A Minimal Medium for the Cultivation of Infective *Trypanosoma cruzi* Epimastigotes

By JOSÉ L. AVILA,* ROBERTO PÉREZ-KEPP AND ANTONIO BRETANA

Instituto Nacional de Dermatología, Apartado 4043, Caracas 1010A, Venezuela

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In a culture medium containing bovine liver catalase, but lacking exogenous free amino acids, only two vitamins (choline and folic acid) were found to be essential for the continuous *in vitro* multiplication of seven different infective strains of *Trypanosoma cruzi*. The provision of additional vitamins or sugars had no stimulatory effect under these culture conditions. These, and previous studies, have allowed the design of a trypanosomal minimal medium composed only of bovine liver catalase, choline, folic acid, glucose and inorganic salts. This was able to support the continuous cultivation of *T. cruzi* for more than 12 consecutive passages (i.e. about 160 d of culture). By several criteria, namely morphological features as seen by electron microscopy, infectivity for vertebrate and invertebrate hosts, glucose utilization and protein biosynthesis, this medium (which is the simplest so far described) proved to be nutritionally adequate for *Trypanosoma* spp. In addition, the medium appeared to be relatively specific for *T. cruzi*, as it did not support the growth of *T. rangeli* and American isolates of leishmania.

INTRODUCTION

In a previous communication (Avila *et al.*, 1979*a*) the continuous cultivation of infective epimastigotes of *Trypanosoma cruzi* in a totally defined culture medium was reported. This liquid medium contained a single purified protein, 8 vitamins, 4 nucleotides, 13 amino acids, glucose and inorganic salts. Among 12 different commercial proteins tested as nutrients, only BLC, horseradish peroxidase, bovine haemoglobin and lactoperoxidase supported continuous growth, at relatively high yield, of mouse-infective *T. cruzi*, while other haemoproteins were inactive. BLC had the best *T. cruzi* growth-promoting activity of all the proteins tested; indeed it provided all the amino acid requirements of actively growing parasites and thus eliminated the need for the addition of exogenous free amino acids to the culture medium.

The composition of this medium was based upon observations suggesting an inability of *T. cruzi* to undertake *de novo* synthesis of purines (Fernández & Castellani, 1958; Gutteridge & Gaborak, 1979; Ceron *et al.*, 1979) and various vitamins (Citri, 1954; Boné & Parent, 1963). Using defined culture medium, however, it was demonstrated (Avila & Avila, 1981) that *T. cruzi* epimastigotes do not require exogenous nucleotides for continuous cultivation, suggesting that this parasite has the capacity for *de novo* purine and pyrimidine synthesis. Also, only choline and folic acid, among the eight vitamins provided in the original culture medium, were necessary for discrete multiplication of *T. cruzi*.

The present paper describes an extension of this approach to the study of the continuous cultivation of seven different strains of infective epimastigotes of *T. cruzi* in a minimal medium composed only of BLC, choline, folic acid, glucose and inorganic salts. A number of features (multiplication rate, electron microscopic morphology, infectivity for vertebrate and inverte-

Abbreviations: BLC, bovine liver catalase; EPPS, 4-(2-hydroxyethyl)-1-piperazine propanesulphonic acid; LIT, liver infusion tryptose; MCRM, micromolecular component rich medium; TMM, trypanosomal minimal medium.

brate hosts, glucose utilization and protein biosynthesis) were examined to test the nutritional quality of this new medium for supporting *T. cruzi* growth.

METHODS

Parasites. Studies were performed with strains Y, Ma, Fl, Bertoldo, A-35, Ya and EP of *T. cruzi*, as previously described (Avila *et al.*, 1979a, 1981a, b). The cultures were maintained serially in BLC-containing medium, and were used after the nineteenth passage in these experiments.

Culture media. MCRM was prepared as previously described (Avila *et al.*, 1979a) and compared with the simplified medium for *T. cruzi*, TMM, the composition of which is described in Table 1. In some experiments, choline was excluded from TMM. The initial pH of both media was 6.4 and each culture flask (Falcon no. 3012, Fisher Scientific Co., Pittsburgh, Pa., U.S.A.), containing a total volume of 10 ml, received 0.3–0.8 ml inoculum containing 5×10^6 parasites from the preceding subculture.

Parasite counts. At 2 d intervals 0.5 ml volumes were aseptically withdrawn from the cultures at each subpassage and the parasites counted in a haemocytometer at $\times 400$ magnification.

Electron microscopy. This was carried out as previously described (Bretaña & O'Daly, 1976).

