Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 44 (1987)
Heft: 3

Artikel: "Trypanosoma congolense" : differentiation to metacyclic trypanosomes in culture depends on the concentration of glutamine or proline

Autor: Ross, C.A.

DOI: https://doi.org/10.5169/seals-313854

Acta Tropica *44*, 293–301 (1987)

Centre for Tropical Veterinary Medicine, University of Edinburgh, Midlothian, Scotland

Trypanosoma congolense: differentiation to metacyclic trypanosomes in culture depends on the concentration of glutamine or proline

C. A. Ross

Summary

The effect of glutamine on the number of metacyclic trypanosomes produced in insect form cultures of *Trypanosoma congolense* TREU 1457 was investigated. When cultured in the absence of glutamine, trypanosomes did not develop to metacyclic forms. While metacyclics were produced in all cultures maintained with glutamine in the concentration range 2–20 mM, optimum numbers were obtained between 4 and 12 mM glutamine. This pattern was constant in flasks cultured for over two months. The substitution of glutamine by proline in culture medium did not influence the number of metacyclic forms produced. Removal of proline or glutamine from cultures producing metacyclics caused a rapid fall in production of metacyclic forms which could be reversed by re-introduction of either nutrient. Cultures of another stock of *T. congolense* responded similarly when maintained in different concentrations of glutamine, showing that the effect may be a general one.

Key words: *Trypanosoma congolense;* in vitro cultivation; glutamine; proline; metacyclics.

Introduction

Several cloned stocks of *Trypanosoma congolense* have been adapted to continuous culture, in which all the life cycle stages from the tsetse vector can be found (Gray et al., 1984, 1985). In the absence of a supporting cell monolayer, epimastigote trypanosomes adhere to the surface of the culture flask, grow and differentiate into infective metacyclic forms, which retain the morphological,

Correspondence: Dr. Carole A. Ross, Centre for Tropical Veterinary Medicine, University of Edinburgh, Easter Bush, Roslin, Midlothian, EH25 9RG, Scotland

biological and antigenic characteristics of the metacyclic trypanosomes in the proboscis of the tsetse fly (Luckins et al., 1981; Crowe et al., 1983). Continuous efforts have been made to improve the culture system and to produce the highest possible yields of functional metacyclic trypanosomes which are used in this laboratory in a wide variety of immunological, biochemical and epidemiological investigations.

In a preliminary study, it was found that doubling the glutamine concentration used in culture medium from 2 mM to 4 mM increased the number of metacyclic forms produced while causing no other obvious changes to the cultures. Proline, structurally related to glutamine, is the most abundant amino acid in the haemolymph of *Glossina morsitans morsitans* (Cunningham and Slater, 1974) and probably freely available to insect forms of *T. congolense* in vivo. Accordingly, the effect of both proline and glutamine concentrations on the number of metacyclic forms harvested from culture was examined systematically. The results of this study are given below.

Materials and Methods

Trypanosome stocks

Two cloned stocks of *Trypanosoma congolense* were used in these experiments. *T. congolense* TREU 1457 was derived from the Nigerian isolate Zaria/67/LUMP/69. TREU 1881 is a derivative of the Zambian isolate TRPZ 105.

Culture methods

Cultures of *T. congolense* producing infective metacyclic forms were maintained and passaged according to the methods of Gray et al. (1981, 1984). Culture medium was prepared from powdered Eagles Minimal Essential Medium (MEM) ("Autopow", Gibco Europe Ltd. Cat No. 11–100–26), an autoclavable medium which contained no glutamine or proline. Liquid medium was supplemented with sodium bicarbonate (2.2 g/l) and heat inactivated foetal bovine serum (20% v/v). Medium was also routinely supplemented with glutamine. A standard concentration of 4 mM was regarded as the control against which changes in morphology and in timing of differentiation could be compared.

Separation of metacyclic forms

To estimate the number of metacyclic forms produced in the cultures, a 0.5 ml sample of freshly harvested supernatant was placed on 2 ml of diethylaminoethyl cellulose DE-52 (Whatman) equilibrated with phosphate buffered saline pH 8.0 containing 1% (w/v) glucose (PSG) and packed in small polystyrene columns (Pierce, U. K.). Metacyclic forms were eluted with 2.5 ml PSG and their number per ml in the total eluate (3 ml) obtained by haemacytometer count. Although these separations were carried out at room temperature, all solutions and reagents used were ice-cold.

