Rev. Biol. Trop., 19(1, 2): 197-210, 1971

Cultivation and transformation of hemoflagellates. A review*

by

Rodrigo Zeledón**

(Received for publication, July 23, 1971)

Although the rat trypanosome, *Trypanosoma lewisi,* was obtained in pure culture about 70 years ago, and in spite of valuable information accumulated during all these years concerning the nutrition and cultural characteristics of several hemoflagellates, we have to recognize that there are still many gaps on the subject and that a great deal of knowledge is lacking in order to understand and solve several problems concerning these organisms.

DEFINED MEDIA

In general, the nutritional requirements of hemoflagellates are poorly understood and only a few monogenetic organisms of the family Trypanosomatidae *(Crithidia fasciculata, C. oncopelti, Blastocrithidia culicis, Leptomonas* spp.) and three digenetic species *(Leishmania tarentolae, Trypanosoma ranarum* and *T. mega)* have been cultured in completely defined media with reproducible results (TRAGER, 53; BONÉ *et al.,* 7). The only mammalian trypanosome cultivated in a partially defined medium with some success is *Trypanosoma cruzi* (CITRI & GROSSOWICZ, 13; BONÉ & PARENT, 5). Even Citri & Grossowicz's medium does not seem suitable for all strains of the parasite, and contradictory results have been obtained in different laboratories; they claim that the medium is suitable for *Leishmania tropica* but not for other species of *Leishmania.* PITTAM'S medium (41) is a good start towards a synthetic medium for the cultivation of *T. rhodesiense* and *T. brucei,* but the yields are too small and the flagellates

* Supported in part by U.S. Public Health Service Research Grants AI-05938 and TW00148 from the National Institute of Allergy and Infectious Diseases.

** Department of Parasitology, University of Costa Rica, and Louisiana State University International Center for Medical Research and Training (LSU-ICMRT), San José, Costa Rica.

cannot be maintained indefinitly, indicating that more efforts must be made along this line. In all these cases of cultures of the digenetic members of the family, the only forms obtained are those corresponding to the morphological types found in the invertebrate vectors.

NUTRITIONAL DIFFERENCES

The data accumulated permit establishment or prediction of some gradation on the nutritional complexity of these organisms, beginning with the insect monogenetic forms, the least exacting of them all. Even in this case some of the most primitive types have different requirements and a compound, such as hematin, almost universally needed by the members of the group, is apparently synthesized by *Crithidia oncopelti* (LWOFF, 37). The peculiar ability of this organism to synthesize certain compounds has been attributed to the presence of bacterial endosymbionts. However, NEWTON (39) is of the opinion that neither the bacterial identity of the "polar bodies", nor their biosynthetic ability have been demonstrated unequivocally. It has been hypothesized that members of the genus *Leptomonas* are probably phylogenetically ancestral to the other forms of the family (BAKER, 4). However, on a nutritional basis, they are very similar to *Crithidia* and *Blastocrithidia* and in some instances slightly more exacting than these (GUTTMAN, 27). Those flagellates, either of the genus *Leishmania* or *Trypanosoma,* that parasitize cold-blooded animals, probably occupy the next step in the nutritional evolutionary trend. A step further will be represented by a primitive mammalian trypanosome, such as *T. cruzi,* followed by other members of the Stercoraria group and by some of the mammalian species of *Leishmania,* and the sloth parasite *Endotrypanum.* Finally, the most exacting forms of all are the Salivaria group, in which some species cannot be cultured, apparently because they cannot revert to the more primitive invertebrate forms.

T. cruzi will grow in an autoclaved liquid medium where *T. rangeli* will not (ZELEDÓN, 59). When a dialysis sac containing whole blood is suspended in this medium *T. rangeli* will grow, indicating that this organism requires some thermolabile dialyzable factors. Furthermore, *T. cruzi* and its close relative *T. vespertilionis* have been cultivated by Krassner at least for a few transfers in Trager's defined medium for *L. tarentolae,* to which stearate has been added (TRAGER, 53).

We are not going to consider here the known growth factors for some of the members of the group and their probable nutritional role, since the subject has been reviewed recently by TRAGER (53), and by NEWTON (39).

