report he lays stress upon the appearance in the erythrocytes of various bodies which were observed after the acute stage had passed. These bodies, he considers, are stages in the life cycle of the spirochæte gallinarum. In order to confirm his observations I have carefully gone through the whole series of blood smears taken daily from the various fowls, commencing at the date of inoculation and continued for a long period after the disappearance of spirochætes from the peripheral circulation, also smears from organs *post-mortem*, but have failed to discover anything corresponding to the bodies he describes and pictures in his report.

TRYPANOSOMA AMERICANUM NEW SPECIES: A TRYPANOSOME WHICH APPEARS IN CULTURES MADE FROM THE BLOOD OF AMERICAN CATTLE.[1] (PRELIMINARY NOTICE).

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Historical.

IN a postscript to his celebrated article on the blood parasites of the little owl, Schaudinn (1904) details some information received from Kossel and Weber in a personal communication. The last-named investigators, in Finland, in 1900 had made a smear of blood, taken from a cow dying of hemoglobinuria. In this smear, in addition to examples of a piroplasma, which they identified as piroplasma bigeminum, there was found a trypanosome, considerably smaller than trypanosoma evansi. Schaudinn examined this preparation, and after comparing the morphology of the trypanosome with that of piroplasma bigeminum states that it probably constitutes a stage in the life-history of piroplasma bigeminum similar to that taken by the trypanosome of halteridium in the life-history of that blood parasite. His belief was based on the size, character of the cytoplasm, and nuclear relations of the two phases found in Kossel and Weber's preparations.

The year before Kossel and Weber had made smears of the intestinal contents of ticks taken from sick cattle. On account of the above discovery these smears were re-stained and studied, and in them were found trypanosome-like stages similar to those found in the blood of the cow. Hence, with Kossel and Weber, Schaudinn accepts as a working hypothesis that the evolution of piroplasma bigeminum and piroplasma canis takes place in a manner similar to that of halteridium.

Bowhill and Le Doux (1904), in a paper on malignant jaundice of the dog, refer to endoglobular parasites which show long, flagella-like processes. It is also stated that one of them (Bowhill) has observed flagellated forms of a piroplasma, which they call piroplasma bovis. They write: "The parasite consists of an enlarged elongated

[1] Reprinted from Bulletin 119 of the Bureau of Animal Industry, United States Department of Agriculture, 1909.

portion running into a delicate 'flagellum,' upon which, on close examination, may be seen two minute bulbous protuberances."

Bowhill (1905), studying equine piroplasmosis, finds, free in the blood stream, parasites which "consist of a delicate, pear-shaped head, possessing a clearly defined red-stained karyosome, and a long flagellum ending in a bulbous protuberance." He adds that these flagellated forms may be within a red blood cell, or half in and half out. The flagellum may be 3·5 μ in length or even more.

A glance at the figures given by these authors shows, however, that they are not dealing with flagella at all, but with processes resembling pseudopodia. This is pointed out by Minchin (1909), who writes (p. 89):—

"I suggest that the 'flagellum' is probably to be interpreted as a pseudopodium, by means of which the parasite may quit a corpuscle, and by means of which it may, when free, attack and enter another corpuscle. It is not possible, however, to give a final judgment upon this point until the so-called flagellum has been seen and studied in the living condition."

Miyajima (1907) gives what seems to be excellent evidence in support of the doctrine that trypanosomes and hæmosporidia are but phases in the life-history of the same organisms. Cultures made in common bouillon of blood from Japanese cattle parasitised with a species of piroplasma presently developed trypanosomes. The history of such cultures is as follows:—

First day. No motile forms are seen.

Second day. A number of motile cells appear which occupy the upper layer of sedimented corpuscles and which show macroscopically as whitish dots.

Third day. A few motile forms are seen.

Fourth day. Motile forms, typical trypanosomes, appear in numbers, multiply rapidly, and reach their maximum in from ten to fourteen days.

In cultures at room temperature the trypanosomes remain motile for forty-five days. At from 10° to 20° C. they live for three months. Subcultures are also readily made.

