutamic Acid	1.0	gm
Glucose	10.0	gm
Distilled Water	1.0	liter

Adjust final pH to 7.0 before autoclaving

a Sorvall refrigerated centrifuge (RC2-B)[7] at 10,000 x g for 30 min. The supernates were then pooled, filtered through an ultra-fine sintered glass filter[13], and dialyzed against 4 changes of distilled water for 4 days at 4 C. The filtrate was concentrated 10 X by dialysis against dry polyvinylpyrrolidone[14] (PVP) at 4 C and then stored at -20 C in 10 ml portions. This material constituted the cryptococcal culture filtrate (C.F.).

Preparation of Typhoid "H" Antigen

Salmonella typhosa, strain number 2-763, obtained from the stock culture collection, Department of Microbiology, University of Oklahoma Medical Center, Oklahoma City, was used for the preparation of typhoid "H" antigen.

A 1 liter flask containing 500 ml of Difco tryptic soy broth[2] was inoculated with 1.0 ml of a culture of actively motile organisms and incubated at 37 C for 18 hr. An equal volume of 0.6 per cent formalin containing 0.85 per cent NaCl was then added. After incubation at 25 C for 48 hr the bacteria were removed by centrifugation in an International refrigerated centrifuge (Model PR-1)[6] for 30 min at 1400 x g. The bacterial cells were washed 3 times with 100 ml volumes of 0.3 per cent formalin containing 0.85 per cent NaCl, resuspended in the formalinized saline, adjusted to match a number 4 McFarland nephelometer standard, and stored at -20 C until used.

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[13]Corning Glass Works, Corning, New York.

[14]General Aniline and Film Corporation, Calvert City, Kentucky.

Bovine Serum Albumin Antigen

In certain of the experiments, a 1.0 per cent bovine serum albumin[15] (BSA) solution in 0.85 per cent NaCl was used for immunization.

Immunization Procedures

Immunization by the Intravenous Route

The particulate antigens used for the production of antisera in rabbits consisted of formalinized whole cells with and without capsule, cell walls, and the "zeolite ghosts". The suspensions were adjusted tubidimetrically to equal a number 10 McFarland standard. The soluble antigens, sonicate with and without capsule and cytoplasmic material, were adjusted to contain approximately 1.16 to 1.26 mg protein and 0.6 to 1.0 mg carbohydrate. Another series of rabbits was immunized with the whole nonencapsulated cells suspended in a solution of 360 ug of crude capsular polysaccharide per ml and with the same concentration (360 ug/ml) of crude capsular polysaccharide antigen alone.

Each rabbit received 3 ml of the appropriate antigen intravenously per day for 5 days, followed by a 10 day rest period. This constituted 1 series of injections. Each rabbit received 5 such series. Trial bleedings were made by cardiac puncture 5 days after the last injection of antigen during each series and at the end of the final series.

In order to evaluate the rise and decline of the antibody titer the immunization and bleeding schedules were modified as follows:

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[15]Mann Research Laboratories, Inc., New York, New York.

rabbits were immunized by 1 series of injections and then blood samples were drawn every other day for 15 days. Each rabbit received 2 such series.

To study the effect of cryptococcal capsular polysaccharide on the immune response to typhoid "H" vaccine and to bovine serum albumin (BSA) the following immunization schedule was used. Control rabbits received 0.5 ml typhoid "H" antigen intravenously on day 1 of the immunization schedule, 1.0 ml on day 4, 2.0 ml on day 8, and 3.0 ml on day 12. In the experimental rabbits, 6.25 mg capsular polysaccharide was administered with each "H" antigen injection, for a total of 25 mg. Another group of rabbits received a single injection of 5.0 mg capsular polysaccharide on day 1 of the immunization schedule. They then received the "H" antigen as described above. All bleedings were made by cardiac puncture, 6 days following the last injection of antigen.

Control rabbits receiving BSA were given 2.0 of a 1.0 per cent BSA solutior in 0.85 per cent NaCl intravenously every other day for a total of 9 injections. In the experimental group, each rabbit received 2.78 mg capsular polysaccharide with each injection of BSA for a total of 25 mg capsular polysaccharide. All trial bleedings were made 6 days following the last injection of antigen.

Immunization by the Subcutaneous and Toe Pad Routes

Rabbits were injected, by the subcutaneous route, with formalinized whole cells suspended in 1.0 ml 0.85 per cent NaCl and 1.0 ml Freund's complete adjuvant[2]. The mixture was homogenized in a Sorvall Omni-Mixer[7] for 3 minutes. Each rabbit received 1.0 ml of the whole

cell-adjuvant mixture (approximately 1.1 x 10^8 cells), given as 4 subcutaneous injections in the flank. Trial bleedings were made by cardiac puncture after 2 weeks and were followed by a booster injection of 1.1 x 10^8 cells as described above. Post-booster trial bleedings were made after 2, 4, and 6 weeks.

A second group of rabbits received the same series of injections, except that the Freund's complete adjuvant was omitted. The trial bleeding schedule was the same as described above.

Cell wall antigen in Freund's complete adjuvant was prepared in the following manner. To 2.0 ml of 0.85 per cent NaCl were added 8.0 mg of cell wall and 2.0 ml of Freund's complete adjuvant. The mixture was homogenized in a Sorvall Omni-Mixer[7] for 3 min. Initially, each rabbit received a total of 2.0 mg of cell wall given as 2 subcutaneous injections of 0.4 ml each in the flank and 2 injections of 0.1 ml each into the toe pads. Trial bleedings were made by cardiac puncture after 2 weeks. A booster injection of 2.0 mg cell wall was then given as described above. Post-booster trial bleedings were made after 2, 4, and 6 weeks.

Crude capsular polysaccharide antigen in Freund's complete adjuvant was prepared in the following manner. To 0.5 ml of a stock polysaccharide solution (1 mg/ml) were added 0.5 ml 0.85 per cent NaCl and 1.0 ml Freund's complete adjuvant. The mixture was homogenized in a Sorvall Omni-Mixer for 3 minutes. Each rabbit received 2 toe pad injections and 2 subcutaneous injections in the flank consisting of 0.1 ml each. By this method, each rabbit received, on initial injection, a total of 100 ug capsular polysaccharide. Trial bleedings and booster

injections were carried out according to the schedule described above for the cell wall antigen.

In another antigen-adjuvant study, rabbits received, by toe pad injection, one of the following quantities of crude capsular polysaccharide given as a single dose: 0.36, 3.6, 36, or 360 ug. The antigen-adjuvant (Freund's complete) mixture was made by appropriate dilution of a stock polysaccharide solution (3.6 mg/ml) and was homogenized in a Sorvall Omni-Mixer for 3 min. Each rabbit received 2 injections of 0.1 ml each into each of 2 toe pads. Trial bleedings were made by cardiac puncture at weekly intervals for 4 weeks and then a final bleeding was made after 8 weeks.

Immunization by the Intraperitoneal Route

Each rabbit was injected intraperitoneally with 7×10^8 formalinized whole cells suspended in 5.0 ml 0.85 per cent NaCl. Trial bleedings were made at weekly intervals for 2 weeks. This constituted 1 series; each rabbit received 3 such series.

Collection and Storage of Serum Samples

All blood samples were obtained by cardiac puncture according according to the method described by Campbell, et al., (1964). The blood samples were allowed to clot at 25 C for 2 hr and they were then stored overnight at 4 C for complete clot retraction. Serum samples were separated from the clots by centrifugation at 4 C and they were stored at -20 C until ready for use.

Antibody Adsorption Procedures

Absorbtion of Antisera with Whole Cryptococcal Cells

Antisera to cryptococcal whole cells and cell fractions were analyzed by adsorbtion techniques. Formalinized whole cells to be used for adsorbtion were first suspended in 0.85 per cent NaCl and incubated at 37 C for 2 hr. The suspension was then incubated at 4 C for 18 hr, centrifuged at 10,000 x g for 30 minutes in a Sorvall[7] superspeed refrigerated centrifuge (Model RC2-B), and the supernate was decanted. The cells were then washed 3 times with 0.85 per cent NaCl. Antiserum was added to the packed cells to make a 20 per cent cell suspension. The antiserum-whole cell mixture was incubated for 2 hr at 37 C and then for 18 hr at 4 C. The agglutinated cells were removed from the mixture by centrifugation, as described above. This procedure was repeated 3 times to assure complete removal of agglutinins. After adsorbtion, each antiserum was assayed for agglutinating activity by the tube agglutination technique.

Adsorbtion of Antisera with Mannan

The optimal proportion of antibody-antigen for each antiserum was determined by the block titration method (Campbell, et al., 1964). Each antiserum was then absorbed with its appropriate antigen concentration. The antibody-antigen mixture was incubated for 2 hr at 37 C and then for 18 hr at 4 C. The precipitate was removed by centrifugation at 10,000 x g for 30 min at 4 C. This procedure was repeated until no more precipitate was formed and the supernatant fluid was negative for antibody by the ring precipitin test.

Serological Tests

Tube Agglutination Test

Agglutinating antibody titers were obtained by using a standard agglutination procedure. Each test was performed by placing 0.5 ml of 0.85 per cent NaCl into each of 10 serological test tubes and then adding 0.5 ml of antiserum to the first tube. The 2 fold serial dilutions of antiserum were made beginning with transfer of 0.5 ml of the NaCl-antiserum mixture from tube number 1 to tube number 2, etc. After each transfer the contents of the tubes were mixed thoroughly by drawing the mixture up and down at least 5 times with 1.0 ml serological pipette. The serial procedure was continued through tube number 9, and the last 0.5 ml was discarded from the pipette. The last tube was retained as the antigen-saline control tube. A 0.5 ml sample of a suspension of formalinized whole cells, adjusted to match a number 2 McFarland nephelometer standard, was added to each of the 10 tubes. Tubes were incubated for 2 hr at 37 C and then for 24 hr at 4 C. The highest dilution of serum causing agglutination of cryptococcal cells was defined as the tube agglutinating antibody titer (TAA).

Ring Precipitation Test

The ring precipitation test was performed according to the method of Campbell, et al., (1964). With a Pasteur pipette, antiserum was introduced into the bottom of the 6 x 50 mm tubes. The antigen solution was then layered carefully over the antiserum to form an interface. The tubes were allowed to stand for 30 min at 25 C, at which time final readings were recorded. Reactions were observed by holding

the tubes in front of a light, against a black background.

Immunodiffusion

Immunodiffusion methods similar to those of Ouchterlony (1949) were used. A 1.0 per cent solution of highly purified agar (Ionagar No. 2, Oxoid)[16] was prepared barbital buffer, pH 7.4, I.S. 0.15, containing 0.01 per cent merthiolate as a preservative. The solution was heated on a Pyro-Magnestir[17] hot plate until the agar was completely dissolved. The hot agar was pipetted in 15 ml quantities into sterile, standard size (13 x 100 mm), disposable, plastic Petri dishes. The plates were allowed to stand overnight at 25 C with their lids agar. For further drying, the plates were inverted and allowed to stand for 24 hours with the covers in place before being stored at 4 C in an air tight plastic bag. They were allowed to harden at least 1 week before use.

Unless otherwise specified, the wells cut in the agar plates were 10 mm apart in any direction. After the wells were cut with a cork borer the agar plugs were removed with a capillary pipette attached to a suction apparatus. The appropriate wells were then filled with approximately 0.1 ml ot antigen or antiserum by means of a capillary pipette. The plates were incubated at 25 C and examined after varying lengths of time for the development of precipitation bands. In all cases, readings were recorded after 2 and 5 days. Certain of

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[16] Consolidated Laboratories, Box 234, Chicago Heights, Illinois.

[17] Lab-Line Instruments, Inc., Melrose Park, Illinois.

the plates were photographed with a Polaroid 800[18] camera using Type 42[18] black and white film or with a Pentax[19] "spotmatic" camera.

Passive Cutaneous Anaphalaxis Test

Male albino guinea pigs, weighing 250-300 gm, were used for the passive cutaneous anaphalaxis (PCA) tests. Each guinea pig was shaved with Oster[20] animal clippers approximately 18 hr before sensitization with antibody. This procedure avoided trauma and minimized excitement of the animals. Ovary (1958) reported that trauma may cause variation in the reactions and that an excited animal may become anergic.

All antisera used for sensitization were diluted with pyrogen-free 0.85 per cent NaCl[21]. For the purpose of sensitization, intradermal injections were made using 1.0 ml tuberculin syringes fitted with short bevel, 26 gauge, 0.5 inch, disposable needles[22]. A 0.1 ml volume of antiserum, or its appropriate dilution, was introduced with the bevel of the needle turned downward. A bleb approximately 8 mm in diameter was thus produced at the site of injection.

Three hr after sensitization all guinea pigs were challenged by intravenous injection with 1.0 ml of an antigen solution containing

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[18]Polaroid Corporation, Cambridge, Massachusetts.

[19]Honeywell Photo Products, 5200 E. Evans Ave., Denver, Colorado.

[20]Oster Johnson Manufacturing Company, Milwaukee, Wisconsin.

[21]Cutter Laboratories, Berkeley, California.

[22]Becton, Dickinson, and Company, Columbus, Nebraska.

1.0 mg crude capsular polysaccharide and 5.0 mg Evan's blue dye, according to the technique of Kabat and Mayer (1961). For injection, guinea pigs were immobilized on a guinea pig board made as described by Campbell, et al., (1964). The upper surface of a hind foot was shaved with Oster[20] animal clippers, and a small rubber band was placed above the ankle which was held tightly by a slip knot. The needle was then introduced into the small vein between the outer and the middle toes. The tourniquet was removed and the injection of the antigen-Evan's blue mixture was completed.

All PCA readings were made 45 min after antigen injection and were recorded as diameter (mm) of colored area at the skin test site. In some animals the skin was removed to facilitate photography of the reaction sites. Since no difference was found in the size of reactions on the outside and on the underside of the skin, all subsequent measurements were made on the intact animal. A standard reactive antiserum was included when there was the possibility of false negative reactions. The standard reactive antiserum was diluted 1:32 in 0.85 per cent pyrogen-free NaCl[21].

Quantitative Precipitation Test

The quantitative precipitation test (QPT) was used to evaluate the effect of crude capsular polysaccharide on antibody response to bovine serum albumin (BSA). Precipitin titration followed the alpha procedure, in which the quantity of antigen was kept constant, and it was a slight modification of the one described by Campbell, et al. (1964). In order to obtain accurate quantitative data, care was taken

in making dilutions and in the use of pipettes. Also, analytical technique was used in each step of the procedure.

Before use, each antiserum was clarified by centrifugation at 10,000 x g at 4 C for 30 min. For the purpose of determining the appropriate dilution of antiserum for the concentration of antigen (BSA), the qualitative precipitation test was employed. A series of eleven, 6 x 50 mm test tubes was set up and into the last tube was pipetted 0.2 ml of borate-saline buffer, pH 8.4. The buffer was prepared by mixing 5 parts of a stock buffer (boric acid, 6.184 gm; sodium tetraborate, 9.536 gm; sodium chloride, 4.384 gm; and distilled water to 1 liter) and 95 parts of 0.85 per cent NaCl. With a pipette, 0.2 ml of antigen dilution (containing 31, 62, 93, 125, 187, 250, 375, 500, 750, 1,000 ug BSA/ml) was transferred to its appropriately labeled tube. To each of the above tubes was then added 0.2 ml of undiluted antiserum and the contents were mixed by flicking the tube. Tubes were observed immediately and the optimal proportion (OP) zone was indicated by the tube first showing flocculation. In the BSA-anti-BSA system the OP zone should occur in the tube containing 250 ug BSA/ml (Campbell, et al., 1964). Therefore, all antisera not having an OP zone in the 250 tube were diluted appropriately. For example, if the OP zone was found to be in the 500 ug tube the antiserum was diluted two-fold for the quantitative test.

In the quantitative precipitation test, duplicate rows of 11 Kahn (12 x 75 mm) serological tubes were appropriately labeled. The eleventh tube served as an antiserum control, into which was placed 0.25 ml of borate-saline buffer. Into the remaining tubes 0.25 ml of

the appropriate antigen dilution and 0.25 ml of the appropriately diluted antiserum were dispensed. The tubes were shaken, incubated at 37 C for 2 hr, and then stored at 4 C for 1 week. During this period the tubes were agitated daily. They were then centrifuged at 3,400 RPM for 30 min at 4 C in a Adams SeroFuge[23]. The supernatant fluids were removed with a capillary pipette and placed into a clean, correspondingly labeled test tube. The OP zone of the supernate was determined immediately to avoid precipitation due to temperature change.

Tubes containing the precipitate were filled with cold (4 C) borate-saline buffer from a wash bottle, directing the fluid first as a jet against the precipitate in order to dislodge it from the bottom of the tube and to disperse it into fragments. Tubes were centrifuged as described above and the supernates were discarded. The precipitates were then washed 3 times with borate-saline buffer. Protein content of the precipitates and the various antigen dilutions were determined according to a modification of the Lowry, et al. (1951) method.

Chemical Analyses

Lowry Protein Determination

A modification of the method of Lowry, et al. (1951) was used for the determination of total protein in certain cryptococcal antigens and in the quantitative precipitation tests. All samples to be analyzed were diluted in 0.85 per cent NaCl to contain approximately 25 to 200

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[23]Clay-Adams, Inc., New York, New York.

ug protein per ml. A 6.0 quantity of reagent C, consisting of 50 ml of reagent A (10 gm of sodium carbonate made up to 500 ml with 0.1 N NaOH) and 1 ml of reagent B (1.0 gm of sodium tartrate and 0.5 gm of copper sulfate made up to 100 ml with distilled water), was added to 1.0 ml of diluted sample. Tubes were allowed to stand for 10 min at 25 C. A 0.6 ml quantity of reagent D (1 N Folin-Ciocalteu reagent)[3] was added rapidly, with immediate agitation, and the tubes were held at 25 C for 30 min. The optical density of the solutions was read in a Coleman Junior Spectrophotometer[24] at a wave length of 500 mu after standardizing the instrument with a reagent blank. A standard curve was prepared using bovine serum albumin[15]. Serial dilutions of the albumin standard were made in 0.85 per cent NaCl to give a concentration range from 20 to 200 ug of protein per ml and determinations were made by the method described above. The optical density of the unknown solutions was converted to concentration of protein by reference to the standard curve.

Total Nitrogen Determination

Total nitrogen determinations were made using the micro-Kjeldahl method of Lang (1958). Into each standard Kjeldahl digestion tube was placed either a dry weight sample of less than 5 mg or a sample of 1.0 ml or less in volume. To each tube were added 0.2 ml of digestion mixture (40 gm potassium sulfate, 20 ml selenium oxychloride, distilled water to 250 ml, and 250 ml concentrated sulfuric

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[24]Coleman Instruments, Inc., Maywood, Illinois.

acid), 0.2 ml of 30 per cent hydrogen peroxide, and distilled water to bring the total volume to 2.0 ml. Tubes were then held on a Kjeldahl heating apparatus[3] until the contents were evaporated to about 0.1 ml, and they were then cooled to 25 C. The acid digest was diluted to 10.0 ml with distilled water, after which a 3.0 ml aliquot was transferred to a Coleman cuvette[24] set in crushed ice. To these tubes were added 2.0 ml ice cold Nessler reagent[25] and 1.0 ice cold distilled water. After thoroughly mixing, the tubes were allowed to stand at 25 C for 10 min for color development. Optical densities were then determined at 500 mu in a Coleman Junior Spectrophotometer[24]. A standard curve was prepared using an ammonium sulfate standard solution, (1.179 gm ammonium sulfate in 250 ml 0.2 N sulfuric acid). Serial dilutions of the ammonium sulfate solution were made in distilled water to give a concentration range from 50 to 200 ug nitrogen per ml and determinations were made by the method described above. The optical density of the unknown solutions was converted to concentration of nitrogen by reference to the standard curve.