CULTIVATION AND TRANSFORMATION

At present, not even in complex media with whole blood or with several of its elements, have we found a way to maintain and reproduce indefinitely most of the hemoflagellates in their vertebrate parasitic forms. A better knowledge

of the complex nutrients required by the parasites and of the physicochemical and physiological conditions related to, or involved in transformation, as well as of the particular contributions by the host, is needed in order to achieve this goal. An interesting step along this line has been taken by STEINERT (49, 50) who determined that urea, present in toad serum is responsible, in great part, for the transformation of the cultured forms of a toad trypanosome, *T mega,* into typical blood stream trypomastigotes. Some physicochemical factors apparently enhance the effect of urea on this transformation. The problem is even more complex with trypanosomes of warm-blooded animals. In *T. theileri* and *T. conorhini,* from the cow and rat respectively, and probably in others, high temperature plays an important role in the maintenance or appearance of blood stream forms *in vitro,* as shown by RISTIC and TRAGER (42) with the first species and by DEANE and DEANE (15) and DESOWITZ (19), with the second. Repeated cultivation of these forms of *T. conorhini* by succesive transfers was not achieved; on the other hand, low temperature alone, in the absence of whole blood or red cells, is not enough for the transformation of blood stream forms into epimastigotes (DEANE & KIRCHNER, 16). SPLITTER and SOULSBY (48) cultured and subcultured *T. theileri* continuosly at 37 C, and observed that epimastigotes increased proportionately as cultures aged beyond six days. This was associated with a marked fall of the pH. Starting with epimastigotes obtained in cultures at 27 C they were able to produce trypomastigotes when subcultured and incubated at 37 C. More recently, SÁNCHEZ and DUSANIC (44) mimicked the blood stream phases of *T. lewisi* in a monophasic culture medium at 27 C. Hemoglobin and peptone were essential for a growth that, in any event, could not be maintained after the third transfer. Optimal laboratory temperature constitutes a striking physiological difference as compared with that of true blood stream forms.

Perhaps with the exception of *T. cruzi,* the agent of Chagas' disease, no other mammalian trypanosomes have been obtained in their blood stream forms *in vitro* on a more or less continuous basis, in spite of different efforts by several investigators. GORDON and WILLET (22) were able to produce slender blood stream trypomastigote types at 37 C in glucose-Ringer solution plus 56 C inactivated rabbit serum, from stumpy metacyclic trypanosomes of *T. rhodesiense* from tsetse flies. Even in tissue cultures, *T. gambiense* will produce the insect forms and a rise in temperature to 37 C will kill the flagellates (DEMARCHI & NICOLI, 18; FROMENTIN, 20). An interesting advance along this line has been offered by LE PAGE (36) who was able to maintain blood forms of *T. brucei* for 20 to 30 hours in a tissue culture system at 37 C. Young parasites from mice were able to multiply three-fold *in vitro* only in the presence of cells. When the temperature is raised to a certain level in tissue cultures, *T. cruzi* trypomastigotes from common cultures will penetrate the cells and become amastigotes, since this is an essential phase in the cycle of this species. The same happens with the closely related species *T. vespertilionis* from the bat. In the former species, according to NEVA *et al.* (38), intracellular trypomastigotes cannot appear at 38 C but occur at 33 C and at this latter temperature a cyclical infection is established when the flagellates invade the cells and become amastigotes again.

On the other hand, TREJOS *et al.* (54) found that amastigotes transform into stout trypanosomes at 37 C, whereas at 26 C slender forms are produced both outside and inside the cell. The physiological identity of these trypanosomes with the trypomastigotes obtained in vertebrate hosts should be confirmed.

