Rev. Inst. Med. trop. São Paulo
6(3):93-100, maio/junho, 1964

UDC 593.161.3
576.983.161.3

GROWTH AND DIFFERENTIATION IN *TRYPANOSOMA CRUZI*

I. Origin of metacyclic trypanosomes in liquid media

E. PLESSMANN CAMARGO

SUMMARY

Growth and differentiation of crithidiae of *T. cruzi* into metacyclic trypanosomes, are studied in two different media, one complete, supporting sub-cultures, the other incomplete; results are expressed as counts of the total numbers of flagellates and of metacyclic forms.

Crithidia — metacyclic differentiation occurs, in both media, only at the end of the exponential growth phase. In crithidiae maintained by daily sub-culturing in permanent exponential growth, differentiation does not occur. Transference of cultures at the beginning of differentiation to fresh complete medium stops the process; this does not occur when transfers are made to fresh incomplete medium, where differentiation proceeds without interruption. Depletion of one or more factors of the medium or of internal pool of crithidiae are suggested as required for differentiation.

Morphological data about the crithidia-metacyclic transformation are presented.

INTRODUCTION

In the biological cycle of trypanosomes there is a succession of generations or steps that differ as to their morphology and behaviour. It may be assumed that this is due to some modification occurring in their environment such as the nature of the host, variation in temperature and so on.

In *Trypanosoma cruzi* we find only crithidia and trypanosoma-like flagellates in the hind gut of the invertebrate host. The former live in the higher portions of this segment of the digestive tract, where they actively multiply, while metacyclic trypanosomes — which represent the infective stage of the parasite — evolve in the terminal part of the digestive tube.

Metacyclic trypanosomes, once introduced into the vertebrate host, penetrate a number of cells and are transformed into leishmaniae. They multiply as such very actively and are released into the bloodstream after differentiating into trypanosome forms. If ingested by the insect vector these forms revert again to the crithidial stage.

From a strictly morphological point of view the various stages differ as to the presence or absence of a free flagellum, a shorter or longer undulating membrane, the relative position of the kinetoplast, etc.

Concerning biological differences among the various forms, there is scant and indirect evidence, such as the different sensibility of crithidial and trypanosome forms towards the lytic action of normal serum [10] and the ability to penetrate cells.

It is well-known that in trypanosomes environmental factors can trigger the morphological transformation from one stage to the other; for instance, in *T. cruzi*, a shift in temperature causes blood type trypanosomes to change into crithidiae (for details and references see SILVA [13]). STEINERT [14] showed in vitro that for *T. mega* the change from crithidial to bloodstream trypanosomes can

Faculdade de Medicina, São Paulo — Dept. de Parasitologia, Cx. Postal 2921, São Paulo, Brazil.
This work was supported by a grant from Fundação de Amparo à Pesquisa do Estado de S. Paulo.

be induced by adding urea to the nutrient medium; TRAGER [17] obtained the development of *T. vivax* trypanosomes to the infective stage in tsetse-fly tissue culture when the temperature was increased from 30-32°C to 38°C. In *T. conorrhini* it has been demonstrated [3, 4, 5] that the development from crithidial or metacyclic forms to blood type trypanosomes, in a proper medium, depends on an increase of temperature, from 28° to 37°C.

Concerning *T. cruzi*, very little information is available on the morphogenesis of different stages of development. This is partially due to the difficulties involved in cultivating this organism; with techniques of mammalian tissue culture some morphological aspects of the change from intracellular leishmaniae to free blood type trypanosomes could be better described [9, 13] but, so far, the mechanisms involved remain unknown.

The present paper is an attempt to study the factors involved in the "in vitro" metamorphosis of crithidial to metacyclic trypanosomes; morphological aspects are presented, discussed and related to previous knowledge.

MATERIAL AND METHODS

Organisms — The human strain "Y" of *T. cruzi* isolated by SILVA & NUSSENZWEIG [12] and maintained in our laboratory by mouse passages and subcultures in diphasic blood-agar medium, was utilized.

