A COMPARATIVE STUDY OF FOUR STRAINS OF ORGANISMS ISOLATED FROM FOUR CASES OF GENERALIZED BLASTOMYCOSIS.*

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THE organisms which form the basis for this study were isolated from the following cases:

No. 1. Disseminated Blastomycosis—Bassoe.[1]

No. 2. Generalized Blastomycosis—Irons and Graham.[2]

No. 3. Generalized Blastomycosis, Case I—Christensen and Hektoen.[3]

No. 4. Generalized Blastomycosis, Case II—Christensen and Hektoen.[3]

For the sake of brevity I shall refer to them throughout this paper as organisms Nos. 1, 2, 3, and 4, respectively. The clinical and pathological details of the several cases are given in the original papers. A second recital would be mere repetition.

This work was undertaken with the hope that progress might be made toward a definite classification in a natural botanical system.

TECHNIQUE.

The usual cultural methods were used—growth on various liquid and solid media at room and incubator temperature under aerobic and anaerobic conditions, etc. In addition, methods employed in botanical research were used, such as the Van Tieghem cell, Wolbach's agar slides, and liquid paraffin cells.

The Van Tieghem cell consists of a hollow cylinder of glass a centimeter in height and diameter. The edges may be ground to secure firm union with glass slide and cover-slip which complete the cell. The glass ring and cylinder are cemented to a slide, and liquid media or distilled water are filled in to about one-third the height of the cylinder. A cover-slip is prepared as in a hanging-drop preparation and inverted over the upright cylinder. It may be fixed with paraffin or balsam. The purpose of the preparation is to afford means of watching day by day, for a period varying at the will of the investigator, the growth and development of an individual cell or group of cells. If the hanging-drop be broth, media of the same density should cover the bottom of the cell. If it be agar, distilled water should be used. By diffusion the inoculated portion remains constant in concentration, evaporation and absorption going on equally.

* Received for publication December 10, 1906.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 91.

[2] *Ibid.*, p. 666.

[3] *Jour. Amer. Med. Assoc.*, 1906, 47, p. 247.

Wolbach's[1] agar slides are made by flowing sterilized microscopic slides with melted agar, allowing the agar to harden, and inoculating by streaks with the platinum loop. A cover-slip is then placed over the inoculated surface, and the edges are sealed with a hot wire. I found it more serviceable to seal the edges with paraffin, particularly when the preparation is to last a week or so. The slides and cover-slips may be conveniently sterilized in the hot-air oven by placing them in Petri dishes.

Paraffin chambers were employed for watching cells submerged in distilled water, physiological salt solution, etc., and for anaerobic growths in nutritive liquid media. They are made by painting squares slightly smaller than the cover-slip on sterile slides with liquid paraffin. After hardening, a drop of the solution or medium is placed in the center of the square, inoculated, a sterile cover-slip dropped on, and its edges paraffined down. The preparation is useful in the same way as the Van Tieghem and Wolbach cells, enabling the observer to watch individual cells in liquid environment with a magnification as high as 1,000.

Dehydration with gypsum blocks was not attempted because of the negative results of Busse[2] and Ricketts.[3] A number of inoculations of white mice were made to assure continued pathogenicity.

COMPARATIVE STUDY OF THE ORGANISMS IN CULTURES.[4]

GROSS CULTURAL APPEARANCES.[5]

A. Solid media.

1. Glucose-agar stabs.

a) At room temperature.

No. 1. Fine white, delicate, aerial and submerged hyphae, apparent in 48 hours. Growth increases rapidly so as completely to cover the surface with a white, downy layer and to outline triangular area (base uppermost) along stab, in 7 to 14 days.

No. 2. Same. Firmly adherent to substratum.

No. 3. Same. Aerial hyphae not so abundant. Surface growth consists of waxy, crater-like elevation.

No. 4. Same. Aerial hyphae more prominent. Hyphae coarse and long.

b) At incubator temperature.

