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# ON THE CULTIVATION OF AN ACID-FAST ORGANISM FROM LEPROUS LESIONS IN TISSUE CULTURES AND OTHER MEDIA *

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### INTRODUCTION

Many positive results of attempts to cultivate the organism of leprosy have been reported, but it is doubtful if anyone has brought forth unquestionable evidence for the cultivation of the true etiological agent. Some have isolated acid-fast rods only, while others have cultivated pleomorphic organisms with an acid-fast stage; it is doubtful if any two workers have cultivated the same organism.

The work here reported deals with the isolation of a diphtheroid having an acid-fast as well as a non-acid-fast stage. The literature bearing on this problem includes several reports of isolation of non-acid-fast diphtheroids.

Bordoni-Uffreduzzi isolated an organism that was alcohol-fast but not acid-alcohol-fast. Babes isolated twelve cultures, at no time acid-fast, which he believed to be the true *Myco. leprae* which had lost their acid-fastness upon culture media. Levy, Spronck, Czaplewski, Bayon, Williams, and Duval obtained similar diphtheroids. Dean isolated one from rat leprosy. McCoy obtained several strains that were acid-fast on amoebae agar and non-acid-fast on plain or glycerin agar. A pleomorphic filamentous organism was isolated by Reenstierna which varied considerably in morphology and staining reactions.

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## EXPERIMENTAL WORK

### MEDIUM

Tissue cultures were used, employing the well-known methods of Carrel (1, 2). The medium consisted of chick tissues, guinea pig plasma, Tyrode solution and diluted chick embryonic fluid contained in 3.0 cm. Carrel flasks.

*Tissue*—Tissues from 7 to 10-day chick embryos were used.

*Tyrode solution*—To each liter of Tyrode solution 20 cc. of plain broth was added to serve as source of food and facilitate detection of any contaminating organisms. The solution was filtered (Berkefeld), tubed and incubated for 48 hours; contaminated tubes were discarded.

*Plasma*—Guinea pig blood was mixed with sterile 1-1000 heparin solution, 1 cc. of the solution being drawn into a 20 cc. syringe followed by the blood to the 11 cc. mark. The mixture was quickly forced out into a sterile tube in ice water, and immediately centrifugalized in cups filled with ice water. The heparin plasma was removed with a pipette, expelled into a test tube, and kept in the ice water.

*Embryonic fluid*—Minced chick embryo was diluted seven times with Tyrode solution and centrifugalized. The supernatant fluid was pipetted off and distributed in test tubes, 2.5 cc. to a tube, and 0.5 cc. removed from each, planted in plain broth and incubated for 5 days. If the tubes remained clear the fluid was ready for use.

### LEPROTIC MATERIAL

Human nodules were removed aseptically and ground with a glass rod in a test tube containing bits of glass which had been sterilized previously. The material was diluted with Tyrode, the coarse particles allowed to settle out, and the supernatant fluid removed to a small Petri dish. Rat granuloma was treated in the same manner. The broken-down nodular material required no preliminary treatment; it was aspirated from the lesion, diluted with Tyrode, and transferred to a Petri dish.

### METHOD

The fragments of chick tissue (0.5 to 1.5 mm. diameter) were immersed in the leproma suspension for a few minutes, removed with a Pasteur pipette, and embedded in plasma previously diluted with three parts of Tyrode, in Carrel flasks. The plasma was heavily inoculated with tissue. After washing the coagulated plasma with Tyrode the embryonic fluid was added, the flasks stoppered with rubber caps, and incubated at 37°C.

The tissue fragments grew luxuriantly, forming an almost continuous growth throughout the plasma. Visible evidences of bacterial growth occurred between the fifth and tenth days.

## RESULTS WITH HUMAN MATERIAL

The results with human material are tabulated in Table I. In every case control plants were made on glycerin potato and coagu-

lated egg media. In none of these was any growth noted after two months.

