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Trypanosoma brucei: Differentiation of in Vitro-Grown Bloodstream Trypomastigotes into Procyclic Forms[1]

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ABSTRACT. *Trypanosoma brucei* strain 366D trypomastigotes grown at 37°C in the presence of a human fibroblast cell line formed foci underneath the feeder cells whereas trypanosomes grown in the presence of a human epithelial cell line grew only in the culture supernatant. A culture system was developed to study the differentiation of bloodstream trypomastigotes grown in the epithelial cell system into procyclic trypomastigotes at 27°C. The morphological differentiation into the procyclic form was complete by 48 h. Cell division did not occur until 30–40 h after transfer to 27°C. Various characteristics of this system were examined, including the effect of the feeder layer, the type of medium, the presence of the metabolites cis-aconitate and citrate, the preadaptation period, and the trypanosome cell concentration. The respiration of the recently differentiated procyclic cells was less sensitive to inhibition by CN^- than that of established procyclic forms, implying a delayed appearance of complete mitochondrial oxidative pathways. This trypanosome differentiation system has the advantage that the animal host is not needed and the entire process is carried out in in vitro culture.

THE differentiation of the bloodstream trypomastigote form of *Trypanosoma brucei* into the procyclic culture form, which survives in the tsetse midgut, involves some external morphological change combined with several dramatic internal ultrastructural and physiological changes. The long slender bloodstream trypomastigote has a glycoprotein surface coat and contains a single mitochondrion that lacks well-developed cristae and the associated mitochondrial enzymes. Within the bloodstream, the initial event in the differentiation process is the appearance of the 'stumpy' trypomastigote form, which is a non-dividing cell fixed in G_0 that contains an active mitochondrial lipoyl dehydrogenase enzyme activity but does not contain mitochondrial cytochromes. The next step in the process, the differentiation of the stumpy form to the procyclic form, occurs in nature in the insect midgut. It involves a loss of the surface coat and the establishment of a functional mitochondrion containing cristae.

Cultivation of animal-infective monomorphic and pleomorphic bloodstream forms of African trypanosomes at 37°C in the presence of mammalian feeder cells has been described (7, 11, 12, 15, 17) as has the in vitro differentiation of bloodstream forms into procyclic culture forms. Most work has involved the use of bloodstream forms from the animal although Brun et al. (7) noted, without providing data, that a small portion of cultured polymorphic bloodstream forms transformed within three days into procyclic forms in SDM-79 medium. Brun & Schonenberger (6) observed that the addition of the Krebs cycle metabolites, citrate and cis-aconitate, markedly stimulated this process. Simpson et al. (15) confirmed the stimulatory effect of citrate and cis-aconitate on the differentiation process and also showed that continued exposure of the bloodstream trypomastigotes to these metabolites in culture at 37°C was toxic. The mechanism of action of citrate and cis-aconitate is unknown.

Overath et al. (13) examined the repression of surface glycoprotein synthesis and the loss of the surface coat in *T. brucei* stock 427 during the procyclic differentiation process. Bienen et al. (2–4) found that *T. brucei* LUMP 1026 bloodstream trypomastigotes from the rat underwent a reproducible differentiation into the procyclic form without cell death in modified Cunningham's medium in approximately 72 h at 27°C. They also examined antimycin A, CN^-, and SHAM-sensitive respiration as well as the appearance of cytochromes by spectral analysis. They concluded that the morphological differentiation to the procyclic form occurs independently of the biochemical differentiation that progresses through the initial appearance of mitochondrial dehydrogenases by day 5 to the establishment of a complete cytochrome complement by day 20. Ultrastructural correlates of the biochemical changes occurring during this life cycle change have also been presented (10). The single mitochondrion undergoes a gradual change from the "narrow tube with only few short cristae" (10), characteristic of the long slender bloodstream form, to the extended and branched structure containing abundant cristae found in the established procyclic form. The finding that these morphological changes are incomplete even after 11 days of culture at 27°C (10) is in agreement with the biochemical studies.

In this paper we examine various factors affecting the in vitro differentiation of cultured bloodstream trypomastigotes of *T. brucei* strain 366D into procyclic forms. A preliminary account of this work was reported previously (15).

