NOTES ON BACILLUS BOTULINUS

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The production of heterologous toxins by different strains of *B. botulinus* was first recognized by Leuchs, in 1910. He produced antitoxins in horses for both the Ellezelles and Darmstadt strains and found that the antitoxin of the Ellezelles strain would protect against the toxin of its own strain but not against the toxin of the Darmstadt strain, and vice versa.

In 1917 Dr. E. C. Dickson of the Stanford Medical School, San Francisco, produced antitoxins in three goats, which were immunized against his strains III, IV and VI, respectively. He found that strains III and IV, which he had isolated from outbreaks of botulism in California and Oregon were homologous. Strain VI, however, which was isolated in Albany, New York, from cheese which had caused the death of several persons, produced a toxin which was not neutralized by the antitoxin of either strain III or strain IV. Neither would the antitoxin of strain VI neutralize the toxins of strains III or IV.

By means of toxin-antitoxin tests I have identified twelve strains of *B. botulinus* from material obtained in different parts of California and one from an outbreak of food poisoning in Seattle, Washington. Five of the California strains came directly from outbreaks of poisoning from home-canned vegetables and fruits. One came from hay suspected in a case of forage poisoning in horses. The other six had no direct connection with cases of poisoning. (Burke, 1919 b). These twelve strains fall into two distinct types as shown by the toxin-antitoxin

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experiments. The antitoxin of one type neutralizes the toxin of its own type but not of the heterologous type. I have designated the two as *B. botulinus* Type A and *B. botulinus* type B. The type A strains usually produce a stronger toxin in broth than the type B strains—but aside from this their cultural characteristics on 2 per cent glucose beef infusion agar, double strength beef infusion broth (2 per cent glucose), brain and cooked meat media are identical. There is no difference in morphology.

Dr. Dickson's strains III and IV, to which reference has been made above, are type A strains. The strain which was isolated from cheese by Dr. Nevins in Albany, New York, and which Dr. Dickson refers to as strain VI in his series, is a type B strain. In September 1918 Dr. Robert Graham of the University of Illinois sent me eight cultures containing *B. botulinus* which he had isolated from material from forage poisoning cases among horses and mules in Illinois and Kentucky and from a human case of botulism, traced to canned beans, in Decatur, Illinois. I have tested these cultures against the two types of antitoxin and have found that Dr. Graham's strains are all homologous with my botulinus type B.

It is a point of interest that all of the above nine strains which were isolated in Illinois, Kentucky and New York State should belong to type B, whereas in the Pacific Coast States Dr. Dickson and I have found type A to be predominant. Out of a total of fourteen strains from eight different localities we have isolated nine type A strains and only five type B strains.

PRODUCTION OF BOTULINUS ANTITOXIN FOR LABORATORY PURPOSES

Goats are the best animals to use in producing antitoxin for laboratory tests. As they are very resistant to the toxin of *B. botulinus*, the dosage can be rapidly increased and a good antitoxin produced within a period of two months.

Where goats are not available a weak antitoxin may be produced in rabbits. This can be satisfactorily used in identifying

strains of *B. botulinus* if the toxin is first carefully titrated to determine the minimal lethal dose.[2] I succeeded in carrying two rabbits, one for type A and one for type B toxin, through the eighth injection. The initial dose was about one-fourth of a minimum lethal dose. The dose was increased very gradually each week. At the eighth week each rabbit received 10 minimum lethal doses. After this injection one of the animals developed severe symptoms of botulism and it was thought unwise to carry the injections further as five rabbits had already been lost. It was found that the serum of each rabbit contained a weak antitoxin which would neutralize from six to ten minimum lethal doses of the homologous toxin.

TECHNIQUE FOR ISOLATING B. BOTULINUS

B. botulinus may be readily isolated from agar transplants made direct from the contents of cans of spoiled food because there are, as a rule, few other bacteria present.[3] When however, materials such as manure and intestinal contents are to be tested, it is difficult to recover the organism from the original agar transplants because material of this sort is rich in strict anaerobes and facultative anaerobes and the number of botulinus bacilli is comparatively small.

