THE CHEMICAL, SEROLOGIC AND SKIN TEST ACTIVITIES OF POLYSACCHARIDES EXTRACTED FROM HISTOPLASMA CAPSULATUM AND BLASTOMYCES DERMATITIDIS

by

Ralph A. Knight

A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Bacteriology

University of Utah

August, 1958

This Thesis for the Ph. D. degree

by

Ralph A. Knight

has been approved by

Chairman, ervisory Committee

er, Committee

Head, Major Department

The author wishes to express his appreciation to Dr. Stanley Marcus for his guidance and encouragement, and Dr. Louis P. Gebhardt, Dr. Paul S. Nicholes and Dr. John G. Bachtold for their suggestions and criticisms.

Grateful acknowledgement is made to the Veterans Administration Hospital, Salt Lake City, Utah, for the use of hospital facilities necessary to conduct this investigation.

This investigation was supported in part by research grant E-922 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service and in part by a research grant from the Office of Naval Research.

TABLE OF CONTENTS

TABLES AND FIGURES

Tables

Figures

REVIEW OF LITERATURE

I. Introduction

The skin test is a widely employed method for the demonstration of past or present infection with respiratory or systemic mycotic invaders. The terms histoplasmin and blastomycin are currently used to indicate the filtrate derived from the prolonged growth of the mycelial phase of *Histoplasma capsulatum* and *Blastomyces dermatitidis* respectively in liquid media. Unfortunately, it is not clear from reports of many investigations on histoplasmin and blastomycin skin tests, whether the product and the given dilutions are comparable to any other histoplasmin or blastomycin preparations. The National Institutes of Health have partially resolved this problem by preparing standard histoplasmin for comparative studies and by publishing regulations that must be satisfied before a histoplasmin product may be marketed. However, similar standardization of blastomycin and coccidioidin has not been achieved. The working hypothesis was proposed, that if a known weight of a mycotic skin test antigen could be employed diagnostically, this substance would have the same advantages that P.P.D. has in tuberculosis.

In the following literature review, investigations are considered which were designed to isolate and study serological, skin test and/or chemical properties of polysaccharides derived from *H. capsulatum* and *B. dermatitidis*.

II. Histoplasmosis

A. *Introduction.* Histoplasmosis is a fungus infection caused by *Histoplasma capsulatum*. Manifestations of this disease range from minute localized lesions to chronic or acute generalized systemic disease. Since the organisms, once phagocytized, persist in the phagocytic cells of the systemically infected individual, the infection is said to have a predilection for the cells of the reticulo-endothelial system. Asymptomatic pulmonary involvement is not unusual. The disseminated disease yields a mortality rate of 50 to 98 per cent. The fungus is widely distributed and histoplasmosis is particularly prevalent in the central part of the United States.

B. *History.* Darling (1) described the first case of histoplasmosis in 1906. At the time of this discovery, Darling was searching for leishmaniasis in Panama. He observed, in necropsy specimens from a Martinique negro, small, round, capsulated organisms. Darling recognized that these organisms were not Leishman-Donovan bodies. He concluded, from his morphological study, that these organisms were protozoa and proposed the name

Histoplasma capsulatum. In 1908, and again in 1909, Darling reported (2) similar organisms which he observed in tissues from another negro and a Chinese shopkeeper.

The mycotic, rather than the protozoan, nature of this organism was reported by DeMonbreun (3), in 1934. He was able to culture the organisms from a human infection, and also from an infected dog.

C. Epidemiology. For a long period after the recognition of the disease by Darling, histoplasmosis was believed to be a rare tropical disease. Following Darling's investigation, further cases were not reported until 1926.

The studies of C. E. Smith, et al., on coccidioidomycosis and particularly on the use of coccidioidin (4) for epidemiologic surveys, aroused the interest of investigators in other parts of the United States. Christie and Peterson (5), in 1945, were conducting tuberculosis surveys among children in Tennessee. During the course of the survey, they found 181 children with negative tuberculin skin tests, but radiologic evidence of lung lesions. These investigators tested the 181 children with coccidioidin and found no markedly positive reactions. The same children were also skin tested with a broth filtrate from the growth of H. capsulatum, and a high percentage of reactors were noted. Christie and Peterson concluded from this study that there was an immunogenic relationship between the observed pulmonary calcifications and histoplasmosis. During this same year, Palmer (6) reported a study of tuberculin and histoplasmin skin tests on 3,000 nurses in Detroit, Kansas City, Minneapolis, and Philadelphia. He concluded from these studies that: (1) histoplasmosis was a widespread subclinical infection, (2) persons from some states had a higher rate of infection than persons in other states, (3) that many of the lung calcifications were due to H. capsulatum and not Mycobacterium tuberculosis. Many other investigators (7-17) have extended the knowledge of the epidemiology of human histoplasmosis. This infection has been reported from many parts of the world (18-21), but an endemic focus of histoplasmosis was found in midland United States, extending the length of the Mississippi River Valley.

Investigations of local outbreaks of histoplasmosis, involving family size units, or small groups of persons, have been reported (22-23). The isolation of H. capsulatum from these families and the indirect incrimination of dust as the source of the infection added to the possibility that soil might harbor H. capsulatum. The missing link in the life cycle of H. capsulatum, the isolation of the mycelial spores from soil, was reported by Emmons (24), in 1949. Others (25-32) have confirmed and extended these results. These investigators have been most successful in isolating *H. capsulatum* spores from soil samples in or near chicken coops. Chickens, however, are not the reservoir of *H. capsulatum*. Many hypotheses have been suggested to explain histoplasmin sensitivity. Recently, Zeidberg (33) reported a correlation between a world occurrence of red-yellow podzolic soils and histoplasmin sensitivity. The ability of the red-yellow podzolic soil to support the growth of *H. capsulatum* has not been reported.

Evidence of a natural animal reservoir has never been clearly shown, although *H. capsulatum* has been isolated from dogs, cats, skunks, cattle, rats, mice, horses, foxes, and woodchucks (34-40). The dog, and possibly the cat, have been examined in sufficient numbers to indicate that they might act as a reservoir for the infection. Since these animals live in close association with man, it is conceivable that dogs and cats may be infected from humans. It is also possible that man and his domestic animals are infected from a common source, such as soil. The association of *H. capsulatum* with soil in and around chicken coops, even though chickens cannot be shown to act as carriers of the disease, suggests rats or mice as a possible animal reservoir. Apparently, however, less than one percent of mice and rats have been found infected. A study of rodents indigenous to chicken coops might be of value. Until further studies on the animal or insect reservoir and growth factors within the chicken manure are made, histoplasmosis must be assumed to be contracted from the inhalation of spores of *H. capsulatum* that are present in dust from chicken coops. How these spores get into the soil, and the length of time these spores remain viable in these soils is not yet known.

There is no significant evidence to suggest that pulmonary histoplasmosis is transmissable between humans.

D. Experimental Histoplasmosis. Investigations in the field of experimentally induced *H. capsulatum* infections have revolved around the following three basic problems:

(1) Search for susceptible laboratory animals;
(2) Methods for quantitating the number of organisms injected into these animals;
(3) Search for a route of inoculation that would yield reproducible morbidity and mortality.

Obviously, these problems are interrelated.

Dogs, rats, hamsters, guinea pigs, monkeys, and mice have been used as experimental animals (41-44). White mice appear to be the most useful experimental animal. Variations in the susceptibility of different mouse strains have been reported (45), thus the choice of strains or species of mice is important in experimental work.

Usually, the dose of organisms injected is reported as a dilution of the original suspension. The assumption made is that one dilution of 1-100 equals any other dilution of 1-100, with respect to the number of viable organisms. Howell, _et al._, (46) have published a method in which viable plate counts are made from an aliquot sample of the injecting suspension. A more direct method for standardization of _H. capsulatum_ suspensions has been most recently described by Marcus and Rambo (47) and Rowley and Huber (48). As is usual by the method employed, counts are made of the numbers of cells in a hemocytometer. The total number of _H. capsulatum_ per ml is then calculated.

Massive numbers of _H. capsulatum_ are necessary to kill mice when they are injected intraperitoneally. Gastric mucin has been employed as an adjuvant for inducing reproducible infection via the intraperitoneal route in mice (49). The intracerebral route of challenge has been reported as a useful method of inoculating mice (50). The small volume of inoculum (.02 ml) limits the number of organisms that may be injected intracerebrally, but these induce chronic histoplasmosis in the brain tissues, without further extension of the disease. Only occasionally do experimentally infected animals show macroscopic lesions of histoplasmosis at autopsy, but microscopic and cultural examination of liver, spleen, and other tissues of these animals, usually reveal the yeast phase organisms of _H. capsulatum_. The intravenous route of injection of white mice with known numbers of _H. capsulatum_ is the most dependable and reproducible method for the study of experimental histoplasmosis.

E. _Laboratory Confirmation of Histoplasmosis._ _Histoplasma capsulatum_ belongs to the Moniliacae of the Fungi Imperfecti. This fungus is a diphasic organism that grows in the yeast phase in the mammalian host, or on complex organic media at 37°C. It grows in the mycelial phase at 25°C. On Sabouraud's dextrose agar at 25°C the colony is at first small and cottony in appearance, but rapidly enlarges in size. As it enlarges, the colony slowly becomes tan to dark brown in color. Slide mounts (Henrici preparations) after two to three weeks, when viewed microscopically, show the following characteristics:

(1) Small undifferentiated spores;

(2) Larger, round or ovoid, smooth spores, often on short stalks;

(3) Tuberculate, spherical to pear shaped chlamydospores which measure 10 to 25 microns in diameter. These spores constitute a major diagnostic characteristic of H. capsulatum;

(4) H. capsulatum grows in the yeast phase on 10 per cent blood agar at 37°C. The colonies appear grey in color, small, firm and vary in texture from a creamy consistency to a leathery type of colony, at first resembling colonies of staphylococci.

The mycelial phase of H. capsulatum is the infective stage. The usual portal of entry is probably the respiratory or the gastrointestinal tract. The lymphadenopathy frequently seen in histoplasmosis must be differentiated from that seen in the malignant lymphomas (51), tuberculosis and other fungus infections. Splenomegaly, hepatomegaly, and ulcerative enteritis also occur. Widespread disease due to hematogenous dissemination is usually rapidly fatal. Mild cases may be asymptomatic. A benign primary infection caused by H. capsulatum may result in calcifications and roentgenographic findings which are indistinguishable from primary tuberculosis (52). Anergy can often be demonstrated in the moribund patient. The histologic lesions produced, as a result of fungus infections, are typically granulomatous, and may be mistaken for those of tuberculosis (53-55) unless the invading organisms are identified. Since fungi produce subacute or chronic infections which mimic tuberculosis, an accurate diagnosis of such disease must depend on careful laboratory studies of specimens obtained from infected patients. Such laboratory studies should include direct microscopic examination of sputum, cultures of sputum or biopsy material, tests of pathogenicity of organisms isolated, skin and serologic tests. Culture of blood and bone marrow specimens (56) are sometimes helpful in confirmation of a diagnosis of histoplasmosis.

Sabouraud's agar has for many years been used as the medium of choice for the isolation of fungi. Recently, the use of media containing blood and antibiotics have been reported (57,58) as most efficacious for the isolation of pathogenic fungi. Bacterial and saprophytic fungus inhibiting agents, such as cycloheximide (acti-dione) have also been utilized for isolating pathogenic fungi (59). Chemically defined media have been used largely for nutritional studies (60-62).

The acquired immunity in histoplasmosis is not clearly defined. Much of the evidence available suggests that this disease occurs in primary infection and reinfection types, similar to those observed in tuberculosis. The hypersensitive state in histoplasmosis is associated with some degree of acquired immunity. The lesions in the hypersensitive patient are usually not as numerous or widely distributed, but a more violent inflammatory reaction is noted when the fungus manages to establish itself (63).

The previously mentioned parallelisms between tuberculosis and histoplasmosis extend to skin testing and serologic diagnostic aids. As in tuberculosis, skin tests for histoplasmosis have been the most widely accepted method for demonstrating past or present H. capsulatum infections. Histoplasmin was originally defined by Zarafonetis and Lindberg (64), as the antigenic substance or substances of H. capsulatum. The term histoplasmin is now used to indicate the filtrate derived from the growth of the mycelial phase of H. capsulatum in a liquid medium. Histoplasmin must be capable of producing a positive skin reaction in sensitized animals and man. Since 1945, an ever-widening interest in the use of histoplasmin has resulted in the publication of many reports on its epidemiologic, laboratory and clinical applications. Histoplasmin has been produced by the following: (1) Emmons, and also Howell, at the National Institutes of Health; (2) Christie and Peterson, at Vanderbilt University; (3) Eli Lilly and Company, and (4) the Parke Davis Company. Unfortunately, it is not clear, in many investigations dealing with histoplasmin sensitivity studies, whether the product and the given dilution are comparable to any other histoplasmin. The National Institutes of Health have recently prepared standard histoplasmin for comparative studies, and published biologic regulations that must be satisfied before a histoplasmin product can be sold.

An ideal antigen for skin testing, at a critical dilution, would give a minimum number of heterologous skin reactions, and a maximal number of homologous responses. The question of the specificity of fungus skin test antigens is of prime importance, because it has been shown that cross reactions occur among histoplasmin, blastomycin, and to a lesser degree with coccidioidin and haplosporangin.

Complement fixation, precipitin, collodion particle agglutination, and hemagglutination tests have been used for the detection of humoral antibodies in mammals infected with H. capsulatum. Histoplasmin has been employed as the antigen in H. capsulatum complement fixation tests. However, the antigenic variability of different lots of histoplasmin (65) has made this antigen unsuitable for complement fixation studies. The most widely accepted complement fixing antigen is the cell free extract from yeast phase H. capsulatum. Several methods for preparing this antigen have been reported (66-68). Histoplasmin adsorbed on collodion particles agglutinates in the presence of antibody from rabbits and humans. Sheep erythrocytes, sensitized with histoplasmin, agglutinate when mixed with homologous antiserum from immunized rabbits (69). The use of the precipitin test, as an aid in the diagnosis of human histoplasmosis, was investigated, after C. E. Smith reported the value of the precipitin test in the early detection of antibodies in coccidioidomycosis. Salvin and Furcolow (70) reported the precipitin test to be of value in demonstrating H. capsulatum humoral antibodies that were not detected by concomitant complement fixation tests. The complement fixation test appears to be the most useful single serologic test presently available for the detection of humoral antibodies in histoplasmosis.

The current interest in antigen-antibody reactions in gels is largely due to the work of Oudin (71), and Ouchterlony (72). The principle of these tests is the formation of a visible precipitate of antigen and antibody in a solid medium, usually agar. Oudin has described a simple precipitin tube diffusion technic in which the antibody is mixed with agar. The antigen diffuses through the agar producing visible products of the antigen-antibody reaction. If the antigen contains more than one component, these components migrate through the agar at different rates. Each component reacts with the antibody forming zones of precipitate.

Ouchterlony (72) used the same methods, except that he performed the test in petri dishes instead of precipitin tubes. Ouchterlony also devised a double diffusion reaction, by placing the antigen and antibody a few cm apart in wells in the agar. The reactants diffused through the agar, and when optimal proportions of antigen and antibody were achieved a line of precipitate formed. If there were more than one reacting component in the antigen, multiple lines of precipitate formed. He further found that he could place a number of antigens around the periphery of the agar plate, with the antibody in central well. If the antigens contained dissimilar reacting components, the lines of precipitation would cross over each other, while similar components formed continuous lines around the antibody well.

Diphtheria toxin, pure by all physical and chemical methods, produces three lines of precipitate when reacted with antidiphtheria toxin. The implication was that this method of reaction in agar was more sensitive than the most elaborate physical and chemical tests.

F. Treatment. The antibiotics cyclohexamide (Actidione), Ascosin, Amphotericin B and the chemical agents; ethyl vanillate, atabrine, methyl testosterone, sulfonamides and stilbamidines have been reported as useful in experimental or human histoplasmosis (73-80). The results, however, are not conclusive. At the present time, histoplasmosis must be considered resistant to treatment with available chemotherapeutic agents.

III. North American Blastomycosis

A. Introduction. North American blastomycosis is a specific infection caused by *Blastomyces dermatitidis*, a diphasic fungus that exists in tissue as a budding yeast, but which produces hyphae with conidia when grown at 25°C to 30°C on most laboratory media.

B. History. Gilchrist (81) first described the cutaneous lesions of this disease in 1894. A few months later, Busse, in Germany, described a similar organism. Busse coined the name *Saccharomycosis hommis*. Previously, the name blastomyces had been used to identify a species of fungus isolated from dung (82), but Gilchrist used the name *Blastomyces dermatitidis* to indicate a fungus similar to a yeast found in skin lesions. Actually, the organism described by Busse, and many other European investigators, was *Cryptococcus neoformans*. Gilchrist and Stokes, in 1896, (83) reported the successful cultivation of *B. dermatitidis* on blood serum, glycerin agar, and potatoes. They found this organism fermented grape sugar to produce alcohol and carbonic acid. It is interesting that Flexner conducted the first successful animal infection by the intra-arterial injection of a *B. dermatitidis* culture into a dog. White mice were also successfully infected, but much difficulty was experienced in the experimental infection of guinea pigs. The report of Gilchrist and Stokes was followed by an era of much confusion. For example: Gilchrist cited a number of papers in which "oidium fungi" were reported to cause a number of diseases such as sarcoma (84), adenocarcinoma (85), and myxomatous tumors of man (86). The European authors, during this time, were adding to the confusion by reporting many cases of European "blastomycosis" or *Cryptococcus neoformans* infections.

The following excerpt from Gilchrist's paper in 1896 (83) clearly shows that he had cultured *B. dermatitidis*. "Young cultures consist of large refractive oval or round bodies showing a double contoured membrane, suggesting an episporium and endosporium and containing numerous refractive granules resembling fat drops. These bodies often showed increase by budding and in time form short hyphae, numerous small knob-like projections of the limiting membrane which gradually enlarge and at last form large, round cells or conidia."

Many names were proposed for the cutaneous and/or systemic form of this disease, however, Martin and Smith (87), in 1939, finally showed that North American blastomycosis was different from the European disease, and that Blastomyces dermatitidis had priority as a suitable name for the disease occurring in North America.

C. Epidemiology. Martin and Smith, in 1939, (87) found 98 percent of 340 proved cases of blastomycosis had their origin within the United States. Two cases presumably originated outside this country, one in Canada (88) and the other in England (89). Presumptive cases have been reported from many other countries.

The majority of the patients who developed blastomycosis in the United States came from the southeastern and midwestern states. Schwarz and Goldman (90) sent a questionnaire to 1,569 dermatologists and 405 thoracic surgeons throughout the United States. This survey reported 101 cases of North American blastomycosis diagnosed, and/or treated during the first 6 months of 1953. These authors considered the disease common throughout the United States.

The precise mode of infection is not yet understood. It is generally presumed to be exogenous with the infective saprophytic form of the organism existing in the nature. However, Blastomyces dermatitidis, unlike Coccidioides immitis, Histoplasma capsulatum, Cryptococcus neoformans and Sporotrichium schenkii has not been isolated from any sources in nature.

In a series of 16 canine cases collected from the literature by Ramsey and Carter (91), the disease was studied at necropsy in 13 of the dogs, and in all 13 there was extensive involvement of the lungs. Four of the animals also had cutaneous lesions. The massive nature of the pulmonary disease, with few and in some cases no other lesions, suggested the possibility of aerial transmission. In their study of canine histoplasmosis and blastomycosis, Menge, et al. (92), showed a striking similarity in the epidemiology of the two diseases, and the evidence points quite uniformly to the lungs as the primary site of infection. Of the 7 dogs studied by Menges, 4 were examined histologically and 1 of the 4 was believed to have both histoplasmosis and blastomycosis.

Schwarz and Baum (93, 94) have expressed the opinion that blastomycosis occurs primarily as an infection of the lungs, which later disseminates, giving rise to lesions in the skin, mucous membranes, bones, prostate gland, kidneys, and other tissues. This would make the disease similar to histoplasmosis and coccidioidomycosis, with regard to mode of infection. Such characteristics also serve to distinguish the disease from sporotrichosis. In the latter disease, it is well known that the organisms are present in the soil and on certain plants, and that the infection is the result of traumatic injury which gives rise to cutaneous lesions, usually without pulmonary involvement. The frequency of pulmonary lesions, when no special attempt was made to demonstrate them, speaks strongly in favor of aerial transmission for the initial infection. It is assumed, as a working hypothesis, that the organisms growing in soil are in a filamentous form with the production of conidia which are disseminated in the air. If the evolution of the epidemiologic knowledge of North American blastomycosis is to follow that of coccidioidomycosis and histoplasmosis, this disease is essentially at the stage where only the rare, fatal disseminated disease is readily recognized. In an attempt to demonstrate the existence of a prevalent benign North American blastomycosis, Furcolow and associates (17) conducted a skin test survey with tuberculin, histoplasmin, and blastomycin among 7,194 school children in Hamilton County, Ohio. No supporting evidence was obtained. The 12 per cent positive reactions to blastomycin were regarded as cross reactions in persons showing a positive histoplasmin skin test. One child was a blastomycin reactor, with a negative histoplasmin reaction. The possibility of the occurrence of mild infections can not be dismissed on the strength of negative skin test surveys.

A verified epidemic of blastomycosis was observed by Harris and his coworkers (95) in Grifton, Pitt County, North Carolina. The sudden appearance of 10 pulmonary cases in the locality provided an excellent opportunity for epidemiologic investigations. A survey of the population of the town revealed a number of individuals with positive blastomycin and negative histoplasmin skin tests. The source of the infection was not traced.

D. Some Clinical Features. The clinical manifestations obviously are determined by the extent of the disease and organs involved. While the idea of primary pulmonary infection has much evidence to support it, there probably are sporadic cases without recognizable lesions in the lungs (96,97). This is to be expected, since most of the fungi are opportunists and are not as a rule highly virulent agents dependent on a particular route or mode of spread.

In the skin, the lesions are heaped up, nodular, firm, worty, and likely to have small abscesses at the periphery (98). As the lesions spread peripherally, a firm fibrotic plaque may be left in the center, but many times there is no central healing and the lesions take on the gross appearance of a large fungating mass (99,100). The lesions may be solitary and persist for years with gradual extension, or they may be dissiminated with many nodules appearing on various parts of the body (101). At times, the skin manifestation is a form of subcutaneous abscess that erodes through the surface (102).

The systemic form of the disease can involve any organ of the body, but probably begins most frequently in the pulmonary tract (103-105). The presenting signs and symptoms depend upon the organ or organs affected and the extent of the involvement (106-108).

E. Laboratory Confirmation. Clinical diagnosis of blastomycosis can only be presumptive. The diagnosis is established by microscopic demonstration and cultural identification of the causative organisms. The following examinations should be attempted for the bacteriologic demonstration of the *B. dermatitidis* (109-113):

(1) Direct microscopic examination; wet mounts of all specimens submitted for fungal examination should be prepared by immersing a small portion of the specimen in 10% potassium hydroxide, in order to clear the specimen for microscopic examination. These organisms appear as yeasts with double contoured walls and some cells show budding daughter cells. It is of diagnostic significance that the point of attachment between the mother and daughter cell is very wide, while other yeast, such as *Candida*, have a very narrow area of attachment between the two cells. Air bubbles, oil droplets, and other cellular elements may be confused with yeast phase cells of fungi. Yeast phase cells showing multiple budding rather than the single daughter cell are suggestive of *Blastomyces brasiliensis*. The failure to demonstrate organisms in the wet mount does not rule out mycotic infections.

