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STUDIES ON THE LIFE CYCLE OF *TRYPANOSOMA CONORRHINI*. "IN VITRO" DEVELOPMENT AND MULTIPLICATION OF THE BLOODSTREAM TRYPANOSOMES

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SUMMARY

Hoping to clarify the life cycle of *Trypanosoma conorrhini* of which reproductive forms have never been encountered in the vertebrate host, the authors tried to infect mammals' cells "in vitro" with the flagellate. Intracellular forms of the parasite were not found, but in the nutrient liquid used for the tissue culture, large trypanosomes, identical with the mature bloodstream form of the species, had developed and were multiplying by binary fission.

In trying to determine the factor, or factors, responsible for this "in vitro" proliferation of the blood trypanosome, the authors made a series of experiments and verified that:

1) The presence of living tissue is not necessary, since the same development occurred when only the "nutrient medium" (Hanks' saline, lactoalbumin and calf serum) was used, provided the cultures were incubated at 37°C; in such conditions after several days, an almost pure culture of mature and dividing trypanosomes was obtained;

2) If the same "nutrient medium", with or without a tissue substrate, was maintained at 28°C, development of *T. conorrhini* was the same as in the invertebrate and in conventional blood agar media: abundant multiplication in the crithidial stage and production of the small characteristic metacyclic trypanosomes;

3) In blood agar medium incubated at 28°C, the flagellate developed as usual (crithidias and metacyclic trypanosomes), but if the tubes were incubated at 37°C, the cultures died within a short time.

The authors discuss their observation under different aspects, specially the "in vitro" development and multiplication of the bloodstream form in a non cellular medium, and the factors involved, among which temperature seems to be very important.

INTRODUCTION

Under the name of *Crithidia conorrhini*, a flagellate found in the gut of *Triatoma rubrofasciata* (De Geer) (*Hemiptera, Reduviidae*) was described by DONOVAN, in 1909 [5, apud 11]. Later recognized as a blood parasite of mammals, it was placed in the genus *Trypanosoma*.

The natural host of *Trypanosoma conorrhini* is probably the house rat, *Rattus rattus*, which has been found infected in Java [1] and in Belém, State of Pará (Brazil) [3]. In both cases infection of the rat was detected through the xenodiagnosis method.

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White rats and mice, guinea-pigs and monkeys have been repeatedly infected in laboratory. Whatever the point of inoculation, trypanosomes soon appear in circulation, but are seldom numerous. Direct detection of the parasite in blood smears is usually possible for only a few days. Nevertheless, positive xenodiagnosis proves that, at least in many cases, infection may last for months [3, 11]. Sub-inoculations have produced very light infections on the first passage and negative results beyond that [11]. Up to now, reproductive forms of the flagellate have not been met with in the organism of vertebrates [3, 4, 9, 11].

On the other hand, infection of the invertebrate host is usually both frequent and heavy with abundant multiplication. *Triatoma rubrofasciata* has an almost world-wide distribution (in coastal areas) and the proportion of specimens found naturally infected in different places has been very high [1, 2, 4, 6, 7, 9, 11].

The above mentioned facts have suggested [2, 11] that the vertebrate host represents a recent and not entirely successful venture of the protozoon, this being apparently unable to reproduce in the new environment. *T. conorrhini* should then be capable of perpetuating itself as a species through direct transmission from bug to bug — a hypothesis that has yet to be proved.

Another interesting feature about *T. conorrhini* is the remarkable difference in size and shape, between the "metacyclic" and the blood trypanosomes. In the insect, the "metacyclic" forms are represented by two types: one, more common, short and stumpy, averaging less than 10 μ (individuals with less than 5 μ being not infrequent), with an inconspicuous undulating membrane and a nucleus that is very often kidney shaped; the other is elongated, measuring from 12 to about 15 μ, with a narrow and slightly wavy undulating membrane and a more rounded nucleus. Both types present a large kinetoplast in a terminal or subterminal position.

If a laboratory animal is inoculated with the intestinal contents of an infected bug, trypanosomes appear in its blood within a few hours. These first, immature trypanosomes look like a stretching-out of the larger and elongated metacyclics. But in smears made 48-72 hours later, one finds practically only large trypanosomes that may reach more than 60 μ in body length (minus the free flagellum), with a long, finely pointed posterior extremity and a broad, conspicuous and wavy undulating membrane. These mature trypanosomes have, in front of the kinetoplast, "a peculiar pot-shaped structure", represented by a halo or a slightly stained area, such as described by MORISHITA [11] (Fig. 1, upper half).