MATERIALS AND METHODS

The human fetal lung fibroblast cell line (F2000) was obtained from Flow Labs. The human skin epithelium cell line (NCTC 3075) was obtained from the American Type Culture Collection (ATCC CCL 19.1). A human epithelial tumor cell line (SCaBER) was obtained from Dr. Ahmed of the UCLA Dermatology Department (1); this line was originally derived from a human bladder cancer. All cell lines were maintained in T25 flasks at 37°C in RPMI 1640 medium with 10% (v/v) fetal calf serum (heat-inactivated). Gamma irradiation of the F2000 cell monolayer was performed using a cobalt source. Gamma irradiation of the CCL 19.1 cell line often led to extensive cell sloughing and therefore was not generally employed.

The *T. brucei* strain 366D cells were obtained from Dr. Leo Jenni of the Swiss Tropical Institute and were routinely maintained in culture at 37°C in RPMI medium with 10% (v/v) fetal calf serum in the presence of the F2000 fibroblast cell line. Trypanosomes were transferred to the CCL 19.1 culture at least one week prior to use for differentiation. Established procyclic cells were maintained at 27°C in modified REI medium (16), also known as REIA medium (14).

Measurement of percentage of procyclic forms was performed by mixing 100 μl of culture with 100 μl of 10% (v/v) formalin in 0.15 M NaCl–0.015 M Na citrate (pH 7) and scoring cell types in a hemocytometer under phase contrast microscopy at 400× magnification. Cell concentration was also measured in a hemocytometer. At least 200–600 cells were counted for each point. The data in Figs. 4–9 are representative results from experiments that were repeated several times with similar results.

Respiration studies were performed using a Clark oxygen electrode with a 2-ml water-jacketed chamber. Cells were resuspended in REIA medium for respiration measurements at 37°C

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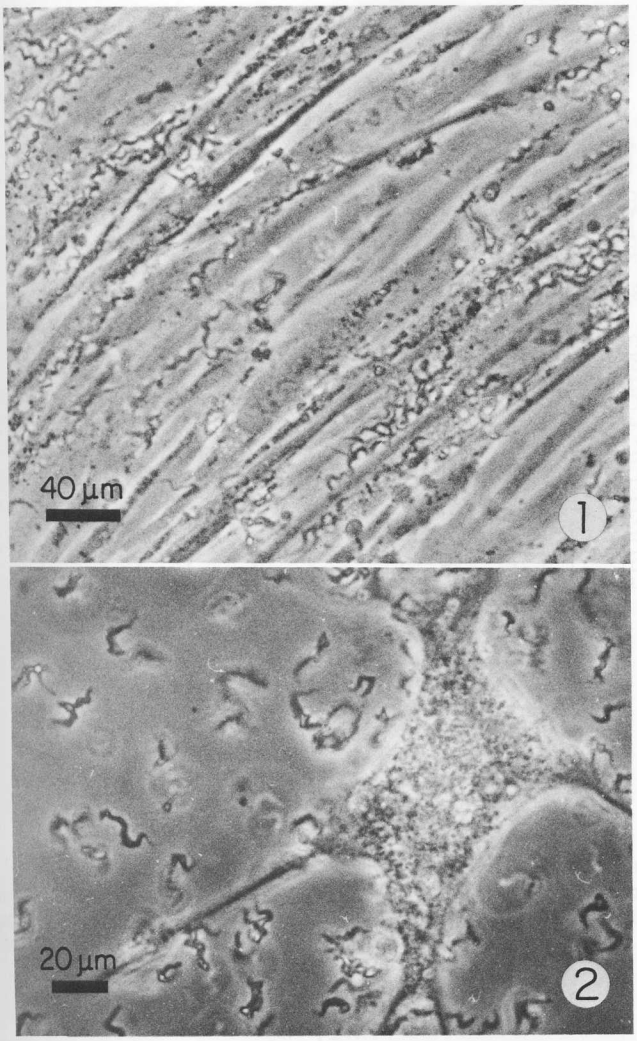

Fig. 1. Phase contrast micrograph of cultured bloodstream forms of *T. brucei* strain 366D grown in the presence of F2000 fibroblast cell line.

Fig. 2. Phase contrast micrograph of cultured bloodstream forms of *T. brucei* strain 366D grown in the presence of CCL 19.1 epithelial cell line.

(bloodstream forms) or 27°C (procyclic forms). Cyanide inhibition of respiration was measured after the addition of 0.001 M KCN.

Photomicrographs were taken with phase contrast at 200–400× magnification.

All chemicals were reagent grade. All culture media were purchased from GIBCO.