TABLE 1.—*Results of cultivation experiments using human material.*

| Patient | Nodule | Cultures prepared | Number of flasks | Number positive | Days of incubation |
|---|---|---|---|---|---|
| A | Nerve, hard | 1- 5-32 | 8 | 0 | 32 [a] |
| B | Hard nodule | 1-23-32 | 8 | 0 | 23 [b] |
| C | Broken down | 3-18-32 | 9 | 2 | 6 |
| C | Broken down | 4- 8-32 | 9 | 9 | 6 |
| C | Broken down | 4-21-32 | 8 | 8 | 5 |
| D | Broken down | 4-19-32 | 8 | 8 | 7 |

[a] During this time six transplants were made; effort discontinued 2-6-32.
[b] During this time three transplants were made; effort discontinued 2-15-32.

Positive growth occurred only from broken-down nodule material, from two patients. No attempt was made to continue the isolations; the time was devoted to a study of the cultured organisms. The positive cultures showed dense masses of acid-fast rods on or near pieces of proliferating chick tissue. (Figs. 1 and 2.) There were some blue-staining organisms present. On the other hand the supernatant embryonic fluid showed only an occasional acid-fast organism and masses of diphtheroids. The acid-fast organisms gradually disappeared from day to day, finally leaving only diphtheroids.

SUBCULTURES

Transfers were made approximately every seven days to fresh tissue cultures. Normal chick tissue cultures were prepared as before except that the bacterial suspension was omitted. The cultures were incubated for 24 hours, then inoculated with a piece of tissue from the previous lot of cultures, this being lifted from the plasma by means of a platinum spatula. After another 24 hours the plasma became turbid, indicating bacterial growth.

In each new lot of tissue cultures there was a heavy growth of acid-fast organisms on or near the fragments of tissues and an almost complete absence of them in the embryonic fluid. Also, by the seventh day the growth was almost a pure culture of blue-staining diphtheroids.

Sub-cultures to laboratory media gave only growths of the diphtheroid. At no time were acid-fast organisms seen. All attempts to separate and obtain the acid-fast organisms in pure culture failed.

Therefore, it was believed that they were different stages of one and the same organism. Proof of this will follow later in the paper.

After carrying the organisms through 12 transplants on tissue cultures, they were finally transferred to minced chick embryo medium, prepared as follows:

To minced chick embryos (7 to 12 days old) 6 cc., and Tyrode solution 24 cc., is added a heparin plasma (as described) 4.0 cc., and sodium citrate, 5 per cent in saline, 1.6 cc. This medium was distributed, 3 cc. to a tube, and these were placed on ice for 24 hours. The plasma coagulum was then broken up thoroughly with a sterile glass rod and inoculated.

The best growth of acid-fast organisms took place in approximately 48 hours, when they were usually as numerous as diphtheroids. After this they gradually disappeared, and on about the seventh day ordinarily all were blue. On transfer to fresh medium the above cycle was repeated. These cultures are now in the 29th generation and behave the same.

### RESULTS WITH RAT MATERIAL

The method outlined above was followed for the cultivation of the organism of rat leprosy. The results are given in Table 2. Controls as before were negative.

TABLE 2.—*Results of cultivation experiments using rat material.*

| Rat | Material | Cultures prepared | Number of flasks | Number positive | Days of incubation |
|---|---|---|---|---|---|
| 1 | Subcutaneous granuloma | 2- 8-32 | 8 | 0 | 29 [a] |
| 2 | Subcutaneous granuloma | 6-30-32 | 5 | 4 | 10 |

[a] During this time four transplants were made; effort discontinued 3-8-32.

One of two trials proved positive. (Figs. 3 and 4.) The first smears showed practically all acid-fast rods, with occasionally a few diphtheroids, but from day to day the former gradually decreased while the latter increased, until finally after 10 to 15 days only an occasional acid-fast rod was discernible.

