ETIOLOGY OF YELLOW FEVER.

VI. Cultivation, Morphology, Virulence, and Biological Properties of Leptospira icteroides.

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Plates 1 to 3.

(Received for publication, May 2, 1919.)

Cultivation.

As the nature of the causative agent of yellow fever was unknown, it was necessary at the beginning of these experiments to formulate a special method of cultivation. The methods employed have been similar to those recommended[1] for the cultivation of *Leptospira icterohæmorrhagiæ* (Inada and Ido). Instead of the serum and citrate plasma of the rabbit or other animal, serum and plasma from non-immune persons were used during the early stage of the cultivation experiments. The principal medium consisted of a mixture of 1 part of the serum and 3 parts of Ringer solution, used in a combination of the liquid form and a form made semisolid by adding melted neutral agar (0.3 per cent), the liquid half (8 cc.) of the medium being superimposed on the semisolid half (8 cc.) in a tall culture tube such as that used in the cultivation of spirochetes.[2]

The first step in the inoculation of the medium was to mix about 0.5 to 1 cc. of the citrate blood, drawn from the median basilic vein of the patient, with the lower or semisolid portion of the medium, while the latter was still in the fluid state (42°C.), and allow the mixture to solidify by cooling. The serum-Ringer dilution was then poured on the semisolid portion and about 0.5 to 1 cc. more of the same blood introduced. A layer of paraffin oil was finally added to

[1] Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 575.

[2] Noguchi, H., *J. Exp. Med.*, 1912, xvi, 199.

cover the surface of the medium. When making a culture with the citrate blood from a patient no human citrate plasma was added to the liquid portion of the medium, as the plasma contained in the blood was sufficient to form a loose fibrin throughout that portion. When subsequent subcultures were set up, however, 0.5 to 1 cc. of citrate human or rabbit plasma was introduced into the liquid portion after inoculation. The presence of a loose cobweb fibrin in the culture medium seems to favor the growth of certain organisms.[1] The conditions provided for in this form of culture medium would allow the growth of various microorganisms requiring different degrees of oxygen tension. In a later period 2 to 3 cc. of the citrate blood were used for each of two or three large flasks (50 cc.) with correspondingly larger quantities of culture medium (25 cc.).

Direct Cultivation from Yellow Fever Patients.

Cultures were made from eleven cases of yellow fever, with only three successful isolations of the leptospira. In the first instance (Case 1) I failed to detect the organisms under the dark-field microscope, but in a culture 3 days old (kept at 26°C.) a few active leptospiras were seen. This was inoculated into four guinea pigs, all of whom died later of the typical experimental infection. One of the guinea pigs had epistaxis and melena in addition to intense jaundice, advanced degeneration of the liver, acute parenchymatous nephritis, ecchymoses in the lungs, stomach, and intestines.[3] This culture was presently lost through a secondary fungus contamination, which was difficult to avoid under the conditions in which the work had to be carried on.

The second positive growth was obtained with the blood derived from Case 4 on the 3rd day of the disease. The organisms were readily detected in the culture after 5 days at 30°C. A few leptospiras were present in the blood when carefully examined in the stained preparations, and the guinea pigs inoculated with this specimen came down with the typical symptoms in 8 days. The culture proved to be pathogenic for guinea pigs.

[3] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 565.

The third successful direct cultivation of the organism from yellow fever patients was obtained with the blood from a fatal case (Case 6). The blood was drawn on the 5th day of the disease and put immediately into six tubes containing the culture medium (October 19, 1918). On October 26 one of the inoculated tubes showed the presence of the leptospira under the dark-field microscope. The culture proved to be capable of producing the typical symptoms and lesions in guinea pigs, pups, and marmosets.[4]

Cultivation from Experimental Animals.

The method employed for obtaining a culture of *Leptospira icteroides* was the same as that outlined for direct cultivation from human blood, except that normal rabbit serum and citrate plasma were used instead of human. The blood was obtained from the heart before the death of the animal. The method was not always successful in the first generation, but it was nevertheless the most reliable of the various combinations tried. In a later subculture the addition of the citrate plasma becomes unessential, although a better growth is had when it is added.

Six strains (Cases 1 to 6) of yellow fever leptospira have been maintained to date by passage in guinea pigs. No direct culture was obtained with the blood from Cases 2, 3, and 5, although cultures were finally obtained from the blood of guinea pigs or marmosets inoculated with the passage strain. These cultures, whether obtained directly from the blood of yellow fever patients or indirectly by way of animal inoculation, were found to be uniform in their characteristics and could be maintained in culture for many months. The leptospira isolated from yellow fever cases is extremely sensitive to any alien microbic intrusion, not surviving the slightest contamination in culture, and the failure to obtain a culture directly from yellow fever cases can in every instance be explained through the occurrence of secondary contamination.

When the guinea pigs reach the stage of collapse, with intense jaundice, it is seldom possible to detect the leptospira in the circulation, and a positive transfer to normal guinea pigs becomes uncertain.