The most reliable technique is to inoculate from the original material into cultures of Van Ermengem's broth[4] and chopped meat media.[5]

[2] One minimum lethal dose of botulinus toxin is that amount which will kill a 250 gram guinea-pig in forty-eight hours.

[3] In one case I have isolated *B. botulinus* from a jar of beans which showed signs of spoilage but in which no toxin could be demonstrated by animal inoculation. The strain was a typical type B strain, producing a moderately strong toxin in Van Ermengem broth culture. This demonstrates the importance of making cultural as well as toxin tests on canned food sent in to the laboratory for examination.

[4] 1000 grams of lean beef to the liter, 5 grams NaCl, 10 grams Witte's peptone, 2 per cent glucose. The medium oil stratified in the tubes. (See Van Ermemgem, E. 1912.)

[5] Finely chopped beef heart or lean meat is mixed with two volumes of water and brought slowly to the boil, being stirred constantly. It is neutralized, tubed and autoclaved at 15 pounds pressure for 30 minutes.

The broth and meat cultures are richly inoculated with the suspected material. Duplicate cultures are made, one set being heated to 60°C. for one hour to destroy the non-spore bearing organisms. The cultures are then incubated at 28°C. after which portions are filtered and tested for toxin by injecting 1 cc. of the filtrate subcutaneously into a 250 gram guinea-pig. If the pig dies within four days the filtrate is tested against the antitoxins of *B. botulinus* types A and B to determine the presence of specific botulinus toxin. For this purpose the filtrate is diluted with saline so that 1 cc. will kill a 250 gram guinea-pig in approximately forty-eight hours. Three 250 gram guinea-pigs are selected. The first pig receives 1 cc. of diluted filtrate mixed with antitoxin for *B. botulinus* type A. The second pig receives 1 cc. of the diluted filtrate mixed with antitoxin of *B. botulinus* type B. The third pig is the control and receives 1 cc. of the diluted filtrate but no antitoxin. Pure cultures of *B. botulinus* type B produce usually a little more than one minimal lethal dose of toxin per cubic centimeter after three days growth in meat or Van Ermengem broth medium. Type A strains produce a stronger toxin in the same time. In mixed cultures, however, the toxin production may be much delayed and a negative diagnosis should not be made until the cultures have been incubated for three weeks.

The organism is isolated from the broth or meat culture in which specific toxin is demonstrated. In place of the usual anaerobic plating methods it is the practice in this laboratory to use thinly seeded agar shake cultures. After incubation these cultures are examined and fished by transmitted light on the stage of a dissecting microscope such as is used in Zoological laboratories. A lens magnifying 10–20 diameters is most satisfactory. (See E. C. Dickson and G. S. Burke, 1918.)

The development of this technique was necessary because the hydrogen plating methods used at first were too slow to permit of the examination of large amounts of material. Shake cultures are easily and quickly made and there is little chance for contamination. The magnification of 10 to 20 diameters afforded by the dissecting microscope lens gives a clear and detailed

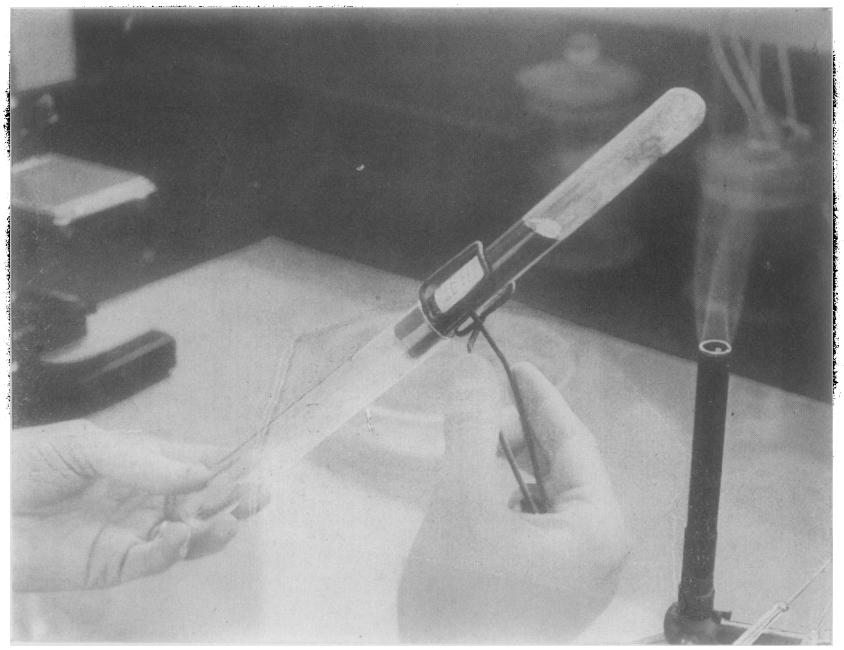
colony picture which is much more satisfactory for the observation of deep agar colonies than that given by the compound microscope. The method has proven so accurate and economical both of time and materials that it seems worth while to give a more detailed description than was given in the former paper.