(2) Cultural procedures; regardless of the results of the wet mount microscopic examination, the clinical specimens should be cultured. The growth and identification of *B. dermatitidis* on laboratory media constitutes indisputable evidence of the infection. Clinical specimens may be cultured on Sabouraud's dextrose agar at 37°C and room temperature. Blood agar containing penicillin and streptomycin has been

used with much success (58). The inclusion of antibiotics in this medium prevents bacterial contamination, but allows the fungi to grow uninhibited. Fungal colonies may appear as early as the third day after inoculation or at any time thereafter up to about 1 month.

The diaphasic fungi (B. dermatitidis, H. capsulatum, and S. schenkii) grow in the yeast phase at 37°C and in the mycelial phase at room temperature. C. immitis, although diphasic, grows in the mycelial state at 37°C, but appears in the yeast phase in the infected animal. Typical oval or pyriform conidia of B. dermatitidis can best be demonstrated by the use of Henrici preparations. This method is the most useful mycological procedure for the positive identification of B. dermatitidis. Pathology laboratory reports on biopsy specimens, taken in conjunction with the cultural results, frequently establish a diagnosis with more assurance than can either procedure alone.

(3) Animal inoculation; fulfillment of Koch's postulates by the inoculation of experimental animals such as guinea pigs and/or mice (114,115) is an often employed procedure. The intravenous or intraperitoneal inoculation is followed by the appearance of suppurative nodular lesions in the liver, spleen, lungs, and lymph nodes. Material from these lesions, as well as peritoneal washings, reveal typical yeast like tissue forms of B. dermatitidis.

(4) Skin tests; cutaneous hypersensitivity in a patient with North American blastomycosis may be demonstrated by a skin test using a vaccine of the killed yeast phase organism or a culture filtrate called blastomycin (116-118). The blastomycin skin test is akin to the tuberculin test involving similar technique of performance, reading and significance. The intradermal injection of 0.1 ml of a standardized antigen in dilution of 1:10 to 1:100,000 depending upon the degree of sensitivity, results in the appearance of maximal induration and erythema in 24 to 48 hours. A positive blastomycin skin test generally indicates a past or present infection. Although this test is only presumptive diagnostic evidence, the test is particularly helpful in mild, obscure cases, or in cases where material for biopsy or culture can not be obtained. The full significance of the blastomycin

skin test has not been established, as yet, because of lack of opportunities to investigate the disease in a large survey. The results obtained with the blastomycin skin test are not as consistant as skin tests for histoplasmosis or coccidioidiomycosis. The test is negative in the earliest stages of the disease, in mild localized cutaneous cases, and in the overwhelming or terminal phases when anergy occurs. An important misleading factor is the occurrence of cross-reaction with histoplasmin, coccidioidin, and the antigen of Blastomyces brasiliensis (119), which are apparently due to a common antigen. Cross-reactions between histoplasmin and blastomycin are frequent. To some extent mistakes of interpretation arising from these cross-reactions are lessened if coccidioidin, histoplasmin, and blastomycin skin tests are performed simultaneously on all patients suspected of these mycotic infections because the skin reactions to homologous antigens are more pronounced.

(5) Complement fixation test: the sera of patients with blastomycosis are able to fix complement with suspensions or extracts of B. dermatitidis used as antigens (120). Like a positive blastomycin skin test, a positive complement fixation test is only of presumptive value, but it has been found useful in detecting cases and spurring greater efforts aimed at more definite diagnosis by demonstration of the organism through mycologic or histopathologic studies. The complement fixing titer is generally negative or low in early, mild or subclinical infections, or in well localized cutaneous blastomycosis (121). The presence of a high antibody titer in the complement fixation test is an indication of a poor prognosis for the patient. Martin (120,122) has emphasized the importance of adequate serologic tests on patients with deep mycotic infections. He advocates the desensitization of hypersensitive patients before therapy, especially iodide therapy, is started. The suggestion was made that iodide therapy in hypersensitive patients causes a further extension of the disease, presumably due to an acute inflammatory process, with hematogenous spread of the organisms to other parts of the body.

F. Treatment. Untreated cases of cutaneous or systemic blastomycosis ordinarily do not spontaneously regress. The cutaneous lesions have been treated with many therapeutic preparations. Iodides appear to be the drug of choice. Pulmonary or systemic blastomycosis has been treated with iodides with variable success. The diamidines, especially stilbamidine, has been reported by Schoenback, et al., (123) and others (124-134) as useful in experimental and human blastomycosis. However, the number of human cases treated with the aromatic diamidines has been small. Final conclusions concerning the therapeutic value of this product in the treatment of blastomycosis awaits further extensive clinical evaluation.

IV. Bacterial and Fungal Polysaccharides

A. Introduction. The name polysaccharide implies a long chain molecule containing more than four molecules of simple carbohydrates. Burger (135) has further characterized these substances as: soluble in water, thermostable, impervious to the action of trypsin and urease, non-dializable, precipitable by acetone, ether and alcohol.

B. Methods of isolating bacterial polysaccharides. Heidelberger, and associates, have published a series of studies (136-140) on the isolation of polysaccharides from *Mycobacterum tuberculosis* varieties human and bovis, and *Diplococcus pneumoniae*. The method devised by these investigators was based on the principle that the polysaccharides should be extracted in such a manner that the least amount of physical change should occur. They believed that even though the polysaccharides were heat stable and could be precipitated by strong chemicals that these methods caused distortion of the chemical configuration of the polysaccharide, therefore, they were not antigenically similar to the native polysaccharide. Their method makes use of the precipitation of the polysaccharide by alcohol, care being taken to keep the preparation at refrigerator temperatures. This method has become the basis for the extraction of polysaccharides from many bacteria and fungi.

Raistrick and Topley (141) and Palmer and Gerlough (142) have reported the isolation of polysaccharide substances from *Salmonella*, using 80 per cent phenol. The dried organisms were suspended in the phenol. This mixture was centrifuged and the supernate decanted. The cellular debris was treated several times with phenol. The supernates from these extractions were pooled and ethanol added. This procedure precipitated the polysaccharides. The phenol (infinitely soluble in alcohol) remained in the alcohol. These authors reported that immunization with 4×10^{-7}mg of Salmonella polysaccharides protected 50 per cent of 88 mice when they were challenged with 5×10^{4} organisms. The phenol extraction method has been used by Nicholes (143) and Alexander, Wright and Balwin (144) for the extraction of polysaccharides from *Pasteurella tularensis*. Oeding (145) compared various methods of isolating polysaccharides. He concluded that, with *Staphylococcus aureus*, formamide extraction at 150°C produced the greatest yield of polysaccharide. Vogel (146) autoclaved broth cultures of *Candida albicans* and isolated serologically active polysaccharides.

C. *Methods of isolating fungal polysaccharides*. The apparent lack of specificity and sensitivity of histoplasmin, blastomycin, and coccidioidin has been the stimulus for investigators to search for a more efficacious diagnostic substance to be used for the detection of deep mycotic infections.

Hirsch and Benson and Hirsch and D'Andrea (147-149) isolated, by electro-dialysis, a polysaccharide-like substance from filtrates of *Coccidioides immitis*. Using this substance they were able to produce skin reactions in patients with coccidioidomycosis infections. Van Pernis, *et al*., (150) using acetone, precipitated a polysaccharide substance from histoplasmin. This polysaccharide produced a skin test reaction in persons known to be infected with *H. capsulatum*.

Hassid, *et al*., (151) isolated a polysaccharide from coccidioidin. They precipitated the polysaccharide with alcohol. When hydrolyzed, this polysaccharide yielded galacturonic acid and glucose. Peck, Martin, and Hauser (152) obtained a polysaccharide from both the mycelium and broth filtrate of *B. dermatitidis*. They employed alcohol precipitations and obtained, from the broth filtrate, a 0.01 per cent yield. A 0.7 per cent yield of polysaccharide was obtained from the cells. The antigenic relationship of these polysaccharides was not studied. Using these substances they were able to produce typical skin reactions in the forearms of two patients with blastomycosis. Scheff (153) employed Heidelberger's methods to obtain a polysaccharide from the mycelial phase of *H. capsulatum*. He reported that the polysaccharide produced stronger reactions than did the protein fraction, when these substances were employed for skin tests of animals. Cross and Howell (154) treated histoplasmin and blastomycin with the chemical methods of Peck, *et al*., (152), and obtained a polysaccharide from each of these substances. In a preliminary report these investigators stated the hypothesis that with critical concentrations of the polysaccharide, a specific skin reaction could be obtained despite the cross-reactivity noted when larger amounts of the test substances were used as skin test antigens. However, no experimental evidence to support this view has been presented. Hill (155) modified the methods of Heidelberger and obtained a polysaccharide from the yeast phase of _H_. _capsulatum_. He reported that a positive skin reaction was induced by this substance in _H_. _capsulatum_ infected guinea pigs. Dyson, _et al_., (156) reported the isolation of polysaccharides from the filtrates and yeast phase cells of _H_. _capsulatum_ and _B_. _dermatitidis_. These workers used sodium acetate, acetic acid and alcohol to precipitate the polysaccharides. They had the impression that a better skin reaction was obtained from the filtrate polysaccharide than from material obtained from cells when these were injected into rabbits.

MATERIALS AND METHODS

I. Strains of Fungi Investigated

The production of polysaccharide capsular material is known to influence the pathogenicity of some micro-organisms, such as *Diplococcus pneumoniae*. The possibility of differences in the nature and amount of polysaccharides in *H. capsulatum* and *B. dermatitidis* was considered as a corollary of this investigation. Three strains each of these fungi, of known mouse virulence, were chosen as the organisms for investigation. The following strains of *H. capsulatum* and *B. dermatitidis* were used:

H. capsulatum, strain G17, was received from C. C. Campbell, Army Medical School, in May, 1950. This strain was isolated from a fatal case at Walter Reed Hospital. The LD_{50} for mice by intravenous challenge with yeast phase organisms is approximately 1×10^6 (47).

H. capsulatum, strain G17(M), was isolated from a superficial infection of the finger of a laboratory worker in the Department of Bacteriology of the University of Utah. The LD_{50} for mice by intravenous challenge with yeast phase organisms is approximately 1×10^6.

H. capsulatum, strain 6651, was obtained from Dr. C. W. Emmons of the National Institutes of Health. The LD_{50} for mice by intravenous challenge with yeast phase organisms is approximately 4.5×10^6.

B. dermatitidis, strain 410, was obtained from Dr. M. Littman, Tulane University in December, 1949. The organism was originally isolated from lung tissue of a pneumonectomized patient in 1948. The LD_{50} for mice by intravenous challenge with yeast phase organisms is approximately 170 "population units" (58).

B. dermatitidis, strain 380, was obtained from Dr. M. Littman, Tulane University. This organism was originally isolated from a fatal case in 1947. The LD_{50} for mice by intravenous challenge with yeast phase organisms is greater than 1000 "population units."

B. dermatitidis, strain 6052, was obtained from Dr. C. W. Emmons, National Institutes of Health, in 1956. The LD_{50} for mice by intravenous challenge with yeast phase organisms is greater than 1000 "population units."

Polysaccharides were extracted from each of these three strains of *H. capsulatum* and *B. dermatitidis*.

II. Study of Media for the Growth of *H. capsulatum* and *B. dermatitidis*.

The studies of Hill (155) revealed that *H. capsulatum* grew, in the yeast phase, in an enriched tryptose phosphate broth. The growth of *B. dermatitidis* in this medium had not been determined. An objection to the enriched tryptose phosphate broth might be that the medium could contain polysaccharide substances. The growth of *H. capsulatum* and *B. dermatitidis* in the chemically defined medium of Smith (4) and the enriched tryptose phosphate broth were compared. It was observed that the chemically defined medium produced scant granular growth, while the enriched tryptose phosphate broth produced abundant yeast phase growth. Although the enriched tryptose phosphate broth contained a small amount of non-antigenic polysaccharide, it was found that the superior growth of yeast phase organism was necessary for the production of the maximum amount of skin test active polysaccharide. Thus, the medium used throughout this investigation contained the following purchased (Difco) ingredients:

(1) Tryptose phosphate broth . . . 29.5 g
(2) Yeast extract . . . . 4.0 g
(3) Maltose . . . . 10.0 g
(4) Cystine . . . . 0.5 g
(5) Distilled water . . . . 1000 ml

Initial pH 7.4
pH after autoclaving 6.8

The following procedure was utilized for the growth of each of the three strains of *H. capsulatum* and *B. dermatitidis*. Seed cultures of the strain of fungi under investigation were prepared by the inoculation of 10 ml amounts of the enriched tryptose phosphate broth. This culture was incubated at 37°C until heavy growth of yeast phase organisms was apparent; that is, about one week. Microscopic examination of the cultures was then employed as a control designed to reveal only yeast phase fungi and no contaminating bacteria. The seed cultures were inoculated in 1 ml amounts into each of 6 low form flasks. Each of these flasks contained 1 liter of the enriched tryptose phosphate broth. The low form flask cultures were placed on a slowly moving mechanical shaker and incubated at 37°C. After approximately 7 days gross and microscopic examination of the material in each flask revealed a heavy growth of yeast phase organisms without bacterial contamination. The organisms growing in each flask were killed by the addition of

1.0 per cent (final concentration) formalin. After 2 days additional incubation, tests for viable fungi were made by the inoculation of 1 ml from each of the flasks into 50 ml of the original type of broth. These tubes were incubated for 2 weeks before being discarded.

The yeast phase cells were separated from the broth by filtration through a Seitz filter. The cells were harvested from the filter pad and resuspended in 200 ml of saline, and then ground in a ball mill at 4°C for 3 days. Frequent microscopic examination of the ground cells revealed that maximum rupture of the cells occurred within 3 days of grinding. The ground cells were centrifuged to remove the cellular debris and the cell free extract of supernatant fluid was used for chemical extraction of the polysaccharide substances. The 6 liters of filtered broth (histoplasmin) were poured into dialysis tubing. Evaporation of water on the outer surface of this semipermeable membrane was used to concentrate the 6 liters of filtrate to 600 ml of concentrated histoplasmin.

Mycelial phase organisms of H. capsulatum, strains G17, G17(M) and 6651, were grown in the tryptose broth for 60 days at 20°C. The histoplasmin and mycelial mat were used as additional sources for the extraction of polysaccharides.

III. Methods of Extracting Polysaccharides

The cell free extracts and concentrated histoplasmin were treated by the polysaccharide extraction method of Heidelberger, et al., (136-140). This method was modified by local circumstances; essentially, the procedure consisted of the following steps:

A. Sodium acetate is added to a final concentration of 10 per cent. Acetic acid is added to adjust the pH to 5.0 and 2.5 volumes of cold 95 per cent ethanol is then added slowly. The resulting precipitate is allowed to settle overnight in the refrigerator.

B. The supernate is decanted and the precipitate dissolved in 250 ml of 10 per cent aqueous sodium acetate, pH 5.0.

C. To the "2.5 volume supernate" is added 10 per cent sodium acetate and an additional 2.5 volumes of cold 95 per cent ethanol. The resulting precipitate is allowed to stand overnight in the refrigerator and then treated as in step B. The polysaccharide obtained was called the "5 volume precipitate."

D. The fluid separated from the "5 volume precipitate" was further treated with sodium acetate and 5 volumes of cold 95 per cent ethanol. The resulting precipitate was then treated as described in step B. This precipitate was called the "10 volume precipitate."

E. Protein is removed from the aqueous polysaccharide fractions by the chloroform extraction method of Sevag (157). This method consists of the addition of an equal volume of chloroform to each aqueous polysaccharide fraction. The chloroform causes the protein to precipitate at the interface between the chloroform and the aqueous solution of polysaccharide. The aqueous polysaccharide fraction is separated and the protein chloroform mixture discarded. The usual experience was that 6 chloroform treatments resulted in no further appearance of precipitate at the interface. After removal of the protein, these fractions were dialyzed against daily changes of distilled water until sodium ions could not be detected in the dialysate by flame photometery. The dialyzed fractions were filtered through Seitz filters, and precipitated with an equal volume of anhydrous ethanol. The resulting precipitates were collected in tared weighing vials and dried *in vacuo*. The total weight of dried polysaccharides was obtained for each fraction.

Subsequent studies of the skin test activity of the 2.5, 5, and 10 volume precipitates, showed each of these fractions to be of equal antigenic activity, therefore, in the definitive investigations the total polysaccharide content was precipitated by the addition of 10 volumes of cold ethanol.

The polysaccharide method outlined in the previous paragraphs, produced good yields of polysaccharides from the histoplasmin, but lesser yields were obtained from the cell free extracts. Investigation of two other methods of polysaccharide extraction were attempted to determine if some other method would produce greater yields of polysaccharide from the cellular material.

A saline suspension of cells of *H. capsulatum* was autoclaved at 121°C for 10 minutes. The coagulated protein was separated from the heat stable polysaccharides by centrifugation. The supernatant polysaccharide solution was removed and the protein debris washed twice with sodium acetate solution, pH 5.0. These washings were added to the supernatant solution and the polysaccharides precipitated by the cold ethanol process outlined previously.

Two hundred ml of a saline suspension of _H. capsulatum_ cells were dissolved in 500 ml of an 80 per cent phenol solution. The mixture was shaken and placed in the refrigerator overnight. The cellular elements were separated by centrifugation. Microscopic examination of the cellular debris revealed that approximately half the cells were still intact. Two more phenol extractions were necessary before all of the yeast cells were ruptured. The supernates from the three extractions were pooled and 2.5 volumes of cold 95 per cent ethanol was added to precipitate the polysaccharides. These polysaccharides were then purified using the method of Heidelberger (136-140) as previously described.

IV. Methods for the Chemical Characterization of _H. capsulatum_ and _B. dermatitidis_ Polysaccharides

A. Paper Chromatography. Paper chromatographic methods are of great value in the analysis of carbohydrate constituents of polysaccharides. Procedures have been described which enable an investigator, by means of one or two dimensional paper chromatograms, to separate the unit constituents of polysaccharides. In general, the polysaccharides are first hydrolyzed and the hydrolysates are treated as mixtures of monosaccharides. The reducing properties of most monosaccharides has been used as the basis for the development of specific colors on the chromatogram.

The following method was used to determine the carbohydrate nature of _H. capsulatum_ and _B. dermatitidis_ polysaccharides:

(1) Hydrolysis of the polysaccharides. Miya (158) has investigated the conditions necessary for optimal hydrolysis of polysaccharides. He concluded that the greatest yield of monosaccharides was obtained by hydrolyzing with 6 N HCL for about 6 hours, followed by neutralization with barium carbonate. Twenty ml (20 mg/ml) of _H. capsulatum_ and _B. dermatitidis_ polysaccharides were hydrolyzed by this procedure.

(2) The chromatogram. The neutralized (pH 7.0) hydrolysates were spotted, in .02 ml amounts, 6 cm from the bottom of Whatman #1 filter paper strips (30 x 45 cm). Equal amounts of xylose (pentose), dextrose (hexose) and maltose (disaccharide) were spotted, as controls, on each chromatogram. A

large glass tank (50cm x 20cm x 40cm in dimensions) was used as the chromatographic chamber. The inside of the tank was lined with large sheets of filter paper, so that the area within the tank was saturated with the solvent. An ascending type of procedure was utilized, therefore, the filter paper sheets were lowered into the tank and suspended so that the bottom of the filter paper was in contact with the butanol, acetic acid and water (40:20:22) solvent. The lid of the tank was sealed to produce an air tight chamber and the tank was placed in the refrigerator to avoid temperature variations. The solvent then moved up the paper by capillary attraction carrying the carbohydrate test substances and controls. The distance these substances move up the paper depends on the size and mobility of the molecules. The solvent front usually reaches the top of the paper in 72 hours.

(3). The development of carbohydrate colors on the chromatogram. The chromatograms were removed from the tank and dried at room temperature. The standard method for the development of reducing sugars on a chromatogram is the spray application of silver nitrate. Silver nitrate reacts with the reducing substances to produce black silver oxide. Excess silver oxide can be removed with sodium thiosulfate (x-ray film fixer) however, some constituents of the filter paper also react with silver nitrate. This lack of specificity made the procedure unsatisfactory. The monosaccharide color developing method of Stattler and Zerbon (159) was investigated and found to be specific for pentose (xylose) and hexose (dextrose) sugars. This method of color development was utilized, the following is essentially the procedure: the dried chromatogram is sprayed with a 0.3 per cent solution of para-amino hippuric acid which is dissolved in 70 per cent ethanol. After lightly spraying, the chromatogram is dried in an oven at 140°C for 8 minutes. This procedure seems to selectively bind the para-amino hippuric acid to the hexose and/or pentose in such a manner that resonating bonds are produced. These bonds then fluoresce when exposed to ultraviolet light. All of the monosaccharides and to a lesser extent the disaccharides fluoresce when subjected to this treatment. Pentoses produce a visible pink color when tested by this method and appear to fluoresce more intensely than do equal amounts of other sugars. The polysaccharide hydrolysates and controls on each chromatogram were developed by this method. A comparison of the position of the spots of unknown (hydrolysate) and control preparations was made by measurement of the distance each ascended the paper.

B. Spectrophotometric Determination of the Pentose and Hexose content of *H. capsulatum* and *B. dermatitidis* Polysaccharides. Dische and Shettles (160) have reported a method for the quantitative estimation of hexose and/or pentose content of polysaccharide solutions. The test is performed in the following manner:

(1) A solution of 1 part water and 6 parts concentrated sulfuric acid is prepared.

(2) A 0.3 per cent solution of cysteine is prepared.

(3) To tubes containing 1 ml of specimen, immersed in an ice bath to prevent excessive heating, are added 4.5 ml of the sulfuric acid reagent. After dissipation of the heat, the tubes are removed from the ice bath and allowed to come to room temperature.

(4) Subsequent heating of these acid carbohydrate solutions for 3 minutes or 10 minutes produces two distinct absorption patterns. However, heating for 10 minutes may result in carmelization of the sugar solution and interfere with the absorption spectrum; therefore, the tests in this work were heated for 3 minutes.

(5) After heating and cooling, 0.1 ml of the cysteine solution is added to each tube. A greenish yellow color develops within a few minutes and remains stable overnight.

(6) The absorption spectrum is determined for each specimen in a Beckman model D.U. spectrophotometer.

(7) One ml of the following solutions was tested:

a. 10.0 mg per cent dextrose

b. 5.0 mg per cent dextrose

c. 2.5 mg per cent dextrose

d. 5.0 mg per cent rhamnose

e. 5.0 mg per cent of unknown H. capsulatum or B. dermatitidis polysaccharides

f. H_2O blank

C. Other Chemical Determinations

(1) Reducing substances, as a measure of true dextrose, were determined by the standard clinical Folin Wu technic, as outlined by Fester (161).

