

CYSTEINE ELIMINATES THE FEEDER CELL REQUIREMENT  
FOR CULTIVATION OF *TRYPANOSOMA BRUCEI*  
BLOODSTREAM FORMS IN VITRO

By MICHAEL DUSZENKO, MICHAEL A. J. FERGUSON,  
GRETEL S. LAMONT, MARY R. RIFKIN, AND GEORGE A. M. CROSS

*From The Rockefeller University, New York 10021*

The pathogenic African trypanosomes, which cause sleeping sickness in man and severe livestock diseases, undergo a developmental cycle between their mammalian hosts and the insect vector (*Glossina spp.*). Cultivation of each developmental stage in vitro is a cherished ambition of parasitologists. This problem was solved for the procyclic insect form some years ago (1–3), but cultivation of hematozoic trypomastigotes remained a challenge. A major breakthrough was achieved when Hirumi and colleagues (4) introduced the use of mammalian fibroblasts as a feeder layer for cultivating hematozoic trypanosomes. Brun and colleagues (5) improved this cultivation system, which is now in widespread use (6). However, the technique is time-consuming and the cell yield is relatively low ( $\sim 5 \times 10^6$  trypanosomes/ml), compared with yields of up to  $1 \times 10^9$  trypanosomes/ml in acutely infected laboratory rodents. Moreover, the presence of feeder cells can cause difficulties in differentiating between parasite and feeder cell metabolism. Efforts have been made to determine the action of the feeder layer but, so far, no explicit function has been described. Media preconditioned by feeder cells were ineffective and growth could not be sustained in vitro when trypanosomes were separated from the feeder cells by membranes permeable to macromolecules but not to cells (7). In contrast, *T. congolense* and *T. equiperdum* can grow (8, 9), and *T. brucei* can grow and undergo antigenic variation (10) inside Millipore filter chambers implanted in mice. During studies of variant surface glycoprotein synthesis in *T. brucei*, we made an observation that prompted us to review literature on the role of thiol compounds in mammalian cell cultivation (11–16) and to explore the possibility that cysteine might be the essential factor supplied by feeder cells during the growth of *T. brucei* bloodstream forms in vitro.

### Materials and Methods

*Incorporation of [<sup>35</sup>S]Methionine into Acid-precipitable Material.* Trypanosomes (MITat 1.4) were isolated from infected rats as described (17), washed twice, and diluted to a concentration of  $3 \times 10^7$ /ml in a modified minimum essential medium (MEM)<sup>1</sup> (18) containing no cystine or methionine, but containing adenosine (12 mg/l), Hepes (7.14 g/l), bovine serum albumin (BSA) (1 g/l), and glucose (33 mM). The cells were preincubated for 10 min at 37°C, before [<sup>35</sup>S]methionine was added to each suspension (17  $\mu$ Ci/ml).

<sup>1</sup> Abbreviations used in this paper: BSA, bovine serum albumin; MEM, minimum essential medium.

After another 6 min, different concentrations of cysteine were added to samples 2–5. 50- $\mu$ l samples were taken and precipitated immediately in ice-cold 5% wt/vol trichloroacetic acid (TCA). Precipitation was completed overnight with BSA (50  $\mu$ g/ml) as carrier. The samples were filtered (glass microfiber filters GF/C; Whatman Chemical Separation, Inc., Clifton, NJ), washed three times with ice-cold 5% TCA and once with ice-cold 1% acetic acid, air dried, and counted in a liquid scintillation counter. All samples were taken in duplicate.