No. 1. Raised, waxy, irregularly spherical, yellowish-white, single colony in 3 to 5 days. No aerial or submerged hyphae visible at any time (30 days).

No. 2. Same. Few coarse, short, aerial and submerged hyphae at fifth day. Hyphae increase slowly, together with enlargement of waxy mass. "Porcupine" growth.

No. 3. Same as No. 1. On thirty-first day hyphae were noted. Were not present on seventeenth day.

No. 4. Same as No. 1. No hyphae at any time.

[1] *Jour. Med. Res.*, 1904, 8, p. 53.

[2] *Virchow's Arch.*, 1896, 144, p. 360.

[3] *Jour. Med. Res.*, 1901, 6, p. 293.

[4] Two complete sets of cultures were made with an interval of one month to note changes, should any occur, because of growth on artificial media. The data here given are a combination of both cultures as no marked variations were noticed.

[5] Duplicate inoculations on all media were made simultaneously, one tube placed in the room (temperature varying between 16° and 24° C.); the other in the incubator at 37° C.

2. Glucose-agar slants.
   a) At room temperature.
      No. 1. Elevated, moist, white, pin-point colonies along the streak at the end of 24 hours. Abundant submerged hyphae at end of fourth day. A few delicate aerial hyphae at end of seventh day; abundant submerged and aerial, such flattened and applied to surface at end of two weeks.
      No. 2. Long, coarse, aerial hyphae and abundant submerged at end of fifth day. In two weeks abundant, white, fine, downy, aerial growth covering agar and extending along glass.
      Nos. 3 and 4. Same as No. 1. No aerial hyphae at any time. Pin-head, nodular colonies on surface.
   b) At incubator temperature.
      No. 1. Delicate, elevated, moist, white, pin-point colonies along streak at end of 24 hours. Increase in density at end of two weeks; surface now covered with white, pasty growth, with some evidence of spherical colony formation. No aerial or submerged hyphae at any time.
      No. 2. Same as No. 1. Colonies more discrete, raised and firmly adherent to substratum.
      Nos. 3 and 4. Same as No. 1. Submerged, short, compact hyphae present on fifth day.
3. Glycerin-agar slants.
   a) At room temperature.
      No. 1. Beginning hyphal growth noticed on third day; submerged prominent on fifth day, both increase uneventfully till thirtieth day (last observation).
      No. 2. Same as No. 1. Growth apparent on second day. Aerial hyphae, coarse and vigorous; submerged, fine, and delicate.
      No. 3. Same as No. 1, except that no aerial hyphae are produced at any time (30 days). On surface white, waxy, discrete colonies.
      No. 4. Same as No. 3. No aerial; abundant submerged hyphae.
   b) At incubator temperature.
      No. 1. Elevated, waxy, nodulated growth on fifth day. Submerged hyphae on seventh day, slight. No aerial hyphae at any time. Growth increases slowly; on seventeenth day waxy growth elevated to 3 mm. above surface.
      Nos. 2, 3, and 4. Same as No. 1. Waxy, wormlike mass; crater-like in places.
4. Blood (ox) serum slants.
   a) At room temperature.
      No. 1. Flat, slightly elevated, spherical, pin-head growths. Few coarse aerial hyphae at end of first week.
      No. 2. Same as No. 1. One peculiar, long, coarse aerial hypha 8 mm. long extending from media to glass.
      Nos. 3 and 4. Same as No. 1. Colonies yellow-white, nodulated, more prominent. Apparent on second day.
   b) At incubator temperature.
      No. 1. Delicate, elevated, white, pin-point colonies along streak at end of

24 hours. Growth increases rapidly to form streak of raised, white, starchlike colonies.

No. 2. Same as No. 1. Colonies more spherical and discrete. Two coarse, short, aerial hyphae noted on fourteenth day.

Nos. 3 and 4. Same as on No. 1.

5. Gelatin stabs.

a) At room temperature.

No. 1. Fine, white, delicate, aerial and submerged hyphae apparent in 48 hours. Growth increases completely to cover surface with characteristic, white, downy mass. No liquefaction.