In detail the technique is as follows. A series of agar shake cultures (5–8) are made from the contaminated Van Ermengem broth cultures, jars of food, etc. (Beef infusion agar, plus 2 per cent glucose, is used, with a reaction as near neutral as possible.) Just before the inoculation is made the agar is boiled for twenty minutes and cooled to approximately 47°C. It is essential to the success of the technique that the agar cultures be thinly seeded in order that the colonies shall be of good size and well separated. Successive dilutions may be used as in plating. As soon as the agar tube is inoculated it is thoroughly shaken and plunged into cold water to harden, after which it is incubated. One very good method of mixing the material in deep agar tubes is to hold the tube in a vertical position grasping it by its upper end. By twisting the wrist the lower end of the tube is then rotated about the vertical axis while the upper end remains practically stationary.

Botulinus colonies may appear in twenty-four hours at 37½°C. or in thirty-six hours at 28°C. Under some circumstances they require a much longer time. (Burke, 1919 a.) In mixed cultures the presence of antagonistic organisms may inhibit the growth. One set of agar cultures should be incubated at 37½°C. and another at 28°C., since *B. botulinus* grows almost equally well at either temperature while some of the contaminating organisms are distinctly inhibited by one or the other temperature. After incubation the colonies in the agar tube are observed and fished under the dissecting microscope by transmitted light.

There are two methods of preparing the agar culture for fishing. In the first, the cotton plug is removed from the culture tube and the tube inverted, with the open end pointed downward into a sterile tube of greater diameter than that of the culture tube. The closed end of the agar culture tube is then passed slowly into the flame of a bunsen burner. The expansion,

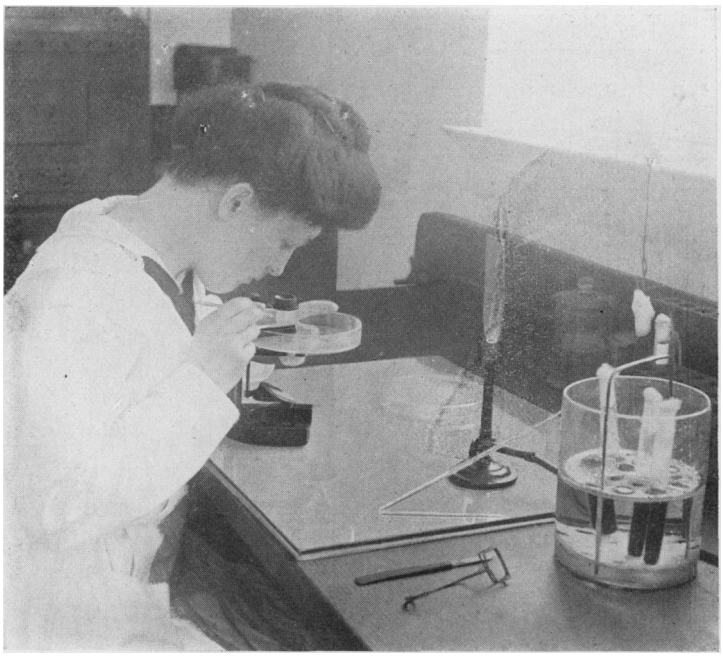
caused by the heat, drives the agar column out of the culture tube into the large tube, from which it is emptied into a petri dish for fishing. In the second method a sharp file mark is made around the culture tube about one-half inch from the bottom. The tube is then placed in a large petri dish and broken by applying a piece of red hot glass rod to the file mark. By driving the cotton plug down into the culture tube, the agar



column is pushed out of the broken end so as to expose any desired colony.

