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THE USEFULNESS OF THE PASSIVE CUTANEOUS ANAPHYLAXIS (PCA) REACTION FOR THE DEMONSTRATION OF ANTI-CRYPTOCOCCAL ANTIBODIES IN HUMANS

by

Dorothy Boyd Prest

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Dorothy Boyd Prest

entitled The Usefulness of the Passive Cutaneous Anaphylaxis (PCA) Reaction for the Demonstration of Anti-Cryptococcal Antibodies in Humans.

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

Howard Evans Taber — Dissertation Director

May 6, 1966 — Date

After inspection of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

Kenneth J. Vertman	5/6/66
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W. Perrin	5/6/66

*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.

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SIGNED: Dorothy B. Prest

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ABSTRACT

The occurrence of Cryptococcus neoformans as a saprophyte in nature and in the air we breathe suggests that infection by the organism may be more common than is evident from the number of clinical cases of cryptococcosis. Proof of this is lacking since methods for the detection of sensitizing antibodies have not yet been developed. This investigation was concerned with the use of the passive cutaneous anaphylaxis (PCA) test with guinea pigs as the test animal for the detection of such antibodies.

Nine rabbits were injected with strains of C. neoformans, two with a strain of C. diffluens and two with a strain of C. laurentii. None of the animals developed signs of illness and none of those tested exhibited pre-infection sensitivity by skin test. Post-infection, some animals reacted to intradermal injection of the cell wall antigen. The antigens used here and in the early PCA testing included whole cell, ruptured cell, crude cell wall, capsular, crude polysaccharide and extracellular starch preparations.

For the PCA tests, the extracellular starch antigen proved to be the most effective. Only one rabbit serum reacted strongly and positively but six others showed some degree of activity when tested with the homologus antigen.

The serums of mice inoculated with C. neoformans failed to react.

Five hundred human serums including three from cases of cryptococcosis were tested for evidence of hypersensitivity. One serum only gave a strongly positive reaction in a titer sufficiently high to suggest previous subclinical infection. Serums from clinical cases of cryptococcosis gave negative results.

While the usefulness of the PCA test for the detection of sensitizing antibodies is not fully established, it appears to be worthy of further investigation.

INTRODUCTION

The fungi that produce disease in man and animals are apparently not parasites in the strict sense since they are not dependent on living material for existence and only rarely is disease due to them spread from man to man or animal to man or vice versa. They are incidental parasites that exist as saprophytes in nature. Soil, for example, appears to be a reservoir for several fungal pathogens including Histoplasma capsulatum (2, 22, 23), Coccidioides immitis (14, 70), Allescheria boydii (2, 17, 19) and many of the dermatophytes (2, 48). Sporotrichum schenckii and Phialophora verrucosa have been isolated from mine timbers (8) and boards from the side of a barn (2), respectively. Cryptococcus neoformans has also been isolated from non-living sources such as fermenting peach juice (65), milk (9, 37), soil (2, 3, 18, 27), air (44, 66), pigeon droppings (5, 20, 21, 27, 42, 60, 73), avian nests (35), and fresh guano of the Mexican freetail bat (34). All of these appear to be vehicles of the yeast rather than the ultimate source. In addition, C. neoformans has been recovered from habitats such as the cecal contents of a healthy horse (75) and the bark beetle (Dendroctonus sp.) as well as from the slime flux of the mesquite tree, Prosopis juliflora (24). The intestinal tract of man and animals, the bodies of insects, and the exudate, bark, leaves and flowers of

living trees or plants are all recognized as natural habitats for yeasts. However, while C. neoformans has been reported only once from the gastro-intestinal tract of a mammal and once from the body of an insect, Evenson and Lamb (24) found C. neoformans in 45% of the samples of mesquite slime flux which they examined. The occurence of this yeast in slime flux was confirmed by Westerlund (76) who also isolated it from bark samples and from twigs of mesquite. The rather common occurrence of C. neoformans in avian dung, slime flux, and bark suggests that these are some of the habitats in which the yeast might be able to proliferate and from which the yeast could be disseminated by air currents, by insects or by other means.

Since primary cryptococcosis is thought to be a pulmonary disease (12), it would appear that C. neoformans is, at times, airborne. It might be expected that an individual who inhales this yeast and develops clinical or subclinical infection would become sensitized in a manner similar to that occurring in the case of Coccidioides immitis and Histoplasma capsulatum. In the case of C. neoformans, evidence for this is lacking. Individuals who recover from asymptomatic or symptomatic coccidioidomycosis or histoplasmosis exhibit a positive skin reaction of the delayed type which is associated with complete immunity to the particular disease. In the case of cryptococcosis, no reliable skin test has been developed.

Jellison, Glesne and Owen (30) recently reported the use of the passive cutaneous anaphylaxis (PCA) reaction in the diagnosis of past or present adiaspiromycosis in experimentally infected animals and in humans. Their success with this method suggested that perhaps this test might be employed for the detection of sensitizing antibodies to C. neoformans. This study was undertaken therefore for the purpose of investigating the use of the PCA reaction as an indicator of past or present infection by C. neoformans in susceptible animals and in relatively resistant animals including humans.

HISTORICAL REVIEW OF THE LITERATURE

The demonstration of hypersensitivity of either the immediate or delayed type to Cryptococcus neoformans in man or experimental animals has been observed infrequently. Nearly all of the cases of cutaneous hypersensitivity reported have been of the delayed type and the majority of these have been concerned with cases of cryptococcosis.

As early as 1927, Berghausen (6) injected a boiled extract of C. neoformans culture subcutaneously into a patient with cryptococcosis and produced a marked local skin reaction at the site of injection. Urbach and Zach (74) found a specific skin allergy to the intracutaneous injection of a filtrate of C. neoformans which they designated "blastomycin." These findings were confirmed by Bernhardt, Zalewski and Burawski (7) using a similar antigen. In 1935, Kessel and Holtzward (36) introduced a broth culture filtrate which had been heated at 60 C for two hours into the skin of a patient with cryptococcosis. They noted a delayed reaction, 2 cm in diameter, which reached its maximum intensity in 24 hours and persisted for five days. Dienst (16) used 0.1 ml of a saline suspension of killed organisms containing one billion cells per ml for skin-testing a human case of cryptococcosis. An erythematous area, 2 cm in diameter, developed in 24 hours and disappeared in 48 hours. In 1953, Carton (10) obtained delayed skin

reactions in a patient using two types of antigens prepared from a strain of C. neoformans. One of these antigens consisted of a 1% suspension of cells heated at 100 C for 10 minutes. The other antigen was made by acid decapsulation of the cells by exposure to HCl for $1\frac{1}{4}$ hours at room temperature after which the cells were washed twice with saline, resuspended and heated at 100 C for 10 minutes. In a patient with both pulmonary moniliasis and cryptococcal osteomyelitis, Leopold (40) was able to induce a delayed skin reaction by the intradermal injection of killed C. neoformans cells. A wheal, 4.5 x 5 cm in size, was produced in 48 hours. Prochnow, Benfield, Rippon, Diener and Archer (60) observed a delayed type of reaction to cryptococcal antigens (from Salvin, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory) when introduced intradermally into a patient with cryptococcal hepatitis. Delayed tuberculin type reactions in individuals with no history of cryptococcosis were reported by Muchmore and his associates (49) who used Salvin's antigen. Eighty six long-time residents of Kingfisher, Oklahoma, a small community in which three cases of cryptococcal meningitis had occurred in one year were tested. Delayed skin reactions with induration greater than 5 mm were observed in 26 individuals.

Experimental studies in animals have shown that hypersensitivity, if it occurs, is of the delayed type. In an attempt to obtain evidence of possible immunity against C. neoformans in a highly susceptible animal, Hoff (29) infected mice with 25 million living organisms. After 12, 16 and 20 days, tests for hypersensitivity were done by the intradermal injection of a C. neoformans peptone broth culture filtrate, a synthetic medium (6% ammonium lactate, 0.2% K_2 HPO_4 and 0.5% NaCl) filtrate or a concentrate of the latter. Positive reactions were not observed in the mice nor were they noted in a rabbit which he had attempted to immunize by a series of six intravenous injections of heat killed cells.

Kligman (38) injected rabbits with 50 million living "Torula histolytica" organisms and tested them two to three months later for evidence of hypersensitivity. The test antigens prepared from three different strains of "T. histolytica" included phenol killed cells, decapsulated cells, living cells, purified capsular material and a Berkfeld filtrate of a two months old culture. Positive skin reactions were not observed. Similar experiments performed with rats as the test animal also gave negative results.

Salvin and Smith (63) were concerned with the production of an effective skin-testing antigen which might be used to demonstrate hypersensitivity to C. neoformans and serve as an indicator of subclinical cryptococcosis. In 1961, they described a soluble cell wall antigen

which, when injected intradermally in 0.1 ml amounts into guinea pigs that had, three weeks to two months before, received living C. neoformans cells intraperitoneally, induced a delayed skin reaction.

A delayed type of hypersensitivity was also noted by Lomanitz and Hale (45) following the intravenous injection of decapsulated yeast cells and whole cells into guinea pigs and rabbits previously sensitized by the administration of heat-killed cells. No immediate type reactions were ever observed. The sensitivity to the decapsulated cells and cell wall antigens could be passively transferred by using a mixture of cell suspensions from peritoneal exudates with antigens and introducing this combination into normal guinea pigs. However, when testing was done using the Prausnitz-Küstner method, hypersensitivity was not demonstrated.

In 1964, Bennett, Hasenclever and Baum (4) confirmed the work of Salvin and Smith (63) and provoked delayed reactions by the intracutaneous injection of 0.1 ml amounts of a soluble cell wall antigen into guinea pigs that had previously received 10^5, 10^6, or 10^7 cells of the same strain of C. neoformans used to make the antigen. The same antigen in 0.1 ml amounts was also used to skin-test a series of hospitalized patients. Areas of erythema and induration exceeding 5 mm diameter in 24 hours were noted in 14 of the 16 patients with treated inactive cryptococcosis, 4 of the 9 patients with active cryptococcosis, 15 of the 22 normal volunteers and 8 of the 17 patients with

diagnoses of mycotic disease. Baum (4) later tested another lot of the cryptococcal skin-test antigen in patients at the Government Hospital in Israel. Delayed reactions occurred in 5 of the 107 adults and children tested.

Abrahams, Gilleran and Weiss (1) made use of the passive cutaneous anaphylaxis test and cryptococcal polysaccharide antigen in their preliminary studies to determine the sensitivity of the method for the detection of cryptococcal antigen produced as a result of active infection. When rabbit anti-cryptococcal serum containing as little as 0.04μg of antibody nitrogen was injected intradermally into normal guinea pigs, cutaneous reactions were produced after a 3 hour latent period in 7 of 10 animals following the intravenous administration of 0.05 to 0.5 mg of cryptococcus polysaccharide. This appears to be the first time that the test designated by Ovary (52) as the passive cutaneous anaphylaxis was used for the detection of anticryptococcal antibodies.

Ovary has made a number of contributions to the development of the PCA test and has used it for various purposes. Ramsdell (61) noting that trypan blue accumulated at the test site employed it in the demonstration of hypersensitivity. Other investigators used dyes such as India ink, Pontamine Sky blue, Evans blue or Geigy blue 536. Ovary (53) preferred to use Evans blue because it was nontoxic to the experimental animal and was more easily prepared than India ink. In

collaboration with Bier (55), it was observed that, in the PCA test, the latent period was inversely proportional to the amount of antibody present provided an excess of antigen was used. Ovary (54) later demonstrated that a reciprocal relationship exists between the amounts of antibody and antigen used in eliciting the PCA reaction. One microgram of rabbit-anti-egg albumin nitrogen and 0.8μg egg albumin nitrogen would provoke a maximal reaction. However, if only 0.02μg of antibody nitrogen was given, 10μg of egg albumin nitrogen were required. According to Leskowitz and Ovary (41) the amount of antigen required was also directly proportional to the size of the antigen molecule.

Ovary and Biozzi (56) used the PCA test to demonstrate that serum from patients who had recovered from either typhoid fever or Malta fever would confer passive cutaneous anaphylactic reactivity to guinea pig skin. The antigens consisted of saline extracts of acid treated cells of Salmonella typhi and Brucella melitensis. He reported also that a non-precipitating as well as a precipitating human diphtheria antitoxin serum was able to sensitize guinea pig skin when diphtheria toxoid was used as the antigen. According to Biozzi, Benacerraf and Ovary (53) the serum from tuberculous patients would produce positive PCA reactions in guinea pigs in conjunction with antigens consisting of extracts of killed tubercle bacilli or of BCG. In another investigation, Ovary (53) reported that the serum from patients with a clinical history of allergy (asthma or hay fever) gave equivocal results. Positive

reactions were obtained in some instances but not in others. Thyroid specific autoantibodies were demonstrated in rabbit anti-thyroid serum, guinea pig anti-guinea pig thyroid serum and in serums from patients with chronic thyroiditis by Ovary, Randall, Witebsky, Shulman and Metzgar (58). Deicher, Holman, Kunkel and Ovary (15) found antibodies against desoxyribonucleic acid (DNA) in 10 of 12 serums from patients with disseminated lupus erythematosus. Ovary and Janoff (57) observed that ferritin could be detected in the circulation of normal guinea pigs by using a modification of the PCA test. According to Josephson, Franklin and Ovary (32), penicillin could elicit a PCA reaction in the guinea pig only if it were conjugated to proteins such as human albumin, α_2, β and γ globulin. Using skin homogenates as the antigens, PCA activity in serum from patients with psoriasis was not demonstrated by Harber, Marsh and Ovary (28).

