CULTIVATION OF RICKETTSIA-LIKE MICROORGANISMS FROM THE ROCKY MOUNTAIN SPOTTED FEVER TICK, DERMACENTOR ANDERSONI.

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PLATES 15 TO 18.

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A large number of microorganisms have been reported in recent years under the name of *Rickettsia*, or as *Rickettsia*-like microorganisms. The majority of these minute, for the most part intracellular, Gram-negative forms have been found in insects and arachnids.[1,2] Only in Rocky Mountain spotted fever,[3,4] typhus,[5] and heartwater disease[6] have pathogenic *Rickettsiæ* been definitely found in mammalian tissues.

Only a few of the *Rickettsiæ* have been isolated in pure culture. Nöller[7] reports successful cultivation of *Rickettsia melophagi*, Kuczynski[8] of *R. prowazeki*, and Sikora[9] of *R. pediculi*. By means of tissue culture technique, Wolbach and Schlesinger[10] obtained virulent cultures of *R. prowazeki* and *Dermacentroxenus rickettsi* from the tissues of experimentally infected animals.

Since the various *Rickettsiæ* thus far described are defined for the most part only by their host species and their morphological features, it is difficult to decide, in the lack of a comparison under controlled conditions, whether the forms found in different insects, notwithstanding their morphological similarity, are specific in each instance for the

[1] Cowdry, E. V., *J. Exp. Med.*, 1923, xxxvii, 431.

[2] Hertig, M., and Wolbach, S. B., *J. Med. Research*, 1923–24, xliv, 329.

[3] Wolbach, S. B., *J. Med. Research*, 1919, xli, 1.

[4] Nicholson, F. M., *J. Exp. Med.*, 1923, xxxvii, 221.

[5] Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

[6] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231, 253.

[7] Nöller, W., *Arch. Schiffs- u. Tropen-Hyg.*, 1917, xxi, 53.

[8] Kuczynski, M. H., *Med. Klin.*, 1920, xvi, 706, 733, 759.

[9] Sikora, H., *Arch. Schiffs- u. Tropen-Hyg.*, 1921, xxv, 123.

[10] Wolbach, S. B., and Schlesinger, M. J., *J. Med. Research*, 1923–24, xliv, 231.

insect species, or whether the same form or group of forms infests several insects. Cultivation on artificial media would undoubtedly furnish more information with regard to their morphological and biological properties.

The present investigation has to do with the microorganisms of the spotted fever tick, *Dermacentor andersoni*, and represents the outcome of an attempt to cultivate the causative microorganism of spotted fever from infected ticks. A suspension of the viscera of an infected tick yielded on cultivation in a certain medium[11] a growth of minute, slightly motile, Gram-negative diplobacillary forms morphologically similar to the forms found in smears of the suspension; and 0.5 cc. of the culture (72 hours old) induced typical spotted fever, after 12 days, when intraperitoneally inoculated into the guinea pig. The fever lasted 6 days, and the animal showed the characteristic scrotal lesions of spotted fever. Transfers with blood taken at the height of fever induced typical spotted fever in other guinea pigs. Subcultures from the original culture, however, failed to induce either infection or immunity, as did also the original culture when 2 weeks old. Other immunological tests proved that the organism in question was in no way related to the virus of spotted fever, which had survived for a time in the original culture. The strain of spotted fever recovered from it was carried in guinea pigs for some time. The organism that had been cultivated resembles *Dermacentroxenus rickettsi* morphologically; it grows best at temperatures ranging from 15–26°C., and not at all at 37°C.

The incident showed that from the tissues of an infected tick a non-pathogenic microorganism closely resembling *Dermacentroxenus rickettsi* may be cultivated on special media. Through the generosity of Dr. R. R. Parker, of Hamilton, Montana, in keeping me supplied with adequate numbers of ticks, I have been able to carry out a systematic study of a large amount of material, both infective and non-infective.

[11] To 9 parts Hiss serum water containing various carbohydrates in series was added 1 part of 2 per cent nutrient agar. N/10 HCl was added to give a pH range of approximately 4.5 to 7.4.

Material and Mode of Investigation.

A number of ticks collected in the Bitter Root Valley, and termed "drag" ticks because of the method of collection, were fed on guinea pigs infected with a strain of spotted fever virus known as Barlow (which was given to me by Dr. R. R. Spencer, of Hamilton, Montana, in 1922) for 72 to 96 hours. Those which became engorged were kept at 12–15°C. during the period before use, which was never less than 7 days. Some drag ticks were fed on normal guinea pigs to determine the presence of natural infection, and in this way three new strains of spotted fever virus were obtained; these were termed Parker, Sawtooth, and Blodgett. The three guinea pigs which became infected from the naturally infected ticks were used for infecting other drag ticks. There were acquired, in this way therefore, four lots of drag ticks, each lot infected with a different strain of the virus.

Most of the ticks fed on normal guinea pigs did not induce infection, and these were regarded as non-infective ticks. The infectivity or non-infectivity of a given tick was determined in normal guinea pigs by feeding and by inoculation of emulsified viscera, or, when these procedures gave negative results, by immunity tests.

A tick which had become well engorged by feeding 72 to 96 hours was sterilized as far as possible on the outside with alcohol, ether, and sterile saline solution, and then cut into halves longitudinally with a sterile knife. One half was put into Regaud's fixative for sectioning and staining with Giemsa's and Gram-Weigert's stain. The other half was emulsified in a sterile mortar with 3 cc. of sterile saline solution. 1 cc. of the emulsion was injected intraperitoneally into a male guinea pig (450 to 500 gm.), and the remainder inoculated into various media. Smears were always made with the emulsion and sometimes also with the cut edge of the half tick before it was put into the fixative. This latter method often gave the best results. The smears were stained with Giemsa's and Gram's solutions.

All guinea pigs injected with the emulsions, unless they had typical spotted fever, were subsequently tested for immunity with blood from infected guinea pigs.

Cultural Methods.

In order to obtain comparable results, nearly equal numbers of infective and non-infective ticks were studied at the same time and under parallel conditions. Whether or not a given tick was infective was of course still unknown at the time when it was killed for cultivation purposes.

