STUDIES ON THE ETIOLOGY OF SPONTANEOUS CONJUNCTIVAL FOLLICULOSIS OF RABBITS

II. Bacteriological Investigations

By PETER K. OLITSKY, M.D., JEROME T. SYVERTON, M.D., and JOSEPH R. TYLER

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

Plates 16 and 17

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Spontaneous conjunctival folliculosis, a disease widespread among rabbits, has been described in a preceding article (1) as occurring in two clinical forms, Type I and Type II. The former is characterized by a dormant chronicity with discrete lesions, whereas the second type, a more actively progressive disease, presents an inflammatory follicular reaction involving all the palpebral conjunctival surfaces. It was found that the transition from one type to the other, an infrequent occurrence in nature, could more often be induced artificially by experimental procedures. From our studies a conclusion was drawn that the causal factor of the affection is not an ultramicroscopic virus but an infectious agent behaving after the manner of bacteria of low grade pathogenicity.

In this second paper the results of our bacteriological investigations will be presented.

Methods

The methods employed in cultivation tests were similar, with the exceptions to be noted, to those in bacteriological studies on spontaneous monkey folliculosis (2). The exceptions consisted in the use of: (*a*) defibrinated rabbit blood instead of horse blood in the preparation of the blood agar medium, a substitution made merely for the sake of convenience; (*b*) dextrose instead of five carbohydrates, of which a 5 per cent solution was filtered through a Berkefeld V candle, and 5 cc. of the sterile filtrate were added to 95 cc. of the blood agar; (*c*) agar containing 2 per cent Witte's peptone, adjusted to pH 7.4, cooled to 45°C., and into this sterile, defibrinated, rabbit blood was introduced to make 8 per cent by volume.

Although plain dextrose agar readily supported the growth of the several species of bacteria that were cultivated, the employment of blood agar was found to be preferable, especially for initial cultures, in that it afforded a more profuse growth of fragile organisms and facilitated their recovery. For each test six plates of blood agar and four tubes of leptospira medium, prepared as already described (2), were seeded with saline solution suspensions of excised conjunctival tissue.[1] The excised tissue, rather than expressed follicular, or grattage material, was found to be more advantageous for bacterial cultivation.

Cultures of Affected Tissue and Control Materials

The bacterial flora of the conjunctival tissue obtained from normal rabbits was not unlike that from monkeys (2). Staphylococci and diphtheroids predominated, whereas the microorganisms less frequently encountered were the Gram-negative, motile or non-motile, chromogenic or non-chromogenic bacilli of indeterminate species; organisms of the *subtilis* group; yeasts and moulds. The relative incidence of these bacteria in normal tissue was low, and in affected conjunctivae much higher. *Bacterium granulosis* and *Bacterium simiae*, associated with human or experimental trachoma (3), and with simian folliculosis (2), respectively, were not found. Nor could we isolate *Bacterium monocytogenes* (3, 4), the incitant of fatal epizootics among rabbit breeding stocks.

Thirty-eight strains of the indeterminate group of organisms were collected, and of these, sixteen different cultures were injected into 46 rabbits in the manner already described (1). It should be noted that characteristic folliculosis was not induced thereby, although in some instances transitory, diffuse abscess formation resulted and in others localized pustules at the points of scarification. In all cases healing was prompt and normal conditions were restored within a week or two after inoculation.

The different species of ordinary or indeterminate bacteria, as mentioned, were not the only ones which were isolated from the rabbit folliculosis tissue. In addition to these, we have cultivated from affected conjunctivae a new species of microorganism which, after inoculation into rabbits, induced a conjunctival follicular reaction indistinguishable from the spontaneous disease as it occurs in nature. This special bacterium has been isolated from seventeen of 24 rabbits

[1] All operations on animals were performed with the aid of ether anesthesia.

showing fully developed, diffuse (Type II) folliculosis. There was, however, considerable difficulty in cultivating it from animals having the Type I disease, which is less readily transmissible to normal rabbits (1), and from those showing a mild degree of the diffuse form of the affection. For example, only five positive cultures were obtained from eighteen such animals.

