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With **6** ***figures*** *in the* ***text***

Cultivation of *Trypanosoma brucei* sspp. in semi-defined and defined media

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SUMMARY

Semi-defined and defined media for the growth of culture forms of *Trypanosoma brucei* sspp. have been developed by enrichment of tissue culture medium **199** with additional vitamins, amino acids, salts and other compounds. The semi-defined medium contains an acid hydrolysate of casein: in the empirically devised defined medium the casein requirement has been circumvented by inclusion of additional vitamins and amino acids. Both media are very hypertonic. Control of pH was found to be particularly critical for growth. The optimum temperature for growth in the semi-defined medium was between **25** °*C* and **28** °*C*, but cells would undergo one or two division cycles at **37** °*C*.

INTRODUCTION

Our eagerness to investigate the molecular basis of the biochemical and morphological changes which occur during the cyclical development of the salivarian trypanosomes (Vickerman, **1971**; Newton, Cross & Baker, **1973)** has been tempered by problems associated with the cultivation of these organisms.

The Salivaria are among the most difficult trypanosomes to cultivate *in vitro*. Of the various cell types occurring during the natural life-cycle, only those trypomastigote forms which are believed to correspond to the invertebrate midgut or 'procyclic' (Newton *et al.* **1973)** trypomastigotes may be maintained indefinitely in culture. Even these midgut forms could previously be grown only in complex blood-containing media such as the widely used biphasic media of Tobie, von Brand & Mehlman **(1950)** and Weinman **(1960)**' and such media are not well suited to biochemical studies on any scale. **A** monophasic blood-containing medium developed by Pittam **(1970)** has recently found a wider application in investigations of trypanosome lipid and sterol composition (Dixon & Williamson, **1970;** Dixon, Ginger & Williamson, **1972)** and for studies on the development of respiratory pathways during the transformation of *T. brucei* from haematozoic trypomastigotes to culture or procyclic trypomastigotes (Evans & Brown, **1971).** Pittam's medium has also been used in our laboratory, but in common with others we have found that it is not an ideal medium for cultivation of *T. brucei* sspp. for biochemical investigations. Most of the problems encountered, such as unpredictable variations in enzyme and respiratory activities, may be attributed to the intrinsically variable and undefined chemical constitution of such a medium. **A** more specific problem

arises in cytochrome spectral measurements since it is difficult to eliminate beyond dispute the possibility of haemoglobin contamination of cell preparations, and a further general problem is presented by the high levels of proteolytic activity which appear to be present in extracts of organisms grown in complex media.

Few defined media have been described for the cultivation of members of the Trypanosomatidae and salivarian trypanosomes have apparently defied all previous attempts at growth in synthetic media in the absence of blood components (Taylor & Baker, 1968). We have investigated this problem further because of the potential usefulness of a defined medium for the cultivation of these organisms.

METHODS AND MATERIALS

Trypanosome strains

The history, as far as it can be documented, of the trypanosome strains used may be important for the evaluation of results presented in this paper.

Trypanosoma rhodesiense 261 was isolated in south-east Uganda in 1959 from a late human infection in relapse following Melarsoprol (B.P.) treatment. It was first transferred into culture by Dr W. E. Ormerod after one rat passage and probably had a maximum of six intermittent rat passages between 1961 and 1964, since when it can be fairly certain that the strain used by us has been maintained in serial subculture, initially by Dr M. D. Pittam in his monophasic medium and later in biphasic medium (Tobie *et al.* 1950) by Dr J. F. Ryley from whom we obtained the strain in September 1969. It has since been maintained by us by twice-weekly subculture in monophasic medium.

Trypanosoma brucei S 42 was isolated in 1966 from an old male warthog in Tororo, Uganda, and preserved as a stabilate. We acquired it in 1969 from the Nuffield Institute of Comparative Medicine, London. It is very obviously pleomorphic and is infective to *Glossina* (S. Lanham, personal communication).

Trypanosoma brucei 427 was isolated in 1960 from an early sheep infection in Uganda (Cunningham & Vickerman, 1962) and, so far as can be ascertained, it was maintained by regular syringe passage in mice at the Lister Institute, London, from 1961 to 1967 when samples were transferred to liquid nitrogen storage. A sample was subsequently transferred to the Nuffield Institute of Comparative Medicine, London, from where we obtained it in 1969. It has the appearance of a monomorphic strain. In contrast to *T. brucei* S 42 it causes a non-relapsing fulminating infection in rats and mice. It is not known whether it is infective to *Glossina*.

Culture forms of these two strains of *T. brucei* were first isolated in January 1970 (as described below) and have been maintained by twice weekly subculture in monophasic medium.

Preparation of monophasic culture medium (MCM)

The procedure used in the preparation of this medium varies slightly from that described by Pittam (1970). It is prepared in three parts.

(a) Phosphate buffer

The solution contains **0.050** M KH_2PO_4 adjusted to pH **7.4** with NaOH.

(b) Blood lysate

Human blood **(4** weeks old) was used in the early stages but it was replaced by fresh horse blood during most of the work described.

About **4** l of fresh horse blood were collected in a bucket containing 500 ml **6%** (w/v) trisodium citrate and transported to the laboratory. The blood was transferred to a **6** l jar and, after allowing the cells to settle during a period of **1** h, the excess plasma was removed and the volume of loosely settled cells was noted. The jar was transferred to a water bath at 56 °C where it was held for **1** h with occasional stirring. Two volumes of cold **(4** °C) distilled water and **1** volume of cold double-strength phosphate buffer were added and the lysate was centrifuged for **1** h at **14000 g.** The supernatant liquid was decanted and filtered in turn through a Carlson–Ford filter (grade **4),** a cellulose nitrate filter **(1.2** µm pore size) and finally through a Carlson–Ford HP/EK filter. All filtrations were performed under non-sterile conditions using a **142** mm filter holder (Sartorius, V. A. Howe & Co. Ltd.). Only one filter of each type was generally necessary to process this volume of lysate. This was attributed to dilution of the lysate following heat treatment which ensured that most particulate material was sedimented during centrifugation. The concentration of the filtered lysate was adjusted to give an absorbance of **41** at 578 nm, equivalent to an oxyhaemoglobin concentration of 50 mg/ml (Haurowitz & Hardin, **1954).** The lysate was stored at **−15** °C.

