STUDIES ON THE FLAGELLAR ATTACHMENT OF AFRICAN TRYPANOSOMES

by

KAY ALEXANDRA KIDD HENDRY B.Sc.(St. Andrews)

DEPARTMENT of ZOOLOGY

UNIVERSITY of GLASGOW

Presented in submission for the degree of Doctor of Philosophy.

March 1987.

DECLARATION

The results presented in this thesis are those of my own original work except where otherwise stated.

ACKNOWLEDGEMENTS

I would like to thank the following for help and support throughout this project:-

The MRC for financial support in the form of a studentship , my supervisor , Professor K. Vickerman for instigating the research and for helpful discussion, particularly in the writing stage and Professor R.S. Phillips for providing facilities.

Professor A.S.G. Curtis, Department of Cell Biology, University of Glasgow, and his technical staff for allowing me to use the interference reflection microscope, providing polypropylene sheeting and BHK cells and for helpful discussion.

Dr.J. Kusel, Department of Biochemistry, University of Glasgow, for the gift of lectins and general help and enthusiasm.

Professor K. Gull and his laboratory, University of Kent, Canterbury, in particular Trevor Sherwin, for allowing me to invade their domain for a month and teaching me to prepare cytoskeletal material and 2D-gel electrophoresis.

Dr. A.G. Luckins, Dr. C.A. Ross, Sandra Taylor and Iain Frame ,CTVM, University of Edinburgh for providing epimastigote cultures and teaching me tissue culturing techniques.

Dr. Laurence Tetley for aid with EM work, in particular immunogold detection techniques and Helen Hendry for technical assistance with EM and photographic reproduction.

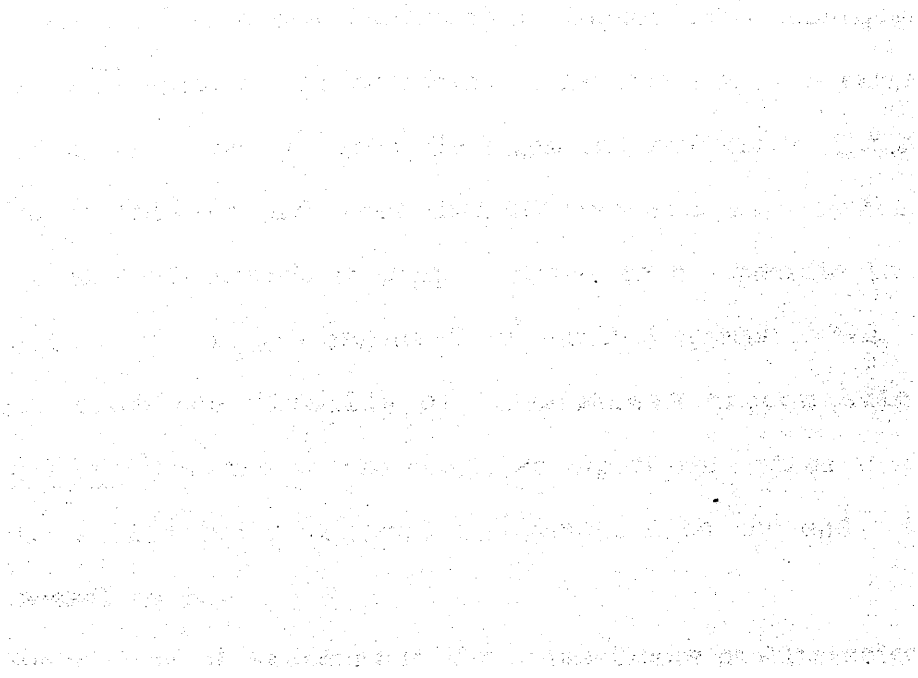
The staff of the Tsetse Research Laboratory, University of Bristol, for Tsetse pupae.

Dr. E.B. Lane, ICRF, Sutherland McIver, University College London and Professor K. Gull for cytoskeletal antibodies. T.M. Preston, University College London, for charged Sephadex beads and Dr. C. Skerrow, Department of Dermatology, University of Glasgow, for the photographs of desmosomes and hemidesmosomes in chapter 1.

Mr. and Mrs. M.L.N. Murthy for wordprocessing and P. Rickus and the Photographic department, Glasgow University for some photographic work.

To Mike Turner, Cathy Cameron, David Strachan, Alan Scott, Dave Killen and Dave Barry, special thanks for help, support, encouragment and, above all laughter throughout the three year sentence.

Lastly, and by no means least, my parents and above all Derek, for "financial support" and mostly "putting up with it all", thank you.



ABSTRACT

Attachment of trypanosomes by the flagellum to a host internal surface is believed to represent an important stage in the development of these parasites in their vectors.

This thesis is concerned with an investigation into various aspects of the flagellar attachment of Trypanosoma congolense epimastigotes.

The morphology of the attachment is described in both the tsetse fly labrum and in vitro where the epimastigotes attach to the plastic wall of the culture flask. Epimastigotes attach to the substratum at one or more sites along the length of the flagellum. At the attachment site the flagellar membrane is extended from the axis and an electron dense, hemidesmosome-like plaque (approx. 35nm wide) forms on the cytoplasmic face of the membrane. 5nm filaments converge on the plaque and associate with similar filaments emanating from flagellum-cell body attachment plaques. Filaments form at the attachment site soon after contact with the substratum. Plaques form subsequently and become more compact with advancing epimastigote age. An extracellular gap (15nm) is present between flagellum and substratum. Ruthenium red binding in this gap indicates that glycoproteins are situated there.

Differentiated attachment plaques appear to be specific to attached epimastigotes and are absent from detached epimastigotes. They are absent from the flagella of bloodstream trypomastigotes of T.congolense even though the flagellar tip of this stage in the trypanosome's life cycle attaches the parasite to the endothelium of bloodvessel walls.

The necessity of attachment for epimastigote proliferation and for differentiation of the mammal-infecting metacyclic stage from the epimastigote has been examined. Prevention of epimastigote attachment by shaking cultures or growing trypanosomes on a polypropylene substratum does not affect the rate of epimastigote division.

Metacyclogenesis does not occur in unattached trypanosomes, however, suggesting that, in this species, attachment is a necessary prerequisite for this developmental step.

Interference reflection microscopy has been used to visualize the effect on extracellular gap width of agents added to the culture medium. An increase in gap width following trypsinization ,on addition of Concanavalin A, Lentil lectin and Wheatgerm Agglutinin indicate that protein, D-mannose and N-acetyl-D-glucosamine are present in this location. External serum concentration, monovalent ion concentrations and the presence of tunicamycin have little effect on the interferometry pattern. Tunicamycin insensitivity reflects a lack of glycoprotein turnover at the site. Divalent cation removal decreases the gap width. The binding of epimastigotes to positively charged Sephadex beads indicates a net negative surface charge. This charge maybe less negative in the region of the attachment as binding to positively charged beads is reduced at the flagellar tip.

Indirect immunofluorescence and indirect immunogold electron microscopical cytochemistry indicate that the proteins actin, vinculin, filamin, intermediate filament proteins and desmoplakins (found associated with morphologically similar metazoan cell attachment structures - hemidesmosomes and focal adhesions) are absent from the epimastigote attachment. Immunofluorescence reactions suggest that actin and intermediate filaments are present in these cells but not localized in their distribution. Confirmation of the presence of these proteins can be obtained with SDS-PAGE and associated Western blotting experiments of both whole cell and cytoskeleton preparations. Comparative 2D-gel electrophoresis of attached and unattached whole epimastigotes and cytoskeletons shows the presence of a novel group of proteins (approx. Mr=70kd) in attached preparations. It is suggested that these proteins maybe involved in the attachment complex.

CONTENTS

ABBREVIATIONS

BHK	Baby hamster kidney cells
BSA	Bovine serum albumin
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol-bis-(beta-amino-ethyl ether)N'-N'-tetra -acetic acid
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GTP	Guanosine tri-phosphate
GUPM	Glasgow University Protozoology Monoclonal (antibody)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	Horse radish peroxidase
HST	High salt Tris-buffer (10mM Tris-HCl, pH 7.4, 1M NaCl, 0.5% (v/v) Tween 20)
IRM	Interference reflection microscopy
LUMP	London University Medical Protozoology (stabilate)
MAb	Monoclonal antibody
MEM	Minimum Essential Medium
MOPS	3-(N-Morpholino)propanesulphonic acid
NP40	Nonidet P40 detergent
PBS	Phosphate buffered saline (0.017M, pH 7.2)
PEG	Polyethylene-glycol
PFR	Paraflagellar rod
PIPES	Piperazine-N,N'-bis(2-ethane-sulphonic acid)
RPMI	Rothwell Park Memorial Institute medium
SAPU	Scottish Antibody Production Unit, Law Hospital, Carluke.
SDS	Sodium dodecylsulphate
TBS	Tris buffered saline (10mM Tris-HCl ,pH 7.4, 140mM NaCl)
TREU	Trypanosome Research Edinburgh University (stabilate)

TRIS	Tris(hydroxymethyl)aminoethane
TTGO	Tris, 20mM, pH 7.2 plus 0.01% Tween 20, 0.1% Gelatin and 1% Ovalbumin
VAT	Variable Antigen Type
VSG	Variant Surface Glycoprotein

CHAPTER 1

INTRODUCTION

1.1. TRYPANOSOMES AND TRYPANOSOMIASIS

1.1.1. The trypanosomatid flagellates

The trypanosomatids are flagellated protozoans and members of the order Kinetoplastida (Honigberg, 1963). The members of this order are distinguished by possessing a kinetoplast - a mass of mitochondrial DNA usually situated in close proximity to the base of the flagellar apparatus. The family Trypanosomatidae are distinguished from the other family in this order, the Bodonidae, by possessing a single flagellum, the Bodonidae have two flagella.

1.1.2. Pathogenic trypanosomatids

Trypanosomatids are parasitic in a wide variety of hosts. Many are the cause of human disease, e.g. Leishmaniasis, Chagas' disease and sleeping sickness. Although these diseases affect a large number of the world's human population (1.2 million by Leishmaniasis and 25 million affected by trypanosomiasis, Schofield, 1985, based on WHO figures), the socioeconomic constraint imposed on the development of Africa by tsetse fly-transmitted animal trypanosomiasis represents one of the biggest trypanosomatid-related problems (recently reviewed by Murray & Gray, 1984).

1.1.3. Trypanosomiasis of domestic livestock in Africa

Trypanosomiasis of livestock is the most widespread disease of domestic animals in Africa south of the Sahara and represents the major obstacle to the agricultural development of the continent. Three main species of livestock-infecting trypanosome are transmitted by tsetse fly vectors - Trypanosoma congolense, T.vivax and T.brucei. The cattle disease caused by infection with these trypanosomes is, in general, known as Nagana. The most common manifestations of the disease are an intermittent fever, anaemia and wasting. It must be mentioned, however, that not all breeds of domestic cattle are equally

susceptible to trypanosomiasis. Native breeds, in particular the West African short horn and N'Dama breeds, have been found to have a high degree of trypanotolerance. It is the introduced breeds and crossed breeds which are highly prone to the disease (Murray et al., 1982). Unfortunately the native N'Dama, and shorthorn breeds have been considered to have a lower productivity than introduced breeds - a dogma which may be unfounded (Murray et al., 1982).

Most cases of cattle trypanosomiasis are due to T.congolense or T.vivax infection; mixed infections of these two species are also common. T.congolense is the predominant parasite in small ruminants in East Africa (Murray et al., 1982). T.congolense can be transmitted by the majority of Glossina species and, thus, can be spread in all habitats in the tsetse belt.

Tsetse flies infest approximately 10 million km^2 of Africa - 37% of the continent, covering 38 countries (FAO-WHO-OIE, 1982). This area, despite the existence of potentially effective control measures (chemotherapy, chemoprophylaxis, tsetse eradication and the establishment and selective breeding of trypanotolerant livestock) is increasing, particularly due to savanna tsetse expansion. The greatest and most active advance of modern times is probably occurring in southern Angola (FAO, 1979; MacLennan, 1980). Of the 10 million km^2 of Africa infested with the tsetse fly, around 7 million km^2 is suitable for mixed agriculture and livestock development. Much of the best watered, fertile land is tsetse fly-infested whilst large areas of good grazing could be immediately utilized for agriculture if free from tsetse infestation (MacLennan, 1980). Many areas of Africa are severely affected by trypanosomasis. West and central Africa contains 26% of the continent's human population but only 9% of the livestock (ILCA, 1979). The FAO (1974) have estimated that the average potential

carrying capacity of this region was 20 cattle/km^2 rather than the then current 3.4 cattle/km^2, with similar figures for sheep and goats. East and southern Africa fare little better, in some countries e.g. Tanzania, up to 70% of the land is infested with the tsetse fly. The FAO-WHO-OIE (1982) have estimated that approximatly 30% of the 147 million cattle in countries affected by the tsetse are exposed to trypanosomiasis; the situation with respect to sheep, goats, horses, donkeys and camels is probably similar but is less well documented.

The annual loss of meat production due to trypanosomiasis was valued at $5 billion in 1963 (FAO-WHO-OIE, 1963), as a result of this and the additional loss in milk production and mixed agriculture due to loss of traction animals and manure (only 6% of farmland in Africa has access to tractors, Jordan, 1985); Africa produces 70 times less animal protein per unit area than Europe (FAO, 1975). Recent reviews estimate the cost of animal diseases to African agriculture at $50 billion (Allsopp et al., 1985). Added to this are the constraints on African rural development by human trypanosomiasis; it is estimated that 50 million people are at risk from the disease. The consequent increasing pressure on tsetse-free pasture will eventually lead to pasture degradation, fall in output and increased production costs.

There are many reasons for the currently deteriorating situation, these include:-

a) A paucity of suitable drugs. Although the existing drugs can be effective against animal trypanosomiasis, no new drugs have been developed for over 25 years. Of the 5 main compounds used (homidium, diminazene, pyrithidium and isometamidium salts and suramin), the trypanosomes are becoming resistant to at least three (pyrithidium and homidium salts and suramin). The estimated cost, $20-30 million, of developing new trypanocidal compounds is not a commercial propostion for drug companies.

b) The absence of a vaccine against trypanosomiasis. This situation is unlikely to improve as the trypanosome surface antigens can vary greatly during an infection. The antigenic identity of a trypanosome is located in its 12-15nm surface coat (Vickerman & Luckins, 1969). This is composed of a single glycoprotein - the variant surface glycoprotein (VSG) (Cross, 1975). Within a rising parasitaemic peak the trypanosome population is composed of a mixture of variable antigen types (VATS), each type being a different VSG (Van Meirvenne et al., 1975), each stimulating a VAT-specific antibody response. The trypanosome infection is characterised by successive peaks of parasitaemia (Ross & Thomson, 1910). Each remission in parasitaemia is due to elimination of a population of trypanosomes by a VAT-specific antibody; each recrudescence is due to the growth of new antigenic types which have arisen de novo within the trypanosome population. Capbern et al. (1977) reported 101 different VATS within a single rabbit infection. Each VSG is the product of a different gene (Borst & Cross, 1982); Van der Pleog et al. (1982) have calculated that there are 100-1000 different VSG genes per trypanosome. As the only protective immunity developed in Nagana (or sleeping sickness) is VAT-specific, the possibility of vaccinating potential hosts is unrealistic.

c) Lack of funds for vector control. The main problem with vector control programmes is the lack of adequate financial backing; often due to failure of multinational cooperation. Civil insurrection is also a problem when large scale control measures are being applied. The war of independence in Mozambique drastically reduced the cattle population of the country due to a breakdown in control programmes. The lack of personnel trained to

plan and implement control measures contributes to this problem.

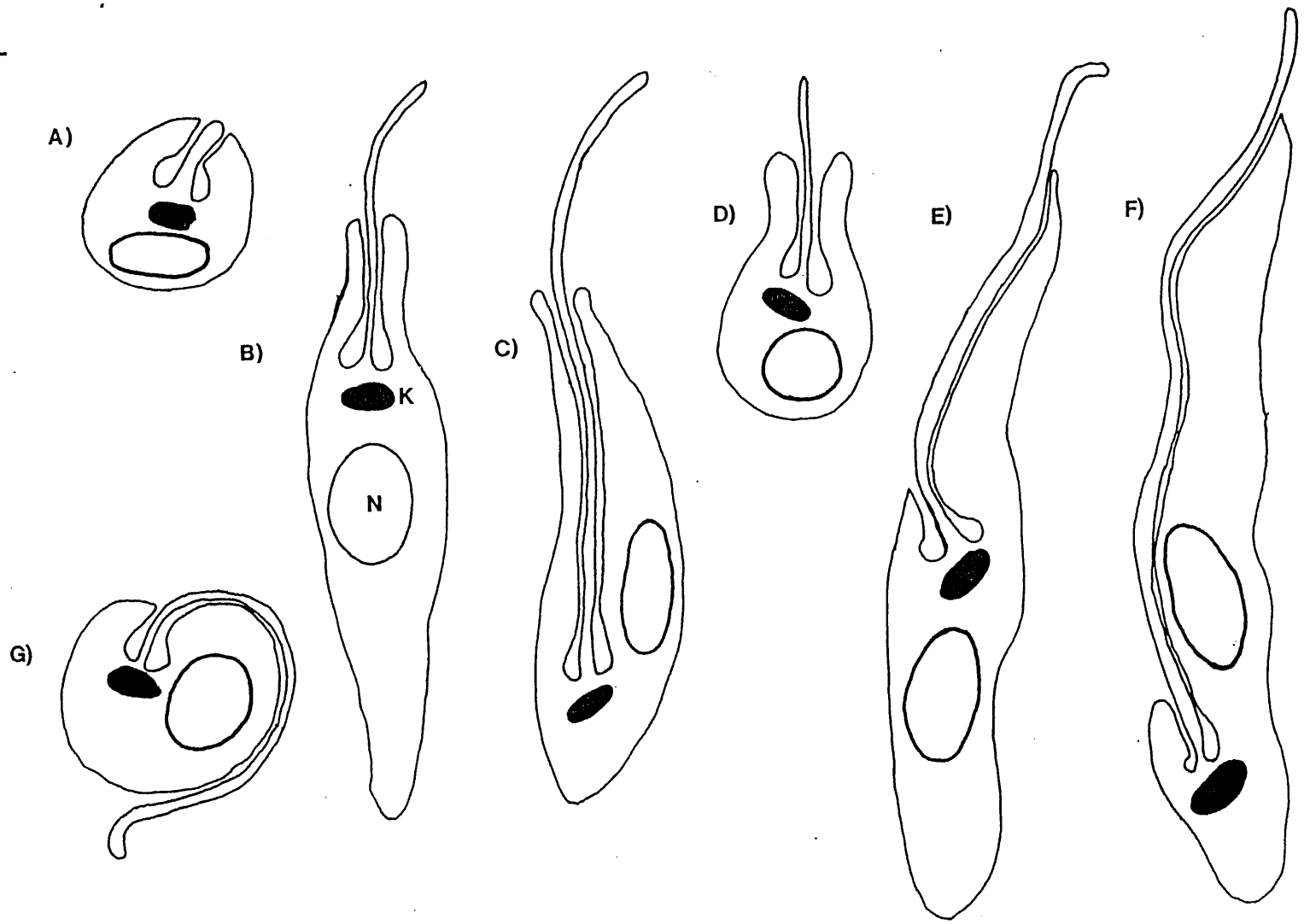
d) The existence of a wild animal reservoir. Animal and human infective trypanosomes have reservoirs in wild animals, especially the ungulates of the continent (Baker, 1968; Burridge et al., 1970; Murray et al., 1982).

In combination, however, all the control measures can be effective. Tsetse fly eradication measures by the use of ground application of residual insecticide combined with drug treatment of cattle, have been shown to work (Jordan, 1979). Aerial spraying of insecticide is also effective if applications are strictly applied. A large 15 year, 5 country project using aerial spraying and drug treatment is currently in progress in Eastern Africa (Jordan, 1985). Anti-tsetse measures, chemotherapy and the introduction of trypanotolerant breeds of livestock to tsetse-infested areas can boost the rural economy of the continent and keep the countries trypanosome free. As an example of the results of effective trypanosomiasis control measures, Zimbabwe recently signed an agreement with the EEC to export a quota of beef to Europe, Zimbabwe can easily fulfil this quota and also export to the rest of Africa and the Middle East, as long as the cattle herds are kept trypanosome free by continued use of control measures (Jordan, 1985).

Resistance of both trypanosomes and tsetse to drugs and insecticides is a continual problem in control programmes. Recent developments in tsetse control have tended to concentrate on new low cost methods of attracting and capturing the tsetse flies (recently reviewed by Vale et al., 1985), or sterile male tsetse release (Langley & Hall, 1986) rather than chemical killing of the flies. New methods of tsetse and trypanosome control must depend upon increased knowledge of the basic biology of both these organisms and the relationship between them.

FIG.1:1. Diagram depicting trypanosomatid morphological types.
(A) Amastigote (B) Promastigote (C) Opisthomastigote
(D) Choanomastigote (E) Epimastigote (F) Trypomastigote
(G) Sphaeromastigote.
(Terms A-F defined by Hoare and Wallace 1966, G defined by Brack 1968). Diagram after Vickerman 1976.
N- nucleus , K- kinetoplast.

1



1.2. PARASITE LIFE CYCLES

1.2.1. Life cycle terminology

All known trypanosomatids are parasitic, the variety of stages within their life cycles is probably due to living in differing environments at different times during their life cycle and thus having to adapt to the ambient conditions. The life cycles are characterized by morphological changes involving repositioning of the flagellum origin and kinetoplast with respect to the nucleus and the ends of the body. The names given to the stages of the life cycles reflect the position of the flagellum base/kinetoplast (Hoare & Wallace, 1966). These stages are defined as follows (Fig. 1.1). (a) Amastigote - round or oval body with the kinetoplast alongside the nucleus and no emergent flagellum; (b) Promastigote - the kinetoplast lies towards the anterior end of the body from which the flagellum emerges free of bodily attachment. (c) Opisthomastigote - the kinetoplast is situated posterior to the nucleus but the flagellum emerges at the anterior end after travelling in a long flagellar canal. (d) Choanomastigote - small, bulbous forms in which the kinetoplast lies just anterior to the nucleus and the flagellum emerges anteriorly from a broad flagellar pocket, (e) Epimastigote - the kinetoplast lies anterior to the nucleus, the flagellum emerges laterally and is attached laterally to the body forming an undulating membrane; (f) Trypomastigote - the kinetoplast is posterior to the nucleus and the flagellum again emerges laterally and forms an undulating membrane.

The term Sphaeromastigote (Brack, 1968) is sometimes used to describe spherical shaped trypanosomes with an emerging attached flagellum.

1.2.2. Monogenetic trypanosomatids

Many of the trypanosomatid genera are monogenetic in their life cycles, i.e. have a single host. These genera are Leptomonas, Crithidia, Blastocrithidia, Herpetomonas and Rhynchoidomonas. Species of these genera exhibit only one or two of the above life cycle stages and are parasites of insects and other invertebrates, usually in the gut. Where the mode of transmission is known it is usually by faecal contamination. In the case of some, Leptomonas and Blastocrithidia species, this is by amastigote 'cysts' created by unequal division of attached promastigotes or epimastigotes. At some stage all these groups exhibit forms attached to the gut of their host, usually to a cuticle-lined hindgut or microvilli-lined midgut. The attached and unattached forms, even if both are the same form as defined by Hoare and Wallace (1966) may differ. In the case of Crithidia or Blastocrithidia at least, the attached choanomastigotes and epimastigotes, respectively, have far shorter flagella adapted for attachment (see section 1.3.1) attached forms are known as haptomonads and the free swimming forms as nectomonads. Herpetomonas species display both promastigote and opisthomastigote stages in different regions of the insect host's gut; some midgut attached forms have been described. The mode of transmission of these flagellates is unclear.

1.2.3. Digenetic trypanosomatids

Species of the genus Phytomonas are digenetic, their two hosts being a plant, usually of the Euphorbiaceae, Asclepiadaceae, Moraceae or Palmae and a phytophagous hemipteran. The flagellates maintain the promastigote form throughout the life cycle. They infect their plant host via the insect salivary glands, having arrived there after migrating through the gut wall and haemoceol.

Species of Leishmania are also digenetic and display amastigote and promastigote stages in their life cycle. In their mammalian host, including man, these organisms multiply as amastigotes within mononuclear phagocytes. Sandflies (Diptera, Phlebotominae) are the vectors of these organisms. In the insect these flagellates are found as free or attached promastigotes. The attached forms are normally found in the region of the oesophageal valve. When attached in this area (and others depending on the species of Leishmania) the flagellates display the characteristic flagellar attachment as described in section 1.3.1.

1.2.4. The genus Trypanosoma

The members of the genus Trypanosoma are basically digenetic though a few have eliminated the vector to become monogenetic. Almost all species display trypomastigote and epimastigote types and a few also display amastigotes. These organisms go through a series of developmental stages in their invertebrate (leech or arthropod) host and often also a series of stages in their vertebrate host. All classes of vertebrate are parasitized by a haematozoic trypomastigote stage.

Hoare (1964) divided the species of Trypanosoma which parasite mammals into two groups: (1) the Stercoraria, in which the developmental cycle in the insect host is completed in the hindgut and thus infective metacyclic trypomastigotes are present in the faeces; transmission is therefore by contamination. (2) The Salivaria, in which development in the insect host is completed in the mouthparts or salivary glands so that the infective metacyclic trypomastigotes are present in the saliva; transmission is, therefore, by inoculation.

A) Stercoraria

The three subgenera that compose this group, Megatrypanum e.g.

T.melophagum, Herpetosoma e.g. T.lewisi and Schizotrypanum e.g. T.cruzi, vary in their life histories but in the main conform to the following pattern.

Metacyclic trypomastigotes are deposited on the skin of the mammalian host in the invertebrate host's faeces during feeding. These trypomastigotes penetrate the epithelium and in the case of members of the subgenus Schizotrypanum penetrate cells e.g. muscle cells, and transform into amastigotes which multiply inside these host cells. On rupture of these cells trypomastigotes are released which may either reinfect mammalian host cells or may be ingested by the haematophagous invertebrate host (reduviid bugs). In the invertebrate host gut the stercorarian trypomastigotes transform into epimastigotes. These forms attach to cuticle lined areas of the hindgut (often the rectal glands) and multiply in this attached or in a free swimming state. The epimastigotes transform into the infective, metacyclic, trypomastigotes in the insect hind gut, these unattached forms are then voided with the faeces.

Members of the subgenus Herpetosoma do not undergo an amastigote intracellular phase in the mammalian host. T.lewisi, for example, divides in the vascular system of its rodent host as epimastigotes.

B) Salivaria

The members of the four subgenera comprising the salivaria, Duttonella e.g. T.vivax, Nannomonas e.g. T.congolense, Trypanozoon e.g. T.brucei and Pycnomonas e.g. T.suis display a similar overall pattern of development. The differences in the subgenus members life histories are mainly evident in the location of the epimastigote attached phase and metacyclic development in the invertebrate host's mouthparts or salivary glands. All species of these subgenera which exhibit cyclic development are transmitted by Glossina sp., the

exceptions to this are that T.vivax is also transmitted mechanically by haemophagous insects and T.equiperdum (Trypanozoon) is transmitted during coitus in horses. T.evansi (Trypanozoon) has no true intermediate host but is normally transmitted by mechanical inoculators e.g. bloodsucking Diptera.

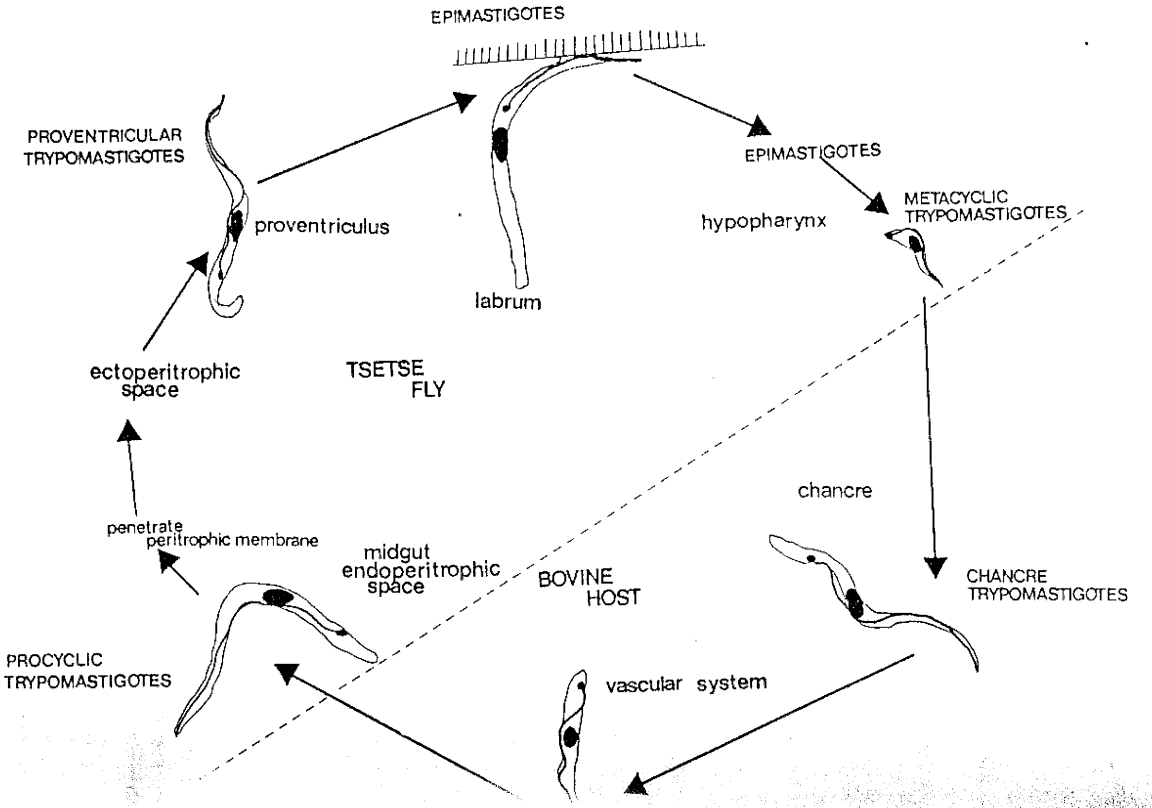
Apart from the last two species the majority of the members of the Salivaria undergo a cycle of development in both the tsetse fly host and possibly in the mammalian host. As the life cycle of T.congolense will be described in detail in the following section, I will give only a brief description of the salivarian type life history here. The infective metacyclic trypomastigotes are injected into the mammal where they transform into more elongate bloodstream trypomstigotes, this transformation may be via an intermediary phase in the connective tissue at the site of the tsetse bite, the so-called 'chancre'. The bloodstream trypomastigotes rapidly divide and build up their population in the mammalian host. Some species are restricted to the vascular system but others are also found in extravascular sites as well as lymph. On being reingested in a tsetse fly blood meal the bloodstream trypomastigotes of subgenera Nannomonas and Trypanozoon become elongate procyclic trypomastigotes. These multiply in the insect midgut endo- and ectoperitrophic spaces, reaching the ectoperitrophic space after penetrating the peritrophic membrane (Evans & Ellis, 1983). After a period of ectoperitrophic development the procyclic trypomastigotes migrate anteriorly to the proventriculus where they repenetrate the peritrophic membrane at its origin and enter into the endoperitrophic cavity. Proventricular trypomastigotes are longer than procyclic trypomastigotes and do not divide. From the proventriculus these trypomastigotes migrate anteriorly to the oesophagus and mouthparts. The members of the Nannomonas subgenera

attach here and transform to epimastigotes and after a period of multiplication, differentiate to the small metacyclic trypomastigotes. Members of the subgenera Trypanozoon, in particular the T.brucei group of flagellates, are thought to migrate from the mouthparts to the salivary ducts and thence to the salivary glands where the epimastigotes become established and attached to the epithelial cells microvilli border. After a period of multiplication unattached metacyclic trypomastigotes are found, these develop from epimastigotes through a series of intermediary stages (Tetley & Vickerman, 1985). Some established procyclics appear to pass into or through the midgut cells (Evans & Ellis, 1983) but it is doubtful that migration to the salivary glands occurs in this fashion as the tsetse heamolymph contains trypanocidal factors (Croft et al., 1982). T.vivax (Duttonella) although having a similar development in the mammalian host to that described above does not have a phase of cyclic development in the midgut of the tsetse host. Bruce et al. (1910) were the first to demonstrate that the entire developmental cycle of T.vivax in Glossina is confined to the proboscis. No trypanosomes survive in the gut, infection in the proboscis is apparently established directly by trypanosomes which anchor themselves to the walls of the food canal on ingestion. The trypanosomatids transform to epimastigotes in the proboscis and then multiply attached to the labrum and labium, some epimastigotes detach and invade the hypopharynx where the infective metacyclic trypomastigotes develop.

1.2.5. Trypanosoma (Nannomonas) congolense Broden 1904

1.2.5.1. General description of the species: Trypanosoma congolense is a small salivarian trypanosome, this organism varies in length between 8-25μm. In the mammalian host this flagellate is monomorphic and sluggish with a marked tendency to stick to other T.congolense (Hoare,

FIG.1:2. Diagram of life cycle of *Trypanosoma congolense*.


EPIMASTIGOTES
PROVENTRICULAR TRYPOMASTIGOTES
proventriculus
labrum
EPIMASTIGOTES
hypopharynx
METACYCLIC TRYPOMASTIGOTES
ectoperitrophic space
TSETSE FLY
chancre
penetrate peritrophic membrane
midgut endoperitrophic space
BOVINE HOST
CHANCRE TRYPOMASTIGOTES
PROCYCLIC TRYPOMASTIGOTES
vascular system

1936), blood cells and blood vessel walls (Büngener & Müller, 1976; Banks, 1978,1979,1980). The undulating membrane is usually inconspicuous and in the majority of cases no free flagellum exists. The nucleus is central, the kinetoplast medium sized and marginal.

1.2.5.2. Development in the mammalian host: Metacyclic trypomastigotes are inoculated by the tsetse fly into an animal. At the bite site a skin reaction, the chancre, develops and within this skin reaction chancre forms of T.congolense are found (Gray & Luckins, 1980). In morphology these are trypomastigotes (posterionuclear kinetoplast) but are larger than metacyclic forms and have an elongate post-kinetoplast region to the body (Roberts et al., 1969). These chancre forms divide in situ and migrate to the bloodstream (Fig. 1.2).

The small trypomastigote trypanosomes divide in the bloodstream of the host and are largely confined to the vascular system. Many reports of their association with capillary walls and bloodcells have been published (Büngener & Müller, 1976; Banks, 1978,1979,1980). The question of whether there is more than one morphological form in the bloodstream has not been effectively answered, there are reports of non-dividing forms within the bloodstream (Nantulya et al., 1978; ILRAD, 1984) in addition to the rapidly dividing forms, but unlike T.brucei, there is no true manifestation of slender and stumpy forms. By analogy with T.brucei stumpy forms, the reported non-dividing T.congolense bloodstream forms are possibly preadapted for survival in the insect host.

1.2.5.3. Development in the vector: After a tsetse fly has fed on an animal with trypanosomes in the blood, the parasite undergoes multiplication and development in the insect digestive tract culminating in the production of metacyclic forms. This may take from 19-53 days. Only a small percentage of tsetse flies produce infective

forms after feeding on an infected animal, Maudlin and Dukes (1985) have provided evidence that some tsetses may be genetically resistant to parasite infection and also that blood serum factors might determine infectivity (Maudlin et al., 1984). The development of the ingested trypanosomes begins in the endoperitrophic space in the midgut where they transform into elongate trypomastigotes, the procyclic forms, these then divide in the midgut for several days.

At days 7-12 (Evans et al., 1979; Ellis & Maudlin, 1985), trypanosomes are found penetrating the peritrophic membrane. In wild caught flies this penetration occurs in the mycetome area of the tsetse midgut whilst in laboratory reared tsetse the penetration appears to occur more posterior in the midgut. The movement to the ectoperitrophic space is thought to take approximately 2 days (Evans & Ellis, 1983). There are reports of trypanosomes found entering midgut cells in this region a few days after reaching the ectoperitrophic space (Ellis & Maudlin, 1985; Kaddu & Mutinga, 1980) but why they should do this is unknown, these reports are brief and undetailed. In the ectoperitrophic space the trypanosomes continue to divide. From the ectoperitrophic space the procyclics migrate anteriorly to the proventriculus and transform into proventricular forms. The proventriculus is normally invaded by the 20th day after infection. These forms still possess a posterionuclear kinetoplast but the posterior end of these cells is very elongate and is often said to be "scroll-like". These forms are non-dividing. They penetrate into the endoperitrophic space of the proventriculus. From the inner lumen of the proventriculus the flagellates migrate forwards via the oesophagus to the proboscis. In the proboscis the trypomastigotes transform into epimastigotes. Whether the migrating forms are preadapted, pre-epimastigotes or true proventricular forms is unclear. Gray et al.

(1981,1984) have observed that the forms which migrate from isolated proboscides to initiate an attached epimastigote culture have a proventricular morphology and that the differentiation to epimastigotes occurs after the proventricular forms have adhered. These observations suggest that proventricular forms are migrating to the tsetse mouthparts in T.congolense. The epimastigotes which have an anterionuclear kinetoplast attach to the cuticle in the labrum and divide. At some stage some epimastigotes appear in and attach to the hypopharyngeal cuticle but whether these are the result of epimastigotes in the labrum detaching and swimming to the hypopharynx and reattaching (cf. nectomonad and haptomonad behaviour among the lower trypanosomatids) or due to a fresh wave of colonization from the proventriculus is unclear. In the labrum and hypopharyx the epimastigotes attach and divide, after a few days metacyclic trypomastigotes are observed free in the lumen (Lloyd & Johnson, 1924; Hoare, 1972). These very small trypomastigotes (9-11μm long, Hoare, 1972) have a posterionuclear kinetoplast situated at the posterior terminus of the cell. It is not known if the epimastigote to metacyclic transformation is by differentiation only or whether one or more cell divisions are necessary as in T.brucei (Tetley & Vickerman, 1985). In culture, however, many intermediate forms are found, therefore a progression of intermediate stages is probably the case in this species also (personal observations, see Chapter 4).

Metacyclic trypanosomes do not divide and are infective to the mammalian host. Infectivity to the mammal is associated with possession of a monomolecular glycoprotein surface coat (Vickerman & Luckins, 1969; Cross, 1975; Rovis et al., 1978); the variation in the glycoproteins making up this coat is genetically controlled (Majiwa et al., 1985) and change within a strain during an infection in a host (Wilson & Cunningham, 1970; Masake et al., 1983). This mechanism is

used by the trypanosomes to evade the mammalian immune response. Thevenaz and Hecker (1980) noted that some attached trypanosomes in the tsetse fly labrum were coated; this also suggests that metacyclogenesis in T.congolense is similar to T.brucei (Tetley & Vickerman, 1985). Metacyclic and bloodstream trypomastigotes possess the surface coat which protects them from attack by the mammalian host's non-specific defense mechanisms in a similar way to that shown for T.brucei (Ferrante & Allison, 1983). The coat is absent from all other forms found in the tsetse.

Changes in cytoplasmic morphology occur in the trypanosomes throughout their life cycle. These changes have been documented thoroughly for T.brucei but similar research has not, in the main, been undertaken for T.congolense.

In T.brucei the most dramatic change is that of mitochondrial morphology. In the bloodstream forms the cristae are tubular in shape and the mitochondrion appears unbranched (Vickerman, 1962), in the uncoated insect forms the cristae appear plate shaped and the mitochondrion branched. This change in morphology also occurs in T.congolense (Vickerman, 1969b; Evans & Ellis, 1979). Activation of the mitochondrion in T.brucei occurs during bloodstream to procyclic transformation or possibly in the short stumpy (non-dividing) bloodstream forms and regression occurs during the epimastigote stage. The changes in the mitochondrion reflect respiratory changes also. T.brucei bloodstream trypomastigotes obtain energy from glycolysis alone and do not oxidise pyruvate in the mitochondrion but excrete it. Glycolysis in trypanosomes is undertaken in discrete organelles, the glycosomes. Oxidation of reduced pyridine nucleotides generated in the glycosome is via a linked glycerol-3-phosphate dehydrogenase-glycerol-3-phosphate oxidase system, the dehydrogenase being located in the

glycosome, the oxidase in the outer mitochondrial membrane (Opperdose, 1985). In the insect, an amino-acid based energy metabolism and cytochrome-mediated terminal respiration occurs (Bowman & Flynn, 1976). In T.congolense repression of the mitochondrion in the bloodstream phase is probably not as complete as in T.brucei (Bowman & Flynn, 1976). The glycosomes also change morphology during the trypanosome life-cycle being mostly bacilliform in the bloodstream trypanosomes and spherical in the insect phases (Böhringer & Hecker, 1975). Microtubule rearrangement in the pellicular sheath must also occur as the trypanosome changes morphology and size throughout its life history. The form these rearrangements take is unknown.

1.2.6. Life cycle patterns - programmed or plastic?

At different stages in its cycle of development the trypanosome must be expressing different genes. The expression of some of these genes is probably due to control by the ambient environment e.g. during the transition from bloodstream to procyclic forms in T.brucei and T.congolense at 28°C. During this transformation variable antigen gene expression is switched off. All organisms in the midgut must express the appropriate genes to enable them to utilize the available substrates for respiratory purposes. However, at some points in the trypanosome life cycle the environment cannot be controlling the trypanosome gene expression as trypanosomes with differing morphologies are found occupying the same environment. The possible existence of a programmed series of developmental stages, therefore, must also be considered. The development of the metacyclic form of T.brucei in the salivary glands of the tsetse (Tetley & Vickerman, 1985) through a series of intermediate stages from the epimastigote form whilst all in the same environment is strong evidence for programmed development. In the same vein, Contreras et al. (1985)

detected that metacyclic stage proteins were being synthesised before morphological metacyclic forms were present suggesting that the epimastigote forms expressing metacyclic genes were preprogrammed to develop into metacyclics and not bloodstream trypomastigotes or amastigote forms. The co-existence of slender and stumpy forms of T.brucei bloodstream trypanosomes would also suggest this change (from rapidly dividing forms to non-dividing, fly adapted forms containing an activated mitochondrion, but with no environmental change) is a programmed series of events inherent in the trypanosome.

It is not known whether the developmental cycles of trypanosomes are unidirectionally programmed i.e. stage A —> stage B —> stage C or if stage C can transform to stage B --> stage A; or whether every step in the developmental cycle is absolutely necessary - i.e. can stage B be omitted and development from A to C occur directly without the intervening form. Aberrations to life cycle pattern direction are occasionally found in culture systems. The in vitro system developed by Gray et al. (1981) for the cultivation of T.congolense proboscis stages appears to mimic the situation in the tsetse proboscis extremely well, however, in cultures established for a few days, and which were initiated from epimastigote forms, morphologically proventricular or procyclic forms are often found, this intriguing observation raises the possibility that the pattern of differentiaion might be more plastic than is thought, at least in the insect phase of development.

1.3. FLAGELLAR ATTACHMENT: AN IMPORTANT ROLE IN TRYPANOSOMATID LIFE CYCLES?

One characteristic common to the majority of the trypanosomatid life histories outlined in the preceding sections is that during their development the flagellates attach to their invertebrate host. In many

cases this attachment phase precedes the developmental form involved in transmission to a new host. This is particularly evident in species of Trypanosoma; both stercorarian and salivarian trypanosomes attach as epimastigote forms from which the infective metacyclic trypomastigotes develop. Infective stages of Leptomonas and Blastocrithidia species, amastigote cysts or 'straphangers', also differentiate from an attached form.

The constancy of the attachment and the similar morphology of the attachment in all cases (section 1.3.1) suggests that attachment is an important feature of development within this group of organisms. The reason for the importance could possibly be due to a need for surface contact for nuclear division and cytokinesis as has been noted in other protozoa e.g. the marine amoeba Paramoeba pemaquidensis (Martin, 1985). This would seem unlikely, however, in view of the fact that trypanosomatids can also divide unattached in other phases of their life cycle. Another reason for attachment could be to act as a trigger for development of the next morphological stage e.g. epimastigote to metacyclic differentiation. Attachment could act as an environmental trigger for any programmed development occurring at this point in the life cycle (section 1.2.6). Attachment, however, does not appear to be necessary for metacyclic differentiation in the stercorarian subgenus Schizotrypanum; T. (Schizotrypanum) cruzi epimastigotes are able to multiply and differentiate into metacyclic forms whilst unattached in liquid phase cultures (Camargo, 1964). Attachment playing a developmental trigger role would also appear to be unnecessary in genera such as Crithidia which do not produce different morphological forms; both attached and unattached choanomastigotes of this genera can replicate and attached nectomonads and unattached haptomonads are interchangeable.

TABLE 1:1. ELECTRON MICROSCOPICAL STUDIES ON TRYPANOSOMATID ATTACHMENT

SPECIES	INVERTEBRATE HOST	LOCATION OF ATTACHMENT	COMMENTS	REFERENCE
LOWER TRYPANOSOMATIDS				
Crithidia fasciculata	Anopheles gambiae (Diptera)	Rectum.		Brooker,1971b.
Herpetomonas muscarum	Chrysomyia chloropyga (Diptera)	Hindgut.		Brun,1974.
Leptomonas oncopelti	Oncopeltus fasciatus (Hemiptera)	Rectal glands.		Lauge and Nishioka,1977.
Leptomonas species	Siphonaptera sp.	Hindgut, rectal ampullae.		Molyneux and Ashford,1975. Molyneux et al.,1981.
Blastocrithidia triatomae	Triatoma infestans (Hemiptrea)	Hindgut, rectal glands.		Mehlhorn et al.1979.
B.gerridis	Gerris sp. (Hemiptera)	Hindgut, rectal glands.	SEM study.	Tieszen et al.,1983. Schaub and Böker,1986.
B.familiaris	Lygaeus phaderus	Rectal glands.		Tieszen et al.,1986.
LEISHMANIA				
L.mexicana amazonensis	Lutzomyia longipalpis	Oesophageal valve, pharynx, cibarium		Molyneux et al.,1975.
L.b.braziliensis	" "	Pylorus,hindgut.		Killick-Kendrick et al.,1977.
L.infantum	Phlebotomus craisi	Oesophageal valve.		Killick-Kendrick,1979.
TRYPANOSOMES OF AMPHIBIANS				
T.rotatorium	Batracobdella picta (leech)	Gastric caeca epithelium.		Desser,1976.
TRYPANOSOMES OF REPTILES				
T.grayi	Glossina tachanoides	Hindgut.		Molyneux,1980.
TRYPANOSOMES OF BIRDS				
T.avium	Simulium rugglesi (Diptera)	Hindgut,rectum.		Desser,1977.
TRYPANOSOMES OF MAMMALS				
STERCORARIA				
T.lewisi	Nosopsyllus fasciatus (Siphonaptera)	Rectum.		Molyneux,1969.
T.melophagium	Melophagus ovinus (Diptera)	Pylorus,hindgut, rectum.		Molyneux,1975.
T.cruzi	Dipetalogaster maxima (Hemiptera)	Rectum.	Flagellum not expanded	Zeldon et al.,1977.
	Triatoma dimidiata	Rectum.		"
	T.infestans	Rectum.	SEM study.	Böker and Schaub,1984.
SALIVARIA				
T.vivax	Glossina fuscipes (Diptera)	Labrum.		Vickerman,1973.
		Labrum.		Tetley et al.,1981.
T.congolense	G.m.morsitans	Labrum.		Evans et al.,1979. Thevenaz and Hecker,1980.
			SEM study.	Molyneux et al.,1979. Molyneux,1980.
		Labrum and hypopharynx.		Gray et al.,1981.
T.brucei	G.m.morsitans	Salivary glands.		Steiger,1973.Tetley and Vickerman,1985.
		Labrum.		Jenni et al.,1980.

TABLE 1:1:B.

IN VITRO STSTEMS

SPECIES	SUBSTRATUM	REFERENCE
Crithidia fasciculata	Millipore filters.	Brooker 1971a.
Trypanosoma blanchardi	Fal plastic flask and BHK cells.	Hommel & Robertson 1976.
T.congolense	Tissue culture flasks.	Gray et al. 1981.

Attachment could, of course, simply be a preventative measure against being removed from the invertebrate host in the faeces or saliva, by attaching the parasites can be assured of remaining within their invertebrate host and therefore the infection can continue. Attachment could simultaneously function as a holdfast and developmental stage trigger, one does not exclude the other.

The consistent morphology of trypanosome attachment and the possible relationship of this attachment to infective stage development, at least in the Salivaria, suggests that disruption of attachment may lead to the loss of production of infective flagellates. If this is indeed the case then a halt to mammalian infection could be achieved by preventing attachment. Very little is known as to the components involved internally and externally at the trypanosome attachment site. If unique molecules were to be found located in the attachment area, then the possibility of targeting drugs against these and thus preventing attachment would exist.

1.3.1. The structural basis of Trypanosomatid attachment

1.3.1.1. Flagellum-substratum attachment: Adherence of the flagellum to the body surface is found in several flagellate groups, however the ability of the flagellum of many parasitic kinetoplastids to attach to host surfaces and to other flagella appears to be unique. Many workers have studied the ultrastructure of trypanosomatid flagellates during development in their insect hosts (reveiwed by Molyneux, 1977,1983, see table 1.1). The ultrastructure of the attachment site in all salivarian epimastigote trypanosomes, regardless of whether attached to invertebrate cuticle or epithelia conforms to an overall pattern. Details of the attachment morphology vary between species but, in all cases transmission electron microscopy shows that the site of attachment is marked by an electron dense, filamentous plaque of

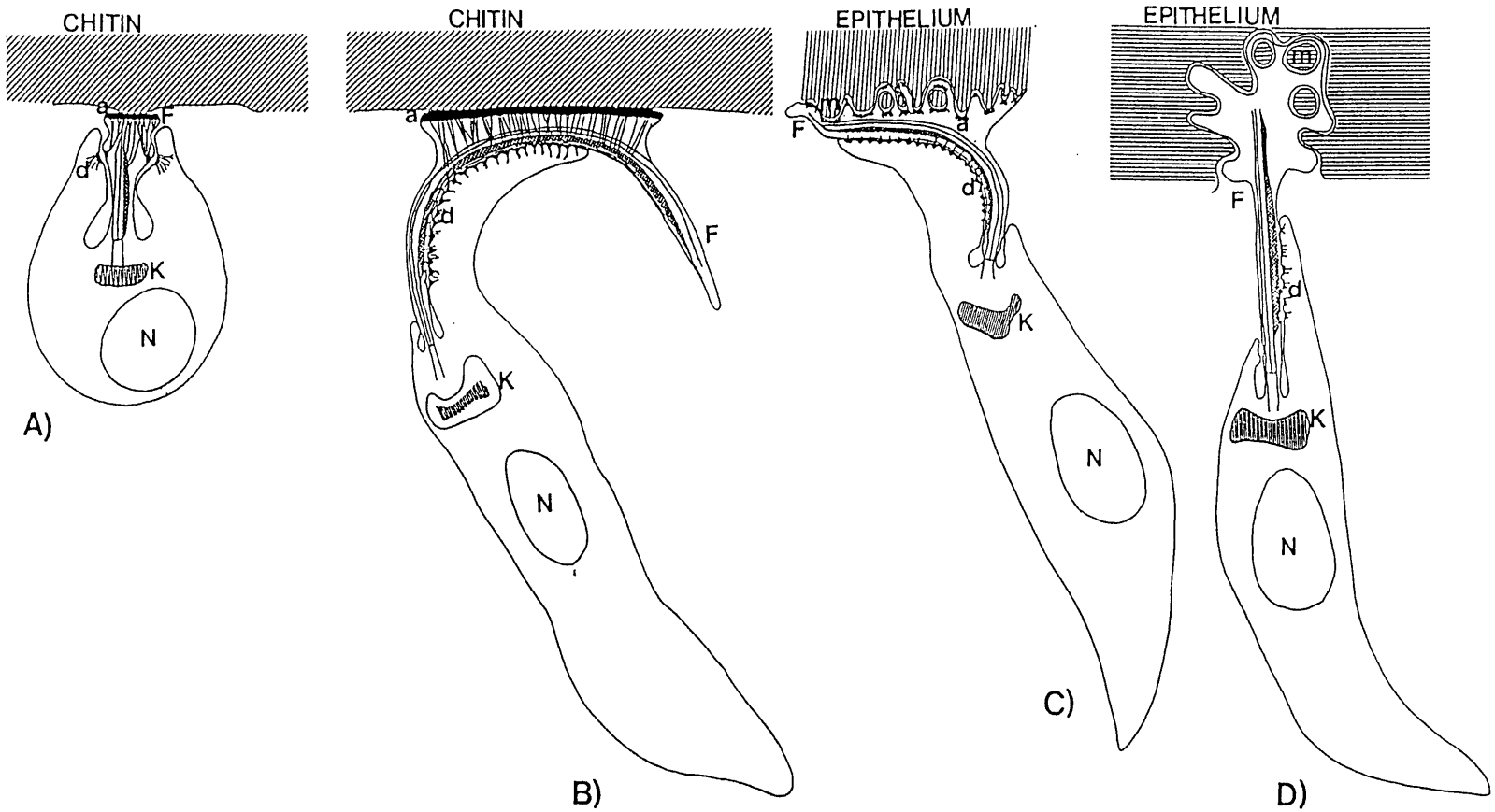

CHITIN
a
F
d
K
N
A)
CHITIN
a
d
F
K
N
B)
EPITHELIUM
m
F
a
d
K
N
C)
EPITHELIUM
m
F
d
K
N
D)

FIG.1:3. Diagram of attached trypanosomatids depicting the different forms of attachment found in this order.

A) Crithidia fasciculata attached to the rectal chitin of Anopheles sp.

B) Trypanosoma vivax epimastigote attached to the labrum of Glossina sp.

C) Trypanosoma brucei epimastigote attached to salivary gland epithelium of Glossina sp.

D) Trypanosoma cobitis attached to the crop epithelium of Hemiclepsis marginata (leech).

F-flagellum , N-nucleus , K-kinetoplast, a-attachment plaques in A) , B) and C) ,absent in D); filaments are noticably associated with the attachment plaques in A) and B).

d- flagellum-cell junctional densities and associated filaments, m-microvilli enclosed by flagellar outgrowths in C) and D), ▒-paraflagellar rod in all.

A) Adapted and modified from Brooker (1971 a&b)

B) After Vickerman (1973)

C) After Tetley and Vickerman (1985)

D) Adapted and modified from Lewis and Ball (1979)

material formed on the inner leaflet of the flagellar membrane in contact with the host surface (Fig. 1.3). Extending from this dense attachment plaque are fine-calibre filaments, these are generally arranged so that they associate with similar filaments extending from the cell body/flagellum junctions (these are generally called desmosomes although there is no evidence available that these are related to the metazoan epithelial cell-cell junctions of that name). The filaments have been called tonofilaments (Molyneux et al., 1981) (the term used for filaments found associated with vertebrate desmosomes and composed of prekeratin) and also micro-filaments (Thevenaz & Hecker, 1980) (the term normally reserved for actin-containing filaments) but there is no evidence for homology between vertebrates and trypanosome systems. These terms are best reserved for particular types of cytoskeletal elements composed of defined proteins. The size of the trypanosome filaments is 3-5nm in diameter (Brooker, 1971a&b; Vickerman, 1973). A gap between the host surface and the flagellar membrane is invariably present. In all species, this gap is in the order of 12-20nm wide and contains extracellular material of a dense filamentous nature. This small gap size appears to be critical for attachment plaque formation; at points of larger flagella-substratum gap distance the plaque is not present. Where an attachment plaque exists the flagellar membrane follows closely the contours of the underlying substratum, maintaining the small regular gap distance.

The attached part of the flagellum is usually swollen at the region forming the attachment. The position of the attachment complex [illegible] flagellum varies, for example in T.lewisi (Molyneux , 1969) Crithidia fasciculata (Brooker, 1971b), Herpetomonas sp. (Brun, 1974) and T.melophagium (Molyneux, 1975), the distal tip of the flagellum is the region of contact; the swollen attached tip

contains filamentous material originating from the dense attachemnt plaque. The microtubular axoneme and paraflagellar rod (PFR) do not change in diameter; the PFR maintains its position next to the flagellar membrane as in unattached cells. T.cruzi, on the other hand, shows no flagellar expansion although attachment plaques are present (Zeldon et al., 1977). In other trypanosome species, in particular the salivarian trypanosomes, it is the mid region of the flagellum (long axis) with which the attachment is made. The free flagellum at the distal tip of the flagellate often does not contain attachment complexes nor does the area close to the flagellar pocket in the cell body. The morphology of these attachment areas varies between subgenera. T.(Duttonella) vivax (Vickerman, 1973) epimastigotes attach to the chitinous lining of the tsetse food canal (labrum). One continuous attachment plaque, 35nm thick is formed at the attachment site. The attached region of the flagellum has an increased width compared with unattached flagella. The plaque can be broken by areas where the trypanosome-labrum gap is greater than 12nm. If the epimastigote is positioned so that more than one surface of the flagellum is opposed to the chitinous surface at a distance of 12nm, more than one attachment plaque may be present. Plaques are never found on the membrane adjacent to the PFR however. T.(Trypanozoon) brucei epimastigotes attach not to cuticle but to the microvillar border of the tsetse fly salivary gland epithelium. The flagellar membrane and the underlying periaxonemal sheath are greatly expanded (Steiger, 1973) and contain arborescent outgrowths which penetrate mbrace them. These outgrowths arise from the periaxonemal sheath including the area around the PFR (Tetley & Vickerman, 1985). Small electron dense attachment plaques (<100nm in diameter) are found within these arborizations. No continuous

attachment plaque (c.f. T.vivax) is present, only the small cup like plaques. There appears to be very little filament association with these small attachment plaques. Desser (1976) also noted that the attached flagella of T.rotatorium (an amphibian infecting trypanosome) epimastigotes attaching to gastric caecal microvilli in the leech Batracobdella picta produced membrane indentations from the swollen flagellar sheath between the epithelial microvilli, these indentations were not extensive arborizations as found in T.brucei. The T.rotatorium indentations also contained attachment plaques where they came into contact with the microvilli at 20nm or less distance. These plaques did have filaments associated with them.

The 3-5nm attachment-associated filaments have been reported by many researchers to extend from the attachment-associated plaque. Often these aggregate into a loose bundle a short distance from the plaque (Brooker, 1971 a&b, Thevenaz & Hecker, 1980) and then associate with morphologically indistinguishable filaments originating from the cell-flagellar "desmosomes". In trypanosomes these filaments have been noted to connect with certain microtubule doublets of the axoneme (numbers 6,7,8,9 and 1 according to the numbering system of Afzelius, 1959), i.e. the ones on the opposite side to those doublets associated with the PFR (Vickerman, 1973). The filament bundle can be noticeable as in C.fasciculata or merge with the axoneme cytoplasm filamentous structure (T.vivax) or virtually absent (T.brucei). In some cases (including Leptomonas sp. (Molyneux et al., 1981) Herpetomonas sp. (Brun, 1974) and Crithidia fasciculata (Brooker, 1971b) vesicles and omega profiles in the flagellar membrane have been found associated with the attachment. These vesicles are composed of flagellar membrane. In C.fasciculata, Brooker noted these to be present when he treated the cells with distilled water which caused the cells' detachment. In C.fasciculata, therefore, vesicle formation, appears to

be a method of detachment. Other authors have postulated these vesicles to be part of an uptake endocytotic mechanism due to the normal region for this (the flagellar pocket) being reduced in size by the flagellar expansion (Molyneux 1977).

1.3.1.2. Flagellum-flagellum attachment: In many cases where neighbouring epimastigotes or haptomonads are very close to each other, flagellar-flagellar 'desmosomes' have been reported. Vickerman (1973) found these in T.vivax as did Evans et al. (1979) in T.congolense, however Thevenaz and Hecker (1980) could not find these in this organism. Brooker (1970, 1971 a&b) reported these inter-flagellar attachments occurring in Crithidia fasciculata, this form of mutual flagellar attachment is the method used to maintain rosettes of these flagellates in the hindgut lumen of their Anopheles host. Molyneux et al. (1981) reported flagellar-flagellar attachments in Leptomonas species also. Desser (1976) however, noted their absence in T.rotatorium. Flagellum-cell body contact is found in some species but no attachment plaques are found in the cell body at the contact point. Plaque formation appears to be a peculiarity of flagellar attachment only.

1.3.1.3. Flagellum-body attachment: The number of cell-flagellar 'desmosomes' and their locations vary in different species. The variations are mainly due to the presence or absence of an undulating membrane. The undulating membrane is the area of the cell body which parallels the flagellum and is connected to it (Fig 1:1). Where this [illegible] these 'desmosomes' only occur around the circumference of the flagellum emerging from the reservoir. Brooker (1970) found 3-8 skewed anterior-posterior lines of these, 3-5 per line along the flagellar canal. In epimastigotes of T.vivax

(Vickerman, 1973) several parallel rows of spot 'desmosomes' occur along the proximal part of the flagellum-body junction, the number of rows is reduced distally. Thevenaz and Hecker (1980) reported up to 12 rows of these 'desmosomes' in the attached flagellar area of T.congolense. The number of rows of 'desmosomes' appears, therefore, to be increased in the region of cell-host attachment; Vickerman (1973) reported that trypanosomes unattached in the lumen of the tsetse labral canal contained only a single row of flagellar-cell body 'desmosomes'.

On the cell-body side of the trypanosome the small dense 'desmosomal' plaques (35-60nm across and conical shaped in T.vivax) are present in a gap in the microtubule pellicle, replacing a microtubule. Often a reduced diameter microtubule is located alongside. A quartet of microtubules and adjoining flagellum-associated endoplasmic reticulum associated with these run parallel to the flagellum within the cell body area of the undulating membrane (Vickerman, 1969a).

In the many studies of attached trypanosomatid flagella (Table 1.1) there is no information other than electron microscopy ultrastructure. Brooks (1978) and Vickerman (1969a) applied ruthenium red staining to trypanosomes and found positive staining within the body-flagellar gap area indicating the presence of glycoproteins in the area, but this is the only apparent venture into the realms of cytochemistry or any other type of study on the attachment of trypanosomes. No information as to proteins, carbohydrates, glycoproteins or any other possible constituent components of the

e.

1.3.1.4. Scanning electron microscopical studies of attachment: Scanning electron microscopical studies have been performed in a few

TABLE 1:2. TRYPANOSOMATIDS ATTACHING WITHOUT ATTACHMENT PLAQUE FORMATION.

SPECIES	HOST	SITE	REFERENCE
Blastocrithidia gerridis	Gerris	Ventriculus	Tieszen et al. 1983.
Blastocrithidia familiaris	Lygaeus pahderus	Midgut.	Tieszen et al. 1986.
Herpetomonas ampelophila	Drosphila melanogaster	Midgut, malphigian tubules.	Rowton et al. 1981.
Leshmania m.amazoniensis	Lutzomyia longipalpis	Midgut.	Killick-Kendrick et al. 1974.
Trypanosoma cobitis	Hemiclepsis marginata (Leech)	Crop.	Lewis and Ball 1979.
Trypanosoma melophagium	Melophagus ovinus	Midgut.	Molyneux 1975.

cases on attached epimastigotes of both stercorarian and salivarian trypanosomes (Molyneux et al. 1979, T.congolense and T.vivax; Gray et al., 1981 T.congolense; Böker & Schaub 1984, T.cruzi). Schaub and Böker (1986) also carried out SEM observations on Blastocrithidia triatomae. Schaub and Böker reported an enlargement of the flagellar axis at the point of attachment in Blastocrithidia triatomae (1986) but not in T.cruzi (1984), consistent with TEM studies by Zeldon et al. (1977). An expanded flagellar axis can also be found in T.congolense (Gray et al., 1981). SEM studies have not provided any further detail than these points.

1.3.1.5. Flagellar attachment without structural modifications: It should be mentioned that there are some species of trypanosomatids that form flagellar attachments to the substratum without developing attachment plaques. Some species appear to attach to the midgut epithelium of their invertebrate host by an interdigitation of their flagella and the host cells' microvilli. In some cases expansion of the flagellum fascilitates this. The same trypanosomatids may adhere to the chitinous surfaces of their invertebrate host by conventional, differentiated, attachments as described above. (see Table 1.2).

Trypanosoma congolense bloodstream forms have also been reported to attach to blood cells and capillary vessel endothelium (Banks, 1978, 1979, 1980; Büngener & Müller, 1976). Büngener and Müller (1976) reported that some degree of interdigitation occurred between T.congolense flagella and the blood vessel membrane. No ultrastructural differentiation was found in the trypanosomatid attachment points (see Chapter 3).

1.4. THE STRUCTURAL BASIS OF ATTACHMENT IN AMOEBAE AND METAZOAN CELLS

The structural basis of cell-substratum and cell-cell attachments have been well documented in multicellular animals. The main

FIG.1:4. A) Transmission electron micrograph showing desmosomes in bovine snout epithelium.

B) TEM of epithelial hemidesmosomes attaching the cells to basal lamina.

C) TEM of chick heart fibroblast focal adhesions (arrow) *in vitro*.

A) and B) kindly provided by Dr. C. Skerrow , C) from Heaysman *etal.* (1982).

t- tonofilaments, d- desmosome, h- hemidesmosome

junctional types resemble those of trypanosomes and are (a) focal adhesions found in cultured fibroblasts, amoebae and amoeboid cells of metazoa, (b) desmosomes found in epithelia and (c) hemidesmosomes, attaching epithelial cells to their supporting basal lamina. The ultrastructural morphology of these structures has been extensively investigated and their protein constituents characterized in relation to function. As these studies are relevant to the subject of this thesis a summary of their structure, function and components is presented here (see Fig. 1.4).

1.4.1. Focal adhesions

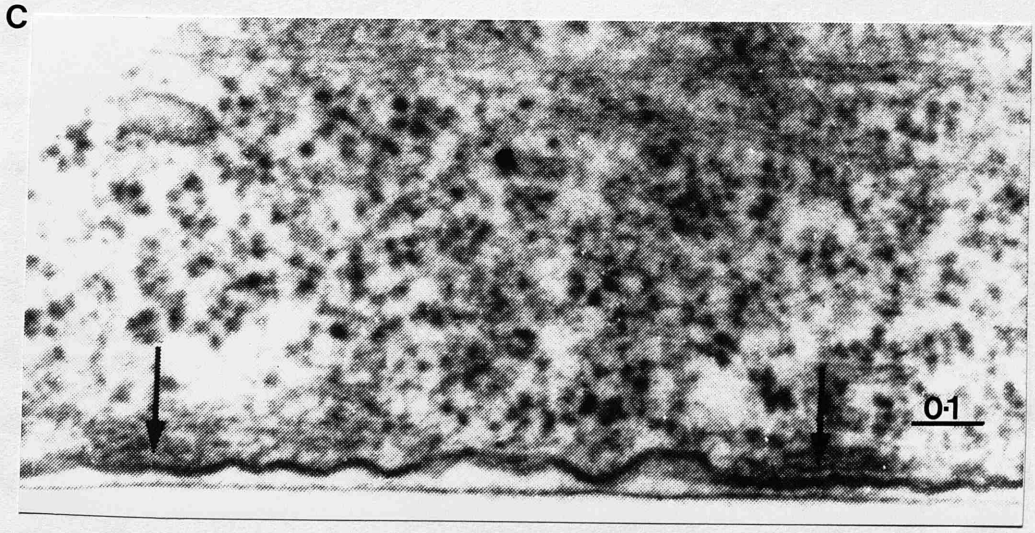
Interference reflection microscopy studies of attached fibroblasts in culture show points of close attachment to the substratum as black, close (10nm gap) focal areas of an arrowhead shape (Curtis, 1964; Izzard & Lochner, 1976; Abercrombie & Dunn, 1975). Immunohistochemical studies and electron microscopy have shown these focal contact points to be the termination sites of actin-containing stress fibre bundles (Abercrombie et al., 1971; Heath & Dunn, 1978; Wheland et al., 1979; Geiger et al., 1980). Transmission electron microscopy shows a dense staining plaque of material located on the cytoplasmic side of the membrane contact points. These plaques have an approximate area of 1 μm^2 and actin bundles insert into them.

Focal adhesions are particularly abundant under the leading lamellae of moving fibroblast type cells, the plaque of dense material forms soon after contact of the lamellipodium to the substrate and subsequently becomes associated with the termini of stress fibres. If chelating agents are added to the medium or the cells are trypsinized, the cells detach and round up and focal adhesion plaques disappear from the membrane. Focal adhesions therefore have a role in cell movement and shape maintenance. Focal adhesions in culture cells would

TABLE 1:3. MAIN FOCAL ADHESION CONSTITUENTS

COMPONENT	MOLECULAR WEIGHT ON SDS GELS	LOCATION	REFERENCE
F-actin	43 kd	filament associated domain	Abercrombie et al. 1971. Willingham et al. 1977. Badley et al. 1978.
Alpha-actinin	100 kd	"	Lazarides & Burridge 1975. Burridge & Fermisco 1981 & 1982.
Fimbrin	68 kd	"	Bretscher & Weber 1980. Bretscher 1981.
Vinculin	130 kd	membrane bound domain	Geiger 1979 , Geiger et al. 1980. Burridge & Fermisco 1980.
-	200 kd	"?	Maher & Singer 1983.
Talin	215 kd	"	Burridge & Connell 1983.
glycoprotein FC-1	60 kd	extracellular gap	Oesch & Birchmeier 1982.
Uvomorulin	120 kd	"	Boller et al. 1985.

4



appear to be the culture cell equivalent of cell-cell zonula adhaerens type junctions. These structures share certain proteins in their electron dense plaques (Geiger et al., 1981). Kartenbeck et al. (1982) showed these junctions to be EGTA-sensitive.

Immunofluorescence studies have located several proteins in the focal adhesion plaque (Table 1.3). There would appear to be two domains within the focal adhesion plaque. One of these, the filament associated domain, contains F-actin, the actin crosslinking protein filamin, and the actin-binding protein alpha-actinin. The second domain, the membrane-bound domain, has been shown to contain vinculin (Geiger, 1979; Geiger et al., 1980; Burridge & Fermisco, 1980) and talin (Burridge & Connell, 1983). Double labelling immunofluorescent studies have shown vinculin to be much closer to the plasma membrane than actin (Geiger et al., 1981; Tokuyasu et al., 1981). Using actin severing proteins e.g. fragmin, Avnur et al. (1983) showed that vinculin and talin remained at the membrane after treatment whereas actin, alpha-actinin and filamin were removed.

Other actin binding proteins e.g. spectrin, tropomyosin and myosin would appear to be absent from the focal adhesion area or in very low concentrations (reviewed by Mangeat & Burridge, 1984). More recently other proteins e.g. a 200kd protein isolated from cardiac muscle fasica adherens (Maher & Singer, 1983), the microvilli microfilament associated protein, fimbrin (Bretscher, 1981), and a 190kd protein isolated from chicken gizzard (O'Halloran & Burridge, 1986) have also been located in the focal attachment plaques. The roles played by each of these proteins within the focal adhesion plaque is largely surmisery drawn from their relative locations within the plaque and their binding properties for other members of the group.