Several culture media were always used, and duplicate series were placed at 26°C. and 37°C. The special media used were: (1) leptospira medium (semisolid) plus carbohydrate (glucose, saccharose, maltose, mannose, levulose, mannitol, dextrin, xylose, inulin, salicin, dulcitol, and rhamnose) in a concentration of 0.2 per cent; (2) the same, containing a piece of fresh rabbit kidney; (3) the same as (1), but with an agar concentration of 1 per cent and slanted; (4) Novy and McNeal's medium containing the same sugars as the foregoing. Plain broth, agar slants, and yolk slants were always employed in addition to the special media. In the earlier experiments ordinary leptospira medium with and without fresh tissue was used, but it was soon omitted as less suitable than other media, notwithstanding the fact that *Leishmanias*, as well as leptospiras, grow vigorously at 15–26°C. on it, as they do also on media (1), (2), and (4). The quantity of tick suspension used for inoculation of the media was 0.1 cc.

As was to be expected, some of the emulsions contained more than one kind of microorganism. In such instances the growth obtained on semisolid medium was plated on the No. 4 medium to separate the organisms.

RESULTS.

Several varieties of bacteria have been isolated from emulsions representing 75 different ticks. The one most frequently encountered was very similar to that isolated in the preliminary experiment. Two others often grew on the special media, sometimes together. All three of the varieties cultivated have certain morphological features which simulate the pleomorphic phases of *Dermacentroxenus rickettsi* in spotted fever ticks, as described by previous investigators. For the sake of convenience, I shall designate the first type *Bacillus rickettsiformis*, the second *B. pseudoxerosis*, and the third *B. equidistans*.

Bacillus rickettsiformis, N. Sp.

Growth.—Initial cultures were obtained only on the special media, and at a temperature of 26°C., as minute, yellowish gray, semitransparent, shining, oval or round, slightly raised colonies near the condensation water. Consistency somewhat mucoid; after several days the colonies show a tendency to coalesce. The condensation water is slightly turbid and contains a grayish, rather sticky sediment. In a few instances a grayish surface growth was obtained on leptospira medium. Old strains grow well on broth, which shows slight opalescence, with surface pellicle and deposit, and on ordinary agar, on which some strains form a light yellowish pigment after several days. No growth is obtained under anaerobic conditions. One strain by gradual adaptation was made to grow at 37°C. Gelatin is not liquefied.

Morphology.—In young cultures the organisms are fairly uniform in size, measuring 0.3μ in width and 0.75μ in length; some of them are moderately motile. Single organisms are lanceolate, fusiform, or rod-shaped, and measure 0.25 to 0.35μ in width at the widest portion and 0.5 to 1μ in length, the longest about 1.4μ. Diplobacillary forms consist of two lanceolate forms joined end to end and measure about 2μ in length. The long rods do not occur in diploid form. In old cultures there is considerable pleomorphism, some individuals becoming oval, fusiform, or filamentous; the filamentous forms may show unevenness in width throughout the length of the body; some, especially those which settle down to the bottom of the fluid medium, have swollen ends. Vigorously growing individuals take a better stain than old ones. The organism is Gram-negative; it does not take Loeffler's methylene blue well. Staining with Giemsa's solution shows a light reddish violet body, of bipolar or diplobacillary shape, the middle portion being less distinctly stained. In bipolar elements the ends are abruptly pointed and show no stricture at the middle portion of the organism. Under certain conditions almost coccoid shapes may be assumed.

Strain Variations.—Variations in size exist among different strains, some being decidedly coarser or finer than the average type. In old surface colonies of some strains a light lemon-yellow color may appear.

Photomicrographs of the organisms found in smears and sections of infective and non-infective ticks, and of cultures of *B. rickettsiformis*, are shown for comparison (Figs. 1 to 23). Figs. 1 and 2 are dark-field views of one of the richest smears I have ever seen from non-infective ticks. The organisms are diploid and appear in paired refractile oval dots close together. In the center of Fig. 1 is a large round mass of organisms which look as if they were packed in a sac. This appearance is characteristic of rich emulsions.

When one compares carefully the forms found in the ticks and those assumed by *B. rickettsiformis* under various cultural conditions, one is tempted to regard them as identical. The causative agent of spotted fever, *Dermacentroxenus rickettsi*, is indistinguishable morphologically from the forms found in infective ticks, which again are similar to those found in non-infective ticks and those assumed by *B. rickettsiformis;* the only points of differentiation in the case of *Dermacentroxenus rickettsi* are its pathogenicity for certain mammalians and the fact that it is uncultivable by the methods employed in this investigation. Since ticks in general harbor in their tissues so called *Rickettsiæ*, the non-pathogenic tissue parasites in some of the wood ticks may likewise be regarded as *Rickettsia*, according to the present day usage

of the term, which is based on morphological characteristics. Should similar organisms be found in ticks collected in the eastern parts of the United States, we shall have an explanation of the presence, in non-infective ticks caught in spotted fever regions, of these forms which are morphologically so confusing when studied in connection with the spotted fever organism.

The foregoing morphological description applies to tick smears equally as well as to cultures of *B. rickettsiformis*. Some very minute (0.25 by 0.5μ) and some rather large (0.4 by 1μ) individuals occur in smears as well as in cultures, as do also coccoid forms (0.35 by 0.4μ).

In tick sections (salivary glands, etc.) single lanceolate individuals (0.25 to 0.35μ to 0.5 by 1μ) predominate; in some sections thinner forms are found, owing to different conditions of fixation and differentiation (Figs. 14, 19, 20). This appearance is also observed in cultures (Figs. 21, 22). Some coarser individuals are found in muscle (Fig. 18).

The organism of Rocky Mountain spotted fever in mammalian tissues presents striking resemblances to *B. rickettsiformis*, notwithstanding the fact that the latter is not related to it in any way. Smears show somewhat coarser organisms (Figs. 24 to 26) than sections (Figs. 27 to 29), owing to flattening of the former and greater decolorization of the latter in differentiation. Similar forms are seen in the smears of cultures of *B. rickettsiformis* (Figs. 30 to 34). Rather coarser individuals but with similar morphological characteristics are seen in Fig. 35.

Bacillus pseudoxerosis, N. Sp.

Growth.—Initial growth was obtained on the special media (slanted and semi-solid) at 26°C. The colonies are light yellowish gray, shining, mucoid, and confluent. Subcultures grow fairly well on plain agar and broth, producing in the latter slight turbidity, with surface scum and stringy sediment. Optimum temperature for growth, 26°C. No growth under anaerobic conditions. Gelatin not liquefied.

Morphology.—Non-motile, slender, pleomorphic bacilli, measuring, in young surface cultures, 0.3 to 0.5μ in width and 0.4 to 2μ in length (Fig. 36). There is some resemblance to *B. xerosis* in arrangement and appearance. In somewhat older cultures groups of short rods are mingled with longer, often considerably thicker, rather curved individuals, 0.5 to 0.55μ by 3 to 4μ (Figs. 37, 38). The organism is somewhat resistant to Gram's stain. Bifurcated forms are seen in some cultures (Fig. 38).