Infectivity tests

Titrations were carried out using parasites which had been eluted from DE-52 columns to determine the proportion of infective parasites present in the metacyclic population. Random bred, female CF1 mice were infected by intraperitoneal injection and bloodsmears from the mice were examined 2 or 3 times weekly for 21–30 d for presence of trypanosomes. Calculation of the number of infective organisms was by the method described by Lumsden et al. (1963).

Results

1. Metacyclic trypanosomes production in culture initiated and maintained in medium containing different concentrations of glutamine

a) Without glutamine. Two cultures of TREU 1457 were initiated by placing 0.5 ml of freshly harvested supernatant from an infective insect form culture, containing 10^7 trypanosomes, into 25 cm^2 flasks. The medium contained no glutamine. Epimastigote trypanosomes attached to the floor of the flasks, grew, divided and formed adherent bundles. After two weeks, at which time epimastigote forms maintained under standard conditions with glutamine begin to differentiate into metacyclic trypanosomes, only elongated and sluggish epimastigote trypanosomes in bundles could be seen. The cultures remained uninfective to mice until the flasks were discarded after 21 days.

b) In cultures containing 2 mM to 20 mM glutamine. A series of 14 flasks was given initial 0.5 ml inocula of trypanosomes and 3.5 ml fresh medium added to each. Cultures in duplicate flasks were maintained in MEM culture medium containing concentrations of glutamine between 2 mM and 20 mM. Over the next two months the metacyclic production in each culture was measured and the numbers obtained shown graphically in Fig. 1.

Adherence of epimastigotes to the surface of new flasks during the first 2 days after passage is crucial in the establishment of these cultures. Initially, trypanosomes grown in 10 mM glutamine or more adhered less well than when grown with 2–8 mM. In consequence, epimastigote bundles in cultures maintained with 10 mM glutamine matured more slowly and metacyclic forms appeared in the supernatant later than trypanosomes grown in lower glutamine concentrations. After one month in culture, however, concentrations of 4 to 8 mM glutamine supported production of similar numbers of metacyclic forms (Fig. 1). Cultures maintained in concentrations of glutamine less than 4 mM consistently produced fewer metacyclic forms although supernatants contained similar numbers of epimastigotes compared to cultures maintained in 4 mM glutamine, showing that the effect was specific to metacyclic production and not due to general reduction of numbers of culture forms.

One hundred and three days after initiation of these cultures, infectivity titrations were carried out, using metacyclic forms obtained from flasks maintained in 4 mM, 8 mM and 20 mM. No difference was found in their infectivity to mice (data not shown).

2. Production of metacyclic forms of T. congolense TREU 1457 on removal and re-introduction of glutamine or proline

A preliminary experiment showed that when 4 mM proline was substituted for 4 mM glutamine in culture medium the number of metacyclic forms produced in cultures of TREU 1457 was unchanged (data not shown). The following experiment was therefore carried out with two parallel series of flasks initially supplemented with 4 mM glutamine or 4 mM proline.