RESULTS

In vitro culture of T. brucei *strain 366D. Trypanosoma brucei* strain 366D cells were cultured routinely at 37°C as slender bloodstream forms in the presence of a 5000 R gamma-irradiated human fibroblast cell line (Flow, F2000) in RPMI 1640 medium with 10% fetal calf serum (Fig. 1). The same irradiated feeder layer could be used for up to three months before the fibroblast cells began sloughing off from the flask. In one experiment, in which the feeder layer was trypsinized to recover all of the trypanosomes, 12% of the total trypanosomes in the culture were present in the large foci beneath the fibroblast cells.

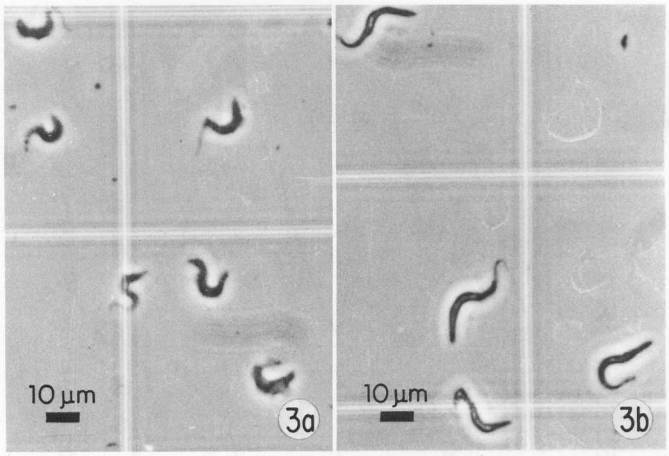

Fig. 3. Phase contrast micrographs of (a) bloodstream forms and (b) procyclic forms within a hemocytometer chamber.

In the presence of a feeder layer of human epithelial cells (ATCC CCL 19.1), the trypanosomes grew in the culture supernatant and did not form the large intercellular foci characteristic of the F2000 culture (Fig. 2). In addition, trypanosomes grown for at least several days in the presence of the CCL 19.1 feeder cells appeared somewhat more 'intermediate stumpy' in shape than the trypanosomes grown in the F2000 culture.

Penetration of SCaBER tissue culture cell line by trypanosomes. In a survey of the suitability of various cell lines for use as feeder cells, we noticed an unusual phenomenon with a human epithelial tumor cell line (SCaBER). The trypanosomes grew beneath these cells as in the F2000 system but occasionally penetrated or were engulfed by the tumor cells and survived for some time as extremely motile forms apparently free in the cytoplasm. The tumor cells that contained motile trypanosomes were rounded and poorly attached to the flask. This phenomenon was not studied further but may have implications for a possible cryptic phase in the life cycle of *T. brucei* in the mammalian host (9).

Standard culture system for differentiation to procyclic forms. The CCL 19.1 bloodstream culture system was used to eliminate the problem of inaccessible intercellular trypanosomes. Trypanosomes were transferred from the stock F2000 culture into the CCL 19.1 culture and allowed to grow at least one week prior to use. Preadaptation for differentiation was accomplished by addition of 5 mM citrate or cis-aconitate to the RPMI medium for 17 h at 37°C. The trypanosomes were then harvested by centrifugation and resuspended at 27°C to a concentration of approximately 1.3×10^6 cells/ml in REIA medium (REI medium with 0.2% glycerol in place of glucose and 10% dialyzed fetal calf serum) with 5 mM cis-aconitate, in the presence of the same CCL 19.1 feeder cell layer. Samples were removed at intervals, and the percentage of procyclic forms measured by phase microscopy in a hemocytometer at 400× magnification (Fig. 3). The trypanosomes could be removed from the feeder cells after 24 h to establish continuous procyclic cultures (data not shown); in the experiments presented in this paper, however, the feeder layer is present throughout the entire differentiation process. Morphogenesis to the procyclic form was complete by 48 h; cell division began approximately 30–40 h after transfer to REIA at 27°C. Justification for the experimental details of this standard procedure is given below.