### SUBCULTURES

When transplants were made to fresh tissue cultures, acid-fast organisms were found on or near the proliferating tissue fragments but almost none in the embryonic fluid. On the tenth day the

growth was almost a pure culture of blue-staining rods. This cycle was repeated with each transplant. After five generations the organism was transplanted to minced chick embryo medium. It is now in the 15th generation and shows the same picture as the human cultures. As with the human cultures, no growth occurred from the original suspension planted on laboratory media.

### MORPHOLOGY

The diphtheroid and acid-fast organisms are very pleomorphic, this varying with the age of the culture and the medium. The diphtheroids appear singly, in pairs and sometimes in short chains. They may appear solid, granular, or beaded, with occasional vacuolated forms. Some are almost coccoid, while others are very long and slender. Except in their staining reactions no morphological differences were noted between the acid-fast and the diphtheroid organisms; the same form could be found stained either red or blue. Solid-staining forms predominated in young cultures, while the granular and beaded forms were more prominent in the older cultures.

### ISOLATION OF DIPHTHEROIDS

A culture of the diphtheroid from a human culture was obtained by plating on plain agar, separate colonies appearing after 48 hours. Individual colonies were replated a total of eight times.

Rough and smooth variants were noted on the plates. These were cultured on coagulated egg medium, where heavy growth occurred in 48 hours. Both produced a deep orange pigment in the presence of air, but in sealed tubes this gradually disappeared. The rough organism produced a dry, wrinkled growth not unlike that of the tubercle bacillus, the smooth one a moist, glistening growth. The same results were gotten with the diphtheroid from a rat culture. Rough and smooth colonies having similar chromogenic appearances were isolated.

### CULTURAL REACTIONS OF THE DIPHTHEROIDS

The reactions in sugar media were determined for the rough and smooth variants from both rat and human sources.† The smooth forms from both sources gave identical reactions; the rough variants were also alike, but the two forms from the same source were slightly

† Limitations of space necessitate omitting the tables showing these reactions.—EDITOR.

different. Therefore, the two diphtheroid organisms isolated from different sources were both morphologically and culturally identical.

Pinkish colored pellicles were produced on the surfaces of the media. These sank on shaking, but new ones were produced. The smooth forms produced a granular deposit, the rough a very flaky sediment. The smooth produced a thin, smooth pellicle with moderate tendency to creep up the sides of the tubes, the rough produced a very wrinkled growth with marked tendency to creep up the sides (Fig. 7).

### CONVERSION OF DIPHTHEROIDS TO ACID-FAST ORGANISMS

Minced chick embryo medium was inoculated with the pure smooth diphtheroids isolated from both human and rat sources. After 48 hours the tubes showed masses of acid-fast rods, the maximum number appearing within the following 48 hours. After that they gradually decreased, leaving finally a culture of pleomorphic diphtheroids. Transfers were made every 5 to 6 days, with the same result. These cultures are now in the eighth generation.

### DISCUSSION

Mixed cultures of acid-fast organisms and diphtheroids were isolated four times out of six trials from human material, and once from two specimens of rat granuloma. All isolations were made with chick tissue cultures.

It is believed that this is the first serious attempt to employ this method. Zinsser and Carey (3) claimed to have cultivated the organism of rat leprosy in tissue cultures, but inoculation of media with the isolated organisms gave negative growth. Apparently no attempt was made to pursue the work beyond the first generation.

The organisms were acid-fast in tissue and non-acid-fast on laboratory media, the tinctorial characteristics varying with the condition of the tissues. With vigorous, actively growing tissue cells they were strongly acid-fast. As the tissues became less vigorous the acid-fast property was less pronounced until finally, when the tissues died and autolyzed, only diphtheroids were seen. The same picture was noted in minced chick embryo cultures.

