ULTRASTRUCTURAL STUDIES ON PARASITIC FLAGELLATES

by

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Statements on collaboration and publication.

The Introduction and Section 2 are unpublished and were composed by myself.

Section 3 is accepted for publication in Acta Tropica and is my own work.

Section 4 was published in J.Cell Sci. All electron microscopy including the freeze-fracture interpretation was done by me. Interpretation of the stages in trypanosome differentiation was by Prof. K.Vickerman.

Section 5 was published in Z.Parasitenkunde and was entirely my work apart from contributions to the Discussion suggested by Dr.G.H.Coombs and Prof. Vickerman.

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Section 7 is in press in Exp. Parasitol. My contribution was the electron microscopy including the cytochemistry and immunocytochemistry and the production of the immunogold reagents plus interpretation of the relevant results.

C O N T E N T S

The figures and tables in this thesis are numbered sequentially in each section, and each section is followed by its own list of references.

Abbreviations

AnTAT - Antwerp Trypanozoon Antigenic Type

AnTAR - Antwerp Trypanozoon Antigenic Repertoire

ATPase - Adenosine triphosphatase

BA - British Association thread type

bSA - Bovine serum albumen

DEAE - Diethylaminoethyl

DMSO - Dimethylsulphoxide

DNA - Deoxyribonucleic acid

DNase - Deoxyribonuclease

DTT - Dithiothreitol

EATRO - East African Trypanosomiasis Research Organization

EDTA - Ethylenediaminetetra acetic acid

EF - Exoplasmic face

ETAR - Edinburgh Trypanozoon Antigenic Repertoire

F_c - part of the immunoglobulin molecule containing the sites for complement fixation

FC - Filipin-induced Complex

FCS - foetal calf serum

FIL - Filipin-Induced Lesion

HDL - High Density Lipoprotein

IMP - Intramembrane Particle

k-DNA - kinetoplast deoxyribonucleic acid

NAD - Nicotinamide adenine dinucleotide

NADPH - Nicotinamide adenine dinucleotide, reduced form

PF - Protoplasmic face

PSGEMKA - buffer comprising; 20mM sodium phosphate, 104mM sodium chloride, 10mM magnesium chloride, 10mM potassium chloride, 0.5 mM EDTA, 5.5mM D-glucose, 0.02%($^{W}/_{V}$) BSA.

PVP - Polyvinyl py rolidone

RB - Respiratory burst

RNase - Ribonuclease

SDS - Sodium dodecyl sulphate

SEM - Scanning Electron Microscopy

TEM - Transmission Electron Microscopy

VAT - Variable Antigen Type

VSG - Variable Specific Glycoprotein

WHO - World Health Organization

PLAN OF INVESTIGATION

A series of ultrastructural studies on the function of Trypanosoma brucei and Leishmania species using, principally, the freezefracture technique with transmission electron microscopy are presented. Few ultrastructural studies on trypanosomatids have been attempted using the freeze-fracture technique and those which have been reported deal largely with parasites separated from blood or cultures.

In order to investigate the invertebrate host/parasite relationships in the salivary glands of the tsetse fly in situ a number of modifications to a basic freeze-fracture apparatus have been undertaken. Development of the capabilities of the apparatus to handle small tissue fragments have been extended to the production of complementary replicas to provide additional information regarding intra-membrane organization.

Freeze-fracture cytochemistry of membrane sterol distribution in various life-cycle stages of both T.brucei and L.mexicana was followed using the modified apparatus. Parallel studies of transmission electron microscopy of sectioned specimens and the application of cytochemical and immunocytochemical methods employing conventional heavy metal precipitation and gold markers respectively, were applied. Such investigations were to illustrate a structural basis for biochemical findings of unusually high proteinase activity in the intracellular stage of L.mexicana in which numerous lysosome-like organelles are present.

The complete reconstruction of the intracellular leishmanial parasite from serial sections was undertaken to extend knowledge of the structural details of this stage to complement the foregoing studies.

SUMMARY

(1) Modification to the construction and operation of a basic Nanotech/Polaron type freeze-fracture module have been made to examine stages in the trypanosome life-cycle in insect tissue samples using the knife-fracture method for freeze-fracture replication. Construction and installation of a complementary replica device further improved the specimen handling capabilities to permit the production of complementary fracture faces of tissue and suspension samples. Details of vacuum system, cold stage and evaporation source improvements are described which result in more reliable production of freeze-fracture replicas. The reduction in size of the specimen support (increasing the cooling rate of the sample) and adoption of liquid propane/ isopentane as coolant allowed the examination of non-cryoprotected suspension samples.

(2) The surface membranes of bloodstream long slender ,short stumpy and procyclic stages of Trypanosoma brucei brucei were compared with respect to freeze-fracture electron microscopy, intramembrane particle (IMP) distribution and β-hydroxysterol content as shown by the characteristic intramembrane lesions induced by the polyene antibiotic filipin. Little difference was observed between IMP density of long slender and short stumpy form body membranes: IMPs were more abundant on the protoplasmic face (PF) than on the exoplasmic face (EF). The procyclic culture form body membrane showed an increased density of PF IMPs and a decreased density of EF IMPs over their bloodstream short stumpy form predecessors. Flagellar membrane fracture faces displayed higher IMP densities than body membrane fracture faces of the same trypanosome. The numbers of filipin -induced lesions (FIL) indicated an increased level of β-hydroxysterols in the short stumpy forms relative to the level in the long slender blood forms.

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FIL density was further increased in the body membrane of the procyclic culture forms. FIL density was higher in the flagellar membrane than in the corresponding body membrane and FIL were excluded from flagellum to body attachment zones of the flagellar membrane of all stages. The polarity of the FIL in the surface membranes was reversed on transforming from bloodstream to culture procyclic stages. These observations indicate qualitative differences between the surfaces of the three stages, independent of the presence or absence of the surface coat.

(3) Acquisition of the variable antigen-containing surface coat of Trypanosoma brucei occurs atthe metacyclic stage in the salivary glands of the tsetse fly vector. The differentiation of the metacyclic trypanosome in the gland has been studied by scanning electron microscopy and by transmission electron microscopy of thin sections and freeze-fracture replicas. The uncoated epimastigote trypanosomes (with a prenuclear kinetoplast) divide while attached to the salivary gland epithelium brush border by elaborate branched flagellar outgrowths, which ramify between the host cell microvilli and form punctate hemidesmosome -like attachment plaques where they are indented bythe microvilli. These outgrowths become reduced as the epimastigotes transform to uncoated trypomastigotes (with postnuclear kinetoplast), which remain attached and capable of binary fission. The flagellar outgrowths disappear but the attachment plaques persist as the uncoated trypomastigotes (premetacyclics) stop dividing and acquire the surface coat to become 'nascent metacyclics'.

Coat acquisition therefore occurs in the attached trypanosome and not, as previously thought ,after detachment. Coating is accompanied by morphological changes in the glycosomes and mitochondrion of the parasite. Freeze-fracture replicas of the host-parasite junctional complexes show membrane particle aggregates on the host membrane but not on the parasite membrane.

It is suggested that disruption of the complex occurs when maximum packing of the glycoprotein molecules has been acheived in the trypanosome surface coat, releasing the metacyclic into the gland lumen.

(4) The freeze-fracture replica technique has been used to compare the plasma membranes of amastigote and promastigote stages of Leishmania mexicana mexicana with respect to IMP distribution and to β-hydroxysterol content as revealed by the distribution of lesions induced by FIL. IMP density was greater in promastigote than in amastigote plasma membranes. IMPs were more abundant in the protoplasmic face (PF) than in the exoplasmic face (EF) of promastigotes, but this situation was found to be reversed in amastigotes. FIL in glutaraldehyde-fixed parasites indicated higher levels of β-hydroxysterols in the amastigote than in the promastigote plasma membrane, and in the promastigote flagellar membrane than in the body membrane. Amphotericin B (a related polyene antibiotic used in chemotherapy of leishmaniasis) induced IMP aggregation in the PF of unfixed amastigotes but did not appear to influence sterol distribution as demonstrated by freeze-fracture of subsequently-fixed and filipin-treated organisms.

(5) Computer-aided reconstruction from serial sections has been used to analyse the 3-dimensional structure of entire amastigotes of Leishmania mexicana mexicana and to determine the number, arrangement and volume of each organelle. In two reconstructions the lysosome-like 'megasomes' were the most numerous organelles, there being 34 in one amastigote, and they comprised 15% of the total cell volume. In contrast, as few as 9 glycosomes were present, accounting for less than 1% of the cell volume. The unitary nature of the mitochondrion was confirmed and its complex basket-like structure was revealed.

(6) Leishmania mexicana mexicana (M379) amastigotes were found to contain much higher activities than cultured promastigotes of five putative lysosomal enzymes: cysteine proteinase; aryl-sulphatase (EC 3.1.6.1.); β-glucuronidase (EC 3.2.1.31.); DNase (EC 3.1.22.1.), and RNase (EC 3.1.27.1.). The release profiles of the first three of these enzymes from digitonin-permeabilized amastigotes suggests that they are located in organelles. Cytochemical staining for cysteine proteinase, using gold-labelled antibodies and arylsulphatase, showed that both were present in large organelles previously named 'mega-somes'. Comparative studies with L.mexicana amazonensis (LV 78), L.donovani donovani (LV 9), and L.major (LV 79) revealed that L.mexicana amazonensis was similar to L.mexicana mexicana in possessing both high amastigote cysteine proteinase activity and large numbers of megasome organelles in amastigotes, whereas the other two species lacked both these features. The results suggest that the presence of numerous lysosome-like organelles in the amastigote is a characteristic of the L.mexicana group of parasites.

Section 1.

Introduction.

INTRODUCTION

1.1. The trypanosomatid flagellates.

The Trypanosomatidae are a family of flagellate Protozoa in the Order Kinetoplastida Honigberg 1963. The possession of a kinetoplast - a mass of mitochondrial DNA usually located close to the base of the flagellum - distinguishes this Order from other zooflagellates. Members of the Family Trypanosomatidae are recognised by the possession of a single flagellum; members of the other family, the Bodonidae, have two flagella.

Trypanosomatid species of the genera Trypanosoma and Leishmania may be pathogenic to man or to his domestic animals. Species of Trypanosoma cause human sleeping sickness in the African continent, Chagas' disease in South America and cattle trypanosomiasis mainly in Africa, while species of Leishmania cause visceral and a variety of skin afflictions in man. A recent report cited 1.2 million persons infected and 1000 deaths per year from Leishmaniasis and 1 million persons infected and 5000 deaths per year from African trypanosomiasis (based on W.H.O. figures, Walsh, 1984). In Africa the indirect effects on man of trypanosomiasis of domestic stock are possibly more serious, as the consequent deprivation of protein considerably exacerbates the hardship of rural existence.