Culture media — Liver infusion tryptose serum medium ("LIT"): This liquid monophasic culture medium was formerly devised by YEAGER * and contains: liver infusion, tryptose, calf serum, hemoglobin solution, glucose and various salts. Before adding hemoglobin the mixture is filtered through a fine Seitz filter pad and heated at 68°C for one hour.

Calf serum and liver infusion broth are stored frozen. Throughout the present experiments, we employed these components from the same original batch. For different batches of the liver infusion broth, which is prepared at our laboratory from fresh calf livers, the adequate volume to be employed should be previously established, by testing the growth response of the cultures. The medium is not autoclaved as originally suggested by YEAGER because results are more reproducible this way.

Lactoalbumin serum medium ("LAS"): This medium consists of: sodium chloride 4.0 g.; potassium chloride 0.4 g.; dissodium hydrogen phosphate 8.0 g.; glucose 2 g.; lactoalbumin hydrolisate * 5.0 g.; calf serum 100 ml; bidistilled water q.s.p. 1,000 ml. The pH is adjusted to 7.2 with HCl and then the mixture is filtered through a fine Seitz filter pad, heated to 68°C for one hour, and frozen. Before use, the medium was enriched with deoxyadenosin ** at a final concentration of 10^{-5}.

Cultures — Cultivation was performed in 250 ml. Erlenmeyer flasks containing 50 ml. of the "LIT" medium; when "LAS" medium was used, ordinary 25 ml. capacity bottles with 5.0 ml. of medium were employed.

To keep cultures in a steady logarithmic multiplication rate, daily inoculations were made; new cultures were seeded to give original densities of 15×10^6 organisms per ml. of fluid. When the flagellates were transfered from the old medium to the fresh one with same composition, a small volume of the original medium was brought in with the inoculum. When, however, a fresh medium of different composition was to be used or when any amount of the former culture medium was undesirable, the sample was subjected to 10 minutes centrifugation at 400 g. and twice washed in the medium before being seeded.

Throughout the experiments the culture flasks were incubated at 28°C and agitated for one minute at ten minute intervals in a mechanical shaker.

Flagellate counts — Samples of the cultures were diluted in citrate saline solution, carefully homogenized and immediately brought to a Coulter Electronic Counter (model A with a 100 μ orifice), threshold 5, for determination of the total number of organisms present.

Metacyclic forms were counted differentially in a Neubauer counting chamber, after diluting the samples in cold physiological solution.

* Personal communication through Dr. J. F. Fernandes, 1962.

* Nutritional Biochemicals Corp., USA.

** California Foundation for Biochemical Research, USA.

RESULTS

Experiments in "LIT" medium — A typical growth curve of *T. cruzi* in "LIT" medium under intermitent shaking at 28°C, is represented in Figures 1 and 2. In cultures with an initial count of 7.5×10^6 flagellates per ml. of medium, the growth phase covered a period of four days, merging then to a stationary phase where the population density attained 1.5×10^8 individuals per ml., the slope of this curve being similar to those obtained by FERNANDES & CASTELLANI *. Usually, during the logarithmic phase of multiplication, the bulk of the population was made up by crithidial forms, metacyclic trypanosomes being very rare. The number of metacyclic forms eventually introduced with the inoculum remained initially constant (Fig. 1) but its relative proportion gradually decreased due to the high multiplication rate of the crithidiae (Fig. 2). However, when the culture attained its final phase of exponential growth, the number of metacyclic trypanosomes increased steadily, reaching after 72-96 hours its highest level with a total number of $3.0-4.0\times10^7$ flagellates per ml., or about 25% of the total count.

