2nd
EDITION

Practical Laboratory Mycology

ELMER W. KONEMAN, M.D.
Executive Director
Colorado Association for Continuing
Medical Laboratory Education
Denver, Colorado

GLENN D. ROBERTS, Ph.D.
Assistant Professor of Laboratory Medicine
and Microbiology
Consultant in Section of Clinical Microbiology
Mayo School of Medicine and Mayo Clinic
Rochester, Minnesota

SARA E. WRIGHT, MT, ASCP

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Preface to the Second Edition

The Second Edition of *Practical Laboratory Mycology* serves not only to update certain sections of the First Edition, but also provides a more effective stand alone text for self-study and student teaching. The First Edition was designed as a supplement to the Medcom Famous Teachings in Modern Medicine slide series, *Clinical Laboratory Mycology, Parts I and II*, and was dependent in large degree on the availability of the color slides for maximum learning.

The First Edition sections on specimen collection, selection and use of media, culture processing, and identification of yeasts have been largely rewritten. Over 80 photomicrographs to supplement the many line drawings of microscopic morphology and 5 color plates including 40 color prints of frequently encountered fungal colonies have been added. A supplemental appendix listing the formulas for commonly used media, stains, and reagents should also prove of value.

The basic organization of the First Edition has been retained in the Second Edition. The logical step by step discussion of specimen collection and processing, media selection, methods for inoculation and incubation of cultures, and the practical approach to the identification of fungi as presented in the Second Edition follows the natural flow of work in clinical mycology laboratories. Thus, instructors of medical mycology students are not only provided with a practical curriculum guide, but one that follows closely the future work patterns in the laboratory.

The authors of this Second Edition continue to find in their teaching programs and workshop sessions that the subgrouping of the 75 to 100 species of fungi of medical importance based on readily observably gross culture and microscopic characteristics materially aids in making the initial study of mycology less complex for new students.

The design and content of the manual remain as an introductory text in laboratory mycology. It should serve well as a prerequisite course of study for a better understanding of more advanced textbooks in mycology, or as a beginning for interested students and laboratory technologists to pursue a career in mycology or engage in research and advanced studies.

Acknowledgments

The authors express gratitude to the American Society of Clinical Pathologists and the authors listed below for permission to reproduce the black and white photomicrographs and the color photographs as follows:

Atlas of Clinical Mycology: Dolan, CT; Funkhouser, JW; Koneman, EW; Miller, NG, and Roberts, GD. American Society of Clinical Pathologists, Chicago, 1975.

Volume I: Systemic Mycoses—Yeasts: Figures 181–191.

Volume II: Systemic Mycoses—Deep Seated: Figures 1, 2 and 5 in Color Plate V.

Volume III: Systemic Mycoses—Opportunistic: Figures 4, 5 and 8 in Color Plate IV.

Volume IV: Superficial Mycoses: Figures 138, 139, 145–148 and 159; Figures 1–4 in Color Plate III.

Volume V: Subcutaneous Mycoses: Figure 170.

Volume VI: Saprobic Fungi: Figures 65, 71, 74, 76, 80, 82, 92, 110, 126, 131, 169; Figures 1–4 in Color Plate I and Figures 4, 6 and 7 in Color Plate II.

Atlas of Medical Mycology: Jones, JW; McFadden, HW Jr.; McWhorter, CA; and Miller, NG. American Society of Clinical Pathologists, Chicago, 1971–1972: Figures 143, 158, 162, and 177.

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Contents

1 Clinical Diagnosis of Mycotic Disease

The diagnosis of a fungal infection begins with the patient who, experiencing certain symptoms, consults a physician. After taking a history and performing a physical examination, the physician must suspect the presence of mycotic disease and procure appropriate cultures.

The skin is probably the most frequent site of fungal disease because of the high incidence of superficial dermatophyte (ringworm) infections. Most individuals have experienced the itching, scaling, weeping manifestations of athlete's foot or "jock itch," the focal loss of hair in scalp infections, or the typical circular, red ring-like lesions of the skin.