Total Carbohydrate Determination

Total carbohydrate determinations were made by use of the anthrone test as modified by Seifter et al., (1950). Samples to be analyzed were diluted in distilled water to contain approximately 20 to 200 ug carbohydrate per 5.0 ml. The tubes were placed in crushed ice and a 10.0 ml quantity of anthrone reagent (0.2 gm anthrone in

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[25] Banco Laboratories, Inc., Fort Worth, Texas.

100 ml 95 per cent sulfuric acid) was added from a fast flowing pipette. The tubes were shaken, stoppered with glass marbles, and heated for 10 min in a boiling water bath. They were then placed in running tap water until cool (approximately 10 min) and the optical densities were determined in a Coleman Junior Spectrophotometer[24]. A standard curve was prepared using a stock glucose solution (200 ug/ml). Serial dilutions of the glucose standard were made in distilled water to give a concentration range from 4 to 40 ug glucose per ml and determinations were made by the method described above. The optical density of the unknown solutions was converted to concentration of carbohydrate, as glucose, by reference to the standard curve.

Paper Chromatography for Monosaccharide Units

Samples to be examined chromatographically were dialyzed for 48 hr against 2 changes of distilled water. Hydrolysis of the samples was accomplished by heating, 1.0 mg of carbohydrate and 1.0 ml of 1.5 N HCl for 3 hr at 97 C in a sealed tube. The material was then dried in vacuo over NaOH pellets and the residue was reconstituted in 0.1 ml distilled water.

The hydrolyzed samples were spotted on 9" x 23" Schleicher and Schnell[26] chromatography paper (No. 2043-B) and chromatographed for 24 hr, in a descending system, in a solvent consisting of 5 parts butyl alcohol, 3 parts pyridine, and 2 parts 0.1 N·HCl. The chromatograms were then air dried and dipped in a solution containing 1.69 gm 2-aminobiphenyl, 5.0 ml glycerol, 0.90 gm oxalic acid, 10.0 ml distilled water,

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[26] Carl Schleicher and Schnell Company, Keene, New Hampshire.

and 84.0 ml acetone (Gordon, 1956). The papers were air dried and heated for 5 min at 100 C for the development of the spots. Standard solutions containing 1.0 mg of glucose, glucuronic acid, galactose, mannose, xylose, ribose, and glucurono-lactone were also spotted as controls. The color scheme consisted of pentoses-red, hexoses-greenish brown, and uronic acids-purple. The sensitivity of the reaction is 0.01 mols/square cm and the procedure used was patterned after the methods of Block (1964) and Trevelyan et al. (1950).

Ultra-Violet Analysis of Cell Fractions

Whole cells, cell walls and zeolite cell ghosts were analyzed by the trichloroacetic acid (TCA) method of Webb (1958). To 4.0 mg (wet weight) of cell walls, cell ghosts, or whole cells was added 1.0 ml of 5.0 per cent TCA and the materials were heated in a boiling water bath for 30 min. They were then diluted with 1.0 ml of 5.0 per cent TCA (unheated), centrifuged at 1,500 RPM to clarify, and diluted with distilled water to a final volume of 10.0 ml. The blank consisted of 1.0 ml of 5.0 per cent TCA (heated for 30 min) and 1.0 ml of 5.0 per cent TCA (unheated), diluted to a final volume of 10.0 ml.

The distilled water and sucrose supernates obtained from the zeolite extraction procedure were examined directly without TCA treatment. The supernates were diluted 1 to 10 with distilled water and appropriate blanks were also prepared from distilled water and 0.25 M sucrose in the same manner described above. Ultra-violet analysis was performed on a Cary 14 Recording Spectrophotometer[27] set at a slit width

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[27]Applied Physics Corporation, Monoronia, California.

of 0.06 mm.

Treatment of Cell Walls with Chitinase

To determine the presence or absence of chitin in the cell wall of C. neoformans, the following experiment was performed as summarized in Table 3. In 10.0 ml screw cap test tubes was placed 8 mg of cell wall or finely ground chitin[28]. A 1.5 ml quantity of 0.08 M acetate buffer, pH 5.0 was then added to each tube, together with 0.1 ml of 0.25 M magnesium chloride and/or 0.1 ml of 0.85 per cent NaCl. To selected tubes was added 1 drop of a 1.0 per cent bovine serum albumin[15] solution. Finally, to each tube was added 400 ug pure chitinase[29] contained in 1.0 ml of the acetate buffer. All tubes were stoppered tightly and incubated at 37 C for 10 days. At 0, 3, 5, 7, and 10 days, 0.2 ml samples were removed and assayed qualitatively for N-acetylglucosamine by the Morgan and Elson reaction as modified by Tracey (1955). The 0.2 ml samples were placed in graduated test tubes. A 10 ug standard and a water blank were included. The volume of all tubes was brought to 1.0 ml with distilled water and 0.3 ml of saturated sodium borate solution was added to each tube. The tubes were shaken, stoppered with glass marbles, heated in a boiling water bath for 7 min, and then cooled in running tap water. Glacial acetic acid was added to the 8.7 ml mark of each tube and this was followed by the addition of 1.0 ml Ehrlich reagent (2.0 gm p-dimethylaminobenzaldehyde in 100 ml glacial acetic acid containing 5.0 ml concentrated hydrochloric

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[28]Eastman Organic Chemicals, Rochester, New York.

[29]Nutritional Biochemical Corporation, Cleveland, Ohio.

TABLE 3

EXPERIMENTAL DESIGN FOR CHITINASE HYDROLYSIS[a] OF CELL WALL ISOLATED FROM CRYPTOCOCCUS NEOFORMANS

Tube Number	Cell Wall mg	Chitin mg	$MgCl_2$ ml	BSA drops	NaCl ml	Chitinase ug	Acetate Buffer (ml)
1	8	-	0.1	1	---	400	1.5
2	8	-	---	-	0.1	400	1.5
3	-	8	0.1	1	---	400	1.5
4	-	8	0.1	-	---	400	1.5
5	-	8	---	1	0.1	400	1.5
6	-	8	---	-	0.1	400	1.5
7	-	-	---	1	---	400	1.5

[a] Incubate at 37 C for 10 days.

acid). The tubes were shaken and allowed to stand for 45 min. The color intensity was matched against the standard and recorded qualitatively from 0 to 4+.

Histological Studies

At autopsy, representative tissue samples from the heart, lung, brain, kidney, liver, and spleen, obtained from rabbits that had received decapsulated cryptococcal whole cells mixed with 360 ug cryptococcal capsular polysaccharide, were fixed in neutral 10 per cent formalin. Tissues were placed in an automatic tissue processor (Auto-technicon)[30] for dehydration through changes of 80, 95, and 100 per cent ethanol, clearing with xylene, and infiltration of tissue with paraffin. Paraffin tissue embedding was done with standard Tissue-Tek embedding equipment[31]. Tissue sections were cut on a rotary microtome[32] at a thickness of 6 u. Sections were stained with Harris's hematoxylin-eosin and Mayer's mucicarmine stains according to the methods outlined in the Manual of Histologic and Special Staining Technics (1960).

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[30]The Technicon Company, Chauncey, New York.

[31]Ames Company, Inc., Elkhart, Indiana.

[32]American Optical Company, Buffalo, New York.

CHAPTER III

RESULTS

Cell Disruption

The early phase of this investigation involved preparation of the various cell fractions which would subsequently be studied chemically and immunologically. Treatment of cryptococcal cells by sonic oscillation resulted in approximately 80 per cent breakage of the cells as determined by direct microscopic examination. This method of cell disruption was found to be superior to Braun homogenization, which resulted in only 20 to 30 per cent cell disruption. With these techniques, however, crude cell walls were isolated in rather small amounts due to autoagglutination of the cell wall fragments, which were inseparable from the unbroken whole cells and glass beads. For this reason, the synthetic zeolite method was evaluated. The effect of zeolite treatment on cryptococcal whole cells is shown in Figures 6 and 7.

When viable whole cells of C. neoformans are gram stained they retain the crystal violet-iodine complex and are seen as deeply staining, Gram positive, budding yeast cells (Figure 6). However, when the cells were treated with zeolite they became gram negative, were devoid of cytoplasmic staining, and had the appearance of cell ghosts (Figure 7). When direct wet mount preparations of zeolite treated cells and

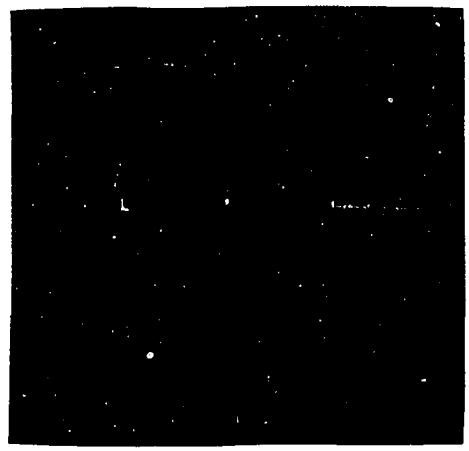


Figure 6. Gram stained smear of Cryptococcus neoformans before treatment with synthetic zeolite. Magnification x 970.

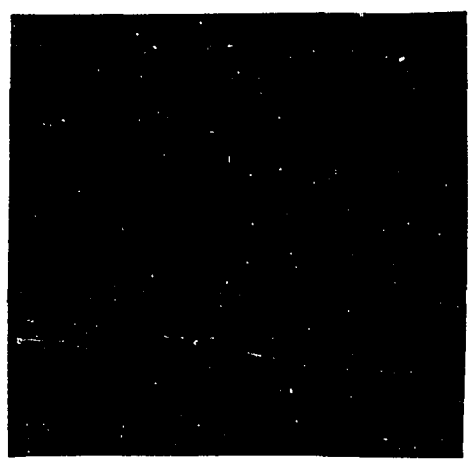


Figure 7. Gram stained smear of Cryptococcus neoformans after treatment with synthetic zeolite. Magnification x 970.

the crude cell walls isolated by sonic oscillation were compared, the zeolite ghosts resembled erythrocyte ghosts, whereas the crude cell wall preparation contained small half-moon particles of broken cells. In no instance were broken particles of cells seen in the zeolite preparations.

Ultraviolet absorbtion spectra were obtained on the zeolite ghosts and whole cells, as well as the water and sucrose supernates. The zeolite ghosts were relatively free of contaminating nucleic acids as compared to the whole cells. The sucrose supernate contained the largest percentage of proteins, absorbing at 280 mu, and the water supernate contained the largest percentage of nucleic acids, absorbing at 260 mu. It appears that the major portion of the soluble cell protein is removed by the sucrose treatment of the isolated cell ghosts. Isolated crude cell walls gave an absorption spectrum similar to that seen with the cell ghosts. The results of this experiment are presented in Figure 8.

Chemical Analysis

Results of the chemical analysis of the antigens which were used for the production of antisera are shown in Table 4. The formalinized whole cell with capsule contained 0.96 mg total carbohydrate/ml. For comparative purposes, the sonicated antigen with capsule was adjusted to approximately the same carbohydrate content, i.e. 1.0 mg/ml. The total carbohydrate content of the whole cell antigen without capsule was only 0.60 mg/ml. Accordingly, the homologous sonicated antigen was adjusted to approximately the same concentration (0.64 mg/ml). Cell wall antigen contained 0.60 mg/ml of carbohydrate, while the zeolite

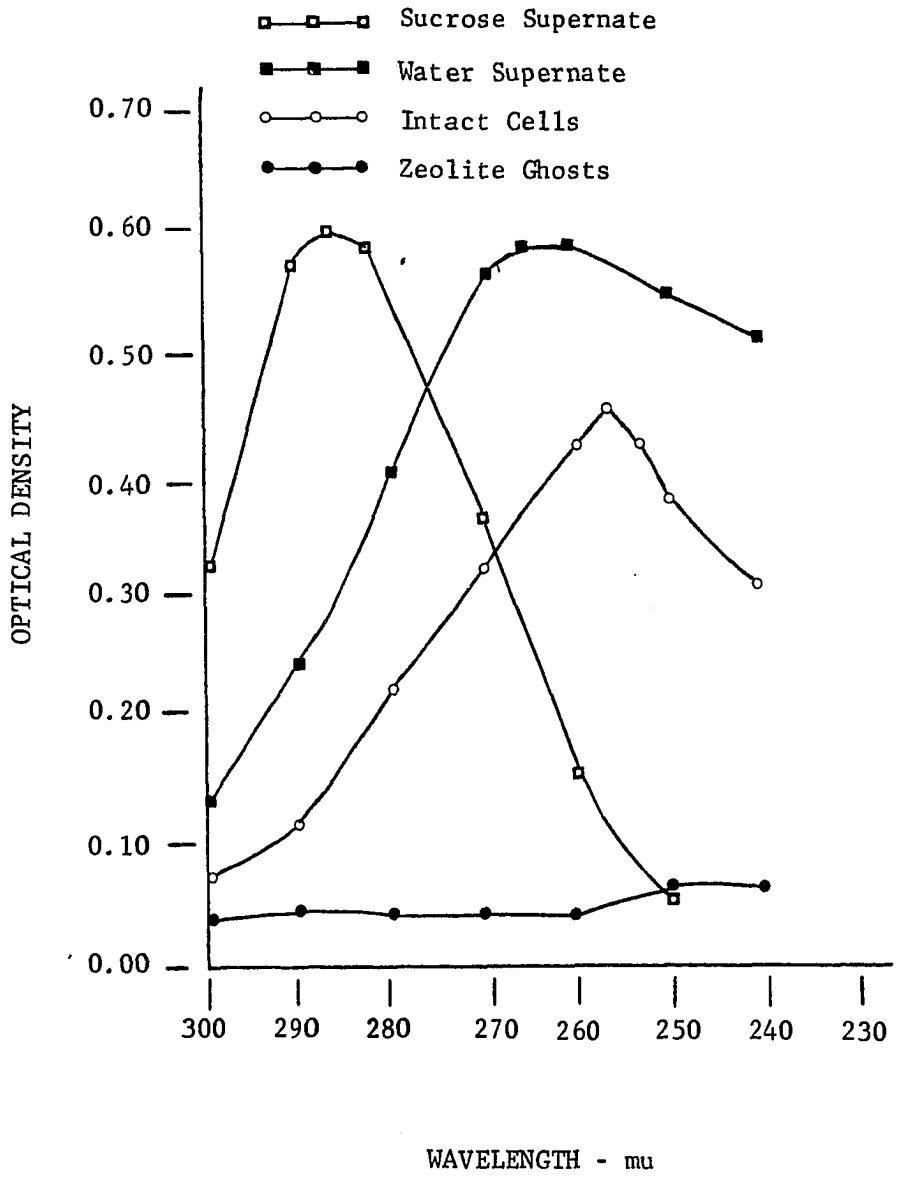


Figure 8. Ultraviolet absorbtion spectra of cell fractions isolated from Cryptococcus neoformans by zeolite treatment.

TABLE 4

TOTAL CARBOHYDRATE AND PROTEIN CONTENT OF ANTIGENS FROM *CRYPTOCOCCUS NEOFORMANS* USED FOR INTRAVENOUS IMMUNIZATION

Antigen	Carbohydrate[a] mg/ml	Protein[b] mg/ml
Whole Encapsulated Cells	0.96	0.46
Sonicated Encapsulated Cells	1.0	1.26
Whole Decapsulated Cells	0.60	0.60
Sonicated Decapsulated Cells	0.64	1.16
Cell Wall	0.60	0.15
Cytoplasmic Material	1.20	0.68
Zeolite Ghosts	0.57	0.42

[a]Anthrone method.

[b]Lowry method.

ghosts contained 0.57 mg/ml. Cytoplasmic material contained 1.20 mg carbohydrate/ml, which is slightly more than was seen with the sonicated antigens.

The 4 particulate antigens, i.e. whole cells with and without capsule, cell walls, and zeolite ghosts, had been adjusted to match a number 10 McFarland nephelometer standard before being analyzed chemically. The total carbohydrate contents of these antigens were in good agreement except for the encapsulated whole cell.

Protein analysis showed a wide range of variability between whole cells and sonicated antigens. However, there was good agreement between whole cells with and without capsule, which had protein contents of 0.46 and 0.60 mg/ml, respectively. The sonicated antigens exhibited similar agreement, with 1.26 mg protein/ml for sonicated encapsulated cells and 1.16 mg protein/ml for the sonicated nonencapsulated cells. Crude cell walls and zeolite ghost antigens contained 0.15 and 0.42 mg protein/ml respectively, while the cytoplasmic antigen contained 0.68 mg/ml.

The total carbohydrate and protein content of the soluble antigens used for agar gel diffusion tests are shown in Table 5. The protein content of these antigens was relatively consistent, ranging from 3.00 to 3.78 mg/ml, except for the culture filtrate which had a protein content of only 0.65 mg/ml. The carbohydrate content ranged from 1.92 mg/ml for the sonicated nonencapsulated cells to 4.20 mg/ml for the zeolite soluble fraction. Three of the antigens, i.e. sonicated cells, with and without capsule, and the cytoplasmic material, were three-fold concentrates of the antigens used for immunization.

TABLE 5

TOTAL CARBOHYDRATE AND PROTEIN CONTENT OF SOLUBLE ANTIGENS FROM CRYPTOCOCCUS NEOFORMANS USED FOR AGAR DOUBLE DIFFUSION

Antigen	Carbohydrate mg/ml[a]	Protein mg/ml[b]
Zeolite Soluble Fraction	4.20	3.12
Sonicated Encapsulated Cells[c]	3.00	3.78
Sonicated Decapsulated Cells[c]	1.92	3.48
Cytoplasmic Material[c]	2.04	3.60
Braun Homogenate	2.68	3.00
Culture Filtrate	2.40	0.65

[a]Anthrone

[b]Lowry method

[c]3-fold concentrate of antigen used for immunization

In the analyses described above, carbohydrate and protein were determined on the hydrated antigens and the values were principly as a means of comparing the relative amounts of each constituent administered during the immunization schedule. In addition, certain of the isolated fractions were brought to a constant weight in vacuo and the percentage protein and carbohydrate were determined. These data are presented in Table 6. The percentage of total carbohydrate of the 3 particulate fractions, i.e. whole cells with capsule, cell walls, and the zeolite ghosts, was found to be 76.8, 64.2 and 69.4, respectively. The total percentage of protein (N x 6.25) in the zeolite ghosts was 22.31, or approximately twice that found in whole cells and cell walls. The phenol extract (water phase), the mannan isolated from whole cells, and the mannan isolated from the zeolite ghosts contained 97.8, 99.2, 98.6 per cent total carbohydrate, respectively, and were relatively free of contaminating nitrogenous material. The crude capsular polysaccharide contained 82.2 per cent carbohydrate and 16.9 per cent protein (Lowry), as compared to 9.1 per cent by the micro-kjeldahl method.

Whole cells with capsules and zeolite ghosts were quantitatively extracted by the mannan extraction method of Peat (1961). The whole cells with capsules contained 15.7 per cent mannan, while the zeolite ghosts contained 7.9 per cent mannan.

All of the various fractions used in this investigation were analyzed by descending paper chromatography for detection of sugar residues. This method was used to detect the 4 monosaccharide elements (glucose, galactose, xylose, and glucuronic acid) known to be present in the capsular polysaccharide isolated from C. neoformans.

TABLE 6

CHEMICAL ANALYSIS OF CRYPTOCOCCUS NEOFORMANS WHOLE CELLS AND CELL FRACTIONS

Fraction	Percent[b] Total N	Percent Protein (N x 6.25)	Percent Protein (Lowry)	Percent Total[c] carbohydrate
Whole Encapsulated Cells	1.68	10.50	[a]	76.8
Zeolite Ghosts	3.57	22.31	[a]	69.4
Cell Walls	0.70	10.62	[a]	64.2
Crude Capsular Polysaccharide	1.47	9.19	16.92	82.2
Phenol Extract Water Phase	0.21	1.31	0.52	97.8
Mannan (Whole Cells)	<0.02	<0.16	<0.06	99.2
Mannan (Zeolite Ghosts)	<0.02	<0.12	<0.04	98.6

[a]Not done

[b]Micro-Kjeldahl

[c]Anthrone method

The results of this experiment are shown on Figures 9 and 10. From the chromatogram shown in Figure 9 it can be seen that the zeolite ghosts contained 3 of the 4 capsular monosaccharides, namely glucuronic acid, mannose, and xylose. However, a relatively large amount of glucose was also present, plus an unidentified spot near the top of the chromatogram. This unidentified spot, along with a large amount of glucose, was also seen in the hydrolytic products of the cell. No other monosaccharide residues were detected in the cell wall hydrolysate. All other fractions analyzed in this manner (Figures 9 and 10) contained glucuronic acid, mannose, xylose, and galactose, suggesting the presence of all of the capsular polysaccharide components. One notable exception is the absence of galactose from the mannan fraction isolated from whole cells (Figure 9, column 6).