(c) 6x *broth concentrate*

90 g tryptose, **60** g casein acid hydrolysate, **60** g liver digest and **12** g glucose were dissolved in phosphate buffer to give a final volume of 1000 ml. The solution was centrifuged for **1** h at **11000g** and the supernatant liquid filtered through a non-sterile HP/EK filter. The filtered solution was stored at -15°C.

The final medium was prepared by combining 300 ml blood lysate, **170** ml **6x** broth and **530** ml phosphate buffer. The pH was adjusted to **7.4** and the medium was pre-filtered through **a** non-sterile HP/EK filter prior to sterile filtration through a cellulose nitrate filter (pore size 0.2µm) attached to a sterile reservoir from which the medium was dispensed using a peristaltic pump controlled by an automatic timing device (G. A. M. Cross unpublished design). The sterile medium was stored at **−15** °C, and was preincubated for **24** h at **25** °C prior to inoculation.

Maintenance of cultures in MCM

All three strains were routinely maintained by twice-weekly transfer of **0.6-** 1.0ml quantities of culture into **5** ml quantities of fresh medium contained in **25** ml screw-capped glass bottles.

Preparation of semi-defined and defined media

Water-insoluble vitamins were individually dissolved at a concentration of 2 mg/ml in ethanol. The required quantities were added to an empty beaker and the ethanol allowed to evaporate. 100 ml distilled water were added and the mixture was stirred at 40 °C for 15 min to redissolve these vitamins before adding the remaining media constituents. The other constituents were dissolved in fresh glass-distilled water, except for haemin, L-glutamic acid, adenine, guanine and folic acid which were dissolved in small volumes of O-~N-N~OH. Media were sterilized by filtration (0.2 pm cellulose nitrate filters) and were stored at −15°. Culture vessels were washed with detergent 7X (Linbro Chemical Co. Inc., New-haven, Connecticut, U.S.A.) or, when necessary, with chromic or nitric acid. After thorough rinsing in deionized water the bottles were soaked for several hours in 0.10 M sodium phosphate pH 7.0, after which they were rinsed in deionized water and dried. This treatment prevented pH changes occurring in stored uninoculated media.

Preparation of linoleic acid – albumin complex

The procedure used was essentially that described by Chen (1967). Activated charcoal was washed in 1 N-HCl followed by distilled water and was then air dried. 10 g albumin were dissolved in 100 ml distilled water and 5 g charcoal were mixed into the solution which was then cooled to 0 °C and brought to pH 3 by addition of 0.2 N-HCl. The solution was stirred magnetically at 4 °C for 1 h. Charcoal was removed by centrifugation for 30 min at 10000 g followed by filtration through a nitrocellulose filter (pore size 0.45 pm). The pH was raised to 7.0 using 1 N-N~OH and the solution was dialysed against distilled water. The volume was adjusted to give a protein concentration of 50 mg/ml and the solution was stored at −15°.

The linoleic acid–albumin complex was prepared by adding 30 ml defatted albumin (previously warmed to 37 °C) to 42 mg linoleic acid in a 100 ml beaker and stirring the solution for 1 h at room temperature. The initially cloudy solution clarifies during this time. The solution was filtered and stored at −15 °C.

The molar ratio at which the linoleic acid and albumin were reacted was 7:1, since the saturation binding ratio of fatty acids to albumin has been found to be between 6 and 7 moles free fatty acid per mole of albumin (Rodbell, 1965).

To confirm the efficacy of the foregoing procedures, fatty acids were extracted, methylated and analysed by gas chromatography. The linoleic acid–albumin complex was found to contain only linoleic acid and the molar ratio of linoleic acid to albumin was 7. The defatted albumin contained no detectable fatty acids (limit of detection was equivalent to 0.01 moles fatty acid per mole of albumin).

Culture conditions for growth in synthetic media

Growth tests were routinely performed using 5 ml volumes of medium contained in 25 ml screw-capped glass bottles fitted with disposable plastic caps which gave a tight seal. Triplicate cultures were used and when negative results (poor growth or no growth) were obtained the tests were repeated twice by reinoculation of

further samples of the test media from stock cultures in MCM, or from a previously successful synthetic medium.

For routine maintenance of cells in the finally evolved semi-defined medium (HXl2V) and the fully defined medium (HX25), transfer of **1** ml volumes of culture to fresh medium at alternating **3**- and 4-day intervals has proved satisfactory. Under these conditions a final cell density of **7–8** x 10^6/ml was normally obtained in 3 days and **1–1.5** x 10^7/ml in 4 days.

Evaluation of growth

Cell samples were examined under the phase contrast microscope and their condition noted. Cell concentration was routinely determined using an electronic cell counter (Coulter Electronics Ltd., Coulter Counter Model B, **70** μm aperture) except when it was desired to distinguish between live and dead cells, or when cell aggregation made manual counting necessary : in these cases an Improved Neubauer counting chamber was used.

Sources of important materials

Casein acid hydrolysate, tryptone, liver digest : Oxoid Ltd., London. HEPES : Hopkin & Williams, Chadwell Heath, Essex. Tissue culture medium 199: Wellcome Reagents Ltd., Beckenham, Kent (10x concentrate, product number TC22). Bovine serum albumin, Cohn Fraction V powder: Sigma London Ltd (product number A4503). Basal Medium Eagle Vitamins (see Table 3) : Biocult Laboratories Ltd., Paisley, Scotland (100x concentrate, product number BCL-420). Charcoal, activated untreated powder: Sigma London Ltd. All other reagents used were obtained in the purest grades available from Sigma London Ltd., or BDH Chemicals Ltd., Poole, Dorset.

