Issued January 17, 1912.

U. S. DEPARTMENT OF AGRICULTURE,

BUREAU OF ANIMAL INDUSTRY.—BULLETIN 145.

A. D. MELVIN, CHIEF OF BUREAU.

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...MMON BLOOD PARASITE OF AMERICAN CATTLE.

BY

HOWARD CRAWLEY,
Junior Zoologist, Zoological Division.

WASHINGTON:
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LETTER OF TRANSMITTAL.

U. S. Department of Agriculture,
Bureau of Animal Industry,
Washington, D. C., August 31, 1911.

Sir: I have the honor to transmit, and to recommend for publication in the bulletin series of this bureau, the accompanying manuscript entitled "*Trypanosoma americanum*, a Common Blood Parasite of American Cattle," by Howard Crawley, of the Zoological Division.

A preliminary description of this trypanosome was prepared in 1909 by Mr. Crawley and published in Bulletin 119 of this bureau. The present paper presents a more extended study of the organism, which is shown to be harbored by about 75 per cent of adult cattle.

Respectfully,

A. D. Melvin,
Chief of Bureau.

Hon. James Wilson,
Secretary of Agriculture.

CONTENTS.

ILLUSTRATIONS.

[All figures made from camera outlines and all on the same scale.]

TRYPANOSOMA AMERICANUM, A COMMON BLOOD PARASITE OF AMERICAN CATTLE.

INTRODUCTION.

In Bulletin 119 of the Bureau of Animal Industry there was described, under the name of *Trypanosoma americanum*, a flagellated organism which appeared in bouillon cultures of the blood of American cattle. At the time of writing, although but 7 cows had been tested, trypanosomes had appeared in the blood cultures of all of them, thus indicating that a large proportion of cattle are infected.

The study therein described had been made primarily on stained smears, and based on what was observed in these it was stated that the earliest stage noted was a round or oval body, occurring in clusters, and, while it showed a kinetonucleus, it lacked a flagellum. This last observation is incorrect, the mistake being due to the fact that in these early stages the flagellum fails to stain. A more extended study, based partly on fresh material, has shown that the organisms in the culture tubes are always flagellated. It is desired to emphasize the fact that this observational error was made, since the precise nature of the earliest organism to appear in the culture tubes is a matter of some moment, bearing as it does on the problem of what is the nature of the element present in the circulating blood of the cow. It may also be stated here at the outset that the results of this more extended study show that this latter undoubtedly is a trypanosome like that present in the culture tubes, because trypanosomes have been found in the blood itself within a few hours after its removal from the cow.

RÉSUMÉ OF LITERATURE.

There is a very considerable literature on the trypanosomes of cattle, and a number of species have been described as occurring in them. It will not be necessary here, however, to do more than consider that which bears directly on the problem in hand, that is, which treats of trypanosomes presumably similar to *Trypanosoma americanum*.[1] Some of this literature was reviewed in Bulletin 119, but one paper which should have been noted was overlooked, and several have since come to hand. It may, however, be advisable again to

[1] References to literature are given in the bibliography at the end of this bulletin.

refer to Miyajima's work, since this is the point of departure of all the studies on this peculiar trypanosome.

The organism found by Miyajima in cultures made from the blood of Japanese cattle is apparently the same as that found in the United States. Unfortunately Miyajima does not give a detailed description of his parasite, and his figures are not particularly clear. They show, however, that he was dealing with a trypanosome possessing only a very short undulating membrane, and his figure 10, of a division form, shows two granules lying at the same level and in the anterior part of the body. From this it may be concluded that the Japanese form has the characteristic of a kinetonucleus lying very close to the trophonucleus. Further, the length, given as five times the diameter of a red blood cell, or say 30 microns, is in general agreement with what is found here.

Martini (1909), working at Manila, repeated Miyajima's work, and obtained trypanosomes in bouillon culture tubes inoculated with the blood of a calf. He used 2 c. c. of blood to 10 c. c. of bouillon. In one case the trypanosomes appeared in 33 hours. As a rule, however, the period was from 43 to 48 hours. The tubes were kept at a temperature of 26 to 27° C.[1] The trypanosomes, while in general about the size of *Trypanosoma lewisi*, showed great variation in length. Some were only one and one-half to three times as long as the diameter of a red blood cell, but as the cultures aged they became longer, and on the fifth and sixth days forms were present 20 to 25 times the length of the calf's red blood cell.[2] The smallest forms were morphologically the same as the largest. The anterior end of the flagellum showed a little club-shaped swelling. The kinetonucleus lay transversely, the trophonucleus longitudinally, and the former was generally in front of the latter. The parasites grew indifferently in acid or alkaline bouillon. In cultures in acid media, the cytoplasm contained chromatin granules. A chronological transition was not observed, and rudimentary forms were always to be found alongside of those fully developed. Attempts to inoculate monkeys failed.

Commenting on the above, there can be no question that Martini was dealing with the same trypanosome as Miyajima, which is also, in all probability, the one which appears in cultures made from the blood of American cattle.

Wrublewski (1908)[3] describes a trypanosome found in the blood of the Lithuanian bison. He remarks that this species of *Bos* is not a

[1] Cultures made in the Zoological Laboratory of the Bureau of Animal Industry on July 13, 1909, showed trypanosomes July 15, the mean temperature of the laboratory for this period being 27.4° C.

[2] It is to be regretted that Martini has not furnished us with actual measurements. According to his own figures, the calf erythrocytes range in diameter from 4 to 6½ microns, and this would make the largest specimens say 100 to 150 microns long, clearly a gross exaggeration. His figure 12 shows an animal 40 to 45 microns long; that of figure 14 is perhaps 50, and these are the largest which he figures.

[3] This paper was missed at the time of writing Bulletin 119.

favorable subject for experimentation and hence his material consisted of smears taken from dead individuals. The trypanosomes found ranged in length from 30 to 50 microns. The posterior end is elongated, but terminates bluntly. In some, the middle portion is broad and rounded (compare figs. 13 and 14, this bulletin). In this broad portion are to be found the kinetonucleus and the trophonucleus, the former in front of the latter. The root of the flagellum is broad and may show a knoblike expansion. The free end of the flagellum is also knobbed. Around the trophonucleus are masses of deeply staining granules, and granules may also be found scattered throughout the entire body.

In some cases Wrublewski obtained blood sufficiently fresh to show living trypanosomes. They are very energetic, and in hanging drops dash through the red cells with extraordinary velocity, hurling these to one side. The parasite moves with the flagellum in front. The movement is rotatory.

Vladimiroff and Yakimoff (1908) name this trypanosome *Trypanosoma wrublewski.*

Of the several bovine trypanosomes described as occurring in the circulating blood, this comes by far the closest to *T. americanum.* Wrublewski's figures 3, 4, and 5 would answer for specimens of the American species. The most noteworthy distinction is in the motility, *T. americanum* rarely showing a rapid translatory movement. There is no especial reason why the two might not be the same, the parasite properly belonging to the bison and transferred from it to domesticated cattle by natural means. The fact that the parasite is apparently quite abundant in the circulating blood of the bison, and very scarce in that of ordinary cattle, is no argument against their identity. Moreover, the sluggishness of *T. americanum* in culture tubes is no criterion as to how it may behave in the circulating blood, regarding which there is, unfortunately, no information at hand.

Recent studies have shown trypanosomes to be present in a large proportion of cattle in Germany, or at least capable of appearing in cultures made from their blood.

Knuth and Rauchbaar (1910) tested 17 adult cattle and 2 calves, and found 10 of the adults to be parasitized. Later, out of a lot of 9 cattle, 6 were found infected.

Knuth, Rauchbaar, and Morgenstern (1910) found trypanosomes in culture tubes from 7 out of 25 cattle. They note the presence in the tubes of developmental forms and of agglomerations.

Behn (1910 β), culturing cattle blood in the same manner, found flagellated forms on the second day. Furthermore, in smears made from cultures 1 to 2 days old, he found round bodies, in many cases

with alveolar protoplasm, which stained an intense blue with Giemsa. These showed one or more large, round nuclei, variable in size and staining red. The bodies varied in size, the largest being nearly as large as a leucocyte. Some were free, others inclosed in white blood cells. Behn appears to consider that the intracellular forms, growing, break open the leucocytes and escape. He bases this view on the fact that in smears from cultures 2 days old the bodies then had attached to them the remnants of disintegrated leucocytes. He proposes the query as to whether he is dealing with a case of phagocytosis or of an evolution of the parasites within the leucocytes, and promises a more detailed study.

No bodies such as the above have been seen in smears made from American cattle, and it is perhaps possible that Behn was dealing with moribund or dead leucocytes.

The same author (Behn, 1910 α) refers to the seven animals mentioned by Knuth, Rauchbaar, and Morgenstern. He states that whereas the cultures from these were always positive from the beginning of August until the end of September, after cool weather set in they no longer developed trypanosomes. One of these cows failed to show parasites after September 11. But in a smear of her blood taken August 8 there was found a single trypanosome, remarkable for its unusual breadth.

Behn gives the following measurements:

	Microns.
Posterior end to middle of kinetonucleus	13
Middle of kinetonucleus to posterior end of trophonucleus	4
Trophonucleus	2
Anterior end of trophonucleus to anterior end	24
Length of body	43
Length of free flagellum	12
Total length	55
Maximum width	12

The trypanosome had the body sharply bent. The cytoplasm showed clear spaces and abundant granules. The granules were almost all of the same size and stained reddish to a blue-violet. Around the nucleus the cytoplasm was freer from granules than elsewhere. The kinetonucleus stained a black violet, and lay in a clear region. The nucleus lay transversely, filling the entire width of the body. It stained pale red.