An attempt was made to identify the unknown substance found in cell walls and the zeolite ghosts. On paper chromatography it did not appear to be a hexosamine. However, in view of the reports of the presence of chitin in the cell wall of certain yeasts, the isolated cell walls of C. neoformans were subjected to hydrolysis by purified chitinase. The results of this experiment are shown in Table 7. It will be seen that N-acetylglucosamine was readily liberated from chitin, however none was liberated from the isolated cell wall fraction, indicating the absence of chitin.

Serological Tests

The serological methods used in this study consisted of whole cell tube agglutination, agar gel diffusion, and passive cutaneous

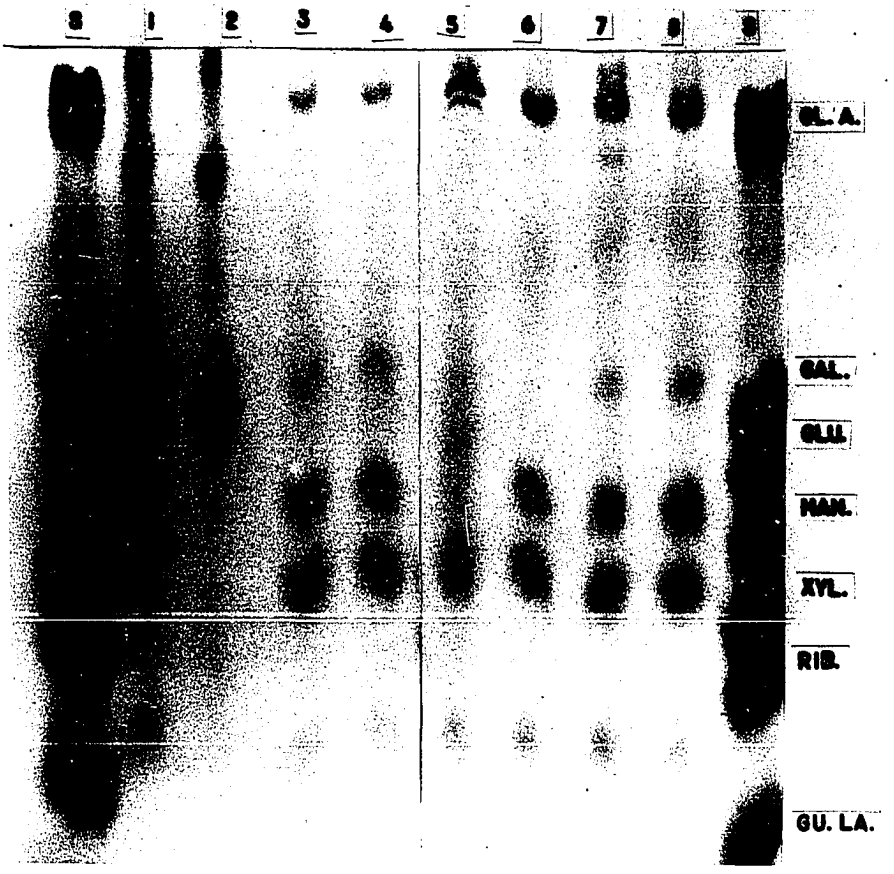


Figure 9. Chromatogram comparing hydrolyzed whole cells and cell fractions from Cryptococcus neoformans: (1) zeolite ghosts, (2) cell walls, (3) encapsulated whole cells, (4) decapsulated whole cells, (5) mannan (zeolite ghosts), (6) mannan (whole cells), (7) capsular polysaccharide, (8) phenol extract (water phase). The two outside columns (S) are the sugar standards: in descending order, Gl.A=D-glucuronic acid, GAL.=D-galactose, GLU.=D-glucose, MAN.=D-mannose, XYL=D-xylose, RIB.=D-ribose, Gu.LA.=D-glucuronolactone.

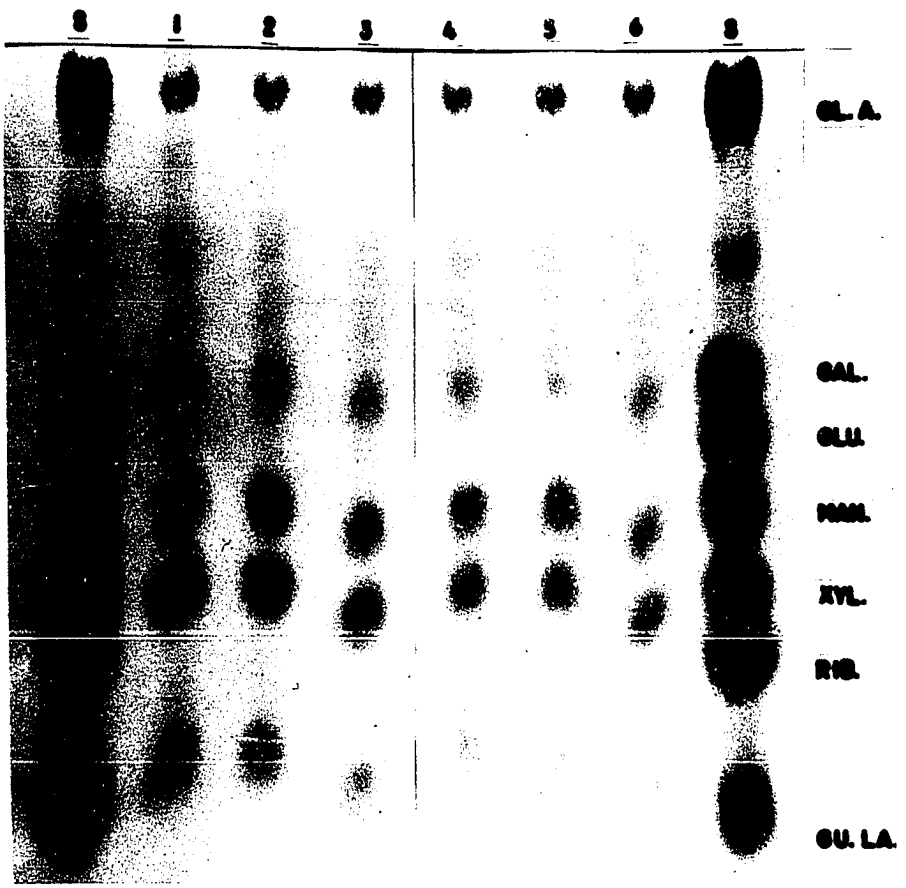


Figure 10. Chromatogram comparing hydrolysates of soluble fraction isolated from Cryptococcus neoformans: (1) zeolite supernate, (2) Braun homogenate, (3) culture filtrate, (4) cytoplasmic material, (5) sonicated encapsulated cells, (6) sonicated decapsulated cells. The two outside columns (S) are the sugar standards: in descending order, GL.A.=D-glucuronic acid, GAL.=D-galactose, GLU.=D-glucose, MAN.=D-mannose, XYL.=D-xylose, RIB.=D-ribose, GU.LA.=D-glucuronolactone.

TABLE 7

QUALITATIVE DETERMINATION OF N-ACETYLGLUCOSAMINE AFTER CHITINASE HYDROLYSIS OF CELL WALL ISOLATED FROM CRYPTOCOCCUS NEOFORMANS

Tube Number	Substrate (8.0 mg)	Reaction at:			
		3 Days	5 Days	7 Days	10 Days
1	Cell Wall	0	0	0	0
2	Cell Wall	0	0	0	0
3	Chitin	2+	3+	3+	4+
4	Chitin	1+	1+	1+	3+
5	Chitin	2+	2+	2+	2+
6	Chitin	2+	2+	2+	2+
7	-	0	0	0	0
Standard[a]	-	4+	4+	4+	4+

[a] 10 ug N-acetylglucosamine/0.2 ml.

anaphylaxis. In the agar gel diffusion method, use of a 1.0 per cent agar in barbital buffered NaCl (pH 7.4) and incubation of the charged petri dishes at 25 C proved to be optimal for the development of precipitin bands. When phosphate buffered NaCl was used, the precipitin bands were more diffuse than those formed with barbital buffer. Furthermore, in certain cases, a non-specific precipitin band appeared with the phosphate buffered agar, whereas it was eliminated by the use of the barbital buffer.

In order to determine whether use of the passive cutaneous anaphylaxis test (PCA) was feasible for the detection of antibody, the following preliminary experiments were performed. To test for toxicity of the antisera and for any non-specific affects of the Evan's blue dye, 2 guinea pigs were sensitized by the method described in Chapter II. After a latent period of 3 hr they were injected intravenously with 5.0 mg Evan's blue contained in 1.0 ml of 0.85 per cent NaCl. After 30 min the reactions were recorded. The animals were then immediately injected intravenously with 1.0 mg capsular polysaccharide antigen and 45 min later the reactions were recorded. The results of this experiment are shown in Table 8. No reactions occurred after challenge with Evan's blue alone. However, when challenged with antigen the same guinea pigs demonstrated typical PCA reactions. These data indicated that the antisera and the Evan's blue dye were non-toxic and caused no non-specific reactions.

It was necessary to determine the optimal antigen concentration necessary to elicit the maximum PCA reaction. The data presented in Table 9 summarize the results of this experiment. There

TABLE 8

RESULTS OF TESTS FOR TOXICITY AND NON-SPECIFIC REACTIONS IN THE PASSIVE CUTANEOUS ANAPHYLAXIS TEST

Antiserum[a] Dilution	Diameter (mm) of Reaction			
	Evan's Blue Dye[b]		Capsular Polysaccharide[c]	
	Guinea Pig No. 1	Guinea Pig No. 2	Guinea Pig No. 1	Guinea Pig No. 2
1:16	0	0	13	14
1:32	0	0	14	12
1:64	0	0	11	10
1:128	0	0	9	8
1:256	0	0	8	6
1:512	0	0	6	5
0.85% NaCl	0	0	0	0
NRS-1:10[d]	0	0	0	0

[a]0.1 ml, intradermally (anti-cryptococcal whole cell without capsule).

[b]Readings made 30 min after injection.

[c]Readings made 45 min after injection.

[d]Normal rabbit serum

TABLE 9

EFFECT OF ANTIGEN CONCENTRATION ON THE PASSIVE CUTANEOUS ANAPHYLAXIS TEST

Antiserum[a] Dilution	Diameter (mm) of Reaction					
	0.5 mg Capsular Polysaccharide		1.0 mg Capsular Polysaccharide		2.0 mg Capsular Polysaccharide	
	Guinea Pig No. 1	Guinea Pig No. 2	Guinea Pig No. 3	Guinea Pig No. 4	Guinea Pig No. 5	Guinea Pig No. 6
1:16	12	11	13	14	13	13
1:32	12	11	14	14	13	13
1:64	10	9	11	11	11	11
1:128	8	8	9	8	8	8
1:256	6	6	7	7	7	6
1:512	4	5	5	6	5	5
NRS-1:10[b]	0	0	1	0	1	0
0.85% NaCl	0	0	0	0	0	0

[a]0.1 ml, intradermally.

[b]Normal rabbit serum.

would appear to be little difference in the reactions observed with the 3 antigen concentrations used. In the guinea pigs that received 0.5 mg capsular polysaccharide, the reactions were slightly smaller than those elicited by the larger doses. However, this difference is probably of doubtful significance. There seemed to be no difference in the results with the 1.0 mg and 2.0 mg dosages. Based on these results, in subsequent experiments a 1.0 mg dose of antigen was used since it appeared to be the lowest concentration which elicited the maximal PCA reactions.

In order to make comparisons between individual antisera it was necessary to establish a standard unit of measurement for the PCA test. Thus, the PCA unit was defined as the reciprocal of the antiserum dilution which elicited a PCA reaction of 4 mm. This value was chosen on the basis of the data presented in Table 10. Undiluted, pooled, normal rabbit serum (NRS) gave an average reaction of 1.0 mm and in no instance did the reaction exceed 3 mm in diameter. Saline (0.85 per cent NaCl) and NRS diluted 1:10 gave average reactions of 0.2 mm and 0.5 mm, respectively, with the majority showing no reaction. A standard reactive antiserum was included to preclude misinterpretation of false negative reactions. The standard antiserum was diluted 1:32 and, in 27 trials, it gave an average reaction of 12.6 mm, with a range of 11 to 14 mm. A typical titration is shown in Figure 11 and illustrates the results obtained with an antiserum containing 512 PCA units.

The second phase of this investigation was concerned with the immunization of rabbits with various antigen preparations in order to

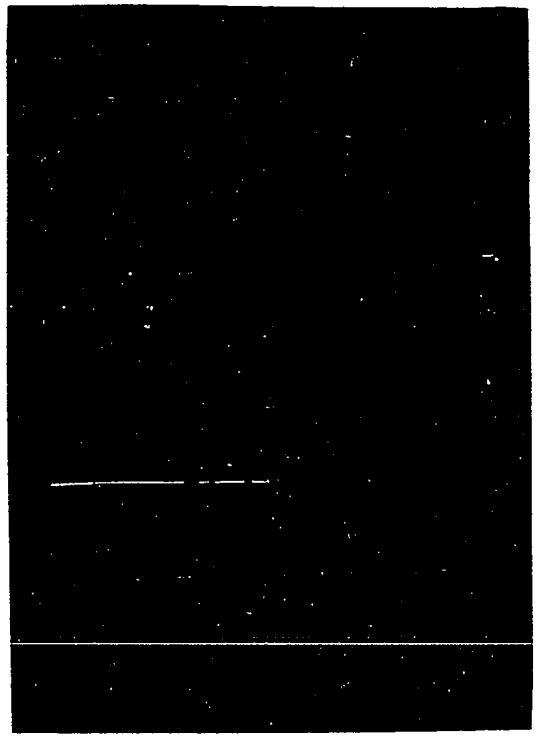
TABLE 10

SUMMARY OF CONTROL TESTS USED IN PASSIVE CUTANEOUS ANAPHYLAXIS

Diameter (mm) of Reaction	No. of Samples Showing Designated Reactions			
	NaCl (0.85%)	NRS[a] 1:10	NRS[a] Undiluted	Standard[b] Antiserum
0	21	24	3	0
1	7	15	6	0
2	0	3	2	0
3	0	0	1	0
11	0	0	0	2
12	0	0	0	9
13	0	0	0	11
14	0	0	0	5
Total	28	42	12	27
Average Diameter	0.25	0.5	1.0	12.6

[a]Pooled normal rabbit serum.

[b]1:32 dilution of antiserum to cryptococcal whole cell without capsule.



1:8	1:16
1:32	1:64
1:128	1:256
1:512	1:1024
Standard	NRS-1:10

Figure 11. A typical passive cutaneous anaphylaxis test in the guinea pig using crude capsular polysaccharide as antigen and rabbit antiserum to Cryptococcus neoformans.

obtain suitable antisera for use in subsequent work. Four major considerations were involved in this phase of the work: (1) the optimal time for obtaining maximal antibody titers, (2) the number of antigen injections necessary to reach maximal antibody titers, (3) the route of immunization and the effect of complete adjuvant on the immune response, and (4) the types of antibody formed during the immunization schedule.

The agglutinating antibody titers following immunization of individual rabbits with C. neoformans whole decapsulated cells are presented in Figure 12. Some variation occurred in the maximal antibody titers. In each rabbit the antibody titer began to decline 6 to 8 days after the last injection of antigen. From these data it was concluded that the optimal bleeding time was 6 days post-injection. The results illustrated a typical "negative phase" and an anamnestic response to injection of homologous antigen. Another notable feature was the rapid decline of agglutinating antibody titers after cessation of antigen injections.

A comparison of the average agglutinating antibody titers following immunization of rabbits with whole cells with and without capsule, zeolite ghosts, and cell walls is shown in Figure 13. Each line represents an average of results obtained from 3 rabbits immunized intravenously. In order to stimulate maximal antibody titers, it was necessary to use at least 5 injection series, i.e. 25 injections of antigen over a 10 week period. The antibody response to the zeolite ghosts was much higher than those to the whole cells or to cell walls. Sera from rabbits immunized with zeolite ghosts had an average

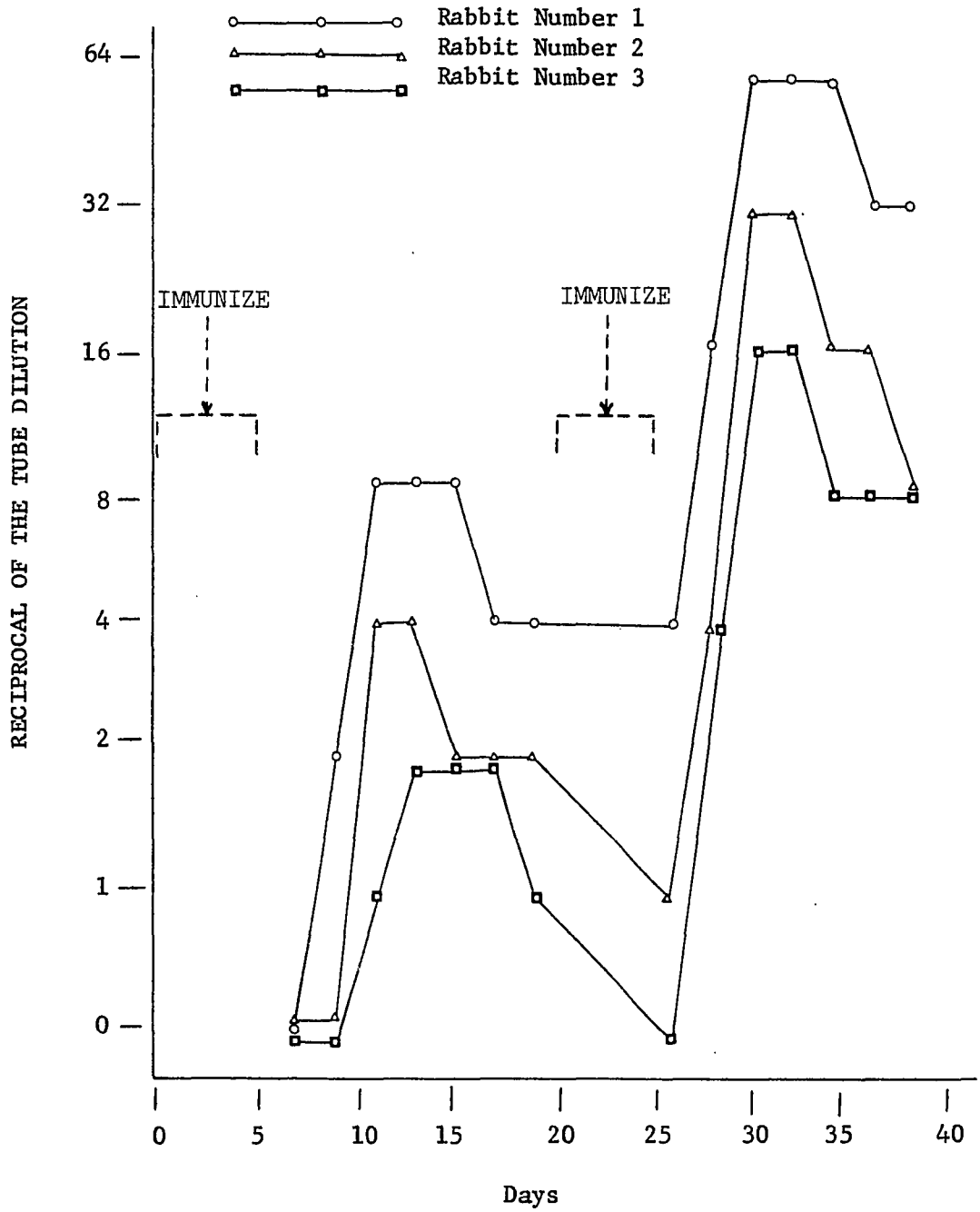


Figure 12. Agglutinating Antibody Titers Following Immunization of Rabbits with Cryptococcus neoformans Whole Cells without Capsule.