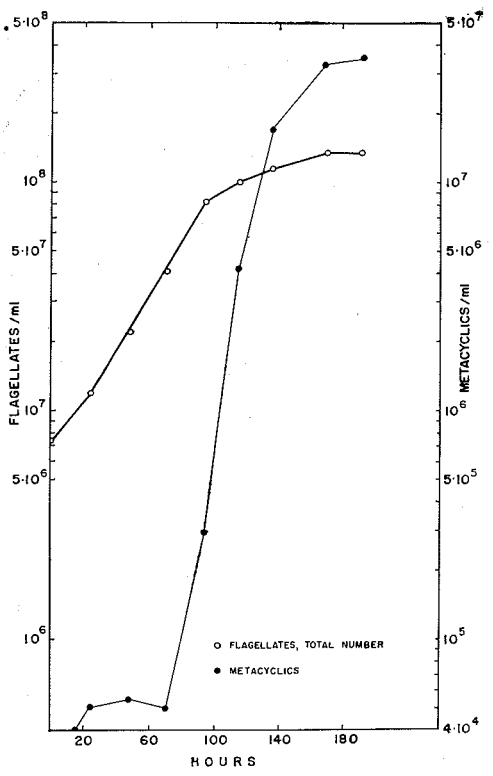

Fig. 1

Fig. 1 — Curves of growth and differentiation of *T. cruzi* in LIT medium. Open circles represent total number of flagellates. Solid circles those of metacyclic trypanosomes.

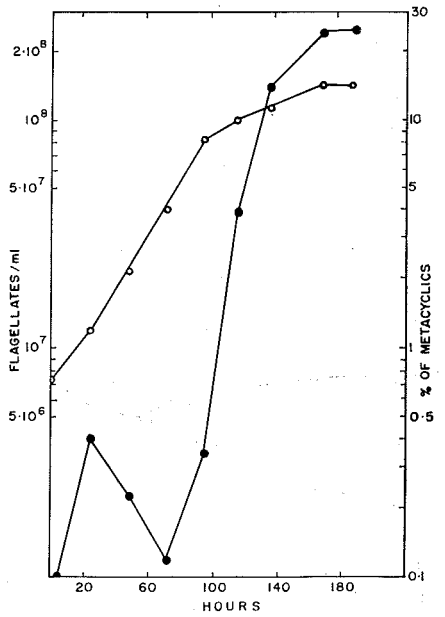

Fig. 2

Fig. 2 — The same surves as in Fig. 1, but with metacyclic trypanosomes expressed in %.

Cultures with original densities ranging from 3 to 20×10^6 organisms per ml. had similar growth curves, the metacyclic forms quickly increasing in number after the logarithmic growth phase which, however, in such instances could cover shorter or longer periods of time. Identical results were observed when cultures of different ages, taken at different stages of the growth curve, were employed as inocula.

When culture were maintained in permanent logarithmic growing rate by daily subculturing, a metacyclic peak never occurred (Fig. 3), the amount of metacyclic forms being held insignificant. If, on the other hand, crithidiae were transferred to fresh culture medium at the end of the exponential phase but before evolution to metacyclic forms had taken place, a small peak of metacyclic forms could be found in the new medium. Nevertheless, after this first upturn, production of

* Personal information.

metacycles stopped (Figs. 1 and 2), so their proportion decreased.

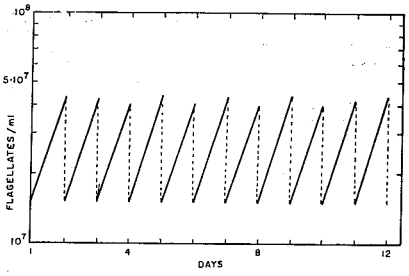

Fig. 3

Fig. 3 — Permanent exponential growth of *T. cruzi* in LIT medium by daily subculturing. Initial concentration of flagellates: 15×10^6/ml.