Continuing his studies Behn (1910 γ) inoculated a calf with blood from a cow whose blood gave positive cultures, but was negative to direct examination. Eleven days later the blood of this calf was positive to direct examination. For the first few days the trypanosomes seen were of the *Trypanosoma franki* type, but later they took on the appearance of *T. theileri*. Cultures made from the blood of this calf, after the trypanosomes had appeared, were positive.

Knuth (1910) tested by the cultural method the blood of 41 cattle, with the following results:

	Total number tested.	Positive for trypanosomes.
Adult cattle	31	21
Young cattle	7	1
Sucking calves	3	0

Schmitt (1910) also found trypanosomes of the *theileri* type in a Pomeranian cow suffering from Texas fever. These were found in the blood, were rare, and were present for 10 days.

Sergent, E. and E., (1911) made cultures of the blood of 82 cattle killed at the abattoir at Algiers. Trypanosomes were obtained nine times. Division forms were abundant, and subcultures were made. The medium used was bouillon.

Delanoë (1911), at Alfort, made cultures of cattle blood, using 3 c. c. of defibrinated blood in 10 c. c. of bouillon. Six out of the ten animals tested gave positive results, but not all of the cultures made from parasitized animals were positive, indicating the great rarity of the trypanosomes in the blood. The elements found in the cultures were crithidia-like, averaged 50 microns in total length, and the free tip of the flagella was broadened. Chromatoid granules appeared in the cytoplasm only in the old cultures. In a subculture in Nicolle's medium, there were seen very small forms without flagella, flagellated elements 35 microns long, and trypanosomes with a posterior kinetonucleus.

Knuth confirms Behn in the respect that, after the onset of cold weather, animals having given positive results cease to do so. He suggests that this may be due either to the disappearance of the insect carriers or to the failure of the trypanosomes to grow in the tubes in cold weather. Finally, by private correspondence, Knuth has been advised that blood cultures of cattle have been made in a number of laboratories in Germany, Denmark, and Sweden, with the subsequent finding of trypanosomes.

Dudukalov and Dudukalova (1910) report the results of an experimental study on the trypanosome of the cow. The culture media used were (a) bouillon; (b) equal parts of bovine blood and 0.8 per cent NaCl solution;[1] (c) agar. Each tube was inoculated with a few drops of blood. After 3 to 4 days there appeared in the tubes round forms of the parasite, about the size of leucocytes, in many cases occurring in great clusters. On the sixth to seventh days elongated bodies appeared among these round bodies, and on the tenth to twelfth days there were present great numbers of motile

[1] The text says 0.08 NaCl solution, doubtless a printer's error.

trypanosomes provided with long flagella. After 2 to 3 weeks the trypanosomes were present in enormous numbers and were of various sizes and forms. They remained alive in the tubes for 2 to 4 months. Subinoculations were made and carried to the fourth generation. The optimum temperature for the cultures is 10 to 15° R.

With reference to the above papers, the observation of the German authors that the trypanosomes no longer develop in the tubes after the onset of cold weather is confirmed by the studies made here, as will be pointed out later. As to that of the Dudukalovs, it would be of interest to know if the round bodies first found were flagellated. If so, the Russian authors were dealing with a process altogether like that displayed by *Trypanosoma americanum*. The more leisurely development can be explained by the rather low temperature to which their cultures were subjected. It should also be noted that the figures accompanying their original article—the résumé given above having been taken from a review in a less difficult language than Russian—show that they were dealing with a trypanosome morphologically identical with *T. americanum*. On the other hand, it was not possible to get the American trypanosome to develop in salt solution, even though an equal bulk of blood had been added.

Stockman (1910) found trypanosomes in the blood of 6 out of 10 cattle which had been inoculated with *Piroplasma* to immunize against piroplasmosis. In one case the trypanosomes were present for eight days; in the other five for only a day or two. The trypanosomes were morphologically indistinguishable from *T. theileri*, and would not grow in culture tubes.

Stockman makes no comment, but there is no reason for regarding what he found as anything but *T. theileri*, rendered more abundant as a result of the weakened condition of the cattle.

METHOD OF THE EXPERIMENTAL WORK.

The procedure followed was to prepare cultures of the blood of the cattle, and to examine fresh or fixed preparations made from these. The method of drawing the blood, making the cultures, etc., has been described in Bulletin 119, and need not be repeated here. The study falls easily into two parts, (1) that of the cultures themselves, or what may be called the experimental work, and (2) that made with the microscope on fresh or stained material. The work with the cultures will be detailed first.

EXAMINATION OF CULTURES.

In all, several hundred cultures were made. The media used were beef bouillon, mutton bouillon, bouillon made from extract of meat, and salt solution. In some cases the cultures were made the

same day as the blood was drawn; in others an interval of from one to several days was allowed to elapse, the blood meanwhile being kept either in the ice chest, in the laboratory, or in the incubator. Further, the blood was sometimes used in measured quantities; in other cases there was merely added to the medium what was considered to be a sufficient amount.

At first the cultures were in some cases placed in the incubator, but the behavior of *Typanosoma americanum* is here the same as that of any other trypanosome in culture, and cultures kept at incubator temperature quickly deteriorate. The explanation has been advanced that the preference of a "cultural" form for a moderate temperature is due to the fact that the biting arthropod which removes it from the mammalian host has the temperature of the surrounding air. Without dogmatizing as to whether or not this may be true, it is perfectly evident that the reason *T. americanum* can not live in cultures in the incubator is because these cultures spoil. Apparently, without reference to the presence or absence of bacteria, the hemoglobin leaches out of the red cells and goes into solution in the medium, and there can be no doubt that the liquids in the tube undergo profound chemical changes. Hence, in a very great majority of cases, the cultures were kept at room temperature, which varied from 20 to 27.5° C., according to the time of year.

In the greater number of cultures the medium used was neutral beef bouillon, prepared in the laboratory. The tubes were charged the same day the blood was drawn, and the quantity of blood, while not measured, varied from 1 to 4 c. c. The quantity of bouillon was not measured accurately, but ranged from 3 to 6 c. c. It was mainly from such cultures as these that the material used in the microscopical studies was obtained.

It is not necessary to tabulate the results obtained from this lot of cultures, as such. The more interesting results were obtained from those experiments in which the quantity of blood was measured and from those carried out with reference to the season of the year. The former are necessarily an entirely different set from those in which the blood was not measured, and the latter set was made up of both measured and unmeasured cultures. Tables for both of these sets of tubes are given later.

It is evident that in order to determine the number of days required for the trypanosomes to appear in a tube, the tube must be examined daily from the time of making until the trypanosomes appear. This was done for 53 tubes, taken throughout the year, and it was found that the average time required was $3\frac{1}{2}$ days. It is understood that this means the time required for the presence of trypanosomes on the top of the column of red cells to be determined or at least

suspected, merely by the use of a hand lens, although in almost all cases this diagnosis was confirmed by the use of the microscope. This average of 3½ days is based on cultures made the day the blood was drawn and which were kept at room temperature.

In all 30 animals were used, and 64 separate tests were made of their blood. Of these animals 27 were yearlings or adults and 3 were young calves. The calves, one of which was but 1 day old, were all negative. The mother of the 1-day calf, tested at the same time, was positive, an indication that trypanosomes can not pass the placenta.

Of the 27 adult animals, 7 gave negative results, hence 74 per cent were infected. The actual figure, however, is probably higher than this, since a single test may be negative merely by accident, and of these 7 cattle, 5 were tested but once. Moreover, as will be shown, the time of year must be taken into account.

The following were six of the cattle which proved negative, with the month when the test was made; the seventh, No. 685, is dealt with below: Nos. 666 and 668, tested in March; No. 667, tested in April; No. 739, tested in May and also in October; No. 536, tested in July; and No. 738, tested in October.

By October the trypanosomes have become much less abundant in the blood, and an animal negative in that month might readily have been positive in July or August. Nevertheless, certain cattle do not harbor the trypanosomes, as the following history of cow No. 685 shows:

Date.	Number of cultures made.	Result.
1909.		
July 13	5	All negative.
July 20	4	Do.
Aug. 18	3	Do.
Aug. 26	Received in the jugular vein 20 c. c. of blood from cow No. 689, known to carry trypanosomes.	
Aug. 30	3	All negative.
Sept. 2	3	Do.
Sept. 8	3	Do.
Nov. 17	6	Do.

This is apparently a case of natural immunity. The inoculation might have failed, but the constant negative findings for five months, including the greater part of the hot season, show that the animal was able to resist natural infection. There was nothing in the history of this animal to differentiate it from any other, and it was in precisely the same environment as all of the other cattle at the Experiment Station. In fact, both it and No. 689, from which it was inoculated, were kept for a part of the summer in the same pen.

SEASONAL VARIATION IN NUMBER OF TRYPANOSOMES IN BLOOD.

The seasonal variation in the abundance of trypanosomes is very well shown by the history of cow No. 218, as follows:

Appearance of trypanosomes in cultures from cow No. 218.

No. of culture.	Date.	Quantity of blood.	Time required for trypanosomes to appear.	Remarks.
244	Aug. 18	Not measured.[1]	4 days	
245	do	do	5 days	
294	Aug. 31	do	6 days	Blood kept on ice 1 day.
295	do	do	4 days	Do.
299	Sept. 1	do	5 days	Blood kept on ice 2 days.
303	Sept. 2	do	do	Blood kept on ice 3 days.
309	Sept. 3	do	4 days	Blood kept on ice 4 days.
330	Sept. 8	do	3 days	
331	do	do	2 days	
334	do	do	do	
420	Nov. 17	1 c. c.	Negative	
421	do	2 c. c.	do	
422	do	3 c. c.	do	
423	do	4 c. c.	do	Contaminated.
424	do	5 c. c.	do	
425	do	6 c. c.	6 days	
426	do	7 c. c.	Negative	
427	do	8 c. c.	6 days	
428	do	9 c. c.	do	
429	do	10 c. c.	do	
436	do	1 c. c.	Negative	
438	do	3 c. c.	do	
440	do	5 c. c.	6 days	
441	do	6 c. c.	Negative	
442	do	7 c. c.	10 days	
443	do	8 c. c.	Negative	
445	do	10 c. c.	5 days	

[1] From 1 to 4 c. c., probably averaging 3 c. c.

