A PRACTICAL CLASSIFICATION OF THE MONILIAS

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Received for publication March 2, 1937[1]

The bacteriologist is frequently confronted with the problem of identifying a yeastlike fungus isolated from some part of the human body. Mycelia-producing yeastlike organisms have long been known to play an important rôle in disorders such as thrush, but only recently has etiologic significance been given to these fungi in certain dermatoses and pulmonary diseases. These fungi have also been found frequently in association with certain debilitating diseases such as carcinoma and tuberculosis and have been isolated from individuals with no demonstrable pathologic lesions. It is obvious that little progress will be made in the understanding of the relation of these organisms to disease processes until there is established a practical method for their identification and classification. Since the bacteriologist isolates these fungi from the patient the methods of identification should be adapted to the resources of the bacteriologic laboratory, and the differential criteria upon which identification is based should be easily recognizable.

Benham (1931), Shrewsbury (1934) and Langeron and Talice (1932) have discussed the complicated story of the attempts to place these organisms in their proper botanical position. Berkhout (1923), Ota (1924 a, b), Redaelli and Ciferri (1929), Langeron and Talice (1932) and others have carried out laborious and detailed morphologic studies of this group, but no two authors have agreed on the proper taxonomic position of the fungi con-

[1] Presented before the Society of American Bacteriologists, Indianapolis, Ind., December, 1936.

cerned. It is recognized by all medical mycologists that it is incorrect to use the term *Monilia* as a generic name for these yeastlike organisms because of the prior use of this term as a designation for a wholly unrelated group of fungi. Shrewsbury (1934) has called attention to this fact and proposed that the precise taxonomic position be decided at a meeting of the International Congress of Mycologists. We, however, agree with Benham (1931) that the name *Monilia*, established by usage in the medical literature, should be retained as long as proof of a sexual phase completing the life cycle of the fungus remains undiscovered.

Attempts to classify such a primitive group of organisms as the Monilias on a purely morphologic basis are greatly handicapped by the fact that reproduction occurs only by budding and the formation of mycelial threads. On the other hand a classification based purely on biologic reactions such as the fermentation of various and rare carbohydrates, as advised by Castellani (1919), is not practical. By this method Castellani (1916) isolated seven different species of *Monilia* from eleven cases of thrush in one locality, London. Such a finding suggests that either the method of classification is inaccurate or that there is little hope of correlating the clinical picture with the species of fungus isolated from the lesion. Benham's (1931) careful study of the "medical" *Monilias* represents a logical and careful approach to the problem of classification and she was able to separate these fungi into definite species by correlating macroscopic and microscopic morphology, fermentation reactions and serologic differences. Stovall and Bubolz (1932) compared more than two hundred strains isolated from sputum and recognized three different types by their characteristic colonies on malt agar, their carbohydrate fermentations and their action on milk. The dependence of the carbohydrate fermentation reactions upon the conditions under which the tests are carried out is well illustrated by minor differences in reactions obtained by different observers with fungi which are obviously members of the same species.

In attempting to classify 124 strains of yeastlike fungi isolated

from patients in Duke Hospital we frequently encountered organisms which we were unable to classify by the methods described by Stovall or Benham. A technique was developed, however, which was satisfactory in our hands for classifying the yeastlike fungi isolated from the vaginal tract (Jones and Martin, 1937) and the same procedures were found applicable to the identification of organisms found in other parts of the body. In addition to the 124 strains isolated by us, 29 strains of *Monilia* from various parts of this country and England were compared by our method with 19 representative strains obtained from Benham (1931), Stovall and Bubolz (1932), Langeron and Talice (1932) and Reed and Johnstone (1935).

The method to be described has the advantages of using only simple, easily prepared media and the criteria upon which separation is based are sufficiently distinctive to make identification of these organisms reasonably certain for the bacteriologist without previous mycologic training.

SOURCES OF CULTURES

The unknown strains were obtained from the following sources:

	Isolated at Duke Hospital	*Isolated elsewhere*[2]	*Total*
Sputum	47	20	67
Vagina	59	0	59
Feces	8	0	8
Skin	3	1	4
Lungs (post-mortem)	3	1	4
Liver (post-mortem)	1	0	1
Bile	0	1	1
Throat	0	2	2
Tongue	1	1	2
Pleural fluid	0	1	1
Dog feces	2	0	2
Lymph node	0	1	1
Source unknown	0	1	1
	124	29	153

[2] These strains were obtained through the courtesy of various physicians in this country. Six strains were sent by Dr. Haler of London. The geographical distribution is rather wide: North Carolina 8 strains, New York 4 strains, Massachusetts 3 strains, 2 strains from Arizona, Alabama and Virginia, and 1 strain from New Jersey and Colorado, respectively.

The following 19 "known strains" were studied comparatively by the same methods used for the identification of the cultures listed above:

From Dr. Benham
- 87 *Monilia albicans* Benham's no. 1755
- 103 *Monilia albicans* Benham's no. 1773
- 69 *Monilia Krusei* Benham's no. 1763
- 88 *Monilia parapsilosis* Benham's no. 1735
- 114 *Monilia candida* Benham's no. 1956, ATCC no. 2113

From Drs. Stovall and Bubolz
- 91 *Monilia* type I
- 92 *Monilia* type II
- 93 *Monilia* type III

From Drs. Langeron and Talice
- 46 *Mycotorula psilosis* Their no. 340
- 44 *Mycotoruloides ovalis* Their no. 296
- C-70 *Geotrichoides Krusei* Their no. 683
- 49 *Candida tropicalis* Their no. 255
- C-76 *Candida parapsilosis* Their no. 341
- 171 *Blastodendrion intermedium* Their no. 493
- 47 *Mycocandida mortifera* Their no. 516

From Drs. Reed and Johnstone
- 238 *Monilia* type II
- 239 *Monilia* type III
- 240 *Monilia* type IV
- 242 *Monilia* type VI

METHODS OF IDENTIFICATION

Although the methods proposed in this paper may be found applicable to the identification of other types of yeastlike fungi, only data pertaining to the genus *Monilia* are presented in this paper. We are using the generic name *Monilia* as it has been generally interpreted in the medical literature; i.e., as including yeastlike fungi which reproduce by budding, by the formation of mycelial threads under certain conditions, and which never form asci or aerial hyphae. The 169 identified strains of *Monilia* were divided into 6 species, including the 4 species described by Benham (1931).

No single procedure can be relied upon for identification of all species and all the steps outlined should be performed. Since it has been found that most variations can be accounted for by

slight differences in composition and pH of the various substrata, the methods of making the necessary media are included in the appendix.

