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Two simple media for biochemical experimentation with cultured procyclic *Trypanosoma brucei*

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Summary

Two simple media are presented which are particularly well suited for biochemical experimentation with procyclic culture forms of *Trypanosoma brucei*. ME-83 is a simple semi-defined medium which supports active cell growth, and from which individual components can conveniently be deleted or replaced. HHP-84 is a fully defined minimal medium, which allows vigorous cell motility over extended times, but in which cell proliferation is not occurring.

Key words: procyclic *T. brucei brucei*; culture medium; minimal medium; high specific activity labeling.

Introduction

The propagation of *Trypanosoma brucei* in culture has developed into a well established technique (Hirumi et al., 1980). Bloodstream trypanosomes can be cultivated in vitro using mixed culture techniques originally developed by Hirumi et al. (1977) and later cogently refined and extended by others (Hill et al., 1978; Brun et al., 1981).

For large scale cultivation of procyclic trypomastigotes, a number of different media have been developed over the years (Tobie et al., 1950; Weinman, 1960; Pittam, 1970; Dixon and Williamson, 1970; Cross and Manning, 1973). The latest development in this area was the construction of a semi-defined growth medium which supports rapid cell growth to high densities, and which permits a direct adaptation of bloodstream forms to growth in culture (Brun and Jenni, 1977; Brun and Schönenberger, 1979).

Over the last few years, trypanosomes have increasingly come into the focus of molecular and cellular biology. In this context, the need for more com-

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pletely defined and less complex media has arisen. A particular problem inherent in most conventional media is their high concentration of amino acids, which makes high specific activity labeling of proteins with individual amino acids very difficult. We have developed a simplified medium (ME-83), which is based on the standard growth medium SDM-79 (Brun and Schönenberger, 1979), but which facilitates the selective omission of individual compounds, as e.g. amino acids. The suitability of this medium has been demonstrated in a recent study of the posttranslational tyrosinylation of trypanosomal alpha-tubulin (Stieger et al., 1984).

In growing cells, an experimental discrimination between cellular functions involved in, or correlated with, cell growth, and functions unrelated to it, as e.g. cell motility, is difficult. We have now developed a fully defined minimal medium (HHP-84), which supports cell motility but not cell proliferation.

Materials and Methods

Culture of procyclic trypanosomes

The stock STIB 366 was originally obtained from R. Brun of the Swiss Tropical Institute in Basel. Cultures were routinely maintained in SDM-79 medium containing 5% fetal bovine serum (Gibco), and they were split twice weekly. Cultures were grown in plastic tissue culture flasks at 26°C in the dark without agitation.

Cell counting

Cells were fixed by mixing with an equal volume of 150 mM NaCl – 10 mM Na-phosphate-buffer, pH 7.0–10% formaldehyde. Cell counts were determined either in a hemocytometer or in a Coulter Counter. No changes in cell counts were observed when fixed preparations were kept at 4° for up to one week.

Determination of incorporation

Trypanosomes from rapidly growing 5 ml cultures were collected, washed twice by centrifugation in the appropriate medium and were finally resuspended in 5 ml. After 30 min preincubation at 26°, radioactive precursors were added to 4 µCi/ml. The kinetics of incorporation were determined by TCA-precipitation of duplicate 50 µl aliquots at different times of the incubation period.

Preparation of ME-83

A 500 ml batch of ME-83 is prepared from the following components: 50 ml of 10× Hanks balanced salt solution (Gibco); 50 ml of 0.1 M HEPES, pH 7.3; 5 ml of 100× non-essential amino acids (Gibco); 5 ml of proline (100 mg/ml in H₂O); 20 ml of tyrosine (0.2 mg/ml in H₂O); 10 ml each of the following amino acid stock solutions (mg/ml in H₂O): arginine (6.4), cysteine (1.2), glutamine (14.6), histidine (2.2), isoleucine (4.8), leucine (4.8), lysine (3.6), methionine (0.8), phenylalanine (1.6), threonine (2.4), tryptophane (0.6), valine (2.4). Distilled water is then added to 500 ml and the medium is sterilized by filtration. Before use, the medium is completed by adding 5 ml hemin (5 mg/ml in 1% NaOH), 5 ml Penicillin/Streptomycin solution (Gibco: 10,000 u/ml) and 25 ml heat-inactivated fetal bovine serum (FBS).

