STUDIES ON THE IN VITRO CULTIVATION OF TRYPANOSOMES AND THE EFFECTS OF SOME DRUGS SHOWING TRYPANOCIDAL ACTIVITIES

A thesis submitted to the University of Lagos, in fulfilment for the Degree of Doctor of Philosophy, in Medical Parasitology.

By

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November, 1987.

DEDICATION

This thesis is dedicated to my husband (Dr. Christopher Otigbuo), and children (Kanu, Chris Jnr., Ndidi and Chinyere) whose love and understanding nas helped me through so many hard times.

To Professor Tolu Odugbemi and Dr. J.P.O. Oyerinde, without whose help, I will still be building castles in the air.

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EXTERNAL EXAMINER

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ACKNOWLEDGEMENT

Nobody could hope to write a thesis without the assistance of many people. I have drawn shamelessly on the expertise of numberous colleagues, peers and friends, whose experience have exceeded mine in certain specialized areas: Experts who have contributed their time, patience and special knowledge to this thesis are too numerous to list. But I specially wish to thank the Professor and Head, Department of Medical Microbiology and Parasitiology, Tolu Odugbemi during whose tenure of office I made a break-through. Dr. J.P.O. Oyerinde, my Supervisor and friend, who has guided me right from the first day I joined the Department.

To Professor Patrick T.K. Wco, I say a big thank you for his guidance and help in the aspect of the work carried out at the Department of Zoology, University of Guelph, Canada. I also wish to thank Professor Ken Wright of the Department of Zoology, Ramsay Wright Zoological Building, University of Toronto, for giving me laboratory space to carry out some of the experiments, and also for making his personal library available to me.

I thank Dr. A.O. Onabanjo who transformed my grammar and made sense out of nothing. Mr. Cyprian Tasie typed the draft,bringing order to the chaotic copy that I presented him. Thank you all from the bottom of my heart.

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ABSTRACT

A modified medium for the *in vitro* cultivation of trypanosomes has been developed. The medium comprised of Eagle Minimum Essential Medium (MEM) base and various components of medium 199, not found in MEM. *T.b. brucei* *T.b. gambiense*, and *T. vivax* were grown in modified medium ME-99 and in four other media (RPMI 1640, MEM, BCM and 199) for culturing trypanosomes. The growth, survival and infectivity of these parasites were compared in these media, as well as in ME-99. *T.b. brucei* was grown over Baby Hamster Kidney (BHK) cells in MEM and supplemented with various animal sera (horse, mouse, and fetal calf serum) as well as Nu-Serum (an artificial medium supplement). The effects of the various sera and the Nu-Serum on the culture system were compared. Parasites grew best in medium utilizing Nu-Serum, were still infective to mammalian hosts and could be sub-cultured. Nu-Serum was found to be a suitable replacement for the traditional animal sera used in culture. This artificial supplement is simple, low in protein content and makes results easily reproducible and consistent.

After the exposure of a monomorphic slender *T.b. brucei* to the Nu-Serum culture, they were observed to have reverted to pleomorphic forms. It was noticeable that Nu-Serum was responsible for this phenomenon.

Cat blood and cat embryo fibroblast-like cells have been isolated. *T.b. brucei*, *T.b. gambiense* and *T. vivax* were cultivated over these new cell lines, as well as other

cell lines for comparative purposes. An attempt at culturing trypanosomes axenically was made. This new system grew the parasites but not as well as the cell systems.

Blood stream forms of T.b. brucei were grown over BHK cells in MEM with various concentrations of anti-protozoal drugs, such as metronidazole, chloroquine and mefloquine. Drugs inhibited the multiplication of the parasites in vitro. The least effective in vitro concentration for metronidazole and chloroquine were 0.003 mg/ml and 0.0024 mg/ml respectively and for mefloquine 0,002 mg/ml. Groups of female mice were treated with 2 of the drugs (metronidazole and chloroquine) individually at 24, 48, and 72 hours after T.b. brucei infection. Each of the drugs, when administered once to four times daily, was observed to reduce the number of parasites in the mice but did not effect a cure; they prolonged the survival period of the animals. A different case was obtained with mefloquine and when this was administered daily at 0.03 mg/kg body weight for 4 consecutive days, a permanent cure was recorded. The combination of metronidazole (0.1 mg/kg) and chloroquine (0.08 mg/kg) given daily for similar number of days, cleared the parasites from the blood stream. No trypanosomes were detected in these mice for 90 days and over.

Mice inoculated with parasite-free culture medium containing a combination of both drugs (chloroquine and metronidazole) at the above concentrations, confer

protection against infection by trypanosomes for 14 days. Alaos, mice inoculated with parasite-free culture medium containing mefloquine alone at 0.002 mg/ml confered protection against subsequent infection for more than 4 months. Individually. metronidazole and chloroquine did not confer such protection. Mice inoculated with washed trypanosomes from cultures containing a combination of metronidazole and chloroquine, were protected against infection for 3 weeks.

Mice initially inoculated with pre-treated and washed trypanosomes from drug combined culture medium, and later given a combination of both drugs intraperitoneally, were protected against infection for a period of 5 weeks.

Mice injected with 0.03 mg/g mefloquine for 3 days consecutively were protected against subsequent T.b. brucei infection for 90 days and over.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

Trypanosomiasis is a serious, frequently fatal disease caused by parasites of the genus Trypanosoma. Various species of the parasite infect domestic livestock, wild animals and man. The most important species in terms of economics are those which affect cattle in Africa, namely : T. vivax, T. congolense and T.b brucei and the two subspecies which cause human sleeping sickness, namely, T.b gambiense and T.b. rhodesiense. There is T. cruzi, which is South African in origin, it also affects humans.

Trypanosomes are highly complex parasites. They pass through several distinct stages in the course of their life cycle (Plate 1.1) and have developed effective mechanisms for survival in their tse-tse fly vectors and mammalian hosts.

(i) MORPHOLOGY AND LIFE CYCLE OF TRYPANOSOMES

The life cycle of African trypanosomes is complex and involves at least 5 different developmental stages (Plate 1.1) in both the mammalian host and insect vector (Glossina species). So far there is no intracellular developmental stages involved in the life cycle but T. brucei from which T. b. rhodesiense and T.b. gambiense are believed to have evolved, infects the blood and tissue fluid of mammals (Losos and Ikede, 1972).

When parasites are taken by the insect vector with a blood meal from a mammalian host, the parasites multiply for 10 days in the posterior section of the mid-gut of the fly as trypomastigote forms. At the end of this period, these long slender trypomastigote forms migrate through the fore-gut (12-19 days) to the oesophagus, pharynx and hypopharynx and then enter the salivary glands of the susceptible fly. Once in the salivary glands, the slender forms transform into the epimastigotes. These epimastigotes in turn differentiate into the short stumpy forms called the metacyclics trypomastigotes (Plate 1.1). These metacyclics do not multiply and they are the only forms infective to mammalian hosts, and only at this stage does the insect vector become infective (Vickerman, 1965). The short stumpy metacyclic trypanosomes lack free flagella. In the mammalian host, T. brucei multiplies as trypomastigotes in the blood and lymph, although Soltys and Woo (1969, 1970) reported multinucleated and amastigote forms in the spleen of experimentally infected animals. The occurrence of amastigotes and multinucleated forms of T.b. brucei in the host was confirmed by Woo and Soltys (1969), who found them in the lumen of a choroid plexus vessel. The parasites invade all organs, starting from the blood and lymph.

The life cycle of the parasite is completed when an infected fly feeds on the infected mammalian host.

(ii) ECONOMIC IMPORTANCE

Human Trypanosomiasis:

This disease lowers the productivity in infected

people and if left untreated for a long time, depending on the infecting species, causes mortality.

The other factors to contend with are costs of treatment (drugs and hospital facilities of infected individuals), cost of maintaining surveillance groups (labour and equipment) to examine people in high risk areas, at regular intervals.

Animal Trypanosomiasis:

The major economic impact of African trypanosomiasis is in cattle production and the countries mostly affected are those with the least number of veterinarians (Braend, 1979). However, the full economic consequences of trypanosomiasis go far beyond figures on disease occurrence or livestock mortality. In areas affected by the disease, it is difficult for the local farmers to keep livestock at all. There are often very few dairy or beef cattle, and no draught animals to carry loads or help farmers produce their crops.

Other economic problems are :

(a) Meat and dairy products have to be imported. They are generally more expensive consequently, and less readily available to the population.

(b) The importation of meat and dairy products, may affect the financial reserves of the country and its balance of trade with the neighbouring countries.

The other factor is cost of treatment, the planning and execution of control of the insect vector. For instance, the clearing of bushes which harbour the vectors, is an

expensive venture. Also, the use of insecticide requires manpower, which may cost a lot of money. Use of insecticides may make the tse-tse flies more chemically resistant (Hadjuck 1981 thereby making it imperative for more and more insecticides to be used before a good result is achieved. This may also be rather expensive, and the insecticides, though important, may give rise to environmental hazards.

Scientists have been working for decades in order to develop effective, economic and safe ways to protect livestock and people against trypanosomiasis. The task proved extremely difficult. Yet, given the wide prevalence of the disease and its major economic importance, the potential reward for success is enormous.

(iii). Pathogenesis

Trypanosomes live in the blood, spleen, cerebrospinal fluid and lymph nodes of the vertebrate hosts.

Animals like horses, mules, dogs, and some ruminants, experience the acute disease and may survive from 5 days to 4 months, and die thereafter. Symptoms observed in these animals include anaemia, fever, running nose and eyes, and oedema. Paralysis due to central nervous system involvement, occurs shortly before death. Cattle often survive for up to several months after the onset of disease before they die whereas pigs almost always recover from the infection.

In man, the infection caused by T.b. rhodesiense and T. b. gambiense starts with a small sore or chancre at the

site of inoculation of the parasite and disappears after a while (1 - 2 weeks). The organisms multiply fast and cause parasitaemia, while invading almost all organs of the body.

T.b.rhodesiense causes an acute illness that results in faster death as opposed to infection caused by T.b. gambiense, where a chronic pattern is observed and drags on for quite sometime (several months - a year) before death ensues. In patients suffering from this disease, the lymph nodes become swollen and is located below the skull in the neck region, and is known as the "Winterbottom's sign".

T.b. gambiense invade the central nervous system and leads to comatose state which gives the disease the name "sleeping sickness". This is because, the patient sleeps even while standing or eating. Often times, death occurs from other complications associated with the disease. These include malnutrition, pneumonia, cadiac failure, secondary infections like other parasitic infections.

(iv) Epidemiology and Control

Factors responsible for the transmission of trypanosomiasis varies locally, since it depends on the coincidence of the parasite and the suitable vector species being available at the same time, and the habits of the human population which is likely to bring them into contact with tse-tse flies because of the non-uniformity in the distribution of sleeping sickness in the tse-tse fly belt of Africa.

However, various tse-tse flies are restricted in certain foci. For instance, the Ugandan shore of Lake Victoria and the Niger-Benue confluence and most parts in the North of Nigeria, harbour T. b. rhodesiense and T. b. gambiense respectively.

The vectors of T.b.gambiense are found at the riverine, moist and shady areas while vectors of T.b.rhodesiense and T.b.brucei are found in the open dry earth.

Majority of infections with trypanosomes are zoonotic in origin, though there may be important circumstances where man-to-man transmission has developed. For instance, in blood transfusion (Gutteridge, 1985).

Presently, control of African trypanosomiasis is still inadequate (Williamson, 1970; Meshnick, 1983), partly because antigenic variation of trypanosomes in mammalian blood has made all attempts at immunization in the field impossible (Hadjuk, 1984). However, vaccine development in the field is underway (W.H.O., 1984).

The control programmes so far employed include chemotherapy, eradication or control of the insect vector with insecticides, and chemoprophylaxis (Meshnick, 1983). So far the results of all these programmes have not been very satisfactory. The reasons are based on the fact that the more than 20 species of Glossina responsible for the transmission of the disease are adapted to a wide range of habitats, hence this also accounts for the widespread of the disease.

So many problems are associated with the control measures presently in use. These are :

(a) Drugs available for the chemotherapy and chemoprophylaxis of this disease are very expensive, and sometimes repeated therapy is necessary while diagnosis is essential to ensure correct and adequate therapy.

(b) When certain drugs fail, trials of other trypanocides may be necessary.

(c) Incorrect treatment may lead to drug resistance and moreso when antitrypanosomal drugs available in the market presently, are limited (W.H.O., 1984). However, in some parts of Nigeria, Zambia, Botswana eradication of the disease has proved successful (McLennan, 1981). But the environmental hazards created by use of some of these insecticides are not to be ignored.

(d) So far, there are no vaccines currently in use for prevention of this disease (Hadjuk, 1981). Therefore, chemoprophylaxis is the most common method currently in use (Toro et al, 1983). Recently, many investigators have directed their efforts towards developing a vaccine against African trypanosomiasis as one of the control measures (Murray et al. 1979, 1980). Their attempts were unsuccessful because of the mammalian blood parasites ability to change their surface coats (antigens). Hence, these trypanosomes evade the hosts' immune response by antigenic variation (Tuner, 1983; Vickerman, 1974) and the heterogeneity of metacyclic variable antigen types (Barry et al., 1979; Nantulya et al., 1983).