An outline of the procedures necessary for identification is as follows:

(1) The fungus is isolated on Sabouraud's glucose agar slant, transplanted to (2) Sabouraud's glucose acid broth and incubated at 37°C. for 48 hours. After noting the type of surface growth, the tube is then shaken to suspend the sedimented organisms and (3) streaked on a beef-extract blood agar plate of pH 7.4, which is incubated at 37°C. for 10 days; the type of colony is noted, and (4) a well-isolated colony is picked and transplanted to a Sabouraud's glucose agar slant. This is incubated at room temperature or 37°C. for 24 or 48 hours. Some of the growth is transplanted to a carrot plug which is kept at room temperature and subsequently examined for asci. The rest of the material is streaked (5) on the surface of a beef-extract agar slant pH 7.4. The growth is subcultured on this medium for 2 or 3 generations and (6) a loopful is streaked on a corn-meal agar slide culture which is incubated at room temperature in a moist sterile chamber for several days. The slide is then fixed, stained and examined microscopically for details of mycelial growth. (7) Four beef-extract broth tubes, containing 1 per cent of glucose, sucrose, lactose and maltose respectively, are inoculated with a pipette containing a saline suspension of the last transplant of the fungus on the beef extract agar slant.

GROWTH ON SABOURAUD'S GLUCOSE AGAR AND SABOURAUD'S GLUCOSE BROTH

All of our strains of *Monilia* grew luxuriantly on Sabouraud's glucose agar and this medium has proved entirely satisfactory for the isolation of these fungi from all parts of the body. The organism develops quickly at room temperature or 37°C. and in all species except *M. Krusei* the colonies are raised, white and appear moist and "creamy." Microscopic examination of the unstained organisms shows round or oval yeastlike budding cells with an occasional short strand of mycelium. Langeron and

Talice (1932) have elaborated on the size and shape of these cells (blastospores) and the position of the buds. In our experience the irregularities of these features are so great that no diagnostic significance can be attached to them, with the possible exception of *M. Krusei*, in which the blastospores are long and narrow. Direct microscopic examination, however, should not be omitted since a young colony of *Geotrichum* may resemble a *Monilia*, but can be differentiated easily by the characteristic oblong square-ended spores of the former fungus.

Growth in glucose broth, especially in hanging-drop preparations, has been frequently used to study the mycelial development of the various species. In our scheme of identification, the Sabouraud's glucose acid broth (see appendix) is used only for test tube culture and serves three important purposes: (1) Purification of the culture in instances where bacteria occur as contaminants in the Sabouraud's isolation slants. The acidity and high carbohydrate content of this medium apparently inhibit bacterial growth and favor the development of the fungus. Bacteria, if present, are detected subsequently by plating the broth culture on blood agar. (2) Certain species of *Monilia* present a very characteristic type of surface growth in this medium and (3) this broth culture must be the inoculum of the blood agar plate if characteristic colonies are to be obtained on this medium.

All broth tubes are incubated for 48 hours at 37°C. and examined for surface growth (plate 1). Irregularities may occur if the tubes are incubated for a longer time and the procedure loses diagnostic value. In cultures of *M. Krusei* a dry-appearing surface film is formed which extends up the sides of the tube to a distance of 5 to 6 mm. above the surface of the medium. A similar surface growth occurs with *M. candida* but the film is broken up by numerous small bubbles of gas and the extension of the growth along the sides of the tube is less (2 to 3 mm.). No surface growth occurs in the other 4 species within 48 hours. Characteristic growth may or may not be obtained if bacterial contaminants are present and the procedure should be repeated with a pure culture if bacteria are found on the blood agar plate inoculated from the broth.

COLONY FORMATION ON BLOOD AGAR

Benham (1931) and Langeron and Talice (1932) have attached diagnostic significance to giant colony characteristics. We attempted a differentiation of species by noting the gross characteristics of 50 strains grown on Sabouraud's honey agar medium for 1 month. Although many of the strains showed differences which might be regarded as characteristic by one very experienced in this type of study, irregularities occurred so frequently that this procedure has been omitted from our scheme of classification.

In the early experiments blood agar plates were streaked from the Sabouraud's broth culture to detect bacterial contamination. The different fungus species, however, grew so differently that the type of colony produced has been used subsequently as a valuable procedure in species differentiation. The plates are streaked from the Sabouraud's broth culture after shaking the tube to resuspend the sedimented organisms. If the plates are streaked from Sabouraud's glucose agar or beef extract agar, colony formation may or may not be characteristic and we believe that the glucose present in the broth inoculum may serve to stimulate growth. All plates are incubated at 37°C. for 10 days. The following descriptions of these colonies present only those features which have proved to be the most constant and of most value in species diagnosis (plate 2).

Monilia albicans. Well-isolated colonies are approximately 1.5 mm. in diameter, are more or less circular in outline and have a smooth border. The surface is slightly convex and the whole colony is dull grayish-white in color.

Monilia parapsilosis. Well-isolated colonies are smaller than those of *M. albicans* (0.6 to 0.8 mm. in diameter), the outline is smooth and circular and the surface is quite convex. The color is a definite pearly white.

Monilia candida. The fungus on blood agar produces large colonies which may attain sizes of 2 mm. and over. The colonies are circular but the most characteristic feature is the mycelial fringe which surrounds the entire colony. These mycelia grow beneath the surface of the medium and form a zone approxi-

mately 1 mm. in width. The color of the colony is grayish-white but is less dull in appearance than the colonies of *M. albicans.*

Monilia Krusei. The growth of these organisms is characterized by the great variations in size and shape which occur in colonies which are equally well isolated. The colonies vary from 0.2 to 1.0 mm. in size and the borders may be round and smooth or irregular. The surface may be smooth, ridged, or nodular and heaped up in the center or flat.

Monilia mortifera. Only four strains were studied but all of these produced colonies approximately 0.5 mm. in diameter which in general resembled the smaller colonies of *M. Krusei.*

Monilia stellatoidea. This fungus produces large colonies which are very characteristic. The colony is composed of a small elevated central zone from which thick tapering "arms" radiate in an irregular manner. These projections are made up of budding cells on the surface of the medium. Between the surface projections mycelial threads can be seen extending beneath the surface of the medium. The colony is readily distinguished from that of *M. candida* by the thick radiating "arms" on the surface which give the whole colony the appearance of a "star in the sky."

Although the colony appearance on blood agar is a distinguishing characteristic of most species, too much importance should not be attached to this feature alone until some experience is acquired in the identification of numerous strains. A single colony on this medium is transplanted to a Sabouraud's glucose agar slant preparatory to subculture on the glucose-free medium. This transplant is necessary to obtain sufficient material to start growth on the beef-extract agar. Carrot plugs (see appendix) are inoculated from the pure culture on the Sabouraud's slant, incubated for several weeks at room temperature and are examined for the presence or absence of large spore-containing cells (asci). Asci could not be found in any of the 172 strains although the cultures were kept for 60 days and examined repeatedly.