(2) Nitrogen content of the polysaccharides was determined by the micro-Kjeldahl procedure.

(3) The phosphorous content of each polysaccharide was determined utilizing the method outlined by Fester (161).

(4) Certain other standard qualitative tests for carbohydrates, that is, Molisch and iodine tests were performed.

(5) Qualitative biuret tests were performed for the detection of protein in each polysaccharide preparation.

V. Serologic Procedures

A. The Oudin and Ouchterlony Methods. The formation of visible antigen-antibody precipitates in agar is a simple yet highly sensitive method for the determination of homogeneity of serologically active substances. Experiments were performed by the modification of the Ouchterlony procedure described by Halbert, Sweck and Sonn (162). Agar plates were prepared by pouring 0.6 per cent barbital buffer agar into clear petri dishes. These plates were incubated at 37°C for 24 hours to remove excess moisture. After the moisture had evaporated, a central well and 3 or more peripheral wells were made utilizing a sterile cork borer about 8 mm in diameter. After exploratory efforts, the first definitive experiment was conducted as follows: diphtheria antitoxin was placed in the central well and the following antigens were placed in the outer wells: diphtheria toxin, normal serum and saline. Duplicate plates were incubated at 20°C for 14 days before the precipitates were clearly visible.

Other experiments were performed to observe the reactions of blood group A and B polysaccharide substances with anti-A serum. Triplicate plates containing anti-A serum in the central well and the A and B substances and a saline control in the outer wells were incubated at room temperature, in the refrigerator or at 37°C for one month. A third series of experiments involving the extracted fungal polysaccharides were carried out as follows: anti _H. capsulatum_ serum, prepared by the injection of rabbits with yeast phase cells was placed in the center well. The following _H. capsulatum_ antigens were placed in the peripheral wells:

(1) _H. capsulatum_ strain G17(M) broth polysaccharide, 2 mg/ml.

(2) _H. capsulatum_ strain G17(M) cell polysaccharide, 2 mg/ml.

(3) _H. capsulatum_ broth filtrate (histoplasmin) undiluted.

(4) _H. capsulatum_ strain 6651 broth polysaccharide, 2 mg/ml.

(5) _H. capsulatum_ strain 6651 cell polysaccharide, 2 mg/ml.

(6) Saline control.

Triplicate plates were prepared and sealed. One plate was incubated at 37°C, another at 20°C and the third at 4°C. Whenever the wells were empty, the contents were replaced. The tests were incubated for 14 days before precipitates were clearly visible and unchanging.

A fourth experiment was conducted using the Oudin agar precipitation technic. A portion of the same anti _H. capsulatum_ serum used in previous experiments was diluted 1:2 with buffered 1.2 per cent agar. While the agar was still liquid, 2 sets each of 6 precipitin tubes were half filled with the agar-antiserum mixture. When the agar had solidified, the following amounts of _H. capsulatum_ G17(M) broth polysaccharide contained in 0.5 ml of saline were added to duplicate tubes: 2.5 mg, 1.25 mg, 0.62 mg, 0.31 mg, 0.15 mg and saline control. These tubes were sealed; one set was incubated at 37°C and the other set at 20°C for 1 month.

Another experiment, the fifth in the series, was performed to investigate the cross reactions of _H. capsulatum_ strain G17(M) polysaccharide with _C. immitis_, _B. dermatitidis_ and H. capsulatum antisera. In the center well of each of three agar plates was placed the following concentrations of H. capsulatum strain G17(M) polysaccharide: 5.0 mg/ml, 2.5 mg/ml and 1.25 mg/ml. The outer wells of each plate received the rabbit antisera and a saline control. Duplicate tests were incubated for 20 days at 20°C before the results were clearly apparent.

The sixth experiment was performed to compare the reactivity of a cell free extract (CFE) of H. capsulatum strain G17(M) and H. capsulatum strain G17(M) polysaccharide against H. capsulatum antiserum. The center wells of duplicate plates were charged with the antiserum. One of the outer wells received CFE and the other peripheral well received the polysaccharide. These duplicate plates were incubated for 13 days at 20°C, after which the lines of precipitate were unequivocal and unchanging.

The seventh experiment was performed to investigate the agar precipitation reactions of guinea pig sera known to contain complement fixing antibodies. Seventeen guinea pig sera containing complement fixing antibody were tested against H. capsulatum strain G17(M) polysaccharide. The 17 guinea pig sera, positive and negative control sera and saline were placed in groups of 5 on the 4 plates. The test was prepared in duplicate and the plates incubated at 20°C for 20 days before the precipitation lines were clearly apparent and unchanging.

The eighth experiment in this series was performed to investigate the cross reactions of H. capsulatum strain G17(M) polysaccharide with pneumococcal capsular antisera. Types 1 through 32 pneumococcal typing sera (Wyeth, lot 35R) were placed into the peripheral wells on 6 agar plates, while the central cell of each plate was filled with H. capsulatum strain G17(M) polysaccharide. Due to the limited supply of the pneumococcal antisera this test was not run in duplicate. The plates were incubated at 20°C for 20 days.

The ninth experiment was designed to determine the agar plate reactions of three strains of B. dermatitidis polysaccharide with B. dermatitidis antiserum which was placed in the center well. The following strains and concentrations of B. dermatitidis polysaccharides were placed in the 4 peripheral wells: B. dermatitidis, strain 380 polysaccharide, 2 mg/ml; B. dermatitidis strain 410 polysaccharide, 2 mg/ml; B. dermatitidis strain 6052, 2 mg/ml and blastomycin (broth filtrate undiluted). This test was set up in duplicate and incubated at 20°C for 20 days before precipitates were definable.

The tenth experiment was performed to investigate the cross reactivity of B. dermatitidis polysaccharide with C. immitis, H. capsulatum and B. dermatitidis antisera. The B. dermatitidis strain 410 broth polysaccharide, 2 mg/ml, was placed in the center well, while the fungal antisera and a normal serum were placed in the 4 outer wells. This test was set up in duplicate and incubated at 20°C for 20 days before the results were clearly apparent.

B. Complement Fixation Methods. Smith has emphasized the value of the standard Kolmer complement fixation test in the laboratory diagnosis of coccidioidomycosis (164). In this investigation the Kolmer complement fixation test was used (165).

Complement titration: complement was titrated by adding 1.0, 1.5, 2.0, 2.5 and 3.0 ml of a 1:50 dilution of pooled guinea pig serum in Kahn tubes. The volumes were equalized with buffer. Amboceptor, which had been previously titrated, 1.0 ml of a 1:1000 dilution and 0.5 ml of a 2 per cent suspension of sheep red blood cells were added and the volume of each tube was equalized with buffered saline. The tubes were placed in a 37°C water bath for up to 60 minutes. One unit of complement was taken as the least amount that gave "sparkling" hemolysis. The dilution of complement that was used in the tests was calculated to contain 2 units in 1.0 ml.

Antigen titration: the capacity of the H. capsulatum and B. dermatitidis polysaccharides as complement fixing antigens was unknown; therefore, optimal dilution antigen titrations were designed. The antigenic unit was considered to be the least concentration contained in 0.5 ml which gave complete fixation with the smallest amount of antiserum. The optimal dilution of antigen in every case was found to have no determinable anticomplementary activity. The titrations were set up using 0.5 ml of serial dilutions of antigen and antibody. Two units of complement were added and the system was kept overnight at 5°C. Hemolysin, 1:1000 and the 2 per cent suspension of sheep red blood cells were then added in 0.5 ml amounts of each. The tubes were incubated in a water bath at 37°C until controls cleared, usually within 30 minutes. The endpoint of the test was the last tube containing easily visible unhemolyzed cells, i.e., about a one plus reaction in the Kolmer system (165).

In all tests, antigen and antibody controls for anticomplementary activity, as well as complement controls, were included. All complement, hemolysin and sheep cells were freshly titrated before being used in a test series.

C. Hemagglutination Methods. Norden (69) has reported that histoplasmin could be used as the antigen for hemagglutination tests with *H. capsulatum* antiserum. The suitability of *H. capsulatum* polysaccharides as hemagglutination antigens, was investigated using the hemagglutination procedures of Middlebrook and Dubos (166). An optimal proportions type of test was carried out according to the scheme shown in Table I.

A control test similar to the test described in Table I was set up using histoplasmin adsorbed sheep red blood cells and normal serum controls for nonspecific agglutinations were included. These tests were incubated in a water bath at 37°C for 2 hours and then overnight at room temperature. The patterns of the red blood cells formed in the bottom of the tubes were noted and macroscopic agglutinations were also observed in the usual manner.

VI. Skin Test Methods

A. Introduction: General Methodology. A critical objective of this investigation was to determine if a defined amount, by weight, of *H. capsulatum* and/or *B. dermatitidis* polysaccharides, when injected intracutaneously, could produce a specific skin test reaction in *H. capsulatum* or *B. dermatitidis* infected animals.

The guinea pig was chosen as the test animal because this animal is known to be susceptible to the infection when large inocula of the fungi are injected intraperitoneally. Infected animals surviving for one month usually exhibit skin hypersensitivity. Albino guinea pigs are more easily observed for skin test reactions, therefore, in this work the test animals were albino or lightly colored guinea pigs. These animals were injected intraperitoneally with a suspension of *H. capsulatum* or *B. dermatitidis*. The number of organisms injected was estimated from hemocytometer counts. One month after the injection, the animals were used for skin tests. The flanks of the guinea pigs were clipped and the area marked off as sites for the injection of the various antigens and controls. In all skin test experiments separate tuberculin syringes, fitted with 26 gauge needles, were used for each antigen. Bias in the observation of skin test reactions was lessened by random number selection of the syringes to be used for injections into the skin test sites of the flanks of the guinea pigs. The average diameter in mm of erythema and induration of each reaction was recorded at 24 and 48 hours. Normal guinea pigs were also included as controls in each experiment.

TABLE 1

OPTIMAL PROPORTIONS, HEMAGGLUTINATION TITRATION OF HISTOPLASMIN OR _H._ _CAPSULATUM_ G17(M) POLYSACCHARIDE AND RABBIT ANTI-HISTOPLASMIN SERUM

<table>
<tr><th rowspan="2">Dilutions of antigen adsorbed to S.R.B.C.</th><th colspan="10">antiserum dilution 1:</th><th rowspan="2">Saline control</th></tr>
<tr><th>10</th><th>20</th><th>40</th><th>80</th><th>160</th><th>320</th><th>640</th><th>1280</th><th>2560</th><th>5120</th></tr>
<tr><td>1:2</td><td colspan="11" rowspan="5">Antigen dilutions, 0.4 ml, mixed with antibody dilutions, 0.4 ml.</td></tr>
<tr><td>1:4</td></tr>
<tr><td>1:6</td></tr>
<tr><td>1:16</td></tr>
<tr><td>Saline control</td></tr>
</table>

In the first experiment in this series, H. capsulatum, strain G17, broth polysaccharide was compared with commercial histoplasmin and other H. capsulatum antigens. Seventeen guinea pigs were infected intraperitoneally with yeast phase H. capsulatum, strain G17. Ten of these guinea pigs were alive after 1 month. The fur on the flanks of these animals was removed with clippers. The 15 test antigens, listed in Table 2, were injected intracutaneously; 8 injections were made in one side and 7 injections on the other. Three normal guinea pigs were also injected with these antigens. The average diameter in mm of erythema and edema was recorded at 24 and 48 hours.

The second series of experiments involved determination of the skin test reactions of polysaccharides tested in guinea pigs infected with either yeast phase or mycelial phase H. capsulatum. The animals were infected by either intraperitoneal or intracardial injection. One month after the injection of the organisms, these animals were skin tested with the polysaccharides derived from the 3 strains of yeast phase organisms and control histoplasmin. The amounts of the different antigens injected and the reactions among the animals used in this experiment are given in Table 13.

The third group of experiments on skin tests were carried out to compare the effect on skin sensitivity of employing polysaccharides derived from strains of H. capsulatum with different virulence characteristics. These experiments were designed to explore the possibility of strain differences in skin reactivity of the polysaccharides derived from the yeast phase and mycelial phase materials. The organisms, H. capsulatum, strain 6651 (low virulence), and G17(M) (high virulence) were injected intraperitoneally into three groups, each containing 9 guinea pigs. Two weeks after this injection, these animals were skin tested with the polysaccharides obtained from the yeast phase and mycelial phase of the three strains of H. capsulatum. The skin tests were repeated again 4 weeks later. Control injections of saline, National Institutes of Health histoplasmin, and a commercially available histoplasmin (Eli Lilly and Co.) were also employed. For control purposes, 3 normal guinea pigs were injected with these skin test antigens.

For the fourth series of skin test experiments, 6 monkeys were obtained from the University of Utah, Department of Bacteriology, Division of Poliomyelitis Research. These animals were survivors of poliomyelitis investigations and were donated. The 6 monkeys were injected intraperitoneally with a heavy suspension of H. capsulatum strain G17(M). One

TABLE 2

METHOD FOR SKIN TESTS FOR FIRST SERIES OF INVESTIGATIONS

Antigen type	Source	Amount used
Histoplasmin	P. D. and Co.*	1:100
Histoplasmin HF16	University of Utah**	1:100
Histoplasmin HF16	University of Utah	1:1000
Cell polysaccharide	H. capsulatum, G17 yeast phase	5.0 mcg
Cell polysaccharide	H. capsulatum, G17 yeast phase	0.5 mcg
Cell polysaccharide	H. capsulatum, G17 yeast phase	0.05 mcg
Broth polysaccharide	H. capsulatum, G17 yeast phase	5.0 mcg
Broth polysaccharide	H. capsulatum, G17 yeast phase	0.5 mcg
Broth polysaccharide	H. capsulatum, G17	0.05 mcg
Histoplasmin, y-1	University of Utah***	1:10
Histoplasmin, y-1	University of Utah	1:100
0.9% NaCl control		

* Parke, Davis and Company

** Prepared by Miss Llonas Allred, Department of Bacteriology, University of Utah, in 1948.

*** Prepared by Mr. Gilbert A. Hill, Department of Bacteriology, University of Utah, in 1952. This is broth filtrate from yeast phase growth.

month later the hair on both flanks of these monkeys was removed and the animals were skin tested with the preparations noted in Table 17.

The next experiment (fifth) involved rabbit skin tests. Dyson and Evans reported rabbits as suitable experimental animals for histoplasmin skin tests (67). The use of rabbits for skin tests offers the following advantages: (1) albino rabbits are more readily available than are albino guinea pigs; and (2) the rabbit is a larger animal and more skin test area is available per animal. In order to further explore the feasibility of using rabbits, the following experiment was performed: one albino rabbit was injected with H. capsulatum, strain G17(M), in an amount calculated to be about an LD50 dose. One month after the injection, the fur on the flanks of this rabbit was removed and the test antigens listed in Table 18 were injected intracutaneously. The skin test reactions were observed 24 and 48 hours after the injections.

The results of skin tests described to this point indicated that the three strains of H. capsulatum yielded polysaccharide antigens which produced specific and reproducible reactions. However, it was desirable to determine how a second lot of polysaccharides, extracted from each of the 3 strains of H. capsulatum, would compare in terms of skin test reactivity with the original preparations. Furthermore, the effect of trypsin on these polysaccharides would yield further information concerning the possibility that trace amounts of protein were responsible for skin activity. For this study, the sixth experiment in this series, 15 albino guinea pigs were infected with H. capsulatum, G17(M), yeast phase organisms by intraperitoneal injection. One month later, the flanks of these animals were clipped and the animals were tested with the antigens listed in Table 19 The trypsin treated antigens were prepared by overnight incubation at 37°C of 5 ml of saline containing 10 mg of each strain of polysaccharide with 1 ml of 1 per cent trypsin (Difco), pH 7.5-8.0.

The seventh experiment in this series was performed to determine the skin test activity of the polysaccharide extracted from B. dermatitidis, strain 380. Furthermore, the cross reactivities of H. capsulatum polysaccharides in B. dermatitidis infected guinea pigs were investigated.

Seven albino guinea pigs were infected intracardially with 1000 yeast phase B. dermatitidis, strain 380, cells. After 1 month, 6 of the 7 infected guinea pigs were alive. The 6 infected animals and 1 normal guinea pig were clipped free of hair and the flanks used for skin testing. The test antigens, in the concentrations indicated in Table 21 were injected intracutaneously into the flanks of the B. dermatitidis infected guinea pigs. The skin test reactions were measured at 24 and 48 hours.

The eighth experiment in this series was performed to measure the homologous and heterologous skin test reactions of H. capsulatum polysaccharides and B. dermatitidis polysaccharides in 6 guinea pigs infected with H. capsulatum and 6 guinea pigs infected with B. dermatitidis.

Six guinea pigs were infected intraperitoneally with yeast phase H. capsulatum, strain G17(M) organisms. Another group of 6 guinea pigs were infected in the same manner using yeast phase B. dermatitidis, strain 410, organisms. One month after the infection of these animals, their flanks were clipped and .05 ml of the skin test antigens listed in Table 20 were injected intracutaneously.

The Division of Biologic Standards of the National Institutes of Health has stipulated the requirements to be satisfied before marketing commercial histoplasmin. Unfortunately, similar regulations for the standardization of blastomycin and coccidioidin have not been published. The regulations necessary for the standardization of histoplasmin were followed in the preparation for human use of H. capsulatum G17(M) and B. dermatitidis 380 polysaccharides. The following tests were performed to comply with the N.I.H. regulations:

(1) The B. dermatitidis and H. capsulatum polysaccharides, 1 mg in 1 ml, were each inoculated by streaking over two tubes of Sabourouds medium. This concentration of polysaccharide represents 100 times the amount injected in the skin tests. B. dermatitidis and H. capsulatum polysaccharides were each inoculated into duplicate tubes of thioglycollate broth. One thioglycollate and one Sabourouds culture from each of the two fungal skin test cultures was incubated at 37°C, while the other set of cultures was incubated at room temperature. These cultures were observed for 20 days before they were declared negative.

(2) The same amount (1 mg/ml) of the H. capsulatum G17(M) and B. dermatitidis 380 polysaccharides were each inoculated intraperitoneally into a group of 20 mice. These mice were observed for toxic effects and deaths. After 30 days of observation, the mice were sacrificed. Spleen and liver cultures, using antibiotic blood agar were performed on all the mice.

(3) The H. capsulatum G17(M) and B. dermatitidis 380 polysaccharide solutions, in a concentration of 10 mcg/.05 ml, were prepared and filtered through an ultrafine sintered glass filter. Each of the skin test antigens was inoculated into two tubes of Sabourouds and two tubes of thioglycollate. One set of tubes was incubated at 37°C and one set at room temperature. This procedure was necessary to determine the possible bacterial or fungal contamination of the solutions to be injected into humans.

Bacterial or fungal growth was not observed on any of the cultures. Mouse deaths were not observed, and the macroscopic and cultural examination of the mice tissue failed to reveal the presence of H. capsulatum or B. dermatitidis. These tests were sufficient to comply with sterility regulations of the National Institutes of Health. The only other stipulation of these regulations was that the skin test preparation should be equal in skin reaction to the standard Lot #2 N.I.H. histoplasmin, when tested in H. capsulatum infected guinea pigs. The previous guinea pig skin tests had shown the H. capsulatum G17(M) polysaccharide to be equal to N.I.H. Lot #2 histoplasmin, while the B. dermatitidis polysaccharide produced skin reaction equal to the only available commercial blastomycin. Thus, according to the National Institutes of Health regulations, these products were safe for injection into humans.

The object of the human skin test experiment was to compare the reactivity of H. capsulatum G17(M) polysaccharide and B. dermatitidis 380 polysaccharides with histoplasmin and blastomycin.

The skin test reactions were performed on patients at the Veterans Administration Hospitals, Salt Lake City. A group of persons from the University of Utah, School of Medicine, also volunteered for these injections. The left and right volar surfaces of the forearms of each person were injected as follows:

Left forearm;

(a) upper portion
0.1 ml of 1-100 dilution of histoplasmin

(b) lower portion
.05 ml containing 10 mcg of H. capsulatum G17(M) polysaccharide

Right forearm;

(a) upper portion
0.1 ml of 1-100 dilution of blastomycin

(b) lower portion
.05 ml containing 10 mcg of B. dermatitidis polysaccharide

Observations of the reactions were made at 24 and 48 hours. The extent of induration and erythema of each positive reaction was measured in mm and recorded. History of geographical areas of residence was obtained from each person exhibiting a reaction of 5.0 mm or more of induration.

VII. Polysaccharide Immunization of Mice

The injection of microgram amounts of bacterial polysaccharides into mice, other experimental animals and man has produced enhanced resistance (141-144). Experiments on immunization with fungal polysaccharides have not been reported.

After a series of exploratory experiments involving small numbers of animals, a definitive immunization experiment was performed with mice. The *H. capsulatum* G17(M) broth polysaccharide was injected intraperitoneally into 60 albino mice at a four day interval, according to the following protocol:

I.P. Injection at 4 day interval	Mg. of *H. capsulatum* polysaccharide
1st	0.005
2nd	0.01
3rd	0.1
4th	1.0

A second group of 60 mice were injected intraperitoneally with 1 mg of *H. capsulatum* G17(M) broth polysaccharide at the time the 4th injection was given to the first group of mice. A third group of 60 mice was immunized by 2 weekly intraperitoneal injections of 1 ml of *H. capsulatum* G17(M) whole cell antigen. A fourth group of 60 mice was used as non-immunized controls. Ten days after the last injection, the 4 groups of mice were challenged by the intravenous injection of 0.5 ml of viable H. capsulatum G17(M) yeast phase cells. These organisms were harvested after growing for 1 week on antibiotic containing blood agar slants. The yeast phase cells were removed from the blood agar slants by suspension in brain heart infusion broth. The number of cells in the suspension was estimated from hemocytometer cell counts. The immunized and control groups of mice were divided into 3 sub-groups of 20 and challenged with 1×10^6, 2.5×10^6 and 5.0×10^6 of H. capsulatum G17(M) yeast phase organisms. The deaths were recorded for each group of mice for 21 days.

EXPERIMENTAL RESULTS

I. Weight of Polysaccharide Extracted from *H. capsulatum* and *B. dermatitidis*.

The polysaccharide extraction method of Heidelberger, as described in the section on methods, produced greater yields than did the other methods that were employed. Larger yields of polysaccharide were obtained from broth in which the fungus was grown, than from the cellular elements. Table 3 presents a summary of the yields of polysaccharide extracted from each of the two species of fungi. It is evident from Table 3 that greater quantities of polysaccharide were obtained from the broth than were obtained from the cellular material. Furthermore, 2.5 volumes of cold ethanol precipitated the major portion of the *H. capsulatum* and *B. dermatitidis* polysaccharides. Although the 2.5 volumes of cold ethanol precipitated the major part of this polysaccharide, the maximum yields of polysaccharides were obtained when 10 volumes of cold ethanol were used as the precipitating agent. In other words, there were still minor amounts of polysaccharide present in the ethanol broth mixture after the 2.5 volume of precipitate was removed. The second lots (Lot #2) of *H. capsulatum* polysaccharides were prepared from two strains of *H. capsulatum* by precipitating with 10 volumes of ethanol. The greater yields of polysaccharide obtained from the Lot #2 of *H. capsulatum* and the *B. dermatitidis* polysaccharides probably represents improvement in the technic of preparing these substances, rather than actual differences in the amount of polysaccharide produced by the two fungi.