From this table we see that in August and September the cultures were all positive, and some showed trypanosomes as early as the second day. On the other hand, of the 17 cultures made in November, only 7 were positive, with 5 days as the minimum time. Moreover, while the average quantity of blood used in August and September was probably about 3 c. c., and in no case exceeded 4. c. c., the least quantity to develop trypanosomes in November was 5 c. c., and one each of the cultures containing 7 and 8 c. c., respectively, were negative.

In the case of cow No. 697, from which many cultures were made, it was found that a larger proportion of tubes were positive, and trypanosomes appeared more quickly in spring and summer than they did in the autumn.

The table following shows all the cases where the time of appearance of the trypanosomes was determined. It gives the number of cultures so determined, the maximum and minimum time required for the trypanosomes to appear, in days, and the average of the whole number. There is also given the mean temperature of each month, as obtained at the Washington, D. C., station of the United States Weather Bureau, and the mean temperature of the zoological laboratory.

Time of appearance of trypanosomes in laboratory cultures, by months.

Month.	Number of tubes.	Time of appearance.			Temperature.	
		Maximum.	Minimum.	Mean.	Washington.	Laboratory.
1909.		*Days.*	*Days.*	*Days.*	°F. °C.	°F. °C.
April	15	4	3	3.3	54.2=12.3	72.5=22.5
May	12	3⅔	1⅔	3.0	64.4=18.0	74.3=23.5
June	1	3	3	3.0	73.4=23.0	78.7=25.9
July	11	4	2	3.3	74.7=23.7	81.5=27.5
August	13	6	3	4.4	73.0=22.8	79.1=26.2
September	6	6	2	3.3	66.4=19.1	75.2=24.0
October	12	8	7	7.2	53.2=11.8	70.4=21.4
November	15	9	3	6.2	50.8=10.4	71.7=22.1
1910.						
March	8	6	2	4.1	51.2=10.7	74.4=23.6
April	15	4	3	3.3	57.9=14.4	74.1=23.4

The reason why the laboratory temperature is always higher than that of the open air is doubtless obvious enough, although attention may be called to the fact that in hot weather the closing of the laboratory windows at 4.30 p. m. prevents any such nightly fall of temperature as is usual in the open. The temperature as recorded by the Weather Bureau, although taken in the city itself, may be assumed to correspond to that of the Experiment Station of the Bureau of Animal Industry, the home of the cattle. Accordingly, the one column shows the fluctuations of temperature to which the animals harboring the trypanosomes were subjected, the second that in which the tubes were kept. The former can hardly be supposed to have any influence, since a mammal maintains its own temperature without reference to that of the surrounding air. Therefore in making comparisons between the rates of growth of the trypanosomes at different times of the year, it is the temperature of the laboratory which should be taken into account and not that of the open country.

In the following, taken from the table given above, the months are in the first two cases grouped with reference to uniformity of temperature and in the last with reference to the time required for the cultures to develop.

1. *Variation in time of appearance of trypanosomes although monthly temperatures are closely uniform.*

Month.	Laboratory temperature.	Mean time of appearance.
	°F.	*Days.*
April, 1909	72.5	3.3
November, 1909	71.7	6.2
October, 1909	70.4	7.2

2. *Uniformity in time of appearance of trypanosomes in spring months.*

Month.	Laboratory temperature.	Mean time of appearance.
	°F.	Days.
March, 1910	74.1	4.1
April, 1910	74.4	3.3
May, 1909	74.3	3.0

3. *Uniformity in time of appearance of trypanosomes although variation in monthly temperatures is considerable.*

Month.	Laboratory temperature.	Mean time of appearance.
	°F.	Days.
April, 1909	72.5	3.3
May, 1909	74.3	3.0
July, 1909	81.5	3.3
September, 1909	75.2	3.3
April, 1910	74.1	3.3

In the first group it is seen that although the temperatures are very close, the time required in one case is more than double that of another. In the second group the maximum difference in time is only 1.1 days, but here the comparison is between the three spring months. In the third, although the times are so near alike that the difference is negligible, there is a maximum difference of 9° F. in the temperature.

In the statement below the entire time during which the study was being carried on is divided according to the three seasons—spring, summer, and autumn. The average time required for the cultures to develop during each season is computed, due weight being given to the number of cultures used, and the mean temperature, both of the open country and the laboratory, are given.

Time of appearance of trypanosomes according to seasons.

Season.	Time of appearance.	Mean temperature.	
		Outside.	In laboratory.
	Days.	°F.	°F.
Spring	3.39	56.9	73.8
Summer	3.80	73.7	79.8
Autumn	6.04	56.8	72.4

There is seen here a considerable seasonal difference in the time required for the trypanosomes to appear in the tubes. This is taken to be an index to their abundance. The mode of examination of the cultures was throughout much the same, namely, a preliminary observation of the top of the column of blood cells with a hand lens, followed, when necessary, by the examination of a drop of the culture

under the microscope. The latter was undertaken only when the former left the question as to the presence or absence of trypanosomes in doubt.

As we shall see later, multiplication of the trypanosomes begins as soon as the blood containing them is removed from the cow. It therefore seems reasonable to regard their discovery in the culture tubes by means of the procedure indicated above as evidence of greater abundance rather than a mere rapid multiplicative rate. Hence it is believed that these figures furnish satisfactory evidence to show that whereas the trypanosomes are less abundant in autumn than in spring or summer, this difference is independent of temperature. The figure for spring is also lower than that for summer, but this difference is not large enough to warrant any conclusions.

EFFECT ON THE TRYPANOSOMES OF KEEPING BLOOD BEFORE CULTURES WERE MADE.

The following table gives the results obtained when the blood was kept one or more days before the cultures were made. The several columns show, in order, the number of the cow; date culture was made; date blood was drawn; interval in days; number of tubes used; number of tubes giving positive results; and place where blood was kept.

Result of examination of laboratory cultures for trypanosomes.

No. of cow.	Culture made.	Blood drawn.	Interval (days).	Number of tubes.	Number of positive cases.	Place blood was kept.
	1909.	1909.				
688	Apr. 2	Mar. 31	2	3	0	On ice.
696	May 7	May 2	5	5	0	Do.
688	Aug. 19	Aug. 17	2	4	0	In laboratory.
689	Aug. 27	Aug. 26	1	2	0	Do.
218	Aug. 27	Aug. 26	1	2	0	Do.
689	Aug. 28	Aug. 26	2	2	0	Do.
218	Aug. 28	Aug. 26	2	2	0	Do.
689	Aug. 29	Aug. 26	3	2	0	Do.
218	Aug. 29	Aug. 26	3	2	0	Do.
689	Aug. 30	Aug. 26	4	2	0	Do.
218	Aug. 30	Aug. 26	4	2	0	Do.
689	Aug. 31	Aug. 26	5	2	0	Do.
218	Aug. 31	Aug. 30	1	2	0	In incubator.
218	Aug. 31	Aug. 30	1	2	2	On ice.
218	Sept. 1	Aug. 30	2	2	0	In incubator.
218	Sept. 1	Aug. 30	2	2	1	On ice.
218	Sept. 2	Aug. 30	3	2	0	In incubator.
218	Sept. 2	Aug. 30	3	2	1	On ice.
218	Sept. 3	Aug. 30	4	2	0	In incubator.
218	Sept. 3	Aug. 30	4	2	1	On ice.
696	Sept. 4	Sept. 3	1	2	0	In laboratory.
697	Sept. 4	Sept. 3	1	2	2	Do.
696	Sept. 5	Sept. 3	2	2	0	Do.
697	Sept. 5	Sept. 3	2	2	0	Do.
696	Sept. 6	Sept. 3	2	2	0	Do.
697	Sept. 6	Sept. 3	3	2	1	Do.
218	Sept. 14	Sept. 13	1	2	0	On ice.
218	Sept. 15	Sept. 13	2	2	0	Do.
218	Sept. 16	Sept. 13	2	2	0	Do.
688	Nov. 4	Nov. 3	1	4	1	In incubator.
696	Nov. 4	Nov. 3	1	4	0	Do.
697	Nov. 4	Nov. 3	1	4	0	Do.
	1910.	1910.				
730	Mar. 11	Nov. 9	2	2	2	On ice.
730	Mar. 29	Nov. 16	13	4	0	Do.

The following data, taken from above, give the place where the blood was kept, the length of time kept, the total number of cultures made, and the number which developed trypanosomes:

Blood kept on ice.

Time kept.	Culture tubes.	Positive cases.
1 day...	4	2
2 days...	9	3
3 days...	4	1
4 days...	2	1
5 days...	5	0
13 days...	4	0

Blood kept in laboratory.

Time kept.	Culture tubes.	Positive cases.
1 day.....	8	2
2 days....	12	0
3 days....	8	1
4 days....	8	0
5 days....	2	0

Blood kept in incubator.

Time kept.	Culture tubes.	Positive cases.
1 day.....	14	1
2 days....	2	0
3 days....	2	0
4 days....	4	0

Thus, eleven cultures developed trypanosomes, and the following shows the time required in each case for the organisms to appear in the tubes:

Blood on ice.

Time kept.	Time of appearance.	Remarks.
1 day.... a	6 days.....	Good growth.
1 day.... b	3 days.....	Do.
2 days... a	5 days.....	Do.
2 days... b	4 days.....	
2 days... c	6–4 days...	
3 days.....	5 days.....	Poor growth.
4 days.....	4 days.....	Moderate growth.
Mean.....	4½ days....	

Blood in laboratory.