[4] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 585.

Cultures set up with this blood usually remain sterile, and the leptospira is not found in the liver and kidneys. In experimental infectious jaundice, on the contrary, the leptospira was almost always found in the later stage of the infection. The extreme lability of *Leptospira icteroides* may account for the negative animal inoculation and microscopic findings in so many cases of yellow fever.

Morphology.

The organism which occurs in the blood and tissues in yellow fever patients in Guayaquil, as well as in those of animals experimentally infected with the blood or tissue of yellow fever patients, is an extremely delicate filament measuring about 4 to 9 microns in length and 0.2 of a micron in width along the middle portion. It tapers gradually toward the extremities, which end in immeasurably thin sharp points. The entire filament is not smooth but is minutely wound at short and regular intervals, the length of each section measuring about 0.25 of a micron. The windings are so placed as to form a zigzag line by the alternate change of direction of each consecutive portion at an angle of 90°.

The organism is unrecognizable by translucent light but becomes quite visible under a properly adjusted dark-field illumination. It possesses an active motility, consisting in vibration, rotation, rapid bipolar progression, and sometimes twisting of parts of the filament. When it encounters a semisolid substance it penetrates the latter by a boring motion, and while passing through it the body assumes a serpentine aspect with few undulations, the elementary windings undergoing no modification.

The organism manifests remarkable flexibility to almost any angle while changing its course of progression in a semisolid medium. In a fluid medium it has fewer and quite characteristic movements. One end is usually bent in the form of a graceful hook, and, while rapidly rotating, the organism proceeds in the direction of the straight end, the hooked end apparently serving as a sort of rear propeller. When extricating itself from an entanglement, however, the same hooked end seems to act like the front propeller of an airplane. Many specimens are seen with both ends hooked, the organism then ro-

tating in a stationary position unless one hook is larger and more powerful as a propeller than the other. The rapid rotation makes the organism appear like a chain of minute dots. From the dynamic point of view the portions which include the several windings from the extremities represent the motor apparatus of the organism. I have never seen a specimen that doubled at the middle portion of the body while lying in a free liquid medium. The motor or terminal portions may be regarded as comparable with the flagella or terminal filaments seen in a spironema or treponema.

The organism is difficult to stain with ordinary aniline dyes, but can be made distinct by osmic acid fixation and one of the Romanowsky stains (Giemsa, Wright, Leishman). When stained with Fontana or carbolized gentian violet solution after mordanting with 5 per cent tannin plus 1 per cent phenol the organism appears as a moderately heavy, slightly undulated filament without a clear elementary indentation. The peculiar forms resembling the letters C and S are quite characteristic. Specimens fixed with methyl alcohol seldom retain the elementary spirals. The beauty of the organism as it appears by dark-field illumination is never well retained in a stained preparation, even in the best specimens. In the latter it appears almost as a totally different organism.

From the findings described it is evident that the present organism falls in the general order of so called spirochetes, but in the strict sense of the term it is neither a bacterium, a spirochete, a spironema, nor a treponema, but belongs to the genus *Leptospira*, of which *Leptospira icterohæmorrhagiæ*, *Leptospira hebdomadis*,[5] and *Leptospira biflexa* have already been described.[1]

The study of the strains of *Leptospira icteroides* obtained from yellow fever cases in Guayaquil showed the organisms to be of somewhat smaller dimensions than the various strains of *Leptospira icterohæmorrhagiæ* in my possession (six strains), as is readily seen from the photographs of the organisms shown in Figs. 1 to 9. The difference is striking when the pictures of the two organisms are compared, particularly in the case of Strain 6 of *Leptospira icteroides*, which is considerably smaller than any of the other strains.

[5] Ido, Y., Ito, H., and Wani, H., *J. Exp. Med.*, 1918, xxviii, 435.

The strains of *Leptospira icterohæmorrhagiæ* isolated from wild rats caught in Guayaquil are also shown (Figs. 10 to 13). These are seen to be similar to the other strains of *Leptospira icterohæmorrhagiæ*, shown in Figs. 5 to 9, and are coarser than the organism obtained from yellow fever cases.

The photographs shown in the three plates were taken for the purpose of comparison at the same time, under similar conditions, and with the same magnification.

Cultural Properties.

Leptospira icteroides does not multiply in a medium in which there is no access to oxygen. In a dense solid medium it grows well within the zone or layer to which a trace of oxygen can still penetrate, but no deeper. It grows best when the supply of oxygen is not excessive, as when a thin layer of liquid paraffin is poured over the surface of the culture medium. A certain amount (above 10 per cent) of a suitable blood serum is essential for its growth. Various bacterial culture substances such as peptone, meat extract, various carbohydrates in different forms, or combinations, are unsuitable; their presence in the serum-containing media apparently neither favors nor impedes development. The percentage of sodium chloride (tried as high as 2 per cent) in the medium seems to have but little influence, and either isotonic saline, Ringer solution, or distilled water may be used as diluent. The organism is highly sensitive to the reaction of the medium, the optimum growth being obtained with a reaction slightly alkaline to litmus paper, not stronger than 0.025 N. It grows well in a neutral medium, but not in one with an acid reaction to litmus paper.