FERMENTATION REACTIONS

Numerous investigators have attempted to classify these organisms by determining the ability of the fungus to ferment certain

carbohydrates with or without the formation of gas. Stovall and Bubolz (1932) are the only workers who have been able to obtain consistent results with a large number of strains. Cultures of *M. albicans*, *Monilia richmondi*, *Monilia psilosis*, *Monilia Pinoyi*, *Monilia metalondinensis*, *Monilia* (Zillig) and *Monilia pseudotropicalis* obtained from the American Type Collection, showed carbohydrate fermentation identical with their type II (*M. albicans*) when tested by their method although these fungi previously had been classified as to separate species because of their differences in carbohydrate reactions. These authors used brom-thymol-blue as an indicator and read results after incubation at 37°C. for 7 days. Other workers using apparently the same technique have not been as successful in identifying these fungi. Reed and Johnstone (1935) cultured 19 strains of *Monilia* from the feces of patients with various types of gastrointestinal disturbances and attempted to classify them by the method of Stovall and Bubolz (1932) (fermentation of maltose and sucrose, clotting of milk and mycelial growth on malt agar). Six strains were identified as type II (*M. albicans*) and 2 strains as type III (*M. candida*) but the other 11 strains fell into various groups which were designated as types IV, V, and VI. We obtained cultures from these authors and subjected them to the procedures outlined in this paper. Types IV and VI were both identified as *Monilia parapsilosis* (Type I) and type V was identified as a *Cryptococcus*. Hopkins and Hesseltine (1936a) made the interesting observation that variations in carbohydrate fermentation could be obtained by three different observers inoculating the same batch of medium with the same strain of fungus. For example, one observer reported acid and gas in levulose, another obtained acid only and the third observed no acid or gas in this sugar. These authors showed that some of these differences could be accounted for by variations in the size of the inoculum. Benham (1931) also observed that different strains of the same species (*M. albicans*) gave variable results when tested with certain carbohydrates, and Wachiowak and his co-workers (1934) reported that the same strain will vary from time to time, not only losing the power to ferment certain carbo-

hydrates but also apparently being able to acquire this ability. Langeron and Talice (1932) considered the fermentation reactions to be too inconsistent for use as a method of identification. Lamb and Lamb (1935) proposed a method of determining the carbohydrate reactions by testing for the presence or absence of the carbohydrate after incubation for 1 month at 37°C. The results obtained by our method agree in every instance with those of Lamb and Lamb except that sucrose and galactose are not fermented by *M. parapsilosis* when the broth tubes are sealed with vaseline.

Our first observations on the carbohydrate reactions were as irregular as those obtained by most of the previous observers, variations being especially marked with maltose, sucrose, and galactose. The techniques described by Stovall and Bubolz (1932) and Benham (1931) were followed as closely as possible but neither gave constant results in our hands when tested upon a large number of strains. Consistent reactions were finally obtained by developing the technique described by Jones and Martin (1937). Of 169 strains classified into 6 species and tested with 9 carbohydrates, only 3 strains presented exceptions. One of the 96 strains of *M. albicans* failed to produce gas in dextrin, 1 strain of *M. candida* did not form gas in galactose and 1 strain of *M. Krusei* did not ferment glucose or levulose. If only the four carbohydrates necessary for diagnosis (glucose, sucrose, lactose and maltose) be considered, there is only 1 exception among the 169 identified strains.

The following points in technique should be emphasized:

1. The organism must be a subculture of the "pure" strain picked from the streaked blood agar plate.

2. The inoculum must be taken from the second or third subculture on the glucose-free medium.

3. The organisms should be suspended in 1.0 to 2.0 cc. sterile saline and pipetted into the broth tubes. This procedure is made necessary by the small amount of growth on the beef extract agar.

4. The broth tubes must be sealed with vaseline. *M. parapsilosis* regularly produces acid in sucrose and galactose if the tubes

are incubated aerobically but does not form acid from either of these carbohydrates when sealed. The vaseline seal also prevents fading of the indicator after becoming acid. Reversal of color or fading may occur, however, if the gas pressure in the tube forces the seal high enough to displace the cotton plug, allowing air to come in contact with the culture.

5. The beef-extract broth should be accurately titrated to pH 7.2 before adding the indicator and autoclaving (see appendix). An increase of 0.2 pH will inhibit the fermentation of sucrose and galactose by *M. albicans* and neither acid nor gas will be produced in glucose or levulose by *M. parapsilosis.*

6. Breakdown of the carbohydrates should be avoided, preferably by sterilizing the 20-per cent carbohydrate solution by filtration.

7. The carbohydrate broths should be used within 2 weeks after the carbohydrate has been added to avoid slight changes in pH on standing.

All data presented in table 1 are readings made after 10 days incubation at 37°C.

The fermentation reactions of *M. Krusei* and *M. parapsilosis* are identical and these species cannot be differentiated by this procedure, but the flat dry growth on Sabouraud's agar, the surface growth on Sabouraud's glucose broth and the irregular poor growth on blood agar easily distinguish *M. Krusei.* Four strains of *M. parapsilosis* produced only a small bubble of gas and 1 strain of *M. Krusei* failed to ferment any carbohydrate whatsoever.

All of the vaginal strains have been tested for their ability to clot milk and the results agreed entirely with those obtained by Stovall and Bubolz (1932), the new species *M. stellatoidea* being similar to *M. albicans* in this respect. The test, although quite satisfactory, is not necessary for diagnosis and was omitted from the study of the other strains.

MYCELIAL GROWTH

Langeron and Talice (1932) classify these mycelia-producing yeastlike fungi by criteria based chiefly on differences in the

TABLE 1

NUMBER OF STRAINS	CULTURES STUDIED	DEXTROSE	SACCHAROSE	LACTOSE	MALTOSE	MANNITE	LEVULOSE	GALACTOSE	DEXTRIN	INULIN
	Monilia albicans (96 strains)									
2	*Monilia albicans* (Benham)	AG	A	—	AG	—	AG	A	AG	—
1	*Monilia* type II (Stovall)	AG	A	—	AG	—	AG	A	AG	—
1	*Monilia* type II (Reed and Johnstone)	AG	A	—	AG	—	AG	A	AG	—
1	*Mycotorula psilosis* (Lang. et Talice)	AG	A	—	AG	—	AG	A	AG	—
1	*Mycotoruloides ovalis* (Lang. et Talice)	AG	A	—	AG	—	AG	A	AG	—
1	*Monilia pinoyi* (Castellani)	AG	A	—	AG	—	AG	A	AG	—
58	Strains isolated from sputum	AG	A	—	AG	—	AG	A	AG	—
19	Strains isolated from the vagina	AG	A	—	AG	—	AG	A	AG	—
2	Strains isolated from the lungs (P.M.)	AG	A	—	AG	—	AG	A*	AG	—
2	Strains isolated from human feces	AG	A	—	AG	—	AG	A	AG	—
2	Strains isolated from dog feces	AG	A	—	AG	—	AG	A	AG	—
2	Strains isolated from the skin	AG	A	—	AG	—	AG	A	AG	—
2	Strains isolated from the tongue	AG	A	—	AG	—	AG	A	AG	—
1	Strain isolated from bile	AG	A	—	AG	—	AG	A	AG	—
1	Strain isolated from a lymph gland	AG	A	—	AG	—	AG	A	AG	—
	Monilia parapsilosis (14 strains)									
1	*Monilia parapsilosis* (Benham)	AG	—	—	—	—	AG	—	—	—
1	*Monilia* type I (Stovall)	AG	—	—	—	—	AG	—	—	—
1	*Monilia* type IV (Reed & Johnstone)	AG	—	—	—	—	AG	—	—	—
1	*Monilla* type VI (Reed & Johnstone)	AG	—	—	—	—	AG	—	—	—
1	*Candida parapsilosis* (Castellani)	AG	—	—	—	—	AG	—	—	—
3	Strains isolated from the vagina	AG	—	—	—	—	AG	—	—	—
2	Strains isolated from the throat	AG	—	—	—	—	AG	—	—	—
2	Strains isolated from sputum	AG	—	—	—	—	AG	—	—	—
2	Strains isolated from skin	AG	—	—	—	—	AG	—	—	—

