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COMPARATIVE STUDIES OF HERPETOMONADS AND LEISHMANIAS.

I. CULTIVATION OF HERPETOMONADS FROM INSECTS AND PLANTS.

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PLATES 10 TO 15.

Our interest in the herpetomonads parasitic in insects and plants in various parts of the world lies in the possible relationship of some of them to the parasites of kala-azar, of India, China, and Mediterranean countries, oriental sore, of Palestine, and espundia, of South America. The mode of infection of man by the leishmanias is not understood, and experimental work on the problem of transmission has, until recently,[1] been rendered extremely difficult by the lack of susceptible laboratory animals. The geographic distribution and epidemiology of the various forms of leishmaniasis have indicated that the parasites may be carried by some insect, and many attempts have been made to find evidence of this mode of transmission.

That the leishmanias may be taken up by insects and are capable of surviving in the insect gut was first shown in the case of *Leishmania donovani* by Patton,[2] who fed bedbugs on kala-azar patients and observed the development of the parasites to the flagellate stage in the bugs. Wenyon[3] later reported the ingestion of *Leishmania tropica* from open lesions of cutaneous leishmaniasis by flies, mosquitoes, and bedbugs, and the development of the parasites in some of these insects. The sand fly (*Phlebotomus*) is looked upon at present[4] as the most probable vector of leishmaniasis, but the experimental evidence is fragmentary.

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[1] Young, C. W., and Liu, Pao-Yung, *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 392.

[2] Patton, W. S., *Scient. Mem. Off. Med. and San. Dept. Gov. India, No. 27*, 1907.

[3] Wenyon, C. M., *Parasitology*, 1911, iv, 273.

[4] Sinton, J. A., *Indian J. Med. Research*, 1924–25, xii, 701.

The search for pathogenic varieties of herpetomonads among the flagellates so commonly found inhabiting the intestinal tract of insects was begun in 1913 by Laveran and Franchini, who reported having induced in rats and mice a disease not unlike kala-azar or oriental sore by inoculation of flagellates obtained from the dog flea, mosquito, sheep ked, and house fly, and even of flagellates of plant origin.[5] Pure cultures were used for inoculation in most instances. Later they obtained somewhat similar results with cultures of a flagellate obtained from the sand fly.[6] Fantham and Porter[7] induced symptoms of kala-azar by inoculation of various insect flagellates and believed that the leishmaniases would prove to be insect-borne herpetomoniases, but Hoare[8] repeated their experiments and obtained only negative results. Becker[9] and Drbohlav[10] also failed to infect animals with flagellates of insect origin. Strong,[11] who found flagellate infections in the hemipteran, *Chariesterus cuspidatus*, of Panama, in its plant hosts, and in a lizard presumed to prey upon the insect, was able to cause ulceration of the skin in a monkey by inoculation of the flagellate material from the intestinal tract of the lizard and to demonstrate leishmaniform parasites in the skin lesion; but he found the plant and insect flagellates to be non-infective.

While a very large number of the plant and insect flagellates have been described, the greater part have been studied only under natural conditions and on the basis of morphological features, and they have usually been given specific names designating their hosts. It has been recognized, however, that the same flagellate may live in several hosts, and Becker,[9] having demonstrated by cross-infection experiments that the flagellates of *Musca domestica* could thrive equally well in the intestinal tract of several other species of flies, and that the flagellates inhabiting each of the fly species which he studied showed a similar adaptability, concluded that the herpetomonads of flies all represent the same species. Drbohlav[10] confirmed Becker's experiments with fly flagellates, using a cultivated strain of *Herpetomonas muscarum*, but was unable to infect the dog flea or water strider with any flagellate save the one naturally occurring in these insects. He was unsuccessful in establishing *Leishmania infantum* in any flagellate host studied.

A number of the flagellates of invertebrates and plants have been cultivated, among them *Herpetomonas culicis* from *Culex pipiens* (Novy, MacNeal,

[5] Laveran, A., and Franchini, G., *Compt. rend. Acad.*, 1913, clvii, 744; *Bull. Soc. path. exot.*, 1914, vii, 665; 1919, xii, 379; 1920, xiii, 796.

[6] Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1920, xiii, 569.

[7] Fantham, H. B., and Porter, A., *Proc. Cambridge Phil. Soc.*, 1915, xviii, 39.

[8] Hoare, C. A., *Parasitology*, 1921, xiii, 67.

[9] Becker, E. R., *J. Parasitol.*, 1923, x, 25.

[10] Drbohlav, J. J., *Am. J. Hyg.*, 1925, v, 580, 599.

[11] Strong, R. P., *Am. J. Trop. Med.*, 1924, iv, 1.

and Torrey[12]), *H. ctenocephali* of the dog flea (Tyzzer and Walker,[13] Wenyon,[14] Laveran and Franchini[5]), *H. pulicis* from *Pulex irritans* (Wenyon[15]), *H. ctenop-syllæ*[5] of the mouse flea, *H. phlebotomi*[6] of the sand fly, *H. jaculum*[5] of the scorpion, *Crithidia melophagi*[5] of the sheep ked, and *H. tarantolæ*[16] of the gecko (Laveran and Franchini). Laveran and Franchini obtained pure cultures also from plants.[17] Nöller[18] cultivated a number of insect herpetomonads and several trypanosomes. Drbohlav[10] obtained pure line strains of *H. muscarum*. The medium used has usually been the N. N. N. medium or some modification of it, though Franchini and Laveran made use also of blood bouillon,[16] and Franchini[19] later found that a number of flagellates would grow in a mixture of bouillon and plant latex.

The purpose of the present investigation was to cultivate a number of herpetomonads from different sources and compare their cultural and biological properties. Serological comparison of the leishmanias[20] had already indicated the value of immunological properties for differentiation and had suggested that these and other biological properties might be used for comparative studies of the leishmanias with other herpetomonads. We have been able to supplement this study by comparison of our strains with other cultivated flagellates, *H. ctenocephali*, isolated by Tyzzer and Walker in 1919,[13] and *T. rotatorium*, isolated by Drbohlav,[10] cultures of which were furnished us through the courtesy of Dr. E. E. Tyzzer, of Harvard University Medical School.