Nos. 2, 3, and 4. Same as No. 1.

6. Potato.

a) At room temperature.

No. 1. Fine, white, delicate hyphae beginning on the third day. Increases to cover entire streak at end of 14 days. Nodular colonies increase simultaneously.

Nos. 2, 3, and 4. Same as No. 1.

b) At incubator temperature.

No. 1. White and brownish-white, raised, compact, pin-point to pin-head nodules apparent on fifth day along streak. Increase until, as potato dries up, growth becomes stationary, white and pastelike.

No. 2. Difficult to get growth at end of two weeks. Few, dried, scattered, pastelike patches on surface.

No. 3. Same as No. 1; growth on third day.

No. 4. Same as No. 1; growth not vigorous.

B. Liquid media.

1. Broth.

a) At room temperature.

No. 1. Fluffy, compact, thistle-down growth beginning in 24 hours. Supernatant fluid clear. Growth increases in size and density. Always strongly coherent.

Nos. 2, 3, and 4. Same as No. 1.

b) At incubator temperature.

No. 1. Fine granular detritus and sediment at bottom of tube in 24 hours. Can be shaken up to form cloud. Some evidence of thistle-down growth on seventh day. Same increases slowly till on thirtieth day thistle-down growth is more strongly in evidence.

No. 2. Short, coarse, compact hyphae at end of 24 hours, increasing slowly and inclosing nodular colonies in mycelial mesh-work. On thirtieth day mycelium increased to resemble growth at room temperature.

No. 3. Fine granular detritus. Slight fluffiness later.

No. 4. Same as No. 3.

2. Dunham's medium.

Indol produced in no instance.

3. Litmus milk.

a) At room temperature.

No. 1. Slight acidity at end of second day. Continues without change for two weeks. No coagulation.

No. 2. No change from control at end of first week. Some time between that date and the end of the second week complete coagulation occurred with slight alkalinity.

No. 3. Slight acidity on fifth day. No coagulation.

No. 4. Possibly slightly acid on fifth day. Later (fourteenth day) no acidity. No coagulation.

b) At incubator temperature.

No. 1. Slight acidity on fifth day. At fourteenth day color somewhat fainter, lighter, and pinker than control. Fat globules on surface.

Nos. 2, 3, and 4. Same as No. 1.

SUMMARY OF GROSS CULTURAL APPEARANCES.

1. The four strains of organisms appear nearly identical, so far as growth in test-tubes goes. A few minor differences are summed up below, under 6.

2. The organisms grow vigorously on the usual laboratory media, with perhaps a slightly more abundant growth on faintly acid glucose-agar.

3. Temperature is perhaps the most important factor in varying the gross and microscopic morphology: room temperature favors production of mycelia and aerial hyphae; incubator temperature inhibits production of hyphae and favors coherent, waxy, yeastlike colonies (budding forms).

4. Those cultures which produce yeastlike growths at incubator temperature develop hyphae within 24 hours when withdrawn and placed at room temperature. Likewise the majority of yeastlike colonies will finally (in 17 to 30 days) show evidence of beginning hypha formation even if kept at 37° C.

5. Glucose-agar stabs and broth form the most serviceable culture media if a limited variety is at hand. Duplicates should always be made to control differences in morphology at room and incubator temperature.

6. The minor differences referred to in 1 are:

a) Firm adherence to substratum of all cultures on solid media of organism No. 2. Growths in incubator of organisms Nos. 1, 3, and 4 are readily removed.

b) Production of hyphae in all cultures of No. 2. It was impossible to obtain pure budding forms. Organisms Nos. 1, 3, and 4 may be obtained in pure budding forms in stab growths in incubator.

c) Formation in the case of organism No. 2 of compact mycelial and nodular growths in broth in incubator, while organisms Nos. 1, 3, and 4 form granular detritus (early) with no mycelium.

d) Coagulation of milk with slight alkalinization by organism No. 2, whereas organisms Nos. 1, 3, and 4 do not coagulate and cause but slight acidity.