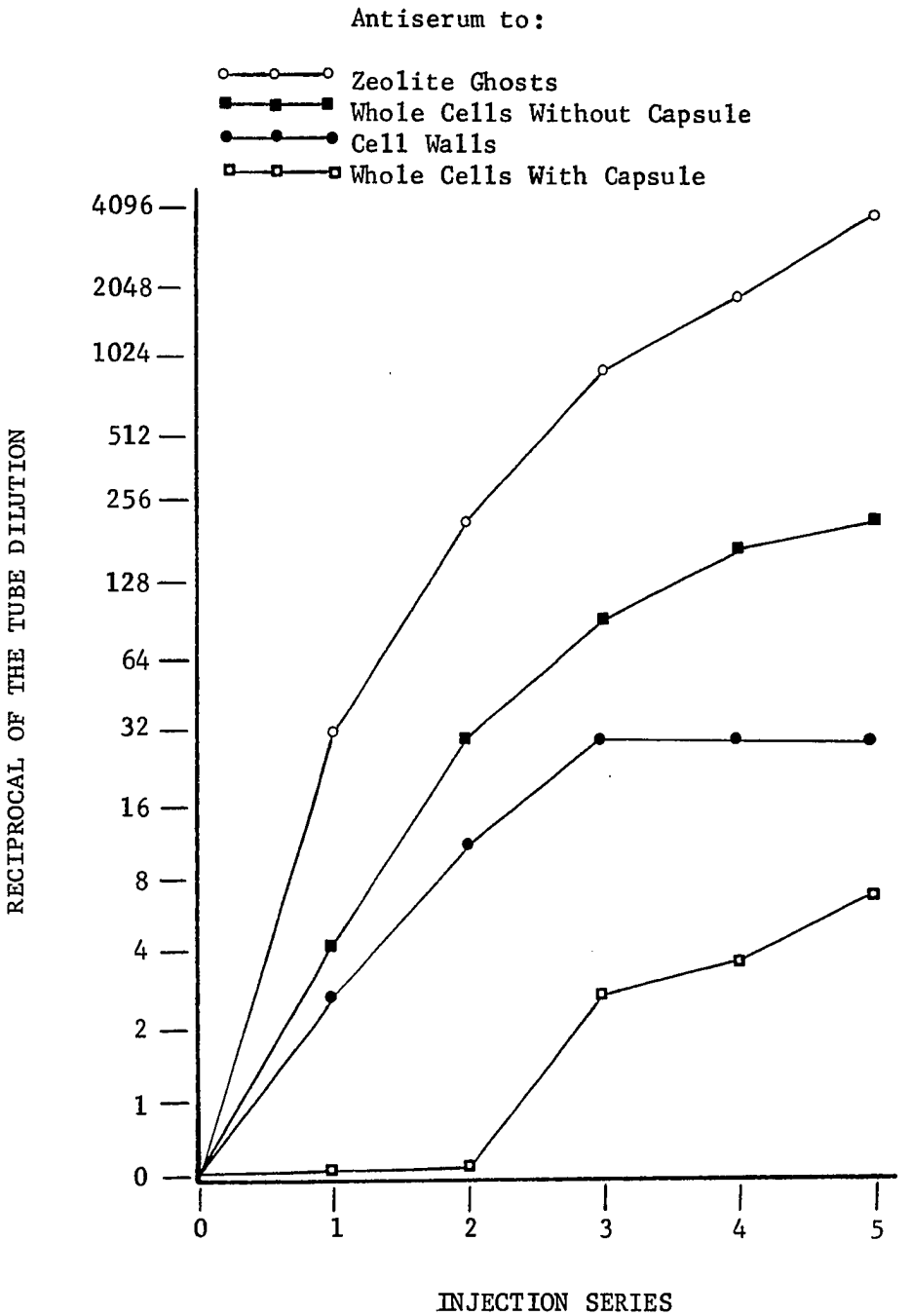


Figure 13. Comparison of the average agglutinating antibody titers following immunization of rabbits with whole cells with and without capsule, zeolite ghosts and cell walls isolated from Cryptococcus neoformans.

agglutinating antibody titer of 1:4,096, whereas rabbits immunized with encapsulated whole cells, decapsulated whole cells, and with cell walls responded with average serum titers of 1:7, 1:160, and 1:32, respectively.

A comparison of the agglutinating and PCA antibody responses to intravenous injection of various antigens is shown in Table 11. A series of 3 rabbits was immunized with each of the antigens, except in the case of decapsulated whole cells where 4 rabbits were used. Immunization of rabbits with zeolite ghosts resulted in agglutinating antibody titers ranging from 1:2048 to 1:8192, whereas with the other particulate antigens titers of only 1:1 to 1:256 were obtained. In general, lower agglutinating antibody titers were the rule with sonicated antigens than with intact cells. Results also showed that in most cases the PCA titers exceeded the agglutinating antibody titers two-to four-fold. This was especially true with antisera to whole cells and to other particulate antigens. There was only a minimal PCA antibody response to the solubilized cell antigens. No PCA or agglutinating antibody was detected in antisera obtained from rabbits immunized intravenously with capsular polysaccharide or cytoplasmic material.

In order to follow the progressive changes in antibody response during immunization, 2 series of antisera were selected for detailed study. One series was from a rabbit (No. E_1) immunized with encapsulated whole cells and the other was from a rabbit (No. G_2) which received decapsulated whole cells. Each antiserum was assayed for both PCA and agglutinating antibody and the results are shown in Figure 14. The rabbit immunized with encapsulated whole cells and the animal immunized with decapsulated whole cells gave final agglutinating anti-

TABLE 11

COMPARISON OF AGGLUTINATING AND PASSIVE CUTANEOUS ANAPHYLAXIS ANTIBODY RESPONSE IN RABBITS IMMUNIZED INTRAVENOUSLY WITH ANTIGENS OBTAINED FROM CRYPTOCOCCUS NEOFORMANS

Antiserum To:	Rabbit Number	Agglutinating Antibody Titer (Whole Cells)	PCA Units (Capsular Polysaccharide)
Zeolite Ghosts	$ZG-_1$	1:2048	-[a]
	$ZG-_2$	1:2048	
	$ZG-_3$	1:8192	
Whole Decapsulated Cells	G_1	1:128	512
	G_2	1:128	512
	G_7	1:128	-
	G_{10}	1:256	1024
Whole Encapsulated Cells	E_1	1:16	64
	E_2	1:4	8
	E_3	1:1	2
Crude Cell Walls	I_1	1:32	256
	I_2	1:16	64
	I_3	1:16	64
Sonicated Encapsulated Cells	F_1	1:2	U[b]
	F_2	1:4	
	F_3	1:2	

TABLE 11--Continued

COMPARISON OF AGGLUTINATING AND PASSIVE CUTANEOUS ANAPHYLAXIS ANTIBODY RESPONSE IN RABBITS IMMUNIZED INTRAVENOUSLY WITH ANTIGENS OBTAINED FROM CRYPTOCOCCUS NEOFORMANS

Antiserum To:	Rabbit Number	Agglutinating Antibody Titer (Whole Cells)	PCA Units (Capsular Polysaccharide)
Sonicated Decapsulated Cells	H_1	1:1	U
	H_2	1:4	U
	H_3	1:2	U
Cytoplasmic Material	J_1	0	0
	J_2	0	0
	J_3	0	0
Crude Capsular Polysaccharide	P_1	0	0
	P_2	0	0
	P_3	0	0

[a]Not done.

[b]Only undiluted antisera gave a reaction of 4 mm or greater.

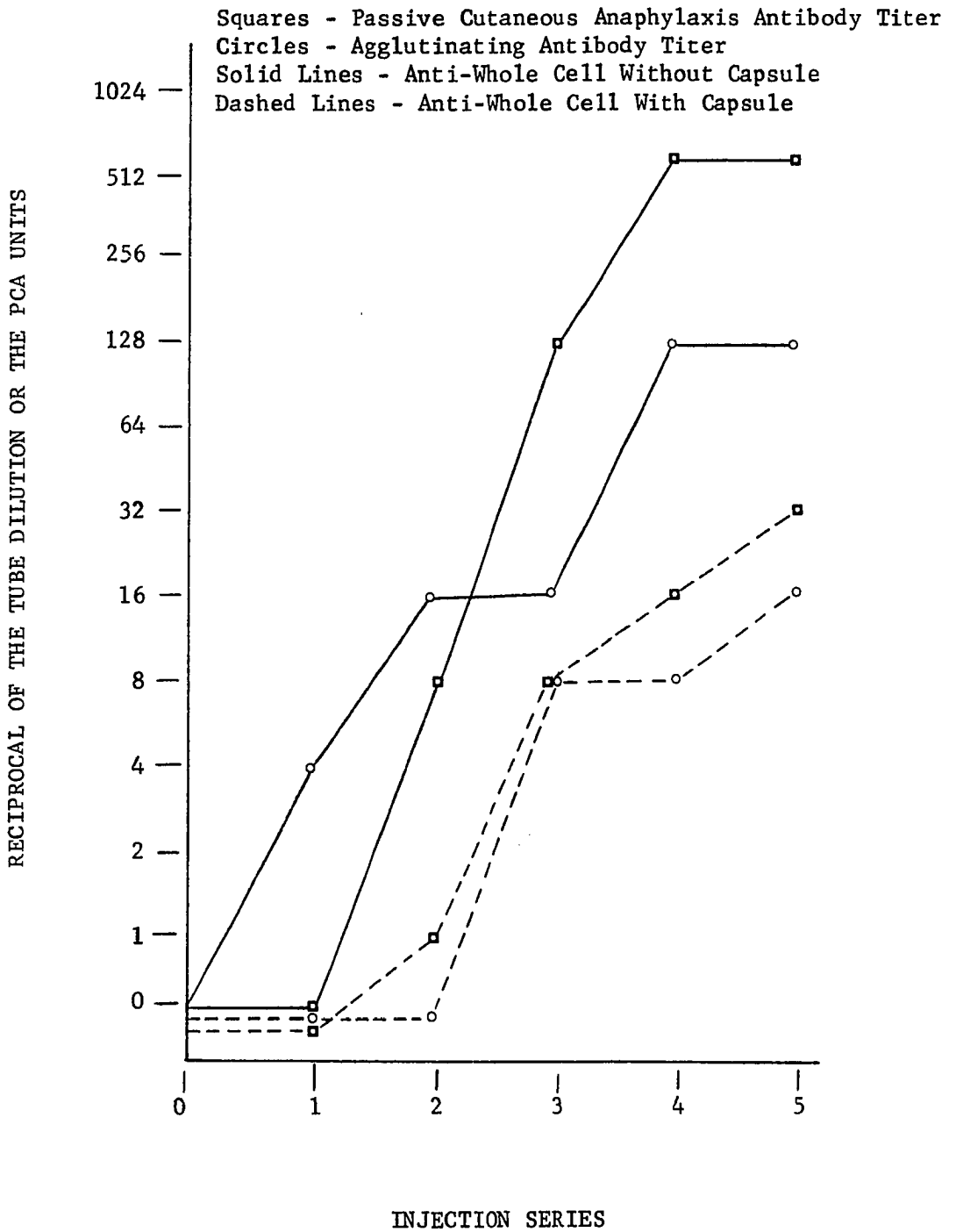


Figure 14. Comparison of agglutinating and passive cutaneous anaphylaxis antibody titers following immunization of rabbits with formalinized Cryptococcus neoformans.

body titers of 1:16 and 1:128, respectively. The final PCA titers were 64 and 512, respectively. Early in the immunization, however, the PCA titer was low as compared to the agglutinating antibody titer in the rabbit immunized with decapsulated whole cells. The PCA titer increased gradually after the second week of the injection series and surpassed the agglutinating antibody titer. In the rabbit immunized with encapsulated whole cells there was essentially no difference in the levels of the 2 types of antibodies.

The agglutinin and precipitin responses to decapsulated whole cells injected subcutaneously, with or without Freund's complete adjuvant, into each of 3 rabbits are shown in Table 12. The agglutinin titers in these animals were lower than those in animals which had received the same material intravenously without adjuvant (Table 11). In no instance did the agglutination titers exceed 1:4. The use of adjuvant did not appear to effect the antibody titers significantly. The number of precipitin bands obtained with these antisera ranged from 2 to 6, and these results are similar to those observed with other fractions injected intravenously (Table 18).

The effect of adjuvant on the immune response to cell wall and capsular polysaccharide of individual rabbits is shown in Table 13. No antibody response to either substance was detected by the agglutination and PCA tests. However, 1 precipitin band was obtained when the antisera to cell walls were reacted with zeolite supernate by the double diffusion technique. No antibody could be detected, by any serological technique, in those rabbits which had been immunized with capsular polysaccharide. In another experiment, each of 3 rabbits

TABLE 12

EFFECT OF ADJUVANT ON THE IMMUNE RESPONSE IN RABBITS IMMUNIZED SUBCUTANEOUSLY WITH FORMALINIZED DECAPSULATED WHOLE CELLS OF CRYPTOCOCCUS NEOFORMANS

Rabbit Number	Weeks After Injection	Agglutinating Antibody Titer (Whole Cells)	Number of Precipitin Bands (zeolite supernate)
	With Adjuvant		
SQA-1	2[a]	0	2
	4[b]	1:1	4
	6[c]	1:2	5
	8[d]	1:2	6
SQA-2	2	0	2
	4	1:1	4
	6	1:1	5
	8	1:1	5
SQA-3	2	0	4
	4	1:1	4
	6	1:2	5
	8	1:4	5
	Without Adjuvant		
SQ-1	2	0	3
	4	1:1	4
	6	1:4	4
	8	1:4	4
SQ-2	2	1:1	2
	4	1:4	4
	6	1:8	4
	8	1:8	5

TABLE 12--Continued

EFFECT OF ADJUVANT ON THE IMMUNE RESPONSE IN RABBITS IMMUNIZED SUBCUTANEOUSLY WITH FORMALINIZED DECAPSULATED WHOLE CELLS OF CRYPTOCOCCUS NEOFORMANS

Rabbit Number	Weeks After Injection	Agglutinating Antibody Titer (Whole Cells)	Number of Precipitin Bands (zeolite supernate)
		Without Adjuvant	
SQ-3	2	0	3
	4	1:1	4
	6	1:4	4
	8	1:8	6

[a]Two weeks after primary injection.

[b]Two weeks after booster injection.

[c]Four weeks after booster injection.

[d]Six weeks after booster injection.

TABLE 13

COMPARISON OF AGGLUTINATING, PRECIPITATING, AND PASSIVE CUTANEOUS ANAPHYLAXIS ANTIBODY RESPONSE IN RABBITS IMMUNIZED SUBCUTANEOUSLY WITH CELL WALLS AND CAPSULAR POLYSACCHARIDE OBTAINED FROM CRYPTOCOCCUS NEOFORMANS

Antiserum To:	Rabbit Number	Agglutinating Antibody Titer (Whole Cells)	PCA Units (Capsular Polysaccharide)	Number of Precipitin Bands (Zeolite Supernate)
Crude Cell Walls	Q_1	0	0	1
	Q_2	0	0	1
	Q_3	0	0	1
Crude Capsular Polysaccharide	S_1	0	0	0
	S_2	0	0	0
	S_3	0	0	0
	S_4	0	0	0

were injected *via* the toe pad with 0.36, 3.6, 36 and 360 ug of crude capsular polysaccharide given as a single dose. Again, no antibody could be detected by any of the serological techniques.

The immune response of 3 rabbits to the intraperitoneal injection of decapsulated whole cells is shown in Table 14. Very low agglutinating antibody titers were obtained.

The foregoing results, particularly those shown in Tables 4 and 11, suggested that, with those antigens containing relatively large amounts of polysaccharide, especially the soluble antigens, the antibody titers were very low as measured by the agglutination and PCA tests. Similarly, the antibody titers induced by encapsulated whole cells were relatively low compared to those induced by the decapsulated cells and the zeolite ghosts (Table 11). These findings prompted the third phase of this investigation, namely, study of the effect of isolated, soluble capsular polysaccharide on the immune response to the homologous decapsulated cells and to heterologous antigens, such as typhoid "H" vaccine and bovine serum albumin. It had been noted previously (Table 4) that the total carbohydrate of encapsulated whole cells exceeded that of decapsulated cells by 360 ug/ml. This difference was also seen in the 2 homologous sonicated antigens. During the complete course of immunization, those rabbits which had received whole encapsulated cells or sonicated encapsulated cells received approximately 25 mg more polysaccharide than those that had received decapsulated antigens. Therefore, an equivalent amount of capsular polysaccharide was added to the decapsulated whole cell antigen such that the total carbohydrate content was 1.0 mg/ml. A series of 7 rabbits was then immunized with

TABLE 14

EFFECT OF INTRAPERITONEAL INJECTION OF ANTIGEN ON THE IMMUNE RESPONSE TO FORMALINIZED DECAPSULATED *CRYPTOCOCCUS NEOFORMANS* WHOLE CELLS

Rabbit Number	Injection Series	Week[a]	Agglutinating Antibody Titer (Whole Cells)	Number of Precipitin Bands (Zeolite Supernate)
IP_1	1	1	0	1
		2	0	3
	2	3	1:1	3
		4	1:2	4
	3	5	1:2	4
		6	1:2	4
IP_2	1	1	0	0
		2	0	2
	2	3	1:1	3
		4	1:2	4
	3	5	1:2	4
		6	1:2	4
IP_3	1	1	0	0
		2	1:1	3
	2	3	1:1	3
		4	1:1	3
	3	5	1:1	3
		6	1:1	3

[a]Weeks during complete course of immunization.

this preparation. The results of this experiment are presented in Table 15.

All 7 of the animals died during the course of the study. Death occurred after the rabbits had received an average of 9 injections of antigen, i.e. an average of 9 mg capsular polysaccharide. Death occurred 16 to 19 days after the first injection of antigen, and approximately 36 hr after the last injection of antigen. However, the individual animals shows considerable variability in both of these aspects.

All of the deaths seemed to follow a similar pattern. The head of the animal was pulled backward and a pink or red exudate appeared around the mouth and external nares. With 5 of the 7 rabbits a considerable quantity of blood was found in the dropping pan. The blood appeared to have come from the nose. Death always occurred after a series of intravenous injections and never after blood collection by cardiac puncture. With these rabbits, only 1 to 3 trial bleedings could be made. In no case did agglutinating antibody titers exceed 1:4 and such levels occurred in only 2 rabbits. Zero titers were obtained with 3 rabbits and titers of 1:2 were observed with the remaining 2 animals.

Shortly after death, each rabbit was autopsied and representative samples of heart, lung, brain, kidney, liver, and spleen were removed and examined histologically. The microscopic lung picture was that of severe, acute, pulmonary edema which seemed to be the primary cause of death. The heart sections showed extensive pathology, but, a complete examination could not be made due to unavailability of the

TABLE 15

EFFECT OF CAPSULAR POLYSACCHARIDE FROM CRYPTOCOCCUS NEOFORMANS ON THE IMMUNE RESPONSE IN RABBITS TO DECAPSULATED HOMOLOGOUS ORGANISMS

Rabbit Number	Capsular[a] Polysaccharide (mg)	Hours After[b] Last Injection	Agglutinating Antibody Titer[c]
1	15	36	1:4
2	11	18	0
3	6	12	0
4	10	40	1:4
5	10	80	0
6	14	20	1:2
7	8	24	1:2
Average	9.1	36	-

[a]Total amount of capsular polysaccharide given up to time of death.