Pure cultures of herpetomonads have been isolated from the following insects and plants: *Oncopeltus fasciatus* (Fig. 1), *Oncopeltus sp.?*[21] (Fig. 2), *Lygæus kalmii* (Fig. 3) (two strains), *Anopheles quadrimacula-*

[12] Novy, F. G., MacNeal, W. J., and Torrey, H. N., *J. Infect. Dis.*, 1907, iv, 223.

[13] Tyzzer, E. E., and Walker, E. L., *J. Med. Research.*, 1919, xl, 129.

[14] Wenyon, C. M., *Tr. Soc. Trop. Med. and Hyg.*, 1914, vii, 104.

[15] Wenyon, C. M., *Arch. Protistenk.*, 1913, xxxi, 1.

[16] Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1921, xiv, 323.

[17] Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1920, xiii, 796.

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

[19] Franchini, G., *Bull. Soc. path. exot.*, 1923, xvi, 41.

[20] Noguchi, H., *Proc. Internat. Conf. Health Prob. Trop. America*, Kingston, Jamaica, British West Indies, July 22 to August 1, 1924, published in Boston, 1924, p. 455.

[21] Specimens of this insect have recently been submitted for identification to Dr. H. G. Barber of the American Museum of Natural History, who was able at once to classify it as to genus. The species has not yet been determined.

tus, Culex pipiens, Musca domestica, bluebottle flies (two strains), *Asclepias syriaca* (two strains), and *Asclepias nivea.* In addition, two impure cultures were obtained, one from *Oncopeltus cingulifer* (Fig. 4), and one from the plant on which it feeds, *Asclepias curassavica.* Attempts to purify these two strains were unsuccessful, and they soon died out.

The specimens of *O. cingulifer* were collected in Tela, Honduras,[20] those of *Oncopeltus sp.?* in the vicinity of Lima, Peru,[22] those of *O. fasciatus* and *L. kalmii* on Long Island and in Shandaken, N. Y., those of *Anopheles quadrimaculatus* in Leesburg, Georgia,[23] and those of *Culex pipiens* near Jersey City. The house and bluebottle flies were collected on the grounds of The Rockefeller Institute. The specimens of *A. curassavica* were brought from Tela,[20] Honduras, and those of *A. syriaca* from Baltimore, Long Island, and Shandaken. For the Haitian milkweeds, *A. nivea,* we are indebted to Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, Yonkers, N. Y., who infected them by allowing infected specimens of *Oncopeltus fasciatus* to feed upon the seed pods.
  For identification of the latex-feeding insects we are indebted to Dr. H. G. Barber, of the American Museum of Natural History. The bluebottle flies were no doubt *Calliphora,* but identification of the species was not possible in these instances.

Method of Cultivation.

For the initial cultivation of the flagellates from insects, solid blood agar plates were employed which contained various carbohydrates and were adjusted to different hydrogen ion concentrations, pH 5, 5.5, 6, 6.5, 7, and 7.5. The proportions of the constituents were as follows:

Defibrinated horse or rabbit blood..........................	300 cc.
2 per cent nutrient agar, melted..............................	500 cc.
Mixed sugar solution, sterilized by filtration through Berkefeld filters:...	25 cc.
Glucose................................. 20 gm.	
Saccharose.............................. 10 "	
Inulin...................................... 5 "	

[22] These specimens were collected by Dr. T. S. Battistini, of Lima.

[23] Dr. Mark F. Boyd, of the International Health Board, was kind enough to inform us of the presence of flagellates in these mosquitoes and also to dissect insects and inoculate culture media.

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Levulose...................................  5 gm.
Raffinose..................................  5  "
Distilled water............................ 100 cc.
0.9 per cent sodium chloride solution........................ 175 cc.
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Various bacteria, molds, and yeasts are usually associated with the flagellates in the insect gut and by their more rapid growth render the isolation of the latter extremely difficult. In several instances impure cultures were obtained by inoculating plates with ascending dilutions of the suspension of teased viscera, and these were purified by transfers to new plates repeatedly until some pure colonies of flagellates appeared. Later, however, in the work with flies, it was found necessary to resort to Barber's technique[24] of picking out one flagellate or a group of them for inoculation of the plates. Theoretically this procedure ought to give fairly constant results, but actually many plates implanted with a single flagellate failed to show any growth, though plates spread gently with several organisms usually yielded some isolated colonies. Probably not every individual was capable of adapting itself to the cultural conditions provided.

It was noticed that whenever an impure culture was obtained by the plate method, the contaminating organisms were usually yeasts, which had apparently suppressed the growth of bacteria to some extent by the production of acidity. The observation suggested the use of culture media adjusted to concentrations of acid which were not sufficient to interfere with the growth of the flagellates, but in which the growth of bacteria was reduced so far as possible. The neutral and slightly alkaline media were not omitted in routine work, however. As a rule, four or five plates of a given hydrogen ion concentration were used; a small drop of the suspension of teased viscera in 0.5 per cent saline was spread on the first plate with a sterile bent pipette, and the other plates were brushed successively with the same pipette. The first plate was usually overgrown by bacteria, but one of the others might yield fairly discrete colonies of bacteria and an occasional pure colony of flagellates.

The cultivation of plant flagellates was much more difficult, not because of the contaminants—for in this instance the only contaminants present are the yeasts and molds from the stem or leaves—but because of the difficulty of adaptation of the flagellates to the media, as shown by the fact that when large numbers of them were introduced they usually lost their motility within a few days and finally degenerated. Pure cultures have thus far been obtained only on leptospira medium,[25] and only three times among many attempts. In one other in-

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[24] Barber, M. A., *Philippine J. Sc., Section B*, 1914, ix, 307.

[25] The formula is as follows: 0.9 per cent NaCl, 800 parts; fresh rabbit serum, 100 parts; 2 per cent nutrient agar (pH 6.5 to 7.0), 100 parts; rabbit hemoglobin (made by laking 1 part of defibrinated blood with 3 parts of distilled water), 10 parts.

stance an impure culture of flagellates from *A. curassavica* was obtained on a medium composed of the usual leptospira medium plus glucose, inulin, and tapioca.

The isolation experiments were carried out at room temperature, which varied from 18°C. to 28°C. during the day and was probably somewhat lower at night. All the plates were sealed with adhesive tape and kept in a moist chamber. Examinations were not made until after 7 to 10 days, experience having soon shown that there was no advantage in earlier examination. The colonies of flagellates develop very slowly, and they are so minute that very close examination is necessary to find them. In initial cultures 10 to 14 days were required in most instances for the colonies to become visible as tiny dew-like spots. When associated with bacteria, the flagellates can only be detected by microscopic examination, and for this purpose the dark-field microscope offers the most convenient and rapid means of detection.

