THE DIAGNOSIS OF FUNGUS DISEASES BY BIOPSY

ROGER DENIO BAKER, M.D.*

DURHAM, N. C.

From the Department of Pathology, Duke University School of Medicine and from the Veterans Administration Hospital, Durham, N. C.

(Received for publication Nov. 26, 1956.)

THE accurate diagnosis of many of the deep fungus infections is possible by the histopathologic examination of tissue removed by biopsy.[1,2] This method is valuable in diagnostic studies of the superficial mycoses and should be used more frequently.

The examination of tissue removed by biopsy permits the observation of the fungus in relationship to the characteristic inflammatory response. This reaction is indication that the fungus is not simply a contaminant but the agent responsible for the lesion, and supplements the demonstration of the fungus in direct preparation or culture.

Although tissue is usually obtained by surgical excision, the use of needle biopsy is valuable to obtain bone marrow and liver. The resection of pulmonary lesions has greatly increased the opportunity of recognizing fungus infections of the lungs.

This paper discusses the use of tissue embedded in paraffin and sectioned by conventional methods. It is unfortunate that often only the histopathologic method may be employed, because all the tissue has been placed immediately in fixative at surgery. A portion should be cultured or preserved unfixed, should the tissue study indicate infection rather than tumor. How many regrets this procedure would save! Moreover, the gross specimen should be preserved until microscopic studies are complete, as additional blocks from other sites may be needed.

Staining by hematoxylin and eosin is adequate for the diagnosis of most of the deep fungus infections. Special stains are applied to additional sections cut from the paraffin block. If these are not available, the hematoxylin and eosin staining of a section may be easily replaced by one of the special stains. The cover slip is removed by placing the slice in warm xylol in the paraffin oven for several hours. After rehydration the special stain is applied. Several stains can be used, one after the other.

The most helpful special stains[3,4] are the Gram[1] (Figs. 1, 2, 5, and 7), the Gridley[5] (Figs. 2 and 3), the periodic acid-Schiff, abbreviated to PAS[6] (Figs.

Aided by Grant E-861, National Microbiological Institute, National Institutes of Health, Department of Health, Education, and Welfare.

*Professor of Pathology, Duke University School of Medicine; Chief, Laboratory Service, Veterans Administration Hospital. Address: Veterans Administration Hospital, Durham, N. C.

1, 2, 4, and 7), the mucicarmine[7] (Fig. 4), and the silver[8] (Figs. 3 and 6). Each has its particular virtue. The Gram stain is indispensable in demonstrating the filaments of Actinomyces and Nocardia; the Gridley stain is ideal to scan microscopic fields for fungi, since the background is neutral; the PAS stain demonstrates sharply nearly all fungi except Actinomyces and Nocardia; the mucicarmine stain is specific for Cryptococcus; and the silver impregnations give sharp delineation of organisms.

Diagnostically the two most useful stains for fungi are the Gram and the Gridley. The acid-fast stain is applied routinely because tuberculosis is often a differential possibility.

Keratin may absorb dyes diffusely and obscure the staining of fungi in the stratum corneum of the skin. Staining with methylene blue (Fig. 8) or by the method of Giemsa obviates this difficulty.

In human lesions the fungus usually fails to present the variety of forms seen in cultures. Rounded forms in tissue characterize some of the mycoses, while filamentous (hyphal) forms characterize others, as is evident in the discussion which follows.

ACTINOMYCOSIS

The Fungus.—Actinomyces bovis occurs as gram-positive, branching filaments 1 μ in thickness (Fig. 1). These are usually collected in mycelial masses called sulfur granules or grains. In hematoxylin and eosin preparations the granules have a diffuse eosin stain, at times with peripheral "clubs" at the terminations of the hyphae. In the PAS preparation (Fig. 1) the granule also stains diffusely. In order to bring out the filaments of the granule the Gram stain *must* be used (Fig. 1).

The Inflammatory Response.—The sulfur granule is found within a minute abscess (Fig. 1), which is, in turn, surrounded by scar tissue containing lymphocytes and plasma cells. At the periphery of the abscess macrophages may be numerous, sometimes in the form of lipophages. Giant cells occur rarely. The disease is a chronic suppurative one.

Clinical Correlation.—The lesions are most frequent in the jaw, oral cavity, tongue, abdominal organs, and lungs. Actinomycotic appendicitis and periappendiceal abscess may be recognized first by histologic study of tissue removed at appendectomy or drainage of abscess. Direct examination of pus for sulfur granules should precede attempts to culture the anaerobic organism. The reason that the lesions are found in the locations indicated is that the infection is endogenous and usually develops via the digestive tract, or, less commonly, the respiatory tract. Hematogenous spread from the region of the appendix to the liver is common, with extension of the hepatic abscess to the subphrenic region, pleura, and lung; generalized systemic infections are uncommon.

NOCARDIOSIS

The Fungus.—Nocardia asteroides is identical to the fungus of actinomycosis, occurring in gram-positive, branching filaments 1 mm. in thickness (Fig.

2). The difference is that these filaments do not form grains or granules, except in mycetomas of the extremities. The filaments are not recognized in hematoxylin and eosin, PAS, or Gridley stains, but are readily demonstrable in Gram stain.

The Inflammatory Response.—The organisms provoke a polymorphonuclear response, with abscess formation, but in some lesions fibrin and caseous necrosis are present. Segments of the hyphae may be engulfed by macrophages. Fibrosis is common in mycetoma, where there are granules, and giant cell response is not infrequent in nocardial mycetoma, the giant cells lying against the granules.