	***Monilia candida* (13 strains)**									
1	*Monilia candida* (Benham)	AG	AG	—	AG	—	AG	AG	AG	—
1	*Monilia* type III (Stovall)	AG	AG	—	AG	—	AG	AG	AG	—
1	*Monilia* type III (Reed and Johnstone)	AG	AG	—	AG	—	AG	AG	AG	—
1	*Candida tropicalis* (Lang. et Talice)	AG	AG	—	AG	—	AG	A	AG	—
1	*Blastodendrion intermedium* (L. et T.)	AG	AG	—	AG	—	AG	AG	AG	—
4	Strains isolated from the vagina	AG	AG	—	AG	—	AG	AG	AG	—
3	Strains isolated from sputum	AG	AG	—	AG	—	AG	AG	AG	—
1	Strain isolated from feces	AG	AG	—	AG	—	AG	AG	AG	—
	***Monilia Krusei* (13 strains)**									
1	*Monilia Krusei* (Benham)	AG	—	—	—	—	AG	—	—	—
1	*Geotrichoides Krusei* (Lang. et Talice)	AG	—	—	—	—	AG	—	—	—
4	Strains isolated from the vagina	AG	—	—	—	—	AG	—	—	—
2	Strains isolated from sputum	AG	—	—	—	—	AG	—	—	—
1	Strain isolated from sputum	—	—	—	—	—	—	—	—	—
3	Strains isolated from human feces	AG	—	—	—	—	AG	—	—	—
1	Strain isolated from liver (P.M.)	AG	—	—	—	—	AG	—	—	—
	***Monilia mortifera* (4 strains)**									
1	*Mycocandida mortifera* (Lang. et Talice)	AG	AG	AG	—	—	AG	AG	—	AG
2	Strains isolated from sputum	AG	AG	AG	—	—	AG	AG	—	AG
1	Strain isolated from human feces	AG	AG	AG	—	—	AG	AG	—	AG
	***Monilia stellatoidea* (29 strains)**									
29	Strains isolated from the vagina	AG	—	—	AG	—	AG	—	AG	—
	Unclassified (3 strains)									
1	Strain isolated from human feces*	AG	A	—	—	—	AG	AG	A	—
1	Strain isolated from lung (P.M.)	AG	A	—	A	—	A	AG	—	—
1	Strain isolated from pleural fluid	AG	A	—	—	—	AG	A	—	—

* One strain did not form acid in this carbohydrate.

type of mycelial growth. In any purely morphologic study the question always arises as to the significance of minor differences in structure. These authors not only separate these fungi into different species but into different genera on the basis of variations in mycelial structure. Two strains of *M. psilosis* from Puerto-Rico were studied by them and classified into two different genera, *Mycotorula* and *Mycotoruloides*. We obtained cultures of type species from these authors and found *Mycotorula psilosis* and *Mycotoruloides ovalis* to be indistinguishable from *M. albicans* by any one of the methods described, including reciprocal agglutinin absorption tests. Their genera *Candida* and *Blastodendrion* were both identical with our species of *Monilia candida.*

Benham (1931) describes the mycelial growth of the various species on corn-meal agar and emphasized chlamydospore formation in cultures of *M. albicans.* Stovall and Bubolz (1932) differentiate types I, II and III by noting qualitative differences in the amount of mycelial growth around colonies grown for 48 hours on malt agar. This method has not been found reliable in other laboratories. Reed and Johnstone (1935) obtained no mycelial growth on malt agar plates with types IV and VI (shown by us to be identical with type I) and Hopkins and Hesseltine (1936) noted that variations could be produced by varying the thickness of seeding of the plates.

Mycelial development in corn-meal agar slide cultures was studied in all of the 172 strains described. The slide culture technique was essentially the same as that described by Benham (1931) except that no coverslip was used and the preparations were fixed and stained with lactophenol and cotton-blue (see appendix). The inoculum in every case was taken from the second or third subculture of the fungus on the glucose free medium and the inoculating loop was streaked heavily the length of the slide, cutting the surface of the agar. The cultures were incubated at room temperature and after the mycelia had developed the slides were dried in the air and stained. In spite of all efforts to maintain a constant technique variations in the amount of mycelial growth occurred. *M. albicans* most frequently develops a "tree-like" branching mycelium with swollen

thick walled cells (chlamydospores) on the tips of the mycelial branches but occasionally only a single mycelial "twig" bearing a few chlamydospores can be found in the entire slide. If the mycelium develops well certain "typical" structures may be regularly found and the following descriptions are based on only the most constant and characteristic features presented by each species (plate 3). This procedure cannot be omitted from our identification scheme as this step is necessary to rule out the yeastlike fungi which do not form mycelia (*Cryptococcus* and *Saccharomyces*).

M. albicans produces a well developed branching "tree-like" mycelium which develops chlamydospores on the tips of most branches. The ball-like structures described by Benham (1931) and Langeron and Talice (1932) occur occasionally but such clumps of spores are rare in corn-meal agar slide cultures inoculated from sugar-free media.

M. parapsilosis produces mycelia with difficulty but such a mycelium, when formed, is fairly well developed and branched. No chlamydospores are found.

M. candida forms mycelia very readily, the hyphal elements projecting for a considerable distance from the line of streak. Numerous spores are found scattered throughout the mycelial branches. There are no chlamydospores.

M. Krusei forms mycelia which, when well developed, appear as naked threads which branch only at wide intervals. The long narrow spores occur in groups which lie in irregular masses resembling a bundle of "crossed sticks." No chlamydospores are found.

M. mortifera produces a branched mycelium very similar to that formed by *M. parapsilosis*.

M. stellatoidea produces mycelia and dense ball-like clusters are formed with great regularity, thus resembling the structures previously described by Benham (1931) and Langeron and Talice (1932) as characteristic of *M. albicans*. Two of the 29 strains studied had single chlamydospores.