Preparation of HHP-84

A 500 ml batch of HHP-84 is prepared from the following ingredients: 50 ml of 10× Hanks balanced salt solution (Gibco); 50 ml of 0.1 M HEPES, pH 7.3; 5 ml proline (100 mg/ml in H₂O)

Table 1. Composition of ME-83 and HHP-84

Compound	ME-83 mg/l	HHP-84 mM
NaCl	8000	136.9
KCl	400	5.4
MgSO ₄ ·7H ₂ O	100	0.4
Na ₂ HPO ₄ anh.	48	0.3
CaCl ₂ ·2H ₂ O	185	1.3
NaHCO ₃	350	4.2
KH ₂ PO ₄	60	0.4
MgCl ₂ ·6H ₂ O	100	0.5
Glucose	1000	5.6
HEPES	2383	10.0
Hemin	50	0.1
BSA	—	—
Proline	1012	8.8
Alanine	9	0.1
Asparagine	15	0.1
Aspartate	13	0.1
Glutamate	15	0.1
Glycine	8	0.1
Serine	11	0.1
Arginine	128	0.7
Cysteine	24	0.2
Glutamine	292	2.0
Histidine	44	0.3
Isoleucine	96	0.7
Leucine	96	0.7
Lysine	72	0.5
Methionine	16	0.1
Phenylalanine	32	0.2
Threonine	48	0.4
Tryptophane	12	0.1
Tyrosin	40	0.2
Valine	48	0.2
Penicillin/Streptomycin		100 u/ml

and distilled H₂O to 500 ml. The medium is sterilized by filtration and stored at 4°. Prior to use, 5 ml hemin-solution, 0.5 g bovine serum albumin and 5 ml Penicillin/Streptomycin solution are added. Since HHP-84 is mostly used for short-term experiments, the antibiotics can be omitted.

Materials

Hanks balanced salt solution (10×), MEM nonessential amino acids (100×), Penicillin/Streptomycin solution and fetal bovine serum were from Gibco. Hemin was from Sigma, and all amino acids and bovine serum albumin were obtained from Fluka, Buchs (Switzerland).

Results

ME-83: growth characteristics

ME-83 is essentially a trimmed-down version of the standard growth medium SDM-79 (Brun and Schönenberger, 1979). Its composition, which is given in detail in Table 1, is relatively simple, and individual components can readily be omitted or replaced to suit the experimenter's needs.

The capability of the complete ME-83 to support cell proliferation has been compared to that of SDM-79. Trypanosomes growing in exponential phase in SDM-79 were centrifuged, washed twice in either SDM-79 or ME-83 containing various concentrations of fetal bovine serum, and were finally resuspended in the respective media at $1-2 \times 10^6$ /ml. Cell proliferation was monitored daily, and the resulting growth curves are given in Fig. 1. The data demonstrate that ME-83 supports vigorous cell growth over a period of several days. The extent of cell growth in ME-83 is strongly dependent on the concentration of FBS, which is in close analogy to the situation previously observed for SDM-79 (Brun and Schönenberger, 1979). In the complete absence of FBS in either medium, the trypanosomes rapidly deteriorate, altering their characteristic cell shape and loosing motility within hours.

Maximal stimulation of cell growth in ME-83 was achieved with 5% FBS, while higher concentrations had no further effect. The final saturation density reached in ME-83 containing 5% FBS is around 1×10^7 /ml, which is slightly inferior to the maximal densities reached in SDM-79 ($2-3 \times 10^7$ /ml). Trypanosomes can be continuously propagated in ME-83. This latter observation demonstrates that the simplified medium supports all functions needed for cell growth and proliferation.