Description of the Microorganism

In the following brief form will be presented the chief morphological and biological characteristics of the special microorganism isolated and cultivated from conjunctival folliculosis of rabbits.

Morphology.—The common form of the microorganism is a slender, thin bacillus, with pointed ends, two or three times longer than its width, and ordinarily smaller in size than *Bacterium granulosis* or *simiae*, or 0.2 to 0.3μ wide and 0.5 to 1μ long. The appearance of the minute, delicate, tenuous bacilli in stained film preparations is not unlike that of *H. influenzae* (Fig. 1). It is pleomorphic; one often sees shorter coccoid or lanceolate structures frequently occurring in pairs, placed end to end. As a rule, smaller organisms preponderate in growths in leptospira medium and larger ones on agar.

Staining Reactions.—The organisms are Gram-negative and non-acid-fast. With stains for capsules, especially those of Muir or Casares-Gil, a faint, delicate envelope can be observed outside of a thin halo surrounding the bacilli or the diplo-lanceolate forms. The capsule is of much finer texture than the membranes surrounding the *granulosis* or *simiae* species, and it is not demonstrable by ordinary tinctorial methods. The Casares-Gil stain also reveals peritrichal flagella which apparently proceed from the capsular material (5). One flagellum is attached to each pole and one, two, or three, usually two, to each side. The thready structures are long, tenuous, and wavy and in stained film preparations often appear matted or thrown to one side (Fig. 2).

Motility.—The multiple flagella contribute to the peculiar and highly active motility of the organism. The bacilli move rapidly in a circular or linear direction with a motion that may be either waddling or tumbling. There are some, however, that revolve about their central points as axes, like a pinwheel, without forward progression. The motility is not lost with the age of a culture, nor is it influenced by the medium employed.

Spores and metachromatic granules are absent.

Agar Plates.—The colonies first appear in 24 to 48 hours as minute, smooth, spherical growths about 1 mm. in diameter, enlarging in 24 to 48 hours to a size of 2 or 3 mm. They are translucent, somewhat convex, moist, and mucoid. The edge is entire; the substance is slightly greyish and homogeneous. There is a tendency of one colony to coalesce with its neighboring one (Fig. 3). When mag-

nified at 10 diameters as with a colony microscope, it exhibits a peaked center and a sharply defined, but slightly raised edge (Figs. 4 and 5). The growth on blood agar is more profuse and the colonies are somewhat less translucent and more greyish. As occurs with *granulosis* and *simiae* microorganisms, cultures of affected conjunctival tissue yield only an occasional colony of the special bacterium among many miscellaneous growths.[2] The colonies are sometimes adherent to, or incorporated within an alien one. In the latter instances, mixed growths can be purified by repeated subplants on fresh media.

Agar slants exhibit within 48 hours a slightly greyish, translucent, coalescent growth, which is glistening, mucoid, homogeneous, and non-spreading. The water of syneresis appears uniformly cloudy or milky, depending on the amount of growth.

Leptospira Medium.—24 hours after inoculation, a faint nebulosity occurs just at the surface. Following this there is an ingrowing, sac-like mass, with its base, 5 mm. across, lying at the center of the under surface and extending for 5 mm. into the medium. The area spreads out laterally so that within a day or two there is a uniformly opaque, whitish layer up to 1 cm. high. A slowly progressive extension of the clouding takes place until the bottom of the tube is reached, usually within 7 days.

Gelatin.—No liquefaction occurs. In stab cultures one observes a tenuous, arborescent, non-spreading line of growth. Colonies on gelatin agar appear greyish, mucoid, and confluent but they are less numerous than on blood agar.

Broth.—In this medium the growth is uniformly turbid without pellicle formation.

Litmus Milk.—Unchanged.

Potato.—Faint, buff colored (changing to brown after 5 days), non-spreading, sparse surface culture.

Indol.—Not produced.

