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ETIOLOGY OF OROYA FEVER.

XI. Comparison of Bartonella Bacilliformis and Bartonella muris. Cultivation of Bacterium murium, N. Sp.

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Plates 11 and 12.

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Mayer,[1] in 1921, discovered, in the red blood cells of a rat which was suffering from severe anemia as a result of trypanosomiasis and chemotherapy, peculiar inclusion bodies, resembling *Bartonella bacilliformis* of Oroya fever, which he designated *Bartonella muris*. Subsequently Lauda[2] reported that splenectomy of white rats brought about a severe anemia which could be transferred to normal rats by inoculating them with the blood or a suspension of liver tissue of the splenectomized animals. The name "infectious anemia of the rat" was introduced to describe the condition. Mayer and his collaborators[3] suspected that the anemia resulting from extirpation of the spleen might be due to *Bartonella muris* and succeeded in demonstrating the inclusion bodies in splenectomized rats. Their assumption was that *Bartonella muris* is present in most rats, and that the invasion of the blood is made possible by the removal of a defense organ, the spleen.

Because of the close analogy between *Bartonella muris* and *Bartonella bacilliformis*—their morphological similarity, their localization in the erythrocytes, and their association with a severe form of anemia, a comparative study has been made of these two bartonellas, the specific subjects of inquiry being: (1) the percentage incidence of appearance of *Bartonella muris* in the blood of splenectomized rats and mice, (2) the result of splenectomy in animals other than rats

[1] Mayer, M., *Arch. Schiffs- u. Tropenhyg.*, 1921, xxv, 150.

[2] Lauda, E., *Arch. ges. Physiol.*, 1925, cclviii, 529.

[3] Mayer, M., Borchardt, W., and Kikuth, W., *Klin. Woch.*, 1926, v, 559.

and mice, (3) the transmissibility of *Bartonella muris* to normal rats, (4) the transmissibility of *Bartonella muris* to other animals, especially splenectomized animals, (5) the susceptibility to *Bartonella bacilliformis* of rats and other animals after splenectomy, (6) the comparative morphological features of *Bartonella muris*, *Bartonella bacilliformis*, and *Grahamella talpæ*, the latter an intracorpuscular parasite of moles described by Graham-Smith,[4] Thomson,[5] and Brumpt,[6] and (7) the cultivation of *Bartonella muris*.

Splenectomy was performed on fifty white rats, three wild rats, three Chinese hamsters (*Cricetulus griseus*),[7] and nine white mice. *Bartonella muris* appeared after splenectomy in the blood of all, in some numerously, in others sparsely. Splenectomy always gives rise to an increase in the number of blood platelets, some of which showed very much more distinct chromatin rods and granules than the platelets of normal animals. These loosely packed or partially liberated thrombocyte bodies are sometimes very easily confused with *Bartonella muris*, especially when lying over or under the erythrocytes.

Splenectomy was carried out also in eight *Macacus rhesus*, two Java monkeys, four rabbits, and four guinea pigs. No intracorpuscular elements resembling *Bartonella muris* or *Bartonella bacilliformis* appeared in the blood of any of these animals, nor did the animals show any anemia comparable to that observed in the splenectomized rats or mice.

Transmissibility of Bartonella muris.

Repeated attempts have been made to reproduce the anemic condition and cause the appearance of *Bartonella muris* in the blood of normal rats and mice by injection of blood or suspensions of liver tissue (Lauda's method) from splenectomized animals, intravenously, intraperitoneally, intradermally, or intratesticularly. Such experi-

[4] Graham-Smith, G. S., *J. Hyg.*, 1905, v, 453.

[5] Thomson, J. D., *J. Hyg.*, 1906, vi, 574.

[6] Brumpt, E., *Bull. Soc. Path. Exot.*, 1911, iv, 514.

[7] I am indebted for these animals (which were obtained primarily for other experiments) to Dr. Carl TenBroeck, who procured them for me in Peking, and to Dr. Amos Wong, who was so kind as to bring them to the United States in his personal care.

ments have always been negative in this respect. Nor has it been possible to transmit the condition to splenectomized monkeys, rabbits, or guinea pigs.

In some instances, however, a quite different condition was induced.