Substitution of individual components of ME-83

In a next step, the effects of omitting individual compounds have been studied.

a) Vitamins and growth factors. In contrast to SDM-79, the ME-83 medium contains no growth factors or vitamins other than those provided by the fetal bovine serum. Addition of a complete MEM vitamin mixture (Gibco) had no discernible effect on cell viability, shape or growth. However, the lack of one or several growth factors and/or vitamins might be responsible for the slightly lower saturation density, which is characteristic for ME-83.

b) Phosphate. In order to explore the growth support capability of a phosphate-free version of ME-83, all phosphate salts were deleted from the basic salt mixture. The buffering capacity of the phosphate buffer was compensated for by the addition of 25 mM HEPES buffer. The total phosphate content of the batch of fetal bovine serum used in these experiments was determined to be 528 mg PO_4^{3-} /l, i.e. 5.6 mM. Hence, the final phosphate concentration

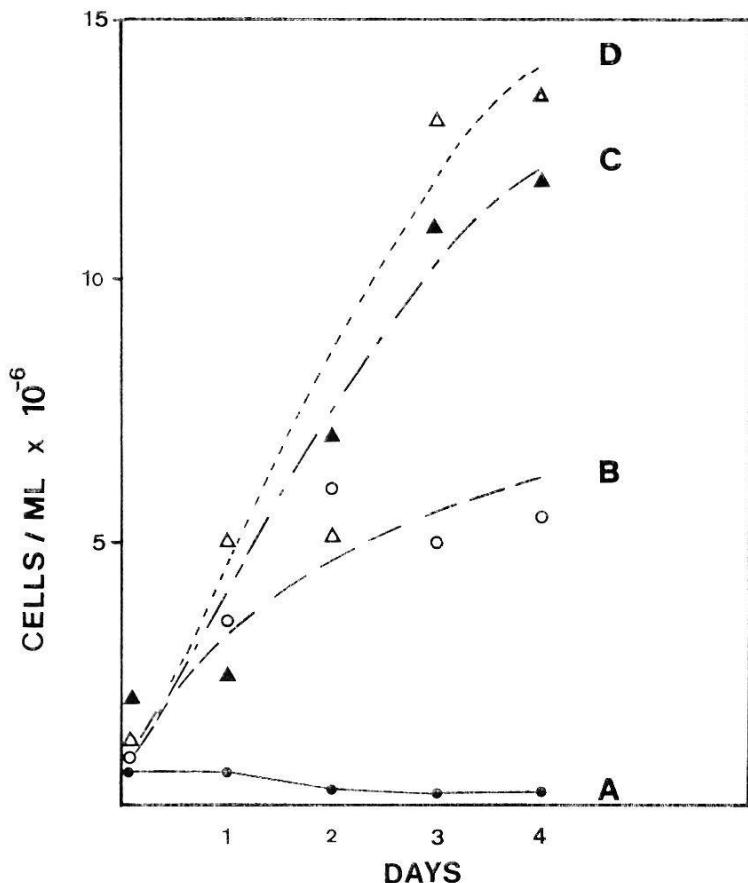


Fig. 1. Growth curves of STIB 366 in SDM-79 and in ME-83. A (●): ME-83 without serum; B (○): ME-83 with 1% FBS; C (▲): ME-83 with 5% FBS; D (△): SDM-79 with 5% FBS.

(organic and inorganic) of phosphate-free ME-83 containing 5% FBS was 26 mg/l, i.e. 0.28 mM.