PLATE 1.1a THE VARIOUS DEVELOPMENTAL STAGES OF TRYPANOSOMES IN THE MAMMALIAN HOST

D - INTERMEDIATE TRYPOMASTIGOTE

E - STUMPY TRYPOMASTIGOTE

F - SLENDER TRYPOMASTIGOTE

I - NUCLEUS

2 - KINETOPLAST

3 - UNDULATING MEMBRANE

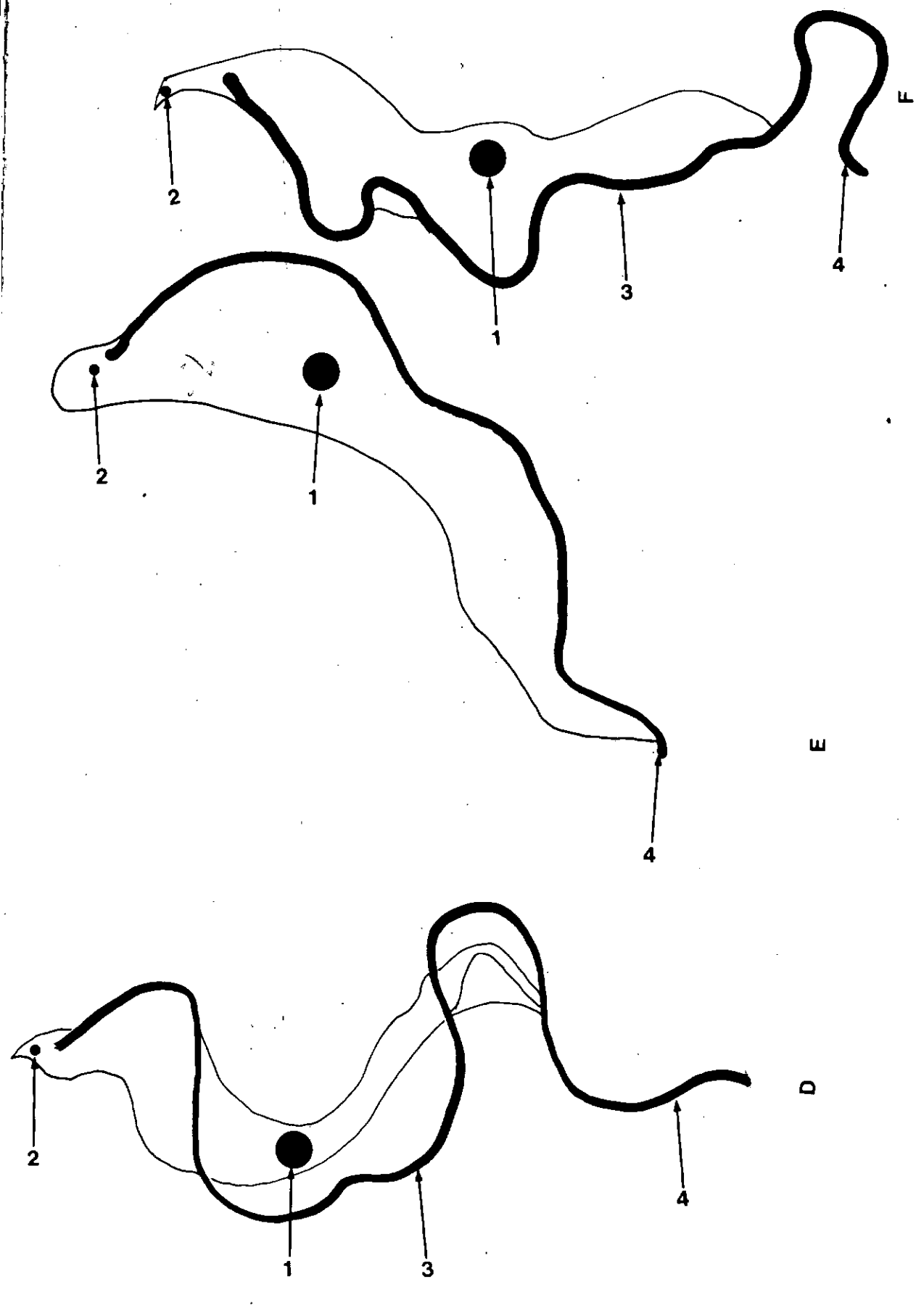

F
2
1
3
4
E
2
1
4
D
2
1
3
4

PLATE 1.1 b THE VARIOUS DEVELOPMENTAL STAGES OF TRYPANOSOMES IN THE INSECT VECTOR

A – METACYCLIC TRYPOMASTIGOTE

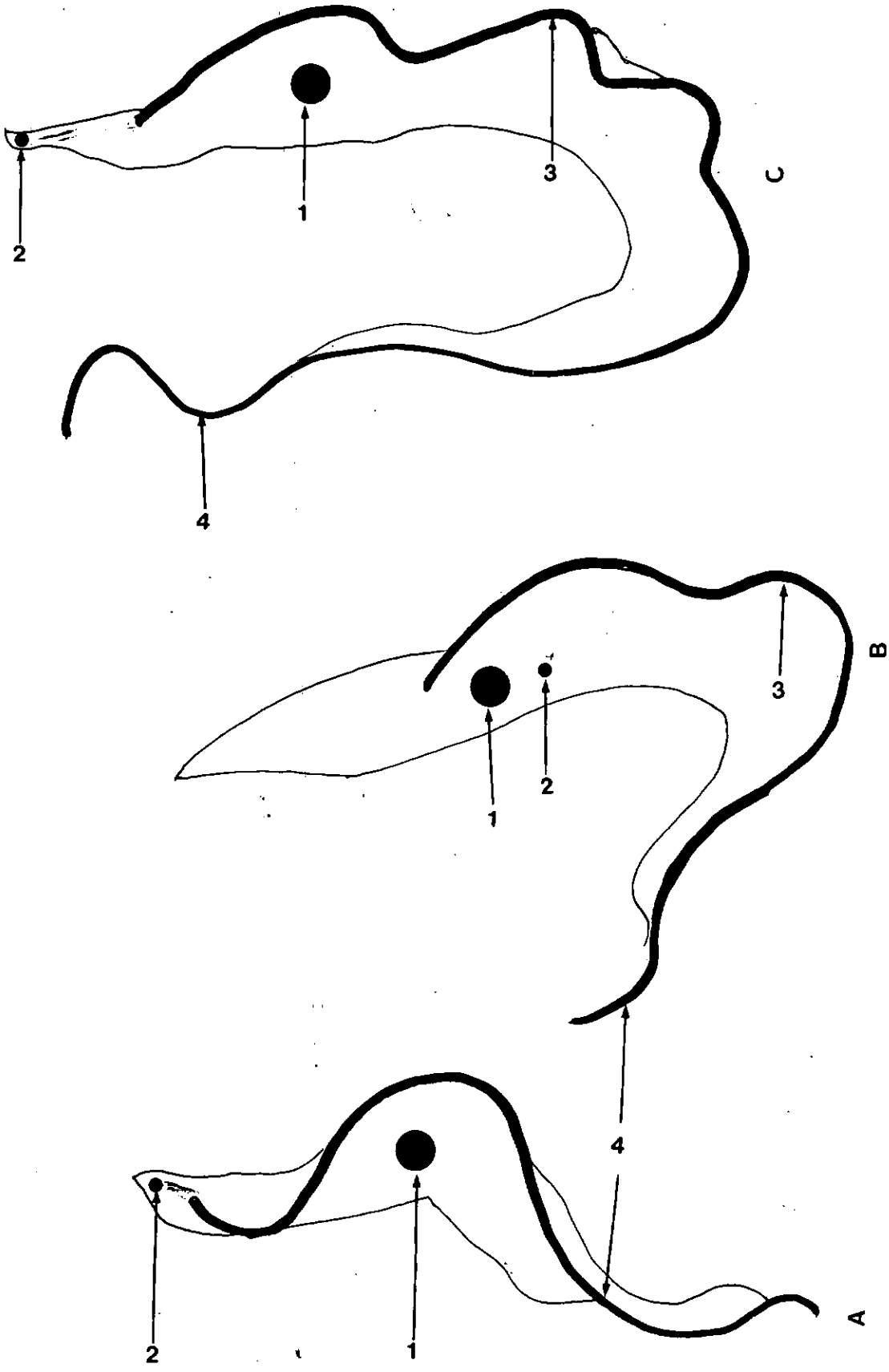
B – EPIMASTIGOTE

C – CULTURE FORM/MIDGUT TRYPOMASTIGOTE

1 – NUCLEUS

2 – KINETOPLAST

3 – UNDULATING MEMBRANE


C
1
2
3
4
B
1
2
3
A
1
2
4

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

The need to cultivate trypanosomes *in vitro* has been a long standing requisite for the following reasons:

(a) For the maintenance of parasites without using an animal model (Balber, 1983; Brun *et al*., 1979; Hirumi *et al*., 1977).

(b) For diagnostic and chemotherapeutic purposes (Raether and Seidenath, 1984; Seebeck and Gehr, 1983; Wirth and Kierszenbaum, 1985).

(c) For experimental studies related to biochemistry (Grady *et al*, 1984), for immunologic studies (Jenni and Brun, 1981, 1982; Doyle *et al*., 1979; Nyindo and Rurangirwa, 1981), and for the biological studies of the parasites (Brun *et al*., 1984; Hirumi and Hirumi, 1984; Joshua and Kayit, 1984).

The necessity for cultivating cells/parasites *in vitro* especially in media of definite composition has received much attention and resulted in an enormous amount of published work (Berens *et al*., 1976; Cross and Manning, 1973).

The first medium produced for the *in vitro* cultivation of trypanosomes still remains widely used. This is the so-called NNN medium (Novy, Nicholle and McNeal, 1903). This is a blood agar medium with a liquid overlay, and has been extensively used for the growth of both stercorarian and salivarian trypanosomes. Earlier media used for

in vitro cultivation of trypanosomes (Novy *et al*, 1903; Tobie *et al*, 1950; Weinman, 1953; Pittam, 1970), were mostly diphasic, and the limitation of their use in experimental procedures caused by the complexity and undefined components are enormous (Cross and Manning, 1973; Dixon and Williamson, 1970). More-defined or semi-defined media are now available for the cultivation of trypanosomes (Cross and Manning, 1973; Berens *et al*, 1976). This advancement in *in vitro* cultivation of trypanosomes has enhanced the understanding of the nutritional requirements and the biochemical make-up of the parasites.

The commercial availability of tissue culture media has led to the development of highly effective culture media for growth of trypomastigotes. One such medium, known as HX-12 (Cross and Manning, 1973) is based on the tissue culture 199, with commercially available salt solutions, amino acids, hepes, caesin hydrolysate and vitamins. Another of such medium is known as HO-MEM (Berens *et al*., 1976). This medium is very effective for cultivation of *L. donovani* promastigotes as well as Costa Rican strain of *T. cruzi*. The HO-MEM medium is based on the Eagle's Minimum (MEM) with commercially available salt solutions, MEM amino acids and non-essential amino acids. The possibilities offered by chemically defined media in quantitative researches, the uniformity and the ability to reproduce experiments, stimulates a more intense research aiming towards an established, clearly defined medium for cell cultures. Recently, Seebeck and Kurath (1985) developed two simple chemically defined media

that suits the need of the biochemist. They were able to maintain T. b. brucei in these media, and the media also made it easy for anyone of the components to be deleted or replaced. However, like many of the other chemically defined media. (Seebeck and Kurath 1985) media did not support parasite growth or cell mutiplication. In this case, there is still a need for the development of a chemically defined medium or a system that will grow and maintain trypanosomes continuously.

Partially successful attempts at cultivating trypanosomes in vitro using mammalian cells as feeder layer was made by earlier investigators (Le Page, 1967; Hawkins, 1971). In the last decade, significant advances have been made in culturing various parasites; in the case of trypanosomes, the most successful methods to date have involved co-cultivation of parasites together with some supportive cell of mammlian origin (Hirumi et al, 1977). These investigators used bovine fibroblast like cells as feeder layer in Rosswell Park Memorial Institute medium (RPMI 1640), supplemented with foetal bovine serum in order to grow and maintain T. brucei of various stock, in continuous culture for long periods of time. This system made cloning possible (Hirumi, 1979). Doyle et al (1979), studied the antigenicity of the parasites in this system. Even though the T. b. brucei S427 used by Hirumi and his co-workers (1977a) has lost the ability for cyclical transmission, it was possible

to cultivate it over other mammalian cells (Hirumi et al., 1980; Brun et al., 1979; Hill et al., 1978a,b). However, all the tissue culture systems so far developed have utilized mammalian cells belonging to bovine (Hirumi and Hirumi, 1982, 1984; Gray et al., 1979, 1981, 1984) and rodents (Hill et al., 1978a,b; Brun et al., 1981) group of animals. In view of this, the need arises to raise other cell lines, for instance from carnivores; to make for variety.

There are several problems encountered with current in vitro culture systems. The problems are as follows:

(a) The system almost always requires use of animal cells and/or sera. Animal sera has been shown to cause inconsistencies in laboratory experiments and also make results non-reproducible (Ham, 1971).

(b) Setting up the culture system is cumbersome and requires a lot of time.

(c) The set up is very expensive.

(d) The results are not consistent; and

(f) Toxic metabolites emit from the cells and pose serious problems in experiments (Duszenko et al., 1985; Brun and Jenni, 1985).

Therefore, a system that eliminates the feeder layer cell requirement and with the animal serum replaced by an artificial medium supplement, will prove very beneficial.

The study of suitable agents for chemotherapy of African trypanosomiasis has been on the increase in recent times (Balber, 1985; Urich and Cerami, 1984;

Meshnick, 1984; Nathan et al, 1984). However, there have not been much progress in that area and results has not been consistent. For instance, Jennings et al, (1983, 1984) and Raether and Seidenath (1983), reported the efficacy of metronidazole and some other imidazoles in T. b. brucei experimental infections, while Keithly and Langreth (1983) reported the inefficacy of the same drug.

From the foregoing it is evident that more research effort is indicated particularly with regards to the in vitro cultivation of the parasites in large quantities and on a continuous basis, parasite materials for research would be ensured.

This study is designed to :

(a) compare the various culture media presently in use to culture Trypanosomes in an attempt to develop a medium that will be most suitable for use in culturing various species of African trypanosomes.

(b) find a replacement for animal serum in the cultivation of trypanosomes, so as to eliminate the problems encountered with the animal sera.

(c) raise a new cell line from a carnivore, so as to make for variety of cell lines to choose from, and to see if cells from a carnivore will grow the parasites better than that of bovine and rodentia, already in use.

(d) culture parasites axenically in an attempt to eliminate use of cells and avoid problems caused by the metabolites emitted from the cells during mitosis,

and to provide useful material for vaccine production.

(e) test the existing and newly synthesized anti-protozoal drugs and study the possibility of using them as trypanocides rather than look for new drugs, since it is unlikely that drug firms are going to produce new trypanocides due to their limited market.

(f) study in vitro and in vivo effects of Metronidazole, Chloroquine and mefloquine. The studies will concentrate on the infectivity level of cultured T. b. brucei and will determine the stage at which the drugs are effective, as well as the duration of protection it might confer on mice against infection.

(g) determine if the drugs have any synergistic effects, so that one may not need to give high doses of these drugs in order to achieve a desirable therapeutic effect.

(h) determine the possibility of attenuating the parasite virulence in drug tested cultures, for possible use as prophylactics.

CHAPTER 3

COMPARATIVE STUDIES OF THE FOUR MEDIA FOR THE IN VITRO CULTIVATION OF TRYPANOSOMES

ABSTRACT

Four media already in use for the *in vitro* cultivation of trypanosomes (Eagle Minimum Essential Medium, Tissue Culture Medium 199, Basal Culture Medium (BCM), and Roswell Park Memorial Institute Culture Medium 1640 (RPMI 1640) were employed to grow the Nigerian strains of *T. b. brucei*, *T. b. gambiense*, and *T. vivax* to find out which is best suitable for their growth and infectivity.

It was found that MEM gave the best result, since parasites grew best in this medium and maintained their infectivity longer in it.

CHAPTER 3

INTRODUCTION

In the early Century, all media employed for the *in vitro* cultivation of trypanosomes did not utilize mammalian or insect cells (Novy *et al*, 1903; Tobie *et al*, 1950; Weinman, 1953; Pittam, 1970). These media were mostly dephasic (blood agar base, with a liquid over lay), and the limitation in experimental procedures caused by their complexity and undefined components were enormous (Cross and Manning, 1973; Dixon and Williamson, 1970). More defined or semi-defined media are now available for the cultivation of trypanosomes (Cross and Manning, 1973; Berens *et al*, 1976). This advancement in *in vitro* cultivation of trypanosomes, has enhanced the understanding of the nutritional requirements and the biochemical make up of the parasites. However, these media are still fraught with complications (Steiger and Steiger, 1976). For instance, most of them have problems in the maintenance of parasites and growth at times became inconsistent. In Cross and Mannings (1973) HXA semi-defined medium, cell yields were never more than 5×10^6 parasites/ml culture medium and the medium could not maintain *T. b. brucei* adequately. For the HX-12 defined medium of Cross and Manning (1973), there were lots of inefficiencies in the maintenance of the parasites for the parasites could not be subcultured. In this HX-12 defined medium, more than one subculture was impossible, and cultures were non-infective to mice

after 3 days.

Seebeck and Kurath (1985) recently developed two simple chemically defined media that suits the need of the biochemist. They were able to maintain T. b. brucei in these media. The media also made it easy for any of the components to be deleted or replaced. However, these media did not support parasite multiplication. Therefore, a medium or media that will grow and maintain most T. b. brucei stocks and species is still needed.

The aim of this study is to grow the 3 Nigerian species of African trypanosomes (T. b. brucei, T. b. gambiense and T. vivax) in various culture media namely;

Rosewell Park Memorial Institute 1640 medium (RPMI 1640).

Minimum Essential Medium (MEM)

Tissue Culture Medium 199

Basal Culture Medium (BCM)

All of the above media were obtained from GIBCO (Industrial Avenue, Canada) in powder form and made up according to Manufacturers' instructions (see Materials and Methods).

Parasites were grown in these media in order to find out which culture medium or media will provide the best growth of the 3 species of trypanosomes.

MATERIALS AND METHODS

MATERIALS

Parasites : - T. b. brucei strain 8/18, T.b. gambiense, and T. vivax, were obtained from Nigerian Institute for Trypanosomiasis Research Center (NITR), Vom. The parasites were maintained in albino mice, by serial passage, at the Departments of Medical Microbiology and Parasitology and Pharmacology (Chemotherapy Unit), College of Medicine of the University of Lagos.

Media : RPMI 1640

BCM

MEM

199

The above media were obtained either from GIBCO, Sussex, England or GIBCO, Industrial Avenue, Burlington, Ontario, Canada.

The media came in powder form and were made up as follows:-

MINIMUM ESSENTIAL MEDIUM (MEM)

To make up 1 litre of Eagle Minimum Essential Medium, 95 ml of double distilled water was measured out, into a mixing container, that is, as close to the final volume as possible. MEM powder 10.7 g was weighed out and added to water at 20°C and gently stirred. Sodium bicarbonate ($NaHCO_3$) 0.35 g was added. The volume of the medium was then made up to 1 litre, with double distilled water and

the mixture was stirred until all traces of solutes were completely dissolved. The pH of the medium was adjusted to 7.4 and filtered. The medium was sterilized and stored until needed.

MEDIUM 199

To make up 1 litre of 199, 95 ml of double distilled water was measured out, into a mixing container, that is as close to the final volume as possible. Medium 199 powder 9.9 g was added at 20°C and gently stirred. $NaHCO_3$ powder, 0.35 g was added. The volume of the mixture was then made up to 1 litre with double distilled water and the mixture was stirred until completely dissolved. The pH of the medium was adjusted to 7.4, filtered, sterilized and stored until needed.

ROSWELL PARK MEMORIAL INSTITUTE 1640 MEDIUM (RPMI 1640)

This medium was made up the same way MEM and 199 were made up. However, 10 g of the medium powder was used.

BASAL CULTURE MEDIUM (BCM)

The method for making up BCM is the same as above, and 10 g of the medium powder used.

Other Tissue Culture Materials

Plastic nunc 25 cm^2 or falcon flasks 25 cm^2 obtained from GIBCO. Foetal calf serum (FCS) obtained from GIBCO and was stored in the freezer in the laboratory at - 4°C.

Examination of cultures was carried out using inverted phase contrast microscope.

Counting of parasites was done by using Coulter Counter (Coulter Electronics Model ZBI, aperture at 70 um) or New Improved Neubauer hamocytometer.

METHODS

Isolation of Mouse Kidney Cells

Mouse was anaesthetized, the chest and abdomen were sterilized with 70% alcohol. Blood was obtained from the mouse by cardiac puncture. The abdominal cavity was opened and the kidneys excised and placed in a sterile petri-dish. The kidney were dissected out, the capsules along with the connective tissues and medullary tissues were discarded. The cortex of each kidney was then minced into 4 mm cubes and phosphate buffered saline added. When the fragments had settled, the supernatant was discarded. The washing with phosphate buffered saline was carried out 3 times. Trypsin, 0.25% was later added and the mixture stirred gently for 10 minutes. The fragments were allowed to settle once more and the supernatant discarded. Fifty millilitre of fresh 0.25% trypsin was again added to the flask containing the chopped mouse kidney. The mixture was stirred with the aid of a magnetic stirrer for 2 hours, until full digestion was achieved. Growth medium MEM supplemented with 20% foetal calf serum was added in order to stop digestion (that is, trypsin action). Straining of cell suspension was done through sterile muslin to remove

coarse tissue debris, and the filterate was centrifuged at 500 rpm for 10 minutes to deposite cells. The deposited cells were resuspended in MEM. Counting and seeding into 25 cm^2 tissue culture plastic flasks at 10^5 cells/flask was carried out.