In spite of unsuccessful trials by earlier workers [4, 11], it has been demonstrated that *T. conorrhini* may be easily maintained in artificial media, such as Senekjie's and NNN, inoculated with the bloodstream forms of the parasite [3, 8]. Sub-cultures can be obtained indefinitely if transfers are made each 15th-20th day.

In the culture tubes the parasite reproduces abundantly in the crithidial stage. As the culture ages, multiplication activity lessens and the characteristic metacyclic trypanosomes become ever more frequent (Fig. 1, lower half).

In the blood agar media, therefore, the parasite behaviour is similar to that it has in the digestive tract of the invertebrate host. The end product of the cycle in culture seems to be the "metacyclic" trypanosome which, it is believed, corresponds to the infective stage for the vertebrate. As it is well known this is the rule for digenetic hemoflagellates: in the conventional culture media these parasites do not go through the same stages as in their vertebrate host.

* * *

In an attempt to clarify the life cycle of the very incompletely known trypanosomes of mammals found in Brazil, one of the authors has isolated and is maintaining some strains in laboratory (in animals, and/or culture media, and/or triatomid bugs). Different approaches are tried for the study of these parasites, one of them being the tissue-culture technique. Here, our obvious aim is to check the possibility of the parasites going through an intracellular stage in their normal mammal hosts.

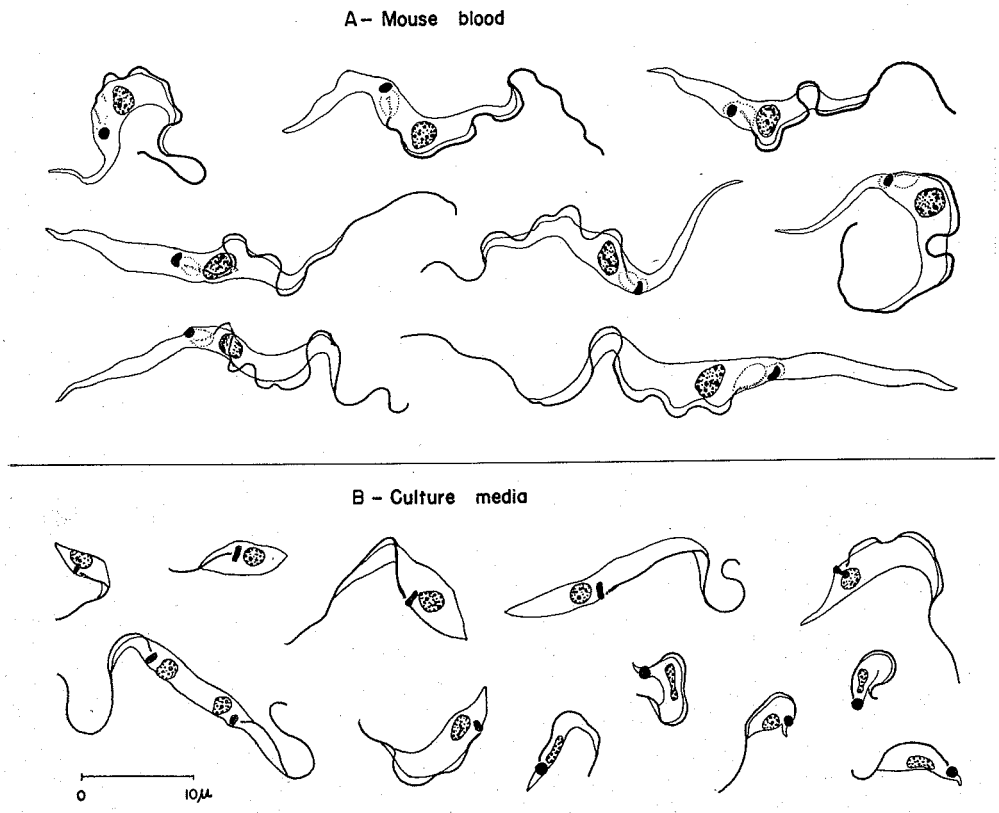

Fig. 1 — *Trypanosoma conorrhini.* Upper half, forms seen in blood of white mice; lower half, forms seen in usual diphasic blood agar media.

The latter method appeared particularly adequate for the study of *T. conorrhini*, since: a) its cycle is in some ways similar to that of *T. cruzi;* b) it seemed unable to multiply in the bloodstream trypanosomose stage; and, c) the previous unsuccessful search for intracellular forms of the parasite in tissues of infected animals could not be taken as definitive proof of the inexistence of such forms.