The parasites are all borne by arthropod vectors: tsetse flies (Glossina spp.) transmit the African trypanosomiases, triatomine bugs Chagas' disease, and sandflies (Phlebotominae) the leishmanias. Traditionally combating these diseases has been by vector control. The difficulties encountered in establishing such control in African trypanosomiases invoke ecologically undesirable, costly and impractical measures requiring insecticide application over wide areas and the subsequent maintenance of these zones tsetse-free (Jordan, 1985). Vector control of Chagas' disease relies on separating bug

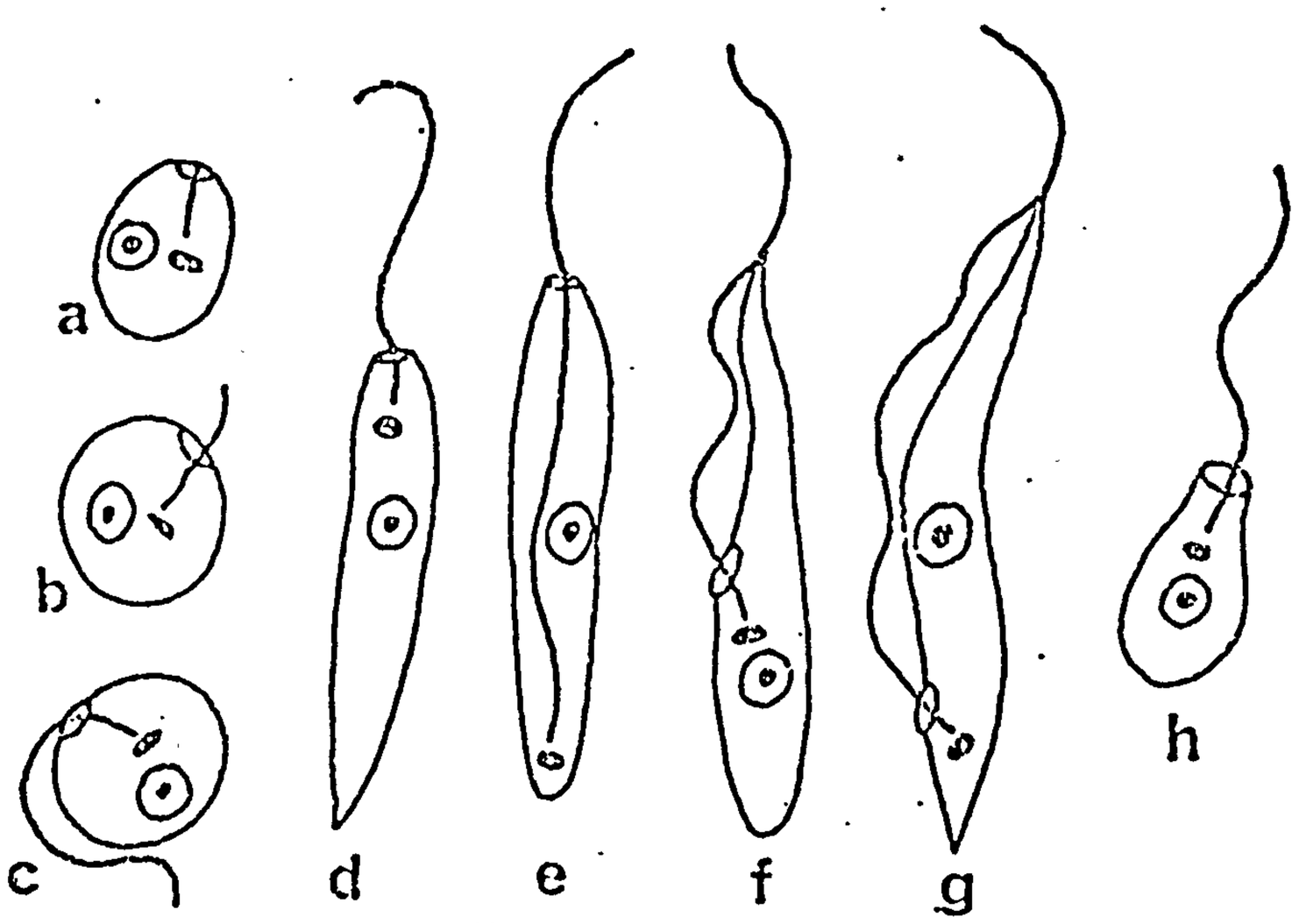
from man by replacing housing with constructions less liable to harbour the vector. Interruption of host contact with sandflies would appear to be the most effective way of breaking the transmission cycle in leishmaniasis. Taken alone however vector control measures have not proved effective. Chemotherapy as a disease control alternative has been vitiated by toxicity of the drugs used against the diseases and by the development of drug resistance (Gutteridge, 1985).

A more comprehensive knowledge of parasite biology is therefore necessary to understand parasite survival strategies. Studies on host-parasite relationships in particular may suggest ways of interrupting the developmental cycle - for example by suppressing development of infecting forms in the mammalian host. Strategic combinations of drug therapy, vector control and immunoprotection founded on understanding of parasite survival mechanisms may provide the best approach to control.

1.2. Patterns of development

During the elaborate developmental cycles of these flagellates in mammal and vector, changes occur in the morphology of the parasite. This cyclical transformation includes changes in the position of the kinetoplast within the body in relation to the nucleus and to the ends of the flagellate's body. The extent of the flagellum and its relationship with the body of the flagellate may also change. A spectrum of morphological stages is recognised and these stages have been given different names (Hoare and Wallace, 1966) as follows (see Fig. 1): amastigote - round to oval body, flagellum short, not emerging from pocket; promastigote - kinetoplast close to anterior end of elongate body, flagellum emerging anteriorly and unattached; opisthomastigote - similar, but kinetoplast is post-nuclear and

Fig. 1. Diagram of stages in Trypanosomatidae. a. Amastigote; b, c. Sphaeromastigotes; d. Promastigote; e. Opisthomastigote; f. Epimastigote; g. Trypomastigote; h. Choanomastigote. (Based on Hoare & Wallace 1966.)


a
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flagellar pocket forms long canal to anterior end of body; choanomastigote - body pyriform, kinetoplast just in front of nucleus, flagellum emerges anteriorly; epimastigote - pre-nuclear kinetoplast, flagellum emerges from pocket part way along body and is attached to body along its anterior portion; trypanomastigote - similar, but kinetoplast and flagellar pocket are post-nuclear. The term sphaeromastigote is sometimes used for a rounded body with an emerging flagellum. The different genera of trypanosomatids are characterised according to which of these stages are present in the life-cycle and whether the life-cycle involves one host or alternates between two different hosts, usually an invertebrate and a vertebrate.

1.3. The genera Leishmania and Trypanosoma

This thesis is concerned solely with species of the genera Leishmania and Trypanosoma. The characteristics of these genera as compared with the other trypanosomatid flagellates are given in Table 1. A classification of the species of Trypanosoma parasitizing mammals and based on the station in the vector where development of the infective form (metacyclic) of the parasite occurs was proposed by Hoare (1964). Two sections of the genus were recognized - Stercoraria and Salivaria - and these were divided into subgenera on the basis of the bloodstream form morphology of the parasite and details of its development in both mammalian host and vector. The African trypanosomes (Salivaria) develop in Glossina species (tsetse) - except those conveyed by mechanical transmission - and enter the mammalian host by inoculation with the vector's saliva after final development in either the mouthparts - Subgenera Duttonella and Nannomonas - or the salivary glands - Subgenus Trypanozoon (see Table 2). The characteristic features of the life-cycles of salivarian trypanosomes are shown in Table 3. The stercorarian trypanosomes such as

TABLE 1.

PRINCIPAL GENERA OF THE TRYPANOSOMATIDAE

GENUS	DIAGNOSTIC CHARACTERS[a]	NUMBER OF DESCRIBED SPECIES	HOSTS & PRACTICAL SIGNIFICANCE
LEPTOMONAS Kent 1880	Monogenetic; *promastigotes* and cysts only in life cycle	c.70	Mainly insects (Hemiptera, Diptera, Hymenoptera, Blattoidea, Lepidoptera, Siphonaptera, Anoplura), rarely other invertebrates and protozoa. Some so called "lizard leishmanias" may belong in this genus Non-pathogenic
HERPETOMONAS Kent 1880	Monogenetic, promastigotes and *opisthomastigotes* in life cycle	c.20	Diptera, possible other insect-orders. Non-pathogenic
CRITHIDIA Leger 1902	Monogenetic; *choanomastigotes* only	c.30	Diptera, Hemiptera, Trichoptera; Hymenoptera. Non-pathogenic
BLASTOCRITHIDIA	Monogenetic; *epimastigotes* and cysts only in life	c.30	Diptera, Hemiptera, Siphonaptera and ixodid ticks. Possibly pathogenic in some species.
RHYNCHOIDOMONAS Patton 1910	Monogenetic; *trypomastigote* stage only (but genus poorly known)	3	Diptera, Lepidoptera. Non-pathogenic
PHYTOMONAS Donovan 1909	Digenetic; *promastigotes*	c.20	Plants (Euphorbiaceae, Asclepiadaceae, Moraceae, Palmae, mainly) and phytophagous Hemiptera. Pathogenic species cause hartrot in oil and coconut palms, and wilt disease in coffee plant
LEISHMANIA Ross 1903	Digenetic; intracellular *amastigotes* (mammal) and promastigotes (vector)	c.20	Mammals (Primates, Rodentia, Edentata, Hyracoidea, Carnivora, Marsupialia) and Diptera (Phlebotominae). Pathogenic species in man cause dermal, muco-cutaneous and visceral leishmaniasis
ENDOTRYPANUM Mesnil & Brimont 1908	Digenetic; intraerythrocytic trypomastigotes and epimastigotes (in mammal); promastigotes and amastigotes (in vector).	2	Edentata (sloths) and Diptera (Phlebotominae, genus *Lutzomyia*). Non-pathogenic
TRYPANOSOMA Gruby 1843	Digenetic; *trypomastigotes* (and more rarely epimastigotes or intracellular amastigotes) in vertebrate, epimastigotes (rarely promastigotes, amastigotes) in vector	c.300	Vertebrates (all classes) and Hirudinea or Arthopoda (insects, mites). Pathogenic species cause sleeping sickness and Chagas' disease in man, nagana and related diseases in domestic animals (see Tables 3 & 4)

[a] The morphological type characteristic of each genus is given in italics. In *Endotrypanum* the type found in the mammal depends on the species.

Based on Vickerman, 1986.

TABLE 2. SUBGENERA OF TRYPANOSOMA FROM MAMMALS. DISTINGUISHING FEATURES AND REPRESENTATIVE SPECIES.