Among the other investigators using the PCA test were Fisher, Middleton and Menzel (26) who were unable to sensitize guinea pig skin with serum from spontaneously allergic patients exhibiting immediate wheal reactions and erythema to pollen antigen, horse serum and horse epithelium. However, they did obtain positive PCA reactions with serums from individuals who had been sensitized to tetanus antitoxin. Rosenberg, Chandler, Gordon and Fischell (62) found the PCA test useful as an indicator of antibody production by cells. When cells from immunized guinea pigs were implanted intracutaneously into

normal guinea pigs, the production of antibodies at the local skin sites could be detected following the intravenous injection of the antigen and Evans blue. Spleen cells were observed to be effective in the production of antibody. Fischer and Connell (25) tested serums taken from patients sensitive to ragweed before and after treatment with ragweed emulsion. Five of 11 patients who received 5-10,000 Protein Nitrogen Units (PNU) of ragweed emulsion developed antibodies which could be transferred to guinea pig skin. Johnson (31) found the PCA test to be an effective method for the quantitative titration of an antibody to chemical allergens. Picryl chloride and 2,4 dinitrofluorobenzene were used alone and as conjugates of bovine gamma globulin, crystalline egg albumin or casein for the production of antibodies and for the test antigen. Sonntag and Marcus (69) noted that the PCA test was of limited practical value for the demonstration of penicillin hypersensitivity. Positive reactions were obtained with 2 of 10 serums from patients who were known to be drug sensitive. In the hands of Terr and McLean (72), insect sting hypersensitivity could be demonstrated by the PCA test. Serum from 13 of 26 patients reacted with a honey bee antigen and 3 of the 26 reacted with the yellow jacket antigen as well as with the honey bee antigen. PCA reactions were not induced with wasp or hornet antigens in any instance. Using serum from patients who had been desensitized to ragweed, Connell and Sherman (13) compared antibody activity in the PCA, skin sensitization,

blocking and hemagglutination tests. They found that PCA reactions were related to high blocking antibody titers (1000 PNU or more) and frequently to high agglutinating titers but not to the skin sensitizing titer.

Jellison, Glesne and Owen (30) adapted the PCA test to the diagnosis of adiaspiromycosis using serums from 585 individuals who had been exposed to animals or environments where infection with either Emmonsia crescens or E. parva could possibly occur. Supernatant fluids from spherule cultures grown for several months at 37 C in beef infusion broth with rabbit blood and glucose were used as the antigens. The serums of two individuals reacted with E. crescens antigens in a titer of 1:8. This titer was considered sufficiently high to indicate possible previous infection. The antibodies have persisted in one individual for 8 years and, in the other, for 10 years, although no symptoms of disease were apparent during this time. Nevertheless, the antibody responses were considered to be the result of a natural infection.

MATERIAL AND METHODS

Organisms

The species and strains of the genus Cryptococcus used in this investigation as well as the source from which each was isolated are listed below:

Species	Source
Cryptococcus neoformans M1000	A patient with cryptococcal meninigitis.(Isolation made 25 to 30 years ago.)
Cryptococcus neoformans 8476	A patient with cryptococcal meninigitis.(Isolated in 1963.)
Cryptococcus neoformans 45_2	Slime flux of a mesquite tree, Prosopis juliflora.(Isolated in 1963 by Lamb and Evenson.)
Cryptococcus neoformans CDC-51	A patient with cryptococcosis. (Obtained from the Communicable Disease Center, Chamblee, Georgia.)
Cryptococcus diffluens	Leaf of the Passion Vine, Passiflora coerulea, in Tucson. (Isolated in 1961 by Thomas & Evenson.)
Cryptococcus laurenti	Slime flux of mesquite tree, Prosopis juliflora. (Isolated in 1963 by Lamb and Evenson.)

Antigens

From each of the strains listed, except C. neoformans CDC-51, formalin-killed whole cell antigen, ruptured cell antigen, crude cell wall antigen, crude carbohydrate antigen, crude capsular polysaccharide antigen and extracellular starch antigen were prepared. The ruptured cell antigen and the crude cell wall antigen were not prepared from C. neoformans CDC-51.

1. The formalin-killed whole cell antigens were prepared according to the method of Neil, Abrahams and Kapros (51) except that a different medium was used for culture and the cells were killed without heating. The particular yeast strain was grown on Wickerham's (77) malt extract- yeast extract agar (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1.0% glucose and 2.0% agar) in Roux-type bottles at 26-28 C for 72 hours. This medium will hereafter be referred to as M-Y agar. The surface growth was washed off with normal saline containing 2% formalin and stored at room temperature for 24 hours. The cells were collected by centrifugation, washed twice with sterile distilled water, and checked for viability by streaking on duplicate plates of M-Y agar which were incubated at 26-28 C for 5 days. The whole cells were resuspended in 0.5% formalin and stored in the refrigerator. Immediately preceding use, the cells were washed twice with normal saline to remove traces of formalin and resuspended in sterile normal saline. The number was determined by direct count

in a Levy-Neubauer hemocytometer.

2. Ruptured cell antigens were prepared by growing the cells on M-Y agar in Roux-type bottles at 26-28 C for 48-72 hours. The surface growth was then washed off with sterile distilled water, collected by centrifugation for 1 hour at 10,000 rpm in the Servall refrigerated centrifuge, washed twice with sterile distilled water and suspended in 50 ml of sterile distilled water. The cells were then twice subjected to a pressure of 16,000 psi in the French press. Wet mount determinations and Gram stained preparations showed that approximately 70% cell rupture was obtained. The ruptured cell antigen was stored at −20 C.

3. The crude cell wall antigen was prepared in part according to the method of Salvin and Smith (63) except that a lower pressure was used in the disruption of the cells and the antigen was particulate in nature rather than soluble. The preliminary steps in the production of this antigen were identical to those for the ruptured cell antigen. The crude cell wall fragments were, however, collected by centrifugation at 10,000 rpm in the Servall refrigerated centrifuge for 1 hour. The supernatant fluid was discarded and the sediment was resuspended in sterile distilled water. The centrifugation and subsequent washing processes were repeated usually 7-9 times, or until the supernatant fluid was clear. The sediment, which consisted of the crude cell wall fragments, was resuspended in 50 ml of sterile distilled water and stored at −20 C.

4. The crude carbohydrate antigen was produced largely by the method of Kabat and Mayer (33) for the preparation of pneumococcal capsular polysaccharide except that Wickerham's (77) M-Y broth (0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.0% glucose) was used as the culture medium. Five flasks each containing 200 ml of M-Y broth were inoculated with 1 ml of a 30-hour M-Y broth tube culture. After 4 days of incubation at 26-28 C during which the flasks were agitated on a New Brunswick Scientific Company shaker at the rate of 200 oscillations per minute, 2 g of phenol crystals were added to each flask. They were then incubated at room temperature for 24 hours. Wet mounts and Gram stained smears were made from each flask to check for possible contamination. The cells were separated by centrifugation in the Servall refrigerated centrifuge for 1 hour at 10,000 rpm and discarded. The supernatant fluid was concentrated almost to dryness by lyophilization and was then resuspended in 200 ml of sterile distilled water. Twenty grams of sodium acetate and 2 ml of glacial acetic acid were added and precipitation of the carbohydrate was accomplished by addition, with constant stirring, of two and a half volumes (500 ml) of 95% ethanol. The mixture was refrigerated overnight and then again subjected to centrifugation in the Servall refrigerated centrifuge for 30 minutes at 10,000 rpm. The supernatant fluid was discarded and the precipitate was dissolved in 200 ml of sterile distilled water to which 20 g of sodium acetate and 2 ml of

glacial acetic acid were added. To remove the protein, 40 ml of chloroform and 8 ml of N-butyl alcohol were introduced. The mixture was homogenized in a Waring Blendor in the cold room for 5 minutes and separated by centrifugation in the Servall refrigerated centrifuge for 1 hour at 10,000 rpm. The supernatant fluid was decanted and saved. The emulsion layer was washed with sterile distilled water and the washings were added to the supernatant fluid. Extraction was continued until a negative Biuret reaction was obtained. Then sufficient alcohol ($2\frac{1}{2}$ - 3 volumes of 95% ethanol) was added with stirring to precipitate the carbohydrate. The mixture was refrigerated overnight and subjected to centrifugation for 1 hour at 10,000 rpm. The supernatant fluid was discarded and the precipitate was redissolved in 100 ml of sterile distilled water to which 140 ml of 95% ethanol was added. This material was again subjected to centrifugation for 1 hour at 10,000 rpm to remove debris. Following the addition of 10 g of sodium acetate and 1 ml of glacial acetic acid to the decanted supernatant fluid, it was refrigerated overnight. The precipitate was collected by centrifugation in the Servall centrifuge for 1 hour at 10,000 rpm, washed twice with 70% ethanol, twice with 80% ethanol, and twice with 95% ethanol before being dried in the frozen state. The Molisch and Bial tests performed on all of the carbohydrate antigens were positive.

5. Crude capsular polysaccharide antigens were prepared from cultures grown on M-Y agar for 72 hours at 26-28 C. The cells were washed off with sterile distilled water, concentrated by centrifugation for 1 hour at 10,000 rpm and resuspended in approximately 50 ml of sterile distilled water. The cells were decapsulated in a Brownwill Biosonick sonicator by exposure to maximum intensity for 20 minutes. By examination of wet mounts made with India ink, it was determined that 90% of the capsules had been removed and that less than 1% of the cells were damaged. The cells separated by centrifugation at 10,000 rpm for 30 minutes were discarded. To precipitate out the protein, trichloracetic acid crystals were added to the supernatant to make a final concentration of 4% by weight. The mixture was refrigerated overnight at 4 C. After centrifugation for 30 minutes at 10,000 rpm, the sediment was discarded. The polysaccharide precipitated from the supernatant fluid by the addition of 3 volumes of 95% ethanol and overnight storage in the refrigator was collected by centrifugation for 30 minutes at 10,000 rpm. The sediment was washed 2 times each with 80% ethanol and 95% ethanol. This was followed by 2 washings with ether. It was then dried over calcium chloride in a dessicator jar in the cold. The dried antigens were stored in screw capped tubes in the refrigerator. All preparations were Molisch and Bial positive.

6. The extracellular starch antigens were prepared according to the method of Mager (46). Each of the strains of Cryptococcus was inoculated into flasks containing 200 ml of Mager and Aschner's (47) medium (0.1% $(NH_4)_2 SO_4$, 0.05% Mg $SO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, 1.0% glucose and 0.02% thiamine). The inoculum consisted of 1 ml of a 24 hour Mager-Aschner broth culture. The flasks were usually incubated at 26-28 C for 10 days with constant agitation. However, in the case of C. neoformans CDC-51, starch antigens were also prepared following 4 days of incubation and in the case of C. neoformans M-1000, starch antigens were made from cultures incubated for 4, 15 and 20 days as well as for 10 days. At the end of the incubation period, smears were made to test for purity. Approximately 3-5 ml were removed asceptically from each flask for the determination of starch production as indicated by the development of a blue color following the addition of 2-3 drops of Lugol's iodine solution. To kill the organisms, the flasks were heated for 30 minutes at 75-85 C in an Arnold steam sterilizer. The killed cells were collected by centrifugation in the Servall refrigerated centrifuge for 1 hour at 10,000 rpm and discarded. The supernatant fluid was concentrated to approximately one-fourth of its original volume by evaporation on a steam bath. To remove the protein, trichloracetic acid crystals were added to a final concentration of 4% by weight and the mixture was refrigerated overnight at 4 C. After centrifugation for 1 hour at 10,000 rpm, the

sedimented protein was discarded. The polysaccharide was precipitated by the addition of 3 volumes of 95% ethanol to the supernatant fluid and, after 24 hours, was separated from it by centrifugation. The precipitate was washed 4 times with 70% ethanol containing a few drops of glacial acetic acid, twice with 95% ethanol, twice with ether and was then dried over calcium chloride in a dessicator jar in the refrigerator at 4 C. The dried material was stored in screw capped tubes in the refrigerator at 4 C.

The methods for the preparation of the six antigens are briefly presented in Tables 1 – 6.

Animals

The purpose of the investigation was to determine the usefulness of the PCA test as an indicator of past or present infection. It was necessary therefore to develop a working PCA system employing serum from animals such as mice that are susceptible to experimental C. neoformans infection and from animals such as the rabbit which are relatively resistant to infection. An attempt was made to produce subclinical or clinical infection in 14 young, white female rabbits obtained locally and weighing 1360 to 2600 grams each. Previous to infection, approximately 5 ml of blood was collected from the ear vein of each rabbit. Following coagulation, the serum was removed and stored at –20 C. Later it was tested for reactivity in the PCA test.