Effect of initial trypanosome cell density. Four differentiation cultures were initiated as described above with different cell

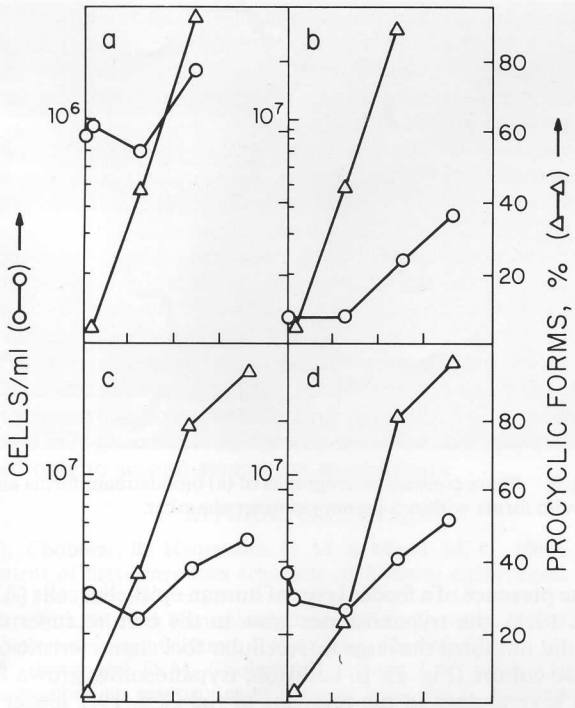

Fig. 4. Effect of initial cell density on the differentiation of *T. brucei* from the bloodstream form to the procyclic form in REIA medium at 27°C with the CCL 19.1 feeder layer present. (a) 0.6 × 10^6 cells/ml. (b) 1.2 × 10^6 cells/ml. (c) 2.8 × 10^6 cells/ml. (d) 3.5 × 10^6 cells/ml.

densities of bloodstream trypomastigotes. As shown in Fig. 4, an initial cell density of 1–3 × 10^6 cells/ml appeared optimal in terms of viability and the rate of appearance of procyclic forms.

Effect of the concentration of cis-aconitate on the differentiation process. Bloodstream trypomastigotes from CCL cultures were preadapted for 17 h at 37°C with different concentrations of cis-aconitate and then allowed to differentiate in REIA medium at the same cis-aconitate concentration. As shown in Fig. 5, 5–10 mM cis-aconitate appeared to be optimal. This agrees well with the optimal concentration of cisaconitate determined by Brun & Schonenberger (6) in the SDM-79 *T. brucei* differentiation system.

Comparison of cis-aconitate and citrate on the differentiation process. As shown in Fig. 6, both citrate and cis-aconitate at 5 mM were separately or jointly effective in stimulation of the differentiation process. Cis-aconitate alone, however, appeared to limit the extent of cell death in the early stages of differentiation.

Necessity for the continued presence of citrate (or cis-aconitate) during the differentiation process. Trypomastigotes were preadapted for 17 h with 5 mM citrate and then transferred to the REIA medium at 27°C, with and without citrate, in the presence of the feeder layer. As shown in Fig. 7, the continued presence of the metabolite decreased the extent of cell death in the early stages of the process.

The effect of the culture medium and the presence of a feeder layer on the differentiation process. The media tested, in addition to REIA, included Cunningham's medium (8) and SDM-79 (5). Cunningham's medium yielded procyclic cells that ap-

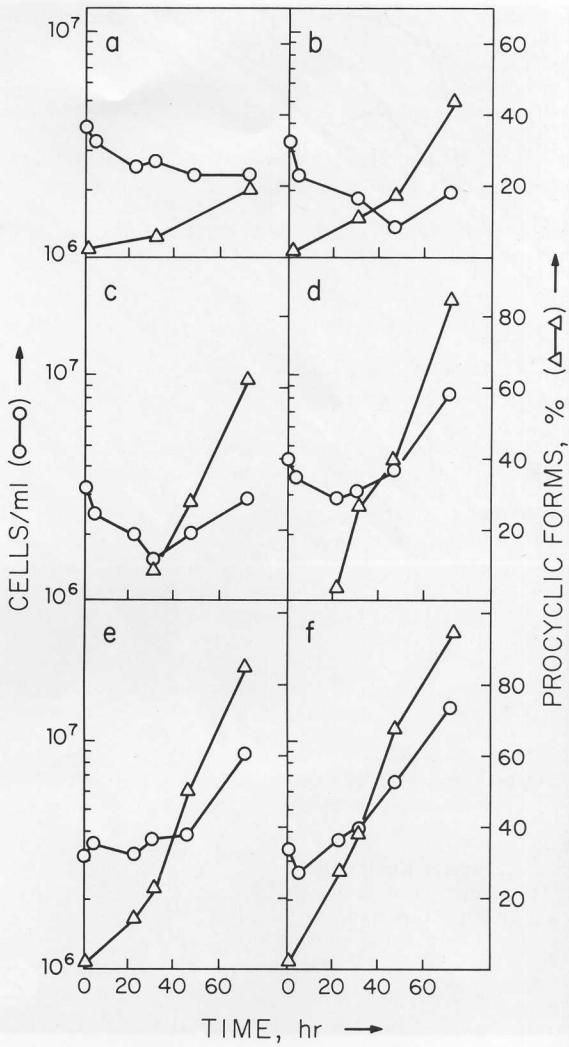

Fig. 5. Effect of the concentration of cis-aconitate on the differe
tion of *T. brucei* from the bloodstream form to the procyclic form.
were preadapted for 17 h at 37°C with the same concentration o
aconitate as used in the REIA medium at 27°C. (a) a mM cis-acon
(b) 2 mM. (c) 3 mM. (d) 4 mM. (e) 5 mM. (f) 10 mM.