The most striking discrepancy found in the studies of mycelial development was the failure of *M. albicans* to produce the dense

ball-like clusters previously reported as characteristic for this species. This constant finding in our 96 strains is due, we believe, to the use of the scanty growth of the organism on the sugar-free medium as inoculum. Both strains of *M. albicans* from Benham and *Mycotorula silosis* and *Mycotoruloides ovalis* from Langeron and Talice developed chlamydospores but no large clusters. It should be noted that chlamydospores do not form regularly if the corn meal agar is inoculated from Sabouraud's agar or Sabouraud's broth cultures. It is conceivable that the transfer of small amounts of glucose from the inoculum may cause irregularities similar to those previously noted in the carbohydrate fermentation reactions. *M. stellatoidea* regularly produces large clusters but only rarely is a single chlamydospore found.

SEROLOGIC STUDIES

The lack of correlation between the morphology and the biologic reactions of these fungi has led several investigators to study the possibility of using a serologic method for differentiation. Immune rabbit sera have been prepared and tested for agglutinins (Benham, 1931, Almon and Stovall, 1934, Hines, 1924) for precipitins (Stone and Garrod, 1931, Kesten et al., 1930, Lamb and Lamb, 1935) and for complement-fixing antibodies (Stone and Garrod, 1931). A study of the results obtained by these authors indicates that antibodies can be obtained in good titer and are of value in confirming an identification established by other methods. The experience of these investigators has shown that *M. albicans* and *M. candida* cannot be differentiated by either precipitin or agglutination tests even after the sera have been absorbed with the appropriate antigen. Cross agglutination among more unrelated species has also been a problem although most of these difficulties can be overcome by the use of specifically absorbed sera. The close antigenic relationship of some of the members of this genus and the variation in titers obtained with various strains of the same species exclude both agglutination and precipitin tests as procedures of practical value in identifying large numbers of strains. For example, in Ben-

ham's paper, table 4, are listed the agglutination reactions of 30 strains of *M. albicans* obtained from various sources and identified morphologically. Eighteen of these strains (60 per cent) were agglutinated to high titer by the homologous serum only, in 4 instances the same titers were obtained with anti-*albicans* and anti-*Krusei* serum and in 8 strains the titer in anti-*Krusei* serum was only slightly less than the titer observed in anti-*albicans* serum. We have run a number of agglutination tests using

TABLE 2

Maximum agglutinin titers obtained with the 6 species when tested with 3 antisera

	TITER	M. ALBICANS—67 TESTS ON 61 STRAINS	M. CANDIDA—15 TESTS ON 11 STRAINS	M. PARAPSILOSIS—16 TESTS ON 11 STRAINS	M. KRUSEI—14 TESTS ON 10 STRAINS	M. STELLATOIDEA—24 TESTS ON 24 STRAINS	M. MORTIFERA—2 TESTS ON 2 STRAINS
Anti-*albicans* serum	1:640	18	5	0	0	0	0
	1:320	29	8	2	0	0	0
	1:160	18	1	6	2	0	0
	1:80	2	1	2	1	5	0
	Less than 1:80	0	0	6	11	19	2
Anti-*parapsilosis* serum	1:640	10	1	12	0	0	0
	1:320	11	3	4	0	0	0
	1:160	15	5	0	3	1	0
	1:80	13	6	0	5	17	0
	Less than 1:80	18	0	0	6	6	2
Anti-*Krusei* serum	1:640	0	0	0	2	0	0
	1:320	1	0	0	1	0	0
	1:160	15	2	0	6	0	0
	1:80	14	3	3	5	0	1
	Less than 1:80	37	10	13	0	24	1

various immune rabbit sera, with essentially the same results. Attempts were made to control the reaction by using organisms grown on sugar-free media for the test suspension and by using sera prepared by injection of organisms grown on the same medium. Attempts were also made to find the optimal dilution of the suspension to be used in the test as well as the effects of variations in temperature on the agglutinating reaction. Consistently higher titers were obtained when the antigen suspension was diluted 1:1000 by volume and the reaction was read after one

hour incubation in the 55°C. water bath instead of shaking at room temperature or incubating at 37°C. Such an increase in titer did not lead to greater specificity. Since many strains form a granular suspension in the control tube, determinations of exact titers are frequently difficult. Complement-fixation tests were used for the purpose of obtaining a more definite end point but no increase in specificity was attained.

Table 2 summarizes the results of 139 tests on 119 strains of *Monilia* all of which have been identified by the above-described procedures. The results indicate that the serologic reactions are of value, in that the previously described criteria of separation into species can be correlated to some degree with differences in antigenic structure, but may lead to erroneous diagnoses if relied upon for routine identification.

PATHOGENICITY EXPERIMENTS

The pathogenicity of *M. albicans* for rabbits has been well established by Benham (1931) and Stovall and Pessin (1933). The latter authors found *M. candida* (type III) to be pathogenic only in enormous doses and *M. parapsilosis* (type I) to be incapable of causing any demonstrable lesion in rabbits. Six strains of *M. albicans* were inoculated intravenously and all the rabbits died in 4 to 5 days with the typical lesions in the kidney described by the above named authors. Three strains of *M. albicans* caused definite abscesses in the skin 48 hours after intracutaneous inoculation. Three strains of *M. stellatoidea* in equivalent doses (approximately one half the growth on a Sabouraud's slant) did not cause death in any of the rabbits. One animal was killed on the seventh day after inoculation and examined carefully for lesions but none could be found. The 2 surviving rabbits were inoculated with *M. albicans* 2 weeks after the initial dose of *M. stellatoidea* and both animals died within 5 days. Three strains of *M. stellatoidea* produced no abscesses after intracutaneous inoculation. Two of the four strains of *M. mortifera* inoculated intravenously and intracutaneously, showed no evidences of pathogenicity. *M. albicans*, therefore, is the only pathogenic species of this genus, if we accept the

ability to produce lesions in the rabbit as evidence that the fungus possesses pathogenic properties for man.

UNIDENTIFIED STRAINS

Three strains of *Monilia* could not be identified with any of the 6 species described above. These organisms differed, not only in carbohydrate fermentation and colony formation on blood agar, but in the type of mycelial growth in corn-meal agar. We have avoided describing them as new species because we feel that at least 4 or 5 similar strains should be studied as a group before significance is attached to the failure of an occasional organism to fall into an empiric laboratory classification.

SUMMARY OF DIFFERENTIAL CHARACTERISTICS

The following summary describes only the most important and constant features upon which species differentiation is based. The fermentation reactions of only 4 carbohydrates, glucose, sucrose, lactose and maltose, are considered.

M. albicans. Creamy colony on Sabouraud's agar; no surface growth in Sabouraud's broth; medium-sized dull gray colony on blood agar; chlamydospores in corn-meal agar; acid and gas formation in glucose and maltose, acid in sucrose.