These trypanosomes Miyajima regarded as derivatives from the piroplasma present, for the following reasons: In the first place, the blood of 200 cattle was examined for trypanosomes, without result, and trypanosomes have never been seen in Japanese cattle. He then selected twenty-one native animals and examined their blood. In nine of these piroplasma was found; in twelve it was not. Cultures were then made of the blood of these twenty-one animals, and trypanosomes appeared in seven cases, all from animals the blood of which had previously shown piroplasma. The remaining fourteen cultures were negative.

Further to prove his results, Miyajima tried the following experiment: Three calves were selected, the blood of which failed to show parasites. These were injected with a culture containing motile trypanosomes in abundance, and later were kept free from ticks. One animal gave negative results, but the other two became infected, as was proved first culturally and later microscopically. Miyajima writes (p. 90):—

"In one instance, eight days after inoculation, the blood of the susceptible animal began to give a growth of the flagellated parasite in culture, whereas seventeen days later, by the aid of the microscope alone, the intracellular parasites were visible in the same animal."

It was found that the quantity of blood used to inoculate the tubes was a matter of indifference. One platinum loopful gave precisely the same results as 1 cc.

Miyajima's conclusions, given on page 90, are here quoted :—

"1. A variety of hæmocytozoa, known as piroplasma parvum, can readily be cultivated outside of the living body.

"2. The parasites undergo the developmental change in blood bouillon, and finally take the form of a typical trypanosoma. This trypanosoma can not be detected in the blood of living animals.

"3. A simple mixture of blood and bouillon is the most suitable medium for the cultivation of protozoa, such as piroplasma parvum and trypanosoma lewisi."

In an earlier communication Miyajima, in collaboration with Shibayama (Miyajima and Shibayama, 1906), brought out the following facts: From 13·6 to 76·1 per cent. of Japanese cattle, according to locality, were found to be parasitised by a piroplasma. It was not possible, by blood injections, to transfer this parasite from one animal to another. There was no evidence to show that this parasite is pathogenic to either native or foreign cattle. In the case of the former the only method whereby the presence of the parasite could be demonstrated was by microscopical examination. As to the latter, the authors examined a herd of twenty cattle imported some time previously from Wisconsin. Although all of these animals had remained in perfect health, fourteen were found to be parasitised. On the other hand, two Japanese calves (age not given) were infested with ticks hatched from infectious eggs received from the Bureau of Animal Industry. The eggs were from ticks collected in Texas. The two calves, each infested with 200 ticks, responded by a mild attack of what was presumed to be Texas fever, but with regard to the blood examination the authors wrote: "Während die ganzen Beobachtungszeit zeigte der tägliche Parasitenbefund bei der Versuchstieren keine Abweichung von dem normalen Kalbes." From which the supposition is that piroplasma bigeminum was not found, but that the Japanese piroplasma remained present as in the control animals.

From the above it is evident that the Japanese piroplasma found by these authors is peculiar in that it is not pathogenic. In this respect it differs from all of the other known species of this genus. Whether this is any warrant for believing that it is really not a piroplasma at all is of course very questionable, but it may be pointed out that it does not follow that all of the pear-shaped parasites of the red blood cells of mammals are genetically connected. How this may be can not be determined until the life-history of each species is known. I call attention to this fact here, since Miyajima's work is quoted as showing that piroplasma has a trypanosome stage in its life-history, a conclusion scarcely warranted.

In addition to the above papers, which are concerned with the attempt to link together trypanosomes and hæmosporidia, there are a considerable number of observations which prove the presence of trypanosomes in cattle where there is no reason to believe them pathogenic.

Frank (1909), in Germany, found living specimens in the spleen pulp of an ox, the animal having died of a disease taken to be either anthrax or blackleg. The bacilli of neither of these diseases being found, Frank was inclined to blame the animal's death on the trypanosome, but no proof was furnished.

Frosch (1909) describes this parasite, which he calls trypanosoma Frank, his material being stained smears from the spleen. It shows a sharply pointed posterior end, often terminated by a flagellum; a round, peripherally placed kinetonucleus, between which and the trophonucleus there is a wide interval. Frosch agrees with Frank in considering the trypanosome to be pathogenic.

Frank and Frosch (1909) restate the proposition of the pathogenicity of this parasite, saying that this is the first case of a bovine dying in Europe of trypanosomiasis.