Cells incubated in this medium fully retained their shape and motility over the entire 24 h observation period. However, no cell proliferation occurred in this medium. This observation suggests that the low phosphate content might be just sufficient to allow the basic metabolism to take its course, but that it is too low to permit any large scale biosynthetic events, which would be a prerequisite for cell proliferation.

c) *Amino acids.* Since the formulation of ME-83 allows the selective omission of individual amino acids, we have explored the effect of omitting either leucine, methionine or tyrosine on the efficiency of labeling cells with the respective radioactive analogues. In both cases, omission of either amino acid had no discernible effect on cell motility and cell proliferation. The incorporation of radioactive analogues was strongly enhanced in the absence of the unlabeled amino acids (Table 2). When the incorporation of ^3H -labeled tyrosine was compared in media containing either 1% FBS or 5% FBS, a slightly higher incorporation ($1.5 \times$) was observed in the lower concentration of FBS, presumably due to the presence of some unlabeled tyrosine in the serum. However, cell growth and motility are improved considerably in the higher serum concen-

Table 2. Comparison of amino acid and nucleotide incorporation in ME-83 and SDM-79. Rapidly growing 5 ml cultures (3×10^6 cells/ml) were prepared and labeled as detailed in Materials and Methods. Section A shows the total amount of radioactive amino acids incorporated per 10^6 cells after 4 h of incubation in either full SDM medium or in ME-83 from which the corresponding unlabeled amino acid had been omitted. Section B presents the incorporation of radioactive nucleotides in either SDM or ME-83 medium. All media contained 5% FBS.

A

Radioactive amino acid	cpm/ 10^6 cells*		Increase in incorporation
	SDM-79	ME-83**	
^3H -leucine	1851	46884	25 \times
^3H -tyrosine	2110	20164	10 \times
^{35}S -methionine	2404	31948	13 \times

* incorporation after 4 h labeling

** ME-83 from which the corresponding amino acid was deleted

B

Radioactive nucleotide	cpm/ 10^6 cells***		Increase in incorporation
	SDM-79	ME-83	
^3H -thymidine	778	890	—
^3H -uridine	777	830	—
^3H -desoxyadenosine	1087	15709	14.5 \times

*** incorporation after 1 h labeling

tration (compare Fig. 1), and 5% serum was therefore routinely used in all labeling experiments.

d) Sugars. The standard formulation of ME-83 contains 1 mg/ml of glucose (5.6 mM). Procyclic trypansomes do not utilize glucose as prime energy source (Haston, 1975). Consequently, we investigated the effect of replacing the glucose with equimolar concentrations of a series of other sugars, such as the disaccharides sucrose and maltose, the hexoses galactose and mannose, the hexitol sorbitol and the pentoses ribose and xylose. With all sugars tested, no differences in cell motility could be discerned, and very similar growth rates and final cell densities were obtained (Fig. 2).

e) Nucleotides. In our standard formulation, ME-83 does not contain nucleosides other than those contributed by the FBS. Labeling experiments with the radioactive pyrimidine nucleotides thymidine or uridine have demonstrated that incorporation of these precursors in ME-83 is not improved over the level obtained in SDM-79. Quite in contrast, labeling with the purine nucleo-