Isolation of Parasites from Mouse Blood and Inoculation of Cultures

The method used for the isolation of trypanosomes from mouse blood is a modification of that of Hirumi et al (1977). In this method the medium employed was MEM instead of RPMI 1640, and the serum used in supplementing the cultures was Nu-Serum instead of foetal calf serum. Mice were inoculated intraperitoneally with infected blood diluted with phosphate buffered saline (pbs) from an infected animal.

To determine the degree of parasitaemia, animals were bled by the orbital sinus puncture and microhaematocrit centrifuged method (Woo, 1969) was used. Wet blood films were examined microscopically in some instances. Syringe rinsed with citrated saline (anti-coagulant) was used to take blood from the heart of the infected mouse and placed in a centrifuge tube. The blood was immediately centrifuged at 900 rpm for 10 minutes. The buffy layer containing the trypanosomes was transferred to a fresh centrifuge tube and mixed with an equal amount of PBS and centrifuged at 1,400 rpm for 20 minutes. The trypanosomes formed a white pellet at the bottom of the centrifuge tube. The upper

solution was discarded and the parasites were washed 3 times with PBS and by sequential centrifugation at 1,400 rpm for 20 minutes each time. The pellet was then suspended in Eagle MEM with 5% foetal calf serum (FCS). About 10^3 parasites were inoculated into each culture flask containing 5 ml of medium and mouse kidney cells were employed as monolayer. Cultures were examined everyday under inverted phase contrast microscope.

Trypsinization of Cells

The media in the culture flasks were decanted and the cells rinsed with pbs solution (pH 7.2). Cold 0.1 ml trypsin/cm^2 cell monolayer was added to trypsinize the cells. It took about 5 minutes for most of the cells to completely detach and round up. MEM was then added to re-suspend the cells. The cells were later counted and seeded into fresh culture flasks with 5 ml of medium.

Counting of Cells and Parasites

The counts were carried out by using a Coulter counter with aperture at 70 µm. In some cases, counts were done using the new improved Neubauer haemocytometer, employing the method for white blood cell count. Prior to the routine application of the electronic counter, a number of counts were made in comparison with the counts made with a haemocytometer. The settings were chosen so as to facilitate accurate and reproducile results.

Preparation of Blood Smears

This blood smears on microscopic slides were made from infected animals, and of flagellates from cultures after centrifugation. The smears were fixed in absolute alcohol for 10 minutes, air dried and dipped in 10% buffered formalin for 10 minutes (Woo, 1969). They were then stained for 40 minutes in Giemsa (Gurr Improved R66 GIBCO, Sussex, England). The slides were examined under oil immersion objective (X 100) and photomicrographs were taken with Zeiss photomicroscope.

Infectivity Tests

Parasites from the following cultures, utilizing the various media were inoculated into 5 groups of animals containing 6 mice each

(a) MEM + MK cells + 20% FCS

(b) M199 + MK cells + 20 FCS

(c) BCM + MK cells + 20% FCS

(d) RPMI 1640 + MK cells + 20% FCS

(e) Mouse Kidney cells + 20% FCS (control).

The study was carried out in duplicate.

RESULTS

GROWTH OF T.B. BRUCEI, T.B. GAMBIENSE AND T. VIVAX IN THE FOUR MEDIA TESTED

T. b. brucei

The results are summarized in Tables 3.1, 3.2 and Figures 3.1 and 3.2.

From the investigation it was found that T.b. brucei grew best in MEM. A marked increase (2×10^2 parasites/ml culture fluid) in the number of parasites between day of inoculation (day 0) and day 2 (Figure 3.1) was observed. Thereafter, there was a gradual but steady increase in the number of parasites uptill day 10, following which the number of parasites in the culture remained constant untill day 16, when the number slightly increased to 2×10^{10} (Table and Figure 3.1).

In tissue culture medium 199 the parasites maintained a gradual but steady increase in growth till day 8. Between day 8 and day 10, no increase was observed. However, an increase was observed on day 12, and no increase was recorded until day 16, when a maximum parasite count of 2×10^9 was observed (Table 3.1).

T.b. brucei growth in BCM was poor as compared to the growth of the same parasite in other media tested. A maximum parasite count of 2×10^6 was recorded on day 8. Thereafter there was a decline in the parasite count to 2×10^5 on day 10. The number remained constant at this level until the experiment was terminated.

In RPMI 1640 medium, T.b. brucei maintained a steady growth from day 2 up till day 8 when a maximum parasite count of 2×10^7 (Table 3.1) was recorded. After that, the parasite number remained constant until the end of the experiment.

T.b. gambiense

The growth of this strain in the various media is summarized in Table 3.2.

In MEM, the parasites grew exponentially between days 2 and 4, with increase in parasite number from 2×10^3 to 2×10^5 (Table and Figure 3.2). Later, the parasite number increased gradually until the 8th day. From day 8 until the experiment was terminated, T.b. gambiense number in MEM remained constant (Table and Figure 3.2). In 199, the parasite increased gradually until the 8th day when the parasite did not increase in number further, but remained constant until the 16th day when the experiment was terminated.

The parasites was observed not to grow well in BCM, although, there was an increase in parasite number between day 2 and 4 (after a latent period of 24 hours), the number of parasites thereby remained constant for sometime between day 4 and day 8. However, from day 10, a steady decline in the parasite number was observed until day 16 when no living trypanosme was recorded.

In RPMI 1640, the parasite count increased markedly from 2×10^3 on day 2 to 10^5 on day 4. The number remained constant from then on, till day 8 and thereafter, a slight decline in the parasite count from 2×10^5 to 2×10^4 on day 10 was obtained. The parasite number then remained constant at 2×10^4 from day 10 till the end of the experiment.

T. vivax

Table 3.3 summarizes the result observed in the growth of T. vivax in the four media tested.

T. vivax grew best in MEM as compared to the rest of the media tested. There was a gradual increase in the number of parasites from 2×10^3 to 2×10^5, between day 4 and day 8. From then on, the parasite number remained constant till the end of the experiment.

In the medium 199, T. vivax maintained a constant number of parasites (2×10^3) from day 2 to day 6. However, the parasite number increased to 2×10^4 on day 8 and maintained this level until day 16.

For the BCM, the parasites number rose to 2×10^3 on day 2 with a slight decline to 10^3 on day 4, and a further fall to 10^1 on day 6. Thereafter, no living parasite was observed till the termination of the experiment.

In the RPMI 1640, the parasite number increased from 10^3 to 2×10^3 on day 2. The number remained constant until day 8 when an increase from 2×10^3 to 2×10^4 was observed. Although the number remained constant at 2×10^4 for a period of 2 days, a decline from day 10 in the number of parasites was noticed until the 16th day when no living trypanosome was recorded in the culture.

Table and Figure 3.3 shows that T. vivax multiplied at the same rate in all but one of the media up to the 4th day of culture. The cultured parasites in BCM started declining in number from the 4th day with no survivor on day 8. However, the cultured parasite in RPMI 1640 and 199 maintained the original constant growth rate for another 2 days after day 8, after which the parasite remained constant in MEM. It was not until day 12, that a decline was recorded for the parasite grown

in RPMI 1640. The decline in number continued until day 14 when the parasite died out in the culture. On the first 4 days of lag phase, there was an increase in number for the following 4 days before the growth remained constant till day 16 when the experiment was terminated.

INFECTIVITY OF T.B. BRUCEI, T.B. GAMBIENSE AND T. VIVAX IN THE FOUR MEDIA FOR IN VITRO CULTIVATION OF TRYPANOSOMES

T. b. brucei from all the media tested was infective to mice for as long as the experiment lasted (Table 3.4 and Figure 3.4).

Trypanosoma brucei gambiense, although infective in some media, the infectivity did not last till the end of the experiment. For instance, in MEM and 199, the infectivity of T.b. gambiense was lost after day 8 (Table 3.4), while the infectivity in RPMI 1640 was lost after day 4. The parasite cultured in BCM was not infective to mice.

T. vivax was infective only in MEM and 199, although the infectivity was lost after day 4 in those two media (MEM and 199).

DISCUSSION

It is evident from the result of this study that of all the 4 media tested, MEM gave the best result in terms of growth response for all the parasites cultured in vitro. A similar conclusion had been reached by Tanner (1980) and Brun et al (1981). Brun et al (1981) showed that MEM was superior to other tissue culture media like RPMI 1640 while

Tanner (1980), a year earlier, reported that RPMI 1640 alone did not allow maintenance of blood forms.

Although, 199 has been shown to contain lower concentrations of vitamins (Cross and Manning, 1973) which have been shown to be essential for growth of certain mammalian cells (Eagle, 1955), the results obtained proved that 199 is equally a good medium for the growth of the parasites. The interpretation that can be adduced, is that the ratios of various amino acids contained in the medium, may possibly play a role in determining the success of any culture system. Basal culture medium (BCM) gave the poorest result in the cultures employed, in terms of parasite yield and infectivity which is in conformity with the work of Tanner (1980). RPMI 1640 has been shown to be a very good medium for trypanosome cultivation (Hirumi et al 1977) however, we found that this medium did not perform well as reported by Hirumi et al (1977), Doyle et al (1979), and Brun et al (1979). A possible reason for the discripancy may be that the medium (RPMI 1640) would only support the growth of the specific strain of trypanosme that is, T. b. brucei stock 427 used by the above-mentioned authors. This contention is supported by the observation that the parasite, T. b. brucei stock 427 has lost the ability for cyclical changes (Hill et al, 1978). Such changes in the strain of the parasite might have been reflected in the growth characteristics of the parasite in culture.

From the outlook, T. vivax which is the most difficult of the salivarian trypanosomes to culture, provided the best growth result in MEM. In BCM, T. vivax died after day 4 (Table 3.3).

It is difficult to find an explanation as to why some parasites were not infective in some of the media within the limits of these experiments.

The growth pattern of T. vivax in the four media is of interest. For the first four days of in vitro cultivation, the parasite maintained a constant growth rate, albeit at a low level in the four media. This seems to suggest that the nutrient requirement of the parasites are the same in the four media. The decline in the growth rate of the parasite grown in BCM may be due to exhaustion of nutrients in the medium and/or of accumulation of metabolic waste in the medium which may have become toxic to the parasite. In the manner, the sudden increased growth rate of the parasite in MEM, following a lag period of 4 days might be that in this particular medium, a lag period is necessary for the trypanosomes to adjust to the medium and without which the parasite could not increase.

From the results of this study, it appears there is room for improvement of the various culture media to achieve a better growth of the parasite. Attention will be devoted to this aspect of in vitro cultuvation of the trypanosomes in the following chapters.

TABLE 3.1

COMPARATIVE STUDIES ON THE GROWTH OF TRYPANOSOMA BRUCEI BRUCEI IN FOUR GROWTH MEDIA FOR THE IN VITRO CULTIVATION OF TRYPANOSOMES

Medium	Initial inoculum/ 5ml culture fluid count	Number of Parasites Harvested/ml culture fluid							
		Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
MEM	10^3	2×10^5	2×10^6	2×10^7	2×10^8	2×10^9	2×10^9	2×10^9	2×10^{10}
199	10^3	2×10^4	2×10^5	2×10^6	2×10^7	2×10^7	2×10^8	2×10^8	2×10^9
BCM	10^3	2×10^3	2×10^4	2×10^5	2×10^6	2×10^5	2×10^5	2×10^5	2×10^5
RPMI 1640	10^3	2×10^4	2×10^5	2×10^6	2×10^7	2×10^7	2×10^7	2×10^7	2×10^7

TABLE 3.2

COMPARATIVE STUDIES ON THE GROWTH OF TRYPANOSOMA BRUCEI GAMBIENSE IN THE FOUR MEDIA FOR THE IN VITRO CULTIVATION OF TRYPANOSOMES

Medium	Initial inoculum/ 5ml culture fluid count	Number of Parasites Harvested/ml culture fluid							
		Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
MEM	10^3	2×10^3	2×10^5	2×10^6	2×10^7	2×10^7	2×10^7	2×10^7	2×10^7
199	10^3	2×10^3	2×10^4	2×10^5	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6
BCM	10^3	10^3	2×10^4	2×10^4	2×10^4	2×10^3	2×10^2	2×10^1	-
RPMI 1640	10^3	2×10^3	2×10^5	2×10^5	2×10^5	2×10^4	2×10^4	2×10^4	2×10^4

TABLE 3.3

COMPARATIVE STUDIES ON THE GROWTH OF T. VIVAX IN THE FOUR MEDIA FOR THE IN VITRO CULTIVATION OF TRYPANOSOMES

Medium	Initial inoculum/ 5ml culture medium count	Number of Parasites Harvested/ml culture fluid							
		Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
MEM	10^3	$2x10^3$	$2x10^3$	$2x10^4$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
199	10^3	$2x10^3$	$2x10^3$	$2x10^3$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$
BCM	10^3	$2x10^3$	$2x10^3$	$2x10^1$	-	-	-	-	-
RPMI 1640	10^3	$2x10^3$	$2x10^3$	$2x10^3$	$2x10^4$	$2x10^4$	10^3	10^1	-

TABLE 3.4

INFECTIVITY OF T.B. BRUCEI, T.B. GAMBIENSE AND T. VIVAX CULTURED IN THE FOUR MEDIA

	DEGREE OF INFECTIVITY Days Post initiation of culture							
Media/Parasite	Day 2	4	6	8	10	12	14	16
MEM/T. brucei	+	+	+	+	+	+	+	+
MEM/T.b. gambiense	+	+	+	+	–	–	–	–
MEM/T.vivax	+	+	–	–	–	–	–	–
199/T.b. brucei	+	+	+	+	+	+	+	+
199/T.b. gambiense	+	+	+	+	–	–	–	–
199/T. vivax	+	+	–	–	–	–	–	–
RPMI 1640/T.b. brucei	+	+	+	+	+	+	+	+
RPMI 1640/T.b. gambiense	+	+	–	–	–	–	–	–
RPMI 1640/T. vivax	–	–	–	–	–	–	–	–
BCM/T.b. brucei	+	+	+	+	+	+	+	+
BCM/T.b. gambiense	–	–	–	–	–	–	–	–
BCM/T. vivax	–	–	–	–	–	–	–	–

KEY :

\+ = Patent

– = non-patent

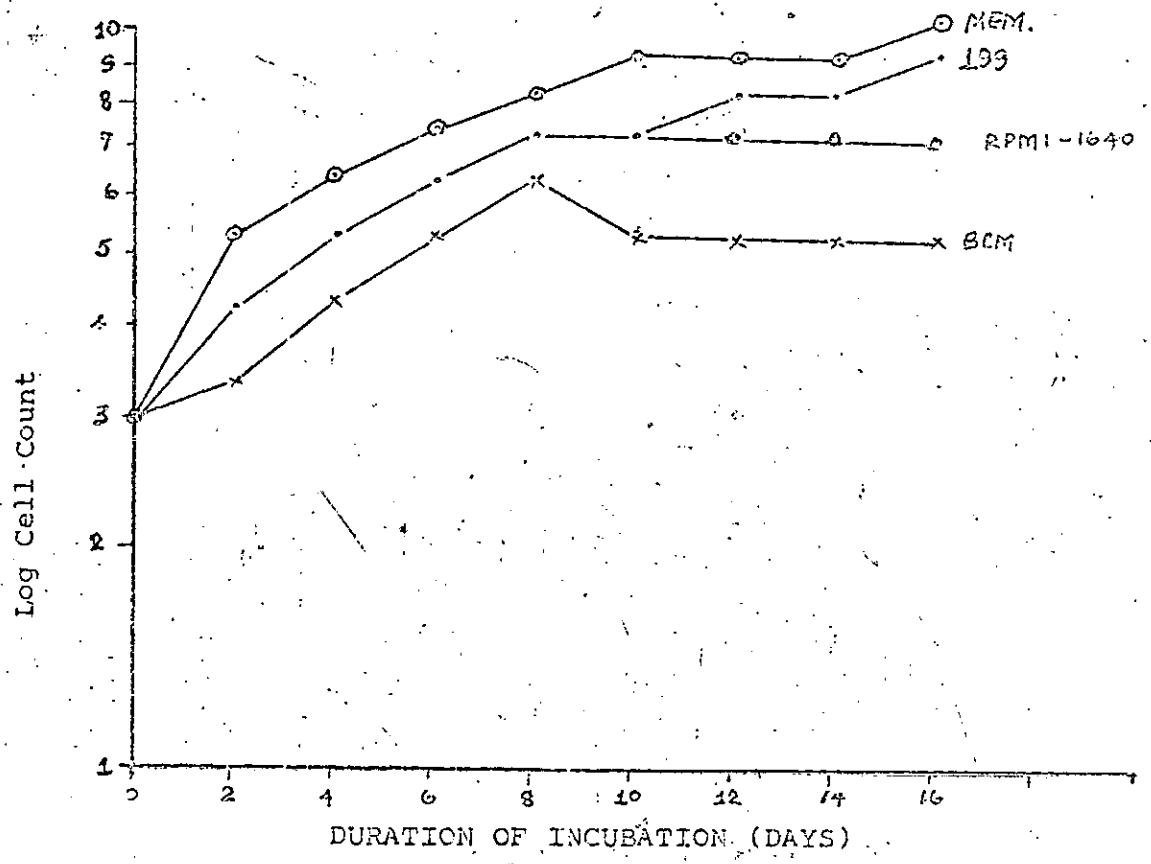


FIGURE 3.1: COMPARATIVE STUDIES ON THE GROWTH OF TRYPANOSOMA BRUCEI BRUCEI IN FOUR GROWTH MEDIA FOR THE IN VITRO CULTIVATION OF TRYPANOSOMES