Vinculin was thought to bind actin but recently it has been shown (Evans et al., 1984; Rosenfeld et al., 1985; Wilkins & Lin, 1986) that this binding is not due to vinculin itself but to an undefined contaminant of vinculin preparations. Vinculin and talin, however, do bind to each other. These two proteins tend to colocalize in the cell and are also found to parallel the distribution of fibronectin on the outside of fibroblasts. Talin has been shown to bind a fibronectin receptor (CSAT receptor) but how relevant this is to focal adhesions is debatable, as many workers have shown that fibronectin is largely absent from focal adhesion areas under normal culture conditions (Avnur & Geiger, 1981; Mattey & Garrod, 1984; Morgan & Garrod, 1984). As talin would appear to be able to bind extracellular proteins, possibly by an intermediary integral protein it is possible that talin is directly anchored to the plasma membrane and then binds vinculin. It is known these two proteins form at newly initiated contact sites and then subsequently actin filaments form - these plaque proteins possibly acting as microfilament bundle organizing centres.

However, vinculin could bind to the membrane independently of talin, zonula adherens, cell to cell junctions in both epithelial and cardiac muscle cells, have been shown to contain vinculin but talin has not been detected (Geiger et al., 1981).

Wilkins and Lin (1982) showed that the 'barbed' end of actin filaments (as viewed when actin filaments are decorated with heavy meromyosin) is bound by the vinculin or vinculin contaminant protein. It is assumed that the alpha-actinin, fimbrin and other actin bundling proteins present at the attachment site serve to bind the associated actin filaments into stress fibre bundles. Control of binding would appear to be partially by calcium ions (alpha-actinin binds actin in a calcium-sensitive manner) and possibly also by vinculin phosphorylation (Sefton et al., 1981).

Integral membrane proteins have not been identified at focal adhesion sites, but Geiger *et al.* (1982) showed by fluorescence recovery after photobleaching (FRAP) that there were two populations of integral membrane proteins, one population freely mobile through the attached membrane region, the other population immobile, possibly due to a cytoskeleton or substratum interaction. Oesch and Birchmeier (1982) showed that antibodies against a glycoprotein (FC-1) inhibited contact and that this integral membrane protein possibly conferred an attachment to the substratum. Badley *et al.* (1978) provided evidence of lectin binding to the outside of contact areas and uromorulin, an adhesive embryonic glycoprotein, is present in the intercellular space of *zonula adherens* (Boller *et al.*, 1985). These results would suggest that an integral membrane glycoprotein binds to an element of the dense-staining attachment plaque, either directly or by intermediary proteins, and in turn the plaque elements bind the cytoskeletal elements of the stress fibre system.

The above description deals with the focal adhesion sites found in fibroblastic cells in culture. Amoeba and amoeboid metazoan cells also display morphologically identical attachment points (Preston & King, 1984), from which actin fibre bundles extend, whether the proteins described above are also present in these sites is not reported in the literature.

1.4.2. Desmosomes

Desmosomes or *maculae adherentes* (Farquhar & Palade, 1963) are cell organelles involved in intercellular adhesion (Overton, 1975). These structures occur in the majority of epithelial types. Desmosome ultrastructure is similar in all vertebrate species (reviewed by Overton, 1975 and Skerrow, 1986). Many invertebrates also possess junctions with an ultrastructure similar to that of vertebrate

desmosomes (e.g. in Rhodnius abdominal musculature, Toselli & Pepe, 1968). Structurally these organelles consist of parallel cell membranes separated by an intercellular space of ~25-35nm. On the cytoplasmic side of the membranes are electron dense plaques 10-25nm thick, these may be separated from the inner leaflet of the membrane by a 3nm gap (Farquhar & Palade, 1963; Kelly, 1966; Overton, 1975; Henderson & Weber, 1981). Tonofilaments (10nm diameter) composed of prekeratin, a class of intermediate filament (IF), converge towards the plaque; many appear to abut laterally in a looping or arching configuration (Staehelin, 1974; Kelly & Shienvold, 1976). Smaller calibre filaments may occupy the plaque-membrane region (reviewed by McNutt & Weinstein, 1973). Kelly & Kuda (1981) and Pribazari & Kelly (1985) have suggested these filaments might serve as mechanical couplers between the intermediate filaments and the membrane, possibly linking to intramembranous proteins. The extracellular space contains extracellular material, this is often organized into an electron dense mid-line halfway between the opposed plaques.

The relationship between desmosomes and intermediate filaments (IFs) is an area of current debate and the contrasting results in the literature may be related to different cell types. Some workers (Franke et al., 1982) suggest that these two sets of structures have an interdependent formation due to concomitant appearance and disappearance of plaque material and tonofilaments in embryonic cells. However, Denk et al. (1985) have shown that the maintenance and position of desmosomal organization is not dependent upon a physical interaction of desmosomes with the IF cytoskeleton. Lentz and Trinkhaus (1971) suggest that the association of IFs with the desmosomal plaque is secondary to formation of the desmosome, the plaque acting as an organizing centre for the IF bundle. Other workers

(Hennings et al., 1980; Jones et al., 1982) however, have found that desmosomes do not act as IF organizing centres or polymerization sites for IF bundles in cultured mouse keratinocytes.

Desmosomal development involves reorganization of insoluble proteins (Jones et al., 1982; Jones & Goldman, 1985) and no de novo protein synthesis is required for desmosome formation in mouse keratinocytes in culture (Hennings & Holbrook, 1983). Desmosome formation appears to be controlled, in culture at least, by the levels of exogenous calcium ions present. If the calcium ion concentration is increased after being kept low, rapid and synchronous accumulations of desmosomal components in regions of intercellular contact are found (Hennings et al., 1980; Jones et al., 1982; Hennings & Holbrook, 1983; Watt et al., 1984). On the other hand a decrease in calcium ions either by placing in low calcium solutions or by chelating calcium ions causes a disruption of the cell adhesion and a movement of the plaque and IF material away from the membrane area into the cell interior (Overton, 1962; Borysenko & Revel, 1973; Dembitzer et al., 1980; Jones et al., 1982; Kartenbeck et al., 1982; Hennings & Holbrook, 1983; Mattey & Garrod, 1986a&b). Trinkhaus-Randall and Gipson (1984) suggested that the calcium-dependent formation of desmosomes is mediated by calmodulin and Tsukita and Tsukita (1985) isolated a 240kd calmodulin-keratin filament binding protein from desmosomal plaque material. They called this protein Desmocalmin.

These findings have led Jones and Goldman (1985) to propose a model for desmosome formation in cultured keratinocytes. They proposed that desmosomal precursors are moved along with associated IFs to sites of cell to cell contact on addition of exogenous calcium. These packages then aggregate to form desmosomes. In this model the association of the desmosome precursor-IF complex with the plasma membrane acts as an initiator of desmosome formation (but the

TABLE 1:4. MAIN DESMOSOME CONSTITUENTS

COMPONENT	MOLECULAR WEIGHT ON SDS GELS	LOCATION	REFERENCE
Desmoplakin I	205 kd	Plaque	Franke et.al. 1981 & 1982.
Desmoplakin II	230 kd	Plaque	Franke et.al. 1981 & 1982.
Desmoplakin III	81 kd	Plaque	Gorbsky et.al. 1985.
-	86 kd	Plaque	Gorbsky & Steinberg 1981.
Glycoprotein triplet	150 kd	? not surface	Gorbsky & Steinberg 1981.
Desmocollin I (glycoprotein)	100 kd	extracellular gap location	Cowin et.al. 1984.
Desmocollin II (glycoprotein doublet)	115 kd	extracellular gap location	Cowin et.al. 1984.

desmosome does not act as a polymerization or initiation site for elaboration of IFs). Transmembrane or extracellular components are then involved in alignment of half desmosome precursor structures present in adjacent cells. It is proposed that when two such sets of extracellular components come into contact they bind to each other and then desmosome formation can be completed.

The isolation of desmosomes from bovine nasal epithelium was achieved by Skerrow and Matoltsy (1974a&b). Gorbsky and Steinberg (1981) adapted the isolation procedure to obtain desmosomal cores, structures consisting of the membrane and intercellular components but with greatly reduced plaques and devoid of tonofilaments. Desmosomal cores consist of five major groups of proteins (Table 1.4) (reviewed by Garrod & Cowin, 1985 and Skerrow, 1986). These consist mainly of two proteins of 205kd and 230kd, these proteins are located in the desmosomal plaque (Franke et al., 1981, 1982) and have been named Desmoplakin I and II, these proteins are closely related and share antigenic sites. Jones and Goldman (1985) provided evidence that Desmoplakin I is associated with the intermediate filament bundle during desmosome formation. Two other proteins of 81 and 86kd are also present in desmosomal cores; the 81kd protein has been located in the plaque and named Desmoplakin III (Gorbsky et al., 1985). The 86kd protein may also have a plaque location (Gorbsky & Steinberg, 1981). Three groups of glycoproteins (Desmogleins - Gorbsky & Steinberg, 1981) are also found in isolated desmosomal cores; their enrichment in desmosomal cores was thought to signify an intercellular location (Gorbsky & Steinberg, 1981). However, antibodies to the glycoprotein triplet of 150kd do not react with the surface of living cells. The two remaining glycoprotein groups - a glycoprotein of 100kd and a glycoprotein doublet of 115kd have been located on the cell surface of

desmosomes (Cowin et al., 1984) and are thought to play a role in desmosomal adhesion (Cowin et al., 1984; Garrod & Cowin, 1985). These glycoproteins have been named Desmocollins I & II (Cowin et al., 1984) and are immunologically related to each other but not to the 150kd glycoprotein group.

These desmosomal constituents are widely conserved in vertebrate cells (Cowin & Garrod, 1983; Cowin et al., 1984,1985; Sruhbier & Garrod, 1986) and mutual desmosome formation between several cell types from different species can also be formed in culture (Mattey & Garrod, 1985).

1.4.3. Hemidesmosomes

Structurally these cell-basal lamina junctions are very similar in appearance to one half of a desmosome. An electron dense ellipsoidal shaped 10-20nm thick plaque with which tonofilaments associate. An extracellular gap between the cell and basal lamina exists, sometimes containing dense material, probably composed at least partially of glycoprotein. These structures function to attach basal cells of stratified squamous epithelia to their substratum, the basal lamina. These junctions, through their associated tonofilaments, probably serve to exert tension, distributing force throughout the cells and thus play a part in the maintenance of cell shape (Kelly, 1966; Krawczyk & Wilgram, 1973). Hemidesmosomes are reported in a wide variety of vertebrate epithelia and also some invertebrate tissues (Toselli & Pepe, 1968).

Very little information other than the purely structural is available about hemidesmosomes, the bulk of their supposed properties and structure being extrapolated from observations on desmosomes. Trinkaus-Randall & Gipson (1984) showed that hemidesmosome formation is dependent on calcium concentration and that this dependence is

mediated by a calmodulin-regulated mechanism. Recent reports (Shellswell et al., 1986) of formation of hemidesmosomes in vitro in the absence of basal lamina anchoring fibrils suggests that intrinsic factors may be important in the control of hemidesmosome formation. Immunohistochemical studies (Franke et al., 1981; Mueller & Franke, 1983) have located the presence of Desmoplakins I and II in hemidesmosome plaques in bovine mucosal and squamous epithelia. No other reports of desmosome components being localized in hemidesmosomes have been found in the literature, so whether or not the desmosome surface glycoproteins (Desmocollins) are present on the hemidesmosome surface is unknown.

1.5. ATTACHMENT AND THE CYTOSKELETON IN TRYPANOSOMATIDS

The attachment mechanism of trypanosomatids appears to be intimately involved with the cytoskeleton. The cytoskeleton of trypanosomes is in parts well researched and in others virtually unknown. Microtubules and tubulin in trypanosomes fall into the former category. The descriptions of arrangements of microtubule structures within trypanosomatids are extensive and have been reviewed by Vickerman & Preston (1976). Basically, the trypanosomes are enclosed by a layer of pellicular microtubules below their cell membrane and have the usual 9+2 arrangement of microtubules in the flagellar axoneme. Intranuclear microtubules are also evident during mitosis of this organism. Recently, molecular studies on tubulin have been the subject of many research studies, particularly using the species Crithidia fasciculata (Russell et al., 1984; Russell & Gull, 1984) and T.brucei (Seebeck et al., 1983a&b; Thomashow et al., 1983; Steiger et al., 1984; Kimmel et al., 1985; Imboden et al., 1986; Schneider et al., 1986; Sherwin et al., 1986). Genetic studies (Seebeck et al., 1983a; Thomashow et al., 1983; Kimmel et al., 1985; Imboden et al.,

1986) have shown that the trypanosome genome contains about 10 alpha and 10 beta tubulin genes in a tightly clustered array of alternating alpha and beta genes. Sather & Agabian (1985) and Imboden et al. (1986) have shown that a single size class exists from each alpha and beta tubulin mRNA.

Two major protein subunits, alpha and beta tubulins, exist in T.brucei (Steiger et al., 1984). Schneider et al. (1986) have shown that the alpha tubulin fraction is composed of two isotypes, $alpha_1$ and $alpha_3$ the relative abundances of which do not change throughout the organisms life cycle. $Alpha_1$ is the primary translation product of the alpha tubulin mRNA and $alpha_3$ is a post-translational acetylation modification of this. $Alpha_3$ is the most abundant alpha isotype in the flagellum (almost the entire alpha-isotype component of the flagellum is $alpha_3$) and subpellicular tubules and in consequence, the most abundant form of alpha-tubulin in the cell. A similar alpha-tubulin isotype distribution was also shown to be present in C.fasciculata (Russell et al., 1984; Russell & Gull, 1984). Beta-tubulin remains as one isotype throughout the life cycle of the organism (Schneider et al., 1986). Sherwin et al. (1986) have shown that the alpha-tubulin can be postranslationally tyrosinylated at their carboxy terminal ends, this modification being associated with growing microtubules. The beta-tubulin isotype also contains a tyrosine carboxyterminal end but this is not due to post-translational changes (Kimmel et al., 1985). Differing microtubule stabilities between trypanosomes and hosts have led Seebeck & Gehr (1983) to suggest that microtubules could be used as a target for antitrypanosome drugs. Nothing is known of the biochemistry or genetics of tubulin in T.congolense.

Actin, in contrast to tubulin, has had very little attention paid to it with respect to trypanosomes. One study (DeSouza et al., 1983) reported its identification by fluorescence immunocytochemistry in

T.cruzi epimastigotes, located throughout the cell and particularly in the flagellum. No reports of intermediate filaments or any actin- or tubulin-associated proteins are available for trypanosomatids.

The lack of studies on the cytoskeleton and attachment in trypanosomes means that little is known as to the presence of components of metazoan cell attachment sites in these protozoa. It is unknown if vinculin, talin, alpha-actinin, filamin or other components of the focal adhesion attachment plaques found in amoeboid metazoan cells are present (reviewed by Mangeat & Burridge, 1984; see Section 1.4.1) or if any desmoplakin-like or desmocollin-like material found in desmosomes or hemidesmosomes (reviewed by Cowin et al., 1984; see Sections 1.4.2. and 1.4.3.) are present. If any of these known proteins are situated in the trypanosomes attachment site then possible functional analogies can be drawn; it is unknown if the attachment is purely anchoring, as with hemidesmosomes/desmosomes or could also have a role in cell movement e.g. in the initial stages of attachment, in an actin/focal adhesion like manner. However if the trypanosome attachment was found to be novel in component structure then this novelty could be used as a drug target site for inhibition of trypanosome attachment and thus metacyclic development (see Chapter 5) at least in the economically important species in this study.

1.6. THE TRYPANOSOMA CONGOLENSE EPIMASTIGOTE CULTURE SYSTEM

1.6.1. History

Cultures of T.congolense have been initiated by a number of researchers, usually at temperatures between 22 and 28^{o}C and using trypanosomes from the bloodstream of the vertebrate host (reviewed Bishop, 1967; Evans, 1978). These bloodstream trypanosomes rapidly transformed in vitro into non-infective procyclic forms. Other workers have serially maintained procyclic T.congolense in the presence of

Glossina tissues and organ explants at 28°C (Trager, 1959; Cunningham, 1973,1977). This organism has also been maintained in the presence of an established Glossina cell line (Steiger et al., 1977) and with mouse lung explants at 37°C (El-on et al., 1977). In very few of these cases did mammal-infective forms develop and even then only occasionally at 37°C (Steiger et al., 1977).

Gray et al. (1979) were able to set up primary cultures of T.congolense in vitro at 37°C with bovine dermal explants excised from local reactions at the sites of tsetse bites. These cultures showed parasite multiplication and retention of infectivity for up to 21 days. However, it was not possible to subpassage these cultures and the extensive cellular outgrowth from the dermal explants contributed to the decline in trypanosome numbers. Gray et al. (1981) overcame this problem by initiating primary cultures with trypanosomes from the mouthparts of infective G.morsitans in the presence of bovine dermal explants and maintaining these cultures at 28°C. Within 48 hours of placing infected proboscides in the culture flask beside the dermal explant, trypanosomes were present in the culture medium in the vicinity of the dermal explant. These numbers steadily increased and by day 40 the base of the flasks were covered with an extensive layer of adherent epimastigotes. Cultures were infective to rodents from approximately day 14 onwards. The proboscides and collagen explants could be dispensed with on subpassaging (Gray et al., 1981); long-term cultures containing rapidly dividing adherent epimastigotes and producing metacyclic trypanosomes from approximately day 10 onwards were established. Hirumi et al. (1982) reported a similar T.congolense culture system for initiating infective cultures from tsetse proboscides or infective blood, however, instead of dermal collagen explants a commercial product, Vitrogen (the Collagen corporation,

Pualo Alto, California) was used to stimulate epimastigote attachment.

Light microscope examination of developmental forms present in these cultures showed that forms present in the culture system were equivalent to those described as present in the tsetse mouthparts infected with T.congolense (Lloyd & Johnson, 1924). Gray et al. (1981) also established by a brief EM morphological study that the attached epimastigotes in culture were equivalent to those in the tsetse fly proboscis.

1.6.2. Morphology of trypanosomes present in the culture system

A variety of morphological types were observed in Giemsa stained smears of culture supernatant. These included forms morphologically similar to procyclic and proventricular forms normally found in the tsetse fly and gut; epimastigote stages of variable lengths from short, rounded epimastigotes to long epimastigotes with drawn out posterior regions and blunt posterior ends. Epimastigotes were frequently found as clusters. Short trypomastigotes with subterminal kinetoplasts and poorly developed undulating membranes were also present, these were identical to metacyclic forms found in the tsetse proboscides. Forms resembling intermediate stages between epimastigotes and metacyclics were also visible.

1.6.3. The use of in vitro grown epimastigotes in experimental work

The advantages of the Gray culture system over in vivo material for studying attachment and metacyclogenesis lie in the large number of both epimastigotes and metacyclics available for experimental study and observation. As many as 1.6×10^6 metacyclics per ml can be generated over a two day period of culture in comparison to the occasional output of 350 metacyclic trypanosomes per infective G.morsitans (Harley & Wilson, 1968). The numbers of metacyclics make molecular studies such as those of Crowe et al. (1983) possible and

the large numbers of attached epimastigotes make possible biochemical/ molecular studies of this phase also.

CHAPTER 2

OBSERVATIONS ON THE BEHAVIOUR OF TWO CLONED STOCKS OF *T.CONGOLENSE* IN EPIMASTIGOTE CULTURE

2.1. INTRODUCTION

The Trypanosoma congolense epimastigote culture system of Gray et al. (1981), described in section 1.6, was used in the present study as a source of organisms to study epimastigote attachment. The importance of the culture system to the research described in this thesis warrants some description of the culture system and that some comparisons are made of the two separate stocks of T.congolense used.

The behaviour of T.congolense epimastigotes in culture has previously been described by Gray et al. (1981), Hirumi et al. (1982) and by Gray et al. (1984), the latter study being carried out on cloned stocks including TREU 1457, also described in the present account. In the following report, the sequence of events has been observed for stocks TREU 1457 and TREU 1627. The observed behaviour was essentially as described by Gray et al. (1984) however, variations in metacyclic number and the time of metacyclic appearance in the cultures was noted between the two stocks.

2.2. MATERIALS AND METHODS

2.2.1. Derivation of T.congolense stocks used in the present study

Two stocks have been used throughout the present study, stock T.congolense TREU 1457 and stock T.congolense TREU 1627.

Stock TREU 1457 is a cloned derivative of TREU 1290 (Luckins et al., 1983). The original isolate code for this Nigerian T.congolense is Zaria/67/LUMP/69 and was isolated from an ox. Gray et al. (1984) describe the use of this clone (TREU 1457) in establishing a epimastigote culture.

Stock TREU 1627 is a cloned derivative of a Gambian isolate, original isolate code is Kantong Kunda/77/LUMP/1794 (Ross et al., 1985), the behaviour of this stock in epimastigote culture has not been previously described in the literature.

2.2.2. The culture medium

After testing several media e.g. RPMl, MEM, Medium 199, Gray et al. (1981) concluded that a minimum essential medium (MEM)-based medium was optimal for reproducing this biological system. The present study used medium of the following composition; Eagles' minimum essential medium (MEM) with Earle's salts (Gibco Europe Ltd., Paisley), to this HEPES (Sigma Ltd.) was added to a final concentration of 20mM; 7% Na H CO_3 was also added and the medium pH brought to 7.2-7.3 with 5M NaOH. This medium was filtered through a 0.22um sterile filter and stored at $4^{o}C$. Before use, complete culture medium was constituted, by the addition of foetal calf serum (FCS) to a final concentration of 10 or 20% and L-glutamine to a final concentration of 20mM, the medium was often refiltered after these constituents had been added. Complete medium was not usually stored for more than one week. No antibiotics were added as these were noted by Gray et al. (1981) to have adverse effects on the trypanosomes. It was found necessary to test individual batches of FCS (Gray et al., 1984) as great variations in the numbers of metacyclics produced in vitro were found with different batches of serum. Sera which supported epimastigote growth without a massive amount of death and produced $1x10^{6}$ metacyclics/ml were selected. MEM batches were not found to vary greatly but Gray et al. (1984) report that batches over 4 months old produce fewer metacyclics in culture.

2.2.3. Culture maintenance

Initially T.congolense epimastigote cultures were obtained from the Centre for Tropical Veterinary Medicine, University of Edinburgh. These cultures contained adherent epimastigotes and epimastigotes and metacyclics in the supernatant.

New cultures were initiated from such cultures by simple subpassaging. Mature cultures containing in the order of $1x10^7$ trypanosomes/ml culture supernatant and including many bundles of epimastigotes in that supernatant were the cultures of choice for passaging. One ml of the supernatant from a well-washed culture was placed in a gassed (5% CO_2) flask ($25cm^2$ Nunc, Gibco, or Falcon), the flask was gently shaken so that the liquid covered the bottom of the flask and then 3ml of 10% FCS MEM culture medium was added. Cultures were maintained at 28^oC. Culture medium was changed completely after 48 hours. After approximately 72 hours in culture, medium containing 20% FCS was used for maintenance of cultures. Once the adherent layer of epimastigotes had covered the culture flask base it was necessary to scrape some of the epimastigotes off the base during culture medium change. This procedure provided room for the attachment of free epimastigotes.

2.2.4 Sampling from culture supernatants.

Observations of cultures were made at 48 hour intervals using an inverted phase contrast microscope (Leitz) (magnification 320x). Smears of culture supernatant were made at each 48 hour medium change. These were air dried and methanol fixed (2min). When dry they were hydrolyzed in HCl (1N) at 60^oC for 9 mins, rinsed in 0.1M phosphate buffer pH 7 and stained in 5% Giemsa's solution in phosphate buffer at 37^oC for 1 hour. Slides were then rinsed in phosphate buffer and left to dry. These slides were observed using a Wild Microscope 100x objective). Numbers of morphological types visualized were counted. 100-1000 trypanosomes were counted per slide and the proportions of each form expressed as a percentage of the total. Arithmetic means plus and minus two standard errors were calculated.

2.2.5 Cloning epimastigote forms

Epimastigotes obtained from culture supernatant were cloned using a pin into the 10µl wells of a Terasaki plate (Lux) with a humid atmosphere. The drops were checked optically and then transferred to the 1.5cm diameter wells of a culture plate (Falcon) containing growth media. Various concentrations of FCS (0-25%) were included in the MEM. Freshly prepared culture medium and medium refiltered after being conditioned for 24 hours in a flask containing a mature epimastigote culture were used.

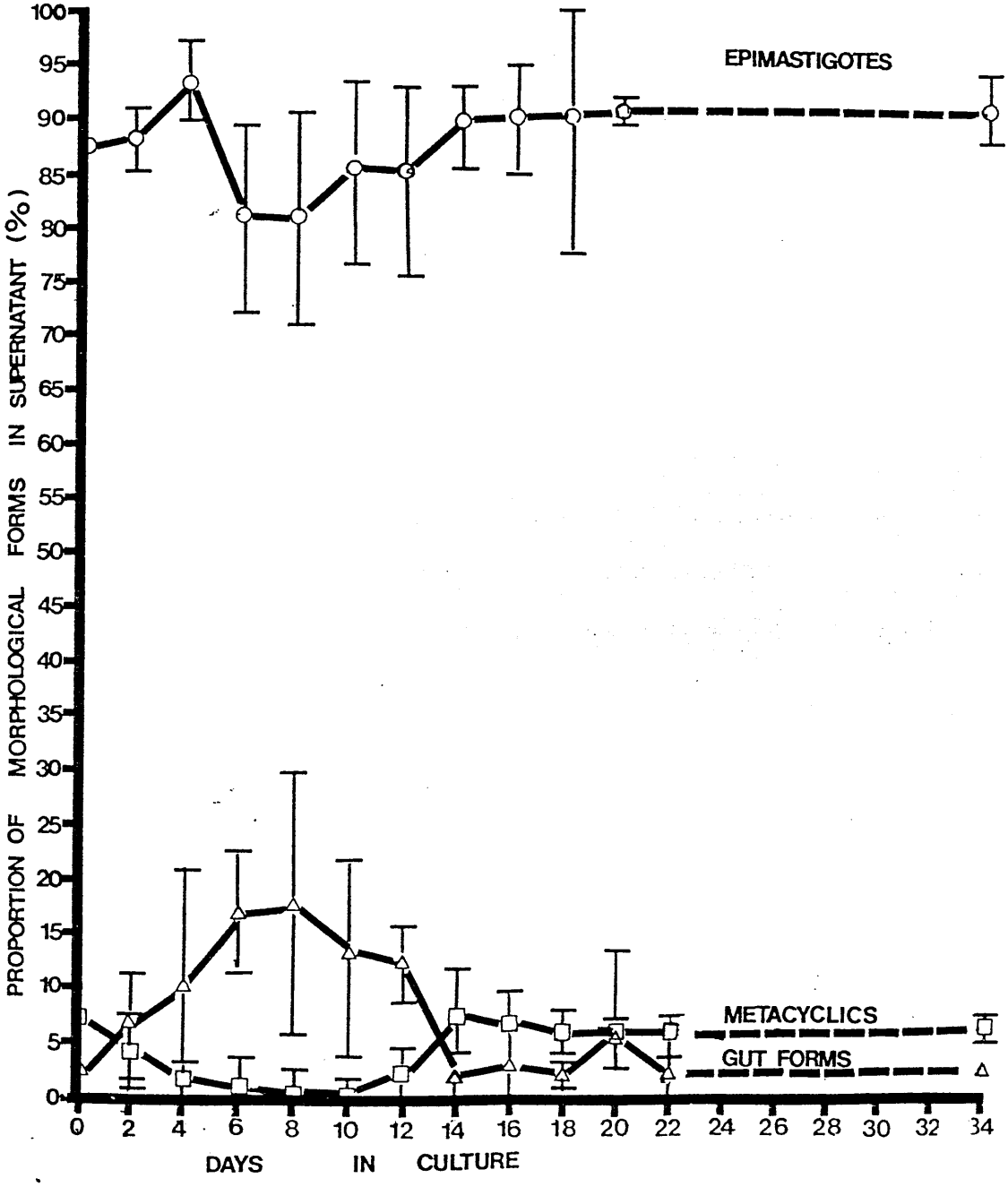
Culture wells to which the epimastigotes were added contained (a) medium alone, (b) a bovine dermal explant (Gray et al., 1981) present in the culture well for 24 hours before the addition of the epimastigote, (c) the base of the culture well precoated with Vitrogen (Collagen Corpn., Paulo Alto, C.A.), (Hirumi et al., 1982). The Vitrogen was rinsed in culture medium several times before use, as per the manufacturers instructions or (d) a single, washed, uninfected tsetse proboscis. As well as epimastigotes, proventicular forms were also used for cloning experiments. These insect gut forms were obtained by dissecting out proventriculi of T.congolense infected Glossina morsitans morsitans (pupae were obtained from the tsetse research laboratory, University of Bristol and infected as described in Chapter 3). The isolated proventriculi were rinsed in sterile MEM several times and then ripped open using fine dissecting needles. The proventricular form T.congolense were thus found free in the surrounding liquid and could be used for optical cloning as described above.

2.3 RESULTS

2.3.1 General observations of cultures

The sequence of events in the development of the cultures was as described previously by Gray et al. (1981,1984). Epimastigotes

FIG.2:1. Proportion of morphological forms present in Giemsa-stained smears of T.congolense stock TREU 1457 culture supernatants expressed as a percentage of the total. Arithmetic means plus and minus two standard errors (n=3). The cultures used to compile this graph were cultures which produced metacyclics (see Table 2:1). Metacyclic forms (□), tsetse gut forms (△), epimastigote forms (○).


EPIMASTIGOTES
METACYCLICS
GUT FORMS
PROPORTION OF MORPHOLOGICAL FORMS IN SUPERNATANT (%)
DAYS IN CULTURE
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100

1

introduced to fresh medium at subpassage, attached and multiplied. 24-48 hours later these forms were noted to have shortened considerably and were found in small groups or singly all over the culture flask base.

Proventricular-like forms were visualized after the first 2-3 days. These forms had post-nuclear kinetoplasts and elongate bodies and were found in both the supernatant and associated with the flask base. These forms were largely absent after the first two weeks. Epimastigotes elongated steadily from day 4 or so onwards until they were observed to have very elongate, blunt posterior ends (10-20μm long) after approximately one week. Metacyclics were found on the flask base adjacent to epimastigote bundles from approximately day 10 in stock TREU 1627 and day 12 in stock TREU 1457. More metacyclics were noted to be present in cultures of TREU 1627 than flasks containing TREU 1457. Metacyclics were noticeable because of their small size and characteristic shape and also due to their motion. They appeared to be attached by the middle region of the cell and to rock back and forwards on this pivot point. Metacyclics were also found swimming free in the supernatant.

Once an adhering layer of elongate epimastigotes was established, free epimastigote bundles were found in the supernatant, new cultures or cryopreserved cultures could be initiated from the supernatant.

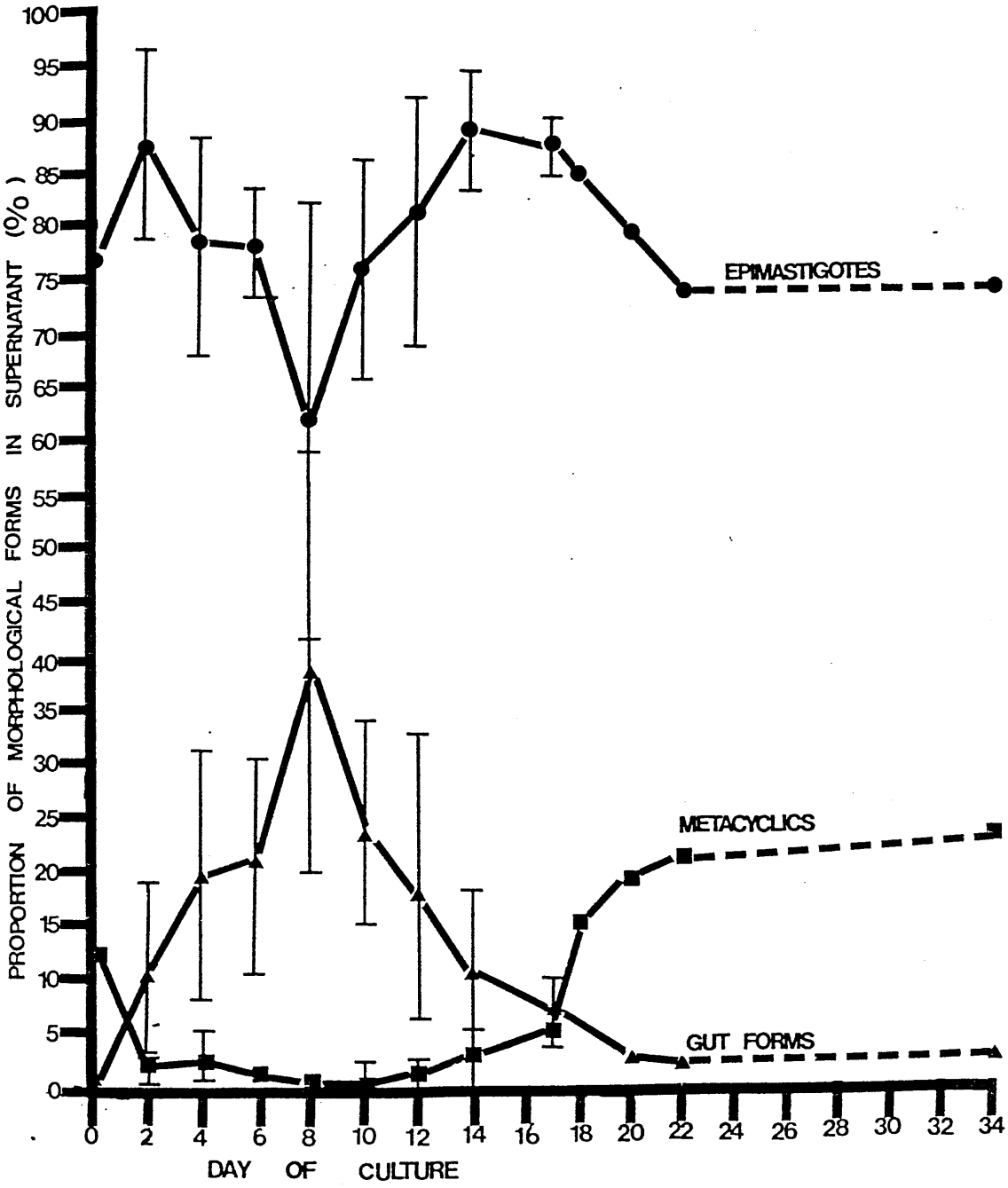
2.3.2 Numerated monitoring of growth in culture

Differences observed in the cultures of the two stocks used were mainly the time of appearance and proportion of morphological forms. Fig 2.1 shows the proportion of morphological types present in Giemsa-stained smears of culture supernatant material for stock TREU 1457. At all times the majority of forms present were epimastigotes. Proventricular-like forms increased in numbers peaking at

FIG. 2:2. Proportion of morphological forms present in Giemsa-stained smears of T.congolense stock TREU 1627 culture supernatants expressed as a percentage of the total.

Data for days 2-18 are presented as arithmetic means plus and minus two standard errors (n=3). Days 18-34 are actual data plotted from one experiment, data from other cultures were not available due to contamination problems.

Metacyclics (■), tsetse gut forms (▲), epimastigote forms (●).


PROPORTION OF MORPHOLOGICAL FORMS IN SUPERNATANT (%)
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
EPIMASTIGOTES
METACYCLICS
GUT FORMS
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
DAY OF CULTURE

2

approximately day 8 after which they declined to a low level. The increase in proventricular-like forms was mirrored by a decrease in the number of epimastigotes present, although this may be an artifact of the counting method as the number of cells present in the supernatant increases over this time period, but a similar number were

cacyclic forms were found noticeably in the supernatant from approximately day 12 onwards, the numbers present rose over the first few days but levelled off quickly remaining at approximately 6% of the total population throughout.

Fig. 2.2 shows the equivalent data for the stock TREU 1627. Again epimastigotes were the most abundant morphological type throughout the whole period of culture. The percentage of epimastigotes present appeared slightly lower than TREU 1457 stock cultures but this difference is not significant. Again the troughs and peaks in this graph are probably largely due to the sampling method used. The presence of proventricular-like forms in this stock followed a similar pattern to that of TREU 1457 cultures but the amounts present differed. Far more proventricular-like forms were found in this stock - double those found in TREU 1457 cultures at the peak although both stocks contained similar levels after the initial two week period. The proportion of metacyclics also differed from that of TREU 1457. The number of metacyclics present in the supernatants of TREU 1627 again rose to noticeable levels after approximately day 12. The graph depicts the increase of TREU 1627 metacyclics initially being slower than TREU 1457, but after approximately day 17 the number of metacyclics found in TREU 1627 cultures was far greater than those found in TREU 1457 cultures. This higher proportion of metacyclics in TREU 1627 cultures was maintained throughout the life of the culture.

The proportion of cultures which produced metacyclic forms at all

TABLE 2:1. METACYCLIC PRODUCTION - Proportion (%) of cultures that produced metacyclics by the days stated.

	DAY 10	DAY 14	DAY 16	DAY 32	NEVER
STOCK					
TREU 1457	0	26.6%	33%	43%	57%
		90%	100%	100%	-

differed widely between the two stocks (see Table 2.1). In cultures initiated, under similar conditions only 43% of stock TREU 1457 cultures produced metacyclics, metacyclics were not visible on the flask base by day 10 in any culture examined, 26% of cultures of this stock contained matacyclic forms by day 14 in culture, 33% by day 16

ilar data from stock TREU 1627 was

cases cultures produced metacyclics and all had done so by day 16, 90% by day 14 and 64% of culures contained metacyclics by day 10.

2.3.3. Attempts to clone epimastigote forms

The cloning of epimastigote forms was attempted in the hope of finding whether proventricular forms could be derived from a single epimastigote, before or after multiplication.

However, clones started from culture supernatant epimastigotes, whether placed in fresh medium, conditioned medium, (regardless of serum concentration) with or without the presence of bovine dermal explants, Vitrogen, or tsetse proboscides never sucessfully grew. All cloned epimastigotes died in less than 48 hours and many before 24 hours.

Attempts to clone from proventricular forms, in the hope that transformation to epimastigote would occur thus allowing the sequence of differentiation to be followed were also tried. Again, however no cells survived long enough to carry out this proce dure. Bacterial contamination was also a problem in these proventricular form clones.

2.4. DISCUSSION

The main difference found between the growth profiles and morphological types of these two T.congolense stocks is in the proportion of metacyclic forms generated. The two stocks also differ widely in their ability to produce metacyclic forms consistently.

Cultures of stock TREU 1627 have always produced metacyclic forms in this laboratory and as such are the stock of choice for experiments on metacyclogenesis (Chapter 5). The difference in the numbers of proventricular forms produced between the two stocks could possibly be related to metacyclic production. These forms may again differentiate metacyclics mimicking forms which move from labrum to hypopharynx in the *in vivo* situation. Despite numerous attempts to clone epimastigote and proventricular forms, both from culture and the tsetse fly no clones were ever successfully grown. Thus the sequence of events an individual organism goes through cannot be described. It is not known if dedifferentiation of epimastigotes to proventricular forms can occur or whether the proventricular forms can divide and thus arise from such forms added in the original passage material. It is generally thought proventricular forms are non-multiplicative so by whichever manner the proventricular-like forms arose is likely to be controversial.

Why the difference in metacyclic production found in these two stocks exists is not obvious. The pattern of epimastigote development in the two appears very similar by direct observation, the number of epimastigotes attaching is similar (see Chapter 5), the cycle of initial shortening and then elongation equivalent, as were the culture methods. The larger numbers of proventricular-like forms would appear to be related to metacyclic numbers but why this should be so is not at all obvious.

2.5. SUMMARY

The main difference between the two stocks of *T.congolense* (TREU 1457 and TREU 1627) in *in vitro* 'proboscis' culture was shown to be the proportion of metacyclic forms present and the percentage of cultures which produced metacyclics. TREU 1457 in all replicates

produced a lower proportion of metacyclics than TREU 1627. Only 43% of TREU 1457 cultures produced metacyclics. Cultures of TREU 1627, however, produced metacyclics in every instance and up to 20% of the total supernatant population could be composed of these forms. Metacyclics were visible after approximately 10 days in culture.

the two stocks was similar. Procyclic/ proventricular-like forms were visible in both stocks after 2-3 days in culture rising to a peak at day 8 (approx. 30% of total supernatant forms in TREU 1627 and 17% in TREU 1457) falling to low levels (<5% of the total population) subsequently.

CHAPTER 3

ELECTRON MICROSCOPICAL OBSERVATIONS ON *TRYPANOSOMA CONGOLENSE* EPIMASTIGOTES, AND BLOODSTREAM TRYPOMASTIGOTES IN THEIR HOST ORGANISMS

3.1. INTRODUCTION

Many electron microscopical studies have been carried out on the insect attached phases of trypanosomatids (Table 1.1) including several on Trypanosoma congolense (Evans et al., 1979; Molyneux et al., 1979; Thevenaz & Hecker, 1980; Molyneux, 1980; Gray et al., investigations have described the epimastigote attachment morphology while concentrating on different facets of it; eg. in relation to other insect stages in the life cycle of this parasite (Evans et al., 1979) location of attachment within the tsetse proboscis (Molyneux et al., 1979, Molyneux, 1980, Thevenaz & Hecker, 1980) while describing the detail of the attachment structure also. Gray et al. (1981) included a brief TEM and SEM study of attached epimastigotes in the tsetse for comparison with one on their in vitro grown epimastigotes. This latter reason is also the main reason for further description of the in vivo attachment in this thesis, as a preamble to the study of these organisms in vitro and to emphasise the similarity of in vivo and in vitro grown epimastigotes and thereby justify the use of in vitro grown organisms throughout the rest of the study.

Previous studies have also described T.congolense in the vasculature of mammalian hosts (Büngener & Müller, 1976; Banks, 1978,1979 & 1980). The latter studies concentrated on the relationship of these organisms to the blood vessel endothelia and blood cells. The present study also briefly examines this relationship in comparison with the attachment in the insect host. The mesenteric microvasculature was the only site examined as Banks (1978) observed that T.congolense is mainly found in the microvasculature in mice, and in particular the mesenteric microvasculature.

The observations on T.congolense in both tsetse and mammal made in this study uphold in the main observations made in the previous

investigations. In that structural differentiation is found at the epimastigote flagellar attachment site but this is absent in trypomastigote attachment to the mammalian host.

3.2. MATERIALS AND METHODS

[illegible]ed proboscides

[illegible] pupae were supplied by the Tsetse Research Laboratory, University of Bristol Veterinary School. Within 24 hours of hatching, flies were infected by feeding on infected BALB/c or CFLP male mice in the first peak of parasitemia. Infection was with either T.congolense TREU 1457 or TREU 1627 stocks. Flies were subsequently maintained by membrane feeding on citrated sheep blood (Gibco Ltd.) three times a week. The saliva of the flies probed onto warm microscope slides (37°C) was checked for trypanosomes from 12 days after the infecting blood meal. G.morsitans containing metacyclic forms in their saliva were selected.

Flies were immobilized by placing in the ice-box of a refrigerator for 4-5 minutes then dissected. The head was cut off and the proboscis dissected according to the method of Lloyd and Johnson (1924). After examining the severed proboscis under phase contrast microscopy for the presence of trypanosomes the parts were separated out under 2.5% glutaraldehyde in 0.1M phosphate buffer. Fixation was carried out for 90-120 minutes at room temperature. Proboscides were post-fixed in 2% OsO_4 in 0.1M phosphate buffer for 2 hours at room temperature then block stained in 0.5% (w/v) aqueous uranyl acetate for 30 minutes. Proboscides intended for transmission electron microscopy (TEM) were dehydrated in a graded alcohol series and embedded in Araldite after transferring through propylene oxide. Blocks were trimmed and thin transverse sections of proboscis (silver and gold) cut with glass or diamond knives on an LKB 1 ultramicrotome.

Sections were picked up on copper grids and stained sequentially in 2% methanolic uranyl acetate and in lead citrate (Reynolds, 1963). Sections were viewed in an AEI 801 TEM operated at 60 KV. Photographs were taken on Ilford EM4 film.

Proboscides prepared for scanning electron microscopy (SEM) were [illegible] into a retaining chamber, comprising two stainless steel meshes separated by a teflon spacer and housed in a brass fitting, to facilitate dehydration in graded steps of acetone and critical point drying from CO_2 as described by Tetley and Vickerman (1985). Dried proboscides were mounted on double-sided sellotape on aluminium stubs and coated with a 30nM gold coat in a Polaron Sputter coater. The spread proboscides (arranged so that the groove of the labral gutter faced uppermost) were examined in a Philips SEM 500. Photographs were taken using Ilford HP4 roll film.

3.2.2. Trypanosomes in blood vessels

BALB/c (male) mice were infected with T.congolense TREU 1457 from frozen stabilates. Approximately five days later the first peak of parasitemia was at a high value (log 8.4-log 8.7) as determined by the method of Herbert and Lumsden (1976). At this time the mice were killed by dislocation of the neck and the mesenteric blood vessels fixed for electron microscopy. These structures were fixed for 2 hours in 0.1M phosphate-buffered 2.5% glutaraldehyde pH7.2, rinsed in several changes of buffer over one hour, post fixed in 2% OsO_4 in the phosphate buffer for one hour, rinsed in water, block stained in 0.5% (w/v) aqueous uranyl acetate for 30 minutes, dehydrated in an alcohol series and embedded in Araldite (48 hours at 60^{o}C) after treating with propylene oxide. Sections were cut and prepared as described above (3.2.1).

FIG.3:1.-3:4. Transmission electron micrographs of T.congolense epimastigotes attached to the labral chitin of Glossina morsitans morsitans.

F-flagellum, E-epimastigote, P-attachment plaques, g-gap between substratum and flagellum , mt-microtubules of axoneme, PFR-paraflagellar rod, d-flagellum-cell attachment junctional complexes, f-filaments.

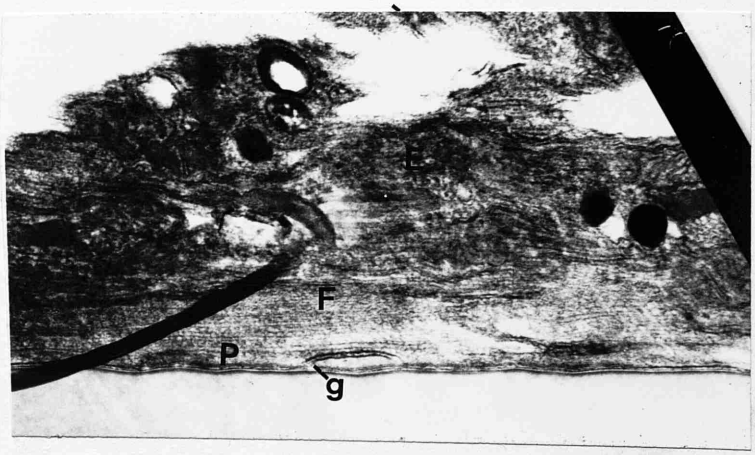
FIG.3:1. Longitudinal section showing an attached flagellum. More than one attachment point to the labrum is present along the flagellum. Attachment plaques are situated at these points.MAG. 75,600x.

FIG.3:2. Attached flagella cut in transverse section. Some of the attached flagella have long thin lateral extensions (LE). Interdigitation between neighbouring epimastigotes is found. One flagellum contains two axonemes plus associated PFRs within a single sheath. MAG. 189,000 x.

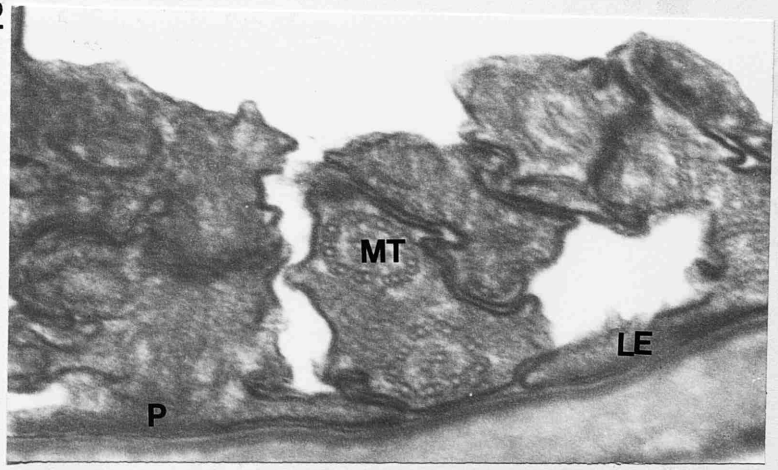
FIG.3:3. Oblique profiles of attached flagella. No interflagellar attachment densities are noted, although interdigitation between neighbouring flagella occurs. MAG. 189,000 x.

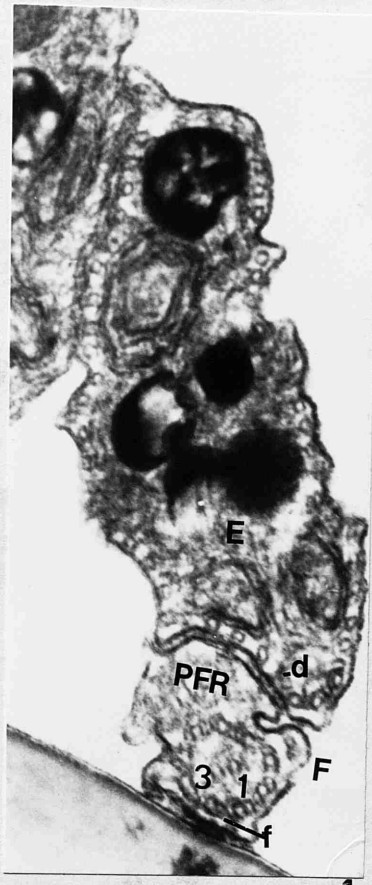
FIG.3:4. Cross-section of attached cell. The flagellum does not contain a very noticeable attachment plaque although filamentous material is present at the point of attachment to the labrum. These filaments associate with microtubule doublets 1-3.
MAG. 189,000 x.

1



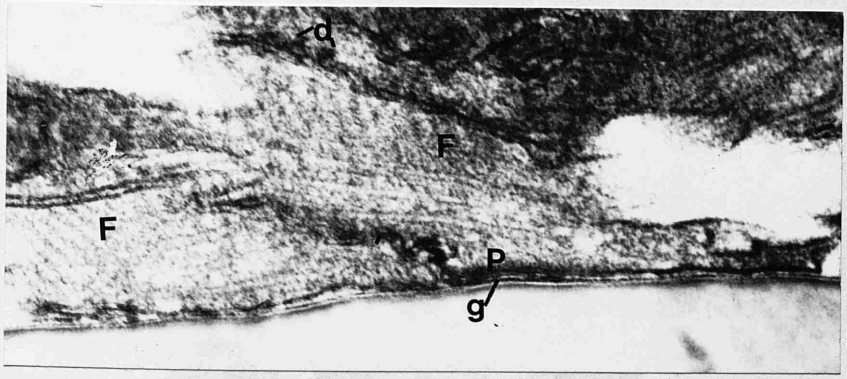
2





4

3



3.3. RESULTS

3.3.1. Transmission electron microscopical observations on infected tsetse proboscides

On examination of sections of T.congolense infected tsetse proboscides bundles of trypanosomes were observed attached by the flagella to the labral chitin. Within the labral gutter the bundles of trypanosomes were found towards the proximal end of the labrum but not at the proximal extremity.

In this location the bundles of trypanosomes were found all around the cross-sectional profile of the labrum but more trypanosomes were found towards the sides of the labrum than on its ventral base. No association between trypanosomes and internal labral stuctures e.g. sensillae was noted. In all proboscides the number of trypanosomes was low (less than 50 per cross section) and no proboscis contained an uninterrupted layer of attached trypanosomes.

A thin (10nm) amorphous layer of stainable material was noted to coat the labral chitin, it was to this layer that the trypanosomes were found attached. The flagella were found lying along the labral surface and at intervals along the flagellar shaft attachment points were observed. More than one attachment point per flagellum was noted to occur (Fig 3.1). At the sites of attachment small distentions of the flagellar shaft were found. In cross-section it was noted that lateral extensions from the flagellar shaft were present. The majority of lateral extensions noted were not large, although very thin, long processes were noted, in some cases (Fig. 3.2) up to 0.5µm long and approximately 0.1um wide. Interdigitation between neighbouring attached flagella was noted (Fig 3.3) also. At the flagellar membrane of the points of attachment, plaques of electron-dense material were found, on average these plaques were 20nm thick but plaques between 10 and 33nm thick were noted. The plaques could be continuous across the

cross-sectional periphery of the flagella or be interrupted so that more than one small plaque of material per cross-section was present. Fine filaments (5nm diameter) were noted emanating from the electron dense plaques. These filaments appeared to associate with the axoneme and paraflagellar rod structures. In attachments where the plaque was less prominent, filament association with axonemal microtubule doublets 1-3 (Afzelieus, 1959) was noted (Fig. 3.4). The filaments were noted to converge on the junctional complexes between the flagellum and cell body. A gap between the flagellum and labrum surface at the attachment points was present. This gap had an average width of 10nm ($\pm 5{\cdot}2 : n=20$) and contained stainable material of an amorphous appearance.

The flagellum-cell body junctional complexes were marked by the presence of small (10nm) conical shaped accumulations of dense staining material. These junctional densities were found on both cell body and flagellar sides of the junction but were more prominent on the cell body side where they were found to be located in a linear row in a gap in the pellicular microtubules. A small gap (10nm) was present between the membranes separating the two halves of the junction. Filamentous material was noted to emanate from these junctional densities. On the flagellar side the filaments associate with both the PFR and axoneme and also with the similar filaments emanating from the flagellar-labral attachment plaque.

The flagellar-cell junctional densities had a longitudinal axis spacing of approximately 40nm (centre-centre) and were found along the length of the cell from the point of flagellar emergence from the flagellar pocket. In cross sections of the attached flagellum two rows of flagellum-cell junctional complexes were observed in some cases.

The trypanosomes found attached appeared to be epimastigotes

FIGS.3:5-3:10. Scanning electron micrographs of T.congolense in the tsetse labrum.

E-epimastigote, M-metacyclic, I-intermediate form, F-flagellum, LCl-LCl receptor, e-expansion from flagellar axis at point of attachment, P-proximal end, D-distal end.

FIG.3:5. Labrum of infected tsetse. One bundle of epimastigotes is present approximately 1/3-1/2 of the distance from the proximal end (arrow). Single trypanosomes are located distal to the bundle. MAG. 188 x.

5

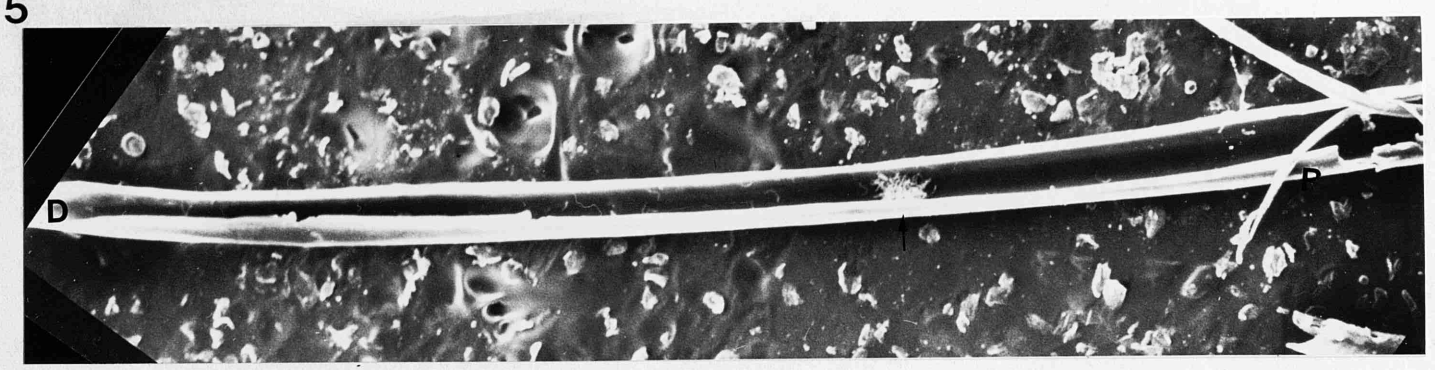


FIG.3:6. Montage of an epimastigote bundle. Approximately 200 trypanosomes are present in this bundle. The forms observed are epimastigotes all are eqivalent in length (20-25μm) and have blunt posterior ends. The epimastigotes are attached by their flagella, all the attached flagella orientate towards the centre of the bundle. LC1 sensory receptors are present in this region of the labrum.

MAG. 3,000 x.

FIG.3:7. LC1 sensilla lying alongside epimastigotes. The mechanoreceptor is free from epimastigote attachment.

MAG. 2,400 x.

6

7

(i.e. the kinetoplast was located anterior to the nucleus). Extensive bundles of these organisms were noted and cell body-cell body, cell body-flagellum and flagellum-flagellum associations were commonly found. Interdigitations between attached cells were found but no cell-cell attachment densities were noted. Some attachment plaques were noted, however, in flagella which attached to other epimastigote cell bodies. Many dividing forms were present and occasionally two axonemes and associated PFRs within one sheath were found attached to the labral chitin (Fig. 3.2). The trypanosome profiles in the labral cross-sections were oblique or longitudinal in the ventral region, suggesting that the organisms located there were lying orientated around the labral gutter rather than along its axis. However, trypanosomes at the edges of the gutter, particularly those at the extreme margins were found to be cut transversely and thus probably orientated so that their attachment was lying along the long axis of the labrum.

3.3.2. Scanning electron microscopical observations on infected tsetse proboscides

SEM observations on infected tsetse labra showed an uneven distribution of attached flagellates . Bundles of epimastigotes were observed at approximately 1/3 of the distance from the proximal end and were never seen proximal to this (Fig. 3.5). More distal parts were found to contain trypanosomes but these occured as single organisms, not as bundles; such single organisms were present at the distal tip of the labrum in some cases.

Very heavy parasitization was not encountered even in longstanding fly infections. Bundles of more than 250 epimastigotes were not observed (Fig. 3.6) and labra with more than three such bundles were not found. The low epimastigote bundle number also existed in tsetse flies whose probed saliva had contained a few

FIG.3:8. A) Montage of the distal end of a lightly infected labrum. Epimastigotes (blunt ended) and intermediate forms (blunt ended but with the flagellar pocket in a posterior position) are present. Some trypanosomes appear attached by their flagella and others have their flagella lying free in the labrum lumen.
MAG. 1,244 x.

B) Distal region of a infected labrum containing a bundle of epimastigotes in a more proximal position. Epimastigotes, intermediate forms and possible metacyclic forms are present.
MAG. 600 x.

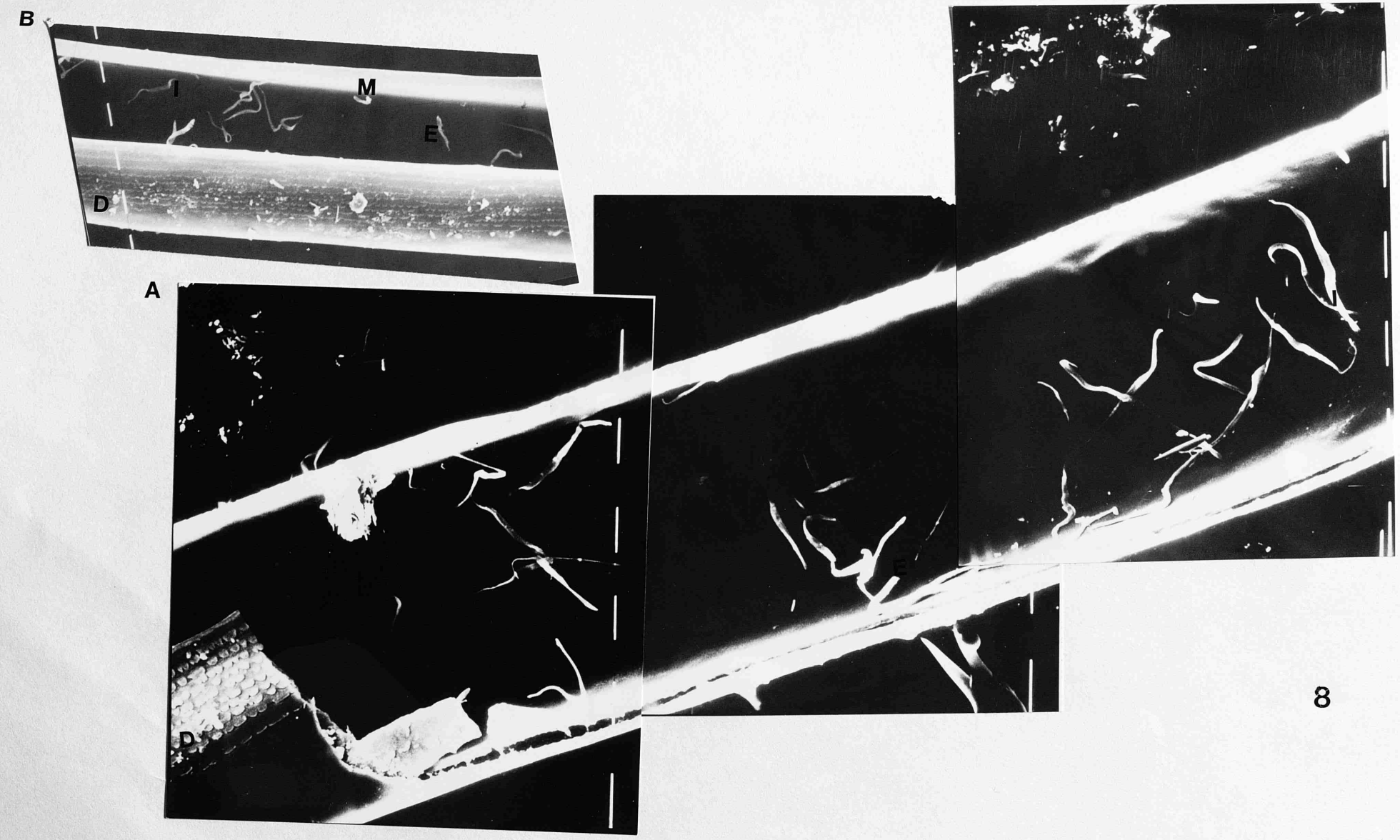

B
I
M
E
D
A
D

FIG.3:9. Part of the mid-portion of a flagellum attached to the labrum. A slight lateral "splaying out" of the membrane from the flagellar shaft is noticable over the attached portion. The attachment lies directly beneath the cell.

MAG. A) 17,300 x. B) 11,600 x.

FIG.3:10. Membranous extensions from the flagellar shaft are visible at the point of attachment of this flagellum.

MAG. 25,600 x.

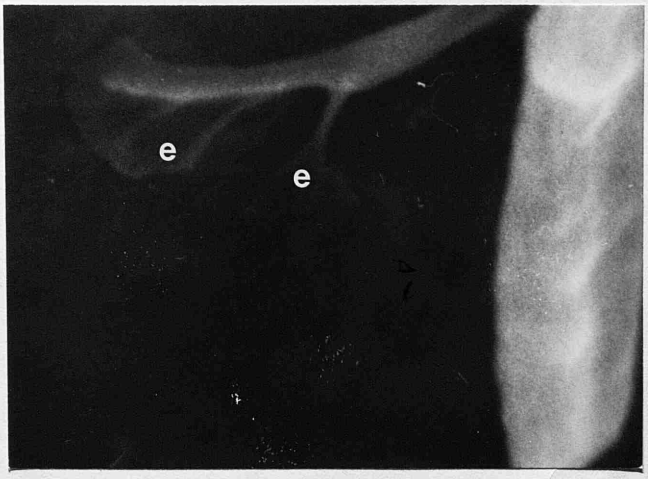
hundred metacyclics. On closer examination of this area it was noted that the location of these bundles of epimastigotes coincided with an area of sensory receptors - LCl sensory receptors (Rice et al., 1973). In cases where no receptor hairs were noted, pits in the labral gutter were found. These pits appeared not to contain any emergent structure. Although the trypanosomes occured in the same region as the mechanoreceptors, some sensillae were found free of trypanosomes despite the parasites being located close by attached to labral chitin (Fig. 3.7). The bundles of epimastigotes were located on the base and sides of the labral gutter although a lateral location rather than a ventral location appeared to be preferred, particularly in very low infections where single organisms were found rather than rosettes. The single trypanosomes were found in regions more distal than the sensilla area (Fig. 3.8).

The attachment, where it was possible to see single flagella, was found to be made with the mid portion of the flagellum leaving the anterior tip free. A foot-like distension from the flagellum appeared to be present at the attachment point (Fig. 3.9), occasionally extensions from the flagellar axis 2 .5µm in size were noted (Fig. 3.10). Attached flagella were arranged so that the attachment lay directly below the cell body. No auxilliary structures were noted associated with the attached flagellum. The cell bodies of attached cells were found in all directions - lying along the labrum and lying free in the lumen. The attachment sites of the majority of the trypanosomes within the rosette of attached organisms were obscured by the mass of parasites. The flagella of bundle members appeared to aggregate forming an intertwining mass, particularly in those epimastigotes at the centre of a bundle. The flagella of cells attached at the outer perimeter of a bundle also pointed towards the

9 A

B

10



centre of the bundle although they were not part of the central entwining mass of flagella. No clear picture could be obtained of whether all the flagella in the attached mass were orientated in any particular direction along the labrum. In one instance all epimastigotes and flagella attachments in a bundle were noted to be lying so that they were longitudinally orientated along the labrum and pointing towards the distal tip of the proboscis. Single trypanosomes in lightly infected proboscides appeared randomly orientated in all directions.

The vast majority of trypanosomes found in the labra examined were epimastigotes. All appeared long and thin, approximately of equal length (up to 25µm) and had noticeably squared, blunt posterior ends. Many appeared twisted along their length. Proboscides from flies which had contained only a few metacyclic trypanosomes in salivary probes were found to contain only a light infection of trypanosomes. These organisms were found towards the distal end of the labrum. Not all these flagellates were epimastigote-like in appearance (Fig 3.8). Some appeared very elongate in their posterior cell body portion and to have pointed not squared posterior ends. These organisms were thinner than the epimastigotes and from the position of the flagellar pocket were possibly trypomastigotes. Possible metacyclic trypanosomes were occasionally noted (Fig 3.8b). Single epimastigotes or at least blunt ended, posterior elongated organisms were also noted in these distal infections. In many cases these types formed the majority of the morphological forms present (Fig. 3.8). Forms with blunt ends but with the position of the flagellar pocket in that of a trypomastigote were also found in the distal portion of proboscides, these have been called intermediate forms. Both T.congolense stocks (TREU 1457 and TREU 1627) examined gave similar infection patterns, however, stock TREU 1627 infected labra consistently (3/3 infected) contained bundles

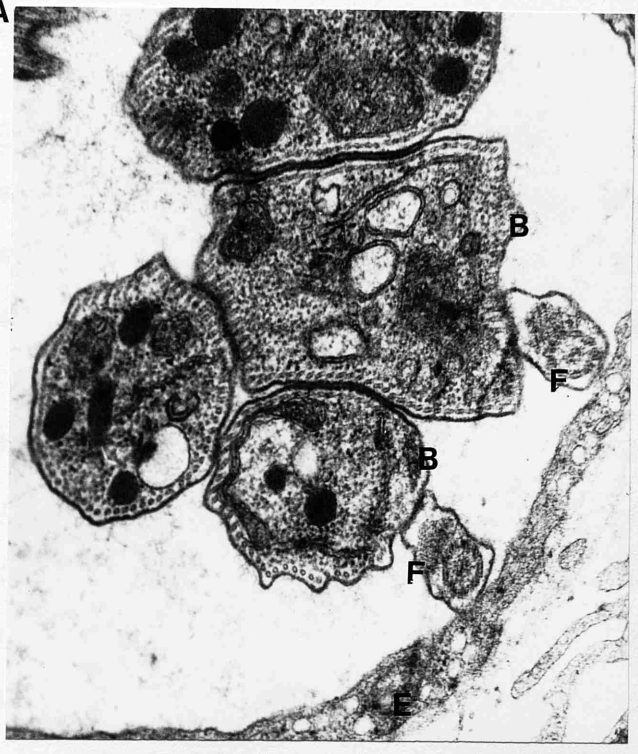
FIG.3:11. and 3:12. Transmission electron micrographs of T.congolense trypomastigotes in the microvasculature of the mouse mesentery.

F-flagellum, B-trypomastigote body, MT-axoneme microtubules, PFR-paraflagellar rod, E-endothelium.

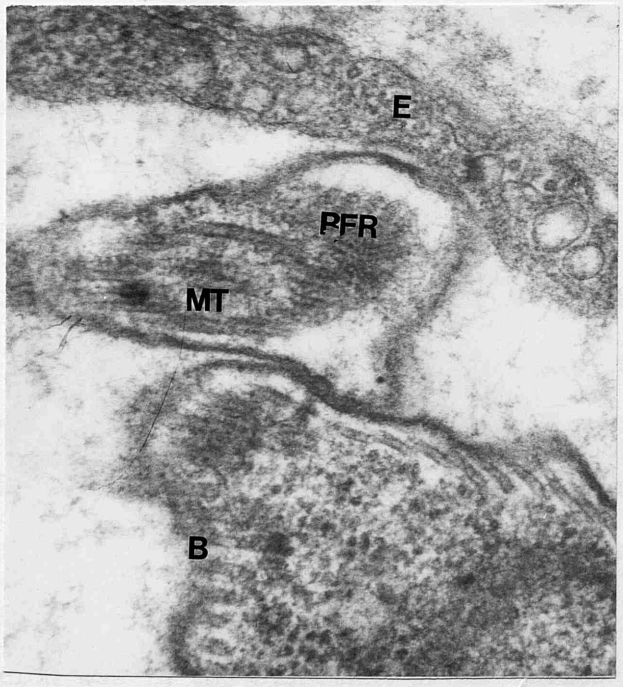
FIG.3:11. Bloodstream trypanosomes associated with the endothelium of the bloodvessel. No modifications of the flagellum are associated with the attachment. Associations with other trypanosomes are also found.

MAG. A) 31,100 x. B) 57,400 x.

11 A



B



of epimastigotes in the sensilla region of the labra whilst TREU 1457 infections were more often light and contained a larger number of single organisms, only 40% of proboscides infected with TREU 1457 contained bundles of epimastigotes.

3.3.3. TEM observations on T.congolense in peripheral blood vessels

In this TEM study many trypomastigotes were found associated with the endothelium of blood vessel walls (Fig. 3.11). The flagella of these trypomastigotes were the main parts of the trypanosomes found adhering to the blood vessel walls, but cell bodies were also found in contact with the endothelium (Fig. 3.12). The flagella associated with the vascular wall however did not appear modified for attachment. No extensions of the flagellar shafts were noted and no dense attachment plaques, nor were filaments noticed in the flagella at the sites of the association with the host vessel walls. The flagella-cell body attachment structures were composed of a single row of attachment densities. No clustering of trypanosomes was noted in this study but trypanosomes were found associated with each other, no attachment plaques or filaments were noted, flagella-flagella, flagella-cell body and cell body-cell body associations occurred.