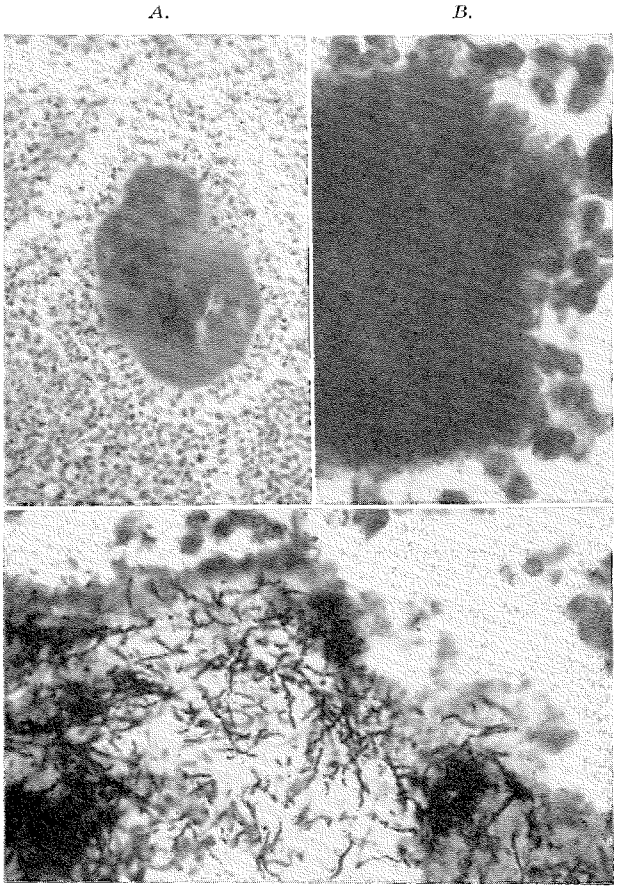


Fig. 1.—*A*, Actinomycosis. Sulfur granule within an abscess. (Hematoxylin and eosin.) *B*, Actinomycosis. Sulfur granule. Note the failure of the PAS stain to demonstrate individual hyphae in the granule. (Periodic acid-Schiff stain.) *C*, Actinomycosis. Sulfur granule. Sharp delineation of the gram-positive, branching filaments composing the granule. (MacCallum bacterial stain.)

Clinical Correlation.—As nocardiosis is an exogenous infection, it develops as a primary pulmonary disease by inhalation or from a puncture wound of an extremity. The pulmonary disease may be extensive and even fatal, with massive, necrotizing pneumonia.[9] Smaller foci may be encountered in pulmonary tissue removed surgically, and here the Gram stain is mandatory.[10] The disease

spreads from the lungs, on occasion, to produce a pyemia or a solitary abscess of the brain or kidney. Aerobic culture and gram-staining should be undertaken.

NORTH AMERICAN BLASTOMYCOSIS

The Fungus.—Blastomyces dermatitidis is a thick-walled sphere, 8 to 15 μ in diameter, with single budding. The organisms are readily seen in hematoxylin, PAS, Gridley, and silver strains (Fig. 2). Gram-staining is capricious, only a portion of the organisms giving the gram-positive reaction. The central portion of the organism is separated by a slight space from the capsule, an important point of difference from developing forms in coccidioidomycosis.

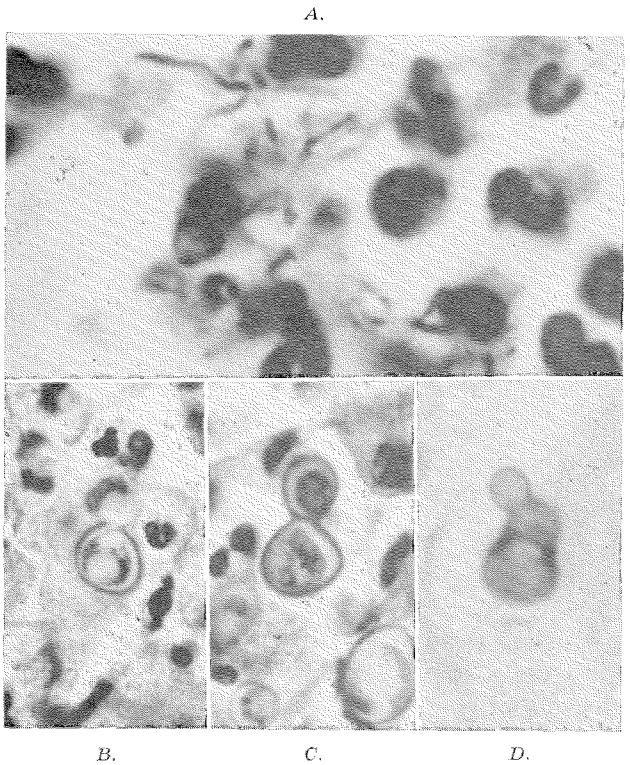


Fig. 2.—*A*, Nocardiosis. Pulmonary lesion. Gram-positive, branching filaments, like those of actinomycosis, but not occurring in granules. (MacCallum bacterial stain.) *B*, North American blastomycosis. Note the space between the thick capsule and the central material. (Hematoxylin and eosin.) *C*, North American blastomycosis. Budding blastomycete in center. (Periodic acid-Schiff stain.) *D*, North American blastomycosis. Budding blastomycete. The neutral background of this stain is helpful in scanning slides for the presence of organisms. (Gridley stain.)

The Inflammatory Response.—The disease is a chronic, suppurative one, with other forms of reaction in varying degree. In acute systemic lesions the organisms may grow massively, interspersed with a few polymorphonuclear neutrophils, giving a yellow, caseous appearance grossly. In older lesions, giant cells contain organisms, and caseous tubercles may be found. Miliary dermal and epidermal abscesses characterize cutaneous blastomycosis.

Clinical Correlation.—Pulmonary lesions alone may be present in some cases, and a nodule of the lung may be subjected to biopsy. The diagnosis can be made by frozen section or by examination of wet mounts of material scraped from the cut surface of the lesion. Cultures should be obtained. Pleural and laryngeal extension from blastomycosis of the lung is common. Blastomycotic tissue from the larynx may resemble squamous-cell carcinoma because of the epithelial hyperplasia caused by the chronic inflammatory response to the fungus. The consequences of an erroneous diagnosis of laryngeal carcinoma are so great, i.e., laryngectomy, that the possibility of blastomycosis or other chronic inflammation must always be considered. If there is any doubt, Gridley and PAS stains should be used.

In systemic blastomycosis, biopsy of lesions of bone, skin, lymph node, and brain may reveal the diagnosis.

Usually cutaneous blastomycosis contains so few organisms that they are difficult to find. The miliary abscesses and the epidermal hyperplasia suggest the diagnosis. The Gridley stain is of most value in searching for the fungus.

SOUTH AMERICAN BLASTOMYCOSIS

The Fungus.—The single cells of *Blastomyces brasiliensis*, from 10 to 30 μ in diameter, are like those of *Blastomyces dermatitidis*. The distinguishing feature in South American blastomycosis is the presence of multiple buds, which may be large or small. Special stains are of great value in demonstrating multiple budding (Fig. 3).

The Inflammatory Response.—This is similar to that of North American blastomycosis and coccidioidomycosis.

Clinical Correlation.—Biopsy material from lymph nodes or oral-cutaneous lesions is frequently encountered.