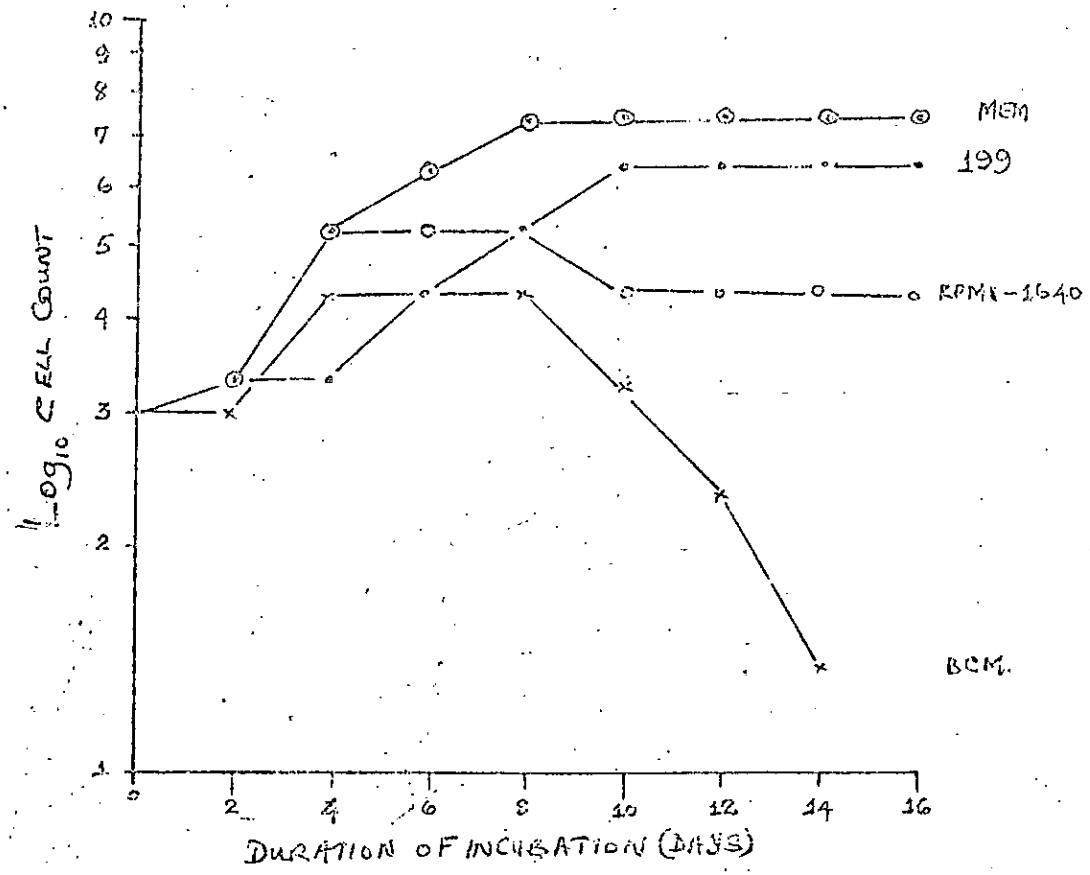


FIGURE 3.2:- COMPARATIVE STUDIES ON THE GROWTH OF TRYPANOSOMA BRUCEI GAMBIENSE IN THE FOUR GROWTH MEDIA FOR THE IN VITRO CULTIVATION OF TRYPANOSOMES

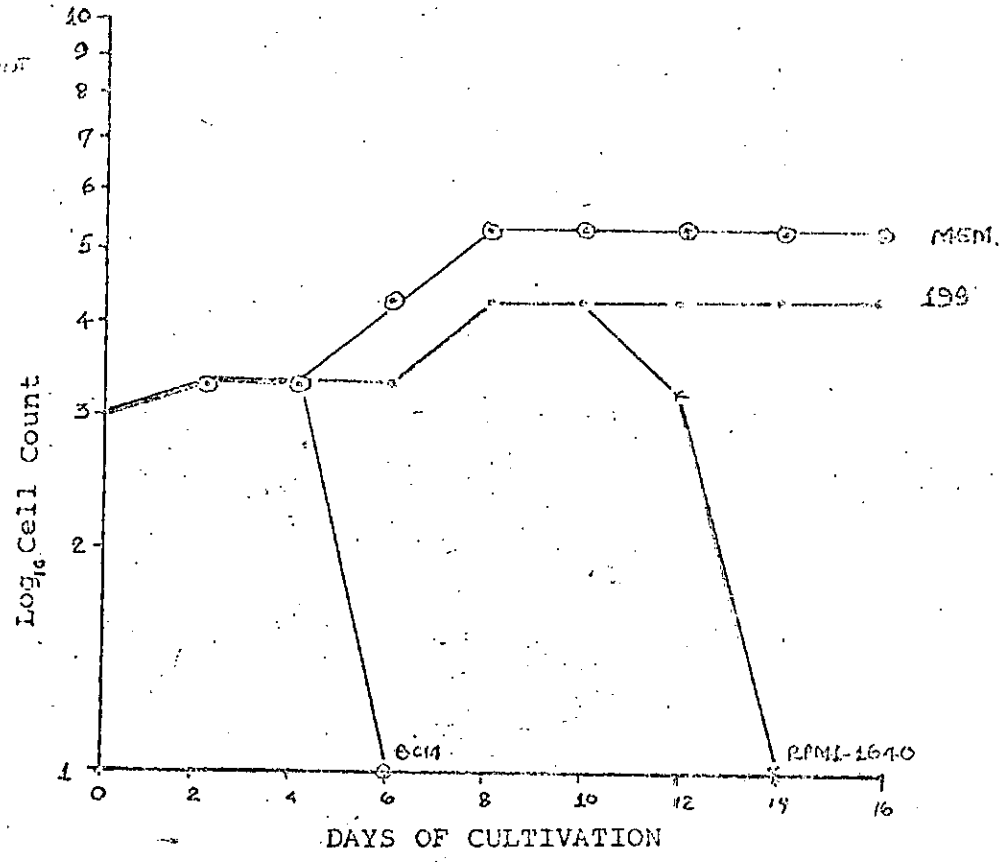


FIGURE 3.3: COMPARATIVE STUDIES ON THE GROWTH OF TRYPANOSOMA VIVAX IN THE FOUR GROWTH MEDIA FOR THE IN VITRO CULTIVATION OF TRYPANOSOMES

CHAPTER 4

A MODIFIED MEDIUM (ME-99) FOR THE IN VITRO CULTIVATION OF TRYPANOSOMES

ABSTRACT

A modified medium (ME-99) for the *in vitro* cultivation of trypanosomes has been developed. The medium comprised Eagle MEM as a base and various components of medium 199 not found in MEM. *T. b. brucei*, *T.b. gambiense* and *T. vivax* were grown in ME-99 and various other media combinations such as MEM/RPMI 1640, MEM/BCM, MEM/199, RPMI 1640/BCM, RPMI 1640/199, BCM/199. Growth and infectivity of these parasites were compared in these media and ME-99.

Parasites grew better and maintained their infectivity better in the ME-99.

CHAPTER 4

INTRODUCTION

Media used for the *in vitro* cultivation of trypanosomes requires either whole or haemolysed red blood cells on biphasic slants or in monophasic media. Although the blood based media have been widely used to support the cultivation of trypomastigotes for many of the studies on the biochemistry and immunology of trypanosomes, most of these media are noted to have serious deficiencies which limit their application to these areas (Hendricks *et al*, 1978). These include cultivation of the parasites with antigens derived from erythrocytes, inconsistent growth of different species and strains of trypanosomes, relatively low yields of organisms, and rate of parasite differentiation. It has been shown that the rate of differentiation of circulating blood *T.b. brucei* parasites from rapidly dividing slender forms to intermediate and stumpy forms (Black *et al*, 1982) influences the parasite population, growth rate, and the kinetics of host antibody responses (Sendashonga *et al*, 1982).

T. vivax causes disease in livestock and is, therefore, of considerable economic importance in Africa. Whereas, the *in vitro* cultivation of *T. brucei* and *T. congolense* have recently been achieved (Hirumi *et al*, 1977, Hill *et al*, 1978; Brun *et al*, 1981; Gray *et al*, 1981), attempts to cultivate animal infective *T. vivax* have been relatively unsuccessful (Trager, 1975; Isoun *et al*, 1974). Rats are usually refractory to infection with

T. vivax. Hence, minimal research has been carried out on it.

Even though so much work has been done on the in vitro cultivation of trypanosomes using various media, the problem still remains that the media so far developed, have not been particularly suitable for culture of the African strains of trypanosomes, and have also not been suitable for growth, survival, and infectivity of T. vivax.

The aim of this study is to develop a medium that is best suitable for the growth of African (Nigerian) trypanosomes with respect to duration of continuous culture, yield of large quantities of the parasites with retained infectivity for long periods of time.

MATERIALS AND METHODS

PARASITES

The parasites used in these experiments were the same as those used in Chapter 3 (page 25)

METHOD FOR ISOLATION OF MOUSE KIDNEY CELLS

The method of isolation of the mouse kidney cells had been described above (Chapter 3 , page 27)

INOCULATION OF CULTURES

Same as above (Chapter 3, page 28)

TRYPSINIZATION OF CELLS

Same as above (Chapter 3, page 29)

COUNTING OF CELLS, LIGHT MICROSCOPY AND MAKING OF SLIDES

Same as above (Chapter 3, page 29)

Preliminary studies were first carried out to test the underlisted media combinations in order to determine what will give the best result in terms of growth and infectivity.

(a) MEM/RPMI 1640

(b) MEM/199

(c) MEM/BCM

(d) RPMI 1640/199

(e) RPMI 1640/BCM

(f) BCM/199

The combination of MEM and 199 being potentially good was modified by the deletion and addition of components and termed "Modified ME-99". The deletion and addition are as follows :

PREPARATION OF THE MODIFIED ME-99

The modified ME-99 comprised Eagle MEM as base, and the various components of 199 not found in MEM. The method involved the preparation of :

(a) MEM

(b) the various components of 199

(c) mixing the two prepared media in a 1:1 ratio.

PREPARATION OF MEM (Base)

Five Stock Solutions were prepared :

Stock solution 1

Component	Amount mg/l
L-Arginin ..	105
L-Histidine ..	31

L-Isoleucine	..	52
L-Leucine	..	52
L-Lysine	..	58
L-Methionine	..	15
L-Phenylalanine	..	32
L-Threonine	..	48
L-Tryptophan	..	10
L-Tryosine	..	36
L-Valine	..	46

L-Tryosine was dissolved in 2 ml 0.1N hydrochloric acid, while the rest of the components were dissolved in distilled water.

Stock Solution 2

L-Cystine	..	24 mg/l

This component was made up in distilled water.

Stock Solution 3

L-Glutamine	..	292 mg/l

This component was dissolved in 20ml distilled water.

Stock Solution 4		mg/litre
Folic acid	..	1
Choline	..	1
Nicotinic acid	..	1
Panthothenic acid	..	1
Pyridoxal	..	1
Thiamine	..	1
Riboflavin	..	1

The above were dissolved in 10 ml bi-distilled water.

Stock Solution 5		mg/litre
Dextrose	..	100
Phenol red	..	20
Calcium Chloride	$CaCl_2 \cdot 2H_2O$	265
Pottasium chloride	Kcl	400
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	200
Sodium chloride	Nacl	6,800
Sodium phosphate	$NaH_2PO_4 \cdot H_2O$	140

The above compounds were dissolved in bi-distilled water.

One litre of Eagle MEM was then prepared by adding the stock solutions, 1,2 and 3 (20 ml each), 4 (10 ml) and 5 (100 ml), into a double neck 2 litre flask and made up to 1 litre to which 10,000 i.u and 10 mg per litre each of penicillin and streptomycin were respectively added to the medium. The pH was adjusted to 7.4 with Hepes. The resulting solution was filtered through millipore and stored at $4^{\circ}C$ for use, later.

PREPARATION OF COMPONENTS OF M199

Eight stock solutions were prepared thus :

(1)

Stock Solution 1		mg/litre
Hypotanthine	..	10
Thymine	..	10
Uracil	..	10
NH_4OH (IN)	..	0.175 ml

The above components were added to 100 ml bi-distilled water and the mixture was warmed (in a warm bath), filtered, and distributed into sterile 30 ml containers.

(2) Stock Solution 2

	mg/litre
Guanine ..	3.0
Guanosine ..	3.0

These two substances were dissolved in 28 ml bi-distilled water and 0.1 ml concentrated NH_4OH was added to aid dissolution. The mixture was heated to 100°C, cooled and brought up to 30 ml with bi-distilled water.

The mixture was prepared fresh before use.

(3) Stock Solution 3

	mg/litre
Biotin ..	1.0

It was dissolved in 7.5 ml bi-distilled water and then 0.1 ml dil Hcl was added, and the mixture was made up to 10 ml with bi-distilled water, filtered and distributed into 10 sterile test-tubes (1 ml/test-tube). The tubes containing the mixtures were stored at 4°C.

(4) Stock Solution 4

	mg/litre
Tocopherol ..	1.0

This component was dissolved in 10 ml bi-distlled water, filtered, and distributed into 1 ml portions in sterile test-tubes and stored at 4°C.

(5) Stock Solution 5

	mg/litre
Menadione ..	10

This vitamin was dissolved in 10 ml bi-distilled water, agitated and left to stand for 18 hours at 37°C.

It was later filtered and divided into 1 ml portions in sterile containers and stored at $4^{o}C$.

(6) Stock Solution 6

		mg/litre
Adenine	..	4.0
Adenosine	..	4.0

This purine and the sugar derivative were dissolved in 10ml bi-distilled water. The mixture was filtered and distributed into sterile containers (5 ml/container) and stored at $4^{o}C$.

(7) Stock Solution 7

		mg/litre
P-Aminobenzoic acid	..	5.0

This component was dissolved in 20 ml bi-distilled water, filtered and distributed into sterile test-tubes, in 2ml portions. The above mixture was stored at $4^{o}C$ and protected from light.

(8) Stock Solution 8

		mg/litre
Calciferol	..	1.0
Cholesterol	..	2.0

The calciferol was dissolved in 2 ml 95% alcohol. Cholesterol (2 mg) was added and the solution was thoroughly mixed. A sterile magnetic stirrer was used for the purpose of stirring thoroughly. 3 ml of 5% tween 80 was added and the final mixture was filtered and distributed into sterile test-tubes in portions of 5 ml/container. A mixture of 1 ml per litre each of the following was prepared:

(a) serine
(b) Aspartic acid
(c) Alanine
(d) Proline; and
(e) Glycine (amino acetic acid).

Stock Solution		Amount
Stock Solution 1	..	30 ml
NaCl	..	80 g
Stock Solution 2	..	30 ml
KCl	..	40 g
$MgSO4 . 7H_2O$	..	2 g
KH_2PO_4 60mg) dissolved in double		
$NaHPO_4$ 60mg) distilled water and made up to 100 ml		
White CaCl (anhydrous) 1.4 g dissolved in double		
distilled water and made up to		200 ml
Stock Solution 7	..	2 ml
Stock Solution 3	..	1 ml
Stock Solution 8	..	5 ml
Stock Solution 4	..	1 ml
Stock Solution 5	..	1 ml
Stock Solution 6	..	5 ml

The volume of the above mixture was brought up to 900 ml with bi-distilled water and the mixture of serine, aspartic acid, glanine, proline, and glycine previously weighed and set aside was added. The final mixture of components of Medium 199 was shaken well and stored at $4^{\circ}C$ for 18 hours, to ensure complete dissolution of the components. At the end of 18 hours period, the mixture was removed from $4^{\circ}C$ temperature and allowed to return to room temperature. Thereafter, it was made up to one litre, with double distilled water, filtered and distributed into sterile bottles (100 ml/bottle) and stored at $4^{\circ}C$. The following components were made up and added before use of medium 199 components.

L-Cysteine .. mg

Glutathione .. 5 mg

These two components were dissolved in 50 ml double distilled water. Vitamin A (10 mg) dissolved in 1 ml ethyl alcohol and tween 80 (5%) 10 ml, were added to the mixture of L-cycteine and glutathione. Finally, adenosine triphosphate 200 mg dissolved and made up to 100 ml with double distilled water, was added to the whole mixture. The mixture was then filtered and stored at 4°C. However, the mixture of L-cysteine, glutathione, vitamin A, tween 80 and adenosine triphosphate was made up fresh, just before use, by adding 100 ml of the stock solution to 800 ml bi-distilled water, 1 ml of the solution containing cysteine, glutathione, tween 80, vitamin A and adenosine triphosphate. The solution was brought up to 1 litre with bi-distilled water. The pH was adjusted with Hepes. The solution was not stored for more than 2 months.

PREPARATION OF ME-99

ME-99 was found to be best when MEM base and 199 component were added thus :

In the tissue culture flask, 2.5 ml of Eagle MEM (already made up as described earlier) was added. To the same flask, 2.5 ml of 199 components mixture was added. 20% foetal bovine serum or 10% Nu-Serum was used as supplement.