*Growth of T. brucei in Axenic Culture.* Trypanosomes were cultivated in a handmade MEM (18) without cystine and  $\text{NaHCO}_3$  but with added adenosine (12 mg/l), Hepes (7.14 g/l), and glucose (33 mM). Immediately before use, 84.5 ml of this medium was supplemented with 3.6 ml  $\text{NaHCO}_3$  (5% wt/vol stock), 10.0 ml fetal calf serum (heat inactivated), 0.1 ml L-ornithine (10 mg/ml stock), and 2.0 ml myristic acid/BSA complex prepared in a molar ratio of 7:1, using myristic acid (1.2 mg/ml) and defatted BSA (50 mg/ml) as described (2). Trypanosomes (MITat 1.2) were initially taken from an irradiated feeder layer-supported culture. The trypanosome concentration in this culture was  $1.8 \times 10^6$ /ml and 400  $\mu$ l was added to 7 ml of fresh modified MEM without feeder cells. The initial parasite concentration was  $1 \times 10^5$ /ml. Cultivation was performed in 6-well plates (Falcon Labware, Oxnard, CA) at 37°C in a 5%  $\text{CO}_2$ /air environment. Immediately after inoculation, cysteine was added to three wells to a final concentration of 0.6 mg/l. After 6 h, cysteine was added to the same cultures to a final concentration of 2.0 mg/l. From then on, cysteine was supplemented to these cultures twice a day to a final concentration of 4.0 mg/l at each addition. For this purpose, a 1,000-fold-concentrated cysteine stock solution was prepared in MEM and sterile filtered each time immediately before use. The cell concentration was counted using a hemocytometer. Trypanosomes from a growing culture were subcultured when the cell density reached between  $1.5 \times 10^6$  and  $2.0 \times 10^6$ /ml. Subcultivation and the time schedule for cysteine addition were repeated exactly as described, starting again with  $1 \times 10^5$  trypanosomes/ml and three different control and experimental cultures.

*Measurement of Cysteine and Cystine Uptake by T. brucei.* Trypanosomes (MITat 1.4) from highly infected rats were isolated as described (17). The parasites were washed twice in Krebs-Ringer solution, pH 7.4, containing 10 mM glucose, and adjusted to a cell concentration of  $4 \times 10^8$ /ml in the same medium. For cysteine uptake experiments, all glassware was prewashed with 10 mM EDTA (11) and all washing and incubation solutions were supplemented with 1 mM reduced glutathione. For each concentration of cysteine and cystine, 1.5 ml of this cell suspension was preincubated in a water bath at 37°C for 3 min, then mixed with the same volume of twofold-concentrated cysteine or cystine solution in the same medium, also preincubated for 3 min. Samples (200  $\mu$ l each) were taken out at four different time points within 90 s and centrifuged through an oil layer directly into perchloric acid, as described (19). All samples were taken in duplicate. Uptake was measured using [ $^{35}\text{S}$ ]cysteine and [ $^{35}\text{S}$ ]cystine and counting an aliquot of the perchloric acid layer. From these figures, the actual uptake in nanomoles per  $10^7$  cells per minute was calculated.

## Results

Bloodstream forms of *T. brucei* are very sensitive to cysteine. As measured by incorporation of [ $^{35}\text{S}$ ]methionine into protein, cysteine had a stimulatory effect at concentrations of 1.5 or 3.0 mg/ml (Fig. 1). At concentrations of 24 mg/l or higher, [ $^{35}\text{S}$ ]methionine incorporation ceased abruptly and trypanosomes died within 10 min, as judged by light microscopy. Cystine, in the same molar concentration (48 mg/l), was not toxic to trypanosomes and had no effect on protein synthesis. The presence of cystine at the same concentration as cysteine did not protect the parasites from the toxic effect of the reduced form (data not shown).

To investigate the possibilities for axenic cultivation of *T. brucei*, we used a

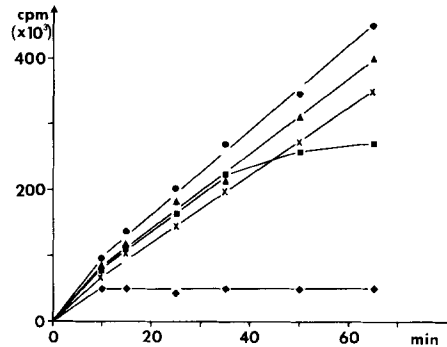


FIGURE 1. Incorporation of [ $^{35}\text{S}$ ]methionine into TCA-precipitable protein in the presence of cysteine. (x) Control minus cysteine or cystine, (●) cysteine 1.5 mg/l, (▲) cysteine 3.0 mg/l, (■) cysteine 12 mg/l, (◆) cysteine 24 mg/l.