RESULTS

Isolation of culture forms of Trypanosoma brucei

Culture forms of the two strains of *T. brucei* were derived by transfer of washed trypanosomes, obtained by centrifugation of blood taken from heavily infected rats, into **5** ml volumes of MCM. Figs. 1A and 1B show the kinetics of outgrowth of stable culture forms from the initial inoculum of haematozoic trypanosomes. The procedure used in the establishment of these culture forms was an empirical one: regular inspections of the cultures were made and the medium was changed occasionally. In the example shown for *T. brucei* S 42, active multiplication was not evident until 30 days after inoculation. Subsequent observations suggested that this stage is reached earlier if the medium is exchanged (by centrifugation and decantation) more frequently in the early stage of the culture. We have no explanation for the observed kinetics of transformation : extrapolation of the final growth rate back to the time of inoculation shows that the final population could have arisen from a single competent organism in the inoculum.

During the first few subcultures of *T. brucei* S 42 approximately 2% of the cells present were smaller than normal and were without nuclei (as judged by acridine

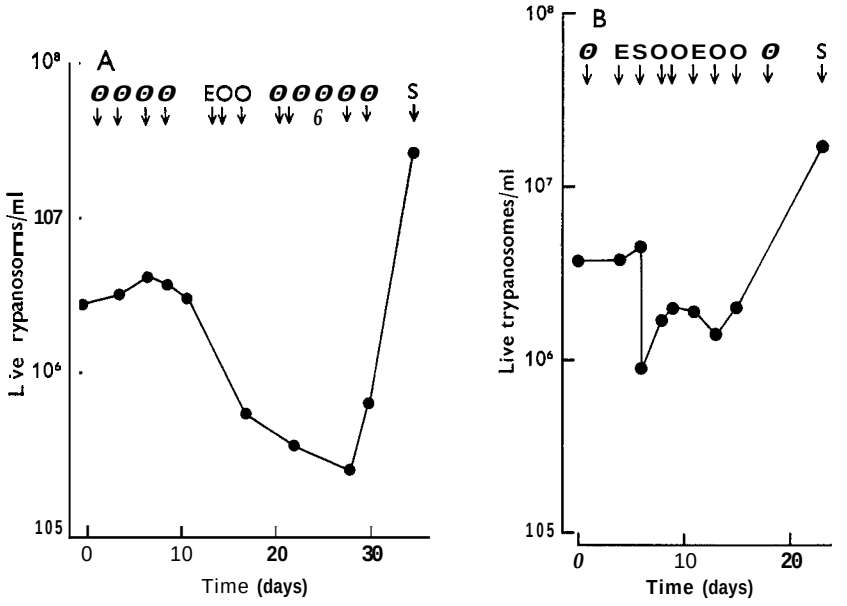

Fig. 1. Kinetics of cell growth during the initial isolation of culture forms of *T. brucei* S 42 (A) and *T. brucei* 427 (B) by inoculation of bloodstream trypanosomes into 5ml volumes of MCM. O, culture bottle opened for inspection or counting; E, exchange of medium; S, subculture.

staining). Their morphology and motility were otherwise normal. These cells had presumably arisen as a result of faulty division.

T. brucei 427 (Fig. 1B) became established more readily than S 42 in the example shown. Extrapolation of the final growth rate would suggest that at least 10% of the inoculum may have been competent to transform. The early period of culture was again characterized by the production of cells with bizarre morphology out of which a population of actively dividing cells of normal shape and size arose,

At the time of isolation of culture forms of *T. brucei* 427 we were under the impression that this was a monomorphic strain and would not therefore be expected to transform readily in culture. Culture forms of *T. brucei* 427 are less readily grown than are *T. rhodesiense* 261 and *T. brucei* S 42 and frequently give unreliable growth in large-scale batch culture. Additionally this strain would not grow in about half of the batches of medium which supported the other strains. This was related to the batch of human blood used in early experiments. No problems have been experienced during the use of 12 different batches of horse blood. Cultures of *T. brucei* 427 gave rise to infections in mice on several occasions during the testing of some early batches of semi-synthetic media (not those which are described in this paper) whereas *T. brucei* S 42 never did.

Since their initial isolation in January 1970 these two culture strains of *T. brucei* have been maintained by twice weekly subculture in MCM.

Development of a semi-defined medium

It was decided to attempt to develop a synthetic medium based on the readily available tissue culture medium 199 (Morgan, Morton & Parker, 1950). To explore the possibility of using medium 199 as a basal medium our initial experiments were designed around the preparation of hybrid media incorporating medium 199 and the various components of MCM in order to determine which constituents of MCM might be replaced by medium 199.

It was found that medium 199 buffered with phosphate and supplemented with adenine, adenosine, guanine, guanosine and methionine (each at a final concentration of 20 mg/l), folio acid (5 mg/l), glucose (2 g/l), casein hydrolysate (2 g/l, later increased to 5 g/l) and blood lysate would support the growth of *T. brucei* S 42 and *T. rhodesiense* 261. The crude blood lysate was later replaced by a soluble high molecular weight erythrocyte fraction prepared by Sephadex G 25 chromatography of a 15000g supernatant fraction obtained from lysed washed erythrocytes. EDTA (400 mg/l) was also added to counteract the precipitation which occurred in the first medium and which was thought to result from incompatibility of phosphate and the divalent cations present in medium 199.