Time kept.	Time of appearance.	Remarks.
1 day....{a.....	3 days.....	Very poor growth.
1 day....{b.....	4–3 days...	Very poor growth; moderate growth.
3 days.....	.....	Not examined until 15th day, when it was positive.

Blood in incubator.

Time kept.	Time of appearance.	Remarks.
1 day.....	12 days....	Very poor growth.

When the blood was kept in the ice chest at a temperature of about 16° C. for 1 to 5 days, only 7 out of 24 cultures developed, or 29 per cent. For blood kept in the laboratory the figure is 3 out of 38, or 8 per cent. With blood kept in the incubator, but one culture out of 22 produced trypanosomes. This shows that keeping the blood before the cultures are made has a destructive influence on the trypanosomes.

As to the time required for the trypanosomes to develop, the average is about 4½ days, yet tubes made from blood kept 4 days developed in 4 days. Furthermore, whereas when the blood was kept in the laboratory only 2 out of 8 tubes developed, the trypanosomes appeared on the third and fourth days. The implication here is that the greater part of the trypanosomes in the blood are either killed or so enfeebled that they are unable to develop, while the growth which takes place is due to the hardier survivors. This is supported by the character of the growth which was eventually brought about in the tubes. In no case was this luxuriant, and it was good in only three of the tubes, pointing to the conclusion that the number of trypanosomes present in the tubes was very much below the average.

In all of the cases considered above, control cultures made promptly after the blood was drawn were positive.

RESULTS OBTAINED WITH MEASURED QUANTITIES OF BLOOD.

The following table gives the results obtained with measured quantities of blood, the medium used being the laboratory make of beef bouillon, and the cultures being made the same day the blood was drawn. Unless otherwise stated, the culture tubes were kept in the room. In all cases given in the table where the quantity of blood was small, and the results negative, controls showed the blood used to contain trypanosomes.

Results obtained with measured quantities of blood.

Culture.	Date.	Cow.	Quantity of blood.	Result (+, positive; −, negative).	Time.
	1909		c. c.		Days.
4	Mar. 29	688	3.0	+	[1] 6
5	...do....	688	1.5	−	(1)
31	Apr. 5	688	2.0	+	3
33	...do....	688	1.5	+	4
38	...do....	688	2.0	−	(1)
39	Apr. 7	688	2.5	+	3
41	...do....	688	2.0	+	3
42	...do....	688	1.5	−	(1)
43	...do....	688	3.0	+	4
53	Apr. 12	688	5.0	+	3
54	...do....	688	4.0	+	4
55	...do....	688	4.0	+	3
56	...do....	688	4.0	+	3
57	...do....	688	4.0	+	4
58	...do....	688	3.5	+	3
59	...do....	688	3.0	+	3
64	Apr. 24	695	2.0	+	4
65	...do....	695	1.5	+	.....
66	...do....	695	.4	+	.....
67	...do....	695	1.5	+	.....
68	...do....	695	3.5	+	.....
70	...do....	695	2.5	+	.....
72	...do....	696	3.0	+	.....
73	...do....	696	5.0	+	4
74	...do....	696	3.5	−	.....
75	...do....	696	5.0	+	.....
76	...do....	696	4.0	−	.....
77	...do....	696	5.0	+	.....
78	...do....	696	3.0	+	.....
79	...do....	696	4.0	+	.....
81	Apr. 26	697	2.5	+	4
82	...do....	697	2.0	+	.....
83	...do....	697	2.0	+	.....
85	...do....	697	1.0	+	.....
86	...do....	697	2.5	+	.....
87	...do....	697	2.5	+	.....
88	...do....	697	1.0	+	.....
89	...do....	697	.5	+	.....
133	May 13	688	2.5	+	3
134	...do....	688	3.0	+	3
135	...do....	688	2.0	+	3
136	...do....	688	2.0	+	.....
137	...do....	688	2.5	+	.....
138	...do....	688	2.0	+	3
139	...do....	688	3.0	+	2
140	...do....	688	2.0	+	3
141	...do....	688	1.5	+	.....
142	...do....	688	1.5	+	.....
144	...do....	688	2.0	+	3
145	...do....	688	2.5	+	3
146	...do....	688	3.0	+	3
147	...do....	688	3.0	+	3
420	Nov. 17	218	1.0	−	.....
421	...do....	218	2.0	−	.....
422	...do....	218	3.0	−	.....
423	...do....	218	4.0	−	.....
424	...do....	218	5.0	−	.....
425	...do....	218	6.0	+	6
426	...do....	218	7.0	−	.....
427	...do....	218	8.0	+	6
428	...do....	218	9.0	+	6
429	...do....	218	10.0	+	6
436	...do....	218	1.0	−	.....
438	...do....	218	3.0	−	.....
440	...do....	218	5.0	+	.....
441	...do....	218	6.0	−	.....
442	...do....	218	7.0	+	.....
443	...do....	218	8.0	−	.....
444	...do....	218	9.0	−	.....
445	...do....	218	10.0	+	5
	1910		Drops.[2]		
532	Apr. 7	697	1	−	.....
533	...do....	697	2	−	.....
550	Apr. 13	697	1	−	.....
551	...do....	697	2	−	.....
552	...do....	697	3	−	.....
553	...do....	697	4	−	.....

[1] Culture in incubator.

[2] 1 drop=0.0675 c. c.

Results obtained with measured quantities of blood—Continued.

Culture.	Date.	Cow.	Quantity of blood.	Result (+, positive; −, negative).	Time.
	1910		*Drops.*		*Days.*
574	Apr. 21	697	5	−	.....
575	...do.....	697	5	−	.....
576	...do.....	697	10	−	.....
577	...do.....	697	10	−	.....
618	July 14	697	5	+	6
			c. c.		
622	...do.....	697	1.0	+	.....
623	...do.....	697	1.0	+	.....
624	...do.....	697	1.0	+	.....
625	...do.....	697	1.0	+	.....

Here it is seen that the smallest quantity of blood to give a positive result was 5 drops, or 0.3375 c. c., and that this result was obtained only once out of three trials. Assuming 6,000,000 red cells and 10,000 leucocytes per cubic millimeter, we find as a possible proportion one trypanosome for 2,022,000,000 red cells and 3,370,000 whites. Hence to find the trypanosome in the circulating blood would be merely a piece of good fortune. Further, culture 444, containing 9 c. c. of blood, was negative, yet this amount of blood contains 90,000,000 leucocytes.

For the most part the bouillon used for the cultures was that made in the laboratory from beef, for ordinary bacteriological work. Some 16 tubes were used in which the medium was made from commercial meat extract. It was not treated with an alkali, and hence was acid in reaction. The growth in the latter medium was in general not so luxuriant as that in the laboratory make of bouillon, and in two cases the trypanosomes failed to appear at all, although controls (in laboratory bouillon) were positive.

Fourteen cultures were made in physiological salt solution, 6 parts per thousand. In one set of experiments with this medium 5 c. c. of salt solution was used in each tube, to which were added 2, 3, 4, and 5 c. c. of blood, respectively. Trypanosomes never developed, although in all cases the controls were positive. These results were at odds with those obtained by the Dudukalovs (see page 9).

In one set of tubes cow's milk was used, the results being negative.

GENERAL RESULTS OF CULTURAL WORK.

The results may be summarized as follows:

(1) *Trypanosoma americanum* grows readily in ordinary bouillon, made from either beef or mutton.

(2) The average time required for the trypanosomes to be readily detected in the tubes is 3½ days.

(3) The smallest quantity which gave positive results was 5 drops, or 0.3375 c. c. The largest measured quantity to give negative results was 9 c. c.

(4) There is a seasonal fluctuation, the trypanosomes being more abundant in the spring and summer than they are in the autumn.

OBSERVATIONS ON FRESH MATERIAL.

The successful cultures were those made by adding the blood of the cow to bouillon of several different kinds. The freshly made culture necessarily consisted of a red column, composed of an admixture of blood and the medium. Upon standing for about 24 hours the blood cells settled to the bottom of the tube, leaving above them a clear fluid composed partly of blood serum and partly of bouillon itself. At first the surface of this column was smooth and of a uniform red, but by the end of the second day it usually began to present appearances which were presumably caused by the rising to the surface of the leucocytes. These appearances, however, were very varied. In some cases the surface remained perfectly flat but showed itself studded with minute whitish specks and motes which, when large enough for their form to be determined, were of irregular contours and closely resembled particles of dust. Frequently, however, a fuzzy deposit appeared upon the surface, consisting of a reddish-white stringy mass, which might be elevated as much as 1 or 2 mm. These fuzzy masses never occupied more than a portion of the surface of the column of red cells. At other times this surface was differentiated into a series of ripples, representing in miniature the surface of wind-blown sand.

But in spite of these various aspects, there was never any difficulty in determining macroscopically (or preferably with the aid of a hand lens) whether the tubes did or did not contain trypanosomes, at least after they had had time, if present, to establish themselves. Frequently by the third day there could be distinguished on the surface of the red cells minute dots, differing from the motes above mentioned in that they were white and circular. When these had reached a certain size it could generally be seen that their top surfaces were not flat but convex, and when as sometimes happened they were supported by the masses of fuzz and thus carried above the surface they showed as more or less spherical bodies. At first these trypanosome colonies were very minute and frequently scattered over the surface in a more or less uniform manner, but as the culture got older they always became larger and often fewer in number. Increase in size is of course a matter of growth, and the diminution in numbers was doubtless a case of fusion of closely lying colonies. In mature cultures there were at times but two or three colonies—enormous white masses 2 or 3 mm. across.

The small colonies were always circular, the larger circular, oval, or irregular. A curved outline, however, was always maintained. The form of the colonies, however, is doubtless merely a matter of surface tension. Fusion of two very small circular colonies results in the production of a large circular one, but when a certain size is reached the surface tension is able only to impart to the compound colony a curved outline. It was at times to be noted that the separate units composing the large compound colonies were very incompletely fused.