Six liters of unseeded tryptose phosphate broth were treated with the ethanol extraction method used for fungal polysaccharide extractions. This polysaccharide did not produce positive skin tests in *H. capsulatum* infected guinea pigs, even when 50 mcg were injected intradermally.

II. Chemical Characterization of *H. capsulatum* and *B. dermatitidis* Polysaccharides.

A. Paper Chromatographic Results. The hydrolyzed *H. capsulatum* and *B. dermatitidis* polysaccharides each contained one ascending migrating constituent. These carbohydrates, when developed into ultraviolet light visible spots, were in the same relative position as the control dextrose spots. The exact location of each spot was verified by measuring the distance each had moved up the paper.

TABLE 3

WEIGHT OF POLYSACCHARIDES EXTRACTED
FROM H. CAPSULATUM AND B. DERMATITIDIS

Organisms		Grams of polysaccharide					
		Cell			Broth		
		2.5 v	5 v	10 v	2.5 v	5 v	10 v
H. capsulatum							
	strain G17	0	0	0	.047 (.009)*	.006 (.0001)	0
Lot No. 1	G17(M)	.059 (.001)			1.60 (.03)	.013 (.0003)	0
	6651	.043 (.0008)	0	0	.61 (.01)	0	0
Lot No. 2	G17(M)	- -	- -	- -	- -	- -	3.69 (.07)
	6651	-	-	-	-	-	1.21 (.06)
B. dermatitidis							
	strain 380	- -	- -	0.51 (.009)	- -	- -	5.74 (0.1)
	410	-	-	-	-	-	1.45 (.02)
	6052	-	-	-	-	-	3.92 (.07)
Broth control		- -	- -	- -	- -	- -	.08 (.001)

* Yield in terms of per cent, i.e., weight per 100 ml of original material.
0 = no yield
- = fraction not prepared

These results are illustrated in Figures 1 and 2. The control pentose (xylose) showed a visible pink color which fluoresced and was separated from the hexose by a significant distance. The disaccharide control was least fluorescent and moved the shortest distance of the three controls.

B. The Monosaccharide Content of the Polysaccharide Preparations. Table 4 records the negative logarithm of the spectrophotometric transmissions of the standards, dextrose (hexose) and rhamnose (pentose), and of the *H. capsulatum* polysaccharides. Figures 3 and 4 are graphic representations of the standard dextrose measured at 4100 A° after 3 minutes and 10 minutes heating with sulfuric acid. The Allen correction (167) was applied to these data to remove interfering light transmissions.

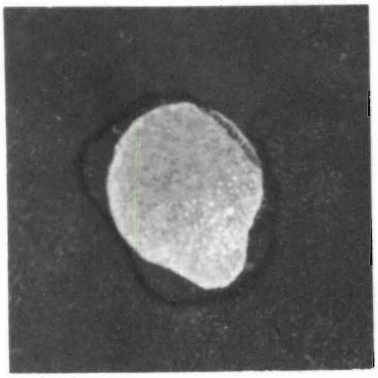
Table 5 records the data from a second experiment to determine the hexose content of three strains of *B. dermatitidis* polysaccharides. Figure 5 is a graphic representation of the standard dextrose transmissions measured at 4100 A° after 3 minutes boiling. Calculating from these data, the following amounts of hexose were present in each of the polysaccharides:

1. *H. capsulatum* G17 . . . . 2.19 mg%
2. *H. capsulatum* 6651 . . . . 2.07 mg%
3. *B. dermatitidis* 6052 . . . . 4.70 mg%
4. *B. dermatitidis* 410 . . . . 4.90 mg%
5. *B. dermatitidis* 380 . . . . 4.85 mg%

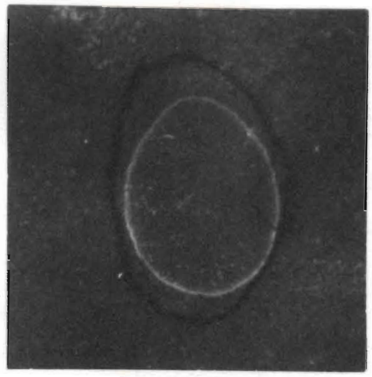
A transmission peak similar to the pentose (rhamnose) was not observed. It is apparent from the different transmission readings for the 3 and 10 minutes tests that heating the polysaccharides for longer periods of time causes a greater hydrolysis of these substances, but this extended heating for 10 minutes also produces a more flat and less accurate curve for the dextrose standard (see Figure 4). Therefore, in the second series of tests with the *B. dermatitidis* polysaccharides, only the 3 minute heating test was performed.

C. Results of Other Chemical Tests. Table 6 records the results of the micro-Kjeldahl nitrogen determinations. Table 7 summarizes the qualitative and quantitative data that

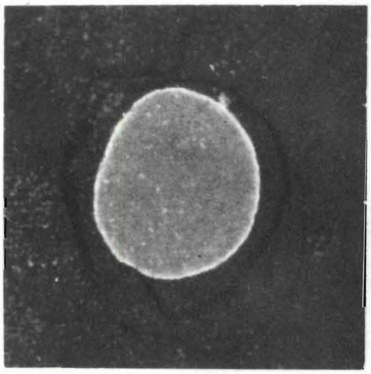
Figure 1. Paper Chromatography. The fluorescent effect of Para-amino Hippuric acid on carbohydrates and polysaccharides.



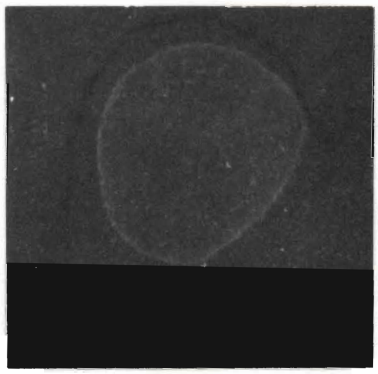
XYLOSE



INULIN



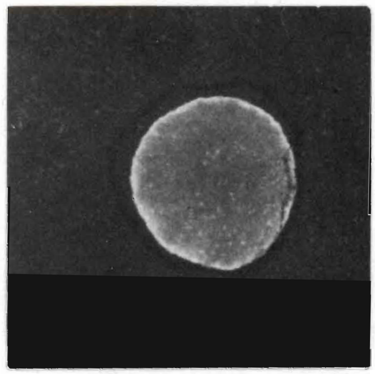
DEXTROSE



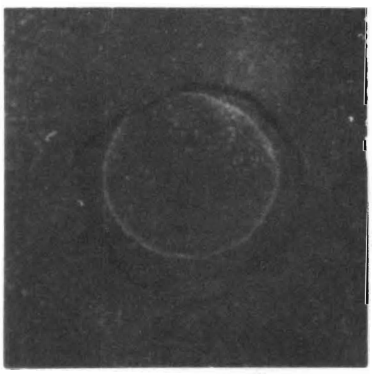
_H_. _CAPSULATUM_

G17(M)

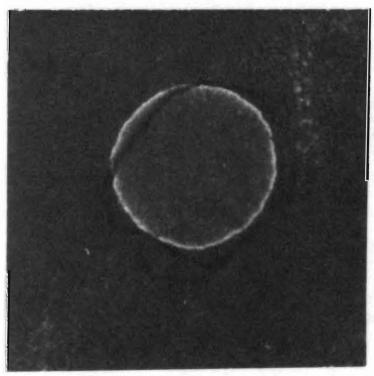
POLYSACCHARIDE



GALACTOSE



SUCROSE

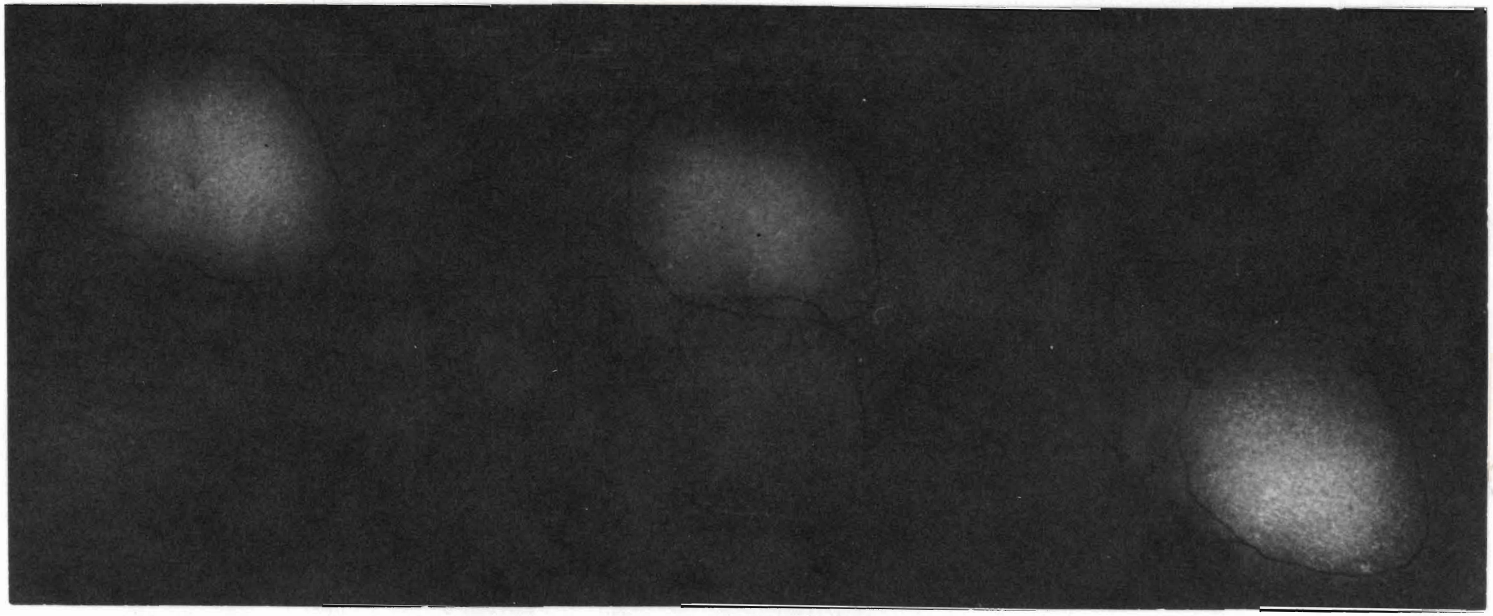


MALTOSE

FIGURE 2

PAPER CHROMATOGRAM OF

DEXTROSE, XYLOSE AND H. CAPSULATUM POLYSACCHARIDE



DEXTROSE CONTROL	H. CAPSULATUM STRAIN G17 (M) HYDROLYSED POLYSACCHARIDE	XYLOSE CONTROL
RF = 0.235	RF = 0.214	RF = 0.129

SOLVENT: BUTONOL, ACETIC ACID AND WATER

TABLE 4

SULFURIC ACID CYSTEINE TEST FOR HEXOSE AND PENTOSE FROM *H. CAPSULATUM* POLYSACCHARIDES

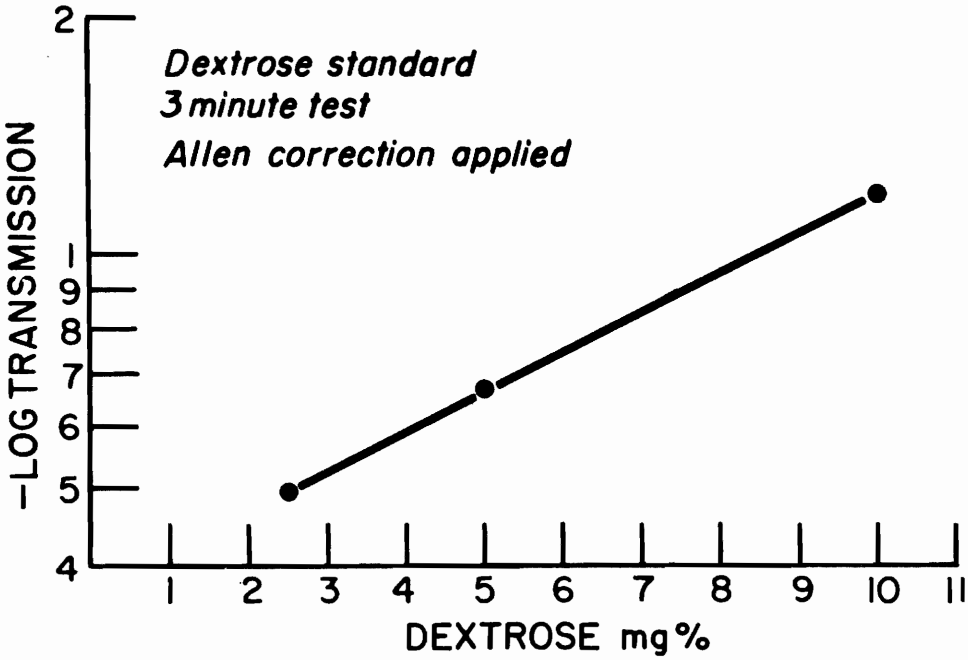
Test Material	Wave Length, Angstrom Units								
	3600	3700	3800	3900	4000	4100	4200	4300	4400
Dextrose 10mg%									
3 minutes*	**.030	.043	.065	.097	.134	.160	.148	.104	-
10 minutes*	.015	.022	.029	.041	.057	.068	.067	.048	.030
Dextrose 5 mg%									
3 minutes	.023	.029	.045	.064	.087	.105	.097	.068	-
10 minutes	.009	.013	.019	.029	.044	.052	.051	.035	.018
Dextrose 2.5 mg%									
3 minutes	.013	.017	.026	.039	.052	.065	.060	.041	-
10 minutes	not measurable		not measurable		not measurable		not measurable		
Rhamnose 5 mg%									
10 minutes	.047	.057	.090	.136	.151	.106	.065	.034	.011
Blank	.000	.000	.000	.000	.000	.000	.000	.000	.000
H. capsulatum G17(M) polysaccharide 5mg%									
3 minutes	.025	.024	.029	.037	.048	.052	.047	.032	-
10 minutes	.035	.046	.062	.085	.110	.123	.110	.079	.048
H. capsulatum 6651 polysaccharide 5mg%									
3 minutes	.010	.013	.019	.025	.031	.036	.031	.023	-
10 minutes	.039	.041	.050	.060	.075	.084	.072	.052	.034

* Indicates time of incubation of test in boiling water

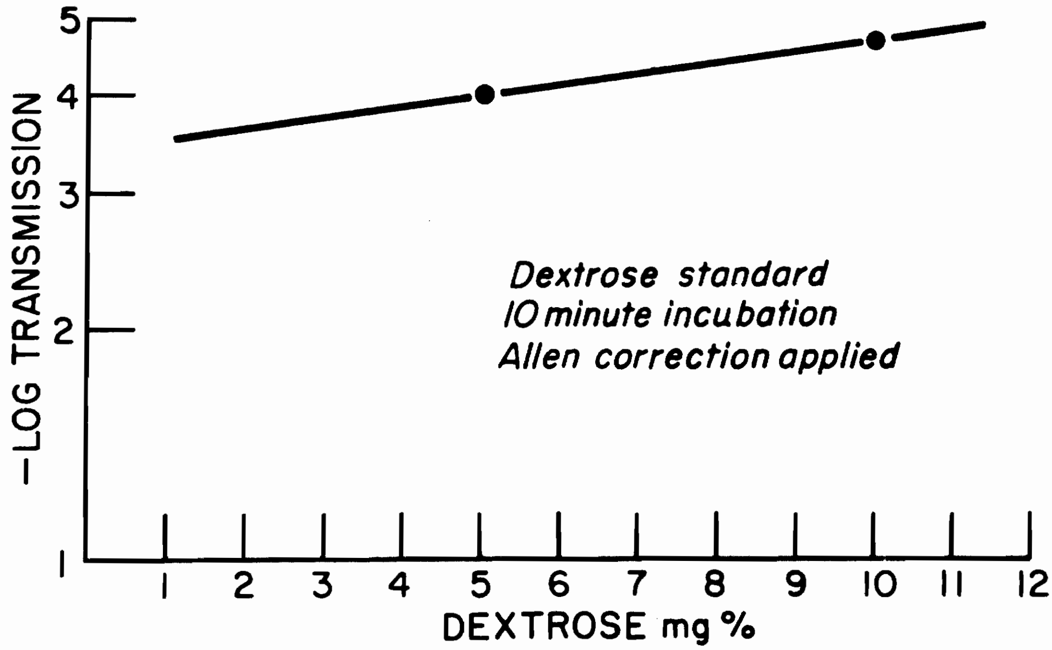
** Negative log of transmission

Figure 3

H. CAPSULATUM TEST



H. CAPSULATUM TEST



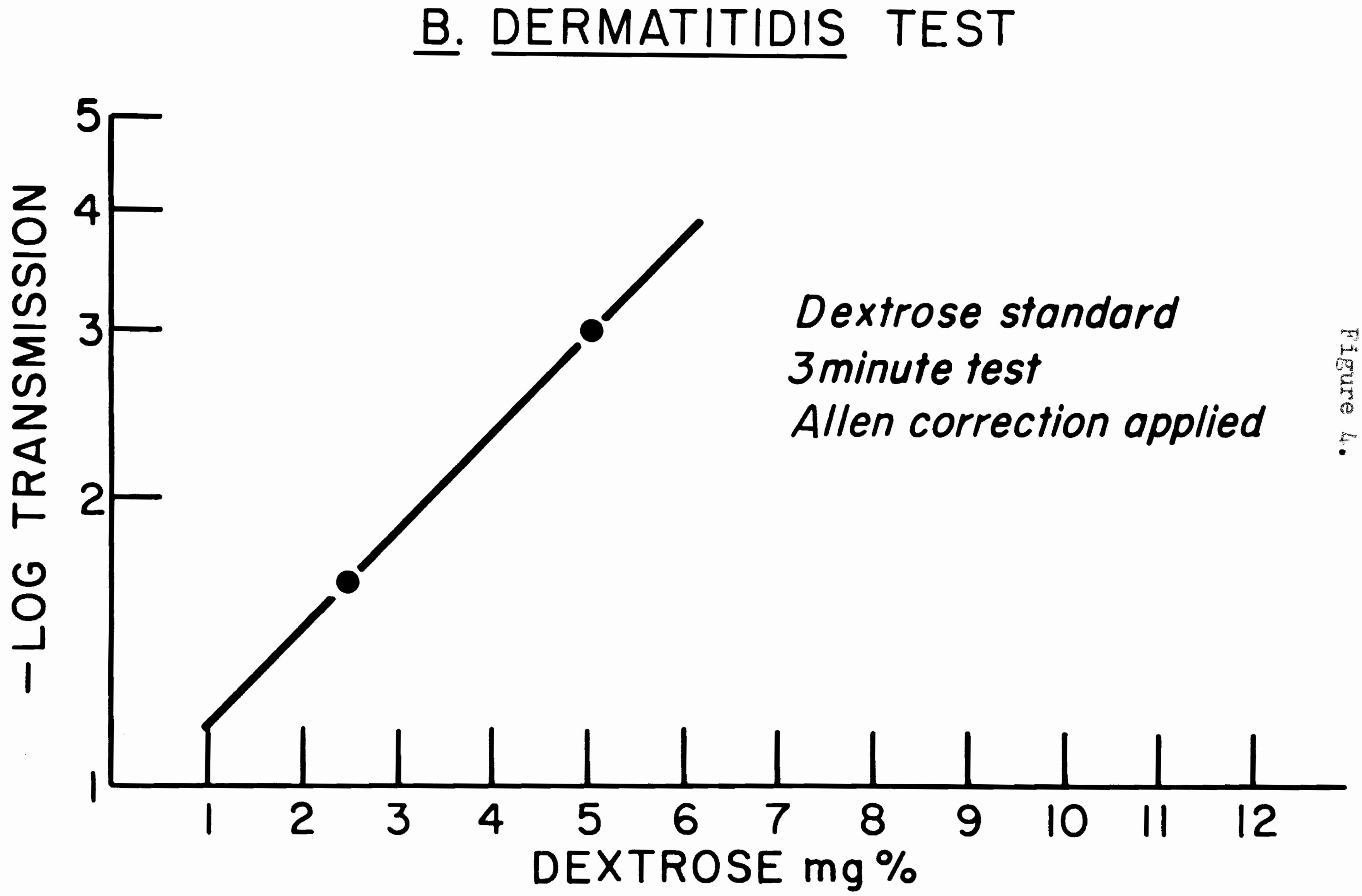

B. DERMATITIDIS TEST
-LOG TRANSMISSION
5
4
3
2
1
Dextrose standard
3minute test
Allen correction applied
1 2 3 4 5 6 7 8 9 10 11 12
DEXTROSE mg %

Figure 4.

TABLE 5

SULFURIC ACID, CYSTEINE TEST FOR HEXOSE AND PENTOSE FROM B. DERMATITIDIS POLYSACCHARIDES

Test Material	Wave lengths, Angstrom Units								
	3600	3700	3800	3900	4000	4100	4200	4300	4400
Dextrose 5 mg% 3 minutes	*.059	.084	.100	.149	.200	.300	.200	.149	.100
Dextrose 2.5 mg% 3 minutes	.067	.071	.079	.104	.136	.152	.136	.104	.079
<u>B. dermatitidis</u> 6052 polysaccharide 5 mg% 3 minutes	.060	.079	.090	.134	.171	.280	.171	.134	.090
<u>B. dermatitidis</u> 410 polysaccharide 5 mg%	.057	.079	.097	.130	.175	.295	.175	.130	.097
<u>B. dermatitidis</u> 380 polysaccharide 5 mg%	.043	.054	.093	.132	.173	.285	.173	.132	.093
Blank	.000	.000	.000	.000	.000	.000	.000	.000	.000

* Negative log of transmission

TABLE 6

KJELDAHL NITROGEN CONTENT OF B. DERMATITIDIS AND H. CAPSULATUM POLYSACCHARIDES

	ml of acid*	Nitrogen mg/100 mg
B. dermatitidis		
strain		
380	4.2	4.2
	3.9	3.9
	3.6	3.6
410	4.5	4.5
	4.5	4.5
	4.0	4.0
6052	4.2	4.2
	4.4	4.4
	4.1	4.1
H. capsulatum		
strain		
G17(M)	4.4	4.4
	3.5	3.5
6651	7.5	7.6
	7.8	7.9
	6.8	6.6
G17	9.4	9.6
	9.4	9.6

* ml of 0.1 N HCl necessary to neutralize the ammonium borate for each duplicate or triplicate test.

TABLE 7

A SUMMARY OF SOME CHEMICAL TESTS ON *H. CAPSULATUM* AND *B. DERMATITIDIS* POLYSACCHARIDES

Test	*H. capsulatum* strain G17(M) Composition		*B. dermatitidis* strain 410 Composition	
	Qualitative	Per cent	Qualitative	Per cent
Iodine	neg.		neg.	
Molisch	pos.		pos.	
Biuret	neg.		neg.	
Nitrogen		4.0		4.2
Reducing substances* (6 hour hydrolysis)		90.0		75.0
Phosphorous		1.07		0.8
Hexose		58.8		50.0
Pentose	neg.		neg.	