SUBGENUS	MAMMALIAN TRYPOMASTIGOTE; DISTINGUISHING FEATURES	REPRESENTATIVE SPECIES	BEHAVIOUR IN MAMMAL
SECTION: STERCORARIA			
SCHIZOTRYPANUM Chagas 1909	Small TPM(15-24μm); short pointed PE; large sub-terminal kinetoplast; long FF.	T.(S.) cruzi	Division as intra-cellular AM in muscle or MNP; ND TPM in blood
		T.(S).dionisii	Division as inracellular EPM in 'pseudocysts' derived from MNP
SECTION: SALIVARIA			
DUTTONELLA Chalmers 1908	Medium TPM(21-26μm); blunt, small (14-17μm) to rounded PE; large terminal kinetoplast; long FF.	T.(D.) vivax	Division in BS as medium TPM; wholly intra-vascular
		T.(D.) uniforme	As T. vivax but TPM smaller
NANNOMONAS Hoare 1964	Small TPM(12-18μm); blunt PE; medium, sub-terminal marginal kinetoplast; no FF.	T.(N.) congolense	Division in BS as TPM attached to endothelia
		T.(N.) simiae	As T. congolense, but long and short forms occur (pleomorphic)
PYCNOMONAS Hoare 1964	Small TPM(8.5-19μm); very short pointed PE; small sub-terminal kinetoplast; short FF.	T.(P.) suis	Division in BS as TPM
TRYPANOZOON Lühe 1906	Pleomorphic TPM; long slender forms(mean 30μm) with long FF and short stumpy forms (mean 18μm) with no FF; small sub-terminal kinetoplast	T. brucei	Dividing slender TPM in blood, lymph, CT Non-dividing stumpy TPM in blood & lymph
		T.evansi T. equiperdum	As T. brucei but monomorphic

Abbreviations: AM-amastigote; BS-bloodstream; CT-connective tissues; EPM-epimastigote; FF-free flagellum; ND-non-dividing; MNP-mononuclear phagocyte; PE-posterior extremity; TPM-trypomastigote

Based on Vickerman, 1986.

TABLE 3.

SALIVARIAN TRYPANOSOMES OF MAMMALS:
HOSTS, TRANSMISSION AND RELATION TO DISEASE OF MAN AND DOMESTIC ANIMALS

SPECIES	MAIN HOSTS/RESERVOIRS	VECTORS/TRANSMISSION	DISEASE	GEOGRAPHICAL DISTRIBUTION
T.(Trypanozoon) brucei brucei	Ruminants, camels, pigs, carnivores	Tsetse flies(C;AS;SG)	Nagana	W., C., E. & parts of S. Africa
T.(T) brucei rhodesiense	Man, cattle, antelopes; carnivores	Tsetse flies(C;AS;SG)	Acute sleeping sickness	E. Africa
T.(T) brucei gambiense	Man, domestic pig, dog	Tsetse flies(C;AS;SG)	Chronic sleeping sickness	W. Africa
T.(T) evansi	Camels, equines, ruminants, Indian elephant, carnivores, vampire bat	Tabanid flies(M); Vampire bats	Surra	N. Africa, S. Asia, E. Indies, Mauritius, C. & S. America
T.(T) evansi equinum	Equines, ruminants, pig, capybara, vampire bat	Tabanid flies(M); vampire bats	Mal de caderas	C. & S. America
T.(T) equiperdum	Equines	Venereal contact	Dourine	N. Africa, S. Europe, S. America
T.(Pycnomonas) suis	Pigs, warthog	Tsetse flies(C;AS;SG)	Acute in piglets	C. & E. Africa
T.(Duttonella) vivax	Ruminants, equines, bushpig	Tsetse flies(C;AS;P)	Nagana	W., C. & E. Africa
T.(Nannomonas) congolense	Ruminants, equines, pigs, carnivores, rarely camels	Tsetse flies(C;P;S;P)	Nagana	W., C. & E. Africa

Abbreviations: Transmission C - cyclical; M - mechanical. Metacyclics formed in: AS - anterior station, P - in proboscis, SG - in salivary glands; PS - posterior station.

Based on Vickerman, 1986

TABLE 4. SPECIES OF LEISHMANIA CAUSING DISEASE IN MAN.

SPECIES	DISEASE/LESION	DISTRIBUTION	RESERVOIR HOSTS
OLD WORLD SPECIES[a]			
L. tropica[c]	Dry cutaneous; chronic; urban OS;(LR)	Europe, Asia, N. Africa	Dogs
L. major	Wet cutaneous; acute; rural OS.	Asia, Africa	Rodents
L. aethiopica	Dry cutaneous OS; (mucocutaneous, DCL)	Ethiopia, Kenya	Hyrax
L. donovani donovani[d]	Visceral Kala azar (PKADL)	Africa, Asia	None known
L. donovani archibaldi[d]	Visceral	Kenya, Sudan	Rodents
L. donovani infantum[c]	Infantile visceral	Mediterranean	Dogs, foxes porcupine
NEW WORLD SPECIES[b]			
L. mexicana mexicana[c]	Cutaneous (Chiclero's ulcer)	Central America	Rodents
L. mexicana amazonensis[c]	Cutaneous (DCL)	Brazil	Rodents, oppossums
L. mexicana pifanoi[c]	Cutaneous (DCL)	Venezuela, Brazil	Rodents
L. braziliensis braziliensis	Mucocutaneous; (Espundia) metastasising	S. America (exc. Argentina, Chile)	Rodents
L. braziliensis guyanensis	Cutaneous (Pian bois); metastasising	Guyanas, N. Brazil	Edentates
L. braziliensis panamensis	Cutaneous; metastasising	Panama	Forest rodents, edentates, procyonids
L. peruviana[e]	Cutaneous(Uta)	Peruvian Andes	Dogs
L. chagasi[c]	Infantile Visceral	S. America	Foxes

Footnotes:-

a Vectors, species of Phlebotomus
b Vectors, species of Lutzomyia
c Incidentally parasites of man
d Primarily parasites of man (anthroponotic)
e Only New World species not associated with forest habitat.

OS - Oriental Sore; (DCL) - disseminative cutaneous leishmaniasis in some individuals in absence of cell-mediated immunity; (LR) - Leishmaniasis recidivans, chronic, nonhealing lesion not responding to treatment; (PKADL) - post-Kala azar dermal leishmaniasis, skin lesions occurring after apparent cure of visceral leishmaniasis. Abbreviations in brackets represent occasional complications.

Based on Vickerman, 1986.

Trypanosoma cruzi, the causative agent of Chagas' disease (Table 2) enter their host by faecal contamination during vector feeding, the parasites maturing in the insect hind-gut.

The genus Leishmania has not yet been divided into subgenera; the characteristics of the various species and sub-species are presented in Table 4.

1.4. The life-cycle of leishmanias

Leishmania life-histories are characterised by the presence of intracellular amastigotes in the mammal and free or attached promastigotes in the sandfly vector. Phlebotomid flies are the only insects capable of transmitting leishmanias (Williams and Coelho, 1978). Infective promastigotes, transmitted by fly bite, transform into intracellular amastigotes in the mammalian host mononuclear phagocyte system (Bray, 1974).

Leishmania species differ in their pattern of development in the sandfly. Three sections of the genus are recognized - Hypopylaria, Peripylaria and Suprapylaria - according to the regions around the pyloric valve colonized by developmental forms of the parasite in the gut of the fly (Lainson and Shaw, 1976). Only part of the promastigote population in the fly mid-gut and proboscis is mammal-infective; these individuals differ from the rest in their surface antigen and lectin-binding properties (Sacks and Perkins, 1984 1985; Sacks et al., 1985). These non-dividing vector forms will proceed to transform into amastigotes and divide after ingestion by mammalian macrophages. They escape to spread the infection in the mammal by repeated rounds of cell invastion and multiplication. Whether new cells are infected by lysis and uptake of parasites by other phagocytes is uncertain: infected cells could farm out their

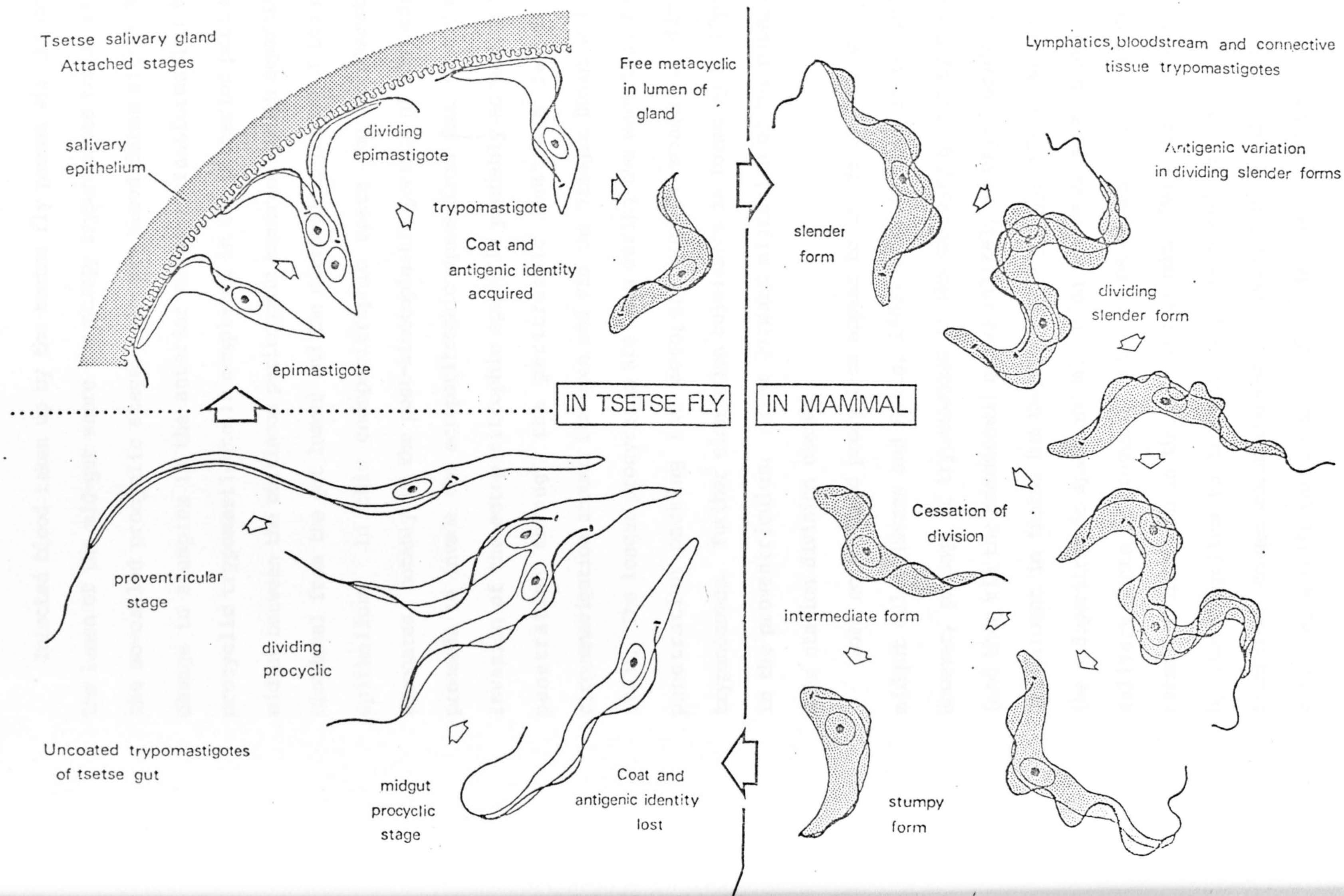
parasites at cell division. Infected phagocytes are taken up during a sandfly bloodmeal to infect the vector again.