The addition of phenol red to culture media in a ratio of 1 cc. of a 0.0025 per cent solution to 10 cc. of medium has no perceptible disturbing effect upon the growth of *Leptospira icteroides*. Growth takes place in culture tubes in which phenol red indicates the values ranging from pH 6 to pH 7.4. In the case of cultures containing rabbit serum phenol red is gradually decolorized to a trace of pink.

Growth is much more rapid at a temperature of 37°C. than at 25–26°C., but the organisms remain viable much longer at the latter

temperature. No growth is obtainable at a temperature above 42°C. or below 10°C.

Erythrocytes present in the culture do not undergo any special alteration that can be ascribed to the growth of this organism, nor does the hemoglobin. The serum proteins seem in no wise modified and remain transparent. No external changes of the culture media, except a light layer of grayish haze over the surface of a solid or semisolid medium observed in richly growing old cultures, take place, and for this reason the growth of the organism can be ascertained microscopically only.

It has been noticed that *Leptospira icteroides* shows a particular preference for a semisolid medium such as is provided by the presence of agar (0.3 per cent) or by loose fibrin. They entangle themselves in the substance in large numbers and move about in it very actively. Continuous multiplication goes on in this type of medium. After a few weeks the growth may become so dense as to render the uppermost layer of the medium faintly grayish. This peculiarity of the organism may partly account for its predilection for the parenchymatous organs such as the liver and kidney.

Leptospira icteroides multiplies through transverse fission.

Virulence.

While the pathogenic properties of *Leptospira icteroides* for different species of animals have not been exhaustively studied, it has been shown that most of the domestic animals, such as the donkey, horse, sheep, pig, and cat are completely refractory to the injection of the organism. Very young dogs, not older than 6 or 7 weeks, are found to succumb to experimental infection. None of the birds so far employed for experiment has been found to be susceptible.[3] Among the mammals the guinea pig appears to be most susceptible and the marmoset somewhat less so. For this reason the guinea pig has been chosen for determining the degree of virulence of several strains of *Leptospira icteroides*.

The mode of inoculation consisted in intraperitoneal injection in descending doses of a culture 2 to 3 weeks old. The guinea pigs used varied from 300 to 350 gm. A 2 to 3 weeks old culture (26°C.) of this organism, grown in a semisolid rabbit serum medium with

0.15 per cent agar, may contain 50 to 100 leptospiras per field (Leitz $\frac{1}{12}$ oil immersion and ocular 4). In order to arrive at an accurate determination of virulence different strains would have to be used in correspondingly comparable concentrations. But this is extremely difficult in the case of an organism which forms entangled masses of many individuals or shows a tendency to congregate in varying numbers about the particles of culture medium. In the present series of experiments suspensions of cultures of different strains were so prepared as to make each contain approximately an equal number of organisms in the suspension from which higher dilutions were prepared. Each strain was used in successive tenfold dilutions, and one or two guinea pigs were inoculated with 1 cc. each of each dilution. Because

TABLE I.

Determination of Virulence of Leptospira icteroides, Strain 1.

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	*days*	
1	1	3	Died in 7 days.
2	0.1	4	Survived (!). No jaundice.
3	0.01	5	Died in 9 days.
4	0.001	$3\frac{1}{2}$	" " 8 "
5	0.0001	4	" " 9 "
6	0.00001	5	" " 10 "
7	0.000001	No fever.	Survived.

of individual variations in resistance among the guinea pigs in all later experiments two animals were used for each dilution.

Four strains of *Leptospira icteroides* were studied in this way. The results are recorded in the following protocols.

Experiment 1.—Aug. 10, 1918 (at the Guayaquil Yellow Fever Hospital) Strain 1. 18 day culture of the second generation, grown on semisolid human serum agar at 30°C. (Table I.)

Experiment 2.—Dec. 2, 1918. Strain 3. 20 day culture of the third generation, grown on semisolid rabbit serum agar medium at 26°C. (Table II.)

Experiment 3.—Jan. 2, 1919. Strain 5. 3 week culture of the third generation, isolated from a marmoset experimentally infected with a visceral emulsion from a guinea pig which died of the typical infection after inoculation with blood from a fatal yellow fever case. The culture was grown on the same medium as that used for Strain 6, at 26°C. (Table III.)

TABLE II.

Determination of Virulence of Leptospira icteroides, Strain 3.

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	*days*	
1	1	$3\frac{1}{2}$	Died in 8 days.
2	1	5	Survived (!).
3	0.1	4	Died in 9 days.
4	0.1	5	" " $8\frac{1}{2}$ "
5	0.01	5	Survived (!).
6	0.01	$5\frac{1}{2}$	Died in 11 days.
7	0.001	4	" " 9 "
8	0.001	5	Survived.
9	0.0001	5	"
10	0.0001	No fever.	"
11	0.00001	7	"
12	0.00001	No fever.	"
13	0.000001	" "	"

TABLE III.