It was anticipated that withdrawal of the erythrocyte lysate would present the greatest obstacle to the development of a defined medium. It was assumed that its removal would necessitate the addition of haemin, but it was thought likely that the lysate would be supplying other essential growth factors. The lysate was found to contain free fatty acids including linoleic acid. Omission of linoleic acid from animal diets produces a wide range of deficiency symptoms (Holman, 1968). Trypanosomes will absorb free fatty acids (Dixon & Williamson, 1970; H. P. Voorheis, personal communication), and so long as there are doubts about the ability of *T. brucei* sspp. to synthesize fatty acids (since the necessary metabolic pathways have not been demonstrated even in culture forms) it seems possible that exogenous fatty acids might be essential for growth. Both haemin and a linoleic acid–albumin complex were therefore added to media when the blood lysate was withdrawn. Oleic acid was already present in tissue culture medium 199.

First blood-free medium

Table 1 gives the composition of the first medium (HXA) in which *T. brucei* S 42 was successfully maintained in the absence of crude serum or erythrocyte fractions. The medium contained defatted serum albumin which was essential for growth. Its main function may have been associated with pH maintenance since the medium became more rapidly acidified in its absence, and it was later found to be unnecessary in media in which the buffering capacity was improved by addition of HEPES (N-2-hydroxyethyl-piperazine-N'-2-aminoethane sulphonic acid).

Fig. 2 shows the growth obtained during 16 serial subcultures following transfer of cells from MCM into medium HXA. Cell yields greater than 5 x could not be achieved in this medium, suggesting that some growth factor was present at a limiting concentration. Maintenance of *T. brucei* in this medium was not easy:

Table 1. *Composition of medium* ***HXA****

	mg/l
KH_2PO_4	**6800**
Casein hydrolysate	**5000**
Defatted albumin	**4000**
Glucose	**2000**
EDTA-disodium salt†	**400**
Adenine	**20**
Adenosine	**20**
Guanine	**20**
Guanosine	**20**
L-methionine	**20**
Haemin	**10**
Folic acid	**10**
	ml/l
Medium **199,** 10x concentrate	**100**
Linoleic acid–albumin complex	8

* Adjusted to pH **7·40** with NaOH. † **Ethylenediaminetetra-acetic** acid.

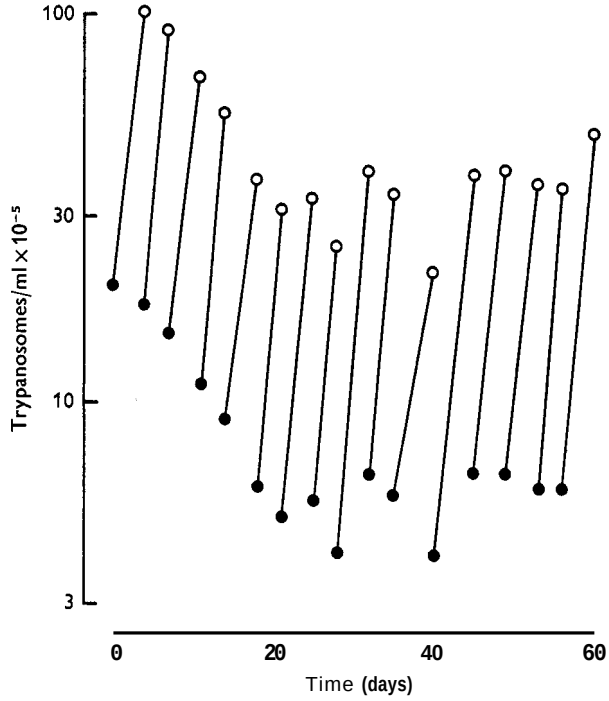

Fig. **2.** Growth of ***T. brucei*** S **42** during routine maintenance by serial subculture in medium HXA, following transfer from MCM on day 0. ● indicates the initial cell density at inoculation; ***Θ*** indicates the final cell density prior to subculture.

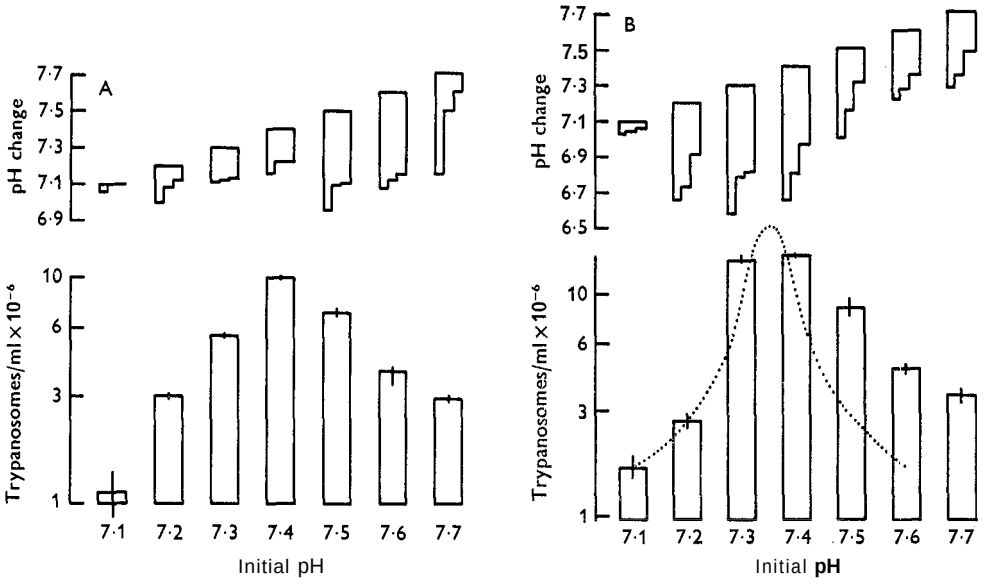

Fig. 3. Effect of pH on the multiplication of T. *brucei* S 42 (A) and *T. rhodesiense* 261 (B) in MCM. The lower histograms relate the average increase in cell numbers during 3 days' growth to the initial pH value. The vertical bars indicate the observed range of counts in the triplicate samples at each pH value. The upper histograms relate the pH changes during growth to the initial pH value. The final pH values are displayed for each individual culture, since the pH variation between the triplicate samples was larger than the variation in cell numbers. The dotted line superimposed on the lower histogram in Fig. 3B is intended to represent the result which might have been expected in the absence of pH changes during the course of growth.

inoculum size was critical and growth was very unreliable if the cell concentration was allowed to fall below 5×10^5/ml. The inclusion of both casein hydrolysate and the linoleic acid–albumin complex appeared to be essential for growth.