1.5. The life-cycle of Trypanosoma brucei and other salivarian trypanosomes

Trypanosomes tend to have a greater number of developmental stages in their life-cycles than do leishmanias; the various stages are also found more widely distributed within both vector and mammal host. The life-cycle of Trypanosoma brucei as a representative salivarian trypanosome is shown in Fig. 2. After inoculation into mammalian dermal connective tissue by a probing tsetse, the T. brucei trypomastigote metacyclic trypanosomes set up a local inflammatory reaction - the chancre - from which they emerge to enter the local lymphatics and subsequently, the bloodstream and connective tissues. During this initial adaptation to the mammal from very different conditions in the tsetse saliva, the trypanosome is in a non-dividing phase and undergoes elongation to the long slender morphology. The establishment of the infection in the mammal is then through rapid binary fission of the slender forms in the blood (doubling time about 6 hours; Seed, 1978) leading to a parasitaemia which then goes into remission. A relapsing parasitaemia with recrudescence following remission is characteristic of salivarian trypanosome infections in the mammal.

During remission the proportion of slender trypanosomes in division is reduced and a non-dividing short stumpy form is observed which persists after destruction of most of the slender bloodstream population (Balber, 1972) by IgM antibody (Seed, 1977). These stumpy forms are recognized by the majority of workers as being responsible for establishing infection in the tsetse after ingestion of an infected bloodmeal (Robertson, 1912, 1913).

Figure 2. Diagram of the life-cycle of Trypanosoma brucei showing developmental and multiplicative stages. Those stages possessing a variable antigen-containing surface coat are stippled.


Tsetse salivary gland
Attached stages
salivary epithelium
dividing epimastigote
trypomastigote
Coat and antigenic identity acquired
epimastigote
Free metacyclic in lumen of gland
IN TSETSE FLY
IN MAMMAL
Lymphatics, bloodstream and connective tissue trypomastigotes
slender form
Antigenic variation in dividing slender forms
dividing slender form
Cessation of division
intermediate form
stumpy form
proventricular stage
dividing procyclic
Uncoated trypomastigotes of tsetse gut
midgut procyclic stage
Coat and antigenic identity lost

Infected blood taken up by the tsetse fly passes via the crop to the lumen of the mid-gut where the stumpy trypanosomes transform into the so-called procyclic stage. Slender blood forms either die or change to stumpies in the anterior midgut; transformation to the procyclic trypomastigote is completed in the posterior part of the midgut between the chitinous peritrophic membrane (which separates the bloodmeal from the gut lumen) and the microvillar border of the midgut epithelium. In this endoperitrophic space the trypomastigote elongates, notably in the post-kinetoplastic portion of the body, and proceeds to invade the ectoperitrophic space from four days onwards. Invasion of the ectoperitrophic space is probably accomplished by penetration through the peritrophic membrane rather than circumnavigation around its open end via the hindgut (Evans and Ellis, 1983). The longer procyclics are seen during the week that follows penetration packing the ectoperitrophic space as dividing trypanosomes. Further elongation culminates in forms up to 60μm long in the proventriculus. After forward migration of the parasite to this station division ceases.

Some established procyclics appear to invade or pass through the midgut cells (Evans and Ellis, 1983) killing them in the process. However, passage of trypanosomes to the salivary glands by this route (and then via the haemocoel) seems unlikely as trypanocidal factors are present in tsetse haemolymph (Croft et al., 1982). Reinvasion of the endoperitrophic space and migration via oesophagus, mouthparts and salivary ducts by proventricular forms seems probable in order to establish infection in the salivary glands. Here the parasites attach by the flagellum to the salivary gland cells: further attached transition stages occur eventually giving rise to the free-swimming, coated metacyclic forms which infect the mammal and thus complete the cycle.

1.6. Structural organization of trypanosomatid flagellates

By transmission electron microscopy of sections, a uniform structural organization is seen to be common to all trypanosomatid flagellates. The principal structures of a Leishmania mexicana promastigote as a representative trypanosomatid in longitudinal section are shown in Fig. 3. Typical eukaryote organelles such as nucleus, Golgi apparatus, endoplasmic reticulum lysosomes and basal bodies are found within a dense cytoplasmic matrix packed with free ribosomes and bounded by a plasma membrane which is continuous with the flagellar membrane.

The ultrastructural features distinctive for trypanosomatids are as follows. The flagellum possesses a paraxial rod in addition to the axoneme and arises from a flagellar pocket or pit. The single mitochondrion typically extends the length of the body and is linear, branched or reticulate; it contains a prominent DNA nucleoid or kinetoplast located close to the basal bodies which insert on (or close to) the mitochondrial envelope. A second barren basal body lies alongside the flagellum base. The mitochondrial cristae are discoid or tubular. The primary enzymes of the glycolytic pathway are housed in glycosomes - microbody-like organelles unique to the family. The cytoskeleton is composed of supporting microtubular elements associated with the surface membrane (pellicular microtubules). A microtubule-surrounded cytopharynx is present in some but is presumably secondarily lost by most trypanosomatids. There are no storage carbohydrate bodies but lipid globules are commonly present. The Golgi apparatus lies typically close to the flagellar pocket, but is not connected to the basal bodies. A pulsatile or contractile vacuole is rarely present, emptying into the flagellar pocket.

Figure 3. The ultrastructural features of trypanosomatids. The diagram on the left shows the principal structural features of the transmission electron micrograph of a sectioned Leishmania mexicana promastigote on the right. An additional flagellum is also present. Scale bar = 0.5 μm

Abbreviations:

ax - axoneme

bb - basal body

d - desmosome

fl - flagellum

fp - flagellar pocket

g - Golgi apparatus

gly - glycosome

kp - kinetoplast

l - lipid globule

m - mitochondrial profile

n - nucleus

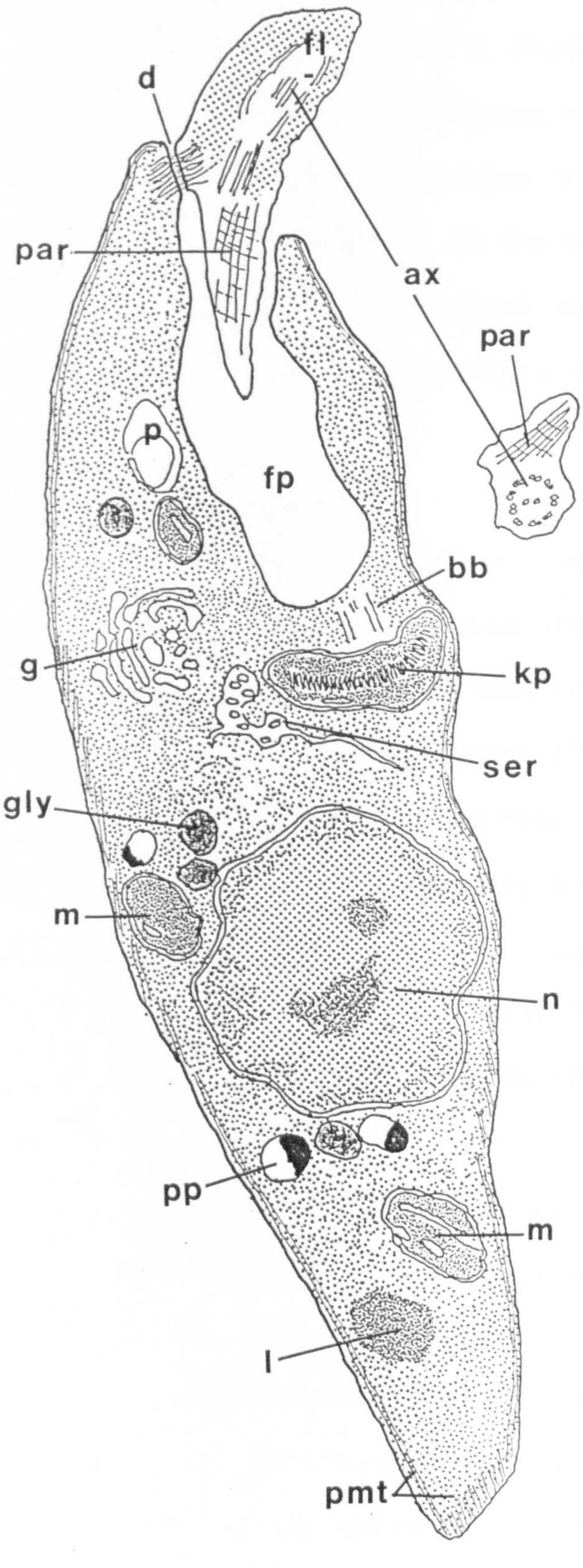
p - pulsatile vacuole

par - paraxial rod

pp - polyphosphate granule

pmt - pellicular microtubule

ser - smooth endoplasmic reticulum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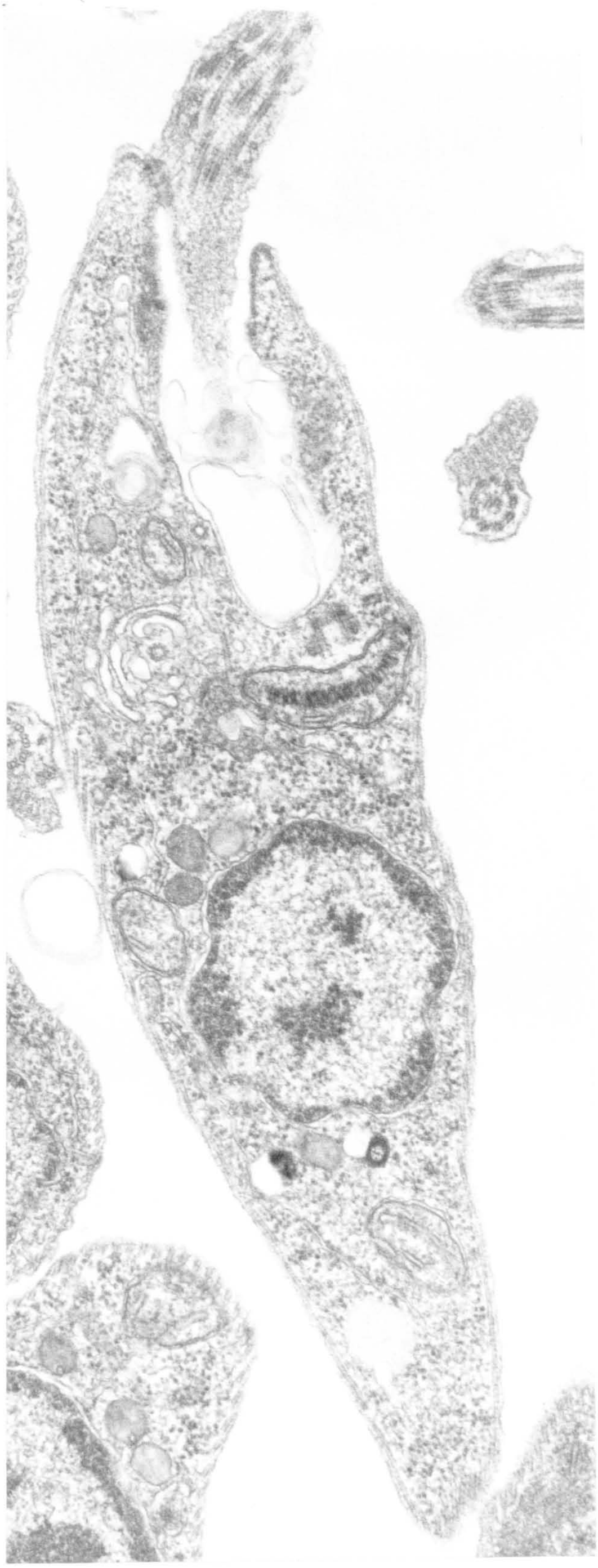

1.7. The kinetoplast/mitochondrion

Trypanosomatids possess a single mitochondrion, which may be extensively branched, containing a mass of DNA in most cases located at the base of the flagellum in a region termed the kinetoplast capsule. The arrangement of the kinetoplast DNA (k-DNA) as a fibrous nucleoid within the kinetoplast capsule region is a constant feature in trypanosomatids and provides a distinguishing taxonomic character.