Trypanosomes were also found attached to blood cells, again associating with the blood cells via their flagella and again no flagellar modification was noted.

Many of the trypanosomes appeared to be orientated in the same direction, as judged by the direction of the 'dynein' arms associated with the A-microtubules of the flagellar axoneme. It is unknown if this orientation was in the direction of blood flow or opposite to it.

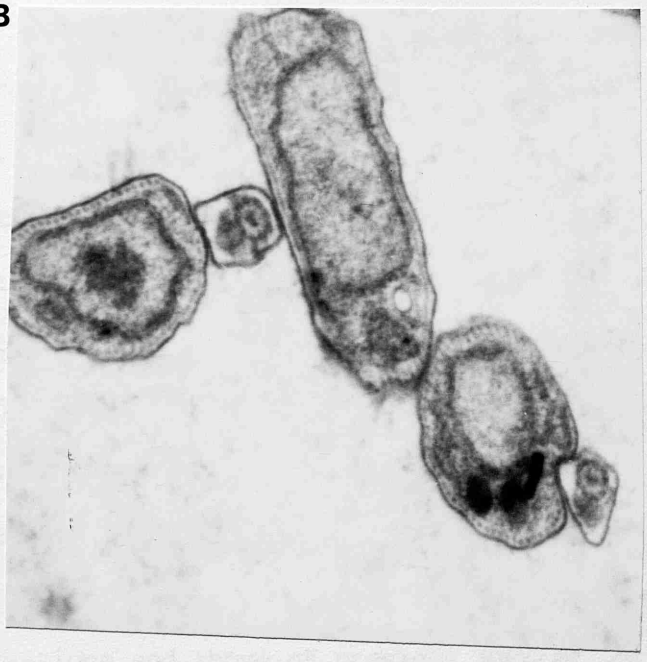
Although reasonably high parasitaemias were measured in the host animals, the number of trypomastigotes found in the blood vessels was not excessive. No blood vessels in which the lumen was entirely

FIG.3:12. A) Body regions of the trypanosome also contact the endothelium. B) Trypanosome body regions are also noted to contact each other.

MAG. A) 20,300 x. B) 29,000 x.

12 A

B



obscured by trypanosomes were observed. Dividing trypomastigotes were commonly found in the blood vessels examined.

3.4. DISCUSSION

3.4.1 Transmission electron microscopical observations in the tsetse labrum

The observations reported in this study in general conform to those previously reported for T. congolense attachment in their insect and mammalian host.

The transmission electron microscope observations of attached epimastigotes in the tsetse labrum are similar to those previously reported both by Evans et al. (1979) and Thevenaz and Hecker (1980) for this species and by Vickerman (1973) for T.vivax attached in the same position. The trypanosomes attach with their flagella, a dense plaque of material forming at the site of the attachment. The dense plaque of material found in the study appears thinner (10-33nm) than those found by previous investigators (30-60nm calculated from Thevanaz and Hecker's micrographs). This may be partially explained in relation to development. No coated forms were found in the TEM study suggesting that metacyclogenesis was occuring at a low level in the labrum although metacyclics had been detected in the probed saliva. These metacyclics probably originated from the hypopharynx (not examined in this study). Thus many of the epimastigote forms were possibly not yet fully mature. In vitro it has been noted that less mature cultures of epimastigotes contain more organisms with a less differentiated attachment plaque (Chapter 4). Other differences noted between the present observations and those of previous authors are also minor. The finding of a maximum of two junction densities per cross sectional area of attached flagellum rather than the twelve found by Thevenaz and Hecker (1980) and six by Vickerman (1973) in T.vivax, but again

this could be attributed to immaturity and development; a fact reinforced by the small numbers of trypanosomes found per cross section in the present examination. The orientation with respect to the long axis of the flagellum of many of the trypanosomes in this study differs from that of Thevenaz and Hecker (1980) who looked at this aspect of the attachment in detail and Vickerman (1973) who suggested that T.vivax epimastigotes lie with their flagellar anterior tip pointing towards the probosis tip. Thevenaz and Hecker (1980) and Evans et al. (1979) also state that T.congolense were orientated longitudinally along the labrum, the majority pointing towards the proboscis tip, but in two flies Thevenaz and Hecker noted the majority of epimastigotes were pointing towards the proximal part of the proboscis. The epimastigotes in the present study appeared orientated around the labral gutter when attached to the ventral base of the labrum and along the proboscis long axis when attached at the lateral edges of the proboscis. Unlike Thevenaz and Hecker (1980) no association with sensillae receptors in the labra was noted.

3.4.2. Scanning electron microscopical observations on the tsetse labrum

The epimastigote location in the labrum, on the whole, is consistent with the observations of Thevenaz and Hecker (1980) and Molyneux et al. (1979). The latter study was also an SEM investigation. These previous authors stressed the importance of the relationship between the attached epimastigotes and the LC1 sensory receptors. These authors stated that the occlusion of the labrum by attached trypanosomes caused the rate of ingested blood flow to be reduced. To keep the blood flow constant an increase in the amount of feeding is therefore required. The LC1 receptors in the labrum are partially responsible for determining the feeding and gorging

responses (Rice et al., 1973), thus, epimastigote binding to the LC1 receptors is reputed to impare sensory function and contribute to the increased probing observed in infected tsetse fly (Jenni et al., 1980). In the present study,however, although both sensillae (or basal pits of these structures; Rice et al., 1973) and epimastigote bundles were found in the same location the relationship between them did not appear as unwavering. Many sensillae were found free of epimastigotes and many epimastigotes were found not associated with sensillae although located near them. Trypanosomes were also noted in regions of the labrum free of sensillae. Thus the steadfast association of epimastigotes and LC1 sensillae put forward by the previous workers (Molyneux et al., 1979; Jenni et al., 1980; Thevenaz & Hecker, 1980; Molyneux & Jenni, 1981) does not appear to hold in the present investigation.

The orientation of observed attached epimastigotes by SEM in this study corresponds with that made in TEM observations, in that epimastigotes were orientated both around the labral gutter and along it and that the direction of cell orientation appears to be towards the centre of an epimastigote bundle rather than any specific orientation with respect to the proboscis.

The only observed morphology of the flagellar attachment in SEM is that of an enlargement of the flagellar axis at the point of attachment. A similar flagellar enlargement at the attachment point was also noted by Schaub and Böker (1986) in their SEM study of Blastocrithidia triatomae attached to the rectal chitin of Triatoma infestans. All the epimastigotes observed in this study and also (from examination of the published micrographs) in the study of Molyneux et al. (1979), were of approximately equivalent length and morphology with noticably blunt posterior ends. The SEM study of Böker and Schaub

(1984) on T.cruzi epimastigotes attached to their Triatomine host's rectum detected two epimastigote forms - a long form and a short form and observed only the short forms dividing. This differentiation of epimastigote forms was not noted for T.congolense despite small epimastigotes being observed in in vitro culture (Gray et al., 1981).

3.4.3. Development of proboscis infection

The observations in the infected proboscides examined of possible non-epimastigotes or intermediate forms between epimastigotes and trypomastigotes particularly at the distal end of the proboscis is interesting in terms of the development of the infection. These forms were observed both in proboscides which contained epimastigote bundles and those without bundles. This observation would tend to suggest that these elongate intermediate forms are perhaps the forms migrating from the proventriculus to initiate epimastigote development in the labrum and that this invasion continues despite parasitization having previously been established. Their presence at the distal end when bundles appeared to be solely established at the proximal end can be accounted for by them being unattached and able to swim up and down the labrum and be washed to the distal end during preparation for SEM. The finding of epimastigote forms in the distal portion also could mean that the intermediate forms transform into epimastigotes before flagellar attachment occurs. The new epimastigotes then swimming to a suitable location in the labrum and attaching.

This hypothesis is reinforced by the presence of intermediate forms in labra where no epimastigote bundles were present and also by the low numbers of metacyclics in the saliva of these flies suggesting these were immature proboscis infections.

Conversely the finding of single epimastigotes in the distal portion of the labrum might suggest that epimastigote forms can detach

and transport themselves to the hypopharynx or that epimastigotes are capable of movement between feeds. This latter suggestion would be interesting in terms of permanence of the attachment. It would seem unlikely that the large, intertwined bundles of epimastigotes would detach periodically, although in culture epimastigote bundles are frequently observed in the supernatant having, presumably, detached, these can be used to initiate new cultures (Gray et al., 1981, and Chapter 2) in vitro. This could at least be possible in vivo also. Very few metacyclics were found in the labra despite these flies having probed out metacyclic forms in their saliva. These forms could have originated from the hypopharynx or any metacyclics free in the lumen could have been washed out during EM preparation. It appears to be assumed, generally that the hypopharynx is infected after the labrum and that metacyclic development occurs primarily in the hypopharynx. Evans et al. (1979) and Thevenaz and Hecker (1980) reported the presence of coated, metacyclic forms, in the hypopharynx but also noted their presence in their labrum. In combination, these observations indicate that the labral infection is initiated by intermediate trypomastigote/epimastigote forms migrating from the proventriculus which transform to epimastigotes in the labrum and subsequently attach in the proximal region of the labrum. Very little metacyclogenesis occurs in the labrum, the epimastigotes possibly detaching and invading the hypopharynx where the bulk of the metacyclogenesis occurs. Differentiating metacyclics were not noted amongst the epimastigote bundles.

3.4.4. T.congolense in the mammalian host

The trypomastigotes in the mesenteric blood vessels of the mice used in this study were often found associated with the endothelia of the blood vessel, blood cells and each other. Flagella were often

observed to contact other trypanosomes, endothelia or blood cells but no attachment plaques or filaments were observed in these flagella nor was any extension of the flagellar sheath noted. The trypomastigotes were observed to bind to endothelia, blood cells and other trypanosomes via non-flagellar areas of the cell also. No true evidence of binding rather than just touching the capillary walls or blood cells can be obtained from this TEM study alone but the examination of living material by Banks (1978,1979,1980) suggests that binding occurs in living organisms and as such the fixed material examined here is probably depicting this same association. Büngener and Müller (1976) also carried out TEM observations on this species in the microvasculature of the mouse. They observed a great deal of interdigitation and dovetailing of both flagella and trypanosome cell bodies with other trypanosomes and the endothelial cells of the blood vessel. These authors also noted an absence of flagellar attachment associated structures. They noted that the accumulations of trypanosomes and their association with the blood vessel walls lead to blockage of blood vessels. The trypanosome aggregations in the present study did not appear to be extensive as noted by Büngener and Müller neither to be causing total blood vessel blockage nor interdigitating with each other and endothelia to the same extent, but appeared to be "sticking" together by cell contact alone. Banks (1978) carried out a light microscopical study on T.congolense in blood vessels and found that the blood vessels of the microvasculature contained the vast majority of the trypanosomes. In live animals he determined that the trypanosomes were attached by their anterior ends and that the unattached posterior end pointed in the direction of the blood flow. This would account for the abundance of cross sectional profiles of trypanosomes noted in the present investigation. He also noted that aggregation of trypanosomes only occurred once the host was dead and

trypanosomes had detached. Thus the large aggregations of trypanosomes observed by Büngener and Müller (1976) may have been due to this post mortem detachment rather than present in the living animal. Banks (1979) examined the attachment phenomenon of trypomastigotes to blood vessels and blood cells further and concluded that the binding was not due to a simple charge interaction, and suggested sialic acid on the trypanosomes might be binding to a sialic receptor on the mammalian cells. Banks (1980) examined the effect of the adherence of T.congolense to blood vessels of its host and noted that injury of the blood vessels was induced by this binding, probably due to incidental action of the immune response and complement-induced destruction of the parasite than through direct injury caused by the flagellate binding to the host itself.

Thus the characteristic attachment morphology of attached T.congolense in the insect host is not found in attached trypanosomes in the mammalian host. This suggests that flagellar adhesion alone is not the trigger for differentiation of attachment plaques but that other factors are influencing the formation of the epimastigote attachment structures.

3.5. SUMMARY

In the labrum of the tsetse fly, T.congolense epimastigotes are attached by their flagella. Epimastigotes occur in bundles, principally in the proximal 1/3 of the labrum; no marked association of the flagellates with the labral sensillae is discernible. An electron-dense plaque is present on the flagellar membrane at the attachment site; 5nm filaments are associated with the plaque and link it to other intraflagellar structures (axoneme, paraflagellar rod). The gap between flagellar membrane and substratum is 10nm.

The presence of proventricular-like forms in labra, particularly those with a light parasitization, suggests that labral infection is initiated by these forms which then subsequently differentiate to epimastigotes. The lack of metacyclic forms in labra, despite their presence in probed saliva, suggests that metacyclogenesis occurs primarily in the hypopharynx.

In the mesenteric blood vessels of the mouse experimental host, trypomastigotes attach by their flagella to host endothelium, but no differentiation of the flagellum at the point of attachment is discernible.

CHAPTER 4

ELECTRON MICROSCOPICAL OBSERVATIONS ON *IN VITRO* GROWN *T.CONGOLENSE* EPIMASTIGOTES

4.1. INTRODUCTION

In contrast to attachment of trypanosomatids *in vivo*, there have not been many ultrastructural studies carried out on attached trypanosomatids *in vitro*. Brooker (1971a) described the structure of haptomonads of *Crithidia fasciculata* attached to Millipore filters, Hommel and Robertson (1976) briefly described the attachment of *T.blanchardi* to the plastic of a culture flask base, and in their 1981 paper Gray *et al.* briefly described the ultrastructure of the attachment of *T.congolense* epimastigotes to culture plastic in order to compare their *in vitro* grown cells with their *in vivo* grown counterparts.

As discussed in Chapter 3, the morphology of *T.congolense* epimastigotes attached to the tsetse proboscis has been examined in previous studies in particular that of Thevenaz and Hecker (1980) with minor observations by Evans *et al.* (1979) and brief SEM observations by Molyneux *et al* (1979). The purpose of undertaking ultrastructural studies on the attached epimastigotes of this species in this thesis has been to describe more fully the attachment site morphology *in vitro* using both TEM and SEM, and to compare the attachment structure with that of their *in vivo* grown counterparts in order to justify the extrapolation of conclusions drawn from research on *in vitro* grown epimastigotes to those attached to the tsetse proboscis.

4.2. MATERIALS AND METHODS

4.2.1. Conventional transmission electron micoscopy (TEM)

Cultures of *T.congolense* TREU 1457 and TREU 1627 were grown on Thermanox plastic culture coverslips (LUX) in Ambitubes (LUX, Flow Labs.) and maintained as described in section 2.2.3.

At various time intervals (3,5,7,13,15 and 17 days) the flagellate monolayer bearing coverslips were removed and fixed for

TEM. The monolayers were rinsed in incomplete MEM and then in 0.1M phosphate buffer, fixed for 1h in 2.5% glutaraldehyde in 0.1M phosphate buffer, rinsed in several changes of phosphate buffer containing 2% sucrose, and post fixed in 2% aqueous OsO_4 for 1h. After rinsing in distilled water and staining in 0.5%(w/v) aqueous uranyl acetate solution for 30min the films were dehydrated in alcohol and embedded in Araldite after transferring through propylene oxide and propylene oxide:Araldite mixtures. The cells were embedded by placing the coverslips, cell side down, on top of the Araldite mixture and then polymerizing the resin for 48h at 60°C. After polymerization the coverslips were removed from the resin blocks by plunging into liquid N_2 for a few seconds and then rapidly returning the blocks to room temperature. The rapid changes in temperature caused the coverslips to split away from the resin blocks leaving the epimastigotes embedded in the Araldite. The Araldite blocks were then re-embedded, cell side down on fresh Araldite and repolymerized at 60°C for 48h. Sections were cut and processed as described in Section 3.2.1.

4.2.2. Tannic acid enhancement procedures

4.2.2.1. Digitonin permeabilization: Mature cultures of T.congolense epimastigotes on Thermanox coverslips were rinsed in MEM before fixing for 1h in 2.5% glutaraldehyde in 0.5M cacodylate buffer pH7.2 containing 2% tannic acid (Sigma), 0.05% digitonin and 50mM $CaCl_2$. This fixation was followed by thorough rinsing in cacodylate buffer, postfixation with 1% OsO_4 for 30min and embedding as described previously.

4.2.2.2. Tannic acid-Glutaraldehyde-Saponin fixation: Mature epimastigote cultures on Thermanox coverslips were rinsed in MEM and then fixed for 30-60min in 2.5% glutaraldehyde in Buffer-A (100mM $NaPO_4$,

50mM KCl, 5mM MgCl) pH7.0 plus either 0.25mg/ml saponin and 1mg/ml tannic acid or 0.5mg/ml saponin and 2mg/ml tannic acid (Maupin and Pollard, 1983). Following fixation the coverslips were rinsed in Buffer-A pH6 and then post fixed in 1% OsO_4 in Buffer-A pH6 for 30min. The coverslips were then rinsed in Buffer-A pH6 and then distilled water and dehydrated in alcohol and embedded as described above.

4.2.2.3. Osmium Ferricyanide - Tannic acid-Uranyl ion fixation: The Osmium ferricyanide-Tannic Acid-Uranium fixation described by McDonald (1984) for use with PtK1 cells was used for the T.congolense epimastigotes.

Mature cultures on Thermanox coverslips were rinsed in MEM. The epimastigotes were fixed for 30min in 2.5% glutaraldehyde in 0.2M cacodylate buffer plus 50mM $CaCl_2$, pH7.4, followed by rinsing in 0.2M cacodylate buffer plus 50mM $CaCl_2$. All the above steps were carried out at room temperature. The culture bearing coverslips were then placed in 0.2M cacodylate buffer plus 0.8% $K_3Fe(CN)_6$ and 0.5% OsO_4 and incubated in this solution for 15min on ice. This incubation was followed by three 5min rinses in buffer at room temperature and then a 1min incubation in 0.5% tannic acid in buffer, followed by a buffer rinse, a distilled water rinse and then 1h block staining in 2% aqueous uranyl acetate, followed by a distilled water rinse and dehydration in alcohol. Embedding procedures were as described in section 4.2.1.

4.2.3. Osmium-Thiocarbohydrazide-Osmium fixation (OTO)

Mature cultures of epimastigotes on Thermanox coverslips were rinsed in MEM then fixed in the following solution for 1h: 2.5% glutaraldehyde in 0.1M cacodylate plus 4.5mM $CaCl_2$ pH7.2. The coverslips were rinsed in buffer then post fixed in 2% OsO_4 in 0.1M cacodylate buffer, without $CaCl_2$ at pH6.8 for 15min. After post

fixation the coverslips were briefly rinsed in cacodylate buffer then distilled water. The coverslips were then incubated for 5min in a saturated aqueous solution of thiocarbohydrazide, briefly rinsed in water and then in cacodylate buffer pH6.8, followed by 15min in 2% OsO_4 and embedded as described above. The method is as described by Aoki and Tavassoli (1981).

4.2.4. Ruthenium red treatment

Epimastigotes grown on Thermanox coverslips in either 0, 10, or 20% FCS MEM were briefly rinsed in 0.1M cacodylate buffer pH7.3. The monolayers were then fixed for 1h at room temperature in a solution composed of 0.1M cacodylate buffered glutaraldehyde (2.5%) and ruthenium red 1500ppm stock solution added in the ratio 5 fixative solution:1 ruthenium red solution (Pate and Ordal, 1967; Vidric, 1973). After this fixation the coverslips were rinsed in cacodylate buffer and then post fixed in a solution comprising 1 volume 2% OsO_4 in 0.1M cacodylate and 1 volume 15000ppm ruthenium red solution. The coverslips were rinsed in distilled water and then dehydrated and embedded as described in section 4.2.1

4.2.5. Detachment of attached cells

Several methods were used, both singly and in combination to detach some of the epimastigotes and observe the resulting detached epimastigotes using TEM and the remaining areas on the coverslips by scanning electron microscopy (SEM). SEM studies of attached epimastigotes were also carried out.

4.2.5.1. Mechanical detachment of epimastigotes for TEM: Cultures of epimastigotes grown on Thermanox coverslips were simultaneously scraped and squirted with MEM, using a pasteur pipette. The resulting suspension of trypanosomes was pelleted by centrifugation (200xg for

10min) and fixed for transmission electron microscopy. Cells were fixed for 1h in 2.5% glutaraldehyde in 0.1M phosphate buffer, rinsed in buffer (3x5min) containing 2% sucrose,and post fixed for 1h in 2% OsO_4 in phosphate rinse buffer. The fixed cells were enclosed in 1.5% low temperature melting agarose (Seaplaque) then block stained in 0.5% aqueous uranyl acetate for 30min and dehydrated and single embedded in Araldite as before.

4.2.5.2. Scanning electron microscopy of coverslips: Coverslips from which flagellates had been scraped were fixed, rinsed and post-fixed as described previously but then dehydrated in acetone. The dehydrated material was then critically point dried from CO_2. The coverslips were mounted and processed as described in Section 3.2.

4.2.5.3. Chemical detachment of epimastigotes: SEM & TEM: Mature epimastigote cultures grown on Thermanox coverslips were rinsed in MEM and then treated with 0.25% or 1.25% Trypsin in MEM for 15mins. Cultures were then flushed with MEM using a pasteur pipette the detached epimastigotes were then processed for TEM as described in Section 4.2.5.1. The coverslips and the material remaining attached to them were processed for SEM as described in Section 4.2.5.2.

4.2.6. Negatively stained whole mount preparations of epimastigote cytoskeletons

Epimastigote cultures were grown on Formvar coated, carbon stabilized (non-charged) gold or nickel (Balzars) EM (3mm diameter) grids. The grids were sterilized before use by overnight ultraviolet irradition in a laminar flow hood.

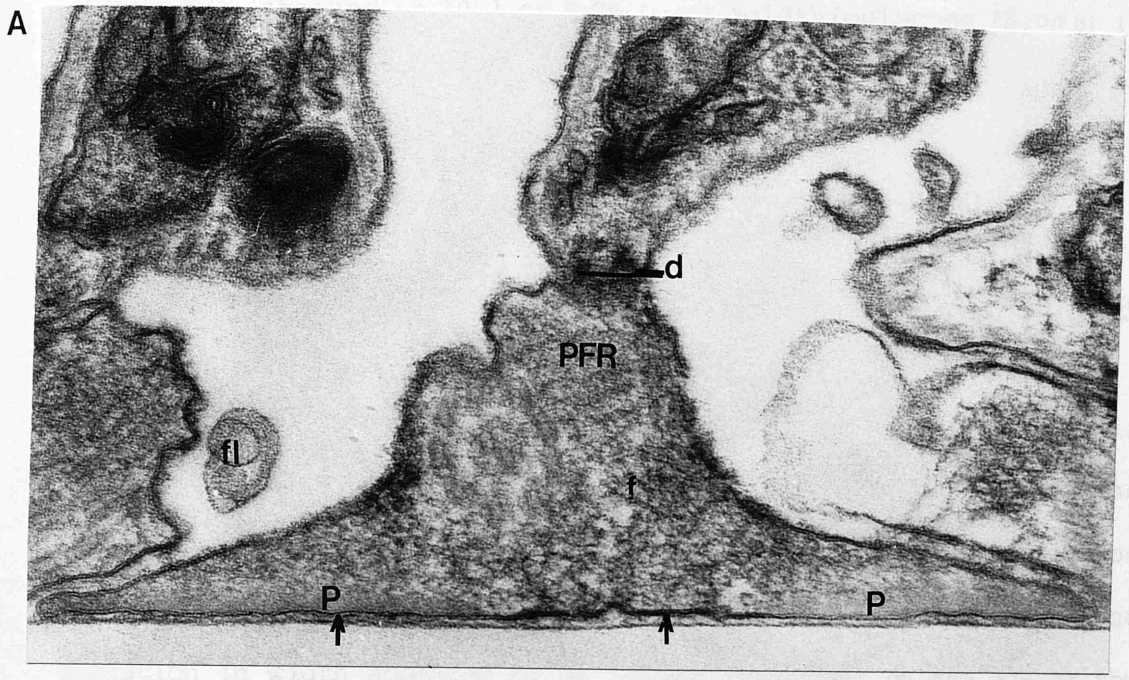
Grids with epimastigotes growing on them were rinsed in 0.1M phosphate buffer then floated on drops of the following solutions: 1% Nonidet P40 in PEME (100mM PIPES (Sigma), 2mM EGTA, 1mM $MgSO_4$, 0.1mM

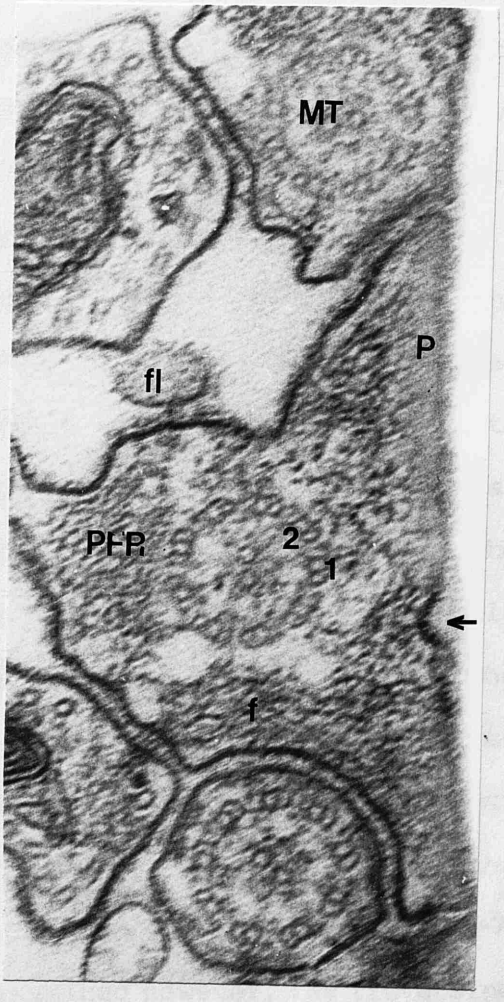
FIG.4:1. Transmission electron micrographs of cross-sections of T.congolense epimastigote flagella attached to Thermanox coverslips.

MAG. all 69,000 x.

P- attachment plaque, PFR- paraflagellar rod, f- filaments, d- flagella-cell body attachment densities, arrow- gap between cell and substratum, fl- filopodia, MT- microtubules.

1





EDTA) for 1-4mins, then transferred to 2.5% glutaraldehyde in PEME for 30 seconds, stained in 20µl of 0.7% (w/v) goldthioglucose (Sigma) in distilled water which was immediately removed by suction. The stained grids were then viewed by transmission EM.

4.3. RESULTS

4.3.1. General description of flagellar attachment in T.congolense epimastigotes

The trypanosomes were found attached to the coverslips by their flagella. At the site of the attachment (Fig. 4.1) an electron dense plaque of material was evident. This plaque had an average width of 42nm but widths varying between 24 and 70nm were observed, the variation in width appeared to be loosely dependent on the age of the culture (see Section 4.3.2). The plaques were found associated with the inner leaflet of the flagellar membrane. These structures had two regions, a more compact zone lying closer to the membrane and a more filamentous zone in the interior region of the plaque.

Fine filaments (4-7nm) in diameter were found associated with the more diffuse region of the attachment plaque. These filaments extended into the flagellar interior and a band of these filaments was noted to traverse the width of the flagellum and associate with the identical filaments extending from flagellum-cell body junctional complexes. The traversing band of filaments in the vast majority (74%) of flagella cross sections examined (n=54) was situated on the opposite side of the flagellum to the paraflagellar rod (PFR). The PFR in this species, as in other trypanosomes, was found associated with microtubule doublets 4-7 (using the numbering system of Afzelius, 1959). Thus, the traversing filaments were located nearest to microtubule doublets 9-7. In 21% of flagella cross sections examined however, the traversing band of filaments crossed the flagellar width nearest to doublets 2-4

FIG.4:2. TEM of longitudinal section of T.congolense epimastigote flagellar attachment.

MAG. 46,650 x.

Labelling as for FIG.4:1.

FIG.4:3. TEM of cross section of T.congolense epimastigote flagellar attachment showing two sets of flagella-cell attachment junctions (d).

MAG. 69,000 x.

i.e. at the same side of the flagellum as the PFR. In approximately 5% of the flagellar examined the traversing band of filaments crossed the flagella on both sides of the axoneme and PFR.

The cross sectional profile of the flagellum shaft was extended laterally at the site of flagellar attachment. In many organisms this extension was found to be in the order of 1μm in width. In longitudinal sections of attached flagella (Fig. 4.2), downward projections extending approximately 1um along the flagellum in an anterior-posterior direction were noted. Thus an extension of $1\mu m^2$ or more from the flagellum was possibly occurring at each attachment point. Longitudinal sections of flagella also showed that more than one extension and attachment plaque could occur per flagellum. In the few flagellar extremities encountered in sections, the attachment areas were not found at the tip but situated towards the flagellar mid-region.

Attachment plaques did not necessarily extend continuously across the entire width of the lateral extension. In many cases a break in the plaque was noted (e.g. Fig. 4.1) so that more than one plaque was then found in the flagellum with the intervening portion devoid of dense material. A gap between the flagellum and substratum was present. In regions where plaque material was present on the inner membrane, the average width of this gap was 14.9nm ($\pm 3, n=97$). Where no plaque material was present, either due to the gap occurring in an intervening region between two plaques or due to no plaque material being present in the flagellum, the width of the extracellular gap was greater than 20nm. In cross sections where a break in attachment plaque material and an associated increase in gap width was found, the membrane was invaginated, recalling endocytotic 'omega' profiles but no vesicles were noted in the interior of any of

the attached flagella.

In the majority of flagella examined the axoneme was found in a central position within the flagellum lateral extension. The extension was not necessarily symmetrically arranged around the axoneme but neither was the axoneme displaced to one side as found in T.vivax (Vickerman, 1973). The structure of the microtubule axoneme and the PFR and their relationship to one another were identical to these found in other trypanosomes (Fuge, 1969; Vickerman, 1973).

Flagella cut in longitudinal section along the zone of adhesion of the flagellum to the cell body displayed a row of cell-flagellum attachments. These attachments were marked by small accumulations of electron dense material on either side of the junction. These junctional complexes were spaced at intervals (centre to centre) of 70nm along the flagellate. A gap of 20nm between the flagellum and cell body halfs of the junction was found. On the cell body side of the junction a space in the pellicular microtubule sheath was noted, the space appeared to be the width of one microtubule (Fig. 4.1) the electron dense material of the junctional complex was found located within this microtubule space. Occasionally it was noted that one of the microtubules in the pellicle adjacent to the space occupied by the junctional density, had a reduced diameter (Fig. 4.1) in comparison with the other pellicle microtubules.

The cell-flagellum junctional densities appeared conical in shape and were more compact on the cell body size of the junction than on the flagellar side. The cell body half of the density was larger in size - 45nm across compared with 22nm on the flagellar side. Fine filaments (5nm in diameter) were found extending from these dense zones on both the cell and flagellar side. On the flagellar side of the junction these filaments were found associated with similar filaments extending from the flagellum-substratum attachment plaque in

attached cells and also with the filamentous connection between microtubule doublet number 7 and the PFR (Fig. 4.1). The filaments on the cell body side of the junction were less distinct in appearance and merged into the filamentous cytoplasm in the cortex of the cell.

Transverse sections of the flagellum-body junction where the flagellum was not attached to the substratum displayed a single cell-flagellum junctional complex however transverse sections of flagella attached to the substratum contained several rows of cell-flagellar junctional densities; the maximum noted was six. Flagella in which the flagellar attachment gave the appearance of being in an early stage of attachment plaque formation contained fewer rows of cell-flagellar junctional densities than did flagella with well formed cell attachment plaques (Fig. 4.1). The increased number of cell-flagellar junction rows resulted in the space in the microtubule pellicle being increased in size in attached epimastigotes.

The extracellular gap between flagellum and substratum and also the gap between the cell and flagellar junction densities contained stainable material of an amorphous nature and similar in appearance in both gaps.

Circular, tubular and oblique tube-like membrane bound profiles were noted in micrographs of attached epimastigotes. These structures had an average diameter of 160nm and were not found to contain much internal structure, occasionally a few wispy strands of filamentous material was evident within them. These structures were noted only in association with the attachment area of attached epimastigotes and probably correspond to the 'filipodia' described in Section 4.3.6.

In general epimastigotes were found attached in clusters. Flagellar extensions of adjacent epimastigotes were found very closely opposed (<20nm in some instances), however no discernible flagellum-

flagellum attachment plaques were noted. No cell body-cell body attachment plaques were found either. However, two sets of cell-flagellar junction densities could be found within the same cell (Fig. 4.3) if growth was such that two areas of cell body attach the flagellum. It is noticeable in Fig. 4.3 that the flagellum of the cell adjacent to the unusual cell, although touching the cell body of the unusual cell does not contain dense material at the site of attachment with the cell body in question.

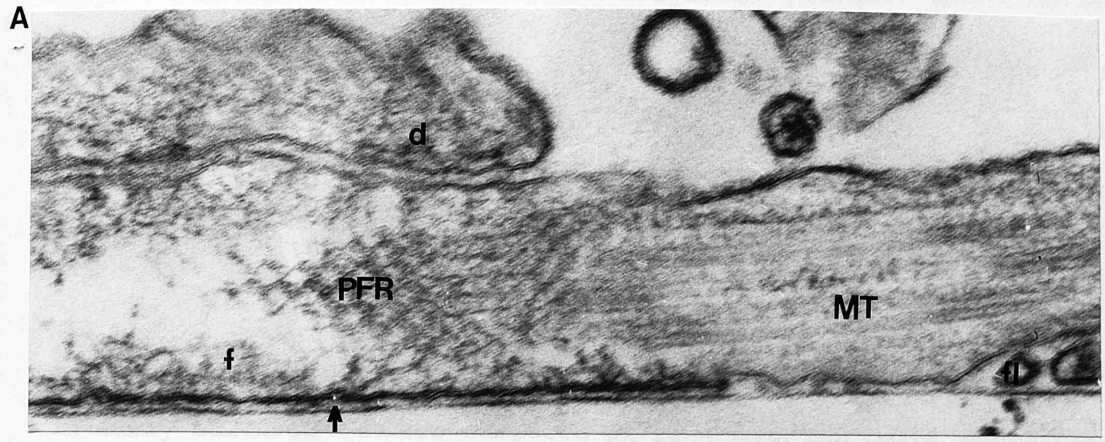
Within a bundle of epimastigotes the flagella of the component cells appeared to be orientated in the same direction. All flagella in a cluster were aligned in a similar orientation with respect to the long axis of the cell although within this orientation a 180^{o} rotation could be found in adjacent flagella (determined by the position of the PFR and microtubule doublets with respect to each other). Thus in a bundle all flagella were found in cross section or all in longitudinal section rather than a mixture of these. Although no intraflagellar attachment junctions were found in epimastigote bundles, flagella were observed to fit into the shape of the space allotted between neighbouring flagella.

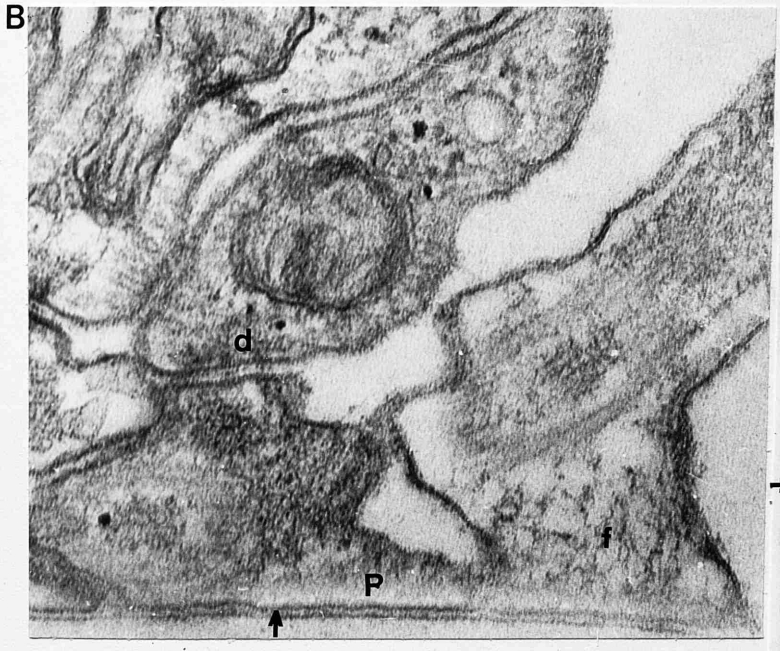
4.3.2. Development of flagellar attachment

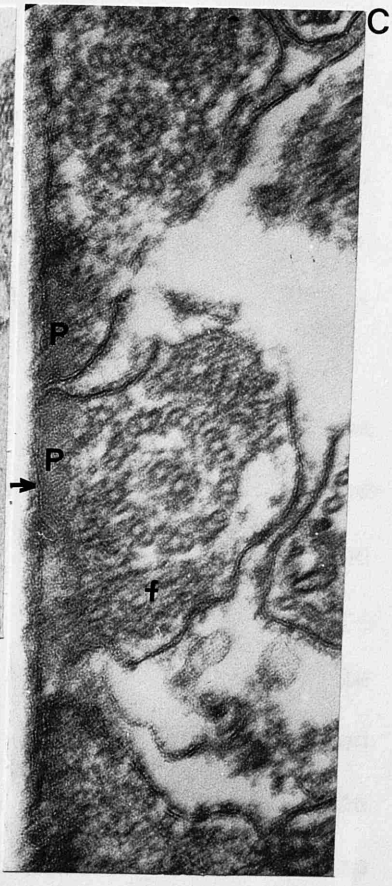
In early cultures, 1-5 days, the majority of the epimastigote attachment sites did not contain a dense attachment plaque (Fig. 4.4). the plaques present appeared more filamentous and less compact than those of older culture epimastigotes. The width of the plaques in early cultures were in the order of 15-20nm. In very early attachment sites filaments were present without any plaque formation. The extent of lateral membrane extension from the flagellar shaft was less pronounced in early attachment profiles than those of mature attachments and the attachment associated filaments appeared less

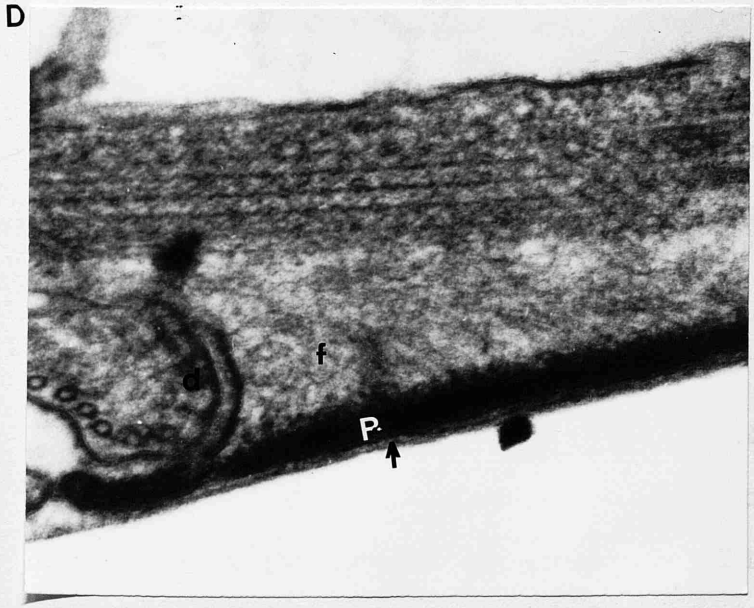
FIG.4:4. TEM micrographs of T.congolense flagellar attachment sites at various times after culture initiation.
(A) 3 days (B) 5 days (C) 7 days (D) 17 days
MAGS. (A) 76,500 x. (B) 76,500 x. (C) 88,200 x. (D) 69,000 x. Labelling as for 4:1.

4









organized, although traversing filament bands were found in some cases. The number of rows of cell-flagellum junction densities also appeared to be fewer in early attached cells than established cultures. Older cultures contained cells with compact, dense attachment plaques with widths of approximately 40nm and increased numbers and organization of associated filaments. An increased number of rows of cell-flagellum junction densities and a general increase in the number of attached cells per cluster was found to occur with age. In early cultures bundles composed of less than ten cells were common. In older cultures bundles with over one hundred members were present. After approximately day 7 onwards (Fig. 4.4), attachment morphology was found to be very similar in cultures of different ages. In all cultures cells with a less pronounced "immature" attachment were noted as well as "mature" attachments, some cells with very dense attachment plaques were also observed (Fig. 4.4).

Dividing epimastigotes were commonly found in sections. The epimastigotes appeared to remain attached whilst dividing and the newly formed epimastigote flagellum attached in an adjacent position to the existing flagellum.

The epimastigotes fixed using enhancing procedures on the whole did not show different morphology from cells conventionally processed although an improved resolution of some structures was observed. The following observations will concentrate on these emphasised areas rather than overall descriptions.

4.3.3. Tannic acid enhancement procedures for TEM

4.3.3.1. Detergent permeabilization: Even at the very low concentrations of digitonin used (0.05%) the disruption of the cells was excessive and as such the amount of tannic acid able to penetrate the cell membrane and bind to cell structures was too great for

FIG.4:5. Oblique section of a T.congolense epimastigote treated with Tannic acid (2%) and Digitonin (0.05%).
There is a great deal of detergent damage and the Tannic acid is at too great a concentration for interpretation of structure.
E-epimastigote body , F-flagellum.
MAG. 126,000 x.

FIG.4:6. TEM of T.congolense treated with Tannic acid (2mg/ml) and Saponin (0.5mg/ml). The detergent damage is too great for structure interpretation.
MAG. 453,600 x.

clarity and structure interpretation (Fig. 4.5). Permeabilization with this detergent was therefore abandoned and another protocol used.

The cells fixed in solutions containing the greater concentration of saponin (0.5mg/ml) were also inclined to be disrupted more than desired for structure interpretation. Much of the cell interiors were found to have been extracted by the detergent treatment - this disruption did not aid interpretation particularly of structure association (Fig. 4.6). Cells fixed in media containing a lower concentration of saponin (0.25mg/ml) did not appear to suffer the wholesale extraction found with the higher detergent concentration. In many cases, however, the tannic acid appeared to have been unable to penetrate the cell membrane fully and was observed to accumulate around the surface of the epimastigotes in electron micrographs (Fig. 4.7). Penetration of the chemical did occur in some instances, however, and resulted in an increase in detail visible in the attached flagella. In favourable cross-sections the band of filaments traversing the width of the flagellum were more clearly visible than in conventionally fixed material and the association of this band with the cell body - flagellum junction densities was evident (Fig. 4.7). Filaments joining the cell-substratum attachment plaque to microtubule doublets 8-2 were also visible (Fig. 4.7). The general ultrastructure of the cell, in particular the membrane organelles, was also noted to be enhanced after this treatment. The gap between the flagellum and substratum also became more apparent due to the greater visibility of the plasma membrane.

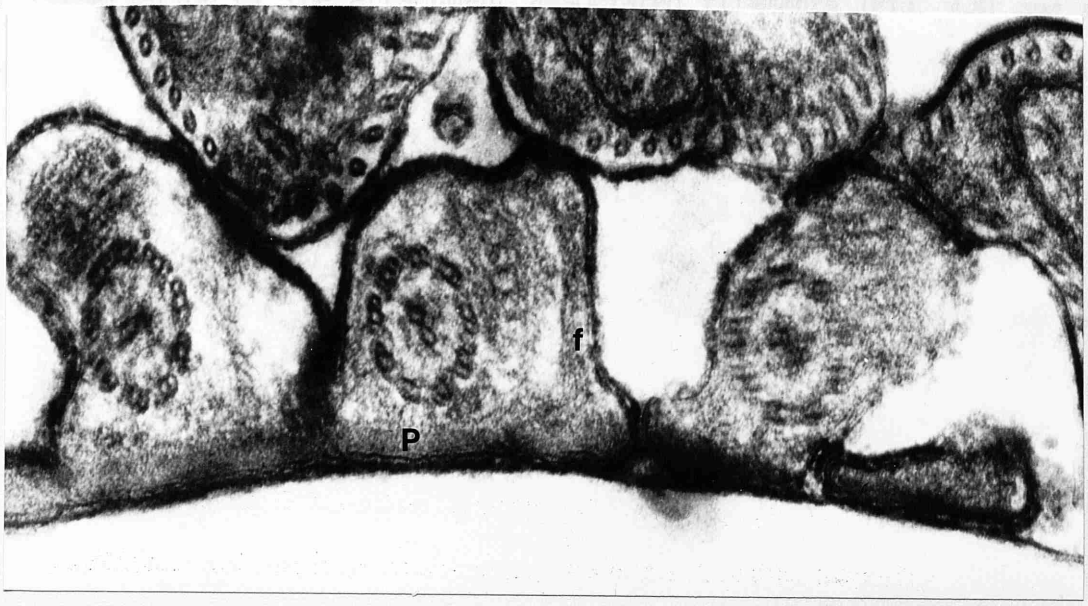
4.3.3.2. Osmium ferricyanide-Tannic Acid: This fixation protocol has previously been shown (McDonald, 1984) to increase membrane contrast and improve the presentation of microfilamentous structures.

FIG.4:7. TEM showing cross sections of attached flagella of epimastigotes treated with Tannic acid (1mg/ml) and Saponin (0.25%). Filament enhancement is found with this treatment.
MAG. 81,000 x.
Labelling as for FIG.4:1.

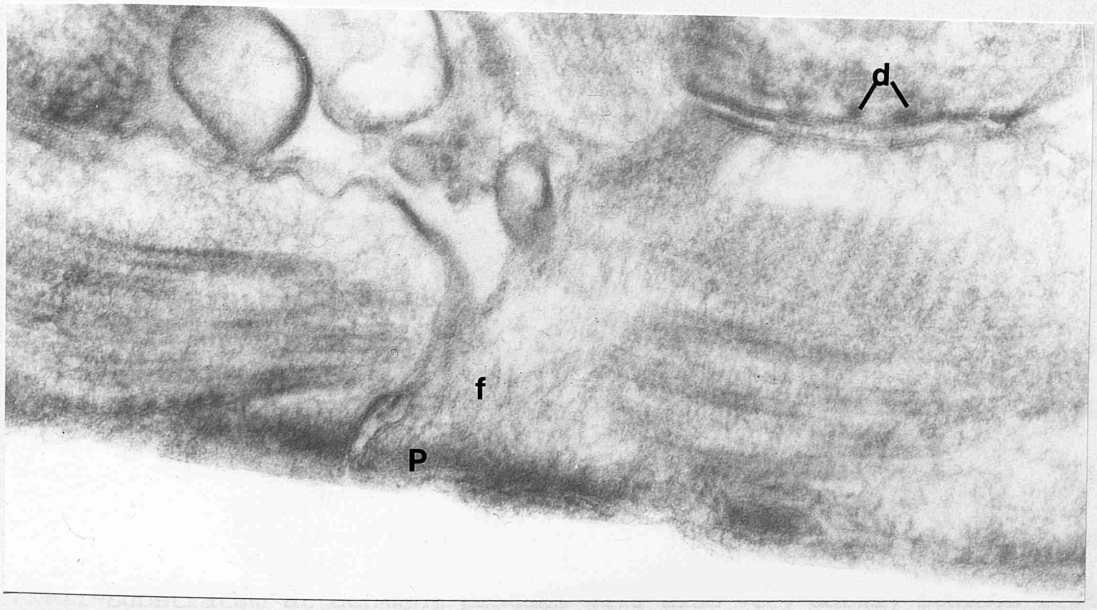
FIG.4:8. Attachment site of T.congolense treated with OSFeCN-Tannic acid. Filaments are evident within the flagellum but the coarse cytoplasmic staining found with the Tannic acid /detergent treated cells is absent.
MAG. 190,000 x.

FIG.4:9. TEM showing cross sections of attached flagella after fixing the cells with Osmium-Thiocarbohydrazide-Osmium. Filament and plaque material are enhanced (c.f. FIG.4:1.)
MAG. 229,000 x.

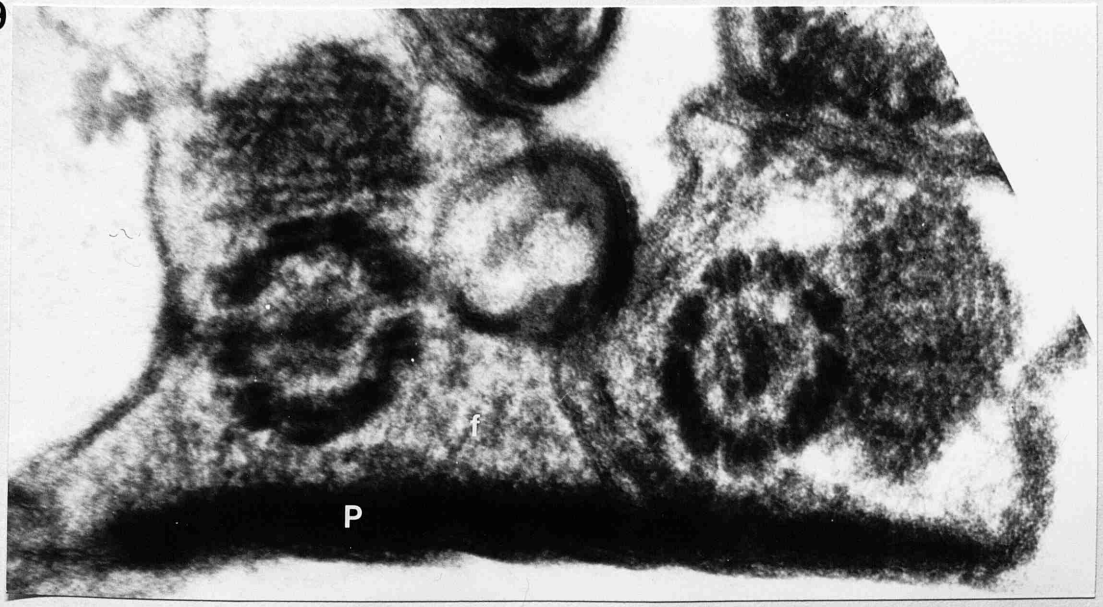
7



8



9



The resulting fixed material appeared to increase the preservation of the attachment associated filaments (Fig. 4.8) and an apparent increase in number of individual filaments was observed within the flagella not only in direct association with the attachment plaque but in the entire flagellar sheath, spanning its width. The general structure of the cell and the attachment zone were noted to be more enhanced than generally observed in conventionally fixed preparations but less than in the fixations including tannic acid in the fixative solution; this was more than compensated for by the removal of the general dark, coarse, appearance of the above fixations; the detergent damage was also removed.

4.3.4. Osmium-Thiocarbohydrazide-Osmium (OTO) treatment

This protocol has also been reported to preserve fine filaments normally prone to osmium damage (Aoki & Tavassoli, 1981). In the epimastigote attachment site the fine filaments were again more evident than in many conventional fixations (Fig. 4.9), however, the visibility of these filaments under this protocol was not so clear as with osmium ferricyanide-tannic acid or tannic acid-saponin. An improvement in the visualization of the accumulation of dense material at the both halves of the cell-flagellar junctions was noted. The cell-substratum attachment plaques were also very darkly stained under this processing regime and became very obvious in all micrographs.

4.3.5. Ruthenium Red

Treatment with ruthenium red is thought to accentuate polysaccharides in cell surface coats (Luft, 1971a&b). The chemical was found to bind to the epimastigotes over their entire cell surface both on the cell body and flagellum (Fig. 4.10) including the areas of the flagellum-substratum attachment gap and the flagellum-cell body attachment gap. In the latter gap a double layer of ruthenium binding

FIG.4:10. and 4:11. TEMs of attached flagella treated with ruthenium red during fixation. Ruthenium red is bound all over the cell surface including the extracellular and flagellar-cell junction gaps.

FIG.4:10. Culture medium included 10% FCS.

FIG.4:11. Culture medium included no FCS.

Inset- Coverslip surface without any cells showing a low level of ruthenium binding.

MAG. 4:10. 286,200 x 4:11. 104,000 x Inset. 189,000 x.

Arrow head shows ruthenium binding.

FIG.4:12. Scanning electron micrograph depicting epimastigote bundles attached to a Thermanox coverslip.

E- epimastigotes M- metacyclics.

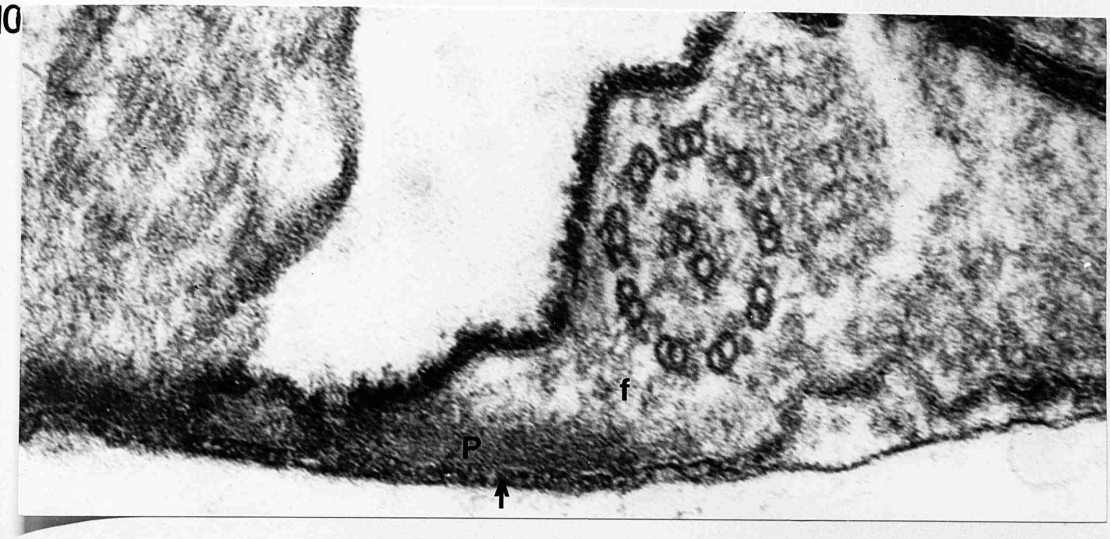
MAG. 1,280 x.

FIG.4:13. SEM of attached epimastigote flagella. Two attachment extensions can be noted in the flagellum nearest the bottom of the micrograph and one in the flagellum parallelling it.

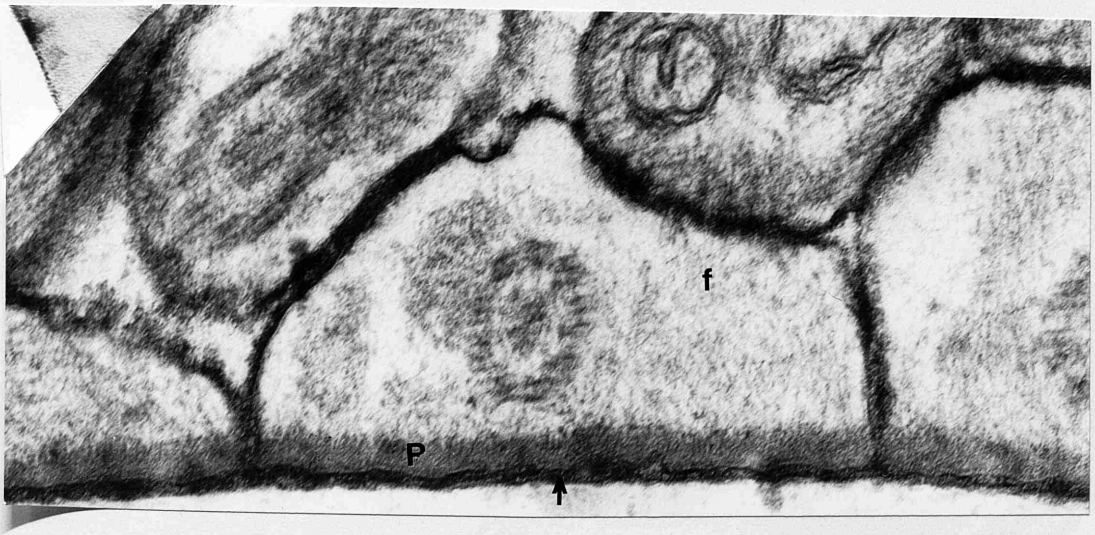
A- attachment extension, F- flagellum.

MAG. 13,000 x.

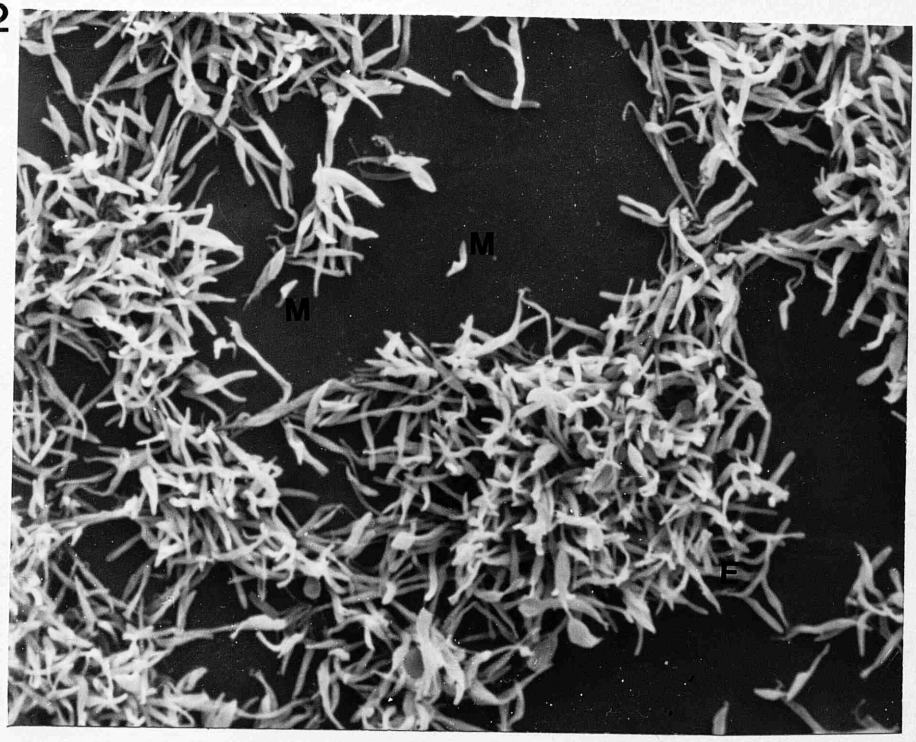
10



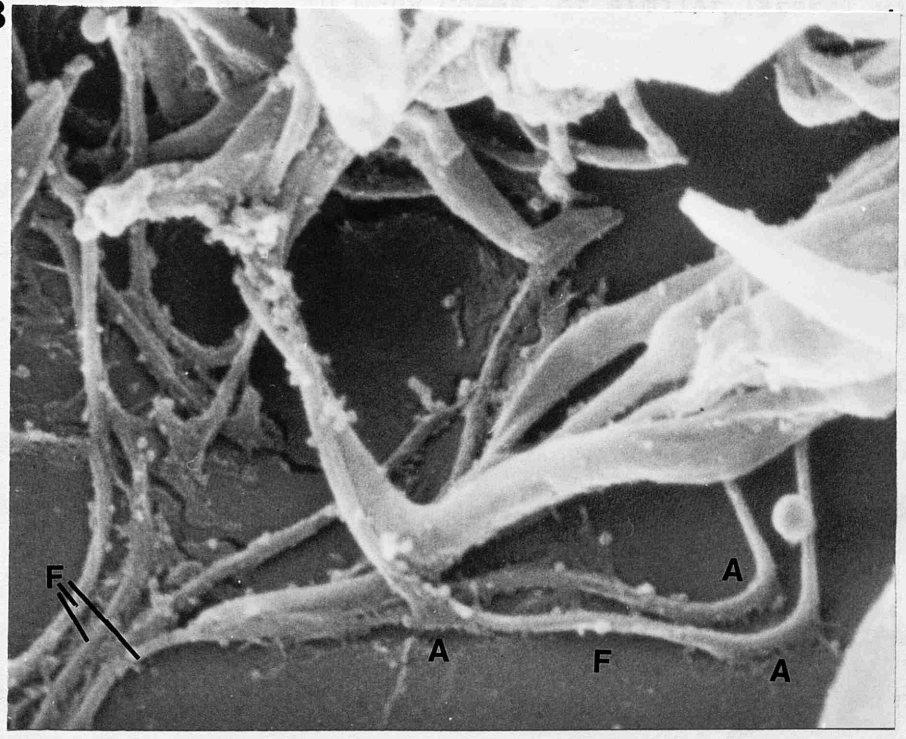
11



12



13



was noted. The ruthenium binding also occurred in the absence of serum (Fig. 4.11) but the amount of ruthenium staining of areas of the coverslip surface where cells were absent appeared reduced in the absence of serum. An enhanced staining of plaque and filament material was also found in the cells treated in this manner.

4.3.6. Scanning electron microscopy of attached flagellates

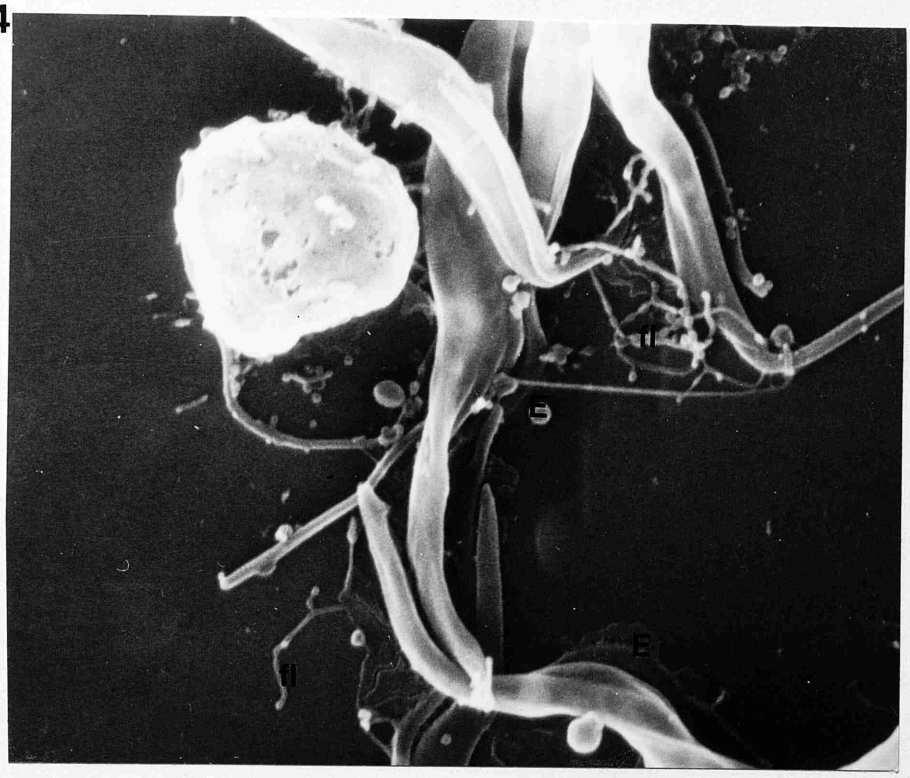
SEM observations of Thermanox coverslips bearing T.congolense epimastigote cultures showed bundles of elongate epimastigotes attached by their flagella and having blunt posterior ends. Between the epimastigote bundles small (approximately 6μm long) metacyclic trypomastigotes were visible (Fig. 4.12).

On closer observation the attached flagella were noted to have both dorso-ventral and lateral 'foot-like' membrane expansions. These expansions were in the order of 1μm in length (Fig. 4.13 - compare with Fig. 4.2) and, in many cases, of similar lateral width (Fig. 4.14). Flagella were often found remaining attached to the substratum when the cell-body had been removed suggesting that the flagellum-substratum attachment is stronger than the cell-flagellum attachment. The attachments were noted to be located in the mid region of the flagellum and not at the anterior tip. More than one attachment could be present per flagellum. When a lateral extension of the flagellum membrane was noted the flagellar axis occupied a central position. Within an epimastigote bundle the flagella were noted to be orientated in the same anterior-posterior orientation, the attachment sites of these flagella occurred in adjacent positions. As well as the lateral extensions of adjacent flagella abutting onto each other, small filopodia-like extensions were located near the points of attachment, intertwining with filopoda extending from neighbouring flagella. The intertwining of neighbouring filopodial outgrowths gave the impression

FIG.4:14. Lateral membranous extensions from the attached flagella are visible in this micrograph (E). Filopodia are also evident (fl).
MAG. 10,300 x.

FIG.4:15. "Footprint" material remaining after mechanical detachment of epimastigotes. This SEM shows parts of flagella (F) remaining in bundles with lateral membrane extensions (E) between them.
MAG. 40,000 x.

14



15

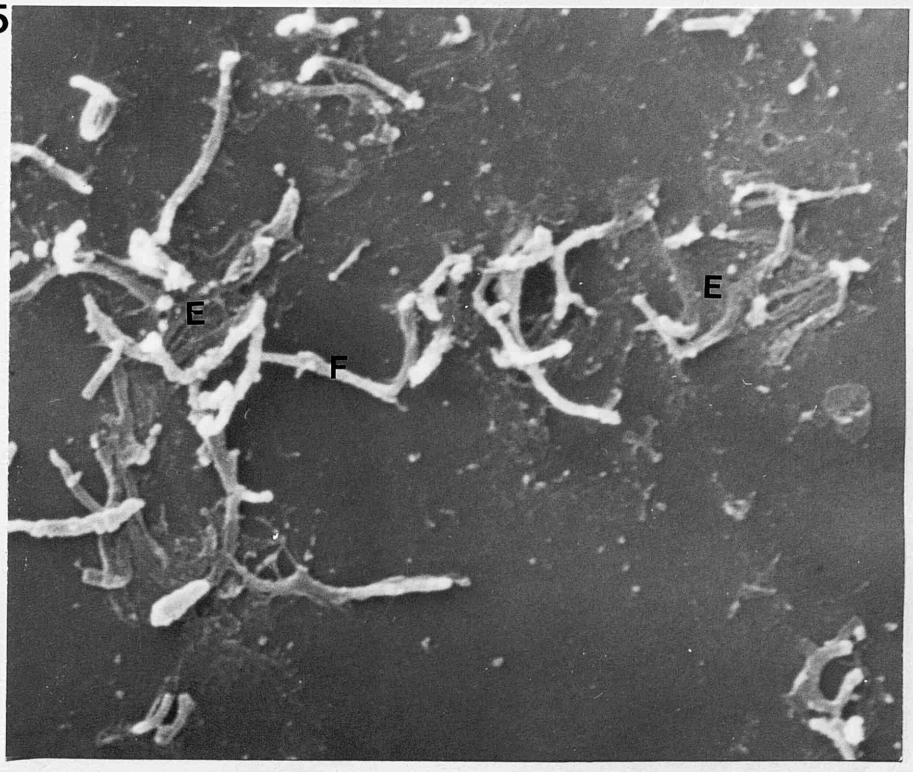
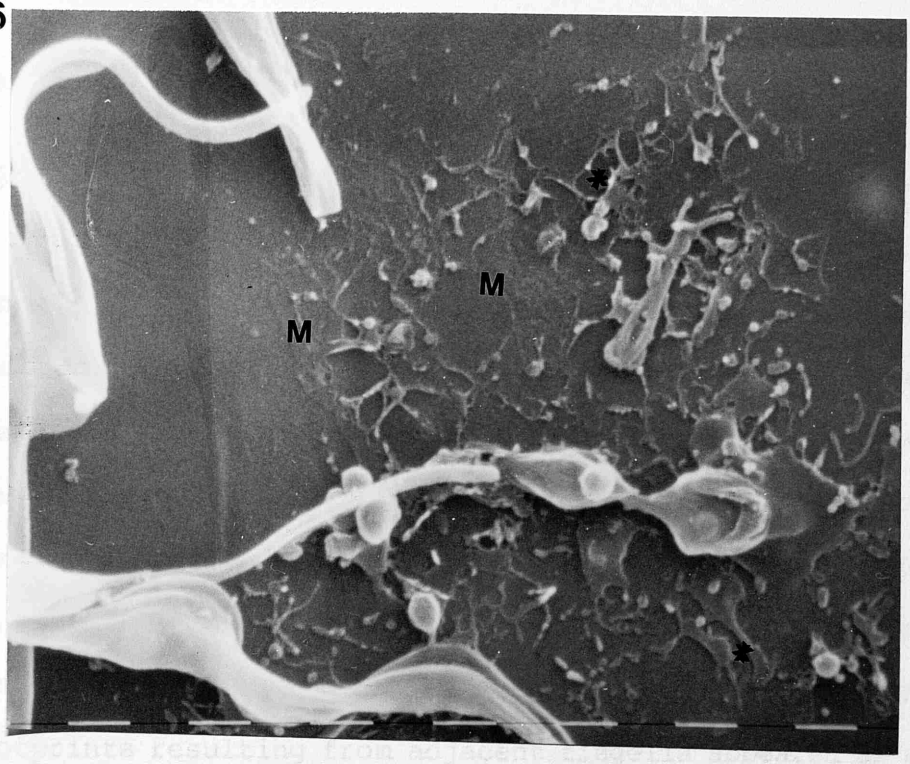


FIG.4:16. and 4:17. "Footprint" material remaining after chemical detachment of epimastigotes.

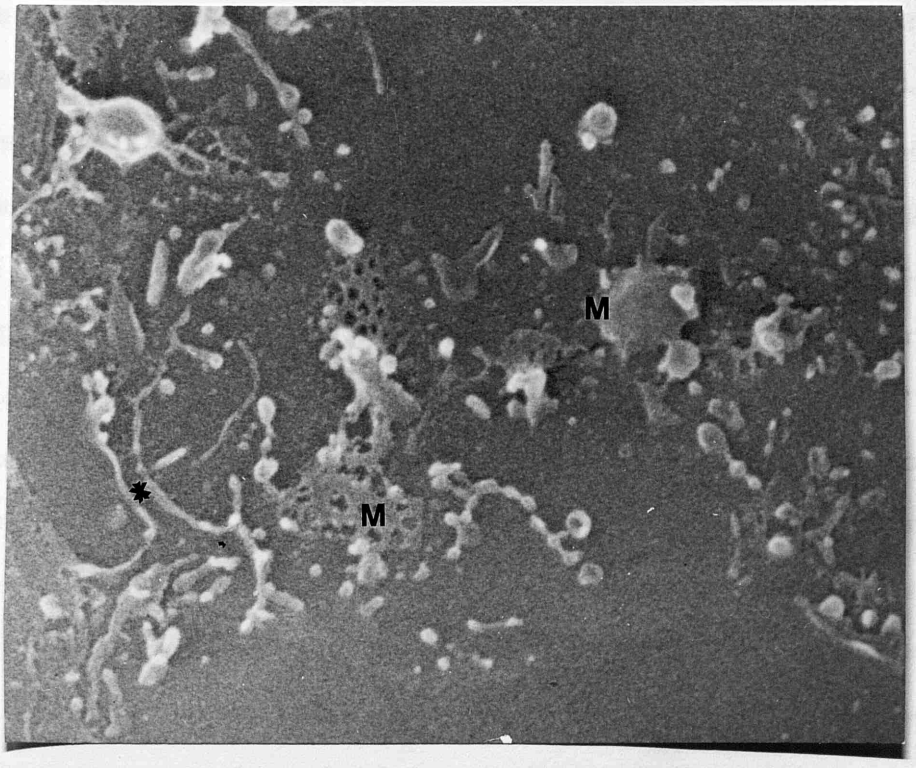
Thin membranous material remains attached to the coverslips (M), many are composed of two parts with a central vacant area possibly where the flagellum has been removed (*). Some of the remaining material has a honeycombed appearance (FIG.4:17).