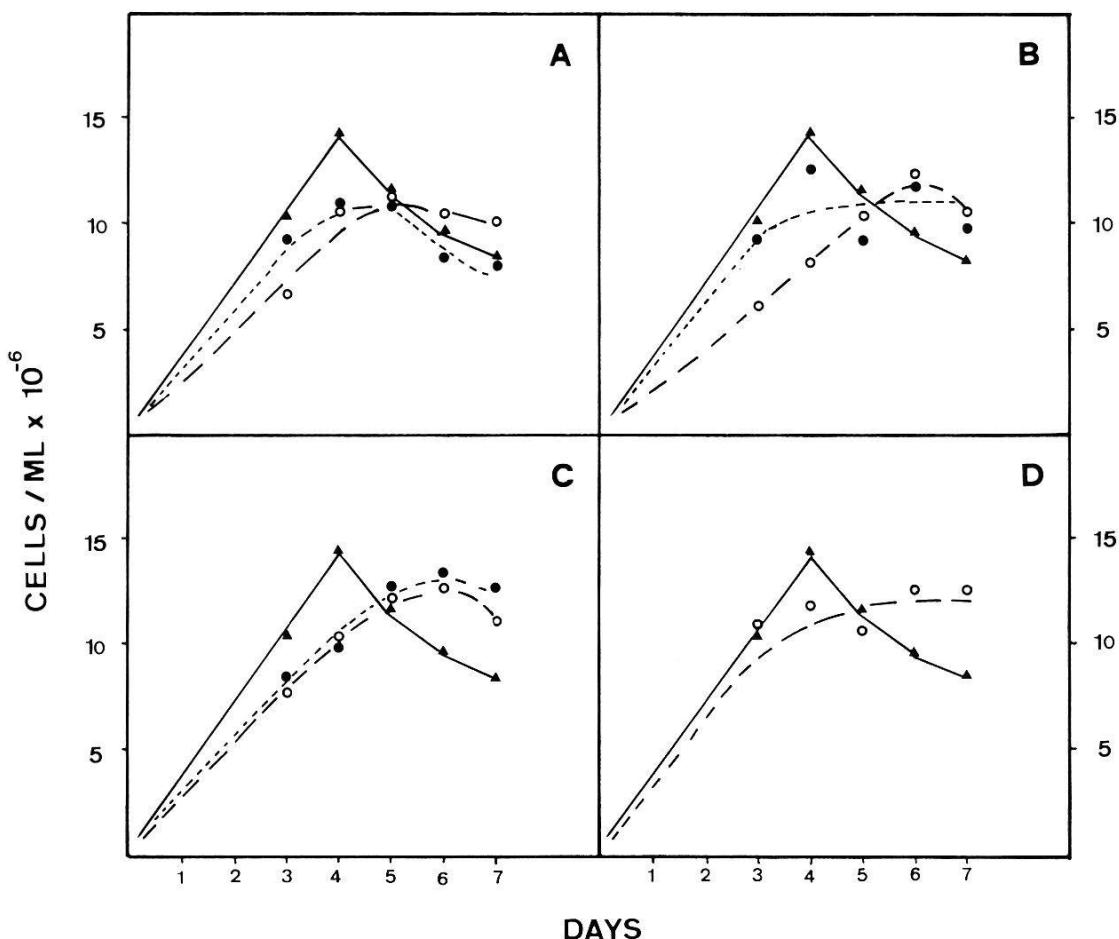


Fig. 2. Growth of STIB 366 in the presence of various sugars. Trypanosomes were seeded at a density of 1×10^6 into ME-83 containing either glucose (standard formulation) or equimolar concentrations of the following sugars: panel A: maltose (●), sucrose (○); panel B: mannose (●), galactose (○); panel C: ribose (●), xylose (○); panel D: sorbitol (○). The growth curve obtained with glucose (▲) is included in all four panels as a reference. All media contained 5% FBS.

tide desoxyadenosine results in a 20–30 fold higher incorporation of radioactivity in cells maintained in ME-83, as compared to controls in SDM-79 (Table 2B).

In addition, significant differences are observed between the labeling kinetics in the two media. In ME-83, desoxyadenosine is incorporated at a high rate with linear kinetics only up to about 1.5 h. After this period, incorporation rapidly levels off. In contrast, incorporation in SDM-79 proceeds linearly over at least 4 h, albeit at a much lower rate. The incorporation values given in Table 2B have been determined at 60 min, i.e. at a time point where both kinetics are still linear.

HHP-84

For a number of experimental approaches, a medium would be desirable which fully supports cell motility, but which does not allow cell proliferation. Such a condition would enable the observer to discriminate between metabolic events correlated with the basic cellular metabolism (i.e. amino acid synthesis) and those involved directly in cell proliferation (i.e. DNA replication).

We have constructed a completely defined minimal medium, termed HHP-84 which fulfills this requirement. Its composition is given in Table 1. HHP-84 consists of Hanks balanced salt solution (usually containing glucose as a sugar), HEPES buffer, and proline as a source of energy. In addition, a minimal concentration of protein is crucial for cell viability. The particular source of protein is apparently not critical, since we have found that fetal bovine serum, purified bovine serum albumin, ovalbumin and bacterial alpha-amylase work equally well. As a standard formulation, we use 0.1% bovine serum albumin as stabilizer protein.