Determination of Virulence of Leptospira icteroides, Strain 5.

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	*days*	
1	1	3	Died in 7 days.
2	0.1	3	" " 8 "
3	0.1	$2\frac{1}{2}$	" " 6 "
4	0.01	$3\frac{1}{2}$	" " 7 "
5	0.01	$4\frac{1}{2}$	" " 9 "
6	0.001	5	" " 10 "
7	0.001	$3\frac{1}{2}$	" " 8 "
8	0.0001	5	" " 10 "
9	0.0001	4	" " 9 "
10	0.00001	6	Survived.
11	0.00001	No fever.	"
12	0.000001	" "	"
13	0.000001	" "	"

Experiment 4.—Jan. 2, 1919. Strain 6. 20 day culture of the third generation, directly derived from human blood (not passed through guinea pigs), grown on semisolid rabbit serum agar at 26°C. There were about 25 organisms per field in the original suspension. (Table IV.)

The foregoing experiments show that the strains of *Leptospira icteroides* possess, on the whole, a strong virulence for guinea pigs. In two cases (Nos. 1 and 6) the minimal lethal dose was 0.00001 cc., in one (No. 5) 0.0001 cc., and in another (No. 3) 0.001 cc. But in the experiments with Strains 1 and 3 some of the guinea pigs receiving as large a quantity as 1 cc. or 0.1 cc., showed only a transient febrile reaction and speedily returned to normal, notwithstanding the fact that 0.0001 part of these doses killed other guinea pigs in the same series of experiments. This is not altogether exceptional,

TABLE IV.

Determination of Virulence of Leptospira icteroides, Strain 6.

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	*days*	
1	1	4	Died in 7 days.
2	0.1	3	" " 10 "
3	0.1	3½	" " 6 "
4	0.01	4	" " 8 "
5	0.01	5 (?)	" " 7 "
6	0.001	3	" " 9½ "
7	0.001	5	" " 10 "
8	0.0001	4	" " 10 "
9	0.0001	Doubtful.	Survived.
10	0.00001	3	Died in 8 days.
11	0.00001	6	Survived.
12	0.000001	4	"
13	0.000001	No fever.	"

because in the higher dilutions there were instances in which a smaller dose induced a fatal infection while a larger one failed to do so. Again, in fatal instances the severity of the infection did not parallel the amount of culture injected. In other words, the susceptibility of guinea pigs varies considerably among different individuals. In another series of experiments, not yet reported, it was noticed that certain guinea pigs possess an almost complete natural immunity to *Leptospira icteroides*. This becomes an important factor in a consideration of the percentage of successful transmissions of this organism from human cases to guinea pigs.

Gradual Loss of Virulence through Cultivation.

All the strains of *Leptospira icteroides* were brought to New York from Guayaquil on semisolid rabbit serum agar. The cultures were kept at ordinary temperature during the journey (about 28°C. in the tropics and 15°C. after reaching the United States). They had been renewed in Guayaquil on October 26, 1918, and were tested for their pathogenicity for guinea pigs in New York on December 2, 1918; that is, 37 days after the transfer into new media. Intraperitoneal inoculations were made into guinea pigs of 1 cc. of Strains 1, 3, 4, 5, and 6, and the animals developed the usual symptoms and lesions characteristic of the infection produced by these strains, showing that under the circumstances described the organism remained virulent for 37 days.

On December 9, 1918, some of the older cultures of Strains 1 and 3, which had stood over 4 months since cultivation, were also tested, with varying success. Strain 1 was still quite virulent, but Strain 3 failed to produce a fatal infection. In a subsequent experiment, however, by using six guinea pigs, each being inoculated with 2 cc. of the culture intraperitoneally, it was possible to obtain a fatal infection in one of the animals. Through this guinea pig the virulence of the culture was again raised to its original height; that is, it again became capable of causing typical infection in guinea pigs in smaller quantities.

It should be noted that examination of the viscera, especially the lungs, of the guinea pigs which escaped death or severe infection from the inoculation of an attenuated strain, by killing them at the end of about 14 days from the time of inoculation, usually revealed the presence of old hemorrhages of greater or less extent in the lungs. Perhaps it may prove a useful procedure for ascertaining the outcome of transmission to inoculate several guinea pigs with the blood of a yellow fever patient and examine the lungs within a period of from 10 to 14 days. In this way, notwithstanding the absence of striking external manifestations, the results of inoculation can be more accurately followed. Unfortunately this fact was not known at the time of the experiments, reliance being placed upon the development of a fatal infection.