Clearly, each separate colony which arose on the surface of the red cells in a tube represented a focus of infection, the later increase in bulk being merely due to the multiplicative activity of the trypanosomes. No accurate counts were made, but such foci probably ranged in numbers, in the various tubes, from half a dozen or less to perhaps 100. These foci of infection must in each case be either a single trypanosome, or some element not a trypanosome but which evolves into one, or two or more trypanosomes or such elements.

In the preliminary notice (Bulletin 119) it was stated that it was not possible to say what this was, that is, whether the element in the circulating blood of the cow was an actual trypanosome or something of an entirely different facies, and unfortunately such observations as have been made since do not settle this point absolutely, yet they leave little doubt resting upon it. Trypanosomes were never seen in fresh blood from the cow, but they finally were found in stained smears of centrifuged blood, the smears having been made 3 or 4 hours after the blood was drawn. It may be stated here that these organisms did not occur singly, but in clusters, and were evidently in a process of rapid multiplication. The probability that they arose from the division of a trypanosome, and not from something else, is so great as to be a practical certainty. The work of Wrublewski and Behn, previously quoted, supports this hypothesis. In that of the former, the trypanosomes described as parasites of the aurochs are so like *T. americanum* that the latter might be regarded as little more than a variety of the former. The failure to find the trypanosome in the perfectly fresh blood of American cattle is of little moment, since if the organism is only a variety of *T. wrublewski* it is in a strange host, and hence might be able to maintain itself in only very limited numbers. Moreover, it is a matter of common knowledge that trypanosomes may exist in the blood of animals in such small numbers as to evade observation with the microscope. A great many such cases have been brought out in the experimental work on the pathogenic trypanosomes of mammals, and it seems to be almost the rule for those of birds. Evidently, when a trypanosome can not be found in the blood with the microscope, it is more or less of a venture to claim that it exists there as

a trypanosome and not as something else. Yet in the case of birds it is known that after failure to find them in the circulating blood they have been picked up in extremely small numbers in the bone marrow. Besides, in spite of the enormous amount of work which has been done on these flagellates, nothing has ever been discovered in the blood but an element instantly recognizable as a trypanosome.

Finally, there is Behn's discovery. But this trypanosome is very different from either *T. americanum* or *T. wrublewski*. It is not only a very much broader animal, but differs in that the nuclei are farther back in the body. It is quite possible, however, that this trypanosome, although the forerunner of the organisms found in the culture tubes by the German investigators, is a wholly different species from *T. americanum*. So far the cultural trypanosome of German cattle has neither been described nor figured, and it is hence impossible to compare it with that from American cattle.

ATTEMPTS TO DISCOVER THE TRYPANOSOME IN FRESHLY DRAWN BLOOD.

The experiments made at Washington to determine this point may now be given.

Freshly drawn blood from cattle was centrifuged, and preparations were thereby obtained in which the leucocytes were as abundant as the red cells. This work was done in June, July, and August, 1910, when the cultural method showed the trypanosomes to be abundant. Six different animals were used, of which four were healthy, one was in an advanced stage of tuberculosis, and one was suffering from a mild case of Texas fever.[1]

A large number of fresh preparations were examined, but nothing in the way of a trypanosome was ever found. In a search of this sort the examination of fresh material was then and still is believed to be the most efficient method, since the flagellate readily betrays its presence by its motility. The work was done with a 16 mm. objective and a No. 12 eyepiece, and a preparation containing as much blood as a large smear could be searched in a few minutes. A thorough test of this procedure led to the view that the trypanosomes could not be found in recently drawn blood, and the examination of stained smears was undertaken rather with the idea of looking for hypothetical "preflagellate" stages than for actual trypanosomes. Yet it was in these that the discovery was made. The trypanosomes were found on two slides, a small group in each case, out of some 25 examined.

Cultures were also centrifuged. The first experiment, made July 15, 1910, with a culture 23 to 24 hours old, revealed trypanosomes,

[1] This cow was tested in October.

singly or in clusters and either associated or not with white cells. Later experiments, made in August, reduced the time to 15 hours. Younger cultures were not tried.

The findings, however, were of considerable interest. In the 15-hour culture only a single trypanosome was found, but this was an organism having a body quite 20 microns in length, and a flagellum as long as the body, making it all of 40 microns long. The undulating membrane was short, and the appearance was typical for *Typanosoma americanum*.

In a culture 18½ hours old made from blood 5½ hours after it was drawn there were found several clusters of trypanosomes, one of which must have been composed of at least 75 individuals. Since in the culture tubes division of the flagellates is by no means a rapid process, requiring from 1 to 2 hours, multiplicative activity must have been going on for some time. At the slower rate, it would take 14 hours for one trypanosome to produce 64. The organisms found on the first day in centrifuged cultures differed in no respect, so far as could be determined, from those found in two and three day cultures, studied without centrifuging.

EVOLUTION OF THE TRYPANOSOMES IN CULTURE.

The history of the evolution of the trypanosomes, as it takes place in a successful culture, may now be traced.

As already stated, there is a certain variability in the rate of evolution, but it may conveniently be considered under three headings—the period of growth, of culmination, and of decline—although the three processes to a certain extent overlap.

In cultures 2 to 3 days old there are present a small number of organisms, say from 1 to 6 to each preparation, a preparation probably containing 1 cubic millimeter of the culture. The animals are ordinarily short and relatively broad, with the anterior half broader than the posterior. At times, however, they are quite slender. A flagellum is always present and in most cases a short undulating membrane can easily be seen. The cytoplasm is hyaline. With ordinary light, granules can not as a rule be seen, but dark-field illumination shows them to be present. It may also here be noted that this method revealed the fact that in the adult trypanosomes the flagella often show a double contour, even to the extreme tip. The organisms are either solitary, in pairs, or in clusters. The clusters are nearly always of few individuals, but this of course depends upon the rate with which multiplicative activity has been inaugurated. The flagellum and undulating membrane are in constant action, and progression in a slow, unsteady manner, with numerous changes of direction, is frequent.

Cultures 3 to 4 days old show all of the phenomena presented by the earlier cultures. There are very many more trypanosomes present, however, and the clusters average much larger. Solitary forms are relatively much rarer, almost all of the animals being in the clusters. The trypanosomes themselves have become longer. The flagella and undulating membranes are in constant motion, and it may here be stated that motility does not seem to fluctuate with age, senile and youthful forms showing it in approximately the same degree.

Evolution now follows the lines already indicated. In the 4 and 5 day cultures solitary forms occur, but are scarce. Nearly all the trypanosomes present are in clusters, which may consist of 3 to 4 individuals, or be of enormous size. One, from a 4-day tube, measured 156 by 190 microns, and must have been composed of thousands of trypanosomes. The individual animals are larger; some long and slender, or crithidia-like; others show a typical trypanosome contour, while still others have become club-shaped. Large, stout divisional forms are frequent. In many the cytoplasm has become very granular.

From the fifth and sixth days onward the cultures begin to present a far more diversified appearance. There are present large and small clusters in which the trypanosomes may be band-shaped or club-shaped. There are many more free individuals, doubtless those which have become separated from the clusters. The free forms are band-shaped, club-shaped, or short oval bodies. Any and all of these may be in division. There also appear in the older cultures very large bodies with two or more flagella, no doubt resulting from a more or less abnormal division process. The club-shaped forms and the giant trypanosomes are marks of degeneracy, and as the culture ages more and more of the trypanosomes begin to undergo senile decay, and the culture eventually dies out.

No accurate data were kept as to how long the cultures generally lasted, but by the end of a month they are very evidently degenerate. In one case a tube charged 63 days previously was observed to show, microscopically, a large number of apparent colonies. A study of these colonies under the microscope showed, however, that they consisted merely of great masses of granular balls, with here and there a feebly motile, misshapen, and intensely granular trypanosome.

The process of evolution of the individual, or of evolution combined with so-called involution, is not, except at the outset, difficult to follow. As given above, a solitary trypanosome having a total length of 40 microns was found in a centrifuged culture 15 hours old. (In the 2 and 3 day cultures the trypanosomes are not so large as this.) It will probably not be far from the mark to look upon a trypanosome of this size as the originator of the clusters which appear

in the tubes. Division is always by longitudinal splitting, but may be equal or unequal. The observations rather favor the view that it is at first unequal, the original mother cell giving off a short, relatively broad bud. Such unequal divisions were seen in the ordinary (not centrifuged) cultures, and were not uncommon in one of the centrifuged cultures of the first day. At all events, beginning the series with a short, broad, flagellated element, the history is as follows: This element divides, and the two daughter cells are also at first short, broad organisms provided with flagella and undulating membranes. The cytoplasm is hyaline, and fine granules are probably always present. An increase in length produces a relatively more slender form, which, in its turn, becomes a band-shaped typical trypanosome. In time, the anterior half becomes broader and rounded, like the head of a club, the posterior half remaining narrow. The flagellum extends forward from the anterior tip of the broad anterior half and carries with it a narrow band of cytoplasm, which is probably composed partly of endosarc, since it is frequently granular. The narrow posterior end becomes narrower; it is reduced to a mere spike, and finally disappears, leaving the monadine forms which show a flagellum either extending free or accompanied for a part of its length by a band of cytoplasm. In certainly a great many cases, however, and perhaps all, before the complete atrophy of the posterior end, the head of the club had undergone a distortion which carries the point of origin of the flagellum around to one side of the body, so that forms are obtained wherein the flagellum extends out at right angles to the longitudinal axis of the body.

Along with the morphological changes there is a conspicuous change in the granularity of the cytoplasm. In the young trypanosomes the granules are small, but there is a gradual increase in size and numbers, and the senile forms are closely packed with coarse granules. The monadine form degenerates into a coarsely granular ball.