In experiments which are not discussed in the present paper, autoclaved media and liver infusion broth of different batches were employed. A certain variation in the final proportion of metacyclic forms could be observed, depending on the liver infusion used and on the conditions of the autoclaving. However, the fundamental characteristics of the process remained unaltered in all instance, a sudden mass production of metacyclic forms always appearing only at the end of the logarithmic growth phase.

Experiments in "LAS" medium — The growth curves shown in Figs. 4 and 5 were obtained when we transferred to "LAS" medium, samples from cultures kept in continuous exponential groth in "LIT" medium. These graphs show that this medium does not yield so abundant growth of organisms if compared to "LIT"; in "LAS" cultures with an original population density comparable to that of "LIT" medium represented in Graph 1, the stationary growth phase was attained at levels always lower. The point at which the logarithmic growth phase merged into the stationary phase was dependent on the original density of the culture: with a larger number of flagellates in the inoculum the number of generations was slightly smaller. On the other hand, as shown in Fig. 5, the progressive production of trypa-

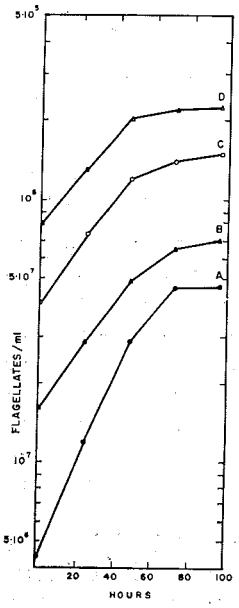

Fig. 4

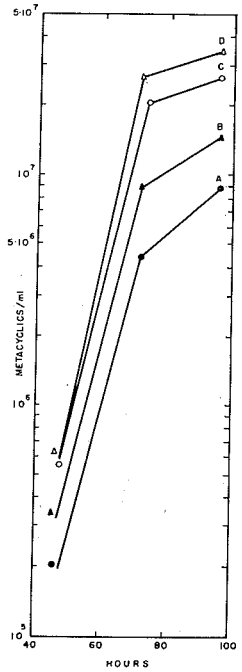

Fig. 5

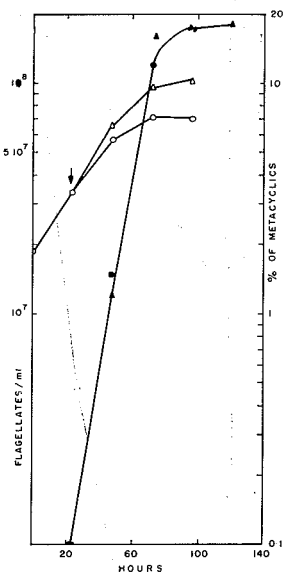

Fig. 6

Fig. 4 — Growth in LAS medium. Initial concentrations (flagellates/ml) in cultures A, B, C and D were respectively 4.5, 16.0, 40.0 and 80.0×10^6.

Fig. 5 — The same cultures as in Fig. 4 but with number of metacyclic trypanosomes plotted against time.

Fig. 6 — Transference (arrow) of a 48 hs LAS culture to fresh LAS medium. Open and solid circles represent respectively flagellates total numbers and metacyclic trypanosomes % in the starting culture. Open and solid triangles represent flagellates total numbers and metacyclic trypanosomes % in the transfered culture.

nosome forms in "LAS" cultures, started 24-48 hours after seeding, reaching its highest levels in 72-96 hours. The number of metacyclic forms at the peaks of the growth curve was higher in cultures started with an originally higher density of organisms but their relative proportion in the population in all instances still averaged 18%.

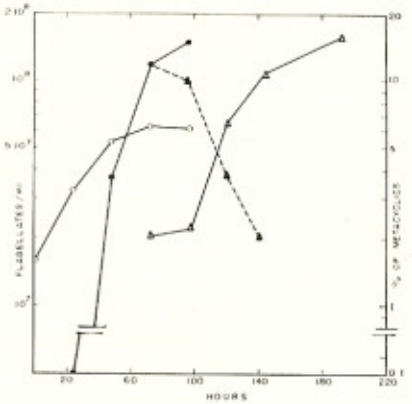

Fig. 7

Fig. 7 — Transference of a 72 hs LAS culture to fresh LIT medium. Open and solid circles represent respectively flagellates total numbers and metacyclic trypanosomes % in LAS medium. Open and solid triangles represent flagellates total numbers and metacyclic trypanosomes % in LIT medium.