Infectivity studies. Two different approaches were used to determine the infectivity of cultured *T. cruzi*: (1) infectivity towards vertebrate hosts using susceptible NMRI mice as previously described (Avila *et al.*, 1979b); (2) infectivity for invertebrate hosts, using nymphs of *Rhodnius prolixus*. The insects were provided by the PAHO/WHO Research and Reference Centre of Vector Biology and Control, Maracay, Venezuela. They were allowed to feed for 60 min on each of the cultured *T. cruzi* strains contained in individual bovine intestinal membrane topped pots containing five nymphs. The insects were then maintained in a humid atmosphere at 25 °C for 15–21 d post-feeding. The nymphs were examined individually for infection with *T. cruzi* 7–21 d after feeding; each nymph was wetted and killed in normal saline and the rectum and posterior intestine dissected out, separated from the rest of the gut and macerated in a small drop of saline on a microscope slide. Each preparation was thoroughly mixed and covered with a 13 mm diameter coverslip. The entire area under the coverslip was examined for trypanosomes using phase-contrast illumination ($\times 10$ objective, final magnification $\times 400$).

Glucose utilization and protein biosynthesis. On withdrawal of samples for parasite counting, the pH was measured at room temperature (20 °C) before centrifugation in the cold (2400 g for 20 min at 4 °C); the glucose concentration was determined in the supernatant using the anthrone method of Jermyn (1975). It has previously been demonstrated, by TLC on kieselguhr G using ethyl acetate/isopropanol/H₂O (65:23:12, by vol.) as eluant, that glucose represents about 91–98% of the sugars present in the culture medium (unpublished observations). Protein biosynthesis was determined after 10 d cultivation by measuring the incorporation of L-[4,5-³H]leucine into soluble proteins using the method of Mancilla *et al.* (1967). Epimastigotes were incubated for a total of 3 h in the presence of L-[³H]leucine [50 µCi (185 kBq) per flask].

Biochemicals. BLC (C-40, lots 60F-7155 and 70F-7050, and twice crystallized C-100), glucose, streptomycin, penicillin, HEPES, EPPS and phenol red were obtained from Sigma. Folic acid, choline, the minimal essential

Table 1. *Composition of TMM for culture of T. cruzi*

The medium was made up to 1 litre with deionized water and the pH adjusted to 6.4 with 1 M-HCl.

Component	Concentration (g l ⁻¹)
NaCl	6.8
KCl	0.4
MgSO ₄ ·7H ₂ O	0.2
NaH ₂ PO ₄ ·H ₂ O	0.14
CaCl ₂	0.2
Choline chloride	0.002*
Folic acid	0.002*
Glucose	1.0
NaHCO ₃	2.2
HEPES	2.26
EPPS	2.34
Phenol red	0.01
Streptomycin	0.1
Penicillin	100000†
Bovine liver catalase	3.0

* From a 100-fold concentrated frozen stock solution.

† Concentration expressed in international units.

medium and the vitamin mixture (100-fold concentrate) were from Grand Island Biological Co. (New York, N.Y., U.S.A.). L-[^3H]Leucine (1.25 Ci mol^{-1} ; $46.25 \text{ GBq mol}^{-1}$) was purchased from Schwartz-Mann (Orangeburg, N.Y., U.S.A.). Inorganic salts were obtained from Merck-Darmstadt.

RESULTS

In MCRM supplemented as described previously (Avila *et al.*, 1979a) the seven *T. cruzi* strains studied reached an average maximum concentration of about 15×10^6 organisms ml^{-1} after 10 d cultivation. Strains A-35 and Ya attained the highest (19×10^6 organisms ml^{-1}) and lowest (7×10^6 organisms ml^{-1}) levels of growth in this medium respectively. These levels of growth amount to 34% of the average maximum growth obtained with a richer, undefined but conventional LIT medium (Pérez-Kepp *et al.*, 1981). The mean growth level reached in MCRM represented a 30-fold multiplication over the initial concentration of cells in the inoculum.

The results in Table 2 show that all seven *T. cruzi* strains grew in TMM in a continuous manner (although at different rates) for at least 13 successive passages. The Bertoldo strain gave the highest levels of growth and Ma strain gave lowest levels by comparison with the extent of growth in the control medium (MCRM). The highest average *T. cruzi* concentrations in TMM during 13 consecutive passages for all seven strains was about 9×10^6 organisms ml^{-1} , which represents an 18-fold multiplication over the inoculum. The growth curves obtained in MCRM and TMM (average for seven strains) are shown in Fig. 1. Maximum growth in TMM was equivalent to 63% of that in MCRM and 20% of that in LIT medium (Pérez-Kepp *et al.*, 1981).

No difference in *T. cruzi* growth-promoting activity was found between BLC added as purified powder (Sigma; C-40) or as twice crystallized enzyme (Sigma; C-100). However, for practical reasons, most of the experiments reported in this paper were carried out with C-40 preparations.