* Includes Hexose

have been determined for the H. capsulatum strain G17(M) and B. dermatitidis strain 410 polysaccharides. The other strains of each of these fungi showed essentially the same results. It may be deduced from these data that the polysaccharides are largely or entirely glucose polymers. The nitrogen content could represent contamination or some structure such as an occassional glucosamine moiety. The insignificant phosphorus content is most readily explained as contamination.

III. Results of Serologic Tests.

A. The Oudin and Ouchterlony Tests. Figure 6 is a photograph of the diphtheria toxin and antitoxin reactions. The white zone around the central antitoxin well is a non-specific reaction (also observed by other investigators) caused by the serum. Four lines of precipitate are visible between the toxin and antitoxin wells, while no precipitate is visible in the area between the saline or normal serum and the antitoxin. These results have been reproduced, for teaching purposes, on a number of occasions.

The second experiment failed to show precipitation between the human blood groups A and B substances and the anti-A serum. This experiment has been repeated several times, using anti-A or anti-B serum, without visible reactions. No explanation is available at present for this result.

The third series of tests involving the reaction of H. capsulatum polysaccharide, produced visible precipitates for all of the antigens, except the saline. The precipitates formed a white line around the center well, broken only by the diffusion of the saline. It is noteworthy, that the broth filtrate (histoplasmin) contains protein and this protein would be expected to diffuse at a different rate than the polysaccharide. However, the continuous band of precipitate included the broth filtrate. There are several possible explanations that may be offered to account for these observations. The protein may have diffused at the same rate of speed as the polysaccharides, or the observed reaction of the broth filtrate may have been due exclusively to the polysaccharide content of the broth filtrate. The precipitate appeared first opposite the broth filtrate and it was the widest band of precipitate. This experiment has been repeated and the same results have been obtained. The experiment has suggested that the polysaccharide antigen contains only one precipitating component and that this component is the only visible reacting substance in the polysaccharides from the three strains of H. capsulatum.

Figure 5. Diphtheria Toxin-Antitoxin Precipitation in agar. Four lines of precipitates in the area between the diphtheria toxin and antitoxin; no lines of precipitate opposite the saline or normal wells.

The titration of the *H. capsulatum* G17(M) yeast phase broth polysaccharide by the Oudin procedure, in the fourth experiment, showed a precipitate at the interface between the polysaccharide antigen, and the agar. Duplicate tubes, containing 0.62 mg/ml of polysaccharide, were the lowest concentrations howing precipitates. It is surprising that a photograph of a *Salmonella typhosa* polysaccharide and anti-typhoid serum, appearing in Oudin's article, showed the same reaction at the interface without lines of precipitate in the agar antibody mixture. This experiment has been repeated and the same results obtained. The optimal temperature for these reactions appears to be about 20°C. It should be noted that the incubation times for these tests are prolonged (14 days), but once the reaction appears, it remains stable for many weeks.

The absence of the cross reactions of *H. capsulatum* G17(M) polysaccharide with other fungal antisera are demonstrated in Figure 7. It should be noted that the only precipitating band appears opposite the well containing the *H. capsulatum* antiserum.

Figure 8 is an enlarged view of the *H. capsulatum* antigen-antibody precipitate and 1 band of precipitate is apparent. No visible antigen-antibody reactions were apparent opposite the wells containing the *C. immitis* or *B. dermatitidis* antisera. For purposes of reference, the following measurements made in the experiments, illustrated by Figures 7 and 8, are recorded at this point:

1. Total distance between wells . . . 32 mm
2. Distance from center well to precipitate band . . . . 22 mm
3. Time . . . . 20 days at 20° C

A comparison of reactivity of the *H. capsulatum* cell free extract (CFE) and *H. capsulatum* G17(M) polysaccharide showed that single bands of precipitate appeared at the same time, opposite both the CFE and the polysaccharide. The following linear relationships, measuring from center of wells containing antigen and antibody yielded these results:

1. CFE, total distance between wells . . 17 mm
2. CFE, distance from central well to band of precipitate . . . . 10 mm

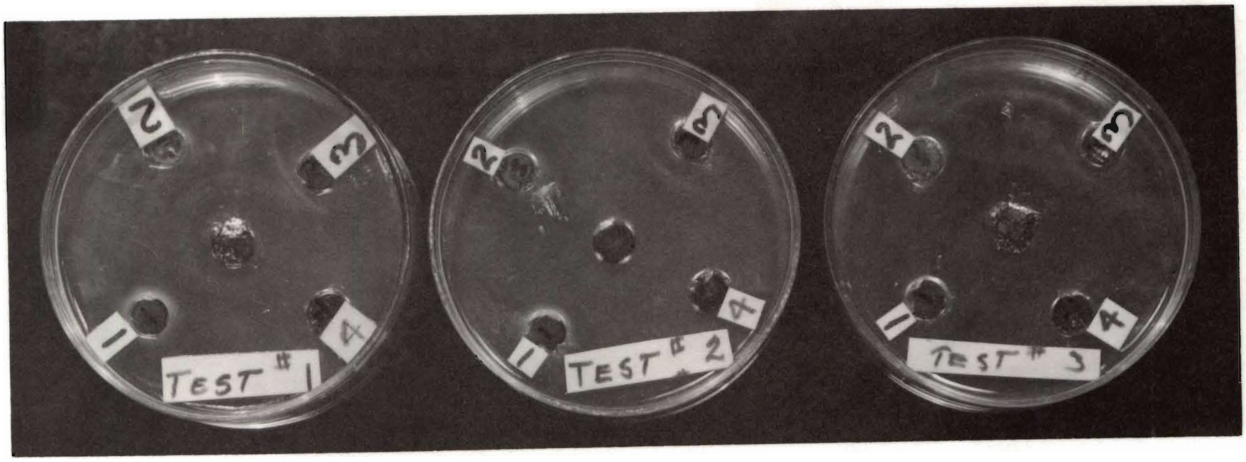


Figure 6. Absence of Cross Reactivity of H. capsulatum G17(M) Polysaccharide with C. immitis, B. dermatitidis, and H. capsulatum antisera.

Test 1. Center well contains 5 mg/ml H. capsulatum polysaccharide antigen.

Test 2. Center well contains 2.5 mg/ml H. capsulatum polysaccharide antigen.

Test 3. Center well contains 1.25 mg/ml H. capsulatum polysaccharide antigen.

Peripheral wells contain the following antisera:

#1 - C. immitis.
#2 - B. dermatitidis
#3 - H. capsulatum
#4 - Saline

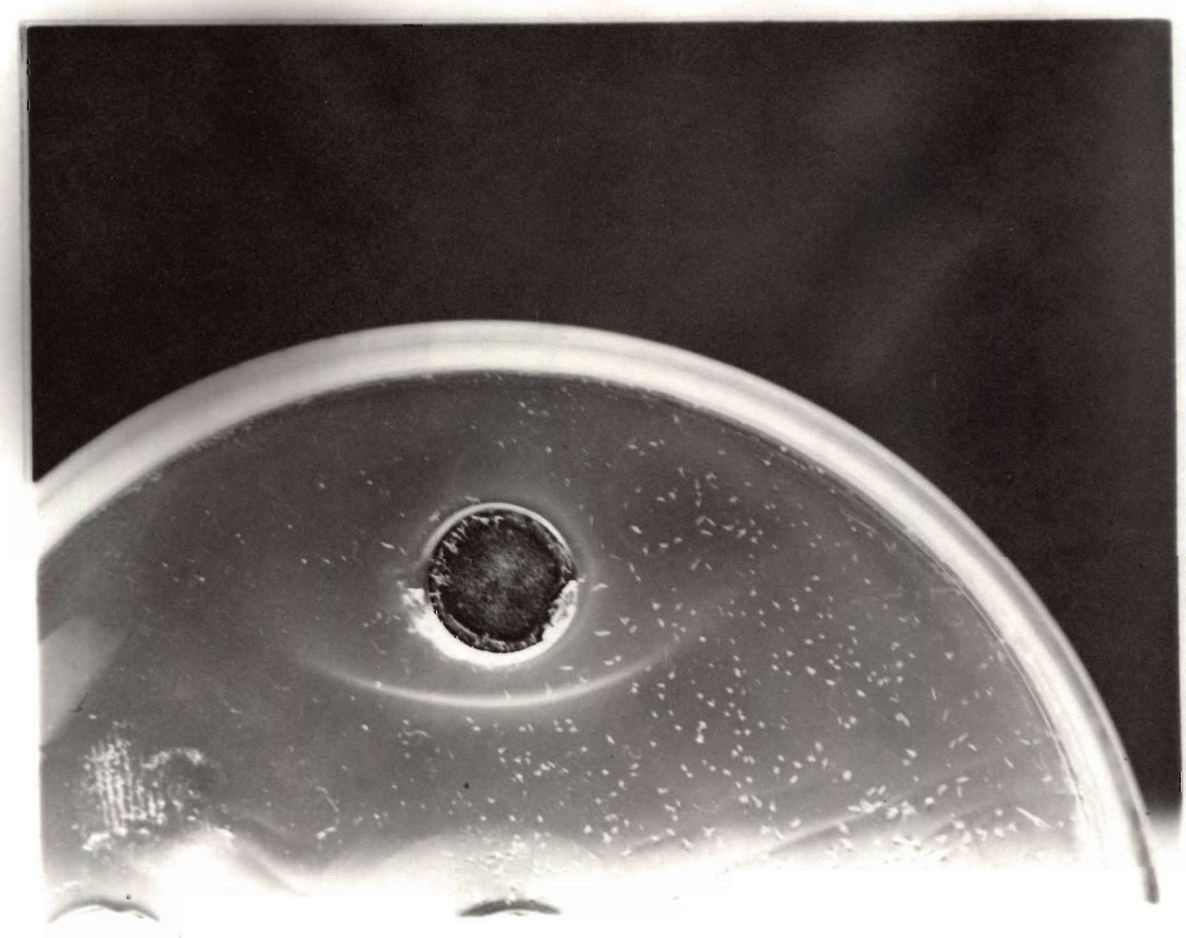


Figure 7. Enlarged view of the Single Precipitating Band Produced by H. capsulatum strain G17(M) Polysaccharide and H. capsulatum antisera (Rabbit).

3. H. capsulatum G17(M) polysaccharide, total distance between wells . . . . . 14 mm

4. H. capsulatum G17(M) polysaccharide, distance from central well to band of precipitate . . . . 8 mm

5. Time . . . . 13 days at 20°C

H. capsulatum complement fixing antibody (low titers, i.e., 1:2-1:4) from infected guinea pigs failed to produce visible precipitates in Ouchterlony agar diffusion tests. A known hyperimmune rabbit control serum (complement fixing titer 1:128) produced a typical band of precipitate with the H. capsulatum G17(M) polysaccharide antigen.

There were no cross reactions when the H. capsulatum G17(M) polysaccharide was mixed with each of 32 pneumococcal typing sera.

The last two experiments involving the reactions of the three strains of B. dermatitidis polysaccharides with B. dermatitidis antiserum and the search for cross reactions of the B. dermatitidis polysaccharide with other fungal antisera, showed reactions that were analogous to the similar experiments conducted with H. capsulatum polysaccharides. The experiments revealed that a specific antigen-antibody reaction could be demonstrated in the agar plates with the homologous anti-blastomyces sera and no cross reactions occurred using H. capsulatum polysaccharide. In other words, the blastomyces polysaccharides produce a specific reaction without cross reactions when tested by the Ouchterlony method.

B. Results of Complement Fixation Tests. In this portion of the investigation, the complement fixation tests were used to determine the suitability of H. capsulatum strain G17(M) polysaccharide as a complement fixing antigen and to investigate the correlation of complement fixing antibodies present in H. capsulatum infected guinea pigs. Table 8 records the anticomplementary titration of the H. capsulatum G17(M) polysaccharide and Table 9 records the results of an antigenic titration of the H. capsulatum G17(M) polysaccharide using known positive serum. Several titrations with similar results indicated that the H. capsulatum polysaccharide antigen was satisfactory for complement fixation tests. In Table 10 are recorded the results of complement fixation tests conducted on sera from 25 guinea

TABLE 8

ANTI-COMPLEMENTARY TITRATION OF H. CAPSULATUM STRAIN G17(M) POLYSACCHARIDE

Antigen Dilution 0.5 ml	Saline 0.85%	Complement 2 units		Hemolysin 1:1000	SRBC* 2%	Results
Undil	0.5 ml	1.0 ml	Incubated at 4°C for 18 hours; then 37°C for 1 hour.	0.5 ml	0.5 ml	2+ **
1:2	"	"		"	"	neg.
1:3	"	"		"	"	"
1:4	"	"		"	"	"
1:6	"	"		"	"	"
1:8	"	"		"	"	"
1:12	"	"		"	"	"
1:16	"	"		"	"	"
1:24	"	"		"	"	"
1:32	"	"		"	"	"

* Sheep red blood cells
** 50% lysis

TABLE 9

COMPLEMENT FIXATION WITH H. CAPSULATUM
STRAIN G17(M) POLYSACCHARIDE

Antigen Dilution 0.5 ml	Anti H. capsulatum*serum 1:2	Complement 2 units		Hemolysin 1:1000	SRBC** 2%	Results
1:2	0.5 ml	1.0 ml	Incubated at $4^{\circ}C$ for 18 hours; then $37^{\circ}C$ for 1 hour	0.5 ml	0.5 ml	4+***
1:3	"	"		"	"	4+
1:4	"	"		"	"	4+
1:6	"	"		"	"	4+
1:8	"	"		"	"	4+
1:10	"	"		"	"	4+
1:12	"	"		"	"	2+
1:16	"	"		"	"	1+
Serum control	"	"		"	"	- ***
Antigen control 1:2	0	"		"	"	-

* Anti *H. capsulatum* hyperimmune rabbit serum

** Sheep red blood cells

*** 4+ = 0% lysis (complete fixation)
- = 100% lysis (no fixation)

TABLE 10

RESULTS OF COMPLEMENT FIXATION OF SERA FROM
H. CAPSULATUM STRAIN G17(M) INFECTED GUINEA PIGS

Guinea pig*** number	Complement fixation titer
1	neg.
2	1:2
3	neg.
4	1:2
5	1:2
6	neg.
7	neg.
8	1:4
9	neg.
10	1:4
11	1:8
12	1:8
13	neg.
14	1:2
15	1:2
16	AC*
17	neg.
18	QNS**
19	AC
20	1:128
21	neg.
22	neg.
23	AC
24	AC
25	neg.
Control: Positive serum (rabbit serum)	1:128

Number pos. 10
Number neg. 10
Number unsatisfactory. . . 5

*AC, anticomplementary serum.
**QNS, quantity of serum not sufficient for test
***One month after the H. capsulatum infection of these guinea pigs, skin tests were performed and blood was obtained by intracardial puncture.

pigs. No definite correlation could be demonstrated between complement fixing antibodies and the extent or severity of the *H. capsulatum* infections in these guinea pigs.

It should be emphasized that the value of the polysaccharide as a complement fixing antigen in human histoplasmosis has not been investigated. Martin (118) has suggested that although fungal polysaccharide antigens can be used successfully in complement fixation with guinea pig sera, human anti *H. capsulatum* sera do not fix complement in the presence of a *H. capsulatum* polysaccharide antigen.

C. Hemagglutination Tests. Table 11 records the results of the optimal proportions hemagglutination titration of *H. capsulatum* strain G17(M) polysaccharide and a similar titration using histoplasmin (product prepared at University of Utah by Gilbert Hill, 1952).

These results confirm the findings of Norden (69) that histoplasmin can be used as a hemagglutination antigen. Polysaccharide hemagglutination tests have been repeated many times since the initial positive results shown in Table 11 were obtained, however, none of the subsequent tests have been positive; therefore, the use of these polysaccharides as hemagglutination test antigens cannot currently be considered for routine use.

IV. Results of Skin Tests.

A. Guinea Pig Skin Test Reactions. Table 12 presents the results of the first series of skin tests. A positive skin test has been defined by early investigators in this field of investigation as an area of induration of at least 5 mm. Most of the skin test antigens used in this experiment produced skin reactions greater than 5 mm. The variations in skin test reactivity of these antigens are apparently due to the hypersensitive state of the individual guinea pigs. For example, anergy of guinea pigs with a critical *H. capsulatum* infection is well documented. In general, the most consistently positive and the larger areas of induration were obtained with the Y-1 histoplasmin preparation of Hill. However, the *H. capsulatum* G17 polysaccharide, in the indicated concentration, produced more consistent skin tests than did the commercial histoplasmin*.

* Purchased from Parke, Davis and Company.

TABLE 11

OPTIMAL PROPORTIONS RESULTS OF *H. CAPSULATUM* STRAIN G17(M) HEMAGGLUTINATION TITRATION EMPLOYING HISTOPLASMIN AND POLYSACCHARIDE AGAINST RABBIT ANTI-HISTOPLASMIN SERUM

Concentration of polysaccharide adsorbed to SRBC	antiserum dilution 1:										Saline control
	10	20	40	80	160	320	640	1280	2560	5120	
1 mg/ml	+	+	+	+	+	+	-	-	-	-	-
100 mcg/ml	+	+	+	+	-	-	-	-	-	-	-
250 mcg/ml	+	+	-	-	-	-	-	-	-	-	-
Dilutions of Y1 (Hill) histoplasmin											
1:2	+	+	+	-	-	-	-	-	-	-	-
1:4	+	+	-	-	-	-	-	-	-	-	-
Concentration of polysaccharide adsorbed to SRBC	Controls Normal rabbit serum										
1 mg/ml	-	-	-	-	-						
500 mg/ml	-	-	-	-	-	Anti-*H. capsulatum* sera + Normal SRBC - neg.					
250 mcg/ml	-	-	-	-	-						
Dilution of histoplasmin											
1:2	-	-	-	-	-						
1:4	-	-	-	-	-						

TABLE 12

H. CAPSULATUM STRAIN G17 SKIN TESTS

GUINEA PIGS INFECTED WITH H. CAPSULATUM STRAIN G17

G.P. #	PSS+	HF+ 16 1:100	HF+ 16 1:1000	P.D.+ 1:100	***Polysaccharide Cell, mcg .05	0.5	5.0	Broth, mcg .05	0.5	5.0	Y-1+ 1-10	1-100
1.	-	±*	3	8	-	10	-	6	4	4	12	7
	-	-**	10	2	5	-	6	12	2	-	-	9
2.	-	-	6	4	-	4	-	5	3	10	4	6
	-	6	9	13	10	10	13	12	5	9	13	5
3.	-	-	±	±	-	-	-	10	-	4	12	8
	-	-	11	11	5	-	-	18	4	9	12	10
4.	-	-	-	-	4	-	-	-	-	10	6	8
	-	-	-	-	-	-	-	-	-	-	9	-
5.	-	-	-	-	4	3	-	4	2	5	6	5
	-	-	-	-	-	-	-	11	2	10	15	-
6.	-	-	5	5	-	3	-	5	10	4	12	8
	-	-	10	10	4	9	6	9	9	3	14	5
Normal controls	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-

\+ See Table 2 for description of antigens

* The upper number in each square indicates the average mm of induration measured at 24 hours.

** The lower number in each square indicates the average mm of induration measured at 48 hours.

*** Cell and broth polysaccharides extracted from H. capsulatum G17

In the second experiment the *H. capsulatum* strains G17 and 6651 polysaccharides were tested in guinea pigs infected with *H. capsulatum* G17; the results are given in Table 13. Reasons for the failure of the *H. capsulatum* strain 6651 polysaccharide, in the indicated microgram amounts, to produce positive skin test reactions are not apparent. It is noteworthy, that 10 mcg of *H. capsulatum* G17 broth polysaccharide produced reactions equal to the National Institutes of Health Lot #2 histoplasmin in the recommended dilution of 1:100. These results also emphasize the difficulties of producing consistent *H. capsulatum* infections in guinea pigs.

Table 14 presents the results of another experiment in this series. These results are similar to those obtained in the two previous experiments. The skin test results conducted thus far indicated that some of the guinea pigs in each experiment were critically infected and anergic, while other animals were able to resist this infection without producing skin hypersensitivity. Accordingly, a third series of experiments were designed to determine the effect of variations in skin reactivity if the route of inoculation is changed from intraperitoneal to intracardial. Table 15 records the results of these tests. It is apparent that animals infected via the intraperitoneal and intracardial routes reacted similarly to the injection of the skin test antigens. Furthermore, the data suggest stronger reactions on the part of the animals infected with mycelial material.

In a second experiment in this third series of experiments, polysaccharides extracted from the yeast and mycelial phases of growth of the three strains of *H. capsulatum* were used as skin test antigens in guinea pigs infected with the "low virulence" *H. capsulatum* strain 6651 and the "high virulence" *H. capsulatum* strain G17(M). These results are presented in Table 16. The polysaccharides extracted from yeast phase organisms produced a larger area of induration in the skin tests, but there was no difference in the skin hypersensitivity of guinea pigs infected with either the "low virulence" or "high virulence" organisms.

For the fourth series of experiments involving skin tests on 6 monkeys, the results are listed in Table 17. These monkeys failed to show skin reactions. Subsequent autopsy of these animals revealed no gross or microscopic evidence of *H. capsulatum* infection.

Table 18 presents the data that were observed when skin tests were performed on a rabbit infected with *H. capsulatum*. No specific positive skin reactions were

TABLE 13

SKIN TESTS WITH *H. capsulatum* STRAINS G17 AND 6651

IN GUINEA PIGS INFECTED WITH *H. capsulatum* STRAIN G17

G.P. #	Polysaccharide 6651, mcg*						Polysaccharide G17, mcg			PSS	P.D.+	NIH++
	From cell			From broth			From broth					
	.01	1.0	10	0.1	1.0	10	0.1	1.0	10		1:100	1:100
1.	-** -***	- -	- -	- -	- -	- -	- -	8 5	- -	- -	- -	7 -
2.	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- 4
3.	- -	- -	- -	- -	- -	- -	7 -	10 3	20 10	- -	4 -	10 6
4.	- -	- -	- -	- -	- -	- -	5 -	7 7	15 11	- -	6 4	14 9
5.	- -	- -	- -	- -	- -	- -	- -	- -	15 3	- -	- -	7 3
6.	- -	- -	- -	- -	- -	- -	- -	5 -	10 5	- -	- -	10 5
7.	- -	- -	- -	- -	- -	- -	- -	- -	14 10	- -	- -	10 5
8.	- -	- -	- -	- -	- -	- -	- -	5 2	10 5	- -	- -	6 2
9.	- -	- -	- -	- -	- -	- -	- -	4 5	15 5	- -	- -	5 5
10.	- -	- -	- -	- -	- -	- -	4 -	6 -	12 9	- -	- -	12 6
11.	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
12.	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
3 G.P. Controls	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

*Mcg of *H. capsulatum* strain 6651 injected intracutaneously into G.P.
**Top number in each square indicates the average mm of induration observed at 24 hrs.
***Bottom number in each square indicates the average mm of induration observed at 48 hrs.
+P.D. = Parke, Davis and Company histoplasmin.
++NIH = National Institutes of Health histoplasmin, Lot #2.