COCCIDIOIDOMYCOSIS

The Fungus.—A developing form of *Coccidioides immitis*, frequently seen in sections stained with hematoxylin and eosin, has a thick wall (Fig. 3) and hematoxylin-stained internal material continuous with the capsule. The lack of a space between the capsule and the internal material differentiates this form from the tissue forms of North American blastomycosis. Sporangia, 20 to 60 μ in diameter, containing endospores 2 to 5 μ in diameter, may be found, as well as free endospores. In some lesions only the empty shells of sporangia remain. The Gridley stain is best for scanning lesions in search of organisms, while the PAS stain demonstrates the cells well. Hyphae with arthrospores may be found in pulmonary lesions.

The Inflammatory Response.—This response is similar to that of blastomycosis. Suppuration is common, and neutrophilic reaction to the released endospores is often prominent. Developing forms are encountered in giant cells, or in fibrotic tubercles.

Clinical Correlation.—Organisms may be found in nodules or cavitary lesions removed surgically from the lungs, even when the lesions are calcified.[12] Biopsy material of this sort may be treated in a variety of diagnostic manners. The

cut surface of the lesion can be scraped with a knife and the material so obtained examined in unstained moist preparations. Material can be inoculated into the peritoneal cavities of mice with sacrifice of the mice after a week. Tissue sections of the lesions of the peritoneal surfaces of the injected mice show the organisms in various developmental forms.[13]

Lesions from other regions may be similarly examined, such as those from the skin, the lymph nodes, or the meninges.

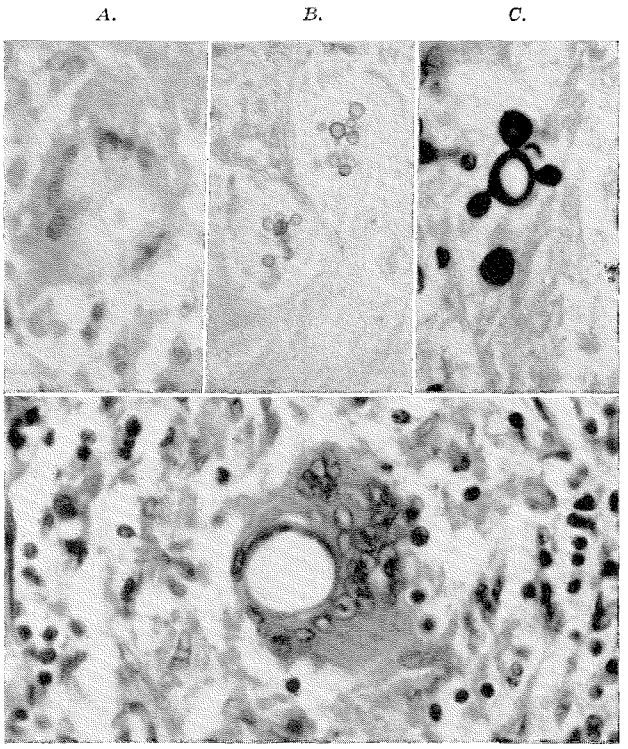


Fig. 3.—*A*, South American blastomycosis. Budding organism in giant cell. (Hematoxylin and eosin.) *B*, South American blastomycosis. Multiple budding. (Gridley stain.) *C*, South American blastomycosis. Multiple budding. Gomori's methenamine-silver nitrate technique. *D*, Coccidioidomycosis. Thick-walled fungus cell in giant cell. Histopathologic diagnosis is possible from this photograph because of the thick wall with a suggestion of inner material beneath the capsule. *Blastomyces dermatitidis* would show a central structure separated from the capsule, as in Fig. 2. Tissue from this case was inoculated intraperitoneally into mice, and all stages of *Coccidioides immitis* demonstrated in sections of the lesions of the peritoneum of the mice. (Hematoxylin and eosin.)

Culture of the fungus in the fluffy, mycelial form may produce infection in the laboratory worker; histopathologic diagnosis or inoculation into mice in such instances may be reasonable substitutes for culture.

HISTOPLASMOSIS

The Fungus.—Histoplasma capsulatum in lesions measures from 1 to 3 μ in diameter (Fig. 4). Hematoxylin-stained material occurs as a mass surrounded

by a capsule. The minute organisms are seen within the cytoplasm of macrophages. The PAS technique causes the capsules to stand out sharply. The Gridley stain is of value, and Gram stains are sometimes excellent.

The Inflammatory Response.—The organism is usually within macrophages. When there are large masses of the fungus-containing macrophages the central portion may become necrotic.[14] Organisms may be demonstrated in old and calcified lesions in histologic section.[15]

Clinical Correlation.—Tissue may come from lesions of the lung, the lymph nodes, the skin, or the mucous membrane of the oral cavity.[16] In systemic cases the bone marrow provides diagnostic tissue.