Organisms of the genus *Leishmania* are rather easily obtained in the parasitic form in tissue culture, but we know practically nothing of their requirements during this phase. Promastigotes are easily killed in common culture media by raising the temperature above 32 C, whereas in tissue culture they might survive a little longer, suggesting some protective mechanism on the part of the cells (BISHOP, 5). HAWKING (28), and more recently HERMAN (30) have shown that in tissue culture supplemented with certain complex substances, transformation of amastigotes of *L. donovani* into promastigotes can be obtained at 37 C without a change in temperature. TRAGER (51) observed that when amastigotes of *L. donovani* from hamster spleen were maintained *in vitro* at 37 C in a medium of human erythrocyte extract and hamster or human serum, most of them transformed into intermediate forms between amastigotes and promastigotes that multiplied and lived for a few days. This led him to suggest that rounded intermediate organisms and promastigotes are more exacting in their nutritional requirements at 37 C than promastigotes grown at 28 C. Furthermore, it is a well known phenomenon that some organisms need additional growth factors at higher temperatures; this seems to be the case with the promastigotes of some of the *Leishmania* (GUTTMAN, 26; TRAGER, 53). SIMPSON (47), studying the transformation process of amastigotes of *L. donovani* in a basic defined medium after a lowering in temperature, was able to observe a need for certain amino acids, an energy source (glucose) and probably other substances during the process. JANOVY (31) and also SIMPSON (47) observed an increase in the respiration rate during this transformation and correlated it with proliferation of new mitochondrial profiles, as shown by RUDZINSKA *et al.* (43) in electron microscopy, and with the appearance of porphyrin-containing compounds (KRASSNER, 32). On the other hand, LEMMA & SCHILLER (35) obtained the transformation of three species of human *Leishmania* promastigotes into "amastogotes" in a cell-free nutrient blood agar medium with Hank's solution, by acclimatization of the flagellates to higher temperatures. Detailed morphological and physiological studies on these round forms will be necessary before concluding that they are equivalent to the parasitic forms, or whether they correspond to the "sphaeromastigotes" observed by BRACK (8) in *T. cruzi.* As discussed by TRAGER (53), infectivity plus Granich's porphyrin test, oxygen uptake and electron microscopy will yield useful information in these cases, in order to determine which forms we are dealing with.

FROTHINGHAM and LEHTIMAKI (21) found that temperature and/or the type of serum affect the form (rounded or elongated) of intracellular *L. donovani* and *L. braziliensis pifanoi* in tissue culture. LAMY (33) and LAMY *et al.* (34), working with *L. donovani* and *L. tropica* in tissue culture, came to the conclusion that transformation of promastigotes into amastigotes and viceversa could be obtained at low temperatures (22-25 C) and that the intracellular

condition is probably the main factor involved in the phenomenon. Nevertheless, higher temperature and other factors will contribute to a more extensive achievement of this transformation. These points have been confirmed in *L. donovani* by AKIYAMA and TAYLOR (1) who have stated that: "temperature appeared to be a selective, rather than an inductive, factor in the transformation process".

By serial passages of *T. cruzi* forms in three enriched cell-free tissue culture media, PAN (40), was able to obtain a predominance of amastigotes, even at temperatures as low as 24.5 C. Higher temperatures were important only when components such as chick embryo extract and chicken plasma were omitted. This again could be an indication that under certain conditions, a transformation of this sort may take place, with physical factors such as temperature, playing a very small role, or none at all. Nevertheless, as mentioned above, whether the rounded organisms obtained under such conditions correspond both morphologically and physiologically to amastigotes, is something that has to be proved. It could very well be that this is only a rounding phenomenon that occurs to the flagellates under certain external conditions not necessarily identical to that which takes place *in vivo*. Very recently DEANE and KLOETZEL (17) were able to show that in tissue culture dyskinetoplastic epimastigotes of *T. cruzi,* that cannot be subcultured, can transform into metatrypomastigotes; from these, they obtained dyskinetoplastic amastigotes that were able to multiply. It is assumed, but apparently not observed, that these amastigotes are able to produce only slender trypanosomes. In the case of *Leishmania donovani,* it has been observed that dyskinetoplastic amastigotes obtained with acriflavin are unable to produce common culture flagellates (HERMAN, 30).

The inability of dyskinetoplastic forms to revert to the more primitive flagellate forms strongly suggests a role of kinetoplast DNA in the synthesis of some important enzyme systems (NEWTON, 39). SILVA and CAMARGO (46) suggested that the transformation processes in hemoflagellates are probably controlled by an operon system of the type propossed by Jacob and Monod, and that different physical or chemical factors act as inductors or co-repressors in the phenomenon.

of the so-called insect forms, and their transformation in the invertebrate vector, would be to study in greater detail the physiology and composition of the vectors,

INSECT TISSUE CULTURE AND TRANSFORMATION

Another important approach to the problem of nutritional requirements and particularly of some of their fluids. The substances that predominate in the alimentary tract, hemolymph and salivary glands of the different invertebrates involved in the transmission of hemoflagellates will no doubt yield very important clues. TRAGER (52) has approached this problem by successfully culturing *T. vivax* (one of the species that cannot be cultured in common media) in an insect cell tissue culture, and under certain conditions, was able to reinfect sheep with it. *T. brucei* was also grown in the same medium, producing similar

forms to those of *Glossina,* but which were not infective for vertebrate animals.