peared abnormal morphologically and which became attac
to the flask surface. These cells did not survive more than
week at 27°C (results not shown).

The SDM-79 and REIA media both allowed a non-opti
differentiation into procyclics even without the presence
feeder layer. The presence of a feeder layer decreased cell d
and increased the rate of the differentiation process. The
cessity for the addition of citrate to SDM-79 medium and
stimulatory effect of a continued presence of the feeder laye
shown in Fig. 8a–d. The results shown in Fig. 8e illustrate
large extent of cell death that occurs in REIA medium in
absence of a feeder layer (even in the presence of citrate),
the results shown in Fig. 8b illustrate that the feeder layer a
cannot substitute for the addition of citrate to the SDM
medium (or REIA).

In REIA medium, the absence of both citrate and a fe
layer leads to massive and rapid cell death without differe
tion (Fig. 9a). Addition of 5 mM citrate to the REIA med
in the absence of a feeder layer does allow some differentia
as noted above but does not inhibit the massive cell death

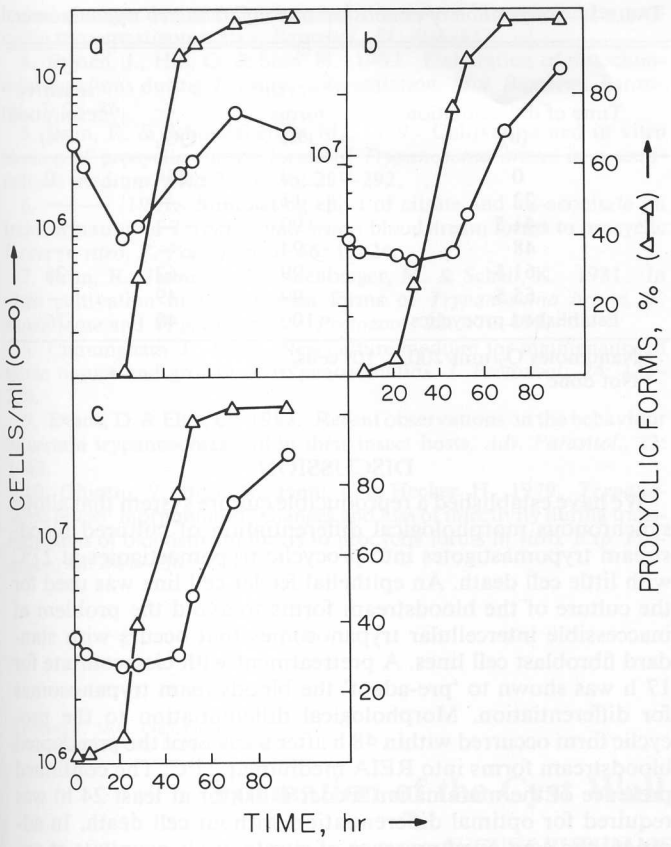

Fig. 6. Comparison of the effects of addition of citrate or cisaconitate on the differentiation of *T. brucei* from the bloodstream form to the procyclic form in REIA medium. (a) 5 mM citrate. (b) 5 mM cisaconitate. (c) 5 mM citrate and 5 mM cisaconitate.