From the pure colonies of flagellates found on plates with well separated colonies, subcultures were readily obtained on similar media (plates or slants), and still more certainly on leptospira medium, with or without carbohydrates. On this medium growth is remarkably rapid and prolific, in marked contrast to that of the first generation. All the strains isolated during the present experiments grow much more rapidly and profusely than any of the leishmanias or the strains of flagellates from dog flea or frog. They grow well at 37°C., as well as 25°C., while for the other flagellates the lower temperature is more suitable.

Description of Flagellates Studied.

Seven of the twelve strains isolated were derived from milkweed-feeding insects and their plant hosts. When the cultures came to be studied with respect to their serological and carbohydrate-fermenting properties, it was found that the seven strains represented in reality only two species of flagellates. Similarly, the two strains from mosquitoes behaved as one serologically. If we were to classify the strains according to host species, we should have to create several names for organisms which by morphology and biological reactions show identical characteristics, while if we were to depend on morphology alone, a number of flagellates which behave differently would have to be classed as a single species. The study of biological relationships, however, made it possible to identify a given flagellate in different environments and to separate different flagellates occurring in the same host. The classification of the strains was accordingly very much simplified. In order to avoid confusion it seems desirable to employ throughout this paper the nomenclature finally adopted,

TABLE I.

Species.	Source.	Morphology in insect or plant.	Morphology in culture on leptospira medium.
H. oncopelti.	Oncopeltus fasciatus. Oncopeltus sp.? Lygæus kalmii No. 1.	Narrow, slender bodies; anterior end rounded, posterior pointed. Flagellum about the length of the body.	Similar to insect forms, but usually smaller, with shorter flagellum. Anterior end sometimes truncated.
	Asclepias syriaca No. 213. Asclepias nivea.	Characteristic ribbon-like bodies, sometimes twisted; flagellum usually short.	
H. lygæorum.	Lygæus kalmii No. 2.	Slightly longer and narrower than flagellates of L. kalmii No. 1; flagellum somewhat longer.	
	Asclepias syriaca No. 46.	Characteristic ribbon-like forms, but somewhat shorter than those of A. syriaca No. 213.	
H. culicidarum.	Anopheles quadrimaculatus Culex pipiens.	Short, truncated forms, with short flagellum.	Similar to forms in insect, but considerably larger.
H. muscidarum.	Musca domestica.	Large forms, many with characteristic long double flagellum.	Similar to H. oncopelti in young cultures; much larger in old cultures, but never as large as H. muscæ domesticæ of von Prowazek.
H. media.	Calliphora sp.? No. 1.	Smaller than flagellates of M. domestica; flagellum long.	Similar to young cultures of H. muscidarum, but smaller and narrower; never become large.
H. parva.	Calliphora sp.? No. 2.	Similar to H. media.	Small, rounded forms, with very short flagellum.

although the manner in which it was arrived at is chiefly discussed in Part II of our report.[26]

Table I is a résumé of the names, source, and morphology of all the flagellates grown in pure culture. Identical strains from two or more sources have been named in accordance with the source from which they were first cultivated (*H. oncopelti, H. lygæorum*).[27] The mosquito flagellates, which proved identical, have been called *Herpetomonas culicidarum*, since the same strain was isolated from two genera of the family *Culicidæ*. As will be clear from the experiments described in Part II of this report, it was not possible to identify this organism with others previously described as occurring in mosquitoes.

The naming of the fly strains was a more difficult problem, inasmuch as each of the three proved to be distinct from the others. The preparations of the flagellates found in the gut of *Musca domestica* show chiefly large forms with double flagellum which conform with the descriptions of *H. muscæ domesticæ*, but the organisms seen in young cultures are not unlike *H. oncopelti* and *H. lygæorum*. As the cultures grow older much larger forms appear, but these do not correspond exactly with the naturally occurring individuals, and it is difficult to exclude the possibility that some other strain than *H. muscæ domesticæ* was simultaneously present in the insect and grew more readily in culture. It was likewise impossible to identify two distinct strains from *Calliphora* as *H. calliphoræ*. The strains were finally given the new species designations, *Herpetomonas muscidarum, H. media*, and *H. parva*.

All the flagellates cultivated have a single anterior flagellum, arising near the parabasal body, which is situated beside, or in front of, the nucleus. No undulating membrane, such as characterizes *Crithidia* and *Trypanosoma*, has been demonstrated in any of the strains, either under natural conditions or in culture. The term *Leptomonas*, while it has priority over *Herpetomonas*, is less well defined, and it seems reasonable, as suggested by Wenyon,[15] to restrict the name to the species for which it was created until it can be proved that *Herpetomonas* is actually a synonym of it. Werner's objection to *Herpeto-*

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[26] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 327.

[27] The name *H. lygæi* has been used by Patton (*Arch. Protistenk.*, 1909, xiii, 1) to designate a flagellate species found in *Lygæus militaris* of India.

monas,[28] on the ground that it was created for a biflagellate genus, has been invalidated by Wenyon's cytological study[15] of *H. muscæ domesticæ*, which demonstrates that the biflagellated forms are actually individuals in a very early stage of division.

Herpetomonas oncopelti, n. sp. (Figs. 5, 6, 9, 10, 13, 14, 17, 18).

Strain 1.—This strain (Fig. 5) was obtained from the intestinal tract of *Oncopeltus fasciatus* (Fig. 1) caught in Shandaken, N. Y. The insect feeds on the latex of the common milkweed, *Asclepias syriaca*, which is of widespread occurrence in the temperate regions of the United States. According to Holmes,[29] the insect is rarely found north of New Jersey, being replaced in the northeastern states by the species *Lygæus kalmii*, which also feeds on the latex of *A. syriaca*. Only three specimens of *O. fasciatus* have been obtained in the course of the present study (one on Long Island, and two in Shandaken), and only one of these was infected with herpetomonads. Farther south, however, in New Jersey and Maryland, Holmes found that the majority of specimens harbored flagellates.