MAG. 4:16. 12,500 x. 4:17. 25,000 x.

16



17



that these were contributing to the integrity of the epimastigote bundle. These filopodia could be extensive, in some cases many micrometers long, and bifurcation did occur, some of these structures had a beaded appearance (Fig. 4.14).

4.3.7. Morphology of material remaining after epimastigote removal

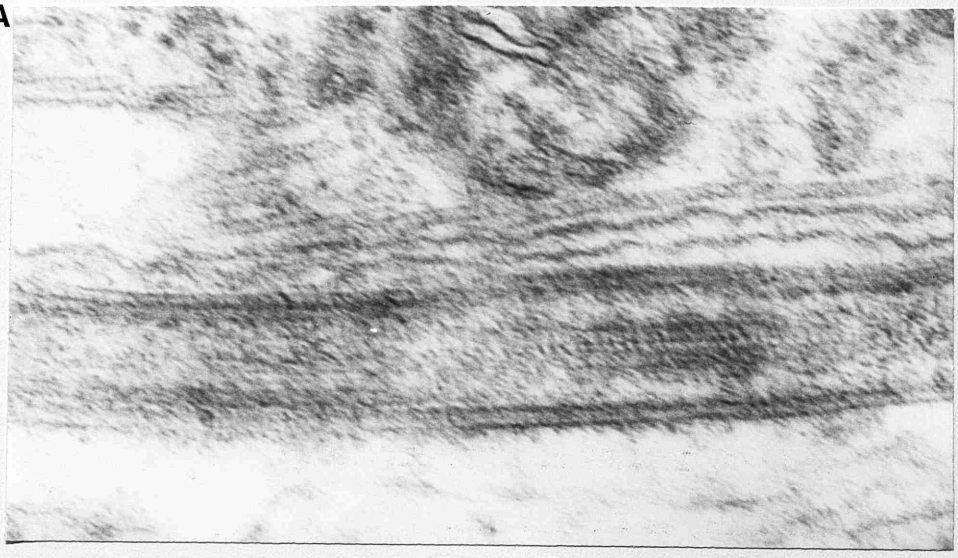
When epimastigotes were removed by mechanical means alone the remaining structures, observed by SEM, in the path of the scraped area were attached flagella, parts of flagella (in the main, areas around the attachment), filopodia and attachment site membrane extensions. I have called the last structures "footprints" using analogy to structures remaining after fibroblast cells are removed (Badley et al., 1978). The "footprint" structures appeared to be composed of thin membrane with irregular lateral edges and irregular shape (Fig. 4.15). Footprints resulting from adjacent flagella appeared as a mass of membranous material. The regular alignment of neighbouring flagella was very noticeable in many micrographs. SEM studies on the material remaining in cultures where cells were removed by a combination of chemical and mechanical means, showed fewer detached flagella than the purely mechanical removal of cells. The remaining material appeared to be composed of either complete epimastigotes with very little damage apparent and very extensive filopodia outgrowths from the area of the flagellar attachment (Fig. 4.14) or footprint material (Fig. 4.16). The footprint material found appeared to be composed of a thin membranous layer apposed to the coverslip. In some instances this material had a perforated, honey-combed appearance (Fig. 4.17). Many of the footprints appeared composed of two similar membranous parts with a central area in which less material was present, the central area was approximately 0.5μm in width - a similar width to the flagellar shaft.

FIG.4:18. Cross section and longitudinal section of flagella of mechanically detached epimastigotes. No attachment plaque material is present.

MAG. (A) 120,000 x. (B) 124,400 x.

Labelling as for FIG.4:1.

A



B

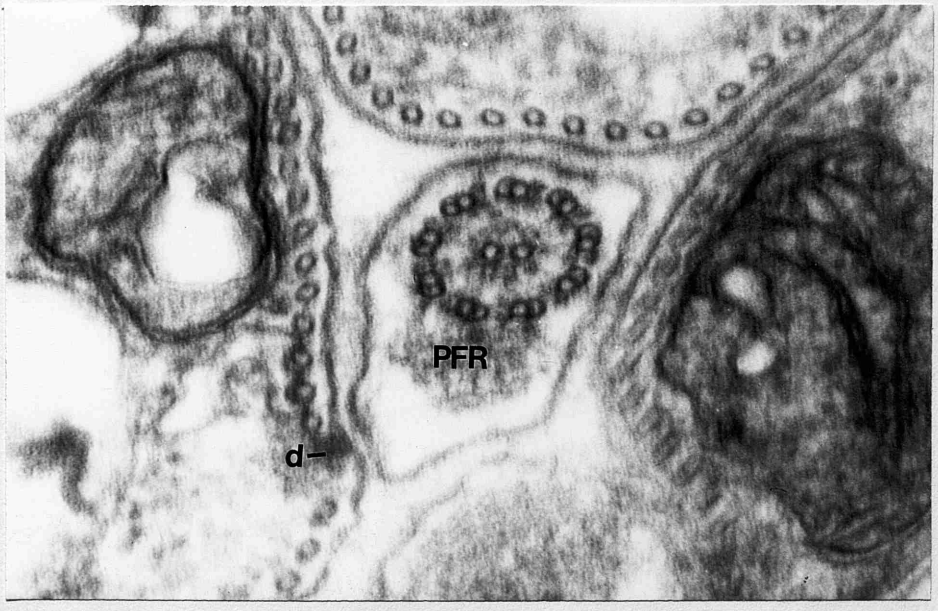
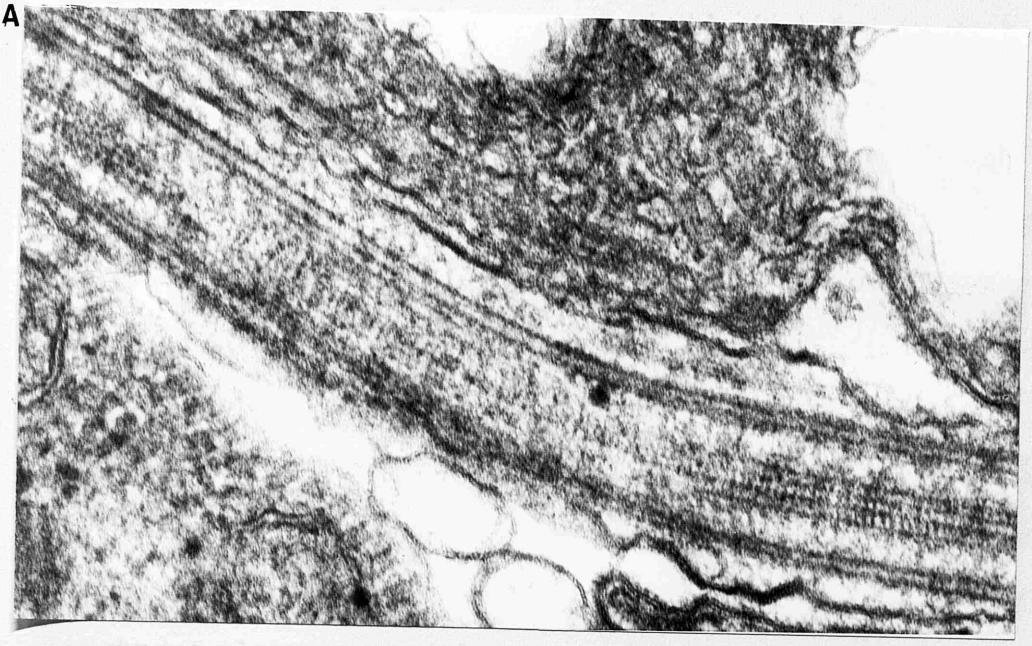


FIG.4:19. As for FIG.4:18. but depicting chemically removed cells.

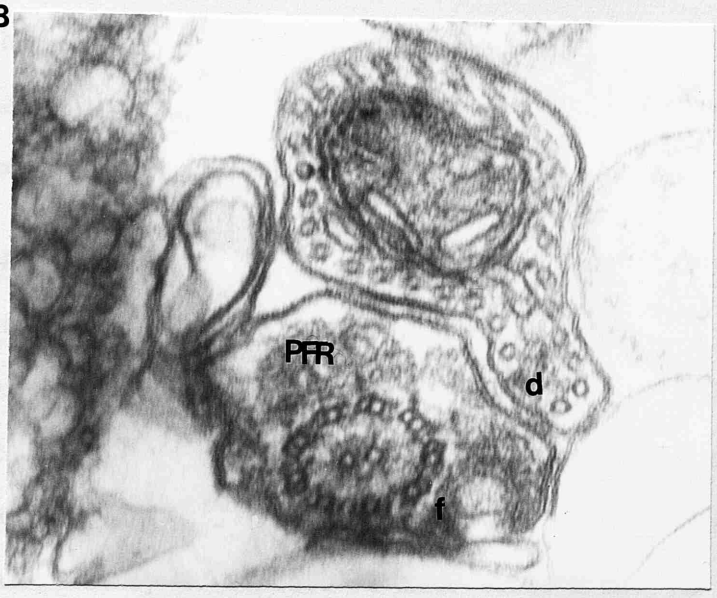
MAG. (A) 142,000 x. (B) 106,000 x.

19

A



B



TEM observations on flagella of cells detached by scraping alone did not show any evidence of very electron dense attachment plaques (Fig. 4.18), some flagellar cross sections did contain filaments and the traversing band of filaments was visible in a few cases. In these later flagella several rows of cell-flagella junction densities were visible. The flagellar surfaces where the attachment plaques would have been expected were irregular. The flagellar cross sectional widths did not show the very great lateral extensions found in some attached cells. Longitudinal sections of flagella also showed an irregular plasma membrane but there was no evidence of very dense attachment plaques (Fig. 4.18a). The ultrastructural morphology of epimastigotes detached by a combination of mechanical and chemical methods was similar to that described above (Fig. 4.19). No very dense attachment plaque material was noted but filaments were present in some flagella. No large extension of the flagellar membrane was found in either cross or longitudinal sections of this material. The flagellar membrane was again noted to be irregular in the area where attachment plaques would have been expected.

4.3.8. Negatively stained whole mount cytoskeleton preparations of attached epimastigotes

Epimastigote cultures grown on coated EM grids and detergent extracted before being negatively stained, remained in bundle formation attached to the coated grids. Cells treated in this manner were found mainly to consist of microtubules and flagellar structures although in many cases the cell membrane had not been fully extracted and was also present to some extent. The epimastigotes retained their general shape including the blunt posterior end. These cytoskeleton preparations showed that this blunt end is formed by microtubules ending at the posterior extremity of the cell without tapering. Cell-

FIG.4:20.-4:23. Negatively stained whole mount preparations of attached T.congolense epimastigote cytoskeletons.

FIG.4:20. Areas of attached flagella showing lateral extensions of attachment material.

F- flagellar shaft, E- extension.

MAG. A) 29,000 x. B) 40,500 x.

FIG.4:21.-4:22. Flagella of attached epimastigotes. Membranous material can be seen between the flagella abutting the flagella of the neighbouring cells (m), stain has become trapped in this area in some cases.

MT- microtubules.

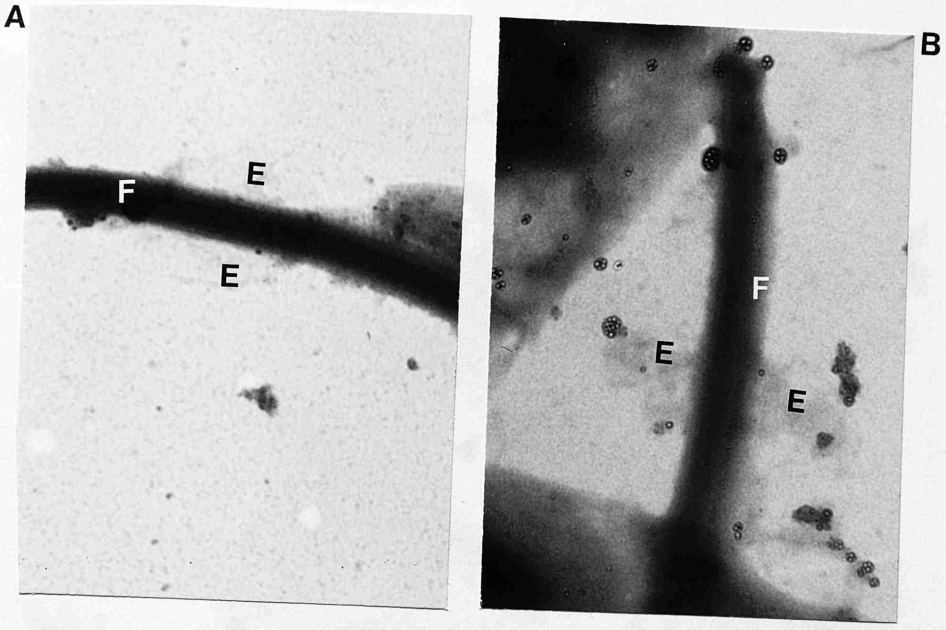
MAG. FIG.4:21. 8,120 x. FIG.4:22. 10,500 x.

FIG.4:23. Bundle of epimastigotes with possible transitional (T) forms, between epimastigote (E) forms and metacyclic forms, present.

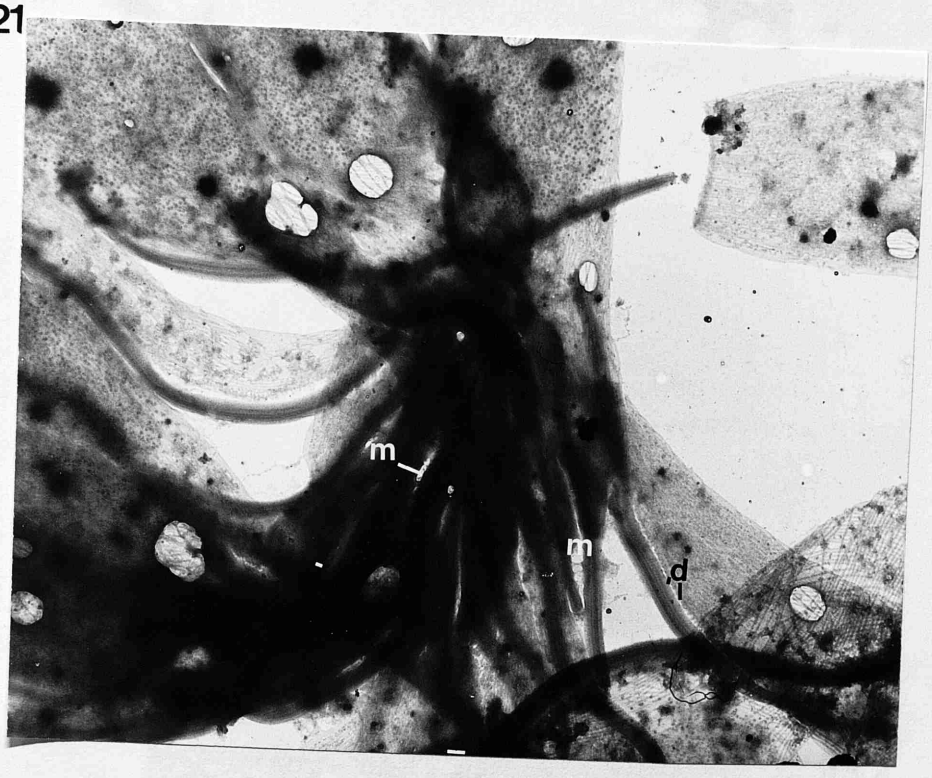
N- nucleus, MT- microtubules.

MAG. 4,100 x.

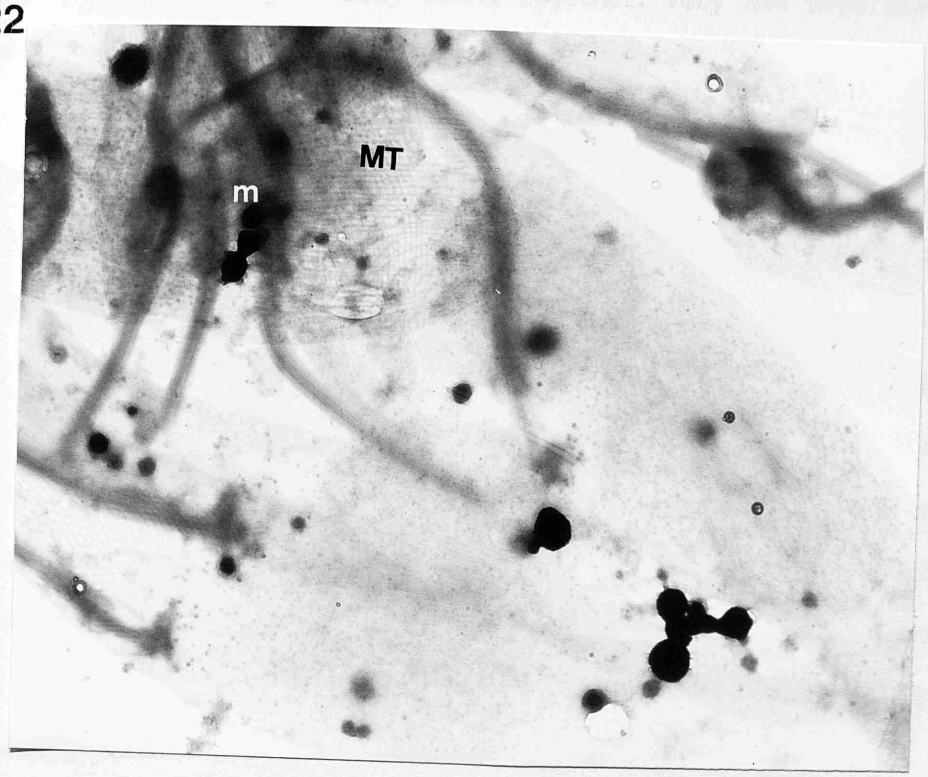
20



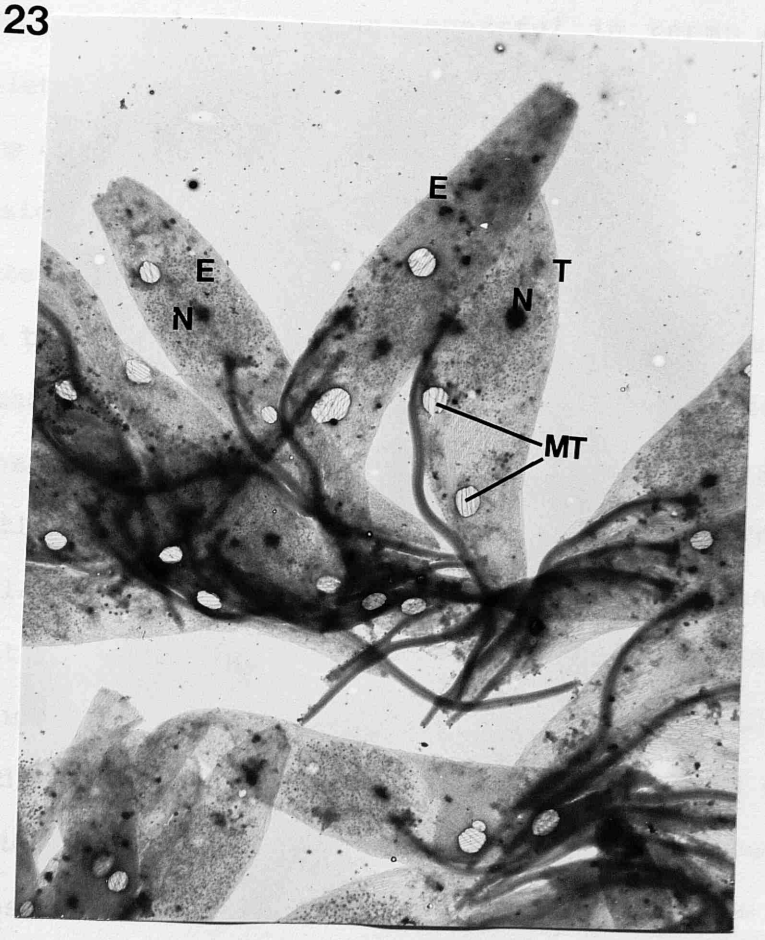
21



22



23



flagellar junctions remained intact (Figs. 4.21-4.23) and flagella and cell bodies were generally found together. Very few separate flagella were noted.

Co-orientation of flagella of the members of an epimastigote bundle was again noticeable in these preparations. Lateral extensions from these flagella were visible but these appeared reduced in size compared with those in SEM preparations, however some extensions of up to 250nm on either side of the flagellar shaft were noted (Fig. 4.20) in cells where the attached flagellum was not bundled into a group. In some bundles a membranous connection between neighbouring flagella was visible (Figs. 4.21 & 4.22). In some groups the stain had become trapped (Fig. 4.21) in the region of the attachment. No filopodia were observed suggesting these structures were membrane associated and removed with the extracted membrane.

This technique was unsuccessful in terms of observing cytoskeletal elements other than microtubules, no filaments associated with the cell attachment could be observed although in a few cases, an impression that the lateral extension material had filamentous structure was obtained.

As the flagellar base and, in most cells, some nuclear material was intact in these preparations the morphological forms of these trypanosomes could be distinguished. Dividing epimastigotes were frequently observed within epimastigote bundles. Some cells had their flagellar base in a more posterior position than those of epimastigotes, as if these were transitional forms between epimastigotes and metacyclic trypomastigotes (Fig. 4.23). These cells remained attached members of the cluster of cells and otherwise had epimastigote morphology. Metacyclic forms were not found but as the cultures used for these preparations were only a few days old (to

TABLE 4.1. COMPARISON OF IN VIVO AND IN VITRO GROWN TRYPANOSOMA CONGOLENSE EPIMASTIGOTE ATTACHMENT SITE MORPHOLOGIES

	IN VIVO	IN VITRO
LOCATION[a]	Flagellum, but not anterior tip.	Mainly mid region of flagellum, not at anterior tip.
FLAGELLAR EXTENSION DIMENSIONS[a]		
Lateral	0.5µm	1µm
Anterior-posterior	0.5µm	1µm
FLAGELLAR-SUBSTRATUM ATTACHMENT PLAQUE THICKNESS[b]	20nm ± 11 sd. (n=26)	42nm ± 25 sd. (n=97)
ATTACHMENT ASSOCIATED FILAMENTS[b]		
Size	5nm diameter	4-7nm diameter
WIDTH OF FLAGELLAR-SUBSTRATUM GAP[b]	10nm ± 5.2 sd. (n=20)	14.9nm ± 3 sd. (n=97)
FLAGELLUM-CELL BODY GAP WIDTH[b]	10nm	20nm
FLAGELLUM-CELL BODY JUNCTIONAL DENSITIES[b]		
Shape	Conical	Conical
Size (width)	10nm	45nm (cell body half) 22nm (flagellar half)
N°.	max. noted = 2	max. noted = 6
Centre-centre spacing	40nm	70nm
ACCSESSORY STRUCTURES[a]	None noted	Filopodia-like structures 160nm diameter, many µm in length found extending from flagellar axis in region of attachment.

[a] From Transmission and Scanning Electron Micrographs
[b] From Transmission electron micrographs

avoid overcrowding of grids so that epimastigote bundles could be discerned) metacyclics would not have been expected to be present.

4.4. DISCUSSION

The morphology of these attached epimastigotes mimics very closely that of trypanosomes attached *in vivo* but appears to portray superficial differences. (see Table 4.1) Both the observations by Gray *et al.* (1981) and this present study noted that filopodial-like extensions are commonly found emanating from the flagella in the vicinity of the attachment site, these thin extensions were not found in *in vivo* grown epimastigotes and may, therefore, be an adaptation to culture conditions where the surface of the plastic appears smoother than that of the labral chitin (compare SEM micrographs in Chapter 3 and in this chapter). The filopodia may help to bind the epimastigotes together on this smooth substratum particularly as no physical junctional continuum was noted between flagella in the present study. Filopodia have also been noticed in living *in vivo* grown epimastigotes by interference reflection microscopy (Chapter 6) and by Nomarski microscopy (unpublished data), and thus are not fixation or dehydration artefacts. Tetley and Vickerman (1985) reported that filopodial-like extensions were found associated with *T.brucei* epimastigotes attached to the Tsetse-fly salivary gland epithelium microvilli. However, unlike these *T.brucei* "flagellipodia" the filopodia of *T.congolense* did not contain any attachment plaques. Only a small amount of filamentous internal structure was noted. These filopodia were removed by detergent treatment again suggesting a membrane origin rather than primarily a junctional or cytoskeletal one.

Attached *T.congolense* *in vitro* also appear to show a greater expansion of the flagellar sheath than those attached *in vivo* although

some lateral expansion is also noted in vivo (Section 3.3.1.1).

By piecing together information obtained from transverse sections, longitudinal sections and SEM pictures a composite image of the expanded flagellum can be obtained. In SEM pictures of in vitro epimastigotes lateral expansions of 1μm or more from the flagellar sheath were noted, similar extensions were also shown in transverse sections of these epimastigotes. Longitudinal sections, however showed the distended areas to also cover a similar length in the posterior-anterior axis so that each attachment area was potentially $1\mu m^2$ or more in area. Longitudinal sections and SEMs also showed that more than one attachment site could occur along the length of the flagellum. Unlike the single adherence plaque per flagellum reported in T.vivax epimastigotes (Vickerman, 1973). Attachment sites were not found at the tip of the flagellum by any form of electron microscopy. Thevenaz and Hecker (1980) also noted this in in vivo epimastigotes of T.congolense unlike the T.brucei attachment area (Tetley & Vickerman, 1985) and many other trypanosomatids e.g. Crithidia fasciculata (Brooker, 1970,1971a). Differences encountered between species are probably not important, reflecting species differences rather than functional differences, as both T.congolense and T.vivax attach to the same material and are often found within the same tsetse but display differences in attachment morphology in terms of numbers of attachments and locations of components of the attachment.

The attachment associated plaque in in vitro T.congolense does not necessarily appear continuous across the width of the extended plaque but can be composed of more than one part, Thevenaz and Hecker (1980) and the present study (Chapter 3) also described this in in vivo grown epimastigotes. Thevenaz and Hecker noted that the area below the axoneme did not contain attachment plaque material, however, their micrographs and those of Evans et al. (1979) show many

trypanosomes with attachment plaque material beneath the axoneme similar to the description in this study. Thevenaz and Hecker (1980) also reported the presence of the plaque in relation to its association with the microtubule doublets 8 to 2. The semicircular shape of some of the non-plaque associated membrane initiates speculation whether these areas are in fact endocytotic "omega" profiles normally associated with the flagellar pocket area in trypanosomes. Molyneux (1977) has suggested that endocytosis occurs at the attachment site in some trypanosomatids, particularly those in which the attachment is formed at the flagellar tip and the flagellum is greatly shortened in length, thus reducing the size of the flagellar pocket and in some cases effectively blocking it so that endocytosis is then decreased in the flagellar pocket. Molyneux (1977) suggests that endocytosis is occurring at the attachment point in compensation for its reduction in the flagellar pocket. Some authors have reported the presence of vesicles in the expanded attached flagellum (Brun, 1974). Brooker (1971b) reported that Crithidia fasciculata detachment, in response to dilution of the culture medium, occurred by endocytosis of attached membrane. No vesicles were noted in T.congolense epimastigote attachment areas in either the present study or others on this species (Evans et al., 1979; Thevenaz & Hecker, 1980; Gray et al., 1981). The appearance and dimensions of the attachment plaque in this species are similar in structure to that previously described for numerous trypanosomatids. The 5-7nm diameter attachment associated filaments have also been described previously, however, a distinct band of these filaments traversing the flagellum has not been mentioned. This band of filaments is more evident in epimastigotes treated with enhancing agents in the preparative procedure as are similar filaments extending from the cell attachment

plaque to microtubule doublets 8-2. The increased number of flagellum-cell junction sites in attached trypanosomes is probably a device to strengthen the attachment between cell and flagellum at the point of cell attachment so that increased stability is enforced at this point to counteract shearing forces caused by the flow of liquid over them in vivo. Thevenaz and Hecker (1980) reported 12 or more rows of these flagellum-cell junction densities in their study of attached T.congolense epimastigotes, a maximum of 6 or so rows were noted in the present study, comparable to the number of rows reported in attached T.vivax epimastigotes (Vickerman, 1973).

The sequence of formation of attachment structures can be inferred from examination of young and mature culture attachment ultrastructure. The observations suggest that flagella attach to the substratum and where the appropriate area of the flagellar membrane comes within a distance of 20nm or less from the surface of attachment plaques begin to form. The first structures formed are filaments and then a filamentous plaque develops which becomes more electron dense with age of culture and, presumably, cell age. Cell-flagellar attachment strength would also appear to increase with time if the formation of more rows of cell-flagellar junction densities are indicative of increase in attachment strength.

It may be questioned as to the validity of measuring gap size in an experimental system in which the substratum has been removed during processing for TEM. The practice of 'double' embedding monolayers is a routine technique in laboratories studying culture cells and is followed because the second layer of resin often remains bound to the original layer after sectioning. Plastic coverslips can be sectioned but the resin always separates from the plastic on cutting and the apposition of two different plastic materials can often cause cutting problems when attempting to cut them simultaneously. The gap width

sizes found under attached flagella *in vitro* (approximately 15nm) is comparable to that measured *in vivo* (Chapter 3, Thevenaz & Hecker, 1980; Vickerman, 1973, *T.vivax* - all in the region 12-20nm). The gaps both *in vivo* and *in vitro* contain amorphous stainable material also. Thus the post-embedding removal of the substratum does not appear to affect the attachment morphology.

Contrary to attachment formation, forced detachment of these cells was also examined. TEM of mechanically and chemically and mechanically detached epimastigotes showed that very few detached flagella contained attachment plaque material. Filaments were present as were multiple rows of flagella-cell junction densities but dense cell-substratum plaques and large lateral extensions were absent. The absence of attachment plaques in some flagella can be accounted for by SEM examination of the coverslips after cell removal. After both mechanical and mechanical and chemical cell detachment attachment site material was found remaining on the coverslip. After mechanical removal many flagella were found remaining attached without the cell body. Thus the flagellum-cell junctions may have been weaker than the cell-substratum attachment when exposed to a shearing force. After trypsin treatment, however, the remaining material tended to be more membranous. Far fewer sheared flagella were found amongst the remaining material. The material remaining after trypsinization appears largely to have been membranous components of lateral extensions and less membranous, honey-combed, material of similar dimensions to the attachment extensions.

Badley *et al.* (1978) carried out a similar SEM study on the 'foot-print' material remaining after detachment of fibroblasts. Fibroblast attachment points are also in the order of $1\mu m^2$ and are the termini of actin filaments associated with cell motility. Mechanical

removal of attached fibroblasts leaves a more or less ordered array of globular units 20-30nm in diameter bound by a fibrillar border. This type of material was not observed in the epimastigote 'footprints' although small fibrillar arrays were occasionally noted. In appearance the substrate attachment material left after epimastigote detachment more closely resembled membranous 'sacs' found remaining after treatment of attached 3T3 fibroblasts with trypsin (Culp & Buniel, 1976) or EDTA and a shearing force provided by a stream of solution being passed over these cells (Rosen & Culp, 1977). Rosen and Culp refer to these structures as 'footpads' and have determined that these comprise the attachment plaque and associated cytoskeleton enclosed within a membrane sac pinched off by the mechanical and chemical action. The honeycomb patterned material noted in trypsin treated epimastigote remains could possibly reflect some of the membrane sacs which have been affected by trypsinization. It is tempting to speculate that these structures may have been partially composed of the actual plaque material but no evidence is available to confirm this.

The finding of filamentous structures remaining in detached flagella would suggest that the attachment plaque material binds more tightly to the membrane than to the associated filaments. The filaments would, therefore, appear to be more tightly bound to the flagella-cell junction material than the cell-substratum plaque. The attachment plaque also appears to be at least partially resistant to brief detergent extraction. Negatively stained cytoskeleton preparations of these attached cells show some attachment associated material although much of the membrane and auxiliary structures e.g. filopodia, are removed. As the flagella remain attached under these conditions, the cytoskeletal, filamentous components of the cell attachment complex must remain as is the case for the flagellum-cell

junction points.

The enhancing procedures used in the TEM studies reported in this chapter deserve some discussion. Three of the protocols used, those involving tannic acid, whether alone or in combination with osmium ferricyanide, and the osmium-thiocarbohydrazide-osmium were primarily aimed at preserving the filamentous and membranous structures so that the relationship between components of the attachment complex could be clearly visualized. In practice, however, although enhancement of filament staining occurred after all these procedures very little information above what had been noticed in conventionally processed material was found. These protocols have been used previously to preserve filamentous structures prone to osmium and dehydration damage, in particular actin filaments.

Tannic acid improves contrast and preservation of material due to both cross-linking protein and also acting as a mordant for OsO_4 and the heavy metals used in TEM staining (reviewed by Fujiwara & Link, 1982). This chemical has been widely used in microtubule research to determine microtubule substructure patterns (e.g. Tucker et al., 1985). This chemical has also been used in other ultrastructural research including demonstration of cell junction material (Van Deurs, 1975) and for visualizing microfilaments (Maupin & Pollard, 1983). The permeabilization required to introduce the chemical into the cells however is a drawback when relationships between components in a cell are being examined. Tannic acid also increases filament diameter which is not useful if measurements are being made. The method of McDonald (1984) which avoided detergent, using primarily osmium ferricyanide to enhance the fixation and tannic acid as an added component was also tried. Osmium ferricyanide is reputed to improve actin filament preservation without altering filament diameter, provide good membrane

contrast and avoid the general cytoplasmic coarseness often observed with tannic acid fixed material. The result of this method when used in fixing the epimastigotes was to provide an increased visualization of filaments avoiding displacement of structures and retaining a clear cytoplasm. Many more individual filaments compared to the other protocols used were visualized spanning the width of the flagellum. The OTO method (Aoki & Tavassol, 1981) is also reputed to preserve fine filaments normally prone to osmium damage. The use of this on epimastigotes did not enhance the filaments as much as that of tannic acid or the osmium ferricyanide methods but did appear to improve the visualization of both attachment plaque material and flagella-cell junction densities allowing their shape to be discerned easily.

These fixations have been previously reported as methods for preserving actin filaments which are prone to osmium and dehydration damage. The size of the filaments found in association with the epimastigote attachment plaque (approximately 5nm) are similar in size to the actin filaments. This similar morphology and the known association of actin with ameboid-like cell adhesion points has led to speculation whether the epimastigote filaments are composed of actin or not (Chapter 7). As a preliminary to such studies, therefore, these structures appear preserved by methods which preserve actin filaments, however this could be coincidental and due to structural rather than compositional similarities.

Ruthenium red is thought to interact with a variety of acid substituted large polymers, including acid mucopolysaccharides and acid polypeptides. In many cells this chemical has been found to bind acid mucopolysaccharides (Luft, 1971b). Cell surface binding of this chemical is commonly found, due to the presence of polysaccharides in the form of glycoproteins, on the cell surface. The binding of this chemical to the entire cell surface including flagella membranes and

within the cell-substratum gap and flagellum-cell junction gap indicates that glycoproteins are present in these gaps as well as over the entire cell surface.

Brooks (1978) reported the binding of this chemical to T.equiperdum. She reported that the ruthenium red occluded the cell-flagellum junction space and intensely stained filamentous material spanning this space. She also noted that the flagella-cell junction densities were increased in contrast as is found for the cell-substratum attachment in the present study although very little improved contrast in the flagella-cell junction dense material was noted. The ruthenium staining in the extracellular gap is not totally due to polysaccharides on the cell surface. The coverslip surface is also stained by this material in the absence of attached cells. Luft (1971b) reported that this phenomenon is due to the absorbance of serum components onto the substratum. These components were shown to stain positively with this chemical. The exclusion of serum from the culture medium in the present study, however, did not totally remove this substratum ruthenium red binding but did somewhat reduce it without removing the staining from the cell-substratum gap suggesting at least part of the material present in the gap is a component of the epimastigote surface.

In conclusion the observations reported in this chapter confirm the findings of Gray et al. (1981) that the ultrastructure of the epimastigote flagellum-substratum attachment site in in vitro grown T.congolense is equivalent to that of epimastigotes grown in vivo, a feature also shown for C.fasciculata haptomonads (Brooker, 1970,1971a). This report expands upon the description of the morphology of the in vitro attachment of T.congolense and is comparable with the descriptions of attachment of T.congolense in the

tsetse proboscis (Evans et al., 1979; Thevenaz & Hecker, 1980, and Chapter 3). The finding that, to all intents and purposes, the epimastigote attachment in vivo and in vitro are identical in so far as morphology would lead to the supposition that the components of the attachment in both growth conditions are identical.

Results of experiments aimed at finding the composition of internal and external components and the properties of the epimastigote attachment site in vitro would therefore be valid for the attachment of these organisms in the tsetse proboscis. The similar ultrastructure found in all trypanosomatids when attached to chitin, and in some cases epithelia also, might mean that extrapolation of information from in vitro grown T.congolense epimastigote attachment to other trypanosomes is also valid. Thus this organism, which can be grown in large quantities in an attached state, can be used in place of other trypanosomes for which no in vitro system for the epimastigote form has been established, e.g. T.brucei.

4.5. SUMMARY

Electron microscopy of in vitro grown T.congolense epimastigotes reveals that they possess similar attachment morphology to their in vivo attached counterparts in the tsetse fly proboscis. An electron dense plaque is present (approximately 40nm thick) in the flagella at the site(s) of attachment to the substratum. More than one such attachment can occur per flagellum, at each attachment a lateral extension from the axis of approximately 1μm^2 exists. An extracellular gap of 15nm is found. Ruthenium red staining suggests the presence of glycoprotein in this gap. 5nm filaments associate with the flagellar attachment plaques, linking this with microtubule doublets 2-8, the PFR and flagella-cell body junction complexes. These last structures resemble desmosomes and in the region of the flagellar attachment

increase in number/cross sectional area from one in unattached flagella to a maximum of 6 in attached. Tannic acid and similar enhancement procedures show the filaments in greater detail. The development of the attachment plaque can be discerned from cultures of different ages. Initially filaments alone are present. Over a few days thin plaques (20nm) develop becoming thicker and more electron dense with time. An increase in flagellum-cell body attachments also occurs with time as do the number of epimastigotes per bundle.

Adjacent flagella line up in the same orientation as their neighbours and attachment sites tend to form at adjacent positions. No interflagellar attachments are present, instead filopodia-like outgrowths (160nm wide, many μm long) from the flagella entwine with those of neighbouring flagella keeping the bundle together. These features are particularly visible in SEM micrographs. Detached epimastigotes, whether chemically or mechanically detached, do not contain attachment plaques in their flagella. Membranous 'sacs' or footprints are found remaining on the coverslip after epimastigotes have been removed. These 'sacs' are susceptible to trypsinization.

Flagellar attachment sites are still discernible in detergent extracted cells although membrane features e.g. filopodia are removed and extensions from the flagellar axis are reduced in size.

CHAPTER 5

THE REQUIREMENT FOR EPIMASTIGOTE ATTACHMENT DURING DIVISION AND METACYCLOGENESIS

5.1. INTRODUCTION

In the cyclically-transmitted salivarian trypanosomes, the mammal-infective, metacyclic, stage develops from the epimastigote after a period of epimastigote replication. The constancy of epimastigote attachment suggests that it plays a necessary part in the continuation of the life cycle in these organisms. The role of attachment may simply be to provide a hold-fast with no other purpose than to retain the epimastigotes in a region where the developing metacyclics can infect their mammalian host, and to prevent the epimastigotes from being dislodged by the incoming blood meal or by saliva. Alternatively, the attachment might have a developmental significance and form an essential part of the programme for (a) epimastigote replication and/or (b) metacyclogenesis to occur.

The following experiments using *in vitro* grown *T.congolense* epimastigotes examine whether preventing attachment, either by shaking the cultures in an orbital shaker or seeding the cultures on a polypropylene substratum, affects (a) epimastigote division and (b) metacyclogenesis. The developmental forms found in the supernatant and attached to the base of the culture flask have been examined, the forms found raise questions as to the degree of programming of the development in the trypanosome life cycle.

5.2. MATERIALS AND METHODS

5.2.1. Trypanosome stocks and their routine cultivation

Stocks TREU 1457 and TREU 1627 were maintained in culture as described in Section 2.2.

5.2.2. Shaking culture experiments

Replicate pairs of flasks ($25cm^2$) were inoculated with equal numbers of trypanosomes at equal densities. One flask of each pair was

immediately placed in an orbital shaker (Gallenkamp) at either 62 r.p.m. or 40 r.p.m., the other was placed in a non-shaking incubator. All experiments were carried out at 28°C.

For stock TREU 1457 two seeding densities were examined approximately $5x10^6$ trypanosomes/ml and approximately $2x10^7$ trypanosomes/ml at a shaking speed of 62 r.p.m..

In all experiments, at 6, 12 or 24 hour intervals (depending on experiment) the number of attached trypanosomes in each flask was counted in 10 randomly selected (using random number tables to select microscope stage coordinates) fields of an inverted microscope (Diavert, Leitz) eyepiece containing a grid graticule. The area measured per field of view was $0.16mm^2$ at the magnification used. From these 10 fields the total number of trypanosomes attached could be estimated. At each sampling time a 10µl sample from the culture supernatant was also removed. From this sample, the density of trypanosomes in the supernatant was counted in an improved Neubauer haemacytometer and the abundance of unattached trypanosomes in each flask calculated. Smears of each culture supernatant were also made at each sampling time; these were air dried, methanol fixed (2 min) and, after acid hydrolysis (1M HCl at 60°C for 9 min), stained in 5% Giemsa's stain (BDH) in phosphate buffer pH 7.0. The smears were examined for the presence of metacyclics and the proportion of metacyclics in the total supernatant population counted and expressed as a percentage. 100-1000 trypanosomes were examined per slide.

Cultures were monitored for either 48 hours or 16 days. In the latter case it became necessary to change the culture medium; for shaken flasks this involved pelleting the supernatant trypanosomes (200xg for 10 min) and returning them to the flask in a volume of fresh medium. The numbers of attached and unattached trypanosomes were then recounted. The metacyclic percentage proved to be very similar

before and after centrifugation, showing the procedure did not introduce any bias to the experiment.

At the termination of one 16 day experiment shaken flasks were transferred to a still incubator and the supernatants monitored for metacyclics for a further 18 day period.

The dynamics of shaken and still culture population numbers were monitored not only by counting cell numbers but also by determining changes in the total protein content of cultures. For each experiment five identical pairs of shaken and still cultures were initiated. At 12 hour intervals trypanosome population numbers in one pair of flasks were enumerated as described above and in an equivalent pair of flasks the total population protein content was estimated.

To assay the total protein concentration of a culture the attached cells were scraped off the flask base with a polypropylene pipette (Sterilin) and the total population centrifuged at 200xg for 10 mins. The cells were resuspended in 100ul of Ca^{2+} and Mg^{2+}-free PBS containing protease inhibitors (Leupeptin 50µg/ml, Chymostatin 5µg/ml and Pepstatin A 5µg/ml (Sigma Ltd.). The cells were then lysed by 3 cycles of rapid freezing and slow thawing. An adaptation (Foster, 1984) of the Bradford (1976) dye binding method was used to estimate the protein content. 2ml of reagent were added to the 100ul lysate. The reagent consisted of 100mg Coomassie Brilliant Blue G 250 (Sigma Ltd.) dissolved in 10% orthophosphoric acid in 15% ethanol. The solution was filtered through Whatman No. 1 filter paper before use. Reagent plus sample mixtures were incubated at room temperature for 5 mins before being read spectrophotometrically at a wavelength of 280nm in a Spectroplus spectrophotometer (MSE). The amount of protein in the sample was calculated from a calibration curve derived using known quantities of BSA.

5.2.3. Polypropylene substratum experiments

Polypropylene sheeting (kindly supplied by Prof A.S.G. Curtis) was cut to fit the bases of individual 3.5cm diameter wells of tissue culture plates (NUNC, Gibco Ltd.). The polypropylene was sterilized in methanol and left in a laminar flow hood to ensure full evaporation of methanol before use. To each polypropylene lined well 0.5ml of culture medium containing a known amount of trypanosomes was added. 0.5ml of culture medium proved to be the largest volume that could be used to maintain the culture completely within the polypropylene without contacting the culture dish sides. Parallel control wells with no polypropylene were also initiated. The cultures were maintained at 28°C in a humid atmosphere for a period of 48 hours. At 12 hour intervals 10ul samples were removed from each well, the number of trypanosomes counted and a smear made. The wells were also examined under an inverted microscope for the presence of attached trypanosomes.

5.2.4. Statistical analyses of results

The number of replicates used in each experiment are indicated in each figure legend. Where changes in total population size have been monitored the results are expressed as geometric means plus and minus 2 standard errors; where proportionate changes have been monitored, results are expressed as arithmetic means plus and minus 2 standard errors.

To calculate population doubling times (PDTs) for populations in phases of logarithmic growth, the slope of the curve, r, was estimated from an exponential regression curve. The PDT equals $2 \times \frac{\log_e}{r}$.

FIG.5:1. T.congolense stock TREU 1457

Shaken and non-shaken cultures of two seeding densities ; group 1 - $2x10^7$, 2 - $5x10^6$ were monitored over a 48 hour period and total numbers of trypanosomes in the culture calculated from counts of attached and unattached trypanosomes in each culture. Shaking speed 62 rpm.

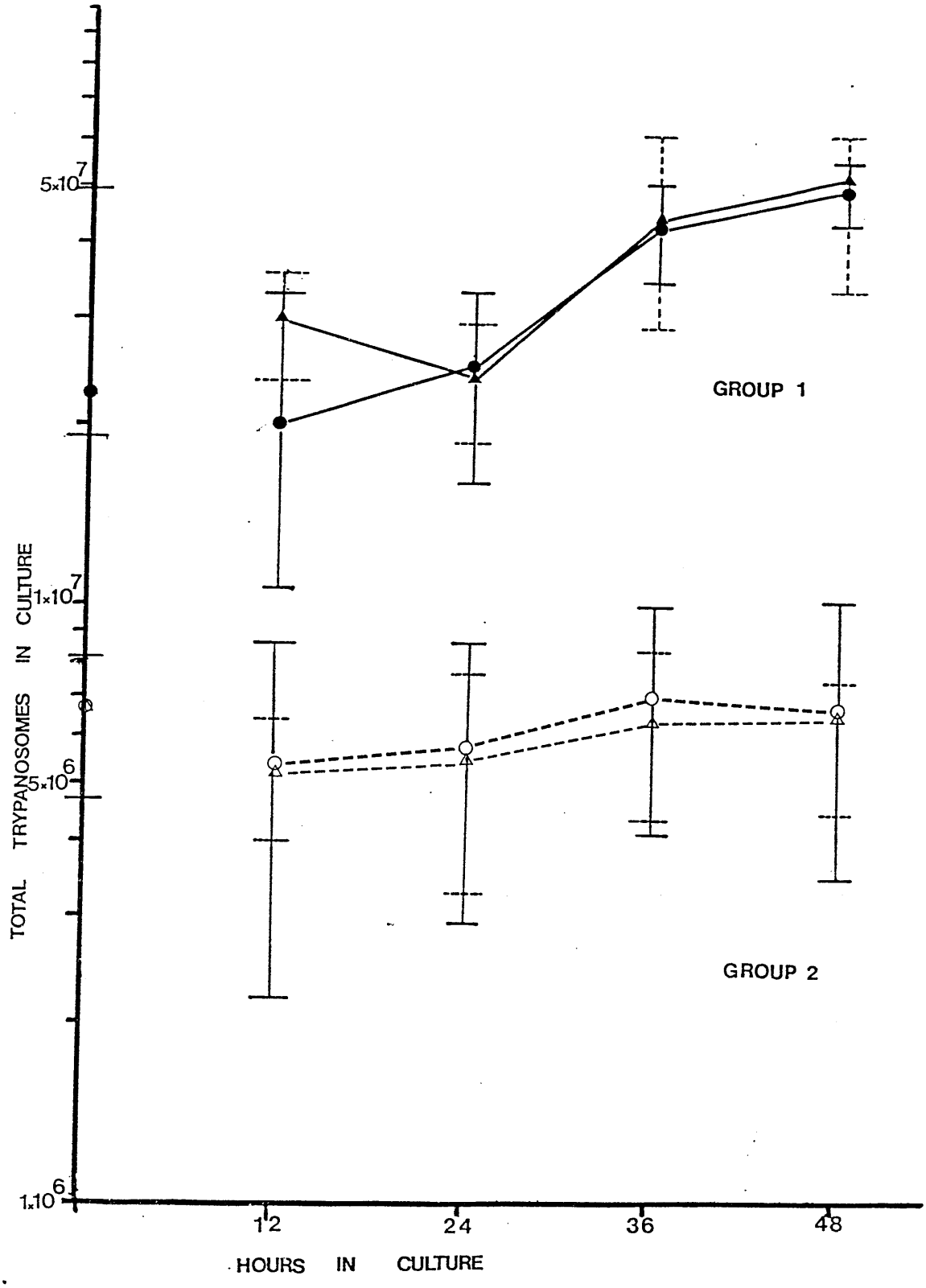
The graph shows geometric means plus and minus 2 standard errors (n=4).

●- non-shaken cultures, group 1

▲- shaken cultures, group 1

O- non-shaken cultures, group 2

△- shaken cultures, group 2

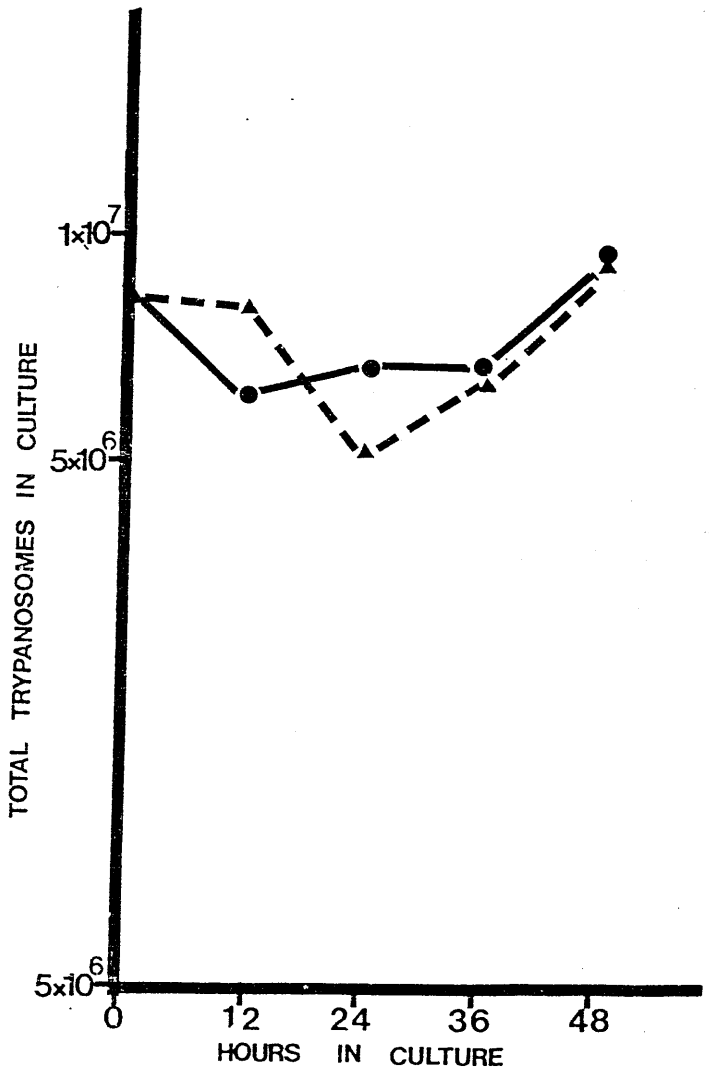

5×10^7
1×10^7
5×10^6
1×10^6
TOTAL TRYPANOSOMES IN CULTURE
GROUP 1
GROUP 2
12
24
36
48
HOURS IN CULTURE

1

FIG. 5:2 T.congolense stock TREU 1627

One example of a growth curve of a pair of shaken and non-shaken cultures monitored as for FIG. 5:1. Actual data plotted. Seeding density 8.5×10^6 trypanosomes/flask. Shaking speed 62 rpm.

●- non-shaken culture ▲- shaken culture

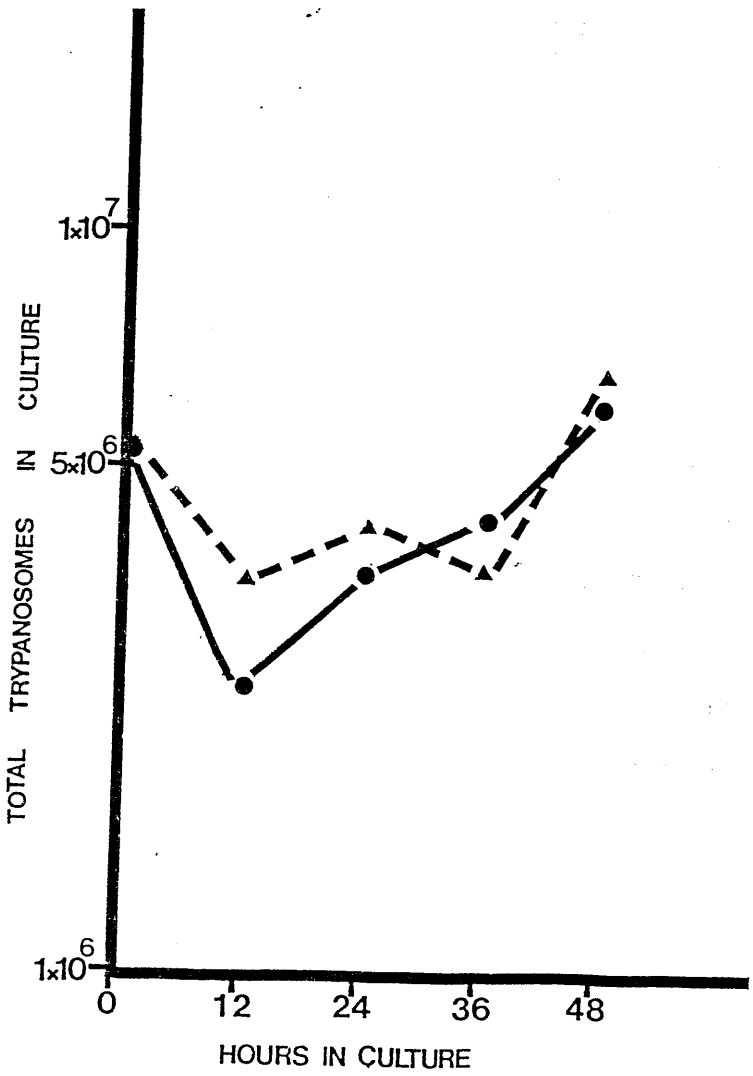

TOTAL TRYPANOSOMES IN CULTURE
1x10^7
5x10^6
5x10^6
0
12
24
36
48
HOURS IN CULTURE

2

FIG.5:3. T.congolense stock TREU 1457.

One example of an experiment depicting the growth curves of one shaken and one non-shaken culture. Seeding density $5x10^6$ trypanosomes/flask. Shaking speed 40 rpm.

●- non-shaken culture ▲- shaken culture


1x10^7
5x10^6
1x10^6
TOTAL TRYPANOSOMES IN CULTURE
0
12
24
36
48
HOURS IN CULTURE

3

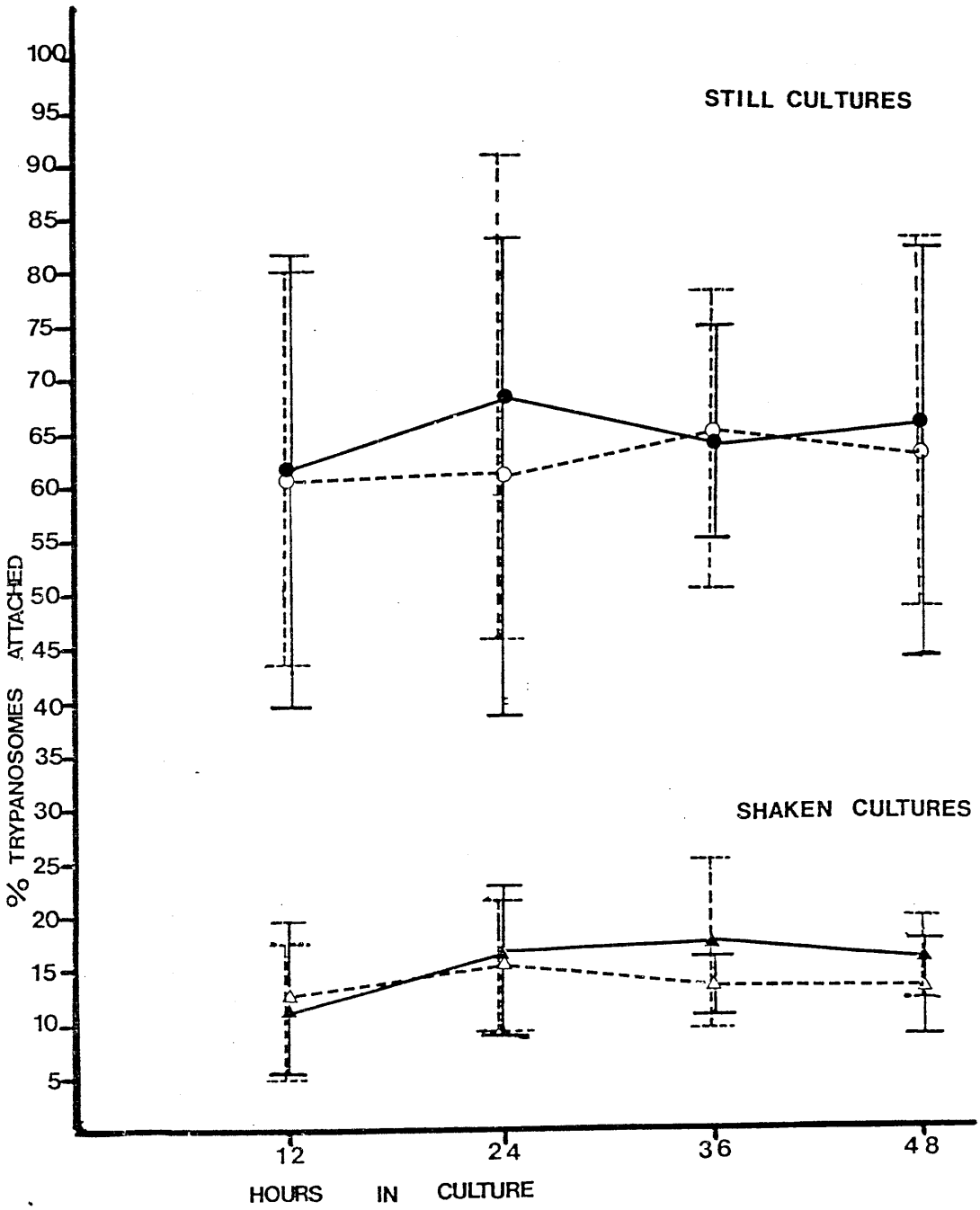
FIG. 5.4. (A) T.congolense TREU 1457. Shaking speed 62 rpm. Proportion of attached trypanosomes expressed as a percentage of the total population in replicate pairs of shaken and non-shaken cultures. Arithmetic means plus and minus two standard errors (n=4).

(B) T.congolense TREU 1627. Shaking speed 62 rpm. One example of the proportion of attached trypanosomes in a pair of shaken and non-shaken cultures.

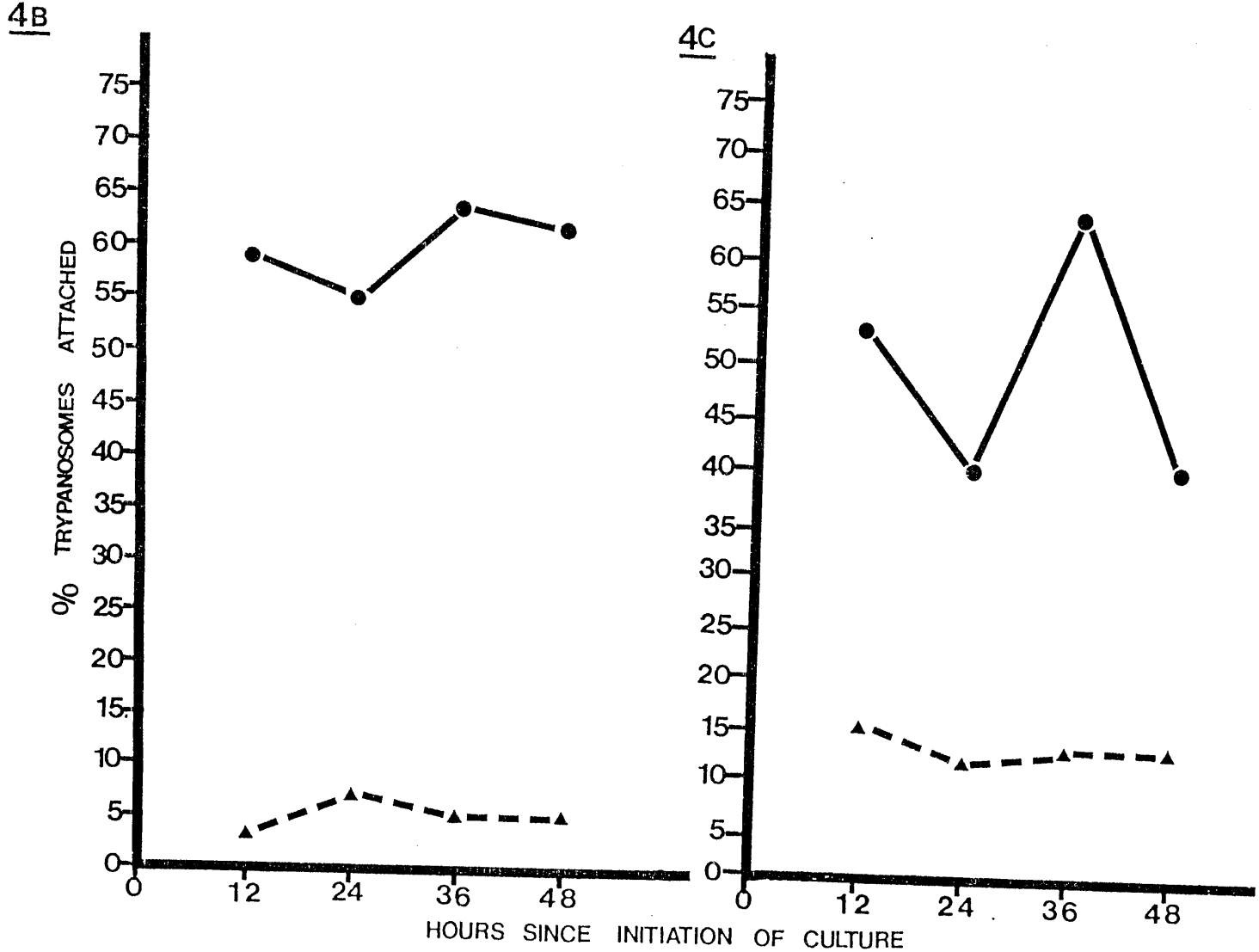
(C) T.congolense TREU 1457. Shaking speed 40 rpm. One example of the proportion of attached trypanosomes in a pair of shaken and non-shaken cultures.

○●- non-shaken cultures

△▲- shaken cultures


STILL CULTURES
SHAKEN CULTURES
% TRYPANOSOMES ATTACHED
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
12
24
36
48
HOURS IN CULTURE

4A


4B
4C
% TRYPANOSOMES ATTACHED
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
12
24
36
48
HOURS SINCE INITIATION OF CULTURE

5.3. RESULTS

5.3.1. Shaking culture experiments

5.3.1.1. Changes in total population sizes: Over a 48 hour period cultures of T.congolense TREU 1457 shaken at 62 r.p.m. displayed growth curves similar to those of their non-shaken counterparts (Fig. 5.1), there was approximately an exponential increase in the total number of trypanosomes in the flask. This observation was true for both seeding densities examined. The growth rates for both groups of cultures, however, appeared to be affected by inoculum size. The population doubling time (PDT) of the high seeding group (group 1) non-shaken cultures was calculated as approximately 21.6 hours, that of the low seeding group (group 2) was approximately 69 hours. The PDTs for the corresponding shaken cultures were 40.6 and 79.3 hours respectively.

Similar results were also obtained for the other stock of T.congolense examined, TREU 1627, (Fig. 5.2), in that both shaken and still cultures showed an overall increase in total population with time. The PDTs of shaken and non-shaken cultures appeared, however to be much more alike than those of TREU 1457. The shaken culture depicted in Fig. 5.2 has a PDT of 46 hours whilst its non-shaken counterpart has a PDT of 47.5 hours.

Experiments performed shaking TREU 1457 cultures at 40 r.p.m. showed similar gross results to those shaken at 62 r.p.m. The total number of trypanosomes, again, increased with time (Fig. 5.3). However, once more the PDTs for both shaken and still cultures were very similar; 59 hours and 57.5 hours respectively.

5.3.1.2. Changes in percentage of attached flagellates: The percentage attachment results for these cultures are shown in Fig. 5.4. In 62 r.p.m. shaken cultures of TREU 1457 (Fig. 5.4a) less than 25% of the

FIG.5:5. T.congolense TREU 1627. Shaking speed 62 rpm.

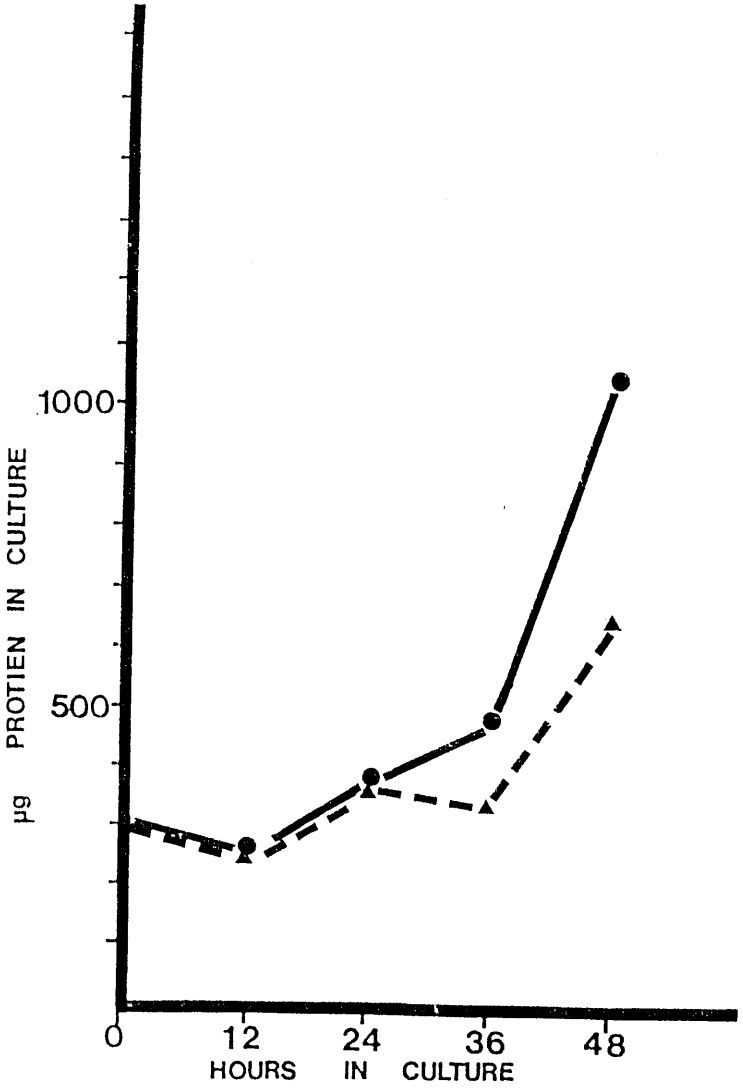
(A) Total protein content (in µg) of a pair of shaken and non-shaken flasks. The protein amount was measured by spectrophotometry and calculated from a calibration curve of known quantities of BSA.

(B) Duplicate flasks to those in (A) counted for total trypanosome number present.

Seeding density 5.8×10^6 total trypanosomes/flask.