This cycle has been observed by others. Klitin (4) isolated an organism that lost its acid-fastness after prolonged cultivation, though inoculated rabbits again gave acid-fast organisms. A diphtheroid was isolated by Kedrowsky (5), but after injection into

rabbits only acid-fast organisms were found. He concluded that the acid-resistant rods and the diphtheroids were different growth phases of the same organism. Williams (6) concluded that leprosy may be caused by several strains of an extremely pleomorphic streptothrix which under certain circumstances may show acid-fast and non-acid-fast filaments, and diphtheroids and acid-fast rods. This same view was held by Bayon (7) and Walker (8).

The strains of diphtheroids isolated from human and rat material presented identical cultural reactions. All have acid-fast rods when inoculated into tissue cultures and chick embryo medium. This indicates that the organisms are the same.

It is believed that they represent the true etiological agent of leprosy. The reason for such a statement is the fact that the same organism was isolated from four human nodules and one rat granuloma. As Bayon said:

> The fact that diphtheroids are ubiquitous should not deter us from trying to identify any such micro-organism which has been isolated from leprous nodules once proper precautions have been taken to avoid contaminations with extraneous bacteria. Acid-fasts are equally ubiquitous as diphtheroids yet this fact does not allow us to deny the etiological significance of the acid-fast microorganisms found in tuberculosis.

On the basis of the above results it must be concluded that human and rat leprosy are caused by one and the same organism. Animal inoculation experiments are now in progress, using material from chick embryo cultures and from glycerin agar slants.

SUMMARY

1. Chick tissue cultures of Carrel were used as the culture medium for the isolation of the organisms of human and rat leprosy.

2. An acid-fast organism together with a diphtheroid were isolated from four human nodules and one rat granuloma.

3. When transfers were made to artificial culture media only the blue-staining diphtheroid multiplied.

4. After the primary isolations on chick tissue cultures, minced chick embryo medium furnished an excellent substrate for the cultivation of the organisms.

5. As with chick tissue cultures, the minced embryo medium gave rise to acid-fast and non-acid-fast organisms.

6. A pure culture of the diphtheroid when inoculated into chick embryo medium gave acid-fast and non-acid-fast organisms.

7. The diphtheroid and the acid-fast rods are apparently different growth phases of the same organism.

8. The organisms were acid-fast in tissues and non-acid-fast on laboratory media. The tinctorial characteristics varied, depending upon the living condition of the tissue. In vigorous, actively growing tissue the organisms were strongly acid-fast. As the tissue became less vigorous the acid-fast property was less pronounced and, finally, as the tissue died, only non-acid-fast diphtheroids were seen.

9. It is believed that human and rat leprosy are caused by one and the same organism.

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## REFERENCES ‡

(1) CARREL, A. Jour. Exp. Med. 38 (1923) 407.
(2) CARREL, A. Tissue Cultivation, in "Filterable Viruses" by T. M. Rivers, et al., Williams & Wilkins Co., Baltimore, 1928.
(3) ZINSSER, H. AND CAREY, E. G. Jour. American Med. Assoc. 58 (1912) 692.
(4) KLITIN, I. I. Wojenno-Medizinsky Jour. St. Petersburg, 1905. Quoted from Wolbach and Honeij. Jour. Med. Res. 29 (1913) 367.
(5) KEDROWSKY, W. Zeitschr. f. Hyg. 66 (1910) 1.
(6) WILLIAMS, T. S. B. British Med. Jour. 2 (1911) 1582.
(7) BAYON, H. Lepra 14 (1914) 187.
(8) WALKER, E. L. Jour. Prev. Med. 3 (1929) 167.

‡ Not including those referred to in the introductory portion of the original article.—EDITOR.

## DESCRIPTION OF PLATES

### PLATE 1

FIGS. 1 and 2. Chick tissues inoculated with human *Mycobacterium leprae*, 24 hours old.