[b]Hours after last injection of antigen that death occurred.

[c]Blood collected prior to death.

proper sections. It was assumed that the heart pathology was secondary to the pulmonary edema. No pathology was observed in the other organs examined. In the section stained for fungi with Mayer's mucicarmine stain, there was no evidence of cryptococcal cells in any of the tissues studied. It was concluded that the pathological picture observed was consistant with that seen in systemic anaphylaxis.

The average agglutinating antibody responses in the rabbits which were injected with decapsulated whole cells plus capsular polysaccharide (Table 15), decapsulated whole cells alone, encapsulated whole cells alone, and capsular polysaccharide alone are shown in Figure 15. No antibody was detectable in serum from rabbits immunized with capsular polysaccharide. Very low titers were obtained in rabbits receiving encapsulated whole cells as compared with those receiving decapsulated whole cells. In rabbits immunized with decapsulated cells plus capsular polysaccharide, intermediate antibody levels were seen. During the first 2 weeks of the immunization schedule, rabbits receiving encapsulated whole cells demonstrated no agglutinating antibody response. In the same period a modest response occurred in those rabbits receiving the whole cell-capsular polysaccharide mixture.

The results of studies designed to measure the effect of capsular polysaccharide on the immune response to typhoid "H" vaccine are summarized in Table 16. The average agglutinating antibody titer obtained with the 3 control rabbits was 1:4,778, as compared with average titers of 1:9,557 and 1:6,826 in the rabbits that received either 5.0 mg and 25.0 mg capsular polysaccharide, respectively. The capsular polysaccharide thus appeared to have no effect.

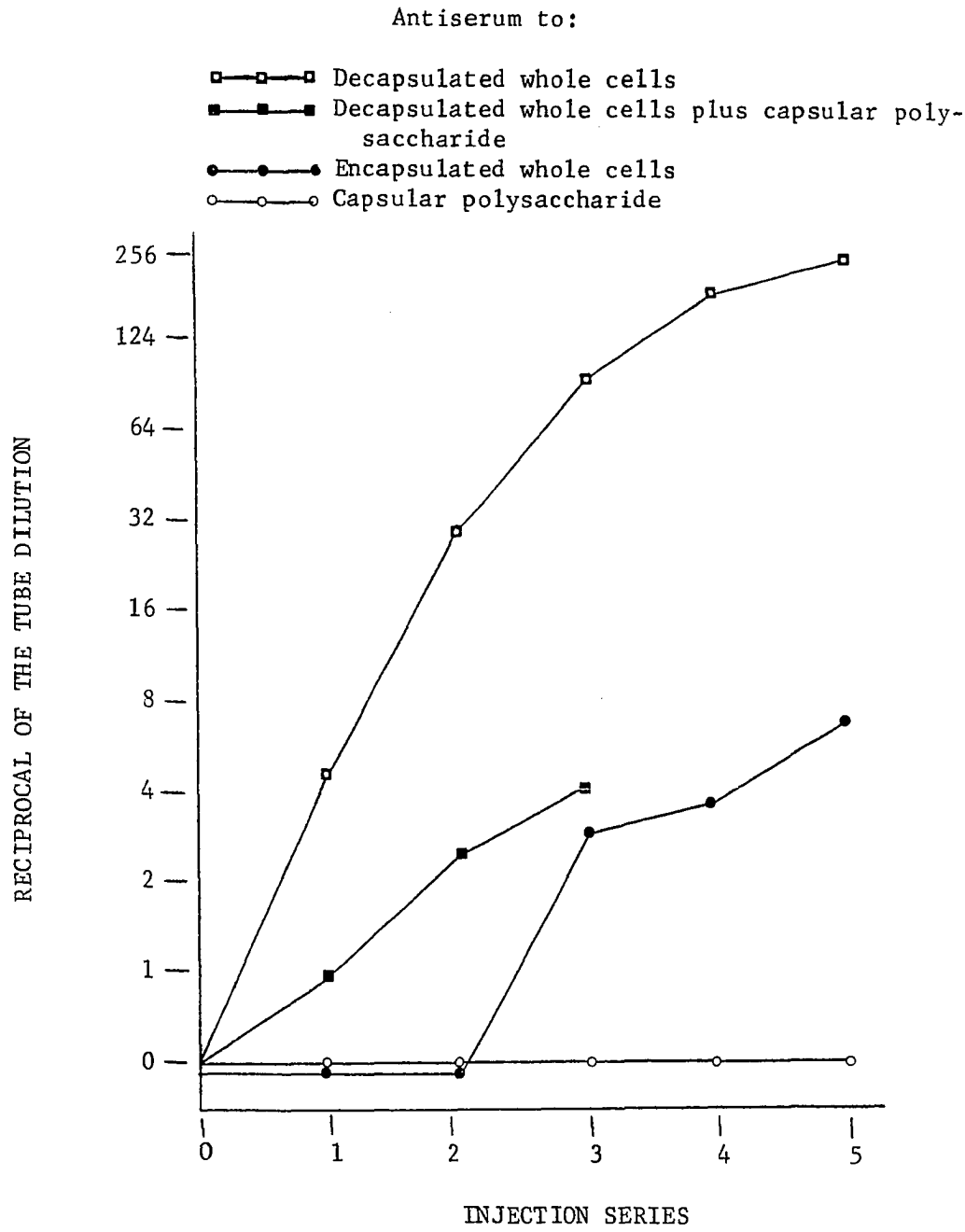


Figure 15. Agglutinating antibody responses in rabbits immunized with encapsulated and decapsulated whole cells, decapsulated whole cells plus capsular polysaccharide, and capsular polysaccharide.

TABLE 16

EFFECT OF CAPSULAR POLYSACCHARIDE FROM CRYPTOCOCCUS NEOFORMANS ON THE IMMUNE RESPONSE IN RABBITS TO TYPHOID "H" VACCINE

Polysaccharide Total mg/rabbit	Rabbit Number	Agglutinating Antibody Titer	Average
0	Typ-1	1:2,048	1:4,778
	Typ-2	1:8,192	
	Typ-3	1:4,096	
5.0	Typ-4	1:8,192	1:9,557
	Typ-5	1:4,096	
	Typ-6	1:16,384	
25.0	Typ-7	1:8,192	1:6,826
	Typ-8	1:4,096	
	Typ-9	1:8,192	

Similarly, capsular polysaccharide appeared to have no effect on the immune response of rabbits to bovine serum albumin (BSA). A series of 3 rabbits was immunized with each concentration (5.0 and 25.0 mg) of polysaccharide. It can be seen from the data presented in Table 17 that in control and test rabbits antibody responses, as measured by the quantitative precipitation test were about the same. Two representative quantitative precipitation curves are presented in Figure 16. The total milligrams of protein precipitated was plotted against milligrams BSA added, using results obtained with 1 control rabbit (BSA-1) and 1 test rabbit (BSA-5). It is clear that the capsular polysaccharide had no effect on the antibody response to BSA and that these antisera demonstrated typical quantitative precipitation curves.

Using the ring precipitin test, capsular polysaccharide was detected in the serum of all rabbits that had been immunized intravenously with capsular polysaccharide. For this purpose, rabbit serum was used as antigen and a high titered (1:4,096) antiserum to zeolite ghosts was used as antibody. Appropriate controls were employed, for example, normal rabbit serum and pre-immune serum were reacted against the anti-zeolite ghosts serum. In these controls, no precipitin bands could be detected. Using this method, capsular polysaccharide was also detected in the sera of rabbits immunized with BSA plus capsular polysaccharide, typhoid "H" vaccine plus capsular polysaccharide, and in those rabbits that received decapsulated whole cells plus capsular polysaccharide. In this latter series of rabbits it was possible to detect, in 5 out of 10 sera, capsular polysaccharide antibody and the capsular polysaccharide existing in the same serum simultaneously. This was

TABLE 17

EFFECT OF CAPSULAR POLYSACCHARIDE FROM CRYPTOCOCCUS NEOFORMANS ON THE IMMUNE RESPONSE IN RABBITS TO BOVINE SERUM ALBUMIN

Polysaccharide Total mg/rabbit	Rabbit Number	Antibody[a] Protein (mg/ml)	Average
0	BSA-1	11.36	7.62
	BSA-2	2.37	
	BSA-3	9.14	
25.0	BSA-4	8.49	6.93
	BSA-5	10.32	
	BSA-6	2.03	

[a]Quantitative precipitin data.

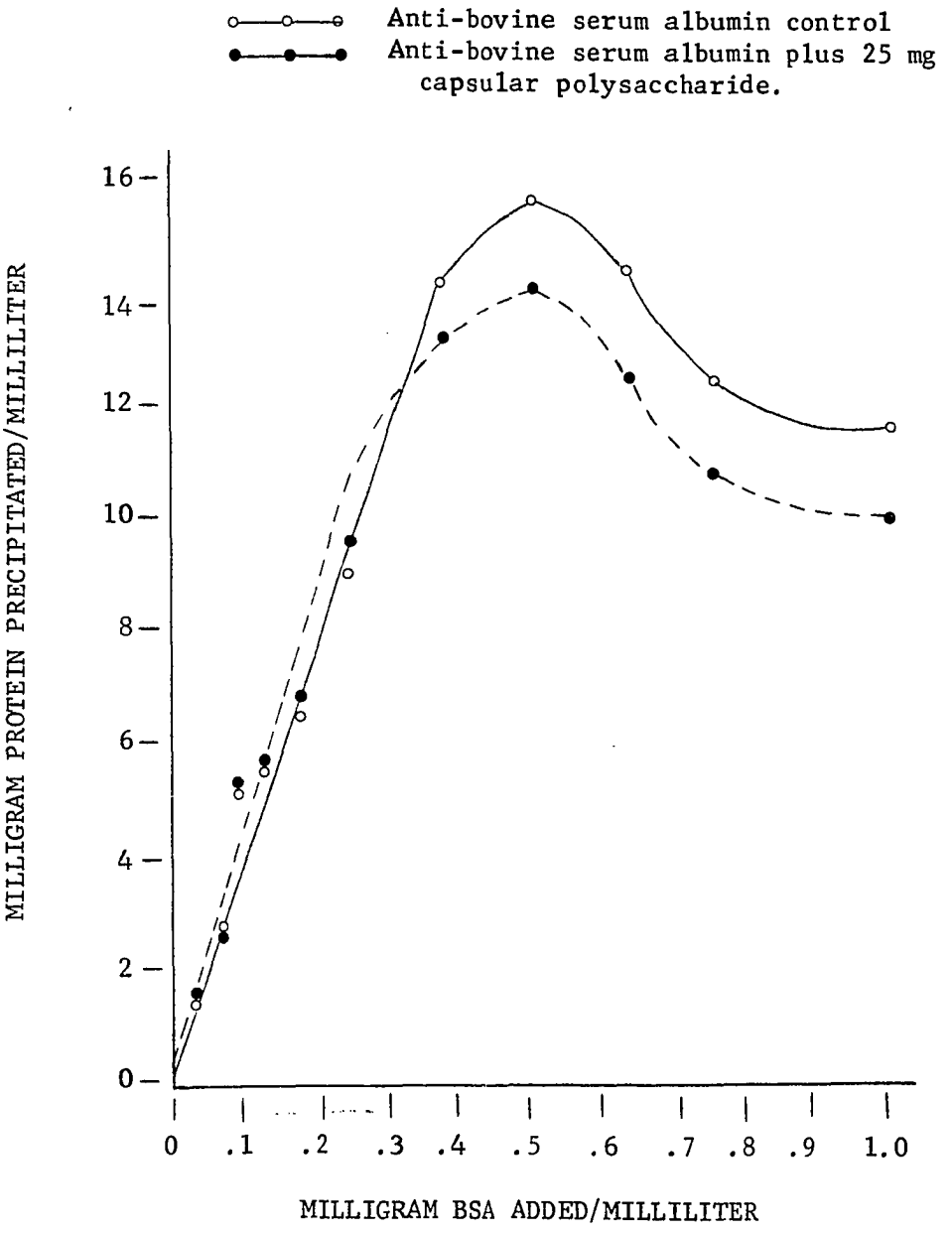


Figure 16. Representative quantitative precipitation curves comparing the antibody responses in rabbits to bovine serum albumin alone and bovine serum albumin plus 25 mg capsular polysaccharide from Cryptococcus neoformans.

especially true in those rabbits whose agglutinating antibody titers were high, for example, rabbits No. 1, 4, 6, and 7 (Table 15).

Antigenic Analysis

The fourth phase of this investigation dealt with an antigenic characterization of *C. neoformans* and its isolated cell fractions and also a study of the effect of various extraction procedures on the serological reactivity of the capsular polysaccharide. However, it was first necessary to obtain a soluble, serologically reactive antigen which would produce the maximal number of precipitation bands by the Ouchterlony double diffusion method. For this purpose, 4 soluble antigens were evaluated for use in the double diffusion tests. The results are presented in Table 18. It was concluded that the zeolite supernate was superior to the sonicate, Braun homogenate, and the culture filtrate. Using this antigen, a maximum of 6 precipitin bands were seen with sera from rabbits immunized with whole encapsulated, decapsulated cells and sonicated decapsulated cells. Serum from a rabbit (ZG_1) immunized with the zeolite ghosts demonstrated 7 precipitin bands. These results are also shown in Figure 17. It is also evident from these data that the particulate antigens were superior in stimulating the production of precipitating antibody in the rabbit. In general, the sonicated antigens were poor stimulators of precipitating antibody production. These results correlate with those seen in Table 11 which shows that the particulate antigens, i.e. whole cells, cell walls, and zeolite ghosts, were superior antigens for stimulating the production of agglutinating and PCA antibody.

TABLE 18

PRECIPITATING ANTIBODY RESPONSES IN RABBITS IMMUNIZED INTRAVENOUSLY WITH VARIOUS SOLUBLE ANTIGENS OBTAINED FROM CRYPTOCOCCUS NEOFORMANS AS MEASURED BY AGAR GEL DIFFUSION

Antiserum To:	Rabbit Number	Number of Precipitin Bands To:			
		Zeolite Supernate	Sonicate	Braun Homogenate	Culture Filtrate
Whole Encapsulated Cells	E_1	6	3	3	2
	E_2	5	2	2	1
	E_3	4	2	2	2
Whole Decapsulated Cells	G_1	6	3	1	3
	G_2	6	3	3	3
	G_7	6	3	1	3
	G_{10}	6	3	2	3
Zeolite Ghosts	ZG_1	7	3	3	3
	ZG_2	6	3	3	3
	ZG_3	6	3	3	3
Crude Cell Walls	I_1	3	3	1	1
	I_2	3	3	1	1
	I_3	3	2	1	1

TABLE 18--Continued

PRECIPITATING ANTIBODY RESPONSES IN RABBITS IMMUNIZED INTRAVENOUSLY WITH VARIOUS SOLUBLE ANTIGENS OBTAINED FROM CRYPTOCOCCUS NEOFORMANS AS MEASURED BY AGAR GEL DIFFUSION

Antiserum To:	Rabbit Number	Number of Precipitin Bands To:			
		Zeolite Supernate	Sonicate	Braun Homogenate	Culture Filtrate
Sonicated Encapsulated Cells	F_1	2	0	0	0
	F_2	1	0	0	0
	F_3	3	1	0	1
Sonicated Decapsulated Cells	H_1	6	0	0	2
	H_2	5	3	0	2
	H_3	3	1	0	1
Cytoplasmic Material	J_1	4	0	0	2
	J_2	4	0	0	2
	J_3	2	1	0	3
Crude Capsular Polysaccharide	P_1	0	0	0	0
	P_2	0	0	0	0
	P_3	0	0	0	0

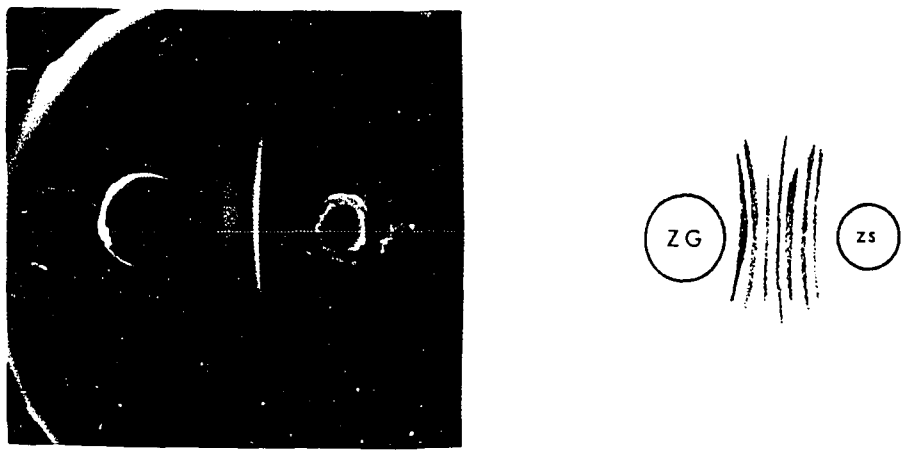


Figure 17. A representative agar gel double diffusion plate demonstrating a typical precipitin band pattern. ZG.=anti-zeolite ghosts, zs=zeolite supernate.

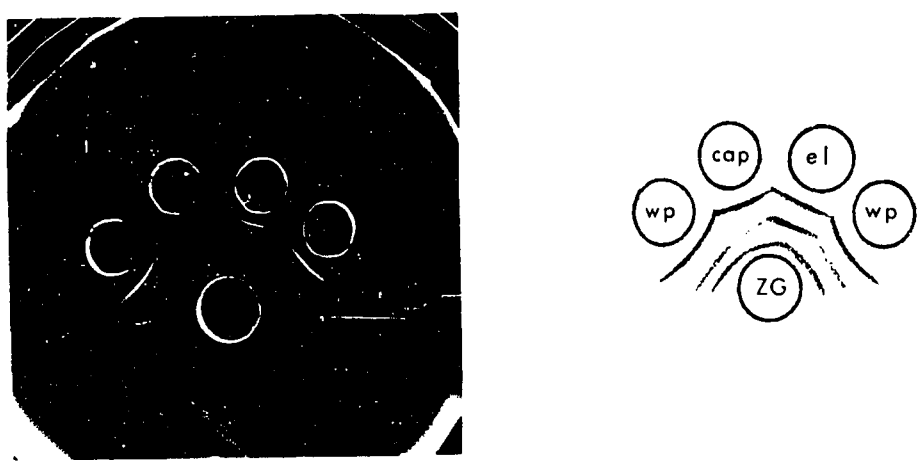


Figure 18. Agar gel double diffusion plate comparing the polysaccharide antigens obtained from Cryptococcus neoformans. wp=phenol extract (water phase), cap=capsular polysaccharide, el=eluted capsular polysaccharide, ZG=anti-zeolite ghosts.

It will also be noted in Table 18 that 2 to 4 precipitin bands were detected with sera from rabbits immunized with cytoplasmic material. It is of interest that in the same antisera no agglutinating antibody could be demonstrated (Table 11). The data presented in Tables 11 and 18 also reveal that no precipitating, agglutinating, or PCA antibody was detected in those rabbits that received capsular polysaccharide intravenously.

Several methods were used to isolate the capsular polysaccharide from C. neoformans, namely, sonication of whole cells followed by precipitation with cold ethanol (Figure 2), phenol extraction (Figure 3), mannan extraction (Figure 4), and simple elution. Elution was accomplished by incubating formalinized whole cells at 37 C for 4 hr and then isolating the polysaccharide by cold ethanol precipitation. As can be seen in Figure 18, there were no differences between the polysaccharides isolated by sonication, phenol extraction (water phase), and elution of whole cells, as measured by the double diffusion technique using antiserum to zeolite ghosts (ZG).