MICROSCOPIC APPEARANCES OF ORGANISMS UNDER CHANGING EXPERIMENTAL CONDITIONS.

Observations were made of particular individual cells and recorded from time to time preliminary to studying the methods of hyphal production. Budding forms were inoculated anaerobically[1] on liquid broth and glucose-agar, using the paraffin and Wolbach preparations as described under "Technique." Slides were placed in the room on the warm (37° C.) stage. Budding and hyphal production occurred irrespective of temperature, though the former was more marked on the warm-stage growths.

It was possible to observe the actual process of growth in a few instances. In those cells which were about to bud or sprout the heavy, refractile outer wall was seen to put forth at one point two short, collar-like projections.[2] This undoubtedly was due to a break in the capsule and a drawing-back of the protoplasm on each side. Gilchrist noted and drew these collar-like projections in tissue, but, so far as I know, they have not been seen in cultures before.

The projection of the protoplasm occurs fairly rapidly, resembling closely the projection of pseudopodia by protozoa. The protoplasm which precedes is homogeneous, the finer and coarser refractile bodies remaining in the mother-cell for some time. The mother- and daughter-cells may easily be differentiated for a long time because of the heavy-contoured periphery of the parent cell.

I could detect no difference in the reproductive process which would suggest why in one case buds formed and in another hyphae developed. In some instances both buds and hyphae were seen to arise from the same mother-cell. In any particular field watched for 24 hours all the cells did not show evidences of reproductive processes. In one case four cells out of a field of 27 showed beginning hyphal buds at the end of 24 hours; in another, four out of 10 showed reproductive changes; in a third, three out of 15, etc. It is interesting to note in this connection that the warm-stage slides showed a higher proportion of growing cells than preparations kept at room temperature.

One constant finding on solid media was the production of bodies which may be one of a number of botanical possibilities. I refer to the formation of clusters of botryoidal bodies at the end of hyphae, at the end of short lateral buds, and, in some cases, within a short distance of a sphere to which they are attached with a long, hypha-like stalk. Figs. 1, 2, and 3 show various types of these clusters. In older cultures growing at room temperature in which these bodies were not present, they

[1] The preparations were at least practically anaerobic. The only oxygen which may have been used must have come from that dissolved in the media. No visible "bubbles" of air were allowed in the preparations.

[2] These projections are doubtless the sides of a cylindrical or funnel-shaped collar through which the protoplasm is extruded. The appearance noted is due to refraction in optical section

could be made to form by placing the culture in the incubator (Fig. 3). Forms similar to the one drawn in Fig. 4 were found in younger growths. Apparently the bulbous enlargement at the tip bursts to form atypical clusters. No such actual bursting was ever observed.

The question at once presents itself: What are these bodies? At the present time one cannot decide positively. In no case was it possible to cause the liberated bodies to bud or produce hyphae, although transfers were made into solid and liquid media under aerobic conditions, at room and incubator temperature, etc. For the present, therefore, I believe it would be in the interest of conservatism to regard them as bulbous hyphae with liberated protoplasmic and food granules produced by the bursting of a turgid protoplasmic mass at 37° C. and in an anaerobic, solid medium.

Hyphae, on solid media, grew apparently in the direction of the oxygen supply, as shown by the prevalence of the growth in the proximity of the paraffin border. In those cultures which had been growing for two weeks unusual forms were produced at the distal end of hyphae. Fig. 5 shows the end of such a hypha. Fig. 6 shows a more advanced stage of the same process. It is not clear just what method of growth is represented. It may be simply a vegetative production of lateral branches or a reproductive growth from spores germinating within a mother-cell. In the absence of more exact botanical proof, I should be inclined to regard the former as the real condition present.