Morphology in Insect Host.—The forms found in the insect (Figs. 5, 9, 17) have relatively narrow bodies, the anterior end is somewhat rounded, and the body gradually narrows to a point at the posterior end. The flagellum originates near the parabasal body, which is round or oval and is situated closer to the anterior end than to the nucleus. In most specimens the flagella are about the length of the body, perhaps longer. When the organisms are moving swiftly in forward motion, the flagellum vibrates rapidly; in less active specimens it has an undulating, serpentine motion and may sometimes be alternately coiled into loops and stretched out. None of the flattened or twisted ribbon-like bodies so characteristic of plant herpetomonads has been encountered. Occasionally short, broad, pear-shaped forms have been observed, some of which have long and others very short flagella.

Cultural Properties.—Once obtained in culture, the organism is easily grown on the semisolid leptospira medium or on any of the blood agar slants, such as the N.N.N. or Nöller's medium, which have been widely employed for the cultivation of leishmanias and insect flagellates. On leptospira medium the growth becomes visible within 48

[28] Werner, H., *Arch. Protistenk.*, 1909, xiii, 19.

[29] Holmes, F. O., *Biol. Bull.*, 1925, xlix, 323.

hours at room temperature as a grayish white, usually homogeneous mass, which gradually extends deeper into the medium. At 37°C. growth can be recognized macroscopically after 24 hours. On blood agar slants grayish, dew-like, moist and shining colonies become visible within 72 hours at room temperature and 48 hours at 37°C. They gradually spread and become confluent, and the layer of growth assumes a light grayish color. The colonies are of a sticky consistency, and a thin grayish pellicle may form on the surface of the condensation water.

The morphological features vary greatly according to the medium on which the organism is grown. In young colonies on the surface of blood agar slants (Figs. 6,a, 10,a) most of the individuals are pear-shaped, oval, or truncated at the anterior end, with a short flagellum which moves constantly from side to side. The flagellum arises far in the interior of the cell, the parabasal body lying at one side of the nucleus. After some days the organisms become aggregated into rosettes of varying size, the flagella being directed inward, the bodies becoming uniformly narrower, pyriform, or spindle-shaped, and the flagella longer. In the condensation water much longer, slenderer forms are found, with flagella as long as the body.

The flagellates grown on leptospira medium are of elongate form, with flagella usually the length of the body (Figs. 6,b, 10,b). Unusually long forms are found in cultures which have been kept at room temperature more than 2 or 3 weeks, and these resemble the specimens of the organisms in the insect host. The parabasal body occupies a position anterior to the nucleus, the anterior portion of the organism is wide and rounded, and the body narrows down to a slender, pointed posterior portion. These long forms with well developed flagella travel about freely and swiftly in all directions, in contrast to the short truncated forms, with vibrating flagella, which rotate swiftly but do not travel out of the microscopic field.

Numerous bizarre forms appear in media having a pH near 5. In some the cytoplasm is divided except at the anterior portion, where a single flagellum arises, the division beginning near the anterior portion or at any point farther toward the posterior end. There may be one, two, or several clefts in a single organism, and the lobes are usually unequal in size. It is difficult to interpret this phenomenon, which

has also been observed under natural conditions in the insect host, but it appears to be an abnormal multiple division.

Strain 2 (from Oncopeltus sp.?).—Figs. 9, 10. Another species of *Oncopeltus* (Fig. 2) was obtained by Dr. Telémaco S. Battistini in the vicinity of Lima. Its plant host is also a species of *Asclepias*. (The specimens of milkweeds which Dr. Battistini collected, however, proved to be free from flagellates.) The initial impure culture of this flagellate was purified by the Barber method. No morphological differences could be detected between this strain and the one derived from *Oncopeltus fasciatus*.

Strain 3 (from Lygæus kalmii No. 1).—Figs. 17, 18. *Lygæus kalmii* (Fig. 3) resembles *O. fasciatus* in appearance, and its geographic distribution is somewhat similar. In the Catskill Mountains (Shandaken), at an elevation of 1000 feet above sea level, *Lygæus kalmii* far exceeded *O. fasciatus* in number. Four of the twenty-four specimens collected harbored flagellates, and in two instances cultivation was successful. While the two strains were morphologically indistinguishable in culture, they proved subsequently to be distinct species, one of which is identical with *H. oncopelti*.

Strain 4 (from Asclepias syriaca No. 213).—The occurrence of herpetomonads in *Asclepias syriaca* (Fig. 30) was first reported by Holmes,[30] who regarded the organisms found by him as identical with *H. elmassiani* of Migone, described by França.[31] Of two strains of flagellates isolated from two infected milkweeds collected in Shandaken, N. Y., one proved to be *H. oncopelti*. The initial growth was obtained on leptospira medium and was not detected until several weeks after inoculation. The culture was purified by plating.

Strain 5 (from Asclepias nivea).—Figs. 13, 14. Through the kindness of Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, a plant of the species *Asclepias nivea*, a Haitian milkweed, was placed at our disposal. It had been infected by allowing infected specimens of *Oncopeltus fasciatus* to feed on the seed pods. The morphology of these flagellates in the latex (Fig. 13) is similar to that of *H. oncopelti* in *Asclepias syriaca* (Fig. 30). They are characteristically ribbon-like, with twisted bodies, clear, almost hyaline protoplasm, and short flagellum. In culture (Fig. 14), however, they cannot be distinguished from the culture forms derived from hemipterans. The initial culture was pure, and, as in the case of all the plant strains, was obtained on leptospira medium. Only one tube of twelve inoculated yielded growth.

Herpetomonas lygæorum, n. sp. (Figs. 11, 12, 19, 20).

Strain 1 (from Lygæus kalmii No. 2).—As has already been mentioned, cultures of herpetomonads were obtained from two insects of the species *Lygæus*

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[30] Holmes, F. O., *Phytopathology*, 1924, xiv, 146.
[31] França, C., *Ann. Soc. belge med. trop.*, 1920–21, i, 245.

kalmii. The preparations made from suspensions of the intestinal tract show that the forms in *Lygæus* No. 2 were a trifle narrower and longer than the majority of those in *Lygæus* No. 1, and had somewhat longer flagella. The morphological characteristics of the two cultures, however, are very much the same (Figs. 18, 20). The two species can be distinguished only by serological and fermentation reactions.