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[2] A probable explanation for the sparse colony distribution of the three species of organisms in initial cultures of affected tissues is that the medium may not be completely satisfactory for primary isolations, or the organisms may be suppressed by the growths of the rapidly developing, concomitant, miscellaneous bacteria present. Indeed, it has been shown by Weiss that the growth of staphylococci *per se* chiefly by lowering the pH of the medium definitely inhibits the development of *Bacterium granulosis*—the degree of inhibition being measurable on a quantitative basis. Diphtheroids also suppress *granulosis* growth but not as markedly (6). As already indicated, staphylococci and diphtheroids are predominating organisms in cultures of affected conjunctival tissues. We have also observed the inhibition, under these conditions, of growths of *granulosis*, *simiae*, and the special rabbit bacteria. (See also Reference 7; and for a recent discussion of the principles underlying bacterial antagonism, Neufeld, F., and Kuhn H., *Z. Hyg. u. Infektionskrankh.*, 1934, **116,** 95.)

Nitrates.—Not reduced.

Carbohydrate Reactions.—This organism is non-fermenting in type. No acid or gas is produced on dextrose, levulose, mannose, mannitol, saccharose, raffinose, inulin, galactose, maltose, salicin, xylose, dextrin, arabinose, amygdalin, lactose, dulcitol, rhamnose, trehalose, sorbitol, or inositol.

Other Properties.—The bacterium is aerobic and facultatively anaerobic. It produces no characteristic odor in cultures and is bile-resistant. The optimum temperature for development is 28–30°C. The thermal death point is at 56°C. for 15 to 30 minutes.

Filtrability.—Cultures suspended in saline solution failed, in ten tests, to pass through single disc Seitz and Berkefeld V filters. Similar material suspended, however, in hormone broth was filtrable through three of the four Berkefeld V filters used but not through Seitz discs. Precisely similar results were obtained when cultures of *Serratia marcescens* (*Bacillus prodigiosus*) were employed. The filtrability of many species of bacteria is possible in a hormone broth suspension and the results of tests with the special bacterium do not necessarily indicate that it has a filtrable stage or that it is a "filtrable" organism. However that may be, we made use of this as a practical finding in selective filtration through pervious Berkefeld V candles of mixtures of the bacterium with larger, non-filtrable organisms, such as staphylococci, so as to facilitate its recovery in a pure state from initial mixed cultures. The methods followed have already been described in similar experiments with *Bacterium granulosis* (7).

Serological Reactions.—Repeated intravenous injections into rabbits of living cultures of the special bacterium yielded antisera with an agglutinin titer of 1:1000 to 1:20,000 for all the available cultures, 22 in number, of the microorganism. The specificity of the agglutination was shown by the negative reactions in 1:10 dilution of the sera in tests with ordinary bacteria and with 37 strains of the miscellaneous, indeterminate bacteria recovered from rabbit conjunctivae. At this point mention should be made of the fact that *Bacterium granulosis* and *simiae* exhibit no cross-agglutination with the special bacterium. Hence the rabbit organism is serologically specific.

No agglutinins against this bacterium were found in 1:2 to 1:15 dilutions of the sera obtained from 29 normal rabbits. On the other hand, thirteen of fourteen animals infected by subconjunctival inoculation of the organism, as will be immediately described, revealed specific clumping in 1:20 to 1:160 dilutions of serum in tests made during the 3rd to 6th week after inoculation. Nevertheless, the organism was not agglutinated by the serum of rabbits having spontaneous folliculosis or the experimental disease evoked by injection of affected tissue. By comparison, no agglutinins were found in trachoma serum against *Bacterium granulosis* (8), nor in monkey folliculosis blood against *Bacterium simiae* (2). Moreover, Weiss (9), in a summary of his own and prior reports on localized infections of the conjunctiva, a class that includes rabbit folliculosis, has concluded that such affections do not induce appreciable antibody formation.