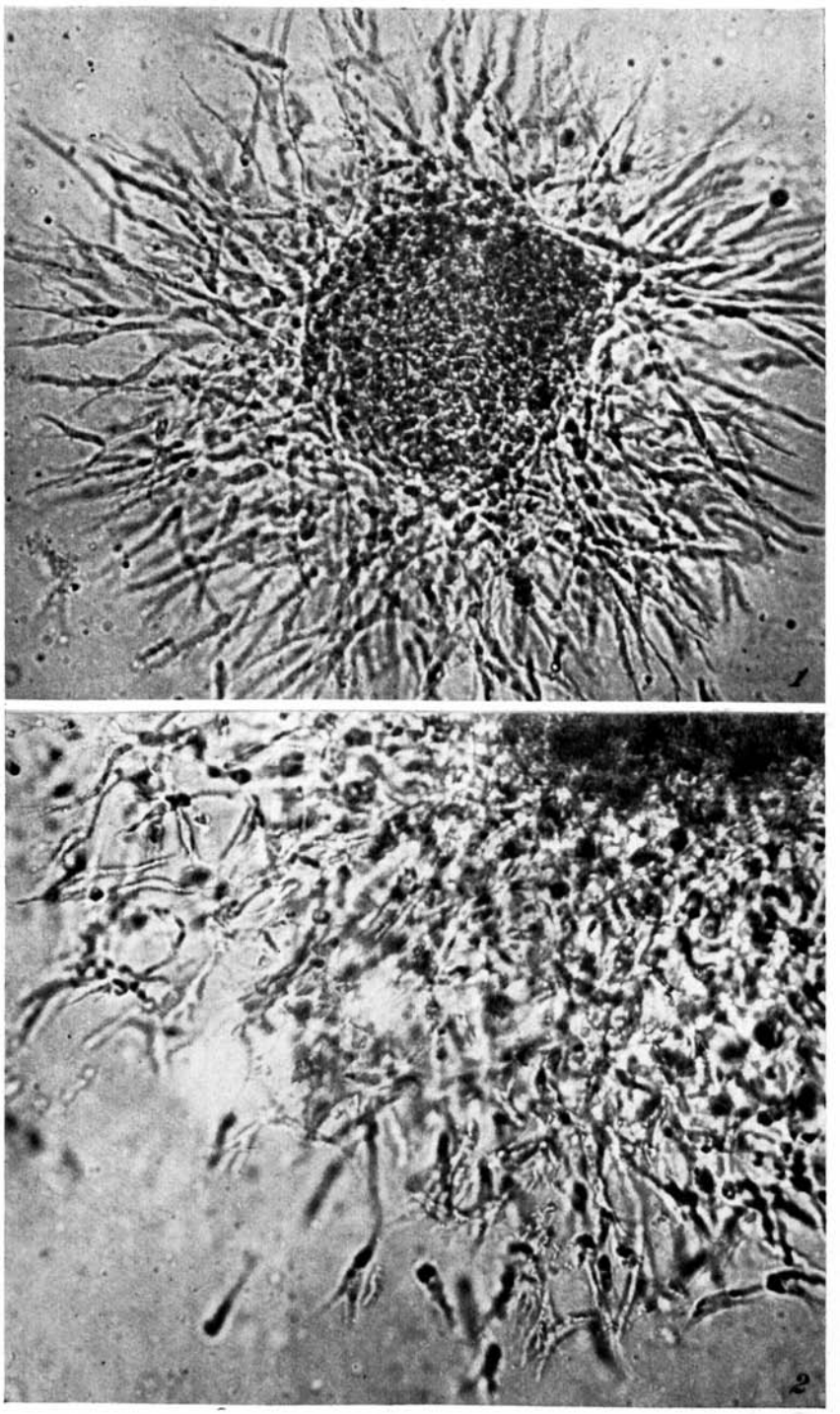


PLATE 1.

PLATE 2

FIGS. 3 and 4. Chick tissues inoculated with rat *Myco. leprosy bacillus*, 24 hours old.