No changes were observed in the growth rate when samples of flagellates raised in "LAS" medium at various stages of development were transferred to fresh similar medium, the production of metacyclic forms proceeding normally (Fig. 6), with a slope similar to that of the original cultures.

However, if samples of cultures originally raised in "LAS" medium were transferred at any stage of development to "LIT" medium, growth was stimulated as shown in Fig. 7, but the number of trypanosomes became steady, its percentage gradually decreasing until the phase of logarithmic increase of the new culture merged into the stationary phase.

In "LIT" medium cultures seeded with organisms originally maintained for less than 24 hours in "LAS" medium, when the metacyclic production had not yet started or was just incipient, the same result shown in Fig. 2 was obtained: there was a slight peak in the metacyclic production rate, their relative proportion gradually decreasing.

Morphological data — Microscopical examination of samples of the culture allowed us

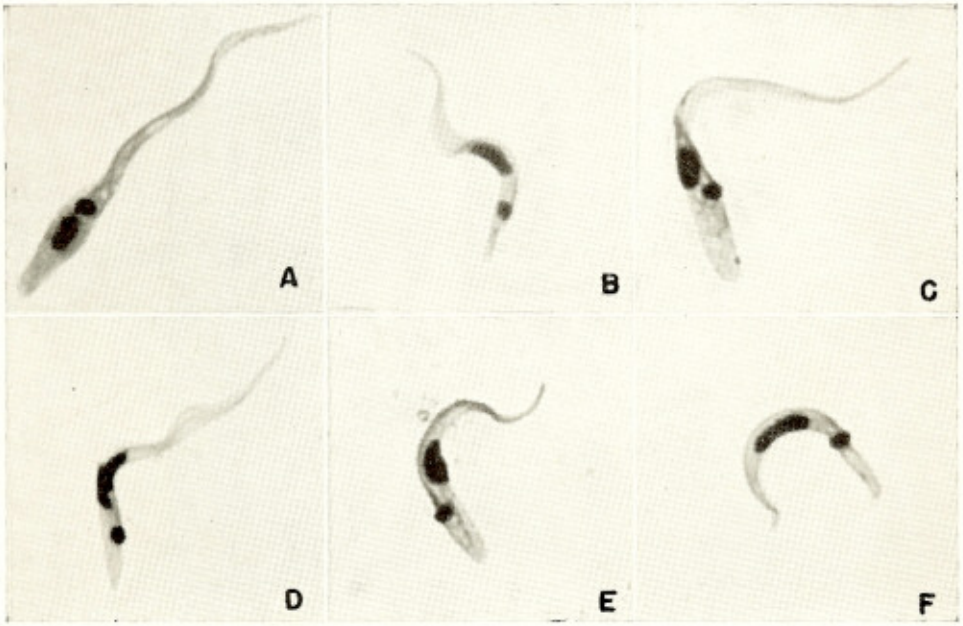

Fig. 8

Fig. 8 — Transitional forms from crithidiae to metacyclic trypanosomes: a — crithidia; b — metacyclic trypanosome; c-f — transitional forms.

to state that during the phase of logarithmic increase, metacyclic forms are very rare, crithidial forms prevailing due to an accelerated process of binary fission. When the phase of logarithmic increase is over, the dividing crithidiae become less frequent while organisms metamorphosing from crithidial forms to trypanosomes began to appear. Many transitional forms showing intermediate characteristics between crithidial and metacyclic forms occurred during this period.