●- non-shaken cultures ▲- shaken cultures

5A


µg PROTIEN IN CULTURE
1000
500
0
12
24
36
48
HOURS IN CULTURE

5B

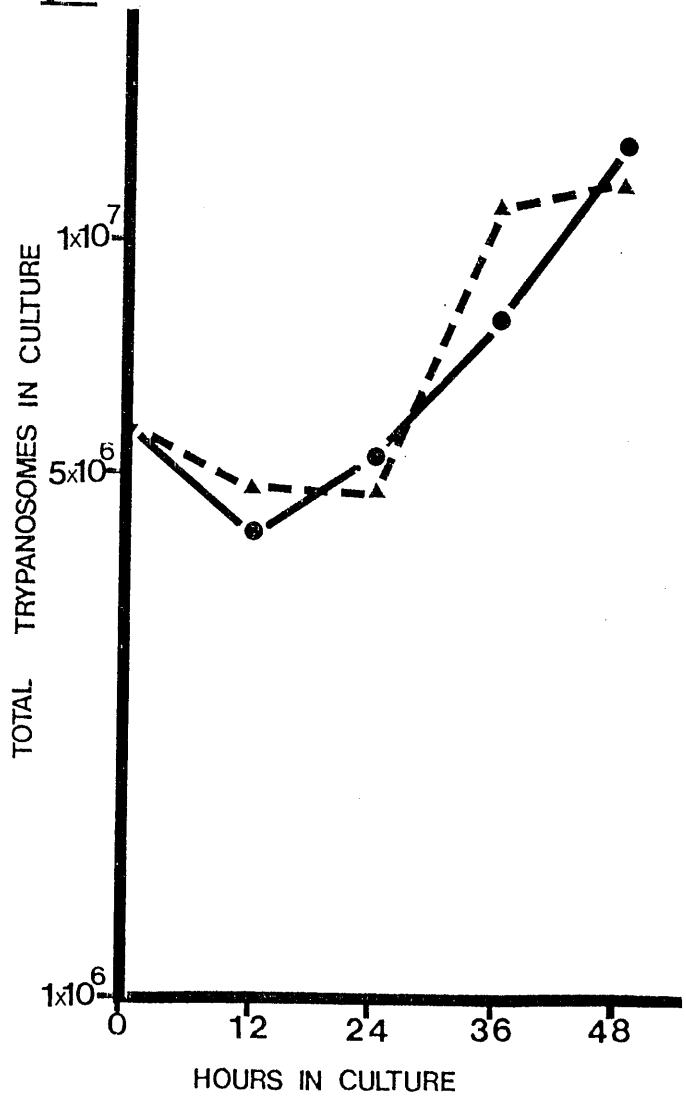
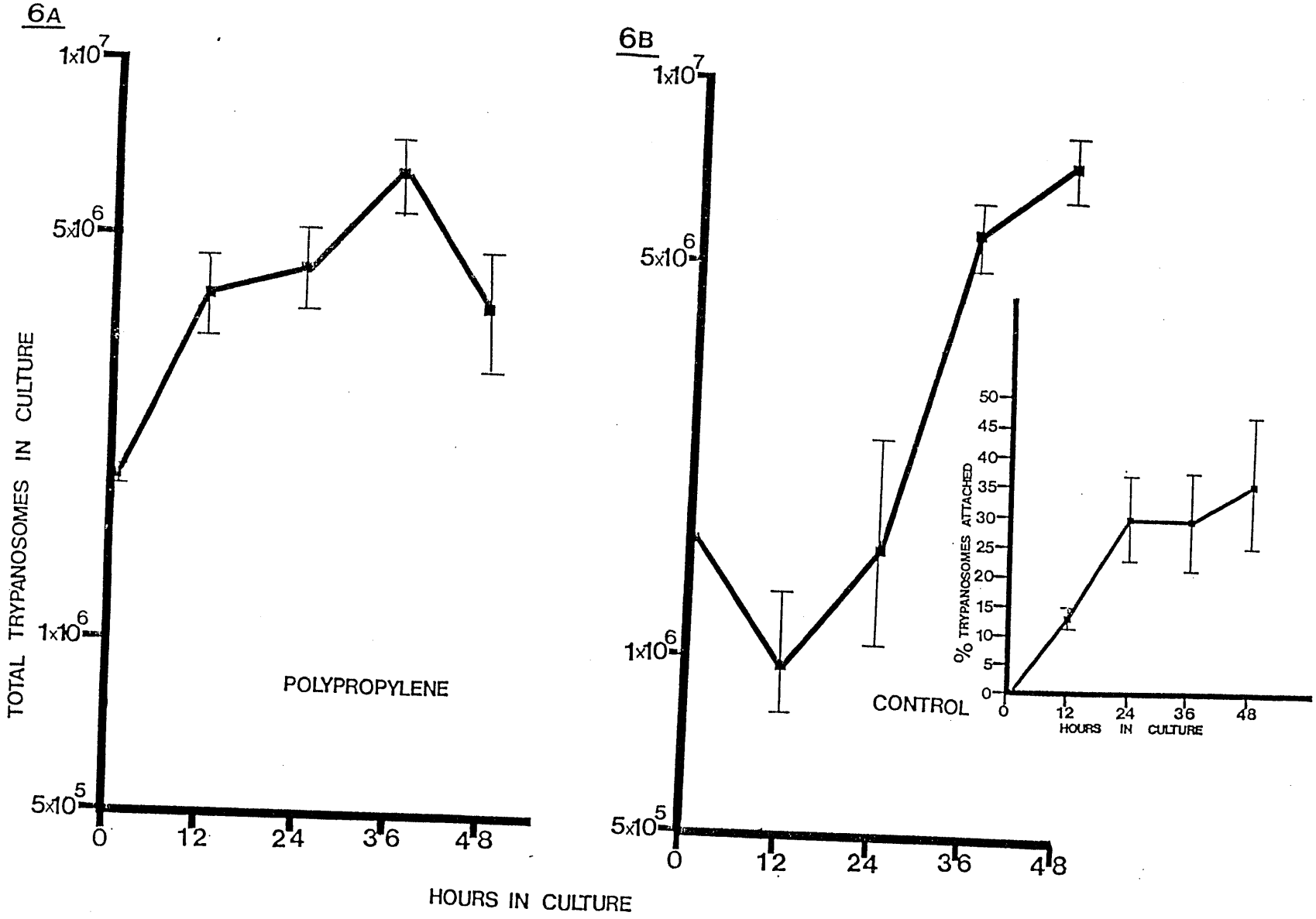

TOTAL TRYPANOSOMES IN CULTURE
1x10^7
5x10^6
1x10^6
0
12
24
36
48
HOURS IN CULTURE

FIG.5:6. T.congolense TREU 1627.

(A) Growth curve of cultures grown on a polypropylene substratum. Total number of trypanosomes/culture counted and expressed as geometric means plus and minus two standard errors (n=8).

(B) Control cultures growth curve of cultures grown on culture plastic.

INSERT Percentage of trypanosomes attached in control wells. (n=4).


6A
1x10^7
5x10^6
1x10^6
5x10^5
TOTAL TRYPANOSOMES IN CULTURE
POLYPROPYLENE
0
12
24
36
48
6B
1x10^7
5x10^6
1x10^6
5x10^5
CONTROL
% TRYPANOSOMES ATTACHED
50
45
40
35
30
25
20
15
10
5
0
0
12
24
36
48
HOURS IN CULTURE
HOURS IN CULTURE

total cell population were found to be attached, whereas in still cultures, between 40% and 90% of the total flask population were attached. Stock TREU 1627 (Fig. 5.4b) shows the same range of attached and unattached trypanosomes as that of TREU 1457 shaken at the same speed. T.congolense TREU 1457 shaken at 40 r.p.m. also shows a similar difference in the percentage of attached trypanosomes in the populations of shaken and non-shaken flasks (Fig. 5.4c).

5.3.1.3. Changes in protein concentration: Temporal changes in the total protein content of shaken and non-shaken cultures are shown in Fig. 5.5a. These results show that the total protein content increased with time in both shaken and still cultures, and correlated with changes in the total cell numbers of identical cultures as shown in Fig. 5.5b.

5.3.2. Polypropylene substratum cultures

Fig. 5.6 shows the growth profile for TREU 1627 cultures grown on a polypropylene substratum. The total cell population increased with time until approximately 36 hours after initiation when the population then decreased. This was probably due to evaporation of culture media. No attachment was noted in polypropylene cultures although control cultures contained a large proportion of attached epimastigotes.

5.3.3. The effect of the control of epimastigote attachment on metacyclogenesis

As metacyclics are not produced in culture until approximately 2 weeks post initiation (Gray et al., 1984) the shaking experiments were extended to cover this time period. The stock TREU 1627 was used as it reliably produces metacyclic forms in culture. Giemsa stained smears of supernatant trypanosomes from duplicate shaken and still cultures were monitored to determine the numbers of metacyclics. These are

FIG.5:7 T.congolense TREU 1627. Shaking speed 62 rpm.

(A) Proportion of metacyclics in the culture supernatants of replicate pairs of shaken and non-shaken cultures.

Metacyclics counted from Giemsa stained smears and expressed as a percentage of the total trypanosomes counted.

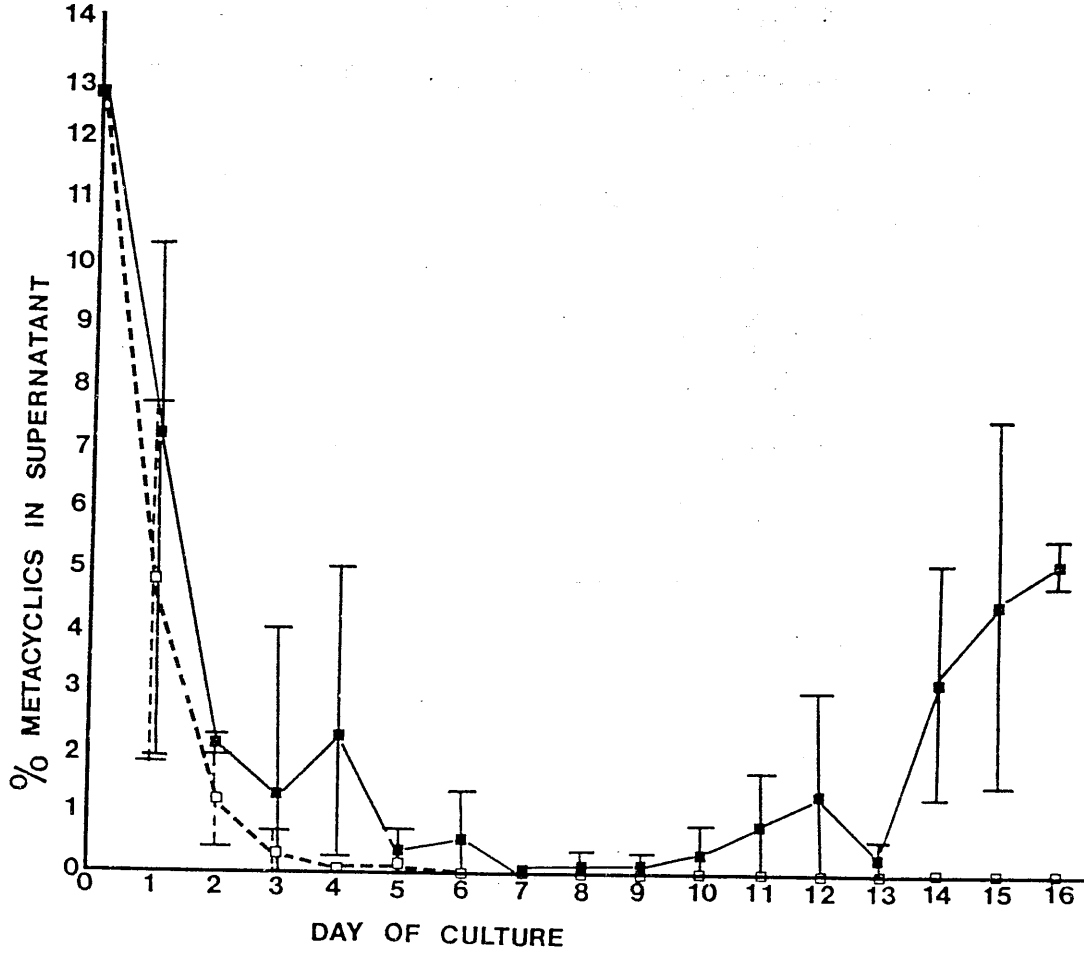
Arithmetic means plus and minus two standard errors (n=3).

■- still cultures

□- shaken cultures

(B) Metacyclic profile of one culture shaken for 16 days and subseqently transfered to a still incubator (arrow) and monitored for a further 16 days. Metacyclics counted as in (A).

7A


% METACYCLICS IN SUPERNATANT
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
DAY OF CULTURE

7B

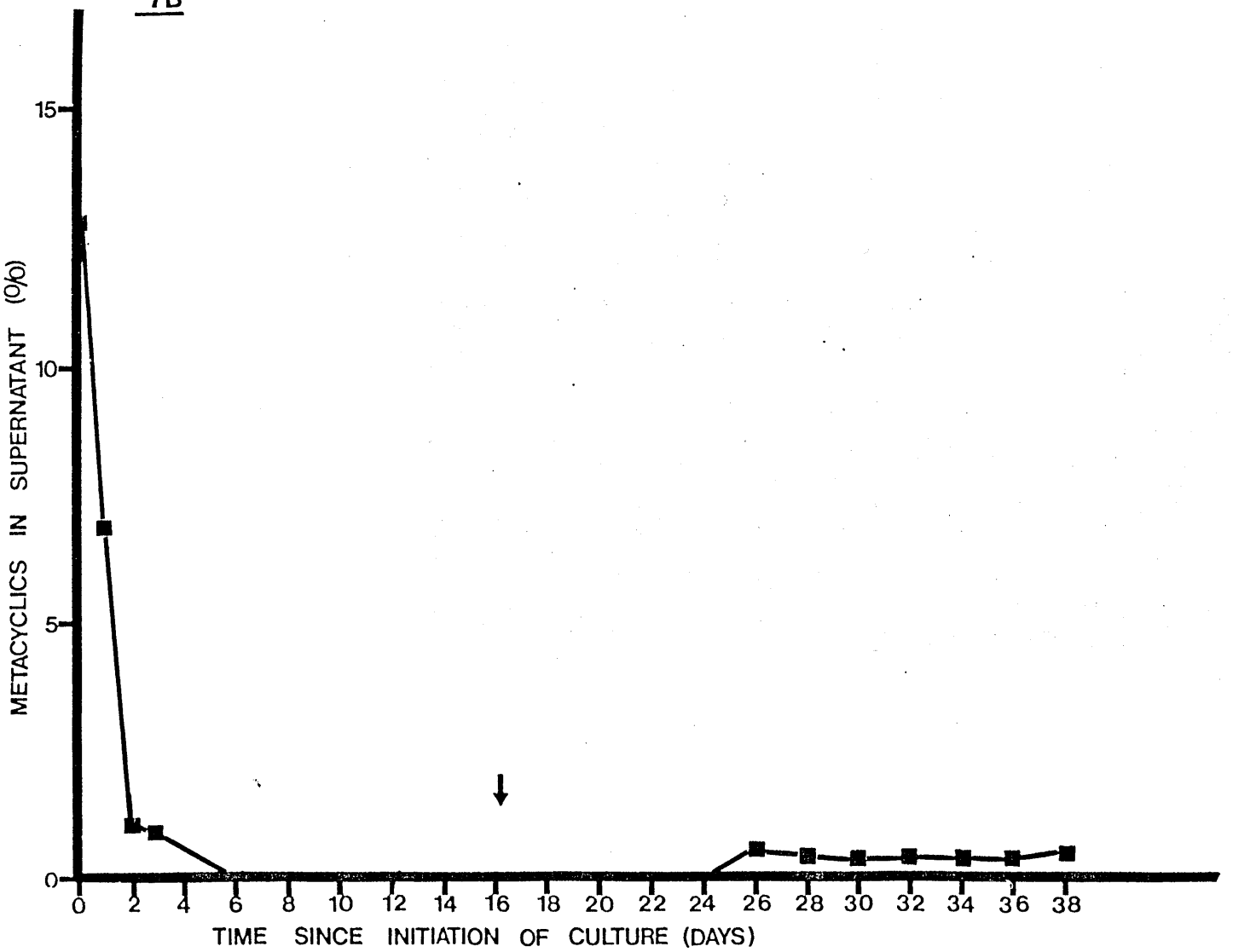
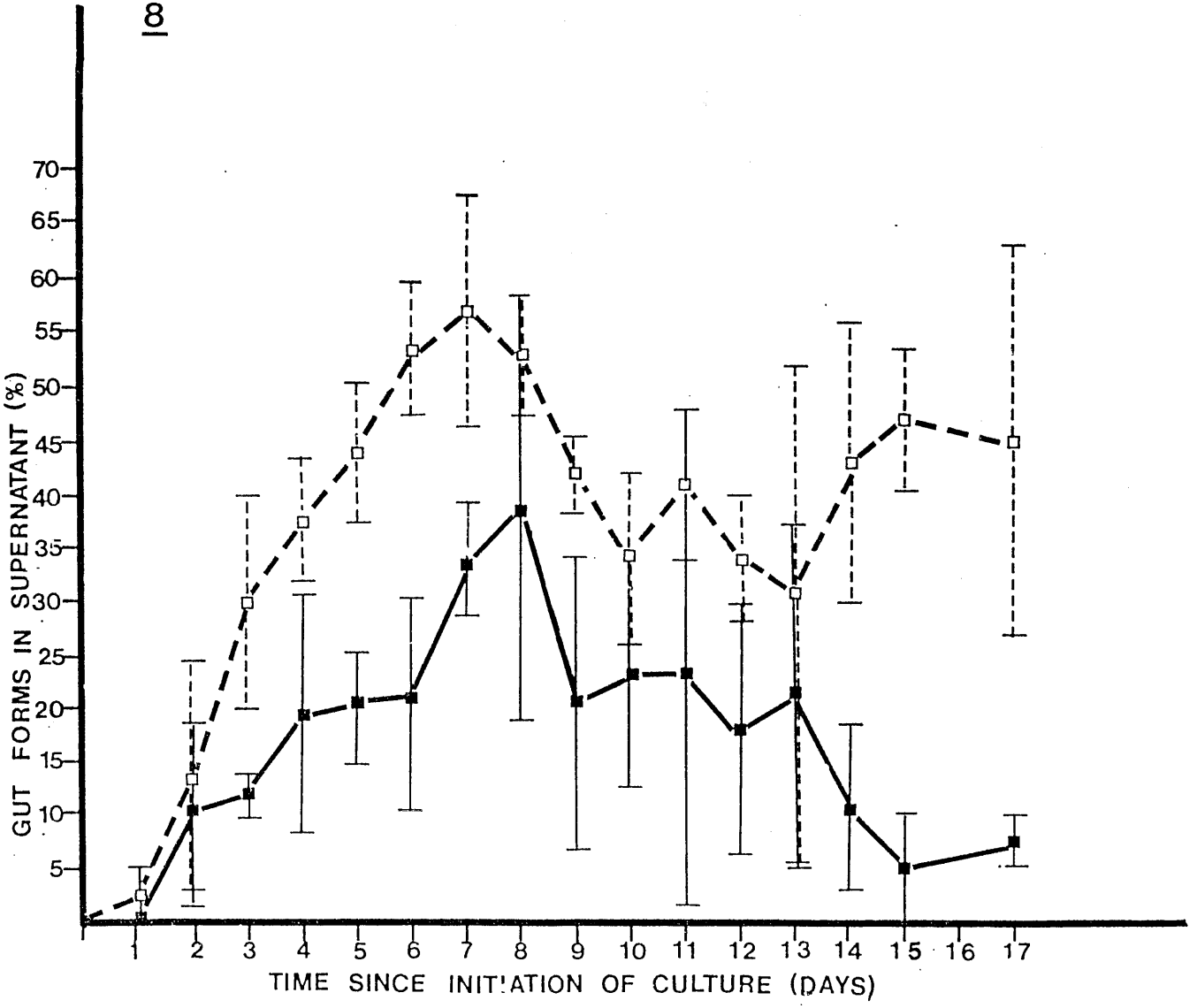

METACYCLICS IN SUPERNATANT (%)
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
TIME SINCE INITIATION OF CULTURE (DAYS)

FIG.5:8. Percentage of tsetse fly gut forms (procyclic and proventricular forms) in the smears examined (FIG.5:7.). Expressed as a percentage of the total cells counted in the smears. Arithmetic means plus and minus two standard errors (n=3).

■ - non-shaken cultures □ - shaken cultures


GUT FORMS IN SUPERNATANT (%)
70
65
60
55
50
45
40
35
30
25
20
15
10
5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
TIME SINCE INITIATION OF CULTURE (DAYS)

expressed as percentages of the total supernatant population in Fig. 5.7. After the decline of inoculated metacyclics, negligible numbers of metacyclics were detected in any flasks until approximately day 10 post-initiation. Thereafter increasing numbers of metacyclics were found in still cultures but none were found in shaken flasks.

Shaken flasks on day 16 were transferred into a non-shaking incubator and the supernatants monitored for metacyclic forms. It was observed that at approximately day 10 post-transfer metacyclics were found in the supernatant (Fig. 5.7b). Unfortunately only data for one flask is presented as the others died off or became contaminated before completion of the experiment.

In the smears of long term shaken culture it was noted (Fig. 5.8) that large numbers of procyclic and proventricular-like forms were present. No procyclic or proventricular forms were detected in the smears of inoculated material. Non-shaken cultures also showed a high percentage of insect gut form trypanosomes in the supernatant (Fig. 5.8) however, differences in the population profile of insect gut form trypanosomes in shaken and non-shaken cultures with time were found. Both conditions showed a rise in gut forms to a peak at approximately the same time (6-7 days post inoculation). Thereafter, the proportion of gut forms in non-shaken cultures receded. In shaken cultures, although a decrease in proportion of gut forms occurred, the numbers remained high.

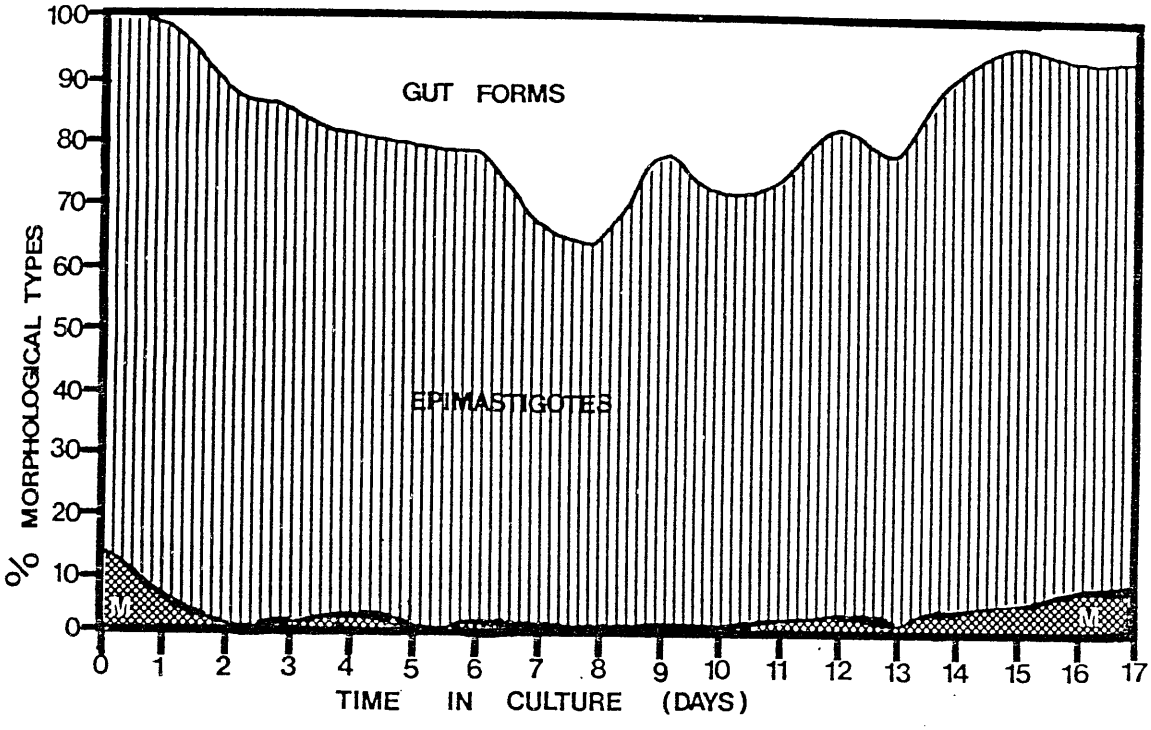
Fig. 5.9 shows the proportions of the developmental forms found in long term shaken and non-shaken cultures. In non-shaken cultures metacyclics reappeared when gut form trypanosome numbers were receding. In shaken cultures metacyclics did not reappear. In these cultures the gut forms remained at a high level. It is noticeable from Fig. 5.9 that metacyclics are not present when 30% or more of the supernatant population is composed of insect gut form trypanosomes, or

FIG.5:9. Proportion of epimastigote, gut forms and metacyclic trypanosomes in the long term shaken and non-shaken cultures depicted in FIG.5:7 and 5:8. T.congolense TREU 1627 . Shaking speed 62 rpm.

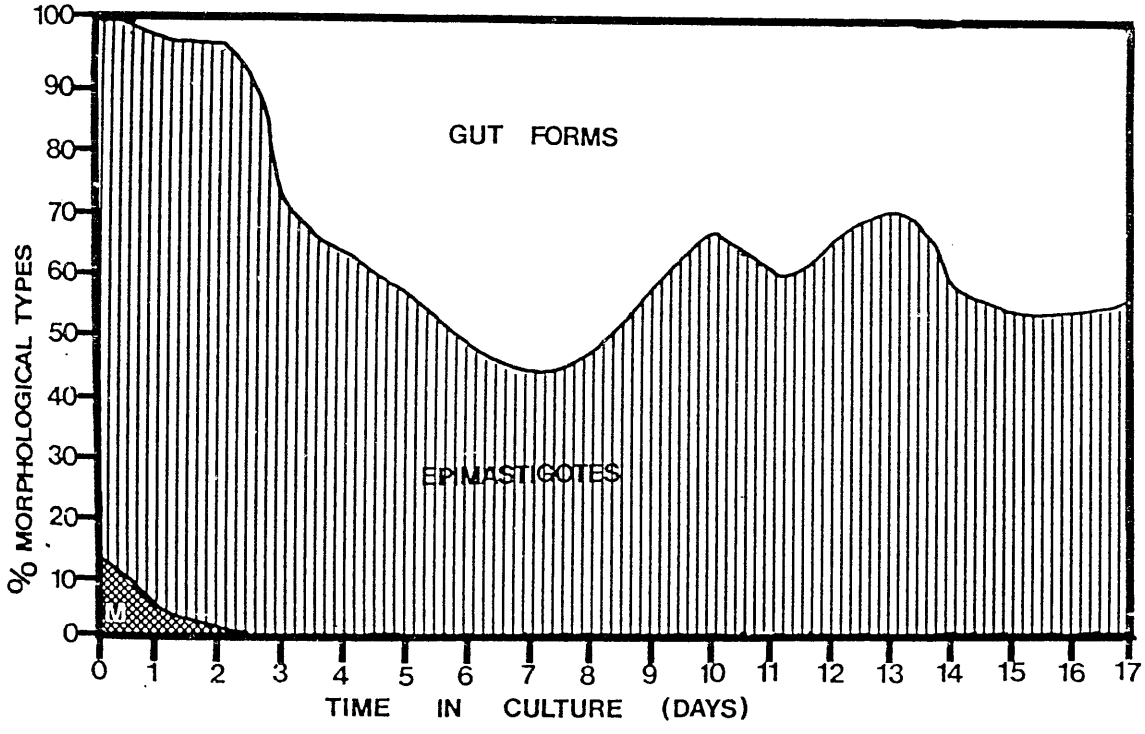
(A) Non-shaken culture (B) Shaken culture

9

A


GUT FORMS
EPIMASTIGOTES
M
M
% MORPHOLOGICAL TYPES
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
TIME IN CULTURE (DAYS)

B


GUT FORMS
EPIMASTIGOTES
M
% MORPHOLOGICAL TYPES
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
TIME IN CULTURE (DAYS)

conversely, less than 70% epimastigotes.

5.4. DISCUSSION

These results showed that regardless of cultures being shaken or still there was an increase in the total population of trypanosomes, whether measured by direct enumeration or total protein content. This phenomenon was independent of seeding density, stock of trypanosomes or speed of shaking. Rates of growth were similar under both shaking and non-shaking conditions (Fig. 5.1) despite the percentage of attached epimastigotes in shaken and non-shaken cultures being very different. In non-shaken cultures 40-90% of the total trypanosome population was attached whereas at a maximum only 25% of the total population was attached in shaken flasks. Thus a similar growth profile was obtained in shaken and non-shaken cultures under very different regimes.

There are two possible ways in which this could have been achieved. The small number of epimastigotes which were attached in shaken flasks could have been dividing far faster than their duplicates in non-shaken flasks. This could conceivably have been due to a greater circulation of media nutrients and, indeed, is a standard tissue culture method for increasing growth in some other cell types (Paul, 1970). Assuming that division occurred only in attached epimastigotes, then, for TREU 1457 cultures at a high seeding density (Fig. 5.2 group 1), approximately 60% of the population divided and doubled the population in 69 hours under non-shaking conditions. Similarly 15% of the population in the shaken flasks (62 r.p.m.) would have divided and doubled the population in 79 hours - a rate of division approximately 4.5 times faster than in still flasks. However, this assumes no division took place when epimastigotes were unattached.

The other possible explanation for shaken and still cultures having similar overall growth profiles is that epimastigotes could divide unattached. The results of the polypropylene substratum experiments (where the total cell population increased but no epimastigote attachment occurred) support this latter view. Thus it would appear that epimastigote proliferation, in this species, is not dependent on attachment. Other protozoans also display this division character; epimastigotes of the South American, Chagas disease causing, Trypanosoma cruzi can multiply in a liquid phase culture (Camargo, 1964). Camargo's culture system also included periods of intermittent shaking. Other kinetoplastids e.g. Crithidia species, also replicate in liquid phase culture media although individuals of these species attach to each other in large rosette formations (Brooker, 1970; Huges et al., 1983). Other protozoans, however, the marine amoeba Paramoeba pemaquidensis for instance (Martin, 1985), require to attach to a surface to undergo division; similarly, James and Byres (1967) found that Acanthamoeba sp. could undergo nuclear division in suspension but needed to attach to a surface for cytokinesis. Attachment does not appear to be necessary in T.congolense epimastigote division.

In the natural situation, epimastigotes are the stage preceding the infective metacyclic forms. Metacyclogenesis also occurs in culture (Gray et al., 1981, 1984) thus the effect of non-attachment on metacyclic development could be examined in this experimental system. The short-term culture experiments discussed above, however, cannot be used to examine the effect of non-attachment on metacyclogenesis as they were only 48 hour cultures whereas metacyclic production only occurs in vitro 10-14 days after initiation. It was therefore necessary to extend the shaking experiments over a longer period of

time to allow the monitoring of metacyclic production under conditions of attachment and non-attachment. The results indicated that attachment is a necessary prerequisite for metacyclogenesis in this species (Fig. 5.7). The lack of metacyclics in shaken cultures was not due to any inherent inability to produce metacyclics as demonstrated by metacyclic production in one of these shaken cultures after being transferred to still conditions, but rather the prevalent conditions were imposing the restriction on the differentiation process. The metacyclogenesis results presented here for T.congolense differ markedly from those of other species. Epimastigotes of T.cruzi both multiply and differentiate to metacyclics in liquid phase culture (Camargo, 1964); under some conditions differentiation can occur in over 90% of the population (Crane & Dvorak, 1982). No attachment appears necessary for this developmental step although attachment and differentiation also appear linked in T.cruzi. In vivo the epimastigotes of this species are found attached to the rectal chitin of their Reduviid vectors, and metacyclogenesis occurs whilst the epimastigotes are in an attached state.

The reason why metacyclogenesis should be dependent upon epimastigote attachment is a different question. Pereira et al. (1981) have postulated that lectins in the guts of Reduviid bugs trigger transformation of T.cruzi. Receptors for these lectins have been found on epimastigotes but not on trypomastigotes. Differentiation of the metacyclic can be blocked by a monoclonal antibody to a 72 kilodalton surface glycoprotein (Sher & Snary, 1982). Sher and Snary suggest that this glycoprotein may be a receptor controlling the transformation. Differentiation receptors in situ are, however, difficult to reconcile with metacyclogenesis of T.cruzi in liquid phase cultures (Camargo, 1964; Crane & Dvorak, 1982) although various nutritional factors have also been suggested to be triggering metacyclogensis e.g. organic

acids (Fernandez et al., 1969); sera factors (O'Daly, 1976, Sher et al., 1983; Contreras et al., 1985). The physiological stress of pH change (Fernandes & Caceres, 1976) and temperature change (Pan, 1971) have also been postulated to be triggering factors. Lopetegui and Miatello (1982) provided evidence that a depletion of some nutrient factors in the medium can induce metacyclogenesis. Metacyclogenesis is generally reported to occur on cessation of exponential growth of epimastigotes for the postulated nutritional reasons and up to 90% of trypanosomes differentiate. This is not the case in these T.congolense experiments where epimastigote division is continuing as well as metacyclogensis with differentiation occurring only in a small population of cells. As the medium was changed every 48 hours regardless of the level of metacyclic production, no long term changes in nutritional levels were being imposed upon the cells. If nutritional stress was occurring then every 48 hours on adding new medium, metacyclogenesis would cease, this does not happen.

The prerequisite for attachment for metacyclogenesis in T.congolense raises the question of what initiates the developmental process in this species. It does not appear to be a simple initiation of differentiation by contact as contact and attachment of trypanosomes in culture occurs from the first minutes of inoculation and is relatively complete within the first hour (Chapter 2) but metacyclogenesis is not evident until approximately 10 days afterwards. This same phenomenon was demonstrated in changing a culture from shaken to still conditions. The natural condition in the tsetse labrum also displays a delay in the time between contact and attachment of epimastigotes and metacyclic production; epimastigotes are found in the labrum days before any metacyclics are found (Lloyd & Johnson, 1924; Hoare, 1972). Thus it would appear that attachment of

epimastigotes is necessary for metacyclic production although not epimastigote proliferation. The necessity for attachment is not, however, an immediate precursor of metacyclogenesis but could possibly be the trigger for a series of processes ultimately leading to metacyclic production a few days later.

It is possible that metacyclogenesis could be dependent on a specific number of epimastigote divisions taking place before hand as has been suggested for T.cruzi (Pan, 1978), but this would not account for the lack of metacyclics in shaken cultures. If metacyclogenesis required a set number of epimastigote divisions after initial attachment then the observed behaviour i.e. a set time after initiation of a culture, transferring a culture to an attached condition from a non-attached one or in situ in the tsetse labrum, metacyclic forms are visible and not before, would fit this hypothesis very well.

It is not known whether the trypanosome cycle is at all stages programmed in one direction or is reversible or even if stages can be omitted totally, for example can the insect gut forms of T.congolense transform directly to trypomastigotes, omitting the epimastigote stage and can epimastigotes differentiate 'backwards' into proventricular or procyclic forms? The observations presented in this study that epimastigote attachment appeared to be a necessary prerequisite for metacyclogensis would make the possibility of direct procyclic to metacyclic differentiation unlikely. The other example raised above, that of epimastigote to procyclic/proventricular forms however may have been occurring in these experimental cultures. Observations made in this study of large numbers of procyclic and proventricular forms in long term shaken flasks, where none were detected in the inoculating material, introduces the possibility that 'backwards differentiation' may have been occurring i.e. epimastigotes

"dedifferentiating" into proventicular and procyclic forms. A small number of proventricular-like forms are regularly found in epimastigote cultures of this species during the initial few days after inoculating a new flask from supernatants containing large numbers of epimastigote bundles (Gray et al., 1981 and Chapter 2). The possibility that a very small number of procyclic/proventricular forms were put in the initial experimental cultures cannot be entirely eliminated; procyclics would rapidly divide under shaking conditions as they readily grow in liquid cultures (Brun, 1982). However, if no gut forms were added, as the smears of the initiating material suggest, then it raises the possibility that stages of the life cycle (at least of the insect forms) are more plastic than originally supposed, the forms prevalent depending upon the ambient conditions, transformation being in either direction.

In conclusion, it appears that for T.congolense, attachment is a necessary prerequisite for metacyclogenesis. Thus prevention of attachment by some means could possibly prevent the development of infectivity to the mammal. However, as the attachment is not specific, in that T.congolense epimastigotes will attach to a variety of surfaces, some internal component of the attachment mechanism would be required to be used as a target for disruption of attachment and subsequent disease control measures.

5.5. SUMMARY

Prevention of epimastigote attachment by either shaking the cultures or growing trypanosomes on a polypropylene substratum did not affect epimastigote division. T.congolense stocks TREU 1457 and TREU 1627 whether shaken at 62 rpm or 40 rpm and monitored by direct enumeration or by monitoring temporal changes in protein content displayed approximately exponential increases in total cell population

equivalent to that of non-shaken replicates. Different proportions of attached epimastigotes in shaken and non-shaken flasks were found; shaken flasks contained low numbers of these (<25%) whereas attached epimastigotes formed the majority of the population (40-90%) in non-shaken flasks. Cultures grown on a polypropylene substratum to which no cells attached also showed exponential population growth. Metacyclogenesis did not occur in situations where no epimastigote attachment was present. Attachment appears to be a necessary prerequisite for metacyclogenesis in this species.

A high proportion of proventricular-like forms were found in the supernatant of long-term shaken cultures (30% of the supernatant population) despite their absence in the culture inoculum.

This observation could mean that epimastigotes are a totipotent stage, differentiating according to the ambient environment rather than an inherent programme.

CHAPTER 6

A MICROSCOPICAL INVESTIGATION OF THE INTERFACE BETWEEN ATTACHED T.CONGOLENSE EPIMASTIGOTES AND THE SUBSTRATUM

6.1. INTRODUCTION

In general three explanations have been proffered in the literature for the mechanism of cell adhesion; (1) that cells attach to each other or to the substratum by bridging mechanisms e.g. cross-linking by glycoproteins; desmosomes have been shown to contain glycoproteins within their intercellular gap (Gorbsky & Steinberg, 1981; Cowin et al., 1984), these are thought to contribute to the cell adhesion. Another suggestion for bridging mechanisms is by calcium-carboxyl bridges (Rappaport et al., 1960; Weiss, 1962; Rosen & Culp, 1977) between cells. (2) Cell attachment has also been suggested to occur via electrostatic interactions and (3) that the main attractive and adhesive force between cells or cells and substratum is due to the balancing of long range Van der Waals - London forces and repulsive forces arising from the cell surface charge (Curtis, 1964,1973; King et al., 1979a).

These three mechanisms are not considered mutually exclusive and combinations of these are visualized as being able to account for different stages in a cell's settling and attaching process.

In TEM studies of sections of attached T.congolense epimastigotes a gap; (~12nm) between the cell and substratum is visible. This gap appears to contain stainable material. The extracellular gap is found in both in vivo and in vitro attachment studies. In fixed material, however, the possibility of the gap being a fixation artifact can never entirely be eliminated. If the extracellular gap is a true structure and present in living attached epimastigotes then it might be possible to cause chemical disruption of trypanosome attachment and thereby deduce the nature of the extracellular components of the organism's attachment.

Two approaches have been utilised in the present study (1) the effect of different reagents on the gap between trypanosome and

FIG.6:1. Diagram showing reflection patterns from surfaces.

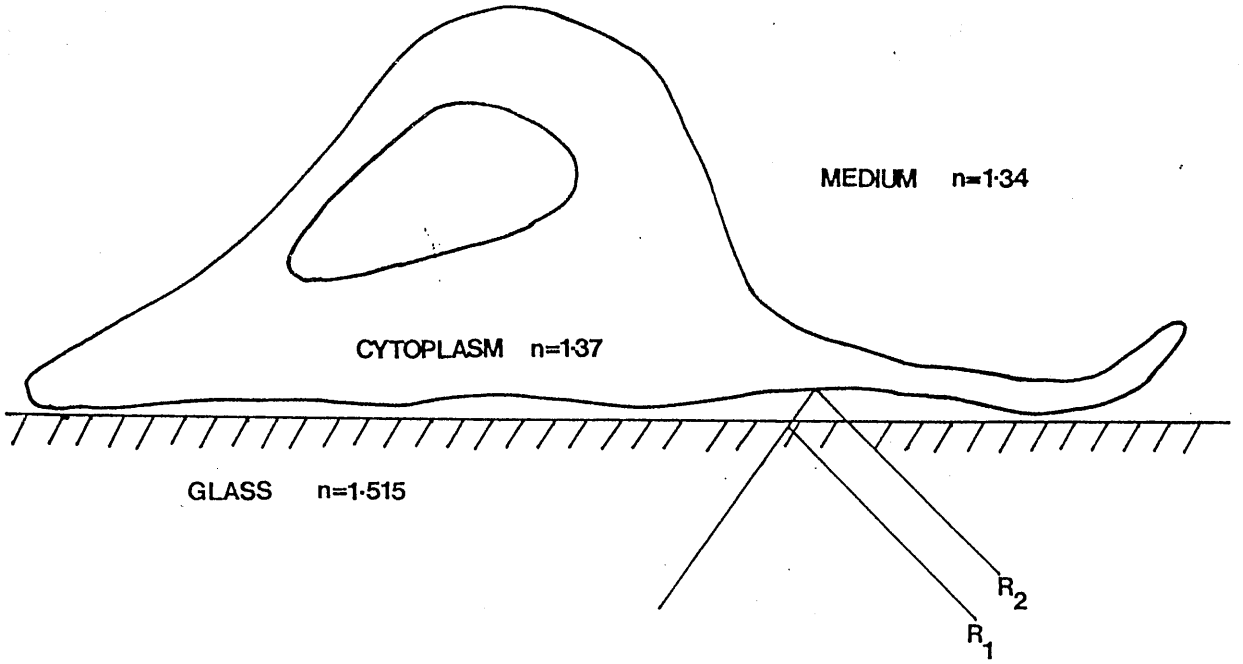
R_1= Reflection from glass/medium interface

R_2= Reflection from medium/cell interface

The refractive index values given are for standard cells and conditions.

After Verschueren (1985).

1



substratum as visualized by interference reflection microscopy (IRM) and (2) the ability of epimastigotes to attach to small beads with different chemical properties. By studying living cells possible fixation artefacts can be eliminated.

6.2. BASIC PRINCIPLES OF IMAGE FORMATION IN INTERFERENCE REFLECTION MICROSCOPY

Under epi-illumination the image of a non-opaque object results from light reflected at interfaces between media of different refractive indices (n), and the intensity of the refracted beam increases with increasing differences between n values. When cells are grown on a coverglass and observed with epi-illumination through an oil-immersion objective lens which also functions as a condenser, the first reflection will occur at the transition from glass to culture medium (R_1) (Fig. 6.1). Owing to differences in refractive index, R_1 will be a relatively strong reflection, therefore the background will be relatively clear. The presence of a cell on the coverglass can then modify this background intensity. At sites where the cell surface is separated from the substrate by a film of culture medium, reflection will occur at both the glass-medium (R_1) and medium-cell (R_2) interfaces. If the thickness of the film of medium is of the same order of magnitude as the wavelength used, then both reflected beams can interfere. The optical path difference, Δ , between the two reflections is given by the cosine law (Tolansky 1973): $\Delta = 2\underline{n}m\underline{d}\cos\theta$ where $\underline{n}m$ is the refractive index of the medium, $\underline{d}$ is the distance between the interface, and θ is the angle of refraction in the medium. A dark fringe will be projected when $\Delta = N\lambda$ a bright one when $\Delta = (N+1/2)\lambda$. The integral number N is the order of interference. With monochromatic incident light these interferences result in bright and dark zones compared with the background; white light yields a

coloured pattern. The intensity of monochromatic light or the colour of white light interference pattern reflected at the cell's underface gives information about the closeness of contact between the cell and the substrate. For incident white light, interference colours occur in a particular sequence directly related to Δ , which can thus be estimated by reference to a standard colour chart. At a value of less than 40nm a black image is obtained. As Δ increases, the image changes through grey to white. For values of Δ greater than 300nm chromatic images of the standard interference colour series will be encountered (Curtis, 1964; Izzard & Lochner, 1976; Preston & King, 1978a&b; reviewed by Verschueren, 1985).

In this way effects of various reagents on the extracellular gap size and therefore the nature of the intervening material can be monitored by observing the attachments by IRM. Any interpretation of results would apply directly only to cell-glass adhesion but as the epimastigote attachment ultrastructure is equivalent whether in vivo or in vitro bound to glass or plastic, it is likely that any in vitro interpretation could be related to the in vivo situation.

Using these techniques electrostatic, protein, glycoprotein and calcium associated components of the epimastigote attachment site exterior have been examined.

6.3. MATERIALS AND METHODS

6.3.1. Trypanosomes and cultures

For interference reflection microscopy in vitro epimastigote cultures of T.congolense were used similar to those described in Section 2.2 but initiated and maintained in 1.5 or 3.5cm diameter plastic tissue culture plates (Falcon, or Nunc). Sterilized glass coverslips (Chance propper, No.1) were placed in the bottom of the wells before adding trypanosomes and culture medium, the epimastigotes

then grew attached to the glass substratum. Cultures of a short duration (1-4 days) and/or low inoculum density (<1x10^5 cells/ml) were used in these experiments, these conditions avoided problems of overcrowding and subsequent mass death of trypanosomes; the low density of trypanosomes also allowed greater clarity of image interpretation. For charge assay experiments, swollen sephadex or sepharose beads were placed in 1.5cm culture plate wells in culture medium. Washed epimastigotes were added to these wells.

6.3.2. Light microscope monitoring of attachment

Routine monitoring of culture wells was carried out using an inverted phase contrast microscope (Diavert, Leitz) fitted with Leitz phaco objectives, the 32 x magnification objective was used. For interference reflection studies the coverslips, with attached cells, were removed from the culture wells and placed on a viewing chamber composed of two pieces of glass coverslip stuck onto a glass slide so that the cell-bearing coverslip was supported above the glass slide. The test medium was placed in the space below the coverslip. The experimental coverslips were then veiwed in a Vickers MI7 microscope adapted for interference reflection microscopy (Curtis,1964). White light from a high pressure mercury lamp (200W) was collimated by additional irises and passed down a Vickers oil (100 x magnification 1.3NA) objective by means of a beam splitter. Cells were photographed using Kodak Tripan-X 400 film or 200 ASA Ektachrome film. The camera tube magnification was 6.3 x.

The attachment of trypanosomes to swollen sephadex or sepharose beads was observed using a Leitz Ortholux II microscope fitted with Leitz Fluoresz 50 or 100 x magnification objectives.

6.3.3. Interference reflection experiments

Culture media containing various substances were tested for their effect on epimastigote attachment. Test solution and trypanosomes were added together to the culture wells, or in some cases the trypanosomes were preincubated in test medium before inoculating the culture wells. The following solutions were tested: (a) MEM (as described in Section 2.2) containing 0, 10 or 20% foetal calf serum (FCS) (b) 0 and 10% FCS MEM containing 250µg/ml trypsin (c) 0 and 10% FCS MEM containing K^+Cl^- at the following molarities; 10µM, 100µM, 1mM, 10mM and 100mM. The osmolarities of the KCl solutions were measured using a Holbrikro osmometer. However the difference between incomplete MEM and MEM containing KCl was no greater than incomplete MEM and MEM plus 10% FCS and the variation in osmolarity between various medium bottles. Therefore, osmotic equivalents to KCl of an non-ionic nature were not found to be necessary. (d) 0 and 10% FCS-MEM containing 1mM or 10mM EDTA at pH 6, 7.3 and 8. Cultures were also initiated in Ca^{2+} and Mg^{2+} free PBS pH 7.2 (170mM NaCl, 5mM KCl, 3mM KH_2 PO4, 8mM Na_2HPO4). These cultures could only be grown for periods less than 24 hours before death of trypanosomes occurred. To some of these cultures 2mM calcium was added and the effect observed after 30mins. (e) The effects of the following lectins (many supplied by Dr J. Kusel) and some competing sugars were also examined. The concentrations used were subagglutinating (if trypanosome agglutination occurred) so that the effect of the lectins on attachment and not surface agglutination could be determined. Lectins were mixed with MEM or PBS at twice the final concentration and then added to an equal volume of washed trypanosomes and incubated for 30 mins before adding to the culture well. The lectins tested were Concanavalin A (ConA) type IV 5-10µg/ml; lentil (Lens culinaris) lectin 50µg/ml; wheat germ (Triticum vulgaris)

TABLE 6:1.

LECTIN in PBS	HUMAN ERYTHROCYTE GROUP	AGGLUTINATION AFTER 30min 20°C
CON A 10µg/ml	O	+/-
CON A 100µg/ml	O	+
WGA 1mg/ml	O	+
Lentil lectin 100µg/ml	O	+
Potato lectin 1mg/ml	O	+
Gorse lectin (UEA type I) 50µg/ml	O	+
Peanut agglutinin 100µg/ml	O	-
PBS alone	O	-
Peanut agglutinin 100µg/ml	Neuraminidase (1.8U) treated O	+
PBS alone	Neuraminidase (1.8U) treated O	-
Soybean lectin 50µg/ml	A	+
PBS alone	A	-

agglutinin (WGA) 500µg/ml, potato (Solanum tuberosum) lectin 500µg/ml, soybean (Glycine max) agglutinin 500µg/ml, gorse (Ulex europeus) (UEA1) lectin 500µg/ml, peanut (Arachis hypogaea) agglutinin 500µg/ml. Peanut agglutinin was also tested on epimastigotes pretreated with neuraminidase (1mg/ml) for 15 mins. Competing sugars for some of the lectins viz. ConA, lentil lectin and WGA were used as controls for effects visualized. The competing sugars at 0.5M in MEM or PBS were incubated with the appropriate lectins for 30 mins and then added to an equal volume of trypanosome suspension incubated and then put into culture wells. Trypanosomes were also incubated with sugars (0.5M) alone. The sugars used were α-methyl -D-mannoside (a binding competitor of ConA and lentil lectin) and N-acetyl-D-glucosamine, a WGA binding competitor. The activity of all lectins was tested by examining their reactions on human erythrocytes of appropriate blood groups. All lectins agglutinated the appropriate erythrocytes (Table 6.1). (f) The effects of tunicamycin at concentrations of 50µg/ml, 5µg/ml and 0.5µg/ml in MEM with 0 or 10% FCS were also examined.

Lectin experiments, lectin inhibition experiments and tunicamycin experiments were performed in parallel on BHK cells (Flow Laboratories) grown in MEM in similar conditions to those of the epimastigotes. Approximately $1x10^4$ BHK cells/well were seeded. All lectins, sugars, neuraminidase and tunicamycin used were Sigma Ltd. products.

6.3.4. Electron microscopy

Electron microscopy was performed on epimastigotes treated with WGA, WGA plus N-acetyl-D-glucosamine, N-acetyl-D-glucosamine, ConA, ConA plus α-methyl-D-mannoside, α-methyl-D-mannoside, Lentil lectin, Lentil lectin plus α-methyl-D-mannoside, tunicamycin and MEM controls. For electron microscopy cells were grown on Thermanox coverslips,

fixed in 1% glutaraldehyde in 0.1M phosphate buffer, post fixed in 1%OsO_4(aq), dehydrated in ethanol, treated with propylene oxide, and embedded in Araldite. Silver and gold sections were cut from blocks, stained in 2% methanolic uranylacetate and lead citrate and examined in a transmission electron microscope (AEl 801).

6.3.5. Charge assay cultures

Beads 40 - 120mm in diameter of positive, negative and neutral charge were tested for epimastigote attachment to them. Negatively charged carboxymethyl (CM)-sephadex and positively charged diethylaminoethyl (DEAE)-sephadex beads (both Pharmacia, a gift from T.M. Preston) were swollen in the following solutions; 145mM NaCl(pH7), 70mM NaCl (pH7), 290mM sucrose solution (pH7) and 140mM sucrose solution (pH7). The beads were incubated in the solutions in a boiling water bath for 2 hours; several changes of the solutions were made over this period. Beads were left in the solutions for a further hour and then autoclaved at 15psi for 15mins and left to cool. Equal volumes of bead suspension and washed trypanosomes in MEM (no FCS) were added to the culture plate wells (1.5cm diameter) and then monitored, using an inverted microscope, over a 2 day period.

Sepharose 4B beads (Pharmacia) (neutral charge) were swollen in boiling MEM for 1 hour, these were subsequently washed twice in MEM and autoclaved. Equal volumes of bead solution and trypanosome solution (MEM) were added and monitored as above. Pre-swollen ConA-sepharose beads (Pharmacia) (Sepharose 4B beads with purified Concanavalin A linked to them) were left for a few hours in MEM, the beads were subsequently washed twice in MEM. Equal volumes of bead solution and trypanosome suspension were added and monitored as above.

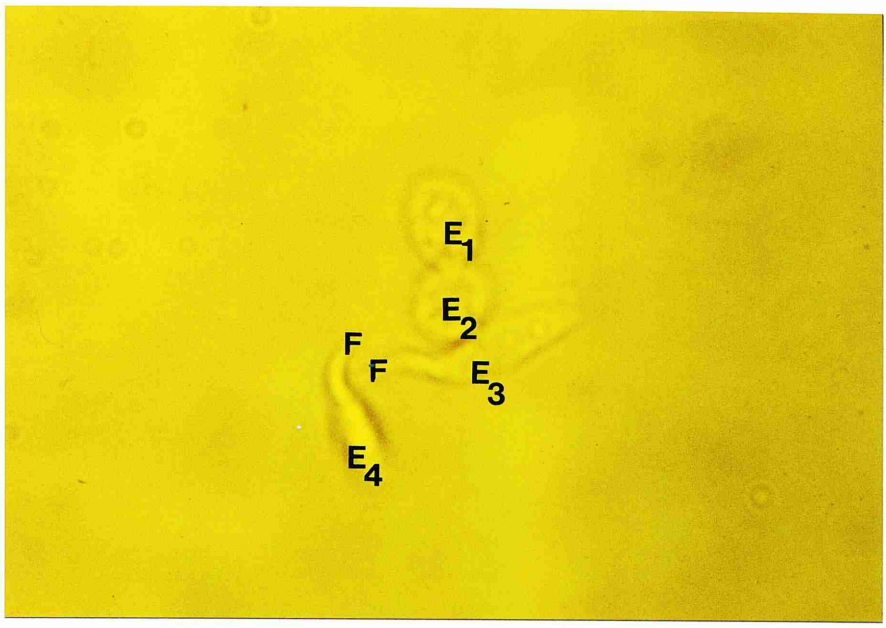
In all experiments the mode of attachment was recorded subjectively according to the epimastigotes movement pattern.

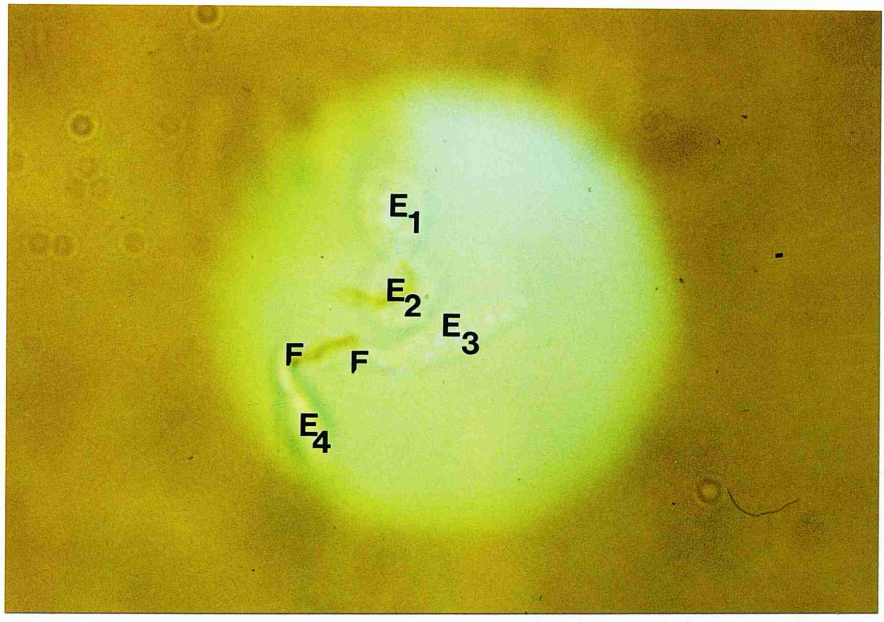
FIG.6:2. Series of photographs showing the same group of T.congolense epimastigotes viewed with A) phase contrast microscopy B) phase contrast plus interference reflection microscopy and C) interference reflection microscopy alone.

F- flagella, E_1-epimastigote 1, E_2-epimastigote 2, E_3-epimastigote 3, E_4-epimastigote 4.

MAG. 6:2-6:10. all 3,500 x.

2





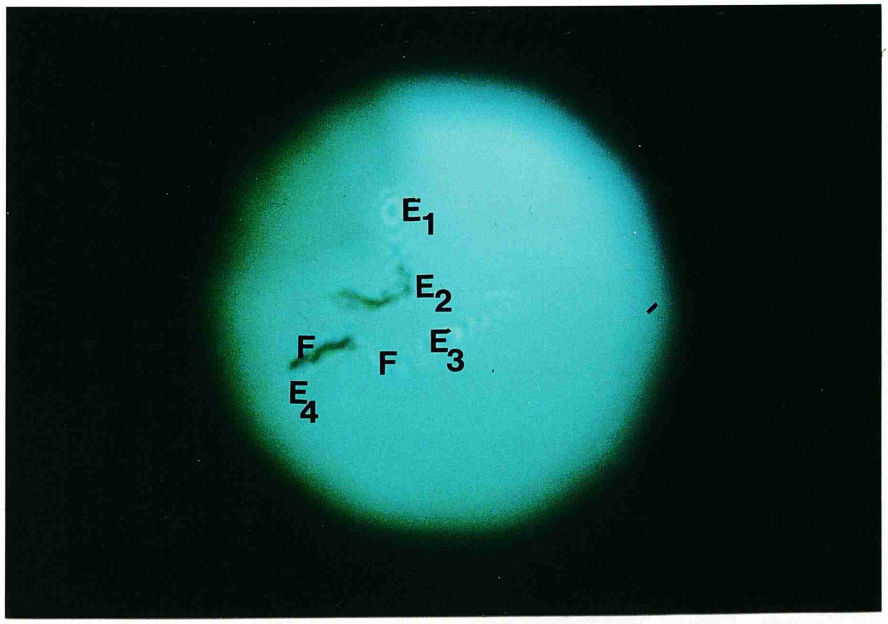
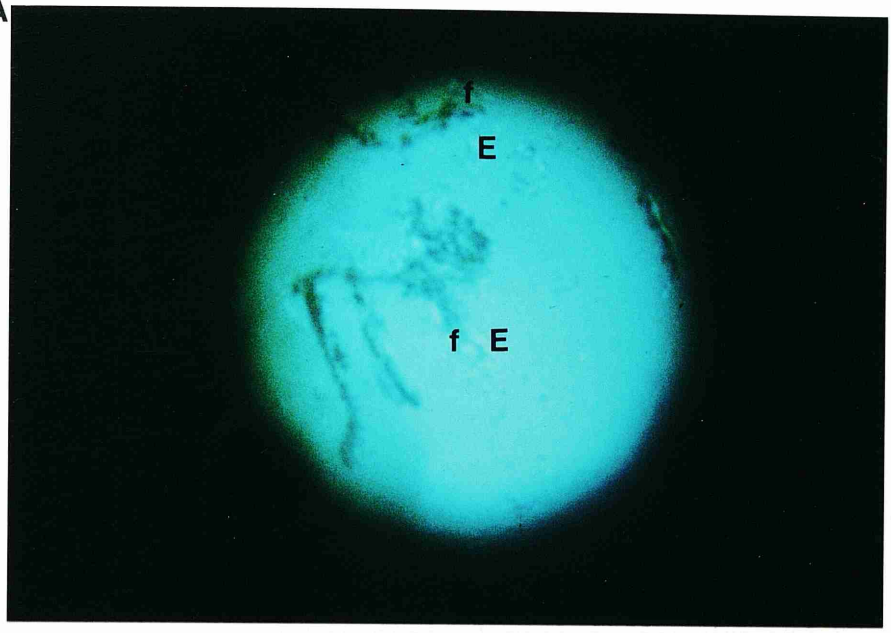


FIG. 6:3. Interferometry patterns of attached epimastigotes grown in media containing different FCS concentrations.

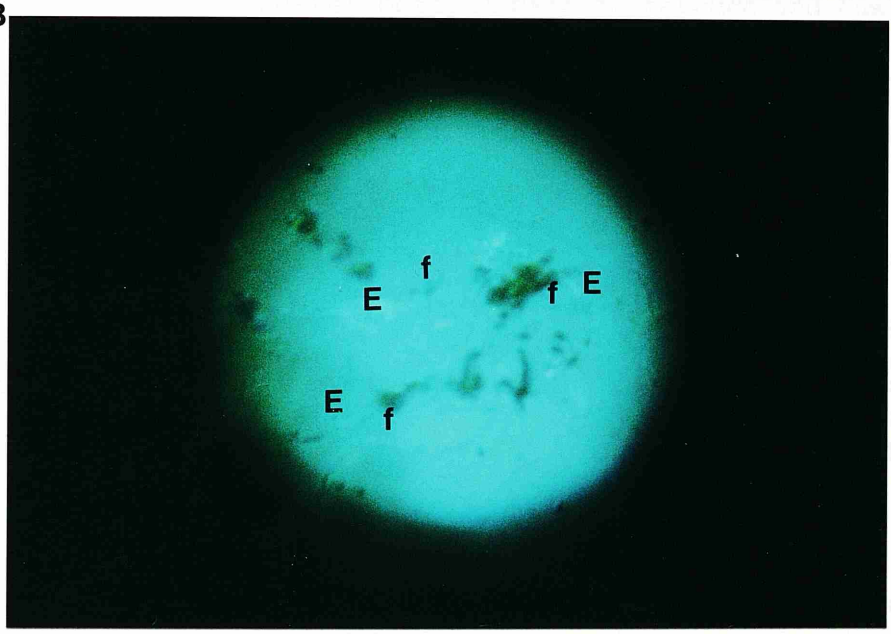
A) 0% FCS B) 10% FCS C) 20% FCS

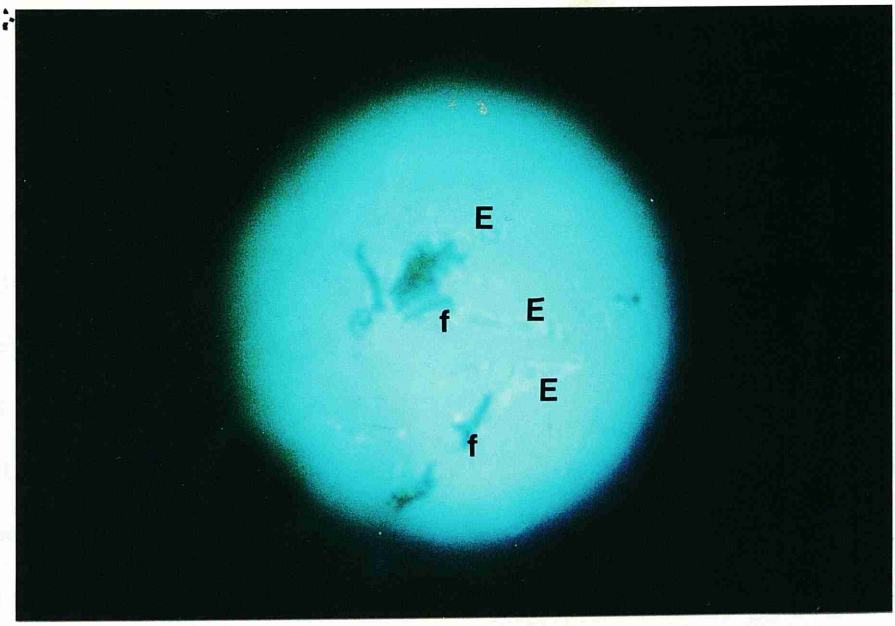
3

A



B





6.4. RESULTS

6.4.1. Interference Reflection Microscope observations

Over part of the epimastigote's flagellum a dark image is observed by interference reflection microscopy (IRM) (Fig. 6.2). This is indicative of a close apposition between flagellum and glass in this area. In all cases the cells showed their points of closest apposition to the coverslips to be present in the flagella. Cell bodies moved in and out of the IRM image plane and appeared white or displayed slight spectra of colour within the fringes visible, these colours signify a greater optical path difference and therefore an increased gap distance between the cell at these points and the coverslip than the darker images found in the flagella. Extensions from the flagella axis were noted in some epimastigotes in all types of cultures. These extensions took the form of dark, thin, hair-like structures emanating from the flagellar trunks and appeared black under IRM conditions. These structures were considered to correspond to the 'filopodia' noted in scanning electron microscopy of T.congolense attached epimastigotes (Chapter 4).

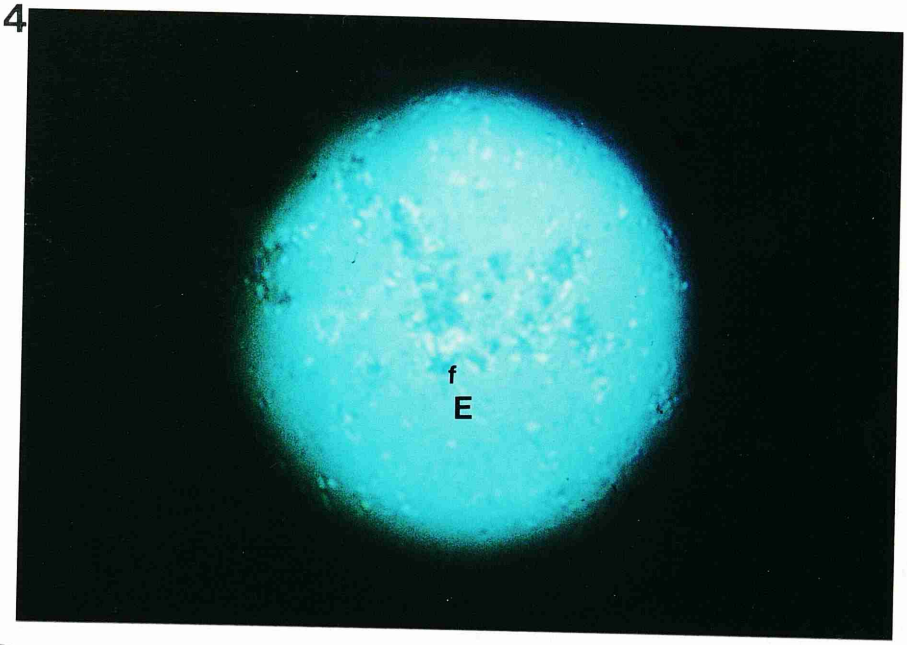
6.4.1.1. Effects of serum concentration: Cells grown in 0, 10 or 20% FCS MEM displayed essentially similar IRM images in terms of gap width size (Fig 6.3). Black images were obtained at sites of close cell-glass interaction, particularly in individual flagella and as a mass in the centre of a group of attached epimastigotes, this colour of IRM image is indicative of a cell-glass distance of about 20nm or less. Epimastigotes in cultures with no serum (Fig 6.3a), however displayed less extensive black fringe areas, the dark areas present were more punctate in pattern and a little less dark than the cells grown in the presence of serum. It was noted that individual flagella could contain more than one black area but, where it was possible to see individual

FIG. 6:4. Reflection interferometry pattern of epimastigotes grown in medium containing 250μg/ml trypsin.No FCS was present in the growth medium. The cells appeared morphologically unaffected by this treatment.

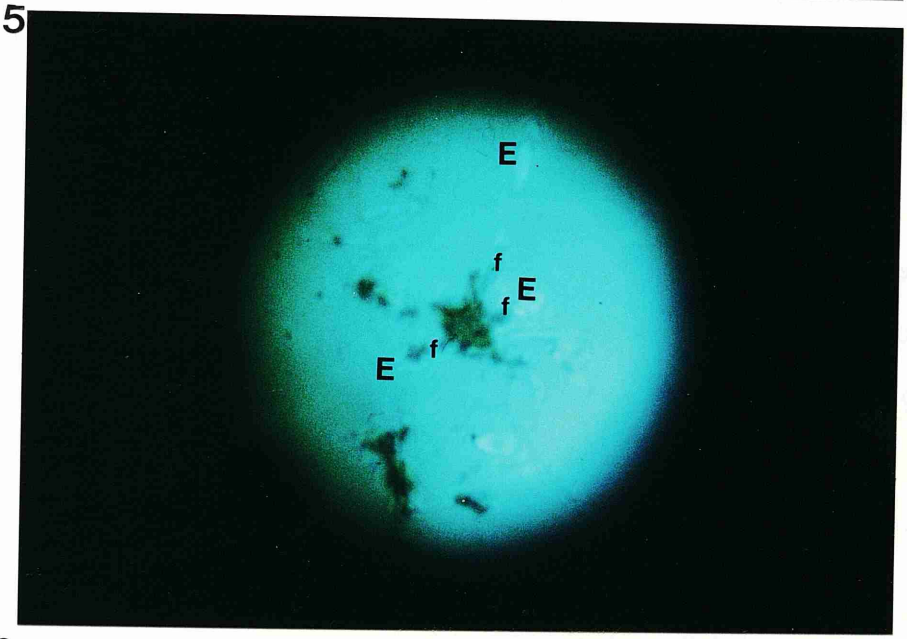
FIG. 6:5. Reflection interferometry pattern of epimastigotes grown in medium containing 1mM $K^{+}Cl^{-}$. 10% FCS was present in in the growth medium.

FIG. 6:6. Reflection interferometry pattern of epimastigotes grown in medium containing 1mM EDTA. No FCS was present in the growth medium. The dark interference colour appears darker and covers a more extensive area (c.f. FIG. 6:3a).

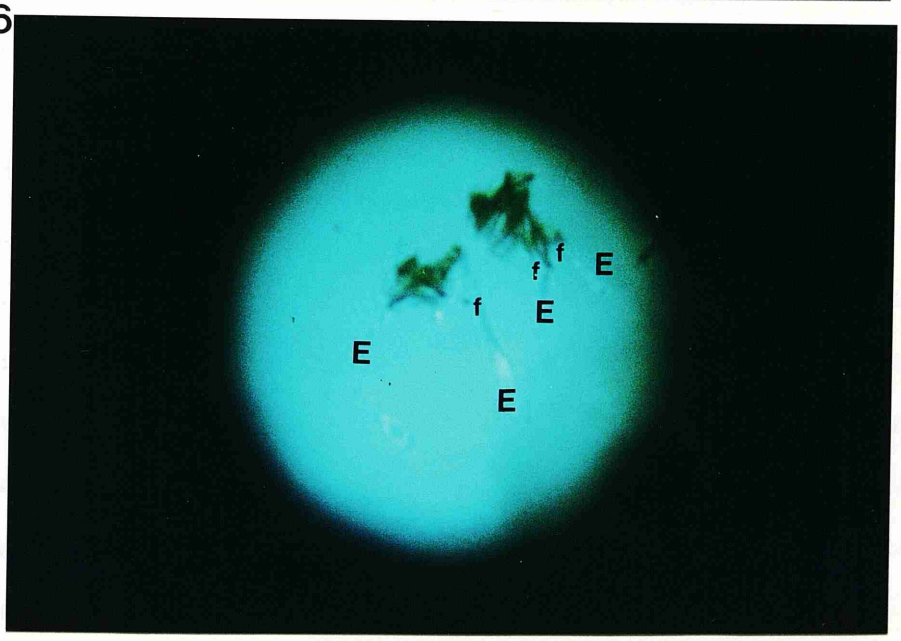
4



5



6



flagella, it was noted no black images were obtained at the tip of the flagellum the majority being towards the point where the flagellum leaves the reservoir area of the cell body.

Very little difference in density of attachment image was noted between epimastigotes cultured in media containing 10% or 20% FCS (Figs. 6.3b + 6.3c).

6.4.1.2. Effects of trypsin: Cultures containing 250 ug/ml trypsin (Fig. 6.4) displayed IRM images which were lighter and less extensive than those in control wells (compare with Fig. 6.3). This occured whether or not serum was present in the medium. The trypanosomes, however were normal in appearance and behaviour. The lighter image indicates the gap width was increased.

6.4.1.3. Effects of monovalent ions: The effect of KCl at molarities ranging from 10 μM to 100mM was examined. No differences between experimental wells and those of controls were detected (Fig. 6.5). The black IRM images present in attached flagella and lighter images over the rest of the epimastigotes were noted to be very similar to those of control cultures.

6.4.1.4. Effects of divalent cation removal: IRM images of attached epimastigotes grown in media containing the divalent cation chelator EDTA at 1mM or at 10mM and at pH6, 7.3 or 8 all displayed an IRM image which appeared darker than that of controls.(compare Figs. 6.6 and 6.3a). Cells grown in Ca^{2+} and Mg^{2+} free PBS also displayed this darker image over the attached flagellar area. When calcium was added to divalent cation free PBS cultures and examined 30 minutes later the IRM pattern image was more akin to that of control cells than that of the dark cation free image. The reversible change in IRM pattern due to cation removal or addition could be noted within one cell.

FIG. 6:7-6:10. Reflection interferometry patterns of epimastigotes grown in media containing lectins. No FCS was present in the growth media. No black interference patterns are found only grey or white.