In Rats 54 and 55, inoculated intratesticularly with a suspension of the liver of Rat 40, which had shown a marked blood invasion by *Bartonella muris*, the testicles became acutely inflamed. Rat 54 was castrated on the 6th day of disease. The condition persisted in Rat 55 for 10 days, but by the 14th day the testicle had healed. *Bartonella muris* was not found in the blood of either of these animals. Histological study of the excised testicle of Rat 54 showed acute infiltration of polymorphonuclear leucocytes into the acini, together with edema, congestion, and focal necrosis. Rather coarse bacilli were present in the lesion, some having been taken up by the leucocytes. From the testicular tissue was obtained a pure culture of a microorganism to be described later (Strain 54).

Two normal rats, 56 and 57, inoculated intraperitoneally and intratesticularly with the suspension of testicular tissue of Rat 54, became very ill within 48 hours, and in the blood of Rat 56, taken on the 3rd and 4th days, were found a few intracorpuscular bodies suggestive of *Bartonella muris*. The animal died before the 5th day. Rat 57 showed similar elements in the erythrocytes on the 3rd day but none thereafter. It died of diarrhea in 10 days. There was only slight induration of the testicles in these two animals.

A similar result was obtained in one of three normal rats inoculated with citrated blood of splenectomized Rat 41 (*B. muris* ++). In this case (Rat 66) a bilateral intratesticular inoculation had been made. The inflammation of the testicles reached its maximum on the 5th day and receded during the 3 following days. The same microorganism as had been isolated from Rat 54 was obtained from the aspirated fluid (Strain 66). In the other two animals (Rats 64 and 65), in which the inoculation was unilateral, there was no induration. Examination of blood films of these three animals failed to reveal the presence of *B. muris*. Three other normal animals inoculated intratesticularly and intraperitoneally with a suspension of the liver of Rat 41 showed neither testicular induration nor invasion of the blood by *B. muris*.

There is apparently present in the blood or liver of some splenectomized rats a microorganism capable of setting up acute orchitis in normal rats. The transient appearance of intracorpuscular bodies in the two rats inoculated with the testicular tissue of Rat 54 was far from convincing evidence of transmission of *B. muris*. The failure of most of the attempts to transmit the organism shows at least that regular transmission is not readily accomplished in American rats.

Mayer,[8] in Hamburg, encountered a similar difficulty. It would, indeed, seem unusual that a microorganism which is unable to invade the blood until the spleen is removed should acquire the power to infect the blood of a normal animal by transfer from the splenectomized one.

It has been found that the intraperitoneal deposition of a small fragment of normal rat or mouse spleen, or the intraperitoneal injection of a saline suspension of the tissue, into splenectomized rats or mice will cause the rapid disappearance of *Bartonella muris* from the blood. Hence *B. muris* is extremely sensitive to splenic substances, even when the spleen itself is not present.

Susceptibility to Bartonella bacilliformis of Splenectomized Animals.

Experiments to be reported elsewhere have shown that the spleen has no important defensive function against the invasion of *Bartonella bacilliformis*. While the blood culture titers of splenectomized monkeys infected with *Bartonella bacilliformis* were usually higher than those of control animals of the same series, yet variations in blood titer are so common in monkeys infected with this microorganism that they cannot be taken as significant in this instance. Rabbits, guinea pigs, and rats were not any less resistant to infection with *Bartonella bacilliformis* after splenectomy than are these animals normally.[9]

Comparative Morphology of B. muris, B. bacilliformis, and Grahamella talpæ in the Blood (Plate 11).

Comparison of blood films stained with Giemsa's solution shows *B. muris* (Fig. 2) to be smaller than *B. bacilliformis* (Fig. 1). There is also less variation in form in *B. muris*, which usually appears as short rods or coccoid forms whose size varies within a very small range. *B. bacilliformis*, on the other hand, though often having an oval or coccoid form similar to that of *B. muris*, frequently occurs in long, slender rods, and *Y* or *V* forms are very common (Fig. 1). The long slender forms usually have square ends, as though the or-

[8] Mayer, M., Borchardt, W., and Kikuth, W., *Arch. Schiffs- u. Tropenhyg.*, 1927, xxxi, 295.