Thus in the general properties as given, the bacterium isolated and cultivated from cases of rabbit folliculosis appears to be distinct from other described microorganisms. It has, however, the same generic characteristics as *Bacterium granulosis* and *Bacterium simiae*. The diagnosis, or characters, of this genus may be stated as follows:

Small, slender Gram-negative rods present in the conjunctiva of man and animals affected by a follicular type of disease; mucoid type of growth which in early subplants takes place with some difficulty in ordinary media; motile, flagellated, and encapsulated; aerobic and facultatively anaerobic, produce no endospores, optimum temperature for growth 28–30°C.

On the other hand, the rabbit microorganism differs in specific characters from those of either the human or simian bacterium, in the degree of motility; in the arrangement of flagella; in the manner of growth on the same medium; in the reactions with carbohydrates, and especially in serological specificity, so that the designation of the rabbit bacillus as a distinct species is indicated.

Pathogenicity Experiments

Sixteen cultures of the special bacterium were available for pathogenicity tests.

Of the sixteen cultures, fourteen individually and two pooled were injected into anesthetized animals. The 48 hour growth on agar slants of a second, or more remote generation, was suspended in 1 cc. of 0.9 per cent saline solution. 0.2 cc. of the suspension was introduced subconjunctivally into young rabbits having smooth lids. These animals were selected and quarantined in the manner already described (1). Only the left lower membrane received the culture and two rabbits were employed for each strain; hence 30 rabbits were employed for the sixteen cultures.

Only one strain was inactive; the remaining fifteen induced conjunctival follicular reactions in all but two of 28 inoculated animals. The resultant experimental disease could not be differentiated either clinically or histopathologically from the natural affection or from the infection evoked by inoculation of folliculosis tissue itself (1). In all instances the disease developed eventually into diffuse, Type II folliculosis (Figs. 6 to 8) and from six of such animals the identical organism was recovered. These latter strains were again shown to be specifically active in normal rabbits.

Instillation of cultures of the special bacterium into the conjunctival sac of normal rabbits, in accordance with the method used previously (1), also brought about the same type of specific infection in two of three animals. These two exhibited characteristic folliculosis, 11 and 18 days respectively after the last instillation. Furthermore, the same microorganism was recovered from the conjunctivae of both, in the 3rd and 7th week of their illness, when folliculosis was fully established.

Microorganisms in Tissues.—The stained sections of lesions induced by the special bacterium and film preparations of expressed follicular material were studied. In the latter instance, the Giemsa- or Gram-stained grattage material obtained from 22 rabbits showed in ten of them miscellaneous bacteria varying in number and consisting chiefly of staphylococci and diphtheroids. In addition, in all the specimens were observed discrete or clumped, free or intracellular, Gram-negative bacteria which closely resembled the special microorganism in morphology (Fig. 9). An examination of sections of over twenty different follicular conjunctivae, stained by the Giemsa, Gram, or eosin-methylene blue methods, yielded similar results (Figs. 10 and 11). The microorganism, however, was found in such material to be smaller and often intracellular. It is of interest that the bacteria were seen in section or film preparations that were derived from rabbits having either the natural disease, or experimental folliculosis induced by inoculation of the special bacterium and of conjunctival tissue. Finally, cellular inclusion bodies that are characteristically associated with the action of many ultramicroscopic viruses were not observed.

SUMMARY AND CONCLUSIONS

Before summarizing the experimental results presented herewith, a brief review is indicated of our study on follicular conjunctivitides existing in the different species of animals thus far examined.

Spontaneous follicular conjunctivitis of monkeys and chimpanzees, which resembles the early, or follicular, stages of human trachoma, has been shown to be an infectious disease, and to arise from the action of a bacterium and not an ultramicroscopic virus (2, 10). A new species of organism, designated tentatively as *Bacterium simiae, n.sp.*, and capable of reproducing granular lids in normal monkeys and apes,

has been recovered from cultures of simian folliculosis tissues. This organism, although of a different species, is of the same genus as *Bacterium granulosis* which is associated with human trachoma (3).