1.7.1. The kinetoplast

The kinetoplast nucleoid is a network composed of thousands of interlocked (catenated) circular DNA molecules concentrated in the one region of the mitochondrion which constitute between 10 and 25% of the total DNA of the cell (Englund, 1981). As seen in section, size and shape of the network varies from a slightly concave disc of around 80nm thickness in leishmanias, to a sphere 0.43μm in diameter in T. cruzi trypomastigotes (data from Simpson, 1972). In vertical section the kinetoplast is seen as a band of fibrils; this single band may be replaced by multi-layered rows in T. cruzi trypomastigotes (Brack, 1968).

Using electron microscopy of spread and shadowed DNA molecules (Kleinschmidt, 1968) the physical characters of k-DNA have been determined. After cesium chloride density centrifugation of lysates the rapidly sedimenting k-DNA networks can be spread on a protein film for electron microscopic examination. Maxicircle and minicircle components of the network can be distinguished on their contour length. The 20,000-30,000 minicircles present per kinetoplast make up 90-95% of the network mass and are each about 0.5μm in contour length (Englund, 1981). A relationship between the thickness of the kinetoplast and the diameter of its minicircles has been suggested

(Simpson, 1972); there is a close correlation between the half circumference of the minicircle and the thickness of the nucleoid as seen in sections. This correspondence has recently been established for the T. vivax kinetoplast (Borst et al., 1985) which has the smallest minicircles found in any trypanosomatid and a corresponding diminutive thickness of the k-DNA nucleoid (55nm as compared with 91nm in T. brucei).

No clear role has emerged as yet for the minicircle; its small size precludes it from encoding large proteins and as minicircles have not been shown to transcribe, their role may be merely a structural one (Borst et al., 1976) in maintaining the network. The other component of the k-DNA, the maxicircle has a contour length of 6-12μm and in T. brucei about 50 copies are present per network. Maxicircles have been characterized in several trypanosomatids and are comparable to mitochondrial DNA of other cells in that they are transcribed (Hoeijmakers, 1981), one of their major products being mitochondrial ribosomal RNA. Maxicircle DNA has also been found to code for several respiratory enzyme subunits including those of cytochrome b, cytochrome oxidase and mitochondrial ATPase (Stuart, 1983).

1.7.2. Dyskinetoplasty

The conspicuous absence of a typical kinetoplast is occasionally noted in certain species of the sub-genus Trypanozoon, especially T. equiperdum, transmitted venerally between horses, and T. evansi transmitted mechanically by fly bite between ungulates outside the tsetse belt of Africa. Trypanosomatids of other genera or sub-genera of Trypanosoma are incapable of survival without the kinetoplast.

In dyskinetoplastic trypanosomes the single k-DNA nucleoid is replaced by clumps dispersed throughout the mitochondrion. This condition is believed to occur by mutation and can be induced by

treatment with DNA-intercalating drugs (e.g. acriflavine) (Hajduk, 1978). The mutants arise due to faulty replication and the condition - dyskinetoplasty (or K^-) - is irreversible, although the total k-DNA content relative to the normal amount (of a K^+ organism) may be unchanged.

In some species, a few maxicircles have been found though minicircles cannot be visualized in spreads and the nature of the mutation expressed seems to depend upon the extent of deletions in the maxicircle k-DNA. Opperdoes (1976) argued that important deletions cause the loss of the necessary part of the maxicircle complement required to obtain a functional mitochondrion. Thus studies on T. evansi and T. equiperdum failed to demonstrate the presence of an oligomycin-sensitive ATP-ase (present in T. brucei and which functions in the mitochondrial phosphorylating system) in these species. T. equiperdum appears to lack maxicircles and this may account for the absence of the enzyme in this species; minicircles networks are, however, apparently normal.

1.7.3. The mitochondrion

The unitary mitochondrion in trypanosomatids is found as a single or branched organelle lying beneath the pellicle. A branched mitochondrion will usually contain typical plate-like cristae although these may disappear as the form of the organelle changes to reduce to a single canal in certain stages having digenetic life-cycles, especially in T. brucei.

Accompanying the slender-stumpy transformation in bloodstream T. brucei, a change occurs in the single mitochondrion from a simple acristate narrow tube, running the length of the body, to a swollen organelle with tubular cristae (Vickerman, 1965). A threefold

increase in the cell volume and around four times increase in mitochondrial volume have been demonstrated using morphometric methods on ultra-thin sections of T. brucei (Hecker et al., 1972) as the organism progresses from slender to stumpy form.

Correlated changes in energy metabolism occur with this transformation (Vickerman, 1965). Glucose, the principal energy source of slender bloodstream forms is replaced by amino acids, and especially proline, present in the fly midgut. The stumpy form is thought to be adapted to survival in the midgut by virtue of its possession of proline and ketoglutarate oxidases, absent from slender forms (Brown, Evans and Vickerman, 1973).

Further development of trypanosome mitochondrial activity occurs in the posterior region of the tsetse midgut lumen. Here the ingested stumpy forms tranform into longer, dividing so-called procyclics. These acquire succinoxidase activity and greatly augment their proline oxidase activity as the swollen mitochondrion becomes extensively branched. Later, the acquisition of cytochrome-mediated terminal respiration and sensitivity to cyanide parallels the development of plate-like cristae in the proventricular forms which possibly represent maximum mitochondrial activation (Brown, Evans and Vickerman, 1973); cyanide sensitivity is absent from blood forms which rely on aerobic glycolysis to provide ATP. Reduced NAD is reoxidised via the cyanide-insensitive glycerophosphate oxidase system for terminal respiration in the mitochondrion, in concert with an extra-mitochondrial dehydrogenase located in a unique trypanosomatid organelle, the glycosome (Opperdoes and Borst, 1977).

Progression through the cycle in the fly to the attached epimastigote salivary gland stage is accompanied by replacement of the discoid cristae by many tubular cristae in a less branched mitochondrion. The cristae are reduced in number and the

mitochondrion becomes a single canal on differentiation to the metacyclic stage. Details of the respiratory metabolism of these stages is unknown (Vickerman, 1966; Steiger, 1973).

T. evansi and T. equiperdum, which are monomorphic and not cyclically transmitted, resemble bloodstream T. brucei slender forms morphologically and biochemically (Vickerman, 1971), whereas other salivarian trypanosomes cyclically transmitted by tsetse resemble T. brucei stumpy forms in their bloodstream stages in that T. vivax and T. congolense possess tubular cristae in an expanded mitochondrion; plate-like cristae occur in the vector stages together with mitochondrial reticulation in all but the metacyclic stage (Vickerman, 1969a, 1973, 1974).

In the case of the leishmanias the existence of a mitochondrial cycle is not very apparent (Rudzinska et al., 1964). A similar network of a branched mitochondrion with plate-like cristae occurs in both amastigote and promastigote stages of the life-cycle (Brun and Krassner, 1976) contrasting with the dramatic changes to the mitochondrion and respiration of T. brucei during its life-cycle. The similarities in structure of the mitochondrion throughout the Leishmania life-cycle is parallelled by a lack of major change in respirable substrate utilization. Amastigotes and promastigotes of Leishmania mexicana mexicana both utilize glucose, albeit at different rates (Hart et al., 1981) and a terminal cytochrome chain similar to mammalian cells is present, carbon dioxide and succinate being the main end products of respiration in both stages.

1.8 Glycosomes and other Microbody-like organelles.

A number of morphologically similar, enzyme containing granules collectively named microbody-like organelles or peroxisomes have been described in trypanosomatids. These resemble the microbodies or peroxisomes of other eukaryotes and are single, 6nm membrane bound, generally spherical structures (0.3 μm diameter in T. brucei) with a dense matrix occasionally including a cystalloid. In the course of the life-cycle of some trypanosomatids these organelles change from spherical to bacilliform structures which may vary in electron density.

Recently in T. brucei, however, the location of a number of glycolytic enzymes of the glucose/glycerol to 3-phosphoglycerate pathway, and also the siting of the glycerol-3-phosphate dehydrogenase, was shown to be within the microbody-like organelle, now designated the glycosome. The cyanide-insensitive glycerol-3-phosphate oxidase, previously thought to have been present in these organelles, was shown to be present in the mitochondrion (Opperdoes and Borst, 1977).

Glucose metabolism in bloodstream T. brucei differs from eukaryote glycolysis in that it terminates with the production of pyruvate which is excreted into the bloodstream. In the stationary phase procyclic T. brucei and in T. cruzi epimastigotes and bloodstream trypomastigotes the oxidation of pyruvate to carbon dioxide, succinate or acetate takes place.

In Herpetomonas samueli promastigotes (Souto-Padron and De Souza, 1980), Crithidia fasciculata (Muse and Roberts, 1973) and T. cruzi culture epi- and trypomastigotes (Docampo et al

1976; Mierelles and De Souza, 1980) the enzyme catalase, considered to be a marker for peroxisomes, has been detected cytochemically by the method of Novikoff and Goldfischer (1969). Specific catalase activity may vary from one species to another and it is probable that some negative results obtained with this method indicate too brief an incubation period rather than the complete absence of the enzyme.

Peroxisomes are known to participate in the formation and breakdown of hydrogen peroxide and in oxidation of amino acids in other eukaryotes; these and other functions are likely in trypanosomatids.

Apart from the biochemical studies on glycosomes in T. brucei (where catalase has not been demonstrated, Steiger, 1973) the enzyme content of similar organelles in other species awaits biochemical investigation.

The glycosome in T. brucei also contains the enzymes of other pathways and not merely those of glycolysis: enzymes of the glycerol metabolism, carbon dioxide fixation, pyrimidine synthesis, purine salvage and ether lipid synthesis pathways have been localized in glycosomes biochemically (Opperdoes, 1985).