Strain 2 (from Asclepias syriaca No. 46).—Figs. 11, 12. The impure culture of this strain was discovered several weeks after inoculation of leptospira medium with the plant latex. Purification was accomplished by the Barber technique. In the plant (Fig. 11) and also in culture (Fig. 12), this organism is indistinguishable from the strain from *A. syriaca* No. 213, except for slight difference in size, but serologically and in fermenting properties it is identical with *H. lygæorum.*

Herpetomonas sp.? from Oncopeltus cingulifer (Figs. 7, 8, 33, 34).

Oncopeltus cingulifer (Fig. 4) feeds on the latex of *Asclepias curassavica,* a Honduran milkweed, in which flagellates were first found by Hegner.[21] The relation between the insect strain and that of its plant host could not be ascertained in this instance, since pure cultures were not obtained. The impure growth was detected 75 days after inoculation on one of several dozen plates which had been similarly inoculated and kept under identical conditions. The flagellates of *O. cingulifer* differed in appearance from *H. oncopelti* in having a considerably longer flagellum.

Herpetomonas sp.? from Asclepias curassavica (Figs. 15, 16, 31, 32).

An impure culture of this organism was obtained on a semisolid (leptospira) medium containing glucose, inulin, and tapioca. The flagellates in the culture (Figs. 16, 32) bore rather slight resemblance to the forms found in the latex (Figs. 15, 31) and were much longer than those of the pure cultures isolated subsequently from American and Haitian milkweeds (Figs. 12, 14). Their morphological features do not correspond with those of the cultural forms (impure) of *H. elmassiani* obtained by Migone from *Aranjia angustifolia* in Paraguay, as described by França.[31]

Herpetomonas culicidarum, n. sp. (Figs. 21, 22).

Through the cooperation of Dr. Mark F. Boyd, of Leesburg, Georgia,[23] we were able to secure an impure culture of a *Herpetomonas* from *Anopheles quadrimaculatus* on a blood sugar slant (pH 5). The strain was purified by plating on similar medium. The original film preparation made by Dr. Boyd (Fig. 21) contained numerous flagellates of truncated and spindle shape, with short flagella. The flagellates in culture (Fig. 22) were considerably longer, in this instance, than those in the original material. In all other instances the reverse was true.

Strain from Culex pipiens.—Small numbers of herpetomonads were found in the intestines of two larvæ of *Culex pipiens*, collected in New Jersey, and from one of them a culture was obtained on acid blood agar plates. The immunological and fermenting properties of this strain are identical with those of the flagellate from *A. quadrimaculatus*.

Herpetomonas muscidarum, n. sp. (from Musca domestica) (Figs. 23, 24).

Many of the house flies collected about the grounds of The Rockefeller Institute harbored herpetomonads, some of them very large forms with long active flagellum, in many instances double, as described by von Prowazek.[32] A section of intestinal tract from one of the infected flies, in which the flagellates were swarming as in culture, was picked out intact with a fine pipette, washed, and the contents suspended in 0.5 per cent saline. Inoculation of acid blood agar with ascending dilutions of this suspension yielded some pure colonies.

While the cultural properties of this organism are very much the same as those of the other cultivated strains of the series, in morphology it is slightly different. In young cultures on leptospira medium (Fig. 24, *a*) the individuals are no larger and the flagella no longer than in the case of *H. oncopelti* and *H. lygæorum*, but after several weeks in this medium extremely long forms appear, with flagella not unlike those of the organisms in the original material (Fig. 24, *b*).

Herpetomonas media, n. sp. (from Calliphora No. 1) (Figs. 25, 26).

This culture, which was obtained by the Barber method, is a pure line strain. Morphologically this organism resembles *H. muscidarum*, but it never attains the size of that strain, even in old cultures, and it is immunologically distinct.

Herpetomonas parva, n. sp. (from Calliphora No. 2) (Figs. 27, 28).

This culture also represents a pure line strain. It is decidedly smaller than the other two strains from flies. On blood agar slants the colonies of *media* and *parva* are light gray, heavier than those of *muscidarum*, and show less tendency to coalesce.

Herpetomonas ctenocephali; Trypanosoma rotatorium; the Leishmanias.

Morphologically, *H. ctenocephali* is quite unlike any of our strains. In its leaf-like form it resembles rather the flagellates found in the plant latices, though the flagellum is much longer. The frog trypanosome is again entirely different from any of the other flagellates studied. On the other hand, the morphological features of the leishmanias, while distinctive, approach those of the culture

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[32] von Prowazek, S., *Arb. k. Gsndhtsamte*, 1904, xx, 440.

TABLE II.

Source of flagellate.	Length of body.	Anterior end to parabasal body.	Parabasal body to nucleus.	Nucleus.	Posterior end to nucleus.	Width at widest portion.	Length of flagellum.	Remarks.
	μ	μ	μ	μ	μ	μ	μ	
Oncopeltus fasciatus............	12–25	1.8–3.5	2–3	2.5–3	12–15	2–3	10–25	Shandaken.
" (culture forms)........	11.5–17	2–4	2–4	2.5–3	1.2–6	2–3.5	6–18	
Oncopeltus cingulifer..........	12–25	2–4	0.5–3	1.8–2.8	6–15	2–3	10–28	Honduras.
" (culture forms)........	7–16.5	1.5–3.5	2.3–4.5	1.5–2.8	0.3–3.5	1.8–8	4–18	"
Oncopeltus sp.? (culture forms)..	7–16.5	1.5–3.5	2.3–4.5	1.5–2.8	0.3–3.5	1.8–5	4–18	Peru.
Lygæus kalmii.................	8–12	1.8–2	1–1.5	1.8–2	5–8	1.8–2.5	6–12	Shandaken.
" " (culture forms)....	7–14	1.8–3.5	0.5–3	1.8–2.2	2–6	2.5–3.5	6–16	Strain 1.
" " "	6–12	2–3	0–2 (some beside nucleus).	1.8–2.2	3–4	2–2.5	4–14	" 2.
Anopheles quadrimaculatus......	6–8	1.8–2	0.5–1	1.8–2	2–2.8	1.8–2.5	4–6	Dr. Boyd's preparation.
" (culture forms)...	6–11	1.8–2	0.5–2.2	1.8–2	2–5	2–3	8–12	
Culex pipiens..................								Too few specimens in preparation. Similar to Anopheles forms.
" " (culture forms)....	8–14	2–3	2–4	2–3	3–5	3–5	8–12	
Musca domestica...............	15–30	2.5–3	2–3	2.5–3	7–10	3–3.5	15–35	
" " (culture forms)...	12–30	3–5	2–6	2.5–4	4–20	3–4	10–30	