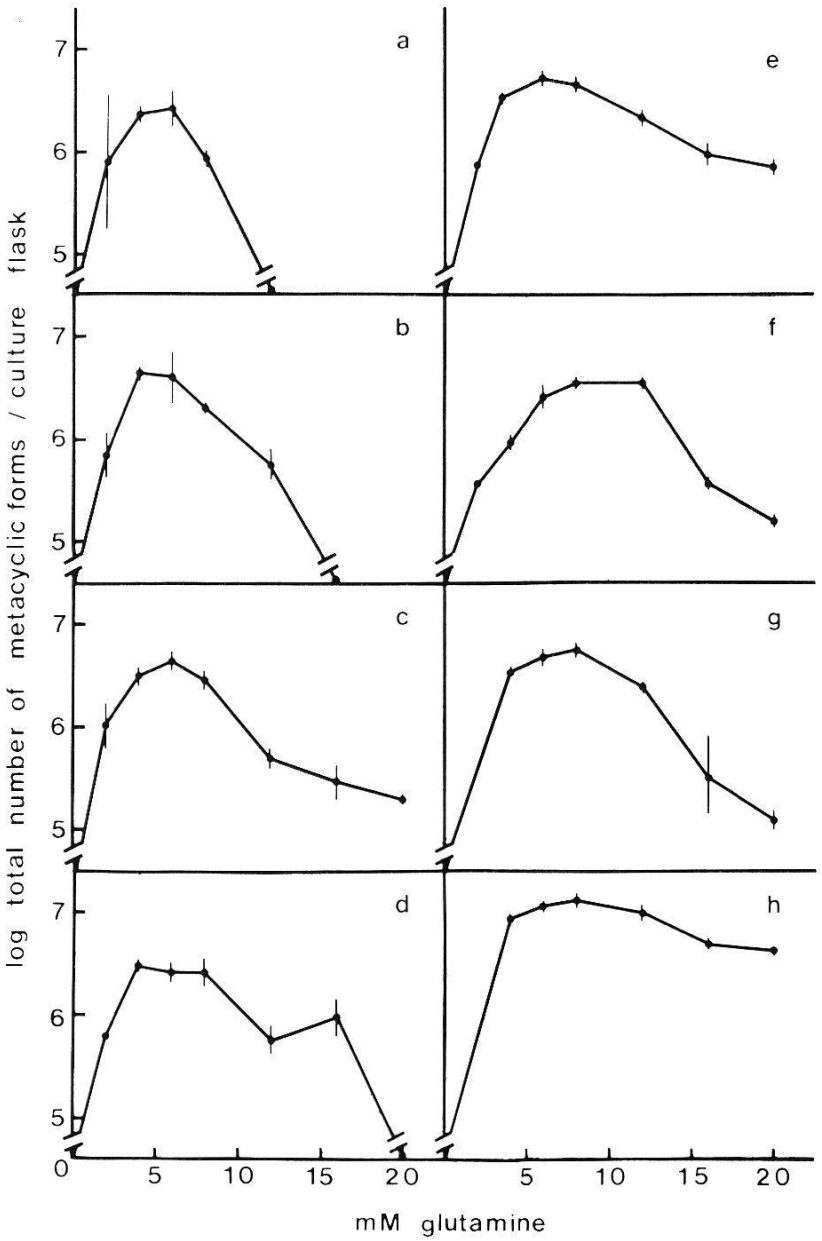

Fig. 1. Number of metacyclic forms produced in cultures of TREU 1457 maintained with different concentrations of glutamine. Results are expressed as metacyclics produced per flask. Culture volume was 4 ml. Metacyclics were counted on days a) 17, b) 22, c) 26, d) 33, e) 36, f) 43, g) 59, and h) 64. Points plotted show the average value of two flasks. The line limits show the value of individual flasks.

For each experimental series, eight flasks of TREU 1457 cultures were initiated and maintained with culture medium, containing 4 mM glutamine or 4 mM proline. Metacyclic forms were observed 12 days after initiation in cultures grown in proline, and after 14 days when grown in glutamine. Metacyclic numbers were first estimated on day 21 for the series grown in glutamine and on day 23 for those grown in proline and every 7 days thereafter for each series. The results of these measurements are presented in Fig. 2.

After five weeks in culture, six flasks grown from each set were given medium which contained no added glutamine or proline, while two flasks