Bacillus equidistans, N. Sp.

Growth.—Initial growth was obtained on the special slanted and semisolid media at 26°C. Light grayish white, somewhat fluorescent, round or oval, shining colonies on the surface of slants, tending to become confluent within several days. Growth of later subcultures obtained on plain broth and agar slants. The broth becomes moderately turbid, and after several days there is a heavy sticky scum and sediment. Colonies on slants are at first delicate but in a few days become heavier, mucoid, and have a light, brownish yellow tint. The organism grows best at 15–26°C.; 37°C. is less favorable for growth. No growth occurs under anaerobic conditions. Gelatin is not liquefied.

Morphology.—The most characteristic feature of this organism is its appearance of uniform dispersion, as if the individuals were purposely placed equidistant from one another. No motility has been observed. In young surface cultures there are many single individuals measuring 0.25 to 0.3μ by 0.4 to 0.8μ (Fig. 39). Diploid forms are sometimes predominant (Fig. 40), and there is an appearance of a narrow bridge between the two cells. In some instances minute coccoid forms, not unlike *Bacterium tularense*, may be assumed. This organism is negative by Gram's stain and stains reddish violet with Giemsa's solution. It appears to have a capsule, as indicated by a clear unstained or lightly stained zone several times wider than the cell itself. In older cultures are found irregularly curved filamentous forms, with a tendency to branch (Fig. 41).

The feces of ticks yielded the same three types of organisms on cultivation, *B. rickettsiformis* being most frequently present. It may be mentioned that the injections of suspensions of feces from twenty ticks which were infective (as shown by production of spotted fever by feeding on guinea pigs) produced some local infiltration and rise of temperature within 48 hours, but no spotted fever symptoms nor any immunity to the disease. This experiment merely shows the infrequency of spotted fever organisms in the feces of infected ticks and does not exclude the possibility of their occasional presence.

The results of fermentation tests with the three varieties of microorganisms cultivated are recorded in Table I. The medium used was Hiss's litmus serum water, and the cultures were kept 7 days at 26°C.

The strains of *B. rickettsiformis* used in this test only slightly attacked dextrose, galactose, levulose, and lactose. In some other experiments, however, strains were encountered which caused slight acidity with dextrose, maltose, xylose, and dextrin. *B. pseudoxerosis* affected the majority of carbohydrates employed but never sufficiently to cause any coagulation. *B. equidistans* fermented dextrose suffi-

ciently to produce marked acidity and coagulation without evolution of gas. Other carbohydrates were either very slightly or not at all fermented. In this respect the organism, though perhaps belonging to the group of *B. mucosus capsulatus*, appears to differ from any of the group so far known.

TABLE I.

Fermentation Reactions with the Microorganisms Cultivated from Ticks.

	B. rickettsiformis.		*B. pseudoxerosis.*		*B. equidistans.*	
	7 days.	30 days.	7 days.	30 days.	7 days.	30 days.
Dextrose.....	Trace acid.	+	Distinctly acid.	+++	Coagulation; acid; no gas.	+++
Saccharose....	−	−	" "	+++	−	−
Galactose.....	Trace acid.	+	" "	−	Slightly acid.	++
Maltose.....	−	−	" "	+++	" "	+++
Levulose.....	Trace acid.	+	" "	+++	" "	++
Xylose.....	−	−	" "	+	−	−
Lactose.....	Trace acid.	+	" "	++	Decolorized; no acid.	−
Mannose.....	−	−	" "	+++	Slightly acid.	++
Mannitol.....	−	−	" "	+++	−	−
Dulcitol.....	−	−	−		−	−
Raffinose.....	−	−	−		−	−
Rhamnose....	±	+	Trace acid.	+++	−	−
Dextrin.....	−	−	" "	+++	Markedly acid; no gas.	+++
Inulin.....	−	−	" "	+++	Trace acid.	−
Salicin.....	−	−	" "	+	−	−

Frequency and Distribution of the Various Organisms among Wood Ticks.

Most of the tick emulsions were sterile as tested with plain media (agar and broth) and with special media at 37°C., but the majority yielded some colonies on the special media after several days incubation at 26°C. Subsequent purification by plating showed that most of the ticks contained *B. rickettsiformis*, some *B. pseudoxerosis* or *B. equidistans* in addition, while a few yielded only one of the latter. In Tables II to VI are recorded the data regarding the findings in the case of each tick, including the results of biting tests, injection of

emulsion of viscera, and subsequent tests for specific immunity against spotted fever, the presence or absence of microorganisms in smears and sections, and finally the varieties of bacteria cultivated.

TABLE II.

Drag Ticks Which Did Not Induce Spotted Fever When Tested on Normal Guinea Pigs 12 Days Previously.

Tick No.	Results of					Bacteria isolated in culture.
	Feeding.	Inoculation of emulsion.	Immunity test.	Smears.	Sections.	
1	−	−	−	−	−	−
2	−	−	−	<+	+	*B. rickettsiformis.*
3	−	−	−	−	−	−
4	−	−	−	−	−	−
5	−	−	−	<+	+	*B. rickettsiformis.*
6	−	−	−	−	−	−
7	−	−	−	+	+++	*B. rickettsiformis.*
8	−	−	−	−	−	−
9	−	−	−	−	<+	*B. rickettsiformis; B. pseudoxerosis.*
10	−	−	−	−	−	−
11	−	−	−	−	−	−
12	−	−	−	−	−	−
13	−	−	−	−	−	−
14	−	−	−	−	−	−
15	−	−	−	+	++	*B. pseudoxerosis.*
16	−	−	−	+	++	" "
17	−	−	−	−	−	−
18	−	−	−	+++	++++	*B. pseudoxerosis.*
19	−	−	−	++	++++	" *rickettsiformis.*
20	−	−	−	−	−	−
21	−	−	−	−?	++	*B. rickettsiformis.*
22	−	−	−	−	−	−
23	−	−	−	−	−	−
24	−	−	−	−	−	−
25	−	−	−	+	++++	*B. rickettsiformis.*

Analysis of Results Shown in Tables II to VI.

Of 25 ticks fed previously on normal guinea pigs, none conferred either infection (as tested by biting experiments or inoculation of emulsified viscera) or immunity; that is, they were non-infective under the present experimental conditions. 15 of these yielded no culture,

5 yielded strains of *B. rickettsiformis*, 3 *B. pseudoxerosis*, and 2 *B. rickettsiformis* and *B. pseudoxerosis*.