Calliphora spp.	16-20	1.5-2	2-2.5	2-2.2	6-16	2.5-3	8-25	Strain 1.
" " (culture forms)	6-18	2-3	2-5	2-2.2	3-5	2.5-3	10-20	" 1.
" " "	5-9	1.8-2	2-2.5	1.8-2.2	2-3	2-2.5	4-8	" 2.
Asclepias syriaca	8-12	1.8-2	1.8-2	2-2.2	4-6	2-2.5	2-10	Shandaken plant 46.
" "	13-22	1.5-2	1-2.5	2-3	5-14.5	2-3.5	6-12	" " 213.
" " (culture forms)	6-12	1.8-3	0.5-2.5	1.8-2.5	3-4	2-2.2	4-14	Strain 46.
" " "	7-17	1.5-3.5	2.2-4	1.8-2.5	1-6	2.8-3.5	6-16	" 213.
Asclepias nivea	13-22	1.5-2	2-3	2-3	5-14.5	2-3.5	6-12	
" " (culture forms)	10-17	2-4	2-4	2.5-3	2-6	2.5-3.5	6-18	
Asclepias curassavica	14-20	1.2-2	1.3-4	2-3	8-14	2-4	3-12	Honduras.
" " (culture forms)	10-18	2-4	2-3	2-2.5	2-6	2.5-3	6-20	Impure culture.
Ctenocephalus canis. H. ctenocephali H.	Round, 3.3-6 Kinetic, 11-16.7					2.5-4.3 1.3-2.5	7.3-21	After Tyzzer and Walker.[13]
" " " (culture forms)	10-15	1-1.2	1-1.5	1.5-1.8	5-10	1.8-2	15-18	H strain. Twisted. Sharply pointed at both ends.
Rana pipiens. Trypanosoma rotatorium (culture forms)	10-15	1-1.2	1-1.5	1.5-1.8	5-10	1.8-2	6-8	Twisted and sharply tapered posterior portion.
Oriental sore. *Leishmania tropica.*	10-12	1.5-2	1.8-2.2	1.8-2.2	6-8	2-2.5	10-15	Slender forms.
Espundia. *L. brasiliensis.*	5-8	1.8-2	0.5-1	1.8-2.2	1-2.5	1.5-3	3-6	Stocky "
Kala-azar (China). *L. infantum.*	9-12.5	1-1.2	1-1.5	1.5-1.8	4-6	2-2.5	3-6	Sharply drawn to ends.

forms of our insect and plant flagellates; they are pyriform and stocky when grown on the surface of blood agar slants, but characteristically longer and slenderer on leptospira medium, and have longer flagella.

The flagellates of the dog flea and frog grow very slowly, 1 to 2 weeks being required for the growth on blood agar slants to become visible. The leishmanias grow somewhat more rapidly, but much more slowly than the plant and insect strains. All the strains studied show more rapid and prolific growth on leptospira medium than on plates or slants.

Table II is a comparison of certain morphological features of the flagellates studied. Preparations of cultures grown on leptospira medium were used in making the measurements of culture forms, except in the case of *Leishmania brasiliensis*; in this instance the stocky forms obtained from a blood slant culture were measured.

The preparations used as a basis of comparison were all made by fixing dried films in methyl alcohol and staining with Giemsa's solution. The early part of the work was not done under laboratory conditions, and this method was the only one which could be conveniently carried out. Later the cultures were studied by cytological methods (wet fixation in Schaudinn's sublimate alcohol, followed by staining with iron-hematoxylin or Giemsa's solution, with subsequent differentiation), but since these preparations furnished no additional information regarding the structure of the organisms and were not available in all instances, they have not been especially mentioned in the present study.

SUMMARY.

Nine strains of herpetomonads have been isolated in pure culture from eight varieties of insects, and three strains from two species of plants. Four of the cultures were derived from latex-feeding insects (*Oncopeltus fasciatus, Oncopeltus sp. ?, Lygæus kalmii*) and three from latex plants (*Asclepias syriaca, Asclepias nivea*); two from mosquitoes (*Culex pipiens* and *Anopheles quadrimaculatus*), one from the house fly (*Musca domestica*), and two from bluebottle flies. In addition impure cultures have been obtained from *Oncopeltus cingulifer* and from its plant host, *Asclepias curassavica*.

The flagellates cultivated, all of which belong to the genus *Herpetomonas*, have been identified chiefly by their biological relationships,

which will be described in detail in Part II of this report. The seven strains from latex-feeding insects and latex plants represent two distinct species, which have been designated *H. oncopelti* and *H. lygæorum*. The two strains from mosquitoes proved to be the same organism and have been called *Herpetomonas culicidarum*. The culture obtained from *Musca domestica* contained larger individuals than those of any other strain, and the organism is morphologically distinct from either of the *Calliphora* strains. None of the fly flagellates cultivated could be identified with the species *H. muscæ domesticæ* or *H. calliphoræ*, and hence they have been given new names, *Herpetomonas muscidarum, H. media,* and *H. parva.*

Blood agar plates were used for initial cultivation of the strains from insects and the semisolid leptospira medium for isolation of the plant flagellates. A number of the strains were purified by plating on acid blood agar, a procedure which reduces considerably the growth of bacterial contaminants. The Barber technique was utilized for isolation of the flagellates from flies, because of the very large number of bacteria found with them in these insects, and, in one or two instances, for the purification of impure cultures. Once they have been obtained in culture, all the strains grow well on leptospira medium, as well as on blood slants. Growth takes place both at 26°C. and at 37°.

The morphology of the organisms is considerably modified by cultivation. This is especially true of the plant flagellates. In the latex they have ribbon-like bodies, often twisted, and comparatively short flagella; the protoplasm is clear, almost hyaline. The flagellates seen in the gut and feces of insects are usually large, slender organisms, with flagella as long as or even longer than the body, which contains numerous volutin granules in the cytoplasm. In cultures under parallel conditions the flagellates from both these sources become shorter and thicker, the plant forms no longer appear flat and ribbon-like, and in general the organisms approach one another in morphological features. Even in the case of the least modified insect flagellates, *i.e.* those from flies, there is never exact correspondence between the natural and the cultivated forms.