Knuth (1909), studying the same material, made measurements of the flagellates present. He gives the length as from 20 to 40 μ, which includes the flagellum; the breadth as 2 μ. According to this author, it more closely resembles trypanosoma theileri than any other species.

Mayer (1909) also discusses this same trypanosome, and after going over its morphology says that it has the characters of a certain group of trypanosomes, typified by trypanosoma theileri, which are found exclusively in cattle, hitherto only in tropical or subtropical countries.

Mayer then quotes a number of authors who have found trypanosomes occurring sporadically in cattle. Such mostly show the characters of this group. These discoveries were made in Asia and Africa. In some cases the trypanosomes were in healthy cattle; in others in those suffering from various diseases other than trypanosomiasis. Among these may be mentioned piroplasmosis and rinderpest. In no instance was there any reason to look upon the trypanosomes as pathogenic.

Hence Mayer regards the animal discovered by Frank to be harmless. Finally, he calls it trypanosoma franki, a valid name.

Methods.

Miyajima's work was repeated in this laboratory with entirely satisfactory results. Bovine blood, cultured in common beef bouillon, develops trypanosomes in from two to four days, dependent upon temperature. They also appeared in cultures of cow's blood in mutton bouillon, either acid or alkaline, and, furthermore, they developed in the case of every cow tested.

The methods were as follows: Blood was drawn from the jugular vein of the cow by means of a syringe, and transferred to flasks of 100 cc. capacity. About 30 to 50 cc. of blood was taken in each case. In each flask were placed six to eight facetted beads of rough glass, such as those found in shops, strung together, for sale as very cheap necklaces. A few minutes shaking of the flask serves to collect the fibrin into a solid clot, embedded in which will be the beads. In these operations suitable precautions were taken to prevent contamination.

To take the blood from the flasks, pieces of glass tubing were used, one-fourth inch in diameter and eight to ten inches long. Over one end of each of these a piece of rubber tubing was pulled to serve as a mouthpiece. The quantity of blood desired may then be drawn up into the glass tube by suction. The tubes with the rubber tubing attached were sterilised by boiling for five to ten minutes and then used at once. Owing to the poor conductivity of glass they do not cool readily, and it was considered possible that they might heat the blood sufficiently to kill any animal life present. Accordingly, the precaution was usually taken of allowing the first filling of the glass tube to drop back into the flask and of using only the second.

As soon as the glass tube was filled with the blood to be used, the rubber tube was closed by pinching and the contents transferred at once to a culture tube. This operation was always performed by two persons, one to handle the blood, the other the culture tubes. In this way the latter were not exposed to the possibility of contamination for more than a second or two.

This procedure gave entirely satisfactory results. Bacterial contamination, when it occurs, appears to be the result either of getting the blood contaminated during the process of drawing it or in the later handling of the tubes. Moreover, it is not quite so destructive to the growth of the trypanosomes as some authors maintain. While the contaminating of cultures is a reflection on the operator's technique, the trypanosomes seem able to endure a certain amount of plant life, and may even be found alive in very foul cultures. It was also at times noted that bacteria would start in a tube and later die out.

Observations.

As a rule, the trypanosomes appeared on the third day. In one case, however, the "room temperature" being high, from 80° to 90° F., they were found in forty hours. After a few days the microscope is not necessary to demonstrate their presence, since they collect in little colonies on the surface of the column of blood cells. These colonies show as small white plates, and may be 3 or 4 mm. in diameter. They are readily distinguishable from bacterial contamination, in that they are flat and sharply circumscribed, whereas the masses of bacteria are always more or less diffuse and tend to cloud the bouillon.

The ratio of the quantity of blood to bouillon was varied within wide limits, but, as Miyajima found, this makes no difference as far as the appearance of trypanosomes is concerned.

So far as the study has yet gone, the flagellates have appeared in the blood of all the cows tested, seven in number. Of these, the first used was a cow born at the Experiment Station of the Bureau of Animal Industry at Bethesda, Md., on 15th November 1900. This animal had twice been injected with blood from cattle known to be parasitised with piroplasma bigeminum. It was hence assumed that her blood contained this parasite, and it was for this reason that she was chosen for the experiment.