The genesis of the colonies takes place as follows: A cluster is started, probably by a single trypanosome. This, by division, produces a pair, joined by their posterior ends. A second division produces a group of four, but the arrangement is not radial, but linear. The four are united, in common, by their posterior ends, but one of the pairs is slightly in advance of the other. In further divisions this linear arrangement is maintained, so that the clusters do not consist of rosettes, but of strings. Since, however, any given pair of trypanosomes is very nearly at the same level as the pair next to it, the linear disposition is more or less completely masked, and rounded clusters are produced. The actual arrangement may, however, be nicely demonstrated by observing a rounded cluster

by dark-field illumination. As the cluster becomes heated by the rays it spreads out, becoming larger and looser, and then it can be easily seen that such a cluster consists of a number of closely related strings of trypanosomes. It may also be seen when trypanosomes have multiplied in a sealed preparation on a slide. Here, if the blood film be sufficiently thin, the groups are constrained to develop within one plane, and the linear arrangement is often very plain. Accordingly, the rounded cluster is a group of the second order, made up by the strings, which are in their turn made up of individual trypanosomes. Occasionally such strings (or perhaps a better term is bouquets) of trypanosomes were seen which were bipolar; that is, there were two strings extending from a central point in opposite directions. The strings may possibly also give off lateral branches.

In view of the organization of the clusters, as just indicated, there is no objection to the view that each has arisen from a single trypanosome. If this be so, we should get, as a very rough average, say 25 to 50 trypanosomes per culture tube, or perhaps 10 per cubic centimeter of blood. This gives one trypanosome to 1,000,000 white cells. The data given on page 20 show, however, that the trypanosomes are not so abundant as this. Such figures are little more than guesses, however, although they show that the trypanosomes are very rare in the circulating blood of the cow.

It may be of interest to give certain of the observations in detail: Fresh preparations from culture tubes 2 to 3 days old, examined under the microscope, show in abundance clumps composed partly of red cells and partly of leucocytes. These clumps stand out quite sharply from the film of red cells and evidently possess a considerable degree of consistency. Their production is probably in part due to the fact that as the leucocytes rise to the surface they tend to entangle a certain number of red cells; but there seems to be some other influence at work, because at times these clumps consisted entirely of red cells, and the red cells were always in the majority. In all cases the red cells, as a result of mutual pressure, were more or less misshapen.

It was very quickly learned that the place to look for the first trypanosomes was in these clumps. In the early cultures one or two trypanosomes could usually be found in association with one of them, either in the midst of the blood cells or lying at the periphery, in the narrow clear space which usually separated a clump from the surrounding film of erythrocytes. A day later there might be a considerable number of trypanosomes in such a situation, mostly in little clusters around the edges of the clump. In a number of cases, where preparations were obtained showing trypanosomes in association with the clumps, the position of these was determined by means

of the mechanical stage, and the preparation was set aside. Examinations on the following day always showed a conspicuous increase in the number of trypanosomes, and it was in such cases as these that the growth of the trypanosomes in strings or bouquets was at times demonstrated.

The appearance of trypanosomes in association with these clumps of blood cells suggested at once that it was here that the origin of the foci of infection was to be found, and, since the clumps were small, that the problem of whether it was an actual trypanosome or some resting stage which gave birth to the trypanosomes in the cultures could easily be solved. In the endeavor to elucidate this point the dark-field illumination was at first used and some very deceptive appearances were noted.

As stated above, these clumps are composed largely or wholly of red cells, the peripheries of which, with dark-field illumination, show as bright bands of a quite appreciable width, inclosing a space which does not reflect the light and is accordingly clear. Since the cells are closely packed together, the appearance is that of a region marked out with very irregular polygons. When the preparations studied were from cultures in an appropriate stage of development, that is, when the trypanosomes were still scarce, observation would at times appear to show the edges of one of these red cells to be in motion. This motion at once recalled that of the flagellum of a trypanosome, as it indeed proved to be, for presently it could be seen that it was not the edge of the red cell that was in motion, but a thread, a flagellum, and by careful observation the flagellum could be traced to a faintly appearing trypanosome. The curious point about this phenomenon was, however, that although a point might be selected where the most careful scrutiny failed to reveal the presence of a trypanosome, the serpentine movement would presently manifest itself, and then the trypanosome itself could be discerned. It was as if the animals sprang into being full fledged.

The mystery was solved by a study of the clumps by transmitted light. It is probably a matter of common experience that fresh preparations are not very satisfactorily observed with an oil-immersion lens with daylight. The usual method was then somewhat modified. The small arc lamp belonging to the dark-field apparatus was used, its light being first passed through a piece of "euphos" glass, and then through a vessel containing a solution of methylene blue. Euphos glass cuts out the destructive ultra-violet rays; the methylene-blue solution is for the benefit of the observer's eyes, and can be made of whatever density desired. Finally—and this is of importance—immersion oil must be placed between the top of the condenser and the slide. This procedure permits the use of the highest-power eyepieces with a 2 mm. apochromatic lens, and the

trypanosomes may be studied under powers of 1,500 to 2,250 diameters.

It was by this means learned that the trypanosomes which appeared so mysteriously were already present in the mass of red-blood cells, and had merely shifted their position. But, unfortunately, no light was shed on their origin. In a great many cases round cells, the appearance of which was not wholly that of normal leucocytes, were individually kept under observation for several hours, but nothing was ever seen to take place. The first trypanosomes ever seen in preparations from any culture were elongated, flagellated organisms.

In the early cultures the trypanosomes occurred either singly or in groups of two or three. As has already been pointed out, the pairs arise by division, but there is another way whereby paired animals and clusters of very few might take origin. This would be by agglutination. Besides being associated with the clump of red and white cells, the trypanosomes are found wandering through the preparation. They create little or no disturbance amongst the red cells, since they travel at a slightly higher level and may indeed be creeping on the under surface of the cover glass. Their movements are slow and unsteady and changes of direction are frequent. The following detailed observations are typical of what may be seen:

MOVEMENTS OF TRYPANOSOMES IN CULTURES.

In a culture two days old, studied March 5, 1910, two young trypanosomes came together at 4 p. m., and for 55 minutes what was seen can best be designated as a wrestling match between the two. Movements of the flagella and undulating membranes were practically continuous, and the two kept their bodies constantly curved, each looped around the other. At 4.55 they both straightened out and lay side by side, bodies in contact, the flagella pointing in the same direction. This position was, however, maintained but for a minute or two, after which they again bent their bodies, looped themselves together, and resumed the apparent struggle. At 5.30 the observation was interrupted, to be resumed at 7.15 p. m., when the same condition of affairs was found.

In another case, in a 3-day culture, two slightly more mature flagellates were seen to come into contact and to remain so for several hours. Here, as in the case described above, the flagella and undulating membranes were in constant movement, and the animals also spent a part of the time each looped around the other. But, in addition, they would frequently partly separate and then each, with straight longitudinal axis, appose its body to that of the other. When so disposed, they were oriented indifferently in the same or

opposite directions, and sometimes the end of one was opposite the middle of the body of the other. Under such circumstances it was generally quite impossible, even with the very favorable conditions under which the observations were made, to trace any line of demarcation between the two; but neither here nor in any other of the cases observed was there any reason to suspect conjugation. Neither is it believed that unions of this sort were permanent, since contact between the two was never by any specified portion of the body, and might be instantaneously broken, whereas the pairs arising by division remained attached, and always by their posterior ends.

When the trypanosomes were not rare in the mount, it was not at all unusual for a solitary individual to leave the edges of the cell clumps and wander alone in the preparation. There might be quite a few trypanosomes wandering through the preparation, but there was never any evidence of any influence exerted at a distance. But in a number of cases it was noted that as soon as two individuals came into contact each at once became more energetic.

THE PROCESS OF MULTIPLICATION.

The actual process of multiplication was not seen many times, but the following observation probably epitomizes the process:

In a 4-day culture, at 2 p. m., March 7, 1910, there were found a large and a small trypanosome in contact. The former was a wholly normal animal of adult aspect, the latter egg-shaped but with a pointed posterior end and a short flagellum. It was rather less than half as long as the adult animal, and lay in contact with the anterior half of it. In a very few minutes it was seen that the flagellum of the egg-shaped element was much longer, and a little later the element itself was seen to be split halfway from the anterior to the posterior end. Unless the appearance of singleness of this body when first seen was deceptive, and due to the angle from which it was observed, the division into a partly separated element, with the two anterior ends each provided with a flagellum, did not require more than 15 or 20 minutes.

At 2.35 p. m. the large trypanosome broke away from the paired element, but some 10 minutes later joined it again, wrapping its body around the pair in such a way that there was obtained an oval body provided with three flagella at one end. The trio remained in intimate contact until 4.30 p. m., and during all of this time it was possible only momentarily to see any line of demarcation between the three elements composing it. At 4.30 the large trypanosome again separated from the pair, and then it was possible to see that although the two small cells were still in contact by their posterior ends, they had increased very considerably in length, and were now much like the young forms seen in the young cultures.

When the original pair was first seen it was taken to be a case of unequal division, but the subsequent behavior of the large trypanosome is rather against such an interpretation. The little trypanosomes, in which the body is oval, and may or may not be pointed at the ends, were frequently found, either singly or paired, in the clusters occurring in the young cultures, and other cases of division like that above described were at times seen. In these, so far as the observations go, the pairs arising from division remained in contact, but it is not impossible that separation may sometimes take place before growth is completed, thus giving rise to solitary oval forms. It is also theoretically possible that they arise from unequal division, as in *Trypanosoma lewisi*, but unfortunately neither my preserved material nor my observations on fresh material enable me to settle this point.

Besides the oval form, there was occasionally found in the early cultures a round element, about as large as a red blood cell, provided with a long flagellum. Such are presumably to be regarded as modifications of the oval form.

MOTILITY OF THE TRYPANOSOMES.