Our studies on the "in vitro" infection of tissue cultures by *T. conorrhini* led to some unexpected observations which make the subject of this paper.

MATERIAL AND METHODS

The strain of *T. conorrhini* originated from the intestinal contents of a naturally infected specimen of *Triatoma rubrofasciata* caught in the city of Rio de Janeiro. In our laboratory it is being maintained for more than one year in mice, laboratory-bred triatomid bugs, and in blood agar media.

The blood agar medium consists in a modification of the original Nöller's: beef broth — 1,000 ml; peptone — 10 gr; sodium chloride — 5 gr, and agar — 12 gr. The medium is prepared, distributed in tubes, sterilized and mixed with about 10% fresh, defibrinated rabbit blood according to the usual technique.

For the experiments of tissue infection "in vitro" we used a line of monkey heart cells which is being maintained at the Parasitology Department for some time *. Some trials were also made with HeLa cells.

* These cells were first isolated by Salk *et al* in 1954. They were obtained through Dr. R. Piza de Carvalho, of the Microbiology Department, Faculdade de Medicina de São Paulo.

Tissue infection was tried in stationary test tubes with coverglass, to which a transplantation of cells had been made 2-3 days before. This was calculated so as to allow for a reasonable layer of cells on the coverglass (without overgrowth) at the time of infection.

The nutrient medium was made of: Hanks' saline solution (with phenol red as indicator) — 100 ml; enzymatic hydrolysate of lacto-