For the past 4 months horse serum has been used for culture media, since it was easily obtainable at much less expense and in larger quantities than rabbit or sheep serum. It is far less satisfactory, however, for the cultivation of *Leptospira icteroides*, than sheep serum, which in turn is much inferior to rabbit serum. In testing out the virulence of different strains of the organism recently, rather rapid loss of virulence has been encountered. The reason was not at first clear, the period of time being comparatively short, but it soon became evident that cultivation of *Leptospira icteroides* on sheep or horse serum media leads to rapid diminution of virulence, since the cultures grown on rabbit serum media remained pathogenic. By following the course of development of these strains in media containing rabbit serum and phenol red it was found that the original pinkish color of the indicator gradually becomes paler until it fades to a trace. The color may be brought back to a deeper hue by the addition of disodium phosphates, but it never reaches the original grade. Apparently a change takes place in the media as well as in the indicator through the growth of the organism. Such a change has not been observed in the cultures grown on media containing sheep or horse serum. Whether the persistence of virulence of the organism in the media containing the rabbit serum has any relation to the phenomenon just described or whether they are two unrelated coincidental phenomena has not been further studied.

Resistance and Viability.

Leptospira icteroides is a non-spore-bearing organism and offers little resistance to the action of heat, desiccation, putrefaction, or disinfectants.

Heating to 55°C. for 10 minutes or freezing and thawing kill the organism, and complete desiccation promptly destroys its vitality.

In the presence of various bacteria, such as *Bacillus coli*, *Bacillus aerogenes*, *Bacillus subtilis*, *Bacillus mesentericus*, *Bacillus pyocyaneus*, pneumococcus, staphylococcus, *Streptococcus hæmolyticus*, etc., *Leptospira icteroides* is destroyed within a short time. The more numerous the bacteria the quicker the disappearance of the leptospira; hence in decomposing excreta or urine, sewer or stagnant water, or in con-

taminated foodstuffs, no leptospira can be found 24 hours after being introduced into them.

On the other hand, certain contaminating fungi or non-putrefactive and non-acid-producing bacteria, sometimes bacilli and sometimes cocci, have been found growing in the cultures of *Leptospira icteroides* without seriously interfering with the viability of the latter, which are actively motile among the intruding fungi or bacteria. In such a contaminated culture these intruders do not cause any perceptible modification of the culture medium, except that their discrete colonies may be found imbedded here and there in the medium.

When a pure culture of the organism was poured into a cup of sterile distilled water and left unprotected from the air or dust the leptospiras survived several days, but finally disappeared, partly because of the lack of nutrition and partly because of bacterial growth. The leptospira intentionally added in large quantity to fecal matter kept at room temperature disappeared within a few hours.

On several occasions attempts were made to infect the larvæ of *Stegomyia calopus* by introducing emulsions of liver or kidney containing a large number of the organisms into the receptacle with the larvæ, but no leptospira could be found in such a mixture after 2 hours. In this respect the virus of yellow fever is one of the least resistant of all pathogenic organisms which have been obtained in culture. In my experience there has seldom been an impure culture of this organism.

Another interesting phenomenon in connection with the organism in question is that it soon dies out; it may degenerate within 12 hours in a piece of liver or kidney removed from an infected guinea pig and kept at a temperature of about 10°C. In hundreds of instances a leptospira was found only rarely in the liver, kidney, or blood from guinea pigs which had died of typical experimental yellow fever several hours before autopsy. In this respect *Leptospira icteroides* differs considerably from *Leptospira icterohæmorrhagiæ* isolated from the cases of infectious jaundice in Japan or Europe, the latter being still easily recoverable from animals kept over night after death.

With regard to the resistance of the organism to the action of various ordinary disinfectants the work is still incomplete. It has been found, however, that it is readily killed within 5 minutes by 2 per

cent phenol or 0.1 per cent bichloride of mercury. In a 10 per cent solution of sodium taurocholate, sodium glycocholate, or sodium cholate the organism promptly disintegrates, but saponin has no injurious effect upon it. Human or animal bile dissolves the organism rapidly when used in concentrations stronger than 30 per cent.

Filterability.

A noteworthy characteristic of *Leptospira icteroides* is its ability to pass through the pores of filters. Some experiments to determine this point were carried out as early in the investigation as the transmission of Strain 1 from the human case to guinea pigs. By the use of Berkefeld filters V and N with suction by means of a water pump it was possible to filter an emulsion of the liver and kidney of a guinea pig experimentally infected with the passage strain 7 days previously. The clear filtrates, which were bacteriologically sterile, were inoculated intraperitoneally into normal guinea pigs in doses of 10 cc. each on August 8, 1918. Both animals came down with typical symptoms after $7\frac{1}{2}$ and 8 days respectively. In the blood of these animals a small number of leptospiras were demonstrated 24 hours before death. In the emulsions of the liver and kidney the organisms were also present, and upon further passage to normal guinea pigs the emulsion proved to be infectious.

Possibility of the Existence of a Granular Phase in the Life of Leptospira icteroides.