TABLE 1

FORMALIN-KILLED WHOLE CELL

ANTIGEN PREPARATION

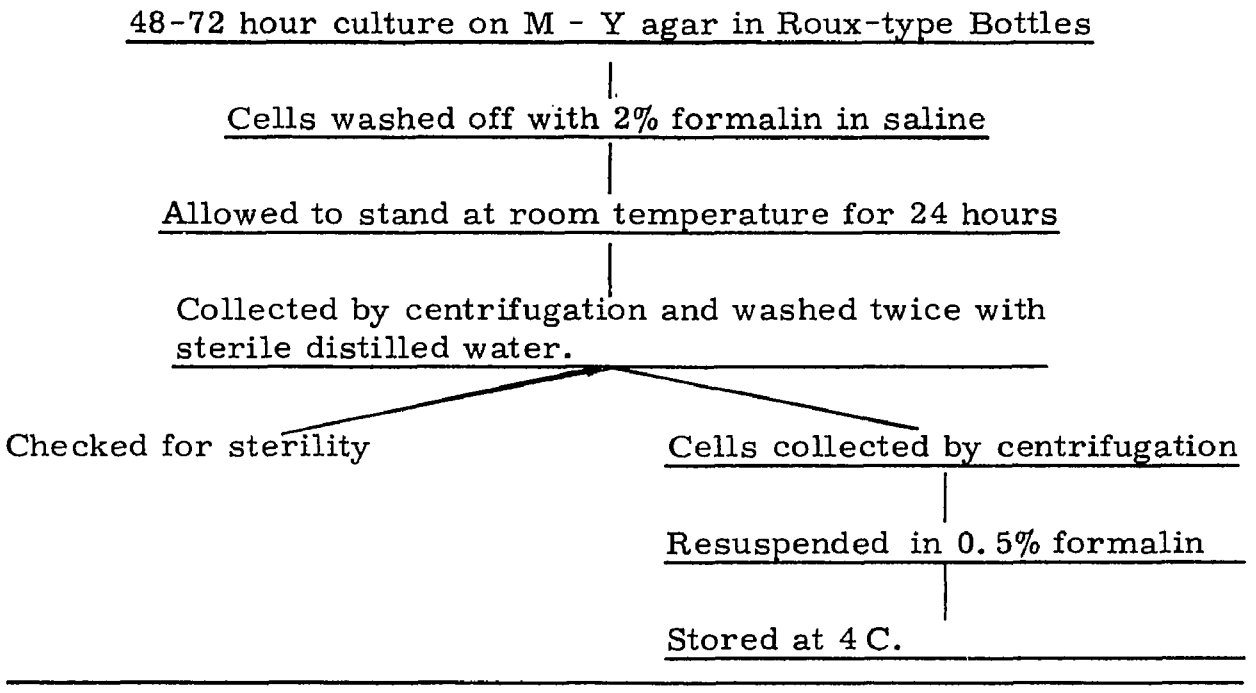


TABLE 2

RUPTURED CELL ANTIGEN PREPARATION

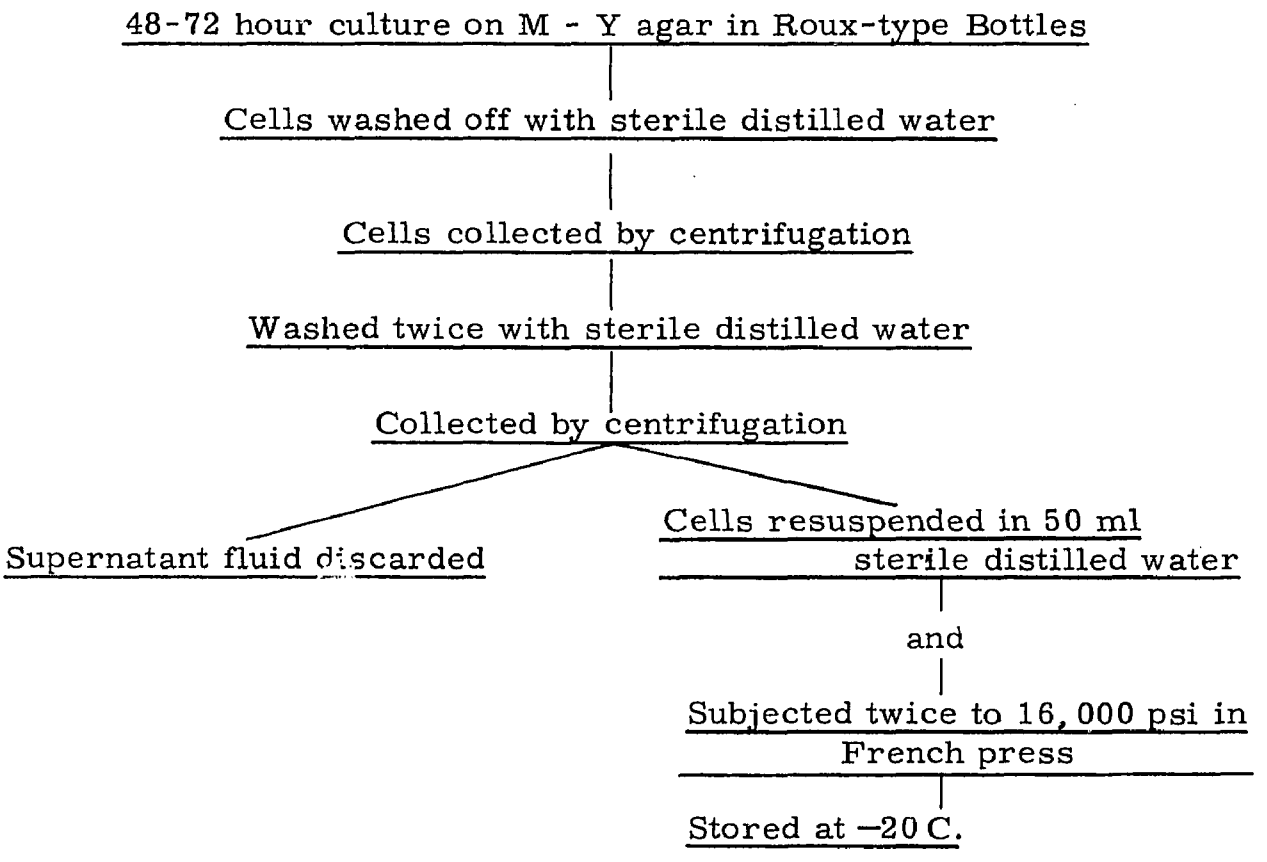


TABLE 3

CRUDE CELL WALL ANTIGEN PREPARATION

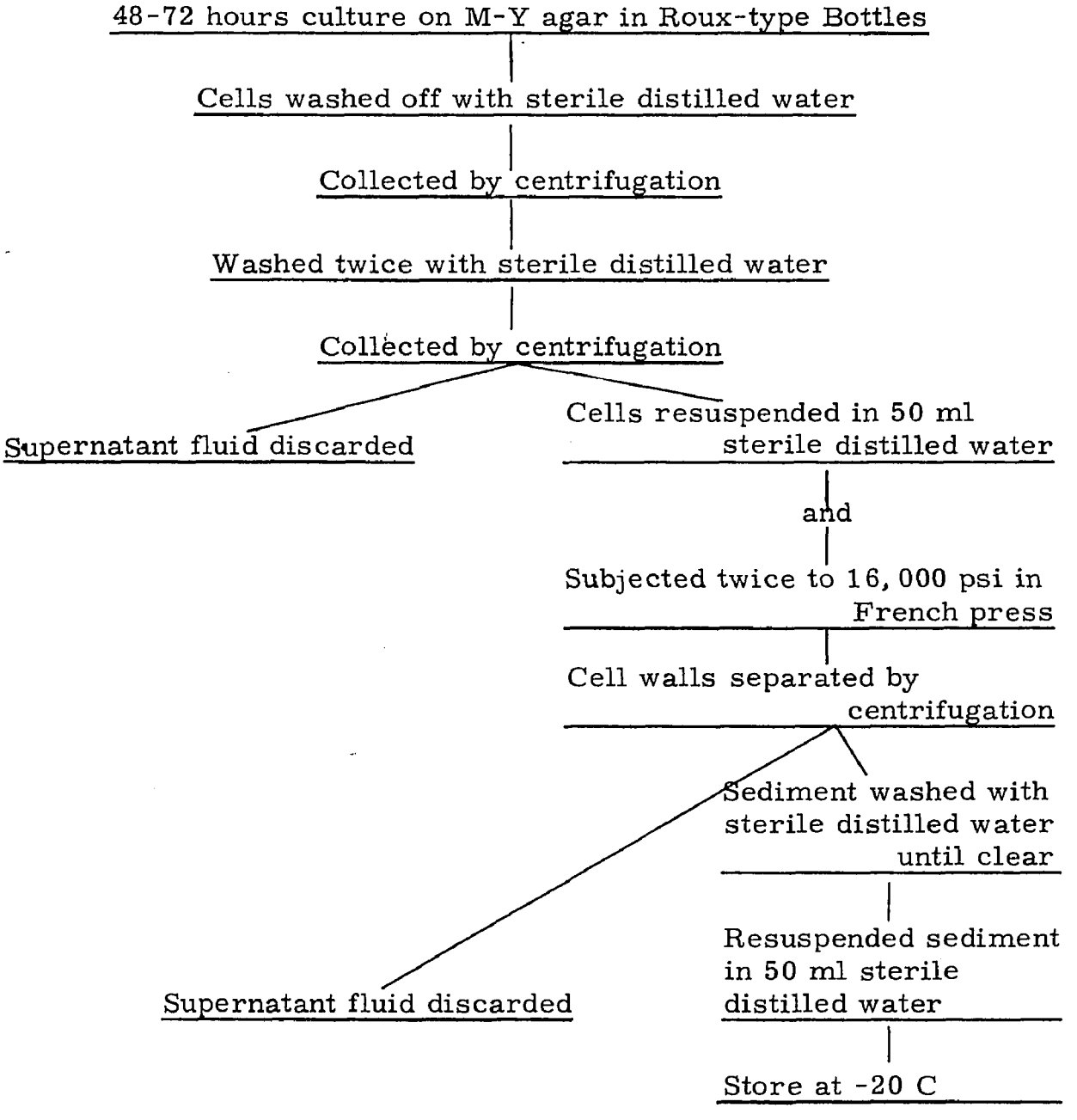


TABLE 4

CRUDE CAPSULAR POLYSACCHARIDE ANTIGEN PREPARATION

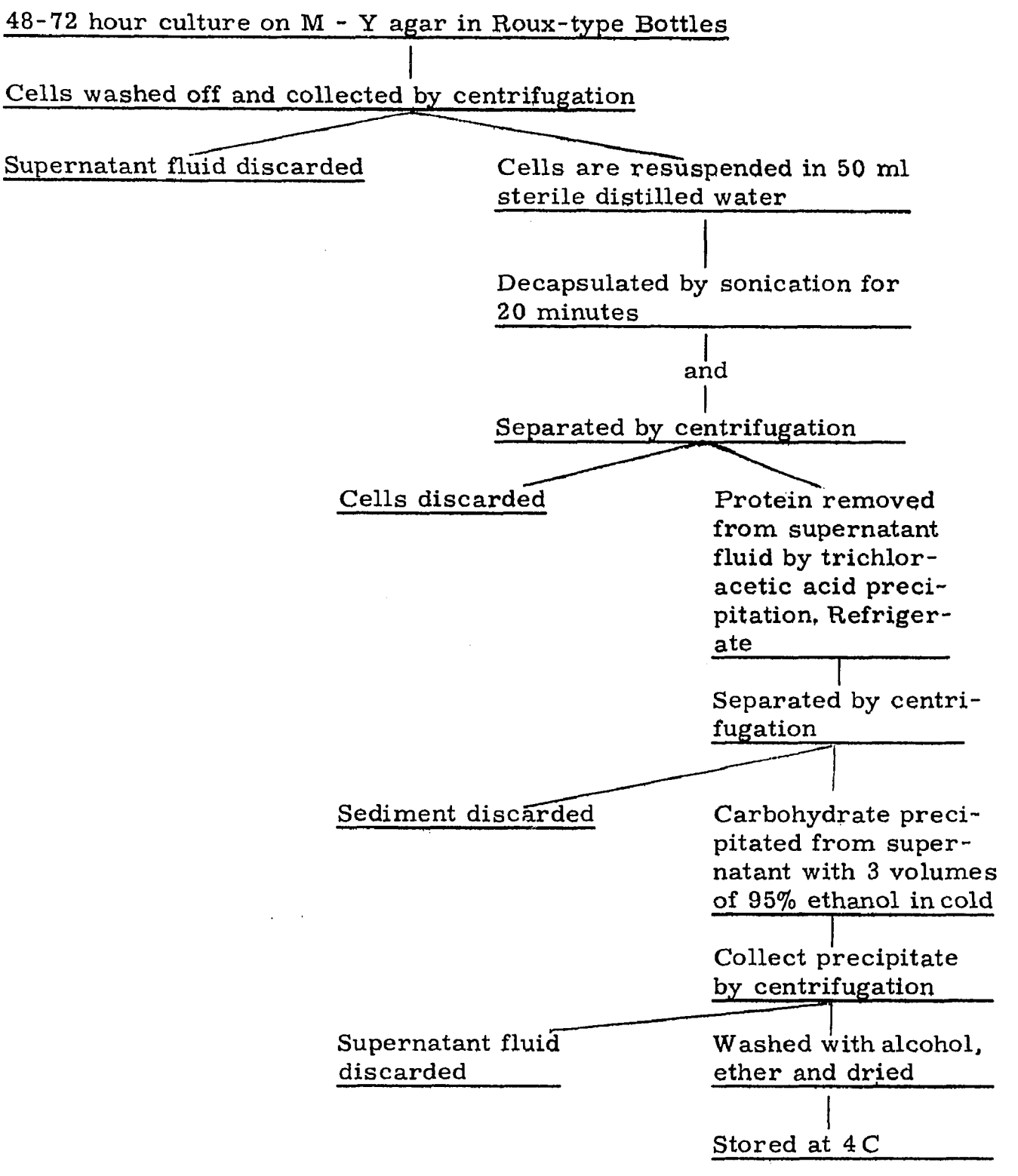


TABLE 5

CRUDE CARBOHYDRATE ANTIGEN PREPARATION

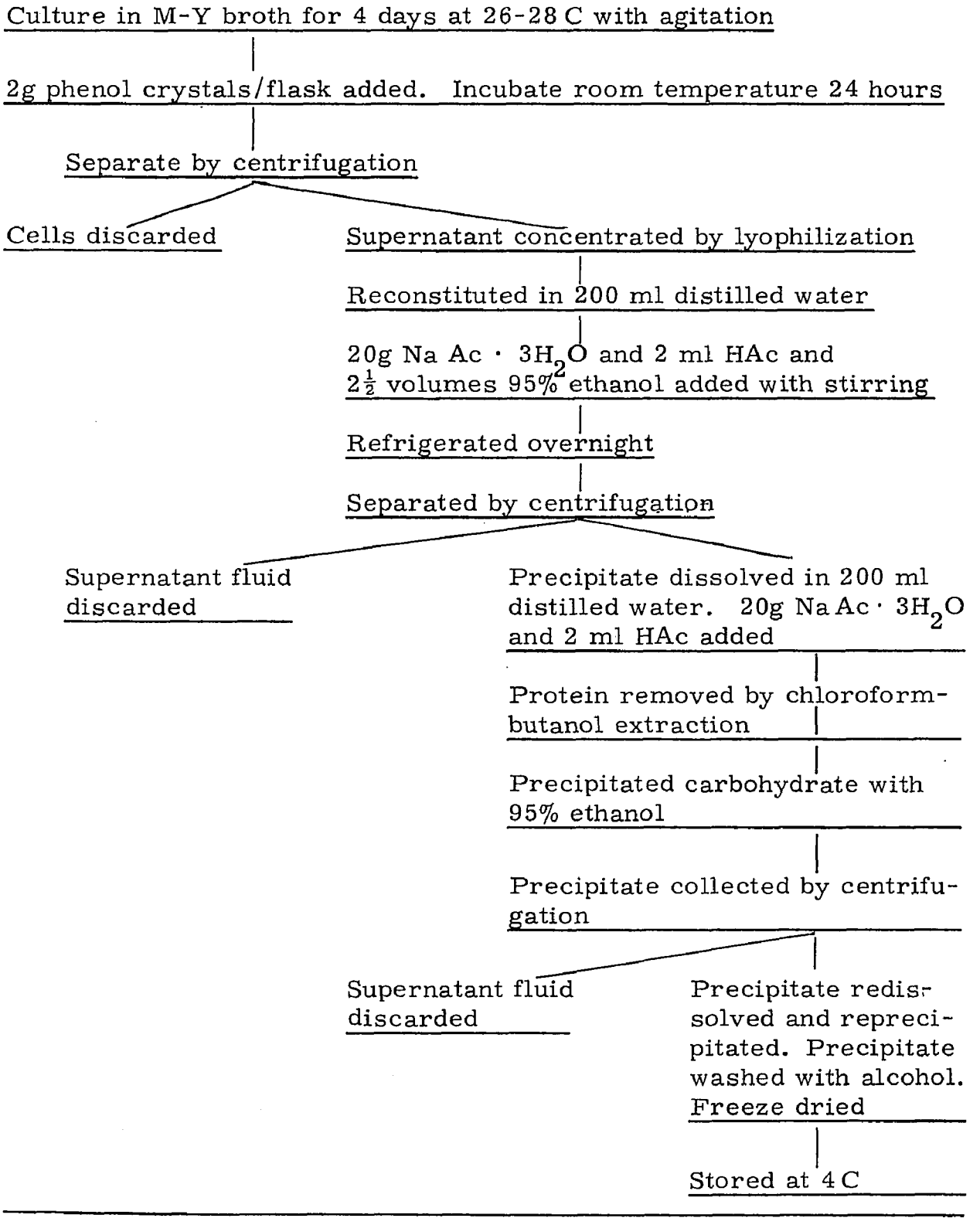
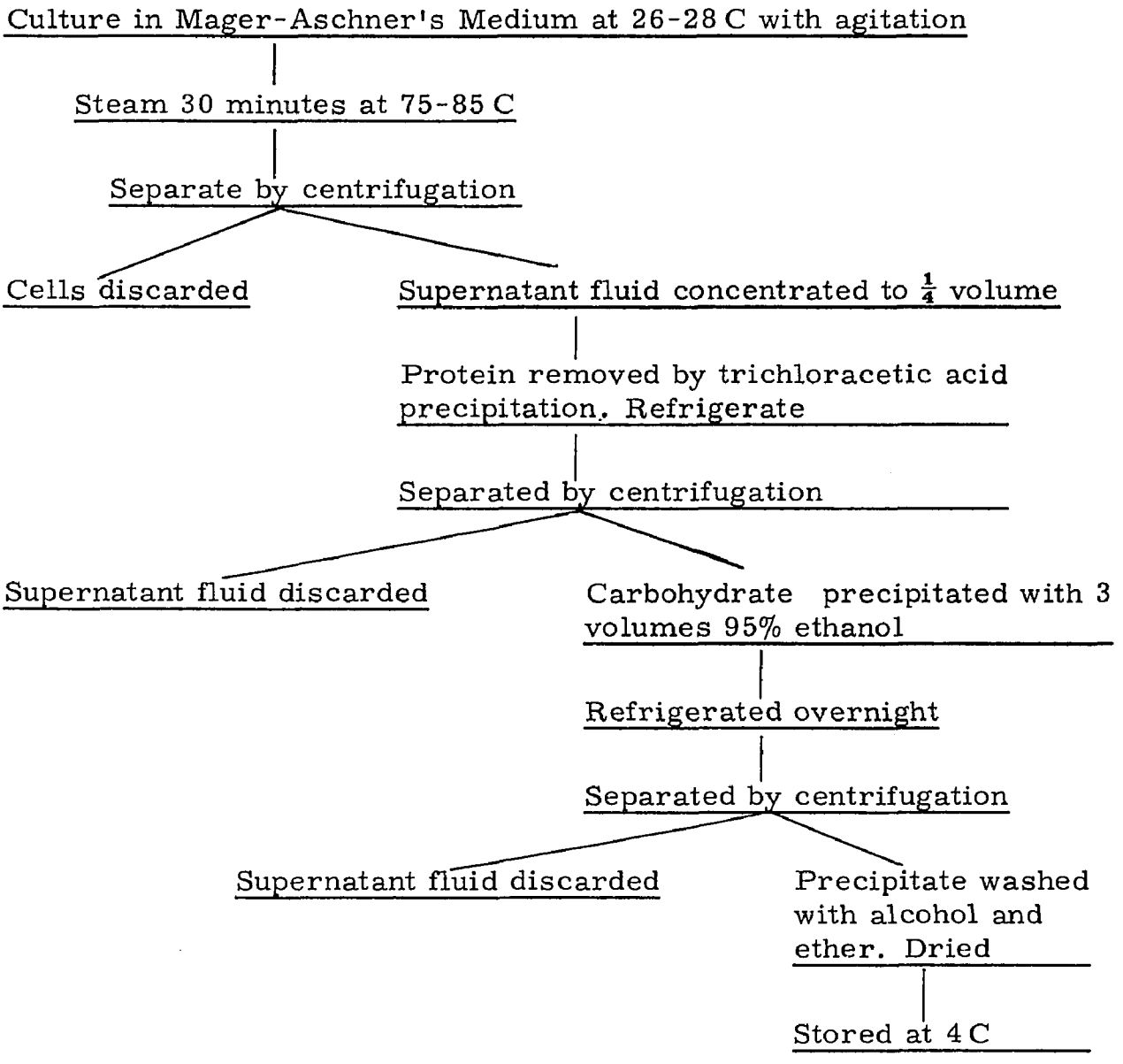


TABLE 6

EXTRACELLULAR STARCH ANTIGEN PREPARATION



Four of the rabbits were tested for previous natural sensitization. Using electric clippers, the fur over the back area was cut short twenty-four hours before injection to minimize any non-specific reaction due to irritation. Each animal then received a series of intradermal injections consisting of 0.1 ml amounts of the following C. neoformans M-1000 antigens : 5,000 whole cells in sterile saline, the ruptured cell antigen diluted 1:50 with sterile saline, the crude cell wall antigen diluted 1:50 with sterile saline, 400μg of crude carbohydrate in saline, 400μg crude capsular material in saline and 400μg of extracellular starch in saline. Readings were made at intervals of 15 minutes, 30 minutes, 1, 2, 4, 6, 24 and 48 hours to check for immediate, as well as delayed tuberculin type reactions.

In an attempt to produce infection, each of the 14 individual rabbits was inoculated either with a strain of Cryptococcus neoformans or with a related species. The inoculum consisted of cells grown for 24 hours on an M-Y agar slant, incubated at 26-28 C. The surface growth was washed off with sterile physiological saline and the total number of cells injected was determined by direct count in a Levy-Neubauer hemocytometer and by the pour plate method of culture using M-Y agar. The size of the inoculum for each rabbit is given in Table 7.

All animals were observed daily and weighed weekly for a period of 4 weeks. At the end of this period, six of the rabbits were

TABLE 7

SUMMARY OF EXPERIMENTS CONCERNED WITH RABBITS