TABLE 14

SKIN TESTS WITH <u>H</u>. <u>CAPSULATUM</u> POLYSACCHARIDES STRAINS G17, G17(M) AND 6651 IN GUINEA PIGS INFECTED WITH <u>H</u>. <u>CAPSULATUM</u> STRAIN 6651

G.P.	H. capsulatum polysaccharide, mcg											NIH+	T.P.++ Poly	PSS
	G17*		6651*				G17(M)*							
	Broth		Cell		Broth		5V Broth		2.5V Broth		Cell			
#	10	1	10	1	10	1	10	1	10	1	10	1:100	50mcg	
1.	15** 10***	5 5	- -	- -	5 5	- -	- -	- -	- -	- -	- -	8 -	- -	- -
2.	10 10	6 4	5 5	7 10	- -	- -	- -	- -	- -	- 10	- -	8 10	3 -	- -
3.	13 13	- 3	- -	- -	- 4	- -	- -	- -	- -	- -	- -	- 10	- -	- -
4.	7 -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	5 5	- -	- -
5.	10 12	- 6	- -	- -	- -	- -	- -	- -	- -	- -	- -	- 5	- -	- -
6.	7 10	- 7	- -	- -	- -	- -	- -	- -	- -	- -	- -	5 6	- -	- -
7.	10 10	5 6	- -	- -	- 4	- -	- -	- -	- -	- -	- -	4 5	- -	- -
Control 2 G.P.	- -	- -	4 -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

*G17, 6651, G17(M) indicates the strain of <u>H</u>. <u>capsulatum</u> used for polysaccharide extraction

**The number in the top of each square indicates the average mm of induration measured at 24 hours after skin test.

***The numbers in the bottom of each square indicate average mm of induration measured at 48 hours after skin test.

\+ NIH = National Institutes of Health histoplasmin Lot #2

++ T.P.= Control tryptose phosphate broth polysaccharide.

TABLE 15

RESULTS OF GUINEA PIG SKIN TESTS

G.P. Infected with *H. capsulatum* G17(M)	Yeast phase polysaccharides							NIH
	G17(M) Broth		6651 Broth		G17(M) Cells		G17 Broth	
	10mcg	100mcg	10mcg	100mcg	10mcg	100mcg	100mcg	1:100
I.P.* Yeast**	1.67+	7.33	2.17	2.00	0.0	1.0	10.00	8.17
I.C.* Yeast	0.67	4.67	0.83	4.33	0.0	0.0	7.0	6.50
I.C. Mycelial**	1.24	12.26	0.0	6.0	0.0	2.0	11.24	10.50
I.P. Mycelial	4.00	10.00	2.60	8.60	0.0	0.0	15.40	10.80

*I.P. = Intraperitoneal infection
*I.C. = Intracardial infection
**Yeast = Yeast phase of *H. capsulatum*
**Mycelial = Mycelial phase of *H. capsulatum*
+ Each number represents the mean mm of induration obtained from readings made on 4-6 guinea pigs. All guinea pigs were skin tested 30 days after the infecting dose of *H. capsulatum*.

TABLE 16

RESULTS OF GUINEA PIG SKIN TEST IN ANIMALS INFECTED WITH "HIGH VIRULENCE" AND "LOW VIRULENCE" STRAINS OF H. CAPSULATUM

Guinea pigs infected with H. capsulatum strain #	S.S.6651++ YP+ Broth 100mcg	S.S.6651++ MP+ Broth 100mcg	S.S.G17(M) YP Broth 100mcg	S.S.G17(M) YP Cell 100mcg	S.S.G17(M) MP Broth 100mcg	S.S.G17 YP Broth 100mcg	S.S.G17 MP Broth 100mcg	NIH 1:100	Lilly** 1:100	PSS
6651	7.2*	3.0	5.0	3.5	4.0	10.0	3.8	2.9	0.5	0
G17	5.0	3.0	7.2	7.2	2.8	7.0	2.8	4.6	1.0	0
G17(M)	10.0	5.0	12.0	8.0	5.0	14.0	2.0	7.2	6.2	0
Mean	7.5	3.7	8.1	6.2	3.9	10.3	2.9	4.9	2.6	
Standard error	1.28		1.34		1.48	2.54	0.44	0.77		

\+ YP = yeast phase polysaccharide
MP = mycelial phase polysaccharide

++ S.S. refers to polysaccharide extracted from the three strains of fungi.

* Mean in mm of induration observed on 5 or more guinea pigs.

** Lilly = Eli Lilly and Company histoplasmin

TABLE 17

RESULTS OF MONKEY SKIN TESTS

Six monkeys injected intraperitoneally with
<u>H.</u> <u>capsulatum</u> strain G17(M) yeast phase cells

Monkey	PSS	*NIH	**P.D.	<u>H.</u> <u>capsulatum</u> polysaccharide broth	
		1:100	50 mcg	10 mcg	100 mcg
1.	-	-	-	-	-
2.	-	-	-	-	-
3.	-	-	-	-	-
4.	-	-	-	-	-
5.	-	-	-	-	-
6.	-	-	-	-	-

*NIH = National Institutes of Health Lot #2 standard histoplasmin
**P.D.= Polysaccharide extracted from broth without previous fungal growth

TABLE 18

RESULTS OF RABBIT SKIN TESTS

Rabbit infected with *H. capsulatum* strain G17(M) yeast phase cells

Skin Test Antigen	Results of skin tests
H. capsulatum G17(M) broth polysaccharide	
(a) 20 mcg	-
(b) 200 mcg	-
H. capsulatum 6651 broth polysaccharide	
(a) 20 mcg	-
(b) 200 mcg	-
National Institutes of Health Lot #2 histoplasmin	
(a) 1:100	-
Saline	-

observed. Another objection to the use of rabbits was that the fur on these animals had to be removed by depilation and this procedure caused much skin irritation; in skin reactive rabbits, such irritation might confuse observations to be made.

In the sixth series of investigations, the effect of trypsin on the H. capsulatum polysaccharide skin test activity and the comparison of two lots of H. capsulatum polysaccharide were investigated. These results are recorded in Table 19. These results revealed that the chemical method used for the extraction of polysaccharide was satisfactory for the duplication of skin test active polysaccharides. In other words, comparable skin test results were obtained from two separate lots of H. capsulatum polysaccharide. These results also demonstrate that trypsin does not affect the skin test activity of the polysaccharide and is suggestive evidence that protein does not act as the antigenic substance in these skin tests. It is also noteworthy that 100 mcg of each polysaccharide produce uniformly positive skin tests.

In the seventh series of experiments the skin test reactions of B. dermatitidis strain 380 polysaccharides were investigated. In addition, the heterologous reactions of H. capsulatum polysaccharide in B. dermatitidis infected guinea pigs were investigated. These results are tabulated in Table 20. The results indicate that 10 mcg of B. dermatitidis strain 380 polysaccharide produced skin test reactions equal to or greater than the 5mm of induration chosen as the customarily positive reaction in guinea pigs inoculated with B. dermatitidis; the polysaccharide antigen was superior to the sample of commercial blastomycin used for skin testing. A standard blastomycin is not available from the National Institutes of Health.

Cross reactions were obtained with 100 mcg of H. capsulatum G17(M) broth polysaccharide when this amount was injected into the skin of B. dermatitidis infected guinea pigs. Using 10 mcg of this polysaccharide, however, in B. dermatitidis infected guinea pigs, only minimal skin test reaction were observed.

Further investigations of the homologous and heterologous reactions of H. capsulatum and B. dermatitidis polysaccharides were conducted in the eighth series of experiments and the results of these skin tests are recorded in Table 21 and 22.

TABLE 19

RESULTS OF GUINEA PIG SKIN TESTS

(100 mcg of each polysaccharide)

Comparison of 2 lots of polysaccharide and the effect of trypsin

Skin Test Materials	G17(M) Infected Guinea Pigs															Normal Controls	
NIH 1:100	10*	8	10	12	10	12	8	5	15	15	12	15	15	10	12	-	-
Saline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6651 Lot #1 Trypsin	12	10	15	12	15	10	12	8	15	20	10	15	20	15	15	±**	±
6651 Lot #1	10	8	12	15	12	10	10	8	15	15	15	15	15	15	15	-	-
6651 Lot #2 Trypsin	12	10	20	17	20	15	12	10	20	20	12	10	20	17	16	±	±
6651 Lot #2	12	12	15	12	15	15	-	10	15	15	15	15	20	12	18	±	±
G17(M) Lot #1	12	15	20	22	20	15	10	15	20	20	15	15	20	20	15	-	-
G17(M) Lot #1 Trypsin	10	11	15	15	15	12	10	20	10	20	10	15	18	12	12	±	±
G17(M) Lot #2 Trypsin	15	12	20	5	10	20	12	15	10	15	18	12	12	12	10	±	±
G17(M) Lot #2	12	15	15	12	20	20	15	15	15	20	15	15	15	15	17	-	-
G17	11	15	20	15	15	15	15	10	20	15	17	17	18	15	14	-	-

* The number in each square refers to the average mm of induration observed 24 hours after the intracutaneous injections.

** ± = Reaction of 2 mm or less.

TABLE 20

SKIN TEST RESULTS WITH H. CAPSULATUM AND B. DERMATITIDIS POLYSACCHARIDES IN GUINEA PIGS INFECTED WITH B. DERMATITIDIS

G.P.	B. dermatitidis*** 380 Cell 10 mcg	Cell 100 mcg	Broth 10 mcg	Broth 100 mcg	H. capsulatum G17(M) Broth 10 mcg	Broth 100 mcg	Blastomycin P.D.** 1:100	Histoplasmin NIH 1:100	Saline
1.	10*	15	15	20	±	6	6	-	-
2.	7	7	10	14	-	5	-	-	-
3.	15	20	10	17	-	4	4	-	-
4.	7	9	6	20	5	5	-	-	-
5.	7	15	4	10	-	-	4	-	-
6.	7	10	5	15	±	±	5	-	-
7. Control	±	-	±	±	-	-	-	-	-

*Number refers to diameter of reaction in mm (edema) measured at 24 hours

**Parke, Davis and Company blastomycin

***Polysaccharide antigens

TABLE 21

SKIN TEST REACTIONS OF GUINEA PIGS INFECTED WITH B. DERMATITIDIS

STRAIN 410: HOMOLOGOUS AND HETEROLOGOUS SKIN TEST REACTIONS

G.P.	H.capsulatum 6651			H.capsulatum G17(M)			Histo-plasmin	Blasto-mycin	Blasto-poly**	Blasto-poly**	Saline
	50 mcg	15 mcg	10 mcg	50 mcg	15 mcg	10 mcg	1:100	1:100	380	410	
1.	12*	8	4	12	6	4	-	-	8	9	-
2.	14	8	4	14	4	-	-	-	8	4	-
3.	9	7	4	8	6	4	4	3	12	12	-
4.	14	4	-	12	4	-	-	3	6	8	-
5.	4	4	3	-	-	-	-	4	4	4	-
6.	8	6	3	6	4	-	-	3	8	8	-
Controls											
7.	-	-	-	-	-	-	-	-	-	-	-
8.	-	±	±	-	-	-	-	-	-	-	-
9.	-	-	-	-	-	-	-	-	-	-	-

* The numbers refer to the average mm of induration measured at 24 hours after the intracutaneous injections.

** Blastopoly = B. dermatitidis polysaccharide from strains 380 and 410 respectively.

TABLE 22

SKIN TEST REACTIONS OF GUINEA PIGS INFECTED WITH <u>H. CAPSULATUM</u> STRAIN G17(M): HOMOLOGOUS AND HETEROLOGOUS SKIN TEST REACTIONS

G.P.	Polysaccharide <u>H.capsulatum</u> 6651			Polysaccharide <u>H.capsulatum</u> G17(M)			Histoplasmin NIH	Blastomycin P.D.**	Blastopoly 380	Blastopoly 410	Saline
	50 mcg	15 mcg	10 mcg	50 mcg	15 mcg	10 mcg	1:100	1:100	10 mcg	10 mcg	
1.	10*	5	6	9	6	5	7	4	-	-	-
2.	10	8	8	9	8	6	8	4	-	-	-
3.	12	6	6	12	7	6	8	-	-	-	-
4.	14	7	5	8	6	6	8	-	-	-	-
5.	6	10	7	9	7	6	7	-	-	-	-
6.	7	6	6	9	7	6	8	-	-	-	-
Controls	-	-	-	-	-	-	-	-	-	-	-

* Number refers to the average mm of induration observed 24 hours after the intradermal injection.

** P.D. = Parke, Davis and Company blastomycin

Non-infected controls for both experiments listed in Table 21.

These data show that a specific reaction can be elicited by the B. dermatitidis strains 380 and 410 polysaccharide in a concentration of 10 mcg. The results of the H. capsulatum polysaccharide skin tests reveal a degree of cross reaction with 10 mcg of the strain H. capsulatum G17(M) polysaccharide, but these heterologous reactions were less than the homologous reactions and, more significantly, they were less than the minimum reaction of 5 mm.

B. Human Skin Test Reactions. The human skin tests were conducted in the ninth series of experiments. In this investigation 134 persons were skin tested. Table 23 shows a comparison of the histoplasmin and the H. capsulatum G17(M) polysaccharide reactions. It is noted that 20 positive reactions were obtained. Of these reactions, 16 were obtained with both histoplasmin and H. capsulatum broth polysaccharide. In 2 cases either polysaccharide or histoplasmin yielded a positive result. Table 24 is a summary of the comparative reactivity of blastomycin and B. dermatitidis strain 380 polysaccharide. The only reactions were apparently of a non-specific nature. Table 25 reveals the mean mm of induration for each of the reactants. The results are considered in the discussion.

V. Results of Mouse Immunization

Animal losses during immunization and within 24 hours after the intravenous challenge reduced some of the subgroups of 20 mice to smaller numbers. Table 26 suggests that some degree of protection is evident. The magnitude of the differences and the significance of these data can only be ascertained by statistical analysis, accordingly the data were analyzed by the method of Wilcoxon and Litchfield (156).

Table 26 also presents the calculated LD_{50} values and the 95% confidence limits for each group of animals. These data indicate that there is significant protection of mice immunized with either whole cells or polysaccharide. The LD_{50} of the mice receiving 4 immunizing injections of polysaccharide and mice receiving 1 immunizing injection of polysaccharide were so similar that no difference could demonstrated by the method used. The relative efficacy of the immunization procedures when compared with the non-immunized control group was further investigated by the calculation of potency ratios. Table 27 presents these results.

TABLE 23

HUMAN SKIN TEST REACTIONS

Comparison of *H. capsulatum* strain G17(M) polysaccharide with commercial histoplasmin

Histoplasmin pos.,	Polysaccharide pos.,	16
Histoplasmin pos.,	Polysaccharide neg.,	2
Histoplasmin neg.,	Polysaccharide pos.,	2
Histoplasmin neg.,	Polysaccharide neg.,	114
Total positive reactions		20
Total number of tests		134
Per cent positive		15.7

All persons with a positive skin test have a history of living in an *Histoplasma capsulatum* endemic area.

TABLE 24

HUMAN SKIN TEST REACTIONS

Comparison of <u>B. dermatitidis</u> strain 380 polysaccharide with commercial blastomycin

Blastomycin pos.,	Polysaccharide pos.,	0
Blastomycin pos.,	Polysaccharide neg.,	0
Blastomycin neg.,	Polysaccharide pos.,	5
Blastomycin neg.,	Polysaccharide neg.,	129
Histoplasma polysaccharide pos.,	Blastomyces polysaccharide pos.,	5
Histoplasmin pos.,	Blastomyces polysaccharide pos.,	0

TABLE 25

HUMAN SKIN TEST REACTIONS
AMONG 20 POSITIVE REACTORS

	24 hours $\overline{X}$ mm of induration	48 hours $\overline{X}$ mm of induration
Histoplasmin	6.9	12.4
H. capsulatum G17(M) polysaccharide	12.8	14.0
Blastomycin	0.33	0.0
B. dermatitidis 380 polysaccharide	9.2	7.0

TABLE 26

MOUSE DEATHS AND LD_{50} AFTER CHALLENGE WITH
H. CAPSULATUM STRAIN G17(M) YEAST PHASE ORGANISMS

Method of Immunization of mice	Challenge dose of H.capsulatum G17(M) yeast phase cells 1×10^6	2.5×10^6	5.0×10^6	LD_{50}	95% Confidence Limits
Whole cell	1/16*	2/16	9/16	8.0×10^6	15.6 - 4.1**
4 injection polysaccharide	5/18	2/17	13/18	4.5×10^6	6.75 - 3.0
1 injection polysaccharide	3/12	2/12	8/15	4.5×10^6	9.0 - 2.25
Control Non-immunized	11/19	12/20	20/20	1.05×10^6	1.83 - 0.6

* Numerator = number dead; denominator = number challenged

** All numbers raised to 10^6.
Example:
$15.6 - 4.1 = 15.6 \times 10^6 - 4.1 \times 10^6$

TABLE 27

POTENCY RATIOS OF *H. CAPSULATUM* WHOLE CELLS AND POLYSACCHARIDE IMMUNIZED MICE

Method of Immunization of mice	Potency Ratio	95% Confidence Limits
Whole cell antigen	$\frac{8 \times 10^{6}*}{1.05 \times 10^{6}} = 7.62$	(3.18 - 18.2)
4 injection polysaccharide or 1 injection polysaccharide	$\frac{4.5 \times 10^{6}}{1.05 \times 10^{6}} = 4.28$	(1.76 - 10.4)

* 8×10^{6} = LD_{50} of whole cell immunized mice.
1.05×10^{6} = LD_{50} of non-immunized control mice.

The 4 injection and 1 injection polysaccharide immunized mice reacted equally to the challenge doses, and a significant degree of protection was induced. The animals receiving polysaccharide were able to withstand 1.76 to 10.4 times the number of organisms which killed 50 per cent of the controls. The whole cell immunized mice were able to receive 3.18 to 18.2 times more organisms than the control mice. It is apparent, therefore, that the skin test polysaccharide preparation was able to induce a significant degree of protection in mice when given before the challenge dose of organisms. One immunizing injection of 1 mg of polysaccharide material gave protection equal to that obtained by 4 injections with this polysaccharide. The protection, while significant was apparently not as extensive as that induced by whole cell vaccine.

DISCUSSION

The extraction method used in this investigation has produced polysaccharide substances from each of 3 strains of H. capsulatum and B. dermatitidis. Although the per cent yield of polysaccharide appears to be small (1 per cent or less), it should be emphasized that 3 grams of skin test active polysaccharide, when used in 10 mcg doses, amounts to 300,000 skin test doses. In addition, the use of this method of extraction from yeast phase organisms eliminates the hazards of pulmonary infections contracted in the laboratory that may occur when the mycelial phase of growth is used to produce histoplasmin or blastomycin. There is no apparent reason why the methods of growth and extraction presented in this investigation could not be employed for the commercial production of fungal skin test polysaccharides.

It is surprising that a greater quantity of polysaccharide was found in the broth than was isolated from the cells. This may indicate that the polysaccharide diffused out of the cells during growth, or that the formalin added to the culture prior to the extraction of the polysaccharide caused changes in the cell wall permeability and allowed the intracellular polysaccharide to diffuse from the cells into the medium. In any case, the data presented indicate that these polysaccharides are antigenic while the polysaccharide naturally present in the growth medium are not antigenic.

The chemical studies of the H. capsulatum and B. dermatitidis polysaccharides were not intended to be definitive studies to learn the structure of these polysaccharides; rather, they were qualitative and quantitative analyses designed to yield information concerning constituents and contamination of the preparations.

The paper chromatographic procedures, using the para-amino hippuric acid method of developing the spots into ultraviolet light visible spots, provided a method for the separation of the polysaccharide components. The chromatographic results have shown that the hydrolyzed polysaccharide migrates up the paper essentially as one component and this hydrolysate appears in the same relative position as the dextrose control. An example of the chromatograms obtained with H. capsulatum G17(M) hydrolyzed polysaccharide has been presented (Figure 2). Studies of the other three strains of H. capsulatum polysaccharides and the B. dermatitidis polysaccharides have produced similar results. It would be possible to further quantitate the results of the paper chromatograms by eluting the spots of hydrolyzed polysaccharide from the paper and then performing chemical analyses on the eluates.. This type of procedure is invaluable when extremely small amounts of the original test substance are available. When larger quantities of test substances are available it is more convenient to conduct standard macro chemical studies. For this reason, standard chemical studies of the constituents of the polysaccharides were conducted.

A strictly chemical test for the determination of the components of the polysaccharides was the cysteine, sulfuric acid tests. It should be emphasized that this method does not yield the absolute hexose content. This is apparent from the fact that when the unhydrolyzed polysaccharides were exposed to the longer heat treatment of 10 minutes in sulfuric acid, the results obtained suggested greater amounts of hexose. Although the mechanisms of color development in this test have not been explained, it may be assumed that the cystiene forms some sort of bond with the free hexose groupings of the polysaccharide. When the polysaccharide is heated for 10 minutes rather than 3 minutes, more hydrolysis occurs and consequently, more reactive groups are available for reaction with the cystiene In this investigation, there were no transmission peaks similar to those reported for pentose, however, the same transmission peak of 4100 A^{o} reported by Dische and Shettles (160) was observed for the hexose standard and the H. capsulatum and B. dermatitidis polysaccharides. Calculating from these data, it was found that the B. dermatitidis polysaccharide contained twice as much hexose as the H. capsulatum polysaccharides. The difference between these two polysaccharides cannot be readily explained. It is evident from this study that hexose appears to be a major constituent of the polysaccharide.

Other chemical studies have been conducted to further study the constituents of the polysaccharide. The Folin Wu determinations for reducing substances revealed that the H. capsulatum and B. dermatitidis polysaccharides contain 90 per cent and 75 per cent respectively of reducing substances, assumed to be dextrose (see Table 7). The small, but significant nitrogen content is envisioned as an occasional glucoseamine residue in the polysaccharide moiety. The extremely small phosphorous content can best be explained as a contaminant.

The Oudin and Ouchterlony agar precipitation tests are simple, yet effective, methods for determination of the antigenic homogeneity of substances. In the first series of H. capsulatum polysaccharide reactions with H. capsulatum, B. dermatitidis and C. immitis antisera, it was shown that the H. capsulatum polysaccharides produce one antigenic component against the homologous anti H. capsulatum serum. The B. dermatitidis polysaccharide showed the same specificity of reaction, that is, the B. dermatitidis polysaccharides produced only one band of precipitate against the anti B. dermatitidis serum without cross reactions with other fungal antisera. These results are considered additional evidence that the polysaccharides do contain one antigenically active constituent. It is assumed, of course, that the observed precipitates were due to the interaction of the polysaccharide and antiserum. The chemical studies seem to preclude the possibility that these antigen-antibody reactions were due to any substances except the polysaccharide.

