A CULTURAL STUDY OF THE LIFE-CYCLE OF HISTOPLASMA CAPSULATUM DARLING 1906

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Darling (1906, 1907, 1908, 1909) first described the disease histoplasmosis and its etiologic agent *Histoplasma capsulatum* in a series of papers from the Ancon Hospital, Canal Zone. His description was based on three cases, two Martinique negroes and one Chinese, from which he pictured the disease as one of emaciation, splenomegaly, leukopenia, anemia, and pyrexia. Encapsulated, round or oval bodies, 3 μ in diameter, were seen in large endothelial cells in lesions of the lungs, liver, spleen, intestines, and lymph nodes. These bodies were thought to be protozoa and the resemblance of this new disease to kala-azar was noted.

Da Rocha-Lima (1912), however, after a comparative study of material obtained from Darling and material from a case of kala-azar, came to the conclusion that *Histoplasma capsulatum* was more closely related to the fungi than to the protozoa. Although the fungus nature of the organism causing histoplasmosis was suspected, no attempts were made to culture the material from cases reported prior to 1934, because a diagnosis of histoplasmosis in these cases was made only after a microscopic examination of killed and fixed tissues obtained at autopsy.

Cultures were obtained in 1934, however, from two cases in which an invading yeast-like organism was known to be present during life. One case was reported by Hansmann and Schenken (1933) at the Washington meeting of the American Association of Pathologists and Bacteriologists, May 9, 1933, and published

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in November, 1934. The fungus was seen in, and cultured from, biopsies of the skin, inguinal lymph nodes, and buccal mucosa. Although the yeast-like organism found later, at autopsy, in the endothelial cells in lesions of the lungs, adrenals, skin, and lymph nodes were suggestive of *Histoplasma capsulatum* of Darling, the case was reported as a new disease caused by a yeast-like fungus because the spleen was not involved. The fungus isolated from this case was placed in the genus *Sepedonium* because the culture developed as an aerial mold-like growth producing tuberculate chlamydospores.

The other case was reported by Dodd and Tompkins (1934) at the Richmond meeting of the American Society of Tropical Medicine, November 15–17, 1933 and was published in March, 1934. This case was diagnosed as histoplasmosis, before death, by finding the oval bodies in the mononuclear cells of peripheral blood smears. De Monbreun (1934) studied the fungus obtained from blood cultures taken two days before death and from the spleen at autopsy. He described the fungus as *Histoplasma capsulatum* of Darling.

The fungi isolated from these two cases, although placed in different genera, *Histoplasma* and *Sepedonium*, were identical in every respect (Redaelli and Ciferri 1937). Each, in the filamentous form on artificial media at room temperature, produced large, thick-walled, round and pyriform tuberculate chlamydospores on aerial hyphae. Both strains also produced the disease experimentally in animals (dog and rat by Hansmann and Schenken; monkey, mouse, and puppy by De Monbreun) with small oval yeast-like parasites 1–3 μ in diameter in the endothelial cells of characteristic lesions in the liver, spleen, lungs, lymph nodes, and adrenals.

Since these reports, cultures of *Histoplasma capsulatum* have been isolated from reported cases of histoplasmosis by Clemens and Barnes (1940), Reid, Scherer and Irving (1940), and Negroni (1940); and, from two unreported cases by Dr. Frank Forry and Dr. Parsons (cited by Meleney (1940)). A culture of this fungus has also been isolated by De Monbreun (1939) from a natural case occurring in a dog.

The fungus to be described in this paper was isolated (Glesne and Conant 1940) from the blood stream of a three-months-old infant 24 hours before death. At autopsy, the small (1–3 μ) oval cells were seen in blood smears of the liver and in sections of the spleen (Plate I, figs. 1 and 2).