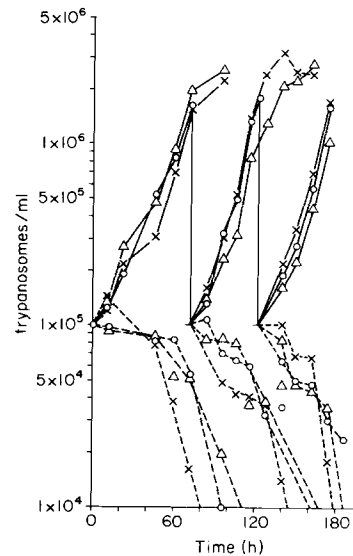


FIGURE 2. Growth of *T. brucei* in axenic culture. The growth kinetics are indicated for each of the six samples with (solid lines) or without (dotted lines) cysteine, during three subcultures.

modified MEM supplemented with cysteine twice a day. To minimize autoxidation, cysteine was always added from a freshly prepared stock solution (1,000-fold concentrated) to a final concentration of 0.6 mg/l at the time of subculture (when the initial cell density was  $1 \times 10^5/\text{ml}$ ), 2 mg/l after 6 h, and 4 mg/l at each 12 h interval thereafter. If 4 mg/l cysteine was added initially, at a trypanosome concentration of  $1 \times 10^5/\text{ml}$ , >90% of the trypanosome population was killed within 24 h, but resumption of growth was evident if low levels of cysteine were added subsequently. Thus, it appears that the cysteine-to-cell ratio is critical. Omission of cysteine at any subcultivation caused the death of the cultures (Fig. 2). Under these conditions, it was possible to grow bloodstream forms of *T. brucei* strain 427 (2, 4, 17) continuously, axenically. The population doubling time for trypanosomes in this cultivation system was  $\sim 12$  h, compared

with ~7 h for the same clone in feeder layer-supported cultures. To show that the cultivation conditions were not causing differentiation of the bloodstream forms, cell samples were taken after three subcultivations (12 generations) and examined by Giemsa staining and immunofluorescence. Only trypomastigote forms were observed. Incubation of formaldehyde-fixed trypanosomes with rabbit antiserum prepared against the purified homologous variant surface glycoprotein, followed by fluorescein isothiocyanate-coupled goat anti-rabbit IgG, showed specific and uniform fluorescence of all cells. No antigenic switching or loss of the surface coat was apparent and these trypanosomes were still infective for mice (data not shown). Growth as procyclic forms would have been unexpected, since the culture temperature of 37°C is nonpermissive for these non-infective forms. Epimastigotes have only been observed in vitro during cultivation in the presence of *Glossina* salivary gland explants (20).

Cultures could also be initiated with infected mouse blood, at a starting density of  $5 \times 10^3$  trypanosomes/ml. Pyruvate (1 mM) or catalase (1.75 µg/ml) were added to eliminate the toxic effect of cysteine (21), which is due to H<sub>2</sub>O<sub>2</sub> produced during autoxidation. Whereas the trypanosomes died at cysteine concentrations of 5 or 30 µM repeatedly added in media without pyruvate, a density of  $1 \times 10^6$  trypanosomes/ml was reached after 102 h in the presence of pyruvate. There was no growth in the absence of cysteine.

Growth of procyclic trypanosomes at 27°C either in SDM-79 (3) or in MEM supplemented with proline (600 mg/l), hemin (7.5 mg/l), pyruvate (100 mg/l), and fetal bovine serum (10%), occurred with a population doubling time of 15 h and yield of  $1 \times 10^7$  trypanosomes/ml in the presence or absence of cysteine. Interestingly, we were able to subculture procyclic trypanosomes indefinitely in MEM with almost the same kinetics and yield as in the more complex SDM-79 medium.

Uptake experiments using [<sup>35</sup>S]cysteine and [<sup>35</sup>S]cystine showed that bloodstream-form *T. brucei* can effectively transport only cysteine and not cystine (Fig. 3). Whether the small amount of apparent uptake of cystine that is evident from the enlarged scale insert in Fig. 3 is real or artifactual is unknown. We have no satisfactory explanation for the curious kinetics of this apparent uptake. For reasons related to its maximum solubility of  $4.7 \times 10^{-4}$  M in aqueous solution at 37°C (22), cystine uptake was only measured at concentrations  $<2 \times 10^{-4}$  M. At  $1 \times 10^{-4}$  M, the physiological concentration of cystine in human serum (23), the rate of cysteine uptake was 37-fold higher than that of cystine. The cysteine concentration in human serum is  $\sim 1.3 \times 10^{-5}$  M (23). The  $K_m$  for cysteine uptake was  $4 \times 10^{-4}$  M and the  $V_{max}$  was 3 nmol/min per  $10^7$  cells.