Of 49 ticks fed previously on spotted fever guinea pigs, 14 conferred neither infection nor immunity; of these 2 yielded no cultures, 5 yielded *B. rickettsiformis*, 2 *B. pseudoxerosis*, 2 both of these organisms, 1 *B. equidistans*, 1 *B. pseudoxerosis* and *B. equidistans*, and 1 an undetermined Gram-negative bacillus.

TABLE III.

Drag Ticks Which Became Engorged When Fed on Spotted Fever Guinea Pigs (Barlow Strain) 12 Days Previously.

Tick No.	Results of					Bacteria isolated in culture.
	Feeding.	Inoculation of emulsion.	Immunity test.	Smears.	Sections.	
1	+	+		<+	+	*B. rickettsiformis.*
2	+	+		<+	+	" "
3	+	+		<+	+	−
4	+	+		+	++	−
5	+	+		+	+	−
6	+	+		+	++	*B. rickettsiformis.*
7	+	+		+	++	" "
8	+	+		±	+	" "
9	+	+		±	+	" "
10	−	−	+	+	+	" "
11	+	+		++	+++	" "
12	−	+		<+	++++	" "
13	+	+		<+	<+	" " *B. pseudoxerosis.*
14	+	+		++	+++	*B. rickettsiformis.*
15	−	+		+++	+++	" *pseudoxerosis.*
16	−	+		++++	+++	" *rickettsiformis.*
17	+	+		+++	+++	" "

Of the remaining 35 ticks, 16 (Group 1) caused typical spotted fever, both by biting and inoculation of emulsions, 13 (Group 2) failed to infect by biting, but the inoculation of emulsions conferred infection, 4 (Group 3) caused slight fever which proved by subsequent immunity test to have been due to spotted fever infection, and 2 (Group 4) were negative, but the animals which received the emulsified viscera proved to be immune to spotted fever; that is, all of these 35 ticks

carried the virus of spotted fever, 16 of them in highly virulent form. 10 of the ticks of Group 1 yielded *B. rickettsiformis* in culture, 2 *B. rickettsiformis* and *B. pseudoxerosis*, 1 *B. equidistans*, and 3 no growth. Of Group 2, 1 was sterile, and 12 yielded cultures, 4 *B. rickettsiformis*, 2 *B. pseudoxerosis*, 2 both, 3 *B. rickettsiformis* and B. *equidistans*, and 1 *B. equidistans*. Of Group 3, 1 was negative and 3 positive, 1 yielding *B. rickettsiformis*, 2 *B. pseudoxerosis*. One tick of Group 4 yielded *rickettsiformis* the other *rickettsiformis* and *pseudoxerosis*.

TABLE IV.

Drag Ticks Which Became Engorged When Fed on Spotted Fever Guinea Pigs (Parker Strain) 12 Days Previously.

Tick No.	Results of					Bacteria isolated in culture.
	Feeding.	Inoculation of emulsion.	Immunity test.	Smears.	Sections.	
1	−	−	−	++	+++	*B. rickettsiformis; B. pseudoxerosis.*
2	−	−	−	−	−	−
3	−	−	−	−	−	−
4	−	+	+	+	++++	*B. rickettsiformis; B. pseudoxerosis.*
5	−	+		+	+++	*B. rickettsiformis.*
6	−	−	−	+	++	" "
7	−	+	+	<+	<+	" "
8	−	−	−	−	−	−
9	+	+		++++	+++	*B. rickettsiformis; B. pseudoxerosis.*
10	+	+	+	++++	++++	*B. rickettsiformis.*
11	−	+	+	<+	+++	" " *B. equidistans.*

The percentage of non-infective ticks which yielded cultures was 40 per cent, of infective ticks 85 per cent. *B. rickettsiformis* occurred alone in 27 of the 52 ticks from which cultures were obtained (51 per cent), and in association with one of the organisms in 11, the total incidence of this organism being 73 per cent. *B. pseudoxerosis* occurred alone in 9 instances, and in association with one of the others in 9, the total incidence being 33 per cent, while *B. equidistans* occurred alone in 3 and with one of the others in 4 instances, the total incidence being 13 per cent.

TABLE V.

Drag Ticks Which Became Engorged When Fed on Spotted Fever Guinea Pigs (Sawtooth Strain) 12 Days Previously.

Tick No.	Results of					Bacteria isolated in culture.
	Feeding.	Inoculation of emulsion.	Immunity test.	Smears.	Sections.	
1	−	+	+	+++	+++	*B. pseudoxerosis.*
2	−	−	−	+	+	" "
3	−	−	−	±	<+	" *rickettsiformis.*
4	−	+	+	±	<+	" " *B. pseudoxerosis.*
5	−	+		<+	+	" *equidistans.*
6	−	−	+	−	+	" *rickettsiformis; B. pseudoxerosis.*
7	−	Fever.	+	−	+	" *pseudoxerosis.*
8	−	−	−	++	++++	" " *B. equidistans.*
9	−	+	+	+	+	*B. rickettsiformis; B. equidistans.*
10	−	+		++	+++++	*B. rickettsiformis; B. equidistans.*
11	−	Fever.	+	+++	+++++	*B. rickettsiformis.*

TABLE VI.

Drag Ticks Which Became Engorged When Fed on Spotted Fever Guinea Pigs (Blodgett Strain) 12 Days Previously.

Tick No.	Results of					Bacteria isolated in culture.
	Feeding.	Inoculation of emulsion.	Immunity test.	Smears.	Sections.	
1	−	−	−	<<+	+	*B. rickettsiformis; B. pseudoxerosis.*
2	−	−	−	±	+	*B. pseudoxerosis.*
3	−	−	−	±	+	" *rickettsiformis.*
4	−	−	−	<+	+++	Minute, motile, hemolytic, Gram-negative bacillus.
5	−	−	−	<+	+++	*B. equidistans.*
6	−	−	−	?	+	" *rickettsiformis.*
7	−	−	−	?	+	" "
8	−	Fever.	+	?	+	" *pseudoxerosis.*
9	−	"	+	+	+	−
10	+	+	+	++	++	*B. equidistans.*
11	−	+	+	±	+	−

Comparison of the cultural findings with the microscopic examination of the smears and sections shows that 17 ticks which conferred neither spotted fever nor immunity to the infection were negative both microscopically and by culture. All those ticks which yielded cultures of one organism or the other were positive also by microscopic examination, containing minute Gram-negative organisms in varying numbers in smears and sections, in some instances in enormous numbers. On the other hand, in 5 instances in which the ticks were definitely infective, the smears and sections showed the familiar Gram-negative organisms, yet no cultures were obtained from the emulsions. These forms may have been the true spotted fever organism, *Dermacentroxenus rickettsi*, and, like similar organisms in tissues from infected animals, could not be cultivated by the methods employed in the present investigation. The most striking point, however, is the morphological and tinctorial resemblance between the non-pathogenic organisms and the spotted fever parasites.