The morphological features of the cultivated flagellates vary according to the medium on which the organisms are grown and the age

of the culture. The flagellates grown on the surface of blood slants are pyriform, with truncated anterior portion, and short flagellum; in the condensation water, however, the individuals are elongated and have long active flagella. On the leptospira medium the slender active forms with long flagella predominate. In the presence of fermentable carbohydrate, or in medium containing considerable acid, peculiar bifurcated or multifurcated individuals are seen. Similar forms have been seen under natural conditions. Cultures of *Leishmania* behave in the same way under the conditions described.

There is a striking difference in rapidity of growth between the organisms isolated by us and the leishmanias, *H. ctenocephali*, and *T. rotatorium*. While the stock cultures of the group first mentioned multiply rapidly at 37°C., growth becoming visible within 24 hours, the latter group grow scarcely at all at 37° and only slowly at 25°, 1 to 2 weeks being required for growth to become macroscopically demonstrable.

While the flagellum of the leishmanias, as also of *H. ctenocephali*, is long, serpentine in its movements, and heavy, having the appearance of being enveloped by a sheath throughout its entire length, that of the recently isolated strains is thin, less flexible, and without the sheath-like appearance. The only exceptions to this rule are the flagellates from *Musca domestica* and *Calliphora* No. 1, which have a long flagellum not unlike that of the leishmanias.

As the foregoing observations indicate, morphological differentiation of the flagellates studied, while not impossible, is subject to error by reason of the variations due to age and cultural conditions. The flagellates of the latex-feeding insects, the plants, the flies, and the mosquitoes can readily be distinguished from *Leishmania* by their rapid growth at 37°C., but their differentiation from one another is possible only by serological and fermentation reactions.

EXPLANATION OF PLATES.

PLATE 10.

FIGS. 1 to 4. Latex-feeding insects, natural size. Fig. 1, specimen of *Oncopeltus fasciatus* caught on Long Island. Fig. 2, *Oncopeltus sp.* from Lima, Pera. Fig. 3, *Lygæus kalmii*, specimen from Long Island. Fig. 4, *Oncopeltus cingulifer* from Honduras.

PLATES 11 TO 14.

FIGS. 5 to 28. Herpetomonads of insects and plants, as they appear under natural conditions and in culture. Drawn from preparations stained with Giemsa's solution after fixation by methyl alcohol. Magnification × 1200. The species name and source are indicated in each instance. *a*, forms from slant cultures. *b*, forms from cultures on leptospira medium.

PLATE 15.

Magnification × 1200.

FIG. 29. *Herpetomonas davidi*, original preparation made by Lafont,[33] presented by Professor Emile Brumpt. Giemsa's stain.

FIGS. 30 to 34. Drawings from preparations fixed in methyl alcohol and stained with Giemsa's solution. Fig. 30, flagellates from a specimen of *A. syriaca*, a plant of the group found by Holmes[30] in Maryland and studied by him. Fig. 31, flagellates from the latex of *Asclepias curassavica* (Honduras). Fig. 32, impure culture of flagellates from *Asclepias curassavica*. Fig. 33, flagellates from the gut of *Oncopeltus cingulifer*, which feeds on *Asclepias curassavica*. Fig. 34, impure culture of flagellates of *Oncopeltus cingulifer*.
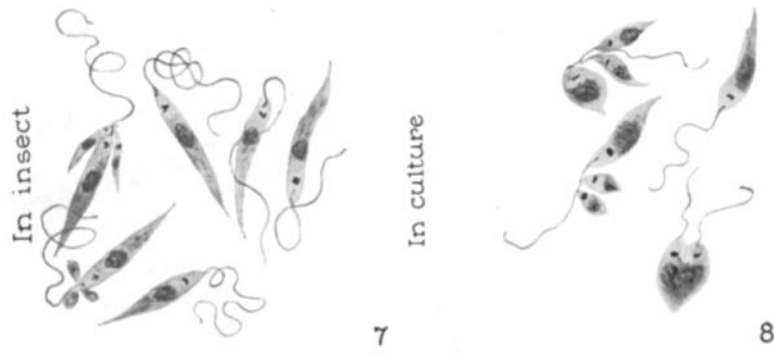
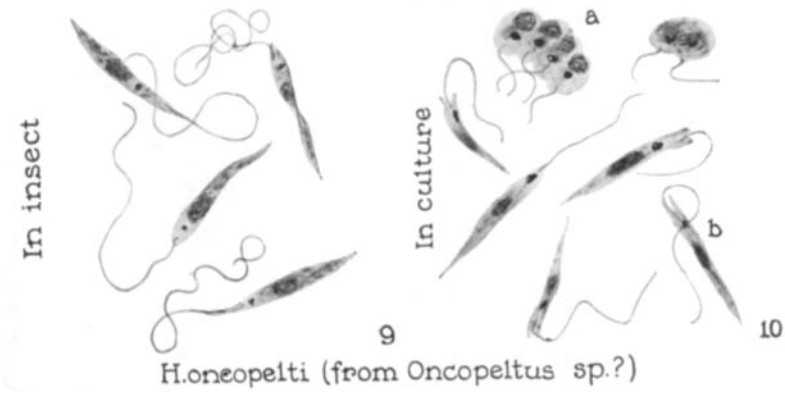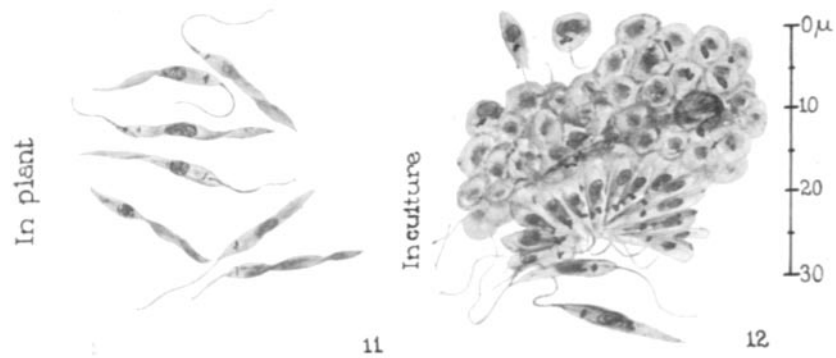[33] Lafont, A., *Compt. rend. Soc. biol.*, 1909, lxvi, 1011.