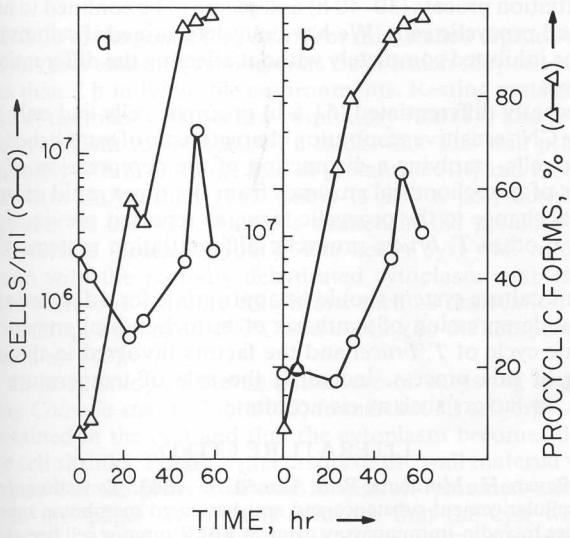

Fig. 7. Effect of the addition of citrate to REIA medium during the differentiation of *T. brucei* from the bloodstream form to the procyclic form. (a) Preadaptation for 17 h with 5 mM citrate. No addition of citrate or cisaconitate to REIA medium at 27°C. (b) Preadaptation as in (a). Addition of 5 mM citrate to REIA medium at 27°C.

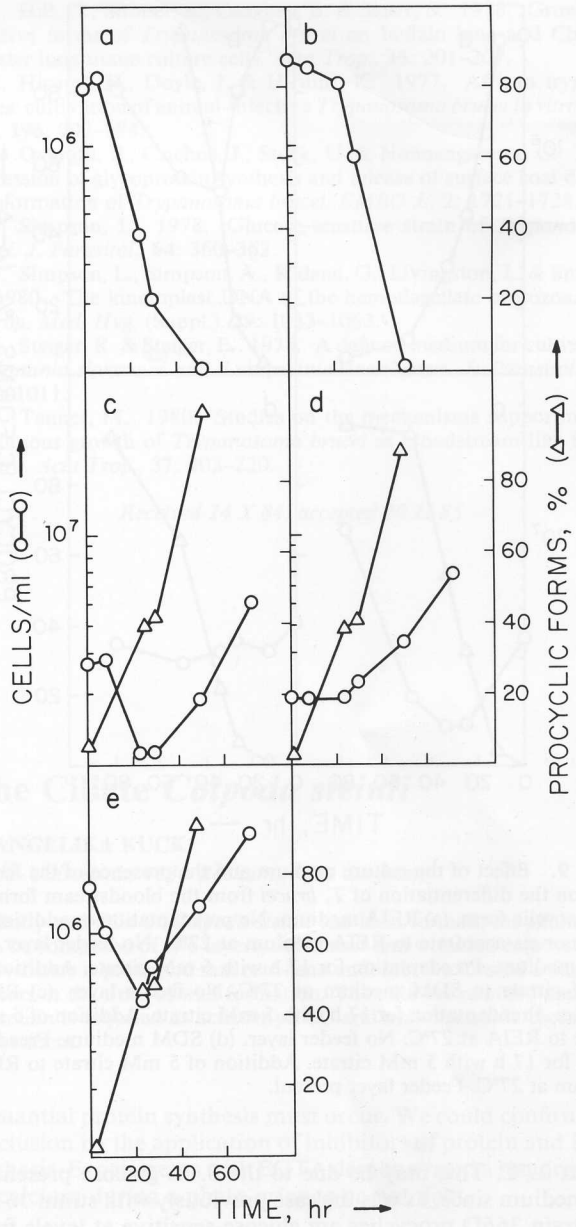

Fig. 8. Effect of the culture medium and the presence of a feeder layer on the differentiation of *T. brucei* from the bloodstream form to the procyclic form. (a) SDM medium. No addition of citrate or cis-aconitate. No feeder layer. (b) SDM medium. No addition of citrate or cis-aconitate. Feeder layer present. (c) SDM medium. Preadaptation for 17 h with 5 mM citrate. Addition of 5 mM citrate to SDM at 27°C. No feeder layer. (d) SDM medium. No preadaptation with citrate or cis-aconitate. Addition of 5 mM citrate to SDM medium at 27°C. Feeder layer present. (e) REIA medium. Preadaptation for 17 h with 5 mM citrate. Addition of 5 mM citrate to REIA medium at 27°C. No feeder layer.

9c). A similar effect of citrate is seen with cells in SDM-79 medium (Figs. 8c, 9b). The continued presence of the feeder layer in addition to citrate decreases the cell death in both REIA (results not shown) and SDM-79 media (Fig. 9d).