The source of the non-pathogenic microorganisms is unknown. I have never encountered any of them in mammalian bacterial flora, and they do not grow at body temperature. The optimum temperature of 12–26°C. or lower clearly indicates their adaptation to insects. For comparison, 5 drag ticks which had never been fed on guinea pigs were studied culturally. One of these contained *B. rickettsiformis*, the others were sterile. The result shows, however, that *B. rickettsiformis* does occur under natural conditions in the wood tick. The high incidence of these non-pathogenic parasites in the wood ticks used for the present study may not indicate their presence in wood ticks in general, for ticks collected in different localities and at different periods may not yield the same results.

Pathogenicity and Immunological Properties of the Bacterial Strains.

Guinea pigs, rabbits, and monkeys (*Macacus rhesus*) were inoculated with young cultures of *B. rickettsiformis*, *B. pseudoxerosis*, and *B. equidistans* grown on serum-containing media. In no instance were any lesions of spotted fever induced. There were a number of instances in which a continuous high temperature developed and lasted 2 to 5 days, but the spleen was usually normal, and most of the

animals recovered within a week. They were later tested for their susceptibility to the spotted fever virus, but in only two instances among 69 animals was any immunity present. In the two instances in which the animals became resistant to spotted fever, no significance was attached to the observation, because other animals which had received the same culture developed typical spotted fever.

Several rabbits were immunized with *B. rickettsiformis*, *B. pseudoxerosis*, and *B. equidistans*, respectively, by intravenous injection of cultures, and the immune serums were tested for neutralizing properties for the spotted fever virus. The results were negative. The action of spotted fever immune serums from hyperimmunized rabbits[12] and from recovered human beings[13] was tested *in vitro* upon each of the three organisms, but no specific agglutination or complement fixation was demonstrated. There was a tendency to the formation of clumps in some instances with human or rabbit serums (including normal ones) upon 24 to 48 hours standing at room temperature, owing to slow multiplication of the organisms.

The three organisms were also tested for agglutination and complement fixation with anti-*tularense* immune serum kindly furnished by Dr. Edward Francis, but the results were negative.

With regard to the use of tick emulsions as antigens in specific complement fixation tests with immune serums prepared in rabbits by repeated injections of virulent blood from infected guinea pigs, it was found that ticks fed on guinea pigs 7 days previous to the test still contain enough guinea pig blood to give strong fixation; of 20 ticks fed 40 days previously, fixation was still complete with the emulsions of 3 (females). The immune serum used for this work was of a titre such that 0.00001 cc. of guinea pig serum still completely fixed complement. To avoid this difficulty, human and guinea pig convalescent serums were tested with infective and non-infective tick emulsions. The results were uniformly negative, except in the case of two human (one normal, one convalescent) and two rabbit (one normal, one immune) serums, which fixed complement.

[12] Noguchi, H., *J. Exp. Med.*, 1923, xxxvii, 383.

[13] The specimens of serum from persons recovered from Rocky Mountain spotted fever were sent me through the kindness of Dr. W. F. Cogswell and Dr. John X. Newman, of the State Board of Health of Montana.

SUMMARY.

A systematic study of 74 ticks, the infectivity or non-infectivity of which was determined by biting experiments, inoculation of emulsions, and specific immunity tests, showed the presence in some instances of several types of microorganisms morphologically resembling the inciting microorganism of spotted fever. The most frequently isolated was *B. rickettsiformis, n. sp.*, those less commonly encountered were *B. pseudoxerosis, n. sp.*, and *B. equidistans, n. sp.* These organisms are non-pathogenic for the guinea pig, rabbit, and *Macacus rhesus*. In morphological features they resemble the forms found in smears and sections of the ticks, yet their presence had no relation to infectivity. Immunologically they are not related to the spotted fever virus. All three are pleomorphic under cultural conditions, and the question arises whether or not the minute non-pathogenic *Rickettsia* forms and the somewhat coarser symbionts found in *Dermacentor andersoni* are morphological variations due to variations in nutrition, oxygen tension, tissue reactions, etc., in the different tissues and cells in which the organisms are embedded. At all events, the differentiation of the non-pathogenic *Rickettsia*-like organisms from *Dermacentroxenus rickettsi* is extremely difficult.[14] In definitely infective ticks of the present study intranuclear forms were not constant.

A point of special interest is that these non-pathogenic microorganisms from ticks grow best at room temperature, in this respect resembling culturally certain flagellates inhabiting the alimentary tract of insects.[15] The difficulty of obtaining initial growth on artificial media and the gradual adaptation to less specialized media are other notable characteristics of these organisms. The possibility that *B. rickettsiformis* is a non-pathogenic phase of the spotted fever organism, comparable with the avirulent flagellate culture forms of

[14] Dr. E. V. Cowdry has been kind enough to look through the preparations described in this report without being informed whether they came from infective or non-infective ticks, in order that he might give an objective opinion concerning them. The sections of non-infective ticks contained forms which Dr. Cowdry regarded as *Dermacentroxenus rickettsi;* that is, forms indistinguishable from those found in sections of infected ticks. The organisms occurred in the salivary glands in both infective and non-infective ticks.

[15] Experiments to be reported upon later.

Leishmanias, seems remote in view of the negative immunological findings.

Hereditary transmission of *B. rickettsiformis* is clearly indicated by its presence in large numbers in ovaries and egg cells, a characteristic also of the spotted fever organism[16] and of other insect-borne *Rickettsiæ*.[1,2]

EXPLANATION OF PLATES.

PLATE 15.

FIGS. 1 and 2. Dark-field views of an impression film preparation from a non-infective tick. Film fixed in methyl alcohol, stained with Giemsa's solution, and photographed by dark-field illumination. Note the large unresolved mass of organisms in the left half of Fig. 1. × 900.

FIG. 3. Dark-field view of *B. rickettsiformis* cultivated for 6 days at 26°C. on Hiss serum water containing 1 per cent salicin. Note several agglomerates of the organism from which some individuals have become partly dissociated. × 1000.

FIG. 4. Same film preparation as Figs. 1 and 2, photographed in the ordinary way. × 1000.

FIG. 5. Smear preparation of an infective tick emulsion, fixed with buffered formalin. Gram (negative) counterstained with saturated alcoholic solution of fuchsin. × 1000.