LIFE-CYCLE OF HISTOPLASMA CAPSULATUM

De Monbreun (1934) noticed that colonies appearing in broth cultures of the primary isolation of his fungus contained both mycelial and yeast-like forms. When this material was transferred to 10-per-cent rabbit-blood agar slants, sealed and incubated at 37°C., the parasitic yeast-like phase developed and could be maintained by repeated transfer every 4–5 days. On blood agar and Sabouraud's glucose agar at room temperature, however, only the saprophytic mycelial phase of the fungus developed. The investigator was able to convert this mycelial phase to the yeast-like phase with his two strains, human (1934) and dog (1939), only by inoculating susceptible animals and then streaking blood agar slants with material obtained from subcutaneous abscesses or the peritoneal cavity. He believed the mycelial form to be fixed and conversion to the yeast-like form impossible by cultural methods. Ciferri and Redaelli (1934), however, were able to convert De Monbreun's strain to the yeast-like form by growing it on blood agar at 37°C. With the strain studied in this paper and described below, it was found possible to complete the life-cycle of the fungus by cultural methods without resorting to animal inoculation.

Parasitic yeast-like form

After several transfers on Sabouraud's agar at room temperature to insure the complete mycelial development of *Histoplasma capsulatum*, the mycelial form was streaked heavily on blood agar slants and incubated at 37°C. Close-growing moist mycelial colonies developed in these cultures with much of the growth beneath the surface of the agar. No yeast-like forms were seen when this material was examined microscopically. When blood agar slants were inoculated with the mycelial-form

and sealed with paraffin, however, the yeast-like form appeared. The paraffin seal served to keep the agar slants moist for long periods of time, and by doing this, the slants were maintained in an optimal condition for the conversion of the fungus to the yeast-like form. The colonies appeared as small, white, moist growths, few in number and scattered over the surface of the agar. They were similar to bacterial colonies (*Staphylococcus albus*) and did not become apparent for 7–10 days. Occasionally a bacteria-like white growth developed from the side of a large piece of inoculum on the slant and spread a short distance over the surface of the agar. This material and the small colonies were pasty in consistency and easily picked up by a loop. Transfers to fresh blood agar slants sealed with paraffin and incubated at 37°C. produced a white pasty growth made up entirely of small budding yeast cells (Plate I, figs. 3 and 4).

In fresh preparations these yeast cells appeared as thin-walled oval bodies, 1.5–2 x 3 μ, which reproduced by a single bud from the pointed end (Plate I, fig. 4; Plate III, fig. 1). As the bud approached the size of the parent cell, both became round, losing their characteristic oval shape. The cells contained a large protoplasmic granule showing active brownian movement and a large vacuole. A clear halo was visible around these cells and was thought to be due to either refracted light or capsular material, such as is found surrounding these cells in tissue. Smears stained with Hiss' capsule stain and dilute India ink preparations, however, failed to demonstrate a true capsule.

Mycelial form of the fungus

The early development of the mycelial form of *Histoplasma capsulatum* from the yeast was studied by the use of slide cultures and Van Tieghem cell cultures at room temperature. Slide cultures were made by supporting microscopic slides on "U" shaped tubing in petri dishes which were then sterilized. The slides were flooded with a thin layer of Sabouraud's glucose agar, inoculated with the yeast from blood agar slants, and sterile water added to the petri dish. These preparations were removed from time to time and examined microscopically by

putting a drop of water or stain and a coverslip on the surface of the growth. Van Tieghem cell cultures were prepared by inoculating hanging drops of Sabouraud's glucose agar, beef-infusion agar, Sabouraud's glucose broth and 2 per cent peptone broth on sterile coverslips which were cemented to the ring with vaseline. These preparations were examined microscopically at various intervals without disturbing the development of the fungus. By using these two methods the complete development of the fungus was followed from the yeast to the mycelial form with its characteristic large round or pyriform, thick-walled tuberculate chlamydospores.

