THE PERIODIC-ACID-SCHIFF STAIN FOR THE DEMONSTRATION OF FUNGI IN ANIMAL TISSUE[1]

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The routine histopathologic stains used for the demonstration of fungi in animal tissue are inadequate. The fungi are differentiated poorly and sometimes not at all. This is a limitation in the diagnosis of the mycotic granulomas in which the organisms may be rare. A definitive fungus stain, therefore, is needed, particularly by the pathologist and mycologist. During an attempt to stain the capsules of certain fungi, brilliant staining with the periodic-acid-Schiff-reagent technique (Hotchkiss-McManus stain) was observed. A survey of many different types of mycotic tissue revealed the general usefulness of this reaction in mycologic histopathology. The present communication presents the method and briefly reviews the pertinent literature.

The chemistry of this and related reactions has been recently reviewed by DeLamater, Mescon, and Barger (1950). The various applications of the Schiff reagent have in common the requirement that the tissues must be hydrolyzed in such a manner as to release substances (usually aldehydes) that will recolorize and react with the Schiff reagent. The best known use of the Schiff reagent is in the Feulgen reaction, which is presumably specific for aldehydes released from desoxyribonucleic acid by acid hydrolysis of the tissue. Under optimal conditions the nuclei only stain.

The Bauer stain calls for prolonged hydrolysis of tissue in chromic acid (Lillie, 1947*a*). Under these conditions glycogen stains intensely red with the Schiff reagent. The Bauer reaction is an empirical procedure (Hotchkiss, 1948). Potassium permanganate or other oxidizing agents can be substituted for the chromic acid in the hydrolyzing process. How this may affect the chemical specificity of the reaction is not yet clear. It is not possible to predict from the chemical structure which substances will recolorize with the Schiff reagent.

The chemistry of the periodic-acid-Schiff technique is fairly well understood (Hotchkiss). The substances stained by this procedure belong to the general class of carbohydrates. These must contain the 1,2-glycol grouping in unsubstituted form or the equivalent structure in which OH groups are replaced by amino or alkylamino groups. Jeanloz (1950), however, has stated that he believes structure has no definite relation to a positive reaction. Periodic acid

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attacks some carbohydrates containing this grouping with the conversion of 1,2 glycols to 1,2 aldehydes. Substances such as glycogen, hyaluronic acid, gastric mucin, chitin, and glycerol, were found by Hotchkiss to give strong reactions with the Schiff reagent following periodic acid hydrolysis. Cellulose, glucose, maltose, sucrose, etc., give weaker reactions, whereas cellobiose, α-D-glucopyranoside, etc., give no reaction. Hotchkiss mentions, incidentally, that fungi and plant tissue are strongly stained.

Using acidified sodium periodate as a hydrolyzing agent, Lillie (1947*a*) recorded that the capsule (cell wall?) of certain yeasts and molds stain purplish red. This investigator conducted extensive studies with the Bauer stain on various pathogenic fungi in tissue and concluded that this technique has definite advantages over the Feulgen, Gram, and Gram-Weigert procedures (Lillie, 1947*b*). For purposes of diagnosis the organisms are stained various shades of red.

DeLamater's studies on fungi with the aldehyde-mordanted basic fuchsin stain and the Feulgen reaction (routine and modified) have been directed primarily toward revealing intranuclear components (1948*a*,*b*, 1949). The purpose of the present study is to present a technique by which the presence or absence of fungi in granulomatous tissue can be decisively decided.

METHODS

The procedure used in these studies is that described by McManus (1947, 1948). The fungi have been studied both in tissue and in culture. The tissues have been routinely fixed in 4 per cent formaldehyde or Rossman's fixative. Both were satisfactory and undoubtedly many other routine fixatives are adequate. Most of the tissues were taken from animals experimentally infected for this purpose. The procedure is as follows:

1. Fix.
2. Dehydrate, embed, and section.
3. Deparaffinize sections and dehydrate through absolute alcohol.
4. Wash in distilled water.
5. Immerse in 1 per cent periodic acid for 5 minutes.
6. Wash in running tap water for 15 minutes.
7. Stain in Schiff reagent for 10 to 15 minutes.
8. Transfer directly to two changes of either of the following solutions for 5 minutes each:

(1) 10 per cent potassium metabisulfite	5 ml
1 N hydrochloric acid	5 ml
Distilled water	100 ml
(2) Thionyl chloride	5 ml
Distilled water	100 ml