The phenomena noted thus far have been those observed for the most part on solid media. Liquid preparations in paraffin cells were also productive of results. Notably the relationship of budding forms to the oxygen supply was well shown in the case of an eight-day preparation of organism No. 3 growing at room temperature in broth. Through the accidental chipping-off of a small particle of the paraffin border, air was allowed to get into the preparation, with a resulting gradual evaporation of the medium. Fig. 7 was made of this slide, *c* to *b* being the part of the slide from which the broth was evaporated; *a* to *b*, the remaining medium and cells. The amount of hyphal production is seen to be dependent on the proportion of dissolved oxygen and proximity to incoming air.

Various experiments of submersion under distilled water and salt solution, etc., were not productive of anything new. In some instances the organisms were killed without change; in others plasmolysis occurred; in others the peripheral theca was seen to be disrupted, liberating contained bodies; in others large chlamydospores (resting cells) were formed (Figs. 8, 9, and 10).

The results of observations of the growing organism may be grouped as follows:

1. It is possible, by appropriate methods, to watch the germination of fungi from hour to hour and day to day for a period of a month or longer. Only by such methods can one hope to arrive at the complete life-cycle.

2. The collar-like projections, observed by Gilchrist in tissue, indicative of a rupture of the capsule, may be observed in artificial media.

3. A temperature of 37° C. favors production of budding forms

(as in gross aerobic cultures) as well as stimulating both reproductive and vegetative germination.

4. Involution forms are produced in from three to eight days or longer in anaerobic glucose-agar cultures. These should be regarded, for the present, as burst hyphal enlargements. It cannot be said that the enlarged, ramified enlargements of older (two weeks) hyphae are asci.

5. The organisms are facultative anaerobes on glucose-agar, but obligate aerobes in broth.

6. Immersion in distilled water, KOH, etc., resulted in death of cells, with or without plasmolysis, rupture of cells in some cases, formation of large resting cells in others.

FERMENTATION EXPERIMENTS.

Eight sugars were used as a basis for these experiments: dextrose, lactose, levulose, nutrose, galactose, maltose, mannit, saccharose; and also *inulin*. Fermentation tubes were inoculated with the four organisms (nine each) and placed in the incubator for 16 days. In no case was carbon dioxide generated. A few of the tubes showed gas bubbles at the top. These were submitted to the KOH absorption test for CO_2. Alcohol and acetic acid were not tested for. In view of the negative results, we must conclude that the four organisms are not sugar-inverters and fermenters and may be classed with similar organisms in Ricketts' group, of which the fungus from Case 12 may be taken as a type.

ANIMAL EXPERIMENTS.

White mice were used as hosts. Because of lack of time a few only were injected, and these simply to assure pathogenicity. The injections, consisting of a 0.5 to 0.8 c.c. of a salt suspension of budding forms, obtained from agar colonies growing at 37° C., were made intraperitoneally with the usual precautions. All of the mice died in from four to 11 days after the injection. Typical subcutaneous abscesses formed in the cases of mice inoculated with organisms Nos. 1 and 4, and characteristic budding forms were found in the pus. Organisms were not recovered from the other mice.

SUMMARY AND CONCLUSIONS.

1. Four strains of organisms isolated from four cases of generalized blastomycosis appear identical.

2. Pronounced variations in the gross and microscopic morphology of the organisms are produced by variations in temperature. As a routine for purposes of study cultures should be grown at both room and incubator temperatures.

3. Botanical methods for watching the development of individual cells are the most useful for studying the life-processes of fungi. By

PLATE 5.

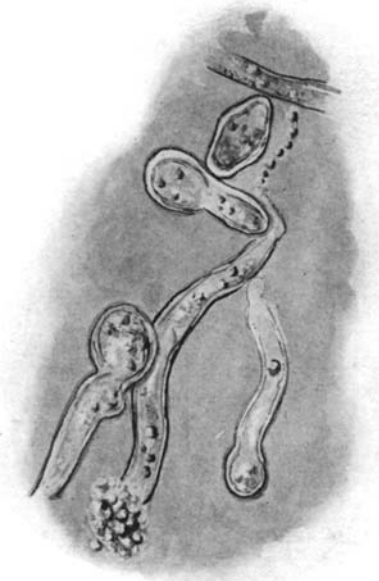

FIG. 1.