That there may exist a granular phase of life in various members of the family of spirochetes has been repeatedly suggested by investigators. Balfour, Fantham, Leishman, and Todd[6] advanced the idea that the spirochetes of relapsing fevers in man and fowls pass through a granular stage at some time in their life. The following observation seems strongly to suggest the possibility that this phenomenon also occurs in the life of *Leptospira icteroides.*

[6] Balfour, A., *Internat. Congr. Med.*, 1913, xxi, 275. Fantham, H. B., *Ann. Trop. Med. and Parasit.*, 1914, viii, 471. Leishman, W. B., *Internat. Congr. Med.*, 1913, xxi, 282. Todd, J. L., personal communication.

The various cultures of *Leptospira icteroides* made at Guayaquil on October 26, 1918, were brought back to New York on November 24 without special accident. But on examination no leptospira could be found in any of the tubes containing cultures of Strain 5, although the other cultures were growing well. A thorough examination of the eleven tubes of Strain 5 was continued for several days without success.

Six culture tubes which were made on October 18, 1918, with the blood from Marmoset 4, severely infected with Strain 5, and which had been showing a fairly good growth on October 26, were also examined. These tubes showed no spiral organisms. There were large numbers of refringent granules imbedded in the culture medium in which the leptospiras had been abundantly present a month previously. These granules appeared to be the degenerated remains of the leptospiras. The hope of recovering the strain from these cultures was almost abandoned, but as a last resort a dozen guinea pigs were inoculated with 1 cc. of the contents of each of these tubes. Some of these animals in due time came down with typical symptoms. The spiral forms of the leptospiras were found in varying numbers in the blood, liver, and kidneys of these animals, and a culture of the strain was regained.

It is of course possible that these old culture tubes contained the spiral leptospira in such small numbers that they escaped microscopic detection, but it is also possible that they existed in a granular phase under certain conditions.

SUMMARY.

By the employment of methods designed to promote the growth both of aerobic and anaerobic organisms, particularly those belonging to the class of spirochetes, it was possible to obtain a pure culture of a delicate organism, the morphological features of which place it in the genus *Leptospira*. On three occasions, that is, from three out of eleven cases of yellow fever, the organism was directly cultivated. These three strains were found to induce the characteristic symptoms and lesions when tested on guinea pigs. The organism was designated *Leptospira icteroides*.

Leptospira icteroides was also obtained in pure culture from the blood of guinea pigs which succumbed to infection after being inoculated with the blood or organ emulsions from patients suffering from yellow fever. These cultures also proved to be virulent when tested on susceptible animals.

The morphological characteristics and certain biological properties of the organism were considered in detail. It is invisible under translucent illumination and is difficult to stain by most aniline dyes. It is highly sensitive to the presence of bacteria and is rapidly destroyed in a medium in which certain other organisms are present. The presence of blood serum (man, sheep, horse, rabbit, etc.) seems to be essential for its growth. It grows well at a temperature of about 25–26°C. and more quickly at 37°C., though at the latter temperature it dies out within a few weeks. At 25°C. under favorable conditions and in suitable culture media it remains viable for several months without losing its virulence. *Leptospira icteroides* multiplies by transverse division.

The virulence attained by some strains was such that 0.00001 cc. of a culture could induce typical fatal infection in guinea pigs. There exists a considerable variation among guinea pigs in their susceptibility to *Leptospira icteroides*.

The organism is killed within 10 minutes at a temperature of 55°C. and is also destroyed by complete desiccation or freezing and thawing. Bile and bile salts dissolve it in certain concentrations, but not saponin.

Leptospira icteroides passes through the pores of Berkefeld filters V and N, and there is a possibility of its having a granular phase of life under certain conditions.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icteroides*. Strain 6 (Case 6). × 3,000.

FIG. 2. The same. Strain 4 (Case 4).

FIG. 3. The same. Strain 5 (Case 5).

FIG. 4. The same. Strain 3 (Case 3).

PLATE 2.

FIG. 5. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icterohæmorrhagiæ*. Japanese strain. × 3,000.

FIG. 6. The same. British strain.

FIG. 7. The same. French strain.

FIG. 8. The same. American Strain 1.

FIG. 9. The same. American Strain 2.

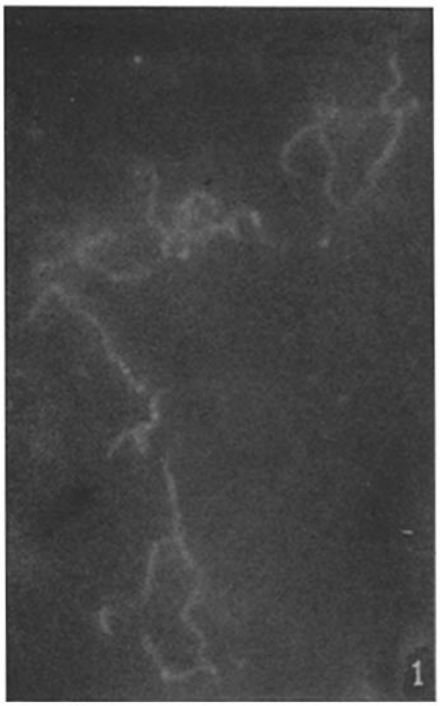
PLATE 3.