Accordingly, the appearance of the trypanosomes was taken to be an exact confirmation of Miyajima's results, to the effect that piroplasma has a flagellate stage in its life-history. But whereas the

Japanese biologist had to depend upon the tedious and uncertain method of examination to determine whether or not he was dealing with animals parasitised by piroplasma, this point can in the United States be settled without difficulty and with almost the certainty of a mathematical demonstration. The cattle in the northern parts of the United States, outside of the tick-infested region, are free from piroplasma. This can be proven in individual cases by placing infectious ticks on the animal, *i.e.*, ticks which have hatched from eggs laid by ticks taken from southern cattle. A northern cow thus treated will sicken of Texas fever and usually die, whereas no effects will be noticed in the case of a cow in the blood of which piroplasma was already present.

Therefore, four animals were obtained from north of the Federal quarantine line and cultures made of their blood. The trypanosomes appeared with due promptness. Since the chances were all against these animals being parasitised by piroplasma (and, further, since one of them, as noted below, after being infested with ticks contracted Texas fever with fatal results), it was seen that there was a serious discrepancy between Miyajima's results and mine. My Japanese colleague got trypanosomes from blood in which he demonstrated the presence of a piroplasma, and failed to get them in blood in which piroplasma could not be found, whereas my experience is that the trypanosomes appear without reference to the presence or absence of hæmosporidia.

As an additional test, two cows from the herd at the Experiment Station of the Bureau of Animal Industry were tried, and the flagellates developed in cultures from their blood. The animals of this herd are assumed to be, and in all probability are, free from piroplasma bigeminum. Finally, one of the four cows purchased for the experiment was infested with infectious ticks, and in due course died of Texas fever—practically absolute proof that at least one animal from the blood of which trypanosomes were cultured did not originally harbour the hæmosporidian. It was not considered necessary to sacrifice the other animals used in the study.

The first point which suggests itself is whether the trypanosomes which appear in culture tubes arise from actual trypanosomes originally present in the blood or from some element of a wholly different facies. While this point can not be regarded as finally settled, the probabilities are in favour of the second hypothesis. The blood of American cattle, in both health and disease, has been studied for many years, and with a single exception (Bowhill, 1909) trypanosomes have never been reported. If they were normally present in any numbers, it seems unlikely that they should so far have escaped notice. Moreover, while many of the cultures were examined daily from the first, flagellates were never found on the first day, and but once on the second.

But the most direct evidence that they evolve in the tubes from some unrecognised form is the fact that at first we do not find typical trypanosomes, or even flagellates, but round or oval bodies. Miyajima figures bodies which he regards as the forerunners of the flagellates, but it is not certain that these are not white blood cells. Neither his figures, unfortunately made by photography, nor his description permit of a final judgment.

In my own preparations I have now and then come across an isolated spherical body, of much the size of a large leucocyte, which does not exactly resemble any of the various types of white cells. It may be that such belong to the cycle of the trypanosomes, but to settle this requires further investigation. At present I am only able to say that I have not been able to get hold of anything which can