INFECTED WITH *CRYPTOCOCCUS* SP.

Rabbit Number	Pre-infection Skin-tests	Infecting Organisms	Number of viable Organisms	Post-infection Skin-tests	Response of serum in PCA test with ESA
1	negative	*C. neoformans* M-1000	5.8×10^{7} [a]	Cell Wall +*	++
2	____	*C. neoformans* M-1000	5.8×10^{7} [a]	Cell Wall +*	++++
3	negative	*C. neoformans* M-1000	5.8×10^{7} [a]	Cell Wall -* Ruptured Cell +	+
4	____	*C. neoformans* M-1000	5.8×10^{7} [a]	Cell Wall +* Ruptured Cell +	0
5	negative	*C. neoformans* M-1000	1.12×10^{9} [a]	Negative	0
6	negative	*C. neoformans* M-1000	1.12×10^{9} [a]	Negative	+
7	____	*C. neoformans* CDC-51	7.25×10^{7} [a]	____	+
8 x	____	*C. neoformans* CDC-51	7.25×10^{7} [a]	____	____
9	____	*C. neoformans* CDC-51	5×10^{7} [a]	____	+
10	____	*C. diffluens*	5.4×10^{7} [a]	____	0
11	____	*C. laurentii*	9.6×10^{7} [a]	____	0
12	____	*C. laurentii*	9.0×10^{7} [b]	____	0
13	____	*C. diffluens*	8.7×10^{7} [b]	____	0
14	____	*C. neoformans* M-1000	4.95×10^{7} [b]	____	+

– not done x died 18 hours after injection a Intravenous route of inoculation

* other antigens negative b Intraperitoneal route of inoculation

skin tested using the same concentrations of C. neoformans M-1000 antigens previously described and observing the same reading intervals. Then the rabbits were lightly anesthetized with ether, exsanguinated by cardiac puncture using the flask technique and autopsied for evidence of infection. Sections of liver, spleen, kidneys, heart, lungs and brain were minced with sterile scissors and cultured in M-Y broth. Following coagulation of the blood the serum was removed and stored at −20 C until it could be tested for PCA reactivity. The record for the handling of each rabbit is summarized in Table 7.

Serum was also available from mice that had been inoculated 10 days to 16 weeks earlier with various strains of C. neoformans. The mice were bled by cardiac puncture. Following coagulation, the serums from related groups were pooled and stored at −20 C until use. The essential data concerning the mice whose serums were tested for PCA reactive antibodies are listed in Table 8.