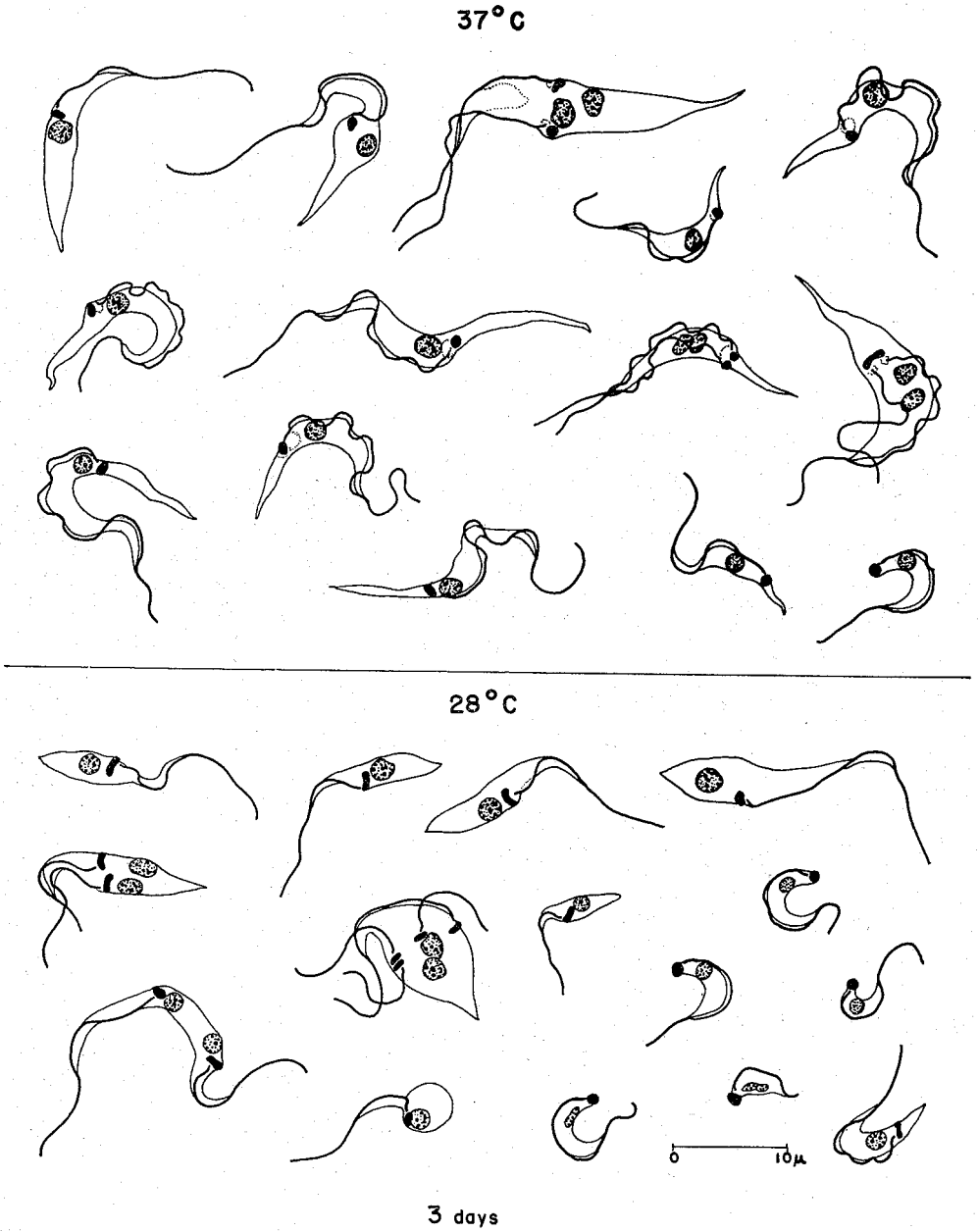

Fig. 2 — *T. conorrhini* in tissue culture nutrient medium (Hanks' saline, lactoalbumin and calf serum), 3 days after inoculation. Upper half, incubated at 37°C; lower half, incubated at 28°C.

albumin (2.5 percent in Hanks' saline) — 10 ml; calf serum — 20 ml.

To infect the cells, slants of 15-20 days old cultures of *T. conorrhini* on blood agar medium were flushed with Hanks' saline, washed twice by centrifugation and the final sediment was resuspended in an amount of clean nutrient medium calculated according to the number of tissue tubes (1.5 ml per tube) to be inoculated. In these tissue tubes the old nutrient medium was then substituted by the new one containing the cultural forms