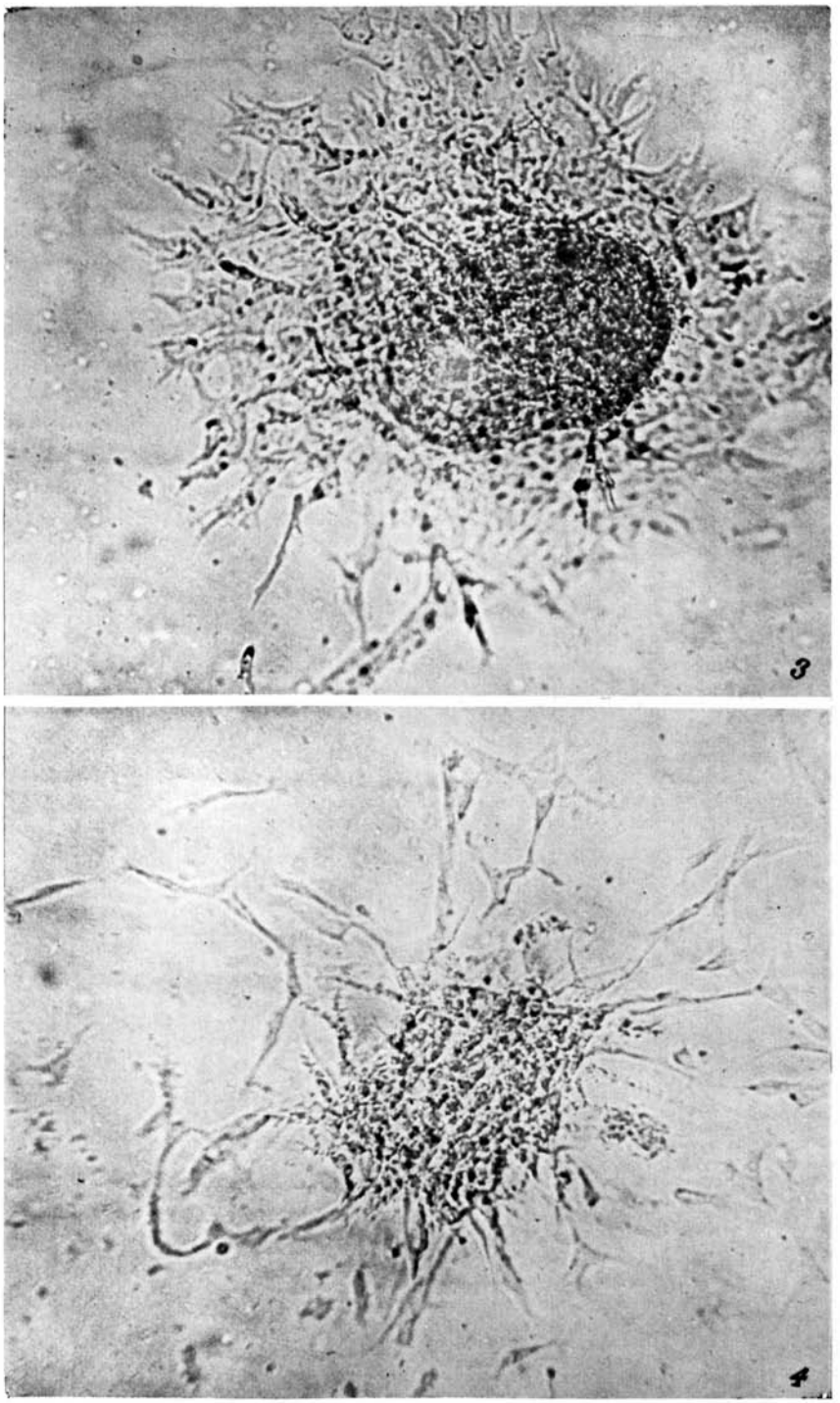


PLATE 2.

PLATE 3

FIGS. 5 and 6. Acid-fast organisms cultivated from human material in minced chick embryo medium.

FIG. 7. Growths of the diphtheroids on broth: (a) Smooth form from rat granuloma, (b) rough form from rat granuloma, (c) smooth form from human nodules, (d) rough form from human nodules.