Deeper skin or subcutaneous fungal infections are less commonly encountered. However, mycotic disease must be suspected with any progressive, nonhealing ulcer of the skin or mucous membranes, or in the presence of deeply penetrating sinuses that exude purulent material. One should particularly be suspicious of fungal infections if these subcutaneous or ulcerative lesions follow traumatic wounds contaminated with soil or vegetative matter. Skin penetration with thistles, thorns, burrs, or other similar materials while one is working in gardens or walking barefoot through the brush is particularly conducive to the development of cutaneous or subcutaneous mycotic infections. The physician must remember, however, that these types of cutaneous lesions may not represent primary disease, but rather the skin extension of a serious, systemic fungal infection. A thorough examination of the patient, including cultures of sputum, urine, or other body secretions and chest X-rays is always indicated when fungi are recovered from skin or mucous membrane lesions.

Certain skin lesions, such as the painful erythematous areas seen in erythema nodosum or the symmetrical macular, papular, or vesicular eruptions of the extremities in erythema multiforme should alert one to the possibility of fungal infections. Symmetrical vesicular skin lesions of the hands or feet, representing a so-called "id" reaction, represent allergic manifestations to bacterial or fungal infections that may involve other parts of the skin or deep viscera. Although the lesions of the id reaction are not infected, their presence should alert the physician to the possibility of a generalized fungal infection.

Because fungal spores or hyphal fragments easily become airborne and can be readily inhaled, patients with mycotic infections commonly exhibit pulmonary involvement. Chronic cough, with or without sputum production, chest pain, dyspnea and shortness of breath are common symptoms. The persistance of a pulmonary infiltrate on X-ray, or the presence of a cavity, "fungus ball," or "coin lesion" may represent either an inactive, latent, or slowly progressive mycotic infection that requires further investigation.

Wheezing, asthma-like attacks or expectoration of thick mucous plugs are common manifestations of allergic bronchopulmonary mycosis. Such symptoms may not indicate an endogenous fungal infection, rather they may be caused by inhalation of dust containing spores or mycelial fragments to which a hypersensitive host reacts.

General systemic reactions such as fever of unknown origin, weight loss, and night sweats are seen with disseminated mycotic infections. Many systemic fungal diseases at one time or another in their development may closely simulate tuberculosis.

The current renewed interest in clinical mycology has resulted largely from the increased incidence of fungal diseases in patients with compromised host resistance. Physicians must remain aware of the potential presence of mycotic disease in all immunosuppressed patients and procure appropriate cultures as described below.

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2

Specimens for Study of Fungi

COLLECTION OF SPECIMENS

The specimen must be adequate. Spit is not sputum; a sparsely inoculated dry swab is next to worthless, and a few casually collected skin scales or randomly plucked hairs may be totally unrepresentative.

Specimens must be delivered promptly to the laboratory. Overgrowth with bacteria or with rapidly growing strains of saprophytic fungi may compromise the recovery of more slowly growing pathogenic species of fungi.

If sputum samples must be transported via the mail, overgrowth with contaminating microorganisms may be controlled by adding 50,000 units of penicillin, 100,000 μg of streptomycin, or 0.2 mg of chloramphenicol for each milliliter of material.

The specimen must be labeled completely. Both the culture source and the fungal disease clinically suspected must be identified on the label. Special media or more rigid culture procedures may be required to recover the species of certain pathogenic fungi, such as *Histoplasma capsulatum, Coccidioides immitis,* and *Blastomyces dermatitidis.*

PROPER LABEL

Patient name _____
Hospital # _____
Room No. or address _____
Physician _____
Culture source _____
Organism suspected _____

SPECIFIC SPECIMEN SOURCES

Lower Respiratory Tract

Sputum should be collected from a deep cough early in the morning soon after arising, after the mouth is rinsed with water. Samples should be transported to the laboratory as soon after collection as possible. Twenty-four-hour collections are not acceptable, not only because of the inconvenience to the patient, but because samples may become overgrown with bacteria or saprophytic fungi.

Direct Gram's stain grading systems by which the quality of sputum samples is assessed by counting the relative number of oropharyngeal squamous epithelial cells and polymorphonuclear leukocytes do not apply to mycotic pulmonary infections since the inflammatory reactions commonly are not suppurative in nature.

Sputum may be collected by induction from patients who are not coughing. Nebulized saline or Isuprel® may be forced into the bronchial tree through the use of intermittent positive pressure breathing equipment, which generally results in deep spasmotic coughing.

Transtracheal aspiration may be helpful in obtaining specimens from patients who are debilitated and cannot produce sputum or are too ill to withstand the induction procedure.