INFECTIVITY TESTS

Parasites from the following cultures, utilizing the various media combinations and the modified ME-99 were

inoculated into groups of animals consisting of 6 mice each.

(a) MEM + RPMI 1640 + MK cells + 20% FCS

(b) MEM + BCM + MK cells + 20% FCS

(c) MEM + 199 + MK cells + 20% FCS

(d) RPMI 1640 + BCM + MK cells + 20% FCS

(e) RPMI 1640 + 199 + MK cells + 20% FCS

(f) BCM + 199 + MK cells + 20% FCS

(g) ME-99 + MK cells + 20% FCS

(h) MK cells + 20% FCS (control)

RESULTS

The results are summarised in Tables and Figures 4.1, 4.2 and 4.3. A comparison of growth and infectivity of T.b. brucei, T.b. gambiense and T. vivax in the various media combinations of the four media, was carried out.

GROWTH OF T.B. BRUCEI IN THE VARIOUS MEDIA COMBINATIONS

T.b. brucei grew best and maintained its infectivity in all the various media combinations (Table 4.1) when compared with the other trypanosome species tested.

The pattern of growth of T.b. brucei in the various media combinations involved a gradual but steady increase of the parasites from Day 2 until Day 8 (Table and Figure 4.1). The growth of the parasites thereafter, remained constant until Day 16. The infectivity was not lost throughout the study (Table 4.5). The maximum parasite count recorded for T.b. brucei in MEM/RPMI 1640 media combinations was 2×10^7, MEM/BCM 2×10^6, for MEM/199 it was 2×10^9, while for RPMI 1640/BCM it was 2×10^4 and for

RPMI 1640/199 a maximum parasite count of 2×10^8 wa recorded. For BCM/199, the maximum parasite count recorded was 2×10^6.

GROWTH OF T.B. GAMBIENSE IN THE VARIOUS MEDIA COMBINATIONS

The pattern of growth of this specie of trypanosome in the media combinations involved an initial lag of 96 hours post-inoculation. The parasites did not begin to multiply until the 6th day (Table 4.2, Figure 4.2). After the 8th day, the parasite growth tapered off. There was no more growth until the 16th day. However, for media combination MEM/199 an increase in the parasite number was observed on day 2, and thereafter, there was no increase in the parasite number until day 6 and an increase in day 8. After the latter day, increase in parasite number tapered off. And for media combination RPMI 1640/BCM, a decrease in the parasite number from day 2 to day 8 when increase occurred in the parasite number. Thereafter, from day 10, the increase in parasite number became constant (Table and Figure 4.2). The infectivity of the parasite was lost after day 6 in all the media combinations except MEM/199 (Table 4.6). The maximum parasite count observed in MEM/RPMI 1640 was 2×10^5, for MEM/BCM 2×10^5, for MEM/199 2×10^6, for RPMI 1640/BCM 2×10^4, for RPMI 1640/199 2×10^5, for BCM/199 2×10^5.

GROWTH OF T. VIVAX IN THE VARIOUS MEDIA COMBINATIONS TESTED

T. vivax did not grow very well in the various media combinations when compared with the growth responses obtained from T.b. gambiense and T.b. brucei. The pattern

of growth of T. vivax involved a gradual increase from Day 2 until Day 4, when no further increase was observed (Table and Figure 4.3). The level tapered off until Day 16. The parasite number decreased in number after Day 6. However, for media combination MEM/BCM, RPMI 1640/BCM and BCM/199 the parasites in the media started dying from Day 9 and by Day 12, no living parasites could be seen in the media.

The maximum parasite count recorded for T. vivax in MEM/RPMI 1640 media combination was 2×10^4, for MEM/BCM 2×10^4, for MEM/199 2×10^5, for RPMI 1640/BCM 2×10^4 for RPMI 1640/199 2×10^4, for BCM/199 2×10^4.

T. vivax in MEM/RPMI 1640 maintained its infectivity in culture up till Day 12 and lost it thereafter (Table 4.7). Parasites cultured in MEM/BCM lost their infectivity after Day 10. In MEM/199 the infectivity was maintained up to Day 16. In RPMI 1640/BCM media combination, infectivity was lost after 6 days of culturing while in RPMI 1640/199 infectivity of the parasite was lost after Day 8 (Table 4.7). In BCM/199 the infectivity of the parasites was lost after Day 8.

Microscopic examination of the 3 species of trypanosome cultured in medium ME-99 showed that the morphology of the cultured parasites (Plates 4.1, 4.2 and 4.3) is identical to those maintained in vivo in laboratory animals (Plates 4.4, 4.5 and 4.6).

GROWTH OF T.B. BRUCEI IN ME-99

The results are summarised in Table 4.4. Growth of T.b. brucei in ME-99 was found to be very good. There was

a 2-fold increase every 2 days and the parasites kept multiplying until the 8th day when the parasite number levelled. The parasites also remained infective throughout the study. The maximum parasite count recorded was 2×10^{10}.

GROWTH OF T.B. GAMBIENSE IN ME-99

This parasite like the T.b. brucei, gave the best result in ME-99 (Table and Figure 4.4). There was a 2-fold increase every 2 days until Day 6 when the parasite number became constant and the maximum parasite count recorded was 2×10^{9}. The parasites also remained infective throughout the study.

GROWTH OF T. VIVAX IN ME-99

Growth of T. vivax in ME-99 was found not as good as T.b. brucei and T.b. gambiense, but better in ME-99 than in the other media combinations (Tables 4.1, 4.2, 4.3 and 4.4). A one-fold increase was observed every 2 days until Day 8 wnen the parasite number levelled. The parasite remained infective throughout the study. The maximum parasite count recorded was 2×10^{6}.

DISCUSSION

The results of the preliminary experiments showed that MEM, in combination with any other medium investigated in this study is ideal for the in vitro cultivation of the parasite. In like nammer, Brun et al, (1981), Duszenko et al (1985), Ross et al (1985) and Gray et al (1985) reported on the suitability of MEM for the in vitro cultivation of trypanosomes. These observations form the basis for the selection of MEM as a base medium for

modification in the present study, in the hope that an improved and a more reliable medium will emerge than those previously used.

The superiority of ME-99 was manifested when the growth rate of parasites in the medium was compared to that of other media combinations. Whereas a maximum parasite count of not more than 2×10^4 with T. vivax was recorded in all the other media combinations (Table 4.3), a 2×10^6 maximum parasite count was obtained in ME-99 with T. vivax (Table 4.4) which has been shown to be very difficult to culture (Isoun, 1979). Although infectivity of T.b. gambiense and T. vivax was lost after sometime (Tables 4.6 and 4.7) in culture in the other media, but in ME-99 the infectivity was retained until Day 16 (Table 4.8) when the experiment was terminated. Brun and Shonenberger (1979) had reported good growth with SDM-99 medium; they attributed this improvement to a number of additives (Brun and Shonenberger, 1979) contained in the medium. In the present study, similar additives with the inclusion of cysteine, were incorporated in the medium ME-99 being studied. It is possible that the presence of these additives accounted for the good result obtained. More recently Seebeck and Kurath (1985) had shown that the additives were necessary and essential for trypanosome growth. The possibility that some of the additives such as the amino-acids, chemically react with the parasite metabolic waste and as a result of this, some constituents. was reduced in the modified ME-99. Duszenko et al (1985) showed that the involvement of cysteine may

possibly stimulate the progressive growth and provide retention of the infectivity of the parasite for longer periods as observed in this study.

It is also possible that undefined medium - supplements such as serum and tissue extracts almost certainly contain inhibitory as well as stimulatory substances, which may cause problems in in vitro studies. This is subject for investigation in the next chapter.

TABLE 4.1

GROWTH OF TRYPANOSOMA BRUCEI BRUCEI IN VARIOUS COMBINATIONS OF GROWTH MEDIA EVALUATED

Media Combination (50/50 v/v)		Number of Parasites Harvested/ml culture fluid								
Medium 1	Medium 2	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 15	Day 16
MEM	RPMI 1640	2×10^4	2×10^5	2×10^6	2×10^7	2×10^7	2×10^7	2×10^7	2×10^7	2×10^7
MEM	BCM	2×10^3	2×10^4	2×10^5	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6
MEM	199	2×10^5	2×10^6	2×10^7	2×10^9	2×10^9	2×10^9	2×10^9	2×10^9	2×10^9
RPMI 1640	BCM	2×10^3	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4
RPMI 1640	199	2×10^4	2×10^5	2×10^7	2×10^8	2×10^8	2×10^8	2×10^8	2×10^8	2×10^8
BCM	199	2×10^4	2×10^5	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6

TABLE 4.2

GROWTH OF TRYPANOSOMA BRUCEI, GAMBIENSE IN VARIOUS COMBINATIONS OF GROWTH MEDIA EVALUATED

Media Combination (50/50 v/v)		Number of Parasites Harvested/ml culture fluid							
Medium 1	Medium 2	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
MEM	RPMI 1640	$2x10^3$	$2x10^3$	$2x10^4$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
MEM	BCM	$2x10^3$	$2x10^3$	$2x10^4$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
MEM	199	$2x10^4$	$2x10^4$	$2x10^5$	$2c10^6$	$2x10^6$	$2x10^6$	$2x10^6$	$2x10^6$
RPMI 1640	BCM	$2x10^2$	$2x10^2$	$2x10^3$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$
RPMI 1640	199	$2x10^3$	$2x10^3$	$2x10^4$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
BCM	199	$2x10^3$	$2x10^3$	$2x10^4$	$2x10^6$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$

TABLE 4.3

GROWTH OF TRYPANOSOMA VIVAX IN VARIOUS COMBINATIONS OF GROWTH MEDIA EVALUATED

Media Combination (50/50 v/v)		Number of Parasites Harvested/ml culture fluid							
Medium 1	Medium 2	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
MEM	RPMI 1640	$2x10^3$	$2x10^4$	$2x10^4$	$2x10^3$	$2x10^3$	$2x10^3$	$2x10^3$	$2x10^3$
MEM	BCM	$2x10^3$	$2x10^4$	$2x10^4$	$2x10^3$	$2x10^2$	0	0	0
MEM	199	$2x10^4$	$2x10^5$	$2x10^5$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$	$2x10^4$
RPMI 1640	BCM	$2x10^3$	$2x10^4$	$2x10^4$	$2x10^2$	0	0	0	0
RPMI 1640	199	$2x10^3$	$2x10^4$	$2x10^4$	$2x10^3$	$2x10^3$	$2x10^3$	$2x10^3$	$2x10^3$
BCM	199	$2x10^3$	$2x10^4$	$2x10^3$	$2x10^3$	$2x10^2$	0	0	0

TABLE 4.4

GROWTH OF T.B. BRUCEI, T.B. GAMBIENSE AND T. VIVAX IN ME-99

Specie of Parasite	Number of Parasite Harvested							
	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
T.b. brucei	$2x10^5$	$2x10^7$	$2x10^9$	$2x10^{10}$	$2x10^{10}$	$2x10^{10}$	$2x10^{10}$	$2x10^{10}$
T.b. gambiense	$2x10^5$	$2x10^7$	$2x10^9$	$2x10^9$	$2x10^9$	$2x10^9$	$2x10^9$	$2x10^9$
T. vivax	$2x10^3$	$2x10^4$	$2x10^5$	$2x10^6$	$2x10^6$	$2x10^6$	$2x10^6$	$2x10^6$

TABLE 4.5

INFECTIVITY OF TRYPANOSOMA BRUCEI BRUCEI IN VARIOUS COMBINATIONS OF GROWTH MEDIA EVALUATED

Media Combination (50/50 v/v)		Day 2	4	6	8	10	12	14	15	16
Medium 1	Medium 2									
MEM	RPMI 1640	+	+	+	+	+	+	+	+	+
MEM	BCM	+	+	+	+	+	+	+	+	+
MEM	199	+	+	+	+	+	+	+	+	+
RPMI 1640	BCM	+	+	+	+	+	+	+	+	+
RPMI 1640	199	+	+	+	+	+	+	+	+	+
BCM	199	+	+	+	+	+	+	+	+	+

KEY : + = PATENT

TABLE 4.6

INFECTIVITY OF TRYPANOSOMA BRUCEI GAMBIENSE IN VARIOUS COMBINATIONS OF GROWTH MEDIA EVALUATED

Media Combination (50/50 v/v)		Days 2	4	6	8	10	12	14	16
Medium 1	Medium 2								
MEM	RPMI 1640	+	+	+	-	-	-	-	-
MEM	BCM	+	+	+	-	-	-	-	-
MEM	199	+	+	+	+	+	+	+	+
RPMI 1640	BCM	+	+	+	-	+/-	+/-	-	-
RPMI 1640	199	+	+	+	-	-	-	-	-
BCM	199	+	+	+	-	-	-	-	-

KEY : + = PATENT

\- = NON-PATENT

TABLE 4.7

INFECTIVITY OF TRYPANOSOMA VIVAX IN VARIOUS COMBINATIONS OF GROWTH MEDIA EVALUATED

Media Combination (50/50 v/v)		Days 2	4	6	8	10	12	14	16
Medium 1	Medium 2								
MEM	RPMI 1640	+	+	+	+	+	+	−	−
MEM	BCM	+	+	+	+	+	−	−	−
MEM	199	+	+	+	+	+	+	+	+
RPMI 1640	BCM	+	+	+	−	−	−	−	−
RPMI 1640	199	+	+	+	+	−	+	−	−
BCM	199	+	+	+	+	−	−	−	−

KEY: + = PATENT

− = NON-PATENT

TABLE 4.8

INFECTIVITY OF T.B. BRUCEI, T.B. GAMBIENSE AND T. VIVAX IN ME-99

Specie of Parasite	Number of Parasite Harvested							
	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
T.b. brucei	+	+	+	+	+	+	+	+
T.b. gambiense	+	+	+	+	+	+	+	+
T. vivax	+	+	+	+	+	+	+	+

KEY :