After several consecutive passages, *T. cruzi* strains obtained directly from MCRM were cultured in TMM under three different experimental conditions: (1) in the presence of the complete vitamin mixture used in MCRM, (2) in the presence of choline and folic acid plus only one of the other vitamins present in MCRM, (3) in the presence of folic acid alone. It was found that the individual addition of pantothenate, inositol, nicotinamide, pyridoxal, riboflavin and thiamin at the concentrations used in MCRM had no effect on parasite yield in TMM, although the addition of the total vitamin mixture allowed a marked increase in multiplication, growth being comparable to that obtained for *T. cruzi* strains routinely maintained in MCRM. Omission of choline lowered the parasite yield by about 50%. These results confirmed the excellent viability of *T. cruzi* epimastigotes grown in TMM, and the importance of choline for maintaining high rates of cellular multiplication. After demonstrating the continuous multiplication of *T. cruzi* epimastigotes in TMM, we then studied the infectivity for vertebrate and invertebrate hosts of these culture forms. All ninth passage *T. cruzi* strains obtained from TMM were infective for NMRI mice and nymphs of *R. prolixus*.

Table 2. Continuous growth of *T. cruzi* in TMM

The composition of TMM is shown in Table 1. Control values refer to growth obtained in MCRM, after 10 d cultivation at 28 °C. Values in parentheses are maximal parasite concentrations (10^6 cells ml^{-1} obtained in each passage in TMM).

Strain	Growth as percentage of control at passage:					
	1	2	3	6	8	13
Ma	41 (9.1)	52 (14.2)	65 (10.9)	52 (11.3)	46 (10.2)	58 (10.0)
EP	50 (9.9)	78 (14.5)	85 (7.0)	82 (14.1)	100 (11.0)	102 (15.0)
Fl	45 (8.3)	51 (12.5)	100 (13.9)	56 (11.2)	70 (13.9)	41 (8.8)
Y	24 (6.4)	53 (12.2)	78 (11.2)	71 (13.8)	55 (12.2)	62 (12.0)
A-35	56 (12.5)	36 (9.4)	100 (10.4)	36 (9.0)	74 (16.3)	69 (14.0)
Ya	82 (6.7)	56 (5.9)	95 (9.9)	59 (5.1)	38 (2.9)	38 (2.9)
Bertoldo	100 (20.0)	100 (10.6)	100 (14.4)	62 (9.4)	55 (12.2)	90 (9.0)
Mean	57%	61%	89%	60%	63%	66%

Fig. 1. Growth of *T. cruzi* in catalase-containing media: ○, MCRM; ●, TMM. Bovine liver catalase was at a concentration of 3 mg ml⁻¹. Growth values are means from seven *T. cruzi* strains studied. The bars represent S.D.

Fig. 2. Electron micrograph of longitudinal section through the kinetoplast region of *T. cruzi*, cultured in: (a) MCRM, (b) TMM. Features shown are: N, nucleus; f, flagellum bases in flagellar pocket; g, Golgi apparatus; m, pellicular microtubules; v, inclusion vacuoles of unknown significance; and k, kinetoplast. Bovine liver catalase aggregates can be seen outside the parasite (arrows), but also in pinocytotic vacuoles (white asterisk). The bar markers represent 1 μm.

The electron micrographs in Fig. 2 show some ultrastructural features of *T. cruzi* epimastigotes cultured in TMM and, for comparative purposes, in MCRM. No significant differences were observed in the ultrastructural morphology between epimastigotes cultured in either medium as compared with those grown in LIT medium (deSouza *et al.*, 1978).

Fig. 3. pH changes (○, ●) and glucose utilization (△, ▲) in *T. cruzi* epimastigotes cultured in MCRM (open symbols) and TMM (filled symbols) as a function of the time of cultivation at 28 °C. Values are means from seven *T. cruzi* strains studied. The bars represent S.D.

Table 3. L-[³H]Leucine incorporation into soluble proteins of *T. cruzi*

[³H]Leucine incorporation is expressed as d.p.m. (mg protein)⁻¹ h⁻¹. Each result is the mean of 10 determinations ± S.E. The numbers in parentheses represent the total leucine incorporation [nmol (mg protein)⁻¹ h⁻¹] based on the specific radioactivity of the leucine.

Leucine concn (mM)	[³ H]Leucine incorporated		Ratio of incorporation TMM/MCRM
	MCRM	TMM	
5	10500 ± 790* (5.39)	22900 ± 800* (9.45)	2.18
50	17400 ± 680 (7.56)	26500 ± 700 (12.01)	1.52

* These values are significantly different ($P < 0.005$).