[9] Noguchi, H., *J. Exp. Med.*, 1927, xliii, 851.

ganism had been cut transversely. Shorter rods may be bent at a sharp angle at one or two places, showing that they are composed of two or three individuals. These branching forms are not found in preparations of *B. muris*, which resembles *Grahamella talpæ* more than it does *B. bacilliformis*, though differing from *Grahamella* in its manner of distribution. I am indebted to Dr. A. C. Coles, of Bournemouth, England, for Giemsa-stained preparations of the blood of two English moles showing *Grahamella* infection (Fig. 3). The grahamellas are often found in such numbers in a single cell as to fill the stroma completely; *B. muris* is usually scattered among many cells, and many individuals are found outside the cells. The preparations show two types of *Grahamella*, one distinctly and uniformly thick, as compared with *B. muris*, and suggesting a bacterium, the other more delicate and showing beaded formations. No branched forms or long threads have been seen in the films of *Grahamella*.

B. muris, *B. bacilliformis*, and *G. talpæ* have similar staining reactions with Giemsa's solution, but basic fuchsin, while giving sharp definition of *B. bacilliformis*, fails to stain *B. muris*.

B. muris of the rat is indistinguishable from the similar elements which appear in the blood of wild rats or Chinese hamsters after splenectomy. The forms found in the corpuscles of splenectomized mice are more granular and show beaded formations.

Cultivation of B. muris.

The etiologic rôle of *B. muris* in the anemia of splenectomized rats is difficult to establish because of the fact that no animal has so far been found which is susceptible to the organism under natural conditions, *i.e.*, when the spleen is present. Once the spleen is removed, *B. muris* appears in the blood, hence splenectomized animals cannot be used for demonstration of the pathogenic effects of a microorganism which has been isolated.

In the course of repeated attempts at cultivation, I have isolated two different organisms, both of which resemble *B. muris* in morphology. The first (Strain 28 A, Figs. 7 and 8) came from the blood of a splenectomized rat and grew on leptospira medium as a grayish layer at the surface. It did not grow on ordinary culture media. It was

subsequently found that this organism had the features of a minute diphtheroid. It had no pathogenicity whatever for normal rats.

From the blood of the same animal (Rat 28) another minute, apparently non-motile, Gram-negative bacterium was isolated on a blood agar plate (Strain 28 B, Figs. 4 to 6). This organism grows on blood agar in very minute colonies but not on leptospira medium or on ordinary culture media. On a blood agar slant it grows more readily, and after 3 or 4 days at 30° or 37°C. the dew-like discrete surface colonies gradually spread to a diameter of 3 mm. or more and, when the surface is densely seeded, coalesce to a shiny, faintly bluish gray, moist layer of growth. The condensation water becomes slightly turbid. This organism is not an acid-producer like the first, but is hemolytic. Morphologically, especially when stained for a short time (20 minutes) with Giemsa's solution it appears very much like *B. muris*. The most interesting feature of the organism is its ability to set up an acute orchitis in normal rats when intratesticularly injected.

The first experiment was made on Rat 42, into which a 24 hour culture grown on blood slants at 30° and 37°C. was injected intraperitoneally and intratesticularly. *B. muris* did not appear in the blood, but the testicle became indurated within 6 days, when the animal was killed for transfer. A suspension of the testicular tissue was used for cultivation experiments and for intraperitoneal and intratesticular inoculation of two normal rats, 52 and 53. A pure culture of the organism was isolated from the testicular tissue. No induration followed the intratesticular injection of the tissue suspension, but in the blood of Rat 52 a few forms resembling *Bartonella muris* were detected 8 days after the inoculation.

The virulence tests of Strain 28 B were repeated on two normal rats, 58 and 59, the cultures being injected intratesticularly only. An acute orchitis developed in both animals, and they died 10 and 9 days after inoculation, respectively. *B. muris* was not found in the blood at any time. From the testicles of Rat 59 pure cultures of the organism were recovered.

Pure cultures of Strain 28 B from Rat 42 were tested on two normal rats, 60 and 61, the injections being made directly into the testicles. Rat 60 reacted more vigorously than Rat 61, the inflammation of the testicle reaching its maximum in 3 days and disappearing in 9 days, while Rat 61 showed only a slight induration on the 3rd day. Bodies resembling *B. muris* were found in very small numbers in the blood of Rat 61, but not in Rat 60. A saline suspension of the testicular tissue of Rat 42, kept at 4°C. for 7 days, was injected into two normal animals, 62 and 63, but in neither was there more than a very slight induration of the testicles. *B. muris* was not found in the blood.

Strain 28 B proved, therefore, to be identical in pathogenicity and cultural properties with the strains isolated from Rats 54 and 66. All three pathogenic strains are evidently the same microorganism. The culture forms are small, measuring 0.4 to 0.8 μ in length and 0.2 to 0.3 μ in width, and appear very much like *Bartonella muris*. The organisms found in the indurated testicular tissues were coarser

TABLE I.