FIG. 6. Smear preparation of an infective tick emulsion, stained in the same way as Fig. 5. × 1000.

FIG. 7. A cell from the hypoderm of an infected tick. Zenker's fixation followed by Giemsa's stain. Note the numerous rod-shaped organisms filling the cytoplasm of the cell. × 1000.

FIG. 8. Film preparation of the brain of an infected tick. Giemsa's stain. × 1000.

FIG. 9. Culture of *B. rickettsiformis* grown on ascites agar slant for 6 days at 26°C. Giemsa's stain. × 1000. Note the striking resemblance to the forms shown in Fig. 4.

FIG. 10. Culture of *B. rickettsiformis*. Blood slant 8 days old (26°C.). Giemsa's stain. × 1000.

FIG. 11. A young culture of *B. rickettsiformis* on blood sugar slant 48 hours old (26°C.). Giemsa's stain. × 1000.

FIG. 12. *B. rickettsiformis* on Nöller's medium (slant) grown 48 hours at 26°C. Giemsa's stain. × 1000. The forms shown in Figs. 11 and 12 bear a remarkable resemblance to those described by Wolbach (Fig. 34, Plate VII, *J. Med. Research*, 1919, xli, 1–197).

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[16] Ricketts, H. T., *J. Am. Med. Assn.*, 1907, xlix, 1278.

PLATE 16.

Magnification × 1000. Giemsa's stain.

FIGS. 13 and 14. *Rickettsia*-like organisms in the cells of the salivary gland of a non-infective tick. Regaud's fixation.

FIGS. 15 and 16. Similar organisms in the salivary ducts of a non-infective tick. Regaud's fixation.

FIG. 17. A cell of the hypoderm of a non-infective tick. Regaud's fixation.

FIG. 18. Similar organisms in the muscle fibres of a non-infective tick. Regaud's fixation.

FIG. 19. *Rickettsia*-like organisms in the epithelial wall of the rectal sac of an infective tick.

FIG. 20. Similar organisms in the brain of an infective tick (*Dermacentroxenus rickettsi?*). While these forms are decidedly more minute than some of the organisms found in sections of non-infective ticks, yet the minute forms shown in Fig. 14 (non-infective tick) are not morphologically and tinctorially distinguishable from those in the sections of infective ticks.

FIG. 21. *B. rickettsiformis* grown on slant agar for 8 days at 26°C.

FIG. 22. Same organism grown on serum agar slant for 8 days at 26°C.

FIG. 23. Same organism grown on Carrel's tissue culture medium for 3 days at 37°C.

All the forms found in the sections of ticks are to be observed also in these cultures.

PLATE 17.

Magnification × 1000. Giemsa's stain (except Fig. 31).

FIG. 24. Impression smear of the spleen of a guinea pig experimentally infected with the spotted fever virus. Buffered formalin fixation.

FIG. 25. Film preparation of teased lesions of tunica of a guinea pig infected with spotted fever. Buffered formalin fixation.

FIG. 26. Impression smear of the ear lesion of a guinea pig infected with spotted fever, showing an unusually large clump of spotted fever organisms in characteristic arrangement.

FIGS. 27 to 29. Spotted fever organisms in the endothelial cells of the small blood vessels at the sites of spotted fever lesions in guinea pigs. Regaud's fixation.

FIG. 30. *B. rickettsiformis* grown on ascites agar slant for 5 days at 37°C. (after gradual adaptation to this temperature).

FIGS. 31 and 32. Same organism grown 4 days at 37°C. on blood sugar slant. Fig. 31 Gram's stain (negative) counterstained with fuchsin; Fig. 32 Giemsa's stain.

FIG. 33. Same organism grown in serum broth at 37°C. for 6 days.

FIG. 34. Same organism grown on ascites broth at 37°C. for 3 days.

FIG. 35. Same organism grown on tissue broth at 37°C. for 8 days.

These photographs show the close resemblance of the forms found in cul ıres of *B. rickettsiformis* grown under different conditions to the spotted fever orga ism in mammalian tissues, although *B. rickettsiformis* is not pathogenically r immunologically related to the causative organism of spotted fever.

PLATE 18.

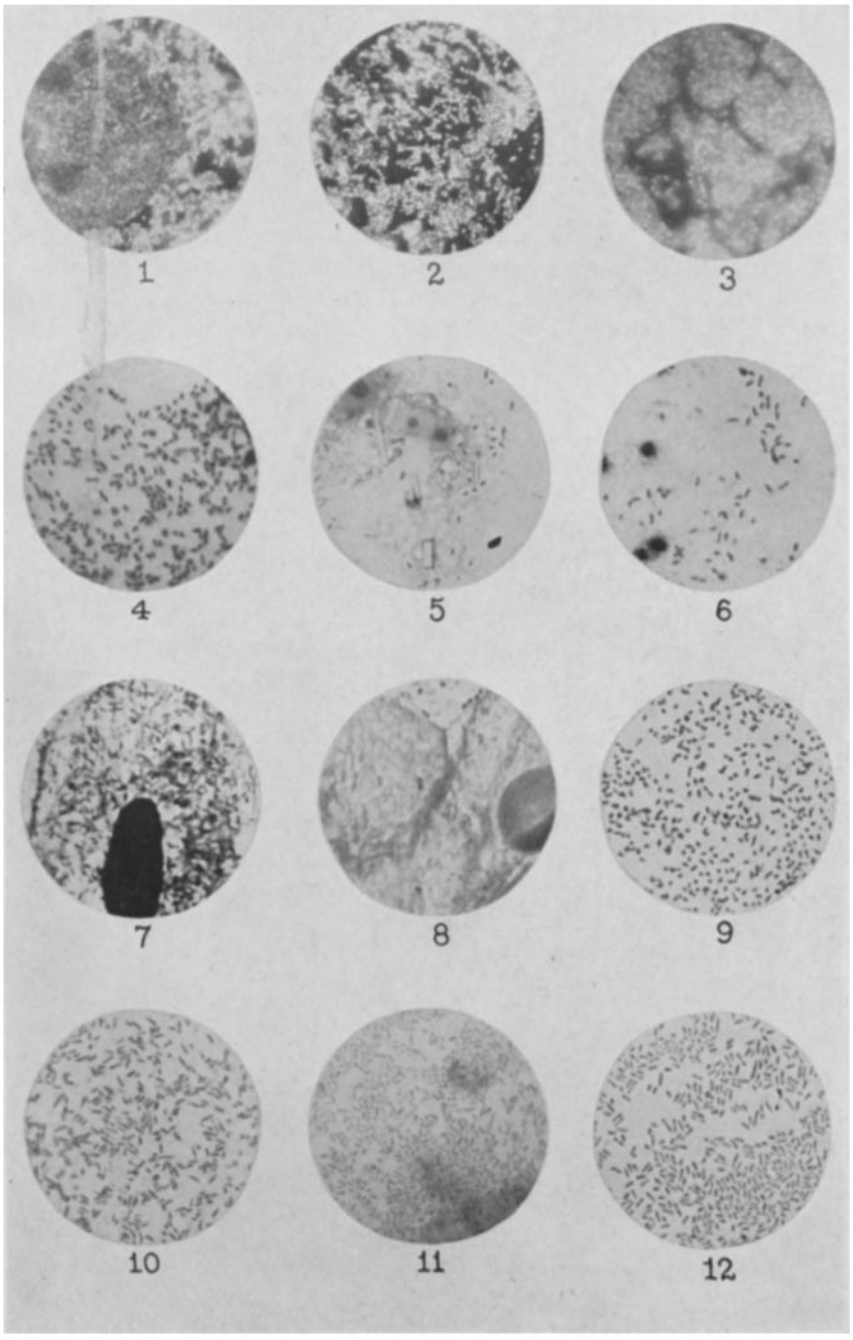
Magnification × 1000. Giemsa's stain.