Procyclic cells transformed in SDM-79 medium with citrate and with or without a feeder layer appeared abnormal morphologically and could not be continuously maintained in cul-

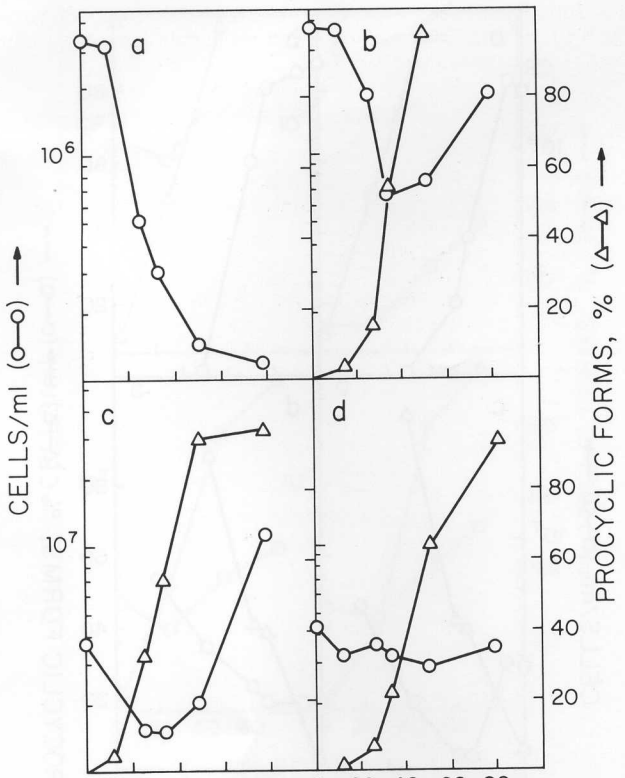

Fig. 9. Effect of the culture medium and the presence of the feeder layer on the differentiation of *T. brucei* from the bloodstream form to the procyclic form. (a) REIA medium. No preadaptation or addition of citrate or cis-aconitate to REIA medium at 27°C. No feeder layer. (b) SDM medium. Preadaptation for 17 h with 5 mM citrate. Addition of 5 mM citrate to SDM medium at 27°C. No feeder layer. (c) REIA medium. Preadaptation for 17 h with 5 mM citrate. Addition of 5 mM citrate to REIA at 27°C. No feeder layer. (d) SDM medium. Preadaptation for 17 h with 5 mM citrate. Addition of 5 mM citrate to REIA medium at 27°C. Feeder layer present.

ture at 27°C. This may be due to the 0.1% glucose present in this medium since, as was the case previously with strain 367H, the strain 366D procyclics are glucose-sensitive at levels from 0.2–0.5% glucose and must be grown in glucose-free medium with dialyzed fetal calf serum. The addition of 0.5% glucose to the REIA medium (with citrate and feeder layer) completely inhibited the differentiation process and also inhibited cell division of established procyclics, producing abnormal, enlarged cells (results not shown).

Level of CN^- sensitive respiration of recently differentiated procyclic cells. The respiration of bloodstream forms grown in the F2000 and the CCL 19.1 culture system and of recently differentiated and established procyclic forms in REIA medium was measured using an oxygen electrode. As shown in Table I, oxygen uptake per cell decreased 5.4× after 65.5 h of differentiation, and the extent of CN^-sensitive respiration increased to 26% after 61.5 h of differentiation. The respiration of established procyclic forms showed a CN^-sensitivity of 76%. This implies, as established previously in other systems (3, 4), that the development of a complete mitochondrial oxidative system in this developmental system does not parallel the rapid morphological change to the procyclic form of the parasite.

TABLE I. *Respiration of established and differentiated trypanosomes.*

Time of differentiation (h)	Procyclic forms (%)	QO_2[a]	CN^--sensitive respiration (%)
0	0	264	0
22	11	—[b]	—
41.5	90	—	—
48	94	—	—
61.5	99	62	26
65.5	99	49	—
Established procyclics	100	40	76

[a] Nanomoles O_2/min/200 × 10^6 cells.
[b] Not done.

DISCUSSION

We have established a reproducible culture system that allows synchronous morphological differentiation of cultured bloodstream trypomastigotes into procyclic trypomastigotes at 27°C with little cell death. An epithelial feeder cell line was used for the culture of the bloodstream forms to avoid the problem of inaccessible intercellular trypanosomes that occurs with standard fibroblast cell lines. A pretreatment with cis-aconitate for 17 h was shown to 'pre-adapt' the bloodstream trypanosomes for differentiation. Morphological differentiation to the procyclic form occurred within 48 h after transfer of the preadapted bloodstream forms into REIA medium at 27°C. The continued presence of the mammalian feeder cells (for at least 24 h) was required for optimal differentiation without cell death. In addition, the continued presence of citrate or cis-aconitate at approximately 5 mM was highly beneficial, both in increasing the rate of differentiation and in decreasing cell death in the early stages. Several culture media were compared, with the best differentiation and growth obtained using modified REI medium (14) of Steiger & Steiger (16). As reported previously with *T. brucei* strain 367H (14), the replication of strain 366D procyclic cells was inhibited by 0.2–0.5% glucose in REIA medium.