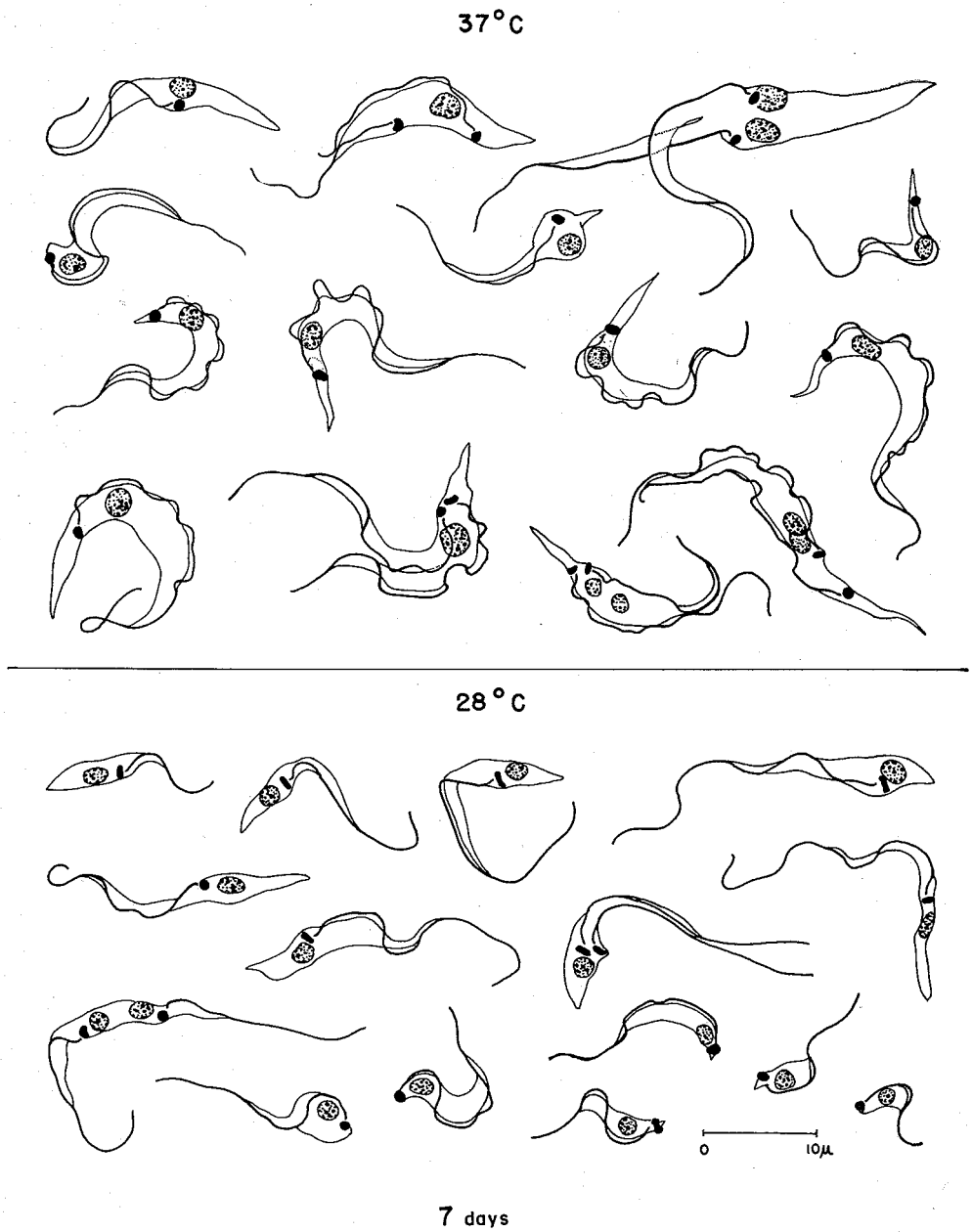

Fig. 3 — *T. conorrhini* in same medium as in Fig. 2, 7 days after inoculation. Upper half, incubated at 37°C; lower half, incubated at 28°C.

of *T. conorrhini*. The tubes were kept at a slanted position in a 37°C incubator.

Each 2nd or 3rd day, according to the fall in pH level, the tissue was refed by substitution of the nutrient medium. In some experiments, the old medium was centrifuged and the sediment resuspended in the new medium, so as to offer the flagellates repeated chances for cellular penetration.