9. Wash in running tap water for 10 minutes.
10. Counterstain with light green.
11. Dehydrate, clear, and mount.

The Schiff reagent is prepared by either of the following methods:

1. Dissolve 0.5 g of basic fuchsin by pouring over it 100 ml of boiling distilled water. Cool to 50 C. Filter and add 10 ml of 1 N hydrochloric acid and 0.5 g

of anhydrous potassium metabisulfite to the filtrate. Allow the solution to stand in the dark overnight. The solution should become colorless or pale straw color. If not completely decolorized, add 0.25 to 0.50 g of charcoal, shake thoroughly, and filter immediately. This solution will keep for several weeks in a tightly stoppered bottle.

2. Dissolve 0.5 g of basic fuchsin by pouring over it 100 ml of boiling distilled water. Cool to 50 C. Filter and add slowly 5 to 8 ml of thionyl chloride. Clear with charcoal, as above (Barger and DeLamater, 1948).

The Bauer reaction has also been investigated in this study for purposes of comparison. The Hotchkiss-McManus stain has proved so superior for diagnostic purposes that the Bauer technique need not be considered in any detail. After chromic acid hydrolysis, the Schiff reagent is seen to be recolorized by constituents in the cell wall. The color varies from pink to purple red. It is less intense than that which occurs following periodic acid hydrolysis. With the Bauer procedure, *Coccidioides immitis*, *Blastomyces dermatitidis*, and *Candida albicans* in tissue stained moderately well. The results with *Histoplasma capsulatum*, *Sporotrichum schenckii*, and *Cryptococcus* (*Torula*) *neoformans* were poor. The capsule of the latter organism failed to stain, whereas it was beautifully demonstrated when stained by the Hotchkiss-McManus technique.

RESULTS

The *fungi* that cause human infection have been arbitrarily divided into (1) the superficial, which cause ringworm diseases; and (2) the systemic, which cause generalized and often fatal infections. Organisms of both groups stain equally well with the Hotchkiss-McManus procedure.

The cell walls of the fungi, unlike those of animal cells, are rigid and fairly thick. They are composed principally of chitin and chitin-cellulose mixtures. Both of these substances have been demonstrated by Hotchkiss to recolorize following periodic acid hydrolysis. In some instances components within the cytoplasm and nucleus have been observed to stain, but in every case it was the cell walls that stained predominantly. When periodic acid hydrolysis is omitted, the fungi do not stain.

In normal skin only a small band of Schiff-positive material is seen around the sebaceous glands, the sweat glands, the blood vessels, and at the dermo-epidermal junction (Stoughton and Well, 1950). The collagen of the cutis stains a weak pink. Liver glycogen stains more intensely. All of the fungi, however, stand out brilliantly with marked contrast in all tissues in which they have been found.

Sporotrichosis. In the tissue of experimental animals the organisms are polymorphic, appearing as ovoid, pyriform, fusiform, rod-shaped, and club-shaped forms (figure 2). Some of the forms are unfamiliar to us and have not been visualized and described by techniques previously used. This will be the subject of a later study. Both the wall and the cytoplasm stain, but nuclear details by present methods are not apparent. In the chorioallantoic membrane of the chick embryo the organisms are intracellular; by other methods they appear