The media usually used for in vitro cultivation of bloodstream trypanosomes (RPMI 1640 or MEM) contain cystine at a concentration of 24 mg/l and no cysteine. As shown by Toohey (12),  $2.5 \times 10^{-3}$  M cysteine, added to MEM, is oxidized within 4 h. On the other hand, it has been shown (14, 15) that human fibroblasts, used as a feeder layer for mouse lymphoma cells, take up cystine and constantly excrete thiol compounds, especially cysteine, which accumulates in the medium. Lymphoma cells could be grown without a feeder layer when either cysteine or 2-mercaptoethanol was added repeatedly at low concentrations to the medium, which contained cystine. As we report here for trypanosomes, high

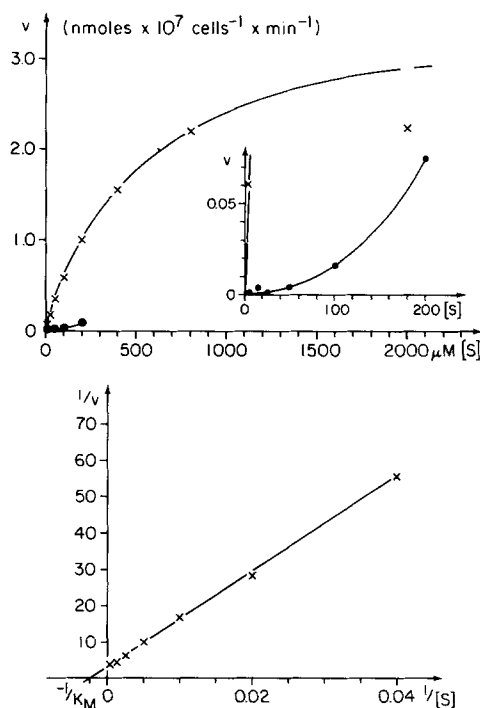


FIGURE 3. Uptake of cysteine and cystine by bloodstream forms of *T. brucei*. The inset shows the apparent uptake of cystine on an enlarged scale. Due to the toxicity of high cysteine concentrations, the uptake indicated for 2 mM cysteine is artifactually low. In the Lineweaver-Burk plot, the ordinate is calibrated in arbitrary units.

concentrations of cysteine ( $3 \times 10^{-4}$  M) were toxic for this lymphoma line, which was also inefficient in cystine uptake. Intracellular levels of glutathione were greatly enhanced in lymphoma cells incubated in the presence of cysteine (14). It is unknown why high concentrations of cysteine are toxic. However, hydrogen peroxide is produced during the autoxidation of cysteine, which is catalyzed by ubiquitously occurring  $\text{Cu}^{2+}$  ions (11). Trypanosomes appear to be rather susceptible to damage by activated oxygen species, which may be related to the absence of protective enzymes, including catalase and glutathione peroxidase (24).

To show whether externally generated hydrogen peroxide causes cell lysis, we performed an experiment with twice the toxic amount of cysteine in the presence of catalase. Trypanosomes ( $3 \times 10^7/\text{ml}$  in the modified MEM described above) were incubated at  $37^\circ\text{C}$  in the presence of  $10 \mu\text{g}/\text{ml}$  ( $\sim 110$  U/ml) catalase before cysteine (48 mg/ml) was added. Under these conditions no cell death was observed within  $2 \frac{1}{2}$  h after cysteine addition and  $\sim 50\%$  of the initial population remained alive after overnight incubation. In contrast, in the control experiment without catalase, virtually all the trypanosomes were killed within 5 min of cysteine addition.

### Discussion

Since this manuscript was originally submitted for publication, Baltz et al. (25) grew *T. brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense*, and *T. gambiense* axenically

in media supplemented with 2-mercaptoethanol or thioglycerol. Although their results agree with the data presented here, we consider it most likely that, as suggested for mouse lymphoma cells (14) and murine lymphocytes (16), 2-mercaptoethanol sustains trypanosome growth by reducing cystine and cysteine. Using MEM containing cystine (24 mg/ml), we have found that addition of 2-mercaptoethanol at 12-h intervals to a final concentration of 5 or  $10 \times 10^{-6}$  M gave growth rates and cell yields comparable to those obtained with cysteine (data not shown). Because of the presence of cystine in fetal calf serum in our studies and those reported by others (14, 16), an absolute dependence of the mercaptoethanol effect on the presence of cystine cannot easily be demonstrated. Various sulphhydryl reagents tested by Toohey (12) gave similar growth rates and yields of P388 leukaemia cells in vitro. The optimal concentrations of cysteine, thioglycerol, and 2-mercaptoethanol were about  $10^{-3}$ ,  $3 \times 10^{-6}$ , and  $10^{-6}$  M, respectively. These compounds were autoxidized with half-lives of about 2, >60, and 40 h, respectively. Thus, thioglycerol, which we have not tested, might be the most convenient thiol regulator for trypanosome cultivation. However, in some cases, including the testing of potential trypanocidal drugs, the presence of potent nonphysiological reducing agents may be inadvisable.