For the testing of animal and human serums for evidence of hypersensitivity, albino guinea pigs of either sex and weighing 200-476 g apiece were employed. They were obtained from Horton's Laboratory Animals, Las Gatos, California, and were permitted a brief period (2-5 days) of adjustment in our laboratory before the testing procedures.

TABLE 8

SUMMARY OF EXPERIMENTS CONCERNED WITH MICE INFECTED WITH C. NEOFORMANS

Mouse Serum Pool Number	Infecting organisms	Number of viable cells injected intraperitoneally	Length of period of infection	Autopsy findings	Response of serum in PCA test with C. neoformans M-1000 starch antigens
1	C. neoformans M-1000	1.25×10^8	10 weeks	negative	0
2	C. neoformans M-1000	1.25×10^5 - 1.25×10^7	10 weeks	negative	0
3	C. neoformans M-1000	1.25×10^4 - 7.25×10^7	10 weeks	negative	0
4	C. neoformans M-1000	7.5×10^3 - 7.5×10^5	10 weeks	negative	0
5	C. neoformans M-1000	1.1×10^7 - 1.1×10^8	10 weeks	negative	0
6	C. neoformans M-1000	2.1×10^8 - 2.1×10^9	8 weeks	negative	0
7	C. neoformans M-1000	2.1×10^7 - 2.1×10^9	8 weeks	negative	0
8	C. neoformans M-1000	2.1×10^5 - 2.1×10^6	8 weeks	negative	0
9	C. neoformans M-1000	2.1×10^7 - 2.1×10^9	8 weeks	negative	0
10	C. neoformans M-1000	2.1×10^6 - 2.1×10^7	8 weeks	negative	0
11	C. neoformans M-1000	1.0×10^4 - 1.0×10^6	10 weeks	negative	0
12	C. neoformans CDC-51	1.1×10^5	7 weeks	infection	0
13	C. neoformans M-1000	1×10^8	16 weeks	negative	0
14	C. neoformans M-1000	2.43×10^5 - 6.6×10^8	10 days	negative	0
15	C. neoformans M-1000 with mucin	7.5×10^4 - 7.5×10^5	12 weeks	negative	0
16	C. neoformans M-1000 with mucin and kaolin	5.5×10^6 - 5.5×10^7	12 weeks	negative	0
17	C. neoformans M-1000	7.5×10^3	12 weeks	negative	0
18	C. neoformans M-1000	5.5×10^3 - 5.5×10^5	12 weeks	negative	0

The Passive Cutaneous Anaphylaxis (PCA) Test

The method developed by Ovary (53) and sucessfully used by Jellison et al (30) was followed. Albino guinea pigs were prepared for skin-testing by clipping the hair from the back area 24 hours prior to the injections. Occasionally a depilatory compound was also employed. Its use was discontinued when it was noted that it offered no distinct advantage and that it introduced the hazard of non-specific irritation. Using a 26 or 27 gauge needle and a tuberculin syringe, a row of intradermal injections of serum (diluted or undiluted) in 0.1 ml amounts was made on each side of the back approximately 1.5 to 2.0 cm from the midline and about 1.5 to 2.0 cm from each other (Fig. 1). Physiological saline alone as well as known negative and positive serums served as controls. Serum dilutions were made with normal saline. After a latent period to allow the fixation of antibody to the cells, 1 ml of the antigen-dye mixture consisting of 0.5 ml of antigen and 0.5 ml of a 1% Evans blue solution in saline was injected usually by the intracardial route but occasionally by the intravenous route. The sites of serum injection were observed for evidence of blueing. Thirty minutes after the injection of the dye-antigen mixture, the animals were sacrificed. They were then skinned and the degree of reactivity, if any, was measured in millimeters on the inner skin surface. The skins were dried and maintained in the refrigerator at 4 C for further comparative studies.

Fig. 1. Pattern of intradermal inoculation of guinea pig prepared for PCA testing.

The preliminary experiments were concerned with finding a reactive serum which would permit development of a working PCA system adaptable for use with human serum. The serums from the 13 rabbits which had received massive injections of living cells were examined for evidence of sensitization detectable by the PCA test. For these studies, diluted and undiluted serum from each of the first 6 rabbits was tested with the 6 different C. neoformans M-1000 antigens. The serum from rabbit #2 was found to be the most highly reactive and this reactivity was especially pronounced with the extracellular starch antigen. The activity of this serum was then titrated to determine the end-point using the starch antigen. The PCA response of each of the other 7 serums was determined with the homologous extracellular starch preparation and, when reactivity was demonstrated, with the C. neoformans M-1000 starch antigen.

Various routes of inoculation of the dye and antigen separately and combined were tried. It was found more satisfactory to consistently use intracardial injection of the dye-antigen mixture.

The 6 hour latent period between the injection of the serum and the introduction of the antigen-dye mixture was found by Jellison and his co-workers (30) to be satisfactory. In this investigation, it proved less satisfactory since the skins frequently failed to return to their normal condition in that time and the results were ambiguous and inconsistent. Since Chase (11) found that latent periods of 5 to 12 hours might be

necessary with different systems, the next step in the development of a consistently operating system in this study was the determination of the optimal length of the latent period. To accomplish this, a sequence of intradermal injections of the reactive serum from rabbit #2 were made at hourly intervals over a period of 6 hours. Ten hours after the initial injection, a mixture of C. neoformans M-1000 extracellular starch antigen and the Evans blue solution was inoculated intracardially. When the inner surfaces of the skins were examined, it was apparent that a latent period of more than 6 hours was needed. The 9 hour latent period was therefore chosen for subsequent testing.

Although a serum highly reactive with the extracellular starch antigen had been found and a suitable latent period had been established, the most effective amount of starch antigen remained to be determined. To accomplish this, PCA tests were performed using the serum of rabbit #2 with 2 different extracellular starch antigens (C. neoformans M-1000 and CDC-51) in amounts of $\frac{1}{2}$, 1 and $1\frac{1}{2}$ mg. A reading of the degree of reactivity on the inner surfaces of the skin, as previously described, indicated that the 1 mg quantity of the starch antigen was more effective than either of the others.

In the preceding tests, the test antigen was the extracellular starch preparation made from culture of C. neoformans M-1000 incubated for a 10-day period. The effectiveness of this antigen was compared with that prepared from cultures of the same strain incubated

for 4 days, for 10 days, for 15 days and for 20 days. All were used in 1 mg amounts and all were found to be equally effective.

Inconsistencies occurred from time to time which appeared to be related to the size of the guinea pig. It had been previously recognized and recorded by other investigators that guinea pigs differ in their sensitivity to this type of testing. An attempt was made to determine the most satisfactory size of guinea pig. It appeared that somewhat better reactions occurred when the test animal weighed about 350 to 400 g and it also became apparent that even animals in the desirable size range varied in their reactivity. In consideration of these individual differences, all PCA testing henceforth was done in triplicate using three guinea pigs. This plan was followed in the re-testing of the 13 rabbit serums for which 1 mg of the extracellular starch antigen and the 9 hour latent period was consistently employed.

Extracellular starch antigen had been prepared by identical methods from four strains of C. neoformans, from one strain of C. diffluens and from one strain of C. laurentii. Each of these was tested in the PCA test with serum from rabbit #2 which had been injected with C. neoformans M-1000.

Pooled serums from related groups of mice that had been injected with either C. neoformans M-1000, C. neoformans CDC-51

or a saprophytic strain of C. neoformans were examined by the PCA method for sensitizing antibodies. A summary of the essential experimental data is given in Table 8.

This investigation was undertaken with the hope that the PCA test could be used to indicate recovery from clinical or subclinical infection due to C. neoformans. The information gained from experience with the animal serums was therefore applied to the testing of 500 human serums. The samples tested were collected from three diagnosed cases of cryptococcal meningitis and from five individuals who had had laboratory contact with C. neoformans for long periods of time. In addition, serums were obtained from hospital patients with various types of illnesses. In every case, preliminary tests were run with undiluted serum. Whenever a sample gave a doubtful reaction, it was re-examined. When there was any indication of a positive reaction, the serum was diluted with physiological saline and the end-point of reactivity determined. Table 9 lists the types of serums subjected to PCA testing.

TABLE 9

SUMMARY OF THE SERUMS TESTED FOR PCA ACTIVITY AND EXTRACELLULAR STARCH ANTIGENS WITH WHICH EACH WAS TESTED.

I.	Rabbit Serums	Homologous ESA	Heterologous ESA
	1 with	C. neoformans M-1000	-0-
	2 with	C. neoformans M-1000 and	C. neoformans CDC-51 C. neoformans 8476 C. neoformans 45_2 C. diffluens C. laurentii
	3 with	C. neoformans M-1000	-0-
	4 with	C. neoformans M-1000	-0-
	5 with	C. neoformans M-1000	-0-
	6 with	C. neoformans M-1000	-0-
	7 with	C. neoformans CDC-51 and	C. neoformans M-1000
	9 with	C. neoformans CDC-51 and	C. neoformans M-1000
	10 with	C. diffluens	C. neoformans M-1000
	11 with	C. laurentii	C. neoformans M-1000
	12 with	C. laurentii and	C. neoformans M-1000
	13 with	C. diffluens and	C. neoformans M-1000
	14 with	C. neoformans M-1000	-0-
II.	Mouse Serums (18 groups)	Infecting Agent	Extracellular Starch Antigen
	16 groups	C. neoformans M-1000	C. neoformans M-1000
	1 group	C. neoformans CDC-51	C. neoformans M-1000
	1 group	C. neoformans from slime flux	C. neoformans M-1000
III.	Human Serums (500)		Extracellular Starch Antigen
	3 from known cases of cryptococcal meningitis		C. neoformans M-1000
	5 from laboratory personnel who had contact with C. neoformans		C. neoformans M-1000
	492 from patients hospitalized with various diseases		C. neoformans M-1000

RESULTS

Thirteen of the 14 rabbits inoculated with strains of Cryptococcus neoformans or with other species of Cryptococcus continued to gain weight and appeared well at all times. The one exception was rabbit #8 which survived only 18 hours after the injection. Rabbits #1, 2, 3, 4, 5 and 6 were injected with 5.8×10^7 to 1.12×10^9 cells of C. neoformans M-1000. In the pre-inoculation skin-testing of rabbits #1, 3, 5 and 6 with cell wall, ruptured cell, whole cell, extracellular starch and crude polysaccharide antigens, no evidence was found of previous exposure to the yeast. Neither immediate nor delayed type reactions occurred. However, similar skin-testing with the same antigens 4 weeks after infection resulted in some degree of reaction in 48 hours with the crude cell wall antigen and the ruptured cell antigen but not with the others. This reactivity was observed in rabbits #1, 2, 3, and 4, two of which had given earlier negative reactions.

No evidence of infection due to C. neoformans was found at autopsy in any of the rabbits. The liver, spleen, kidneys, lungs, heart and brain appeared normal on gross examination. Cultures made from these organs remained negative for C. neoformans.

The serums of seven of the rabbits exhibited some degree of reactivity with the extracellular starch antigens but only that of rabbit

#2 reacted intensely. Although there was some reaction with both the capsular and the crude carbohydrate antigens, the reactive area was in the form of a ring characterized by a circle of blue dye surrounded by a non-colored central portion. In contrast, the reactive area induced by the extracellular starch antigen was not ring-shaped but was rather deeply and uniformly colored. These results are outlined in Table 10 and the response of rabbit serum #2 with the extracellular starch antigen is shown in Figure 2. In the titration of rabbit serum #2 with the extracellular starch antigen, the end point of reactivity was found to be 1:16 (Fig. 3). The data for this experiment which utilized three animals and was done in triplicate is listed in Table 11.

Table 12 summarizes the findings in the experiment concerned with the determination of the optimum latent period. It is apparent from the examination of this table that possibly the 7, 8, 9 or 10 hour period could have been used satisfactorily. The nine hour period was, however, arbitrarily chosen because the area and intensity of reactivity appeared to be maximum at that time.