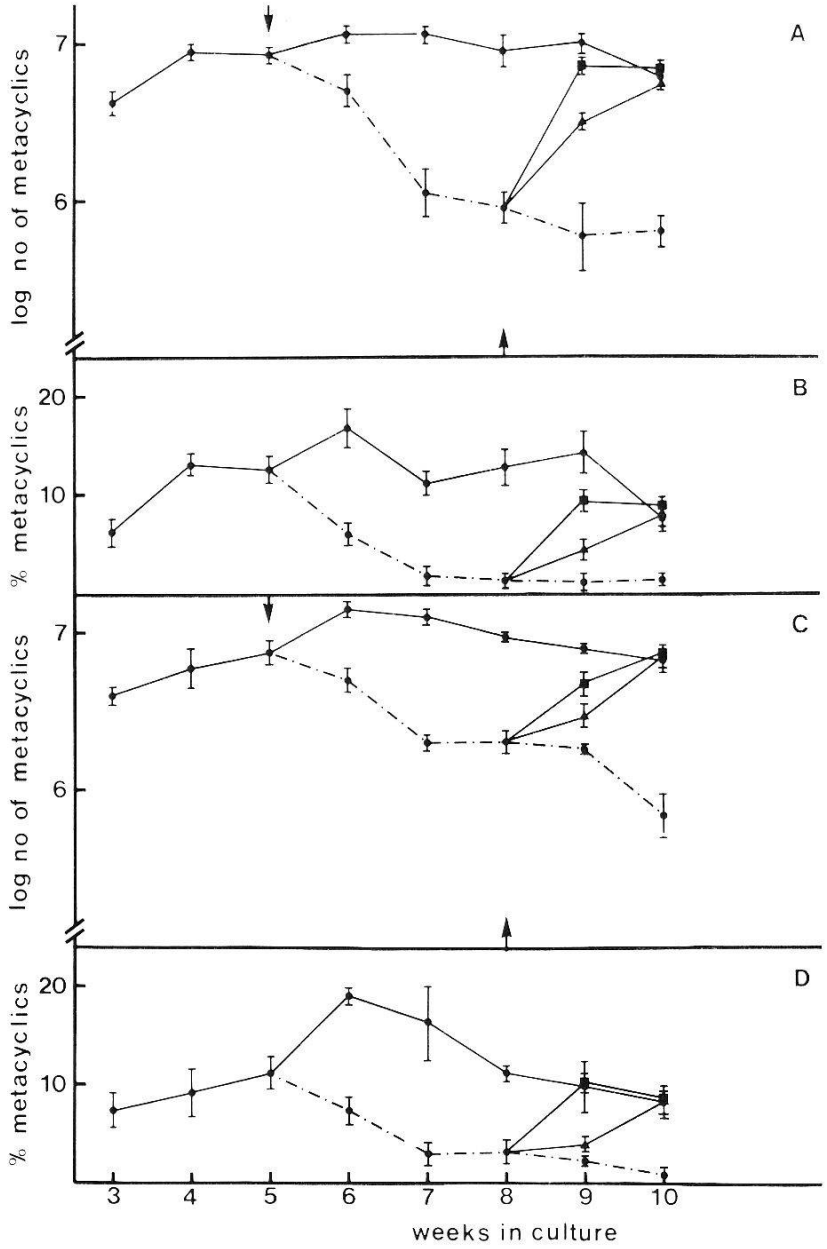

Fig. 2. Number of TREU 1457 metacyclic forms produced on removal and reintroduction of glutamine or proline; A) log of total number of metacyclics in 25 cm^2 flasks initially maintained in 4 mM glutamine; B) percentage of metacyclics in supernatants in flasks initially maintained in 4 mM glutamine; C) log of total number of metacyclics in flasks initially maintained in 4 mM proline; D) percentage of metacyclics in flasks initially maintained in 4 mM proline.

↓ removal of glutamine or proline

↑ readdition of glutamine or proline

●—● 4 mM glutamine (A, B)

●-·-● 0 mM glutamine (A, B)

■—■ 4 mM glutamine re-introduced

▲—▲ 4 mM proline re-introduced

●—● 4 mM proline (C, D)

●-·-● 0 mM proline (C, D)

Standard deviation values are calculated from at least four determinations per point plotted.

continued in the original culture medium. A reduction in the number of metacyclic forms in both sets of six flasks could be observed microscopically after 2–4 days compared to the relevant controls. In cultures from which glutamine had been removed, the number of metacyclic forms recovered fell to only 10% of that obtained from control flasks. Where proline had been removed, the cultures produced 15% as many metacyclic forms as controls but then maintained this level of production. The epimastigotes in all 12 cultures retained their normal morphology, the only difference in appearance being the lack of free-swimming metacyclic trypanosomes between bundles.

To test if the reduction in ability of these cultures to differentiate to metacyclic forms was reversible, nutrients were reintroduced into the medium after three weeks' maintenance without glutamine or proline (and eight weeks after the start of the experiment). For each set of six flasks, two were now given medium containing 4 mM glutamine, two were given medium containing 4 mM proline and two were left without either amino acid. There was an immediate increase in the number of metacyclic forms produced in cultures supplemented with glutamine or proline once again (Fig. 2). Irrespective of the initial supplement, however, cultures maintained in glutamine supplemented medium at this stage of the experiment showed a greater increase in the number of metacyclics harvested compared to those supplemented with proline. After another two weeks, the number of metacyclic forms in all these flasks had recovered to control levels. Cultures remaining without either amino acid in their medium continued to produce far fewer metacyclics, the number of epimastigote trypanosomes harvested fell and trypanosomes were slightly sluggish and elongated.

The percentage of metacyclics in each culture supernatant was also calculated. Graphs showing these profiles (Figs. 2 B, D) demonstrate that the effect of amino acid concentration on this parameter closely paralleled the effect on total metacyclic numbers.