Further work will be necessary to optimize conditions for axenic cultivation of bloodstream forms. Automated infusion or continuous generation of cysteine is an obvious requirement that we have been unable to optimize conveniently using simple osmotic minipump techniques. The maximum yield currently achieved in axenic culture appears to be limited by factors other than cysteine concentration. After 3 d, pyruvate levels reached  $\sim 6$  mM and the level of glucose was not accurately distinguishable from the starting level (33 mM). At the time of each subculture, the pH of the medium had only dropped to a value of 7.3. However, Cross and Manning (2) noted that growth of procyclic *T. brucei* was extremely sensitive to pH change, in defined medium lacking serum. Adenosine, L-ornithine, and myristic acid were added to media to ensure that these nutrients were available in excess for purine (26, 27), polyamine (28), and variant surface glycoprotein biosynthesis (29). Development of this axenic cultivation technique for hematzoic trypomastigotes will assist investigations of antigenic variation, trypanosome metabolism, nutritional requirements, the effect of trypanocidal drugs, and the automation of large scale cultivation.

### Summary

In all previous studies, bloodstream forms of *Trypanosoma brucei* could be grown in vitro only when supported by a feeder layer of mammalian fibroblasts. We have axenically cultivated bloodstream *T. brucei* by adding L-cysteine at regular intervals and appropriate concentrations. The optimum cysteine concentration depends on cell density and is close to physiological serum levels. At concentrations  $>24$  mg/liter ( $2 \times 10^{-4}$  M), cysteine was acutely toxic to trypanosome concentrations of  $3 \times 10^7$ /ml. Toxicity was prevented by addition of pyruvate or catalase, which neutralize  $H_2O_2$  produced by cysteine autoxidation. In uptake studies using [ $^{35}S$ ]cysteine and [ $^{35}S$ ]cystine, *T. brucei* efficiently incorporated only cysteine. The  $K_m$  for cysteine uptake was  $4 \times 10^{-4}$  M. Cystine

supported axenic growth if low concentrations of 2-mercaptoethanol were added at regular intervals.

We thank Carolyn Strobos for assistance with the uptake experiments, Alan Fairlamb for helpful suggestions and critical reading of the manuscript, and Lori Olf for manuscript preparation.