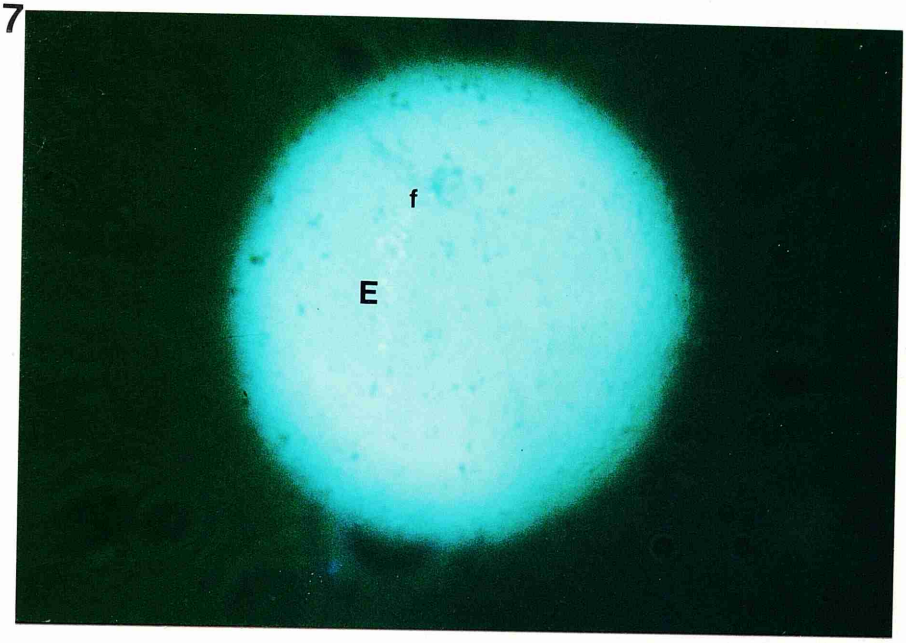
FIG.6:7. Potato lectin, 500µg/ml.

FIG.6:8. Lentil lectin, 100µg/ml.

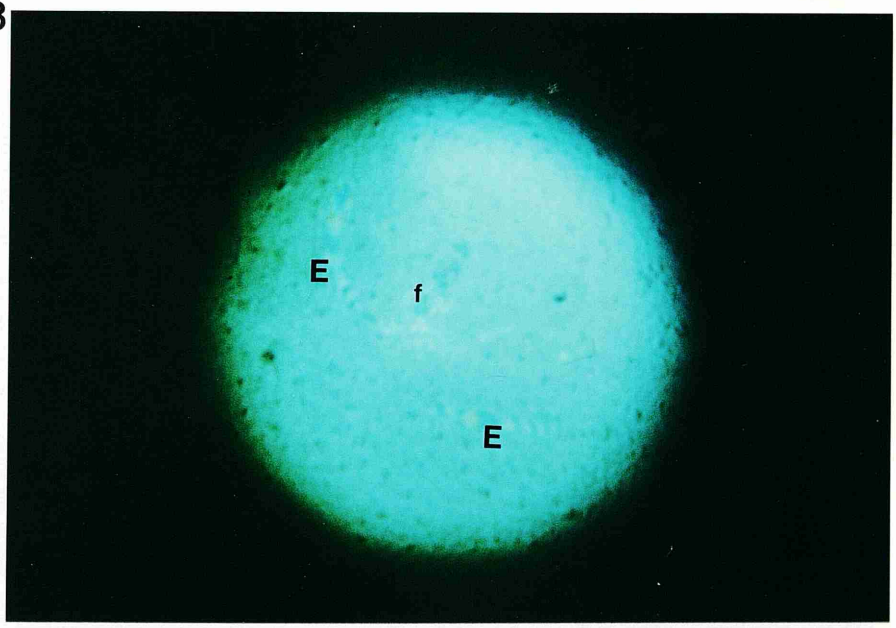
FIG.6:9. Wheat germ agglutinin, 500µg/ml.

FIG.6:10. Concanavalin A, 10µg/ml.

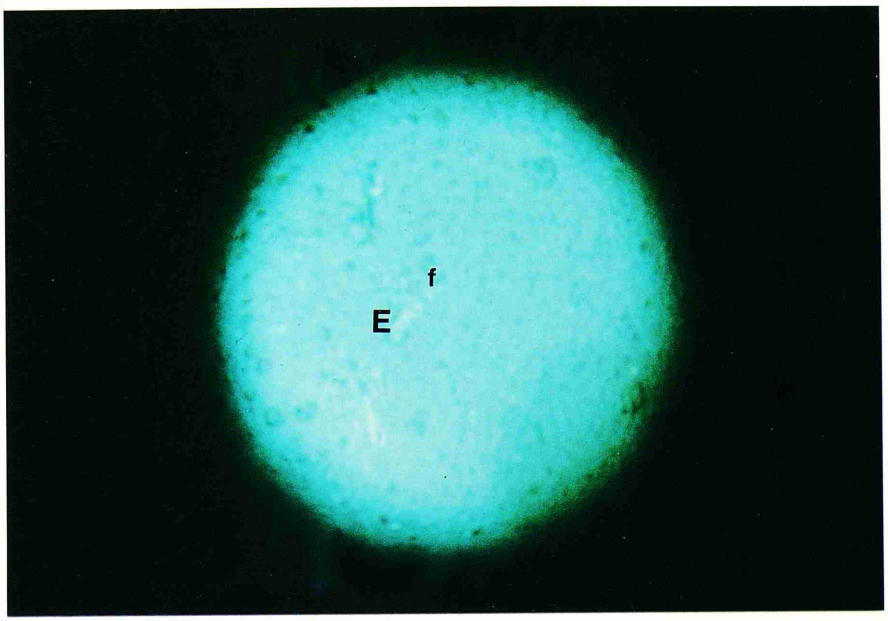
7



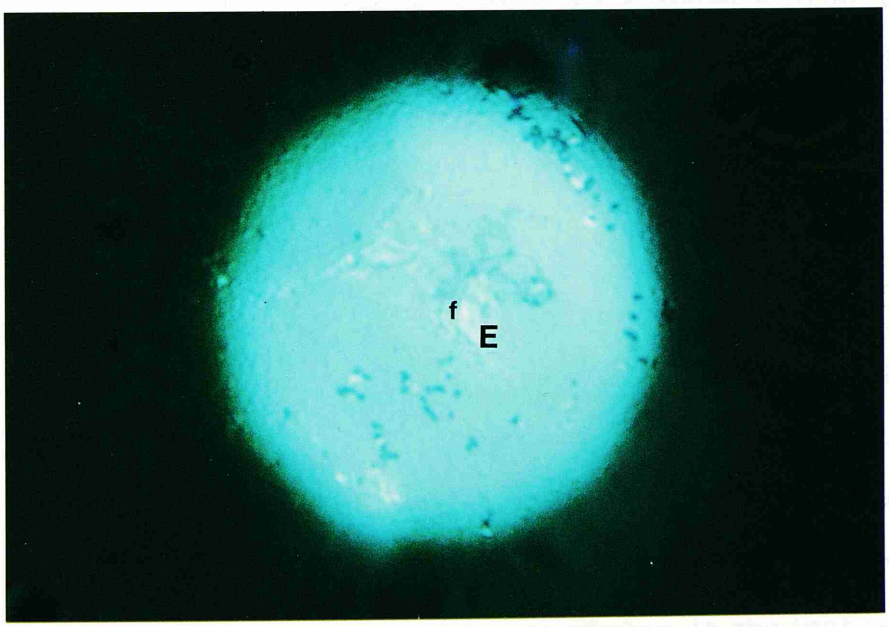
8



9



10



6.4.1.5 Effects of lectins: IRM images of cells treated with gorse lectin, soybean lectin and peanut agglutinin appeared very similar to those of control cultures. Phase contrast observations of these cultures indicated that a very similar number of epimastigotes to that of control cultures were attached. Epimastigotes pretreated with neuraminidase and subsequently with peanut agglutinin still resembled control cells under IRM conditions. Potato lectin (Fig. 6.7) treated cultures gave images that appeared slightly lighter at the point of closest contact between cells and glass than those of control cultures. These sites, located on the flagella, were grey rather than black in colour although punctate patterns were observed. The surface of the glass in cultures treated with potato lectin also appeared affected by this substance, a mottled effect was noted on the glass even in areas lacking trypanosomes. In appearance and behaviour and in the number of cells attached epimastigotes appeared similar to control wells. Lentil lectin (Fig. 6.8) treated cultures again displayed IRM images in which the darkest fringes present in the image (again in the flagella) appeared grey rather than black. In behaviour and appearance, lentil lectin-treated flagellates appeared similar to those grown in MEM alone. WGA treated cells (Fig. 6.9) also displayed grey rather than black images in the flagellar regions indicating a larger gap between cell and glass than in control cells. This lectin, like that of potato lectin, also appeared to affect the glass surface, portraying a mottled effect in IRM images but under phase contrast microscopy there were no visible structures at the 'mottled' sites. Slightly fewer epimastigotes appeared to attach to the glass in these cultures than in controls but in behaviour and morphology those present appeared normal. After 24 hours, however, a large proportion of dead or immobilized trypanosomes were noted in these cultures. Con A

(Fig. 6.10) treated epimastigotes under IRM displayed light grey coloured fringes at the flagellar close apposition regions indicating an increase in gap width at these points. In appearance and mobility attached epimastigotes appeared similar to MEM control epimastigotes but fewer trypanosomes were found attached and even at very low ConA concentrations a slight agglutination of cells occurred.

Cells treated with α-methyl-D-mannoside before adding to either Con A or lentil lectin solutions displayed IRM similar to those of MEM controls as did cells treated with α-methyl-D-mannoside alone; a dark fringe was obtained over the flagella area. Similarly, cells pretreated with N-acetyl-D-Glucosamine and then with WGA showed IRM patterns similar to controls as did cells treated with this sugar alone. In all these cultures appearance was normal.

BHK cells were treated in a similar manner to that of T.congolense epimastigotes in some of the lectin treatments. BHK cells were grown in the presence of Con A, lentil lectin, WGA, Con A plus α-methyl-D-mannoside, lentil lectin plus α-methyl-D-mannoside, WGA plus N-acetyl-D-glucosamine, α-methyl-D-mannoside, N-acetyl-D-glucosamine and MEM alone. These cultures were observed by phase contrast microscopy over a 48 hour period. The BHK cells initially began to flatten and spread but in cultures containing WGA and lentil lectin alone the cells did not fully flatten, remaining partially rounded. In Con A-treated cells no spreading occured, the cells remained rounded and did not attach. In all other cultures, those with lectin or sugar alone or in MEM controls, the BHK cells flattened and divided forming confluent cultures in the wells over the 48 hour period.

6.4.1.6. Effects of tunicamycin: Cells were treated with 0.5, 5 and 50μg/ml tunicamycin and then observed by inverted phase contrast microscopy and IRM over a period of 48 hours. No differences to either

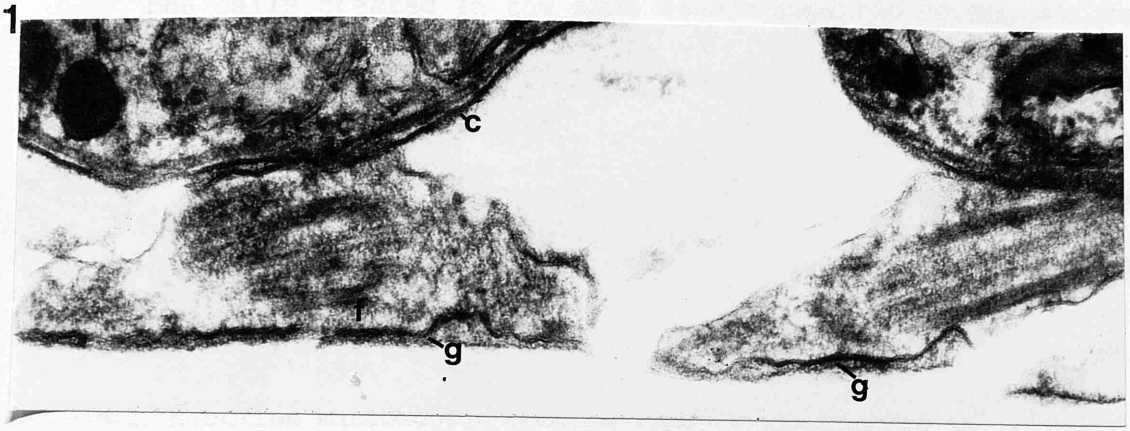
FIGS. 6:11-6:17. Transmission electron micrographs of T.congolense epimastigote attachment sites after growth of cultures in media containing lectins or lectin plus competing sugars or sugar alone for 48 hours.
F-flagellum, PFR-paraflagellar rod, f-attachment associated filaments, g-cell-substratum gap, P-attachment plaques.

FIG. 6:11. Epimastigotes treated with W.G.A. (500μg/ml). The flagellar axis has extended into a foot-like projection. Attachment associated ultrastructure is present in a rudimentary manner where a small cell-substratum gap exists. c-coating on trypanosome.
MAG. 100,800 x.

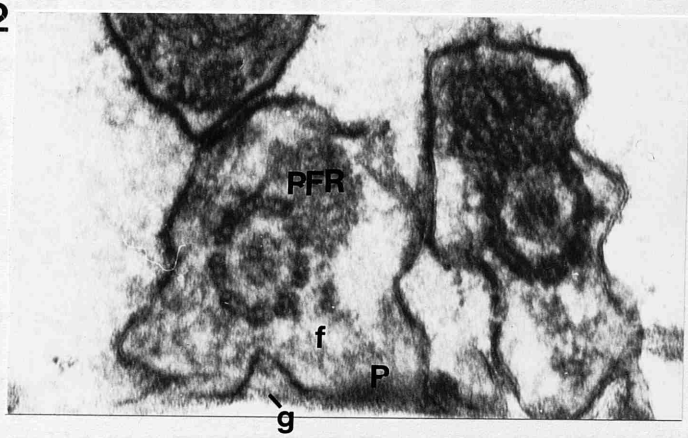
FIG. 6:12. Cross-sections of W.G.A. (500μg/ml) plus N-acetyl-D-glucosamine (0.5M) treated epimastigotes. Attachment associated ultrastructure is present in the flagellum.
MAG. 147,000 x.

FIG.6:13. Oblique section of attachment sites of cultures treated with N-acetyl-D-glucosamine (0.5M).
MAG. 126,000 x.

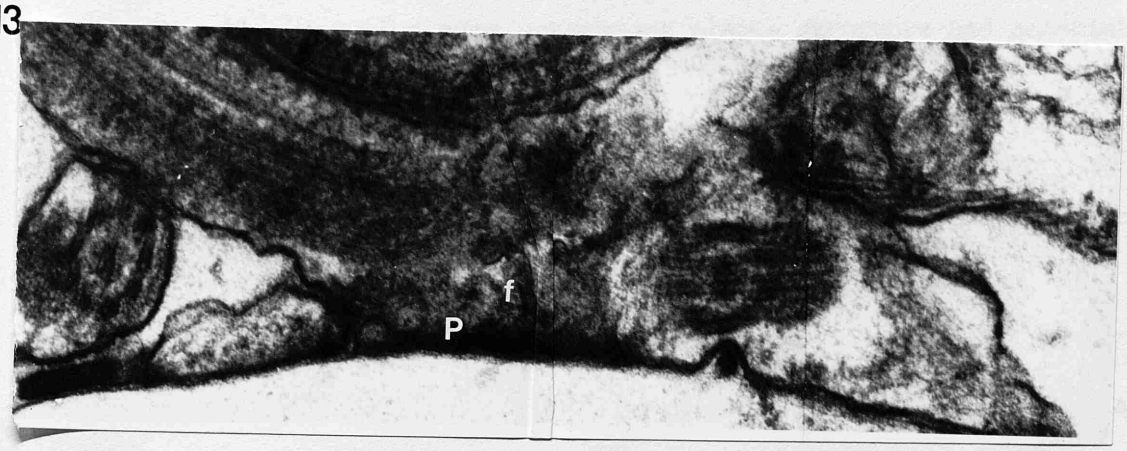
11



12



13



the behaviour, appearance or IRM pattern from those of MEM controls were observed on any T. congolense cultures so treated.

BHK cells treated in the same manner appeared normal for the first few hours, settling, attaching, flattening and becoming confluent, however after approximately 36 hours BHK cells rounded up and detached at all tunicamycin concentrations used. The effect was more pronounced in the cultures containing higher concentrations of tunicamycin.

6.4.2. Electron microscopic observations

Transmission electron micropscopy (TEM) of sectioned T.congolense epimastigotes treated with WGA, Con A and lentil lectin and also the appropriate control sugars showed attached epimastigotes to be present in all cultures. The attachment site morphology in some cases differed from that of control cells.

Cells treated with WGA displayed a range of differences from cells grown in MEM alone. In some cases, attachment sites had an almost normal morphology for those found in a two day culture (Fig. 6.11). The cells had an extended foot-like widening from the flagellar axis and this contained the normal axoneme structures and paraflagellar rod plus some attachment plaque structure and associated filaments. However, these apparently 'normal' attachment complexes contained irregularities; the gap width between cells and substratum was often uneven. In general, dense plaques and their associated filaments were present only where small gap widths were found. At points of a wider gap (>20nm) no attachment complex was present. In other cases there was very little associated area between cell and substratum and no disernible attachment complex ultrastructure was visible - many of the cells appeared to have a surface coating of wispy material (Fig. 6.11). This wispy material appeared concentrated

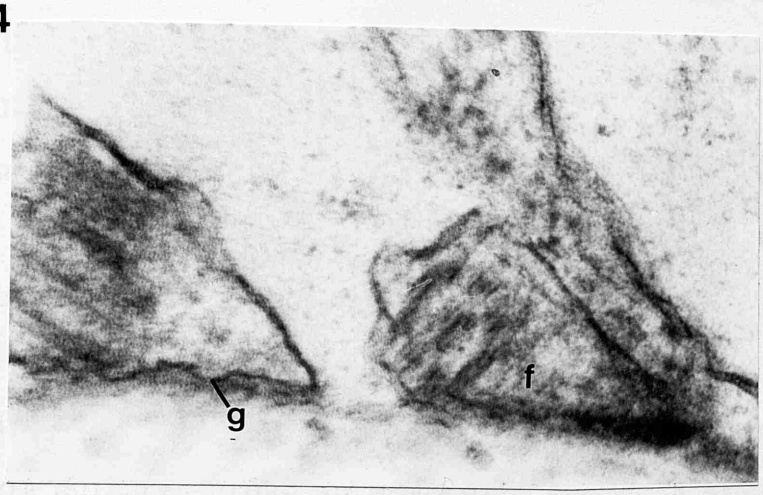
FIG.6:14. Oblique section through epimastigote attachment sites after 48 hours in medium containing Lentil lectin (100 μg/ml). The cell-substratum gap is irregularly wide and attachment associated ultrastructure is rudimentry.

MAG. 135,000 x.

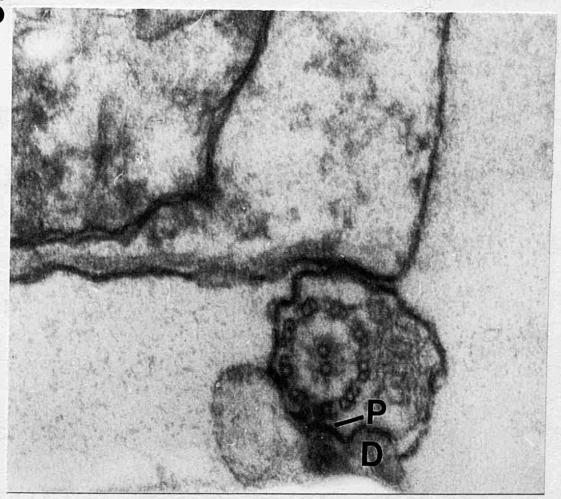
FIG.6:15. Cross-section through an attached flagellum after treatment with Lentil lectin (100 μg/ml) plus α-methyl-D-mannoside (0.5M) for 48 hours. Small attachment plaques are visible where the cell is bound to debris (D) on the substratum.

MAG. 138,000 x.

14



15



within the extracellular space. Some of this amorphous material was also present on the substratum surface when no cells were present, this could be the source of the mottled effect found in IRM studies with this lectin.

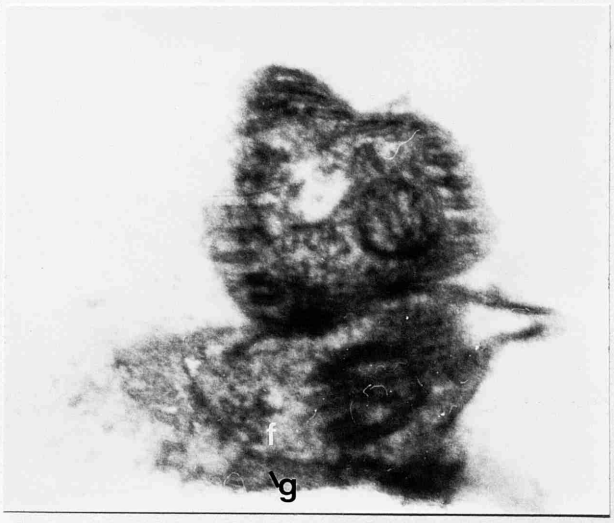
Cells treated with WGA plus N-acetyl-D-glucosamine (Fig. 6.12) contained more cells with recognizable 2 day culture attachment complex morphology than those treated with WGA alone. More filaments emanating from attachment associated electron dense plaques were present. Cells treated with N-acetyl-D-glucosamine (Fig. 6.13) but no lectin displayed attachment ultrastructure morphology indistinguishable from cultures grown in MEM alone. Electron dense attachment plaques and associated filaments were present and a small extracellular gap found between cell and substratum.

T.congolense epimastigote cultures grown in the presence of lentil lectin (Fig. 6.14) like WGA containing cultures, also portrayed a range of attachment ultrastructure morphologies. The majority of attachment sites appeared to have irregular cell-substratum gap widths, again internal ultrastructure at the attachment site was only present when regular, small gap widths close to the substratum were found. Very few electron dense plaques were noticed, filamentous internal structure was however present at a low density in many cases. Cultures containing lentil lectin plus α-methyl-D-mannoside (Fig. 6.15) contained small electron dense attachment plaque structures at points of close attachment. The flagellum in the figure is attaching to debris rather than the coverslip surface but small attachment plaques are visible at the points of close association. Con A treated T.congolense epimastigotes (Fig. 6.16) cultures were found to contain very few attached cells after electron microscopy preparation. The coverslips initially appeared to have similar numbers of trypanosomes as other lectin treated cultures but after processing very few cells

FIG.6:16. Oblique section through an attached epimastigote after 48 hours treatment with Con A (5µg/ml). Rudimentry attachment ultrastructure is present in the form of a few filaments.
MAG. 113,400 x.

FIG.6:17. Longitudinal section through an attached epimastigote after 48 hours treatment with Con A plus α-methyl-D-mannoside (0.5M). Attachment associated ultrastructure is present in the flagellum within a foot-like extension.
MAG. 132,300 x.

16



17

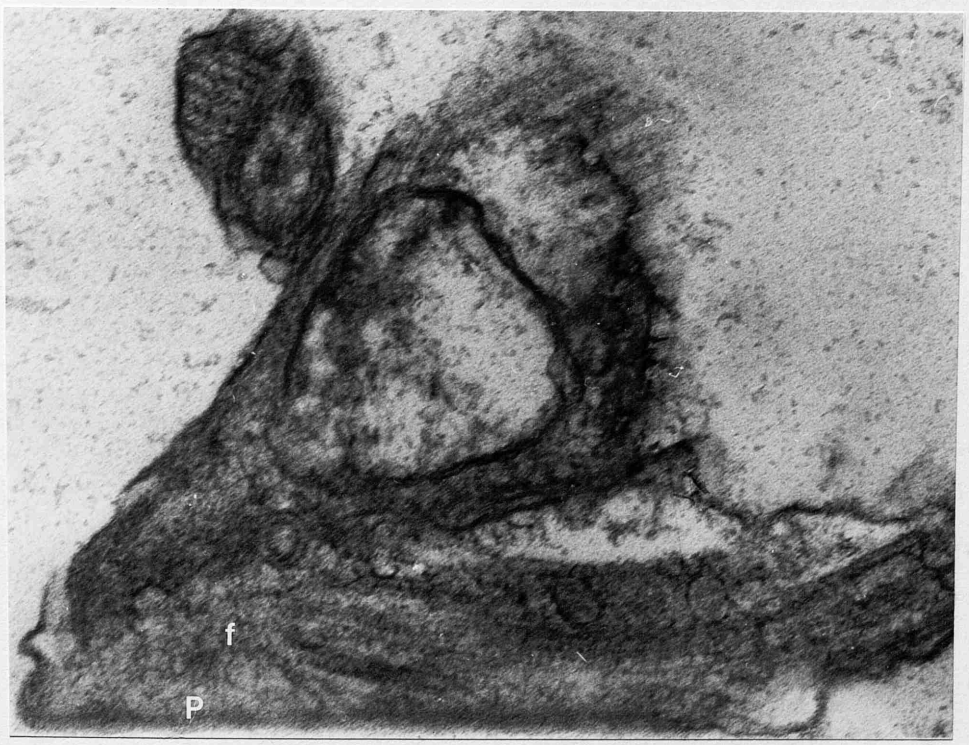
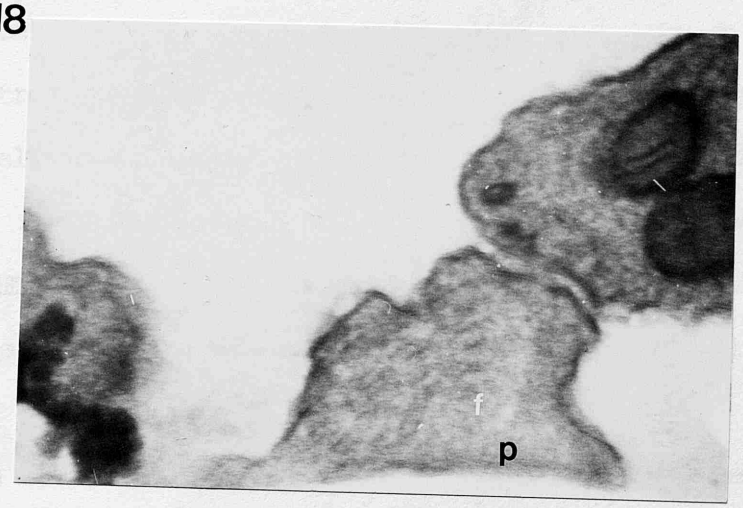


FIG.6:18. Cross-section of a T.congolense epimastigote cultured in the presence of 50µg/ml Tunicamycin for 48 hours. Attachment associated ultrastructure is present in the flagellum.

Labelling as for FIG.6:11.

MAG. 170,100 x.

18



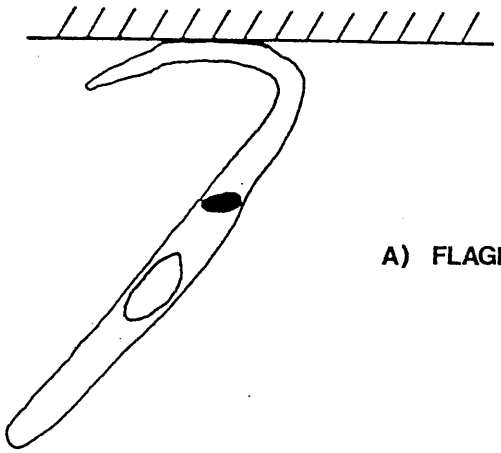
remained. The occasional cells found in TEM sections had a recognizable attachment morphology in that a 'foot like' spreading of the attached flagella had occured. The cells appeared coated by a wispy material which was also present within the cell-substratum gap in greater concentration. This material, however, was not found on the coverslip surface where no cells were present. A larger gap between flagella and subtratum than in control culture cells was present in these attached cells. The internal attachment site ultrastructure appears less pronounced than in control cells although small electron dense plaques and some associated filaments were seen in some cases. In cells treated with α-methyl-D-mannoside plus Con A many more attached cells were found than in those treated with Con A alone. The attachment site morphology (Fig, 6.17) resembled closely that of control, MEM grown, cells and contained the electron dense attachment site plaque, associated filaments and regular, small cell-substratum gap.

Cells treated with tunicamycin for 48 hours and then processed for electron microscopy in general had attachment site morphologies very similar to those of control cells (Fig. 6.18). The cell-substratum gap width was small and regular and electron dense plaques were located on the inner membrane leaflet at the attachment site. Filaments were associated with these attachment plaques and other intraflagellar structures.

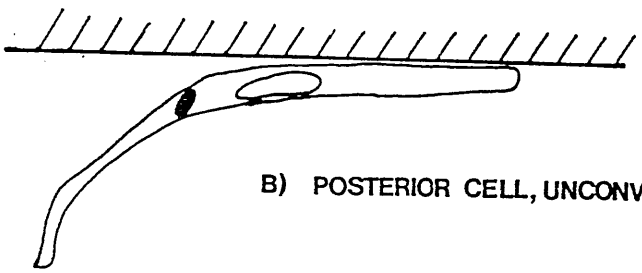
6.4.3. Bead attachment experiments

In the following observations three types of attachment to the beads have been recognized: (1) conventional attachment where the epimastigotes attached by the mid portion of their flagella and could move the flagellum freely in the medium, (2) partial body attachment, an unconventional attachment, where the posterior region of the cell

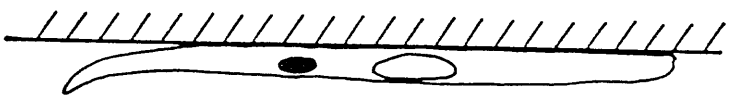
FIG.6:19. Diagram of attachment patterns of epimastigotes to charged Sephadex beads.



A) FLAGELLAR, CONVENTIONAL, ATTACHMENT



B) POSTERIOR CELL, UNCONVENTIONAL, ATTACHMENT



C) WHOLE CELL & FLAGELLUM IMMOBILIZATION

was attached and the anterior end and flagellum could move freely and (3) whole body and flagellar attachment where the entire cell was immobilized and attached to the substratum. Occasionally the very anterior tip of the flagellum could move under these immobilization conditions but no other area (see Fig. 6.19).

6.4.3.1. Sepharose 4B beads: Epimastigotes were observed to attach to these neutral beads by their flagella. Single epimastigotes and bundles of epimastigotes were found attached. No visible preference for the beads or the culture dish base was noted.

6.4.3.2. Concanavalin A - Sepharose 4B beads: Some attachment of epimastigotes to these beads occurred. The majority of attachment was not by the flagellar mode, however. Entire cell bodies and flagella appeared to attach the beads and very little movement of trypanosomes occurred.

6.4.3.3. CM-Sephadex beads: Both molarities of NaCl (70mM and 140mM) gave similar results. Very few trypanosomes were found to attach to these beads by a flagellar mode although a very small number of trypanosomes per experiment did attach in this manner. The majority of trypanosomes in the culture were found attached to the culture dish base or swimming free in the supernatant.

No difference was noted between cultures containing different molarities of sucrose. The vast majority of trypanosomes in the cultures did not associate with the negatively charged beads. Very occasionally flagellar attachment was observed but the majority of epimastigotes either attached to the culture dish base or swam free in the supernatant.

6.4.3.4. DEAE-Sephadex beads: Regardless of whether 70mM or 140mM NaCl was used to swell the beads the same observations were made. In all cases the majority of trypanosomes associated with the beads were immobilized by their whole cell surface. Some immobilized trypanosomes could move the anterior tip of their flagella but only one or two trypanosomes were attached in a flagellar mode. Trypanosomes attached to the culture dish base moved normally in a flagellar mode attachment.

No differences were noted between the cultures of the two sucrose concentrations used. The vast majority of trypanosomes that were associated with these positively charged beads appeared immobilized, some flagellar anterior tip movement was observed in a few trypanosomes but the posterior parts of the cells were immobile. No flagellar mode attachment to beads was noted in any of these cultures. Trypanosomes were attached by their flagella to the culture base and moved normally.

6.5. DISCUSSION

Interference reflection images in these results consistently show the closest cell-substratum gap region to be located in the flagellar region of attached epimastigotes. These black fringed areas are punctate in appearance. They are located within the anterior region of the epimastigote flagellum but not at the tip which moves freely. The dark images appear to be of a greater width than the flagellum shaft and appear to spread from the flagellar axis. The size of the cell-substratum gap deduced from the interference image colour is in the order of 20nm or less. Occasionally thin processes emanating from the flagella can be visualized. When bundles of epimastigotes are observed using IRM, the black, closely opposed cell-glass area is large and appears composed of parts of the flagella of the whole bundle of

cells. All these observations would suggest that these black fringe areas are the IRM visualization of the flagellar attachment complex area described in Chapters 3 and 4. The punctate nature of the attachment sites can be observed in SEM micrographs as can 'filopodia' and mass attachment areas composed of the intertwining flagella and filopodia of all the epimastigotes within the bundle. The location of attachment sites on the anterior portion of the flagella but not at the anterior tip can also be noted in SEM micrographs. The gap size found in the close attachment areas by IRM is in the same order as those measured in TEM micrographs in vitro (Section 4) and in vivo (Section 3 and Vickerman, 1973 for T.vivax). The foot-like spreading outwards from the flagellum trunk at the attachment points can be observed in TEM and SEM micrographs. Bearing in mind all these corresponding points I conclude that the IRM black image areas of close oppostion between cell and glass represent the flagellar attachment areas and therefore, this method of light microscopy can be utilized as a method of monitoring the properties of the attachment of living trypanosomes. The effects of substances added to the basic culture medium on the attachment of T.congolense epimastigotes can therefore be studied. The degree of disruption caused by the added substances has been estimated by the closeness of the flagellar attachment site after treatment.

The presence of serum in the medium does not appear to be necessary for the formation of the attachment complex. The black, close attachment pattern is still found in cells grown in the absence of FCS. Serum may play a part in the attachment however, as the extent of the attached, close apposition area is larger in cells containing either 10 or 20% FCS in their growth medium but serum does not appear to be essential for attachment to occur. In some mammalian cells grown in culture, serum components have been found to be deleterious to cell

attachment and some components can be inhibitory to the process (Curtis and Forrester, 1984). Thus serum is not an essential constituent of medium for *in vitro* grown cell attachment in general. The action of trypsin on the attachment site would suggest that there is a proteinaceous element within the attachment site extracellular gap. Not all this protein can be of an extraneous source as the gap size increase following trypsinization also occurs in cells grown in the absence of serum. The effect noted did not appear to be a secondary consequence of general cell damage as the trypanosome appearance and behaviour seemed normal. Trypsinization of cells has been noted previously to increase the extracellular gap size in cultured mammalian cells. Curtis (1964) also monitored this by IRM. Trypsinization is used as a routine procedure for detaching culture cells from the substratum (Badley *et al.*, 1978) thus epimastigote attachment appears to share this aspect with cells attached by focal adhesions. Protein cannot be the only component of epimastigote attachment, however as trypsinization was not found to be a useful method of detaching epimastigotes (Chapter 4) some material was found to be remaining on the coverslip after trypsinization of cells (SEM studies Chapter 4). This may account for the few dark close attachment images found in IRM experiments (Fig. 6.4). Material is also left attached to the substratum after other culture cells have been removed by trypsinization (Badley *et al.*, 1978). Despite this similarity the epimastigote attachment differs from that of mammalian culture cells and free living amoebae in its reaction to increased KCl concentrations. Over the wide range of concentrations used in this study no differences in the IRM image of the epimastigote attachment site were noticed. This contrasts with results of similar experiments performed on *Naegleria* amoebae by Preston and King (1978, a&b). These

workers noted that concentrations of 400μM KCl or greater substantially increased the area of cell which gave a black IRM image of close attachment. In the absence of KCl black images had been confined to small focal contacts 0.4μm in diameter. The increase in area of close attachment was reversible. Further studies by these authors (King et al., 1979a) showed that NaCl, $CaCl_2$ and $MgCl_2$ caused a similar effect to that of KCl but the changes were unrelated to any change in tonicity or refractive index. Preston and King (1984) also reported similar results with Acanthamoeba castellanii and with fibroblast cells (King et al., 1979b) to those obtained with Naegleria. These authors concluded that the observed effects were due to modulation of charge density on opposed like-charged surfaces. Unlike the amoebae, however, the trypanosomes used in the present study were not moving over a negatively charged surface but were stationary. The effects of KCl addition on these organisms are unlikely, therefore, to be similar to those on the motile amoebae. The result of KCl addition to the epimastigotes would suggest that no ionic charge is being modulated within the cell-substratum gap and therefore causing no increase or decrease in gap width. King et al. (1979a) noted that the presence of electrolytes increased the number of amoeba focal contacts. This does not appear to be the case for T.congolense but cannot be directly measured as the number of IRM contact images differed between cells in this organism.

The lack of effect on the epimastigote attachment might be related to their natural environment in the insect mouthparts. The composition of tsetse fly saliva is unknown but that of other Diptera e.g. Calliphora (reviewed by House & Ginsborg, 1985) contains a large amount of ions and, the salivary gland epithelium has ion pump properties. If this is also true of Glossina saliva then it is perhaps not surprising that epimastigote attachment is maintained in an ionic

environment.

The involvement of ionic charge in epimastigote attachment was further investigated in this study by the use of charged sephadex beads. Total epimastigote immobilization by positively charged (DEAE) beads would indicate that T.congolense epimastigotes have a net negative surface charge as has previously been reported by Hollingshead et al. (1963) for bloodstream forms of T.congolense and by De Souza et al. (1977) for epimastigotes of T.cruzi. The observation in the present study that the anterior tip of the flagellum was, unlike the rest of the cell, freely mobile would suggest that this area may not have a net negative surface charge. Due to the cell immobilization it was impossible to tell whether the attachment complex was formed or not on positively charged beads. The fact that a few trypanosomes attached, apparently by the flagellar mode, to negatively charged beads (CM) and thus overcame the like-charge repellent force would lead to speculation that a positive or neutral net charge is located on the cell surface at the point of attachment. Neutral beads readily supported attachment of epimastigotes, thus the lack of trypanosomes attaching to negatively charged beads would not appear to have been due to any effects associated with a curved surface.

The removal of divalent cations with either EDTA or by the use of Ca^{2+} and Mg^{2+} free PBS as a medium, caused the IRM pattern of the attachment area to darken visibly. This effect could be reversed by the readdition of Ca^{2+} ions. The effect was not due to any anomalies of pH on EDTA action as the same effect occurred at pH's ranging from 6 to 9. This noticeable decrease in gap size found in T.congolense is the direct opposite to results found by other workers using a variety of cell types, mammalian and protozoan. King et al. (1979a) using

Naegleria amoebae and similarly Gingell and Vince (1982b) using Dictyostelium amoebae noted that the addition of Ca^{2+} or Mg^{2+} reduced the cell-substratum gap size. The effects of calcium ions on cell attachment in other cells, although not involving IRM studies, are well reported. With reference to focal adhesion in epithelial cells, these structures are disrupted by the removal of calcium with EGTA, subsequent detachment of cells is noted (Kartenbeck et al., 1982). Similarly, desmosomes, also in epithelia and reported in several different cell types, are disrupted by calcium ion removal, the plaque structure detaching from the membrane and the cells detaching from each other (Borynshenko & Revel, 1973; Kartenbeck et al.. 1982, Hennings & Holbrook, 1983; Watt et al., 1984; Mattey & Garrod, 1986,a&b). Hemidesmosome formation is also dependent on calcium concentration, the number of hemidesmosomes increasing in proportion to the calcium concentration. However, after a period in low calcium hemidesmosomal formation occurs at a faster rate than in control media. This calcium dependent hemidesmosome formation appears to be mediated by a calmodium regulated mechanism (Trinkhaus-Randall & Gipson, 1984). These morphologically similar structures to the trypanosome attachment site therefore display the opposite reactions to that of calcium removal in trypanosomes. This result is difficult to explain. The effects noted by Trinkhaus-Randall & Gipson (1984), of latent increase in hemidesmosome formation in low calcium cannot be related to trypanosome attachment either as the effect in T.congolense was the same within minutes after being placed in the experimental medium or 48 hours later.

The trypanosome reaction could, of course, be a peculiarity related to the natural life cycle of trypanosomes. The epimastigotes attach in the labral canal and hypopharyngeal region of the tsetse fly proboscis, and thus must be able to withstand the flow of saliva

moving down the hypopharynx. As tsetse saliva must contain anti-coagulant factors, which often work by chelation, epimastigotes must be naturally able to remain attached in a solution containing divalent ion chelators, therefore a stronger attachment occurring in the presence of such substances would be advantageous for epimastigote survival.

Initial studies with ruthenium red (reported in Chapter 4) indicated that carbohydrate groups could be present within the extracellular gap at the attachment site. These compounds were detected in the absence of serum. The lectin studies in the experiments reported here confirmed the presence of carbohydrate groups at the cell surface at the attachment site. No fucose or D-galactosamine binding groups were located on the epimastigotes. Similar findings for T.congolense procyclics were reported by Jackson et al. (1978). Potato lectin did not cause cell agglutination even at a high lectin concentration, but the IRM pattern of epimastigote attachment with this lectin did differ slightly from control cells. Possibly, therefore, some (N-acetyl-D-glucosamine)$_3$ residues may be present at this site. Wheat germ agglutinin also increases the gap width at the attachment site. This lectin, however, causes some agglutination of epimastigotes. Agglutination of the entire cell surface would again suggest that N-acetyl-D-glucosamine or N-acetyl neuraminic acid residues are present all over the cell surface including the attachment area. The inhibition of agglutination and the presence of a black IRM pattern at the attachment site in N-acetyl-D-glucosamine containing control cultures would suggest that this is the residue being bound by WGA on the trypanosomes. The irregular width of attachment site-substratum gap in electron micrographs of the attached cells in the presence of WGA compared with the control cells also

suggests that WGA binds sugar residues on the surface of the cells at the attachment area. WGA has been previously reported to agglutinate both procyclic and bloodstream forms of T.congolense (Jackson et al., 1978) and epimastigotes of other trypanosome species (T.lewisi, Dwyer, 1976; T.cruzi, Vivas and Urbina, 1982; Schottelius, 1976; Pereira et al., 1980). It would appear therefore that N-acetyl-D-glucosamine residues or N-acetyl neuraminic acid residues are also present on the surface of the epimastigote stage of T.congolense. The fact that treatment with potato lectin resulted in a similar effect on T.congolense epimastigote attachment would suggest that there are N-acetyl-D-glucosamine residues present on these cells, particularly as WGA caused cell agglutination that could be inhibited by the addition of N-acetyl-D-glucosamine. These observations do not exclude the possible presence of N-acetyl neuraminic acid also, these residues were the sole receptors for this lectin on T.cruzi epimastigotes (Pereira et al., 1980) and are generally found on the surface of all eukaryotic cells being responsible for the negative surface charge. As epimastigotes were demonstrated to have a negative surface charge in the present study, it is plausible that N-acetyl neuraminic acid residues are also present on epimastigote surfaces and WGA is binding to them also. However, if this carbohydrate residue is the source of the cell's negative charge then negatively charged groups should also be present on the cell surface in the extracellular gap. This hypothesis is difficult to reconcile with the observed effect of KCl addition or the occasional observation of epimastigotes binding to negatively charged sephadex beads. This may suggest that N-acetyl-D-glucosamine residues are present in greater amount than N-acetyl neuraminic acid at the attachment site region of the cell surface. This speculation could be supported by the observations of free movement at the flagellar tip whilst the rest of the epimastigote cells were

immobilized by positively charged beads.

The other lectins that affect the IRM pattern of attached trypanosomes in this study were D-mannose binding lectins, namely Con A and lentil lectin. As with WGA these lectins are known to bind the entire surface of T.congolense procyclic and bloodstream forms (Jackson et al., 1978, Rautenberg et al., 1980). In the present study all combinations of flagella-somatic agglutination patterns were noted and Con A coated beads immobilized the cells. These observations would imply that T.congolense epimastigotes contain D-mannose residues over their entire cell surface including the surface of the flagellar attachment site. The paucity of epimastigotes found after electron-microscopy processing of Con A containing cultures and the disrupted attachment morphology found in electron micrographs of Con A or lentil lectin treated cells supported this suggestion. Numerous reports of Con A agglutination of all stages of the lifecycle in many trypanosomatid species have been previously reported (these include Jackson et al., 1978; Pereira et al., 1980; Vivas & Urbana 1982; Dwyer, 1974 & 1976, Muhlpfordt & Schottelius 1977; Schottelius, 1976). D-mannose residues are common on the surface of many cell types. These residues are also present on mammalian cells and on the surface of cells at attachment points - demonstrated in the present study by the disrupting of BHK cell spreading in the presence of Con A and lentil lectin.

Cell surface sugars may have a possible role in cellular recognition. Glycoproteins have been implicated in cell adhesion for many years (Moscona, 1974; Frazier & Glaser, 1979). Overton (1982) demonstrated that desmosome formation could be inhibited by lectin binding of surface glycoproteins supporting both a recognition and adhesive function for cell surface carbohydrates in cell attachment areas. The disrupted and abnormal attachment formation found in the

present study may indicate that glycoproteins are playing a recognition and adhesive role in T.congolense also. The tunicamycin experiments performed on T.congolense epimastigotes imply, however, that the turnover of glycoproteins is low at the attachment site. At concentrations up to 500 times greater than those required to disrupt vertebrate cell attachment no effect was noted on epimastigote attachment. TEMs of these cells also intimate this conclusion. Overton (1982) reported the disruption of mammalian desmosome attachment with a very low level of tunicamycin. Tunicamycin acts as an inhibitor in the synthesis of an intermediate required in formation of N-glycoside linked oligosaccharides (Tkacz & Lampen, 1975) including D-mannose. Thus if the sugar moieties present at the attachment site surface of T.congolense epimastigotes are involved in the cell adhesion they are not being synthesized either at the time of attachment nor up to 48 hours afterwards. The reports of specific binding of T.cruzi trypanosomes to lectin-like molecules within the digestive tract of their Triatomine hosts (Pereira et al., 1981) and the possible involvement of a specific, 72 kilodalton, glycoprotein binding with a lectin-like receptor to trigger differentiation in this species (Sher & Snary, 1982) point to a role for glycoprotein in trypanosome attachment. However, no attachment site specific lectins for T.congolense epimastigotes have been noted in this present study.

In summarizing the findings of the present study, the extracellular attachment site components would appear to include (a) a proteinacious component, some of this at least of trypanosome origin (b) a carbohydrate component, in particular D-mannose and N-acetyl-D-glucosamine and possibly N-acetyl neuraminic acid residues. These are present in the area, as well as the rest of the cell, however no turnover of glycoprotein appears to be occurring (c) ionic charge does not appear to play any role in the attachment process. The

epimastigotes display a net negative surface charge but the anterior portion of the flagellum may have neutral or positive charges predominating. (d) divalent cations, in particular calcium, appear to affect the attachment in an anomalous manner, the removal of these ions would appear to decrease the extracellular gap width and by implication strengthen the attachment rather than disrupt the attachment as occurs in other cell types.

All the components involved would appear to be derived from the cells themselves, very little components of the attachment complex can be extraneous in origin. The non-specific attachment of T.congolense epimastigotes to plastic, glass, nitrocellulose, millipore filters (unreported data), sepharose and other surfaces as well as the interior of the tsetse proboscis would suggest there is no specific receptor site on the substratum that the flagella surface recognizes (cf. T.vivax Hirumi et al., 1982; ILRAD, 1984; Fish et al., 1987). This may be the reason that no apparent attachment specific carbohydrate group was found in this study - no specific group is to be recognized by the cell! Amastigotes and promastigotes of Leishmania mexicana and L.braziliensis and trypomastigotes but not epimastigotes of T.cruzi have been shown by Bretana et al. (1986) to contain a laminin like protein of protozoan origin on their surface. This substance is postulated to be involved in permitting interaction of parasite and host cells. Ouaissi et al. (1984) reported T.cruzi trypomastigotes contained fibronectin receptors, also possibly playing a role in host cell invasion. The presence of these proteins, known to play a role in vertebrate cell adhesion to basal lamina (Timpl et al., 1979) suggest possible similar attachment mechanisms can operate in trypanosomatid attachment. However the flagellate stages displaying these proteins or their receptors do not include the attached,

epimastigote stage; indeed, the laminin-like protein was not detected in epimastigotes of T.cruzi, only in the stages penetrating mammalian cells. As the epimastigote attachment of many trypanosome species is not to cellular material, proteins normally involved in metazoan cell attachment will probably not be present.

6.6. SUMMARY

Reflection interferometry shows the close apposition of flagellum and substratum at the attachment site of living T.congolense epimastigotes. The gap between flagellum and substratum at these positions is~20nm in normal culture medium. The presence or absence of serum in the culture medium has very little effect on the gap width, however, the addition of trypsin (250µg/ml), lentil lectin (50µg/ml), WGA (50µg/ml), ConA (5µg/ml) or potato lectin (50µg/ml) to the medium increases the size of the extracellular gap without any apparent effect on the epimastigotes. These observations indicate that proteins, D-mannose and N-acetyl-D-glucosamine, are present in an external position at the flagellar attachment site. Tunicamycin (at a concentration 500x greater than affects mammalian culture cell attachment) does not affect the trypanosome attachment suggesting no glycoprotein turnover occurs at the site. The addition of monovalent ions does not affect the gap width but divalent cation removal reversibly decreases the cell-glass attachment gap.

The ability of epimastigotes to attach to both positively and negatively charged sephadex beads also demonstrates the non-involvement of ionic charge in the flagellar attachment. The net negative surface charge on the epimastigotes may be reduced in the region of the attachment; the tip of the flagellum can move freely despite the immobilization of epimastigotes by positively charged beads.

CHAPTER 7

ATTEMPTS TO CHARACTERISE INTERNAL COMPONENTS OF THE ATTACHMENT SITE

7.1. INTRODUCTION

The research described in this chapter attempts to discover if the trypanosome attachment site components are novel or related to other, metazoan, cell attachment components. The techniques used have involved immunocytochemistry at light and electron microscope level, protein separation by one and two dimensional gel electrophoresis and associated "western" blotting using antibodies to known cytoskeletal and attachment proteins.

7.2. MATERIALS AND METHODS

7.2.1 Trypanosomes and BHK cells

Trypanosoma congolense epimastigotes, stocks TREU 1457 and TREU 1627 were used in the following study. Experimental cultures were grown on coverslips as described in Section 6.2.1; glass for light microscopy and as a source of material for electrophoresis samples and Thermanox coverslips or on electron microscope grids (gold or nickel, Balzers) (Section 4.2) for electron microscopy.

BHK cells (Flow labs) were used as control cells in many of the following experiments and were maintained as described in Chapter 6.

7.2.2. Fluorescence microscopy

Cells grown on glass coverslips were rinsed in PBS and then fixed and permeabilized using the following procedure (adapted from DeSouza et al., 1983). Cells were fixed in freshly prepared 4% paraformaldehyde in 0.1M phosphate buffer pH7.2 for 10 minutes, rinsed in PBS, then placed in 0.05M NH_4Cl in PBS to quench free aldehyde groups. The cell bearing coverslips were then placed in 0.1% Nonidet P40 in PBS for 2 minutes, rinsed thoroughly in PBS and then treated with the primary antibody diluted in PBS (Table 7.1) or undiluted if a hybridoma supernatant was used. Primary antibody treatment was carried out for 30-60 minutes at room temperature in a humid chamber. Cells

TABLE 7:1. DILUTIONS OF PRIMARY ANTIBODIES USED FOR IMMUNOFLUORESCENCE

ANTIBODY NAME	PROTEIN DETECTED	FORM OF ANTIBODY	DILUTION	SOURCE
KMX-1	Beta-tubulin	Monoclonal Ascites	1:1000	Prof. K. Gull Kent Univ.
Anti-actin	Actin (F and G)	"	1:50 or <	Amersham Int. plc., Amersham.
Anti-IFA (Pruss)	All intermediate filaments	Monoclonal hybridoma supernatant	Neat	S. McIver, Univ. Coll., London.
LP34	Many Keratins	"	"	Dr.E.B. Lane ICRF, London.
LP1K	Type III Keratins	"	"	"
LE41	Keratin 8	"	"	"
LE61	Prekeratin 18	"	"	"
LE64	Prekeratin 19	"	"	"
LE65	Prekeratin 18	"	"	"
LP2K	Keratin 19	"	"	"
Anti-filamin	Filamin	Monoclonal ascites	1:50 or <	Amersham Int. plc., Amersham.
Anti-vinculin	Vinculin	"	"	"
Anti-desmoplakins I&II	Desmoplakins I&II	"	1:5 or <	ICN Biomedicals Ltd., Bucks.
G3C2	Dot pattern in T.brucei flagella	Monoclonal hybridoma supernatant	Neat	T. Sherwin, Kent Univ.
GUPM 23.1 (control)	T.brucei VSG	Monoclonal ascites	1:200	Protozoology, Glasgow Univ.
GUPM 23.2 (control)	"	"	"	"

were then rinsed thouroughly in PBS (3 x 10minutes) and the second-FITC-conjugated antibody applied. As all antibodies used were mouse monoclonal antibodies the second antibody was an FITC-conjugated anti-mouse gamma globulin (Sigma) diluted 1:50 in PBS plus 0.001%(w/v) propidium iodide (Sigma). Second antibody treatment was carried out for 30-60 minutes in a humid chamber at room temperature. Following this the coverslips were rinsed in PBS (3 x 10minutes) and mounted in PBS : glycerol (Merck, 1:1 dilution).

Fluorescence microscopy was carried out using a Leitz Ortholux II microscope using incident light fluorescence from an HBO 50 high-pressure mercury vapour lamp. 2 x 1 CP490 (exciting), TK 510 (dichroic mirror) and K515 (suppressing) filters were used. Photographs were taken using Kodak Ektachrome 200ASA film, Kodak Tripan X 400ASA film or Ilford XPI 400ASA film. In some cases fluorescence was improved by using acetone fixation. When acetone was used as a fixing and permeabilizing agent it was used at -20^{o}C. The epimastigote bearing coverslips were rinsed in PBS and then plunged directly into the cold acetone for 30 seconds, the coverslips were rinsed in PBS and then primary antibody treatment carried out.

The primary antibodies (all monoclonal antibodies) tested were as listed in Table 7.1. NBD-phallacidin (molecular probes, Junction City) was also used for actin detection. NBD-phallacidin is supplied dissolved in methanol. The methanol was evaporated off and PBS added (a volume equivalent to 3 volumes of the methanol solution) before use. Trypanosomes were fixed and permeabilized as described above and the NBD-phallacidin added in place of the primary antibody. Treatment was carried out at 37^{o}C in a humid chamber for 45-60minutes, coverslips were then rinsed in PBS (3 x 10minutes) and then mounted in PBS:glycerol on glass slides.

Anti-tubulin antibody (KMX-1) was used as a positive control for permeabilization and fixation of cells and antibody binding. PBS was used in place of both the primary and secondary antibodies as a control for autofluorescence, the FITC conjugated anti-mouse gamma globulin (Sigma) used alone was used to test for background fluorescence, Anti-T.brucei VSG monoclonal antibodies 23.1 and 23.2 were used as background controls also. BHK cells were used as positive control cells for cytoskeletal antibodies.

7.2.3. Indirect immuno-histochemistry for the electron microscope

7.2.3.1. Post embedding immunogold labelling: Epimastigote cultures were grown on Thermanox culture plastic coverslips. These coverslips were rinsed in MEM (incomplete) and then fixed in 0.25% glutaraldehyde in 0.1M phosphate buffer for 30minutes or in a mixed fixative of 2% paraformaldehyde and 0.25% glutaraldehyde for 30min. Cells were rinsed in 0.1M phosphate buffer (3x10min) and then treated with 0.05M NH_4Cl in phosphate buffer for 15min. Coverslips were again rinsed in phosphate buffer. All these procedures were carried out at room temperature. Cells were dehydrated in progressively colder alcohol:water mixtures until absolute alcohol at -20°C--35°C. Infiltration of resin was carried out at -20°C--35°C also. Lowycril K4M resin was used as this resin polymerizes in ulraviolet irradiation at -20°C and because of this has a better chance of preserving antigenic sites in a natural state than resins which polymerize at high temperatures. Lowycril K4M: alcohol mixtures were used (1:2 then 2:1) to infiltrate the resin into the cells (1h each mixture) followed by Lowycril K4M for 2 hours. Embedding proceedures were then carried out. Fresh Lowycril K4M was placed in small clear plastic trays (Sterilin lymphocyte migration chambers) and the coverslips, cell side down, placed over the resin, the resin was then polymerized at -20°C

under U.V. light for 24h. At this time the coverslips were removed and the surface of the resin containing the cells re-embedded in fresh Lowycril K4M which was then further polymerized under U.V. irradiation at 20°C for 24h. 24h of U.V. polymerization at room temperature followed this second -20°C polymerization period.

Silver-gold thin sections of these embedded cells were cut using glass knives on a LKB I ultramicrotome. These sections were collected on Formavar coated EM grids.

Grids containing sections were placed on top of 10μl drops of TTGO buffer [20mM Tris pH8.2, 0.01% Tween 20(Sigma), 0.1% gelatin (Sigma, 60 bloom), 1% Ovalbumin (Sigma)] and 5% normal BALB/c mouse serum for 15mins. The grids were then placed on drops containing the primary antibody diluted in TTGO buffer plus 1% normal BALB/c mouse serum. Incubations were carried out for 45-90mins at room temperature in a humid chamber. The grids were then rinsed in several changes of TTGO buffer over a half hour period and then floated on drops containing the gold conjugated second antibody diluted in TTGO buffer plus 5% normal goat serum, for 30-60mins at room temperature in a humid atmosphere. The grids were then rinsed by floating them on drops of TTGO buffer with several changes over 45mins, followed by two 10min changes in distilled water. Sections were then stained with 0.5% (w/v) aqueous, uranyl acetate for 5 minutes, rinsed in water and dried. Grids were observed in an AEI 801 electron microscope operated at 60 KV and photographed on Ilford EM4 film.

The primary antibodies used were as follows: Anti-actin (Amersham) monoclonal antibody ascites used at various dilutions ranging from 1/50-1/1000, the second antibody used for detection of this primary antibody was goat anti-mouse IgM conjugated to 5nm gold particles (EM grade) (Janssen Lifescience). Monoclonal anti-tubulin (KMX-1) diluted 1/1000 in buffer, monoclonal anti-filamin (ascites)

(Amersham) diluted 1/200 in buffer, monoclonal anti-vinculin (ascites) (Amersham) diluted 1/22 in buffer and anti-intermediate filaments (Pruss) (hybridoma supernatant) used neat. The second antibody used to detect these primary antibodies was goat anti-mouse IgG conjugated to 5nm gold particles (EM grade) (Janssen Life Sciences). The second antibodies were used at various dilutions from 1/10-1/250. Appropriate controls were carried out in parallel.

7.2.3.2. Pre-embedding labelling: Epimastigotes of T.congolense TREU 1627 grown on Thermanox coverslips were rinsed in MEM and then PBS. The cells were permeabilized with a 0.1% Triton-X 100 in PBS for 30 seconds and then fixed in 0.5% glutaraldehyde in 0.1M phosphate buffer for 10mins, further permeabilized in 0.1% or 0.5% Triton-X 100 in PBS for 10mins and then treated with 0.1M NH_4Cl in PBS for 10mins. Coverslips were then placed in TTGO buffer containing 5% normal mouse serum for 20mins. Primary antibody application was then undertaken. Anti-actin (Amersham) ascites diluted 1:100 in TTGO buffer containing 1% mouse serum was the antibody used and an anti-T.brucei VSG monoclonal ascites (23.1) used as a control, buffer containing serum alone was also used as a control. Antibody treatment was carried out for 1h at room temperature in a humid chamber. Coverslips were then rinsed in TTGO buffer plus 1% normal mouse serum for 10mins followed by several changes of TTGO buffer over 1h. Cells were then treated with the gold conjugated second antibody (GAMIGMEM 5, Janssen Life Sciences) diluted 1/25 in TTGO buffer for 1h, then rinsed in several changes TTGO buffer for 1h. Cells were then fixed in 1% glutaraldehyde in 0.1M phosphate buffer for 45mins, block stained with 0.5% (w/v) aqueous, uranyl acetate for 30mins, dehydrated in an alcohol series over 35mins double embedded in Araldite and processed as described in Section 4.2.2.

7.2.4. Indirect labelling of anti-actin in extracted whole cell preparations for the electron microscope

EM grids bearing T.congolense epimastigote cultures were rinsed in a cytoskeletal stabilization buffer (S buffer) (0.1M PIPES pH6.9, 1mM EGTA, 1.5mM GTP, 4% PEG 6000) (Webster et al., 1978) then placed in 0.75% Nonidet P40 in S buffer for 2mins, followed by 3 x 1min rinses in S buffer and a rinse in Tris BSA buffer (20mM Tris pH7, 1% BSA) followed by 30mins in 5% normal rabbit serum in Tris BSA buffer. Antibody incubation was carried out for 45mins at 37°C in a humid chamber. The antibody used was an anti-actin antiserum (Miles) diluted 1:5 in Tris BSA buffer. After thorough rinsing the grids were treated with protein A-conjugated to 5nm gold particles (Roth, 1983) diluted in Tris BSA buffer until the solution had only a slight pink hue (approximately =1/100). After thorough rinsing in Tris BSA buffer the cells were rinsed in 0.1M phosphate buffer then fixed for 15mins in 1% glutaraldehyde in phosphate buffer. The grids were block stained in 1% aqueous uranyl acetate (w/v) for 2mins rinsed in water and dehydrated in alcohol. The cells were then critical point-dried from CO_2 and viewed in an AEI 801 transmission EM operated at 60KV. The cytoskeletons were photographed on Ilford EM4 film.

7.2.5. Gel electrophoresis and immuno blotting techniques

7.2.5.1. One dimensional SDS-PAGE separation: T.congolense TREU 1627 were grown on glass coverslips. The coverslips used (12mm diameter circular No1) contained approximately 1 x 10^6 cells when covered completely. Attached epimastigote whole cell preparations were rinsed in PBS containing protease inhibitors (Leupeptin 50μg/ml, Pepstatin 5μg/ml, Chymostatin 5μg/ml). Coverslips were then boiled in SDS reducing sample buffer (62mM Tris-HCl pH6.8, 2%SDS (Sigma), 7.5% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue). The

coverslips were crushed with a glass rod to ensure they were covered by liquid. At least 1 x 10^7 cells were required per preparation, the smallest volume of sample buffer that could be used was chosen. This depended upon the number of coverslips. If no further preparation was to be carried out after the boiling of the cells in the sample buffer, 200-500ul was used.

Whole cell supernatant epimastigote preparations were also made. In 2-4 day cultures of TREU 1627 the predominant morphological forms are epimastigotes (Chapter 2), supernatants from such cultures were collected, centrifuged at 200xg for 10mins, rinsed in PBS plus protease inhibitors, and then boiled in sample buffer (100-200µl).

Cytoskeleton preparations of the above attached and unattached material were also made essentially as described by Schneider et al. (1986); cytoskeletons were prepared by treating cells with 0.5% Triton X 100 in MOPs buffer (10mM MOPs, 1mM EGTA, 1mM MgCl) pH6.9 for 10mins on ice, rinsed in the same solution and then treated as above with sample buffer.

SDS-PAGE was carried out on these treated samples essentially as according to Laemmli (1970). 10% acrylamide slab gels were used. After electrophoresis the proteins were blotted onto nitrocellulose paper (Biorad) using the method of Towbin et al. (1979) as modified by Birkett et al. (1985). Blotting was carried out for 16h using a current of 0.1A. After blotting the nitrocellulose was stained with 0.2% Ponceau S (Sigma) in 3% TCA for assessment of protein transfer. The nitrocellulose, after removal from the blotting apparatus and being cut to the required size was placed in TBS-Tween (Tris-HCl 10mM pH7.4, 140mMNaCl plus 0.1% Tween 20) for 5mins with gentle agitation, this was repeated once more. The first antibody was diluted in TBS-Tween and the nitrocellulose incubated with agitation in the antibody

solution for 45mins at room temperature. Unbound immunoglobulin was removed by washing as follows; 2x5mins in TBS-Tween, 5mins in HST buffer (10mM Tris-HCl pH 7.4, 1MNaCl, 0.5% Tween 20) followed by 2x5mins in TBS-Tween. The second, HRP conjugated antibody (SAPU GAM gamma globulin) was diluted in HST buffer (1:250 or greater) and the nitrocellulose incubated in this solution for 45-90mins with agitation. Unbound conjugate was removed by washing in the following solutions; 3x5mins in TBS-Tween, 10mins in HST buffer, 3x5mins in TBS-Tween, 5mins in TBS. Antibody binding was then visualized by treating the nitrocellulose with a solution of peroxidase containing 4-chloro-1-napthol (0.018% 4-chloro-1-napthol in 6% methanol in TBS plus 0.025ml 40% H_2O_2 per 100ml). The reaction was stopped by rinsing the nitrocellulose in several changes of distilled water. Blots were photographed using Ilford FP4 125ASA plate film.

After detection by HRP conjugated antibodies and photography of the resulting blot, some blots were stained with amido black to display the protein bands present. 0.5% Amido black in a solution of water, acetic acid and methanol (9:1:10) was applied for two minutes followed by destaining in the same solution minus Amido black; the resulting blots were photographed as described above.

The antibodies used to detect possible proteins present were KMX-1 (anti-tubulin) monoclonal ascites diluted 1:1000, anti-actin monoclonal (Amersham) ascites diluted 1:500, anti-intermediate filament (Pruss) hybridoma supernatant used neat or diluted 1:5, anti-desmoplakins I and II (ICN Biomedicals) diluted 1:20, and the anti-T.brucei flagellar 'dot' staining antibody (G3C2) mentioned previously (Table 7.1) hybridoma supernatant used undiluted.

7.2.5.2. Two dimensional gel electrophoresis: Samples of attached and unattached T.congolense TREU 1627 epimastigotes and cytoskeletal

preparations of these were prepared using the following regimes:

a) Whole cell unattached epimastigote preparations were made by rinsing the pelleted cells in PBS plus protease inhibitors. To the washed pellet 50ul of lysis buffer (5mM $MgCl_2$, 20mM Tris pH 7.6, 1% (w/v) Nonidet P40) was added and mixed, then three cycles of rapid freezing and slow thawing followed by vortexing in a whirlimix were carried out. Following this 2.5µl RNAse (1mg/ml, Sigma) and 3.5µl DNAse (2.5mg/ml, Worthingtons) were added and the solution mixed, incubated on ice for 15mins and then 60mg Urea and 100ul solution A [2% (w/v) Nonidet P40, 2% (v/v) Ampholines pH 3.5-10 (LKB), 5% (v/v) 2-merceptoethanol, 9.5 M Urea (Biorad)] were added followed by one cycle of freeze-thaw-whirlimix.

b) Cytoskeletal preparations of unattached epimastigotes were prepared as described in Section 7.2.3 by Triton X extraction in MOPs buffer. The pelleted cytoskeletons were then treated as described above for whole cells.

c) Attached whole cell epimastigote preparations were made as described in Section 7.2.3. The protein components of the boiled sample buffer preparation were then acetone precipitated by adding 9 parts cold acetone to the sample buffer solution and leaving overnight at $-20^{o}C$. The resulting precipitates were pelleted by centrifugation (3000g for 10 min in a Microcentaur (MSE)) and treated as described for the whole cell unattached epimastigote preparation described above.

d) Attached epimastigote cytoskeleton preparations were made as described in Section 7.2.3, acetone precipitated and treated as described above.

The prepared samples were then used for 2 dimensional gel separation essentially using the method of O'Farell (1975) with

adaptions described by Burland et al. (1983). The first dimension separation was by isoelectric focusing in low percentage acrylamide (6.8%) tube gels containing urea and ampholines. 10-30µl of sample were loaded per gel and the gels prefocused at 2mA until 400V was reached. Gels were then electrophoresed at 400V for 16.5h then at 800V for 3h. The upper electrode buffer was composed of 20mM NaCl in boiled and degassed double distilled water. The lower electrode buffer contained 0.1% phosphoric acid in degassed, boiled double distilled water. Tube gels were then removed from the tubes and placed in equilibration buffer (62.5mM Tris pH 6.8, 10% (w/v) glycerol, 5% w/v 2-mercaptoethanol, 2.3% SDS (Sigma) and a few grains bromophenol blue) and left for 30mins. Samples were then electrophoresed in the second dimension, separation this time by molecular weight in 10% acrylamide slab gels using the buffer system of Laemmli (1970). The IEF gels were laid on top of the slab gels and fixed in place with molten 1% agarose (in 1/4 strength upper gel buffer). Once set, the slab gel top and base were covered with running buffer (2.5mM Tris, 192mM glycine, 0.1% SDS, all Sigma products). The current used for this second electrophoresis was 20-30mA per gel, once the dye front reached the bottom of the slab gel (10cm length) the electrophoresis was stopped and the gels removed. The proteins were visualized by silver staining as Coomassie Blue was found to be not sensitive enough.

Silver staining of gels was carried out using the method of Wray et al. (1981). Gels were soaked, with gentle agitation, in 50% methanol for at least 1h. The proteins were then agitated in freshly prepared stain solution (0.8g silver nitrate, dissolved in 4mls water, and added slowly to 21ml of 0.36% NaOH and 1.14ml of 18.1M NH_3OH ensuring thorough mixing, this solution was then made up to 100mls with distilled water) for 15mins. The gels were then washed in

distilled water and developed in freshly prepared developing solution (2.5ml of 1% citric acid plus 0.25ml of 38% formaldehyde made up to 500ml with distilled water). Once protein spots/bands had appeared development was stopped by returning the gels to 50% methanol.

Developed gels were photographed in transmitted light using Ilford FP4 125 ASA plate film.

7.2.5.3. Electron microscopy of attached cytoskeleton material: T.congolense TREU 1627 epimastigotes grown attached to Thermanox coverslips were treated as described in section 7.2.3 for the preparation of attached cytoskeletons; cells were washed in PBS plus protease inhibitors and then extracted with 0.5% Triton X 100 in MOPs buffer pH 6.9. The cytoskeleton bearing coverslips were processed for transmission electron microscopy as described in Chapter 4.

7.2.6. Treatment with Cytochalasins B and D

T.congolense epimastigotes and BHK cells (Flow Labs) were treated with Cytochalasin B or D (Sigma). The Cytochalasins were dissolved in 100% DMSO at a concentration of 1mg/ml. The dissolved Cytochalasin was then added to MEM or MEM plus 10% FCS and then the cells added. The cells were grown in culture plate wells (3.5 or 1.5cm diameter) (Falcon) containing glass or Thermanox coverslips or without coverslips. The cells were observed under phase contrast using an inverted microscope over 48 hours. Cells were exposed to concentrations of Cytochalasin B from 3-50µg/ml and Cytochalasin D from 1-12µg/ml. Control wells containing equivalent amounts of DMSO (minus Cytochalasin) and without DMSO or Cytochalasin were run in parallel to the Cytochalasin wells.