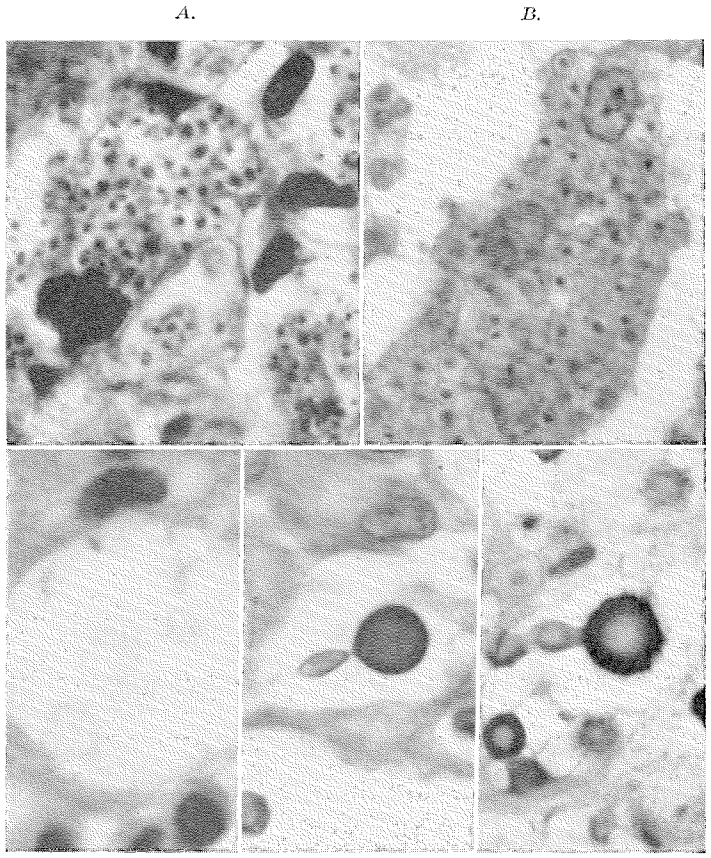


Fig. 4.—*A*, Histoplasmosis. Organisms within macrophages. (Hematoxylin and eosin.) *B*, Histoplasmosis. Organisms within a large macrophage. The capsules of the organisms stain more sharply than in the hematoxylin and eosin preparation. (Periodic acid-Schiff stain.) *C*, Cryptococcosis. Shadowy outlines of organisms surrounded by gelatinous material, in the cytoplasm of a giant cell. Organisms may fail to stain in routine hematoxylin and eosin preparations. Moreover, the organisms have been partially digested in the giant cell and may stain less well on this account. (Hematoxylin and eosin.) *D*, Cryptococcosis. Brilliantly stained budding organism surrounded by gelatinous capsular material. (Periodic acid-Schiff stain.) *E*, Cryptococcosis. Brilliantly stained budding organism surrounded by gelatinous capsular material. This method is specific for *C. neoformans*. (Mucicarmine stain.)

CRYPTOCOCCOSIS

The Fungus.—Cryptococcus neoformans is a round, budding organism 5 to 20 μ in diameter surrounded by a wide, gelatinous capsule (Fig. 4). The organism may fail to stain in hematoxylin and eosin preparations. The PAS, the Gridley, and silver stains are all effective. The gelatinous capsule usually remains unstained. Mucicarmine preparations are specific for the fungus. When the organisms are within the cytoplasm of giant cells they often stain poorly and are much reduced in size, resembling vacuoles. Small forms are easily confused with those of histoplasmosis.

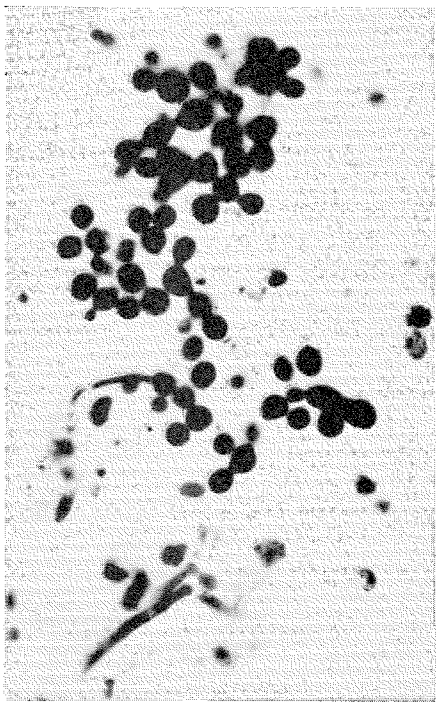


Fig. 5.—Candidiasis. Lesion of esophagus in which rounded fungus cells predominate over hyphae. In other cases the hyphae may predominate. (MacCallum bacterial stain.)

The Inflammatory Response.—The organisms may grow luxuriantly with prominent capsules, causing little inflammation. Often, however, the reaction is chronic, with giant cells, lymphocytes, a few polymorphonuclear cells, and fibrosis.[17] Suppuration is rare and calcification absent.

Clinical Correlation.—Biopsy and excised material comes from the lung or brain, rarely from skin or bone. Pulmonary nodules resected operatively have varied from 3.5 to 7.5 cm. in diameter, have occurred in various lobes of the lungs, and have been solid granulomas except for central necrosis and for cavities in larger lesions.[18,19] In the brain, lesions may simulate primary neoplasms, though the meninges are more commonly affected. When the organisms have

generous capsular material the gross specimen has a gelatinous or jellylike character, similar to that of mucoid carcinoma. Skin lesions occur uncommonly, and contain demonstrable organisms.

CANDIDIASIS

The Fungus.—*Candida albicans* occurs in tissues either in filamentous or in yeast form, or in a combination of the two (Fig. 5). The oval, budding cells measure 2 to 4 μ across. Hyphae may bear spores at points of constriction. Hematoxylin and eosin, Gram, PAS, and Gridley stains are effective. The fungus is gram-positive, but often fails to stain completely.

The Inflammatory Reaction.—Often the lesion, in the form of a white patch, consists largely of the organism in mycelial growth, with loss of epithelium and moderate ordinary chronic inflammation. In deep lesions, abscesses and giant cells form.

Clinical Correlation.—Lesions subject to biopsy are seen most frequently in the oral cavity and on the skin. Growth along the esophagus, in gastric ulcers, and in the cavity of the uterus has been noted. Cutaneous abscesses, endocarditis with septicemia, and meningitis have been reported. Bronchopulmonary lesions are not usually subjected to biopsy.

ASPERGILLOSIS

The Fungus.—In deep lesions the fungus is seen in segmented hyphal form with Y-shaped branchings. The hyphae are usually less than 5 μ thick. Brown spore heads with innumerable brownish spores 2 to 3 μ in diameter may be found in pulmonary cavities, in air sinuses, or in the external ear canal. The PAS and Gridley stains are effective.

The Inflammatory Response.—Abscess formation characterizes disseminated pyemias. Pulmonary lesions may exhibit little inflammatory response, or chronic inflammation and necrosis may be noted. The fungus may invade blood vessels as do the organisms of mucormycosis.

Clinical Correlation.—Aspergillosis often develops secondarily in a predisposing disease. A tuberculous cavity may develop aspergillosis secondarily.

MUCORMYCOSIS

The Fungus.—In tissue, Rhizopus, Mucor, and Absidia form broad, branching, nonseptate hyphae up to 20 μ broad (Fig. 6). The organisms are often inconspicuous but can be demonstrated in hematoxylin and eosin preparations.[20,21] Silver impregnations stain the organisms well. The poor delineation of the organism with other stains is due to the delicacy of the peripheral membrane and the paucity of stainable internal material. In most cases, only hyphae occur, but sporangia have been reported in a case involving an ethmoid air sinus.[21]

The Inflammatory Response.—The inflammatory response is acute, with polymorphonuclears. The organism grows into arteries and veins, causing thromboses and infarcts.

Clinical Correlations.—Mucormycosis is an acute mycosis as a rule. Many cases of cerebral and pulmonary mucormycosis develop in persons who have a predisposing disease or condition, especially diabetes mellitus or leukemia.

Diagnosis by biopsy has been made from tissue from the roof of the mouth,[22] from an air sinus, and from the lung. Rhizopus has been the fungus most frequently cultured.