Trypanosomes maintain their cell shape and motility for at least six days in HHP-84, but no cell proliferation is detectable. Flow cytofluorimetric analyses have demonstrated that the cells are arrested in all stages of the cell cycle. Very similar cell cycle profiles are obtained from cultures actively growing in complete SDM-79 and from cells maintained for 24 h in HHP-84 (unpublished observations). This suggests that exposure of cells to the minimal medium does not cause a block of cell proliferation at any particular stage of the cell cycle. If trypanosomes are kept in HHP-84 for times much longer than 24 h, immotile and dead cells slowly accumulate. This observation clearly suggests that experiments with trypanosomes maintained in the minimal medium should not be extended over more than 20 h.

Discussion

We have developed two simple media which will prove useful for biochemical work with cultured procyclic trypomastigotes of *T. brucei*. ME-83 is a simplified version of the standard growth medium SDM-79 of Brun and Schönenberger (1979). It supports cell growth, albeit to a somewhat lower final cell density, and it is simple enough to permit the selective omission or substitution of individual components. This medium has already proven its suitability for metabolic labeling experiments in a recent study on the exchange of the carboxy-terminal tyrosine of trypanosomal alpha-tubulin (Stieger et al., 1984).

Despite the fact that glucose appears to be utilized by procyclic trypanosomes either during the entire growth phase (Brun and Jenni, 1977; Cross et al., 1975) or only after reaching their stationary phase (Evans and Brown, 1972), the absence of glucose does not affect cell proliferation. Hence, the possibility of constructing ME-media with different sugars offers an attractive experimental system for studying many aspects of sugar metabolism, such as sugar uptake, metabolic pathways of sugar utilization or protein glycosylation, among many others.

Omission of phosphate from ME-83 does not drastically lower the final phosphorus content of the medium (from about 1 mM to about 0.3 mM), since the fetal bovine serum contributes a relatively large proportion of the final phosphate concentration. For practical purposes, the omission of phosphate

salts from the basic formulation of ME to be used for labeling experiments with radioactive phosphorus might not be worthwhile. Nevertheless, it is interesting to note that more phosphate than is provided by the bovine serum alone appears to be necessary for cell growth, since trypanosomes maintained in phosphate-free ME do not proliferate even in the presence of 5% fetal bovine serum. A similar narrowly defined minimal phosphate requirement for cell proliferation has recently also been observed for the yeast *Saccharomyces cerevisiae* (E. Schweingruber, personal communication).

Despite the fact that ME-83 does not contain unlabeled nucleotides or nucleosides, the incorporation of radioactive pyrimidine nucleotides is not higher in ME-83 than in SDM-79. In contrast, a remarkably improved (20–30 fold) incorporation of desoxyadenosine is observed. Such an enhanced uptake of purines in ME-83 is not unexpected, since trypanosomes are purine auxotrophs, and since SDM-79 contains a fair amount of purines and pyrimidines (adenine, adenosine, adenosine-monophosphate, adenosine-triphosphate, guanine, guanosine, hypoxanthine, thymine, uracil and xanthine). With regard to this purine dependence, the vigorous and essentially unlimited growth of *Trypanosoma brucei* in ME-83 is rather puzzling. It either suggests the presence of sufficient purines in the FBS, or else indicates that the purine dependence of trypanosomes is not completely strict.

The second medium presented, HHP-84, is a fully defined minimal medium which supports cellular motility, but not proliferation. This medium is particularly attractive for studies concerning the basic cellular metabolism, as opposed to functions related to cell proliferation. Its use in the study of trypanosomal chemotaxis is presently being explored.

The two media presented in this study will facilitate many aspects of biochemical work with trypanosomes maintained in culture. They complement the well established growth medium SDM-79 and they will hopefully contribute to further establish trypanosomes as convenient laboratory organisms. Furthermore, these new media might enable many experiments to be done with cultured trypanosomes rather than with bloodstream forms, thus considerably reducing the consumption of experimental animals.

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