1.9 Storage bodies

There is no evidence for any form of carbohydrate reserves (e.g. glycogen) in trypanosomatids therefore it must be assumed that protein or lipid might be the respirable substrates. Of the membrane bound inclusions thought to be storage bodies, polyphosphate vacuoles are recognized as comparable in structure and composition to inclusions in unicellular algae (Atkinson, 1974). In trypanosomatids these may be up to 3 µm

in diameter and easily visible by light microscopy. 'Volutin granules' of earlier investigators undoubtedly included polyphosphate vacuoles. The term volutin used to describe the basophilic inclusions in the early literature is misleading in that a heterogeneous collection of organelles may have been stained by the basic thiazine dyes used to detect these granules by light microscopy (Herbert, 1965). In the electron microscope the internal appearance or inherent electron density of some of these inclusions is dependent on the degree of extraction undergone during specimen preparation and often on the elemental composition of their contents.

Lipid droplets can be recognized by the lack of a bounding membrane. Their wide range in size and osmiphilia makes their classification using these criteria difficult. Nevertheless, Steiger (1973) claims to have recognized two classes of lipid in T. brucei, small, deeply osmiophilic droplets in the bloodforms (thought to be reservoirs of lipid for future mitochondrial proliferation) and large, less osmiophilic droplets in the tsetse mid-gut forms (synthesized probably as triglyceride for use in energy metabolism). This supports the contention of Dixon and Williamson (1970) that changes in type and number of lipid storage bodies may relate to trypanosome physiological requirements.

The purpose of triglyceride storage is not clear since the ability of trypanosomatids to utilize fatty acids as an energy source is questionable (Vickerman and Preston, 1976). Hart and Coombs (1982), however, have presented evidence as to the importance of lipids as an energy source for amastigote Leishmania mexicana mexicana. The utilization of fatty acids by amastigotes probably reflects their macrophage intracellular

habitat where triglyceride-rich serum lipoprotein degradation is likely to occur.

1.10 Nucleus, endoplasmic reticulum and Golgi apparatus

In general the trypanosomatid intracellular membrane system is typical of eukaryotes, the nuclear, endoplasmic reticulum and Golgi membranes being interconnected and serving to compartmentalize their associated cellular functions. The molecular composition and function of these endomembrane systems and the changes en route to the plasma membrane remain largely unexplored in trypanosomatids, therefore the following sub-sections are mainly morphological descriptions.

1.10.1 Nucleus

The nucleus of most trypanosomatids is relatively small - a volume of 1 μm^3 having been found in Leishmania mexicana amastigotes for example, (Coombs et al, 1986).

Light microscope observations have been restricted to resolving only gross morphological organization.

A homogeneous single nucleolus is seen, centrally placed in the interphase cell, fragmenting during division in T. brucei (Vickerman and Preston 1976) but dispersing completely to reappear in late division in T. cruzi (De Souza and Meyer, 1974).

The nuclear envelope is composed of two parallel membranes, punctuated by pores, each about 9nm in diameter. The gap between the two membranes is 20-30nm. Pores may vary in density over the nuclear envelope depending on the stage in the cell cycle. The nuclear membrane system is seen in continuity with the endoplasmic reticulum in freeze-fracture

replicas. The outer nuclear membrane is ribosome-studded.

Chromatin is located peripherally on the inner nuclear membrane at interphase and early division and appears to disperse during late division in T. brucei bloodstream forms (Vickerman and Preston 1970). No readily identifiable chromosomes are found during division in trypanosomatids therefore the classical sequence of mitotic events is not observable. Also, the nuclear envelope persists throughout division constricting finally to form two daughter nuclei: its role in partitioning genetic material remains enigmatic. The intra-nuclear microtubule spindle which is believed to elongate the dividing nucleus, is considered in section 1.12.

The apparent lack of both a chromosome condensation cycle and an overt sexual cycle has led to a protracted debate on the ploidy of trypanosomatids. Evidence from nuclear DNA reassociation studies and microspectrofluorimetric measurements of laser-induced fluorescence of DNA indicate that T. cruzi is diploid (Lanar et al, 1981; Castro et al, 1981). In T. brucei electrophoretic variation of 19 enzymes from a series of isolates (Tait 1980) and direct determination of nuclear DNA content by quantitative absorption fluorescence cytophotometry of individual Feulgen-pararosaniline stained cells (Borst et al, 1982) suggest diploidy in this species also. Pulsed field gradient gel electrophoresis, a technique first applied to yeast to fractionate DNA molecules (Schwartz et al, 1983) has provided evidence that, in T. brucei, three chromosomes about 2 megabases long, 6 chromosomes about 200-700 kilobases long and about 100 minichromosomes of size 50-150 kilobases are present (Van der Ploeg et al, 1984). How these mini

chromosomes segregate during nuclear division of the flagellate poses interesting questions particularly in relation to minichromosome association with the spindle microtubules and/or the nuclear envelope during partition.

The basic proteins associated with DNA - the histones - appear in large quantities in the eukaryote nucleus as highly conserved small proteins bound tightly to DNA due to their positively charged arginine and lysine residues, forming the unit particles of chromatin - the nucleosomes. Reaction products deposited using the ethanolic phosphotungstic acid and post-formalin ammoniacal silver techniques at electron microscope level (Gordon and Bensch, 1968) show basic proteins to be present in trypanosomatid nuclei (Souto-Padron and De Souza, 1978).

Work on isolated purified nuclei (Pereira et al, 1978) has enabled closer examination of histone complement in T. cruzi DNA (Astolfi-Filho et al, 1980; Rubio et al, 1980). The lysine-rich class H1 histone, associated with the links of DNA connecting individual nucleosomes, does not appear to be present in the form which is involved in the de-condensation of chromatin in other eukaryotes. This could account for the failure of chromatin to condense during division of T. cruzi (De Souza and Meyer 1974; Solari 1980) and a similar situation may exist in other trypanosomatids.

1.10.2 E.R. and Golgi

Continuous with the nuclear membranes, an extensive network of membrane-bound flattened sacs (cisternae) and tubules are found in eukaryotes comprising the rough and smooth endoplasmic reticulum (e.r.), respectively.

Protein synthesis and glycosylation are the principal functions of rough e.r., the cytoplasmic side of the membrane system being ribosome-studded. Lipid synthesis is one role of the smooth e.r. which also produces the budding transport vesicles required for intracellular transfer of asymetrically inserted membrane proteins to other organelles.

Together with the Golgi apparatus, these systems are located between the nucleus and flagellar pocket (and cytostome, if present) in trypanosomatids, towards the anterior of the organism, forming a connected membrane system for the production and packaging of proteins for distribution throughout the cell.

Usually, there is also a specialised branch of e.r. formed around the cytoplasmic aspect of 3 or 4 pellicular microtubules which are found beside the flagellum/body attachment zone; this e.r. association is a constant feature of salivarian trypanosomes (Vickerman and Preston 1976).

Glycosylation of proteins from the rough e.r. cisternae can take place co-translationally but commonly the Golgi cisternae are the site of glycosylation. Carbohydrates have been detected in the maturing face of the Golgi apparatus in T. brucei (Steiger 1973) and T. cruzi (De Souza, 1976a) using electron microscope cytochemistry (Thiéry, 1967) and thus glycoprotein synthesis would appear to occur in trypanosomes in a way similar to that found in most mammalian cells.

1.11 Lysosomes

DeDuve et al, (1955) first characterized lysosomes as membrane-bound organelles rich in acid hydrolases. These

enzymes have 3 main roles: digestion of ingested material (heterophagy) or of surplus cytoplasm or organelles of the cell itself (autophagy), or as modulators of cell secretory product output (crinophagy). The advent of The Gomori Technique (Gomori 1953) for demonstrating acid phosphatase activity greatly facilitated their identification by electron microscopy. Many other enzymes are present within lysosomes of mammalian cells; their acid pH optima and enzyme latency point to their lysosomal origin.

In trypanosomatids little work has been done in detail to identify and characterize the various lysosomal enzymes except in the cases of T. brucei (Steiger et al, 1980; Opperdoes and Steiger, 1981; Opperdoes and Van Roy 1982), and Crithidia fasciculata (Eeckhout 1972).

Acid phosphatase cytochemistry has been applied to a number of culture form trypanosomatids (reviewed, Vickerman and Preston, 1976) yet studies to detect other lysosomal enzymes by ultrastructural cytochemistry are few.

A detailed study of protein uptake and digestion in T. brucei, using ferritin as tracer, was performed by Langreth and Balber (1975) who also found acid phosphatase activity in the flagellar pocket and in the small spiny-coated vesicles (=acanthosomes) presumed to be primary lysosomes in T. brucei bloodstream forms. Ferritin was found in the large endocytotic spiny-coated vesicles. They noted, however, that contrary to the bloodform, pinocytosis did not occur via the large spiny-coated vesicles in the procyclic (culture) forms and these also lacked demonstrable acid phosphatase activity in the flagellar pocket. Uptake of ferritin was via a smooth membraned

cisternal system which fused with multivesicular structures; all these structures were acid phosphatase positive. However, the comparison between blood stages showed increased pinocytotic and digestive functions as the flagellates transformed from slender to stumpy forms with autophagy prominent in the stumpies. Autophagy is a way of enveloping unwanted cytoplasm (or organelles) with a membrane which then fuses with primary lysosomes; the products of this catabolism are recycled amino acids or phospholipids. The remaining undigestible material is discharged into the flagellar pocket for voiding in a process termed 'cellular defecation' (Brooker, 1971a).

More recent biochemical analysis of hydrolases in T. brucei including enzyme latency data (Steiger et al, 1980) has suggested that acid phosphatase is present mainly in the flagellar pocket and that this is a site of predigestion and protein accumulation (i.e. a storage reservoir). These authors showed that although T. brucei is poor in acid hydrolases compared with higher eukaryotes (Steiger et al, 1979), the enzymes of the trypanosome lysosomes included an acid proteinase, an ∝mannosidase and an acid RNase, their specific activities being lower in culture forms than in blood forms. Further work from the same laboratory (Opperdoes and Van Roy, 1982) has identified a phospholipase having an acid pH optimum, present in lysosomes, probably involved in membrane degradation or perhaps fusion of membranes associated with the lysosome system.

A lower rate of endocytosis compared with mammalian cells was described for T. brucei blood forms using an in vitro continuous dialysis method (Fairlamb and Bowman 1980). Uptake

of radio-iodinated polyvinyl pyrrolidone (I^{125}PVP), which is not degraded by acid hydrolases, was measured with or without albumin present, and it was concluded that I^{125}PVP was taken up by both fluid phase and adsorptive endocytotic mechanisms in the flagellar pocket. This attempt to quantitate protein uptake and to characterize enzymes involved in digestion is directed towards devising lysosomotrophic drugs selectively targeted for the parasite. Suramin and pentamidine, trypanocidal drugs circulating in the plasma bound to protein, are thought to be endocytosed and released within the lysosomes during digestion (Fairlamb and Bowman, 1977).

Apart from acid phosphatase cytochemistry on the intracellular amastigotes of Leishmania donovani (McAlpine 1970) little is known of the lysosomal make up of Leishmania species. Leishmania mexicana mexicana amastigotes have been shown to possess high proteinase activity (Coombs, 1982). The characterization and localization of proteinase activity within the amastigote is now under investigation (Pupkis and Coombs 1984) and is discussed further in Section 7.