3. Effect of glutamine on the number of metacyclic forms in cultures of TREU 1881

To check whether glutamine concentration could affect the production of metacyclic forms in stocks of *T. congolense* other than TREU 1457 a series of 10 flasks containing cultures of TREU 1881 was set up. TREU 1881 was chosen since it consistently produces the highest numbers of metacyclics of all stocks adapted to insect form in vitro culture at the CTVM. Between 2 and 3×10^7 metacyclic forms can be harvested, three times weekly from each 4 ml culture of this stock.

The flasks were maintained with 4 mM glutamine for 31 days and the number of metacyclics being produced were measured on three occasions (Table 1). When medium was changed on day 31, it was replaced in duplicate flasks with medium supplemented with one of four different glutamine concentrations, in the range 2 mM to 16 mM. One pair of cultures was changed to

Table 1. Effect of glutamine concentration on the number of metacyclic forms in cultures of TREU 1881

Glutamine concentration nM	Days in culture	Total number of metacyclics $\times 10^{-7}$ harvested per flask	% metacyclics in supernatant
4	17	1.1±0.3	15.2±4.2
4	24	3.2±0.4	29.6±4.2
4	31	2.5±0.3	23.5±3.2
0	45	0.3±0.08	5.0±1.3
2	45	2.1±0.3	20.4±2.6
4	45	2.7±0.5	28.1±4.6
8	45	2.3±0.2	19.4±3.6
16	45	1.3±0.1	13.3±0.6

maintenance in medium containing no glutamine. After a further 14 days the numbers in each flask were again determined. In general, the results obtained (Table 1) show great similarity to the effects shown on cultures of TREU 1457 (see Fig. 1). There was a dramatic drop in the production of metacyclic forms when glutamine was withdrawn from the culture medium. Between 2 mM and 8 mM the numbers produced remained at the level obtained before day 31, but fell off at higher concentration of the amino acid.

Discussion

The number of *T. congolense* infective metacyclic forms produced in vitro by cloned stocks of this parasite is influenced by the concentration of glutamine present in the culture medium. There is an increase in the number of metacyclic forms harvested with increasing glutamine concentration from 2 mM to 8 mM (Fig. 1). In all cultures maintained with glutamine concentration between 2 mM and 10 mM, trypanosomes showed the normally observed morphological characteristics of adherent epimastigote bundles surrounded by a mixture of detached epimastigotes and free-swimming metacyclic forms. Cultures differed only in the number of metacyclic forms observed and harvested. At concentrations above 12 mM gluamine, metacyclic production was lower. No attempt was made to adjust the ionic balance when trypanosomes were maintained with relatively high levels of glutamine and rounded and distorted trypanosomes were commonly seen in such cultures. Initial adherence of epimastigote forms was also impaired, which contributed to the lower metacyclic production. Adherence in cultures maintained with more than 12 mM glutamine improved in time through natural growth of epimastigote bundles. Accordingly the production of metacyclic forms also increased, and was still increasing after two

months when the experiment was terminated. Glutamine concentration had no effect on the infectivity of metacyclic trypanosomes produced.

When cultures were initiated and maintained in medium containing no glutamine, no metacyclics were produced, although normal epimastigote bundles were established on the floor of culture flasks. If glutamine was removed from cultures already producing many metacyclic trypanosomes, the production of infective forms was drastically reduced but they did not disappear completely. Thus, differentiation of adherent epimastigotes into metacyclic trypanosomes appears to require the presence of glutamine, or a metabolic product of this amino acid. Whether these observations suggest that the effect is a reflection of increased protein synthesis in the formation of metacyclic forms, or a consequence of the existence of a specific factor is unknown. The timing of removal of glutamine causes either no metacyclic forms to be produced at all or a reduction of metacyclic forms harvested. This may imply a requirement for a specific factor, a higher concentration of which is needed for this step in differentiation to proceed, than for continued production of metacyclic trypanosomes.

There is evidence for the stage-specific requirement for glutamine during the life cycles of other parasites. Erythrocytes infected with *Plasmodium falciparum* take up exogenous glutamine at a rate 100-fold that of uninfected erythrocytes, during the period of development of young trophozoites (Elford et al., 1985). In this instance, however, the authors concluded that glutamine was required for the increased rates of parasite protein synthesis and glutathione turnover in the red cell.