In 24 hours the yeast cells became markedly swollen, from 1.5 x 3 to 3.5 x 5.5 μ, and in 48 hours had produced short germ tubes (Plate I, fig. 5; Plate III, figs. 2, 3, 4, 5, 6). These germ tubes were developed from the pointed end, from both ends, and occasionally from the sides of the cells. It was not uncommon to find three germ tubes from a single yeast cell (Plate I, fig. 5; Plate III, fig. 4). These tubes quickly became septate, branched and showed a highly vacuolate protoplasm and numerous oil droplets. A dense mat of mycelium made up of septate branching hyphae, 1.5–2.5 μ thick was formed within a week and a half to two weeks.

On these hyphae, within the medium, could be seen small round to pyriform smooth-walled spores, 2.5–3 μ in diameter as well as large smooth-walled spores, 7–10 μ in diameter, either sessile or terminating short lateral branches (Plate II, fig. 2; Plate III, figs. 12, 13, 14). These spores later developed thick walls and many contained numerous small hyaline bodies which were similar to endospores. When this material was treated with osmic acid and sudan III, however, the small enclosed bodies behaved typically as fat droplets and could no longer be mistaken for ascospores within an ascus. Often, several of these bodies when present in a single chlamydospore, were seen to coalesce and form a large single stained body when treated with sudan III.

In the aerial mycelium the spores were at first smooth-walled but quickly developed into thick-walled chlamydospores with

a tuberculate sculpturing of the outer wall. Some of these chlamydospores were small and pyriform (2–3 x 3.5–5 μ) others were small and round (2.5–3.5 μ in diameter) (Plate III, figs. 16 and 17). In older cultures were seen large round and pyriform chlamydospores (7.5–15 μ in diameter) covered with finger-like protuberances sometimes 6 μ long (Plate II, figs. 3 and 4; Plate III, figs. 9, 10, 11, 15). These chlamydospores were thick-walled and had a peripheral vacuolated protoplasm (Plate III, figs. 18, 21, 22). These large, round or pyriform, tuberculate, thick-walled chlamydospores were the distinguishing morphologic characteristic of *Histoplasma capsulatum* in its mycelial form.

Culturally *Histoplasma capsulatum*, on Sabouraud's glucose agar at room temperature, developed as a white cottony growth (Plate II, fig. 1). As the culture became older the white slowly changed to buff and finally to a light brown. Microscopic mounts of older cultures showed friable broken brownish hyphae and brown tuberculate chlamydospores of varying sizes. This material could be inoculated on blood agar slants, the tubes sealed and incubated at 37°C., and the yeast-like form recovered in 10 days to two weeks.

DISCUSSION

Attempts to classify the human pathogenic fungi have resulted in an increasing list of synonyms for many of the described forms. In the case of *Histoplasma capsulatum*, Darling (1906) described the organism seen in tissue as a protozoan and thought it to be closely related to the Leishman-Donovan bodies seen in tropical Leishmaniasis (kala-azar). Comparative studies of material obtained from Darling, material from a case of kala-azar, and material from epizootic lymphangitis of horses allowed Da Rocha Lima (1912) to consider the organism as a fungus closely allied to *Cryptococcus farcinimosus*. Following this, the fungus has been named *Cryptococcus capsulatus* by Castellani (1919) and *Torulopsis capsulatus* by Almeida (1933). These two genera would place the fungus among the yeast-like forms because of the budding oval cells seen in infected tissue.

In culture, however, this fungus was shown by De Monbreun

(1934) to have a cottony aerial growth characterized by large, thick-walled tuberculate chlamydospores on media at room temperature and a yeast-like growth of small oval budding cells on blood agar at 37°C. when this medium was inoculated with material from experimentally infected animals. He maintained the name *Histoplasma capsulatum* Darling 1906 and added the cultural characters of the fungus to the description of the tissue form. Hansmann and Schenken (1934) also isolated this fungus from a case of histoplasmosis but placed it in the genus *Sepedonium* of the Fungi Imperfecti because the large, thick-walled tuberculate chlamydospores which developed on the aerial hyphae were similar to the chlamydospores described in species of *Sepedonium*.