The colony chosen for fishing is placed in position under the dissecting microscope lens. The surface of the agar above the colony is sterilized by brushing a red hot platinum loop very lightly over it. The agar should be slightly melted by this operation, giving a smooth glossy surface through which a clear image of the colony can be had. A short platinum needle (1 to $1\frac{1}{4}$ inches) is used for fishing. This is sterilized and plunged down through the sterile agar surface into the colony.

Agar shake cultures are made from the material fished from the colonies. These transplants are as a rule thinly seeded. If they are not pure it is an easy matter to find in them well isolated colonies which can be successfully fished without contamination. Final identification of the isolated organism is made by culturing it in broth or meat and testing the filtrate for specific botulinus toxin.



COLONY CHARACTERISTICS OF B. BOTULINUS

Deep agar colonies of *B. botulinus*, as seen under the dissecting microscope with a magnification of 10 to 20 diameters are very characteristic. The description here given is based upon a study of nine pure strains isolated at Stanford University. Seven of the strains were isolated from mixed cultures on the basis of these colony characteristics.

In a young culture the typical Botulinus colony (see plates 1 and 2) is a thin translucent disc, having a small opaque knot or "nucleus" on or near the periphery at one side. The edge of the disc is either flattened or sharply indented at the point nearest the knot. The disc is more or less completely filled with clear spots or "vacuoles" which are in reality gas bubbles caught in the meshes of the bacterial growth as shown by examination of stained smears of the colonies. The outline of the colony is always clear cut and definite.

The translucent appearance of the colonies is due to the comparative thinness of the disc and to the presence of the gas bubbles. The gas bubbles may be of any size. Sometimes they are so large that seven or eight of them fill the disc. No photograph was obtained of such a colony. Again they may be so small as to look like little white specks. The gas bubbles when visible are diagnostic of *Bacillus botulinus*. I have been unable to find any other anaerobe producing this type of colony, although I have examined more than 400 cultures from material rich in anaerobes, and also cultures of the common pathogenic anaerobes.

The knot or "nucleus" in a young culture is very small and dense. In old cultures it develops short stubby processes, which are nevertheless translucent. The diameter of the tuft thus formed, however, is never greater than the diameter of the disc.[6] One of my strains rarely shows any "nucleus" or irregularity of outline, but its gas bubbles are very distinct.

Another very typical feature of botulinus colonies is the transparency of the disc in old cultures after three to six weeks incubation. See plate 3, figures 1 and 2.

In plate 2, figure 3, a very small colony can be seen at the edge of the large colony. The disc is comparatively small and

[6] The pictures of botulinus colonies given by V. Hibler (1908) are similar to the colonies of certain anaerobes which I have encountered as contaminants in my work with *B. botulinus*. Colonies of that type with a heavy hairy tuft at one side of the disc are not found in pure cultures of the strains of *B. botulinus* which we have in this laboratory.

has the appearance of a clear gas bubble. This suggests that the opaque knot is the original nucleus of growth and that as gas is produced the agar is split and the bacteria grow into the space thus formed. The agar is sometimes split in more than one plane (plate 1 and plate 3, figs. 4 and 5).

The colony characteristics of *B. botulinus* are the same on either extract or infusion agar and the reaction of the medium does not affect them. The variations which occur are apparently due to a difference in the stiffness of the medium or to the size of the colonies. In very large colonies it is difficult and sometimes impossible to see the gas bubbles.

GENERAL CULTURAL CHARACTERISTICS OF B. BOTULINUS

The optimum temperature for *B. botulinus* as originally recorded was 22°–28°C. The pure strains isolated at Stanford, however, grow somewhat more rapidly at 37½°C. than at 28°C. At 37½°C. agar shake cultures show well defined and typical growth in 24 hours. At 28°C. 36–40 hours are required for the same growth, and at lower temperatures the rate of development is still slower. Toxin is produced at 37½°C. as well as at 28°C.