1.12 Microtubule Systems and Microfilaments

Uniformly spaced microtubules about 25nm diameter form a corset running longitudinally beneath the plasma membrane of trypanosomatids at all phases of the life-cycle. These elements run for varying lengths in a helical pattern (Angelopolous, 1970) in a clear zone of peripheral cytoplasm and links (inter-tubule bridges) presumably maintain parallel spacing (about 44nm in T. cruzi, Meyer and De Souza, 1976). A structural connection between pellicular microtubules and the plasma membrane is hard to discern in electron micrographs but

persistence of the association through fractionation procedures suggests that connections are present (Hunt and Ellar, 1974; De Souza, 1976b).

Treatment of the pellicular microtubules with disrupting agents (Messier, 1971) has illustrated their resistance to breakdown. Only after reducing the protein disulphide bridges of tubulin subunits composing the microtubules with mercaptoethanol is disruption achieved, suggesting a stable system well suited to a supporting function.

The beating of the attached flagellum causes body shape to deform and this deformation is observed in living flagellates as the undulating membrane. The possibility that pellicular microtubules are concerned with actually generating shape changes seen as elongation and contraction of body form during the life-cycle is akin to the sliding mechanism invoked to explain shape changes in developing sperm nuclei (McIntosh and Porter, 1967).

Microtubule polymerization from a cytoplasmic pool probably occurs during morphogenesis via an organizing centre such as the basal bodies which lie between the flagellar pocket and the kinetoplast. Basal bodies - one associated with the flagellum and one (barren) at right angles to it - are of the usual nine triplet of microtubules construction. One is part of the flagellar apparatus and leads into the axoneme (Vickerman and Preston, 1976; Anderson and Ellis, 1965). Three or four microtubules pass from the flagellum basal body into the pellicular layer in T. brucei to parallel the attachment zone of flagellum to body. These microtubules could also act as reference structures during assembly of the

cytoskeleton.

Trypanosoma brucei tubulins have been recently characterised biochemically as two major protein subunits -α and β tubulins - both showing a degree of microhetero geneity (Stieger et al, 1984). In the three sources in T. brucei - the flagellum, the pellicular microtubules, and the small soluble cytoplasmic pool of tubulin - a varying proportion of two tubulin isotypes have been reported present in both the subpellicular and flagellar microtubules (Schneider et al, 1986). No alterations in this tubulin isotype pattern were found between the bloodstream and culture procyclic stages of T. brucei used in this study.

Microheterogeneity of the α and β tubulins may partially explain the different binding patterns on the flagellar and subpellicular microtubules in T. brucei found using monoclonal antibodies (Gallo and Anderton, 1983; Cumming and Williamson, 1984), and also the phenothiazine drug sensitivity of pellicular but not flagellar microtubules when in vitro tested on procyclic T. brucei and also Leishmania donovani (Seebeck and Gehr, 1983; Pearson et al, 1982). These observations show promise for new avenues of chemotherapy.

A similar distinction between flagellar and pellicular microtubules has been identified by blocking T. cruzi epimastigote replication in vitro with the microtubule stabilizer, taxol (Baum et al, 1981): while organelle replication proceeds, cytokinesis is halted, though flagellar function remains unimpaired.

The classical axoneme configuration is present in the trypanosomatid flagellum (nine peripheral plus two central microtubules) but peculiarities include the unique

longitudinally partitioned B tubules (Anderson and Ellis 1965) of the outer doublets and the association of the axoneme with the filamentous lattice structure, the paraxial rod, within the flagellar sheath (in most trypanosomatids) (Vickerman 1969b; DeSouza and Souto-Padron, 1980).

Comparisons have been drawn between the trypanosome and the euglenid paraxial structure. The latter is a hollow tubular structure of helically arranged 22nm filaments apparently not fixed in position relative to particular doublets of the axoneme (Hyams 1982). In contrast, the T. brucei paraxial structure consists of 5nm filaments in three parallel arrays always located in a region alongside doublets 5 and 6 of the axoneme (terminology of Afzelius 1959), (Fuge, 1969). Such structural differences may underlie important alternatives for the mechanisms of flagellar beating in the two groups: it has been found that the euglenid paraxial structure contains an ATPase, which suggests a role for this component in flagellar movement (Piccini et al, 1975).

Fluorescence studies using an anti-actin antibody reveal binding of the probe in the flagellum of epimastigote T. cruzi and the authors conclude that the paraxial structure is an actin lattice (De Souza et al, 1983). Using SDS-poly acrylamide gel electrophoresis, however, the major two constituents of the paraxial structure in Crithidia fasciculata have been identified as 76 kilodalton and 68 kilodalton proteins (Russell et al, 1983). These are similar in size to the proteins isolated from the paraxial rod of Euglena gracilis (Hyams, 1982) but none have so far been considered to be actin. A common epitope in the isolated paraxial rod proteins from

Euglena and T. brucei has been demonstrated using monoclonal antibodies (Gallo and Schrevel, 1985) suggesting some conservation within these proteins.

Little evidence exists for filaments around 6nm diameter in trypanosomatids except in attached stages of Leishmania and Trypanosoma where such filaments are present within the flagellar sheath, underlying the flagellum to body macular desmosome region and in association with the hemidesmosomes formed during flagellar attachment to substrates (see section 4). Spindle microtubules have been demonstrated in several trypanosomatids (Bianchi et al, 1969; Vickerman and Preston, 1970; De Souza and Meyer, 1974; Heywood and Wienman, 1978; Solari, 1980, 1983) but their lability compared with the flagellar and pellicular systems is reflected by their inconsistent preservation during processing for electron microscopy. Nevertheless, colchicine resistance has been reported in T. brucei (Luckins et al, 1967).

Since there are no centrioles, the nucleating centres for these microtubules are thought to reside on the inner nuclear envelope which persists throughout mitosis. Nuclear division is by elongation and constriction of the organelle to form two daughter nuclei which may possibly carry the genetic information in their envelope-associated peripheral chromatin, as in dinoflagellates (reviewed Dodge, 1973). However, mechanisms for segregation of genetic material continue to be debated (Vickerman and Preston, 1970; Solari, 1980).

The lack of identifiable chromosomes has further hampered studies of nuclear events in trypanosomatids however Solari (1980) has undertaken three-dimensional reconstruction of the T. cruzi epimastigote nucleus, and a complement of ten

chromosomes has been proposed comprising, in part, the kinetochore-like electron dense plaques associated with the spindle, which may prove to be DNA. The function of nuclear microtubules may be to push apart the two halves of the envelope during elongation or to act as a carrier structure transferring the genetic material into newly formed nuclei, as in metazoans.

1.13 Surface membrane specializations

1.13.1 The plasma membrane

In transmission electron micrographs of sections, a trilaminar 7.5nm plasma membrane overlies the subpellicular microtubules, together forming the characteristic pellicle structure of the trypanosomatids. The membrane often co-purifies with the underlying microtubules and these may serve as a convenient marker of plasma membrane fractions (Hunt and Ellar, 1974; De Souza, 1984). Moreover, the persistence of the microtubules with the cytoplasmic aspect of the plasma membrane permits positive identification of this surface.

A reliable enzyme marker for trypanosomatid plasma membrane fractions has yet to be discovered. 5' nucleotidase and Na^+/K^+ ATPase are considered classical marker enzymes for plasma membrane fractions in mammalian cells (Solyom and Trams, 1972) however both are absent in T. cruzi epimastigote surface membranes (Pereira et al, 1978) and the former is absent from T. brucei blood forms (Rovis and Baekkeskov, 1980). Adenylate cyclase has been proposed as a more suitable marker (De Souza 1984). The 3' nucleotidase on the outer aspect of T. brucei (McLaughlin 1982) and Leishmania

donovani promastigote (Dwyer and Gottlieb, 1984) plasma membranes is another possible candidate.

Analysis of plasma membrane fractions biochemically has been undertaken for several trypanosomatids and, as in mammalian cells, phosphatidylcholine and phosphatidylethanolamine have been shown to be the major phospholipids present (bloodstream T. brucei, Voorheis et al, 1979; Leptomonas collosoma, Hunt and Ellar, 1974; T. cruzi epimastigotes, Franco da Silviera, 1981; L. donovani promastigotes, Wassef et al, 1985).

Freeze-fracture, an ultrastructural cryotechnique for the examination of membranes, provides unique information on the disposition of proteins (the intramembranous particles, IMPS) within the membranes. The method has been applied to several trypanosomatids and reveals the existence of specialized domains between the continuous flagellar and body membranes, often separated by an IMP array near the base of the flagellum (Linder and Staehelin, 1977; De Souza et al, 1978). Other regions differing in composition from the neighbouring membrane are the flagellar pocket and, in T. cruzi, the cytostomal field; again these domains may be separated by specific IMP arrays. Protein/lipid ratios may be reflected by IMP densities and as such have been compared in a number or trypanosomatid plasma membranes (Benchimol and De Souza, 1980). Such studies have also shown the variation in particle partitioning that may occur between the inner and outer surface membrane halves in even closely related flagellates and between successive stages in the life cycle (Vickerman and Tetley, 1977). However, this may have only an indirect bearing on the external face of the plasma membrane

as surface components detected by cytochemistry have so far been found to be largely independent of the arrangement of the underlying IMPs.

1.13.2 Surface coats

The bloodstream stages and metacyclics of salivarian trypanosomes exhibit a 15nm thick, uniform surface layer of moderate electron density apposed to the plasma membrane and covering the entire flagellate as seen in electron micrographs of sections. This layer (the surface coat) appears less compact in *T. vivax* than in *T. congolense* or *T. brucei* nevertheless in all three species this has been shown to be the site of the variable antigen (Vickerman and Luckins, 1969). The replacement of one population of trypanosomes in the mammal with another bearing a different variable antigen produces the characteristic relapse and remission pattern of parasitaemias associated with antigenic variation in chronic trypanosome infections.

The surface coat of salivarians is thought to be a parasite adaptation to protect the surface from immune assault, preventing complement components from gaining access to the plasma membrane thus shielding transport and receptor sites (Vickerman and Barry, 1982). The evidence for its endogenous origin was argued by Vickerman (1969) and molecular and biochemical studies have since determined the structure and properties of the coat as a glycoprotein (Cross, 1975; Cross and Johnson, 1976). This 55-65 kilodalton variant specific glycoprotein (VSG) is distributed as ~ 10 million molecules over the plasma membrane (Jackson et al, 1985).

Between 100 and 1000 VSGs, with very different amino acid sequences, can be made by a single trypanosome clone (Capbern et al, 1977) though only one VSG at a time is expressed except during antigen switching (Esser and Schoenbechler, 1985). At the membrane-coat interface carbohydrate moieties are found at the C-terminus and as N-terminal glycosylation sites of asparagine residues. The former are associated with the fatty acid tails responsible for insertion of the molecule into the membrane. The presence of this carbohydrate region had been demonstrated previously by an ultrastructural periodic-acid-silver method (Wright and Hales, 1970). The latter glycosylation sites are variable from one VSG to another and their role is so far unclear (Holder, 1985).