Moore (1934) (1935), after examining these two strains, placed them in the genus *Posadasia* Canton 1898 making two species *P. capsulata* (Darling) Moore (De Monbreun's strain), and *P. pyriformis* Moore (Hansmann and Schenken's strain). For this classification, the tuberculate chlamydospores containing fat droplets were thought to be asci containing ascospores, and the genus *Posadasia* was placed in the family Coccidioidaceae of the Endomycetales along with the genera *Coccidioides*, *Paracoccidioides* and *Rhinosporidium*.

The genus *Posadasia* of Canton 1898, to which Moore would refer *Histoplasma*, was created for a South American fungus found in granulomatous lesions. This fungus as originally described, however, has been shown to be identical with the *Coccidioides* of North America (Almeida 1933). Since *C. immitis* Stiles 1896 had priority of publication, *P. esferiformis* Canton 1898 has been reduced to synonymy. Moore, however, discarded the original description of *Posadasia* for that of da Fonseca and Leao (1928) in which they described the fungus of South American coccidioidal granuloma in the tissue as an endospore-filled body, 5–80 μ in diameter, with the spores liberated through orifices in the cell wall and remaining attached to the parent cell for a long time by small filaments of protoplasm. This structure described by da Fonseca and Leao for *Posadasia* in tissue was apparently thought by Moore to be an erroneous observation in that the

spores remained inside of the cell and tubercles were formed on the surface. This interpretation allowed Moore to place *Histoplasma* in the genus *Posadasia* because he considered the tuberculate asci which he described in cultures of *Histoplasma* to be identical with this tissue form of *Posadasia.*

Almeida (1930), however, had already shown that the South American fungus described as *Posadasia* by da Fonseca and Leao (1928) was, in reality, a new fungus and should not be confused with *Coccidioides* (*Posadasia* of Canton). He created the genus *Paracoccidioides* for this fungus and showed that the tissue form did not produce endospores which migrated through the cell wall but that these structures were multiple buds adhering to the periphery of the cell.

Moore (1935) said that this tissue form of *Paracoccidioides* as described and illustrated by Almeida was also identical with the tuberculate asci which were found in cultures of *Histoplasma.* In this case, *Histoplasma* and *Paracoccidioides* were said to be closely related but not identical because *Histoplasma* produced a yeast-like form in tissue. It would seem, however, that in the previous comparison of *Histoplasma* with the *Posadasia* of da Fonseca and Leao, Moore should have also recognized the fact that *Histoplasma* produced a yeast-like form in tissue.

Ciferri and Redaelli (1934) studied De Monbreun's strain and described three cultural types; type III, or hyphomycetic form, of white cottony growth on media at room temperature, which produced round to pyriform smooth-walled chlamydospores (hypnospores) which later developed into thick-walled tuberculate chlamydospores (stalagmospores); type II, or intermediate form; and type I, or almost yeast-like form on blood agar at 37°C., which contained mycelial elements and yeast-like budding cells identical with those seen in infected tissue. They based the systematic position of *Histoplasma capsulatum* on this yeast-like form, placing it among the anascosporogenous yeasts in the new family Histoplasmaceae of the superfamily Atelosaccharomycetaceae. Redaelli and Ciferri (1934) also placed two other species in the genus; namely, *Histoplasma farcinimosum* (*Cryptococcus farcinimosus* of equine lymphangitis) and *Histo-*

plasma muris (*Cryptococcus muris* of spontaneous splenomegaly of rats). Since *H. muris* has never been cultured and cultures of *H. farcinimosum* did not produce the large tuberculate chlamydospores characteristic of *H. capsulatum*, these two species were placed in *Histoplasma* on the similarity of their budding forms in diseased tissue. Although Ciferri and Redaelli (1935) added *Histoplasma pyriformis* (*Posadasia pyriformis* Moore) to the genus, it was later reduced to synonymy with *H. capsulatum* by Redaelli and Ciferri (1937).