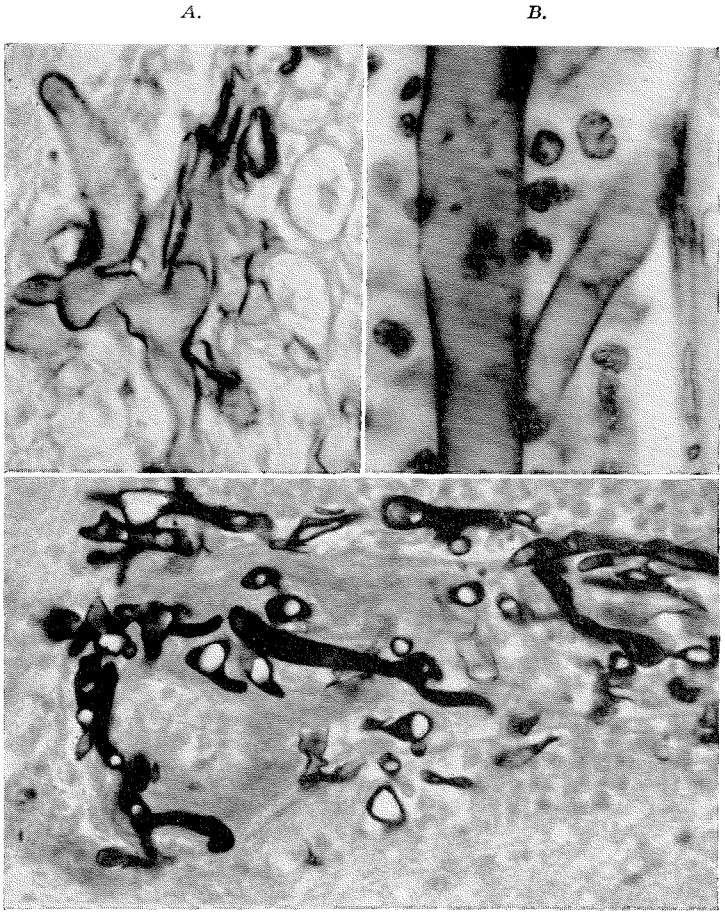


Fig. 6.—*A*, Mucormycosis. Tissue from biopsy of ulcer of palate. Broad, branching, nonseptate hyphae in perineurial lymphatic; nerve fibers to right. *Rhizopus arrhizus* was cultured from this lesion. (Hexatoxylin and eosin.) *B*, Mucormycosis. Branching hypha in blood vessel. (Hematoxylin and eosin.) *C*, Mucormycosis. Hyphae in and around a thrombosed cerebral vessel. Gomori's methenamine-silver nitrate technique.

PENICILLIOSIS

In sections Penicillium is much like Aspergillus. In the one case observed by the writer, as a pulmonary lesion, hyphae were indistinguishable from those of Aspergillus, but Penicillium was demonstrated on culture.

SPOROTRICHOSIS

The Fungus.—*Sporotrichum schenckii*, in tissue, is a cigar-shaped or oval, gram-positive organism 4 to 5 μ in greatest dimension. It is often within the macrophages of lesions of experimentally infected mice. Large asteroid forms, with peripheral radiating spicules, have been reported in human tissues.

The Inflammatory Response.—Tissue from human lesions contains pus, caseation necrosis, or lymphocytic and giant cells. Organisms are seldom demonstrated in human lesions, except in the rare, fatal, generalized cases.

Clinical Correlation.—The clinical picture of a primary ulcer with modules or ulcers along the draining lymphatics is highly characteristic. With a biopsy failing to show organisms, cultures should be obtained; and the diagnosis may be made in a few days, as the organism grows rapidly. To demonstrate the tissue form of the organism, either pus or a suspension of the fungus is injected intraperitoneally into a mouse and sections are made of nodules of the peritoneal wall when the mouse is killed after two weeks.

MADUROMYCOSIS

The Fungus.—Several genera and species are causative agents. The fungus is usually seen in the form of a grain or granule, representing a mycelium or colony of the fungus (Fig. 7). The granules are easily seen with the naked eye, as black, brown, or gray flecks. In tissue sections the granules stain reasonably well with hematoxylin and eosin, and the hyphae and spores of the granule are noted. In contrast to the hyphae of the granules of actinomycosis and nocardiosis, the hyphae of maduromycosis are broad, and stain well with the PAS stain. Occasionally, brown pigment may be seen. In infection with *Monosporium apiospermum* (Fig. 7) the granules are larger than those of actinomycosis, and a central hyphal mycelium presents a peripheral spore-bearing portion. In some infections only spores are present in the granules. Culture is required for the identification of the causative fungus, though the grain of *Monosporium apiospermum* (*Allescheria boydii*) is identifiable in tissue sections.

The Inflammatory Response.—The granule lies within an abscess (Fig. 7) which in turn is surrounded by chronic inflammatory and scar tissue.

Clinical Correlation.—Maduromycosis is usually a disease of a lower extremity, resulting from inoculation of organisms from the soil into the soft tissues. Sinus formation and osteomyelitis commonly develop.

RHINOSPORIDIOSIS

The Fungus.—*Rhinosporidium seeberi* grows as a spore the size of a red blood cell and also as a developing form of larger size. Sporangia may reach a diameter of 350 μ (Fig. 8). Staining with hematoxylin and eosin is satisfactory.

The Inflammatory Response.—This is usually a chronic, round-celled one, rich in plasma cells.

Clinical Correlation.—Tissue obtained by biopsy usually comes from nasal polyps, pharynx, larynx, conjunctiva, skin, penis, vagina, or rectum.

CHROMOBLASTOMYCOSIS

The Fungus.—In lesions, the organism, which may be one of several species, is a rounded brown body, 6 to 12 μ in diameter. Within the organism there are septa for division.[23] The brown bodies often form clusters (Fig. 8). The bodies