Typical crithidiae (Fig. 8a) with long and large body, short undulating membrane starting anteriorly to the nucleus, spherical nucleus, rodlike kinetoplast anterior to the nucleus, and long, free flagellum, are to be found along with typical metacyclic trypanosomes (Fig. 8b), with slender body, long undulating membrane arising near the posterior tip, fusiform nucleus, kinetoplast round and posterior to the nucleus and a short flagellum. The way crithidiae and typical metacyclic trypanosomes move about in fresh preparations is also different: the former present a rigid, oriented type of movement while trypanosomes have wavy and more active movements. During the short period immediately preceding the stationary growth phase of the culture, all possible morphological combinations between crithidial and trypanosome characteristics could be found Fig. 8c, d, e, f).

We could not infer any ordered sequence out of such transformations, which apparently occur without a previous determinism of strictly morphological order.

Although very seldom, metacyclic forms undergoing binary fission could also be seen.

More numerous than these, however, were division processes where one of the daughter cells presented characteristics more or less defined as metacyclical while the other was still a typical crithidia or a transitional form (Fig. 9a, b). Another kind of dividing form seen, was that of metacyclic trypanosomes fully formed but still linked in pairs by their posterior tip as if they were undergoing final binary fission (Fig. 9c).

DISCUSSION

Since BRUMPT [1] proposed his outline on the biological cycle of *T. cruzi*, it has been generally accepted that the so called metacyclic trypanosomes originate from crithidiae by a process where there is essentially stretching of the undulating membrane and migration

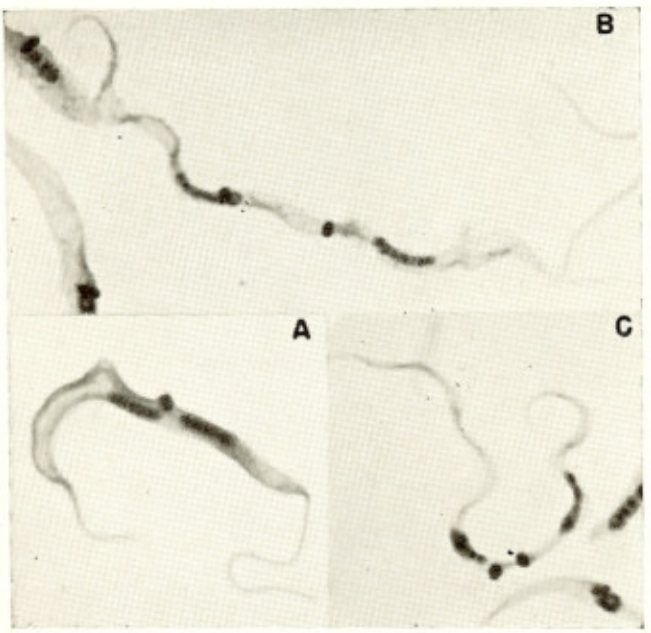

Fig. 9

Fig. 9 — Suggested sequence of dividing and simultaneously transforming crithidiae.

of the kinetoplast to a posterior position in relation to the nucleus. The metacyclic trypanosomes would not undergo binary fission or, at least, this would not be the usual manner through which their number is increased in cultures or in the invertebrate host. Some authors presented different views, mainly ELKELES [6] and NOBLE [12] who ascribed their origin from special "leishmanoid" forms.

Our experiments have shown that the increase in metacyclic forms in the flagellate population of a culture actually results from the metamorphosis of crithidial forms. This is supported by the abundant transitional organisms with intermediate characteristics between crithidial and metacyclic forms present at the end of the exponential growth of the cultures accompanied by an increase in the total metacyclic population. The total absence of leishmaniae, in our media, excludes, at least in this case, the origin of metacyclic trypanosomes from them.