Bronchoscopy biopsies may be helpful in the diagnosis of invasive pulmonary mycoses, particularly if the infiltrates or other lesions are located more peripherally in one or more lobes of the lungs.

Genitourinary Tract

Urine samples may be collected via the midstream, clean-catch technique. The recovery of fungal elements is optimum from the first morning sample, particularly following a 12-hour water fast. Twenty-four-hour urine samples are not acceptable for fungal culture.

Culture of the **uterine cervix** or **vaginal canal** is of questionable value because of the common presence of commensal yeasts that usually overgrow the culture medium.

Cutaneous Samples

Cutaneous samples can be obtained by scraping skin scales or clipping infected nails into a sterile Petri dish. Collection of these materials into a clean envelope for transporting via the mail is also acceptable.

The areas to be sampled should be first wiped with 70% alcohol to remove bacterial contaminants. Skin lesions should be sampled from the erythematous, peripheral growing margins of typical "ringworm" infections. The superficial portions of the nail should be scraped away with a surgical blade before collecting the deeper portions, which have a greater chance of being infected.

Potentially infected hairs can be plucked with a pair of surgical forceps. A Wood's ultraviolet lamp may be helpful in selecting those hairs that are involved if the infection is due to one of the dermatophyte species that produces fluorescence (*Microsporum audouinii*, for example).

Subcutaneous Samples

In the presence of suppurative lesions of the deep skin and subcutaneous tissues, in which pus may be loculated within abscesses or is exuding from deep sinus tracts, aspiration with a sterile needle and syringe should be attempted. If immediate inoculation of the specimen to an appropriate culture medium is not possible, the material should be placed into an anaerobic transport tube. If a swab is used for collection of material, it should be extended into the depths of the wound, without touching the adjacent skin margins. Both anaerobic and aerobic cultures should be performed, primarily to recover the fungus-like bacteria, *Actinomyces* species.

Cerebrospinal Fluid

Cerebrospinal fluid is collected by the physician using the routine lumbar puncture procedure. Most commonly, three separate tubes are used for collection of the fluid: the first used for determination of various chemical constituents, the second for cell-counting procedures, and the third for culture. Approximately 1 to 5 ml are usually available for culture.

Blood

The laboratory should be alerted by the physician if he suspects fungal septicemia because special collection bottles are necessary for optimum recovery of fungi. In smaller laboratories it is permissible to transfer approximately 0.5 to 1.0 ml of blood to the surface of Sabouraud's or brain-heart infusion agar. For the optimal recovery of fungi, 10 ml of blood should be added to a 100-ml brain-heart infusion broth bottle containing a brain-heart infusion agar slant (see Appendix II). Trypticase soy broth or Columbia broth are acceptable alternatives; however, recovery times may be lengthened.

Tissue Samples

Tissue biopsy specimens are obtained either in the operating room or as a minor surgical procedure in the physicians office or clinic. Samples should be placed in a 4 × 4 sterile surgical gauze moistened with sterile saline. Obviously, samples submitted in for-

malin are not suitable for culture, although microorganisms can still be recovered from the center portions of formalinized specimens if the exposure time has been short. Care should also be taken not to place biopsy samples in "saline for injection" solutions as these contain antimicrobic substances.

Bone Marrow

A bone marrow sample is obtained by the physician using sterile technique. Needle aspirations or core biopsy specimens may be obtained from the sternum or preferably the iliac crest. The initial aspirate is generally used for making bone marrow smears; following this an additional 3 to 5 ml of marrow and blood are removed and placed into a sterile vial containing 0.5 ml of 1:1000 heparin. Alternately, the specimen may be put directly into the brain-heart infusion blood culture bottle described above for transport to the laboratory.

Body Fluids

Fluids that accumulate as part of an infectious or inflammatory process are usually obtained by aspiration with a syringe and needle using sterile technique. If the volume of fluid is large, such as the collection of thoracic (paracentesis) or abdominal (ascitic) fluids, sterile gallon-size containers should be used. If there is to be a delay in transport to the laboratory, it is permissible to allow the fluid to settle either at room temperature or in the refrigerator for a few hours. If this is done, care should be taken in transport to see that the sediment is not remixed with the upper fluid layers, as any organisms present in the fluid will be concentrated in the sediment.