M. parapsilosis. Creamy colony on Sabouraud's agar; no surface growth in Sabouraud's broth; small, raised, pearly white colonies on blood agar; mycelium forms with difficulty in corn-meal agar; acid and gas formation in glucose.

M. candida. Creamy colony on Sabouraud's agar; surface growth on Sabouraud's broth characterized by narrow film broken up with bubbles; mycelial fringe around colonies on blood agar; mycelium forms readily in corn-meal agar; acid and gas produced in glucose, sucrose and maltose.

M. Krusei. Flat dry colony on Sabouraud's agar; extensive surface growth on Sabouraud's broth; irregularity in size and shape of colonies on blood agar; mycelium shows few branches with spores arranged like "crossed sticks" in corn-meal agar; acid and gas formation in glucose.

M. mortifera. Creamy colony on Sabouraud's agar; no surface

growth in Sabouraud's broth; small colonies on blood agar; poorly developed mycelium in corn-meal agar; acid and gas formation in glucose, sucrose and lactose.

M. stellatoidea. Creamy colony on Sabouraud's agar; no surface growth in Sabouraud's broth; "star in the sky" appearance of colonies on blood agar; large ball-like clusters of spores in corn-meal agar; acid and gas formation in glucose and maltose.

DISCUSSION

Little progress can be made in the understanding and recognition of fungus diseases until there is developed a practical method of identification of the causative agents. Furthermore, the mycologic flora occurring in normal individuals must be established before too much significance can be attached to the finding of yeastlike organisms in the sputum, vagina, skin or feces of a patient. The burden of routine identification falls on the bacteriologist and the methods of identification, therefore, should be adapted to the resources and training of one trained in bacteriologic technique.

In the scheme of identification proposed in this paper, purely mycologic methods (growth at various temperatures, morphology of the conidia, etc.) have been largely disregarded since even a cursory survey of the literature reveals that there is practically no agreement among mycologists as to terminology, classification or methods of study. In the index of Dodge's (1935) book 103 different species names are listed for the genus *Monilia.* An intensive survey of the literature for the criteria by which these species have been classified has not been attempted, since many species have been renamed and reclassified by investigators who did not study the organisms themselves but relied upon published descriptions. The synonomy presented here (charts 1 and 2) is incomplete but is included for the purpose of illustrating the extremely complicated terminology that results from placing too great emphasis on variously interpreted morphologic details.

We agree with Benham (1931) that the generic name *Monilia* should be retained until a perfect stage on the life cycle of these organisms can be demonstrated. The present concern of the

CHART 1

CLASSIFICATION PROPOSED BY THE AUTHORS	CLASSIFICATIONS PROPOSED BY VARIOUS AUTHORS		CULTURES STUDIED
Monilia albicans (Robin) Zopf 1890	Langeron et Talice	*Mycotorula albicans*	*Mycotorula albicans* Lang. et Talice 1932 *Parasaccharomyces Harteri* Froilano de Mello et al. 1918 "Oidiomycosis" (Sabouraud's collection) *Pityrosporium* of Dowling (China) Strains isolated from various sources
		**Mycotorula psilosis*	*Monilia psilosis* Ashford 1917
		Mycotoruloides triadis	*Monilia sp.* Brocq-Rousseu et al. 1927
		**Mycotoruloides ovalis*	Isolated from a case of rhino-pharyngo-stomatitis
		Mycotoruloides Aldoi	*Monilia Aldoi* Pereira
		Mycotoruloides unguis	*Spicaria unguis* Weill and Gaudin 1919
		Mycotoruloides sp.	*Monilia psilosis* Ashford 1917 *Monilia Ashfordi* Castellani "Oidiomycosis" (Sabouraud's collection) Strains isolated from various sources
	Benham	**Monilia albicans*	*Endomyces albicans* (ATCC 2076) Strains isolated from various sources *Monilia psilosis* Ashford 1917 *Monilia albicans* from thrush (ATCC 2112)
	Stovall	**Monilia* type II	*Monilia richmondi* Shaw 1926 (ATCC 801) *Monilia Pinoyi* Castellani 1913 (ATCC 752) *Monilia* from sprue-Zillig (ATCC 4021) *Monilia pseudotropicalis* Castellani 1913 (ATCC 413) *Monilia metalondinensis* Castellani 1919 (ATCC 753) Strains isolated from sputum
	Authors	*Monilia albicans*	*Monilia macedoniensoides* Castellani 1925 *Monilia Pinoyi* Castellani 1913 87 strains isolated from human sources
Monilia parapsilosis Ashford 1928	Benham	**Monilia parapsilosis*	*Monilia parapsilosis* Ashford 1928 *Monilia parapsilosis* from skin
	Stovall	**Monilia* type I	Strains isolated from sputum
	Authors	*Monilia parapsilosis*	*Candida parapsilosis* Camargo 1934 9 strains isolated from human sources
Monilia stellatoidea Jones and Martin 1937	Authors	*Monilia stellatoidea*	*Monilia stellatoidea* Jones and Martin 1937

* Type species studied by the authors.

CHART 2

CLASSIFICATION PROPOSED BY THE AUTHORS		CLASSIFICATIONS PROPOSED BY VARIOUS AUTHORS	CULTURES STUDIED
Monilia candida Bonorden 1851	Langeron et Talice	**Candida tropicalis*	*Endomyces tropicalis* Castellani 1911 *Monilia tropicalis* Castellani 1913
		Candida parapsilosis	*Monilia parapsilosis* Ashford 1928
		Candida butantanensis	*Monilia butantanensis* Gomes 1924
		Candida sp.	Strains isolated from various sources
		**Blastodendrion intermedium*	*Blastodendrion intermedium* Ciferri and Ashford
		Blastodendrion erectum	*Endomyces albicans* (Sabouraud's collection)
		Blastodendrion Krausi	*Blastodendrion Krausi* Ota 1924
		Blastodendrion Arzti	*Blastodendrion Arzti* Ota 1924
		Blastodendrion Favrei	*Mycelobastanon Favrei* Ota 1925
		Blastodendrion Braulti	*Enantiothamnus Braulti* Pinoy 1911
	Benham	**Monilia candida*	*Monilia candida* (Thom and Church 4719.1)
	Stovall	**Monilia* type III	*Monilia candida* Bonorden 1851 (ATCC 2113) *Monilia candida* Bonorden 1851 (ATCC 1369) *Monilia tropicalis* Castellani 1913 (ATCC 750) Strains isolated from sputum
	Authors	*Monilia candida*	10 strains isolated from human sources
Monilia Krusei Castellani and Chalmers 1913	Langeron et Talice	**Geotrichoides Krusei*	*Monilia Krusei* Castellani and Chalmers 1913
		Geotrichoides cutaneus	*Oidium cutaneum* Beurmann et al. 1909 *Mycoderma cutaneum* Brumpt 1927
		Geotrichoides asteroides	*Trichosporium asteroides* Ota 1926
		Geotrichoides Balzeri	*Parendomyces Balzeri* Gougerot and Burnier 1912 *Monilia Balzeri* Brumpt 1922
		Geotrichoides tumefaciens	*Monilia tumefaciens alba* Foulerton 1900
		Geotrichoides vulgaris	*Candida vulgaris* Berkhout 1923 *Monilia candida* Bonorden 1851 pro parte *Monilia Bonordoni* Vuillemin 1911 pro parte
		Geotrichoides kefyr	*Candida kefyr* Berkhout
		Geotrichoides sp.	Strains isolated from various sources
	Benham	**Monilia Krusei*	*Monilia Krusei* from feces
	Authors	*Monilia Krusei*	12 strains isolated from human sources
Monilia mortifera n. comb.	Langeron et Talice	**Mycocandida mortifera*	*Candida mortifera* Redaelli 1925
		Mycocandida onychophila	*Monilia onychophila* Pollacci and Nannizzi 1926
		Mycocandida sp.	Strains isolated from various sources
	Authors	*Monilia mortifera*	3 strains from human sources