Metacyclic trypanosomes undergoing binary fission did also occur in our cultures but with such a low frequency that this could not surely account for the total increase observed in their number. On the other hand, dividing forms in which one of the daugther cells still kept crithidial or transitional characteristics while the other was almost a metacyclic, occurred more frequently. Metacyclic trypanosomes fully formed but still linked in pairs by their posterior tips, as mentioned, could suggest the metamorphosis of newly divided crithidiae (Fig. 9a, b, c).

It is known that in diphasic culture media the metacyclic forms occur or make their appearance in aged cultures. Unfortunatelly criteria for "aged culture" are not uniform among different workers, but it seems to us that the term is usually applied to cultures where the growth phase came to a halt, i.e., entered its stationary phase. In our experiments, indeed, the highest numbers of metacyclics were always found in cultures at stationary growth phase; they were not detected in appreciable numbers during the log phase or in cultures kept in exponential growth by daily subculturing. One might conclude therefore that under favorable conditions the crithidiae may indefinitely multiply in this stage, their transformation to trypanosomes not being compulsory, but occurring only under special conditions.

Data from other organisms seem to be pertinent here. Thus, in the same way, sporulation in bacilli [7, 8] only occurs in the stationary phase of growth or in conditions of starvation; in *Tetrahymena patula* [16] the differentiation from microstomes to macrostomes occurs only in the stationary phase.

In the case of *T. cruzi* this could be explained either by: a) reduction of some nutrient component necessary for the culture growth in the medium or in the metabolic pool of the organisms or in both, this would bring about differentiation through some sort or metabolic channelling; b) accumulation of some metabolite in the medium of "aged" cultures that would induce differentiation "per se".

Our results have shown that differentiation is prompted by growth in the poorer "LAS" even when the cells were previously grown in the richer "LIT" medium. It was also shown that differentiation is inhibited when cells are transferred from "LAS" to "LIT" medium, but not when transfered from LAS to LAS (Figs. 6, 7). All this is consistent with hypothesis a). However a final choice must await further experimentation, now under progress.

In other trypanosomes the first hypothesis does not explain all the experimental data. In *T. mega* STEINERT [15] has shown that the rate of differentiation is greatest at the stationary phase of growth, but only occurs when cells were transfered to a medium containing serum, or more specifically, urea [14].

In *T. conorhini* [3, 4, 5] differentiation into blood forms occurred only after environmental temperature had been increased.

The last question to be considered here is that of differentiation rates, which are always low in trypanosomes as compared to other organisms. Thus in *T. mega* [15], although diferentiation is proportional to the amount of serum used, its rate is not greater than 10%, only in one instance being around 50%. Similary in *T. cruzi* the rates usually do not exceed 30% (in some instances we can get 50% *).

This could be due to the presence in the cultures of crithidiae with different genetic potentialities; to the occurrence of limited amounts of specific metabolites necessary for

* Umpublished data.

differentiation; or to the incomplete exhaustion of some substance in the metabolic pool of most of the organisms which would thereby not be able to differentiate.

Progress in the this field is difficult because of the complexity and lack of definition of the media used. Development of a defined medium that would support growth and differentiation or at least one of these processes could give answer to some of the questions raised in this paper.

RESUMO

Crescimento e diferenciação em Trypanosoma cruzi; *I. Origem dos tripanosomas metacíclicos em meio de cultura líquido.*

O crescimento de culturas de critídias de *T. cruzi* e a diferenciação destas em tripanosomas metacíclicos são estudados em dois meios líquidos, um completo, suportando subculturas outro incompleto, os resultados sendo expressos em função da contagem de flagelados totais e do número de metacíclicos das culturas.

Em ambos os meios a diferenciação de critídias em metacíclicos ocorre apenas ao final da fase de crescimento exponencial das culturas. A diferenciação não se observa em culturas de critídias mantidas, por repiques diários, em crescimento exponencial permanente. No meio incompleto, que suporta menor crescimento, a diferenciação é mais precoce. A transferência de culturas em início de diferenciação para meio completo, nôvo, interrompe o processo de diferenciação, o mesmo não ocorrendo quando da transferência para meio incompleto nôvo, onde a diferenciação tem prosseguimento sem interrupções. É sugerido que o esgotamento de um ou mais fatôres do meio ou do "pool" interno das critídias seja indispensável para que a diferenciação ocorra.