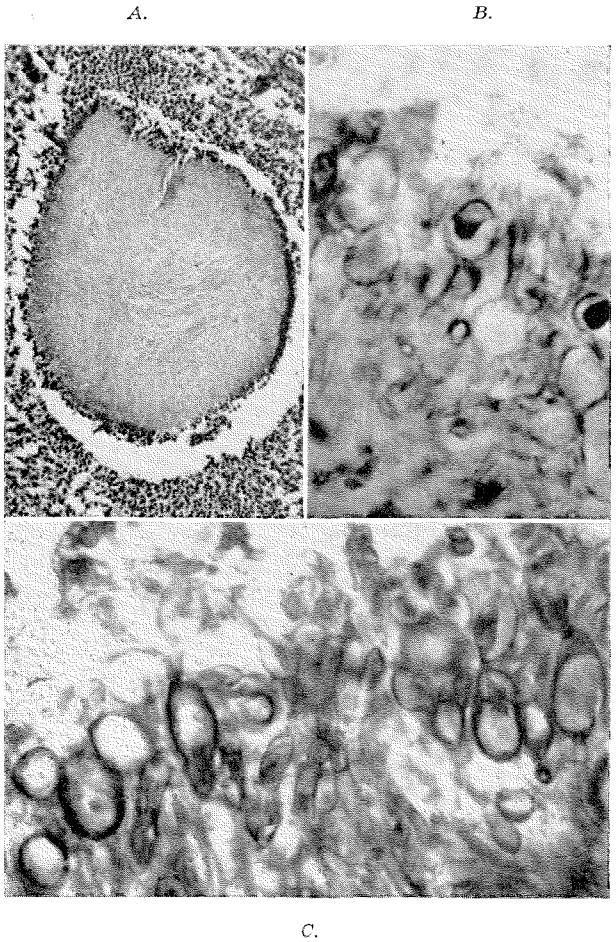


Fig. 7.—*A*, Madurомycosis. Granule surrounded by pus cells. (Hematoxylin and eosin.) *B*, Madurомycosis. Edge of granule. Portions of two rounded cells are gram-positive, but the Gram stain is not helpful with granules of madurомycosis as it is with granules of actinomycosis. Contrast this with Fig. 1. (MacCallum bacterial stain.) *C*, Madurомycosis. Edge of granule. Both spores and hyphae are sharply delineated by this method. (Periodic acid-Schiff stain.) (From Anderson, W.A.D.: Pathology, ed. 2, The C. V. Mosby Co., 1953; in Chapter 15 by Roger D. Baker.)

are so prominently brown in ordinary sections that no special staining is necessary. Culture is required for exact identification of the causative fungus. A related fungus infection has recently been described.[24]

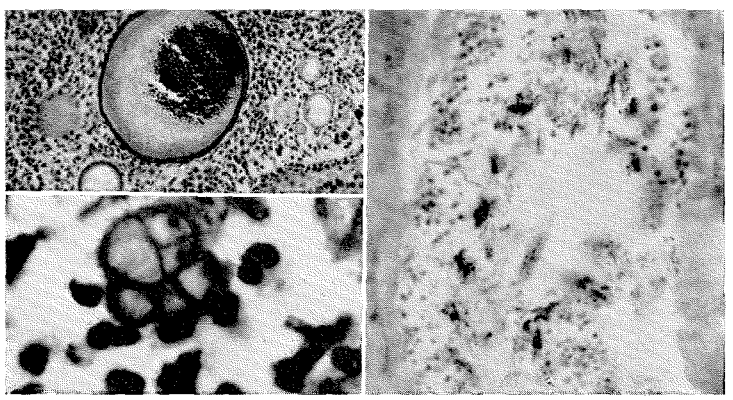
The Inflammatory Response.—The brown bodies are found within giant cells or miliary abscesses of the skin or subcutaneous tissues.

Clinical Correlation.—The infection is usually on an extremity, and the chronic verrucous dermatitis persists for years.

THE DERMATOMYCOSES

The Fungi.—The fungi occur as hyphae or chains of spores. None of the large, complicated spores are seen in tissues such as develop in cultures of Trichophyton, Epidermophyton, and Microsporum. The fungi are visible in hematoxylin and eosin preparations, and in methylene blue (Fig. 8), Giemsa, and PAS stains.

The Inflammatory Response.—The organisms are present in hair or nails without inflammatory response (Fig. 8). In deep infections, which are rare, there may be suppuration and giant-cell response.



A. *B.* *C.*

Fig. 8.—*A*, Rhinosporidiosis. Conjunctiva. Large central sporangium containing endospores. (Hematoxylin and eosin.) *B*, Chromoblastomycosis. A cluster of brown, septate organisms is surrounded by pus cells. (Hematoxylin and eosin.) *C*, Tinea capitis. Minute spores lie between the walls of the hair follicle and the fragmented hair shaft. (Methylene blue stain.) (From Anderson, W.A.D.: Pathology, ed. 2, The C. V. Mosby Co., 1953; in Chapter 15 by Roger D. Baker.)

Clinical Correlation.—Fingernail clippings may be embedded in paraffin, sectioned, and stained for the organisms. In tinea capitis, the organisms lie in the hair follicle and also within the hair shaft. In tinea favosa the scutula are found to consist of abundant mycelial masses. In tinea corporis, organisms are usually not demonstrable in section though rarely they may be seen in the horny layer or within vesicles.

TINEA VERSICOLOR

The Fungus.—Hyphae and rows of spores occur in the stratum corneum, staining with hematoxylin or methylene blue.

The Inflammatory Response.—There is usually none.

Clinical Correlation.—The organism occurs in fawn-colored patches on the skin surface, especially over the chest.

OTOMYCOSIS

The Fungus.—*Aspergillus*, with heads, are readily seen in hematoxylin and eosin and in Gridley stains.

The Inflammatory Response.—There is usually little or none, though scratching may induce chronic dermatitis of the ear canal; and the fungus may be secondary to other conditions, such as eczema.

Clinical Correlation.—In pieces of ear wax embedded and sectioned like tissue, bacteria and fragments of keratinized epidermis are found, in addition to the fungus.

STAINING PROCEDURES

1. *MacCallum-Goodpasture Stain for Gram-Positive and Gram-Negative Bacteria in Tissues.*—Any well-fixed tissue may be used. Cut thin paraffin sections.

SOLUTIONS

Goodpasture's Stain

Basic fuchsin	0.59 Gm.
Aniline	1.00 c.c.
Phenol crystals (melted)	1.00 c.c.
Alcohol, 30 per cent	100.00 c.c.

Gram's Iodine

Iodine	1.00 Gm.
Potassium iodide	2.00 Gm.
Distilled water	300.00 c.c.

Sterling's Gentian Violet Stain

Gentian violet (crystal violet)	5.00 Gm.
Absolute alcohol	10.00 c.c.
Aniline	2.00 c.c.
Distilled water	88.00 c.c.

Picric Acid Solution

A saturated aqueous solution of picric acid.