* Type species studied by the authors.

bacteriologist and clinician is the recognition and treatment of fungus disease in the patient. Increase in our knowledge of these problems cannot be expected until there is a practical method of identification and a terminology that is generally accepted.

SUMMARY

One hundred and fifty-three unidentified strains of *Monilia* isolated from various sources were studied and compared with 19 "known" species types obtained from other investigators. One hundred and fifty of these organisms could be classified in one of 6 species. The methods used in classification are comparatively simple and the criteria upon which identification is based are easily recognizable if the technique described is rigidly followed.

APPENDIX

Sabouraud's glucose agar. Bacto-glucose 40 grams, Fairchild's peptone 10 grams, agar 25 grams, distilled water 1000 cc. Melt in autoclave, filter through cotton, tube and sterilize in autoclave at 15 pounds pressure for 15 minutes. No pH adjustment necessary.

Sabouraud's glucose acid broth. Same formula as for Sabouraud's glucose agar except that no agar is added. No filtration or pH adjustment is necessary.

Beef extract agar. Difco beef extract 3 grams, NaCl 5 grams, Difco peptone 10 grams, agar 25 grams, distilled water 1000 cc. Melt in autoclave, titrate to pH 7.6, filter through cotton, tube and autoclave at 15 pounds pressure for 15 minutes. Final pH 7.4.

Beef extract blood agar. Same basic formula as for beef extract agar with the addition of approximately 10 per cent sterile citrated sheep's blood.

Carbohydrate broth. Difco beef extract 3 grams, NaCl 5 grams, Difco peptone 10 grams. Make up to 900 cc. in distilled water. Heat to boiling and titrate exactly to pH 7.2. Add 100 cc. of indicator solution (see below), filter and tube in 10 cc. quantities. Autoclave at 15 pounds pressure for exactly 15 minutes. Add 0.5 cc. of a 20 per cent solution of the carbohydrate sterilized by filtration through a Seitz filter. The broth should not be kept for more than 2 or 3 weeks because slight changes in pH may occur.

Indicator solution. Brom-thymol-blue 0.04 gram, distilled water 100 cc. Add a small amount of 1 N NaOH to make the solution alkaline. When indicator is in solution, neutralize with 1 N HCl until the exact neutral point is reached and 1 drop of either acid or alkali will cause a complete change of color.

Corn meal agar. 62.5 grams corn meal in 1500 cc. water. Heat to 60°C. for one hour. Filter through paper and make volume up to 1500 cc. Add 19 grams of agar. Arnoldize for one and a quarter hours. Filter through cotton, tube and sterilize. No adjustment of pH necessary.

Carrot plugs. Carrots are cut into cylinders with a cork borer and made into slants by a long diagonal cut. A small wad of cotton is put in the bottom of the test tube before inserting the carrot and the tube is autoclaved. Sterile distilled water is added at intervals to prevent drying.

Fixation and staining of slide cultures. Dehydrate slides by leaving them in air at room temperature for 36 to 48 hours. Stain for 15 minutes with lacto-phenol cotton-blue (see below). Pour off stain and immerse in 70 per cent alcohol for approximately 10 minutes (till agar is almost completely decolorized). Run the slide through 95 per cent alcohol, acetone, a mixture of equal parts of acetone and xylol and finally xylol. Remove from xylol and mount immediately in neutral balsam.

Lacto-phenol cotton-blue. Phenol crystals 20 grams, lactic acid 20 cc., glycerol 40 cc., distilled water 20 cc. Dissolve by gentle heat under hot water tap. Add 1 gram cotton-blue.

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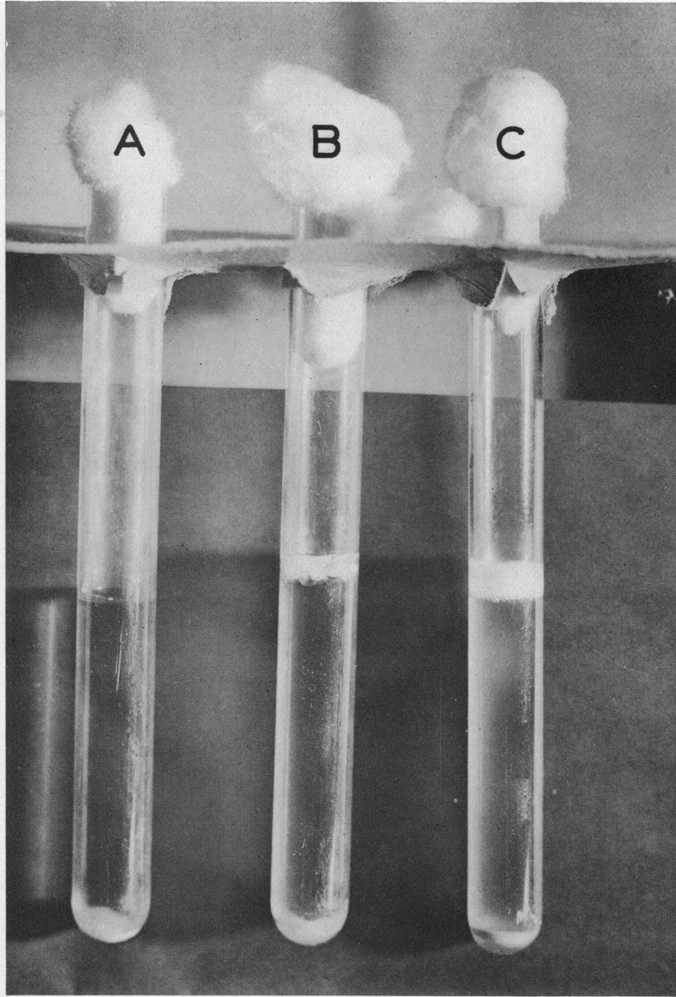
PLATE 1