B. botulinus is a proteolytic anaerobe. It digests and darkens but does not blacken meat and brain media. It produces strong toxin in meat cultures and the organisms remain viable for a much longer period in meat and brain cultures than in broth or agar.

In Van Ermengem broth cultures after 40 hours incubation at 28°C. *B. botulinus* produces a delicate clouding of the medium. If the culture is not shaken the broth just under the oil is usually found to be clear. After incubating for a month or more the cloud disappears leaving the broth clear. In a pure culture there is no noticeable sediment in either the young or old cultures. Other observers have described a heavy sediment in old cultures but in my experience a heavy sediment is a sign of contamination.

My strains of *B. botulinus* do not spore readily in either broth

or agar. In brain media spores sometimes appear in two weeks but usually it requires a much longer period.

In infusion agar, 2 per cent glucose, the anaerobe line for *B. botulinus* is one centimeter below the surface. When extract agar is used, however, the anaerobe line drops to two centimeters. For this reason extract agar tubes should contain at least three inches of agar.

SUMMARY

1. There are two known types of *B. botulinus*, which are here designated type A and type B. They produce heterologous toxins and are easily identified by a toxin-antitoxin test.

2. Antitoxin for laboratory purposes may be produced from either goats or rabbits. Goats are the more satisfactory animals.

3. The most reliable method of demonstrating the presence of *B. botulinus* in contaminated material is to make Van Ermengem broth or meat cultures, incubate for three weeks, filter and test the filtrate for specific toxin.

4. The use of agar shake cultures, examined and fished under a dissecting microscope, is a simple and reliable technique for the isolation of *B. botulinus* from contaminated material or cultures.

5. *B. botulinus* produces colonies in agar which are characteristic and can be readily identified.

6. The strains of *B. botulinus* isolated at Stanford University grow and produce toxin as readily at 37½°C. as at 28°C. They do not produce a heavy sediment in broth and they do not produce spores readily in either broth or agar.

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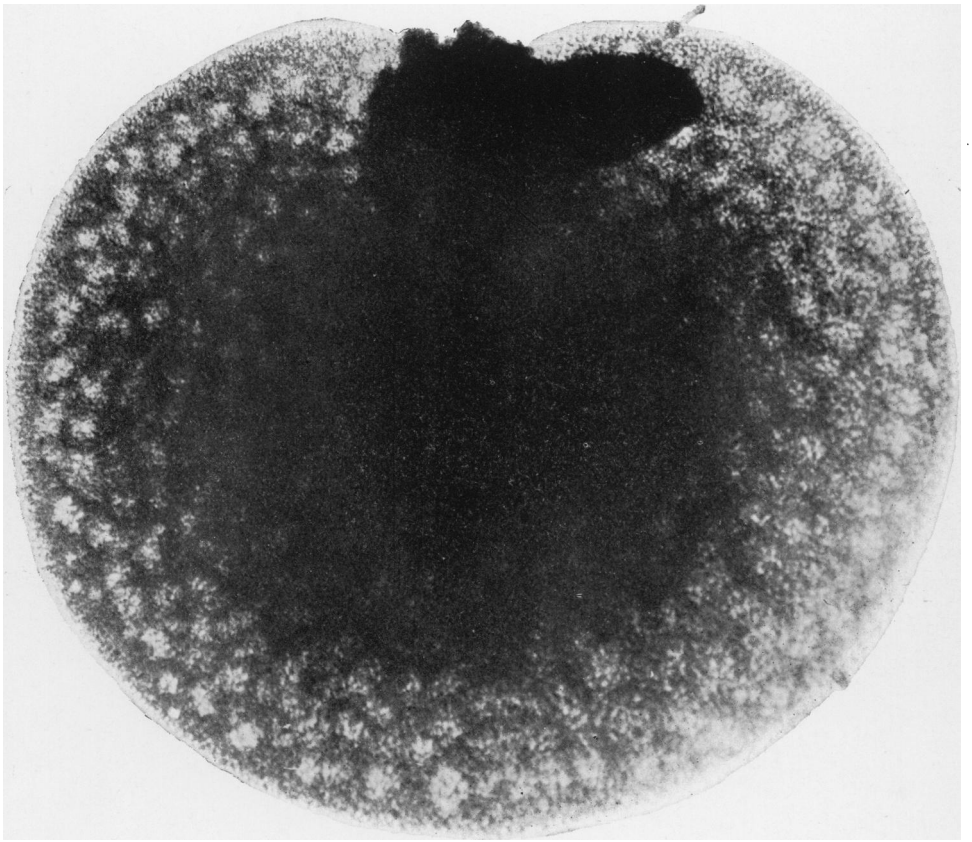
PLATE 1