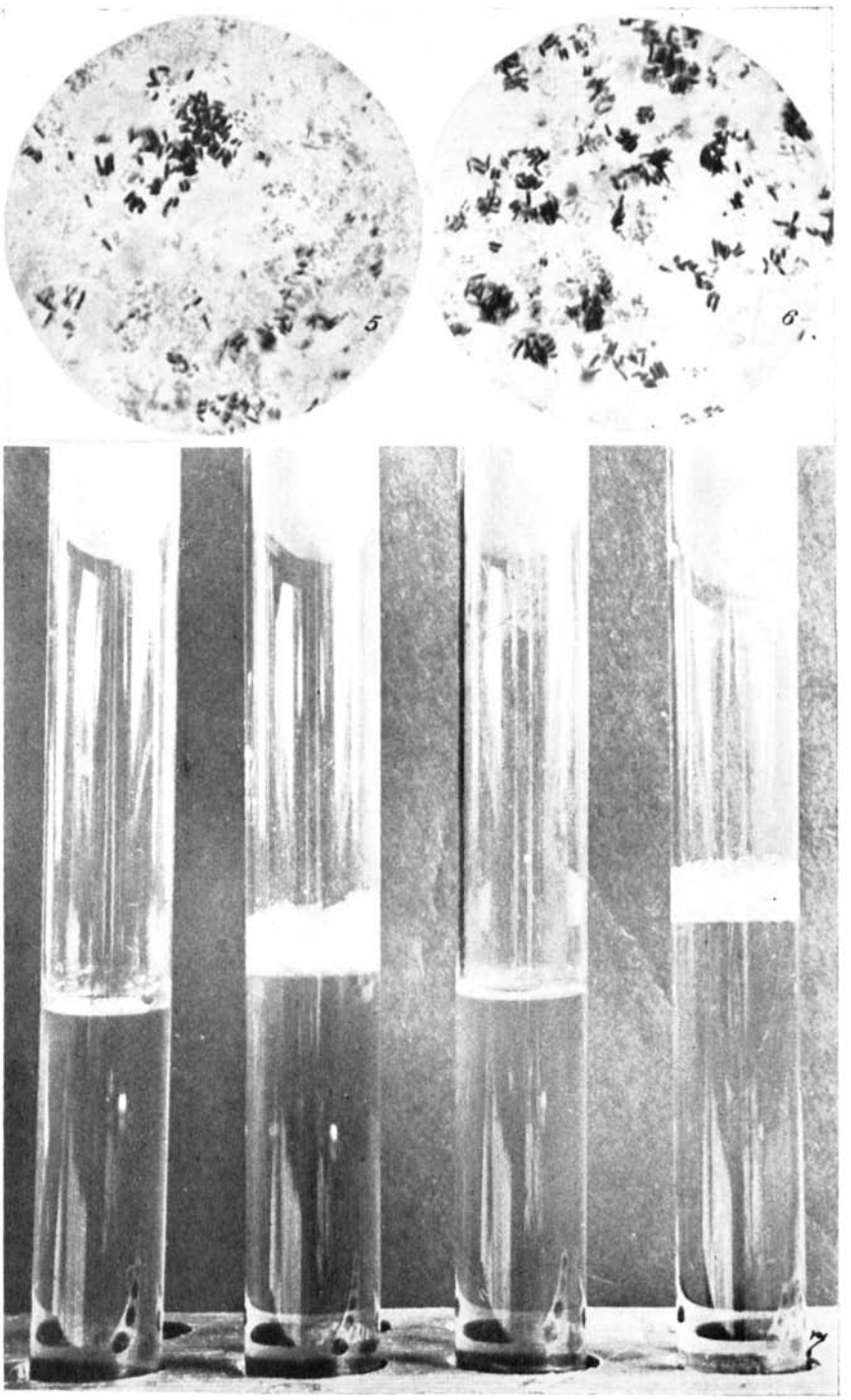


PLATE 3.