FIG. 36. *B. pseudoxerosis* grown on agar slant for 3 days at 26°C.

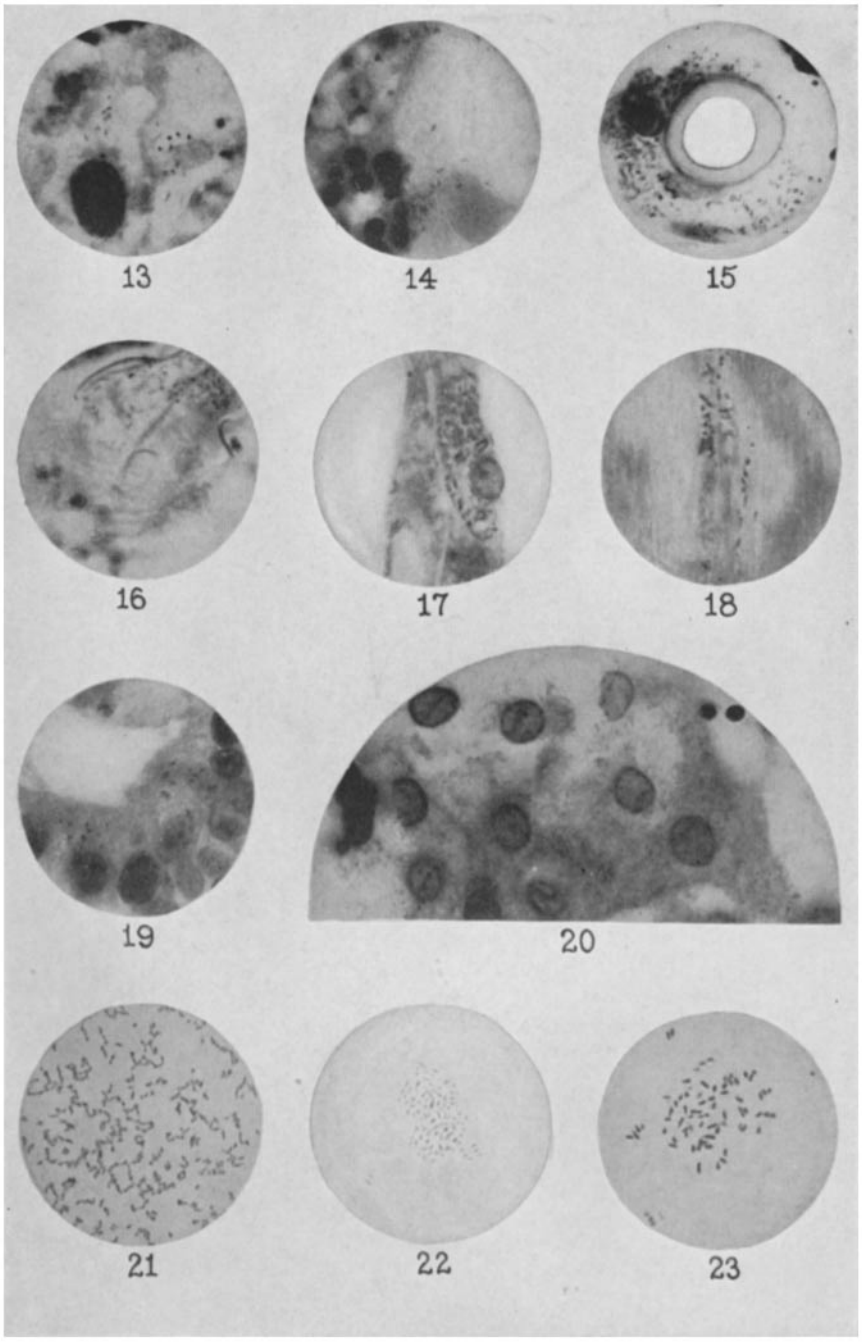
FIGS. 37 and 38. Same organism grown in broth for 5 days at 26°C.

FIGS. 39 and 40. *B. equidistans* grown on agar slant for 3 days at 26°C.