+ = Patent

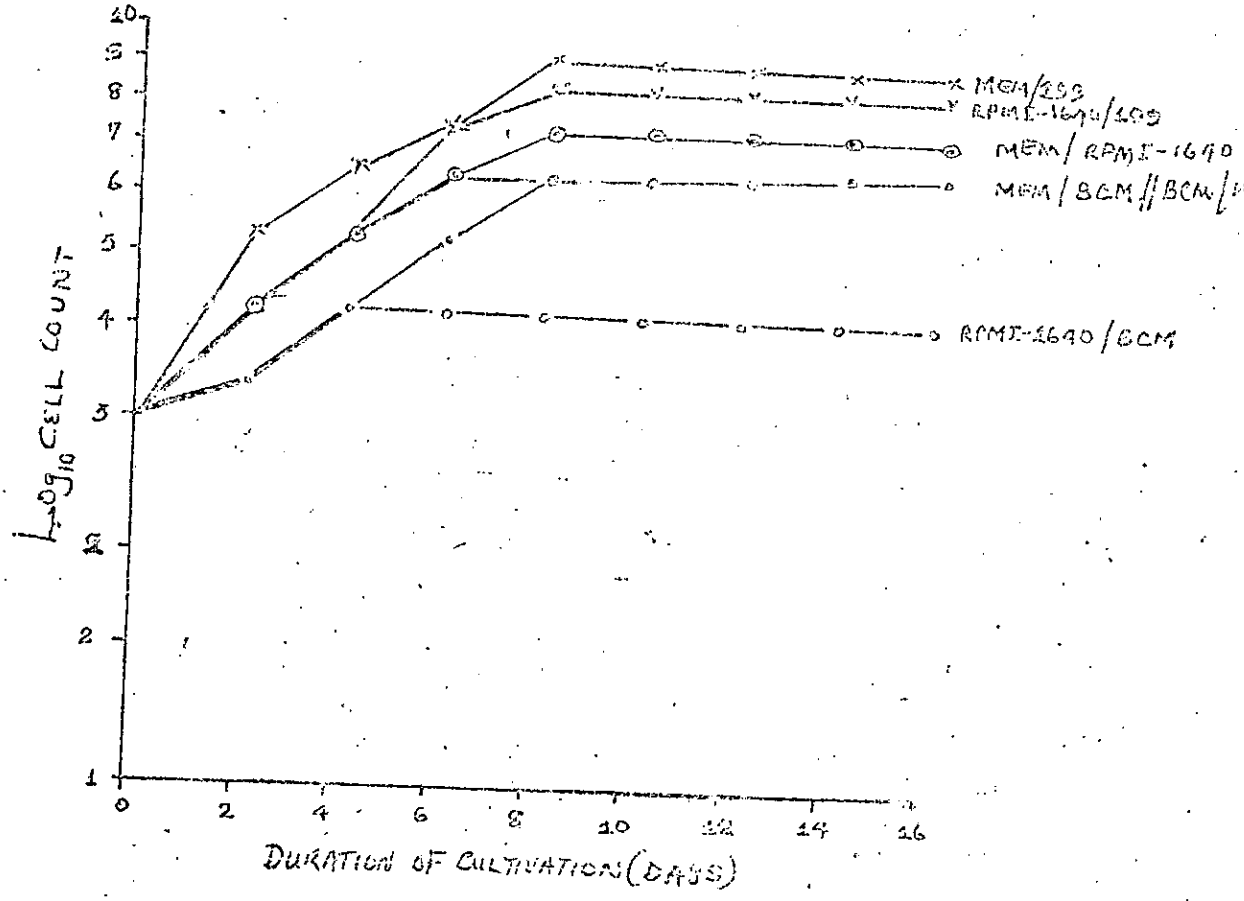


FIGURE 4.1: GROWTH OF TRYPANOSOMA BRUCEI BRUCEI IN VARIOUS COMBINATIONS OF GROWTH MEDIA

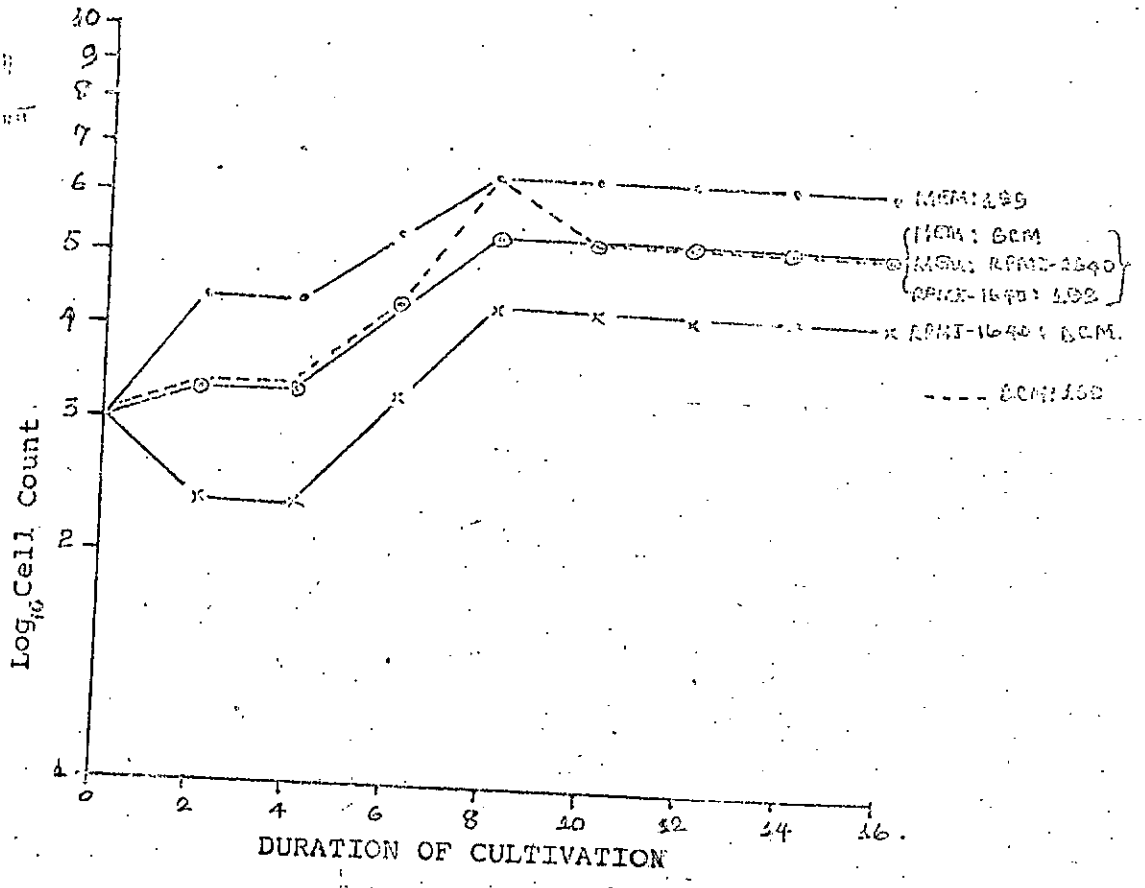


FIGURE 4.2: GROWTH OF TRYPANOSOMA BRUCEI GAMBIENSE IN VARIOUS COMBINATIONS OF GROWTH MEDIA

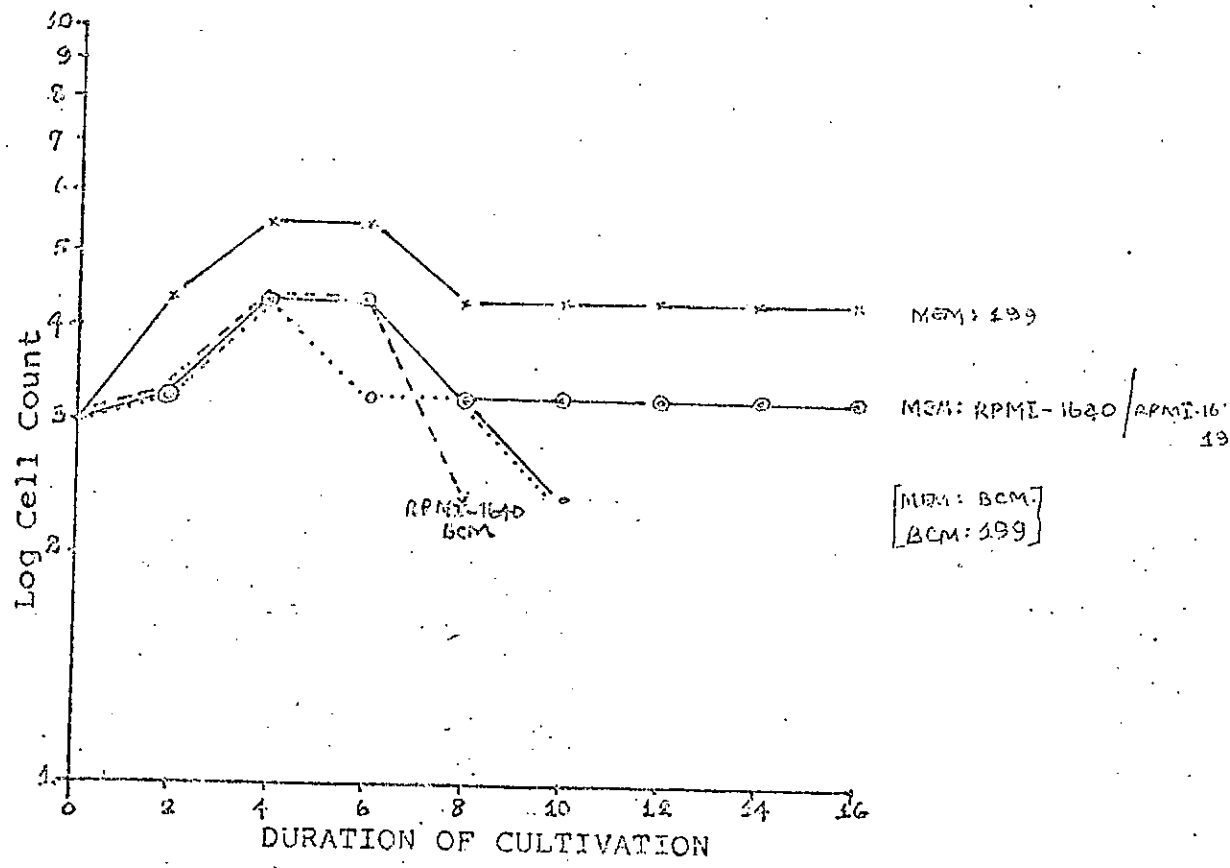


FIGURE 4.3: GROWTH OF TRYPANOSOMA VIVAX IN VARIOUS COMBINATIONS OF GROWTH MEDIA.

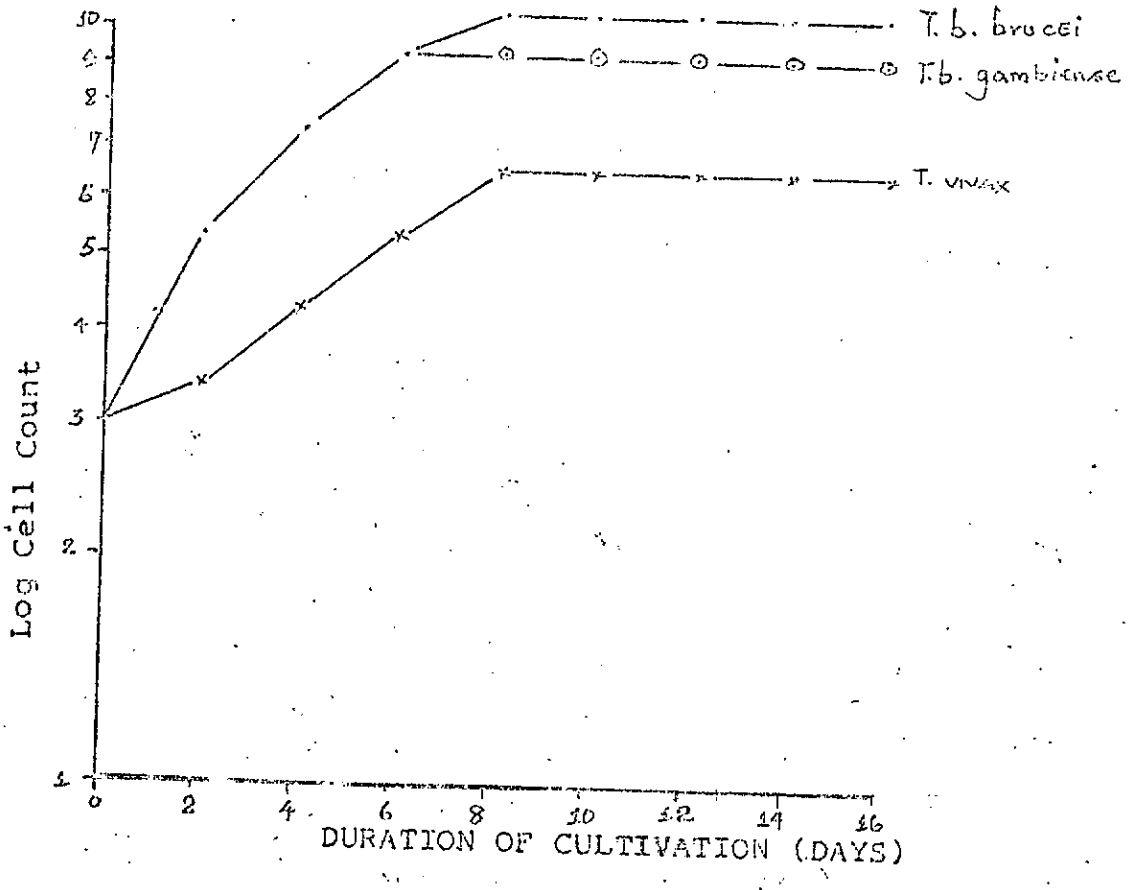


FIGURE 4.4: GROWTH OF T.b. brucei, T. b. gambiense and T. vivax in ME 99

PLATE 4.1 T.B. BRUCEI IN ME-99 CULTURES

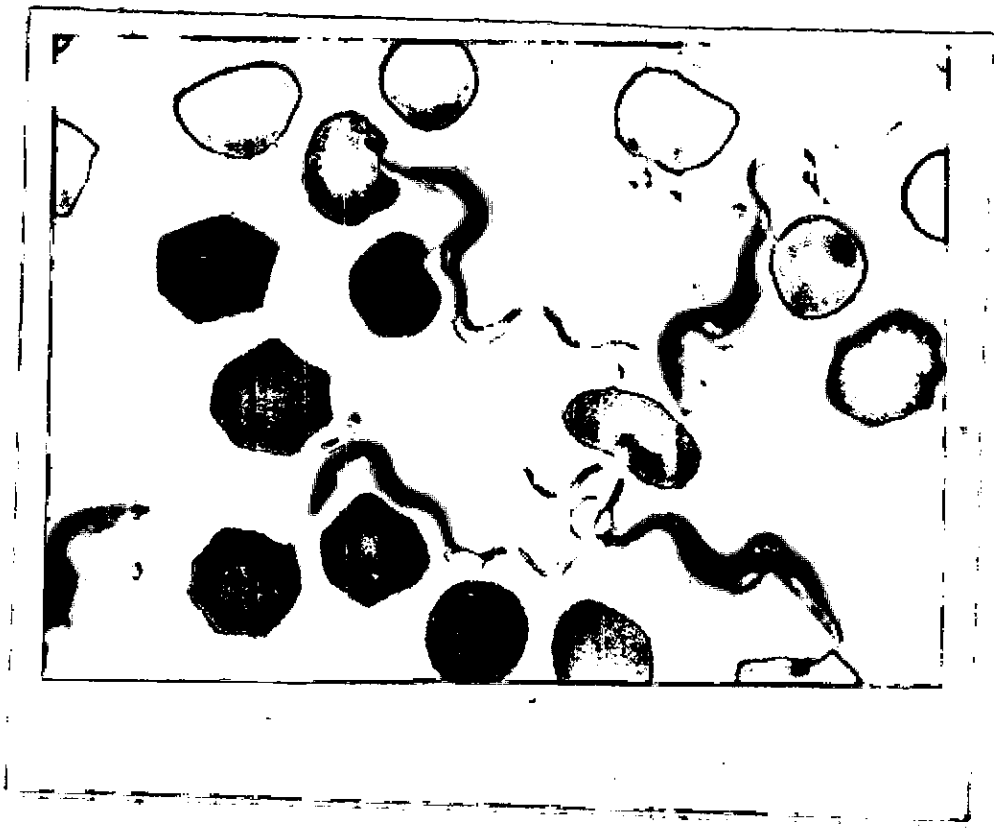


PLATE 4.2 :

T.B. GAMBIENSE IN ME-99 CULTURES

I : NOTE THE PRESENCE OF 2 NUCLEI AND 2 KINETOPLASTS WHICH IS AN INDICATION OF DIVISION

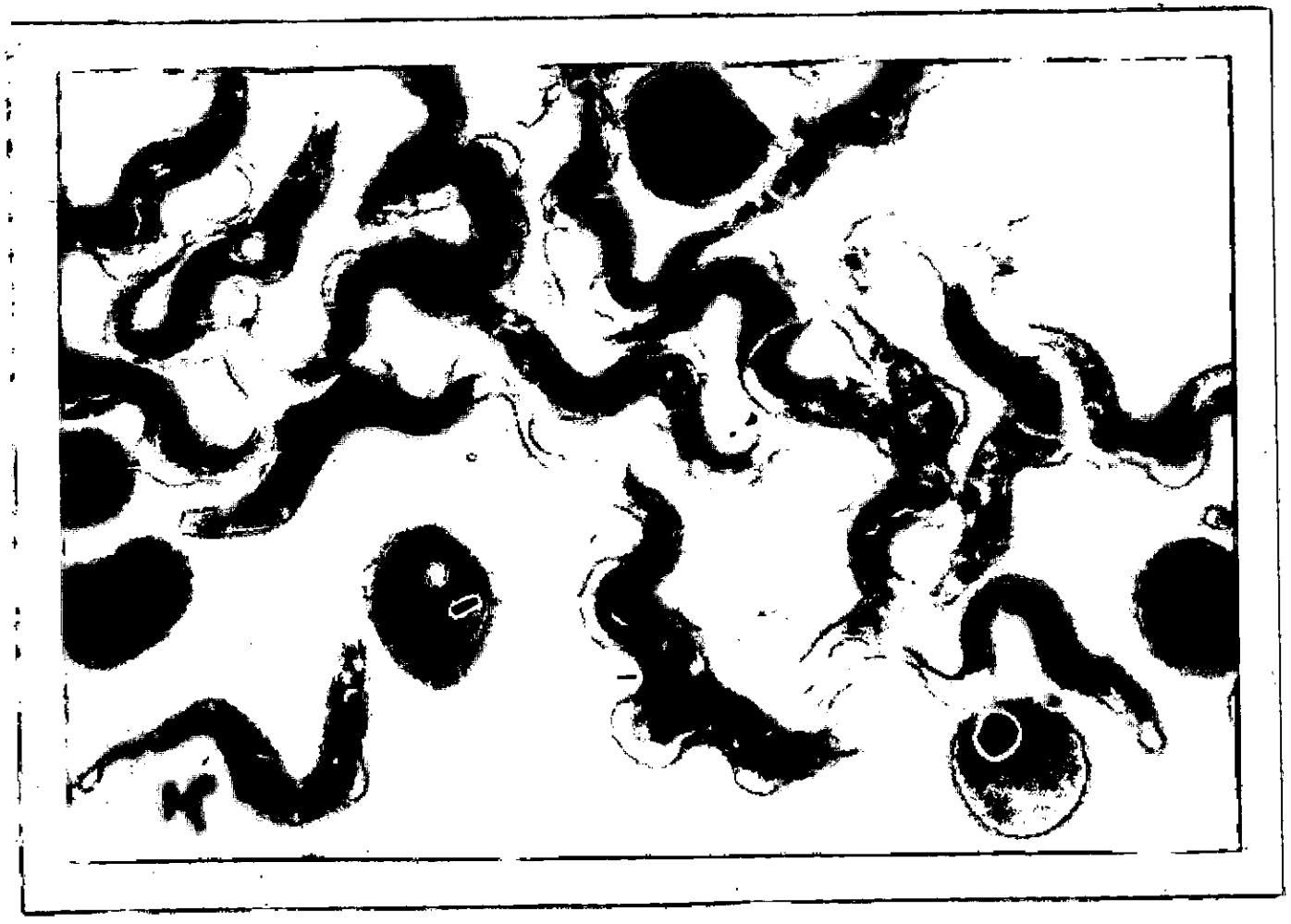
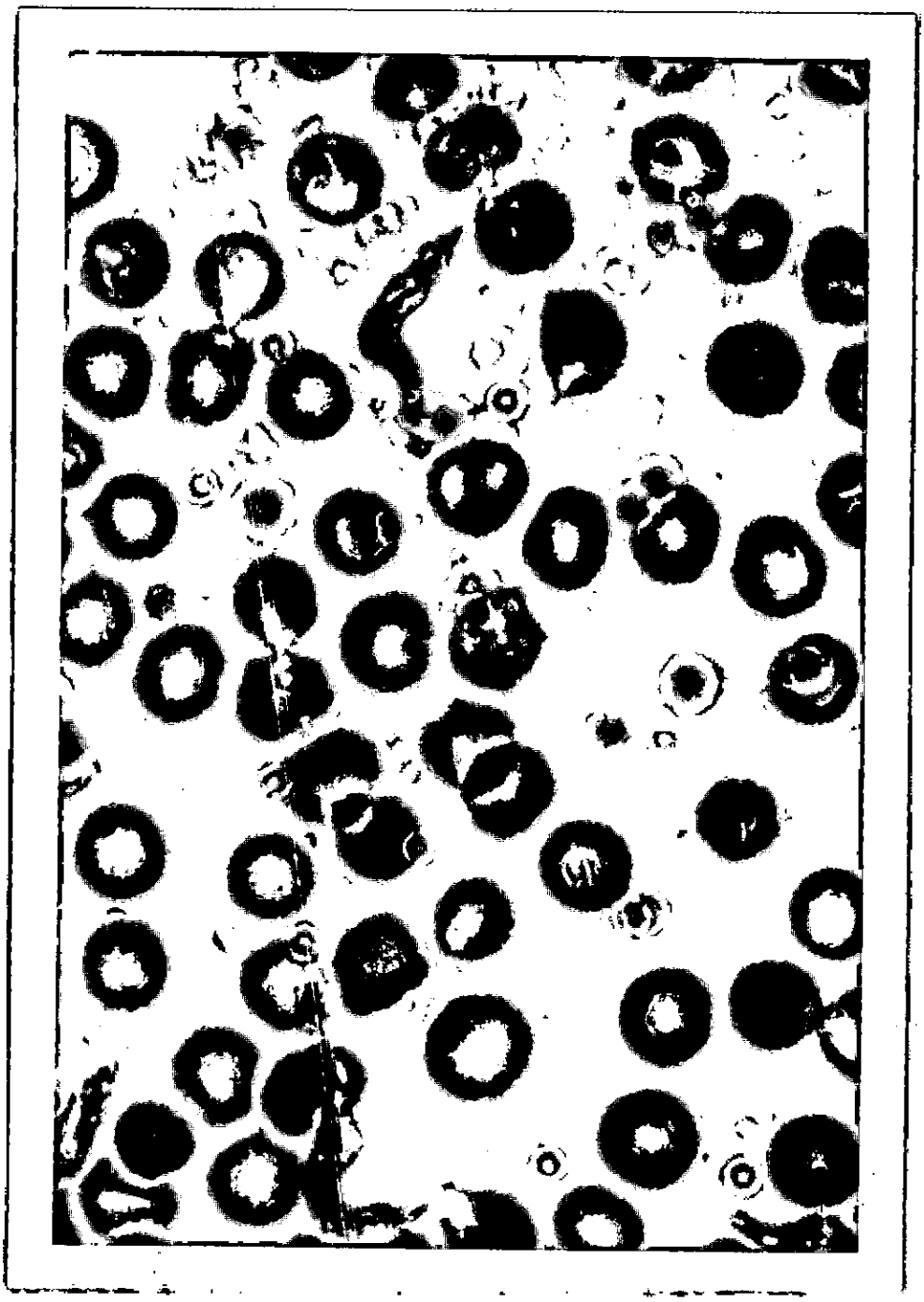