Graber (163) has collected data which indicate that an antiserum must contain between 5 and 10 mcg of antibody nitrogen to produce visible precipitates when mixed with antigen in agar. By contrast, only .03 to .0004 mcg of antibody nitrogen is necessary for the fixation of complement. These data suggest a ready explanation why the low titered (as measured in the complement fixation test) guinea pig sera failed to react in the Ouchterlony agar diffusion test. The H. capsulatum strain G17(M) polysaccharide antigen diluted in saline and stored at 4°C was found to be active for at least one month without loss of antigenicity in complement fixation tests. The antigenic activity of this antigen in complement fixation tests, using human sera, has not been studied.

Skin reactive substances, isolated by chemical extraction from cultures of H. capsulatum, B. dermatitidis, and C. immitis have been reported by other investigators (147-154). There exists a consensus that an ideal skin test antigen, at a critical dilution, should give a maximum number of homologous responses and a minimum number of heterologous responses; that is, the material should be both sensitive and specific. The question of the specificity of fungal skin test antigens is of prime importance, because it has been well established that skin test cross reactions occur with histoplasmin, blastomycin, and to a lesser degree with coccidioidin and haplosporangin (168).

This lack of specificity is the rationale for testing humans suspected of having pulmonary mycoses with the three major systemic fungal skin test antigens (histoplasmin, blastomycin, and coccidioidin).

In this investigation similar skin test reactions were observed with polysaccharide substances derived from either whole cells or broth. These similar skin test reactions obtained with equal amounts of the cell and broth polysaccharides suggest that the greater quantity of polysaccharides obtained from broth was not due to contaminating artifacts but was due to the release of polysaccharide substances into the medium during growth.

In tuberculosis skin tests, the purified protein derivative (PPD) has been accepted as a world wide standard because a known weight of antigen can be injected intradermally and the resultant skin reaction is reproducible and specific. The polysaccharide antigens derived from _H. capsulatum_ and _B. dermatitidis_ also may be injected on a weight basis and, in addition, these antigens contain no protein, therefore non-specific protein hypersensitive reactions can be avoided.

The biologic assay of histoplasmin, as outlined by the National Institutes of Health, requires that the skin test antigen, when injected intracutaneously into guinea pigs, infected 1 month previously with _H. capsulatum_, should produce at least a 5 mm area of induration in each animal and that the observed mean reaction should be equal to the reaction produced by NIH histoplasmin.

The results of the first and second series of experiments produced variable skin test results. The results shown in Table 13 suggested the possibility that there might be a complete strain specificity since the _H. capsulatum_ strain 6651 broth and cellular polysaccharides failed to produce skin tests in animals infected with _H. capsulatum_ G17. However, subsequent experiments ruled out this possibility. The results tabulated in Tables 15 and 16 showed that 100 mcg of three strains of _H. capsulatum_ polysaccharides produced skin tests equal to the National Institutes of Health histoplasmin. Table 15 showed that the skin test reactivity of guinea pigs infected intraperitoneally was equal to the skin test activity of guinea pigs infected intracardially. In addition, there appears to be no difference in skin test activity when guinea pigs are infected with mycelial phase organisms. Table 16 also showed that the polysaccharides extracted from the yeast phase of H. capsulatum G17(M) produced skin tests equal to those produced by polysaccharides extracted from the mycelial phase of the same organism. It is also shown in Table 16 that the polysaccharides extracted from the cells of the H. capsulatum G17(M) and 6651 produced skin tests slightly less than the broth polysaccharides, but the results of other comparative studies, in this investigation, have shown the cell and broth polysaccharides produce equal skin tests.

In Table 19 are shown the results of skin tests comparing two lots of polysaccharides extracted from H. capsulatum strains 6651 and G17(M). It is evident that the second lot of each polysaccharide produced skin tests equal to those produced by the first lot of polysaccharide. The trypsin treated polysaccharide, from 2 strains of H. capsulatum, was no different in skin test activity for each of the 2 strains of H. capsulatum polysaccharide employed. This is further evidence that protein does not act as the skin test active substance present in these antigens.

The skin test reactions recorded in Table 20 showed that the B. dermatitidis strain 380 polysaccharide was capable of producing positive skin test reactions in guinea pigs infected with the same strain of B. dermatitidis. Furthermore, better reactions were obtained with the polysaccharide than were produced by commercially available blastomycin. The B. dermatitidis cell and broth polysaccharides appeared to be equally active. Preference must be given to the broth polysaccharide because greater quantities can be obtained with the same effort of preparation. The 10 mcg test doses of H. capsulatum G17(M) polysaccharide produced an essentially negative reaction in the blastomycotic guinea pigs. However, the 100 mcg H. capsulatum G17(M) polysaccharide showed a degree of non-specific reactivity.

The homologous and heterologous skin test reactions of H. capsulatum strains G17(M) and 6651 polysaccharides, and B. dermatitidis strains 380 and 410 polysaccharides in 6 guinea pigs infected with H. capsulatum and 6 guinea pigs infected with B. dermatitidis yeast phase organisms are shown in Tables 21 and 22. The B. dermatitidis strains 380 and 410 polysaccharides, in a concentration of 10 mcg, produced specific reactions in the homologously infected guinea pigs without non-specific reactions in the H. capsulatum infected guinea pigs. The H. capsulatum polysaccharides in a concentration of 10 mcg produced acceptable skin test reactions in _H. capsulatum_ infected guinea pigs. However, in the _B. dermatitidis_ infected guinea pigs the _H. capsulatum_ polysaccharides skin tests yielded non-specific cross reactions when 10 mcg doses were used. The 2 observed reactions with the 10 mcg of _H. capsulatum_ G17(M) polysaccharide were less than the minimum of 5 mm of induration and about half the size of the skin test reaction in the _H. capsulatum_ infected guinea pigs.

The previous guinea pig studies revealed that 10 mcg of _H. capsulatum_ G17(M) polysaccharide produced a mean homologous skin test reaction of 13 mm. This correlates well with the mean human skin test reaction of 14 mm noted in Table 25 which is concerned with tests on humans. A comparison of the human skin test reactions of histoplasmin and the _H. capsulatum_ G17(M) polysaccharide tabulated in Table 23 shows that the skin test reactions are about equal. It is interesting that the skin test reactions of the polysaccharide antigens were almost maximum at 24 hours and not significantly different from the 48 hour reactions.

All persons exhibiting positive skin tests had spent considerable periods of time in the known endemic areas of the Mississippi and Ohio River Valleys and western portions of the Appalachian Mountains.

Previous guinea pig skin tests with the _B. dermatitidis_ strain 380 polysaccharide showed no cross reactions in guinea pigs infected with _H. capsulatum_, while the commercial blastomycin produced a significant number of cross reactions. In humans, the situation seems to have reversed itself since the blastomycin showed no cross reactions, while a mean of 7 mm of induration of non-specific reaction was noted with _B. dermatitidis_ 380 polysaccharide (see Table 25). However, the commercial blastomycin was inferior to _B. dermatitidis_ polysaccharides in homologous skin tests in _B. dermatitidis_ infected guinea pigs. Human blastomycosis infections, at the present time, appear to be very infrequent in this part of the country, therefore, the chances for a comparative study of blastomycin and _B. dermatitidis_ polysaccharides in humans is not feasible.

The small numbers of persons studied, in this investigation, are not representative of the population of this area, therefore, no conclusions can be drawn concerning the incidence of histoplasmin sensitivity for the local population.

The use of a large number of mice in the *H. capsulatum* immunization experiment, comparing whole cell and polysaccharide antigens, permitted the concomitant determination of the LD_{50} for the immunized and control animals. Statistical analyses of these data permits a numerical evaluation of the immunization procedures. The calculations indicate that these antigens produce a significant degree of protection with potency ratios of 7.62 for the whole cell antigen and 4.28 for the polysaccharide antigens. In other words, 7.62 times the challenge required to kill 50 per cent of normal mice is necessary to kill whole cell immunized mice; the corresponding figure in the case of polysaccharide is 4.28. Furthermore, no difference was noted among animals immunized with 1 or 3 injections of polysaccharide. It will be interesting to further explore the feasibility of immunizing mice and other animals against chronic infectious diseases by the use of corresponding polysaccharide antigens.

SUMMARY

1. *H. capsulatum* strains G17, G17(M) and 6651; and *B. dermatitidis* strains 380, 410 and 6052 have each been grown in enriched tryptose phosphate broth. This medium produced good growth of the yeast phase organisms with significant yields of polysaccharides.

2. Polysaccharides have been extracted from each of three strains of *H. capsulatum* and *B. dermatitidis*. The polysaccharides were obtained by chemical procedures using ethanol and chloroform. This method of polysaccharide extraction produced up to 3 grams of polysaccharide from 6 liters of the fungal broth. Polysaccharides have also been extracted from the cellular elements of several strains of *H. capsulatum* and 1 strain of *B. dermatitidis*.

3. Chemical studies on the hydrolysates of *H. capsulatum* and *B. dermatitidis* polysaccharides polysaccharides have shown that they consist mainly of glucose units with small amounts of nitrogen.

4. Investigations of the serologic properties of the *H. capsulatum* and *B. dermatitidis* polysaccharides, employing the Oudin and Ouchterlony technics have shown specific lines of precipitates opposite the homologous antibody, without evidence of cross reactions with other fungal antisera. The *H. capsulatum* G17(M) polysaccharide has been used as a satisfactory complement fixing antigen, when hyperimmune rabbit or guinea pig sera are used.

5. A major variable in skin testing *H. capsulatum* and *B. dermatitidis* infected guinea pigs was shown to be the degree of skin hypersensitivity of the infected animals. A comparison of the skin test activities of *H. capsulatum* polysaccharide and NIH Lot #2 histoplasmin showed that these polysaccharides produce a degree of induration equal to the NIH standard histoplasmin. Unfortunately, similar comparative studies of *B. dermatitidis* polysaccharides could not be conducted because blastomycin has not been standardized by the National Institutes of Health. However, the *B. dermatitidis* polysaccharides produced more consistant skin test reactions than did a commercial blastomycin. Comparative skin test investigations of polysaccharides derived from the broth and cellular elements of these fungi have shown equal skin test activity. However, preference for the broth polysaccharides was based on the greater quantity of extractable polysaccharide present in

the broth. Polysaccharide extracted from the mycelial phase of _H. capsulatum_ has shown skin test reactions comparable to the polysaccharide extracted from the yeast phase broth of the same strain of fungi. Preference, once again, was shown for the yeast phase organisms because the hazards of pulmonary infections could be avoided.

Quantitative studies of the homologous and heterologous skin test reactions of _H. capsulatum_ and _B. dermatitidis_ polysaccharides have shown that a specific homologous skin test reaction depends on the amount of polysaccharide injected, and that by employing optimum concentrations it was possible to produce specific skin test reactions.

6. Human skin tests have been conducted using _H. capsulatum_ G17(M) polysaccharide and _B. dermatitidis_ strain 380 polysaccharide. One hundred thirty four volunteers were injected intradermally with _H. capsulatum_ and _B. dermatitidis_ polysaccharides and the skin test reactions compared with the skin test reactions produced by the intradermal injection of histoplasmin and blastomycin. Twenty positive skin tests were observed, and a comparison of the skin test reactions elicited by the polysaccharide and the histoplasmin were made. This study has shown that 10 mcg of _H. capsulatum_ G17 polysaccharide produced skin tests equal in sensitivity and specificity to those observed with histoplasmin. No specific _B. dermatitidis_ strain 380 polysaccharide skin tests were observed; however, 10 mcg of the _B. dermatitidis_ polysaccharide produced minimal nonspecific skin test reactions in a few of the persons exhibiting positive _H. capsulatum_ skin tests.

7. The _H. capsulatum_ strain G17(M) polysaccharide was capable of producing active resistance in mice. However, mice immunized with a whole cell antigen showed greater resistance when the _H. capsulatum_ polysaccharide immunized, whole cell immunized and non-immunized control groups of mice were challenged by the intravenous injection of known numbers of viable _H. capsulatum_ G17(M) yeast phase organisms.

REFERENCES

1. Darling, S. T. 1906 A protozoan infection producing pseudotubercles in the lungs and focal necroses in spleen, liver, and lymph nodes. J.A.M.A. 46:1283.

2. Darling, S. T. 1909 The morphology of the parasite H. capsulatum and lesions of histoplasmosis, a fatal disease of tropical America. J. Exper. Med. 11:515.

3. DeMonbreun, W. A. 1934 The cultivation and cultural characteristics of Darling's H. capsulatum. Am. J. Trop. Med. 14:93.

4. Smith, C. E., Whiting, E. D., Barker, E. E., Rosenberg, H. G., Beard, R. R., and Saito, M. T. 1948 The use of coccidioidin. Am. Rev. Tuberc. 57:330.

5. Christie, A. and Peterson, J. C. 1945 Pulmonary calcifications in negative reactors to tuberculin. Am. J. Pub. Health 35:1131.

6. Palmer, C. E. 1945 Nontuberculous pulmonary calcifications in negative reactors to tuberculin. Pub. Health Rep. 60:513.

7. Furcolow, M. L., High, R. H., Allen, M. F. 1946 Some epidemiological aspects of sensitivity to histoplasmin and tuberculin. Pub. Health Rep. 61:1132.

8. Christie, A., and Peterson, J. C. 1946 Histoplasmin sensitivity. J. Pediat. 29:417.

9. Christie, A., and Peterson, J. C. 1946 Pulmonary calcification and sensitivity to histoplasmin tuberculin and haplosporangin. J.A.M.A. 131:658.

10. Sontag, L. W. and Allen, J. E. 1947 Lung calcifications and histoplasmin-tuberculin skin sensitivity. J. Pediat. 30:657.

11. High, R. H., Zwerling, H. B. and Furcolow, M. L. 1947 Disseminated pulmonary calcification; report of 113 cases. Pub. Health Rep. 62:20.

12. Peterson, C. J. and Christie, A. 1948 Histoplasmosis and pulmonary calcifications. Am. Rev. Tuberc. 57:361.

13. Bunnell, I. L. and Furculow, M. L. 1948 Variations in histoplasmin sensitivity in certain cities in eastern Kansas. Pub. Health Rep. 63:1298.

14. Absher, W. K., and Cline, F. Jr. 1949 A correlated study of tuberculin, histoplasmin, and coccidioidin sensitivities with pulmonary calcifications in the Rocky Mountain area. Am. Rev. Tuberc. 59:643.

15. Zahn, D. W. 1949 Pulmonary infiltration associated with sensitivity to histoplasmin: report of case. Am. Rev. Tuberc. 59:636.

16. Sutliff, W. D., Hughes, F., Ulrich, E., and Burkette, L. L. Active chronic pulmonary histoplasmosis. A.M.A. Arch. Int. Med. 92:571.

17. Furcolow, M. L., Schwarz, J., Hewell, B. A. and Grayston, J. T. 1953 Incidence of tuberculin, histoplasmin, and blastomycin reactors among a group of school children. Am. J. Pub. Health 43:1523.

18. Tucker, H. A. 1952 Histoplasmosis on the Isthmus of Panama. Am. J. Trop. Med. 1:462.

19. Inglis, J. A. and Powell, R. E. 1953 Histoplasmosis: A review with report of a fatal Australian case. M.J. Australia 1:138.

20. Edwards, P. Q. and Kier, J. H. 1956 World wide geographic distribution of histoplasmosis and histoplasmin sensitivity. Am. Rev. Tuberc. 5:235.

21. Poles, F. C. and Lavertine, J. D. O. 1954 Acute disseminated histoplasmosis with a report of a case occurring in England. Thorax 9:233.

22. Schwartz, B. and Spitz, L. J. 1952 Histoplasmosis in epidemic form: a follow up study. A.M.A. Arch. Int. Med. 89:541.

23. Loosli, C. G., Procknow, J. J., Tanzi, F., Grayston, J. T. and Combs, L. W. 1951 Pulmonary histoplasmosis in a farm family. J. Lab. & Clin. Med. 43:669.

24. Emmons, C. W. 1949 Isolation of Histoplasma capsulatum from soil. Pub. Health Rep. 64:892.

25. Grayston, J. T., Loosli, C. G. and Alexander, E. R. 1951 The isolation of Histoplasma capsulatum from soils in unused silo. Science 114:323.

26. Furcolow, M. L. and Larch, H. W. 1952 Direct isolation of Histoplasma capsulatum from soil: probable etiological relationship to Camp Gruber pneumonitis. Proc. Soc. Exper. Biol. & Med. 80:246.

27. Zeidberg, L. D., Ajello, L., Dillon, A. and Runyon, L. C. 1952 Isolation of Histoplasma capsulatum from soil. Am. J. Pub. Health 42:930.

28. Gordon, M. A. and Cup, H. B. Jr. 1953 Detection of Histoplasma capsulatum and other fungus spores in the environment by means of the membrane filter. Mycologia 45:241.

29. Ajello, L. and Runyon, L. C. 1953 Infection of mice with single spores of Histoplasma capsulatum. J. Bact. 66:34.

30. Ibach, M. J., Larsh, H. W. and Furcolow, M. L. 1954 Epidemic histoplasmosis and airborne Histoplasma capsulatum. Proc. Soc. Exper. Biol. & Med. 85:72.

31. Zeidberg, L. D., Dillon, A. and Gass, R. S. 1954 Environmental factors influencing the occurrence of Histoplasma capsulatum and Microsporum gypseum in soil.

32. Kier, J. H., Campbell, C., Ajello, L. and Wheelam, D. S. 1954 Acute bronchopneumonic following exposure to infected garden soil. J.A.M.A. 155:1230.

33. Zeidberg, L. D. 1954 A theory to explain the geographic variation in the prevalence of histoplasmin sensitivity. Am. J. Trop. Med. 3:1057.

34. Furcolow, M. L. and Ruhe, J. S. 1949 Histoplasmin sensitivity among cattle. Am. J. Pub. Health 39:719.

35. Randall, C. C., Orr, M. F., Schell, F. G. 1951 Detection by tissue culture of an organism resembling Histoplasma capsulatum in an apparently healthy horse. Proc. Soc. Exper. Biol. & Med. 78:447.

36. Cole, C. R., Farrell, R. L., Chamberlain, D. M., Prior, J. A. and Saslaw, S. 1953 Histoplasmosis in animals. Am. Vet. M. A. 122:471.

37. Rowley, D. A., Haberman, R. T. and Emmons, C. W. 1954 Histoplasmosis pathologic studies of fifty cats and fifty dogs from Soudoun County, Virginia. J. Infect. Dis. 95:98.

38. Emmons, C. W. and Rowley, D. A. 1955 Isolation of Histoplasma capsulatum from fresh and deep-frozen peribronchial lymph nodes of dogs by mouse inoculation. J. Lab.& Clin. Med. 45:303.

39. Emmons, C. W., Rowley, D. A., Olson, B. J., Mattern, C. F. T., Bell, J. A., Powell, E. and Marcey, E. A. 1955 Histoplasmosis: proved occurrence of inapparent infection in dogs, cats and other animals. Am. J. Hyg. 61:40.

40. Furcolow, M. L., Menges, R. W. and Larsh, H. W. 1955 An epidemic of histoplasmosis involving man and animals. Ann. Int. Med. 43:173.

41. Middleton, J. G., McVickar, D. L. and Peterson, J. C. 1950 Experimental histoplasmosis in white rat. Proc. Soc. Exper. Biol. & Med. 25:164.

42. Kotcher, E., Robinson, J. W. and Miller, M. P. 1951 The isolation of Histoplasma capsulatum from tissues of experimentally infected mice. J. Bact. 62:613.

43. Farrell, R. L., Cole, C. R., Prior, J. A. and Saslaw, S. 1953 Experimental histoplasmosis. I. Methods for production of histoplasmosis in dogs. Proc. Soc. Exper. Biol. & Med. 84:51.

44. Salvin, S. B. 1954 Cultural and serologic studies on nonfatal histoplasmosis in mice, hamsters, and guinea pigs. J. Infect. Dis. 94:22.

45. Howell, A. Jr. and Kipkie, G. F. 1951 A comparison of the susceptibility by intracerebral inoculation of six strains of mice with DBA line 1 mice. Am. J. Trop. Med. 31:33.

46. Howell, A. Jr., Kipkie, G. F. and Bruyere, P. T. 1950 Studies on experimental histoplasmosis. Pub. Health Rep. 65:722.

47. Marcus, S. and Rambo, F. R. 1952 Reproducible systemic mycotic infections in mice. Bact. Proc. M34.

48. Rowley, D. A. and Huber, M. 1955 Pathogenesis of experimental histoplasmosis. J. Infect. Dis. 96:174.

49. Campbell, C. C. and Saslaw, S. 1950 Use of mucin in experimental infections of mice with Histoplasma capsulatum infections. Proc. Soc. Exper. Biol. & Med. 73:469.

50. Howell, A. Jr. and Kipkie, G. F. 1950 Experimental histoplasmosis. Susceptibility of male DBA line 1 mice by various routes of injection. Proc. Soc. Exper. Biol. & Med. 75:121.

51. Pinkerton, H. and Iverson, L. 1952 Histoplasmosis. Three fatal cases with disseminated sarcoid-like lesions. A.M.A. Arch. Int. Med. 90:456.

52. Straub, M. and Schwarz, J. 1955 The healed primary complex in histoplasmosis. Am. J. Path. 25:727.

53. Furcolow, M. L. and Brasher, C. A. 1956 Progressive histoplasmosis as a problem in tuberculosis sanatoriums. Am. Rev. Tuberc. 73:609.

54. Pucket, T. F. 1953 Pulmonary histoplasmosis. Am. Rev. Tuberc. 67:453.

55. Silverman, F. N., Schwarz, J., Lahey, M. E. and Carson, R. P. 1955 Histoplasmosis. Am. J. Med. 19:410.

56. Schwartz, S. O. and Barsky, S. 1952 Report of 193 marrow biopsy specimens cultured for Histoplasma capsulatum. Blood 7:545.

57. McVickar, D. L. 1951 Factors important for the growth of Histoplasma capsulatum in the yeast cell phase on peptone media. J. Bact. 62:137.

58. Marcus, S., Nielson, B. D. and Rambo, F. R. 1956 Isolation of systemic mycotic pathogens: qualitative and quantitative aspects. J. Bact. 72:473.

59. Georg, L. K., Ajello, L. and Papageorge, C. 1954 Use of cycloheximide in the selective isolation of fungi pathogenic to man. J. Lab. & Clin. Med. 44:422.

60. Zarafonetis, C. J. D. 1952 Dubos medium with albumin for yeast phase growth of Histoplasma capsulatum. Am. J. Clin. Path. 22:911.

61. Pine, L. 1954 Studies on the growth of Histoplasma capsulatum. J. Bact. 68:671.

62. Rowley, D. A. and Pine, L. 1955 Some nutritional factors influencing growth of yeast cells of Histoplasma capsulatum. J. Bact. 69:695.

63. White, F. C. 1955 Chronic pulmonary disease in histoplasmin reactors. Am. Rev. Tuberc. 72:274.

64. Zarafonetis, C. J. D. and Lindberg, R. B. 1941 Histoplasmosis of Darling: Observations on the antigenic properties of the causative agent. Univ. Hosp. Bull. Ann Arbor, Michigan 7:47.