Summary

Table I lists a number of the more commonly encountered fungal diseases, the optimal sites for culture to confirm the diagnosis, and pertinent comments relative to each site. If fungal organisms are recovered from one or more sites in the absence of overt clinical signs or symptoms, the patient should be followed closely with periodic complete examinations since a latent underlying disease process may be in an early progressive stage.

DIRECT MICROSCOPIC EXAMINATION OF SPECIMENS

The direct microscopic examination of all specimens submitted to the laboratory for fungal culture is highly encouraged. An immediate report can be issued and it may be possible for the physician to institute specific therapy based on the identification of certain diagnostic forms.

Direct mounts are made by mixing a small portion of the material to be cultured in 2 or 3 drops of water, physiological saline, or 10% KOH on a microscope slide. With skin or nail scrapings or potentially infected hairs, 10% KOH is recommended since the mounting fluid clears out background debris and keratinous material, making the hyphal forms easier to see.

A glass cover slip is placed over the liquid mount prior to examination of the slide under the microscope. The mount can be examined with bright field illumination by lowering the condenser to reduce the amount of incident light; or preferably, examination of unstained materials should be made with a microscope equipped with a phase contrast condenser and objectives.

TABLE I
SUSPECTED FUNGAL DISEASES AND OPTIMAL CULTURE SITES

Suspected Diseases	Sites to Culture	Comments
Aspergillosis	1. Respiratory secretions 2. External auditory canal 3. Corneal scrapings	Also culture sputum mucous plugs in suspected cases of allergic bronchopulmonary aspergillosis.
Blastomycosis	1. Respiratory secretions 2. Skin 3. Mucous membranes 4. Bone (osteomyelitis) 5. Urine (rarely positive)	Prepare direct mounts of specimen for microscopic search of characteristic large yeast cells with single broad based buds.
Candidosis	1. Blood 2. Urine 3. Skin 4. Nails 5. Respiratory secretions 6. Oral and vaginal cavities 7. Autopsy tissue (gastroesophageal junction)	*Candida* species are most frequently recovered from respiratory secretions and oral and vaginal mucous membranes; however, their clinical significance from these sites is questionable.
Coccidioidomycosis	1. Respiratory secretions 2. Lung tissue (biopsy) 3. Skin 4. Cerebrospinal fluid 5. Joints (synovitis) 6. Mucous membranes	Tissue submitted to the laboratory for culture contains spherules and endospores, the stage of the fungus not commonly infective to laboratory personnel.
Cryptococcosis	1. Pulmonary secretions 2. Cerebrospinal fluid 3. Urine 4. Blood 5. Bone marrow	More than one site should be cultured in suspected cases. Serological testing for capsular polysaccharide or type-specific antibodies is also recommended.
Dermatophytosis	1. Skin scrapings 2. Nails 3. Hair	Perform direct KOH mounts and search for hyphal segments or typical hair invasion.
Histoplasmosis	1. Respiratory secretions 2. Lung tissue (biopsy) 3. Bone marrow 4. Blood 5. Urine	Direct examination not usually helpful.
Mycetoma	1. Skin 2. Purulent draining sinus tracts	Prepare direct mounts and search for sulfur granules or septate brown grains.
Nocardiosis*	1. Pulmonary secretions 2. Material from sinus tract 3. Blood (rarely positive) 4. Brain abscess	Search for sulfur granules in direct mounts. Demonstrate partially acid-fast filaments in direct acid-fast smears.
Sporotrichosis	1. Lymphocutaneous nodules 2. Respiratory secretions 3. Synovial fluid	Direct examination not helpful.
Zygomycosis (Phycomycosis)	1. Respiratory secretions 2. Nasal sinus material 3. Biopsy of ocular orbit 4. Subdural tissue	Biopsy of infected tissue may be necessary in that fungal elements often do not exfoliate into the secretions.

* Nocardiosis is not a fungal disease but rather a bacterial disease caused by the slowly growing *Nocardia sp.* usually recovered on fungal culture media.

Figures 1 through 16 are photomicrographs which illustrate various fungal forms that may be seen by microscopic examination of direct specimen mounts.

Figure 1 illustrates pseudohyphae and budding yeasts characteristic of *Candida* species. Budding yeasts, with or without pseudohyphae, may be seen in virtually any specimen; however, they are more commonly observed in sputum, oropharyngeal secretions, vaginal secretions, or in the exudates of inflammatory skin infections.