These various attempts to place the fungus of histoplasmosis in a systematic position among the known fungi have been confused not only by a misinterpretation of structures produced in culture (chlamydospores and fat droplets for asci and ascospores) but also by emphasizing the reduced parasitic yeast-like stage to the exclusion of the filamentous form with its characteristic and diagnostic tuberculate chlamydospores. The reduced yeast-like stage was found only in diseased tissue and in cultures at 37°C. Because of the lack of distinguishing morphological characters and the extremely specialized conditions necessary for this type of development, it should not be used for purposes of classification other than to note its occurrence in the life-cycle of the fungus. The natural development of *Histoplasma* on all media at ordinary temperature was its filamentous form producing the characteristic chlamydospores. Since all fungi are identified and classified on the morphology of the spore forms which they produce in culture, this hyphomycetic form should have been used for determining its systematic position.

From the life-cycle study of *Histoplasma capsulatum* discussed in this paper it is felt that this fungus should be considered a member of the Fungi Imperfecti. The large tuberculate cells produced in the aerial mycelium of the filamentous form were chlamydospores and the enclosed hyaline bodies have been shown to be fat droplets by Hansmann and Schenken (1934), De Monbreun (1934), Ciferri and Redaelli (1934), Howell (1939) and the author by using appropriate fat stains. They disappeared when treated with lactophenol, coalesced when treated with fat stains, and were not found in fixed and stained prepara-

tions. *Histoplasma capsulatum* showed many characteristics which were similar to the spiculated, tuberculate chlamydospore-producing fungi of the Fungi Imperfecti, such as *Sepedonium*, *Stephanoma*, *Chlamydomyces*, and *Mycogone* as shown by Howell (1939). Of these genera, the fungus of histoplasmosis was most closely related to *Sepedonium*. Culturally, Howell (1939) has shown a close morphological relationship in that the chlamydospores of both *Histoplasma capsulatum* and *Sepedonium chrysospermum* developed acrogenously on terminal and lateral branches and were similarly sculptured. *Sepedonium chrysospermum* (Bull.) Link, however, has a known ascigerous stage, *Hypomyces chrysospermus* (Bull.) Tul., and an additional spore form, "true conidia," borne from the tips of phialides which were arranged in a verticilliate manner. Since *Histoplasma capsulatum* Darling does not have a known ascigerous stage or "true conidia" which can be so compared with those found in cultures of *Sepedonium*, it should remain, for the present, a distinct genus closely related to, but not identical with, *Sepedonium*. The genus *Histoplasma* and the species *Histoplasma capsulatum* Darling 1906, therefore, should be placed in the Moniliaceae of the Fungi Imperfecti and characterized by a saprophytic filamentous form of loose, cottony, white to light-brown mycelium producing small and large, single-celled, smooth to tuberculate chlamydospores in culture at room temperature, and small budding, yeast-like cells on blood agar at 37°C.; and, by a parasitic yeast-like form causing a fatal systemic infection in man and dog in whose infected tissue the fungus occurs as yeast-like, oval bodies phagocytized by endothelial leukocytes.

SUMMARY

1. A strain of *Histoplasma capsulatum* from a fatal case of histoplasmosis in a three-months old infant is studied.

2. The saprophytic filamentous form developed on Sabouraud's glucose agar at room temperature is characterized by large tuberculate chlamydospores, not asci, as proven by appropriate staining methods.

3. The filamentous form is converted to the yeast-like tissue

form without the use of animal inoculation by cultivation on sealed blood agar slants at 37°C.

4. From a cultural study of the complete life-cycle of *Histoplasma capsulatum* it is shown that the fungus should be placed in the Moniliaceae of the Fungi Imperfecti.