T. congolense cultures can also be affected by addition or removal of proline, which is metabolically derived from glutamine in most animal cells. Proline, therefore, may be more closely connected with the biochemical cause of this phenomenon. Proline is often a main amino acid constituent of insect haemolymph (Bursell, 1970) and occurs at a concentration of 60 mM in tsetse flies (Cunningham and Slater, 1974), i.e. twenty-five times more abundant than any other amino acid except alanine or glutamine. While this is no evidence for the involvement of proline in the regulation of development of *T. congolense* parasites, it is probable that proline is available for ingestion by trypanosomes in natural infections. The effect of concentration of proline greater than 4 mM on *T. congolense* in vitro has not been investigated, but it may induce the production of even higher numbers of metacyclic forms.

Very little is known about the metabolism of *T. congolense* in vivo or in vitro especially for the stages of the life cycle which occur in the insect vector. As free-living, aerobic trypanosomes in the gut and proboscis, the parasites depend on tsetse fluids for supply of essential nutrients and small metabolites. While there have been several reports of a heat-labile anti-trypanosomal factor in tsetse haemolymph (Croft et al., 1982; East et al., 1983), injection of tsetse homogenates into teneral tsetse significantly enhances *T. brucei* infection rates

of flies (Kaaya et al., 1986), suggesting that certain factors capable of enhancing trypanosome development may be present in tsetse tissues.

This is the first demonstration in trypanosomes of induction of differentiation to metacyclic trypanosomes by single metabolites. Considering the importance of metacyclic trypanosomes in the life cycle of this parasite, information on the factors which regulate their appearance and viability may be of practical use. The mechanism which controls this differentiation step requires further investigation.

Acknowledgments

I wish to thank Mrs. M. A. Gray for her interest and encouragement in the early stages of these experiments, and Dr. A. G. Luckins and Dr. A. Tait for critical readings of the manuscript. This work was carried out with financial support from the Overseas Development Administration, London.

Bursell E.: An introduction to insect physiology. Academic Press, London/New York 1970.

Croft S. L., East J. S., Molyneux D. H.: Anti-trypanosomal factor in the haemolymph of *Glossina*. Acta trop. (Basel) *39*, 293–302 (1982).

Crowe J. S., Barry J. D., Ross C. A., Luckins A. G., Vickerman K.: All the metacyclic variable antigen types of *Trypanosoma congolense* identified using monoclonal antibodies. Nature (Lond.) *306*, 389–391 (1983).

Cunningham I., Slater J. S.: Amino acid analysis of haemolymph of *Glossina morsitans morsitans* (Westwood). Acta trop. (Basel) *31*, 83–88 (1974).

East J. S., Molyneux D. H., Maudlin I., Dukes P.: Effect of *Glossina* haemolymph on salivarian trypanosomes in vitro. Ann. trop. Med. Parasit. *77*, 97–99 (1983).

Elford B. C., Haynes J. D., Cunlay J. D., Wilson J. M.: Selective stage-specific changes in the permeability to small hydrophilic solutes of human erythrocytes infected with *Plasmodium falciparum*. Molec. biochem. Parasit. *16*, 43–60 (1985).

Gray M. A., Cunningham I., Gardiner P. R., Taylor A. M., Luckins A. G.: Cultivation of infective forms of *Trypanosoma congolense* at 28° C from trypanosomes in the proboscis of *Glossina morsitans*. Parasitology *82*, 81–95 (1981).

Gray M. A., Ross C. A., Taylor A. M., Luckins A. G.: In vitro cultivation of *Trypanosoma congolense*: the production of infective metacyclic trypanosomes in cultures initiated from cloned stocks. Acta trop. (Basel) *41*, 343–353 (1984).

Gray M. A., Ross C. A., Taylor A. M., Tetley L., Luckins A. G.: In vitro cultivation of *Trypanosoma congolense*: the production of infective forms from metacyclic trypanosomes cultured on bovine aorta endothelial cell monolayers. Acta trop. (Basel) *42*, 99–110 (1985).

Kaaya G. P., Otieno L. H., Darji N., Alemu P.: Defence reactions of *Glossina morsitans morsitans* against different species of bacteria and *Trypanosoma brucei brucei*. Acta trop. (Basel) *43*, 31–42 (1986).

Luckins A. G., Rae P. R., Gray A. R.: Development of local skin reactions in rabbits infected with metacyclic forms of *Trypanosoma congolense* cultured in vitro. Ann. trop. Med. Parasit. *75*, 563–564 (1981).

Lumsden W. H. R., Cunningham M. P., Webber W. A. F., van Hoeve K., Walker P. J.: A method for the measurement of the infectivity of trypanosome suspensions. Exp. Parasit. *14*, 269–279 (1963).