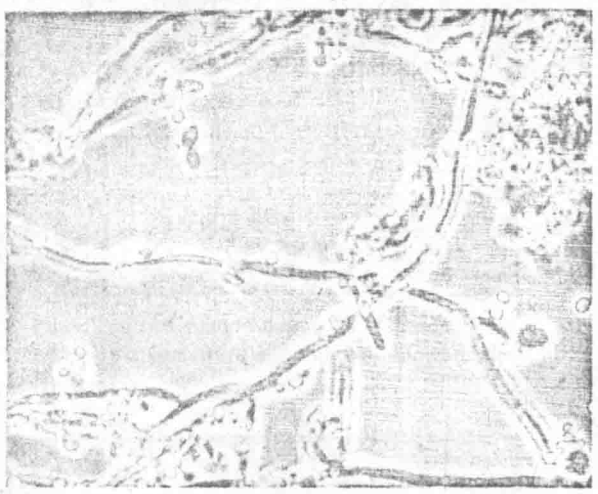

Fig. 1. Phase contrast photomicrograph of pseudohyphae and blastoconidia characteristic of *Candida* species. (high power)

Figure 2 depicts a dense, purulent exudate within which a large (10 to 15 μm) thick walled, broad based budding yeast form of *Blastomyces dermatitidis* is seen (arrow). These characteristic yeast forms may be seen in the sputum of patients with pulmonary blastomycosis or they can be demonstrated in direct mounts made from the exudates of active skin or subcutaneous lesions.

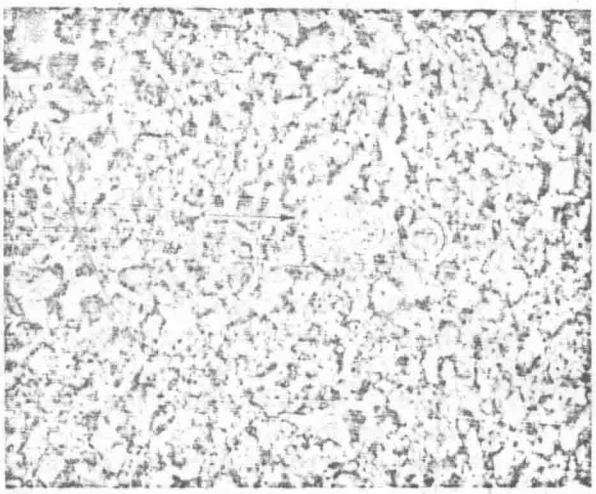

Fig. 2. Phase contrast photomicrograph of a dense purulent exudate including a thick walled, broad based budding yeast form of *Blastomyces dermatitidis* (arrow). (high power)

The large, thick walled spherules of *Coccidioides immitis*, as shown in Figure 3 (arrows), may be confused with the yeast forms of *Blastomyces dermatitidis*; however, they differ because they contain nonbudding endospores and do not form the broad based bud. *Coccidioides* spherules are most frequently observed in tissue sections of infected lung and less commonly detected in direct mounts of respiratory secretions or skin exudates.

Figure 4 is a phase contrast photomicrograph of spinal fluid sediment highlighting the characteristic encapsulated yeast form of *Cryptococcus neoformans* (arrow). In laboratories where phase contrast microscopes are not available, an India ink preparation can be made to aid in the detection of *Cryptococcal* yeast cells.

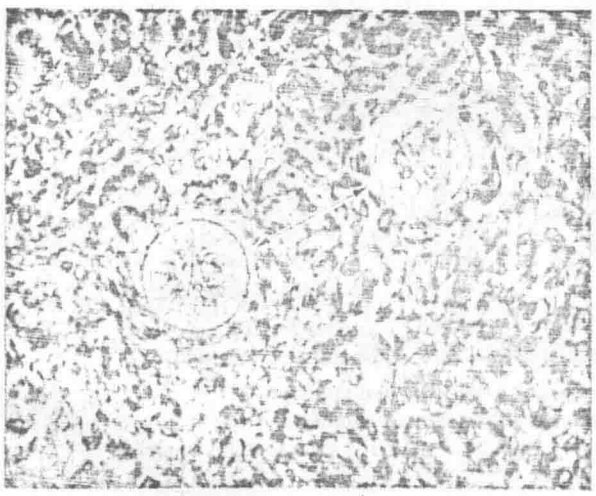

Fig. 3. Phase contrast photomicrograph of purulent exudate including two immature spherules of *Coccidioides immitis.* (high power)