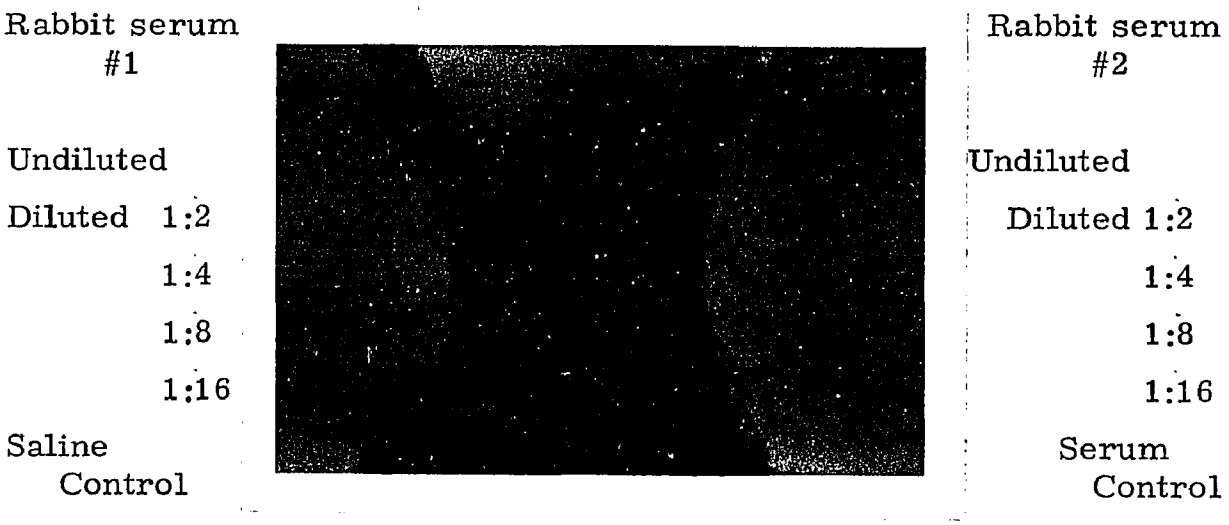
A comparison of the degree of reactivity of serum of rabbit #2 with various amounts of extracellular starch antigen prepared from two strains of C. neoformans is shown in Table 13. The latent period was 9 hours. Maximum reactivity of the serum occurred when the amount of extracellular starch antigen used was 1 mg (Fig. 4). Good reactions of the undiluted serum were also noted with $\frac{1}{2}$ mg of the

TABLE 10

THE PCA RESPONSE OF RABBIT SERUM #2

WITH HOMOLOGOUS ANTIGENS

	Rabbit Serum #2					Controls	
	Dilution						
Antigens	None	1:2	1:4	1:8	1;16	Saline	Negative Serum (Rabbit)
Crude Cell Wall	0	0	0	0	0	0	0
Ruptured Cell	0	0	0	0	0	0	0
Whole Cell	0	0	0	0	0	0	0
Extracellular Starch	4+	4+	3+	3+	2+	0	0
Capsular	3+	3+	3+	2+	2+	0	$\frac{-}{+}$
Crude Polysaccharide	2+	2+	2+	1+	1+	0	$\frac{-}{+}$



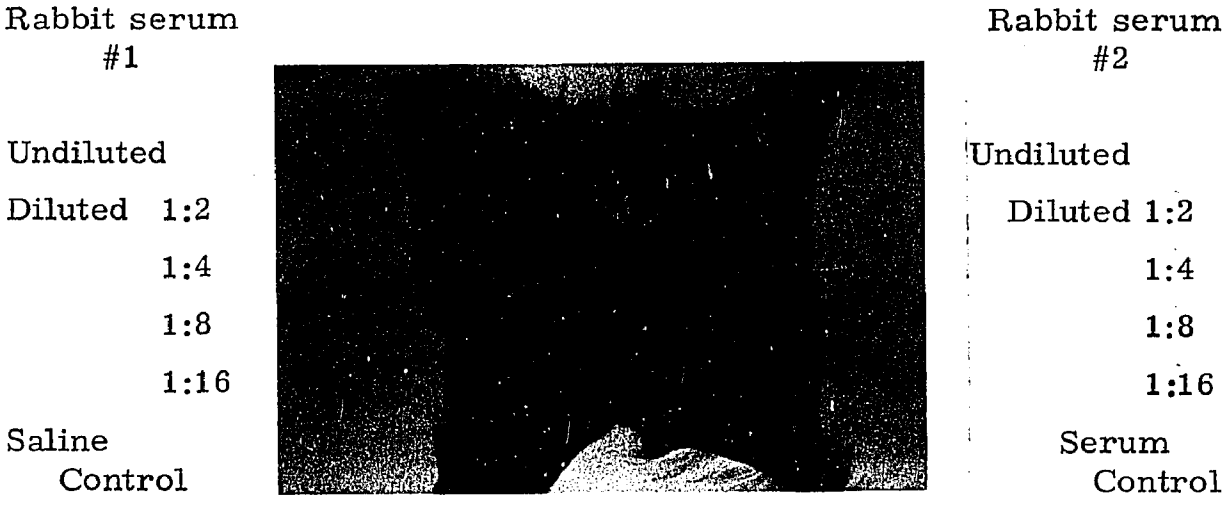


Fig. 2. The response in duplicate of rabbit serums #1 and 2 in the PCA test using homologous starch antigen.

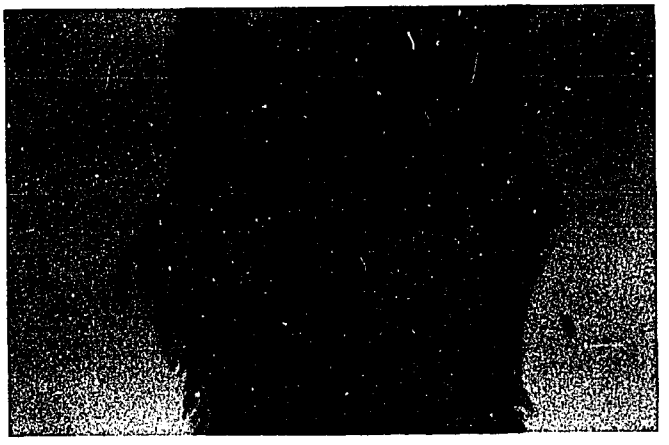


Fig. 3. The titration of rabbit serum #2 in the PCA test using homologous extracellular starch antigen.

TABLE 11

TITRATION OF RABBIT SERUM #2 WITH HOMOLOGOUS EXTRACELLULAR STARCH ANTIGENS*

	Rabbit Serum #2						Controls	
		Dilutions						
E S A	None	1:2	1:4	1:8	1:16	1:32	Saline	Negative Serum (Rabbit)
C. neoformans M-1000	4+	3+	3+	2+	1+	0	0	0
C. neoformans M-1000	3+	2+	1+	±	±	0	0	0
C. neoformans M-1000	4+	3+	3+	2+	1+	0	0	0

* Results in triplicate from 3 animals

TABLE 12

THE DETERMINATION OF THE OPTIMUM LATENT PERIOD FOR THE PCA TEST

	Rabbit Serum #2		Controls	
Time in Hours	Undiluted	Diluted 1:4	Saline	Negative Serum (Rabbit)
5	2+	2+	0	0
6	2+	2+	0	0
7	3+	3+	0	0
8	3+	3+	0	0
9	3+	3+	0	0
10	4+	3+	0	0

TABLE 13

THE DETERMINATION OF THE OPTIMUM AMOUNT OF EXTRACELLULAR STARCH ANTIGEN FOR THE PCA TEST

Amount of ES Antigen	Rabbit Serum #2 Dilutions None	1:2	1:4	1:8	Controls Saline	Negative Serum (Rabbit)
C. neoformans M-1000						
½ mg	2+	2+	+	+	0	0
1 mg	4+	4+	3+	2+	0	0
1½ mg	2+	+	+	+	0	0
C. neoformans CDC-51						
½ mg	2+	2+	+	+	0	0
1 mg	3+	3+	2+	1+	0	0
1½ mg	1+	1+	1+	1+	0	0

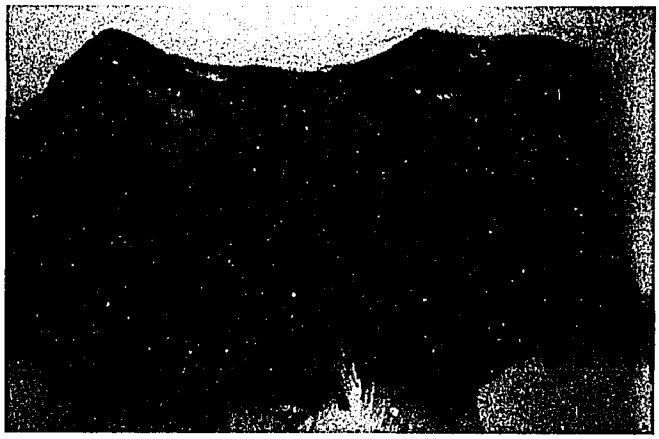
0.5 mg antigen  1 mg antigen

Fig. 4. The effect on the PCA response of rabbit serum #2 with 0.5 mg and with 1 mg quantities of homologous extracellular starch antigen.

extracellular starch antigen although there was a pronounced decrease in response when the serum was diluted 1:4. The larger quantity ($1\frac{1}{2}$ mg) of the extracellular starch antigen also gave less impressive results than the 1 mg amount. It was also noted that the reactions of rabbit serum #2 with either the homologous antigen (C. neoformans M-1000) or the heterologous (C. neoformans CDC-51) antigens were similar but not identical in intensity. Additional testing with this serum indicated that the extracellular starch antigens prepared from cultures of C. neoformans M-1000 that had been incubated for 4, 15 or 20 days were as effective as those incubated for 10 days.

The results shown in Table 14 were obtained by re-testing all of the 13 rabbit serums with the homologous starch antigens in 1 mg amounts and utilizing the 9 hour latent period. Differences in reactivity of the animals were noted but usually two of the three gave similar results. Occasionally only one of the three animals was reactive. At times, a target-like picture in which a blue area at the point of inoculation surrounded by a clear zone and then by a ring of blue dye was encountered. In other instances, only a ring was formed. Serum of rabbit #2 consistently gave strong reactions while lesser degrees of response were noted for serums 1, 3, 6, 7, 9 and 14.

Although serum from rabbit #2 reacted most strongly with the extracellular starch antigen prepared from C. neoformans M-1000 and CDC-51, some degree of cross reactivity was demonstrated with

TABLE 14

THE PCA RESPONSE OF 13 RABBIT SERUMS TO HOMOLOGOUS ESA*

Rabbit Serum #	Homologous Antigens	Test E. S. Antigens	Dilutions				Controls		
			Undiluted	1:2	1:4	1:8	Saline	Negative Serum	Positive Serum
1	C. neoformans M-1000	C. neoformans M-1000	3+	2+	1+	1+	0	+	4+
2	C. neoformans M-1000	C. neoformans M-1000	4+	4+	3+	2+	0	0	—
3	C. neoformans M-1000	C. neoformans M-1000	1+	1+	0	0	0	+	4+
4	C. neoformans M-1000	C. neoformans M-1000	1+	0	0	0	0	0	3+
5	C. neoformans M-1000	C. neoformans M-1000	1+	±	0	0	0	0	3+
6	C. neoformans M-1000	C. neoformans M-1000	2+	+	±	0	0	0	3+
7	C. neoformans CDC-51	C. neoformans CDC-51	3+	2+	+	±	0	∓	4+
9	C. neoformans CDC-51	C. neoformans CDC-51	3+	2+	±	±	0	+	4+
10	C. diffluens	C. diffluens	0	0	0	0	0	0	3+
11	C. laurentii	C. laurentii	+	±	0	0	0	0	3+
12	C. laurentii	C. laurentii	+	±	0	0	0	−/+	+/−
13	C. diffluens	C. diffluens	−/+	0	0	0	0	−/+	+
14	C. neoformans M-1000	C. neoformans M-1000	2+	1+	1+	1+	0	0	4+

* Results in triplicate from 3 animals

similar antigens made from C. neoformans 8476, C. neoformans 45_2, C. diffluens and C. laurentii. The intensity of the reactions was, however, less than that obtained with either C. neoformans M-1000 and CDC-51 antigens and was less apparent with diluted serum. The results shown in Table 15 are maximum responses.

No PCA activity could be demonstrated in the pooled serum from any of the mouse groups that had received massive doses of living C. neoformans cells. Although mice are susceptible to infection with C. neoformans, only one of the groups available for bleeding exhibited any signs of infection. This group had been inoculated with C. neoformans CDC-51.

Of the 500 human serums that were tested for the presence of sensitizing antibodies, only one gave a strongly positive reaction. A 1:4 dilution of the serum reacted just as strongly with the extracellular starch antigens prepared from C. diffluens and from C. laurentii. The three serums from cases of cryptococcal meningitis failed to react at all although suggestive reactions were occasionally observed in serums from cases diagnosed as coccidioidomycosis. Suggestive reactions were also noted in individuals who, over a period of time, had worked with the yeast. Repetition of the doubtful serums failed to substantiate these findings. The results obtained by the titration of the reactive human serum #762 are listed in Table 16 (Figure 5).