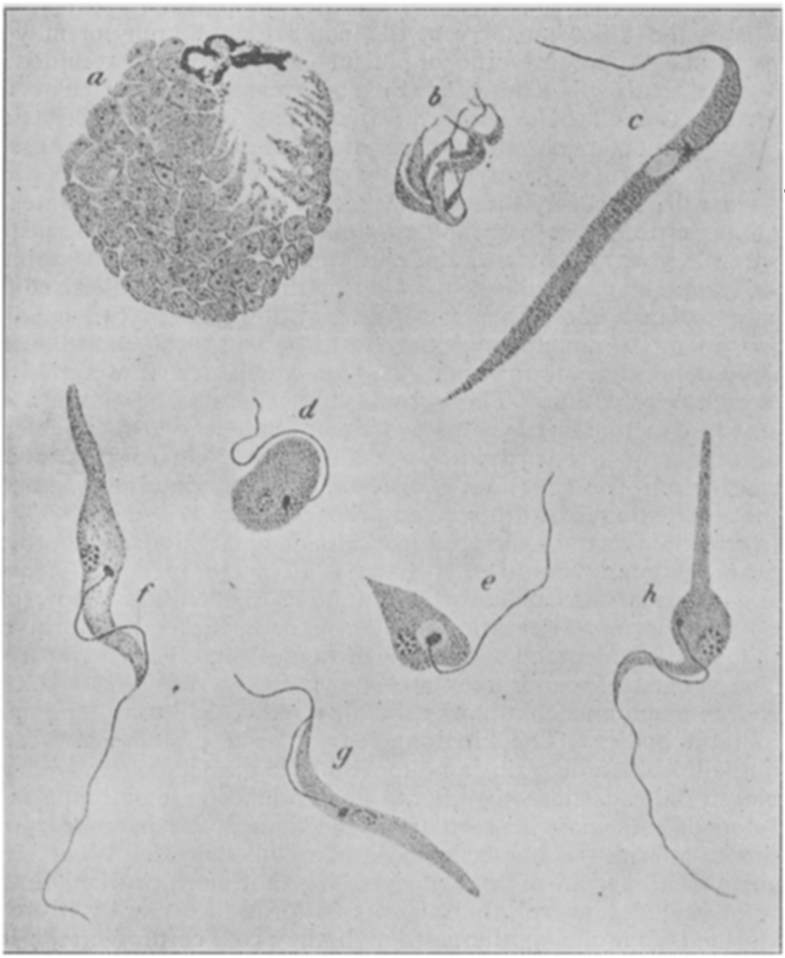

FIG. 1.

Trypanosoma americanum n. sp. *a*, cluster of young forms, 80-hour culture, ×800; *b*, cluster of small flagellated forms, 88-hour culture, ×800; *c*, crithidia stage, 88-hour culture, ×2467; *d*, monadine form, 10-day culture, ×2467; *e*, monadine form—the kinetonucleus lies in a vacuole stained pink by Wright's stain, 10-day culture, × 2467; *f*, typical band-shaped trypanosome, with fully developed undulating membrane, 10-day culture, × 2467; *g*, small band-shaped trypanosome, with fully developed undulating membrane, 10-day culture, × 2467; *h*, club-shaped form, with fully developed undulating membrane, 10-day culture, × 2467. (Figures made from camera outlines.)

safely be put down as a forerunner of the trypanosomes until the body shown in fig. 1, *a*, appears.

This condition may be seen in young cultures. Such masses consist of closely compacted cells which are probably round or oval, but which are sometimes polyhedral on account of mutual pressure. They stain blue in Wright's stain, and each shows a distinct kinetonucleus, but trophonuclei are not to be made out. Clusters of this sort vary greatly in size. They may consist of as few as three or four organisms or as many as several hundreds.

This leads up into the condition shown in fig. 1, *b*. Here is a cluster of little flagellates, of which the cytoplasm is still sharply blue and the kinetonuclei very distinct. Flagella may or may not be present. Apparently the organism evolves from a rounded cell into a lenticular flagellate, but in some cases the lenticular form may be assumed before the flagellum has differentiated. Small isolated lenticular organisms are often seen which do not show flagella.

As a rule, however, these little bodies are typical flagellates, resembling crithidia in morphology, and it is convenient to speak of this developmental phase as the crithidia stage. From this point on, in at least a considerable proportion of the cases, evolution consists apparently in a mere increase in size, till we reach the condition shown in fig. 1, *c*. Here we have a long, band-shaped cell, with a sharply pointed posterior end. The specimen drawn was 28 μ long without the flagellum. The cytoplasm of these stages stains a clear blue, the kinetonucleus is very distinct, while the trophonucleus is represented by a pale, reddish vacuole, and is clearly very poor in nucleic acid. The flagellum is distinct, but an undulating membrane cannot be distinguished in stained preparations.

In addition to the elongated crithidia forms, a monadine form also appears in young cultures, such as is depicted in fig. 1, *d* and *e*. These monads become more abundant as the cultures grow older, but, so far as my observations go at present, they never form more than a small percentage of the trypanosomes present.