Summary of Experiments with the Organism Cultivated.

Source of culture	Strain No.	Result of inoculation
Blood of splenectomized Rat 28	28 A	Non-pathogenic
Blood of splenectomized Rat 28	28 B	Induced testicular lesion in Rat 42. Culture recovered from testicular tissue Suspension of testicular tissue induced slight induration of testicles in Rats 62 and 63
Testicular tissue of Rat 42, inoculated with culture of Strain 28 B	28 B	No testicular lesions in Rats 52 and 53. Few intracorpuscular elements suggestive of *B. muris* seen in blood of Rat 52 Induration of testicles in Rats 58, 59, 60, 61. Culture recovered from Rat 59
Testicular tissue of Rat 54, inoculated with suspension of liver tissue of splenectomized Rat 40	54	Slight induration of testicles in Rats 56 and 57. Intracorpuscular elements in small numbers in blood of both
Testicular punctate of Rat 66, inoculated with citrated blood of splenectomized Rat 41	66	Not tested

(1 to 1.5 μ by 0.3 to 0.4 μ), took a deeper stain, and showed very little resemblance to the delicate *B. muris* of the blood of splenectomized rats.

Several normal rats, rabbits, and guinea pigs were injected intratesticularly with pooled cultures of Strains 28 B, 54, and 66 (from Rats 28, 42, 54, 59, and 66). The rats showed the usual marked induration of the testicles, but the other animals reacted only slightly. *B. muris* did not appear in the blood of any of the animals.

Immunization Experiments.

Several normal rats were inoculated intraperitoneally on several occasions with live cultures of Strains 28 B, 65, and 66, and 9 to 10 days after the last injection the animals were splenectomized. Following the splenectomy *B. muris* promptly appeared in the blood of all, that is, previous active immunization with the microorganism in question conferred no protection against the invasion of *B. muris* on subsequent splenectomy.

An immune serum pepared in a rabbit by repeated intravenous injections with live cultures of different strains of the pathogenic microorganism failed to cause the disappearance of *B. muris* from the blood of splenectomized rats or to prevent invasion of the blood by *B. muris* thereafter when given intraperitoneally immediately before splenectomy.

Although neither active nor passive immunity could be induced in rats against *B. muris*, by means of the microorganism under study, this organism is not definitely proven not to be *B. muris* since according to recent observations of Mayer and his coworkers no immunity develops in splenectomized rats as a result of previous infection, the organisms appearing again in the blood of recovered individuals when blood containing them is intracardially injected. In the case of *B. bacilliformis* killed cultures do not constitute a potent vaccine, and an immune serum prepared in rabbits by repeated intravenous injections of live cultures of the organism confers little or no protection against a virulent strain of the parasite, the incidence of takes among vaccinated and control monkeys being practically the same.

The identity of the pathogenic microorganism cultivated from splenectomized rats cannot be established until suitable experimental animals are found. In the meantime I propose for it the provisional name, *Bacterium murium*.

Bacterium murium was not isolated from the blood of 20 normal rats. Its invasion of the blood of splenectomized animals was perhaps due to the removal of the spleen.

SUMMARY.

Bartonella muris appeared in the blood of all white rats, wild rats, Chinese hamsters, and mice, from which the spleen was removed, but

did not appear in that of splenectomized monkeys, rabbits, or guinea pigs.

It has not been possible to transmit *B. muris* to normal rats, monkeys, rabbits, or guinea pigs, by intraperitoneal, intradermal, or intravenous injection of blood containing *B. muris* from splenectomized rats.

In two instances an acute orchitis was induced in normal rats by injection directly into the testicle of blood or saline suspensions of the liver of splenectomized rats. The intracorpuscular elements occasionally found in the blood of some of the animals could not be definitely identified as *B. muris* or as having appeared as a result of the inoculation. The acute orchitis of rats was transferable to normal rats in series.