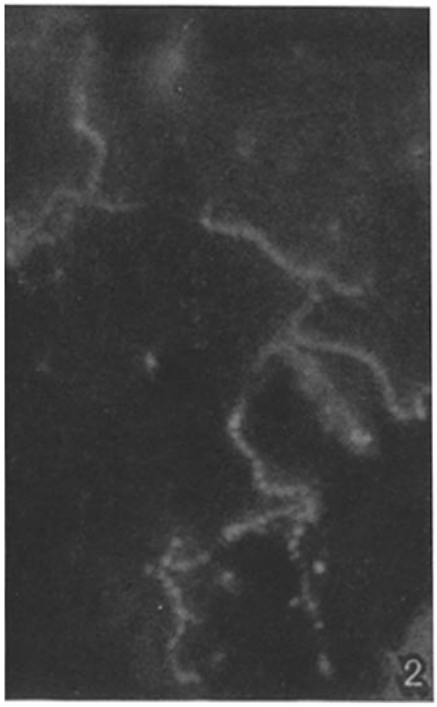
FIG. 10. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icterohæmorrhagiæ*. Group 8 strain obtained from wild rats in Guayaquil. × 3,000.

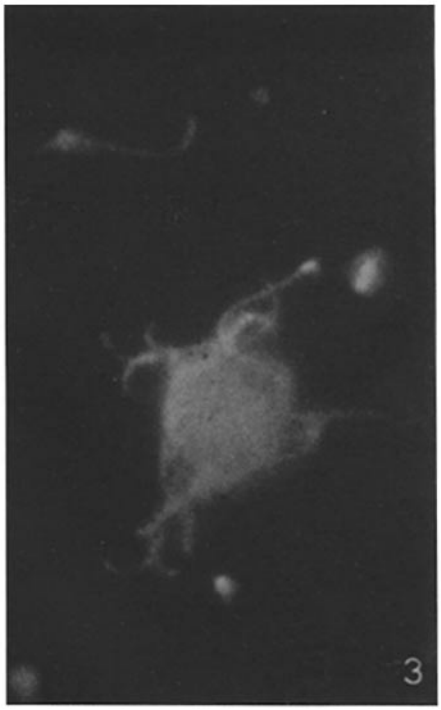
FIG. 11. The same. Group 11 strain.

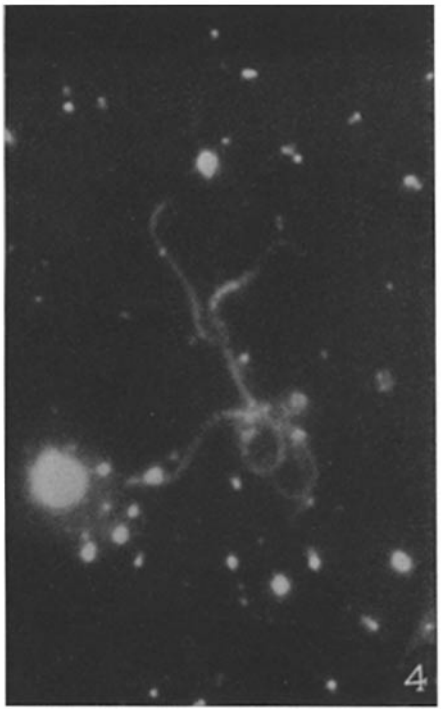
FIG. 12. The same. Group 30 strain.

FIG. 13. The same. Group 30 strain.