The studies were extended beyond folliculosis in monkeys and apes to a similar disease widespread among stock rabbits. Its clinical appearance and microscopic changes resemble the spontaneous malady in simians with some minor differences (1). Like the latter, rabbit folliculosis was also shown to be infectious and contagious.

In the present paper a bacteriological investigation of the rabbit affection is described. In the experiments undertaken we followed the method used by Noguchi in his studies on trachoma, that is, the different organisms recovered from normal and affected conjunctivae were inoculated into the conjunctivae of selected, quarantined rabbits.

Among the bacteria thus isolated from folliculosis tissue a new species was disclosed that brought about conjunctival reactions apparently indistinguishable either from the disease seen in nature or from the experimental infection induced by inoculation of folliculosis tissue. The organism was specifically active in rabbits whether injected subconjunctivally or instilled into the conjunctival sac. Furthermore, the special bacterium has thus far been cultivated only from folliculosis cases and not from other forms of conjunctivitis nor from normal tissues. Finally, specifically pathogenic strains of the identical microorganism have been recovered from rabbits experimentally infected with the bacterium.

A distinctive feature of this bacterium is that it is of the same genus as the microorganisms heretofore designated as *Bacterium granulosis* and *Bacterium simiae*. It is clearly evident then that the three bacteria cultivated to this time form a new genus, for which we propose the name *Noguchia*. The diagnosis of the genus has already been described in this paper, but as was also shown in a foregoing section, certain specific characters differentiate the three. Accordingly, we suggest a change in the names of the *granulosis* and *simiae* species to the proposed binomials, *Noguchia granulosis*, *n.sp.*, and *Noguchia simiae*, *n.sp.* The specific organism, having an evident causal relationship to rabbit folliculosis, can therefore be placed in this classification, with the name of *Noguchia cuniculi*, *n.sp.*[3]

[3] The classification of genus and species follows the recommendations of the Society of American Bacteriologists quoted by Bergey, D. H., in Manual of

In conclusion it would appear that in three types, at least, of follicular reactions in the conjunctiva—in man (the primary lesion of trachoma consisting essentially of follicles), in the simian, and in the rabbit—there is an intimate association of microorganisms having the same generic, but different specific properties.

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EXPLANATION OF PLATES

PLATE 16

FIG. 1. Gram's stain. Agar slant culture. ×1000.

FIG. 2. Casares-Gil stain, showing flagella; the staining fluid has thrown the structures to one side of the bacterium. ×1000.

FIG. 3. 48 hour growth on blood agar plate. Natural size.

FIG. 4. Same colonies photographed by reflected light. ×10.

FIG. 5. Same colonies photographed by transmitted light. ×10.

FIG. 6. Upper and lower conjunctivae of rabbit inoculated with a culture of *Noguchia cuniculi.* The characteristic folliculosis thus brought about should be compared with the condition seen in nature, as described and illustrated in the preceding paper (1). Natural size.

PLATE 17

FIG. 7. Eosin and methylene blue stain. Conjunctiva obtained from a rabbit inoculated subconjunctivally with the microorganism. The induced lesions are

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determinative bacteriology, and by Buchanan, R. E., in General systematic bacteriology, both published by Williams & Wilkins Co., Baltimore, 1925; also the Code of the Nomenclature Committee of the International Society of Microbiology (1930) quoted in Bergey's Manual, as stated, 1934, 4th edition, p. 23.