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PLATE I

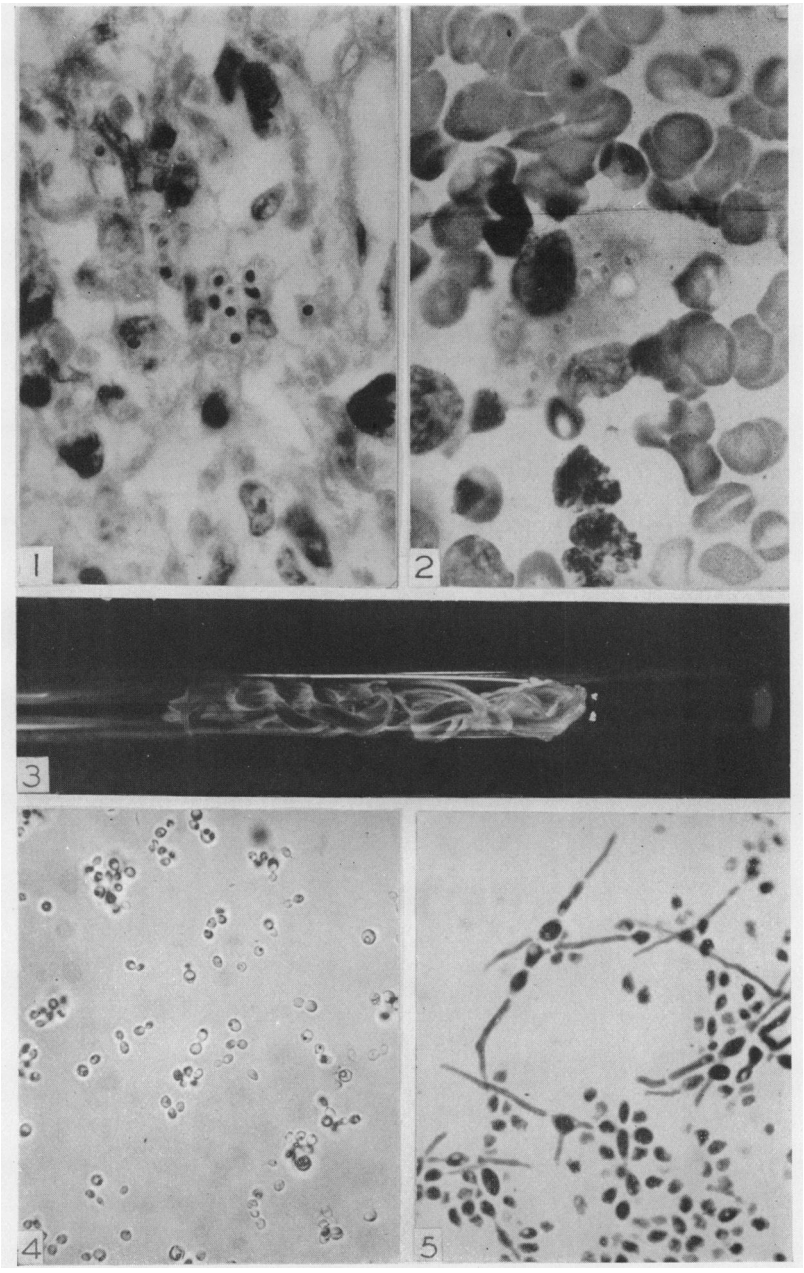
Fig. 1. Section of spleen stained after Bensley's method.

Fig. 2. Smear of blood from liver made at autopsy.

Fig. 3. Five day growth of the yeast-like form of *Histoplasma capsulatum* on sealed blood agar slant at 37°C.

Fig. 4. Fresh preparation of yeast-like form taken from blood agar slants at 37°C.

Fig. 5. Stained preparations of germinating yeast cells on Sabouraud's glucose agar after 48 hours at room temperature.



(Norman F. Conant: Life-Cycle of Histoplasma Capsulatum)