Effect of pH on cell multiplication

It was soon apparent that cell multiplication was very sensitive to pH. Since growth in medium HXA was unreliable, the effect of pH on growth was investigated using MCM. Fig. 3 shows the effect of the initial pH of the medium on the growth of *T. brucei* S 42 and *T. rhodesiense* 261. The experiments were complicated by the change in pH which occurred during cell growth, and allowances must be made because cultures inoculated at the higher pH values actually passed through a region of more favourable pH. The hypothetical line superimposed on Fig. 3B may be a closer reflexion of the true influence of pH on cell multiplication. These results suggested that growth might be sensitive to a variation of as little as 0.050 pH units and that cell multiplication might not occur below pH 7.2 nor above pH 7.5.

Semi-defined medium HX12

Since the pH of cultures in synthetic media fell below 7.2 during growth, we attempted to improve the buffering capacity of the medium. Increasing the

Table 2. *Composition of medium HX12**

	mg/l
HEPESt	19000
Casein hydrolysate	5000
KH_2PO_4	1360
Glucose	1000
$NaHCO_3$	800
L-proline	575
Tri-sodium citrate. $2H_2O$	588
Sodium acetate. $3H_2O$	544
Sodium succinate. $6H_2O$	270
D(+) glucosamine-HCl	220
EDTA, disodium salt	80
Adenine	20
Adenosine	20
Guanine	20
Guanosine	20
L-methionine	20
Haemin	10
Folic acid	10
	ml/l
Medium 199, 10x concentrate	88
Linoleic acid–albumin complex	6

* Adjusted to pH 7.40 with NaOH.
† N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

Table 3. *Medium HX12V: supplementary vitamins*

Component	Final concentration mg/ml
D-biotin	1.0
D-Calcium pantothenate	1.0
Choline chloride	1.0
Folic acid	1.0
L-inositol	2.0
Nicotinamide	1.0
Pyridoxal-HCl	1.0
Riboflavin	0.10
Thiamine-HCl	1.0

concentration of phosphate inhibited growth. Several zwitterionic buffers (Good, Winget, Winter, Connolly, Izawa & Singh, 1966) were tried and HEPES was found to control the pH satisfactorily and to be without apparent ill effect on cell morphology at concentrations up to 0.10 M (the highest concentration tested). HEPES was subsequently incorporated into all media at concentrations in the range 0.060–0.080 M.

Concomitant addition of glucosamine, L-proline, sodium acetate, succinate, citrate and bicarbonate, and reduction of EDTA and phosphate concentrations resulted in medium **HX12** (Table 2). All three strains of T. *brucei* sspp. could be maintained in this medium, but T. *brucei* 427 had some difficulty in adapting to it.

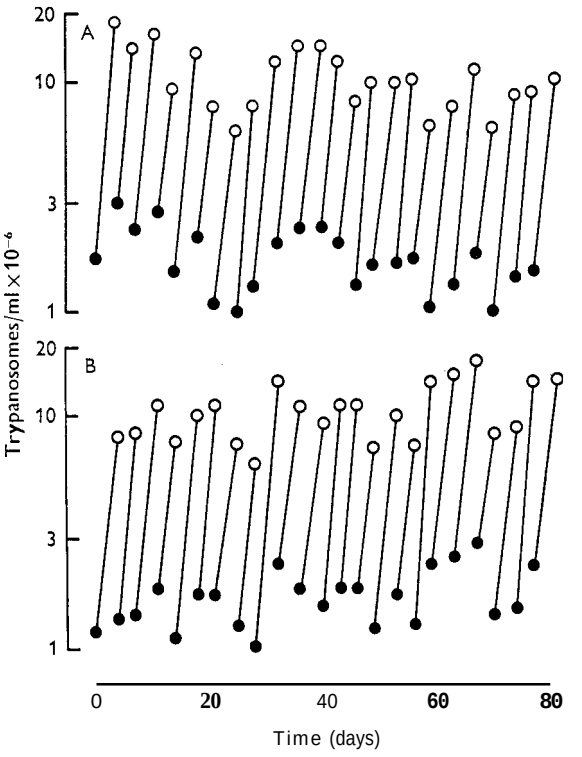

Fig. 4. Growth of *T. brucei* S 42 (A) and *T. rhodesiense* 261 (B) during routine maintenance in medium HX12V.

Medium 199 contains lower concentrations of vitamins than many other tissue culture media and for this reason it was decided to supplement medium HX12 with the vitamins listed in Table 3. These are the vitamins present in the Basal Medium of Eagle (1955a, *b*) and shown by him to be essential for the growth of certain mammalian cell lines. The resulting medium (HX12V) is the final version of our semi-defined medium. It supports good growth of our trypanosome strains (Fig, 4) and also of *T. mega* (R. A. Klein, personal communication). Several benefits were apparent after adding these vitamins to medium HX12. Growth yield was improved twofold with cultures growing consistently to cell concentrations $>10^7$/ml with a doubling time of less than 24 h and there was no difficulty in obtaining growth in larger volumes of medium.

Cells growing in medium HX12V showed a much greater tolerance to pH (Fig. 5) than was expected from the results of the earlier experiments in MCM (Fig. 3). A tenfold increase in cell numbers was obtained during 3 days' growth at all pH values from 7.1 to 7.6. At pH 7.7 most cells were swollen and less motile than normal. The changes in pH during growth were much less than in MCM, testifying to the more effective buffering in the semi-defined medium.