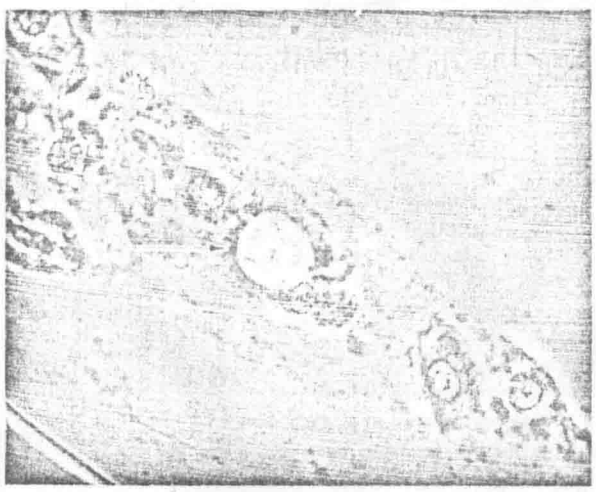

Fig. 4. Phase contrast photomicrograpn of direct mount of spinal fluid sediment including a yeast form of *Cryptococcus neoformans* surrounded by a thick capsule (arrow). (high power)

India ink or nigrosin (available from Harleco, a division of Hartmann-Ledded Co., Philadelphia, Pa.) preparations are made as follows:

1. Place a drop of Pelican® brand India Ink or a drop of nigrosin on a microscope slide (Appendix II).
2. Transfer a small portion (loopful) of the colony to be examined, or a portion of the spinal fluid sediment to the drop of India ink. Mix thoroughly.
3. Place a No. 2 cover slip (18 × 18 mm) over the drop and examine under the low and high power objectives of a microscope.
4. The presence of budding yeast cells surrounded by a thick capsule, easy to see because the capsular material is not penetrated by the ink particles, is diagnostic of *Cryptococcus neoformans* (Figure 5).

Superficial dermatophyte infections can often be diagnosed by demonstrating the characteristic delicate hyphal forms in KOH mounts of skin scales, nail scrapings, or infected hair. Figure 6 demonstrates the delicate hyphal forms against a background of quamous epithelial cells in a positive KOH skin mount. Figure 7 is also a photomicrograph of a positive KOH skin mount, illustrating a variation in which the hyphal forms are breaking up into distinct arthroconidia.

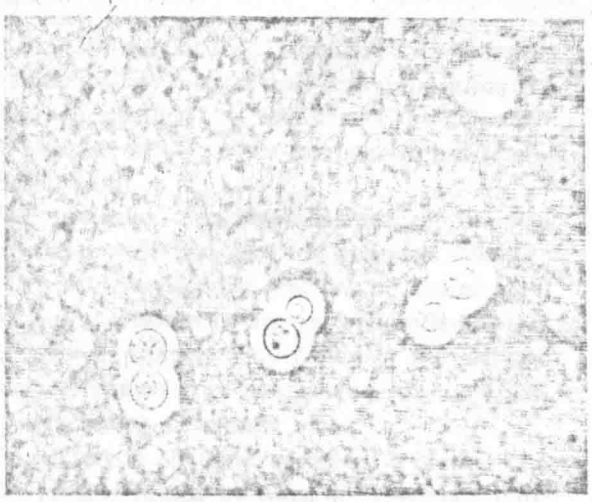

Fig. 5. Photomicrograph of positive India ink preparation showing encapsulated yeast forms of *Cryptococcus neoformans*. (high power)

KOH mounts are prepared as follows:

1. In a drop of 10% or 20% KOH on a microscope slide, add a small quantity of the material to be examined (skin scales, nail scrapings, hairs, etc.).
2. Gently heat the slide in a low flame of a Bunsen burner to facilitate clearing.
3. Place a No. 2 coverslip (18 × 18 mm) over the drop and let it stand at room temperature for approximately 30 minutes. If a greater delay before study of the slide is anticipated, the cover slip can be ringed with Vaseline®.
4. Examine microscopically for the presence of hyphae or other fungal structures (Figures 6 and 7).