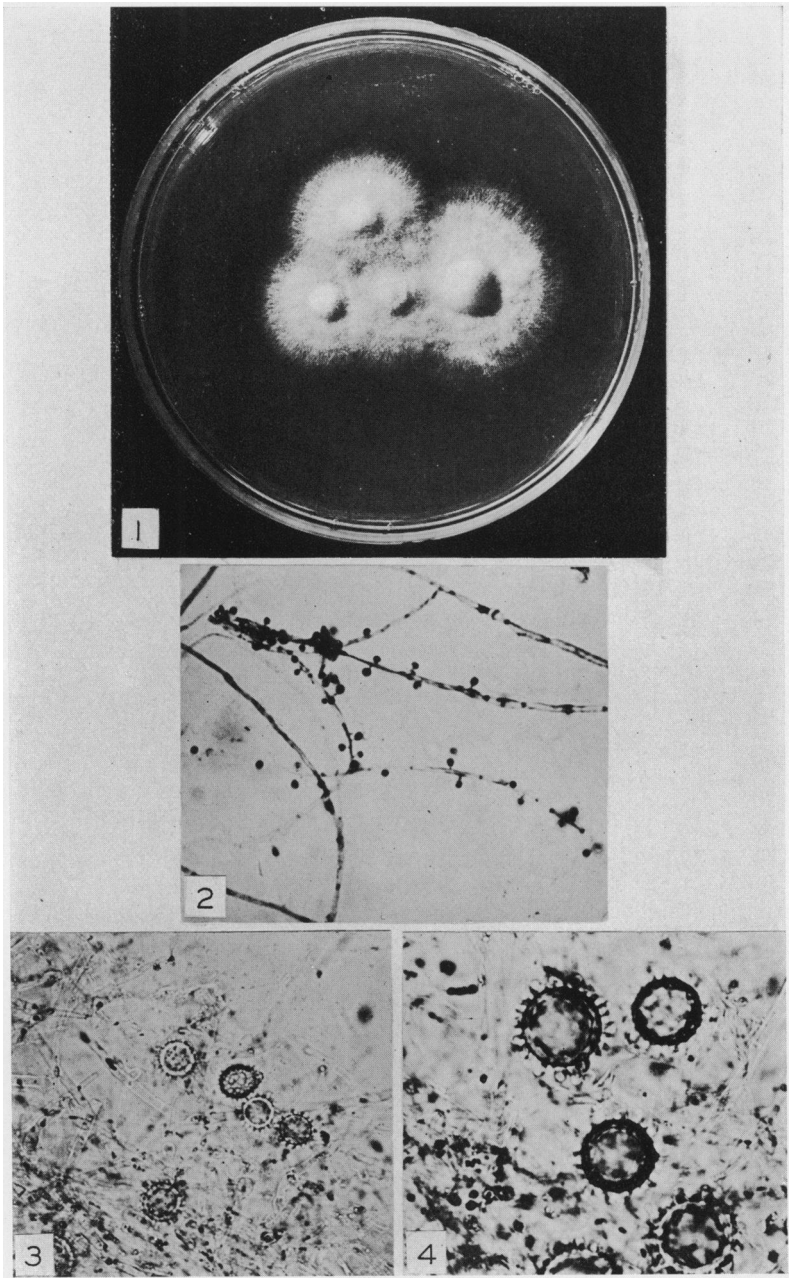
PLATE II

FIG. 1. White cottony growth of *Histoplasma capsulatum* on Sabouraud's glucose agar at room temperature.

FIG. 2. Hyphae and young smooth-walled chlamydospores from Sabouraud's glucose agar.

FIG. 3. Large thick-walled tuberculate chlamydospores from Sabouraud's glucose agar.

FIG. 4. High magnification of tuberculate chlamydospores from Sabouraud's glucose agar.



(Norman F. Conant: Life-Cycle of Histoplasma Capsulatum)

PLATE III

FIG. 1. Yeast cells from blood agar incubated at 37°C.

FIG. 2. Yeast cells germinating with a single tube in 48 hours in Sabouraud's glucose agar cell culture at room temperature.

FIG. 3. Budding yeast cell germinating from each cell on Sabouraud's glucose agar.

FIG. 4. Single yeast cell with three germ tubes on Sabouraud's agar.

FIG. 5. Single yeast cell with two germ tubes on Sabouraud's agar.

FIG. 6. Budding yeast cell which has germinated with three tubes on Sabouraud's agar.

FIGS. 7 and 20. Smooth-walled chlamydospores which have germinated in Sabouraud's broth and produced chlamydospores in 8 days.