Cells grown on glass coverslips for 24 or 48 hours were fixed in 4% paraformaldehyde as described in Section 7.2.2 and treated with either anti-actin monoclonal followed by a FITC-labelled second

FIG. 7:1. Immunofluorescence using anti-actin Mab (Amersham) as a primary antibody on T.congolense attached epimastigotes.

Acetone fixation.

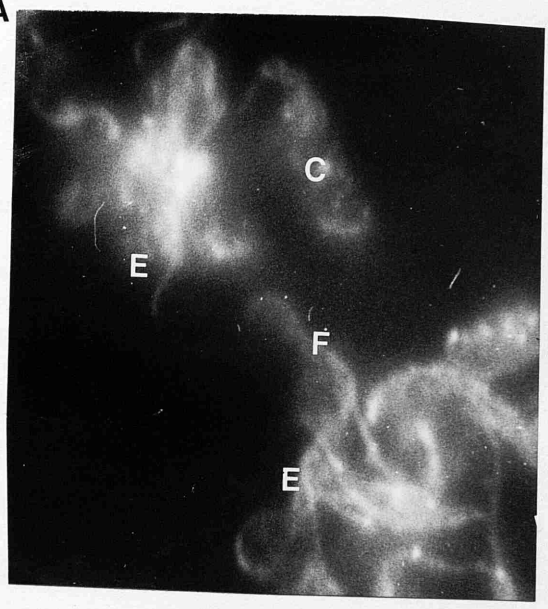
A) Flagellar area in focus B) Cell body area in focus.

E-epimastigote bundle , C-cell body , F-flagellum.

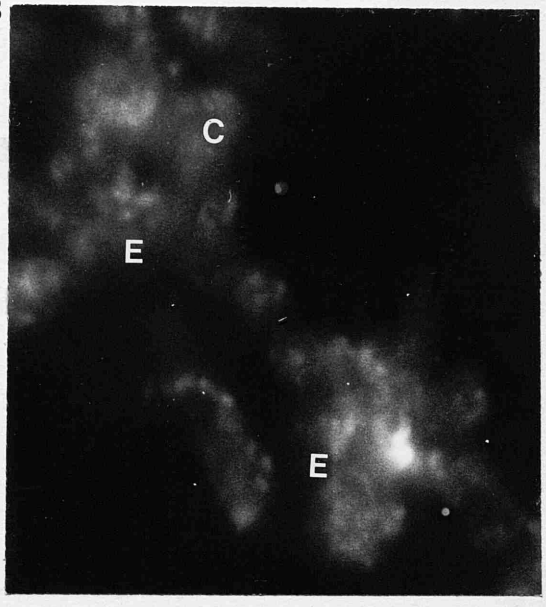
MAG. 960 x.

1

A



B



antibody or NBD-phallacidin and viewed in a fluorescence microscope as described in Section 7.2.2.

Other epimastigote glass coverslips were observed in the interference reflection microscope as described in Chapter 6 and the points of flagellar attachment studied after 24 or 48h in Cytochalasin D or DMSO.

Epimastigotes grown on plastic coverslips and treated in Cytochalasin D were fixed for transmission electron microscopy as described in Chapter 6, DMSO and MEM controls were also fixed for transmission electron microscopy.

7.3. RESULTS

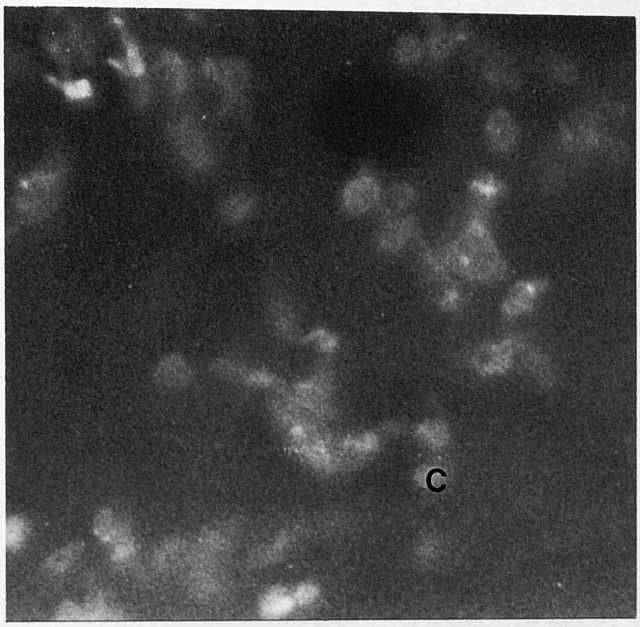
7.3.1. Fluorescence microscopy

Due to the size of the epimastigote attachment associated filaments (5nm in diameter see Chapter 4), the question of whether these were composed of actin was investigated.

Immunofluorescence staining using anti-actin MAbs and also using the F-actin $\overset{\text{binding}}{\rightarrow}$ NBD-phallacidin (Figs. 7.1, 7.2) showed anti-actin fluorescence all over the cell. The fluorescence was faint but above background levels. A low level of fluorescence was found throughout the cell body area and a brighter fluorescence was found in the flagellum. No increase in the brightness of fluorescence was found at the points of flagellar attachment. NBD-phallacidin and antiactin MAb patterns of fluorescence in these cells were similar, thus some of the actin present was probably in the F-actin form.

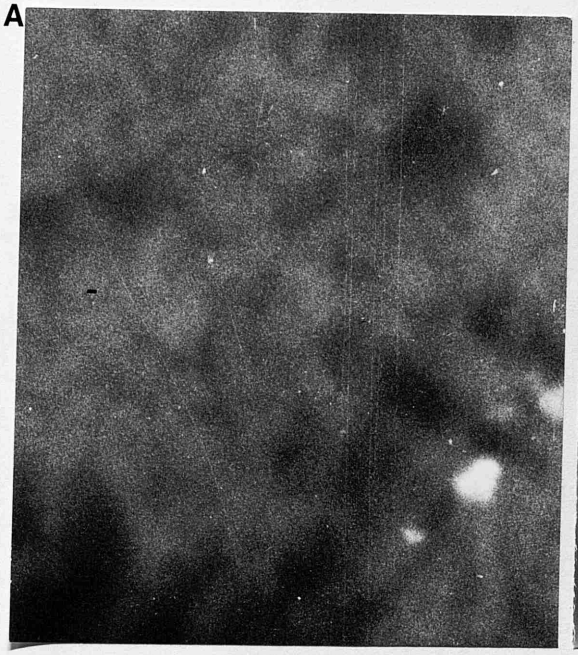
The actin-binding protein vinculin is found in fibroblasts and other moving cell types at the regions of focal adhesions. Anti-vinculin MAb staining in T.congolense epimastigotes varied, in some cases a very faint (but above background fluore scence) was noted all over the attached cells, very similar to the anti-actin fluorescence

2



3

A



B

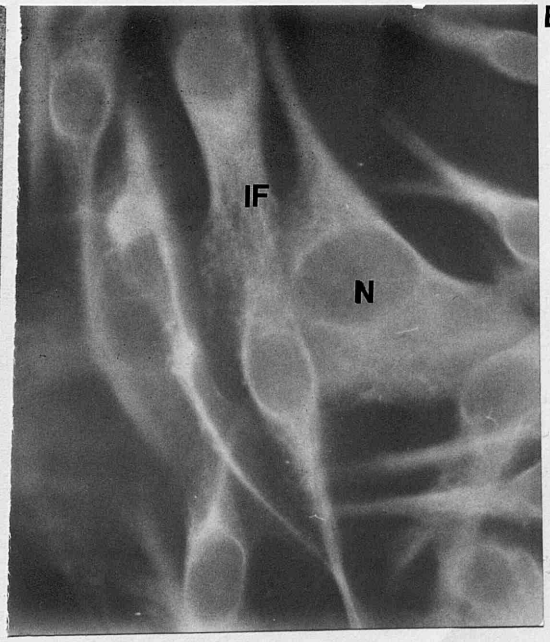


FIG. 7:2. NBD-phallacidin treated T.congolense attached epimastigotes.

Formaldehyde fixation.

Labelling as in 7:1.

MAG. 960 x.

FIG. 7:3. A) Immunofluorescence using anti-intermediate filament Mab (Pruss) as a primary antibody on T.congolense attached epimastigotes.

Labelling as for 7:1.

MAG. 960 x.

B) Attached BHK cells treated with the anti-intermediate filament Mab as a primary antibody.

Formaldhyde fixation.

N-nucleus , IF-intermediate filaments

MAG. 480 x.

FIG. 7:4. T.congolense attached epimastigotes treated with the beta-tubulin specific Mab KMX-1 as a primary antibody.
Formaldehyde fixation.
MAG. 960 x.

FIG. 7:5. Acetone fixed T.congolense attached epimastigotes treated with the G3C2 Mab raised by T.Sherwin.
Labelling as for 7:1.
MAG. 960 x.

4

5

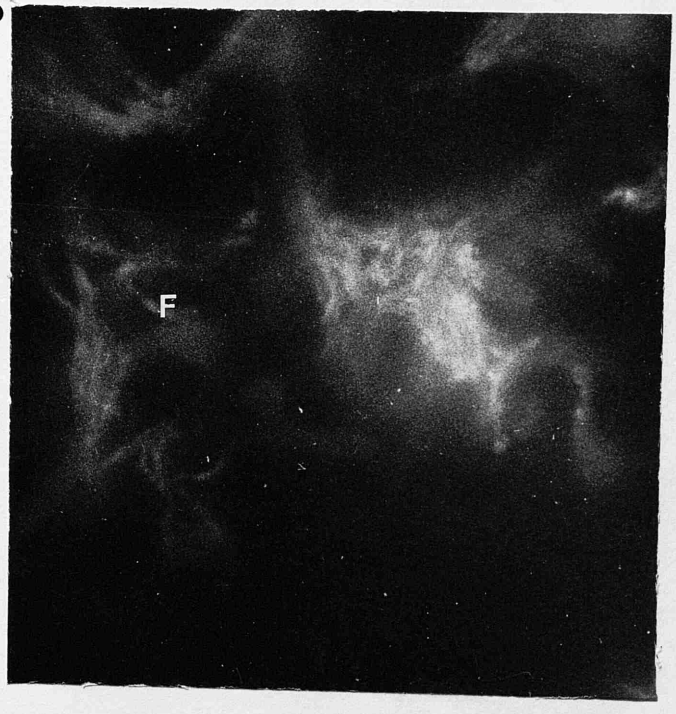
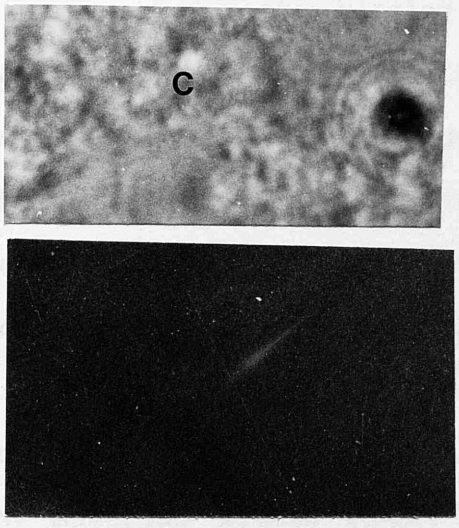


FIG. 7:6. A) Acetone fixed attached T.congolense epimastigotes treated with the, fluorescent (Sheep anti Mouse) second antibody only.

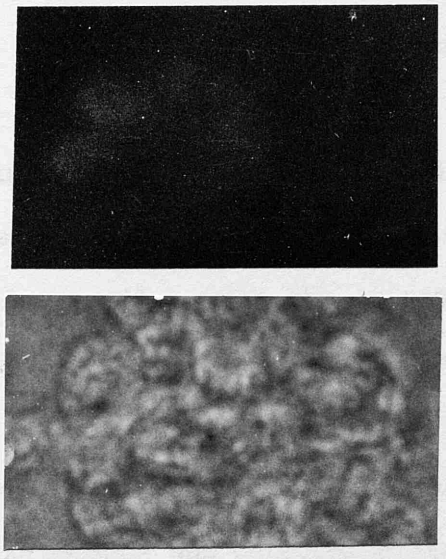
B) Formaldehyde fixed control treated as in A.

6

A



B



distribution; in other cells there was no fluorescence visible at all. There did not appear to be any localization of the fluorescence, when visible, to the attachment area region of the flagellum.

The distribution of the actin binding protein filamin was also examined in T.congolense attached epimastigotes. Negligible positive fluorescence was detected in these epimastigotes. The lack of finding localization of actin and actin binding proteins in the epimastigote attachment area led to testing for the presence of intermediate filaments and desmosome-like attachment proteins. The anti-mammalian keratins antibodies, found by Lane (1982) to bind 'simple epithelia' keratins and prekeratins and be widely species cross reactive, failed to bind to any material within T.congolense epimastigotes. No fluorescence above the background, control fluorescence was noted.

The MAb which binds to all classes of intermediate filament (Pruss et al., 1981) however, did bind to material in T.congolense epimastigotes (Fig. 7.3a). Again no areas of localized fluorescence were found. Faint fluorescence was detected in both flagellar and non-flagellar areas of the cell. The bright, network staining found in BHK cells treated in parallel with the epimastigotes, suggests that the intermediate filament content of the epimastigote cells is low (Fig. 7.3b).

When anti-desmoplakin I & II monoclonal antibodies were applied no positive fluorescence was detected in epimastigotes.

The anti-tubulin MAb (KMX-1) applied to epimastigotes resulted in very bright fluorescence all over the cell, body and flagellar area (Fig. 7.4). In comparison with this tubulin fluorescence, all other positive fluorescence noted in this study were very faint.

The monoclonal antibody raised by T. Sherwin against T.brucei flagellar material (G3C2) and which gives a regular dot staining

FIG. 7:7. Sections of attached T.congolense epimastigotes treated with anti-actin Mab (Amersham) and a gold-conjugated second antibody (SAMGEM5).

Lowycril K4M resin, glutaraldehyde fixation. Gold label is located all over the tissue. High background labelling is also present.

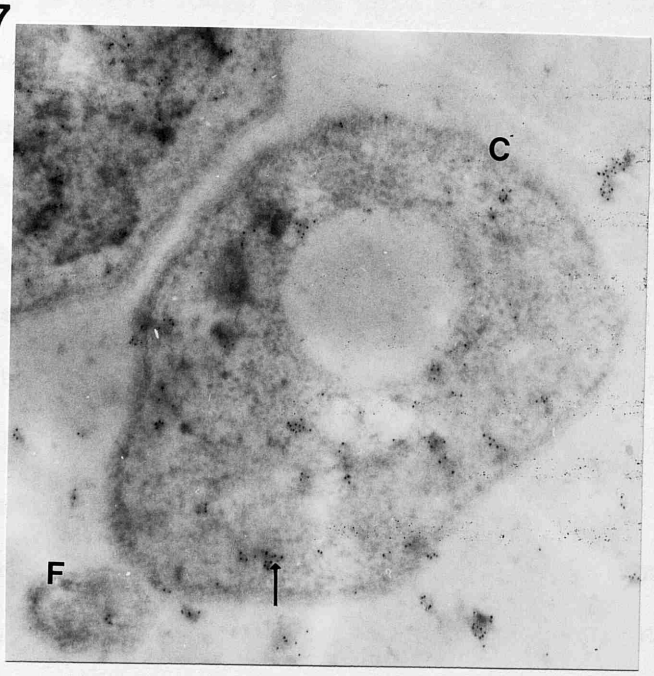
MAG. 75,600 x.

F-flagellum, C-cellbody.

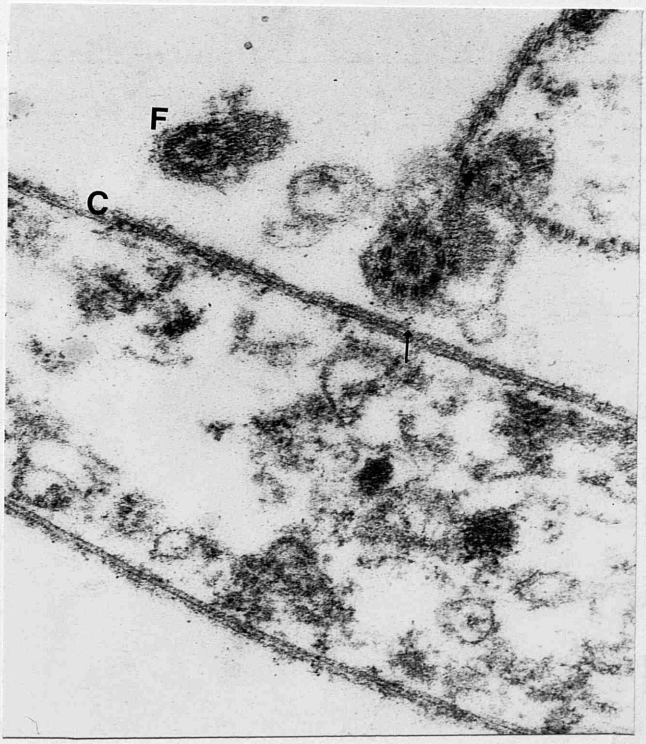
FIG.7:8. Pre-embedding immunolabelling using anti-actin Mab and a gold-conjugated second antibody (as above). Permeabilized cells (0.1%Triton-X 100) were not sufficiently permeabilized to allow the entry of antibody. A small amount of labelling is found within the cell body and all over the flagellar structures present. However , control incubations also showed a similar degree of labelling.

MAG. 88,200 x.

7



8



pattern along the longitudinal axis of T.brucei bloodstream trypomastigotes (personal communication) did not give a similar pattern in T.congolense epimastigotes. In acetone fixed epimastigotes the staining pattern found with this MAb was a long, in some areas double, line of fluorescence, approximately following the flagellum and occasionally a punctate staining pattern was noted (Fig. 7.5). The staining pattern seemed localized to the flagellar area.

7.3.2. Post embedding immunohistochemical electron microscopy

The results obtained using this method, with the intention of increasing the sensitivity of localization above immunofluorescence, were largely unsatisfactory. None of the antibodies tested were localized to any specific point in the cell.

The background staining on areas of the sections without cells was often as high as over the cells when anti-cytoskeletal antibodies were used and control sections, to which a primary antibody had not been applied or a control antibody had been applied, showed similar patterns of gold label to that of experimental sections (Fig. 7.7). The large amounts of nonspecific interaction blocking agents used in the buffer (gelatin, Tween 20, ovalbumin and nonimmune serum) were used in attempts to alleviate this problem of background nonspecific staining, however the problem remained. The different fixation protocols used (paraformaldehyde and glutaraldehyde or glutarldehyde alone) did not alleviate this problem either, nor did using higher dilutions of primary or secondary antibody.

The little information which could be obtained from these electron micrographs suggested that actin was present throughout the epimastigotes with no particular localization site. Filamin and vinculin labelling were also found distributed throughout the cell but no specific localization in the flagellar attachment site was found. Anti-intermediate filament antibody labelling was found within the

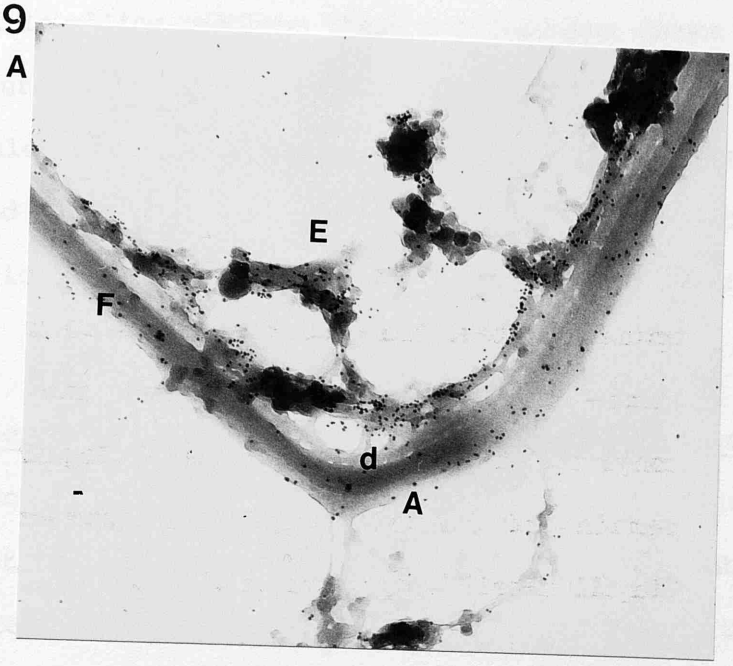
FIG.7:9. A) Flagella of whole mounted detergent extracted epimastigotes. The cytoskeletons were treated with anti-actin antiserum (Miles) and protein A conjugated to 5nm gold particles. F-flagellum , E- the remains of the cell body , A-attachment site , d-flagellum-cell body junctions.

B) Control cells treated with protein A / gold only. Labelling as for A.

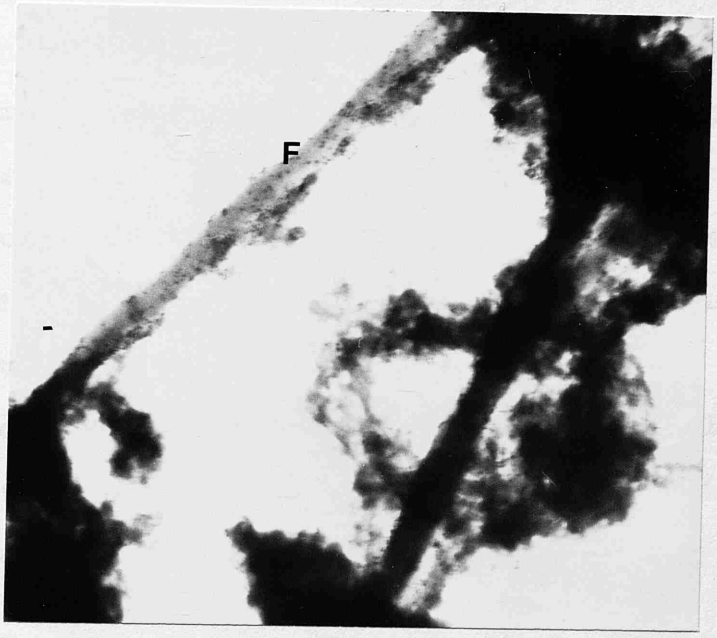
MAG. A) 56,100 x. B) 45,900 x.

9

A



B



epimastigote cell body and not the flagellum. However due to the high background labelling problems these localizations cannot be concluded to be accurate. The anti-tubulin Mab (KMX-1) labelled areas where microtubules were present but again a very high non-specific background labelling was present. Similar high background and nonspecific labelling was found in similar treated sections of BHK cells.

7.3.3. Pre-embedding immunohistochemical electron microscopy

Due to the problems, outlined above, with 'on-section labelling' an attempt to locate actin in the epimastigotes by labelling with antibody before embedding was made. Again, however, problems arose. Using the higher detergent concentration (0.5% Triton X 100) it was found the cells were disrupted to a high degree which, therefore, did not help localization of protein. The lower detergent concentration (0.1% Triton X), however, did not in many cases appear to permeabilize the cells enough, very little internal labelling was found in epimastigote cells, when found, labelling was within the cell body and flagellum (Fig. 7.8). However, control sections had a similar degree of internal labelling.

In both experimental and control cells, the gold label was found on the outside of cells, suggesting the antibodies had not been able to penetrate the cells.

7.3.4. Whole mount immunocytochemistry

Epimastigotes grown on gold, coated EM grids, and detergent extracted before labelling with anti-actin and a gold labelled detection system (protein A conjugated to gold particles) did appear to show an increase in label over the remaining cytoskeleton (Fig. 7.9) although the label in the background was again very high.

FIG.7:10. Western blot of T.congolense cell extracts probed with anti-actin Mab and the corresponding Coomassie stained gel (SDS-PAGE).

Track 1. Whole cell attached preparation.

2. Actin (rabbit muscle, Sigma).

3. Cytoskeletal attached preparation.

10

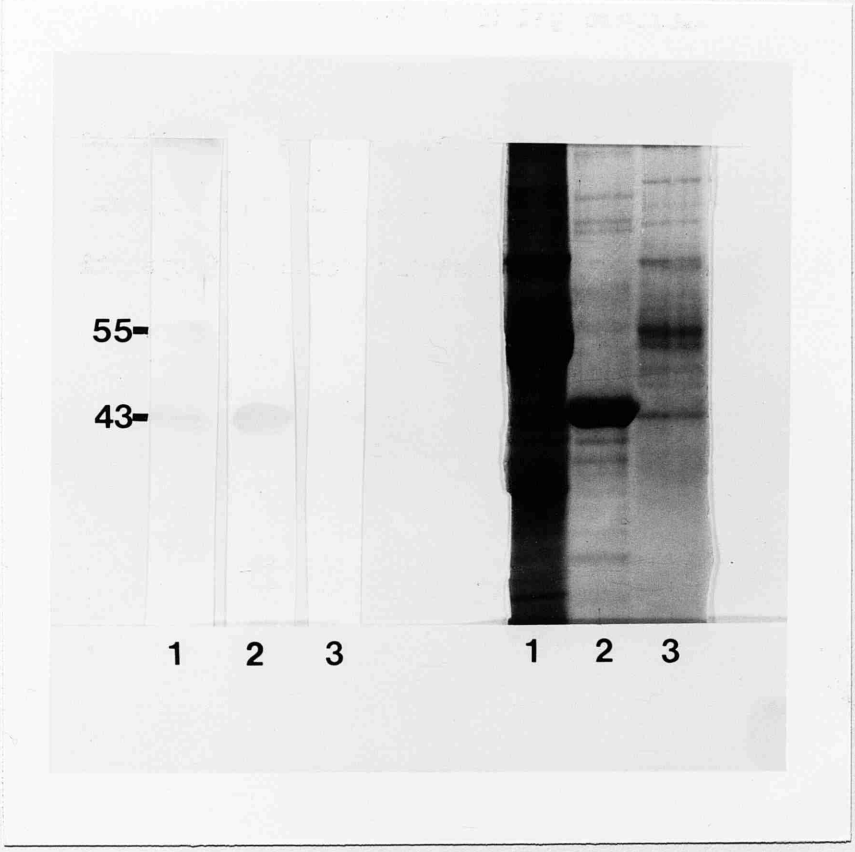


FIG.7:11. A) Western blot of T.congolense epimastigote cell extracts, separated by SDS-PAGE, probed with anti-IF Mab.

B) Amido black staining of the same blot.

PFR- paraflagellar rod proteins (72 and 75 kd).

T- tubulin (55kd). arrow- IF protein.

Track 1. Attached cytoskeletons.

2. Unattached whole cell.

3. Attached whole cell.

4. Unattached cytoskeletons.


A
1
2
3
4
B
PFR
T

Increased labelling was found especially in the areas remaining of the cell-flagellar attachment plaques, particularly on the side of the cell body. A more specific degree of localization cannot be determined due to the extremely disrupted state of these cells.

The increased labelling, however, may not be totally specific as control grids on which no primary antibody was used also showed an increase in label in the regions mentioned above (Fig. 7.9b).

7.3.5. SDS-PAGE and associated immunoblotting of whole cells and cytoskeleton preparations

Immunoblotting experiments, blotting whole cell and cytoskeletal proteins against anti-cytoskeletal antibodies, were attempted to see if differences between attached and unattached epimastigotes exist.

Anti-actin (Amersham) monoclonal antibody did not consistently label any bands in the whole cell or cytoskeletal tracks. In some experiments anti-actin antibody labelled one band which comigrated with muscle actin (Fig. 7.10) in attached and unattached cell extracts but this result was not readily repeatable.

Anti-intermediate filament MAb labelled a high molecular weight band in attached and unattached epimastigote preparations and in both types of cytoskeletal preparation. The band labelled was situated between the positions of the paraflagellar rod proteins (72kd + 75kd) and tubulins (55kd). The apparent molecular weight of this band was 65kd (Fig. 7.11). This antibody appeared to label unattached whole cell and unattached cytoskeletal preparations more than those of attached.

The antitubulin MAb (KMX-1) labelled beta-tubulin bands in all preparations but in unattached whole cells the alpha-tubulin also appeared to be labelled; this is probably due to overloading of these tracks. In trypanosomes the beta-tubulin has an apparent higher

FIG.7:12. Western blot of T.congolense cell extracts probed with KMX-1 (beta-tubulin specific Mab).

Track 1. Brain tubulin.

2. Attached cytoskeletons.

3. Unattached whole cell.

4. Attached whole cell.

5. Unattached cytoskeletons.

FIG.7:13. A) Silver stained two dimensional gel separation of T.congolense unattached epimastigotes .

T-tubulins , P-paraflagellar rod proteins.

B) Silver stained two dimensional gel separation of T.congolense attached epimastigotes.

X- attached cell associated group of proteins.

13

A

B

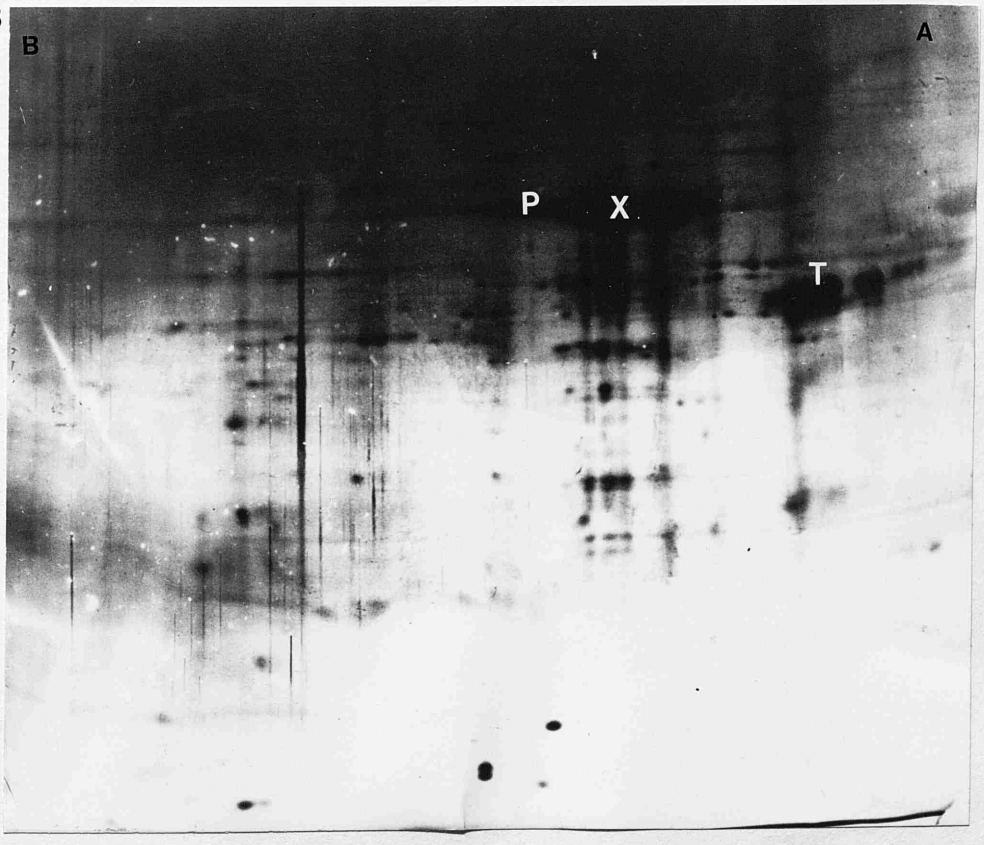


FIG.7:14. Cytoskeletal preparations corresponding to FIG.7:13.

A) Unattached epimastigotes.

B) Attached epimastigotes.

α_1, α_3 - alpha-tubulin isotypes,

β- beta-tubulin isotype(s),

P-paraflagellar rod proteins,

X- unknown attached cell associated group.

14

A

B

molecular weight than alpha-tubulin (Fig. 7.12).

Desmoplakin I and II detection using an anti-desmoplakin I & II MAb failed to detect any bands in the T.congolense samples.

Surprisingly, the 'Dot' anti-T.brucei antibody also failed to detect any bands in these samples; this antibody also failed to label any proteins in T.brucei sample preparations or brain tubulin.

7.3.6. Two dimensional PAGE

Silver-stained 2D gels of T.congolense attached and unattached epimastigotes and the corresponding cytoskeletal preparations displayed differences between attached and unattached preparations.

The whole cell preparations of both attached and unattached cells in general depicted a similar pattern of protein distribution except in the vicinity of the paraflagellar rod proteins (Figs. 7.13a&b). This difference was also reflected in the cytoskeletal preparations (Fig. 7.14a&b). The cytoskeletal gels depicted, in the main, two groups of proteins, the acidic, 55kd, tubulin proteins, and the less acidic, 72kd - 75kd paraflagellar rod proteins, the other protein spots present possibly represented cytoskeleton associated proteins e.g. dynein.

The pattern of the group of spots comprising the tubulin were similar in both attached and unattached preparations. The tubulin group comprised 3 main spots, and by analogy with T.brucei preparations (Schneider et al., 1986) these are the $alpha_1$, $alpha_3$ and beta-tubulins. The more basic two spots are the alpha-tubulins, the more abundant of these the $alpha_3$ isotype and the other the $alpha_1$. The more acidic protein is thus the beta-tubulin. A small spot of protein of slightly less molecular weight and more acidic property was located beside the beta-tubulin spot in some of these T.congolense protein gels. The beta-tubulin spot appeared to be situated at a

FIG.7:15. A) TEM of unattached cytoskeleton material treated as for cytoskeletal preparations for gels.

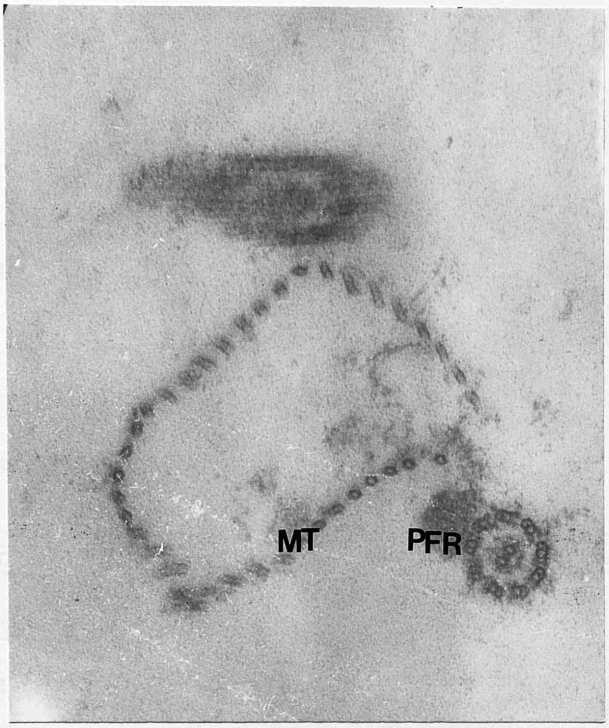
B) TEM of attached cytoskeleton material treated as for cytoskeletal preparations for gels.

PFR-paraflagellar rod , Mt-microtubules of axoneme and pellicle , A-attachment plaque material.

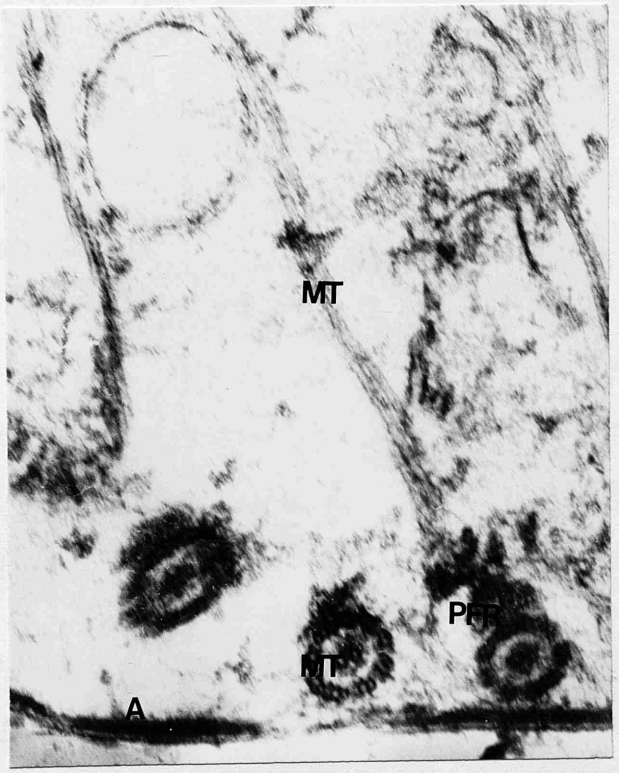
MAG. A) 117,500 x. B) 113,400 x.

15

A



B



position of lower apparent molecular weight than the alpha-tubulin isotypes (Figs. 7.13 and 7.14) in contrast to the one dimensional SDS-PAGE gels (Fig. 7.11). In contrast to the tubulin pattern similarity in both attached and unattached preparations the PFR protein group differed greatly between the attached and unattached state. The PFR group in attached preparations had a large spot of protein situated alongside of similar apparent molecular weight but of more acidic pI. This was present in both whole cell and cytoskeletal preparations (Figs. 7.13b & 7.14b). Nitrocellulose blots of attached cytoskeleton 2D gels were probed with the anti-IF monoclonal antibody, however no spots were found labelled with this.

7.3.7. Electron microscopy of attached and unattached cytoskeleton preparations

Cytoskeletons prepared as for electrophoresis experiments were fixed and processed for electron microscopy. Electron micrographs of this material (Fig. 7.15) show the remaining components of the cells. The microtubules of the pellicular sheath and flagellar axoneme were left plus other components of the axoneme, dynein arms and radial spokes. The PFR was also present. Filamentous material in the region of cell body - flagellar attachment was also found, and some filamentous material within the cell body itself. These structures were found in both attached and unattached epimastigotes. In the attached cytoskeletal preparations, the attachment plaque and some of the associated filaments were present in addition to the other structures (Fig. 7.15).

7.3.8. Cytochalasin B and D experiments

7.3.8.1. Observations on cultures: Cultures of T.congolense epimastigotes containing Cytochalasins (dissolved in DMSO) did not behave any differently from cultures containing no Cytochalasin or

FIG.7:16. Interference reflection micrographs of attached T.congolense epimastigotes treated with Cytochalasin D. All MAG. 4,725 x.

A) Interference reflection pattern of the cells shown in B). The dark area associated with the flagellar attachment sites are visible. A) and B) Cells treated with 9µg/ml Cytochalasin D in 0.6% DMSO. C) Interference reflection image of cells treated with 0.6% DMSO. D) Interference reflection image of cells grown in MEM alone.

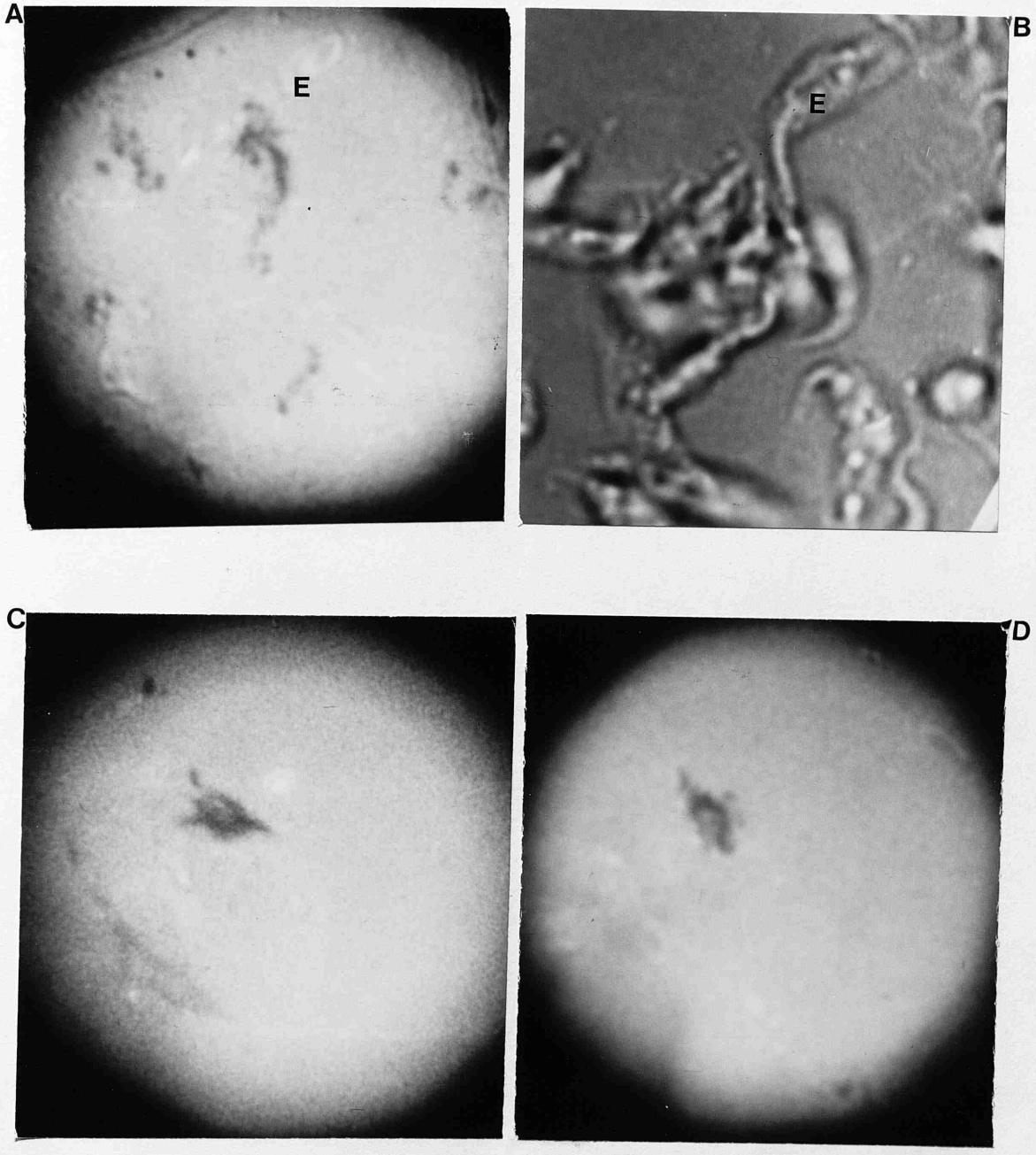

A
E
B
E
C
D

FIG.7:17. TEM of an attached T.congolense epimastigote treated with Cytochalasin D in DMSO.

Filaments and the rudiments of the attachment associated plaque are present as would be expected in a two day culture (see chapter 4).

A- attachment plaque material.

MAG. 162,000 x.

17

DMSO or containing DMSO alone. Cells observed over a 48h period attached and multiplied as in epimastigote controls. BHK cells grown in a similar manner, however, either initially spread and then rounded up and detached or did not spread at all, remaining rounded when grown in the presence of Cytochalasin. BHK cells spread and grew to confluence in cultures containing DMSO alone or in the MEM control.

7.3.8.2. Interference reflection microscopy: Epimastigotes grown in Cytochalasin D when observed by reflection interferometry did not substantially differ from cells grown in the presence of DMSO or MEM alone. A dark interference pattern was found at the point of epimastigote attachment to the substratum when viewed up to 48 hours after placing in culture (Fig. 7.16) in all these conditions.

7.3.8.3. Fluorescence with NBD-phallacidin: No differences in the actin distribution as measured by NBD-phallacidin fluorescence was noted between Cytochalasin-treated epimastigotes and control cells. The fluorescent probe labelled the whole of each epimastigote cell. In contrast to this, BHK cells treated with NBD-phallacidin after being grown in the presence of Cytochalasin D did not have a network of actin filaments in the spread areas of the cell. The rounded BHK cells showed a distribution of actin in the central area of the cell.

7.3.8.4. Electron microscopical observations: Electron micrographs of the epimastigote flagellar attachment area of Cytochalasin D treated epimastigotes appear very similar to micrographs of the attachment sites of control cells. Attachment plaques are present as are associated filaments (Fig. 7.17).

7.4. DISCUSSION

The results obtained in this section, although largely negative or inconclusive, do raise a few interesting points. The results show

that neither actin nor the actin binding proteins tested for (vinculin and filamin) are specifically localized at the epimastigote attachment site as determined by fluorescence immunocytochemistry. By this method of detection, however, it would appear that actin has a general distribution throughout the cell including the flagellum where the fluorescence is increased. The electron microscopical immunocytochemistry results, as far as can be determined, would appear to agree with those of the fluorescence microscopy but no increase in flagellar labelling was found, apart from that found in whole mount detergent-extracted trypanosomes treated with anti-actin and gold conjugated protein A. However due to the extreme detergent damage inflicted on the cell structure and the problems with background protein A/gold labelling it is quite likely this result does not reflect the true distribution of actin. Nitrocellulose blots probed with antiactin antibody also gave inconsistent labelling patterns. They suggest that an actin-like protein is present in these cells but is not associated with the flagellar attachment site. Actin does not appear to increase in attached cells: the lack of any protein which could be actin in 2 dimensional gels of attached and unattached cytoskeletons would agree with this. This does not necessarily mean that actin is absent as the actin could be less resistant to detergent extraction than other cytoskeleton elements. The results of experiments with the actin disrupting agent, Cytochalasin, however also suggest that actin is not intimately involved in epimastigote attachment. The IRM pattern of attachment is not affected by this compound; an increase in the gap size would have been expected if actin was involved in the maintenance of epimastigote attachment, anti-actin fluorescence does not appear to be affected by Cytochalasin either. This would suggest that either the actin-like protein is insensitive to Cytochalasin or the compound was

unable to penetrate into the cell interior. The increased fluorescence noted with antiactin in the flagellum may be simply due to an optical effect of fluorescence in a small rounded structure.

Similarly, labelling with the pan-interactive, anti-intermediate filament antibody (anti-IF) gave an overall cell fluorescence pattern, albeit faint, with no specific localization in the attachment related area. Immunoblotting of whole cell and cytoskeletal material using this antibody also showed the presence of an intermediate filament-like protein in both attached and unattached cells. The amount of this 65kd protein appears to be greater in blots of unattached cells but this is probably an artifact caused by the method used to prepare the samples; although starting with similar amounts of cells the constraints of solubilizing attached material entailed a larger number of preparatory steps, and therefore areas of potential loss of material, than sample preparation of unattached cells.

The lack of labelling by anti-keratin or anti-prekeratin antibodies, compared with the anti-IF antibody would suggest the protein being labelled is not related to the keratin proteins normally associated with desmosomes in vertebrate epithelial cells (Denk et al., 1985) but to the other classes of intermediate filaments located in other areas of vertebrate cells. The lack of fluorescent labelling or labelling of nitrocellulose blots with the anti-desmoplakin I & II antibody would suggest that the epimastigote attachment, although superficially similar to desmosomes and hemidesmosomes is not composed of similar material, nor for that matter are the so-called 'desmosomes' found at the sites of flagellar-cell attachment in trypanosomes as no labelling was found resembling this pattern either. Further to this last point, the antibody which labelled a series of dots along the flagella of T.brucei bloodstream trypomastigotes (T. Sherwin, personal communication) and therefore was considered possibly

to be labelling the 'desmosomal' attachment points did not give a punctate staining pattern in the flagella of T.congolense epimastigotes. This antibody did label the flagellar area but in a single strip rather than a series of punctate dots. No labelling was, however, obtained in blots when probed with this antibody, this could be due to the fact that it was a hybridoma supernatant rather than ascites fluid. Hybridoma supernatant antibodies have been noted in other studies (e.g. that of Cumming & Burgoyne, 1985) not to label blotted protein. If the antibody is labelling the cell-flagella 'desmosomes', the strip staining pattern found could be due to the different arrangement of these structures in attached T.congolense epimastigotes cells compared with bloodstream trypomastigotes (see Chapters 3 and 4). The pattern of fluorescence obtained in attached T.congolense epimastigotes with this antibody is very reminiscent of some IRM patterns obtained when observing attached epimastigote flagella, suggesting a possible relationship between flagellar attachment sites and the epitope labelled by this antibody.

One dimensional SDS-PAGE did not resolve any noticable differences between attached and unattached epimastigotes, however once two dimensional separation was carried out, differences between the two states became apparent. The extra major high molecular weight protein spot present in both whole cell and cytoskeletal preparations of attached epimastigotes is probably not a single protein but a group of associated proteins with relatively insoluble properties. Electron microscopical studies of the material used to prepare the attached cytoskeletal preparation showed that the only structure present in this material which is not present in nonattached trypanosomes treated in the same manner is the attachment plaque material. This would suggest that the extra protein found on 2D gels of attached

epimastigotes is composing the attachment plaque structure. The failing of the anti-IF antibody to bind to blots of this protein would suggest that this is not the same protein labelled in one dimensional SDS PAGE by this antibody and therefore is not related to intermediate filament proteins. The apparent molecular weights of the proteins visualized on the 2D gels is in the order of 70kd similar to those of the PFR proteins but, more acidic than this group. The weight of this putative attachment associated material is not similar to that of proteins found in focal adhesions e.g. vinculin, which has an apparent molecular weight of 130kd (reviewed by Mangeat & Burridge, 1984). Desmosomal proteins vary in weight depending on the protein, the desmoplakins have apparent molecular weights of 250 and 215kd but other desmosomal proteins have smaller masses (Franke et al., 1983).

Further studies, however, will be required to determine the relationship of the protein(s) found on 2D gels of attached epimastigotes to the structure of the attached epimastigotes.

The above findings would suggest that the epimastigote attachment site, although having structural similarities to both focal adhesions and hemidesmosomes, is on immunochemical and biochemical analysis unrelated to either of these. Proteins composing either of these metazoan attachment structures e.g. vinculin (Geiger et al., 1980) and desmoplakin I or II (Mueller & Franke, 1983) or filamentous proteins attaching these, actin and keratins or any intermediate filaments, are not associated with the epimastigote attachment. It would appear, therefore, that the epimastigote attachment system is of a novel type.

Similarity in composition to one of these known structural types might have been expected, particularly as there is such a similarity in morphology between these structures and the trypanosome attachment. Information about the composition of the epimastigote attachment could possibly have resolved whether the trypanosome attachment is a

permanent anchorage point, functionally analogous to the hemidesmosome or whether it is a more temporary structure involved in translation on the substratum, particularly at the time of initial attachment, and therefore functionally more akin to focal adhesions and actin related movement. The lack of similarity in composition leaves this question unresolved.

Although not directly relevant to the attachment site under investigation, the localization of cytoskeletal proteins in T.congolense epimastigotes in this study deserves some discussion. The presence of actin in trypanosomes has been previously reported only by De Souza et al. (1983) who found it located throughout the cell body and particularly in the flagella of T.cruzi epimastigotes. These authors concluded that the PFR might be composed at least partially of actin. However, Russell et al. (1983) showed that the PFR of Crithidia fasciculata was composed of proteins of apparent molecular weights of 68 and 76kd and Gallo and Schrevel (1985) reported that the PFR of T.brucei was composed of proteins of 72 and 75kd and that antibodies against these proteins labelled the PFR of T.cruzi, neither of these groups reported the presence of actin-like proteins in their extracted material. Thus if actin is involved in PFR structure it must play a minor role. Piperno and Luck (1979) reported the presence of actin within the axonemal structure of Chlamydomonas flagella. They suggested that actin may play a role in dynein arm function during microtubule sliding. This functional role could also explain the presence of actin in the trypanosome flagellum.

The presence of intermediate filament-like material has not previously been reported in trypanosomatids. The antibody used in the present study is one which recognizes a conserved epitope within a common structural motif found in all known classes of intermediate

filaments (Pruss et al., 1981). Thus the protein labelled by this antibody within the trypanosome may not be identical to any of the known metazoan intermediate filaments (vimentin, desmin, prekeratin, neurofilaments or glial fibrillary acidic protein, reveiwed by Lazarides (1980) or any of the invertebrate filaments labelled by this antibody, (Bartnik et al., 1985), but also contain the conserved epitope. Pruss et al. (1981) noted that this antibody binds to a protein with an approximate molecular weight of 66kd and suggested that this protein may be a component of all intermediate filaments. The protein labelled in the epimastigote extracts appears to have a molecular weight of approximately 65kd under reducing conditions and be distinct from the PFR proteins and tubulin. It must, however, be borne in mind that the 65kd protein labelled in trypanosomes could be VSG cross reacting with this antibody. The weight of the evidence is against this being the case; over 80% of the cells used to make the unattached cell sample would have been epimastigotes which do not contain VSG protein and this 65kd protein is also labelled by anti-IF in the attached preparation (100% epimastigotes). Intermediate filament-like protein has been previously reported to be present in protozoa. Numata et al. (1985) reported the presence of a 49kd IF-like protein in the ciliate Tetrahymena. This protein is associated with micronuclear movement during meiosis in this ciliate. However, Bartnik et al. (1985) reported a lack of staining with the Pruss et al. (1981) anti-IF antibody in Tetrahymena.

Two major protein subunits, alpha and beta tubulins, exist in T.brucei (Steiger et al., 1984). Schneider et al. (1986) have shown that the alpha tubulin fraction is composed of two isotypes, $alpha_1$ and $alpha_3$ the relative abundances of which do not change throughout the organisms life cycle. On 2-D gels T.brucei tubulin isotypes separate in such a manner that $alpha_1$ is in a more basic position than

alpha$_3$ which has a slightly larger molecular weight. The beta-tub. in appears more acidic and to have a slightly larger molecular weight than the alpha-tubulin isotypes (Schneider et al., 1986, reviewed by Gull et al., 1986).

The pattern of tubulin isotypes in T.congolense appears to differ slightly from that of the T.brucei pattern, the beta-tubulin isotype spot on 2D gels of T.congolense cytoskeletons is at a lower apparent molecular weight than the alpha-tubulin isotypes; the opposite to the pattern found in T.brucei 2D gels. There also appears to be a small spot of protein adjacent to the major beta-tubulin spot on these T.congolense gels, perhaps suggesting the beta-tubulin component of T.congolense is composed of more than one isotype in comparison with T.brucei (Schneider et al., 1986) and Crithidia fasciculata (Russell et al., 1984; Russell & Gull, 1984). Further work will be required on T.congolense epimastigote cytoskeletons to determine if this is the case. In 2D separated gels of T.congolense epimastigote cytoskeleton the spot which corresponds to that of the T.brucei alpha$_3$ tubulin isotype is again the most abundant alpha-tubulin isotype. This tubulin pattern is similar in both attached and unattached epimastigote preparations.

The failure in this study to obtain effective immunolabelling with gold conjugated antibodies for detection of protein localized at the electron microscope level warrants some discussion. Problems have arisen in three areas using this technique (1) high background nonspecific gold labelling, including high labelling in the absence of a primary antibody, (2) lack of specific labelling to any degree and, (3) in the pre-embedding labelling technique, permeabilization of cells whilst retaining a recognizable structure.

The method described in the materials and methods section

(7.2.3.1) was the culmination of trying many methods of preventing high background labelling. The large amount of blocking agents added in the incubation and preincubation buffers should have prevented the background labelling from occurring; many published protocols (e.g. Roth, 1982) require the use of a single blocking agent not four. However four blocking substances are routinely used in other laboratories (Beesley, 1985; Beesley & Betts, 1986). The possibility that the background labelling was due to antibody interaction with free aldehyde groups remaining from the aldehyde fixatives was countered by the incubation with NH_4Cl before labelling, this should, therefore, not have been a problem. The suggestion that the resin used (Lowycril K4M) had become charged was made (Jan De Mey, personal communication, 1986) but on repetition of the technique using a new batch of resin the problem remained suggesting that this was not the case. Similarly an electron charge problem due to charged coated EM grids was not the source of the problem as the practice in this laboratory is not to charge coated, stabilized grids and a high background label was present on using uncoated grids also.

The concentration of both primary and secondary antibody is known to be of critical importance - too high a concentration results in a high background staining. However a wide range of dilutions of both primary and secondary antibodies continued to give an equivalent level of labelling on experimental and control grids.

The high background staining and non-specific binding appears to be a problem inherent to the second, gold conjugated antibody. The nonspecific labelling occurred with two batches of commercially produced gold conjugated antisera. A way of avoiding this problem would be to use direct rather than indirect gold labelling - conjugating the primary antibody to gold (Roth, 1983).

The second problem found in the post-embedding gold labelling technique was the lack of specific antigen binding. This could be due to (a) the absence of the antigen tested for in the tissue, (b) the antigen preservation being inadequate so that the antibody does not recognize the epitope or (c) an inability of the antibody to reach the antigen because of resin or other substances obscuring the antigen.

The first suggestion, the absence of antigen for two of the antibodies tested at least, is unlikely considering the fact that these antibodies labelled material in light microscopical immunocytochemical studies. Thus another reason must be sought. The second possibility, of preservation of antigen is by far the most likely source of the problem. Antigen preservation is affected by the fixation methods used. The type of fixative used, its concentration and the length of exposure are the main aspects to which attention must be paid. The aldehyde fixatives, glutaraldehyde and paraformaldehyde, used in low concentrations (less than 2%) for short periods have been found to enable antigens to retain a reasonable percentage of their activity (Bullock, 1985); Kraehenbuhl et al. (1977) have shown that low concentrations of glutaraldehyde (0.5%) preserve both antigenic properties and tissue fine structure in the majority of cases. Mixtures of aldehydes, paraformaldehyde and glutaraldehyde have been shown to improve antigen fixation (Roth, 1983) but in the present study no improvement was found over glutaraldehyde alone. All aldehydes induce a change in the conformation of protein chains, as the antibodies used in the present detection were all anti-cytoskeletal antibodies and therefore dependant upon tertiary protein structure for function, a slight change in protein chain conformation could have changed the ability of the antigen to be detected. This point is important because all antibodies used were monoclonal antibodies, whose binding affinity is

more sensitive to slight antigen conformation changes than that of polyclonal antisera. Thus the lack of specific binding could have been due to non-preservation of antigens due to fixation problems. Antigen conformation can also be changed by embedding techniques particularly if epoxy resins which require a high temperature for polymerization are used. The use of the acrylic resin Lowycril K4M (Carlemalm et al., 1982; reviewed by Roth, 1982,1983) in this study, however, should have avoided these problems due to the low temperature polymerization required, the low temperature dehydration procedures used prior to this method of embedding should also have lessened conformational changes in proteins known to the induced by organic solvents at room temperature (Singer, 1962). It would appear that the fixation of this material should be examined closely and more methods of fixing the attached epimastigotes for this electron immunocytochemical technique attempted.

The third area where lack of specific labelling in this study could have arisen is an inability of the antibody to reach the antigen due to obscuring of the antigen by resin. This is known to be a problem when epoxy resins are used in which case it is necessary to etch the samples with H_2O_2 before treatment with antibody (reviewed by Roth, 1983 and by Beesley, 1985). However, methacrylate resins such as Lowycril K4M are hydrophilic and therefore should allow a certain degree of antibody penetration and thus antibody-antigen binding should occur easily in thin sections. One solution to the problem may be through the use of ultrathin frozen sections avoiding the use of resins totally. This would bring its own technical problems in attempting to study attached epimastigotes but these might be circumvented.

The pre-embedding immunolabelling technique should in general

avoid many of the above problems with preservation and resins. However penetration of antibody to bind to specific sites and thorough washing to remove nonbound antibody whilst preserving structure causes problems also, particularly if the cells under examination are girded by a tough microtubule pellicle as in trypanosomes. Detergent concentrations which allow disruption of the pellicular microtubule plasma membrane binding generally disrupt the cell interior beyond recognition and therefore probably antigen binding sites also.

As a final point, the antibodies used for detection in electron microscopical immunochemistry must be of a high quality and in some cases polyclonal antibodies provide a greater degree of specific labelling under the adverse conditions imposed on antigen-antibody binding by these techniques. Polyclonal antibodies, however, also introduce more non-specific binding and all the subsequent problems encountered in this study.

Immunocytochemistry for the electron microscope is a technical juggling act between specific and background non-specific labelling, removing the latter without the former and providing compromise conditions between preservation of antigen and penetration of antibody. The juggling in the present study has been on the whole unsuccessful but the preliminary problems have been sorted out and if direct rather than indirect labelling of antibody and possibly cryoultramicrotomy were used the localization of the actin and anti-IF antibodies within the trypanosomes of the EM level should be found.

In summarizing the findings of this chapter I have attempted to look at the trypanosome attachment site in ways not previously explored. All previous studies have been conventional EM studies (reviewed by Molyneux, 1977, 1983). No immunochemical or biochemical analyses have been carried out. The similarities in morphology to other cell adhesion structures do not appear to extend to components

of these known metazoan attachments; in contrast, a novel protein, visualized by 2D electrophoresis, may be a component of the epimastigote attachment. This protein, or group of proteins, will require further analysis for characterization in relation to epimastigote ultrastructure.

During this study the presence of intermediate filament-like components have been detected in trypanosomes for the first time and the arrangement of tubulin isotypes in T.congolense shown to be similar to that of T.brucei, though differences in the number of beta-tubulin isotypes may exist.

SUMMARY

Immunofluorescence studies using anticytoskeleton antibodies and antibodies against metazoan cell attachment proteins show that actin, an intermediate filament-like (but not keratin-like) protein and tubulin are present in attached T.congolense epimastigotes. Vinculin, filamin and desmoplakins appear to be absent from these cells. However, neither actin, nor the intermediate filament protein appears to be localized within the attachment area. Anti-tubulin antibody labels the entire cell very brightly due to the large number of microtubules in these cells. Anti-actin and anti-IF fluorescence are faint in comparison. Further localization of these proteins by indirect immuno-gold labelling of sections of attached epimastigotes for visualization in the TEM has not been possible due to very high background nonspecific labelling.

One dimensional SDS-PAGE and associated 'Western blotting' of whole cell and cytoskeletal preparations of both attached and unattached epimastigotes confirms the presence of an actin like protein and IF-like protein (65kd) in these cells but differences between attached and unattached epimastigotes are not discernible.

Comparative 2D-SDS-PAGE of attached and unattached whole epimastigotes and cytoskeletal preparations show a novel group of proteins (approximately 70kd) to be present in attached flagellates. These proteins have a more acidic pI than the paraflagellar rod proteins and a more basic pI than tubulin. TEM of attached and unattached cytoskeleton material correlates the presence of the attachment plaque with the 70kd group of proteins in gels of the same preparations.

The absence of actin in the attachment has been further demonstrated by the lack of effect of Cytochalasin D on trypanosome attachment in culture. No change in epimastigote attachment as monitored by IRM has been noted. These combined observations suggest that actin, actin binding proteins, IF's and structures present in metazoan cell attachment sites are substantially absent from the epimastigote flagellar attachment. The fibrillar material of the attachment site may be composed of a novel group of 70kd proteins.

CHAPTER 8

GENERAL DISCUSSION

8.1. PROPERTIES OF FLAGELLAR ATTACHMENT

The attachment of Trypanosoma congolense in the tsetse proboscis occurs as the proventricular trypomastigotes arrive in the labrum. These forms, possibly preadapted to become epimastigotes, must attach to prevent being swallowed at the next tsetse blood meal. The first stage of attachment formation begins with the flagellar membrane spreading on the substratum, subsequently cytoskeletal elements form and reinforce contact. Repeated epimastigote division results in bundles of epimastigotes in the labrum. Trypanosome multiplication is followed by trypanosome invasion of the hypopharynx. Whether this is the consequence of epimastigotes detaching from the labrum and then swimming into the hypopharynx or due to premetacyclics undertaking the journey and reattaching to the hypopharynx wall has not been established. The mature (coated) metacyclics eventually detach and become free in the lumen of the hypopharynx. They are then injected into the mammalian host with the tsetse saliva.

The research reported in this thesis has attempted to characterise the unique flagellar attachment mechanism of trypanosomatid flagellates and the attachment of Trypanosoma congolense epimastigotes in particular. As attachment in the vector appears to be a feature of all cyclically-transmitted trypanosomatid life cycles, I have also tried to determine whether it is necessary for continuation of the cycle.

The electron microscopical studies reported in Chapters 3 and 4 extend previous authors' work on this species in vivo (Evans et al., 1979; Molyneux et al., 1979; Thevenaz & Hecker, 1980; Molyneux, 1980) and in vitro (Gray et al., 1981) and establish that the epimastigote attachment in vitro is essentially equivalent to that in the tsetse fly labrum.

The observations on the effect of prevention of attachment on epimastigote division and metacyclogenesis (Chapter 5) establish that, for T.congolense, attachment is unnecessary for parasite replication but necessary for metacyclogenesis to occur. Attachment, therefore, fulfils a developmental role apart from simple anchorage of the parasite to prevent expulsion.

The attempts to determine the nature of adhesive material at the host-parasite interface by light microscopical methods have indicated that proteinacous and carbohydrate material, particularly D-mannose and D-glucosamine-containing saccharides are present in the extracellular space at the site of attachment. Glycoprotein turnover does not appear to occur however. No charge effect appears to be involved in anchoring the cells to the substratum, on the contrary a neutral charge may predominate on the attachment site membrane. Calcium ions do not appear to be necessary either for formation or maintenance of epimastigote attachment, in contrast, a reversible closer cell-substratum attachment was obtained (when viewed by reflection interferometry) on removal of calcium ions.

The final results chapter catalogues the attempts to determine the nature of attachment site internal components. The failure to detect any of the cytoskeletal elements associated with metazoan attachment sites (actin, vinculin, filamin, intermediate filaments or desmoplakins) in the trypanosome flagellar attachment by immunocytochemical methods led me to search for novel elements in this structure.

Comparative 2D-gel electrophoretic separation of pure attached and unattached epimastigote cytoskeletons indicates that a group of proteins of relative molecular mass 70kd and more basic than tubulin are involved in the attachment. Further research is required to determine the cellular location and nature of these molecules and

their relation to the attachment but the EM demonstration of cytoskeletons prepared in the same manner to those used for electrophoretic separation show the same components present in both attached and unattached epimstigotes apart from the presence of attachment plaques on the flagellar membrane of the attached epimastigotes suggesting that the differences in the 2D-gels are attributable to the attachment associated material.

8.2. ATTACHMENT, DIVISION AND METACYCLOGENESIS

The requirement for epimastigote attachment before metacyclogenesis can take place in T.congolense suggests that an intimate relationship exists between these events. The reason for this relationship is unclear. Physiological and morphological transformation of the trypanosomes occurs during the differentiation from non-infective epimastigote to infective metacyclic trypomastigote and must be linked developmentally to these changes. In T.brucei the change from epimastigote to metacyclic takes place via an uncoated trypomastigote (premetacyclic) form (Tetley & Vickerman, 1985) and the development of T.congolense metacyclics is probably similar. The final event in metacyclic production appears to be detachment of the parasite from its host anchorage. Tetley and Vickerman suggested that, in T.brucei, surface glycoprotein acquisition could possibly be responsible for the final stage of detachment by progressively increasing the extracellular gap. If detachment is the final stage of epimastigote to metacyclic differentiation then, conceivably, attachment could be the trigger for initiation of the series of changes involved in the differentiation process.

The elapse of 7-10 days between initial attachment of epimastigotes in culture and the genesis of metacyclics would suggest that the latter is not a direct consequence of the former, but the

result of a series of changes initiated by attachment.

Changes in gene expression can be initiated by cell attachment (Edelman, 1985). It is known from experiments with cultured metazoan cells (3T6 fibroblasts) that protein synthesis is triggered by cell-substratum contact and nuclear macromolecular synthesis by further spreading (Ben Ze'ev et al., 1980). Contact can control the switch to expression of an alternative set of genes e.g. from those associated with primary culture cells to those associated with differentiated cells. Kidney epithelial cells in primary culture express different intermediate filament types in relation to the degree of cell spreading (Ben Ze'ev, 1986). Differential expression of genes could be manifested in epimastigote attachment. Assuming that the additional proteins located in attached epimastigotes, compared with unattached epimastigotes, are plaque proteins or glycoproteins, it seems probable that synthesis of these molecules is initiated by substratum attachment. The absence of attachment plaque components in unattached flagellates as shown in 2D gels suggests that the components require de novo synthesis and not merely assembly. The progressive formation of the attachment plaque noted in TEMs of attached epimastigotes after different periods in culture would support this theory.

The processes of cytokinesis and nuclear division in epimastigotes do not need attachment per se for completion. Metacyclogenesis, however, would appear to require substratum contact by differentiating epimastigotes. This would strongly suggest that an environmental trigger is necessary for the initiation of transformation.

The attachment in vitro of T.congolense epimastigotes to a variety of surfaces suggests that attachment in this species is not mediated by specific receptors. In T.vivax (Hirumi et al., 1984; Fish

et al., 1987) it has been found that epimastigotes will attach in vitro if an array of 9,10-anthraquinone dye molecules are present on the substratum. Fish et al. suggest that T.vivax epimastigotes contain receptors for molecules in the tsetse proboscis with a similar chemical structure to 9,10-anthraquinone, after binding to these moieties differentiation can occur. The lack of any such specificity in T.congolense epimastigote attachment in vitro would suggest that if T.congolense attachment is triggering metacyclogenesis it is the formation of the attachment that initiates the process and not the binding of a specific molecule (e.g. lectin) incidentally associated with flagellar attachment as has been suggested for T.cruzi metacyclogensis (Section 5.4) (Sher & Snary, 1982) or T.vivax (Fish et al., 1987). The trigger could then, by a feedback mechanism, initiate plaque formation and cytoskeletal assembly in the flagellum (in the form of filaments linking the plaque to the microtubules, PFR and cell-flagellar junctions and, via these, possible connections to the rest of the cytoskeleton). Cytoskeletons are thought to play integral roles in cell differentiation in general, being the ideal structures for signal transduction (Ben Ze'ev, 1985) due to their ubiquitous location within cells, the dependence of cell shape and cytoarchitecture on cytoskeletal arrangement and the association of cytoskeletal elements with cell attachment sites and therefore the external environment. Thus, via the cytoskeleton, epimastigote attachment could initiate the changes involved in metacyclogenesis: the inactivation of the mitochondrion, the change in its morphology internally and externally, the initiation of glycosome morphological change and biochemical changes for glycolysis, internal cell reorganization, shortening the length of the cell by over a third and repositioning of nuclei and kinetoplast - properties thought to be associated with mitochondrial shape change, microtubule reorganization

and synthesis of the characteristic monomolecular coat of metacyclics. Cell structure and biochemical changes occur throughout the lifecycle of salivarian trypanosomatids, the majority of changes occur without prior cell attachment. The importance of attachment in the change from epimastigote to metacyclic could possibly be related to the glycoprotein coat synthesis during this transformation. The exact relationship between the two events is unclear. The point that all trypanosomatids attach but only Salivarian epimastigotes differentiate into monomolecular glycoprotein coated metacyclic trypomastigotes might suggest that the two events are not directly related. This controversy is, however, resolved if it is considered that two different events are occurring which are only superficially parts of the same process. Trypanosomatids in general attach to remain in a particular location, whether for nutritional purposes e.g. Leptomonas sp. (Lauge & Nishioka, 1977), to retain a portion of the infective population within the host e.g. Crithidia fasciculata (Brooker, 1971b) or in a location from which the next host can be infected e.g. T.brucei (Tetley & Vickerman, 1985) or to prevent being removed to an area of the insect host in which the parasite would be destroyed e.g. T.vivax (Vickerman, 1973). Keeping the flagellates in one place is one role of attachment, possibly the original role as it is common to all trypanosomatids, but salivarian trypanosomes, and possibly to some extent stercorarian trypanosomes, have 'appropriated' this function and transformed it into a trigger for metacyclogenesis. The first part of the proposed attachment and differentiation sequence i.e. the initiation of plaque component synthesis by attachment should then occur in all attaching trypanosomatids, the rest of the sequence, biochemical changes, morphological changes and surface coat synthesis of salivarian metacyclics, would be by-products of the attachment in

these species only.