More recently WOOD and PIPKIN (57) have been able to study growth and differentiation of *T. cruzi* in Grace's insect cell culture system at 28 C. Blood trypanosomes are transformed into rounded amastigote forms within 48 to 72 hours, and after a period of division, into epimastigotes, and later into metatrypomastigotes. Increasing amounts of hemolymph improve transformation of the epimastigotes into metatrypomastigotes, suggesting that one or more substances influence the process. Further work is necessary along this line.

We do not know what substances and conditions, either in the intestine or salivary glands of these invertebrates, mediate in production of metacyclic trypanosomes. We do know, for instance, that in culture tubes, the appearance of metacyclic trypanosomes of *T. cruzi* require that the culture reach the stationary phase of growth. The reduction of one or more nutrient components, a drop in the pH, age of culture, and the initial size of the inoculum are important in this respect (CAMARGO, 11; CASTELLANI *et al.,* 12). In *T. rangeli* the situation is still less clear. Small metacyclic forms are produced in the salivary glands of species of *Rhodnius* and experimentally in *Triatoma* (A. D'ALESSANDRO, personal communication) and it is possible that the saliva contributes one or more substances that play an important role in morphogenesis. Several years ago it was suggested that one of these substances could be the hematinic pigment stored in the salivary glands of *Rhodnius,* accounting for their typical cherry-color (ZELEDÓN, 58). This pigment seems to become exhausted in heavily parasitized glands, giving them a pink or whitish color.

PHYSIOLOGICAL DIFFERENCES WITHIN THE SAME SPECIES AND TRANSFORMATION

Besides the well-known nutritional and metabolic differences between blood stream and insect forms, especially in some of the African trypanosomes, some variation may be expected between morphologically different blood stream forms in the case of the polymorphic trypanosomes. Short-stumpy and intermediate forms have a more developed mitochondrial apparatus, closely associated with the kinetoplast, that gives a positive reaction to NAD-diaphorase, suggesting, as in the case of insect forms, that the organelle may intervene in electron transport. Slender forms, like monomorphic forms, show no mitochondrial activity. Electron microscopy shows that this organelle is not very well developed (VICKERMAN, 55). Metabolic and nutritional studies on the adequately separated short-stumpy and long-slender forms will be very desirable. For this purpose, it would be necessary to learn to produce the two types of trypanosomes *in vitro.*

Since the flagellates in the tsetse fly are subjected to quite different conditions and are forced to switch to a different physiological pattern, only the short-stumpy forms seem to be capable of evolving in insects and in cultures, apparently due to their preadaptation to a mitochondrial-mediated respiration (WIJERS &WILLET, 56; VICKERMAN, 55). This explains the failure of syringe-

passed trypanosomes to render positive cultures, since in these cases only slender forms are found in the blood. This may also be the case of *Trypanosoma cruzi*, which behaves like a polymorphic trypanosome (SILVA, 45; BRENER & CHIARI, 10). Important biological differences are present in both types of trypanosomes as shown by SILVA (45) and by BRENER (9). Nevertheless, in this case, broad and slender forms are generally found together in the blood of animals, even after multiple passages in the laboratory. The nutritional implications of the phenomenon are obvious and detailed physiological and ultrastructural studies of both types of trypanosomes are desirable.

CULTIVATION AND INFECTIVITY

A phenomenon apparently closely related to nutrition is the loss and reacquisition of infectivity on the part of some cultured forms. As explained by AMREIN *et al.* (3), in the case of trypanosomes of the brucei group, it is not related to the addition of simple substances as previously believed. It is rather a complex problem in which apparently different factors enter into consideration. Besides the age of the culture and the length of storage time of the media before use, it is an interesting fact that blood from different donors, even from individuals within the same species, behaves differently regarding the effect to restore infectivity. AMREIN and HANNEMAN (2), based on the fact that the mixture of suitable and unsuitable bloods does not permit reacquisition of virulence, suggested that inhibitory factors, rather than those of nutrition, are involved in the phenomenon. Considerably more work is necessary to establish the factor or factors related to this phenomenon and it would be desirable to extend the studies to a greater number of strains, maintained in cultures for differents periods, in order to draw more general conclusions. As pointed out before, several years ago TRAGER (52) was able to restore infectivity in *T. vivax* grown in a complex insect tissue culture medium and previously exposed overnight to 38 C; he also stressed the difficulty to define precisely the conditions required to induce infectivity in this case. ZELEDÓN *et al.* (60) found that infectivity of *L. braziliensis* strains in common culture media does not seem to be related to the growth phase of the flagellates; starved epimastigotes showed a noticeable decrease in infectivity.