FIG. 8. Intercalary chlamydospore on Sabouraud's glucose agar.

FIGS. 9, 10, 15. Round tuberculate chlamydospores from Sabouraud's glucose agar.

FIG. 11. Pyriform tuberculate chlamydospore from Sabouraud's agar.

FIGS. 12 and 14. Small, round, smooth-walled sessile and terminal chlamydospores with oil droplets.

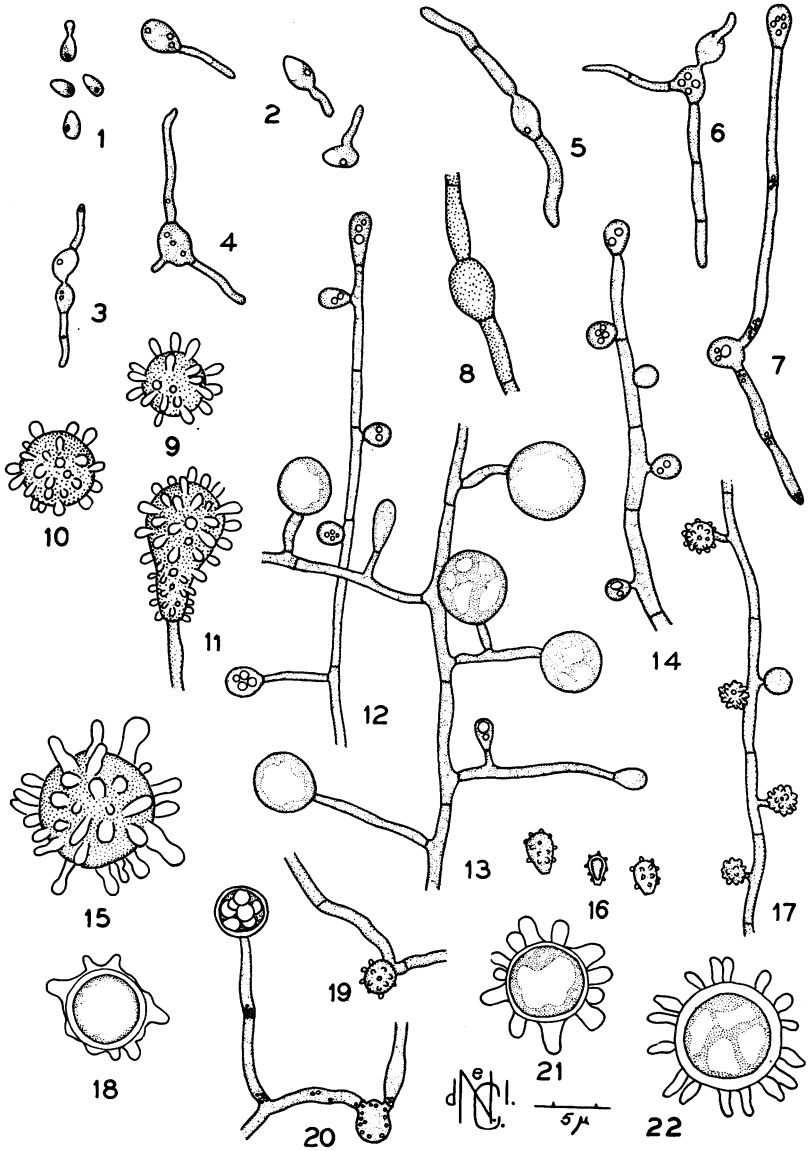
FIG. 13. Large round smooth-walled chlamydospores on Sabouraud's glucose slide culture in 14 days.

FIG. 16. Small pyriform tuberculate chlamydospores from Sabouraud's glucose agar.

FIG. 17. Small round tuberculate chlamydospores on aerial hyphae.

FIGS. 18, 21, 22. Optical view of thick-walled chlamydospores.

FIG. 19. Small tuberculate chlamydospore germinating in cell culture.



(Norman F. Conant: Life-Cycle of Histoplasma Capsulatum)