Effect of temperature on cell multiplication in medium HX12V

Table 4 illustrates the effects observed when cells previously grown at 25 °C were transferred to higher temperatures. The initial growth rate was higher at

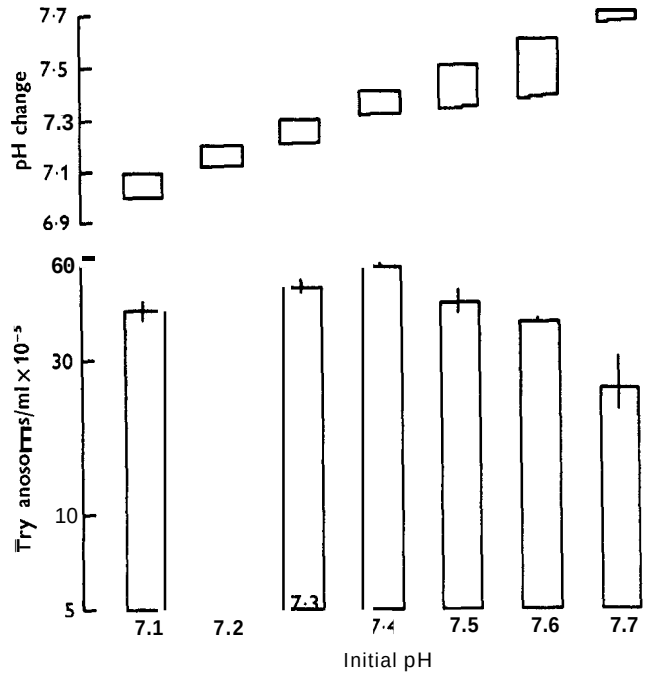

Fig. 5. Effect of pH on the multiplication of *T. brucei* S 42 in medium HX12V (for explanation of diagram see legend to Fig. 3).

Table 4. *Effect of temperature on multiplication of* Trypanosoma brucei *S 42 during successive 3-day subcultures in medium HX12V*

	Increase (n-fold) in cell number. Incubation temperature			
Subculture	25 °C	28 °C	32 °C	37 °C
1	5.0	7.8	5.7	2.0
2	8.1	8.9	1.7	—
3	8.9	7.0	—	—

28 °C than at 25 °C (as is confirmed by the growth curve shown in Fig. 6), but on further subculture at 28 °C the growth rate was no higher than at 25 °C. At 32 °C the cells moved sluggishly and although there was good multiplication during the first subculture there was little growth in the second. At 37 °C there was a twofold multiplication, but 50% of the cells were immotile after 3 days and no attempt was made to subculture further at this temperature. Cultures were non-infective to mice after 3 days at 37 °C. At 28 °C and 32 °C the cells tended to aggregate. This tendency was often observed when cells were first transferred to a modified medium or an alternative physical environment. In a similar experiment using T. *rhodesiense* 261 we observed 3.4-fold multiplication at 37 °C.

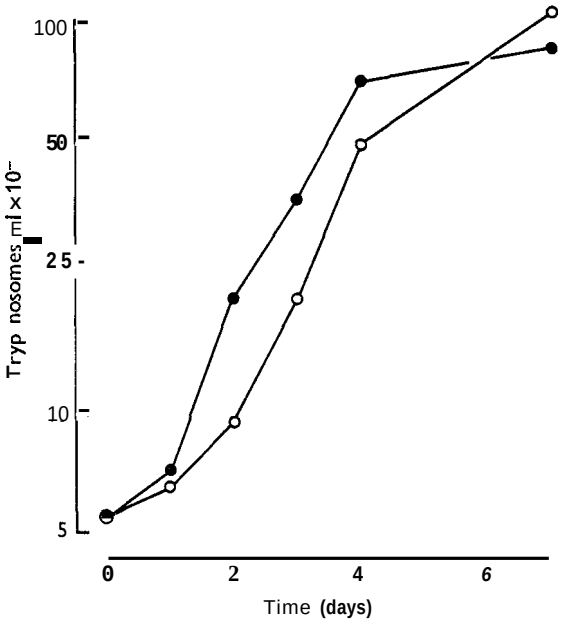

Fig. 6. Growth of *T. brucei* S 42 in 50 ml volumes of medium HXl2V at 25 °C (○) and 28 °C (●).

Development of a casein-free defined medium

The casein hydrolysate present in the media so far described was essential for growth. It could not be replaced by a synthetic mixture of the amino acids found in the casein hydrolysate, nor could it be replaced by a hydrolysate of vitamin and fat-free casein. Furthermore, some batches of 'Oxoid' casein hydrolysate would not support growth. Casein hydrolysate could be replaced by 'Oxoid' tryptone (a tryptic hydrolysate of casein). These observations suggested that the casein hydrolysate was acting as a source of non-amino acid growth factors. As described above, supplementation of medium HX12 with the vitamins listed in Table 3 gave substantial improvements in cell growth. An additional effect of these vitamins was that their presence would permit cell growth when one batch of casein hydrolysate was used which had been ineffective in their absence. However, the growth was only half that obtained with the most competent batch of casein hydrolysate, and addition of these vitamins still did not allow the replacement of casein hydrolysate by a synthetic amino acid mixture.