65. Schubert, J. H., Ajello, L., Cooper, J. S. and Runyan, L. C. 1955 Evaluation of histoplasmin and yeast phase antigens derived from a single strain of Histoplasma capsulatum in the complement fixation test. J. Bact. 69:558.

66. Schubert, J., Ajello, L., Stanford, S. and Grant, V. Q. 1953 Variations in complement fixation antigen production by different strains of Histoplasma capsulatum grown on two media. J. Lab. & Clin. Med. 41:91.

67. Dyson, J. E. and Evans, E. E. 1954 Skin test antigens from yeast phase cultures of Blastomyces dermatitidis and Histoplasma capsulatum. Univ. Michigan Med. Bull. 20:53.

68. Hill, G. A., Marcus, S. and Baker, K. R. 1953 Serologic characteristics of four systemically pathogenic fungi. Bact. Proc. G38.

69. Norden, A. 1949 Agglutination of sheep's erythrocytes sensitized with histoplasmin. Proc. Soc. Exper. Biol. & Med. 70:218.

70. Salvin, S. B. and Furcolow, M. L. 1954 Precipitins in human histoplasmosis. J. Lab. & Clin. Med. 43:259.

71. Oudin, J. 1952 Specific precipitations in gels and its application to immunochemical analysis. Methods of Medical Research. 5:335.

72. Ouchterlony, O. 1949 Antigen-antibody reactions in gels. Acta path. et microbiol. Scandinav. 26:507.

73. Wilson, H. M. and Duryea, A. W. 1952 Pulmonary histoplasmosis: Report of a case of long considered tuberculosis improved by acti-dione. Dis. Chest 21:346.

74. Emmons, C. W. and Haberman, R. T. 1953 Ascosin in the treatment of experimental histoplasmosis in mice. Antibiotics & Chemother. 3:1204.

75. Christie, A., Middleton, J. G., Peterson, J. C. and McVickar, D. L. 1951 Treatment of disseminated histoplasmosis with ethyl vanillate. Pediatrics 7:7.

76. Hansen, A. E. and Beene, M. L. 1951 Sensitivity studies *in vitro* with *Histoplasma capsulatum* to various potential therapeutic agents. Proc. Soc. Exper. Biol. & Med. 77:365.

77. Campbell, C. C. and Saslaw, S. 1951 Atabrine therapy of histoplasma infections in mice. Pub. Health Rep. 66:570.

78. Lamb, J. H. 1954 Combined therapy in histoplasmosis and coccidioidiomycosis. A.M.A. Arch. Dermat. & Syph. 70:695.

79. Curtis, A. C., Bocobo, F., Hannell, E. R. and Block, W. D. 1954 Further studies on antifungal activity of some cinnamic acid deratives and nitrostyrenes. A.M.A. Arch. Dermat. & Syph. 70:786.

80. Baum, G. L., Schwarz, J. and Wang, C. J. K. 1958 Treatment of experimental histoplasmosis with amphoterisin B. A.M.A. Arch. Int. Med. 101:84.

81. Gilchrist, T. C. 1894 A case of *Blastomyces dermatitidis* in man. Johns Hopkins Hosp. Rep. 1:271.

82. Benham, R. W. 1950 Blastomyces: review of literature. Ann. New York Acad. Sc. 50:1299.

83. Gilchrist, T. C. and Stokes, W. R. 1896 The presence of an oidium in the tissues of a case of pseudolupus vulgaris. Johns Hopkins Hosp. Bull. 7:129.

84. Gilchrist, T. C. 1896 Cited the work of Corelli and Frisco. Johns Hopkins Hosp. Bull. 7:129.

85. Gilchrist, T. C. 1896 Cited the work of Porrcali. Johns Hopkins Hosp. Bull. 7:129.

86. Gilchrist, T. C. 1896 Cited the work of Curtis. Johns Hopkins Hosp. Bull. 7:129.

87. Martin, D. S. and Smith, D. T. 1939 Blastomycosis: I. Review of literature. Am. Rev. Tuberc. 39:275.

88. Shepherd, F. J. and Rhea, L. I. 1911 A fatal case of blastomycosis. J. Cutan. Dis. 29:588.

89. Dowling, G. B. and Elworthy, R. R. 1925 A case of blastomycetic dermatitidis. Proc. Roy. Soc. Med. 19:4.

90. Schwarz, J. and Goldman, L. 1955 Epidemiologic study of North American blastomycosis. A.M.A. Arch. Dermat. & Syph. 71:84.

91. Ramsey, F. K. and Carter, G. R. 1952 Canine blastomycosis in United States. J. Am. Vet. M.A. 20:93.

92. Menges, R. W., McClellan, J. T. and Ausherman, R. J. 1954 Canine histoplasmosis and blastomycosis cases in Lexington, Kentucky. J. Am. Vet. M.A. 124:202.

93. Schwarz, J. and Baum, G. L. 1951 Blastomycosis. Am. J. Clin. Path. 21:999.

94. Schwarz, J. and Baum, G. L. 1953 North American blastomycosis (geographic distribution, pathology and pathogenesis). Docum. med. geog. et trop. 5:29.

95. Harris, J. S., Smith, J. G., Humbart, W. C., Conant, N. F. and Smith, D. T. 1957 North American blastomycosis in an epidemic area. Pub. Health. Rep. 72:95.

96. Wilson, W. J., Cowley, E. P., Weidman, E. D. and Gilmer, W. X. 1955 Primary cutaneous North American blastomycosis. A.M.A. Arch. Dermat. & Syph. 71:39.

97. Curtis, A. C. and Bocobo, F. C. 1957 North American blastomycosis. J. Chron. Dis. 5:404.

98. Montgomery, F. H. 1902 A brief summary of the clinical, pathologic and bacteriologic features of cutaneous blastomycosis. J.A.M.A. 38:1386.

99. D'Aunoy, R. and Beven, J. L. 1930 Systemic blastomycosis. J. Lab. & Clin. Med. 16:124.

100. Martin, D. S. and Smith, D. T. 1939 Blastomycosis II. Report of 13 cases. Am.Rev. Tuberc. 39:488.

101. Freidman, L. S. and Signorelli, J. J. 1946 Blastomycosis: A brief review of literature and a report of a case involving the meninges. Ann. Int. Med. 24:385.

102. Stober, A. M. 1914 Systemic blastomycosis: A report of its pathological and clinical features. A.M.A. Arch. Int. Med. 13:509.

103. Gair, C. C. 1925 Systemic blastomycosis. Surg. Gynec. & Obst. 41:490.

104. Feld, D. D. and Codden, A. V. 1949 Systemic blastomycosis. Dis. Chest 16:472.

105. Lowery, C. C., Kraeft, N. H. and Hughes, F. A. Jr. 1951 Blastomycosis of the lung. Am. J. Surg. 81:676.

106. Tuttle, J. G., Lichtwardt, H. E. and Altshuler, C. H. 1953 Systemic North American Blastomycosis. Report of a case with small forms of blastomyces. Am. J. Clin. Path. 23:890.

107. Steen, L. H., Foxworth, D. T. and Baker, L. A. 1954 Acute blastomycotic pneumonia, a report of a fatal case of short duration diagnosed by needle biopsy of lung. A.M.A. Arch. Int. Med. 93:464.

108. Cherinis, E. I. and Waisbren, B. A. 1956 North American blastomycosis: A clinical study of 40 cases. Ann. Int. Med. 44:105.

109. Brown, R. K. and Cummins, W. T. 1915 A differential study of coccidioidal granuloma and blastomycosis. I. Pathology and bacteriology. II. Report of two additional cases of coccidioidal disease. A.M.A. Arch. Int. Med. 15:605.

110. Michelson, I. D. 1928 Blastomycosis. A pathologic and bacteriologic study. J.A.M.A. 91:1871.

111. Kelley, W. A. 1939 A study of the cell and colony variations of Blastomyces dermatitidis. J. Infect. Dis. 64:293.

112. Littman, M. L., Wicker, E. H. and Warren, H. S. 1948 Systemic North American blastomycosis: Report of a case with cultural studies of the etiologic agent and observations on the effect of streptomycin and penicillin in vitro. Am. J. Path. 24:339.

113. Weed, L. A. 1955 North American blastomycosis. Am. J. Clin. Path. 25:37.

114. DeMonbreun, W. A. 1935 Experimental chronic cutaneous blastomycosis in monkeys. A study of the etiologic agent. A.M.A. Arch. Dermat. & Syph. 31:831.

115. Baker, R. D. 1939 The effect of mouse passage on cultural characteristics and virulence for mice of organisms causing blastomycosis. Am. J. Trop. Med. 19:547.

116. Davis, B. F. 1911 The immunologic reactions of oidiomycosis (blastomycosis). J. Infect. Dis. 8:190.

117. Martin, D. S. 1941 The practical application of some immunologic principles to the diagnosis and treatment of certain fungus infections. J. Invest. Dermat. 4:471.

118. Martin, D. S. 1957 Evaluation of skin tests and serologic methods in fungus infections. J. Chron. Dis. 5:580.

119. Perry, H. O., Weed, L. A. and Kierland, R. R. 1954 South American blastomycosis. Report of case and review of laboratory features. A.M.A. Arch. Dermat. & Syph. 70:477.

120. Martin, D. S. 1935 Complement fixation in blastomycosis. J. Infect. Dis. 57:291.

121. Smith, D. T. 1949 Immunologic types of blastomycosis. A report of 40 cases. Ann. Int. Med. 31:463.

122. Martin, D. S. 1953 Serologic studies on North American blastomycosis. J. Immunol. 71:192.

123. Schoenbach, E. B., Miller, J. M., Ginsberg, M. and Long, P. H. 1951 Systemic blastomycosis treated with stilbamidine. J.A.M.A. 146:1317.

124. Heilman, F. R. 1952 Effect of stilbamidine on blastomycosis in mice. Proc. Staff Meet. Mayo Clin. 27:455.

125. Schoenbach, E. B., Miller, J. M. and Long, P. H. 1952 The treatment of systemic blastomycosis with stilbamidine. Ann. Int. Med. 37:31.

126. Praiser, H., Levy, E. D. and Rawson, A. J. 1953 Treatment of blastomycosis with stilbamidine. J.A.M.A. 152:129.

127. Snapper, I. and McVay, L. V. Jr. 1953 The treatment of North American blastomycosis with 2-hydroxy stilbamidine. Am. J. Med. 15:603.

128. Schwarz, J. and Adriano, S. 1953 Failure of stilbamidine to arrest experimental blastomycosis in mice. J. Invest. Dermat. 20:329.

129. Christison, I. B. and Conant, N. F. 1953 Antifungal activity of some aromatic diamidines. J. Lab. & Clin. Med. 42:638.

130. Slaughter, J. C. 1954 Stilbamidine in treatment of widely disseminated blastomycosis (with two year follow up). A.M.A. Arch. Dermat. & Syph. 70:663.

131. Weinberg, B. J., Lawrence, C. H. and Buchnolz, A. 1954 Systemic blastomycosis treated with hydroxy stilbamidine. A.M.A. Arch. Int. Med. 94:493.

132. Stutliff, W. D., Kyle, W. J. and Hobson, J. L. 1954 North American blastomycosis, clinical form of the disease and treatment with stilbamidine and 2-hydroxy stilbamidine. Ann. Int. Med. 41:89.

133. Takaro, J., Hopkins, J. E. T. and Murphy, J. D. 1955 Treatment of pulmonary blastomycosis. Dis. Chest 28:203.

134. Harrell, E. R., Bocobo, F. C. and Curtis, A. C. 1955 A study of North American blastomycosis and its treatment with stilbamidine and 2-hydroxy stilbamidine. Ann. Int. Med. 43:1076.

135. Burger, M. 1950 Bacterial polysaccharides Charles C. Thomas, Publisher.

136. Heidelberger, M. and Menzel, A. 1932 Specific and non-specific polysaccharides of the human type tubercle bacillus. Proc. Soc. Exper. Biol. & Med. 29:631.

137. Heidelberger, M. and Karjala, S. 1939 Specific and non-specific cell polysaccharides of bovine strain of tubercle bacillus. J. Biol. Chem. 127:221.

138. Karjala, S. and Heidelberger, M. 1941 Specific and non-specific polysaccharides of an avian strain of tubercle bacillus. J. Biol. Chem. 137:189.

139. Heidelberger, M., Kendall, F. E. and Scherp, H. W. 1936 The specific polysaccharides of types I, II, and III pneumococcus. J. Exper. Med. 64:559.

140. Heidelberger, M., Macleod, C. M., Markowitz, H. and Roe, A. S. 1950 Improved methods for the preparation of the specific polysaccharides of pneumococcus. J. Exper. Med. 91:341.

141. Raistrick, H. and Topley, W. W. C. 1934 Immunizing fraction isolated from Bact. aertrycke. Brit. J. Exper. Path. 15:113.

142. Palmer, J. W. and Gerlough, T. D. 1940 A simple method for preparing antigenic substances from the typhoid bacillus. Science 92:155.

143. Nichols, P. S. 1946 On the antigenic structure of Bacterium tularense. Ph.D. Thesis, University of Cincinnati.

144. Alexander, M. M., Wright, G. C. and Baldwin, A. C. 1950 Observation of polysaccharide treated erythrocytes agglutinated by tularemia antisera. J. Exper. Med. 91:561.

145. Oeding, P. 1954 Immunochemical studies of staphylococcal polysaccharides. Acta path. et micro. biol. scandinav. 35:484.

146. Vogel, R. 1954 Polysaccharides of Candida albicans. Proc. Soc. Exper. Biol. & Med. 86:373.

147. Hirsch, E. F. and Benson, H. 1927 Specific skin reactions with culture filtrates of Coccidioides immitis. J. Infec. Dis. 40:629.

148. Hirsch, E. F. and D'Andrea, D. 1926 The specific substance of Coccidioides immitis. J. Infec. Dis. 40:634.

149. Hirsch, E. F. and D'Andrea, D. 1927 Sensitization of guinea pigs with broth culture filtrates and killed mycelium of Coccidioides immitis. J. Infect. Dis. 40:638.

150. Van Pernis, P. A., Benson, M. and Holinger, P. H. 1941 Specific cutaneous reactions in histoplasmosis. J.A.M.A. 117:436.

151. Hassid, W. Z., Baker, E. E. and McCready, R. M. 1943 An immunologically active polysaccharide produced by Coccidioides immitis. J. Biol. Chem. 194:305.

152. Peck, R., Martin, D. S. and Hauser, C. 1940 Polysaccharides of Blastomyces dermatitidis. J. Immunol. J. Immunol. 38:449.

153. Scheff, G. J. 1945 Biochemical and immunological properties of Histoplasma capsulatum no. 650. Yale J. Bio. & Med. 18:41.

154. Cross, F. and Howell, A. Jr. 1948 Preliminary report on the isolation of the immunologically active polysaccharide from histoplasmin. Pub. Health Rep. 64:179.

155. Hill, G. A. 1954 The serological characteristics of five systemically pathogenic fungi. Master's Thesis, University of Utah.

156. Wilcoxon, F. and Litchfield, J. T. 1954 A simplified method of evaluating dose effect experiments. J. Pharm. and Exper. Therap. 95:99.

157. Sevag, M., Lackman, D. B. and Smolens, J. 1938 The isolation of the components of streptococcal nucleoproteins in a serologically active form. J. Biol. Chem. 124:425.

158. Miya, F. 1955 The biosynthesis of ribose occurring in a bacterial polysaccharide. Master's Thesis. Brigham Young University.

159. Stattler, L. and Zerbon, F. W. 1952 A new spray reagent for paper chromatography of reducing sugars. Anals. of Chem. 24:1862.

160. Dische, Z. and Shettles, L. B. 1948 A specific color reaction for methyl pentoses and a spectrophotometric micro method for their determination. J. Bio. Chem. 175:595.

161. Fester, H. J. 1950 Manual for standardized procedures for spectrophotometric chemistry. Standard Scientific Supply Corp., New York City, New York.

162. Halbert, S. P., Swick, L. and Sonn, C. 1955 The use of precipitin analysis in agar for the study of human streptococcal infections. II. Ouchterlony and Oakley technics. J. Exper. Med. 101:557.

163. Grabar, P. in Boyd, W. C. 1956 Fundamentals of Immunology. 3rd Edition p. 94 Inter Science Publishers Ltd., London.

164. Smith, C. E., Saito, M. T. and Simon, S. A. 1956 The pattern of 39,500 serologic tests in coccidioidomycosis. J.A.M.A. 7:546.

165. Serologic tests for syphilis, 1955 manual. Public Health Service Publication #411 United States Government Printing Office, Washington, D. C.

166. Middlebrook, G. and Dubos, R. J. 1948 Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. J. Exper. Med. 88:521.

167. Allen, W. M. 1950 A simple method of analyzing complicated absorption curves of use in the colorimetric determination of urinary steroids. J. Clin. Endocrinol. 10:71.

168. Smith, C. E. 1952 Analogy of coccidioidin and histoplasmin sensitivity. Proc. Conf. on Histoplasmosis Public Health Monograph #39, p. 173.

THE CHEMICAL, SEROLOGIC AND SKIN TEST ACTIVITIES OF POLYSACCHARIDES EXTRACTED FROM HISTOPLASMA CAPSULATUM AND BLASTOMYCES DERMATITIDIS

by

Ralph A. Knight

An abstract of a thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Bacteriology

Approved by the faculty committee in

July, 1958

Dr. Stanley Marcus, Chairman, Thesis Committee

University of Utah
1958

Polysaccharides were extracted by the chemical method of Heidelberger, from each of three strains of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. Significantly greater yeilds of polysaccharides were extracted from the culture broth filtrate than were extracted from the yeast phase cells.

Studies of the constituents of these polysaccharides by paper chromatography and cystiene-sulfuric acid tests for pentose and hexose, micro-Kjedhahl nitrogen and other qualitative and quantitative chemical tests suggest that the *H. capsulatum* and *B. dermatitidis* polysaccharides consisted of glucose units with an occassional glucoseamine moiety.

The capacity of the *H. capsulatum* and *B. dermatitidis* polysaccharides to produce visible antigen-antibody precipitates with homologous and heterologous antisera were investigated by the Oudin and Ouchterlony agar precipitation methods. These studies showed that the *H. capsulatum* and the *B. dermatitidis* polysaccharides reacted only with their respective homologous antibody and that each produced a single band of precipitate. These data indicated that each antigen had a specific *in vitro* antigenic action, with one antigenically active component.

Complement fixation and hemagglutination tests were employed using the *H. capsulatum* polysaccharide antigen.

This study showed that the polysaccharide antigen could be used as a complement fixation antigen when hyperimmune guinea pig or rabbit sera were used as antibody. Hemagglutination test results were not reproducible.

The *H. capsulatum* and *B. dermatitidis* polysaccharides were used as skin test antigens. The intradermal injection of microgram amounts of these polysaccharides into guinea pigs infected with either *H. capsulatum* or *B. dermatitidis* produced skin test reactions equal to those given by the standard histoplasmin or blastomycin. A study of the skin test cross reaction of the *H. capsulatum* polysaccharides and *B. dermatitidis* polysaccharides in the guinea pigs infected with *B. dermatitidis* or *H. capsulatum* revealed specific skin test reactions without significant cross reactivity. *H. capsulatum* and *B. dermatitidis* polysaccharide antigens were injected intradermally into 136 human volunteers. Twenty positive reactions were observed and a comparison of the extent of induration produced by histoplasmin and 10 mcg of *H. capsulatum* showed that the *H. capsulatum* polysaccharide produced skin test reactions equal to those observed with histoplasmin. Specific *B. dermatitidis* skin test reactions were not observed; however, minor skin test reactions were observed following injection of the *B. dermatitidis* polysaccharides in a few persons who showed strong reactions with *H. capsulatum* skin test materials. These studies suggest that fungal polysaccharides, isolated by relatively simple chemical methods, may be more suitable for use as skin test antigens than the currently available preparations of histoplasmin and blastomycin.

RESEARCH PROPOSALS

submitted

by

Ralph A. Knight

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Department of Bacteriology

University of Utah

July, 1958

RESEARCH PROPOSALS

1. Definitive studies of H. capsulatum and B. dermatitidis polysaccharides are desirable. For example: counter current distribution procedure could be employed to separate H. capsulatum and B. dermatitidis polysaccharides. This project could be conducted in association with the biochemistry research unit of the Veterans Administration Hospital, Salt Lake City, Utah. The investigation would be designed to determine if the unhydrolysed polysaccharides can be separated or purified by this method. If these polysaccharides could be separated by counter current distribution, the antigenic activity of the various fractions could then be tested by skin test and the Ouchterlony technics.

2. Further serologic studies of fungal polysaccharides are desirable. Further effort would be made to study hemagglutination reactions of these polysaccharides. The reasons for the failure of these polysaccharide antigens to be adsorbed by sheep red blood cells, or rather the failure to obtain hemagglutination of these sensitized red blood cells should be further investigated. Perhaps the treatment of the cells with dilute tannic acid may produce a red blood cell that will adsorb antigen and then react with antibody.

3. Literature has been cited in the thesis to show that fungal polysaccharide antigens can not be used as complement fixation antigens when human sera are tested.

There is no theoretical basis to support such findings. There is indicated a comparative study of whole cell, cell free extract antigens and the corresponding *H. capsulatum* and *B. dermatitidis* polysaccharides used with human sera containing *H. capsulatum* or *B. dermatitidis* antibodies.

4. A significant sample of the residents of Utah should be skin tested to determine the incidence of *H. capsulatum* or *B. dermatitidis* skin hypersensitivity. In conjunction with this approach, an effort should be made to isolate *H. capsulatum* and other fungi from chicken coops and pigeon nests.

5. Better methods of inducing fungal infections in guinea pigs or rabbits may result from lowering the initial resistance of these animals with whole body ionizing radiation or pretreatment with cortisol. Such depression of initial resistance to the fungi might produce a more uniform skin sensitivity among groups of guinea pigs or rabbits and so render these animals more suitable for skin test assay purposes.

6. Polysaccharide skin test antigens should be prepared from *Sporotrichum schenkii* and *C. immitis*. These substances should be examined chemically, serologically and for their value as skin test agents.

7. Further investigations of the immunization of mice with polysaccharides should be made. The basis of

such investigation is the hypothesis that it is possible to enhance resistance against acute or chronic diseases by such immunization.

8. _Toxoplasma_ _gondii_ seems to require living cells for its growth. These organisms are routinely maintained by serial intraperitoneal passage through mice. However, these organisms have been grown in tissue culture. A definitive study of the constituents of tissue culture necessary for the growth of _T._ _gondii_ might reveal a method for the artificial cultivation of this organism.

9. The Sabin-Feldman dye test is not an entirely satisfactory serologic test for the detection of _T._ _gondii_ antibodies because live parasites must be used and the test involves microscopic examination. The use of a nonviable antigen in a serologic hemagglutination test has been reported. Confirmation of this report and further refinement of the method should be attempted.

10. The mechanisms of immunity in toxoplasmosis have not been investigated. A study of the mechanisms of destruction of these parasites and the extent of the resistance produced in experimental animals by infection or by immunization should be attempted.