NUTRITIONAL REQUIREMENTS AND HOST SPECIFICITY

Closely related to nutritional aspects of the blood stream forms of some trypanosomes is the phenomenon of specificity of the parasite to a certain host. Both immunological and nutritional mechanisms seem to act in the definition of the rat and mouse host-parasite relationships with the *T. lewisi* system. Recent work (GREENBLATT & SHELTON, 25) suggests that rat serum is richer in substances that promote growth of *T. lewisi* in vertebrates than mouse serum. Although the substances responsible for this growth are present in mouse serum, unless this animal is supplemented with additional amounts of these factors, im-

munological mechanisms will probably preclude infection. By fractionation of proteins, GREENBLATT *et al.* (23) were able to show that gamma-2 and gamma-M globulin fractions are not responsable for the phenomenon as previously thought (GREENBLATT & LINCICOME, 24). The more purified active protein fractions involved have been separated by physicochemical methods but have not yet been characterized. In any event, it is not clear at present whether this activity is due to a direct effect on the parasites or to an indirect blockage of the mouse defense systems. COSGROVE *et al.* (14) were not able to infect chimaera mice (in which the hemopoietic systems had been replaced by a rat type) unless they were supplemented with rat serum, adding further support to the nutritional explanation of the phenomenon. Experiments seeking suppression of blockage of antibody production in mice by other methods will throw more light on this interesting mechanism.

FINAL REMARKS

Finally, we should state that although there still are considerable gaps in our knowledge of the nutrition of hemoflagellates and their logical implications, the basic work to date represents important steps toward such aspects as a more rational approach to chemotherapy, preparation of valuable vaccines, a better understanding of the transformation processes occuring in these organisms and, as a consequence, toward a better definition of the fascinating phenomenon of parasitism.

SUMMARY

The author reviews the literature on cultivation of several of the family Trypanosomatidae and other biological aspects of these flagellates in relation to external influences.

Although very few species have been cultured successfully in defined media, the known nutritional requirements establish a gradation that ranges from the more simple monogenetic forms (invertebrate parasites) to those of the Salivaria trypanosomes, whose nutritional requirement are more complex and demanding. In view of the transformations of these flagellates during their life cycle, the lack of knowledge on their culture, especially in relation to the characteristics of the vertebrate host becomes more evident. Gaps also exist in our knowledge of nutritional and other factors that influence this transformation. However, physiological and structural changes in certain forms confirm the complexity of this phenomenon. In the few cases in which presumably vertebrate forms have been reproduced *in vitro,* as in certain of the Leishmanias, the identity of the replicates with the original forms must be definitively established.

A more complete biochemical and physiological understanding of the transmitting insects would greatly contribute to the knowledge of the transformation process of the flagellates.

Environmental morphological and physiological influences apparently explain the ability or inability of some polymorphic trypanosomes to infect a new host. Also environmental, and possibly nutritional factors may play an important role in restoring infectivity to certain forms maintained in culture. Finally, the specificity of certain flagellates for a determined vertebrate host can be explained by the possible influence of immunological factors on those of nutrition.

RESUMEN

El autor revisa la literatura sobre el cultivo de los diversos miembros de la familia Trypanosomatidae y sobre algunos otros aspectos de la biología de estos flagelados en relación con su medio exterior. Si bien muy pocas especies han sido cultivadas en medios definidos, las exigencias nutricionales conocidas permiten establecer una gradación de exigencias que van desde las formas monogenéticas más simples (parásitas de invertebrados), hasta las digenéticas cuyas formas más complejas y exigentes se encuentran en el grupo Salivaria de los tripanosomas. En vista de los cambios de forma de muchos de estos flagelados durante su ciclo de vida, se hace evidente la escasez de conocimientos sobre su cultivo, especialmente en cuanto a las características del huésped vertebrado. También existen lagunas en nuestro conocimiento de nutrición o de otro tipo que influyen en este proceso de transformación, haciendo ver nuestro desconocimiento de las causas íntimas del fenómeno y la necesidad de ahondar más en él. No obstante, los cambios fisiológicos y de estructura de estas formas confirman de complejidad del fenómeno. Se insiste en la necesidad de probar con seguridad en aquellos pocos casos que se ha creído reproducir *in vitro* las formas del vertebrado, como sucede con algunas especies de *Leishmania,* que tales formas realmente corresponden a éstas en todos sus extremos. Un mejor conocimiento bioquímico y fisiológico de los insectos transmisores de algunos de estos flagelados permitirá entender mejor las transformaciones que se suceden en los mismos. Los cambios morfológicos y fisiológicos producidos por el ambiente parecen explicar la capacidad o incapacidad de algunos tripanosomas polimórficos de infectar un nuevo huésped. Se señala también que un grupo de factores ambientales, dentro de los cuales podría haber los de nutrición, podrían desempeñar un papel importante en la restauración de la infectividad por parte de algunas formas mantenidas en cultivo. Finalmente se comenta que ciertos factores ligados a la nutrición, posiblemente ayudados por otros de carácter inmunológico, pueden explicar la especificidad de un cierto flagelado para un determinado huésped vertebrado.