*Received for publication 18 June 1985 and in revised form 29 July 1985.*

### References

1. Tobie, E. J. 1958. The cultivation of *Trypanosoma congolense* *in vitro*. *J. Parasitol.* 44:241.
2. Cross, G. A. M., and J. C. Manning. 1973. Cultivation of *Trypanosoma brucei* spp. in semi-defined and defined media. *Parasitology.* 67:315.
3. Brun, R., and M. Schonenberger. 1979. Cultivation and *in vitro* cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Trop.* 36:289.
4. Hirumi, H., J. J. Doyle, and K. Hirumi. 1977. African trypanosomes: cultivation of animal infective *Trypanosoma brucei* *in vitro*. *Science (Wash. DC).* 196:992.
5. Brun, R., L. Jenni, M. Schonenberger, and K. F. Schell. 1981. *In vitro* cultivation of bloodstream forms of *Trypanosoma brucei*, *Trypanosoma rhodesiense* and *Trypanosoma gambiense*. *J. Protozool.* 28:470.
6. Hill, G. C., and H. Hirumi. 1983. African trypanosomes. In *In Vitro Cultivation of Protozoan Parasites*. J. B. Jensen, editor. CRC Press, Inc., Boca Raton, FL. 193-219.
7. Tanner, M. 1980. Studies on the mechanisms supporting the continuous growth of *Trypanosoma (Trypanozoon) brucei* as bloodstream-like form *in vitro*. *Acta Trop.* 37:203.
8. Tizard, I. R., and W. L. Holmes. 1977. The release of soluble vasoactive material from *Trypanosoma congolense* in intraperitoneal diffusion chambers. *Trans. Roy. Soc. Trop. Med. Hyg.* 71:52.
9. Capbern, A., A. N. Pautrizel, P. Mattern, and R. Pautrizel. 1977. *Trypanosoma equiperdum*: multiplication dans les chambres de diffusion implantées chez la souris. *Exp. Parasitol.* 43:1.
10. Ballon-Landa, G., H. Douglas, M. E. M. Colmerauer, D. Goddard, and C. E. Davis. 1985. Growth and antigenic variation of *Trypanosoma brucei*, *T. rhodesiense* and *T. gambiense* in subcutaneous millipore chambers. *Trans. Roy. Soc. Trop. Med. Hyg.* 79:24.
11. Fedorcsak, I., M. Harms-Ringdahl, and L. Ehrenberg. 1977. Prevention of sulfhydryl autoxidation by a polypeptide from red kidney beans, described to be a stimulator of RNA synthesis. *Exp. Cell Res.* 108:331.
12. Toohey, J. I. 1975. Sulfhydryl dependence in primary explant hematopoietic cells. Inhibition of growth *in vitro* with vitamin B<sub>12</sub> compounds. *Proc. Natl. Acad. Sci. USA.* 72:73.
13. Orloff, S., A. B. Mukherjee, J. D. B. Butler, B. Foley, and J. D. Schulman. 1980. Cystinotic and normal fibroblasts: differential susceptibility to cysteine toxicity *in vitro*. *In Vitro.* 16:655.
14. Ishii, T., I. Hishinuma, S. Bannai, and Y. Sugita. 1981. Mechanism of growth promotion of mouse lymphoma L 1210 cells *in vitro* by feeder layer or 2-mercaptoethanol. *J. Cell. Physiol.* 107:283.
15. Bannai, S., and T. Ishii. 1982. Transport of cystine and cysteine and cell growth in cultured human diploid fibroblasts: effect of glutamate and homocysteate. *J. Cell. Physiol.* 112:265.
16. Ohmori, H., and I. Yamamoto. 1982. Mechanism of augmentation of the antibody

- response in vitro by 2-mercaptoethanol in murine lymphocytes. *J. Exp. Med.* 155:1277.
17. Cross, G. A. M. 1975. Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology.* 71:393.
  18. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Wash. DC).* 130:432.
  19. Rifkin, M. R., and A. H. Fairlamb. 1985. Transport of ethanolamine and its incorporation into the variant surface glycoprotein of bloodstream forms of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 15:245.
  20. Cunningham, I., and A. M. Taylor. 1979. Infectivity of *Trypanosoma brucei* cultivated at 28°C with tsetse fly salivary glands. *J. Protozool.* 26:428.
  21. Higuchi, K. 1963. Studies on the nutrition and metabolism of animal cells in serum-free media. *J. Infect Dis.* 112:213.
  22. The Merck Index. 1983. Merck and Co. Inc., Rahway, NJ. 10th ed.
  23. Brigham, M. P., W. H. Stein, and S. Moore. 1960. The concentrations of cysteine and cystine in human blood plasma. *J. Clin. Invest.* 39:1633.
  24. Meshnik, S. R., S. H. Blobstein, R. W. Grady, and A. Cerami. 1978. An approach to the development of new drugs for African trypanosomiasis. *J. Exp. Med.* 148:569.
  25. Baltz, T., D. Baltz, C. Giroud, and J. Crockett. 1985. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. *EMBO (Eur. Mol. Biol. Org.) J.* 4:1273.
  26. Fish, W. R., J. J. Marr, and R. L. Berens. 1982. Purine metabolism in *Trypanosoma brucei gambiense*. *Biochem. Biophys. Acta.* 714:422.
  27. James, D. M., and G. V. R. Born. 1980. Uptake of purine bases and nucleotides in African trypanosomes. *Parasitology.* 81:383.
  28. Bacchi, C. J., C. Vergara, J. Garofalo, G. Y. Lipschik, and S. H. Hutner. 1979. Synthesis and content of polyamines in bloodstream *Trypanosoma brucei*. *J. Protozool.* 26:484.
  29. Ferguson, M. A. J., and G. A. M. Cross. 1984. Myristylation of the membrane form of *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 259:3011.