Initial attempts at fractionation of growth-promoting factors from casein hydrolysate by solvent extraction and ion exchange chromatography were discouraging. Before proceeding further with this approach, several media were tested in which the casein hydrolysate was replaced by an amino acid mixture and further supplemented with several vitamins and other factors which it was thought that the casein hydrolysate might be supplying. Table 5 shows the composition of an empirically devised defined medium (HX25) which supports the growth of *T. brucei* S 42, *T. brucei* 427 and *T. rhodesiense* 261. Analyses of casein hydrolysate suggested that quinones might be present (R. A. Klein, personal communica-

Table 5. *Composition of defined medium* **HX25**

	mg/l		mgll
HEPES	**19000**	L-alanine	**160**
Glucose	**1500**	L-arginine.HC1	**200**
NaCl	**1000**	L-asparagine	**100**
KH_2PO_4	900	L-aspartic acid	**400**
$NaHCO_3$	**800**	L-cysteine.HCl	**100**
Tri-sodium citrate. **2H,O**	**600**	L-cystine	**40**
Sodium acetate. **3H,O**	**540**	L-glutamic acid	**670**
Sodium succinate. **6H,O**	**270**	L-glutamine	**100**
D(+) glucosamine.HC1	**220**	Glycine	**100**
EDTA, disodium salt	**80**	L-histidine.HC1	**160**
Adenosine	**20**	L-isoleucine	**300**
Guanosine	**20**	L-leucine	**460**
Cytidine	**20**	L-lysine.HC1	**400**
Uridine	**20**	L-methionine	**100**
Haemin	**10**	L-phenylalanine	**250**
Polyoxyethylene sorbitan Monopalmitate (Tween 40)	**5**	L-proline	**580**
		L-serine	**160**
Folic acid	**10**	L-threonine	**120**
DL-a-tocophero1	**4**	L-trytophan	**90**
Vitamin B_{12}	**1**	L-tyrosine	**160**
DL-a-lipoicacid (oxidized form)	0.4	L-valine	**360**
Menadione	**0.4**		
Coenzyme Q **6**	**0.4**		
Coenzyme **Q 10**	**0.4**		
Trans-retinoic acid	**0.4**		ml/l
		Medium **199, 10** x concentrate	**88**
		Vitamin solution (Table **3**), **100** x concentrate	**10**
		Linoleic acid–albumin complex	**6**

Adjusted to pH **7.40** with NaOH.

tion). Coenzyme Q **9** has been found in trypanosomes (Vakertzi-Lemonias, Kidder & Dewey, **1963).** Since coenzyme Q **9** could not be obtained commercially coenzyme Q **6** and coenzyme Q **10** were included in the medium. Other vitamins already present in medium **199** at low concentrations and not included in the vitamin supplement (Table **3)** were added in increased amounts and vitamin **B12** was included. Free fatty acids were not present in significant amounts in our growth-supporting batch of casein hydrolysate, but Tween **40** (Polyoxyethylene sorbitan monopalmitate) was nevertheless added to the defined medium since no other saturated fatty acid was present and we had doubts, as discussed earlier, about the capability of the cells to synthesize fatty acids. Cells may be transferred directly from MCM into **HX25** and maintained continuously in this medium by serial subculture of **1** ml into **5** ml of fresh media every **3** or **4** days.

Linoleic acid–albumin requirement

The apparent requirement for linoleic acid and albumin already noted was reexamined using medium HX25. The results (Table **6)** suggested that the inclusion or omission of linoleic acid had no significant effect on growth. Neither was the

Table 6. *Effect of albumin and linoleic acid–albumin complex on growth of* Trypanosoma brucei S 42 *in medium* HX25

Results are expressed as mean growth yields averaged over the stated number of tubes and subcultures.

HX25 variation	No. of sub-cultures	No. of tubes	Growth yield (cells/ml)	Standard deviation
Complete	8	24	7.4×10^6	$\pm 1.2 \times 10^6$
Minus linoleic acid–albumin complex	6	18	4.6×10^6	$\pm 1.2 \times 10^6$
Minus linoleic acid–albumin complex plus defatted albumin (300mg/l)	8	23	7.0×10^6	$\pm 2.1 \times 10^6$

albumin essential, although its inclusion in the medium was apparently beneficial; cell yield was improved and the morphological appearance of the cells was more satisfactory when albumin was included. The final concentration of protein in the medium was 0.30 mg/ml. It seemed unlikely that the albumin was carrying essential low molecular weight growth factors into the medium since it had been treated with charcoal and extensively dialysed. Analyses showed that no fatty acids remained in the albumin after the defatting procedure.

DISCUSSION

Medium HX25 may be regarded as a defined medium by the generally accepted criteria. The major non-synthetic component, serum albumin, appears not to be an obligatory growth requirement and its growth-promoting function remains to be explained. One general problem in defining the composition of culture media arises from the possibility of impurities present in the stated components. Although chemicals of the highest available purity have been used, some are added in such large amounts that very minor impurities could have significant growth-enhancing or inhibitory effects.

The yield of organisms in both media HXl2V and HX25 is not as high as one might hope to achieve in such ostensibly nutritious environments, and this suggests that some unidentified factor may be present at growth-limiting concentration. We have not explored the important area of trace metal requirements and further studies in this direction might be desirable. Alternatively, it may be the accumulation of a toxic metabolite which limits growth in our media.

The defined medium described is not a minimal medium: no attempt has yet been made to simplify its composition. Although the casein requirement has been circumvented, the essential factors supplied by casein hydrolysate in earlier semi-defined media such as HXl2V have not been identified. Medium **HX25** contains a larger range of amino acids and vitamins than the defined media described for *Crithidia fasciculata* (Kidder & Dutta, 1958) and *Leishmania tarentolae* (Trager, 1957). Although the levels of several vitamins and amino acids present in medium 199 have been increased tenfold in medium HX25, the concentration of some vitamins remains substantially lower than in Trager's and Kidder & Dutta's media.

The effect of pH on growth was initially thought to be critical, as judged by the experiments on the growth of *T. brucei* S 42 and *T. rhodesiense* in MCM. However, in medium HX12V there was no significant effect over the same range of pH. Further experiments in MCM suggested that the severity of the pH effect varied between different batches of medium. These observations suggest that the effect of pH on growth may be linked to the concentration of some nutrient in the medium.