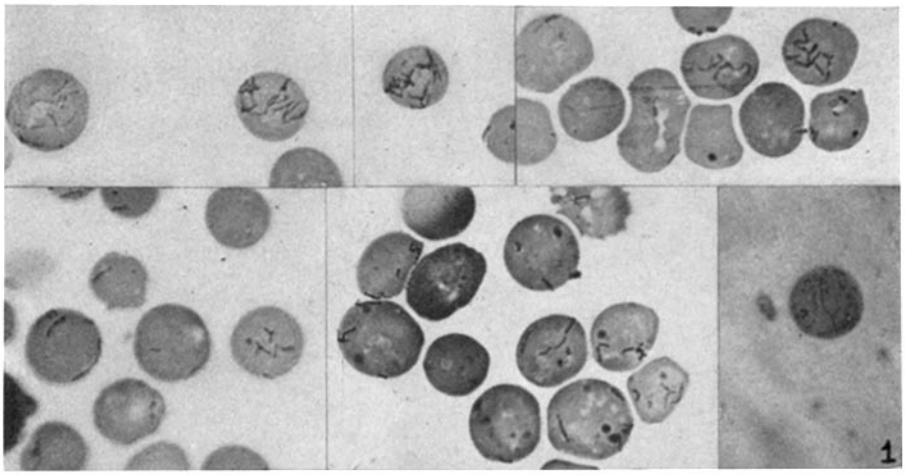
From the testicular tissue, as well as directly from the blood of a splenectomized rat, there was isolated in pure culture a microorganism which induced in the testicles of normal rats an acute orchitis such as resulted from inoculation of the blood or liver suspensions of splenectomized rats. While a few inclusions were found in the erythrocytes of some of the animals, their number was so small and their occurrence so infrequent that they could not be definitely identified as *B. muris*.

In morphological features the cultural forms of the microorganism isolated resemble *B. muris*. The organism found in the testicular tissues, however, is considerably coarser than *B. muris* and takes a deeper stain.

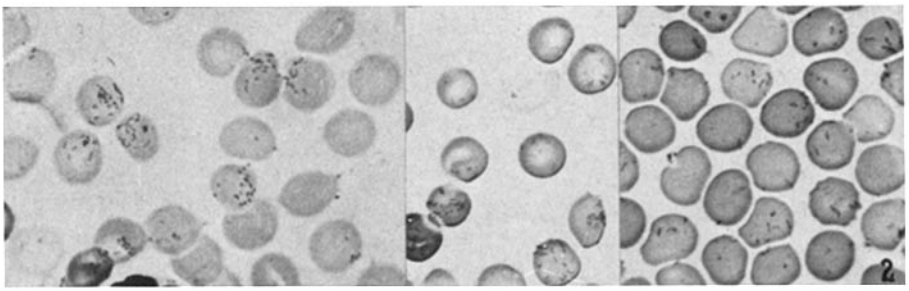
Immunological study failed to settle the question of the relation between *B. muris* and the cultivated organism, which is provisionally called *Bacterium murium*.

Bartonella muris, *Bartonella bacilliformis*, and *Grahamella talpæ* have characteristic individual morphological features.

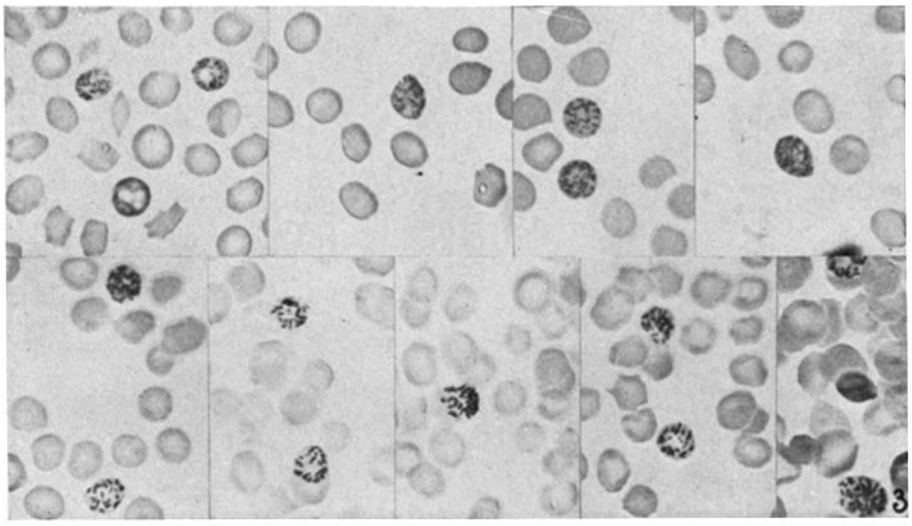
Giemsa's stain. × 1000.



Bartonella bacilliformis in human blood.