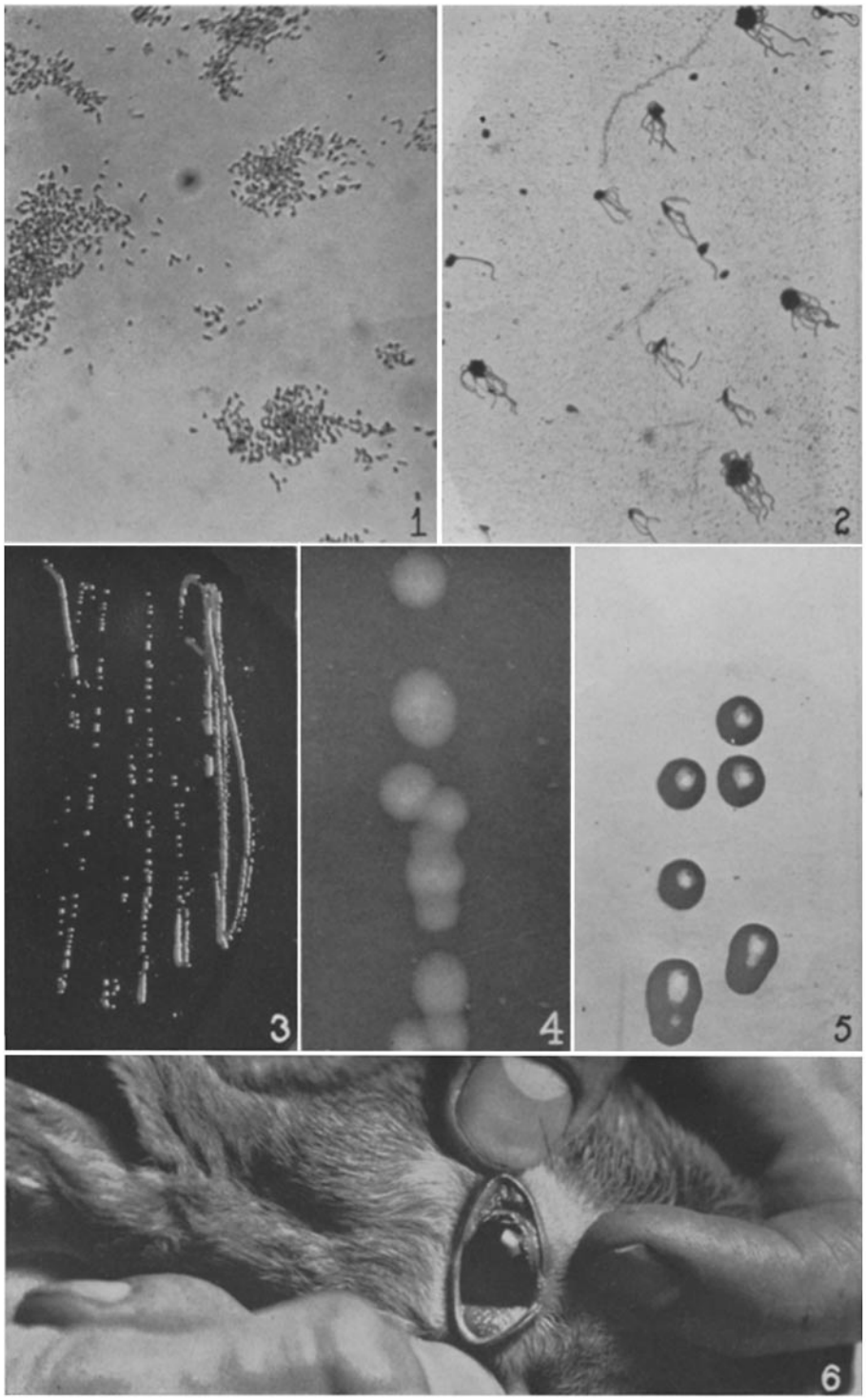
identical with those found in the natural disease or the experimental infection caused by injection of affected tissue, as illustrated in Paper I (1). ×122.

FIG. 8. Eosin and methylene blue stain. Same as Fig. 7. Represents only a part, about a third, of the entire follicle. ×300.

FIG. 9. Giemsa stain. Film preparation of conjunctival grattage material obtained from a rabbit having spontaneous folliculosis. Among miscellaneous microorganisms are seen bacteria with the morphology of *Noguchia cuniculi*. ×1000.