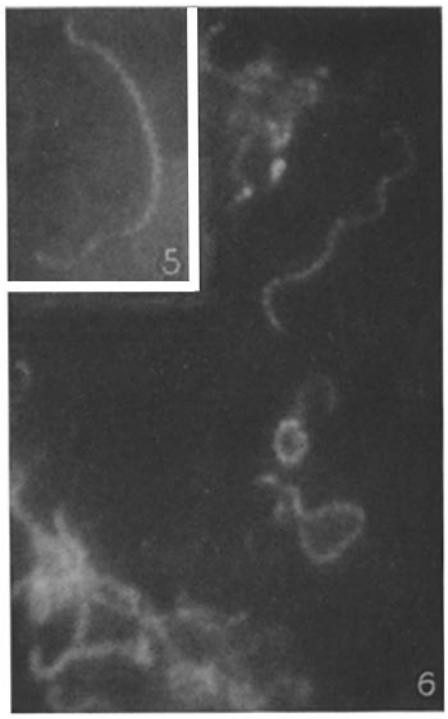


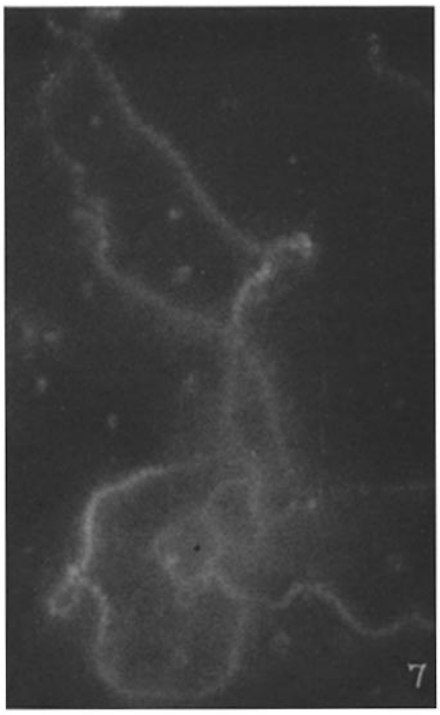


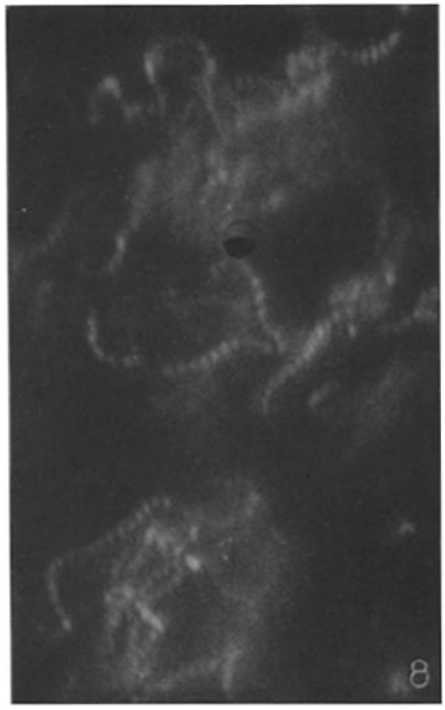




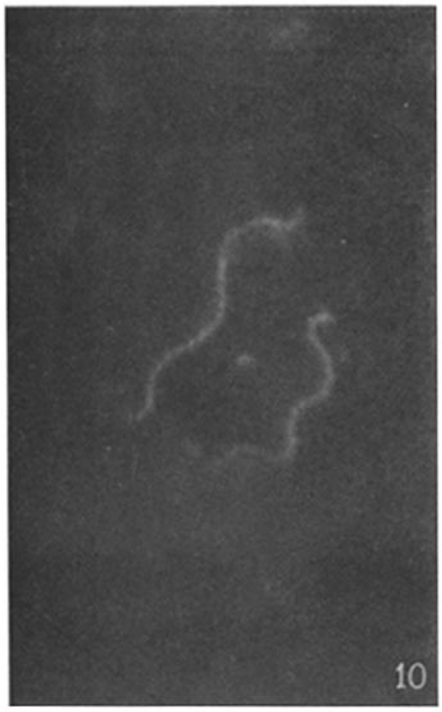
(Noguchi: Etiology of yellow fever. VI.)

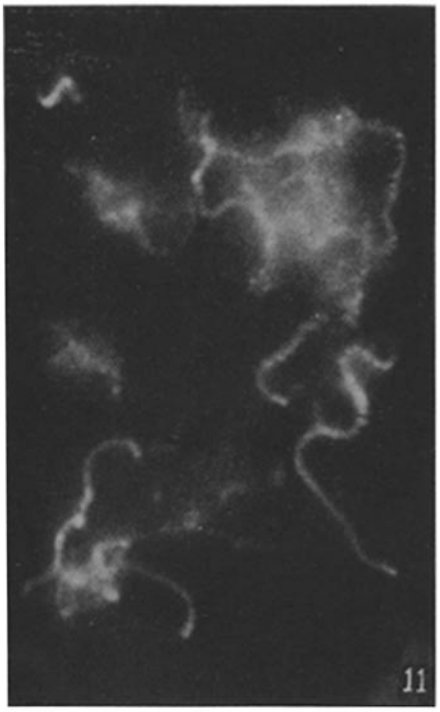


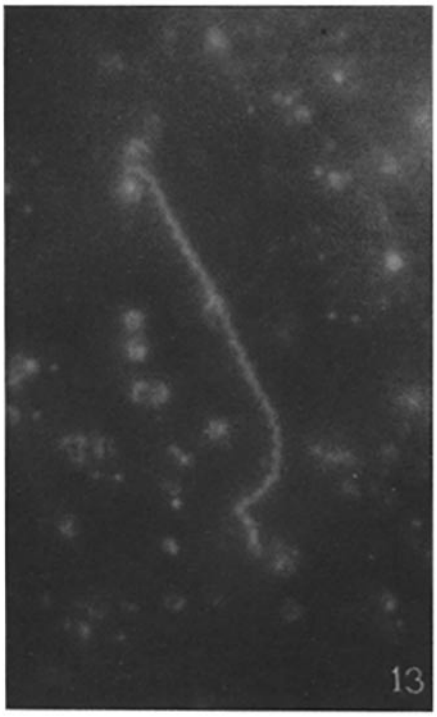




(Noguchi: Etiology of yellow fever. VI.)







(Noguchi: Etiology of yellow fever. VI.)