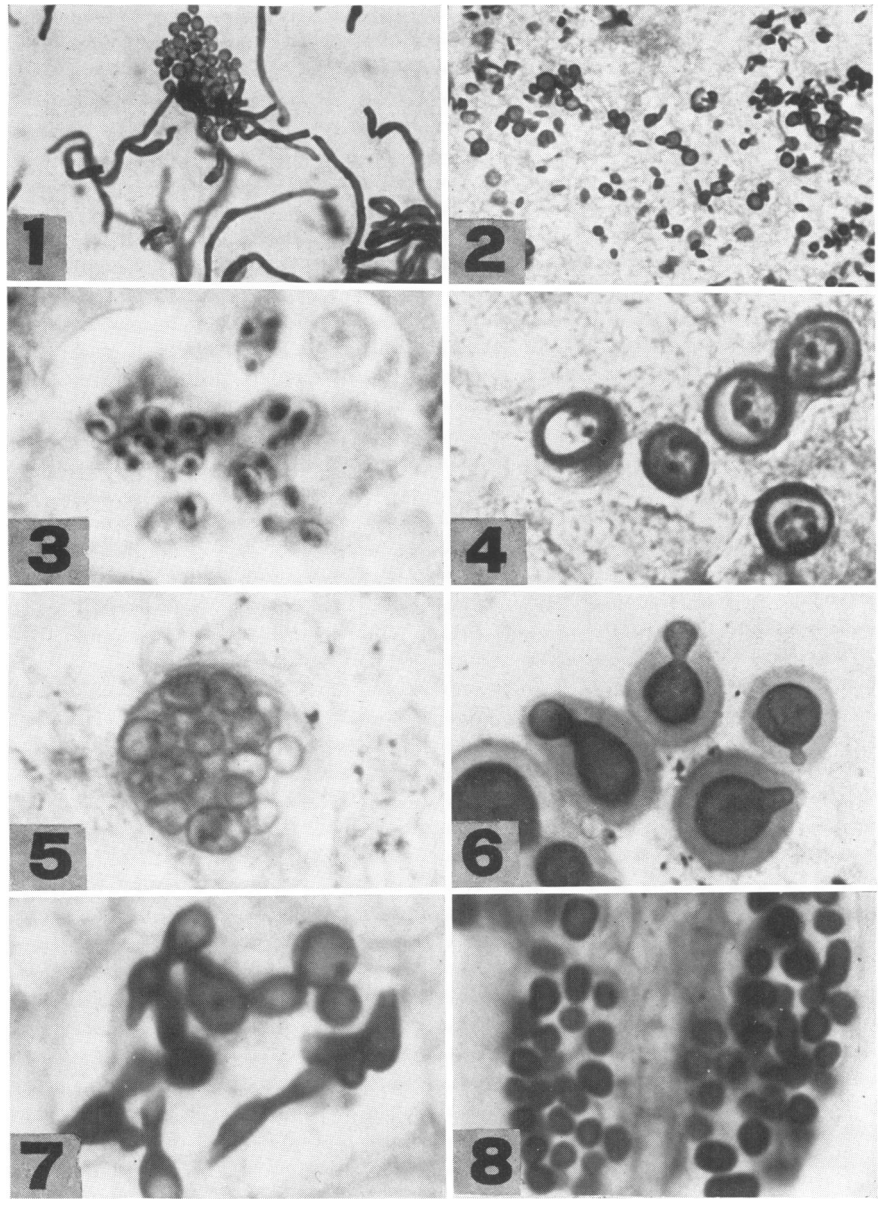

Figure 1. *Malassezia furfur* in skin scrapings from a case of tinea versicolor.

Figure 2. *Sporotrichum schenckii* in mouse tissue. There is great variation in the shapes and sizes of the organisms.

Figure 3. *Histoplasma capsulatum* in mouse tissue. The organisms are within macrophages.

Figure 4. *Blastomyces dermatitidis* in mouse tissue. Note the thick wall and the internal differentiation.

Figure 5. A *Coccidioides immitis* spherule in mouse tissue. The endospores are clearly outlined within the spherule.

Figure 6. *Cryptococcus hominis* in a film from infected chick embryo liver. Note the striking manner in which the capsules surrounding the budding organisms are stained.

Figure 7. *Candida albicans* in rabbit kidney. Filaments as well as budding forms may be seen.

Figure 8. Human hair infected with *Microsporum audouini*. Biopsy specimen. The ectothrix spores surrounding the hair are densely stained.

to be capsulated. Brilliant red staining is obtained with the Hotchkiss-McManus technique, but the "capsule" is not colored.

Torulosis. In both human and animal tissue the cell walls of the yeast stain intensely red and the large capsule characteristic of the species is a lighter pink (figure 6). The cytoplasm is also stained. The capsule, which has been demonstrated to contain hyaluronic acid, was therefore expected to stain. The method has proved especially useful for the demonstration of organisms in films of the sediment of cerebrospinal fluid in cases of torula meningitis.

Histoplasmosis. The color produced by the routine procedure described is somewhat less intense than that observed with the other fungi. The method, however, is as useful as it is in other mycoses. Macrophages containing the organisms, although occasionally rare, are readily visualized. Even under the low power of the microscope one's attention is directed to the presence of organisms, and the usual oil immersion examination for these small organisms is greatly facilitated when the Hotchkiss-McManus stain is employed. As shown by other methods, the fungus is a minute intracellular budding yeast, the cells of which are 2 by 3 microns. The organism has a well-defined purplish ring around the periphery that is separated from the central red mass by a clear zone (figure 3).