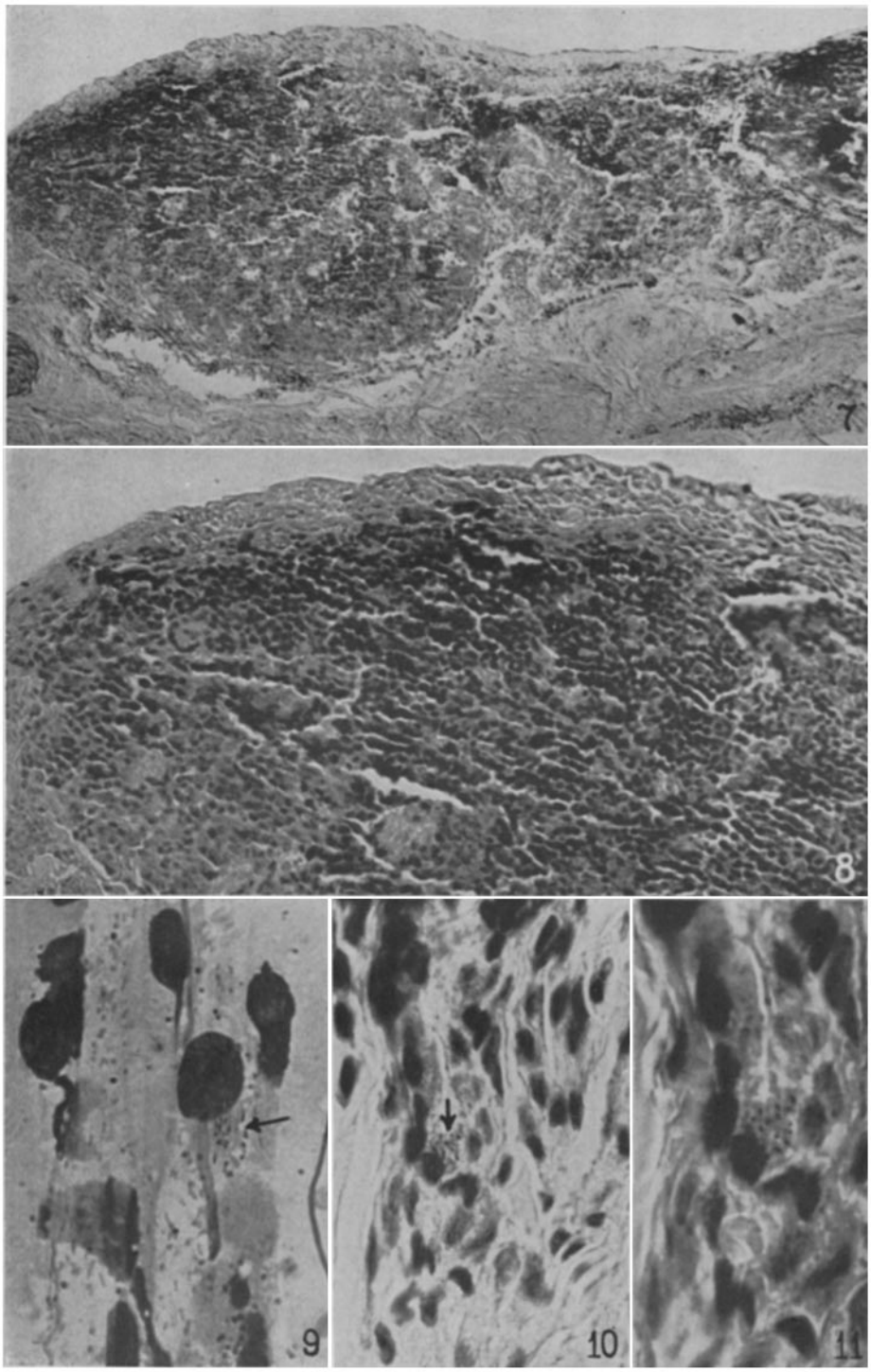
FIG. 10. Eosin and methylene blue stain. Tissue section of spontaneous conjunctival folliculosis in a stock rabbit showing a clump of similar bacteria within a cell. ×1000.

FIG. 11. Same. ×1500.



Photographed by Louis Schmidt

(Olitsky *et al.*: Conjunctival folliculosis of rabbits. II)



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