LITERATURE CITED

1. AKIYAMA, H. J., & J. C. TAYLOR
1970. Effect of macrophage engulfment and temperature on the transformation process of *Leishmania donovani. Amer. J. Trop. Med. Hyg.,* 19: 747-754.

2. AMREIN, Y. U., & R. B. HANNEMAN
1969. Suitable blood sources permitting reacquisition of infectivity by culture-form *Trypanosoma (Trypanozoon) brucei. Acta Trop.*, 26: 70 75.

3. AMREIN, Y. U., R. GEIGY, & M. KAUFFMANN
1965. On the reacquisition of virulence in trypanosomes of the Brucei-group. *Acta Trop.*, 22: 193-203.

4. BAKER, J. R.
1963. Speculations on the evolution of the family Trypanosomatidae Doflein, 1901. *Exper. Parasitol.*, 13: 219-233.

5. BISHOP, A.
1967. Problems in the cultivation of some parasitic protozoa. *Adv. Parasitol.*, 5: 93-138.

6. BONÉ, G. J., & G. PARENT
1963. Stearic acid, an essential growth factor for *Trypanosoma cruzi. J. Gen. Microbiol.* 31: 261-266.

7. BONÉ, G. J., G. PARENT, & M. STEINERT
1966. Le culture de trypanosome en milieu defini, p. 297-298. *In Proc. 1st. Int. Congr. Parasitol.* Rome, 21-26 Sept., 1964.

8. BRACK, C.
1968. Electronenmikroskopische Untersuchungen zum Lebenszyklus von *Trypan. osoma cruzi. Acta Trop.*, 25: 289-356.

9. BRENER, Z.
1969. The behaviour of slender and stout forms of *Trypanosoma cruzi* in the blood-stream of normal and immune mice. *Ann. Trop. Med. Parasitol.*, 63: 215-220.

10. BRENER, Z., & E. CHIARI
1963. Variações morfológicas observadas em diferentes amostras de *Tryjpanosoma cruzi. Rev. Inst. Med. Trop. S. Paulo*, 5: 220-224.

11. CAMARGO, E. P.
1964. Growth and differentiation of *Trypanosoma cruzi.* I. Origin of metacyclic trypanosomes in liquid medium. *Rev. Inst. Med. Trop. S. Paulo*, 6: 93-100.

12. CASTELLANI, O., L. V. RIBEIRO, & J. F. FERNÁNDES
1967. Differentiation of *Trypanosoma cruzi* in culture. *J. Protozool.*, 14: 447-451.

13. CITRI, N., & N. GROSSOWICZ
1955. A partially defined culture medium for *Trypanosoma cruzi* and some other hemoflagellates. *J. Gen. Microbiol.*, 13: 273-278.

14. COSGROVE, G. E., D. R. LINCICOME, & A. WARSI
1969. Development of *Trypanosoma lewisi* in experimental chimaeras. *J. Protozool.* 16: 47-49.

15. DEANE, M. P., & L. M. DEANE
1961. Studies on the life cycle of *Trypanosoma conorhini. In vitro* development and multiplication of the blood stream trypanosomes. *Rev. Inst. Med. Trop. S. Paulo,* 3: 149-160.

16. DEANE, M. P., & E. KIRCHNER
1963. Life-cycle of *Trypanosoma conorhini.* Influence of temperature and other factors on growth and morphogenesis. *J. Protozool.,* 10: 391-398.

17. DEANE, M. P., & J. K. KLOETZEL
1969. Differentiation and multiplication of dyskinetoplastic *Trypanosoma cruzi* in tissue culture and in the mammalian host. *J. Protozool.,* 16: 121-126.