Fig. 1. Deep agar colony of *B. botulinus* from forty-eight hour culture. *d* indicates the edge of a small disc lying in a different plane from the large disc.



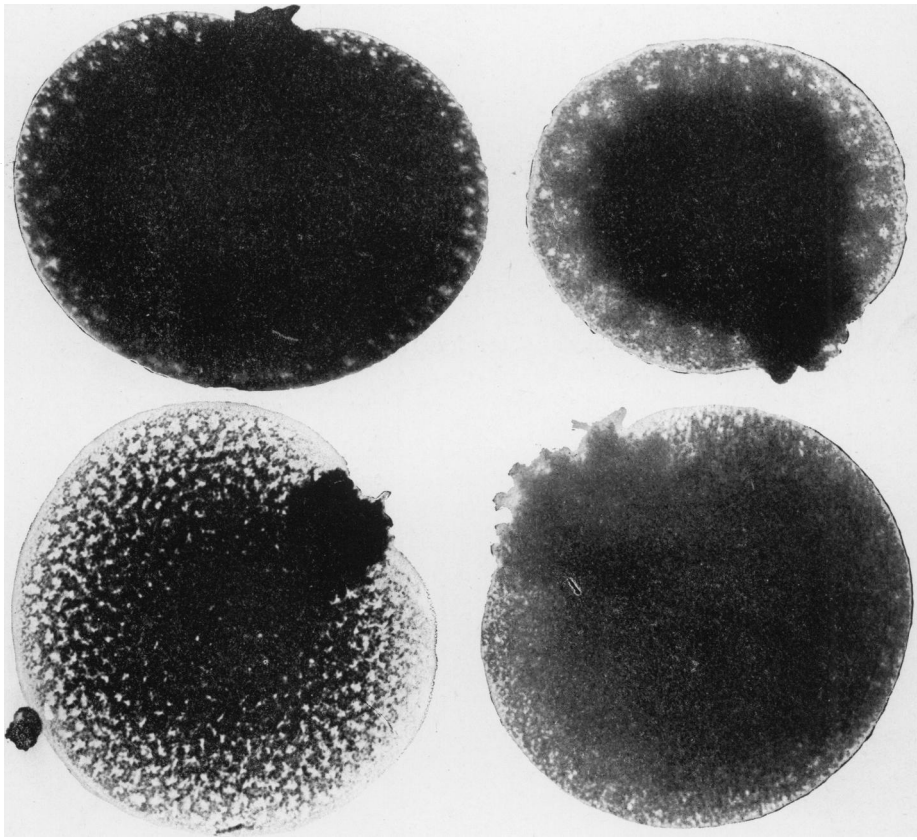
(Burke: Notes on *Bacillus botulinus*)

PLATE 2

Figs. 1 and 2. Deep agar colonies of *B. botulinus* from forty-eight hour culture, showing varying size of gas bubbles.

Fig. 3. Deep agar colony of *B. botulinus* from a week old culture, with a very small colony lying near the edge of the large colony.

Fig. 4. Deep agar colony of *B. botulinus* from a week old culture, showing minute gas bubbles in the disc, and branching processes of the "nucleus."