Examinations were made at different intervals, each time in one or two of the

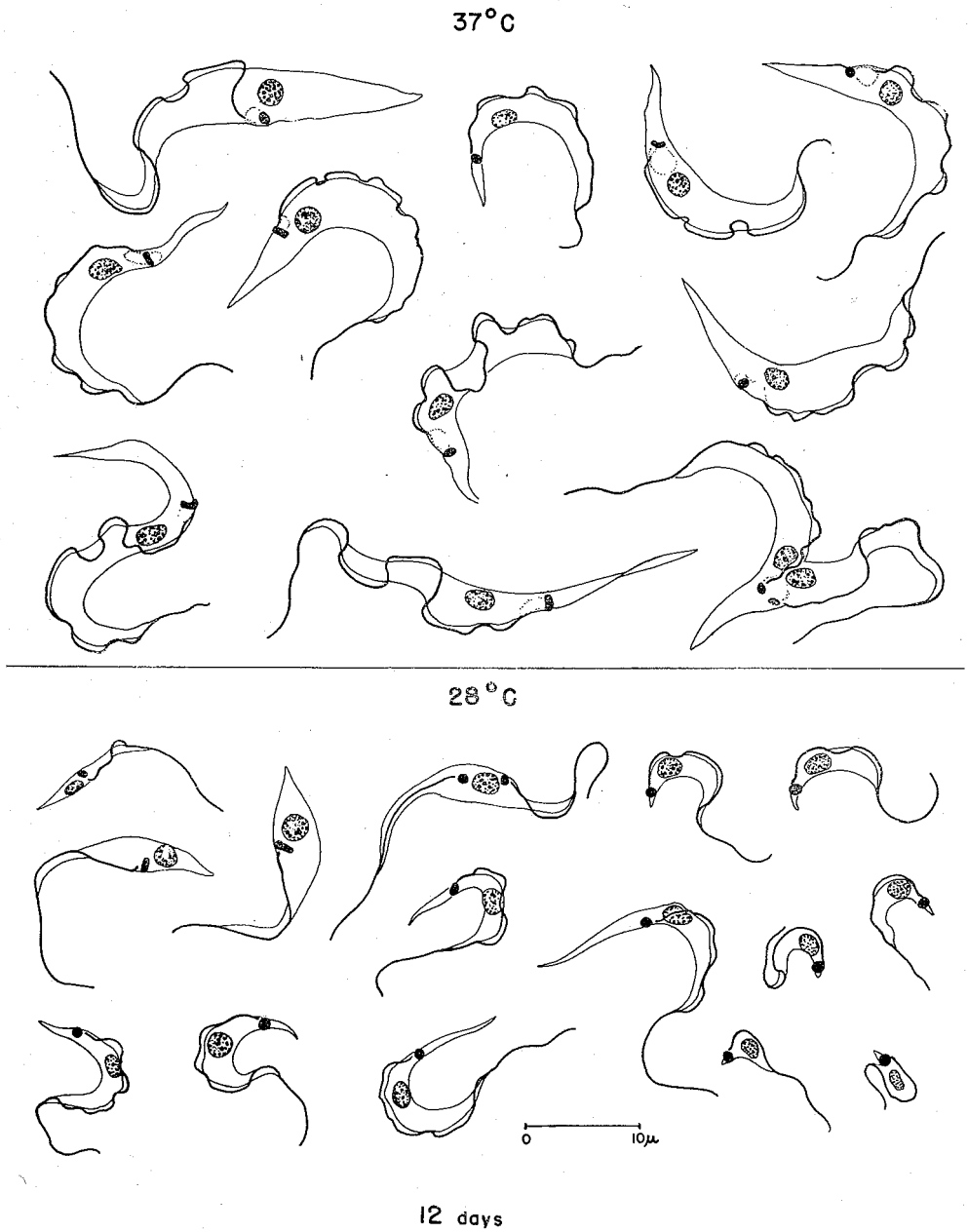

Fig. 4 — *T. conorrhini* in same medium as in Figs. 2 and 3, 12 days after inoculation. Upper half, incubated at 37°C; lower half, incubated at 28°C.

tubes under observation. The coverglass was removed, fixed, stained by Giemsa's and mounted on balsam, for microscopical study. The nutrient medium was centrifuged and the sediment examined in both fresh and stained preparations.

When, in the course of our work, it became evident that we had to test the influence of living cells and of the temperature on the development of *T. conorrhini*, several other experiments were made in which cultural forms of this parasite were inoculated in different groups of tubes, containing:

a) living cells + "nutrient medium", incubated at 37°C;

b) living cells + "nutrient medium", incubated at 28°C;

c) "nutrient medium" incubated at 37°C;

d) "nutrient medium" incubated at 28°C;

e) blood-agar medium incubated at 37°C;

f) blood-agar medium incubated at 28°C.

The above sets of tubes were grouped in different schemes for repeated observations, but the same inoculum was used for all the tubes in each experiment.

The "nutrient medium" was always the same (Hanks' plus lactoalbumin plus calf serum) used for the feeding of monkey heart cells, as already mentioned.

A maximum and minimum thermometer was kept in each incubator and the temperature level was fairly constant throughout the experiments.

RESULTS

We had no evidence of an intracellular stage in the cycle of *T. conorrhini*. The coverglasses examined at various intervals from 18 hours to 11 days after the flagellate had been in contact with the cellular substrate, presented only adherent extracellular forms. Occasionally one isolated leishmanoid parasite was seen within a cell vacuole, and this was interpreted as a phagocytized and degenerating parasite.

However, the liquid phase of the tissue cultures (kept at 37°C) was seen to contain large trypanosomes such as we never encountered in blood agar cultures. They were identical with the bloodstream forms of the parasite and, moreover, some of them were undergoing binary fission.

Aiming to determine the factor, or factors, involved in this "in vitro" development of the mammal stage of *T. conorrhini*, which might or might not be dependent on the presence of living cells, we observed the cycle of the parasite in the various environments previously listed.

It should be emphasized that all experiments were started with the cultural forms of *T. conorrhini* found in 15-20 days old blood agar slants (Fig. 1, lower half).

Results may be thus described:

1) When kept at the same temperature, the tubes containing living tissue supported the development of the same forms of the parasite as did their "controls" which contained the same nutrient medium but no cells.

2) When both above mentioned groups were incubated at 37°C, the sequence of events observed was as follows: a) Within the first 18 hours no quantitative or qualitative modifications were noticeable, except for the appearance of a few trypanosomes resembling the immature bloodstream form. The bulk of the population was made of small or medium-sized, fusiform or pyriform crithidias, extremely active, grouped in great agglomerates and in frequent division. A few longer crithidias and a fair number of "metacyclic" trypanosomes of both the larger and the stumpy types, were seen. b) Between the first and the third days there was a marked fall in population, resulting from mass destruction of the small crithidias and due, probably, to lytic action of the blood serum incorparted in the medium. c) From then on, there was a slow rise in population, this being of a composition entirely different from the initial one. Crithidial organisms were represented almost entirely by the large type with an elongated posterior extremity. Many of them were seen in binary fission, but they seem to

multiply in a slower rythm than the small and medium-sized crithidias previously existing in the cultures. As for the trypanosomes, the "metacyclic" forms completely disappeared, the stumpy type first. Trypanosomes identical with the bloodstream ones became ever more abundant, the smaller or immature form being more frequent at the beginning. After the first week, except for some large crithidias which could still be found, the media contained a pure culture of large, mature, bloodstream type trypanosomes, complete with their "pot-shaped structure". These trypanosomes were often seen in binary division.