Photograph Illustrating Various Types of Growth in Sabouraud's Glucose Acid Broth Incubated at 37°C. for 48 Hours

A. No surface growth (*M. albicans*, *M. parapsilosis*, *M. stellatoidea* or *M. mortifera*).

B. Bubbly surface growth with thin film (*M. candida*).

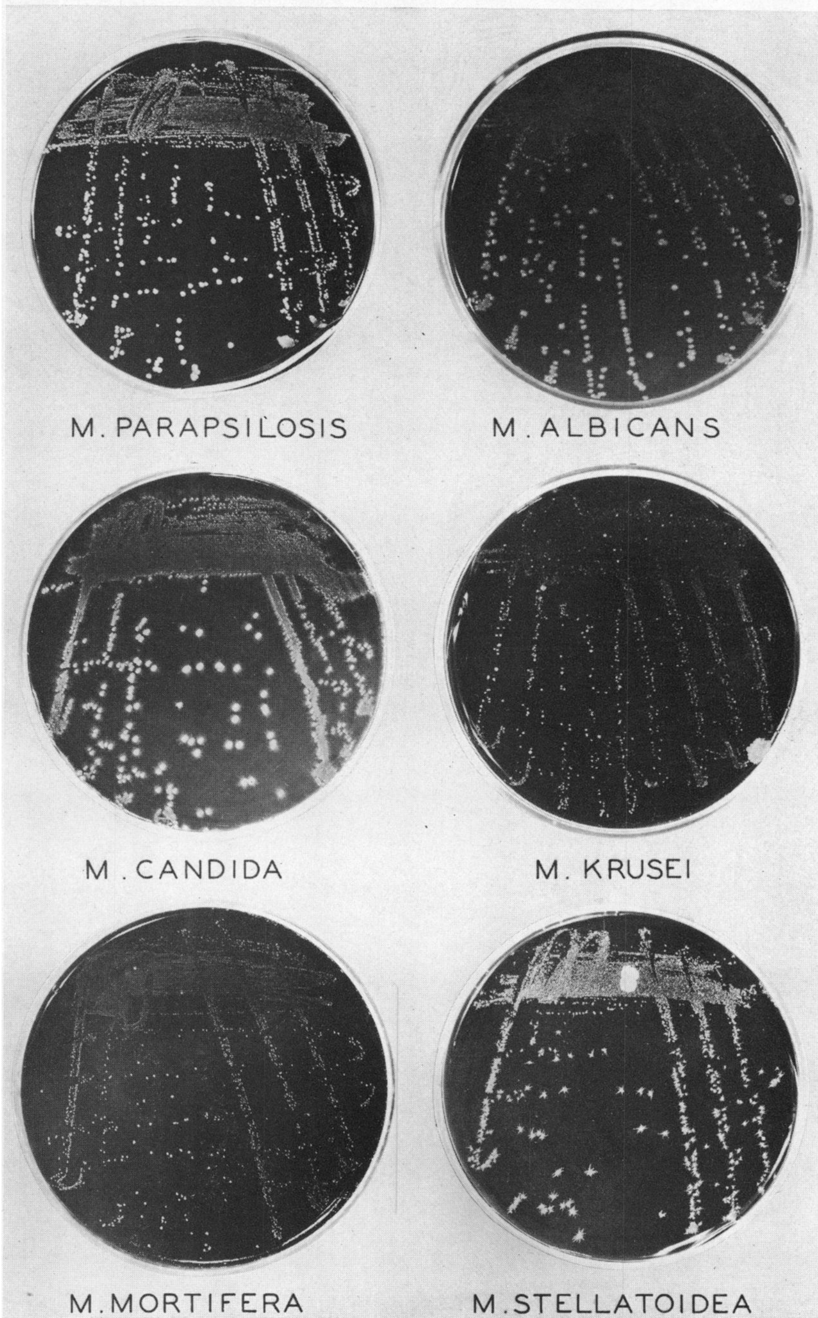
C. Extensive development of surface film (*M. Krusei*).



(D. S. Martin, C. P. Jones, K. F. Yao and L. E. Lee, Jr.: Classification of Monilias)

PLATE 2

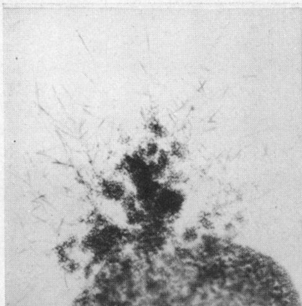
PHOTOGRAPHS OF BLOOD AGAR PLATES SHOWING CHARACTERISTIC COLONIES AFTER 10 DAYS INCUBATION AT 37°C. FOR 10 DAYS



(D. S. Martin, C. P. Jones, K. F. Yao and L. E. Lee, Jr.: Classification of Monilias)

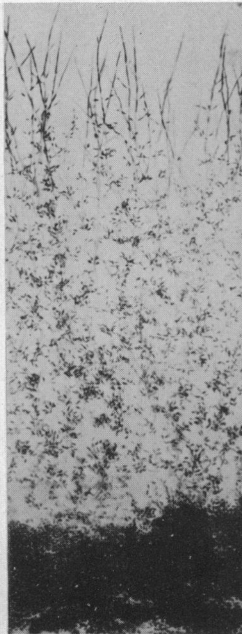
PLATE 3

Photomicrographs Illustrating Types of Mycelial Growth in Corn Meal Agar after Several Days Incubation at Room Temperature

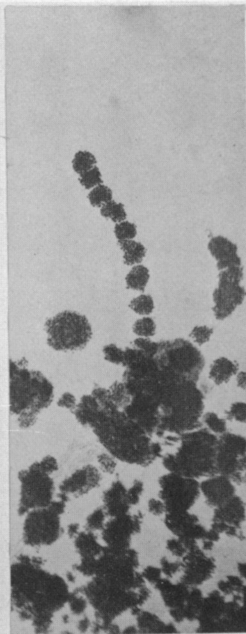


M. PARAPSILOSIS

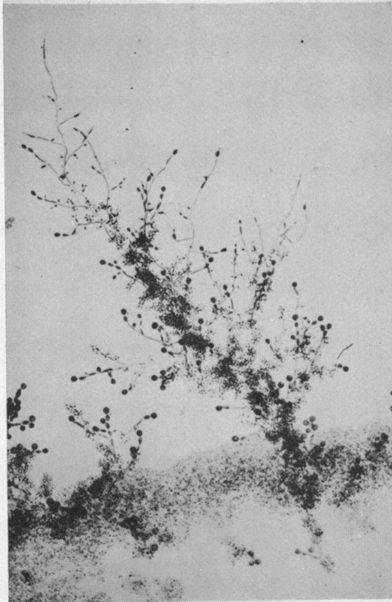
M. MORTIFERA



M. CANDIDA



M. STELLATOIDEA



M. ALBICANS

M. KRUSEI

(D. S. Martin, C. P. Jones, K. F. Yao and L. E. Lee, Jr.: Classification of Monilias)