The typical trypanosomes are shown in fig. 1, *f*, *g*, and *h*. In these the trophonucleus shows as a fair-sized vesicle containing coarse chromatin bodies. The kinetonucleus is usually elongated, forming a long ellipsoid, and stains an almost black garnet colour. The two nuclei are always close together. The undulating membrane is well developed. As may be seen from the figures, the parasites appear under two forms—a band shaped and a club shaped. What significance this dimorphism may possess has not been worked out. It may be stated, however, that these two forms are readily to be distinguished from one another, although they to a certain extent intergrade. The band-shaped forms, or more typical trypanosomes, are more numerous than the others. At first glance the club-shaped phase might be looked upon as a degenerating condition, *i.e.*, as a so-called involution form.[1] But this can hardly be the case. The club-shaped animals appear in the cultures as soon as do the others, and neither their appearance in life nor their staining reactions in smears carries the least suggestion of degeneracy.

The specimens drawn in fig. 1, *f*, *g*, and *h*, were of intermediate size. Larger individuals were seen, and the length over all may

[1] The use of the term "involution" as a synonym for "degenerate" is extremely unfortunate, but it probably has obtained too good a foothold in the literature to permit of the hope of its being dropped.

reach as much as 60 μ. In cases where degeneracy had apparently set in, the posterior end was often greatly prolonged and now and then provided with a flagellum.

Division, in the cases noted, takes place by equal, longitudinal fission.

It will be seen from the above that the trypanosomes which appear in the culture tubes do not arise, so far as all appearances go, from pre-existing trypanosomes, but from larger or smaller clusters of round or oval cells, which in their turn are doubtless derived from some unrecognised form. Since the study is as yet by no means complete, it is necessary to speak with all reserve; but as long as the earlier cultures show only the clusters of rounded cells or flagellates of the crithidia type, we appear to have a development from some condition which bears no resemblance to a flagellate, and which has not yet been recognised. It is only in the somewhat older cultures that typical trypanosomes appear, but these older cultures also show the adolescent stages, suggesting that all of the hypothetical germs present in a tube do not develop at the same rate.

In all the cultures the trypanosomes tend to occur in great clusters. Thus such a condition as is shown in fig. 1, *a*, appears to give rise to a similarly constituted mass of trypanosomes in either the crithidia or trypanosome stage. The trypanosomes occurring free in the liquid are apparently individuals which have escaped from such colonies. These clusters, however, are not to be confounded with agglutination rosettes, which present a wholly different appearance. In the clusters nothing in the way of a definitive orientation can be made out, whereas in all the agglutinations seen the arrangement was radial.

Observations on the living trypanosomes were made with either ordinary light or the dark-field illumination. They perish so quickly with the latter, however, that its use is scarcely to be recommended. The trypanosomes are energetic enough, but seldom display a progressive movement. When they do, however, it is very rapid, and may be either forward or backward. For the most part, however, the movements are confined to vigorous lashings of the flagellum, changes of the form of the body, and a sort of jumping hither and yon, which does not bring about any extensive change of shape. The club-shaped forms were often seen to display a very quick, sudden, jerky movement, accompanied by a shift in the direction of the longitudinal axis. It was like the movement frequently displayed by stylonychia and related ciliates, only much quicker and more abrupt.

When the fresh mounts were of sufficient thickness as not to compress the large clusters, these, in mass, were seen to change outlines like slow amœbas, and to shift their positions. They are covered with a mantle of very lively flagella, and are so compact that they suggest a metazoan as much as they do a cluster of protozoans.

It may be concluded that the trypanosome here described and figured is a common parasite of healthy American cattle. Its morphological peculiarity is that the trophonucleus and kinetonucleus lie very close together. This peculiarity is shown by trypanosoma transvaaliense, taken to be a variety of trypanosoma theileri, and, as well as can be made out from his figures, by the trypanosome found by Miyajima. If this last fact be so, then Miyajima is in error in his conclusion that his flagellate is a phase of piroplasma. At all events, the fact that trypanosomes appear in cultures of blood from healthy cattle is of considerable significance, and is decidedly against the belief that they are stages in the life-history of a hæmosporidian.

Upon the completion of further investigations concerning this trypanosome, for which the name trypanosoma americanum is suggested, a more extended account of its morphology and ontogeny will be given.

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