18. DEMARCHI, J., & J. NICOLI
1960. La multiplication des agentes des trypanosomiasis humaines africanes en culture de tissus. *Ann. Inst. Pasteur Paris,* 99: 120-130.

19. DESOWITZ, R. S.
1963. The development and survival of the blood stream forms of *Trypanosoma conorhini* in culture. *J. Protozool.,* 10: 390-391.

20. FROMENTIN, H.
1961. Entretien de *Trypanosoma gambiense* sur culture de tissu. *Bull. Soc. Path. Exot.,* 54: 1046-1053.

21. FROTHINGHAM, T. E., & E. LEHTIMAKI
1967. *Leishmania* in primary cultures of human amniotic cells. *Amer. J. Trop. Med. Hyg.,* 16: 658-664.

22. GORDON, R. M., & K. C. WILLET
1958. Studies on the deposition, migration, and development to the blood forms of trypanosomes belonging to the *Trypanosoma brucei* group. III. The development of *Trypanosoma rhodesiense* from the metacyclic forms, as observed in mammalian tissue and in culture. *Ann. Trop. Med. Parasitol.,* 52: 346-365.

23. GREENBLATT, C. L., L. A. JORI, & H. J. CAHNMANN
1969. Chromatographic separation of a rat serum growth factor required by *Trypanosoma lewisi. Exper. Parasitol.,* 24: 228-242.

24. GREENBLATT, C. L. & D. R. LINCIOME
1966. Identity of trypanosome growth factors in serum. II. Active globulin components. *Exper. Parasitol.,* 19: 139-150.

25. GREENBLATT, C. L., & E. SHELTON
1968. Mouse serum as an environment for the growth of *Trypanosoma lewisi. Exper. Parasitol.,* 22: 187-200.

26. GUTTMAN, H. N.
1963. Experimental glimpses at the lower Trypanosomatidae. *Exper. Parasitol.,* 13: 129-142.

27. GUTTMAN, H. N.
1966. First defined media for *Leptomonas* spp. from insects. *J. Protozool.,* 13: 390-392.

28. HAWKING, F.
1948. Growth of Protozoa in tissue culture. V. *Leishmania donovani. Trans. R. Soc. Trop. Med. Hyg.,* 41: 545-553.

29. HERMAN, R.
1966. Studies of the numbers and morphology of the intracellular form of *Leishmania donovani* grown in cell culture. *J. Protozool.,* 13: 408-417.

30. HERMAN, R.
1968. Acriflavin-induced dyskinetoplastic *Leishmania donovani* grown in monkey kidney cell culture. *J. Protozool.,* 15: 35-43.

31. JANOVY, J., JR.
1967. Respiratory changes accompanying leishmania to leptomonad transformation in *Leishmania donovani. Exper. Parasitol.,* 20: 51-55.

32. KRASSNER, S. M.
1966. Cytochromes, lactic dehydrogenase, and transformation in *Leishmania. J. Protozool.,* 13: 286-290.

33. LAMY, L. H.
1969. La transformation réciproque des formes mastigotes et amastigotes de *Leishmania* et son déterminisme en présence des cellules vivantes *in vitro. Ann. Inst. Pasteur,* 117: 545-555.

34. LAMY, L. H., T. WONDE, & L. LAMY-ROUX
1967. Influence de la température sur la formation réciproque des formes flagellées et aflagellées de *Leishmania* en présence de macrophages peritonéaux de souris *in vitro. C. R. Acad. Sci., Paris, Ser. D.,* 264: 1889-1891.

35. LEMMA, A., & E. L. SCHILLER
1964. Extracellular cultivation of the leishmanial bodies of species belonging to the protozoan genus *Leishmania. Exper. Parasitol.,* 15: 503-513.

36. LE PAGE, R. W. F.
1967. Short term cultivation of *Trypanosoma brucei in vitro* at 37° C. *Nature (Lond.),* 216: 1141-1142

37. LWOFF, M.
1951. The nutrition of parasitic flagellates (Trypanosomatidae, Trichomonadinae), v.l, p. 129-176. *In* A. Lwoff, ed., *Biochemistry and physiology of protozoa.* Academic Press, New York.

38. NEVA, F. A., M. F. MALONE, & B. R. MYERS
1961. Factors influencing the intracellular growth of *Trypanosoma cruzi in vitro. Amer. J. Trop, Med. Hyg.,* 10: 140-154.