PLATE 4.3 : T. VIVAX IN ME-99 CULTURES

PLATE 4.4 T.B. BRUCEI IN MOUSE BLOOD

PLATE 4.5 : T.B. GAMBIENSE IN MOUSE BLOOD

PLATE 4.6 : T. VIVAX IN MOUSE BLOOD



4.6

CHAPTER 5

NU-SERUM: AN ADEQUATE REPLACEMENT FOR ANIMAL SERUM IN TRYPANOSOMA BRUCEI BRUCEI CULTURES

NU-SERUM: AN ADEQUATE REPLACEMENT FOR ANIMAL SERUM IN T.B. BRUCEI CULTURES

ABSTRACT

Animal sera for cultivation of trypanosomes differ from batch to batch, and often times makes results non-reproducible and inconsistent. T.b. brucei was grown over Baby Hamster Kidney (BHK) cells in MEM and supplemented with various animal sera (horse, mouse, and foetal calf serum) as well as Nu-Serum (an artificial medium supplement). The effects of the various sera and the Nu-Serum on the culture system were compared, using growth, survival and infectivity as criteria for evaluation. The parasites grew well and for a long period of time in the medium utilizing the Nu-Serum and were still infective to mammalian hosts and could be subcultured as compared with parasites cultured in the presence of animal serum. Nu-Serum was, therefore, found to be a suitable replacement for the traditional animal sera used in supplementing cultures. This artificial medium supplement is simple, low in protein content and cheaper than the commercial animal sera. It provides sufficient parasites for experimental studies and also makes results easily reproducible and consistent.

INTRODUCTION

Animal serum is an essential part of many cell culture procedures. However, the use of animal sera causes inconsistencies in laboratory cell culture systems due to contradictions in results (Cross and Manning, 1973; Brun and Jenni, 1985), an alternative to animal serum is thus much needed since in most parasites cultured in these media utilizing animal serum, the infectivity of the parasites was lost after a short period in culture, and subculturing of the parasite was impossible (Cross and Manning, 1973).

In the last decade, semi-defined and chemically defined media have been developed which gave inconsistent yield and maintenance of the parasites (Brun and Shonenberger, 1979; Cross and Manning, 1973) and cannot support the growth or the maintenance of these parasites without the addition of serum (Berens et al, 1976; Cross and Manning, 1973).

It must be stressed that a universal medium, able to stimulate the growth and support the maintenance of any type of cell, without utilizing animal serum, is not yet available.

Up to now, very few media, chemically defined, are able to support cell growth by themselves, without the addition of some factors (Baker and Ebeling, 1939; Brun and Jenni, 1985). Medium 1066, medium NCTC 109, Waymouth's medium 752/1 and Hert-I medium have been successfully used

by many workers without serum in the cultivation and maintenance of L-cells (Baker and Ebeling, 1939; Evans et al, 1956; Evans et al, 1959). A better success has been obtained by simply maintaining some types of cells such as chick embryos, by employing White and TC 199 media, containing various co-factors and intermediate metabolites of some nutritional importance (Morgan et al, 1955; Morton et al, 1950; Pasieka et al, 1956).

The results obtained with these types of media were limited to a discreet survival of the cells, but no increase in their number was achieved.

The present investigation was, therefore, undertaken in an attempt to find a replacement for animal serum in the medium that will support growth, survival and maintenance of the cells as well as support growth, maintenance and infectivity of T.b. brucei in culture and try to eliminate the inconsistencies there are in results encountered with the various batches of animals sera.

MATERIALS AND METHODS

PARASITES

East African Trypanosomiasis Research Organisation (EATRO) Shinyanga III strain of monomorphic T.b. brucei was obtained in the cryopreserved state, in liquid oxygen, from the Department of Zoology, University of Guelph, Canada. The strain was isolated in Uganda in 1946 from a wart hog. The parasites were maintained by serial passage in female Charles River Incorporated (CDI) mice.

ISOLATION OF PARASITES FROM MOUSE BLOOD

The same method as described in Chapter 3 (page 28).

MEDIA AND SERA

Eagle-MEM supplemented by mouse, horse or FCS was obtained from Grand Island Biological Company (GIBCO) or Nu-Serum (Lot No. 85-1211; from Collaborative Research Incorporated, 128 Spring Street, Lexington, Massachussetts, U.S.A.). The constituents of Nu-Serum are given in Appendix I.

CELLS

BHK cell line was obtained from the Department of Microbiology, University of Toronto, Ontario, Canada. The cells were maintained in plastic nunc 25 cm^2 flask with 5 ml of medium. The pH of the medium was adjusted to 7.4 and the following culture combinations were used for the trypanosomes :-

MEM/20% FCS/BHK/CELLS/TRYPANOSOMES

MEM/10% NU-SERUM/BHK CELLS/TRYPANOSOMES

MEM/20% HORSE SERUM/BHK CELLS/TRYPANOSOMES

MEM/20% MOUSE SERUM/BHK CELLS/TRYPANOSOMES

MEM/BHK CELLS/TRYPANOSOMES (CONTROL)

INOCULATION AND MAINTENANCE OF CULTURES

Each culture flask was inoculated with 10^3 trypanosomes, washed with phosphate buffered saline (pbs). Twenty-four-hour-old sub-cultured BHK feeder cells were used for the above culture combinations. All cultures were maintained at 37°C (95% relative humidity with 5% Co_2 and 95% air).

Half of the medium (2.5 ml) in each flask was replaced with fresh medium every 48 hours. Five-day-old trypanosome cultures were inoculated intraperitoneally (i.p) into female CDI mice. Two flasks of each culture combination were prepared and the whole experiment was repeated twice.

PREPARATION OF SLIDES

Blood smears were made from mice inoculated with trypanosomes from various cultures three days post-infection. They were stained with GIEMSA according to the method described in Chapter 3. Photomicrographs were taken with a Zeiss Photomicroscope.

RESULTS

It was found that only the cultures supplemented with Nu-Serum gave consistent results in terms of parasite yield (Tables 5.1, 5.2, Figures 5.1, 5.2) and infectivity (Tables 5.3, 5.4). With cultures supplemented with FCS, horse serum and mouse serum, there were fluctuations in the parasite counts. The pattern involved an initial lag of 24 hours followed by a short period of exponential growth, during which time the number of trypanosomes increased about 2-fold (Tables 5.1 and 5.2). Subsequently, the parasite growth varied between 10^3 and 10^4, although for the first batch of mouse serum (Table 5.2), the parasite number fell below 10^3 after the 4th day.

The growth of parasites in the cultures supplemented with Nu-Serum was found to be the best and the most consistent. There was a marked increase in the number of parasites in the medium from day 1 (24 hours post-inoculation) to day 6 (Tables 5.1 and 5.2) A maximum growth of 2×10^{10} was recorded on day 6 and thereafter, the parasite growth remained constant at 2×10^{10}, until the termination of the experiment. With each new batch of Nu-Serum, an exactly similar pattern of growth yield was observed. Eight such serial transfers were carried out, convering a period of 32 weeks.

Parasites from cultures supplemented with the various sera, were infective to mice. However, parasites from cultures supplemented with mouse serum, lost their infectivity as from the 6th day, when the first batch of sera was used (Table 5.3).

When the second batch of sera was used, the infectivity of the parasites in mouse was lost from the 3rd day (Table 5.4).

DISCUSSION

A new medium supplement (Nu-Serum) in place of animal serum has been found to be suitable for the growth of *T.b. brucei*. MEM was chosen as a base medium because it has been shown to be superior to others like RPMI 1640, and the latter alone had been demonstrated for denying maintenance of blood stream forms (Tanner, 1980). MEM has been successfully used in many experiments involving cultivation

of trypanosmes of several species (Ross et al, 1985; Gray et al, 1985; Brun et al, 1981).

The problems of serum and other undefined supplements have plagued in vitro cell cultivation for years (Ham, 1981). It is known that the composition of serum varies from batch to batch (Honn et al, 1975). It is possible that other factors such as, the diet and health of the animals from which the serum was prepared, along with the methods used to obtain, process, ship (if necessary) and store the serum contribute to the variability of results obtained when different batches of sera are used as culture supplements.

In this study, variable patterns of growth in cultures utilizing animal sera was obtained. For instance, in cultures with 20% horse serum, there were lags on days 2 and 3 which occurred after an initial increase on day 1 (24 hours post-inoculation) with the 1st batch of serum. With the 2nd batch of serum the pattern was different (Table 5.2). Similarly, the cultures utilizing mouse serum had a drop in the parasite count below the inoculum level on days 5 and 6, with the first batch of serum. Concerning the seconf batch of serum, the pattern was different (Table 5.3). With FCS, variable patterns with the two batches of sera were recorded. The variable results (Tables 5.1 and 5.2) obtained while testing different types and losts of sera have been encountered in many other parasite cultures systems that have serum as an essential component (Honn et al, 1975).

Nu-Serum (which comes from the manufacturers in 10% strength) has proved from the present results, to be an adequate replacement for any of the animal sera tested. Apart from being cheaper, it contains less proteins and is chemically semi-defined. Nu-Serum was found to give consistent and superior growth results with T.b. brucei, as opposed to the animal sera tested.

The new culture medium so far described may not be sufficient for studies involving parasite metabolism and establishment of nutritional requirements but it is still of enormous advantage for other studies, (like biochemical studies of trypanosomes) since it is semi-defined and contains only 25% serum. It seems possible that this medium can be further developed to make it 100% serum-free and future investigations will include this.

TABLE 5.1

GROWTH OF TRYPANOSOMA BRUCEI BRUCEI IN THE VARIOUS CULTURE SYSTEMS PREPARED WITH THE FIRST BATCH OF SERA

CULTURE SYSTEM	NUMBER OF PARASITES HARVESTED IN DAYS					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
MEM/20% FCS + BHK CELLS/ PARASITES	2×10^3	2×10^6	2×10^4	2×10^4	2×10^4	2×10^4
MEM/10% Nu-SERUM/BHK CELLS/ PARASITES	2×10^5	2×10^6	2×10^7	2×10^8	2×10^9	2×10^{10}
MEM/20% HORSE SERUM/BHK CELLS/ PARASITES	2×10^3	2×10^6	2×10^6	2×10^4	2×10^3	2×10^3
MEM/20% MOUSE SERUM/BHK CELLS/ PARASITES	2×10^3	2×10^5	2×10^6	2×10^3	2×10^2	2×10^2
MEM/BHK CELLS/PARASITES	2×10^4	2×10^1	0	0	0	0

Starting Inoculum 10^3

TABLE 5.2

GROWTH OF TRYPANOSOMA BRUCEI BRUCEI IN THE VARIOUS CULTURE SYSTEMS PREPARED WITH THE SECOND BATCH OF SERA

CULTURE SYSTEM	NUMBER OF PARASITES HARVESTED IN DAYS					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
MEM/20% FCS/BHK CELLS/PARASITES	2×10^3	2×10^7	2×10^4	2×10^3	2×10^3	2×10^3
MEM/10% Nu-SERUM/BHK/CELLS/ PARASITES	2×10^5	2×10^6	2×10^7	2×10^8	2×10^9	2×10^{10}
MEM/20% HORSE SERUM/BHK CELLS/ PARASITES	2×10^3	2×10^5	2×10^4	2×10^3	2×10^3	2×10^4
MEM/20% MOUSE SERUM/BHK CELLS/ PARASITES	2×10^2	2×10^6	2×10^3	2×10^3	2×10^4	2×10^3
MEM/BHK CELLS/PARASITES	2×10^4	2×10^1	0	0	0	0

TABLE 5.3

INFECTIVITY OF TRYPANOSOMA BRUCEI BRUCEI

IN VARIOUS CULTURE SYSTEMS

Culture System	Duration of parasites in culture (Days)	Infectivity for mice
MEM/20% FCS/BHK CELLS	1	+
	2	+
	3	+
	4	+
	5	+
	6	+
MEM/10% NU-SERUM/BHK CELLS	1	+
	2	+
	3	+
	4	+
	5	+
	6	+
MEM/20% HORSE SERUM/BHK/ CELLS	1	+
	2	+
	3	+
	4	+
	5	+
	6	+
MEM/20% MOUSE SERUM/BHK/ CELLS	1	+
	2	+
	3	+
	4	+
	5	+
	6	-

FIRST BATCH OF SERA

KEY :

+ = Patent

- = Non-patent

TABLE 5.4

INFECTIVITY OF TRYPANOSOMA BRUCEI BRUCEI IN VARIOUS CULTURE SYSTEMS

Culture System	Duration of Parasites in Culture (Days)	Infectivity for Mice
MEM/20% FCS/BHK CELLS	1	+
	2	+
	3	+
	4	+
	5	+
	6	+
MEM/10% NU-SERUM/BHK CELLS	1	+
	2	+
	3	+
	4	+
	5	+
	6	+
MEM/20% HORSE SERUM/BHK CELLS	1	+
	2	+
	3	+
	4	+
	5	+
	6	+
MEM/20% MOUSE SERUM/BHK CELLS	1	+
	2	+
	3	-
	4	-
	5	-
	6	-

SECOND BATCH OF SERA.