TECHNIQUE

1. Deparaffinize to distilled water.
2. Place in Goodpasture's stain for 15 minutes.
3. Wash in distilled water.
4. Differentiate in full strength formalin for a few minutes until section becomes pink.
5. Wash in distilled water.
6. Counterstain in saturated aqueous picric acid for 3 to 5 minutes.
7. Wash in water.
8. Differentiate in 95 per cent alcohol for 3 to 5 minutes.
9. Wash in water.
10. Stain in Sterling's gentian violet solution for 3 minutes.
11. Wash in water.
12. Place in Gram's iodine solution for 1 minute.
13. Blot dry.

14. Place in a solution of equal parts of aniline and xylene, 2 changes.
15. Xylene, 2 changes.
16. Mount in Permount or Clarite.

RESULTS

Gram-positive organisms will be blue; gram-negative organisms, red; background, purplish.

2. *Gridley Fungus Stain.*—Cut paraffin sections at 6 μ from any well-fixed tissue.

SOLUTIONS

Chromic Acid Solution

4 per cent aqueous solution of chromic acid.

Coleman's Feulgen Reagent

Dissolve 1 Gm. of basic fuchsin in 200 ml. of boiling water; filter, cool, and add 2 Gm. of potassium metabisulfite and 10 ml. of normal hydrochloric acid. Let bleach for 24 hours, and then add 0.5 Gm. of activated carbon (Nordit), shake for about 1 minute, and filter through coarse paper. The filtrate should be colorless.

Normal Hydrochloric Acid

Hydrochloric acid, sp. gr. 1.19	83.5 c.c.
Distilled water	916.5 c.c.

Sodium Metabisulfite Solution

10 per cent aqueous solution of sodium metabisulfite.

Sulfurous Rinse

10 per cent sodium metabisulfite	6.0 c.c.
Normal hydrochloric acid	5.0 c.c.
Distilled water	100.0 c.c.

Aldehyde Fuchsin Solution

Basic fuchsin	1.0 Gm.
Alcohol, 70 per cent	200.0 c.c.
Paraldehyde	2.0 c.c.
Concentrated hydrochloric acid	2.0 c.c.

Allow to stand at room temperature for 3 days until solution turns deep blue. Store in refrigerator.

Metanil Yellow

Metanil yellow	0.25 Gm.
Distilled water	100.00 c.c.
Glacial acetic acid	0.25 c.c.

TECHNIQUE

1. Deparaffinize and bring sections to distilled water as usual.
2. Place in 4 per cent chromic acid for 1 hour.
3. Wash in running water for 5 minutes.
4. Place in Coleman's preparation of Feulgen reagent for 15 minutes.
5. Rinse in 3 changes of sulfurous acid rinse.
6. Wash for 15 minutes in running tap water.

7. Place in aldehyde-fuchsin solution for 15 to 30 minutes.
8. Rinse off excess stain with 95 per cent alcohol.
9. Wash in water.
10. Counterstain lightly in metanil yellow solution.
11. Wash in water.
12. Dehydrate in graduated alcohols, clear in xylene, mount in Permount.

RESULTS

Mycelia will be deep blue; conidia, deep rose to purple; background, yellow; elastic tissue and mucin also stain deep blue.

3. *Periodic Acid-Schiff Reaction.*—Cut paraffin sections at 6 μ from formalin or Zenker-fixed tissue.

SOLUTIONS

Periodic Acid Solution

Periodic acid crystals	0.5 Gm.
Distilled water	100.0 c.c.

Schiff's Leucofuchsin Solution

Dissolve 1 Gm. of basic fuchsin in 200 c.c. of distilled water. Bring to boil. Cool to 50° C. Filter and add 20 c.c. of normal hydrochloric acid. Cool further and add 1 Gm. of anhydrous sodium bisulfite. Keep in the dark. The fluid may take two days to become straw-colored; then it is ready for use. Store in refrigerator.

Normal Hydrochloric Acid

Hydrochloric acid, concentrated, sp. gr. 1.19	83.5 c.c.
Distilled water	916.5 c.c.

Sulfurous Acid Rinse

10 per cent sodium bisulfite ($NaHSO_3$)	6.0 c.c.
Normal hydrochloric acid	5.0 c.c.
Distilled water	100.0 c.c.

TECHNIQUE

1. Bring sections to distilled water in the usual way.
2. Periodic acid solution for 5 minutes.
3. Rinse in distilled water.
4. Place in Schiff's leucofuchsin solution for 15 minutes.
5. Rinse in 3 changes of sulfurous acid rinse for 2 minutes each.
6. Wash in running tap water for 10 minutes.
7. Counterstain with Harris hematoxylin for 1 minute.
8. Differentiate in acid alcohol (2 or 3 quick dips).
9. Wash in running tap water until nuclei are clear blue.
10. Dehydrate in graduated alcohols, clear in xylene, and mount in Permount.

4. *Mayer's Mucicarmine Stain.*—Cut paraffin sections at 6 μ from any well-fixed tissue. Use control slide.

SOLUTIONS

Picric Acid Solution

Picric acid, saturated solution	100.0 c.c.
Glacial acetic acid	5.0 c.c.

Weigert's Iron Hematoxylin

Solution A

1 per cent hematoxylin in 95 per cent alcohol.

Solution B

Ferric chloride, 29 per cent aqueous	4.0 c.c.
Distilled water	95.0 c.c.
Hydrochloric acid	1.0 c.c.

Working Solution

Equal parts of solutions A and B. Prepare fresh.

Metanil Yellow Solution

Metanil yellow	0.25 Gm.
Distilled water	100.00 c.c.
Glacial acetic acid	0.25 c.c.

Mucicarmine Stain

Carmine (alum lake)	1.0 Gm.
Anhydrous aluminum chloride	0.5 Gm.
Distilled water	20.0 c.c.

Mix stain in a small flask and heat over small flame until solution becomes deep red (approximately 2 minutes). Add 80 c.c. of 50 per cent alcohol. Filter each time before use. (Stain is usable immediately and is good for several days, giving best results at 24 to 48 hours.)

TECHNIQUE

1. Deparaffinize and bring sections to water as usual.
2. Treat sections for 30 minutes in saturated aqueous picric acid solution.
3. Wash in running water until clear.
4. Place slides on rack and pour on freshly prepared Weigert's hematoxylin for 4 minutes.
5. Wash in tap water.
6. Stain in metanil yellow solution for 1 minute.
7. Rinse in distilled water.
8. Place in mucicarmine stain for 30 minutes to 1 hour or longer, check control slide microscopically for staining time.
9. Rinse quickly in 95 per cent alcohol.
10. Dehydrate in absolute alcohol, clear in xylene, mount in Permount.

RESULTS

Mucin will be deep rose to red; nuclei, black; other tissue elements, yellow.