TABLE 15

THE PCA RESPONSE OF RABBIT SERUM #2 TO THE EXTRACELLULAR STARCH ANTIGENS FROM VARIOUS ORGANISMS

Source of ESA	Rabbit Serum #2 Dilutions				Control	
	Undiluted	1:2	1:4	1:8	Saline	Negative Serum (Rabbit)
C. neoformans M-1000	4+	4+	3+	2+	0	0
C. neoformans CDC-51	3+	3+	2+	1+	0	0
C. neoformans 8476	2+	1+	0	0	0	0
C. neoformans 45_2	2+	+	±	0	0	0
C. diffluens	2+	+	±	0	0	0
C. laurentii	2+	±	±	±	0	0

TABLE 16

THE PCA RESPONSE OF HUMAN SERUM #762*

	Human Serum #762					Controls		
ESA	Undiluted	1:2	1:4	1:8	1:16	Saline	Negative Serum	Positive Serum
C. neoformans M-1000	4+	3+	2+	1+	1+	0	0	4+
C. neoformans M-1000	4+	3+	3+	2+	1+	0	0	3+
C. neoformans M-1000	3+	2+	2+	1+	1+	0	0	3+
C. diffluens	—	—	3+	—	—	0	0	3+
C. laurentii	—	—	3+	—	—	0	0	3+

* Response in triplicate from 3 animals

— not done

Saline Control

Positive Control serum

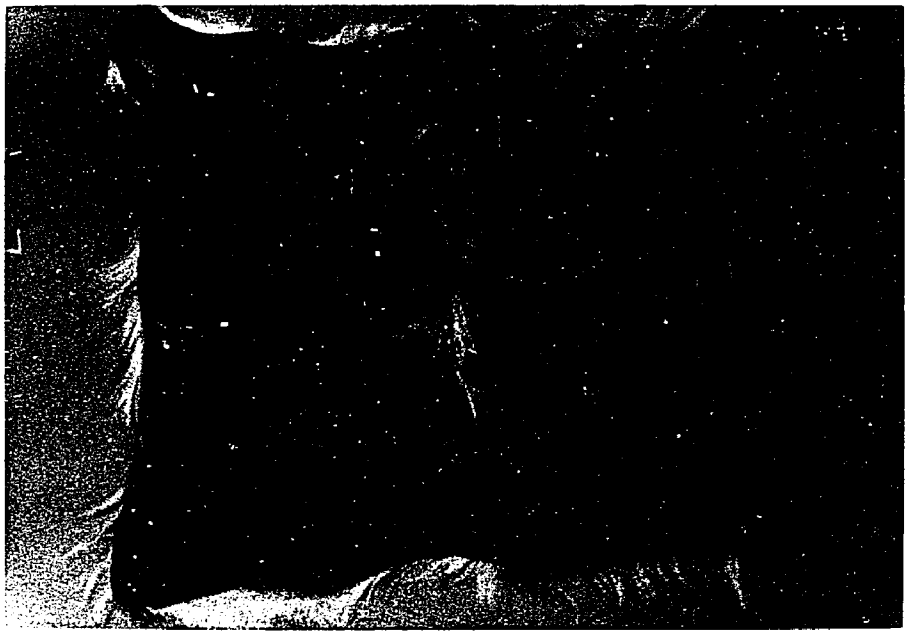
Undiluted #762

Diluted 1:2

1:4

1:8

1:16



Negative Control serum

Undiluted #482

Diluted 1:2

1:4

1:8

1:16

Fig. 5. Response in the PCA test of human serums #762 and #482 using C. neoformans M-1000 extracellular starch antigen.

DISCUSSION

Since rabbits are known to be relatively resistant to infection with Cryptococcus neoformans, it was not surprising that none of the rabbits used in this investigation developed signs of infection, although each had received massive doses of Cryptococcus species. This native resistance may be due in part as suggested by Kuhn (39) to their high normal body temperature (39.5 C). According to Kuhn (39), the optimum temperature for growth of "C. hominis" under laboratory conditions is 29 C. A few investigators, however, have successfully infected rabbits with C. neoformans. Stoddard and Cutler (71) inoculated a rabbit intraperitoneally with 2 ml of a "thick suspension" of "Torula histolytica" and induced meningitis although no other gross lesions were observed. Shapiro and Neal (65) injected four rabbits with a "heavy suspension" of a 48 hour culture of "Torula" isolated from a patient with "Torula meningitis." Two rabbits that had been injected intracranially showed no gross lesions but, four months later, the yeast was recovered from one animal in cultures of the brain, liver, spleen, lungs, and heart. One of the two rabbits injected intraperitoneally appeared ill eleven weeks following inoculation. At autopsy, the internal organs showed no gross lesions, but the yeast was cultured from spinal fluid, skin lesions, liver, spleen and kidney.

The fourth rabbit gave no evidence of infection. On the other hand, Kligman (38) injected 5×10^7 cells of "T. histolytica" intravenously into each of a group of 9 rabbits. Only one rabbit died within a period of four months following the inoculation. A few yeast cells were found in cultures of the brain but death was apparently due to a bacterial pneumonia. Four animals were sacrificed at the end of four months. Typical lesions were found in the lungs, brain, kidney, and spleen of one animal. Smith, Mosberg, Manganiello and Alvarez de Choudens (68) also successfully infected rabbits with C. neoformans, but used the intracranial route.

Nuuttila (personal communication) was unable to produce infection in mice with C. neoformans M-1000 even when the organism was injected with the addition of mucin or mucin plus kaolin. Her findings were unexpected since C. neoformans M-1000 was originally isolated from a fatal case of cryptococcal meningitis. Moreover, mice are considered to be relatively susceptible to infection with this organism (43). Strain C. neoformans CDC-51 did produce infection in mice, but failed to infect rabbits.

The lack of demonstrable gross lesions or symptoms of cryptococcosis in animals that have received massive doses of living cells is difficult to explain. Perhaps a low degree of infection did develop and resulted in the formation of protective sensitizing antibodies. The post-infection skin reaction with the crude cell wall antigen and the

reactivity of the serums from several animals with the extracellular starch, the crude polysaccharide and the capsular antigens suggest that this was the case. It would be of interest to repeat the work and to test the animals with the antigen of Muchmore (49) who obtained positive skin reactions in humans that were thought to have been exposed to natural infection by C. neoformans. The production of some degree of reactivity using the crude cell wall antigen following the attempt to infect rabbits recalls the work of Salvin and Smith (63) and Bennet, Hasenclever and Baum (4), who used a soluble cell wall antigen and produced a delayed skin reaction in previously sensitized guinea pigs. The failure of the crude cell wall antigen to react with rabbit serum in the PCA test could mean that antibodies that react in the skin test are fixed and not circulating. Hence they cannot be passively transferred to guinea pig skin. On the other hand, the failure might be assigned to the antigen itself which, in this investigation, was particulate rather than soluble. However, it might be noted that Terr and McLean (72) observed no consistent relationship between the PCA reaction and the skin test.

Whether or not the smaller size of the #2 rabbit from which the strongly reactive serum was obtained was related to its susceptibility to subclinical infection leading to the formation of antibodies capable of reacting in the PCA test is not known. Other rabbits received the same strain of C. neoformans and all received approximately the same

number of cells. However, their serums exhibited varying degrees of reactivity when tested. These differences may be a reflection of individual differences in the animal's ability to form sensitizing antibodies or in the guinea pig's ability to react to such antigens. If larger numbers of guinea pigs, 8 to 10 for example, had been used to test each of the serums, possibly some improvement in response might have been encountered.

Mice infected with strains of C. neoformans M-1000, CDC-51 or 45_2 did not produce a level of sensitizing antibody that was detectable by the PCA test. In the mice infected with C. neoformans CDC-51, this was not surprising since it is known that in cases of disseminated fungus infection, for example, in disseminated coccidioidomycosis, anergy exists. These findings are similar to those of Hoff (29), who was unable to obtain positive tests for hypersensitivity in mice that had previously been infected with living organisms. Abrahams, Gilleran and Weiss (1) were able to detect cryptococcal antigens in infected mice by the intradermal injection of rabbit anti-cryptococcal serum.

The extracellular starch antigen produced by the method of Mager (46) has apparently never been used in any type of hypersensitivity testing involving C. neoformans. The bases for its effectiveness in this investigation are not clear. The possibility exists that since this material, as well as the crude polysaccharide and capsular

substances, was extracellular in origin, it could be transported to antibody producing cells where it would react as an antigen.

In an investigation of a small farming community in Oklahoma where three cases of cryptococcal meningitis had occurred in one year, Muchmore, Rhodes, Nix, Felton and Carpenter (50) reported culturing C. neoformans from four samples of pigeon or sparrow droppings and from two soil samples. When Muchmore and his associates (49) skin-tested long-time residents of this community, approximately 30% of those tested reacted positively. These findings could mean that these individuals had been infected by inhaling cells of C. neoformans which were disseminated by air currents from bird droppings or soil or possibly from other natural habitats. Evidence that this yeast may be air-borne comes from the work of Shields and Ajello (66) who recently recovered it from air samples.

In the Tucson area, C. neoformans has been found in the slime flux of the mesquite tree and on its bark. Avian dung has not been examined but since this is a source of the yeast in other parts of the United States, one might expect to find it in this material in this area. All these natural habitats are exposed to the air and possibly the yeast could be disseminated by air currents. If this is true, fairly large numbers of individuals in the general population may have had contact with it and become sensitized. Hence, it was surprising that only one of the 500 human serums tested showed a distinctly positive reaction.

The titer of this serum (1:16) is sufficiently high to suggest that a sensitization might have occurred as a result of subclinical infection. Furthermore, the high degree of cross-reactivity with the extracellular starch antigens of C. diffluens and of C. laurentii is interesting. Thomas and Evenson (personal communication) as well as Nuuttila observed that C. diffluens is capable of producing lesions in mice. Although no lesions were observed in mice infected with C. laurentii, Nuuttila recovered it in cultures of lungs from mice injected four weeks previously. It would appear to be possible that these species of Cryptococcus might be able to infect humans as well as mice and that the resulting sensitization would be protective to some degree against infection with C. neoformans. This requires further investigation.

Using the PCA test, Jellison, Glesne and Owen (30) found only two individuals whose serums reacted positively with Emmonsia crescens antigen although serums from over 500 persons who could possibly have been exposed to the organism were tested. In their experience, a titer of 1:8 was accepted as evidence of infection.

While the value of the PCA test for the detection of sensitizing antibodies to C. neoformans is not established, it would appear worthy of further investigation. Modifications of the antigen preparations, for example, might be tried. Possibly a soluble cell wall antigen might have given better results than the particulate one utilized in this study.

Certain refinements of the extracellular starch antigen might either eliminate or enhance the doubtful reactions that were at times observed in the cases of individuals with coccidioidomycosis and in individuals who had been exposed to the organism in the laboratory atmosphere.

SUMMARY

Nine rabbits were injected with strains of Cryptococcus neoformans isolated from cases of cryptococcosis. Two were injected with a strain of C. laurentii, and two with a strain of C. diffluens.

None of the rabbits tested gave evidence of previous sensitization to C. neoformans although some, after injection, reacted to the intradermal injection of the cell wall antigen. None of the 13 rabbits developed observable disease symptoms.

The serum of one of the rabbits injected with C. neoformans gave a strongly positive reaction in the PCA test but the serum of seven others exhibited some PCA activity when tested with the homologous antigen. Of the six antigens used, the extracellular starch antigen was the most effective. The test animal was the guinea pig.

Serums from mice inoculated with C. neoformans failed to react in the PCA test.

Five hundred human serums were tested for PCA activity using the extracellular starch antigen. Only one reacted with a strongly positive reaction and the titer was sufficiently high to suggest previous subclinical infection.

The value of the PCA test in the detection of anti-cryptococcal antibodies in human serums has not been established and should be investigated further.

LITERATURE CITED

1. Abrahams, I., T. G. Gilleran and C. B. Weiss. 1962. Quantitative studies on reverse cutaneous anaphylaxis in mice with progressive cryptococcosis. J. Immunol. 89: 684-690.

2. Ajello, L. 1956. Soil as natural reservoir for human pathogenic fungi. Science 123:876-879.

3. Ajello, L. 1958. Occurrence of Cryptococcus neoformans in soils. Amer. J. Hyg. 67:72-77.

4. Bennett, J. E., H. F. Hasenclever and G. L. Baum. 1965. Evaluation of a skin test for cryptococcosis. Amer. Rev. Resp. Dis. 91:616.

5. Bergman, F. 1963. Occurrence of Cryptococcus neoformans in Sweden. Acta Med. Scand. 174:651-655.

6. Berghausen, O. 1927. Torula infection in man. Ann. Intern. Med. 1:235-240.

7. Bernhardt, R., G. Zalewski and J. Burawski. 1935. Generalisierte torulose (Europaishe blastomykose). Arch. Dermatol. Syphilol. 173:78-90. In Carton, C. A. 1952. Treatment of central nervous system cryptococcosis: a review and report of four cases treated with actidione. Ann. Intern. Med. 37:123-154.

8. Brown, R., D. Weintraub and R. W. Simpson. 1947. Timber as a source of Sporotrichosis infection. In Sporotrichosis infection on mines of the Witwatersrand. Proc. Transvaal Mine Med. Officer's Assoc. Johannesburg, Transvaal Chamber of Mines, 67 p.

9. Carter, H. S. and J. L. Young. 1950. Note on the isolation of Cryptococcus neoformans from a sample of milk. J. Pathol. Bacteriol. 62:271-273.

10. Carton, C. A. 1952. Treatment of central nervous system cryptococcosis: a review and report of four cases treated with actidione. Ann. Intern. Med. 37:123-154.