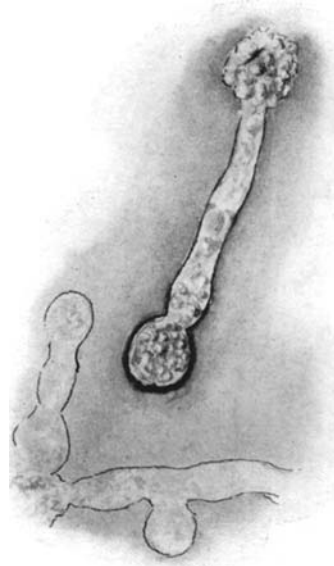

FIG. 2.

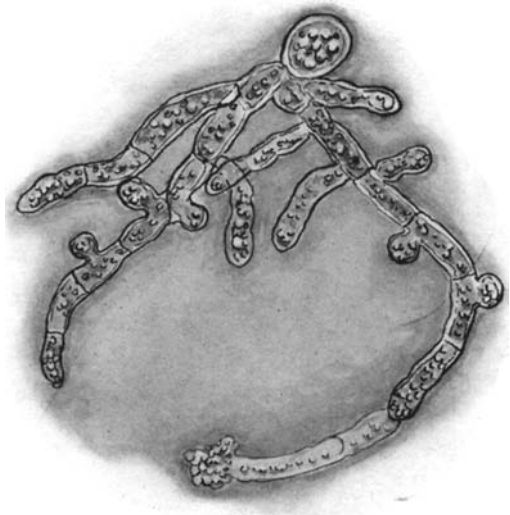

FIG. 3.

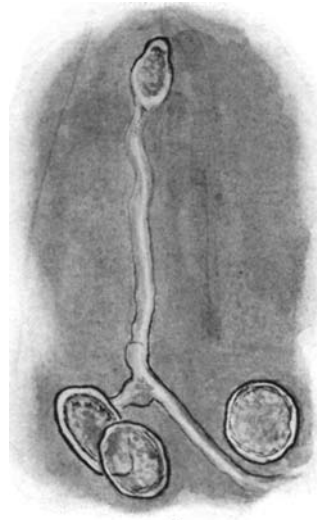

FIG. 4.

PLATE 6.

FIG. 5

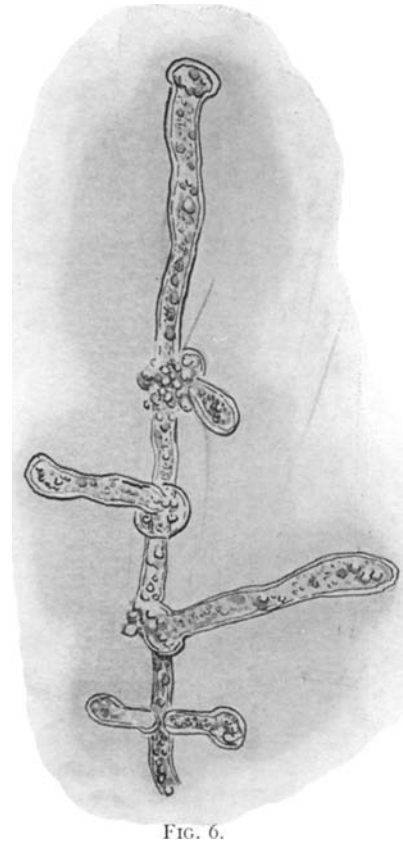

FIG. 6.

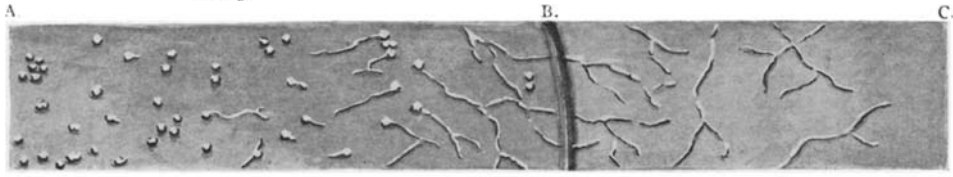

FIG. 7.