The final event in metacyclogenesis, detachment, would be a natural consequence of switching on metacyclic gene expression and switching off epimastigote gene expression, if attachment plaque components are considered epimastigote phenotypic characters. The gradual series described for T.brucei metacyclogenesis (Tetley & Vickerman, 1985) includes a decrease in attachment morphology - fewer flagellipodia, less intertwining with tsetse salivary gland microvilli and a subsequent reduction in number of attachment plaques. These changes can be considered manifestations of a reduction of epimastigote gene expression, including attachment-related genes. Similar changes have been documented for ovarian follicle cells in response to the extracellular signal progesterone. Adhaerens junction associated protein synthesis increases as mitochondrial, endoplasmic reticulum and gap junction protein synthesis decreases and vice-versa, the initiation of one pathway signalling the inactivation of the other (Ben Ze'ev, 1986).

The time delay between the events of epimastigote-substratum contact and metacyclogenesis might appear excessive for purposes of differentiation alone, particularly as Vickerman (1985) surmises that the intermediate premetacyclic stage in T.brucei is brief. The time lag could possibly be partially explained by a requirement for a certain number of epimastigote divisions to occur prior to metacyclogenesis. The trigger for differentiation would then possibly come from neighbouring epimastigotes in a bundle. The alignment of the attachment sites of neighbouring cells and the inability of cloned trypanosomes to transform (Chapter 2) might be consequences of this feature. Signals between epimastigotes could convey the final input to a system primed for metacyclogenesis by attachment.

Whatever the exact reason for epimastigote attachment, it appears to hold a developmental significance related to metacyclogenesis in T.congolense and possibly other African, mammal-infective trypanosomes.

8.3. ATTACHMENT SITE COMPOSITION

The investigation carried out in this study as to the physical properties and components of epimastigote attachment has produced several interesting results. When the epimastigote attachment is compared to hemidesmosomes or focal adhesions of metazoan cells it does not appear to be related to either of these forms of attachment. With regard to the internal components of the epimastigote attachment sites they do not appear to contain either actin or vinculin - fundamental components of focal adhesions nor do they contain intermediate filaments or desmoplakins - hemidesmosome constituents. The group of proteins deduced from 2D-gel electrophoresis of attached cytoskeletons to be attachment site related will require to be analysed further before the relatedness or unrelatedness of the attachment to other cell attachment systems can be elucidated. The lack of action of Cytochalasin D on epimastigote attachment also indicates that actin is not involved with this process.

Examination of external attachment site components by interference reflection microscopy in conjunction with the addition of chemicals indicated the nature of some extracellular components of the epimastigote attachment site. In some respects the epimastigote appeared similar to that of other cell types. Protein and carbohydrate moieties appear to be present in this location - these are also present in focal adhesions and hemidesmosomes. No calcium ions appear necessary for the epimastigote attachment to be initiated or sustained, without divalent cations the cell-glass gap decreased in

size. Similar studies on focal adhesions (King et al., 1979a&b; Gingell & Vince, 1982b) found the opposite effect - gap size increase on removal of calcium ions. Both hemidesmosomes and focal adhesions require calcium ions present for their formation and maintenance (Kartenbeck et al., 1982; Trinkhaus-Randall & Gipson, 1984). Similarly, the addition of monovalent ions appeared to have very little effect on the epimastigote attachment, again unlike focal adhesions (Preston & King, 1978a&b,1984; King et al., 1979a&b).

Thus the only similarity between the epimastigote attachment and focal adhesions and hemidesmosomes is that protein and carbohydrate residues are present in the extracellular gap, even in this respect differences were noted. No turnover of carbohydrate residues appeared to be occurring in the epimastigotes; tunicamycin, at 500 times the concentration found by Overton (1982) to disrupt desmosomes, did not affect the trypanosome attachment over a period of over 48 hours.

The overwhelming conclusion from the findings of this study is that T.congolense epimastigote attachment mechanism is not homologous with that of focal adhesions or hemidesmosomes. The morphological similarity of the attachment among all trypanosomatid attached phases would suggest that the attachment mechanism and composition is similar in all trypanosomatids. The trypanosome attachment may thus be a unique attachment system unrelated to those of metazoan cell types, but morphologically similar due to convergent evolution. On the other hand, the metazoan attachment proteins could be present in the trypanosome system but in a slightly different form and unrecognized by the particular antibodies used in this study. The lack of significant actin detection using a monoclonal antibody, the actin specific binding protein phallacidin and also an antiactin antisera, however, might suggest that actin-like proteins are not involved in the attachment particularly as positive staining was found in other

areas of the epimastigotes. Further biochemical and immunochemical studies require to be carried out on the trypanosome attachment to elucidate its components.

It is not unusual for protozoans to contain filamentous cytoskeletal elements different from those of metazoan cells. There are several well known examples of cytoskeletal elements unique to protozoans. Trophozoites of the parasitic flagellate Giardia have been found to contain unique filaments composed of proteins named giardins (Crossley & Holberton, 1983a&b, 1985). These proteins constitute part of trilaminate microribbons associated with the microtubular cytoskeleton of the ventral disc and its precursor (Crossley et al., 1986). The ventral disc is an adhesive organ enabling the flagellate to hold onto the wall of the small intestine. The giardins are a group of polypeptides with chain Mr values of 30kd. These polypeptides copolymerize with isolated tubulin and thus can be considered a form of MAP (microtubule associated protein). Monoclonal antibodies and antisera raised against the major giardin bands (as observed in SDS-PAGE)-14A, 14B and 15, locate these proteins in the core of the microribbons. The capacity of isolated giardins (band 14A, 14B and 15) to reassemble into 2.5nm diameter ribbon-like structures in vitro has led to them being regarded as the structural proteins of the microtubule-associated microribbons. The faces of the microribbons may be composed of tubulin although in a different orientation from that found in microtubules as anti-tubulin antibodies do not bind these structures although they resemble tubulin monomers in terms of lattice structure.

Other polypeptides that comigrate with the giardins in SDS-PAGE but do not share the antigenic properties of the band 14A, 14B and 15 giardins have been localized within the ventral flagella of Giardia.

These may comprise the paraxial rod found in the emergent portion of these flagella.

The paraflagellar rods of trypanosomatids (Fuge, 1969) and Euglenids (Hyams, 1982) can also be considered as unique protozoan cytoskeletal structures.

These intraflagellar structures are found in the emergent flagella of Euglenids and Trypanosomatids that lack endosymbionts. Structurally differences exist between the PFRs of these two groups of flagellates but recently it has been shown that their constituent proteins share antigenic sites (Gallo & Schrevel, 1985).

The function of the PFR in either group is unknown. It has been shown (Piccinni et al., 1975) that the PFR of Euglena possesses its own ATPase activity unconnected with the dynein ATPase, this finding has led to supposition that the PFR participates in flagellar movement. Another suggestion for function is that the PFR plays a role in controlling the frequency and waveform of the flagellar beat. A comparative study of trypanosomatids that contain a PFR and one that lacks the structure (e.g. Crithidia oncopelti, Burnasheva et al., 1968) could perhaps elucidate whether this is indeed the case. In Euglenoids the PFR is a hollow structure ~200nm in diameter and lies adjacent to the axoneme, no constant orientation with the axoneme has been noted, the PFR lying alongside any of the microtubule doublets. Negatively stained PFRs from E.gracilis (Hyams, 1982) show that the major structural unit is a 22nm filament orientated obliquely to the longitudinal axis of the rod generating a 7 start left handed helix. The filament may have a subunit composition but this has not yet been determined. The major periodicity noted is 54nm at the points of attachment of the PFR to the microtubules of the axoneme. Hyams (1982) showed by comparative gel electrophoresis that the PFR of E.gracilis is composed of two major proteins with apparent molecular weights of

80 and 69kd - PFR_1 and PFR_2 respectively. These proteins are trypsin sensitive.

The structure of the PFR is similar in all trypanosomatids. The 200 by 200nm diameter structure is composed of two electron-dense 'semicircular' plaques separated by 6-7 fibrous elements 25-30nm apart. In trypanosomatids there is a constant association of the PFR with axoneme doublets 4-7. The attachment between the PFR and doublets is formed by 5nm links at a spacing of 25nm. The lattice structure itself appears composed of 5-7nm diameter filaments spaced at 18nm and crossed in 2 directions at an angle of 30-50^{o} to the longitudinal plane the oblique-crossing filaments lie spaced at 25nm intervals (Fuge, 1969). Russell et al. (1983) reported that the PFR of Crithidia fasciculata is composed of two major proteins with apparent molecular weights 76 and 69kd, PFR_1 and PFR_2, respectively. Both these proteins have a pI of 6.1 in 2 dimensional PAGE. Gallo and Schrevel (1985) have shown that a range of trypanosomatids Leishmania, Crithidia, stercorarian and salivarian trypanosomes in many morphological forms (epimastigotes, promastigotes, chanomastigotes and trypomastigotes) contain similar PFR components - 72 and 75kd proteins. Monoclonal antibodies against the PFR constituents of T.brucei bind all trypanosomatid PFR components in western blots of separated protein with a higher affinity for PFR_2 proteins than PFR_1. The monoclonal antibody also has a strong affinity for PFR_1 of E.gracilis and Distigma proteus - weak binding is also found for PFR_2 of Euglenids. Thus at least one common antigenic determinant in PFR's of both Trypanosomatids and Euglenids exist. This is probably a common epitope associated with a functional domain related to a similar assembly in paraflagellar structures in a similar manner to the shared structural motif of intermediate filaments which is recognized by a monoclonal

antibody (Pruss et al., 1981). The similar amounts of PFR_1 and PFR_2 proteins isolated from the cells, as observed in Coomassie stained gels would suggest that the native protein is a dimer. The exact location of PFR_1 and PFR_2 within the PFR has not yet been elucidated.

De Souza et al.(1983) reported the possibility that actin might be a constituent of the PFR in trypanosomes. The above molecular studies on isolated PFRs do not report the presence of an actin-like protein. Thus it would appear that actin is not a major constituent, if at all, of PFR in trypanosomatids. This cytoskeletal structure appears a unique component in the flagella of these flagellate groups.

The other well known example of unique protozoan cytoskeletal elements all relate to non-actin-myosin contractile systems. Several types of these exist which may or may not be related; the spasmoneme found in peritrichous ciliates e.g. Vorticella (Routledge, 1978), the costa of Trichomonas (Amos et al., 1979) and similar organisms and the myonemes of Acantharia (Febvre, 1974; Febvre & Febvre-Chevalier, 1982) all share certain properties in common but are probably distinct structures.

The spasmoneme is a contractile structure situated in the stalk of vorticellid ciliates or in fibres in the pellicle of Stentor. On contraction of the spasmoneme the stalk is rapidly shortened pulling the cell body towards the attached base. The organelle has a striated appearance being composed of individual filaments 2-3nm in diameter with a periodicity of 3.5nm. The spasmoneme is composed of proteins known as spasmins. These proteins vary slightly in molecular weight between species but the majority of species have two major spasmins of 15kd and 20kd - spasmins A and B respectively. These proteins are acidic (pI 4.7). Contraction of the spasmoneme is initiated by calcium ions which bind to the spasmin B component. Unlike actin-myosin contraction, no ATP is required for this process, glycerinated

spasmonemes contract and relax in the presence and absence of calcium (Routledge, 1978).

The costa of Trichomonas species is a rod-shaped intracellular organelle situated in the cytoplasm immediately below the and closely associating with the undulating membrane of these flagellates. At least in some members of the genus (e.g. Trichomonas termopsidis and T.gigantea) this organelle is motile. The motility arises by bending waves, actively produced by the costa itself, being transmitted along the costa. At the points of bending the highly birefringent appearance of the costa is locally reduced when viewed using Nomarski optics. The change in birefringence can probably be accounted for by a change in configuration of the organelle's substructure. The costa is composed of longitudinal lamellae 2-3nm thick at a spacing of 12nm connected by transverse filaments at intervals of 37nm, a change from straight to zig-zag configuration of the lamellae probably accounts for the local reduction in birefringence noted to accompany bending. The principle protein component of the costa, as determined by SDS-PAGE, has an apparent molecular weight of 90kd (Amos et al., 1979). A little evidence exists that the costa is controlled by ATPase/calcium ion activation. The absence of actin, myosin, tubulin and spasmin from the costa indicates a novel molecular basis for motility in this system also.

In both the above cases motility arises from cyclic changes in microfibrillar proteins of novel composition. The myonemes of Acantharia also fall into this category. These organelles are found at the position of the outer cell pellicle in these protozoa. Rapid length changes of the myonemes induce changes in the position of the pellicle. The exact shape, number and size of myonemes vary among the genera, some are long ribbon-like structures e.g. in Acanthoplegma and

in other species a large number of cylindrical myonemes are found. All appear composed of densely packed microfibrils of 3nm diameter. These filaments appear to be twisted around each other in a rope like fashion. The filaments shorten on contraction by varying the pitch of microstrands which cross the organelle, linking the filaments. The cross-linking strands have a periodicity of $\sim$0.8μm depending on the state of the contraction (Febvre & Febvre-Chevalier, 1982). The lack of labelling of myonemes with heavy meromyosin or with NBD-phallacidin (Febvre & Febvre-Chevalier, 1982) suggests no actin is involved in these structures, it is unknown if these structures contain any of the other proteins found in contractile organelles of other protozoa. It is interesting that the point of connection of the myoneme to the plasma membrane resembles a hemidesmosome (Febvre, 1974). Hemidesmosomal-like structures have also been noted in some phytoflagellates e.g. Platymonas tetrathele at the points binding the cell body to the theca. The associated filaments in these protozoa are part of the flagellar rootlet system - possibly another unique protein system and present in many protozoans (Schnepf & Maiwald, 1970).

There are probably many more examples of unique protozoan cytoskeletal elements; those discussed above are the more common and better researched examples. It is interesting that the points of cytoskeleton anchorage to the plasma membrane where a strong contact is required e.g. to counteract contractility in myonemes, to bind an external theca, or to bind organisms to a substratum where the counteracting of shearing forces occurs (trypanosomatids) the organelle found at the site bears strong resemblance to a hemidesmosome. This observation implies that it is function that determines the ultrastructural morphology of these attachments, the similar appearance arising by convergent evolution, and not due to them being composed of the same proteins.

8.4. POSSIBLE CONSEQUENCES

In practical terms, an attachment complex containing trypanosome-specific proteins or glycoproteins might be useful in terms of trypanosome control. The two main findings of the research reported in this thesis (a) T.congolense epimastigotes require to attach to undergo metacyclogenesis, and (b) the attachment may take a unique form, could be used as the basis for control of trypanosomiasis. Mammalian infection requires attachment of epimastigotes therefore if attachment could be prevented by inhibiting the components of the attachment from forming or by destroying them, then infection would not occur.

The practicalities of preventing mammal infection in this manner would probably prove difficult, however. The tsetse flies would have to ingest any anti-attachment substances to enable the substance to work therefore the anti-attachment agent would have to be present within the bloodstream of the bitten animal. The mammal's immunological and nonspecific defense mechanisms would probably destroy the anti-attachment substance. Aerial spraying of anti-attachment substances would appear far fetched and impractical.

Grandiose schemes for trypanosomiasis control apart, the results presented here have provided an insight into the basic biology of epimastigote attachment about which very little information previously existed.

REFERENCES

Abercrombie M and Dunn GA (1975) Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. Expl. Cell Res. 92: 57-62.

Abercrombie M , Heaysman JEM and Pegrum SM (1971) The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. Expl. Cell Res. 67: 359-367.

Afzelius BA (1959) Electron microscopy of the sperm tail. Results obtained with a new fixative. J. Biophys. Biochem. Cytol. 5: 269-278.

Allsopp R , Hall D and Jones T (1985) Fatal attraction for the tsetse fly. New Scientist 108: 40-43.

Amos WB , Grimstone AV , Rothschild LJ and Allen RD (1979) Structure, protein composition and birefringence of the costa: a motile flagellar root fibre in the flagellate Trichomonas. J. Cell Sci. 35: 139-164.

Aoki M and Tavassoli M (1981) OTO-method for preservation of actin filaments in electron microscopy. J. Histochem. Cytochem. 29: 682-683.

Avnur Z and Geiger B (1981) The removal of extracellular fibronectin from areas of cell substrate contact. Cell 25: 121-132.

Avnur Z , Small JV and Geiger B (1983) Actin-independent association of vinculin with the cytoplasmic aspect of the plasma membrane in cell contact areas. J. Cell Biol. 96: 1622-1630.

Badley RA , Lloyd CW , Woods A , Carruthers L , Allcock C and Rees DA (1978) Mechanisms of cellular adhesion III. Preparation and preliminary characterisation of adhesions. Expl. Cell Res. 117: 231-244.

Baker JR (1968) Trypanosomes of wild animals in the neighbourhood of the Serengeti National Park. Symp. Zool. Soc. Lond. 24: 147-158.

Banks KL (1978) Binding of Trypanosoma congolense to the walls of small blood vessels. J. Protozool. 25: 241-245.

Banks KL (1979) In vitro binding of Trypanosoma congolense to erythrocytes. J. Protozool. 26: 103-108.

Banks KL (1980) Injury induced by Trypanosoma congolense adhesion to cell membranes. J. Parasit. 65: 34-37.

Bartnik E , Osborn M and Weber K (1985) Intermediate filaments in non-neural cells of invertebrates: Isolation and biochemical characterization of intermediate filaments from the esopageal epithelium of the mollusc Helix pomatia. J. Cell Biol. 101: 427-440.

Beesley JE (1985) Colloidal gold: A new revolution in marking cytochemistry. Proc. R. Microsc. Soc. 20: 187-198.

Beesley JR and Betts MP (1986) Choosing the correct buffer - a quantitative investigation into the effects of additives in reducing non-specific immunolabelling. Proc. R. Microsc. Soc. 21: 78.

Ben-Ze'ev A (1985) Cell shape, the complex cellular networks and gene expression: Cytoskeletal protein genes as a model system. In: Cell and muscle motility, vol.6. Ed. Shay JW. pp.23-53. Plenum Press, New York and London.

Ben-Ze'ev A (1986) The relationship between cytoplasmic organization, gene expression and morphogenesis. Trends Biochem. Sci. 11: 478-481.

Ben-Ze'ev A , Farmer SR and Penman S (1980) Protein synthesis requires cell-surface contact while nuclear events respond to cell shape in anchorage-dependant fibroblasts. Cell 21: 365-372.

Birkett CR , Foster KE , Johnson L and Gull K (1985) Use of monoclonal antibodies to analyse expression of a multitubulin family. FEBS Lett. 187: 211-218.

Bishop A (1967) Problems in the cultivation of some parasitic protozoa. Adv. Parasitol. 5: 93-138.

Bohringer S and Hecker H (1975) Quantitative ultrastructural investigations of the life-cycle of Trypanosoma brucei: a morphometric analysis. J. Protozool. 22: 463-467.

Boker CA and Schaub GA (1984) Scanning electron microscopic studies of Trypanosoma cruzi in the rectum of its vector Triatoma infestans. Z. ParasitKde. 70: 459-469.

Boller K , Vestweber D and Kemler R (1985) Cell-adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. J. Cell Biol. 100: 327-332.

Borst P and Cross GAM (1982) Molecular basis for trypanosome antigenic variation. Cell 29: 291-303.

Borysenko JZ and Revel J-P (1973) Experimental manipulation of desmosome structure. J. Anat. 137: 403-422.

Bowman IBR and Flynn IW (1976) Oxidative metabolism of trypanosomes. In: Biology of the Kinetoplastida , vol I. Eds. Lumsden WHR and Evans DA. pp. 435-476.Academic Press, London,New York.

Brack C (1968) Electroenmikroskopische Untersuchungen zum Lebenszyklus von Trypanosoma cruzi. Acta Trop. 25: 289-356.

Bradford MN (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utalizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Bretscher A (1981) Fimbrin is a cytoskeletal protein that cross-links F-actin in vitro. Proc. Natn. Acad. Sci. USA. 78: 6849-6853.

Bretscher A and Weber K (1980) Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures.

J. Cell Biol. 86: 335-340.

Bretana A , Avila JL , Arias-Flores M , Contreras M and Tapia FJ (1986) Trypanosoma cruzi and American Leishmania spp.: Immunocytochemical localization of a laminin-like protein in the plasma membrane. Expl. Parasit. 61: 168-175.

Broden A (1904) Les infections a trypanosomes au Congo chez l'homme et les animaux. Bull. Soc. Belge. Etud. Colon. Fevier.

Brooker BE (1970) Desmosomes and hemidesmosomes in the flagellate Crithidia fasciculata. Z. Zellforsch. 105: 155-166.

Brooker BE (1971a) Flagellar adhesion of Crithidia fasciculata to millipore filters. Protoplasma 72: 19-25.

Brooker BE (1971b) Flagellar attachment and detachment of Crithidia fasciculata to the gut wall of Anopheles gambiae. Protoplasma 73: 191-202.

Brooks AS (1978) Ultrastructure of the flagellar attachment site in three species of trypanosomatids. Trans. Am. Microsc. Soc. 97: 287-296.

Bruce D , Hamerton AE , Bateman HR and Mackie FP (1910) Trypanosome diseases of domestic animals in Uganda. III. Trypanosoma vivax Ziemann. Proc. R. Soc. B.83: 15-27.

Brun R (1974) Ultrastruktur und Zyklus von Herpetomonas muscarum, "Herpetomonas mirabilis" und Crithidia luciliae in Chrysomyia chloropyga. Acta Trop. 31: 219-290.

Brun R (1982) Cultivation of procyclic trypomastigotes of Trypanosoma congolense in a semi-defined medium with direct adaptation from bloodstream forms. Z. ParasitKde. 67: 129-135.

Bullock GR (1985) Fixation in immunocytochemistry. Proc. R. Microsc. Soc. 20: 4IM (abst.). Immunocytochemistry Meeting , York, July 1985.

Bungener and Muller G (1976) Adharenzphanomene bei Trypanosoma congolense. Tropenmed. Parasit. 27: 370-371.

Burland TG , Gull K , Schedl T , Boston RS and Dove WF (1983) Cell type-dependant expression of tubulins in Physarum. J. Cell Biol. 97: 1852-1859.

Burnasheva SA , Ostrovskaja MV and Yurzinai GA (1968) The ultrastructure of the flagella in Strigomonas oncopelti. Acta Protozoologica 6: 357-364.

Burridge K and Connell L (1983) Talin: a cytoskeletal component concentrated in adhesion plaques and other sites of actin-membrane interaction. Cell Motility 3: 405-417.

Burridge K and Fermisco JR (1980) Microinjection and localization of a 130K protein in living fibroblasts: a relationship to actin and fibronectin. Cell 19: 587-595.

Burridge K and Fermisco JR (1981) Non-muscle alpha-actinins are calcium-sensitive actin-binding proteins. Nature (London) 294: 565-567.

Burridge K and Fermisco JR (1982) Alpha-actinin and vinculin from non-muscle cells: calcium sensitive interactions with actin. Cold Spring Harbor Symp. Quant. Biol. 46: 587-597.

Burridge MJ , Reid HW , Pullan NB ,Sutherst RW and Wain EB (1970) Survey for trypanosome infections in domestic cattle and wild animals in areas of East Africa. II. Salivarian trypanosome infections in wild animals in Busoga district, Uganda. Br. Vet. J. 126: 627-633.

Camargo EP (1964) Growth and differentiation in Trypanosoma cruzi I. Origin of metacyclic trypanosomes in liquid media. Revta. Inst. Med. Trop. Sao Paulo 6: 93-100.

Capbern A , Giroud C , Baltz T and Mattern P (1977) Trypanosoma equiperdum: etude des variations antigenique au cours de la trypanisomose experimental du lapin. Expl. Parasit. 42: 6-13.

Carlemalm E , Garavito RM and Villiger W (1982) Resin development for electron microscopy and an analysis of embedding at low temperature. J. Microsc. 126: 123-143.

Contreras VT , Morel CM and Goldenberg S (1985) Stage specific gene expression preceeds morphological changes during Trypanosoma cruzi metacyclogenesis. Mol. Biochem. Parasitol. 14: 83-96.

Cowin P and Garrod DR (1983) Antibodies to epithelial desmosomes show wide tissue and species cross-reactivity. Nature (London) 302: 148-150.

Cowin P , Mattey D and Garrod DR (1984) Identification of desmosomal surface components (desmocollins) and inhibition of desmosome formation by specific Fab. J. Cell Sci. 70: 41-60.

Cowin P , Kapprell H-P and Franke WW (1985) The complement of desmosomal plaque proteins in different cell types. J. Cell Biol. 101: 1442-1454.

Crane MStJ and Dvorak JA (1982) Trypanosoma cruzi: spontaneous transformation by a Y strain varient in liquid medium. Expl. Parasit. 54: 87-92.

Croft SL , East JS and Molyneux DH (1982) Anti-trypanosomal factor in the heamolymph of Glossina. Acta Trop. 39: 293-302.

Crossley R and Holberton DV (1983a) Characterization of proteins from the cytoskeleton of Giardia lamblia. J. Cell Sci. 59: 81-104.

Crossley R and Holberton DV (1983b) Selective extraction with Sarkosyl and repolymerization in vitro of cytoskeleton proteins from Giardia. J. Cell Sci. 62: 419-438.

Crossley R and Holberton DV (1985) Assembly of 2.5nm filaments from giardin, a protein associated with cytoskeletal microtubules in Giardia. J. Cell Sci. 78: 205-232.

Crossley R , Marshall J , Clark JT and Holberton DV (1986) Immunocytochemical differentiation of microtubules in the cytoskeleton of Giardia lamblia using monoclonal antibodies to alpha-tubulin and polyclonal antibodies to asociated low molecular weight proteins. J. Cell Sci. 80: 233-252.

Crowe JS , Barry JD , Luckins AG , Ross CA and Vickerman K (1983) All metacyclic variable antigen types of Trypanosoma congolense identified using monoclonal antibodies. Nature (London) 306: 389-391.

Cross GAM (1975) Identification, purification and properties of clone specific glycoprotein antigens constituting the surface coat of Trypanosoma brucei. Parasitology 71: 393-417.

Culp LA and Buniel JF (1976) Substrate-attached serum and cell proteins in adhesion of mouse fibroblasts. Jnl. Cell Physiol. 88: 89-106.

Cumming R and Burgoyne RD (1985) The immunoblotting technique for the characterization of antibodies used in central nervous system immunocytochemistry. In: Techniques in immunocytochemistry, vol.3, pp 55-78. Eds. Bullock GR and Petrusz P. Academic Press, London and New York.

Cunningham I (1973) Quantitative studies on trypanosomes in tsetse tissue culture. Expl. Parasit. 33: 34-45.

Cunningham I (1977) New culture medium for maintainance of tsetse tissues and growth of trypanosomatids. J. Protozool. 24: 325-329.

Curtis ASG (1964) The mechanisim of adhesion of cells to glass. A study by interference reflection microscopy. J. Cell Biol. 20: 199-217.

Curtis ASG (1973) Cell adhesion. Prog. Biophys. Mol. Biol. 27: 315-384.

Curtis ASG and Forrester JV (1984) The competitive effects of serum proteins on cell adhesion. J. Cell Sci. 71: 17-35.

Dembitzer HM , Herz F , Schermer A , Wooley RC and Koss LG (1980) Desmosome development in an in vitro model. J. Cell Biol. 85: 695-702.

Denk H , Lackinger E , Cowin P and Franke WW (1985) Maintainance of desmosomes in mouse hepatocytes after drug-induced rearrangement of cytokeratin filament material. Demonstration of independance of desmosomes and intermediate-sized filaments. Expl. Cell Res. 161: 161-171.

De Souza W , Arguello C , Martinez-Palomo A , Trissi D, Gonzales-Robles A and Chian E (1977) Surface charge of Trypanosoma cruzi: binding of cationized ferritin and measurement of cellular electrophoretic mobility. J. Protozool. 24: 411-415.

De Souza W , Meza I , Martinez-Palomo A , Sabanero M , Souto-Padron T and Meirelles MNL (1983) Trypanosoma cruzi: Distribution of fluorescently labelled tubulin and actin in epimastigotes. J.

Parasit. 69: 138-142.

Desser SS (1976) The ultrastructure of the epimastigote stages of Trypanosoma rotatorium in the leech Batrocobdella picta. Can. J. Zool. 54: 1712-1723.

Desser SS (1977) Ultrastructural observations on the epimastigote stages of Trypanosoma avium in Simulium rugglesi. Can. J. Zool. 55: 1359-1367.

Dwyer DM (1974) Lectin binding studies on a parasitic protozoan. Science 184: 471-473.

Dwyer DM (1976) Cell surface saccharides of Trypanosoma lewisi. II. Lectin-mediated agglutination and fine-structure cytochemical detection of lectin-binding sites. J. Cell Sci. 22: 1-19.

Edelman GM (1985) Cell adhesion and the molecular processes of morphogenesis. A. Rev. Biochem. 54: 135-169.

Ellis DS and Maudlin I (1985) The behaviour of trypanosomes within the midguts of wild-caught tsetse from Zimbabwe. Trans. R. Soc. Trop. Med. Hyg. 79: 867-868.

El-on J , Rosen NL and Paton L (1977) In vitro growth of Trypanosoma congolense in the presence of mammalian tissue at 37°. Proc. of the 5th Internatl. Cong. of Protozool. Abst. no. 124 , New York.

Evans DA (1978) Kinetoplastida. In: Methods of culturing parasites in vitro. pp.55-88. Eds. Taylor AER and Baker JR. Academic Press: London, New York and San Francisco.

Evans DA and Ellis DS (1979) Development of Trypanosoma brucei and Trypanosoma congolense in Glossina. Trans. R. Soc. Trop. Med. Hyg. 73: 126.

Evans DA and Ellis DS (1983) Recent observations on the behaviour of certain trypanosomes within their insect hosts. Adv. Parasitol. 22: 1-42.

Evans DA , Ellis DS and Stamford S (1979) Ultrastructural studies of certain aspects of the development of Trypanosoma congolense in Glossina morsitans morsitans. J. Protozool. 26: 557-563.

Evans RR , Robson RM and Stromer MH (1984) Properties of smooth muscle vinculin. J. Biol. Chem. 259: 3916-3924.

FAO (1974) Expert consultation on the programme for the control of African animal trypanosomiasis. Food and agricultural organization of the United Nations, Rome. AGA/TRYP/74/1E.

FAO (1975) World production of animal protein and need for a new approach. Food and agricultural organization of the United Nations, Rome. AGA: AAAP/75.

FAO (1979) The African Trypanosomiasis. Report of a joint WHO expert committee and FAO expert consultation, Rome, 8-12 November 1976.

FAO-WHO-OIE (1963) The economic losses caused by animal diseases. In:

Animal Health Yearbook 1962. Food and agricultural organization of the United Nations, Rome. pp.284-313.

FAO-WHO-OIE (1982) Animal Health Yearbook 1981 no.18, Kouba V (ed.). Food and agricultural organization of the United Nations, Rome.

Farquhar MG and Palade G (1963) Junctional complexes in various epithelia. J. Cell Biol. 19: 375-412.

Febvre J (1974) Relations morphologiques entre les constituants de l'enveloppe, les myonemes, le squelette et le plasmalemme, chez les Arthracantha Schew (Acantharia). Protistologica 10: 141-158.

Febvre J and Febvre-Chevalier C (1982) Motility processes in Acantharia (Protozoa). I. Cinematographic and cytological study of the myonemes. Evidence for a helix-coil mechanism of the constituent filaments. Biol. Cell. 44: 283-304.

Ferrante A and Allison AC (1983) Alternative pathway activation of complement by African trypanosomes lacking a glycoprotein coat. Parasite Immunol. 5: 491-498.

Fernandes JF , Castellani O and Kimura E (1969) Physiological events in the course of the growth and differentiation of Trypanosoma cruzi. Genetics Supplement 61: 213-226.

Fernandes JF and Caceres O (1976) Intermediary metabolisim, growth and differentiation of Trypanosoma cruzi. Revta. Inst. Med. Trop. Sao Paulo 18: 130.

Fish WR , Nelson RT and Hirumi H (1987) Cell adhesion in Trypanosoma: In vitro studies of the interaction of Trypanosoma vivax with immobilized organic acids. J. Protozool. In press.

Foster KE (1984) Studies on the polymerization of tubulin. Ph.D. Thesis University of London.

Franke WW , Schmid E , Grund C , Mueller H , Engelbrecht I , Moll R , Stadler J and Jarasch ED (1981) Antibodies to high molecular weight polypeptides of desmosomes specific localisation of a class of junctional proteins in cells and tissues. Differentiation 20: 217-241.

Franke WW , Moll R , Schiller DL , Schmid E , Kartenbeck J and Mueller H (1982) Desmoplakins of epithelial and myocardial desmosomes are immunologically and biochemically related. Differentiation 23: 115-127.

Franke WW , Mueller H , Mittnacht S , Kapptell H-P and Jorcano JL (1983) Significance of two desmosome plaque-associated polypeptides of molecular weights 75,000 and 83,000. EMBO. J. 2: 2211-2215.

Frazier W and Glaser L (1979) Surface components and cell recognition. A. Rev. Biochem. 48: 491-523.

Fuge H (1969) Electron microscopic studies on the intra-flagellar structures of trypanosomes. J. Protozool. 16: 460-466.

Fujiwara K and Linck RW (1982) The use of tannic acid in microtubule research. Methods in Cell Biology 24A: 217-232.

Gallo J-M and Schrevel J (1985) Homologies between paraflagellar rod proteins from trypanosomes and euglenoids revealed by a monoclonal antibody. Eur. J. Cell Biol. 36: 163-168.

Garrod DR and Cowin P (1985) Desmosome structure and function. In: Receptors in tumour immunology. Ed. Chadwick CM. Cambridge University Press, Cambridge.

Geiger B (1979) A 130 K protein from chicken gizzard: It's localisation at the termini of microfilament bundles in cultured chicken cells. Cell 18: 193-205.

Geiger B , Tokuyasu KT , Dutton AH and Singer SJ (1980) Vinculin, an intracellular protein localised at specialised sites where microfilament bundles terminate at cell membranes. Proc. Natn. Acad. Sci. USA. 77: 4127-4131.

Geiger B , Dutton AH , Tokoyasu KT and Singer SJ (1981) Immunoelectron microscope studies of membrane-microfilament interactions. The distributions of alpha-actinin, tropomyosin, and vinculin in intestinal epithelial brush border and in chicken gizzard smooth muscle. J. Cell Biol. 91: 614-628.

Geiger B , Avnur Z and Schlessinger J (1982) Restricted mobility of membrane constituents in cell substrate focal contacts of chicken fibroblasts. J. Cell Biol. 93: 495-500.

Gingell D and Vince S (1982a) Substratum wettability and charge influence the spreading of Dictyostelium amoebae and the formation of ultrathin cytoplasmic lamellae. J. Cell Sci. 54: 255-285.

Gingell D and Vince S (1982b) Cell-glass separation depends on salt concentration and valency: measurements on Dictyostelium amoebae by finite aperture interferometry. J. Cell Sci. 54: 299-310.

Gorbsky G and Steinberg MS (1981) Isolation of the intercellular glycoproteins of desmosomes. J. Cell Biol. 90: 243-248.

Gorbsky G , Cohen SM , Shida H , Giudice GJ and Steinberg MS (1985) Isolation of the non-glycosylated proteins of desmosomes and immuunolocalisation of a third plaque protein: Desmoplakin III. Proc. Natn. Acad. Sci. USA. 82: 810-814.

Gray MA , Brown CGD , Luckins AG and Gray AR (1979) Maintenance of infectivity of Trypanosoma congolense in vitro with explants of infected skin at 37°C. Trans. R. Soc. Trop. Med. Hyg. 73: 406-408.

Gray MA , Cunningham I , Gardiner PR , Taylor AM and Luckins AG (1981) Cultivation of infective forms of Trypanosoma congolense from trypanosomes in the proboscis of Glossina morsitans. Parasitology 82: 81-95.

Gray AR and Luckins AG (1980) The initial stage of infection with cyclically-transmitted Trypanosoma congolense in rabbits, calves

and sheep. J. Comp. Path. 90: 499-511.

Gray MA , Ross CA , Taylor AM and Luckins AG (1984) In vitro cultivation of Trypanosoma congolense: the production of infective metacyclic trypanosomes in cultures initiated from cloned stocks. Acta Trop. 41: 343-353.

Gull K , Hussey PJ , Sasse R , Schneider A , Seebeck T and Sherwin T (1986) Tubulin isotypes: Generation of diversity in cells and microtubular organelles. J. Cell Sci. Suppl.5: 243-255.

Harley JMB and Wilson AJ (1968) Comparison between Glossina morsitans, G. pallidipes and G.fuscipes as vectors of the Trypanosoma congolense group: the proportions infected experimentally and the numbers of infective organisms extruded during feeding. Ann. Trop. Med. Parisit. 62: 178-87.

Heath JP and Dunn GA (1978) Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflection high-voltage electron-microscope study. J. Cell Sci. 29: 197-212.

Heaysman JEM , Pergum SM and Preston TM (1982) Spreading chick heart fibroblasts. A correlated study using phase contrast microscopy, RIM, TEM and SEM. Expl. Cell Res. 140: 85-93.

Henderson D and Weber K (1981) Immunoelectron microscopic identification of the 2 types of intermediate filaments in established epithelial cells. Expl. Cell Res. 132: 297-312.

Hennings H , Michael D , Cheng C , Steinert P , Holbrook K and Yupsa SH (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 19: 245-254.

Hennings H and Holbrook KA (1983) Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. An ultrastructural study. Expl. Cell Res. 143: 127-142.

Herbert WJ and Lumsden WHR (1976) Trypanosoma brucei: A rapid "matching" method for estimating the hosts parasitaemia. Expl. Parasit. 40: 427-431.

Hirumi H , Hirumi K and Moloo SK (1982) Simplified methods for propagating Trypanosoma congolense metacyclics in vitro. J. Protozool. 29 : 480 (abst. no.32.)

Hirumi H , Hirumi K , Nelson RT and Moloo SK (1984) Propagation of metacyclic-like forms of East African Trypanosoma vivax. J. Protozool. 31: 7A (abst. no. 24).

Hoare CA (1936) Morphological and taxonomic studies on mammalian trypanosomes. II. Trypanosoma simiae and acute porcine trypanosomiasis in tropical Africa. Trans. R. Soc. Trop. Med. Hyg. 29: 619-645.

Hoare CA (1964) Morphological and taxonomic studies on mammalian trypanosomes. X. Revision of the systematics. J. Protozool. 11: 200-207.

Hoare CA (1972) The trypanosomes of mammals. A zoological monograph. Oxford: Blackwell Scientific Press.

Hoare CA and Wallace FG (1966) Developmental stages of trypanosomatid flagellates: A new terminology. Nature (London) 212: 383-386.

Hollingshead S and Pethica BA (1963) The electrophoretic behaviour of some trypanosomes. Biochemistry 89: 123-127.

Hommel M and Robertson T (1976) In vitro attachment of Trypanosomes to plastic. Experentia 32: 464-465.

Honigberg BM (1963) A contribution to systematics of the non-pigmented flagellates. In: Progress in Protozoology; (Eds. Ludvik J, Lom J and Vavra J), p. 68. Academic Press, New York and London.

House CR and Ginsborg BL (1985) Salivary gland. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol.11. Pharmacology, pp. 196-224. Eds. Kerku GA and Gilbert CI. Pergamon Press, Oxford, New York.

Hughes D , Simpson L and Schneider CA (1983) Flagellar adherence of Crithidia fasciculata cells in culture. J. Protozool. 30: 635-641.

Hyams JS (1982) The Euglena paraflagellar rod: structure, relationship to other flagellar components and preliminary biochemical characterization. J. Cell Sci. 55: 199-210.

ILCA (1979) Trypanotolerant livestock in West and Central Africa. Monograph 2. International Livestock Centre for Africa. Addis Ababa, Ethiopia.

ILRAD (1984) Growing trypanosomes in the laboratory. International Laboratory for Research on Animal Diseases Report 2: 1-4.

Imboden M , Blum B , De Lange T , Braun R and Seebeck T (1986) Tubulin mRNAs of Trypanosoma brucei. J. Mol. Biol. 188: 393-402.

Izzard CS and Lochner LR (1976) Cell-to-substrate contacts in living fibroblasts: an interference reflection study with an evaluation of the technique. J. Cell Sci. 21: 129-159.

Jackson PR , Honigberg BM and Holt SC (1978) Lectin analysis of Trypanosoma congolense bloodstream trypomastigote and culture procyclic surface saccharides by agglutination and electron microscope techniques. J. Protozool. 25: 471-481.

James TE and Byers TJ (1967) The induction of multinuclearity in agitated and in aging cultures of Acanthamoeba spp. Neff. Jnl. Cell Physiol. 70: 53-62.

Jenni L , Molyneux DH , Livesey JL and Galun R (1980) Feeding behaviour of tsetse flies infected with Salivarian trypanosomes. Nature (London) 253: 383-385.

Jones JCR , Goldman AE , Steinert PM , Yuspa S and Goldman RD (1982) The dymnamic aspects of the supramolecular organisation of the intermediate filament networks in cultured epidermal cells. Cell

Motility 2: 196-213.

Jones JCR and Goldman RD (1985) Intermediate filaments and the initiation of desmosome assembly. J. Cell Biol. 101: 506-517.

Jordan AM (1979) Trypanosomiasis control and land use in Africa. Outlook on agriculture 10: 123-129.

Jordan AM (1985) Tsetse eradication plans for southern Africa. Parisitology Today 1: 121-123.

Kaddu JB and Mutinga MJ (1980) Trypanosoma (Nannomonas) congolense in the basement lamina of the anterior midgut cells of Glossina pallipides. Acta Trop. 37: 91-92.

Kartenbeck J , Schmid E , Franke WW and Geiger B (1982) Different modes of internalisation of proteins associated with adhaerens junctions and desmosomes: experimental separation of lateral contacts induces endocytosis of desmosomal plaque material. EMBO. J. 1: 725-732.

Kelly DE (1966) Fine structure of desmosomes, hemidesmosomes and an adepidermal globular layer in developing newt epidermis. J. Cell Biol. 28: 51-72.

Kelly DE and Kuda AM (1981) Traversing filaments in desmosomal and hemidesmosomal attachments: freeze fracture approach toward their characterisation. Anat. Rec. 199: 1-14.

Kelly DE and Sheinvold FL (1976) The desmosome: fine structural studies with freeze-fracture replication and tannic acid staining of sectional epidermis. Cell Tissue Res. 172: 309-323.

Killick-Kendrick R (1979) Biology of Leishmania in phlebotomine sandflies. In: Biology of the Kinetoplastida, vol. 2, eds., Lumsden WHR and Evans DA, pp. 396-460. Academic Press: London and New York.

Killick-Kendrick R , Molyneux DH and Ashford RW (1974) Leishmania in phlebotomid sandflies: I. Modifications of the flagellum associated with attachment to the mid-gut and oesophageal valve of the sandfly. Proc. R. Soc. Lond. B187: 409-419.

Killick-Kendrick R , Molyneux DH , Hommel M , Leaney AJ and Robertson ES (1977) Leishmania in phlebotomid sandflies: V: The nature and significance of infections of the pylorus and ileum of the sandfly leishmaniae of the braziliensis complex. Proc. R. Soc. Lond. B198: 191-199.

Kimmel BF , Samson S , Wu J , Hirschenberg R and Yarborough LR (1985) Tubulin genes of the African trypanosome Trypanosoma brucei rhodesiense: nucleotide sequence of a 3.7 kb fragment containing genes for alpha and beta tubulins. Gene 35: 237-248.

King CA , Heaysman EM and Preston TM (1979a) Experimental evidence for the role of long range forces in fibroblast-substrate interaction. Expl. Cell Res. 119: 406-410.

King CA , Westwood R , Cooper L and Preston TM (1979b) Speed of

locomotion of the soil amoeba Naegleria gruberi in media of different ionic compositions with special reference to interactions with the substratum. Protoplasma 99: 323-334.

Kraehenbuhl JP , Racine L and Jamieson JD (1977) Immunocytochemical localization of secretory proteins in bovine pancreatic exocrine cells. J. Cell Biol. 72: 406-423.

Krawczyk WW and Wilgram GF (1973) Hemidesmosome and desmosome morphogenesis during epidermal wound healing. J. Ultrastruct. Res. 45: 93-101.

Laemmli VK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680-685.

Lane EB (1982) Monoclonal antibodies provide specific intramolecular markers for the study of epithelial tonofilament organization. J. Cell Biol. 92: 665-673.

Langley PA and Hall MJR (1986) Tsetse control by autosterilization. Parisitology Today 2: 125-126.

Lauge G and Nishioka RS (1977) Ultrastructural study of the relations between Leptomonas oncopelti (Noguchi and Tilden) Protozoa Trypanosomatidae, and the rectal walls of adults of Oncopeltus fasciatus Dallas, (Hemiptera Lygaeidae). J. Morph. 154: 291-305.

Lazarides E (1980) Intermediate filaments as mechanical integrators of cellular space. Nature (London) 283: 249-256.

Lazarides E and Burridge K (1975) Alpha-actinin: Immunofluorescent localization of a muscle structural protein in non-muscle cells. Cell 6: 289-298.

Lentz TL and Trinkaus JP (1971) Differentiation of the junctional complex of surface cells in the developing Fundulus blastoderm. J. Cell Biol. 48: 455-472.

Lewis JW and Ball SJ (1979) Attachment of the epimastigotes of Trypanosoma cobitis (Mitrophanow, 1893) to the crop wall of the leech vector Hemiclepsis marginata. Z. ParasitKde. 60: 29-36.

Lloyd LL and Johnson WB (1924) The trypanosome infections of Tsetse flies in Northern Nigeria and a new method of estimation. Bull. Ent. Res. 14: 265-288.

Lopetegui R and Miatello CS (1982) Growth and differentiation of Trypanosoma cruzi in cell-free fluid media. Rev. Latinoam. Microbiol. 24: 125-132.

Luckins AG , Rae PF and Gray AR (1983) Infection, immunity and the development of local skin reactions in rabbits infected with cyclically-transmitted stocks of Trypanosoma congolense. Ann. Trop. Med. and Parasit. 77: 569-582.

Luft JH (1971a) Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanisim of action. Anat. Rec. 171: 347-368.

Luft JH (1971b) Ruthenium red and violet: II. Fine structural localization in animal tissues. Anat. Rec. 171: 369-416.

MacLennan KJR (1980) Tsetse-transmitted trypanosomiasis in relation to the rural economy in Africa-Part 1. Wild. Anim. Rev. 36: 2-17.

Maher P and Singer SJ (1983) A 200-kd protein isolated from the fascia adherens membrane domains of chicken cardiac muscle cells is detected immunologically in fibroblast focal adhesions. Cell Motility 3: 419-429.

Majiwa PAO , Matthyssens G , Williams RO and Hamers R (1985) Cloning and analysis of Trypanosoma (Nannomonas) congolense ILNat 2.1 VSG gene. Mol. Biochem. Parasitol. 16: 97-108.

Mangeat P and Burridge K (1984) Actin-membrane interaction in fibroblasts: what proteins are involved in this association? J. Cell Biol. 99: 95-103.

Martin RE (1985) Population growth in stationary and suspension culture of Paramoeba pemaquidensis Page (Amoebida, Paramoebidae). J. Protozool. 32: 738-739.

Masake RA , Musoke AJ and Nantulya VA (1983) Specific antibody responses to the variable surface glycoproteins of Trypanosoma congolense in infected cattle. Parasite Immunology 5: 345-355.

Mattey DL and Garrod DR (1984) Organisation of extracellular matrix by chick embryonic corneal epithelial cells in culture and the role of fibronectin in adhesion. J. Cell Sci. 67: 171-188.

Mattey DL and Garrod DR (1985) Mutual desmosome formation between all binary combinatins of human, bovine, canine, avian and amphibian cells: desmosome formation is not tissue or species specific. J. Cell Sci. 75: 377-399.

Mattey DL and Garrod DR (1986a) Calcium-induced desmosome formation in cultured kidney epithelial cells. J. Cell Sci. 85: 95-112.

Mattey DL and Garrod DR (1986b) Splitting and internalization of the desmosomes of cultured kidney epithelial cells by reduction in calcium concentration. J. Cell Sci. 85: 113-124.

Maudlin I , Kabayo JP , Flood ME and Evans DA (1984) Serum factors and the maturation of Trypanosoma congolense infections in Glossina morsitans. Z. ParisitKde. 70: 11-19.

Maudlin I and Dukes P (1985) Extrachromosomal inheritance of suceptibility to trypanosome infection in tsetse flies. I. Selection of suceptible and refractory lines of Glossina morsitans morsitans. Ann. Trop. Med. Parasit. 79: 314-324.

Maupin P and Pollard TD (1983) Improved preservation and staining of HeLa cell actin filaments, clathrin coated membranes and other cytoplasmic structures by tannic acid-glutaraldehyde-saponin fixation. J. Cell Biol. 96: 51-62.

McDonald K (1984) Osmium ferricyanide fixation improves microfilament preservation and membrane visualization in a variety of animal

cell types. J. Ultrastruct. Res. 86: 107-118.

McNutt MS and Weinstein RS (1973) Membrane ultrastructure of mammalian intercellular junctions. Prog. Biophys. Mol. Biol. 26: 45-101.

Mehlhorn H , Schaub GA , Peters W and Haberkorn A (1979) Electronmicroscopic studies on Blastocrithidia triatomae Cerisola et al. 1971 (Trypanosomatidae). Tropenmed. Parasit. 30: 289-300.

Molyneux DH (1969) The fine structure of the epimastigote forms of Trypanosoma lewisi in the rectum of the flea, Nosopsyllus fasciatus. Parasitology 59: 55-66.

Molyneux DH (1975) Trypanosoma (Megatrypanum) melophagium: Modes of attachment of parasites to mid-gut, hind-gut and rectum of the sheep ked, Melophagus ovinus. Acta Trop. 31: 65-74.

Molyneux DH (1977) Vector relationships in the Trypanosomatidae. Adv. Parasitol. 15: 1-82.

Molyneux DH (1980) Host-trypanosome interactions in Glossina. Insect Sci. Appl. 1: 39-46.

Molyneux DH (1983) Host-parasite relationships of trypanosomatidae in vectors. Current Topics in Vector Research 1: 117-148.

Molyneux DH and Ashford RW (1975) Observations on a trypanosomatid flagellate in a flea Peromyscopsylla silvatica spectabilis. Annls. Parasit. Hum. Comp. 50: 265-274.

Molyneux DH , Croft SL and Lavin DR (1981) Studies on the host-parasite relationships of Leptomonas species (Protozoa: Kinetoplastida) of Siphonaptera. Jnl. Nat. Hist. 15: 395-406.

Molyneux DH and Jenni L (1981) Mechanoreceptors, feeding behaviour and trypanosome transmission in Glossina. Trans. R. Soc. Trop. Med. Hyg. 75: 160-162.

Molyneux DH , Killick-Kendrick R and Ashford RW (1975) Leishmania in phlebotomid sandflies. III: The ultrastructure of Leishmania mexicana amazonensis in the midgut and pharynx of Lutzomyia longipalpis. Proc. R. Soc. London. B 190: 341-357.

Molyneux DH , Lavin DR and Elce B (1979) A possible relationship between Salivarian trypanosomes and Glossina labrum mechanoreceptors. Ann. Trop. Med. Parasit. 73: 287-290.

Morgan J and Garrod DR (1984) HeLa cells form focal contacts that are not fibronectin dependant. J. Cell Sci. 66: 133-145.

Moscona AA (1974) Surface specification of embryonic cells: Lectin receptors, cell recognition and specific ligands. In: The cell surface in development (Moscona AA ed.) pp.67-99. Wiley, New York.

Mueller H and Franke WW (1983) Biochemical and immunological characterization of desmoplakins I and II, the major polypeptides of the desmosomal plaque. J. Mol. Biol. 163: 647-671.

Muhlpfordt H and Schottelius J (1977) Agglutinationsverhalten von T.cruzi, T.cruzi-Like Stammen T.rangeli und T.conorhini mit dem Lektin von Soja hispida und dem Aaptus papillata Protektin. Tropenmed. Parasit. 28: 1-7.

Murray M , Morrison WI and Whitelaw DD (1982) Host susceptability to African trypanosomiasis: trypanotolerance. Adv. Parasitol. 21: 1-68.

Murray M and Gray AR (1984) The current situation on animal trypanosomiasis in Africa. Prevent. Vet. Med. 2: 23-30.

Nantulya VM , Doyle JJ and Jenni L (1978) Studies on Trypanosoma (Nannomonas) congolense. I. On the morphological appearance of the parasite in the mouse. Acta Trop. 35: 329-337.

Numata O , Sugai T and Wantanabe Y (1985) Control of germ cell nuclear behaviour at fertilization by Tetrahymena intermediate filament protein. Nature (London) 314: 192-193.

O'Daly JA (1976) Effect of calf serum fractions and proteins on division and transformation of Trypanosoma (schizotrypanum) cruzi in vitro. J. Protozool. 23: 577-583.

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

O'Halloran T and Burridge K (1986) Purification of a 190-kilodalton protein from smooth muscle: relationship of talin. Biochim. Biophys. Acta 869: 337-349.

Oesch B and Birchmeier W (1982) New surface component of fibroblast's focal contacts identified by a monoclonal antibody. Cell 31: 671-679.

Opperdose F (1985) Biochemical peculiarities of trypanosomes. Br. Med. Bull. 41: 130-136.

Ouaissi MA , Afchain D , Capron A and Grimaud JA (1984) Fibronectin receptors on Trypanosoma cruzi trypomastigotes and their biological function. Nature (London) 308: 380-382.

Overton J (1962) Desmosome development in normal and reassociating cells in the early chick blastoderm. Devl. Biol. 4: 532-548.

Overton J (1975) Experiments with junctions of the adhaerens type. Curr. Top. Dev. Biol. 10: 1-34.

Overton J (1982) Inhibition of desmosome formation with tunicamycin and with lectin in corneal cell aggregates. Devl. Biol. 92: 66-72.

Pan C (1971) Cultivation and morphogensis of Trypanosoma cruzi in improved liquid media. J. Protozool. 18: 556-560.

Pan SC (1978) Trypanosoma cruzi: Intracellular stages grown in a cell-free medium at 37 C . Expl. Parasit. 45: 215-224.

Paul J (1970) Cell and Tissue culture. (4th. Edition). E. & S.

Livingstone Ltd. Edinburgh and London.

Pate JL and Ordal EJ (1967) The fine structure of Chondrococcus columnaris. III. The surface layers of Chondrococcus columnaris. J. Cell Biol. 35: 37-51.

Pereira MEA , Lourdes MA , Villalta F and Andrade AFB (1980) Lectin receptors as markers for Trypanosoma cruzi. Developmental stages and a study of the interaction of wheat germ agglutinin with sialic acid residues on epimastigote cells. J. Exp. Med. 152: 1375-92.

Pereira MEA , Andrade AFB and Ribeiro JMC (1981) Lectins of distinct specificity in Rhodnius prolixus interact selectively with Trypanosoma cruzi. Science 211: 597-600.

Piccinni E , Albergoni V and Coppellotti O (1975) ATPase activity in flagella from Euglena gracillis. Colocalization of the enzyme and effects of detergents. J. Protozool. 22: 331-335.

Piperno G and Luck DJL (1979) An actin-like protein is a component of axonemes from Chlamydomonas flagella. J. Biol. Chem. 254: 2187-2190.

Pirbazari M and Kelly DE (1985) Analysis of desmosomal intramembrane particle populations and cytoskeletal elements: Detergent extraction and freeze fracture. Cell and Tiss. Res. 241: 341-51.

Preston TM and King CA (1978a) Cell-substrate associations during the ameboid locomotion of Naegleria. J. Gen. Microbiol. 104: 347-351.

Preston TM and King CA (1978b) An experimental study of the interaction between the soil amoeba Naegleria gruberei and a glass substrate during amoeboid location. J. Cell Sci. 34: 145-158.

Preston TM and King CA (1984) Amoeboid locomotion of Acanthamoeba castellanii with special reference to cell-substratum interactions. J. Gen. Microbiol. 130: 2317-2323.

Pruss RM , Mirsky R and Raff MC (1981) All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. Cell 27: 419-428.

Rappaport C , Poole JP and Rappaport HP (1960) Studies on properties of surfaces required for growth of mammalian cells in synthetic medium. Expl. Cell Res. 20: 465-510.

Rautenberg P , Reinwald E and Risse HJ (1980) Evidence for Concanavalin A binding sites on the surface coat of Trypanosoma congolense. Parasitology 80: 113-122.

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Rice MJ , Galun R and Margalit J (1973) Mouthpart sensilla of the tsetse fly and their function III: labrocibarial sensilla. Ann. Trop. Med. Parasit. 67: 109-116.

Roberts CJ , Gray MA and Gray AR (1969) Local skin reactions in cattle at the site of infection with Trypanosoma congolense by Glossina morsitans and G. tachinoides. Trans. R. Soc. Trop. Med. Hyg. 63: 620-624.

Rosen JJ and Culp LA (1977) Morphology and cellular origins of substrate-attached material from mouse fibroblasts. Expl. Cell Res. 107: 139-141.

Rosenfeld GC , Hou DC , Dingus J , Meza I and Bryan J (1985) Isolation and partial characterization of human platelet vinculin. J. Cell Biol. 100: 669-676.

Ross CA , Gray MA , Taylor AM and Luckins AG (1985) In vitro cultivation of Trypanosoma congolense: establishment of infective mammalian forms in continious culture after isolation from the blood of infected mice. Acta Trop. 42: 113-122.

Ross R and Thomson D (1910) A case of sleeping sickness studied by precise enumerative methods: regular periodical increase of the parasites disclosed. Proc. R. Soc. B83: 187-205.

Roth J (1982) The protein A - gold (pAg) technique. A qualitative and quantitative approach for antigen localization on thin sections. In: Techniques in immunocytochemistry, vol 1. pp.107-133. Eds. Bullock GR and Petrusz P. Academic Press, London and New York.

Roth J (1983) The colloidal gold marker system for light and electron microscopic cytochemistry. In: Techniques in immunocytochemistry, vol.2. pp.215-284. Eds. Bullock GR and Petrusz P. Academic Press, London and New York.

Routeledge LM (1978) Calcium-binding proteins in the vorticellid spasmoneme. Extraction and characterization by gel electrophoresis. J. Cell Biol. 77: 358-370.

Rovis L , Barbet AF and Williams RO (1978) Characterization of the surface coat of Trypanosoma congolense. Nature (London) 271: 654-656.

Rowton ED , Lushbaugh WB and McGhee RB (1981) Ultrastructure of the flagellar apparatus and attachment of Herpetomonas ampelophilae in the gut and malpighian tubules of Drosophila melanogaster. J. Protozool. 28: 297-301.

Russell DG , Newson RJ , Palmer GCN and Gull K (1983) Structural and biochemical characterization of the paraflagellar rod of Crithidia fasciculata. Eur. J. Cell Biol. 30: 137-143.

Russell DG , Miller D and Gull K (1984) Tubulin heterogeneity in the trypanosome Crithidia fasciculata. Mol. Cell. Biol. 4: 779-790.

Russell DG and Gull K (1984) Flagellar regeneration of the trypanosome Crithidia fasciculata involves post-translational modification of cytoplasmic alpha-tubulin. Moll. Cell. Biol. 4: 1182-1185.

Sather S and Agabian N (1985) A 5' spliced leader sequence is added in trans to both alpha and beta tubulin transcripts in Trypanosoma brucei. Proc. Natn. Acad. Sci. USA. 82: 5695-5699.

Schaub GA and Boker GA (1986) Scanning electron microscopic studies of Blastocrithidia triatomae (Trypanosomatidae) in the rectum of Triatoma infestans (Reduviidae). J. Protozool. 33: 266-270.

Schneider A , Sherwin T , Russell DG , Gull K and Seebeck T (1986) Subpellicular and flagellar microtubules of Trypanosoma brucei brucei contain the same alpha-tubulin isotypes. EMBO. J. (in press).

Schnepf E and Maiwald M (1970) Halbdesmosomen bei Phytoflagellaten. Experientia 26: 1343-1344.

Schofield CJ (1985) Parasitology Today: an ambitious project. Parasitology Today 1: 2.

Schottelius J (1976) Diewirking von Lektinen, Protekinen und Blutgruppentestseren auf verschiedene Trypanasomendvten. Z. ParastKde. 50: 182-183.

Seebeck T and Gehr P (1983) Trypanocidal action of neuroleptic phenothiazines in Trypanosoma brucei. Mol. Biochem. Parasitol. 9: 197-208.

Seebeck T , Whittaker PA , Imboden MA , Hardman N and Braun R (1983) Tubulin genes of Trypanosoma brucei a tightly clustered family of alternating genes. Proc. Natn. Acad. Sci. USA. 80: 4634-4638.

Sefton BM , Hunter T , Ball EH and Singer SJ (1981) Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. Cell 24: 165-174.

Shellswell GB , Mattey DL and Garrod DR (1986) Hemidesmosome formation by embryonic chick corneal epithelium in vitro. Tissue and Cell 18: 659-666.

Sher A and Snary D (1982) Specific inhibition of the morphogenesis of Trypanosoma cruzi. Science 211: 597-600.

Sher A , Crane MStJ and Kirchhoff LV (1983) Incubation in mice provides a signal for the differentiation of Trypanosoma cruzi epimastigotes to trypomastigotes. J. Protozool. 30: 278-283.

Sherwin T , Schneider A , Sasse R , Seebeck T and Gull K (1986) Distinct localization and cell cycle dependance of C-terminally tyrosinylated alpha-tubulin in the microtubules of Trypanosoma brucei brucei. EMBO. J. (in press).

Singer SJ (1962) The properties of proteins in non-aqueous solvents. Adv. Protein Chem. 17: 1-68.

Skerrow CJ and Matoltsy AG (1974a) Isolation of epidermal desmosomes. J. Cell Biol. 63: 515-523.

Skerrow CJ and Matoltsy AG (1974b) Chemical characterization of epidermal desmosomes. J. Cell Biol. 63: 524-530.

Skerrow CJ (1986) Desmosomal proteins. In: Biology of the Integument. Vol.2; Vertebrates. pp.762-787. Eds. Bereiter-Hahn J , Matoltsy AG and Richards KS. Springer-Verlag, Berlin, Heidelberg, New

York, Tokyo.

Staehelin LA (1974) Structure and function of intercellular junctions. Int. Rev. Cytol. 39: 191-283.

Steiger RF (1973) On the ultrastructure of Trypanosoma (Trypanozoon) brucei in the course of it's life cycle and some related aspects. Acta Trop. 30: 64-168.

Steiger RF , Steiger E , Trager W and Schneider I (1977) Trypanosoma congolense: partial cyclic development in a Glossina cell system and oxygen consumption. J. Parasit. 63: 861-867.

Steiger J , Wyler T and Seebeck T (1984) Partial purification and characterization of microtubular protein from Trypanosoma brucei. J. Biol. Chem. 259: 2596-2602.

Suhrbier A and Garrod DR (1986) An investigation of the molecular components of desmosomes in epithelial cells of five vertebrates. J. Cell Sci. 81: 223-242.

Tetley L , Vickerman K and Moloo SK (1981) Absence of a surface coat from metacyclic Trypanosoma vivax: possible implications for vaccination against vivax trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 75: 409-414.

Tetley L and Vickerman K (1985) Differentiation in Trypanosoma brucei: Host-parasite cell junctions and their persistence during acquisition of the variable antigen coat. J. Cell Sci. 74: 1-19.

Thevenaz PH and Hecker H (1980) Distribution and attachment of Trypanosoma (Nannomonas) congolense in the proximal part of the proboscis of Glossina morsitans morsitans. Acta Trop. 37: 163-175.

Thomashow LS , Milhausen M , Rutter WJ and Agabian N (1983) Tubulin genes are tandemly linked and clustered in the genome of Trypanosoma brucei. Cell 32: 35-43.

Tieszen K , Heywood P and Molyneux DH (1983) Ultrastructure and host parasite association of Blastocrithidia gerridis in the ventriculus of Gerris odontogaster (Hemiptera:Gerridae). Can. J. Zool. 61: 1900-1909.

Tieszen KL , Molyneux DH and Abdel-Hafez SK (1986) Host-parasite relationships of Blastocrithidia familiaris in Lygaeus pandurus Scop. (Hemiptera:Lygaeidae). Parasitology 92: 1-12.

Timpl R , Rohde H , Robey PG , Rennard SI , Foidart J-H and Martin GR (1979) Laminin a glycoprotein from basement membranes. J. Biol. Chem. 254: 9933-9937.

Tkacz JS and Lampen JO (1975) Tunicamycin inhibition of polyisoprenyl N-acetylglucosamine pyrophosphate formation in calf liver microsomes. Biochem. Biophys. Res. Commun. 65: 248-257.

Tokuyasu KT , Dutton AH , Geiger B and Singer SJ (1981) Ultrastructure of chicken cardiac muscle as studied by double immunolabeling in electron microscopy. Proc. Natn. Acad. Sci. USA 78: 7619-7623.

Tolansky S (1973) An introduction to Interferometry. 2nd. edn. Longman, London.

Toselli PA and Pepe FA (1968) The fine structure of the ventral intersegmental abdominal muscles of the insect Rhodnius prolixus during the molting cycle. J. Cell Biol. 37: 445-461.

Towbin H , Staehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natn. Acad. Sci. USA. 76: 4350-4354.

Trager W (1959) Tsetse-fly tissue culture and the development of trypanosomes to the infective stage. Ann. Trop. Med. Parasit. 53: 473-491.

Trinkhaus-Randall V and Gipson IK (1984) Role of calcium and calmodulin in hemidesmosome formation in vitro. J. Cell Biol. 98: 1565-1571.

Tsukita S and Tsukita S (1985) Desmocalmin: A calmodulin-binding high molecular weight protein isolated from desmosomes. J. Cell Biol. 101: 2070-2080.

Tucker JB , Mathews SA , Hendry KAK , Mackie JB and Roche DLJ (1985) Spindle microtubule differentiation and deployment during micronuclear mitosis in Paramecium. J. Cell Biol. 101: 1966-1976.

Vale GA , Bursell E and Hargrove JW (1985) Catching-out the tsetse fly. Parasitology Today 1: 106-110.

Van der Ploeg LHT , Valerio D , De Lange T , Bernards A , Borst P and Grosveld FG (1982) An analysis of cosmid clones of nuclear DNA from Trypanosoma brucei shows that genes for variant surface glycoproteins are clustered in the genome. Nucleic Acids Res. 10: 5905-5923.

Van Deurs B (1975) The use of Tannic acid-glutaraldehyde fixative to visualize gap and tight junctions. J. Ultrastruct. Res. 50: 185-192.

Van Meirvenne N , Janssens PG and Magnus E (1975) Antigenic variation in syringe passaged populations of Trypanosoma (Trypanozoon) brucei. I. Rationalization of the experimental approach. Annls. Soc. Belge Med. Trop. 55: 1-23.

Verschueren H (1985) Interference reflection microscopy in cell biology: Methodology and applications. J. Cell Sci. 75: 279-301.

Vickerman K (1962) The mechanism of cyclical development in trypanosomes of the Trypanosoma brucei sub-group. Trans. R. Soc. Trop. Med. Hyg. 56: 487-495.

Vickerman K (1969a) On the surface coat and flagellar adhesion in trypanosomes. J. Cell Sci. 5: 163-193.

Vickerman K (1969b) The fine structure of Trypanosoma congolense in its bloodstream phase. J. Protozool. 16: 54-69.

Vickerman K (1973) The mode of attachment of Trypanosoma vivax in the proboscis of the tsetse fly Glossina fuscipes: an ultrastructural study of the epimastigote stage of the trypanosome. J. Protozool. 20: 394-404.

Vickerman K (1976) The diversity of kinetoplastid flagellates. In: Biology of the Kinetoplastida, vol.1. pp.1-34. Eds. Lumsden WHR and Evans DA. Academic Press, London and New York.

Vickerman K (1985) Developmental cycles and biology of pathogenic trypanosomes. Br. Med. Bull. 41: 105-114.

Vickerman K and Luckins AG (1969) Localization of variable antigens in the surface coat of Trypanosoma brucei using ferritin-conjugated antibody. Nature (London) 224: 1125-1126.

Vickerman K and Preston TM (1976) Comparative cell biology of the kinetoplastid flagellates. In: Biology of the Kinetoplastida, vol.1. pp.35-130. Eds. Lumsden WHR and Evans DA. Academic Press, London and New York.

Vidric B (1973) Structure and cytochemistry of the Acinar cell in the rat Maxillary gland. Am. J. Anat. 137: 103-118.

Vivas J and Urbina JA (1982) Energy-dependent lectin-induced agglutination in Trypanosoma (Schizotrypanum) cruzi epimastigotes. Acta Cient. Venez. 33: 396-401.

Watt FM , Mattey DL and Garrod DR (1984) Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. J. Cell Biol. 99: 2211-2215.

Webster RE , Henderson D , Osborn M and Weber K (1978) Three-dimensional electron microscopical visualization of the cytoskeleton of animal cells: Immunoferritin identification of actin and tubulin containing structures. Proc. Natn. Acad. Sci. USA. 75: 5511-5515.

Wehland J , Osborn M and Weber K (1979) Cell-to-substratum contacts in living cells: a direct correlation between interference reflection and indirect immunofluorescence microscopy using antibodies against actin and alpha-actinin. J. Cell Sci. 37: 257-273.

Weiss L (1962) Cell movement and cell surfaces: a working hypothesis. J. Theor. Biol. 2: 236-250.

Wilkins JA and Lin S (1982) High-affinity interaction of vinculin with actin filaments in vitro. Cell 28: 83-90.

Wilkins JA and Lin S (1986) A re-examination of the interaction of vinculin with actin. J. Cell Biol. 102: 1085-1092.

Willingham MC , Yamada KM , Yamada SS , Pouyssegur J and Paston I (1977) Microfilament bundles and cell shape are related to adhesivness to substratum and are dissociable from growth control in cultured fibroblasts. Cell 10: 375-380.

Wilson AJ and Cunningham MP (1970) Immunological aspects of bovine

trypanosomiasis. II. Antigenic variation in a strain of Trypanosoma congolense transmitted by Glossina morsitans. Trans. R. Soc. Trop. Med. Hyg. 64: 818-821.

Wray W , Boulikas T , Wray VP and Hancock R (1981) Silver staining of proteins in acrylamide gels. Anal. Biochem. 118: 197-203.

Zeledon R , Alvarenga NJ and Schosinsky K (1977) Ecology of Trypanosoma cruzi in the insect vector. Proc. Int. Symp. Int. Cong. Protozool., 5th. New York. Sci.Publ. 347, pp.59-70. Symp. on Chagas' Disease.