(Burke: Notes on *Bacillus botulinus*)

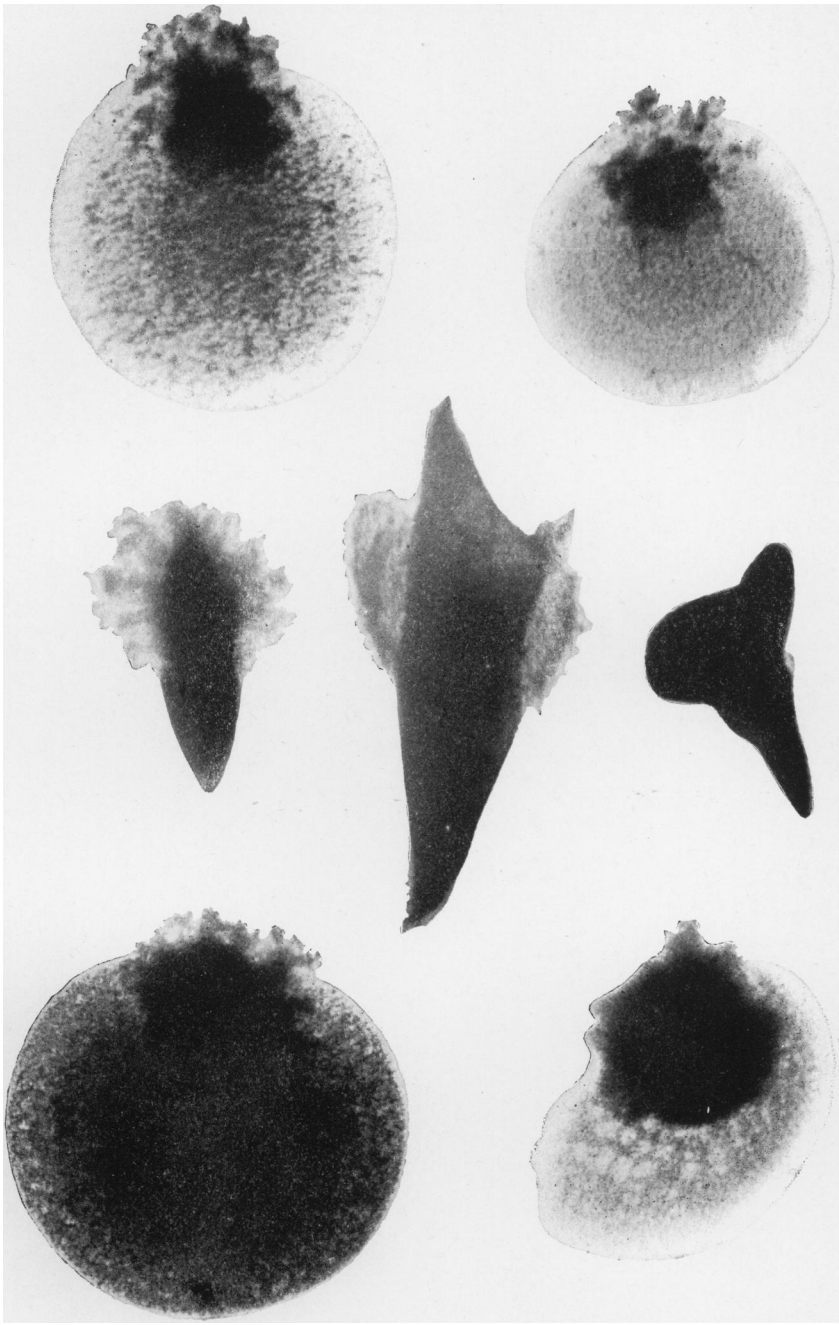
PLATE 3

Figs. 1 and 2. Deep agar colonies of *B. botulinus* from four weeks old culture, showing transparency of disc and branching processes of the "nucleus."

Fig. 3. Deep agar colony of *B. botulinus* from a three weeks old culture. View of the edge of colony.

Figs. 4 and 5. "Composite colonies" of *B. botulinus*, having three discs lying in different planes.

Figs. 6 and 7. Two deep agar colonies from the same week old agar culture. Figure 6 shows the minute gas bubbles in the disc and the branching processes of the "nucleus." In figure 7 the growth in the disc of the colony has diffused into the surrounding medium.



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