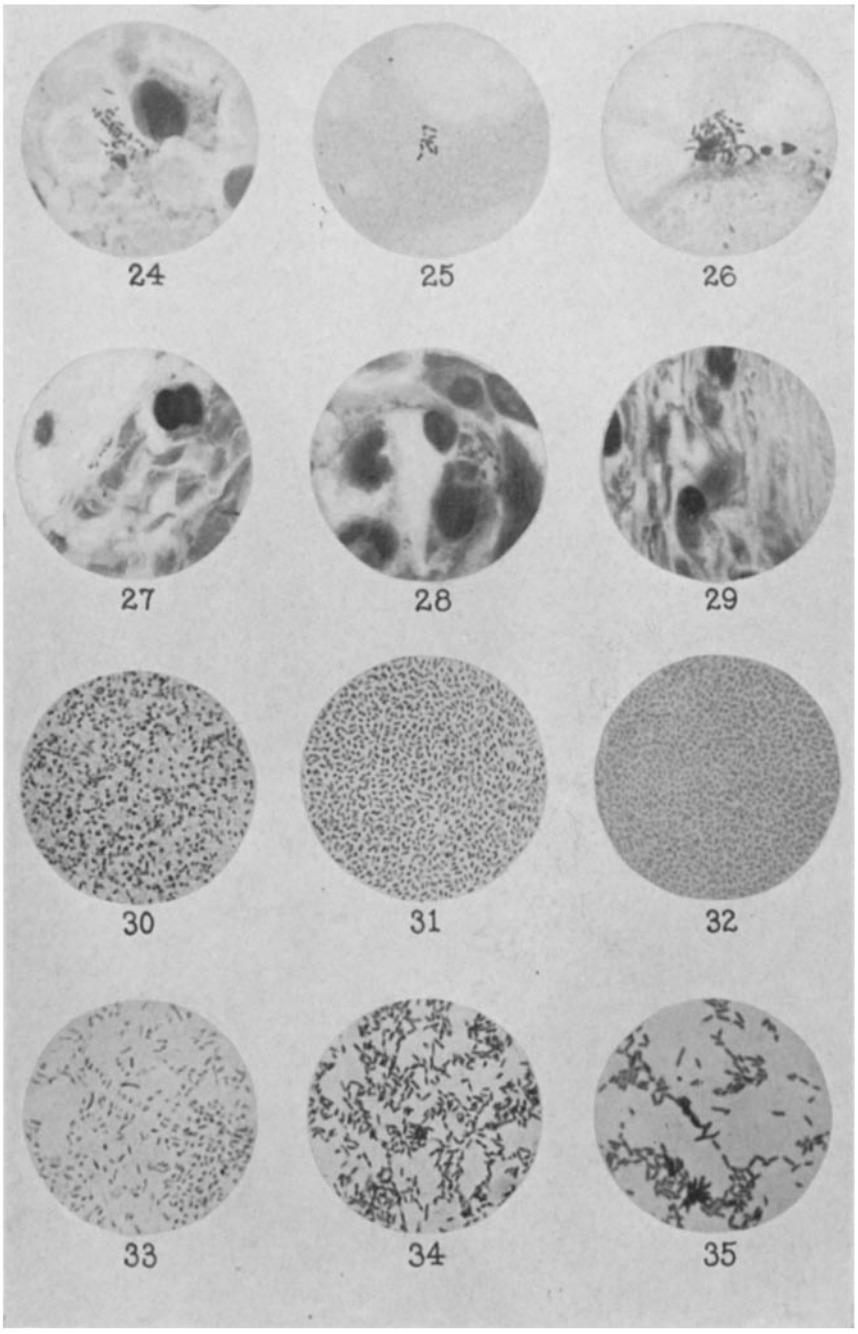
FIG. 41. Same organism grown on broth for 5 days at 26°C.



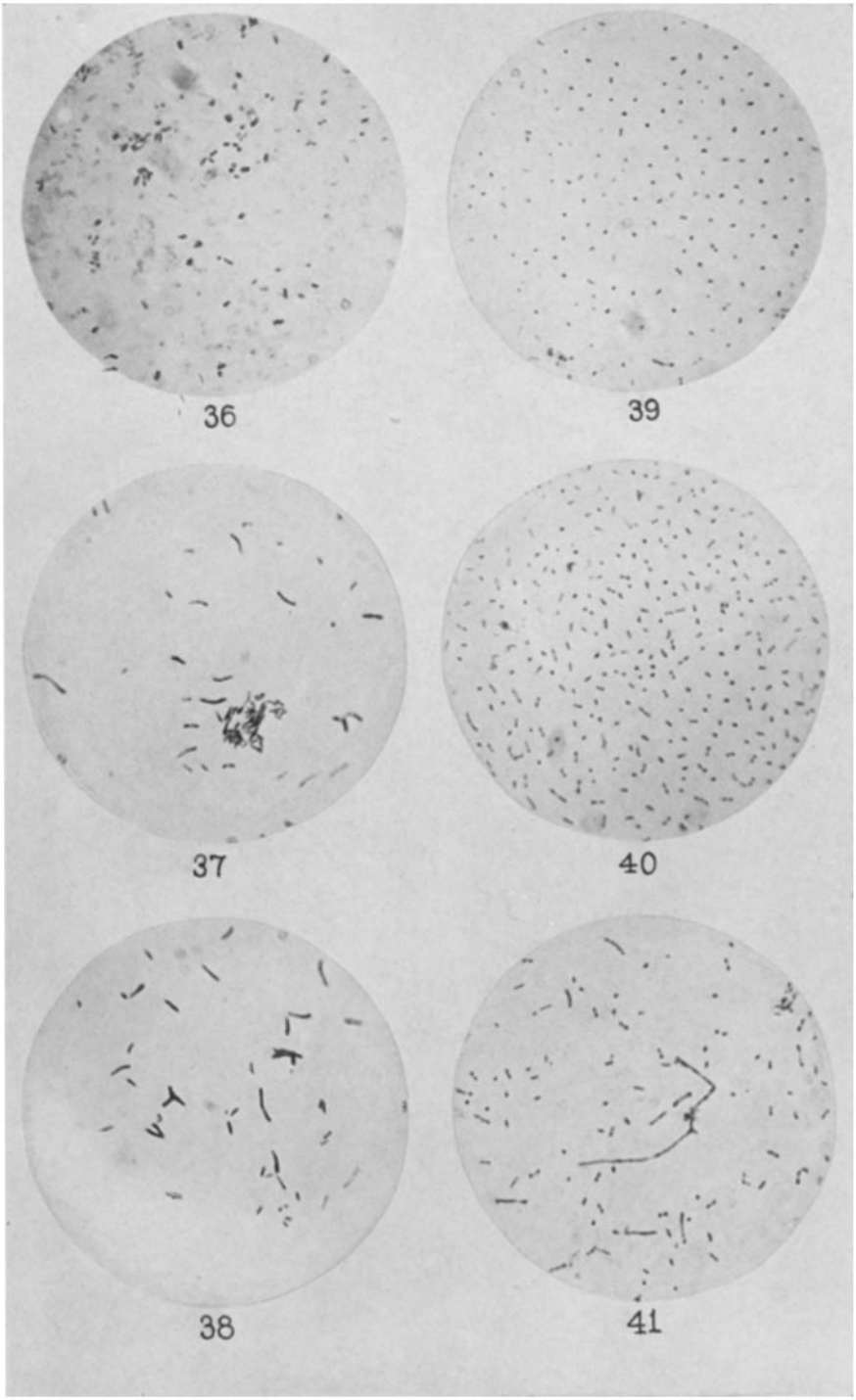
(Noguchi: *Rickettsia*-like microorganisms.)



(Noguchi: *Rickettsia*-like microorganisms.)



(Noguchi: *Rickettsia*-like microorganisms.)



(Noguchi: *Rickettsia*-like microorganisms.)