In insect   In culture
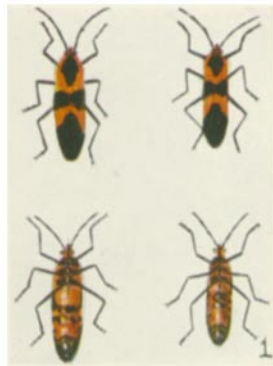
5   6

H.oncopelti (from Oncopeltus fasciatus)

In insect   In culture

7   8

Herpetomonas sp.? (from Oncopeltus cingulifer)

In insect   In culture

9   10

H.oneopelti (from Oncopeltus sp.?)

(Noguchi and Tilden: Herpetomonads and leishmanias.   I.)

In plant

In culture

11 12

H. lygaeorum (from Asclepias syriaca No.46)

In plant

In culture
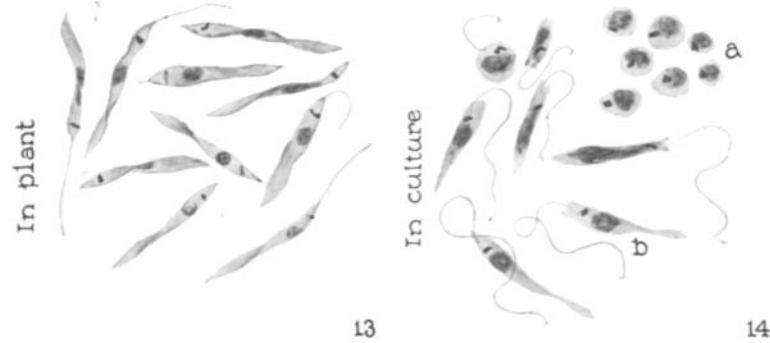
13 14

H. oncopelti (from Asclepias nivea)

In plant

In culture
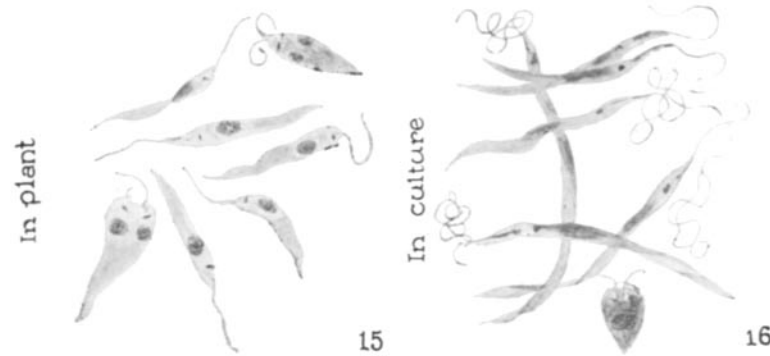
15 16

Herpetomonas sp.? (from A. curassavica)

(Noguchi and Tilden: Herpetomonads and leishmanias. I.)
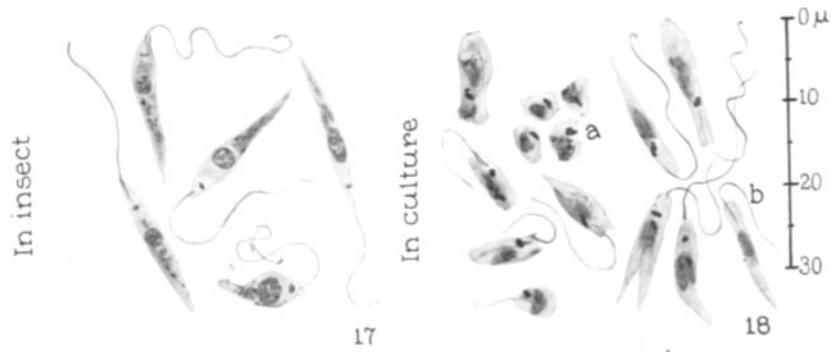
H. oncopelti (from Lygaeus kalmii No.1)
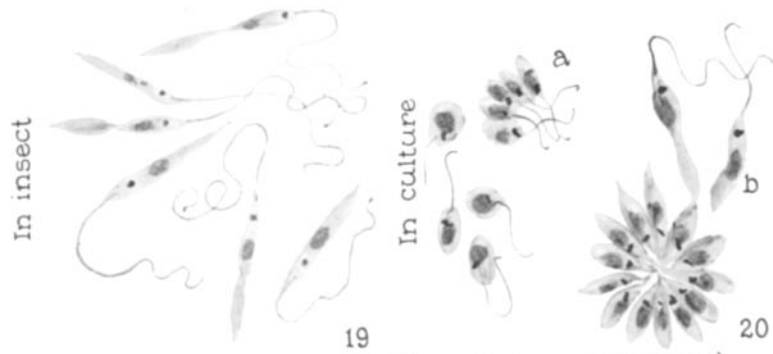
H. lygaeorum   (from Lygaeus kalmii No2)
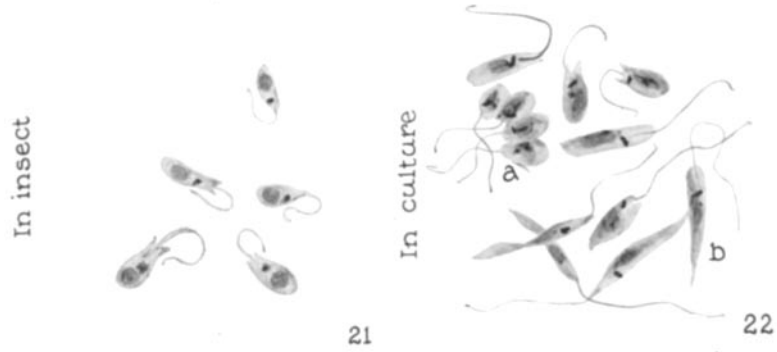
H. culicidarum   (from A. quadrimaculatus)

(Noguchi and Tilden: Herpetomonads and leishmanias.   I.)

H.muscidarum   (from Musca domestica)

H. media   (from Calliphora ? No.1)

H.parva   (from Calliphora? No.2)

(Noguchi and Tilden: Herpetomonads and leishmanias. I.)

(Noguchi and Tilden: Herpetomonads and leishmanias. I.)