Observations were made up to the 18th day, but by the 15th day (or much earlier in the tubes containing living tissue), the cultures began to degenerate.

3) When the same media were kept at 28°C, the cultures were entirely different from the above described. The flagellate population increased steadily due to rapid multiplication of crithidial organisms, specially the small and medium-sized ones which were frequently grouped in large rosettes. Metacyclic trypanosomes of both the stumpy and longer types were common throughout. At about the 10th day some trypanosomes resembling the immature bloodstream form began to appear, but, up to the 18th day of observation they were decidedly less numerous than the "metacyclic". Large, mature bloodstream forms and dividing trypanosomes were never seen.

Degeneration of these cultures maintained at 28°C, specially in the absence of tissue, was much slower than at 37°C.

4) In the diphasic blood-agar medium all the flagellates died within 3-5 days when the tubes were kept at 37°C. It is worth to mention that precautions had been taken against the drying-up of the cultures: Hanks' saline or nutrient broth had been added to increase the liquid phase of the medium and rubber stoppers were used to prevent evaporation.

5) In control diphasic blood-agar media kept at 28°C, development of the parasite proceeded along the path already described. No qualitative difference was noticed between these cultures and those in tubes containing tissue, or with only the liquid phase of the tissue culture and maintained at the same temperature (i.e., 28°C) — except for the mentioned appearance of some immature blood-type trypanosomes in the "nutrient medium". In the blood-agar medium the only trypanosomes seen were of the "metacyclic" types.

Figures 1 to 4 show camera-lucida drawings, on the same scale, of the forms of *T. conorrhini* observed during the present experiments. The accompanying Table gives an idea of the quantitative differences in the incidence of the various developmental stages found in stained smears of tissue culture nutrient medium at different incubation temperatures.

DISCUSSION

Our observations shall be briefly discussed under two aspects:

1) *The life history of* T. conorrhini. Even if we take in consideration the fact that the right cells might not have been used, it seems improbable that the life-cycle of this trypanosome includes an intracellular stage. We have seen that it develops to the mature bloodstream form in the absence of living tissue and that this form is able to reproduce by binary fission.

The fact that dividing trypanosomes have never been found in the blood of vertebrates is probably due to the action of some inhibitory factor that keeps reproduction of the parasite at an extremely low level.

Through the observation of our cultures the impression was gathered that the blood type trypanosome may develop from two different forms, the large crithidia and the longer metacyclic. It seems to us very interesting that not all crithidial organisms are lysed when incubated in the serum saline medium at 37°C, and that the ones which survive are mostly different in size and shape from those that are destroyed. Furthermore, the large surviving crithidias are still able to divide and they may eventually evolve to trypanosomes through posterior migration of the kinetoplast (see drawings, specially

TABLE

Estimation of the quantitative differences in the incidence of developmental stages of *Trypanosoma conorrhini* in tissue culture nutrient medium * incubated at 37ºC and 28ºC.

Form	At 37ºC				At 28ºC			
	18 hours	3 days	7 days	12 days	18 hours	3 days	7 days	12 days
Small crithidia	+++++	+	+	—	+++++	++++	+++++	+++
Large crithidia	+	++	+++	+	—	+	+	+
Dividing crithidia	+++	+	++	+	+++	+++	++	++
"Metacyclic" trypanosome, stumpy	++	—	—	—	+++	++	++	+++
"Metacyclic" trypanosome, slender	++	++	—	—	++	+	+	++
Bloodstream type trypanosome, immature .	+	+++	++	+	—	—	—	++
Bloodstream type trypanosome, mature ...	—	++	+++	++++	—	—	—	—
Dividing trypanosome	—	+	++	++	—	—	—	—

* Nutrient medium consisting of Hanks' saline, lactoalbumin and calf serum, and with or without cellular substrate.

on Plate III, upper half). On the other hand, the longer metacyclic trypanosome seems to be able to develop further through great enlargement of its body and stretching of its posterior end.

However, more study is necessary for a better understanding of the relationships and biological significance of these morphological stages of the flagellate.

2) *The "in vitro" propagation of the bloodstream trypanosomes.* The development and reproduction of *T. conorrhini*, was observed to occur in a liquid medium without a cellular substrate and the one factor that appeared to have a preponderant influence upon the phenomenon was temperature.