Cell division does not occur until the later stages of the differentiation process (30–40 h) and seems to be confined to newly formed procyclic cells. We have not determined if cell division can be inhibited completely without affecting the differentiation process.

Recently differentiated (61.5 h) procyclic cells had only 34% of the CN^-sensitive respiration characteristic of established procyclic cells, implying a disjunction of the derepression of synthesis of mitochondrial enzymes from the more rapid morphological change to the procyclic form, as reported previously for several other *T. brucei* procyclic differentiation systems (3, 4, 10).

This culture system should be appropriate for a detailed study of the derepression of synthesis of mitochondrial enzymes in the life cycle of *T. brucei* and the factors involved in the initiation of this process, including the role of temperature and medium factors such as cis-aconitate.

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Formation of the Cyst Wall of the Ciliate *Colpoda steinii*

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ABSTRACT. After a thin membranous envelope surrounding the cell body and cilia of *Colpoda steinii* has been formed, the main mass of the proteinaceous cyst wall is deposited without exocytosis. It can be composed of two layers, the denser and wrinkled ectocyst and the smooth-walled endocyst; however, the ectocyst may be missing. Evidence is presented that ecto- and endocyst are formed from vesicles derived from abundant rough endoplasmic reticulum which appears at the time of wall formation. The cilia are retained and become embedded in the peripheral cytoplasm. Synthesis of RNA and protein is required as actinomycin C and cycloheximide block cyst formation. Calcium is required during a sensitive phase prior to encystment.

SPECIES of the genus *Colpoda* form thin-walled reproductive cysts and enduring resting cysts from which they can hatch in less than 2 h in favorable environments. Resting cysts are in a state of dormancy with a low respiratory rate (17) and a very dense cytoplasm surrounded by a thick wall. The wall protein is unusually rich in glutamic acid or glutamine (14) and is cross-linked with polyamines (16). There is also a polysaccharide component in the wall, which is stained in the periodic acid Schiff reaction (Kuck, unpubl.). The heavy cyst wall in combination with the partially dehydrated cytoplasm enables the cells to withstand extreme conditions such as desiccation in a high vacuum, heating to 100°C and cooling to the temperature of liquid air (19).

In an early study of the fine structure of encysting and excysting *Colpoda steinii*, Tibbs (15) found that all cell organelles are retained in the cyst and that the cytoplasm becomes dense as the cell shrinks. Possible precursors of the wall material were discussed but the process of wall formation remained unclarified. In this paper we present evidence that the cyst wall is formed from membranes in the cell cortex and not by the exocytosis of vesicles containing precursor material.

Tibbs & Marshall (18) concluded, based on their measurements of glutamic acid in cells settling down for encystment and in cysts, that there is no large pool of coat protein and that substantial protein synthesis must occur. We could confirm this conclusion by the application of inhibitors of protein and RNA synthesis. Experiments with EGTA demonstrated a requirement for calcium during a phase prior to encystment.

MATERIALS AND METHODS

Colpoda steinii was isolated from moss, cloned, and cultured in Schreiber's earth decoct containing 0.09% NaCl, 0.01% $NaHCO_3$, and 0.2% casamino acids (DIFCO) in plastic petri dishes at room temperature. Every 2–3 days 1–2 ml of an old culture was added to 8–9 ml of fresh culture solution. An added grain of wheat supported the growth of sufficient food bacteria. Encystment was induced by washing and resuspension of the cells in fresh culture medium without bacteria. To study the effects of inhibitors, 0.5–100 mM EGTA (ethyleneglycol-bis-(2-amino-ethyl-ether)-*N,N'*-tetraacetic acid), 15–100 μg/ml cycloheximide (Serva), or 12.5–250 μg/ml actinomycin C (Bayer) were added. The usual time of incubation was 12 h. The cultures were then checked for cyst formation, washed free of the inhibitor and examined for their ability to regenerate to the trophic form and to encyst. Excystment was induced by the addition of bacterized culture medium to washed cysts. For electron microscopy, encysting or excysting cells were accumulated by centrifugation, fixed 1 h in 2.5% (v/v) glutaraldehyde in 0.1 M