5. *A Stain for Fungi in Tissue Sections and Smears, Using Gomori's Methenamine - Silver Nitrate Technique.*—Cut paraffin sections at 6 μ from any well-fixed tissue.

SOLUTIONS

1. Five per cent aqueous chromic acid (chromium trioxide, CrO_3).
2. Stock methenamine-silver nitrate solution: Add 5 ml. of 5 per cent silver nitrate to 100 ml. of 3 per cent methenamine, U.S.P. grade $(CH_2)_6N_4$. A white precipitate forms but immediately dissolves on shaking. The clear solution remains usable for months at refrigerator temperature.
3. One per cent aqueous sodium bisulfite ($NaHSO_3$).
4. Five per cent aqueous borax, U.S.P. grade ($Na_2B_4O_7.10H_2O$).
5. One-tenth per cent aqueous gold chloride ($AuCl_3.HCl.3H_2O$). May be used repeatedly.
6. Two per cent aqueous sodium thiosulfate ($Na_2S_2O_35H_2O$).

TECHNIQUE

1. Deparaffinize sections and bring to distilled water as usual. Smears are prepared on albumin-treated slides and fixed in 95 per cent alcohol.
2. Hydrated sections and smears are oxidized in 5 per cent chromic acid for 1 hour, washed in running tap water for 10 minutes, and then treated in sodium bisulfite for 1 minute to remove any residual chromic acid. They are then washed in tap water for 5 minutes and finally in 3 changes of distilled water.
3. Silver at 45° to 50° C. in a working solution prepared by adding 25 ml. of stock methenamine-silver nitrate to an equal portion of distilled water containing 1 to 2 ml. of 5 per cent borax. Fungi and mucin will begin to stain at the end of 25 to 30 minutes and will be adequately stained at the end of an hour. Slides are then rinsed in distilled water 2 or 3 times.
4. Tone in 0.1 per cent gold chloride for 5 minutes. This will also bleach the background. Rinse in distilled water.
5. Remove unreduced silver by treating with 2 per cent sodium thiosulfate for 1 or 2 minutes and, after washing thoroughly, counterstain if desired; using safranin if a red nuclear stain is desired, or a light hematoxylin-eosin combination if tissue detail is important.
6. Dehydrate, clear, and mount as usual.

RESULTS

Fungi are sharply delineated in black with the inner parts of mycelia and hyphae staining an old rose as a result of toning in gold. Mucin also assumes a rose-red color as a result of toning.

REFERENCES

1. Conant, N. F., Smith, D. T., Baker, R. D., Callaway, J. L., and Martin, D. S.: Manual of Clinical Mycology, ed. 2, Philadelphia and London, 1954, W. B. Saunders Company.
2. Baker, R. D.: Fungus Infections, Chapter in Anderson, W.A.D.: Pathology, ed. 3, St. Louis, 1957, The C. V. Mosby Company.
3. Laboratory Manual of Special Staining Technics, Washington, D. C., 1953, Armed Forces Institute of Pathology.
4. Lillie, R. D.: Histopathologic Technic and Practical Histochemistry, New York and Toronto, 1954, The Blakiston Company, Inc.
5. Gridley, M. F.: A Stain for Fungi in Tissue Sections, Am. J. Clin. Path. **23**:303, 1953.
6. Kligman, A. M., and Mescon, H.: The Periodic Acid-Schiff Stain for the Demonstration of Fungi in Animal Tissues. J. Bact. **60**:415, 1950.
7. Mallory, F. B.: Pathological Technique, Philadelphia, 1944, W. B. Saunders Company.
8. Grocott, R. G.: A Stain for Fungi in Tissue Sections and Smears Using Gomori's Methenamine-Silver Nitrate Technic, Am. J. Clin. Path. **25**:975, 1955.
9. Connar, R. G., Ferguson, T. B., Sealy, W. C., and Conant, N. F.: Nocardiosis. Report of a Single Case With Recovery, J. Thoracic Surg. **22**:424, 1951.
10. Weed, L. A., Andersen, H. A., Good, C. A., and Baggenstoss, A. H.: Nocardiosis. Clinical, Bacteriologic, and Pathologic Aspects, New England J. Med. **253**:1137, 1955.
11. Schwarz, J., and Baum, G. L.: Blastomycosis, Am. J. Clin. Path. **21**:999, 1951.
12. Forsee, J. H., and Perkins, R. B.: Focalized Pulmonary Coccidioidomycosis. A Surgical Disease, J.A.M.A. **155**:1223, 1954.

13. Tarbet, J. E., Wright, E. T., and Newcomer, V. D.: Experimental Coccidioidal Granuloma. Developmental Stages of Sporangia in Mice, Am. J. Path. **28**:901, 1952.
14. Silverman, F. N., Schwarz, J., and Lahey, M. E.: Histoplasmosis, Am. J. Med. **19**:410, 1955.
15. Straub, M., and Schwarz, J.: The Healed Primary Complex in Histoplasmosis, Am. J. Clin. Path. **25**:727, 1955.
16. Schulz, D. M.: Histoplasmosis. A Statistical Morphology Study, Am. J. Clin. Path. **24**:11, 1954.
17. Baker, R. D., and Haugen, R. K.: Tissue Changes and Tissue Diagnosis in Cryptococcosis, Am. J. Clin. Path. **25**:24, 1955.
18. Haugen, R. K., and Baker, R. D.: The Pulmonary Lesions in Cryptococcosis With Special Reference to Subpleural Nodules, Am. J. Clin. Path. **24**:1381, 1954.
19. Littman, M. L., and Zimmerman, L. E.: Cryptococcosis, New York and London, 1956, Grune & Stratton.
20. Baker, R. D.: Pulmonary Mucormycosis, Am. J. Path. **32**:287, 1956.
21. Bauer, H., Ajello, L., Adams, E., and Useda Hernandez, D.: Cerebral Mucormycosis. Pathogenesis of the Disease, Description of the Fungus, Rhizopus oryzae, Isolated From a Fatal Case, Am. J. Med. **18**:822, 1955.
22. Harris, J. S.: Mucormycosis. Report of a Case, Pediatrics **16**:857, 1955.
23. French, A. J., and Russell, S. R.: Chromoblastomycosis. Report of First Case Recognized in Michigan, Apparently Contracted in South Carolina, A.M.A. Arch. Dermat. & Syph. **67**:129, 1953.
24. Binford, C. H., Thompson, R. K., and Gorham, M. E.: Mycotic Brain Abscess Due to Cladosporium Trichoides, a New Species, Am. J. Clin. Path. **22**:535, 1952.