Fig. 6. Phase contrast photomicrograph of a KOH mount of skin scales showing presence of delicate hyphal forms characteristic of one of the dermatophytes. (high power)

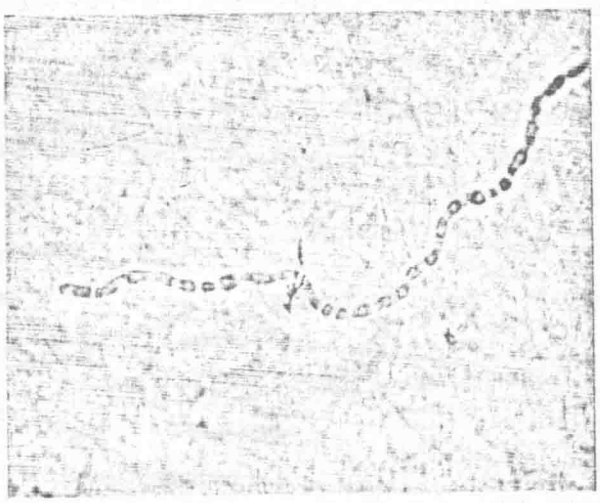

Fig. 7. Photomicrograph of positive KOH preparation of skin scales showing dermatophyte hyphae breaking into arthroconidia. (high power)

Figure 8 illustrates the accumulation of spores on the surface of an infected hair (ectothrix infection), while Figure 9 illustrates invasion of the hair shaft with delicate, arthroconidia-forming hyphae (endothrix infection).

The purulent material obtained from deep, draining cutaneous or subcutaneous ulcers or sinuses should be examined directly for the presence of "sulfur granules." Sulfur granules are aggregates of inflammatory cells, debris, proteinaceous material, and delicate branching filaments or true hyphae of several different fungi causing mycetoma.

Figure 10 is a photomicrograph of a portion of a sulfur granule caused by *Nocardia asteroides*, illustrating the central nidus of necrotic debris surrounded at the periphery by the dark-staining, branching filaments. Figure 11 is a photomicrograph of a direct sputum mount illustrating a cluster of branching filaments of *Nocardia asteroides*, trapped within a dense aggregate of neutrophils and mononuclear inflammatory cells. These filaments are best seen in Gram stains. *Nocardia* species can be differentiated from other members of the *Actinomycetes* because the filaments are partially acid fast, as discussed on page 98.

Figure 12 illustrates the hard appearing, septate bodies (arrow) that may be seen in direct mounts of skin exudations in patients with chromomycosis. These grains are characteristically brown or deep yellow in color.

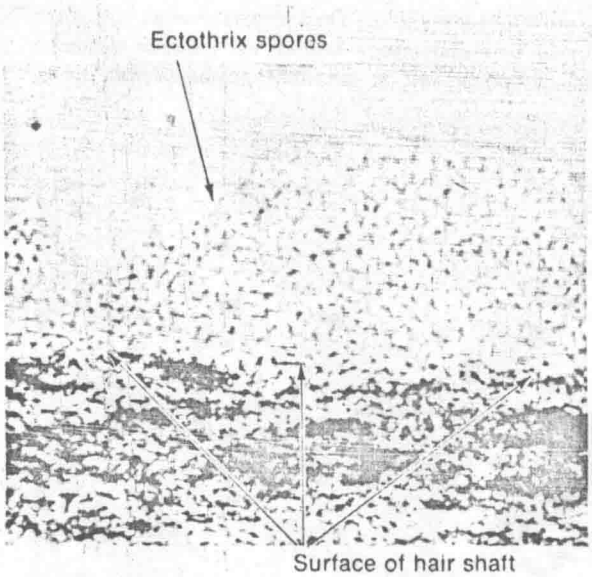

Fig. 8. Photomicrograph of surface of a hair shaft (arrows) showing mosaic accumulation of spores in an ectothrix hair infection. (high power)