Attention has been called to the almost constant motility of the trypanosomes. This was, however, subject to a certain variability, although it was not possible to determine upon what factors this variability depended. The normal movement was an undulation of the flagellum and undulating membrane, accompanied by a certain amount of flexion and torsion of the anterior part of the body. The younger trypanosomes were frequently seen to have their bodies bent into circles, simultaneously displaying energetic movements of the flagella and undulating membranes. Such positions might be maintained as long as the observation lasted. But in the fully fledged parasites the longitudinal axis was usually straight and the postnuclear part of the body rigid.

At times trypanosomes, entangled amongst blood cells or granular débris, acted as though seeking to get free. In such cases periods of violent activity, during which the movements lacked all regularity, alternated with periods of complete rest. In the cases noted above, where two trypanosomes kept in company for considerable periods, the movements also lacked regularity.

On one or two occasions, when the temperature of the laboratory during the night had been unusually low, the trypanosomes observed in the morning seemed rather sluggish. This observation suggested the following experiment: A tube was placed in a vessel containing ice and salt, and kept at a temperature of just above freezing for nearly two hours. A preparation was then brought under the microscope as quickly as possible, and immediately examined, with the

result that the trypanosomes were found to be displaying an activity fully up to normal.

Nor is it certain that heat increases motility. Trypanosomes always become much more active with dark-field illumination, but this may be laid to the ultra-violet or Roentgen rays rather than the heat.

OBSERVATIONS ON FIXED MATERIAL.

The observations made on fixed material confirm and supplement those made on fresh preparations. In one respect, moreover, they carry the study somewhat further, since in two cases smears made from fresh blood show trypanosomes (figs. 1 and 2).

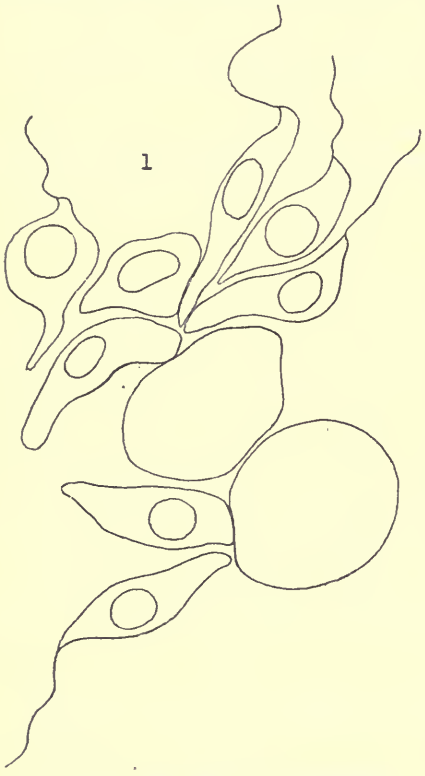


FIG. 1.—Forms of *Trypanosoma americanum* from centrifuged blood.

The blood from which these smears were made was drawn at the Bureau Experiment Station, sent into the laboratory, and centrifuged before the preparations were made from it. The interval was probably about four hours. Hence the blood was not in a strict sense freshly drawn, yet the observation compares with many others made upon trypanosomes. In many cases in the literature where trypanosomes are described as present in the circulating blood, the blood in which they were found had either been removed from the living animal for some time or had even been taken from a dead animal. There had therefore been time for it to cool to the temperature of the air. A case in point is that of *Trypanosoma wrublewski*, found only in blood from dead bison. But in the case in hand it is believed that the removal of the trypanosomes from the circulation had an immediate effect, for the parasites, as seen in figures 1 and 2, are evidently in rapid division. Their extreme scarcity in the cow is practical proof that in such a situation the multiplicative energy is in abeyance.

The only stimuli to which the parasites in the drawn blood were subjected were the lowering of the temperature, the defibrinating of

the blood, and the mechanical disturbance consequent upon centrifuging. It is hardly likely that defibrinating could have any effect, and the centrifuging was carried on for only a few minutes, the preparations being then made at once. Hence no time was given for the number of divisions which the figures show to have taken place. Probably, then, it is the lowering of the temperature which induces multiplication, although it does not necessarily follow that this is in any way correlated with the removal of the trypanosomes by a biting fly. The mere cooling itself might readily furnish all the stimulus needed.

MORPHOLOGY OF THE FORMS IN THE BLOOD.

Taking up now the trypanosomes as they appeared in the centrifuged blood, it will be seen that they are normal in shape, flagellated, and possess a large trophonucleus and a conspicuous vacuole. Unfortunately, the stain used on the two slides on which they occurred was not in proper condition, and stained the cytoplasm so intensely that the situation of the kinetonuclei was obscured.[1]

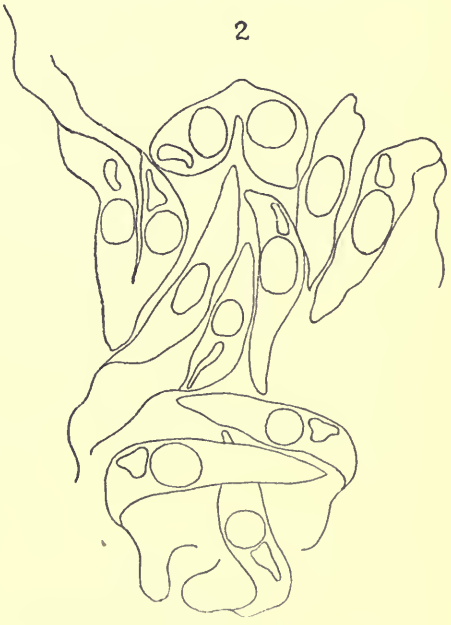


FIG. 2.—Forms of *Trypanosoma Americanum* from centrifuged blood.

For the group shown in figure 1 the average size of the body was 14.7 by 4 microns. In the case of figure 2 the dimensions were: Forms in division, 13.9 by 4 microns; forms not in division, 16.8 by 3.6 microns.

A selection of 14 of the figured flagellates, confined to those which did not appear to have been greatly distorted in fixation, gave an average measurement of 16.8 by 3.8 microns. Newly born daughter cells can not be larger and are probably somewhat smaller than the mother cells from which they were derived. Hence the trypanosome, as it would appear in the circulating blood, probably has a length of at least 20 microns excluding the flagellum, and, as noted on page 24, a trypanosome of this size was found in a culture of the first day.

[1] Efforts to improve the staining of these two slides were futile. The stain resisted a treatment with acid alcohol of sufficient duration to injure the trypanosomes themselves.

MORPHOLOGY OF THE FORMS IN CULTURE.

Figures 3, 4, 5, and 6 show the appearance in smears from the early cultures. The shape is to a large extent artificial; for one thing, the early stages appear to contract on drying, becoming shorter and broader. Primarily, however, the distortion is due to the fact that as the spherical cluster dries it is fixed flat, with results such as are shown very plainly in figure 5. The two cells shown in figure 4 are probably somewhat contracted. In the film they measured 10 microns long. In figure 6 the largest trypanosome has the body 24.7 microns long. The group of six, shown in this figure, give clear indications of the method of their origin, and are probably all derived from a single trypanosome. All but one are oriented in the same direction, and it is evident that this odd member of the group has just been forced out of the place which it had been occupying.

FIG. 3.—Forms of *Trypanosoma americanum* from bouillon culture 96 hours old. The individual members of the cluster are distinguished by the letters *a* to *i*.

Evolution, as already set forth on pages 24 et seq., results in the production of longer and relatively and often actually more slender flagellates. Figure 7 from a culture on the fifth day shows two trypanosomes. They are longer and somewhat more slender than those shown in figures 1 and 2. Figures 8 and 9 are from cultures of the same age. Here the animals are long and slender, that of figure 8 measuring 27.8 microns for the body. The undulating membrane also shows more distinctly in this figure. It was such forms as these which were spoken of as crithidial in the preliminary notice, but so much confusion exists in the literature as to what precisely are the characters of the genus *Crithidia* that the derived adjective is perhaps best let alone.

Figures 11 to 15 show forms from older cultures. Evidently after a certain maximum length is reached the animals again become broader. Finally, by a considerable thickening of the anterior end, the club-shaped form is produced. Very marked, also, is the increased conspicuousness of the undulating membrane.

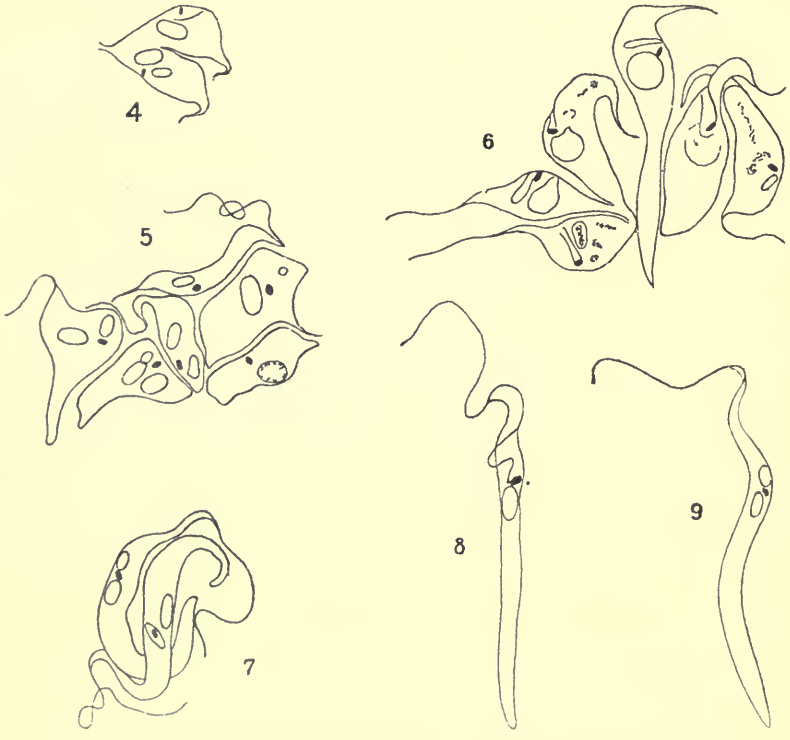
In figures 13 and 14 are shown, among others, forms having a broad, rounded anterior end. Posteriorly the body is rigid and tongue-shaped, and this portion finally degenerates and disappears. Anteriorly the cytoplasm extends out in a long tapering portion, very flexible and mobile in the living animal and bearing the undulating membrane. This portion also becomes absorbed in the gen-

esis of the monadine forms, and trypanosomes having this outline are doubtless more or less senile.