39. NEWTON, B. A.
1968. Biochemical peculiarities of trypanosomatid flagellates. *Ann. Rev. Microbiol.,* 22: 109-130.

40. PAN, C. T.
1968. Cultivation of the leishmaniform stage of *Trypanosoma cruzi* in cell-free media at different temperatures. *Amer. J. Trop. Med. Hyg.,* 17: 823-832.

41. PITTAM, M. D.
1966. *In vivo* culture of African trypanosomes in a partially defined medium, p. 330. *In Proc. 1st. Int. Congr. Parasitol.* Rome 21-26 Sept., 1964.

42. RISTIC, M., & W. TRAGER
1958. Cultivation at 37° C of a trypanosome *(Trypanosoma theileri)* from cows with a depressed milk production. *J. Protozool.*, 5: 146-148.

43. RUDZINSKA, M. A., P. A. D'ALESSANDRO, & W. TRAGER
1964. The fine structure of *Leishmania donovani* and the role of the kinetoplast in the leishmania-Leptomonad transformation. *J. Protozool.*, 11: 166-191.

44. SÁNCHEZ, G., & D. G. DUSANIC
1968. Growth of the blood stream forms of *Trypanosoma lewisi in vitro*. *J. Parasitol.*, 54: 601-605.

45. SILVA, L. H. P.
1959. Observações sobre o ciclo evolutivo do *Trypanosoma cruzi*. *Rev. Inst. Med. Trop. S. Paulo*, 1: 99-118.

46. SILVA, L. H. P., & E. P. CAMARGO
1968. Ciclo evolutivo do *Trypanosoma cruzi*, p. 87-99. *In* J. R. Cançado, ed., *Doença de Chagas*. Belo Horizonte, Brasil.

47. SIMPSON, L.
1968. The leishmania-leptomonad transformation of *Leishmania donovani:* nutritional requirements, respiration changes and antigenic changes. *J. Protozool.*, 15: 201-207.

48. SPLITTER, E. J., & E. J. L. SOULSBY
1967. Isolation and continuous cultivation of *Trypanosoma theileri* in media containing tissue culture fluids. *Exper. Parasitol.*, 21: 137-149.

49. STEINERT, M.
1958. Action morphogenetique de l'urée sur le trypanosome. *Exp. Cell Res.*, 15: 431-433.

50. STEINERT, M.
1965. The morphogenesis of trypanosomes. Progress in Protozoology. *2nd. Int. Conf. Protozool., London. Excerpta Med. Found.*, 91: 40-41.

51. TRAGER, W.
1953. The development of *Leishmania donovani in vitro* at 37° C. *J. Exper. Med.*, 97: 177-188.

52. TRAGER, W.
1959. Tsetse-fly tissue culture and the development of Trypanosomes to the infective stage. *Ann. Trop. Med. Parasitol.*, 53: 473-491.

53. TRAGER, W.
1968. General characteristics of the blood protists. 8. Cultivation and nutritional requirements, v. I., p. 149-174. *In* D. Weinman and M. Ristic, eds., *Infectious blood diseases of man and animals*. Academic Press, New York.

54. TREJOS, A., G. A. GODOY, C. GREENBLATT, & R. CEDILLOS
1963. Effects of temperature on morphologic variation of *Schizotrypanum cruzi* in tissue culture. *Exper. Parasitol.*, 13: 211-218.

55. VICKERMAN, K.
1965. Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. *Nature (Lond.)*, 208: 762-766.

56. WIJERS, D. J. B., & K. C. WILLET
1960. Factors that may influence the infection rate of *Glossina palpalis* with *Trypanosoma gambiense*. II. The number and the morphology of the trypanosomes present in the blood of the host at the time of the infective feed. *Ann. Trop. Med. Parasitol.*, 54: 341-350.

57. WOOD, D. E., & A. C. PIPKIN
1969. Multiplication and differentiation of *Trypanosoma cruzi* in an insect cell culture system. *Exper. Parasitol.*, 24: 176-183.

58. ZELEDÓN, R.
1954. Trypanosomiasis rangeli. *Rev. Biol. Trop.* 2: 231-268.

59. ZELEDÓN, R.
1959. Differentiation of *Trypanosoma rangeli* and *Schizotrypanum cruzi* in a liquid medium, with notes on the nutrition of hemoflagellates. *J. Parasitol.*, 45: 652.

60. ZELEDÓN, R., E. BLANCO, & E. DE MONGE
1969. Comparative infections with Costa Rican strains of *Leishmania braziliensis* Vianna, 1911. *Acta Trop.*, 26: 136-155.