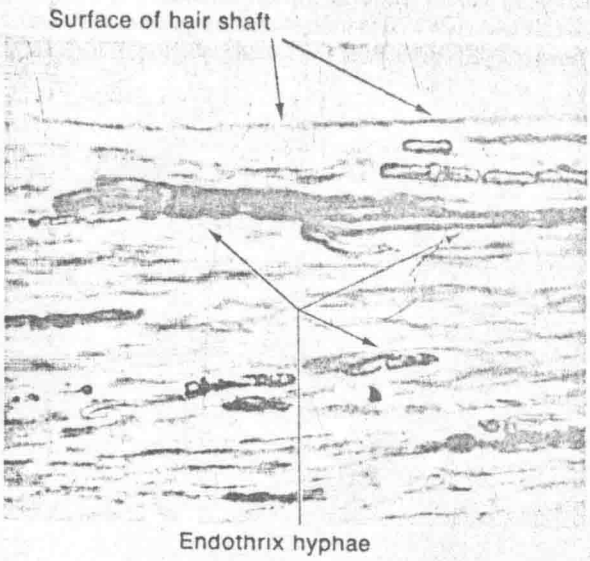

Fig. 9. Photomicrograph of inside of hair shaft (surface shown by arrows) showing arthroconidia-forming hyphae of a dermatophyte in an endothrix-type infection. (high power)

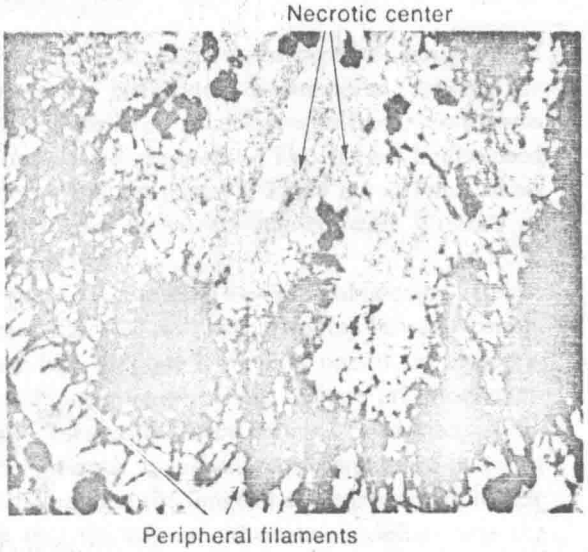

Fig. 10. Photomicrograph of a sulfur granule of *Nocardia asteroides* showing the central accumulation of necrotic debris and branching filaments at the periphery. (high power)

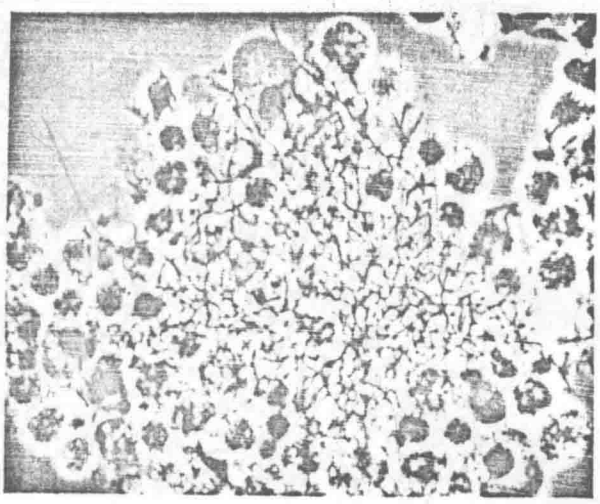

Fig. 11. Photomicrograph of direct mount of purulent material showing branching filaments of *Nocardia asteroides* trapped in polymorphonuclear leukocytes. (high power)

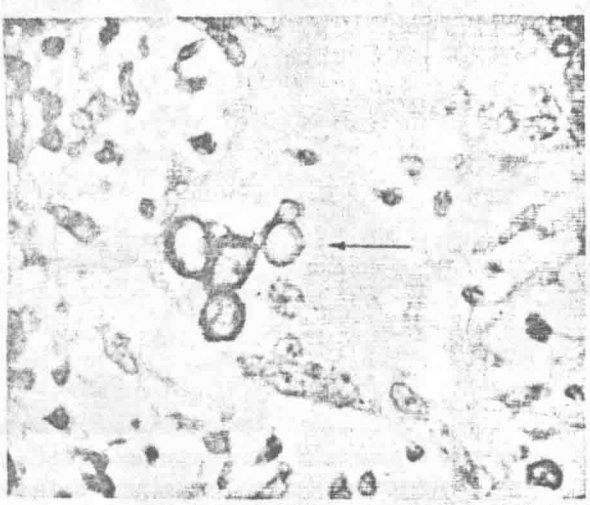

Fig. 12. Photomicrograph of exudate of cutaneous lesion of chromomycosis showing aggregate of sclerotic bodies (arrow). (high power)