Figure 12 shows the largest individual found in the preparations, the body alone measuring 38 microns.

CYTOLOGY OF THE FORMS IN CULTURE.

Along with the changes in size and shape there are conspicuous cytological changes. With reference to these, however, it is desirable to say that the material used was all "dry," and all stained



FIGS. 4 to 9.—Forms of *Trypanosoma americanum* from bouillon cultures 112 hours old.

with Wright's stain. Of late a good deal of the work on trypanosomes has been done with material fixed by the "wet" method, and the dry method has been extensively criticized. But the use of Wright's stain on dried blood is a standard procedure, and the results are so evidently accurate for such delicate structures as leucocytes that they can scarcely be very far wrong for trypanosomes.

In the trypanosomes from the centrifuged blood of the cow the flagella stained, but poorly. In the early cultures, 2, 3, and 4 days, they stain, but often very faintly, this peculiarity being the cause of the error made in the preliminary notice. Later, however, their

affinity for the stain becomes much greater, and in the fully fledged animals the flagella are conspicuous. The color is always red. In nearly all cases the tip is thickened to form a minute knob, as in *Trypanosoma wrublewski*. In the newly born forms the flagellum is short; in the adults it is long; probably on the average it is as long as the body. Frequently, however, in the older cultures, the flagellum is two or even three times longer than the body.

In fresh preparations an undulating membrane is always in evidence, but in stained material made from young cultures it can not as a rule be made out. Later it broadens and becomes conspicuous, but is always short.

The kinetonucleus from the first to the last stains intensely, usually a deep garnet color. It is sometimes round, more usually oval, and appears merely to constrict into two at the time of division. It seems always to be associated with a vacuole, which is at times as large as the trophonucleus. In cultures 2, 3, and 4 days old, when the trypanosomes are in rapid division, this vacuole is obviously merely a cavity within the cytoplasm. It is of irregular outlines, sometimes lobulated, sometimes almost tubular. It presents the peculiarity that in these early stages of evolution it appears to be open to the exterior. The appearance presented recalls what is found in *Euglena*. Some euglenoid type might easily have been the ancestor of the Trypanosomidæ, in which case this vacuole might be regarded as the rudiment of a cytopharynx. The data presented, however, are obviously insufficient to warrant doing more than putting forward the above as a possible suggestion.

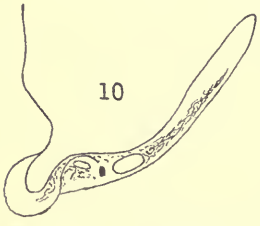
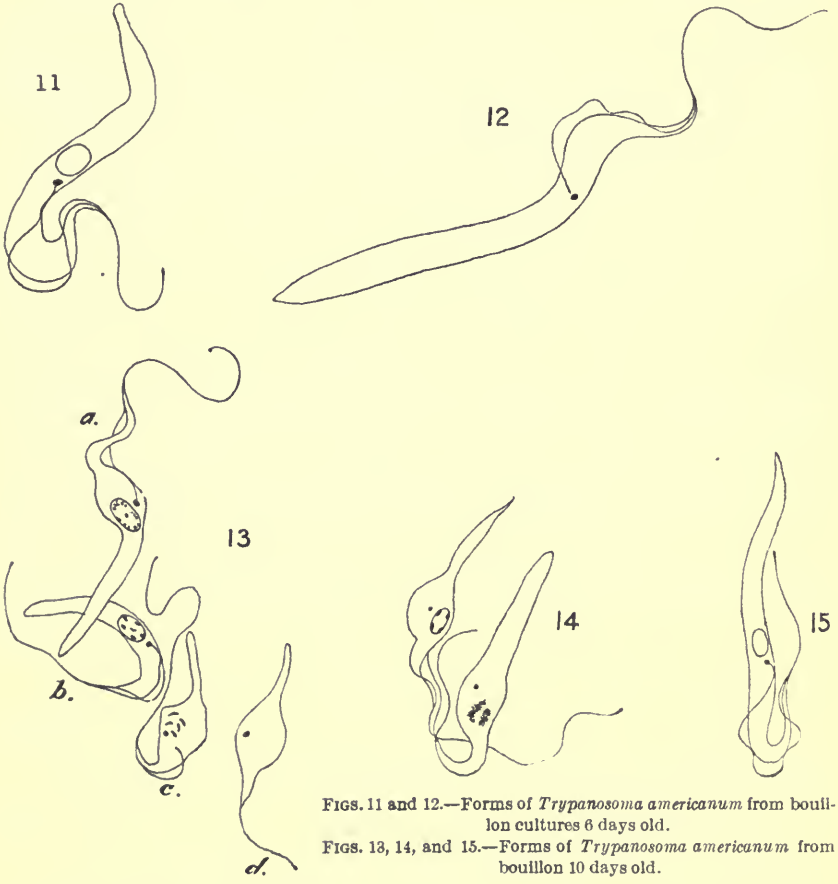


FIG. 10.—*Trypanosoma americanum* from bouillon culture 3 days old.

Later, the vacuole becomes round or oval, ceases to give the appearance of being connected with the exterior, and furthermore absorbs the stain, becoming pink. Although in adult trypanosomes it frequently can not be demonstrated, it is perhaps a constant feature and has been found in the monadine forms, the end terms of the series.

As well as could be made out in the trypanosomes from the freshly drawn blood, the trophonucleus consisted of a large, tense sac, and such is clearly its condition in animals from the young cultures. It stains homogeneously and so faintly that the actual color is often difficult to determine; generally is of a reddish cast. In the very early cultures—2 and 3 days—the trophonucleus is very conspicuous, contrasting sharply with the blue cytoplasm. Later it becomes less homogeneous, presenting a ground substance in which is a quantity of amorphous matter having a different staining reaction.

In the late stages the appearance is that of the conventional trypanosome nucleus, an oval body provided with a number of large, rounded granules. The appearances, however, are manifold, and have not been worked out in detail. Presumably, two factors are at work—the age of the trypanosome and its state with reference to division—and each of these is superposed upon the other.



FIGS. 11 and 12.—Forms of *Trypanosoma americanum* from bouillon cultures 6 days old.
FIGS. 13, 14, and 15.—Forms of *Trypanosoma americanum* from bouillon 10 days old.

The cytoplasm in the early stages stains solidly, but is denser in the middle of the body than at the ends. It is always a clear blue. For the first several days it appears to be provided with a considerable number of small vacuoles of uniform size. Since, however, observations on fresh material show these young trypanosomes to contain granules, and since there is no differentiation of the cytoplasm in the fixed preparations other than these apparent vacuoles, the probability is that they are really granules which refuse the stain. This supposition receives support from the fact that the granules of leuco-

cytes at times appear as holes in the stained material. Later, the cytoplasm loses its solidity and homogeneity and conspicuous blue and violet granules become abundant, and in old cultures the trypanosomes frequently consist of little else than sacs more or less completely filled with coarse granules.

PRINCIPAL CHARACTERISTICS OF TRYPANOSOMA AMERICANUM.

Trypanosoma americanum is a large trypanosome. Figure 12 shows the largest specimen found, the body measuring some 38 microns in length. In this particular case the flagellum was either short or else failed to stain throughout. But since the flagellum is normally as long as the body, a total length of 75 microns is by no means unlikely.

The most marked peculiarity, however, is the very short undulating membrane. The kinetonucleus may be in front of, alongside, or behind the trophonucleus, and the two are always close together. But the reason for the shortness of the undulating membrane is that the nuclear system is pushed forward. The usual situation for the trophonucleus of a trypanosome is near the middle of the body, but in *T. americanum* it is at the junction of the anterior and middle thirds. That is, the distance from the middle part of the trophonucleus to the anterior end of the trypanosome averages 33 per cent of the body length, and in the specimens measured ranged from 25 to 40 per cent. In this respect *T. americanum* differs from the *T. transvaliense* phase of *T. theileri*, in which, according to the data given by Luhs, this distance is about 50 per cent of the whole. Here again it agrees with *T. wrublewski*, although even in the latter the nucleus, according to the published figures, is hardly so far forward.

CONCLUSIONS.

1. *Trypanosoma americanum* lives, in all probability as a typical trypanosome, in the blood of perhaps 75 per cent of yearling and adult American cattle, but is not present in young calves.

2. It comes very close to *T. wrublewski* of the European bison, and may be only a variety of that species.

3. Removal from the circulating blood stimulates multiplicative energy, apparently merely as the result of a cooler environment.

4. Removed from the cow and placed under appropriate conditions, multiplicative energy runs far in advance of growth energy; hence—

5. The trypanosomes divide and redivide very rapidly, and in consequence become smaller than the blood forms.

6. At the end of a few days, multiplicative energy weakening, the organisms have an opportunity to grow and to reach their normal size.

7. The adults are at first very slender, but in time increase in breadth and may become very large.

8. As the cultures reach and pass their maxima the individuals become club-shaped and eventually transform into rounded or oval elements, provided each with a long flagellum.

9. Changes in the morphology of the nuclear system, and in the texture and chemical nature or composition of the cytoplasm, accompany changes in the facies of the entire organism.

10. A distinguishing character is the situation of the trophonucleus, which is normally at the union of the anterior and middle thirds.

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