Despite the lack of exogenous free amino acids in TMM, the glucose consumption and pH changes were similar to those in MCRM (Fig. 3); maximal glucose consumption was reached after 10 d of culture, 80% of total glucose present at the time of inoculation being consumed. This indirectly suggests that glucose utilization by the parasite is similar in both media, and that BLC, after intracellular hydrolysis, provides enough free amino acids to fulfil the nutritional requirements of actively growing epimastigotes.

The [³H]leucine incorporation into soluble proteins was significantly higher in TMM compared with MCRM (Table 3), possibly as a result of competition from other free amino acids present in the MCRM. However, in order to eliminate this possibility, [³H]leucine was also added at 10 × the initial concentration. Incorporation was still found to be significantly higher in TMM, suggesting the possible existence of metabolic differences between TMM- and MCRM-cultured epimastigotes.

Finally, light microscopic analysis of the different cellular forms present in TMM revealed 96% epimastigotes and 4% trypomastigotes in the stationary phase of the culture, which compares closely to the distribution in MCRM.

DISCUSSION

Our main concern in developing a *T. cruzi* minimal medium was to find combinations of nutrient supplements to BLC which will support the continuous growth of infective parasites at a reasonably high rate. The evidence indicates that a medium composed of BLC, choline, folic acid, glucose and inorganic salts is able to support the growth of *T. cruzi* epimastigotes to an average level of about 9×10^6 cells ml⁻¹ for all seven *T. cruzi* strains tested over 13 consecutive passages.

Multiplication was not further improved by addition of other vitamins (*p*-aminobenzoic acid, biotin, pyridoxine, pyridoxamine, ascorbic acid, vitamin B₁₂) or carbohydrates (ribose, xylose,

trehalose) (unpublished results). Also, increased concentrations of the co-factors in TMM did not give increased growth (Avila & Avila, 1981).

Evidence was obtained of the key role of choline in supporting *T. cruzi* multiplication. Our results differed from those obtained with *Crithidia fasciculata* (Palmer, 1974), for which the availability of exogenous choline does not influence the rate of cellular growth. Regarding the possible role of choline in *T. cruzi* multiplication, it is known that this co-factor can serve as a precursor for phospholipid biosynthesis, and is also a labile methyl group donor.

The average maximal growth in TMM is about 20% of that attained in a conventional medium such as LIT, but this was to be expected from a defined and nutritionally poorer medium. On the other hand, the usefulness of TMM would be severely limited if the properties of the parasites, such as infectivity, morphology or metabolism, were radically changed when grown in this minimal medium. However, such problems have not materialized in our studies. Thus, ultrastructural morphology is similar to that previously reported for *T. cruzi* epimastigotes (Bretaña & O'Daly, 1976; deSouza *et al.*, 1978). Also, infectivity is similar to that obtained previously with cultured *T. cruzi* when tested in NMRI mice (Avila *et al.*, 1979a), or when *R. prolixus* were fed with MCRM-derived *T. cruzi* forms. Glucose consumption was the same in both MCRM- and TMM-cultured epimastigotes, although [³H]leucine incorporation differed.

It must be stressed that neither TMM nor MCRM is able to support *Trypanosoma rangeli* multiplication (Avila *et al.*, 1981b) or *Leishmania brasiliensis* and *Leishmania mexicana* proliferation (Avila *et al.*, 1979a), suggesting that these *Trypanosomatidae* have different nutritional requirements. TMM, therefore, represents the first defined minimal trypanosomal medium that is useful for the specific cultivation of infective *T. cruzi*. As previously suggested (Avila *et al.*, 1979a), BLC acted in TMM as an adequate nutritional factor, fulfilling all the amino acid requirements of actively growing parasites. It should be noted that no difference in *T. cruzi* growth-promoting activity was found between BLC used as powder (Sigma; C-40) or twice crystallized enzyme (Sigma; C-100). Furthermore, as previously reported (Avila *et al.*, 1979a), PAGE of both C-40 and C-100 preparations of BLC revealed a major band corresponding to about 90% of the protein added to the gel and a second minor band probably corresponding to denatured protein (Sumner & Gralén, 1938).

The fact that seven different *T. cruzi* strains (six from Venezuela and another from Brazil) grew quite well in TMM also demonstrates that we are not dealing with the cultivation of an unrepresentative 'mutant' strain.

The presence of endosymbionts has been reported in *Crithidia oncopelti*, *Crithidia deanei* and *Blastocrithidia culicis* (Chang, 1974; Mundim *et al.*, 1974). In order to eliminate any possibility of contamination of our *T. cruzi* strains, serial electron microscopic and microbiological studies were undertaken, and no symbionts were found.

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