Coccidioidomycosis. The cell walls of the spherules and the contained endospores stain a brilliant red. There is moderate staining of the cytoplasm of the endospores (figure 5). In animal tissue there is great variation in the size of the spherules. Forms have been seen that confirm a previous observation of budding (DeLamater, 1946). This will be reported in detail elsewhere.

Blastomycosis. The walls of the budding cell are intensely stained and there is some differentiation of nuclear structures as well. The thick, double-walled, round, budding cells described by other methods are sharply delineated (figure 4).

Moniliasis. The organisms have been visualized for present purposes in the experimentally infected chick embryo and in the rabbit kidney. They stain intensely red. In the rabbit kidney filaments as well as budding cells are strikingly illustrated (figure 7).

Chromoblastomycosis. The organisms seen in chick embryo tissue and in mice are stained red. Because of this it has been possible to detect them in sections in which hematoxylin and eosin staining revealed them but poorly.

Actinomycetes. In films made from *Actinomyces bovis* and *Nocardia asteroides* the cytoplasm of the organisms was irregularly stained violet to magenta. In chick embryo tissue and mice *Nocardia asteroides* had a similar appearance. The actinomycetes are well stained by the gram technique, and it is doubtful if the Hotchkiss-McManus stain will prove superior.

Superficial fungi. Biopsy specimens from the scalps of children with tinea capitis (caused by *Microsporum audouini*) have been studied. The ectothrix spores around the hair shafts are stained a brilliant red (figure 8). The intrapiliary hyphae are less intensely stained, but are clearly visible. These sections are particularly striking and the study of such fungi in tissue is greatly facilitated.

Biopsy specimens of guinea pig dermatophytosis due to *Trichophyton mentagrophytes* have also been examined. The mycelia and spores stain bright red. The hyphae in skin scrapings from cases of ringworm due to *Microsporum audouini* and *Trichophyton mentagrophytes* are brilliantly stained. No fixation is required. The slide is first coated with egg albumen to help fix the scraping to the glass. The results are far superior to those obtained with the potassium hydroxide preparation usually employed for the examination of fungi in scales. Scrapings from patients with tinea versicolor were studied without fixation (figure 1). The hyphae and grapelike clusters characteristic of this organism (*Malassezia furfur*) were brilliantly stained. These preparations are extraordinarily exquisite for detail and intensity of color. Further studies on this organism will be reported at a later date.

Fungi in culture. When ordinary films are made from cultural material, fixation is unnecessary. All the fungi we have studied are intensely stained. Cytologic details are obscured because of the deepness of the colorization of the wall. This method should lend itself well to the preparation of permanent slides for teaching and photographic purposes.

In an effort to evaluate the Hotchkiss-McManus stain as a diagnostic method preparations of cultured fungus in body secretions have been studied. Preparations made by suspending cultured organisms in sputum have proved satisfactory. The contrast in this latter menstruum is reduced by the presence of mucopolysaccharides in sputum that take up the stain. The organisms, however, stain more intensely than the background material and can be readily differentiated from debris and cellular elements. These preparations are superior to those afforded by gram staining.

DISCUSSION

The practical advantages of the Hotchkiss-McManus stain as a means for detecting fungi in tissue have been described and demonstrated. The method is superior to any heretofore employed. It is currently in use as a routine and research procedure in our laboratories and has been found to be unparalled as a diagnostic procedure. From a research point of view, it affords an opportunity to visualize and study the pathologic phases of the pathogenic fungi with a clarity that heretofore has not been possible. Detailed studies of individual organisms are in progress.

It seems likely that the procedure can be refined to suit the special study of individual organisms. Such experiments are also in progress. As presently used and described, the method produces an intense staining of the wall of the fungus. The marked contrast between the fungus and the surrounding tissue depends upon this selective staining of the fungus wall. The tissue is counterstained to aid the contrast. The location of fungi in tissue may be readily determined by inspection of the section under the low-power microscope.

SUMMARY

Fungi can be differentially stained in tissue by means of the Hotchkiss-McManus stain. The diagnostic value of the procedure is emphasized. This tech-

nique also permits cellular forms heretofore unrecognized in tissue to be made apparent. Study of the pathogenic fungi is greatly facilitated by this procedure.

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