The polysaccharides isolated by sonication and by mannan extraction of whole cells (mannan) and zeolite ghosts (mannan ZG) are compared in Figure 19. These results suggest that there are distinct differences between the three preparations when they are diffused against antiserum to zeolite ghosts. It is remarkable that only 2 precipitin bands (B and C) were obtained with mannan, while 3 (B, C, E) were obtained with mannan ZG. Three precipitin bands (A, D, and E) were seen when capsular polysaccharide was used as antigen. Bands D and E of the capsular polysaccharide formed bands of identity with band E of the

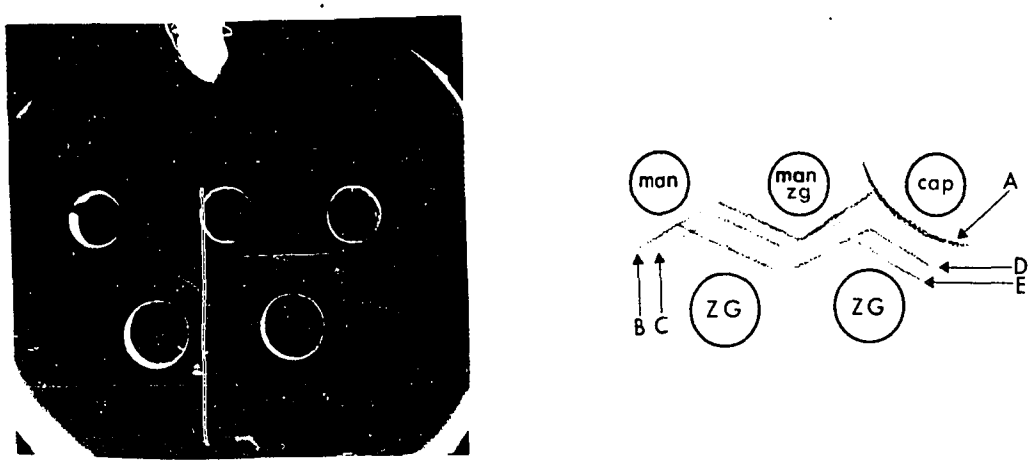


Figure 19. Comparison of the polysaccharide antigens isolated from Cryptococcus neoformans. man=mannan (whole cells), man zg=mannan (zeolite ghosts), cap=capsular polysaccharide, ZG=anti-zeolite ghosts.

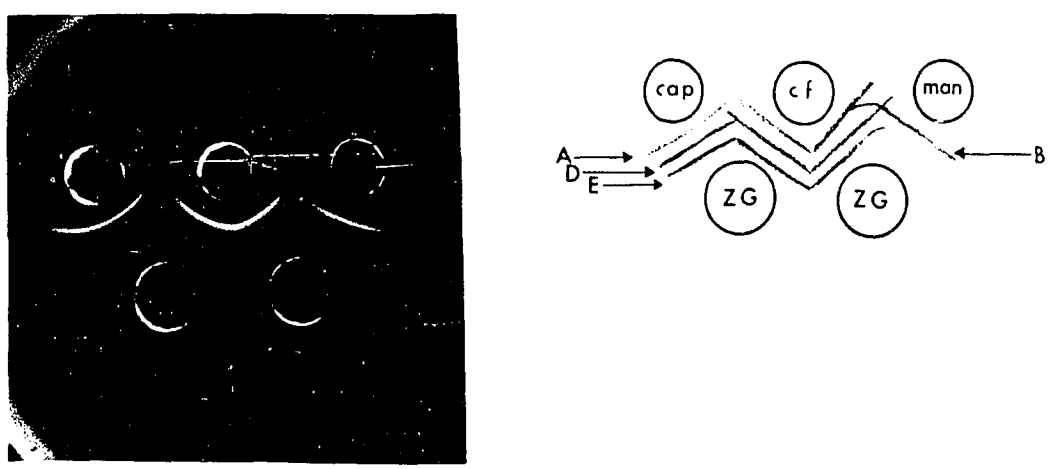


Figure 20. Comparison of polysaccharide antigens isolated from Cryptococcus neoformans. man=mannan, cf=culture filtrate, cap=capsular polysaccharide, ZG=anti-zeolite ghosts.

mannan ZG. Bands B and C formed bands of identity with both mannan preparations. Band A of the capsular polysaccharide formed a band of partial identity with band B of mannan and mannan ZG.

Figure 20 shows that antiserum to the zeolite ghosts produced 3 precipitin bands when diffused against the culture filtrate. It is also apparent that capsular polysaccharide and the culture filtrate are essentially the same, except that a spur was formed with band D. Moreover, band B of the mannan formed a spur with band A of the capsular polysaccharide and culture filtrate. When capsular polysaccharide was subjected to the mannan extraction procedure the number of precipitin bands was decreased from 3 to 1. The remaining band formed a band of identity with the mannan isolated from whole cells. These results are presented in Figure 21.

A major portion of the final phase of this investigation dealt with a comparison of the antisera obtained from rabbits immunized with whole cells, cell walls, and cytoplasmic material. Early in these studies it was found that antisera to encapsulated whole cells and decapsulated whole cells were equivalent in regard to the precipitin bands obtained in agar gel. Therefore, antisera to decapsulated whole cells were used in subsequent antigenic comparisons since they appeared to react more strongly in the precipitating system and, in addition, contained much higher agglutinating antibody levels (see Figure 13). It was also found that antisera from rabbits immunized with the zeolite ghosts were not equivalent to those obtained from rabbits immunized with the whole cell antigens. They were excluded from the antigenic studies to be described, because it was apparent that the zeolite ghosts did

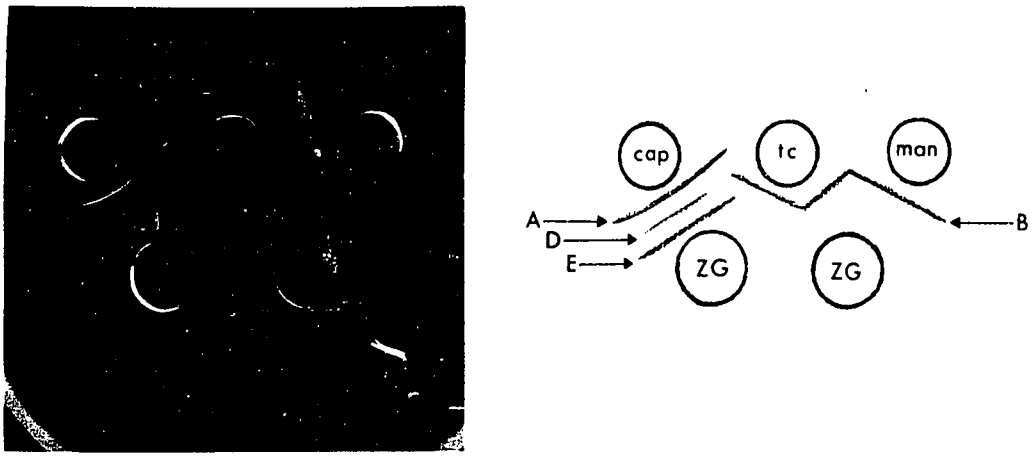


Figure 21. Effect of mannan treatment on capsular polysaccharide. cap=capsular polysaccharide, tc=treated capsular polysaccharide, man=mannan, ZG=anti-zeolite ghosts.

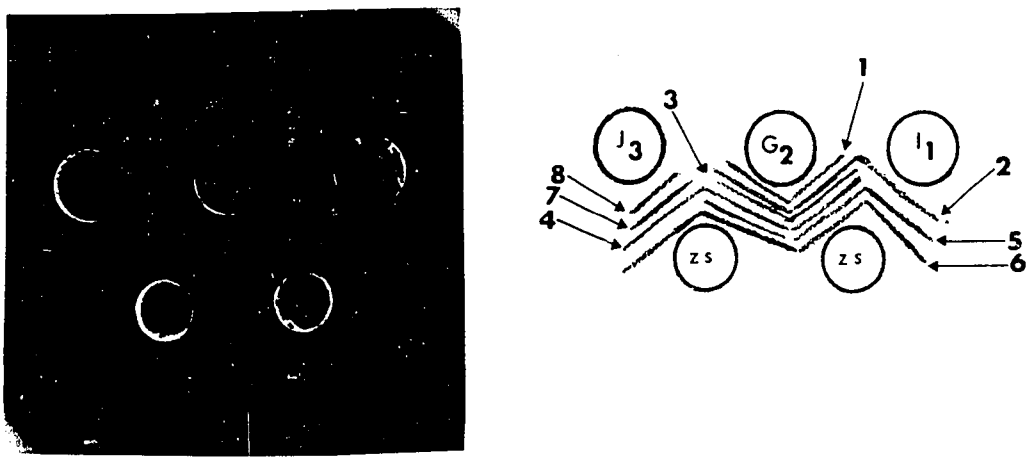


Figure 22. Comparison of antisera to whole cells, cell walls, and cytoplasmic material. zs=zeolite supernate, J_3=anti-cytoplasmic material, G_2=anti-whole cell, I_1=anti-cell wall.

not reflect the true antigenic make-up of Cryptococcus neoformans whole cells.

The first comparison was made between antisera to whole cells, cell walls, and cytoplasmic material using the zeolite supernate as the complete soluble antigen. The results are presented in Figure 22. Six precipitin bands (bands 1 through 6) were obtained with antiserum to whole cells. Bands 2, 5, and 6 formed bands of identity with antiserum to cell wall, and bands 4 and 6 formed bands of identity with antiserum to cytoplasmic material. Band 6 was seen all 3 antisera. However, antiserum to the cytoplasmic material demonstrated 2 additional weak precipitin bands (7 and 8) which did not form bands of identity with antiserum to whole cells or cell walls

After adsorption with whole cells, bands 1, 2, and 5 were removed from the whole cell antiserum and bands 2 and 5 were removed from cell wall antiserum. These data are presented in Figure 23. Adsorbtion with whole cells of antisera prepared against the cytoplasmic material resulted in no change in the precipitin pattern. The data presented in Table 19 demonstrates this and also shows that the precipitin bands obtained when either whole cell antiserum or cell wall antiserum is diffused against capsular polysaccharide are removed by adsorption with whole cells (see Figure 25). The precipitin bands removed by whole cell adsorption were bands 1 and 2 (Figure 22) which formed bands of identity with the cell wall antiserum. It should be noted here that when capsular polysaccharide is diffused against antiserum to the zeolite ghosts (Figure 20) 3 precipitin bands are seen, but when it is diffused against antiserum to whole cells or antiserum to cell

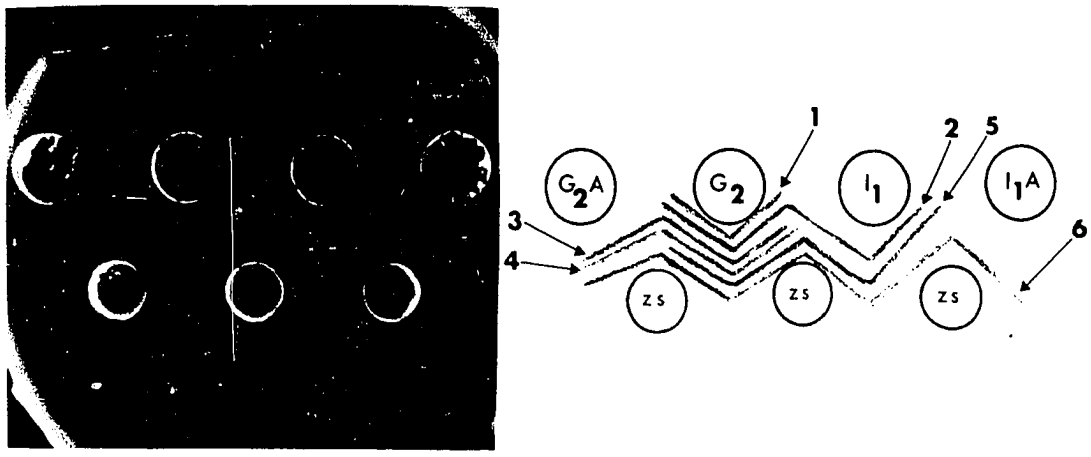


Figure 23. Effect of adsorption with whole cells on the precipitin band pattern seen with antisera to whole cells and cell walls. zs=zeolite supernate, G_2A=anti-whole cells adsorbed with whole cells, G_2=anti-whole cells, I_1=anti-cell wall, I_1A=anti-cell wall adsorbed with whole cells.

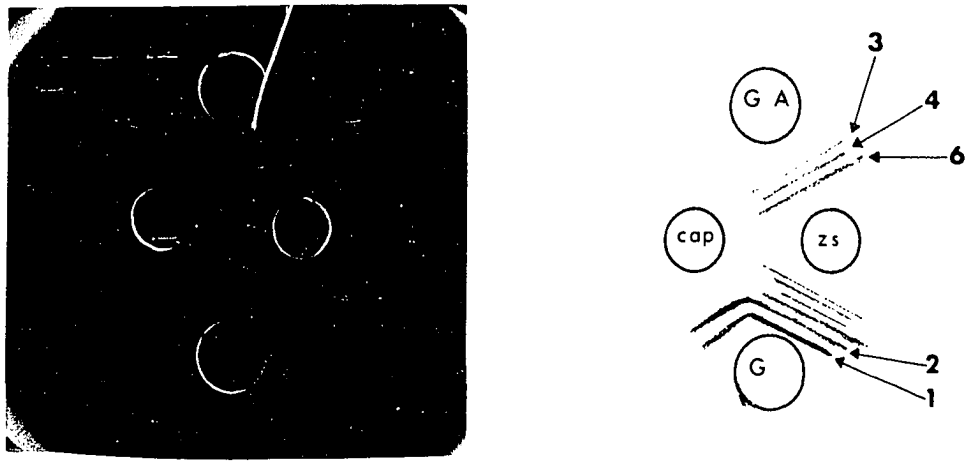


Figure 24. Effect of adsorption with whole cells on the precipitin band pattern seen with an antiserum to whole cells diffused against zeolite supernate and capsular polysaccharide. zs=zeolite supernate, cap=capsular polysaccharide, G_2A=anti-whole cells adsorbed with whole cells, G_2=anti-whole cells.

TABLE 19

EFFECT OF ADSORPTION OF CRYPTOCOCCUS NEOFORMANS ANTISERA WITH WHOLE CELLS ON PRECIPITATING ANTIBODY AS MEASURED BY AGAR GEL DIFFUSION

Antiserum To:	Rabbit Number	Precipitating Antigen					
		Zeolite Supernate		Capsular Polysaccharide		Mannan	
		Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed
Zeolite Ghosts	ZG_1	7[a]	4	3	0	2	0
	ZG_2	6	3	3	0	2	0
Whole Decapsulated Cells	G_2	6	4	2	0	1	0
	G_{10}	6	4	2	0	1	0
Cell Walls	I_1	3	1	2	0	1	0
	I_2	3	1	2	0	1	0
Cytoplasmic Material	J_1	4	4	0	0	0	0
	J_3	4	4	0	0	0	0

[a]Number of precipitin bands.

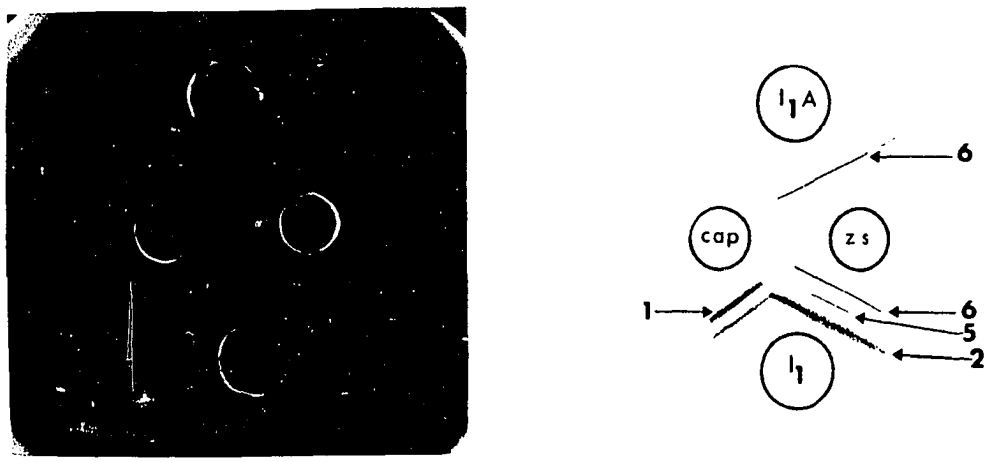


Figure 25. Effect of adsorption with whole cells on the precipitin band pattern seen with an antiserum to cell walls diffused against capsular polysaccharide and the zeolite supernate. cap=capsular polysaccharide, zs=zeolite supernate, I_1A=anti-cell wall adsorbed with whole cells, I_1=anti-cell wall.

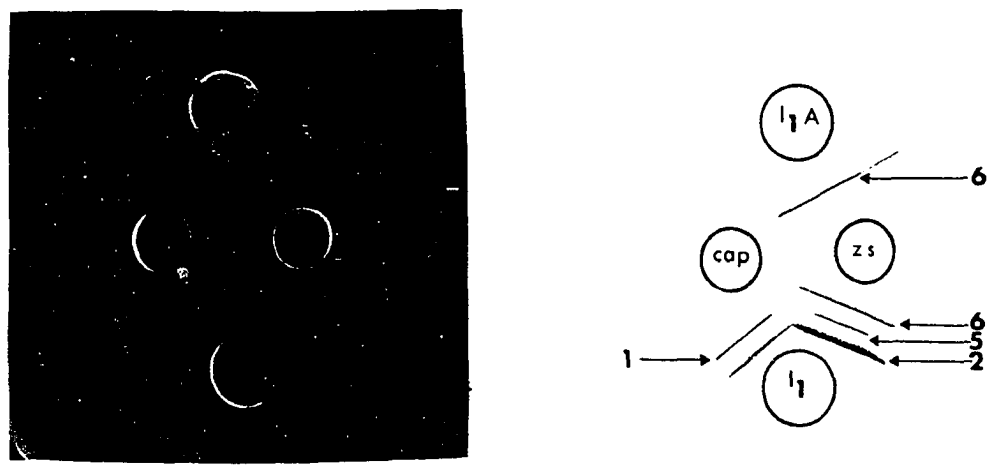


Figure 26. Effect of absorption with capsular polysaccharide on the precipitin band pattern seen with an antiserum to cell walls. cap=capsular polysaccharide, zs=zeolite supernate, I_1A=anti-cell wall absorbed with capsular polysaccharide, I_1=anti-cell wall.

walls only 2 precipitin bands are seen (Figures 24 and 25, respectively).

When antiserum to cell walls is adsorbed with whole cells (Figure 25) or with capsular polysaccharide (Figure 26), bands 2 and 5 to the zeolite supernate are removed, and bands 1 and 2 to the capsular polysaccharide are removed. Bands 1 and 2 form lines of identity with bands 1 and 2 found in the anti-whole cell system (Figures 23 and 24). Band 5, seen in all systems except antiserum prepared against cytoplasmic material, is removed by adsorbtion with whole cells or with capsular polysaccharide. Thus, it appears that at least 3 precipitin bands occur when antiserum whole cells or to cell walls is diffused against capsular polysaccharide, namely, bands 1, 2, and 5. Precipitin band 6 was not adsorbed out with whole cells or zeolite ghosts, nor was it removed with capsular polysaccharide or mannan.