11. Chase, M. W. 1947. Studies on the sensitization of animals with simple chemical compounds. X. Antibodies inducing immediate-type skin reactions. J. Exp. Med. 86:489-514.

12. Cohen, A. A., A. Davis and S. M. Finegold. 1965. Chronic pulmonary cryptococcosis. Amer. Rev. Resp. Dis. 91:414-423.

13. Connell, J. T. and W. B. Sherman. 1965. Relationship of passive cutaneous anaphylaxis to antibodies in the serum of ragweed hay fever patients after injection treatment. J. Immunol. 94:498-501.

14. Davis, B. L., R. T. Smith and C. E. Smith. 1942. An epidemic of coccidioidal infection (coccidioidomycosis). J. Amer. Med. Assoc. 118:1182-1186.

15. Deicher, H. R. G., H. R. Holman, H. G. Kunkel and Z. Ovary. 1960. Passive cutaneous anaphylaxis reactions with a systemic lupus erythematosus serum factor and isolated DNA. J. Immunol. 84:106-111.

16. Dienst, R. B. 1938. Cryptococcus histolyiticus isolated from subcutaneous tumor. Arch. Dermatol. Syphilol. 37:461-464. In Littman, M. L. and L. E. Zimmerman. 1956. Cryptococcosis; Torulosis or European Blastomycosis. Greene and Stratton, New York and London. 205 p.

17. Emmons, C. W. 1950. The natural occurrence in animals and soil of fungi which cause disease in man. p. 416-421. Proc. Seventh Int. Bot. Congr., Stockholm.

18. Emmons, C. W. 1951. Isolation of Cryptococcus neoformans from soil. J. Bacteriol. 62:685-690.

19. Emmons, C. W. 1954. The significance of saprophytism in epidemiology of the mycoses. New York Acad. Sci., Trans., Series 2. 17:157-166.

20. Emmons, C. W. 1955. Saprophytic sources of Cryptococcus neoformans associated with pigeon (Columbia livia). Amer. J. Hyg. 62:227-232.

21. Emmons, C. W. 1960. Prevalence of Cryptococcus neoformans in pigeon habitats. Pub. Health Rep. 75:362-364.

22. Emmons, C. W. 1961. Isolation of Histoplasma capsulatum from soil in Washington, D. C. Pub. Health Rep. 76:591-595.

23. Emmons, C. W. and A. M. Greenhall. 1962. Histoplasma capsulatum and house bats in Trinidad, W. I. Sabouraudia 2:18-22.

24. Evenson, A. E. and J. W. Lamb. 1964. Slime flux of mesquite as a new saprophytic source of Cryptococcus neoformans. J. Bacteriol. 88:542.

25. Fisher, J. P. and J. T. Connell. 1962. Passive cutaneous anaphylaxis in the guinea pig with serum of allergic patients treated with ragweed extract emulsions. Preliminary report. J. Allergy 33:59-61.

26. Fisher, J. P., E. Middleton and A. E. O. Menzel. 1956. Study on passive cutaneous anaphylaxis with serum of allergic patients. J. Allergy 27:256-260.

27. Frey, D. and E. B. Durie. 1964. The isolation of Cryptococcus neoformans (Torula histolytica) from soil in New Guinea and pigeon droppings in Sydney, New South Wales. Med. J. Australia. 51:947-949.

28. Harber, L. C., C. M. Marsh and Z. Ovary. 1962. Lack of passive cutaneous anaphylaxis in psoriarsis. Arch. Dermatol. 85:716-719.

29. Hoff, C. L. 1942. Immunity studies of Cryptococcus hominis (Torula histolytica) in mice. J. Lab. Clin. Med. 27:751-754.

30. Jellison, W. L., L. Glesne and C. Owen. 1962. The passive cutaneous anaphylaxis (PCA) test in adiaspiromycosis. Mycologia 54:466-475.

31. Johnson, C. W. 1962. Hypersensitivity to chemical allergens. II. Quantitative demonstration of antibody by means of passive cutaneous anaphylaxis. Intern. Arch. Allergy 21:279-287.

32. Josephson, A. S., E. C. Franklin and Z. Ovary. 1962. The characterization of antibodies to penicillin. J. Clin. Invest. 41:588-593.

33. Kabat, E. A. and M. M. Mayer. 1961. Experimental Immunochemistry. 2nd. ed. p. 840-842. Charles C. Thomas. Springfield, Ill.

34. Kajihiro, E. S. 1965. Occurrence of dermatophytes in fresh bat guano. Appl. Microbiol. 13:720-724.

35. Kao, C. J. and J. Schwarz. 1957. The isolation of Cryptococcus neoformans from pigeon nests. Amer. J. Clin. Pathol. 27:652-663.

36. Kessel, J. F. and F. Holtzward. 1935. Experimental studies with Torula from a knee infection in man. Amer. J. Trop. Med. 15:467-484.

37. Klein, E. 1901. Pathogenic microbes in milk. J. Hyg. 1:78-95. In Carter, H. S. and J. L. Young. 1950. Note on the isolation of Cryptococcus neoformans from a sample of milk. J. Pathol. Bacteriol. 62:271-273.

38. Kligman, A. M. 1947. Studies of the capsular substance of Torula histolytica and the immunologic properties of torula cells. J. Immunol. 57:395-401.

39. Kuhn, L. R. 1939. Growth and viability of Cryptococcus hominis at mouse and rabbit body temperatures. Proc. Soc. Exp. Biol. Med. 41:573-574.

40. Leopold, S. S. 1953. Pulmonary moniliasis and cryptococcal osteomyelitis in the same patient, p. 1737-1746. In Medical Clinics of North America, Philadelphia Number. W. B. Saunders Co., Philadelphia and London.

41. Leskowitz, S. and Z. Ovary. 1962. The relation between molecular weight of antigen and ability to elicit passive cutaneous anaphylaxis. Immunology 5:1-10.

42. Littman, M. L. and S. S. Schneierson. 1959. Cryptococcus neoformans in pigeon excreta in New York City. Amer. J. Hyg. 69:49-59.

43. Littman, M. L. and L. E. Zimmerman. 1956. Cryptococcosis; Torulosis or European Blastomycosis. Grune and Stratton. New York and London. 205 p.

44. Lodder, J. and N. J. W. Kreger-van-Rij. 1952. The Yeasts, a taxonomic study. North Holland Publishing Co., Amsterdam. 713 p.

45. Lomanitz, R. and J. M. Hale. 1963. Production of delayed hypersensitivity to Cryptococcus neoformans in experimental animals. J. Bacteriol. 86:505-509.

46. Mager, J. 1947. Studies on the polysaccharide of capsulated yeasts. Biochem. J. 41:603-609.

47. Mager, J. and M. Aschner. 1947. Biological studies on capsulated yeasts. J. Bacteriol. 53:283-295.

48. McDonough, E. S., L. Ajello, R. M. Ausherman, A. Balows, J. T. McClellan and S. Brinkman. 1961. Human pathogenic fungi recovered from soil in an area endemic for North American blastomycosis. Amer. J. Hyg. 73:75-83.

49. Muchmore, H. G. et al. 1964. Delayed skin hypersensitivity in man with new Cryptococcus antigen, read before the Annual meeting of National Tuberculosis Association, New York. May 24-27. In Prochnow, J. J., J. R. Benfield, J. W. Rippon, C. F. Diener and F. L. Archer. 1965. Cryptococcal hepatitis presenting as a surgical emergency. J. Amer. Med. Assoc. 191:269-274.

50. Muchmore, H. G., E. R. Rhoades, G. E. Nix, F. G. Felton and R. E. Carpenter. 1963. Occurrence of Cryptococcus neoformans in the environment of three geographically associated cases of cryptococcal meningitis. New Engl. J. Med. 268:1112-1114.

51. Neill, J. M., I. Abrahams and C. E. Kapros. 1950. A comparison of the immunogenicity of weakly encapsulated and of strongly encapsulated strains of Cryptococcus neoformans (Torula histolytica) J. Bacteriol. 59:263-275.

52. Ovary, Z. 1951. A new method for the quantitative determination of anaphylactic antibody in sera of guinea pigs and rabbits. Int. Arch. Allergy Appl. Immunol. 1:315-316.

53. Ovary, Z. 1958. Immediate reactions in the skin of experimental animals provoked by antibody-antigen interaction. p. 459-508. In Progr. Allergy, vol. V. S. Karger, Basel, Switzerland and New York.

54. Ovary, Z. 1959. Passive cutaneous anaphylaxis in the guinea pig: degree of reaction as a function of the quantity of antigen and antibody. Int. Arch. Allergy 14:18-26.

55. Ovary, Z. and O. G. Bier. 1953. Quantitative studies on passive cutaneous anaphylaxis in the guinea pig and its relationship to the Arthus phenomenon. J. Immunol. 71:6-11.

56. Ovary, Z. and G. Biozzi. 1954. Passive sensitization of the skin of the guinea pig with human antibody. Int. Arch. Allergy 5:241-245.

57. Ovary, Z. and A. Janoff. 1961. In vivo detection of ferritin in the normal guinea pig by passive cutaneous anaphylaxis. J. Immunol. 86:79-82.

58. Ovary, Z., H. Randall, E. Witebsky, N. R. Rose, S. Shulman and R. Metzger. 1958. Thyroid-specific autoantibodies studied by passive cutaneous anaphylaxis of guinea pig. Proc. Soc. Exp. Biol. Med. 99:397-401.

59. Partridge, B. M. and H. I. Winner. 1965. Cryptococcus neoformans in bird droppings in London. Lancet 1:1060-1061.

60. Prochnow, J. J., J. R. Benfield, J. W. Rippon, C. F. Diener and F. L. Archer. 1965. Cryptococcal hepatitis presenting as a surgical emergency. J. Amer. Med. Assoc. 191:269-274.

61. Ramsdell, S. G. 1928. The use of trypan blue to demonstrate the immediate skin reaction in rabbits and guinea pigs. J. Immunol. 15:305-311.

62. Rosenberg, L. T., M. H. Chandler, A. S. Gordon and E. E. Fischel. 1958. Antibody production by guinea pig cells demonstrated by the passive cutaneous anaphylaxis reaction. J. Immunol. 81:136-141.

63. Salvin, S. B. and R. F. Smith. 1961. An antigen for detection of hypersensitivity to Cryptococcus neoformans. Proc. Soc. Exp. Biol. Med. 108:498-501.

64. Sanfelice, F. 1894. Contributo alla morfologia e biologia dei blastomiceti che si sviluppano nei suchi di alcuni frutti. Ann. Igiene Sperimentale. 4:463-495. In Ajello, L. 1958. Occurrence of Cryptococcus neoformans in soils. Amer. J. Hyg. 67:72-77.

65. Shapiro, L. L. and J. B. Neal. 1925. Torula meningitis. Arch. Neurol. Psychiat. 13:174-190.

66. Shields, A. B. and L. Ajello. 1966. Medium for selective isolation of Cryptococcus neoformans. Science 151:208-209.

67. Shifrine, M. and H. J. Phaff. 1956. The association of yeasts with certain bark beetles. Mycologia 38:41-55.

68. Smith, G. W., W. H. Mosberg, L. O. J. Manganiello and J. A. Alvarez de Choudens. 1953. Torulosis of the central nervous system in the laboratory animal. Bull. Sch. Med. Univ. Maryland 38:32-41.

69. Sonntag, W. M. and S. Marcus. 1963. Penicillin hypersensitivity demonstrated by passive cutaneous anaphylaxis in the rat. Int. Arch. Allergy 23:239-245.

70. Stewart, R. A. and K. F. Meyer. 1932. Isolation of Coccidioides immitis (Stiles) from the soil. Proc. Soc. Exp. Biol. Med. 29:937-938.

71. Stoddard, J. L. and E. C. Cutler. 1916. Torula infection in man. Monographs of the Rockefeller Institute for Medical Research. No. 6. 98 p.

72. Terr, A. I. and J. A. McLean. 1964. Studies on insect sting hypersensitivity. J. Allergy 35:127-133.

73. Tsubura, E. 1962. Experimental studies on Cryptococcus. I. Isolation of Cryptococcus neoformans from avian excreta and some considerations on the sources of infection. Jap. J. Med. Mycol. 3:50-55. In Excerpta Med. Microbiol. Immunol. Serol. Sect IV, 16:896. Abst. 3628.

74. Urbach, E. and F. Zach. 1930. Generalisierte Torulose. Arch. Dermat. Syphilol. 162:401-421. In Carton, C. A. 1952. Treatment of central nervous system cryptococosis; a review and report of four cases treated with actidione. Ann. Int. Med. 37:123-154.

75. van Uden, N., M. C. Braco-Forte, Jr. and L. do Carmo-Sousa. 1959. The occurrence of Cryptococcus neoformans in the equine intestinal tract.

76. Westerlund, N. C. 1965. The specificity of the slime flux of mesquite for the yeast Cryptococcus neoformans. M. S. Thesis. Univ. Arizona, Tucson, Ariz.

77. Wickerham, L. J. 1951. Taxonomy of yeasts. U. S. Dept. Agr. Tech. Bull. 1029. 56 p.