The influence of temperature on biological phenomena is so widely known that it needs not be discussed here. We only wish to refer, as particularly pertinent, the recent observations by TRAGER [14, 15] on the stimulation of reproductive and infective potentialities of cultural forms of *Trypanosoma vivax*, brought about by modifications in the incubation temperature.

From our experiments with *T. conorrhini*, we think it probable that higher temperature acts indirectly and in two different ways: by allowing or facilitating the lysis of the crithidial stages of the parasite peculiar to the invertebrate host, and by making possible or easier the action of some biochemical factor, or factors, involved in the development and multiplication processes of the trypanosomes.

The observations on which these assumptions are based have been already described but some points should be emphasized. The nutrient medium made of Hanks', lactoalbumin and serum, when incubated at 28°C, allows for some progress in the development of the flagellate towards the bloodstream form, since, after several days, a few immature trypanosomes of this type were found. As we mentioned, however, these trypanosomes did not seem able to develop further if maintained at the same temperature. We may assume then, that the factor, or factors, which would permit progress to full adulthood, is present in this medium but has only partial activity at lower temperatures.

The death of the blood-agar cultures at 37°C might be due both to lethal action of temperature on some stages (e.g. crithidial organisms) of the flagellate and to the absence in the medium of the above mentioned factor, or factors, necessary for the further development of other stages.

In connection with these hypothesis we shall mention the papers by MUNIZ & FREITAS [12, 13] on the "in vitro" development of *Trypanosoma cruzi*. These workers observed the complete vertebrate cycle of *T. cruzi* in a medium consisting of liquid of ascitis artificially produced in guinea-pigs through peritoneal injection of glucose nutrient broth. The medium contained leucocytes and the optimum temperature for the development of the parasite was 37°C. If leucocytes were removed, the metacyclic trypanosomes reverted to leishmanial stage but the cycle was not completed. On the other hand, as stated by the same authors, to start its invertebrate cycle, *T. cruzi* is dependent on two factors, one of which, present in red blood cells, is absorbed by the agar of diphasic media.

There is still much to be learned about the physical and chemical factors involved in the determinism of the life cycles of digenetic flagellates. As far as we are aware, our work with *T. conorrhini* led us to obtain for the first time an almost pure culture of the mature bloodstream form of a mammal trypanosome in a non cellular medium. Further study along these lines might help in the recognition of the above mentioned factors and in the clarifying of the complete life cycle of this and other trypanosomes.

SUMÁRIO

Estudos sôbre o ciclo evolutivo do Trypanosoma conorrhini. *Crescimento e multiplicação dos tripanosomas sangüícolas "in vitro".*

Para esclarecer o ciclo do *Trypanosoma conorrhini* no hospedeiro vertebrado, no qual não tinham sido até agora encontradas formas reprodutivas do parasito, os autores ten-

taram infectar com êsse flagelado, células de mamíferos cultivadas "in vitro". Verificaram que:

1) o *T. conorrhini* não apresentou formas intracelulares;

2) no líqüido nutriente do cultivo de tecido a 37°C, crescera, ao fim de alguns dias, cultura quase pura de tripanosomas idênticos aos que ocorrem no sangue do hospedeiro vertebrado;

3) nessas condições os tripanosomas se reproduzem por divisão binária;

4) a presença de tecido vivo não é essencial, pois que culturas ricas e com formas idênticas foram também obtidas quando se utilizou apenas o meio nutriente (solução salina de Hanks, lactoalbumina e sôro de vitelo), a 37°C;

5) quando as culturas são conservadas a 28°C, seja contendo tecido vivo, seja contendo apenas o meio nutriente, o desenvolvimento do flagelado se processa de maneira semelhante à que ocorre no tubo digestivo do invertebrado;

6) em meio difásico de agar-sangue, a 28°C, o desenvolvimento corresponde também ao que se passa no invertebrado (como é de regra para os hemoflagelados); se são, entretanto, incubadas a 37°C, as culturas neste meio morrem em pouco tempo.

Tecendo comentários sôbre essas observações, os autores concluem que na fase de tripanosoma sangüícola o *T. conorrhini* tem capacidade de multiplicar-se e que seu desenvolvimento independe de um estágio intracelular. Julgando ser a primeira vez que se obtém em cultura o ciclo sangüícola de um tripanosoma de mamífero, os autores fazem ainda considerações sôbre os possíveis fatôres responsáveis pelo fenômeno. Um dos fatôres que parece ter grande importância é a temperatura de incubação.

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