Dados morfológicos mostrando a diferenciação de critídias para metacíclicos são apresentados.

REFERENCES

1. BRUMPT, E. — Le *Trypanosoma cruzi* évolue chez *Conorrhinus megistus, Cimex lectularius, Cimex boueti* et *Ornithodorus moubata*. Cycle évolutif de ce parasite. *Bull. Soc. path. éxot.* **5**:360-367, 1912.
2. CHANG, S. L. & NEGHERBON, W. O. — Studies on haemoflagellates. II. A study of the growth rates of *Leishmania donovani, L. braziliensis, L. tropica* and *Trypanosoma cruzi* in culture. *J. Inf. Dis.* **80**:172-184, 1947.
3. DEANE, M. P. & DEANE, L. M. — Studies in the life cycle of *Trypanosoma conorrhini*. "In vitro" development and multiplication of the blood-stream trypanosomes. *Rev. Inst. Med. trop. São Paulo* **3**:149-160, 1961.
4. DEANE, M. P. & KIRCHNER, E. — Life cycle of *Trypanosoma conorrhini*. Influence of temperature and others factors on growth and. morphogenesis. *J. Protozool.* **10**:391-399, 1963.
5. DESOWITZ, R. S. — The development and survival of the blood-stream forms of *Trypanosoma conorrhini* in culture. *J. Protozool.* **10**:390-391, 1963.
6. ELKELES, G. — On the life cycle of *Trypanosoma cruzi*. *J. Parasitol.* **37**:379-386, 1951.
7. FOSTER, J. W. — Morphogenesis in bacteria: some aspects of spore formation. *Quartely Rev. Biol.* **31**:102-118, 1956.
8. HALVORSON, H. — Physiology of sporulation. In: GUNSALUS, I. C. & STANIER, R. Y., eds. — *The Bacteria*. New York, Acad. Press, 1962, vol. IV, págs. 223-264.
9. MAYER, H. & OLIVEIRA, M. X. — Cultivation of *Trypanosoma cruzi* in tissue cultures: a four-year study. *Parasitology* **39**:91-94, 1948.
10. MUNIZ, J. & BORRIELO, A. — Estudo sôbre a ação lítica de diferentes soros sôbre as formas de cultura e sangüícolas do *Schizotrypanum cruzi*. *Rev. brasil. Biol.* **5**:563-576, 1945.
11. NOBLE, E. R. — The morphology and life cycles of trypanosomes. *Quart. Rev. Biol.* **30**:1-28, 1955.
12. SILVA, L. H. P. & NUSSENZWEIG, V. — Sôbre uma cêpa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folia clin. et biol.* **20**:191-208, 1953.
13. SILVA, L. H. P. — Observações sôbre o ciclo evolutivo do *Trypanosoma cruzi*. *Rev. Inst. Med. trop. São Paulo* **1**:99-118, 1959.
14. STEINERT, M. — Action morphogénétique de l'urée sur le trypanosome. *Exptl. Cell Res.* **15**:431-433, 1958.
15. STEINERT, M. — Études sur le determinisme de la morphogénèse d'un trypanosome. *Exptl. Cell Res.* **15**:560-569, 1958.
16. STONE, G. E. — Polymorphic properties of *Tetrahymena patula* during growth in axenic culture. *J. Protozool.* **10**:74-80, 1963.
17. TRAGER, W. — Tsete-fly tissue culture and the development of trypanosomes to the infective stage. *Ann. Trop. Med. & Parasitol.* **53**:473-491, 1959.

Recebido para publicação em 3 janeiro 1964.