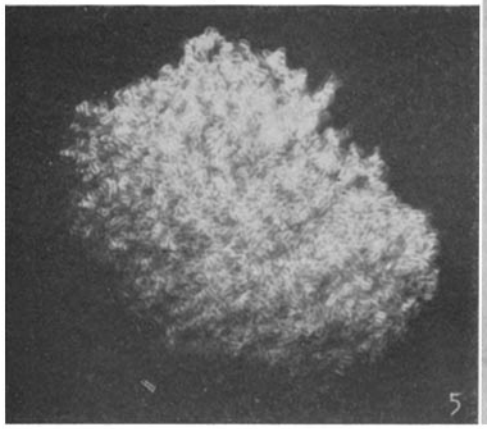
Bartonella muris in blood of splenectomized rats.



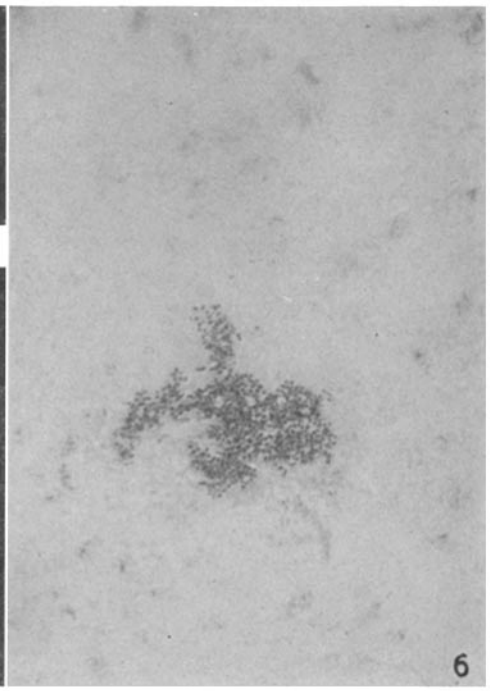
Grahamella talpæ in blood of English moles, Coles Strains 1 (above) and 2 (below).

Cultures from blood of splenectomized rats.

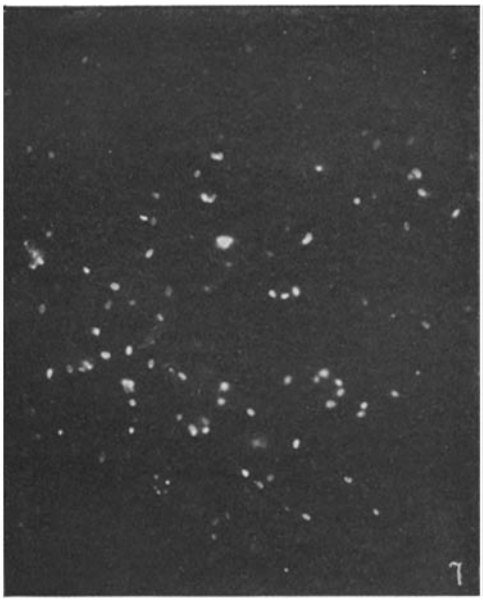
Strain 28B. Blood agar. 48 hrs. Natural size.



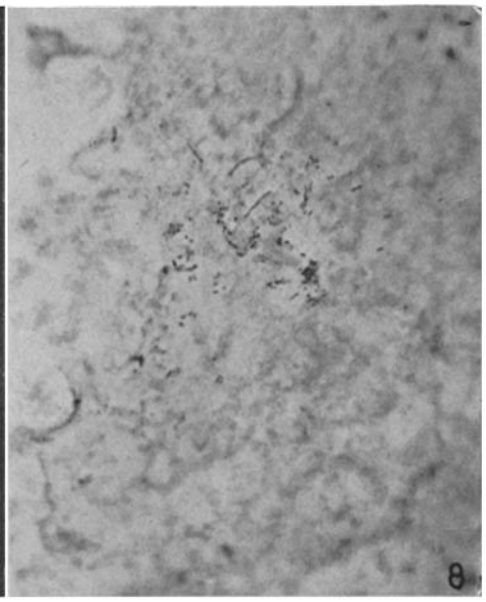
Strain 28B. Blood slant. 24 hrs. Dark field. × 1000.



Strain 28B. Blood slant. 48 hrs. Giemsa's stain. × 1000.



Strain 28A. Dark field. × 1000.



Strain 28A. Giemsa's stain. × 1000.