KEY :

+ = Patent

- = Non-patent

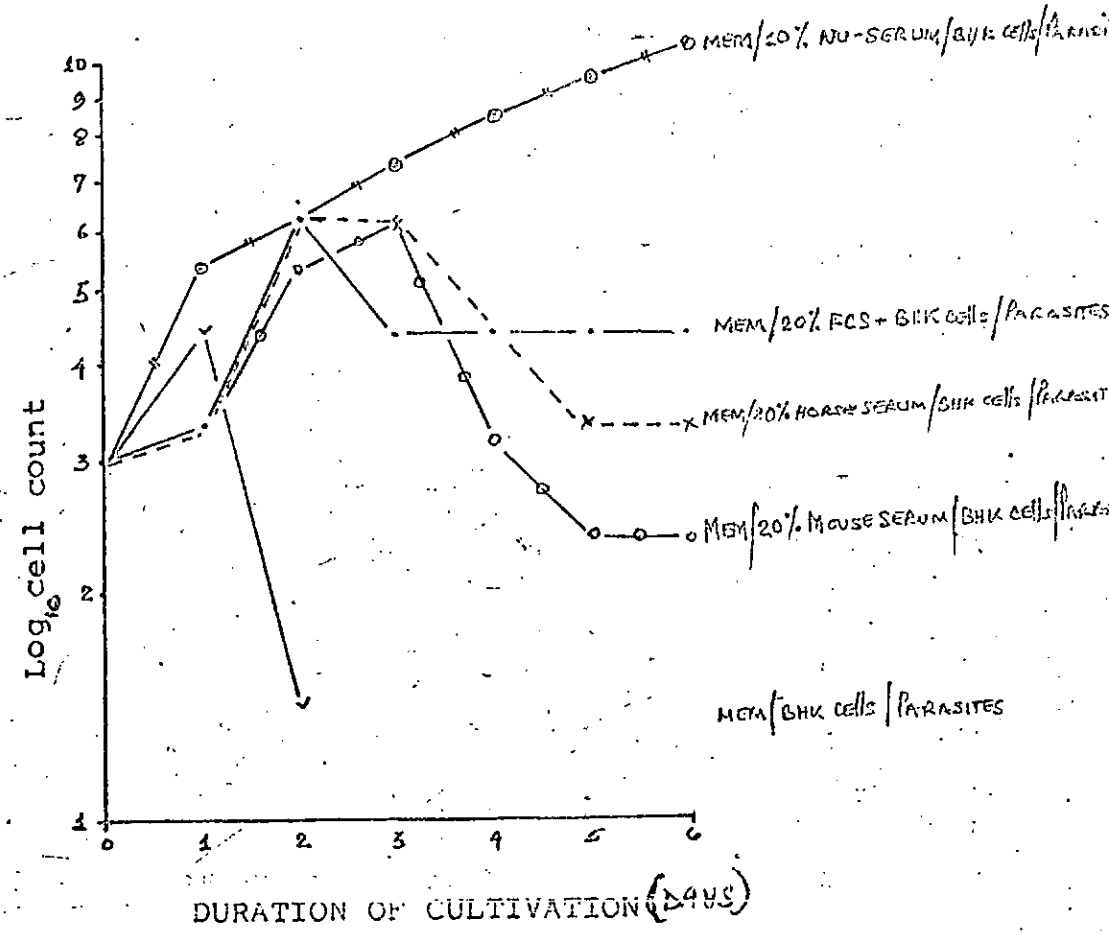


FIGURE 5.1: GROWTH OF TRYPANOSOMA BRUCEI BRUCEI IN THE VARIOUS CULTURE SYSTEMS PREPARED WITH THE FIRST BATCH OF THE SERA.

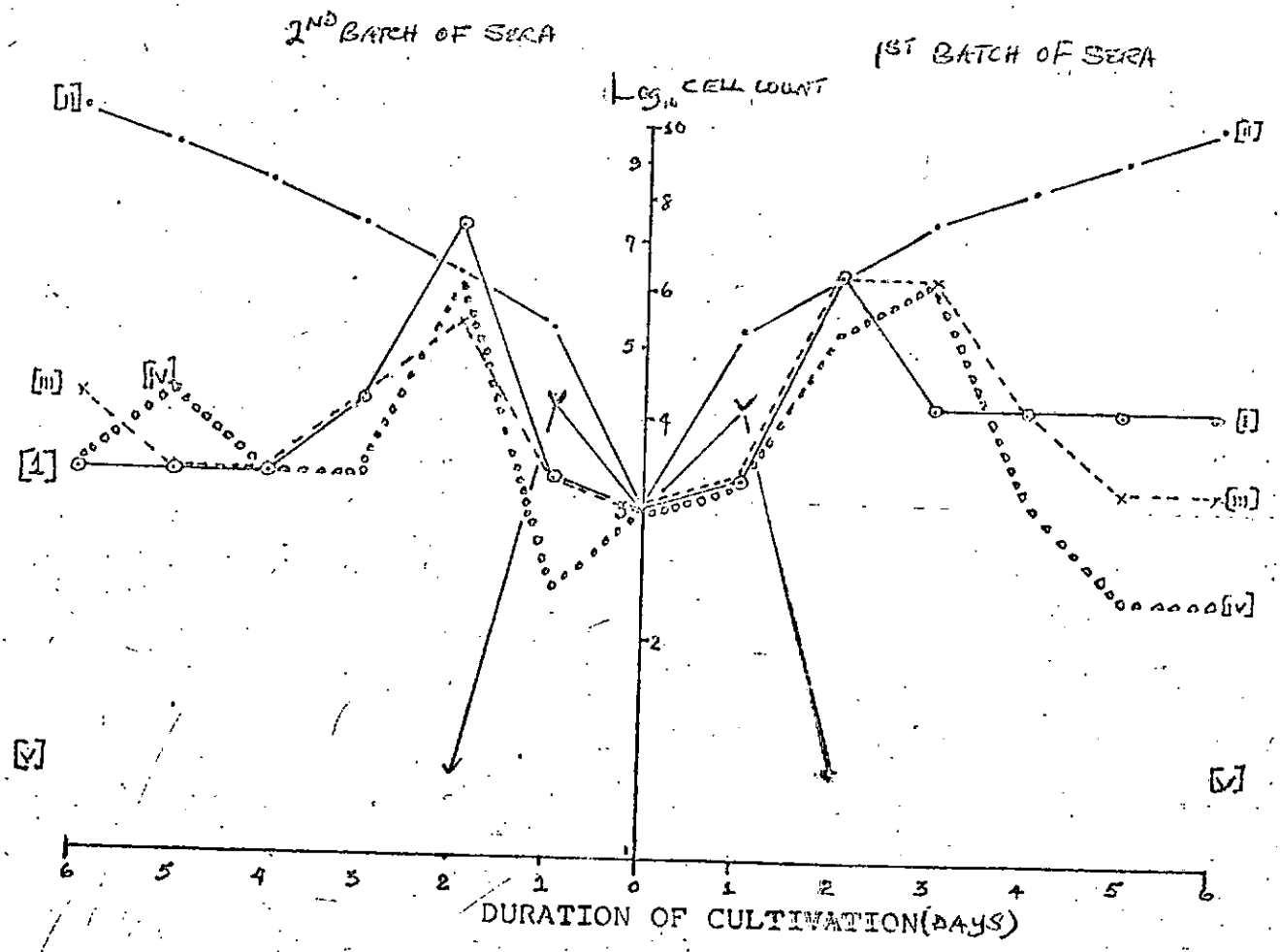


FIGURE 5.1 & 5.2: GROWTH OF TRYPANOSOMA BRUCEI BRUCEI IN THE VARIOUS CULTURE SYSTEMS PREPARED WITH THE 1ST & 2ND BATCHES OF SERA

CHAPTER 6

REVERSION OF MONOMORPHIC *TRYPANOSOMA BRUCEI BRUCEI* INTO PLEOMORPHIC STRAIN IN CULTURE

ABSTRACT

Transformation of T.b. brucei monomorphic forms can be achieved in vitro by the addition of Nu-serum to the culture medium. A monomorphic strain of T.b. brucei which was cultured for 5 days at 37°C in Eagle-MEM (with or without feeder cells) and supplemented with Nu-serum became pleomorphic after inoculation into mice. Dividing stumpy forms were still seen in blood after the parasites have been serially passaged in mice thrice. The strain remained monomorphic when cultured in the medium supplemented with foetal calf serum. This supports the hypothesis that some slender forms, under certain conditions, have the genetic capability of transforming into stumpy forms which can multiply by binary fission in the mammalian host.

INTRODUCTION

Trypanosomes of the brucei group undergo a complex life cycle in the mammalian hosts and the vector, tsetse fly. Blood stream forms taken up by the insect vector transform in the midgut to procyclic forms. Trypanosomes have never been known to transform *in vitro*.

During the routine examination of five-day old cultures of a blood stream form of monomorphic slender, *T.b. brucei*, a few "broad" forms were noticed among the "slender" trypanosomes. This observation prompted a closer look into the trypanosome forms encountered in the culture medium and to study their morphology.

The medium used was supplemented with Nu-serum and the feeder layer was baby Hamster Kidney cells.

MATERIALS AND METHODS

PARASITE

A monomorphic strain of *T.b. brucei* (Shinyanga III) was used. It was initially isolated in 1946 in Uganda and has since been maintained by cryospreservation and serial passages in mice at the University of Guelph since 1967.

ISOLATION OF TRYPANOSOMES AND INOCULATION OF CULTURES

Trypanosomes for culture were isolated from mouse blood according to Hirumi *et al* (1977a,b). The number of parasites in the inoculum was determined using a Coulter counter (aperture at 70 um). MEM was supplemented by either

FCS or Nu-serum (Lot No. 85-121). Nu-serum contains 25% (by volume) of foetal bovine serum and its formulation includes numerous hormones and a series of cell growth promoting supplements (Anon, 1983).

A BHK cell line was obtained from Dr. V.L. Chan (Department of Microbiology, University of Toronto, Toronto, Ontario, Canada) and maintained in plastic Nunc 25 ml flask with 5 ml of medium. The pH in the medium was 7.4 and the following culture combinations were used for the parasites :

(a) MEM + 20% FCS + BHK Cells + Trypanosomes

(b) MEM + 10% Nu-serum + BHK Cells + Trypanosomes

(c) MEM + 20% FCS + Trypanosomes

(d) MEM + 10% Nu-serum + Trypanosomes

(e) MEM + Trypanosomes.

Each culture flask was inoculated with 10^3 washed trypanosomes. Twenty-four old subcultured BHK feeder cells were used in culture systems (a) and (b). All cultures were maintained at $37^{\circ}C$. At least half of the medium in each culture was replaced with fresh medium every 48 hours. Five day old trypanosome cultures were inoculated intraperitoneally into female CDI mice. All cultures were duplicated and the study was repeated twice.

METHOD FOR PREPARING SLIDES, DRAWINGS AND MEASUREMENTS

Blood smears were made 3 days post-infection and after the third passage into mice. They were GIEMSA

stained and the first 50 well-stained trypanosomes seen were drawn under an oil immersion objective with the aid of a drawing tube (Woo, 1969). The drawings were measured using a sonic digitizer (Grafber Model GP-7) interfaced to an IBM Personal Computer with two 360KB diskette drives. The program was written in Advanced Basic for measuring trypanosomes. Photomicrographs were taken at 1100 x magnification and all were printed at the same final magnification.

SCANNING ELECTRON MICROSCOPY (SEM)

Concentrated parasites on coverslips (with the aid of polylysine) were fixed with 2.5% gluteraldehyde for 30 minutes and in Osmium tetraoxide (OSO_4) for 3 hours. The specimens were dehydrated in graded alcohol (30, 50, 70, 90 and 100%) for 20 minutes in each concentration and in Freon, just before critical point drying carried out. The specimens were then mounted on SEM slides and gold coated, examined in Mark 11A Scanning Electron Microscope (accellerating voltage 10kv). Electron micrographs were taken.

RESULTS

Light Microscopy:

Morphological changes during Trnasformation

A trypanosome was scored as transforming from blood stream form to culture form when the following criteria were satisfied; position of the kinetoplast between the

nucleus and the posterior end (that is not subterminal); undulating membrane poorly developed; no or very short free flagellum, whether posterior end was pointed or blunt.

Trypanosomes in mice inoculated with cultures supplemented with Nu-serum were broader, shorter and had shorter free flagella than those in mice inoculated with trypanosomes grown with foetal calf serum (Table and Plate 6.1). Stumpy trypanosomes with very short flagella and blunt posterior ends (Plate 6.1) were seen only in mice inoculated with trypanosomes from cultures that were supplemented with Nu-serum. The stumpy forms multiplied by binary fission (Plates 6.2 and 6.3) and were still present after the third mouse passage. Trypanosomes from mice inoculated with cultures supplemented with FCS remained monomorphic and slender (Plate 6.4). Parasites from the control cultures not supplemented were non-infective to mice.

SEM Parasites photographed with SEM, (Plate 6.5) looked similar to those found in the insect vector.

MEASUREMENTS (IN MICRONS OF TRYPANOSOMA BRUCEI BRUCEI AFTER 3 PASSAGES IN MICE

From Table 6.1, it could be seen that parasites from mice inoculated with trypanosomes from cultures (b) in materials and methods page , supplemented with Nu-serum had a shorter distance (NA : 6.0 - 13.4) from the middle of the nucleus to the anterior tip (NA : 6.0 - 3.4) as opposed to parasites from mice inoculated with trypanosomes from cultures supplemented

with FCS (a) (NA : 7.4 - 13.3). By the same token, the length of free flagellum (AF) of parasites from mice inoculated with trypanosomes from cultures supplemented with Nu-serum was shorter (AF :1.0 - 5.0) as opposed to parasites from mice inoculated with trypanosomes from cultures supplemented with FCS (AF : 2.2 - 5.6). The body length (PA) of parasites from mice inoculated with trypanosomes from cultures supplemented with Nu-serum was also shorter (15.5 - 22.9) than that of parasites from mice inoculated with trypanosomes from cultures supplemented with FCS (16.5 - 32.0). Trypanosomes isolated from mice inoculated with Nu-serum supplemented parasite cultures had greater body width (BW : 2.2 - 3.5) than parasites from mice inoculated with trypanosomes from FCS supplemented cultures (BW: 1.5-2.8)

DISCUSSION

It is believed that the additional components in Nu-serum (not normally found in whole animal serum) triggered the transformation of some slender into stumpy forms. This would explain why previous workers had not seen stumpy forms in their studies. It is possible that Plate 6.3 represents morphological transformation of a slender form in the process of division with subsequent blocking of cytokinesis and Plate 6.6 shows two stumpy forms with their posterior ends touching. This latter interpretation would tend to support the hypothesis that stumpy forms do not divide (Wijers, 1959, 1960). Ashcroft (1957) in one of

his monographs postulated that antibodies inhibit the division of slender forms and transform them into stumpy forms. This postulation cannot explain our observation since the trypanosomes were passaged every three days into mice and no stumpy forms were observed in mice inoculated with the cultures supplemented with foetal calf serum.

Unpredictable and sporadic occurrence of stumpy forms of monomorphic T. evansi in mice is known (Hoare, 1972) and some have short free flagella (Hoare, 1956). Stumpy forms are infective to tse-tse flies (Wijers and Willet, 1960). Future research will include an attempt to determine the susceptibility of tse-tse flies to a monomorphic strain (shown initially to be non-infective to tse-tse flies) after it has been cultured in a medium with Nu-serum supplement. It should be noted however, that a pleomorphic strain of T. evansi was not infective to tse-tse flies (Hoare, 1940) and that Mshelbwala (1967) infected a single fly by feeding it on slender forms.

As far as I know, this is the first report of a monomorphic strain reverting to a pleomorphic strain, although inoculation of a pleomorphic T.b. brucei into turtles (acclimitized to about 35°C) significantly increased the number of stumpy forms (Woo and Soltys, 1969).

The present study supports the hypothesis that some slender forms, under certain conditions can transform into stumpy forms and that these forms multiply by binary fission.

TABLE 6.1

MEASUREMENTS OF TRYPANOSOMA BRUCEI BRUCEI

AFTER THREE PASSAGES IN MICE

Culture Combination	NA* MICRONS ± SD	AF MICRONS±SD	PA MICRONS±SD	BW MICRONS±SD
MEM/20% FCS/BHK CELLS/PARASITES	10.5±1.45 (7.4-13.3)*	2.5±1.04 (2.2-5.6)	20.1±2.51 (16.5-32.0)	2.5±0.45 (1,5-2.8)
MEM/10% Nu-SERUM/ BHK/PARASITES	10.8±1.48 (6.0-13.4)	2.6±0.56 (1.0-5.0)	18.9±2.04 (15.5-22.9)	2.8±0.32 (2.2-3.5)
MEM/20% FCS/ PARASITES	10.5±1.59 (7.5-13.5)	2.9±0.80 (1.3-4.5)	19.1±1.98 (16.6-22.3)	2.6±0.41 (1.8-3.4)

KEY : NA = distance from the middle of nucleus to anterior tip.

AF = length of free flagellum

PA = length of body (excluding free flagellum)

BW = greatest body width

* = Mean with standard deviation followed by range in parenthesis.

PLATE 6.1 STUMPY FORM FROM NU-SERUM SUPPLEMENTED CULTURES

PLATE 6.2 : THE STUMPY FORM FROM NU-SERUM SUPPLEMENTED CULTURES IN DIVISION

PLATE 6.3 : THE STUMPY FORM FROM NU-SERUM SUPPLEMENTED CULTURES IN DIVISION

1 - NOTE THE PRESENCE OF 2 NUCLEI

PLATE 6.4 :

MONOMORPHIC/SLENDER TRYPOMASTIGOTE FROM CULTURES SUPPLEMENTED WITH FCS

PLATE 6.5 : SCANNING ELECTRON MICROGRAPH OF T.B. BRUCEI

PLATE 6.5 : SCANNING ELECTRON MICROGRAPH OF T.B. BRUCEI

PLATE 6. 6 : TWO STUMPY FORMS WITH THEIR POSTERIOR ENDS TOUCHING