Osmolarity measurements using a Fiske Osmometer (Fiske Associates Inc., Uxbridge, Mass., U.S.A.) showed that medium HX12 was very hypertonic. Medium HX25 was made up to the same osmotic strength (460 milliosmolar) since growth appeared to be favoured by the hypertonic conditions. However, the effect of osmotic strength on growth has not been systematically explored. Both these media have a higher osmolarity than either MCM (365 milliosmolar) or HXA (349 milliosmolar). The apparent preference of culture forms for media of high osmolarity may be related to the probable hypertonicity (Bursell, 1970) of the natural environment of the vector midgut forms.

The ability of cells to undergo limited growth in these media at temperatures as high as 37 C offers a possible system for an approach to the problem of the cultivation of bloodstream forms of *T. brucei* sspp. Our observations of two- to fourfold increases in cell numbers at 32 °C and 37 °C, coupled with an inability to subculture at these temperatures, suggests the existence of temperature-sensitive areas in some metabolic pathways. Following transfer of the cells to supra-optimal temperatures, cell division may continue until the exhaustion of existing pools of metabolites which can no longer be synthesized. Identification of such temperature-sensitive pathways in culture forms may indicate likely additional nutritional requirements for the cultivation of bloodstream forms *in vitro*.

REFERENCES

BURSELL, E. *(1970)*. Feeding digestion and excretion (in *Glossina*). In *The African Trypanosomiases* (ed. H. W. Mulligan), pp. *305–16*. London: George Allen and Unwin Ltd.

CHEN, R. F. *(1967)*. Removal of fatty acids from serum albumin by charcoal treatment. *Journal of Biological Chemistry* **242,** *173-81*.

CUNNINGHAM, M. P. & VICKERMAN, K. *(1962)*. Antigenic analysis in the *Trypanosoma brucei* group using the agglutination reaction. *Transactions of the Royal Society of Tropical Medicirte and Hygiene* **56,** *48–59*.

DIXON, H. & WILLIAMSON, J. *(1970)*. The lipid composition of blood and culture forms of *Trypanosoma lewisi* and *Trypanosoma rhodesiense* compared with that of their environment. *Comparative Biochemistry and Physiology* **33,** *111–28*.

DIXON, H., GINGER, C. D. & WILLIAMSON, J. *(1972)*. Trypanosome sterols and their metabolic origins. *Comparative Biochemistry and Physiology* **41**B, *1-1 8*.

EAGLE, H. *(1955a)*. The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies and their cure. *Journal of Experimental Medicine* **102,** *596–600*.

EAGLE, H. *(1965b)*. The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. *Journal of Biological Chemistry* **214,** *839–52*.

EVANS, D. A. & BROWN, R. C. *(1971)*. Cyanide insensitive culture form of *Trypanosoma brucei*. *Nature, London* **230, 251–2.**

GOOD, N. E., WINGET, G. D., WINTER, W., CONNOLLY, T. N., IZAWA, S. & SINGH, R. M. M. *(1966)*. Hydrogen ion buffers for biological research. *Biochemistry* **5,** *467–77*.

HAUROWITZ, F. & HARDIN, R. L. **(1954).** Respiratory proteins. In *The Proteins,* vol. II, part **A,** (ed. H. Neurath and K. Bailey), pp. **279–344.** New **York:** Academic Press.

HOLMAN, **R.** T. **(1968).** Essential fatty acid deficiency. In *Progress in the chemistry of fats and other lipids,* vol. **9,** *Polyunsaturated acids* (ed. R. T. Holman), part II, pp. **279–348.** Oxford: Pergamon Press.

KIDDER, G. W. & DUTTA, **B. N. (1958).** The growth and nutrition of *Crithidia fasciculata, Journal of General Microbiology,* **18, 621–38.**

MORQAN, J. F., MORTON, H. J. & PARKER, R. C. **(1950).** Nutrition of animal cells in tissue culture. **I.** Initial studies on a synthetic medium. *Proceedings of the Society for Experimental Medicine* **73, 1–8.**

NEWTON, B. A., CROSS, G. A. M. & BAKER, J. R. **(1973).** Differentiation in Trypanosomatidae. In *Microbial Differentiation,* the Twenty-Third Symposium of the Society for General Microbiology (ed. J. E. Smith and J. M. Ashworth), pp. **339–73.** Cambridge University Press.

PITTAM, M. D. **(1970).** Medium for *in vitro* culture of *Trypanosoma rhodesiense* and *T. brucei.* Appendix to Dixon & Williamson **(1970).**

RODBELL, M. **(1965).** Modulation of lipolysis in adipose tissue by fatty acid concentration in fat cell. *Annals of the New York Academy of Sciences* **131, 302–14.**

TAYLOR, **A.** E. R. & BAKER, J. R. **(1968).** *The Cultivation of Parasites in vitro.* Oxford: Blackwell Scientific Publications.

TOBIE, E. J., BRAND, T. VON & MEHLMAN, B. **(1950).** Cultural and physiological observations on *Trypanosoma rhodesiense* and *Trypanosoma gambiense. Journal of Parasitology* **36, 48–54.**

TRAGER, W. **(1957).** Nutrition of a hemoflagellate (*Leishmania tarentolae)* having an interchangeable requirement **for** choline or pyridoxal. *Journal of Protozoology* **4, 269–76.**

VAKERTZI-LEMONIAS, C., KIDDER, G. W. & DEWEY, V. C. **(1963).** Ubiquinone in four genera of protozoa. *Comparative Biochemistry and Physiology* **8, 331–4.**

VICKERMAN, K. **(1971).** Morphological and physiological considerations of extracellular blood protozoa. In *Ecology and Physiology of Parasites: A Symposium* (ed. **A.** M. Fallis), pp. **58–91.** Toronto : University Press.

WEINMAN, D. **(1960).** Cultivation of the African sleeping sickness trypanosomes from the blood and cerebrospinal fluid of patients and suspects. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **54, 180–90.**

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