It was shown previously that mannan formed a band of partial identity with the capsular polysaccharide (Figure 20), and that when capsular polysaccharide was subjected to mannan extraction the resulting 2 antigens formed bands of identity (Figure 21). It should be pointed out that these studies were made with antiserum to the zeolite ghosts. When mannan was diffused against antiserum to whole cells or cell walls it formed a band of identity with the capsular polysaccharide (Figures 27 and 28). Mannan formed a band of identity with band 2 seen in both the anti-whole cell- and the anti-cell wall-capsular polysaccharide systems. The data presented in Figures 27 and 28 also demonstrate that adsorption with whole encapsulated cells or capsular polysaccharide removes both bands 1 and 2. When the same antisera were absorbed with mannan, only the precipitin band to mannan was removed. These results

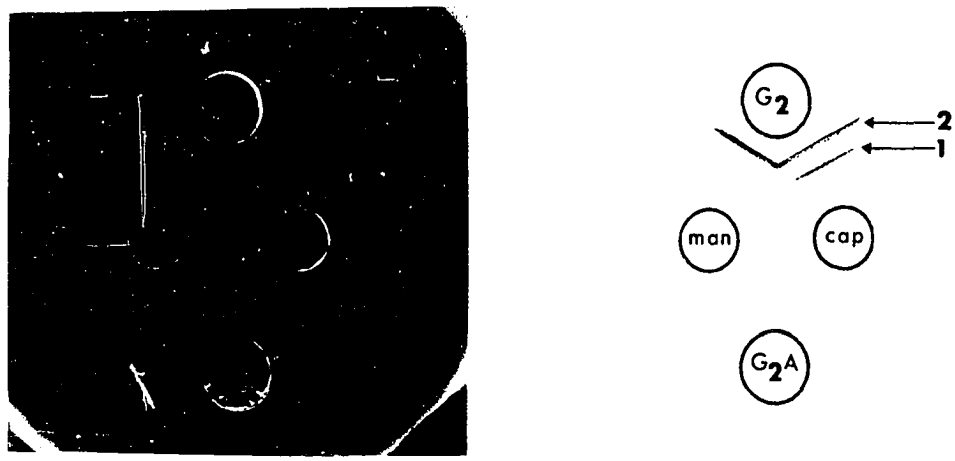


Figure 27. Results of adsorption of antiserum to whole cells with whole cells. cap=capsular polysaccharide, man=mannan, G_2A=anti-whole cell adsorbed with whole cells, G_2=anti-whole cell.

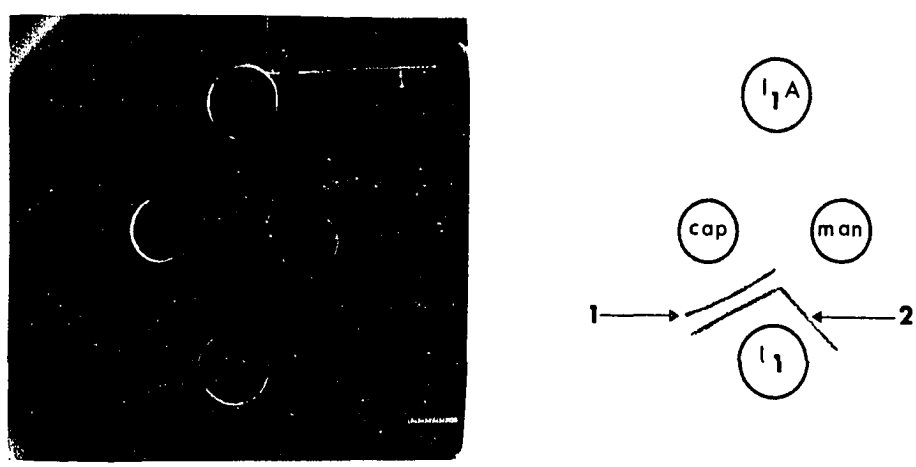


Figure 28. Results of absorption of antiserum to cell walls with capsular polysaccharide, cap=capsular polysaccharide, man=mannan, I_1A=anti-cell wall absorbed with capsular polysaccharide, I_1=anti-cell wall.

are shown in Figure 29. The same result is dramatically demonstrated in Figure 30 which involves experiments using antiserum to the zeolite ghosts. It can be seen that only the band formed with mannan is removed, whereas the bands obtained with the crude capsular polysaccharide and the phenol extract (water phase) are retained. Conversely, as seen in Figure 31, when antiserum to the zeolite ghosts is adsorbed with whole cells all precipitin bands to the polysaccharide antigens are removed. The same precipitin bands are removed by absorption with capsular polysaccharide. The results of adsorption with whole cells and with soluble antigens are summarized in Tables 19, 20, and 21.

The data presented in Table 21 clearly indicates that all of the whole cell agglutinating antibody was removed from serum by adsorbtion with whole cells or with capsular polysaccharide. Both encapsulated and decapsulated whole cells, as well as the zeolite ghosts, were equally effective in removing the agglutinating antibody. When the same antisera were absorbed with mannan, no decrease in agglutinating antibody titer resulted.

In an earlier phase of this investigation it was found that, when rabbits were immunized by the subcutaneous route (Tables 12 and 13) or the intraperitoneal route (Table 14), agglutinating antibody titers were relatively low compared with the levels seen in rabbits immunized by the intravenous route (Table 11). When the same antisera were examined by the agar gel double diffusion method, however, a maximum of 6 precipitin bands could be demonstrated in serum from rabbits immunized by the subcutaneous route. This was true in rabbits that had received decapsulated whole cells either alone or incorporated in Freund's

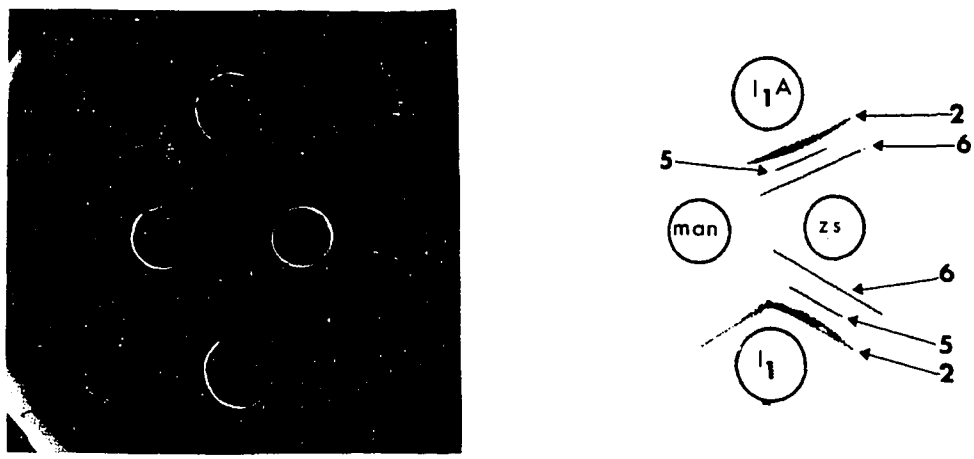


Figure 29. Effect of absorbtion of antiserum to cell wall with mannan diffused against mannan and zeolite supernate. man=mannan, zs= zeolite supernate, I_1A=anti-cell wall absorbed with mannan, I_1=anti-cell wall.

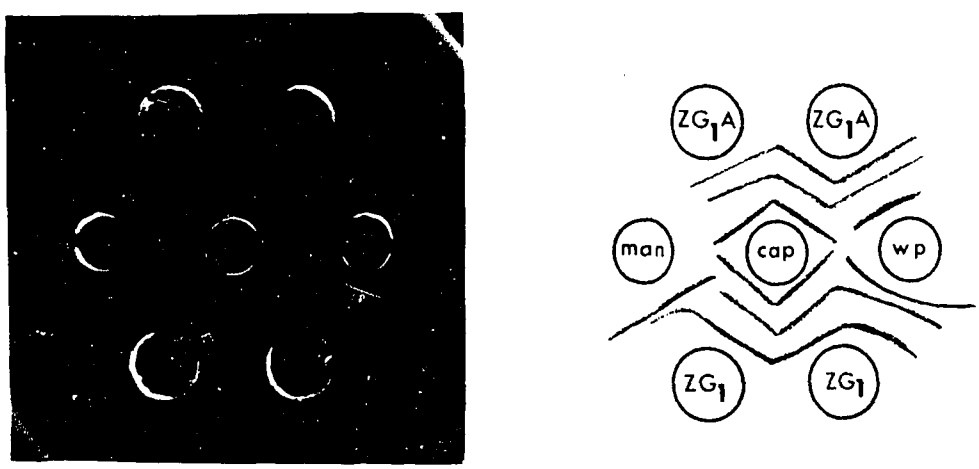


Figure 30. Results of absorption of antiserum to the zeolite ghosts absorbed with mannan. man=mannan, cap=capsular polysaccharide, wp=phenol extract (water phase), ZG_1A=anti-zeolite ghosts absorbed with mannan, ZG_1=anti-zeolite ghosts.

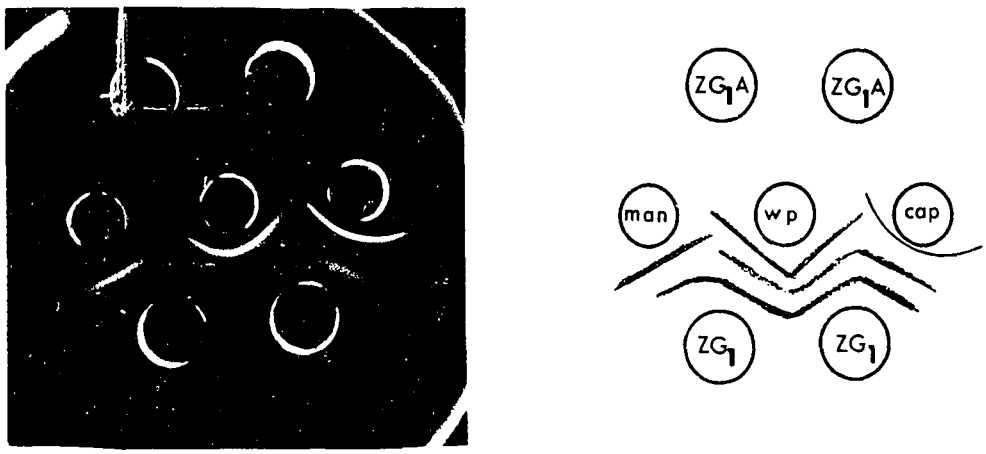


Figure 31. Results of adsorption of antiserum to zeolite ghosts with whole cells. man=mannan, wp=phenol extract (water phase), cap=capsular polysaccharide, ZG_1A=anti-zeolite ghosts adsorbed with whole cells, ZG_1=anti-zeolite ghosts.

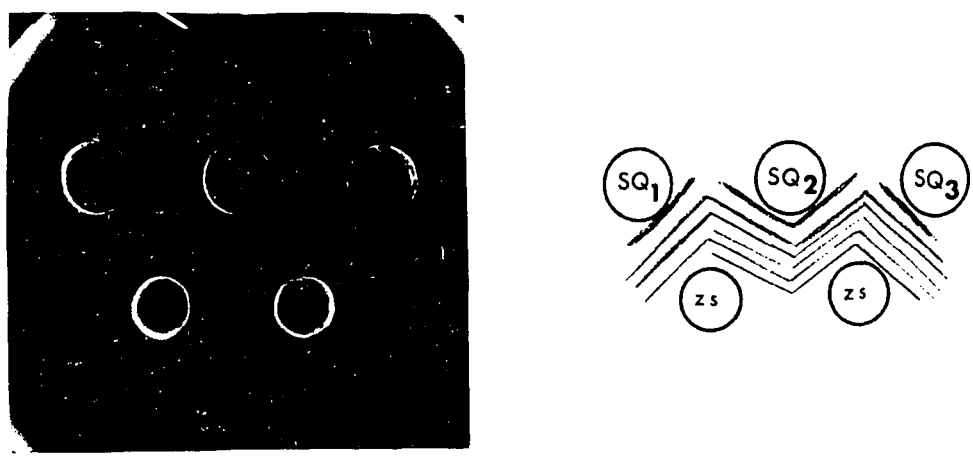


Figure 32. Precipitin band pattern obtained with antisera to whole cells obtained following immunization by the subcutaneous route. zs=zeolite supernate, SQ_1, SQ_2, SQ_3=antisera obtained from three individual rabbits.

TABLE 20

EFFECT OF ABSORPTION OF CRYPTOCOCCUS NEOFORMANS ANTISERA WITH CAPSULAR POLYSACCHARIDE ON PRECIPITATING ANTIBODY AS MEASURED BY AGAR GEL DIFFUSION

Antiserum To:	Rabbit Number	Precipitating Antigen					
		Zeolite Supernate		Capsular Polysaccharide		Mannan	
		Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed
Zeolite Ghosts	ZG_1	7[a]	4	3	0	2	0
	ZG_2	6	3	3	0	2	0
Whole Decapsulated Cells	G_2	6	4	2	0	1	0
	G_{10}	6	4	2	0	1	0
Cell Walls	I_1	3	1	2	0	1	0
	I_2	3	1	2	0	1	0
Cytoplasmic Material	J_1	4	4	0	0	0	0
	J_3	4	4	0	0	0	0

[a]Number of precipitin bands.

TABLE 21

EFFECT OF ADSORPTION OF CRYPTOCOCCUS NEOFORMANS ANTISERA WITH WHOLE CELLS, CAPSULAR POLYSACCHARIDE, OR MANNAN ON THE AGGLUTINATING ANTIBODY TITERS

Antiserum To:	Rabbit Number	Adsorbing Antigen: None	Whole Cells With Capsule	Capsular Polysaccharide	Mannan
Zeolite Ghosts	ZG_1	1:2048[a]	0	0	1:2048
	ZG_2	1:8192	0	0	1:8196
Whole Decapsulated Cells	G_2	1:128	0	0	1:128
	G_{10}	1:256	0	0	1:256
Whole Encapsulated Cells	E_1	1:16	0	0	1:16
	E_2	1:4	0	0	1:4
Cell Walls	I_1	1:32	0	0	1:32
	I_2	1:16	0	0	1:16
Sonicated Encapsulated Cells	F_1	1:2	0	0	1:2
	F_2	1:4	0	0	1:4
Sonicated Decapsulated Cells	H_2	1:4	0	0	1:4
	H_3	1:2	0	0	1:2
Cytoplasmic Material	J_1	0	0	0	0
	J_3	0	0	0	0

[a]Titer

complete adjuvant (Table 12). A representative double diffusion plate showing the precipitin band patterns seen with 3 of these antisera is shown in Figure 32.

As shown in Table 13, when rabbits were immunized with cell wall incorporated in Freund's complete adjuvant, no agglutinating or PCA antibody could be detected. However, 1 precipitin band was seen when these antisera were diffused against the zeolite supernate. This precipitin band formed a band of identity with band 6 of sera from rabbits immunized intravenously with whole cells or with cell walls. Band 6 was the first band to appear, and it could be demonstrated the first week after intravenous immunization. These data are presented in Figure 33.

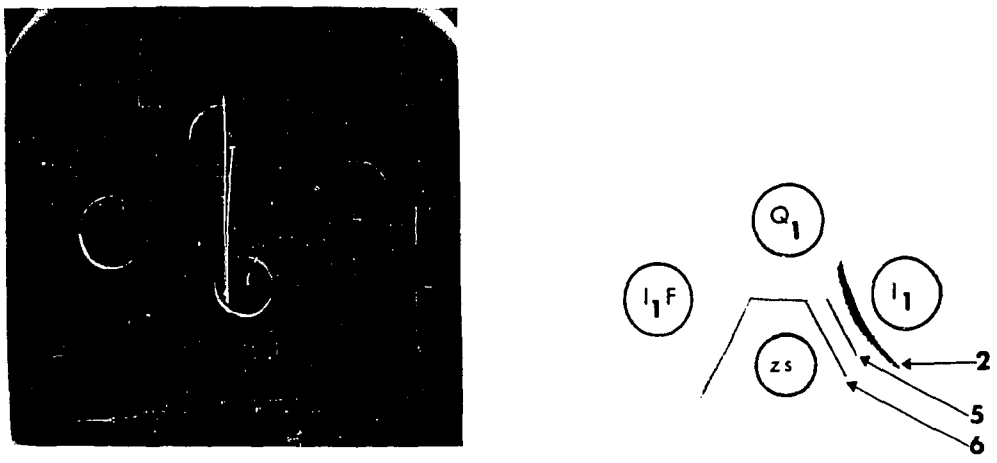


Figure 33. Agar double diffusion plate comparing antisera to cell wall prepared by immunizing rabbits subcutaneously and intravenously, zs=zeolite supernate, I_1F=anti-cell wall obtained during the first week of intravenous immunization, Q_1=anti-cell wall obtained by subcutaneous injection, I_1=anti-cell wall obtained during the last week of intravenous immunization.

CHAPTER IV

DISCUSSION

Several methods have been developed for the disruption of bacterial and yeast cells, for example grinding with powdered glass or alumina, shaking with glass beads, extrusion of cells through a small orifice under high pressure, alternate freezing and thawing, and exposure to ultrasonic energy. Most of these methods have broad, but not universal, application to yeast cells and several of them require rather involved procedures or expensive apparatus. In general, it is not known whether these procedures have any effect on the antigenicity of the isolated cell fractions. The early phases of the present studies were designed to evaluate various procedures for the isolation of cell fractions from C. neoformans.

The first attempts to isolated particulate and soluble antigens from C. neoformans made use of sonic oscillation and Braun homogenization. It was found that these methods were not practical for the isolation of cell walls in large quantities due to autoagglutination of the broken cells which made them inseparable from the whole cells and glass beads. The same problem has been reported with Mycobacterium and Streptomyces after disintegration in the Mickle apparatus (Salton, 1964). With the hope of isolating a cell wall fraction in large quantities and of obtaining a serologically active soluble fraction, the synthetic zeolite

method was evaluated.

The zeolite-water interaction is an exothermic reaction, therefore, the entire procedure was accomplished in the cold in order to minimize any effect of heat on the cryptococcal antigens. Treatment with zeolite appears to be a milder procedure than sonication or Braun homogenization. With the zeolite method, heat is kept to a minimum and violent agitation and foaming are eliminated. With sonication and Braun homogenization, heating and foaming are characteristics difficult to control and probably result in some denaturization of cellular proteins. The zeolite procedure is accomplished at a pH of 10 to 11, which has a stabilizing effect on the extracted proteins by inhibiting the acti n of cellular proteinases usually active at a much lower pH. Auto-agglutination is kept at a minimum, probably due to the cationic exchange properties of zeolite, specifically the removal of divalent cations, and to the fact that the cell walls or ghosts are not broken into small fragments and hydrophobic groupings from the interior of the cells are not exposed.

The mechanism by which zeolite ruptures cell envelopes is unknown. The process is not entirely mechanical since only a small amount of grinding is necessary. Wet zeolite is not effective, even with grinding. The ineffectiveness of wet zeolite suggests that hygroscopic properties of zeolite are involved. It can be postulated that zeolite particles have a shearing affect which causes small ruptures in the cell envelope, and that due to the hygroscopic nature of zeolite, the soluble cell contents are forced out of the cells.

Although considerable work by many investigators has led to

some understanding of the chemical nature of yeasts, especially Saccharomyces cerevisiae. Little is known about the composition of C. neoformans than that its capsule is composed of xylose, glucuronic acid, galactose, and mannose (Evans and Mehl, 1951). Recently Cook, Felton, and Muchmore (1967) reported that a strain of C. neoformans obtained from a soil sample differed from a strain isolated from a human case of cryptococcosis in animal virulence and in cell wall composition. Chemical analysis of the cell wall fractions showed quantitative differences in the hexose, hexosamine, protein, and lipid content of the 2 strains. From these results, the authors concluded that virulence of C. neoformans may be related to cell wall composition.

The cell walls of yeasts contain soluble and insoluble polysaccharide-protein complexes, plus, essentially the same amino acids found in bacteria (Kessler and Nickerson, 1959; Nickerson, 1963). The lipid content of purified cell wall material from 3 strains of Candida albicans and 2 strains of S. cerevisiae was shown to be quite variable, ranging from 1.09 to 10.2 per cent of the dry weight. This variability was also reflected in the total nitrogen and total carbohydrate content of the same strains. The total nitrogen content of isolated cell walls ranged from 1.28 to 9.65 per cent of the dry weight and the total carbohydrate content varied from 60 to 75 per cent of the dry weight. The same investigators reported that glucosamine nitrogen comprised approximately 5 per cent of the total nitrogen content of isolated cell walls. The same variability has been seen in cell walls isolated from the yeast phase of pathogenic strains of Histoplasma capsulatum (Pine, et al., 1966). Thus, the total protein and carbohydrate values for C. neoformans

cell walls detected in these investigations are consistent with those obtained with other yeast cells. The high content of protein in the zeolite ghosts may be due to the presence of contaminating cytoplasmic membranes. Cell ghosts were not as completely disrupted and possibly the cell membrane is retained. It seems probable that contaminating nucleic acids could contribute very little to the high nitrogen values, since ultra-violet analysis demonstrated the absence of any large quantity of material absorbing at 260 mu.