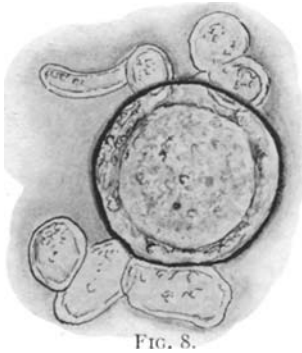

FIG. 8.

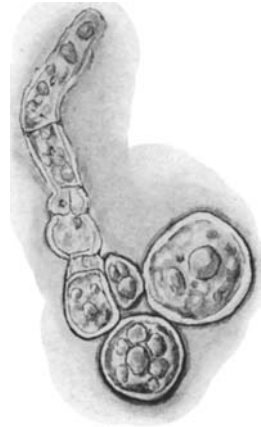

FIG. 9.

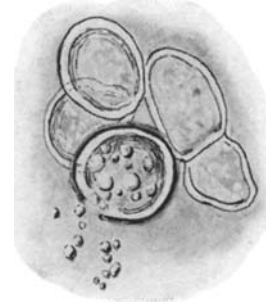

FIG. 10.

means of such methods many unusual morphologic forms may be observed.

EXPLANATION OF PLATES 5 AND 6.

FIG. 1.—Typical ruptured hypha (sporangium (?) ascus (?)) showing free clusters (spores(?), protoplasmic granules (?)). Organism No. 1; 72-hour anaerobic growth at 37° C. on glucose-agar slide. ×1000.

FIG. 2.—Another frequent type of ruptured hypha occurring at short distance from vegetating cell. Organism No. 3; 72-hour anaerobic growth at 37° C. on glucose-agar slide. ×1000.

FIG. 3.—Ruptured hypha occurring in oidial type of growth. This culture at the end of seven days' growth at room temperature had not produced typical group clusters. Accordingly the slide was put into the incubator, whereupon, in 24 hours, the phenomenon occurred. The more lightly shaded, less refracting body with attached clusters was produced during that time. Organism No. 3; 11-day anaerobic growth on slide. ×600.

FIG. 4.—Enlarged bulbous extremity of hypha before rupture. Shows manner of production of group clusters. Organism No. 1; 67-hour anaerobic growth at 37° C. on glucose-agar slide. ×1000.

FIG. 5.—Tip of old (15 days) hypha showing unusual (involution(?)) morphology. Asci (?); a lateral branch formation (?). Organism No. 2; 15-day anaerobic growth at 37° C. on glucose-agar slide. ×1000.

FIG. 6.—Same as Fig. 5. Later period of growth. Organism No. 2; 15-day anaerobic growth. ×1000.

FIG. 7.—Hyphae production in liquid medium and its relation to oxygen supply. No hyphae were produced until, through the accidental chipping-off of the paraffin border, air was allowed to enter, *b* to *c* being the part of the slide from which the broth has evaporated, *a* to *b* the remaining medium and cells. The hyphal production may be seen to be dependent on the proportion of dissolved oxygen and proximity to incoming air. Organism No. 3; eight-day growth at room temperature in parraffin slide of broth. ×80.

FIG. 8.—Chlamydospore (resting cell) occurring after 72 hours' submersion in distilled water. Organism No. 3; 72-hour culture at room temperature in distilled water. ×1000.

FIG. 9.—Plasmolysis occurring after four to eight days' immersion in 0.85 per cent NaCl. Organism No. 4; eight-day culture at 37° C. in physiological salt solution. ×1000.

FIG. 10.—Ruptured sphere and extrusion of cell contents after mounting culture in 1 per cent KOH solution. Organism No. 1. Slide prepared after 72 hours' growth on aerobic glucose-agar slant. ×1000.