Paper chromatography was used to detect capsular polysaccharide in the various antigens. Polysaccharide was detected in all material except the cell wall fraction. It was shown, however, that antiserum to cell walls formed 2 precipitin bands with capsular polysaccharide isolated from whole cells. Thus, it can be concluded that capsular polysaccharide was present in the cell wall fraction, but probably in very small quantities. This finding is consistent with the observation by Gadebusch (1960) that the antigenic material resides in the capsular polysaccharide that is closely bound to the cell wall.

The absence of hexosamine in C. neoformans, as reported in this investigation, is inconsistent with the results reported from studies of other yeasts. However, it is highly possible that the unidentified spot seen on chromatograms might have been glucosamine. It should be pointed out that the mild acid hydrolysis used may not have been vigorous enough to release the hexosamine from the cell or cell wall components. Also, another chromatographic system might have revealed hexosamine.

In the present study, isolated cell walls of C. neoformans were subjected to hydrolysis by purified chitinase. No N-acetylglucosamine

was liberated from the cell wall fraction, suggesting the absence of chitin. However, chitin has been reported to be a cell wall constituent of some yeast cell walls (Kessler and Nickerson, 1959; Nickerson, 1963).

It is known that antigenicity varies with the morphological characteristics or phase of certain fungi. In C. neoformans small capsule strains are more antigenic in rabbits (Kase and Metzger, 1962; Neill, et al., 1950) and induce stronger resistance in mice than do large capsule strains (Abrahams and Gilleran, 1960). An increase in antigenicity is seen when the capsule of a large capsule strain is degraded enzymatically (Gadebusch, 1960). In the present study, decapsulated forms of C. neoformans were more effective immunizing agents than were highly encapsulated forms. It was also found that zeolite ghosts were the most effective antigen for stimulating the production of agglutinating antibody, probably because capsular polysaccharide was eliminated by the zeolite treatment. These observations are consistent with those of Neill et al., (1950) and of Kase and Metzger (1962). Kase and Marshall (1960) reported that 10 consecutive injections of a formalinized, cryptococcal whole cell vaccine would elicit a significant antibody response in rabbits. In contrast, it was found in the present study that at least 25 injections were necessary to obtain regularly antisera of adequate titer. This may have been due to the small quantities of antigen used. In general, optimal agglutinating antibody titers were obtained 6 to 8 days after the last injection of antigen and peak titers fell off quite rapidly, usually within 3 to 5 days. These results are similar to those reported for mice vaccinated with multiple doses of killed C. neoformans in which survival time was

longer when the animals were challenged after 7 to 14 days than when they were challenged after 21 days (Abrahams and Gilleran, 1960). No available data indicates a correlation between agglutinating antibody and immunity to infection.

In general, lower agglutinating antibody titers occurred with the sonicated antigens than with intact cells, regardless of whether the sonicate was prepared from encapsulated or decapsulated cells. This might be explained by the fact that, with the sonicated antigen a large amount of polysaccharide was injected at one time, whereas with the whole cell antigens the soluble polysaccharide was probably released from the cells slowly. Rabbits immunized with cytoplasmic material did not produce any detectable agglutinating antibody, however, these antisera reacted with the zeolite supernate in agar gel diffusion tests. Absence of a significant agglutinating antibody response to the sonicated and cytoplasmic antigens may have been caused by separation of the haptenic polysaccharide from its protein carrier during sonication. Rabbits injected with capsular polysaccharide, intravenously or subcutaneously, in doses ranging from 0.36 ug to 25 mg failed to demonstate antibody response as measured by the PCA and agglutination tests. The finding that capsular polysaccharide is not a complete antigen supports the results of Cozad, et al., (1963) and of Goren (1966, 1967). Goren reported that purified polysaccharide from C. neoformans is antigenic in mice only if it is conjugated to bovine gamma globulin. Gadebusch (1958a, 1963, 1964) however reported that purified capsular polysaccharide is antigenic in mice, rats, and rabbits. It should be pointed out that, the hemagglutinating antibody titers obtained by

Gadebusch were relatively low, i.e. in the range of 1:10 to 1:40. It has been reported by Abrahams (1966) that detection of cryptococcal antibody by the passive hemagglutination test is difficult to duplicate, and proved unsuccessful in his hands.

The relationship of polysaccharide antigens to immunity in cryptococcosis remains to be elucidated. In general, animals immunized with homologous antigens, whether particulate or soluble, containing a high content of capsular polysaccharide elicit minimal antibody responses. Several possible explanations for this come to mind. First, the capsule may inhibit phagocytosis of cryptococcal cells; secondly, the free capsular polysaccharide may combine with antibody in vivo as rapidly as it is formed; thirdly, the capsule may have a toxic effect on the reticuloendothelial system; finally, capsular polysaccharide may cause the host to be in a specific state of immune tolerance. Support for the first suggestion has come from the studies of Bulmer and Sans (1967, 1968). The latter three possibilities have not been thoroughly investigated.

If capsular polysaccharide has a toxic effect on the reticuloendothelial system it seems reasonable to believe that it should inhibit antibody production to both heterologous and homologous antigens. In the present studies, 5 to 25 mg capsular polysaccharide had no effect on the antibody response of rabbits to heterologous antigens such as typhoid vaccine and bovine serum albumin. These dosages of capsular polysaccharide were comparable to those received by rabbits during the course of immunization with encapsulated cryptococci.

All rabbits immunized with decapsulated whole cells plus added

capsular polysaccharide died of systemic anaphylaxis. Anaphylactic shock is ordinarily more difficult to elicit in the rabbit than in the guinea pig. In rabbits several doses of antigen may be required and, even then, many animals may not die. Grove (1932) found that about 25 per cent of rabbits are resistant to sensitization with various antigens and that a prerequisite for anaphylaxis in this species is a high precipitating antibody titer to the homologous antigen. Delayed shock is common and animals undergoing immunization may succumb several hours after an injection of antigen (Cushing and Campbell, 1957; Smith; Conant, and Overman, 1964). It thus appears that the anaphylaxis seen in the present investigation was consistent with that reported previously in the rabbit.

Rabbits injected with the decapsulated whole cell-capsular polysaccharide mixture had significant antibody responses compared with those receiving encapsulated organisms. These results would suggest that soluble capsular polysaccharide, in the dosages used, had no effect on antibody production to the homologous decapsulated organisms. If the mechanism of action of capsular polysaccharide is purely antiphagocytic, it is natural to assume that it functions only in the intact form, i.e. on the encapsulated organism. This assumption, however, is contrary to the results of <u>in vitro</u> studies which showed that both soluble and intact capsular polysaccharides are highly anti-phagocytic (Bulmer and Sans, 1967, 1968). It should be pointed out, however, that the latter system is not entirely comparable with that used in the present study. Also, it has since been found that the antiphagocytic effect of the capsule can be demonstrated in the <u>in vitro</u> phago-

cytic system (Farhi, 1969). For example, polymorphonuclear leukocytes exhibit a 74 per cent phagocytic index (the number of C. neoformans/ leukocyte) with viable nonencapsulated organism, whereas, with formalinized, nonencapsulated cells an index of 91 per cent was obtained. When capsular polysaccharide was added back to the phagocytic system, 22 per cent and 90 per cent phagocytosis were found, respectively. It would appear, therefore, that the present results correlate with those of the in vitro phagocytic studies.

Gadebusch (1964) demonstrated that when rabbits are injected with 25 mg capsular polysaccharide it can be detected up to 6 months later by hemagglutination tests. In the present studies, capsular polysaccharide was detected in sera of rabbits that had received the polysaccharide either alone or together with BSA, typhoid "H" vaccine, or decapsulated cells. With 5 out of 10 of the latter animals it was possible to detect capsular polysaccharide antibody and capsular polysaccharide in the same serum simultaneously. This was especially true in those rabbits with high agglutinating antibody titers.
The most plausible explanation for these results would be that antibody combines with antigen in vivo as rapidly as it is formed. As antibody is formed, it is gradually complexed with capsular polysaccharide until a slight antibody excess is reached, at which time anaphylaxis occurs. If soluble antigen-antibody complexes are present in serum prior to anaphylaxis, it should be possible to detect both antigen and antibody together in the same serum.

It has been shown that, with rabbit antiserum, 0.003 ug antibody nitrogen is sufficient to provoke weak passive cutaneous anaphylax-

is when the latent period is 3 to 6 hr and the serum has a high concentration of antibody (Ovary, 1958). More antibody nitrogen is needed when weaker antisera are employed because the normal gamma globulin competes with the specific antibody gamma globulin in the fixation process. Ovary and Karush (1961) demonstrated that the antibody site necessary for fixation is located on Porter's fraction III of papain digested rabbit antibody. Not all antisera can sensitize guinea pig skin. While rabbit and guinea pig antisera containing as little as 0.003 ug antibody nitrogen in 0.1 ml may sensitize guinea pig skin, about 10 to 25 times more is needed when mouse antisera are used (Ovary and Karusch, 1961) and 50 to 100 times more is needed with rat antisera (Ovary, 1964). Human antisera can also sensitize guinea pig skin (Ovary, 1964). It has been shown that gamma globulin with a molecular weight of 160,000 and a sedimentation constant of 7S, but not 19S gamma globulin, is able to sensitize guinea pig skin (Ovary, Fudenberg, and Kunkel, 1960). Thus, a positive PCA test is indicative of the presence of 7S antibody.

From the data presented in Figure 13, it is evident that, following immunization of rabbits with decapsulated whole cells, there is a differential rise in 7S and 19S antibody. During the first week, there was clearly no PCA detectable 7S antibody, whereas the agglutinating antibody, probably of the 19S type, had reached a titer of 1:4. This difference is even more significant when one considers that the PCA test is approximately 30 times more sensitive than the whole cell agglutination test and that undiluted antiserum gave no PCA reaction. During the second week, there appeared to be a mixture of 7S and 19S

antibodies. When the sensitivities of the tests are considered, it seems quite obvious that the 19S antibody still comprised the major proportion of the cryptococcal antibodies present in the serum. During the third week 7S antibody appeared to surpass that of the 19S type. From these results, it can be concluded that a classical, early 19S antibody response, followed by a later 7S response, occurs in rabbits immunized with cryptococcal whole cells, as evidenced by the agglutinating and PCA antibody tests.

The effect of the route of injection on the response of animals to killed C. neoformans is unknown. Abrahams and Gilleran (1960) reported that the intraperitoneal route was superior to the subcutaneous route, but the vaccinating schedule was not comparable to other reports in the literature, therefore the data are difficult to compare. The effect of adjuvants on the immune response to C. neoformans is relatively unknown. The administration of Bordetella pertusis in conjunction with cryptococcal vaccine has been reported to increase the survival time of mice after challenge with virulent C. neoformans (Abrahams, 1966). Conversely, in the same study, use of Freund's complete adjuvant with killed cells of C. neoformans suppressed the development of immunity. These results were not referrable to any detectable antibody response.

In the present investigation, it was clearly shown that the intravenous route appears to be superior for the production of both agglutinating and precipitating antibody to C. neoformans. Subcutaneous injection, either with or without Freund's complete adjuvant, appeared to be comparable to the intravenous route in stimulating the production of precipitating antibody, but the agglutinating antibody titer was

relatively low. The intraperitoneal route appeared to be inferior to both subcutaneous and intravenous routes with respect to both agglutinating and precipitating antibody. These results are consistent with those reported for Histoplasma capsulatum where an intensive schedule of immunization was found to be necessary for the production of a high titer of precipitating antibody (Markowitz, 1967). Antibody response was poor when rabbits were immunized by the intraperitoneal or subcutaneous route.

A survey of the literature revealed that virtually no characterization of the antigenic components of C. neoformans has been reported. A study by Kaufman and Blumer (1965) revealed considerable variability among rabbits immunized with formalinized cryptococcal whole cells as to agglutinating antibody titers and the number of precipitin bands obtained using a sonicate as antigen. They were able to obtain only 1 to 3 precipitin bands. In the present study, a maximum of 3 precipitin bands was also obtained with antisera to C. neoformans whole cells using a sonicate as antigen. However, when the zeolite supernate was used as an antigen in the double diffusion tests, 6 to 7 precipitin bands were formed as compared with 2 to 3 bands when the culture filtrate or the Braun homogenate was used. As described above the zeolite procedure appears to have a stabilizing effect on the antigens extracted by the grinding procedure.

Capsular polysaccharide isolated from C. neoformans by phenol extraction, sonication, or by simple elution, exhibited the same antigenic specificity as crude capsular polysaccharide. The 3 precipitin bands obtained with culture filtrate appear to be identical with the 3

bands obtained with capsular polysaccharide. Mannan isolated from whole cells formed a band of partial identity with crude capsular polysaccharide. Further studies revealed that when antisera were absorbed with capsular polysaccharide or whole cells the precipitin band to mannan was removed. Absorption with mannan removed the homologous precipitin band, but it did not remove the band of partial identity with the capsular polysaccharide. An explanation for this finding could be that capsular polysaccharide antigen contains at least 1 or more antigenic groupings different from those in the mannan fraction. When antisera to whole cells and cell walls were absorbed with mannan there was no change in the agglutinating antibody titers, but agglutinating antibodies were completely removed by absorption with capsular polysaccharide. These results suggest that mannan is a component of the capsule closely associated with the cell wall, presumably the backbone structure of the capsule. It should be noted that, chromatographically, mannan is composed of all of the elements of the capsule except galactose.

When the zeolite ghosts were subjected to mannan extraction, 3 precipitin bands were obtained. The difference between mannan isolated from the zeolite ghosts and that obtained from whole cells may be related (due) to the zeolite treatment in which only those portions of the capsule which project outward may be removed. When the cells are then subjected to the vigorous mannan extraction, the backbone of the capsule is extracted in fragments. It is also possible that mannan extraction may cause the release of other polysaccharides.

Blandamer and Danishefsky (1966), and Farhi (1969) suggested that the capsular polysaccharide of C. neoformans consists of a mannan

backbone with branches of zylose, glucuronic acid, and galactose. Graded hydrolysis of the capsular polysaccharide revealed that mannose was liberated slowly and zylose, rapidly. This could indicate that zylose was located on the external side chains in the mannan backbone. It is possible that the galactose end groups are turned inward, holding the mannan backbone into the cell wall. This hypothesis would be consistent with the finding that mannan isolated from the zeolite ghosts, but not that from whole cells, contains galactose.

Two precipitin bands (D and E, Figure 21) of the capsular polysaccharide were removed when capsular polysaccharide was subjected to the mannan extraction. The remaining band (A) then formed a line of identity with mannan isolated from the whole cells. After mannan treatment the capsular polysaccharide lost the galactose spot on the chromatogram. Other supporting evidence for the suggestion that the antigenic component of the capsule resides in close association with the cell wall was obtained by adsorption studies. Antisera to zeolite ghosts, cell walls, and decapsulated whole cells all agglutinated encapsulated whole cells equally well. The ability of the antisera to agglutinate encapsulated whole cells was lost upon adsorption with encapsulated or decapsulated whole cells or with zeolite ghosts. These results support the hypothesis that the capsule of C. neoformans is a soluble extension of the antigenic component found in the cell wall.

In the preliminary work dealing with the total antigenic composition of cryptococcal cells, it was found that antisera to zeolite ghosts and antisera to either encapsulated or decapsulated whole cells were not comparable, at least insofar as the number of precipitin bands

was concerned. Therefore, antisera to the zeolite ghosts were not included in the antigenic analysis because they probably did not reflect the true picture of the intact organisms. These antisera were used, however, in part of the work on antigenic specificity.

A total of 8 precipitin bands was obtained in the antigenic analysis. Six of these bands were found in the anti-whole cell system and 2 of them formed bands of identity with anti-cytoplasmic material. Two other precipitin bands (7 and 8) were peculiar to the cytoplasmic material. Bands 3, 4, 7, and 8 were removed from the system when the zeolite supernate was heated for 30 min at 100 C, an indication that they were probably protein in nature. Four precipitin bands were formed with antisera to cell wall. Three bands resulted with the anti-cell wall-zeolite supernate system and 2 bands were seen when capsular polysaccharide was diffused against antiserum to cell wall. However, 1 of these 2 bands (2) formed a band of identity, whereas bands 1 and 5 did not. Thus, it can be concluded that band 5 is peculiar to the cell wall. One precipitin band (6) was common to all antisera diffused against the zeolite supernate. At first it was thought that this band resulted from a non-specific precipitation such as occurs when phosphate buffers are used in double diffusion tests, but this was not the case since it was found that the band was heat stable. It is postulated that, due to the ubiquity of this precipitin band, it most probably represents a soluble component of the cytoplasmic membrane. From these observations it is concluded that the capsular polysaccharide and the cytoplasmic material contributed 3 antigens each and that the cell wall and cytoplasmic membrane contributed 1 antigen each. Nevertheless it is

possible that not all of the soluble antigens, supposedly cytoplasmic in origin, may actually have been derived from the cytoplasm. It is just as likely that some may be solubilized proteins from either the cell wall or cytoplasmic membrane.

CHAPTER V

SUMMARY

Methods of cell disruption evaluated in this study were sonic oscillation, Braun homogenization, and the synthetic zeolite method. Treatment of cryptococcal cells by sonic oscillation resulted in approximately 80 per cent breakage, whereas only 20 to 30 per cent cell disruption was obtained by Braun homogenization. The zeolite procedure was found to be superior, both for disruption of cryptococcal cells and for isolation of antigenically and serologically reactive cell components.

In the present study, decapsulated forms of C. neoformans were more effective immunizing agents than highly encapsulated forms. It was also found that zeolite ghosts were the most effective antigen for stimulating the production of agglutinating antibody. In general, lower agglutinating antibody titers were obtained by immunizing with the sonicated antigens than with intact cells, regardless of whether the sonicate was prepared from encapsulated or decapsulated cells. Also, rabbits immunized with cytoplasmic material did not produce detectable agglutinating antibody, however, precipitating antibody was detected.

Antisera to zeolite ghosts, cell walls, and decapsulated whole cells agglutinated encapsulated whole cells equally well. The ability of the antisera to agglutinate encapsulated whole cells was lost upon

adsorption with encapsulated or decapsulated whole cells or with zeolite ghosts. These results support the hypothesis that the capsule of C. neoformans is a soluble extension of the antigenic component found in the cell wall.

Capsular polysaccharide was removed from encapsulated cells by sonic oscillation and partially purified by ethanol precipitation. This material was found to be haptenic in nature. Capsular polysaccharide appeared to have no toxic effect on the reticuloendothelial system.

Intravenous injection of antigen proved to be superior to that administered by subcutaneous or intraperitoneal routes. Optimal agglutinating antibody titers were obtained 6 to 8 days after the last intravenous injection. A classical, early 19S antibody response was followed by a later 7S response in rabbits immunized intravenously with whole cells.

A maximum of 3 precipitin bands were obtained when antisera to C. neoformans whole cells was diffused against a sonicated cell antigen. However, when the zeolite supernate was used as antigen in the double diffusion tests, 6 to 7 precipitin bands were observed. Use of the culture filtrate or the Braun homogenate antigen resulted in only 2 to 3 precipitin bands.

A total of 8 precipitin bands were obtained in the antigenic analysis. Six of these bands were found in the anti-whole cell system and 2 of them formed bands of identity with anti-cytoplasmic material. Two other precipitin bands were peculiar to the cytoplasmic material and 1 band was cell wall associated. Thus, it was concluded that the capsular polysaccharide and the cytoplasmic material contributed 3

antigens each, while the cell wall and cytoplasmic membrane contributed 1 antigen each.

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