The mobility of the surface glycoprotein in T. brucei has been shown by capping studies (Barry, 1979). The release of coat material in the presence of Ca^{++} (Voorheis et al, 1982) or when blood forms are transformed to culture procyclics (Barry and Vickerman, 1979) suggested that the VSG might be considered a peripheral rather than an integral protein. The intramembrane particle density of the plasma membrane beneath the coat, as revealed by freeze-fracture, is about twenty times less than that of the VSG packing density on the surface (Vickerman and Tetley, 1979) suggesting that the IMPs do not represent VSG molecules. Turner et al (1985) have shown that the VSG is anchored to an amine-cligosaccharide acceptor in the membrane proper and release of the soluble VSG is the result of cleavage of the complex by a phospholipase. This indicates that the VSG may behave more

like an integral protein.

Bloodstream trypanosomes have the ability to shed surface coated membrane as cytoplasmic streamers, or 'plasmanemes' from the body or flagellum *in vitro* - (Vickerman and Luckins,1969; Wright, Lumsden and Hales, 1970) - whether such shedding occurs *in vivo* - is uncertain but the streamers are possibly a source of the 'exoantigen' present in the serum of infected mammals (Weitz, 1963).

Surface coats of trypanosomes other than *T. brucei* have not been so well characterized, though comparative surface charge and lectin studies have been undertaken. The coat in *T. congolense* is reported to have exteriorly exposed carbohydrate residues (Rovis *et al*, 1978) and reaction with Concanavalin A and other lectins results in agglutination (Jackson *et al*, 1978; Rautenberg,1980). The more negative surface charge on *T. vivax* (relative to *T. brucei*) may be due to sialic acid groups as neuraminidase digestion has been shown to abolish iron colloid binding properties at the surface (Vickerman and Preston,1976).

Loss of the surface coat on entering the vector (or on incubation *in vitro* at 26°C) is paralleled by loss of infectivity to the mammal host. The coatless forms are lysed through activation of complement by the alternate pathway (Ferrante and Allison, 1983) in *T. brucei* and are readily taken up by macrophages. Coated forms are unattractive to macrophages unless variant specific antibody is present (Mosser and Roberts, 1982) again demonstrating the shielding function of the coat.

T. cruzi has no distinct surface coat analogous to salivarians and does not undergo antigenic variation. The presence of glycosidic groups on the surface has been shown

by cytochemical means employing Ruthenium red (De Souza et al, 1978), Con A-peroxidase (Chiari et al, 1978), colloidal iron hydroxide (Martinez-Palomo et al, 1976) and cationized ferritin (De Souza et al, 1977). Lectin binding analyses have demonstrated qualitative and quantitative differences in exposed carbohydrate groups among different stages in the life-cycle and between stocks of T. cruzi (Pereira et al, 1980; Schottelius, 1982). Similar lectin studies have so far been undertaken only for promastigote stages in Leishmania species, where differentiation to the infective forms both in vivo and in vitro has been shown to be accompanied by loss of peanut aggultinin binding activity presumably to β-galactose residues (Sacks and Perkins, 1984, 1985).

The surface of Leishmania promastigotes binds polycationic substances and cationized ferritin, the net negative charge being probably due to the phosphate groups of the plasma membrane phospholipids and/or carbohydrate residues present on the surface. As with other cyclically transmitted trypanosomatids, the surface glycosidic groups are considered possible attachment mediators, facilitating adhesion of the flagellates to the insect gut chitin and protecting the parasite against the effects of extracellular digestive enzymes in this location.

The apppearance of stage specific glycoproteins (which may vary from stock to stock) probably having diverse functions, has been reported for T. cruzi. One such glycoprotein- the 90 kilodalton antiphagocytic factor - has glycosidase activity and was shown to be present on all

mammalian stages but absent from metacyclics (Nogueira et al, 1980). The latter have a 75 kilodalton glycoprotein in common with the other vector forms. The glycosidase activity of the former stages is thought to defend the parasite against host humoral responses by affecting lytic activity or phagocytosis by altering the Fc domain of bound immunogobulin (Nogueira, 1983).

Capping of surface bound immunoglobulin occurs in blood-stream T. cruzi (Schmunis et al, 1980), Leishmania enrietti and Leishmania donovani promastigotes (Doyle et al, 1974; Dwyer, 1976) and has been suggested as a means for the parasite to shed antibody or to impede the ingestion of opsonized parasites by macrophages. Entry of different parasites into the macrophages may undoubtedly prove to be by different mechanisms. For leishmanias entry would seem to be a process of phagocytosis involving the progressive surface interaction of parasite ligands with macrophage receptors around the periphery of an eventually internalized parasite - the so-called "zipper" process originally described for macrophage particle ingestion (Silverstein, 1977). An active part played by the parasite during attachment has been suggested for both promastigotes (Aikawa, 1982) and amastigotes (Wyler, 1982) in concert with the microfilament-based macrophage phagocytic mechanism. This was seen by a failure of untreated macrophages to internalize parasites previously treated with cytochalasins.

1.14 Attachment and the flagellum

The characteristic undulating membrane of trypanosomes, as seen by light microscopy of living preparations, is a crest of

cytoplasm drawn up by the beating flagellum and maintained through the presence of junctional complexes between flagellar and body membranes. The complexes are similar in structure and function to the spot desmosomes of epithelial cells and occur throughout the parasitic trypanosomatids as single or multiple rows (Vickerman,1969, 1973, 1974; Brooker,1970; Paulin,1972).

The spot desmosomes appear as 25nm diameter electron dense patches in electron micrographs of thin sectioned T. brucei. Longitudinally arranged 5nm cortical filaments underlie the spot desmonsomes on the body side of the junction and the desmosome roots appear to pass into this structure. A similar arrangement of desmosome roots entering the filaments of the flagellar sheath occurs on the flagellar side of the junction. When examined by freeze-fracture these regions displayed clusters of IMPs presumably representing the junctional proteins involved in cell/body adhesion (Smith et al, 1974; Vickerman and Tetley, 1977; De Souza et al, 1978).

The trypanosomatid flagellum displays a unique ability to form attachments to inert substrates or host cell surfaces at certain stages in the life-cycle. The types of surfaces colonized are either host cuticular or host cell plasma membranes, though artificial substrates have also been demonstrated as suitable (Brooker, 1971b; Hommel and Robertson, 1976).

In the vector the purpose of attachment is to prevent premature discharge of non-infective forms by anchoring the flagellates in their various stations so that they can resist the flow of fluids, for example blood meal, saliva or gut contents.

The hemidesmosome-like attachment has been well documented

for those trypanosomatids adhering to chitinous surfaces (reviewed, Molyneux, 1977, 1984); some of these species have been shown to attach in vitro and the form of attachment seems identical to that in the vector in the case of Crithidia fasciculata and T. congolense (Brooker, 1971; Gray et al, 1981). This would seem to argue against surface specificity for those parasites developing on inert vector surfaces in contrast to those species, such as T. brucei which have developmental stages attached to living host cell membranes. Such attachments are discussed further in section 4 of this thesis.

In Leishmania mexicana amazonensis in the sandfly, however, there is no attachment in the proboscis but flagellates with hemidesmosome-like attachments are seen on the cuticle of the oesphageal valve next to forms with no flagellar attachment plaques and associated with the microvilli of the adjacent gut cells. This might indicate that specific properties of the microvillar surface prevent attachment in this site.

1.15 Adaptations to Intracellular Parasitism

Intracellular parasitism characterizes the mammalian infections caused by leishmanias and T. cruzi. Both parasites initially enter host cells as a result of phagocytosis. Leishmanias multiply within the resulting phagolysosomes as amastigotes which are liberated to continue the infection in other macrophages when the host cell lyses. T. cruzi evades the host cell response by penetrating the vacuolar membrane to lie free in the cytoplasm where amastigote replication ensues: amastigotes transform to the non-dividing trypomastigote which is liberated into the blood. Central to the adoption of an

intracellular existence is the ability to enter the host cell. Entry will depend upon recognition of the prefered host cell by the parasite, and may initially involve some form of chemotaxis to bring the host cell and parasite together; the ability of the parasite to attach to the host cell plasma membrane before internalization is also a prerequisite.

Leishmania promastigotes activate complement by the alternate pathway (Bray, 1983) and in so doing a chemotactic gradient is established attracting macrophages. Complement component C5a is responsible for the chemotaxis whereas a cleaved C3 component binds to the promastigote surface facilitating phagocytosis by the macrophage C3bi receptor, CR3 (Blackwell et al, 1985). In general hosts previously exposed to parasitic protozoans would produce opsonizing antibody to be recognized by Fc receptors on macrophages: cytophilic antibody bound to the host cell via the Fc receptor may present Fab sites which also bind parasites.

T. cruzi bloodstream trypomastigotes appear to have an antiphagocytic surface which does not activate complement and thus does not stimulate uptake by macrophages. This stage, however, is taken up by 'non-professional' phagocytes such as cardiac muscle cells. The antiphagocytic factor is absent from the surface of T. cruzi metacyclics which initiate the infection in macrophages. Where Fc receptors or complement activation are not involved in parasite entry into host cells, other receptor/ligand systems may substitute. Lectin-like glycoprotein receptors on host cell surfaces may mediate attachment of T. cruzi stages to macrophages (Nogueira and Cohn, 1976; Kipnis et al, 1979) and Leishmania donovani

promastigotes have been shown to bind to similar receptors via sugar (n-acetyl-glucosamine) or sialic acid residue ligands on the parasite surface (Chang, 1981).

The mannose-fucose receptor on the macrophage has been demonstrated as important in promastigote, but not amastigote, attachment (Channon et al, 1984) and this observation corresponds with the finding of mannose-rich glycoconjugates on the surface of Leishmania donovani promastigotes (Gottlieb and Dwyer, 1983). Plasma membrane-bound acid phosphatase on L. donovani promastigotes (Gottlieb and Dwyer, 1981) may participate in initial host-parasite interaction since the enzyme has been found to be a mannose-containing glycoprotein (Glew et al, 1982). The macrophage receptors for mannose or N-acetylglucosamine are normally involved in recycling or sequestering host extracellular lysosomal enzymes and thus the parasite could utilize this system to enter the host cell. Other functions for the surface-bound acid phosphatase are discussed later in this section.

Promastigote L. donovani also have plasma membrane phospholipases which may bring about changes in local host membrane structure during attachment or later when the parasite is in the parasitophorous vacuole (Wassef et al, 1985).

Whether entry into the host cell is by induced phagocytosis or by active penetration of the flagellates has been much debated. In vitro treatment of macrophages with cytochalasin B - a microfilament-disrupting agent and therefore an inhibitor of active phagocytosis results in inhibition of entry of T. cruzi epimastigotes and culture trypomastigotes into macrophages (Nogueira and Cohn, 1976, Kipnis, 1979). Similar cytochalasin inhibition (Alexander, 1975; Zenian et al,

1979) and inhibition of host cell glycolysis by iodoacetamide both prevent Leishmania promastigote entry (Zenian, 1981) into macrophages suggesting that parasite entry is by phagocytosis.

Toxic oxygen metabolites such as hydrogen peroxide and free radicals (O_2^-) are the active products of the so-called antimicrobial 'respiratory burst' (RB) initiated by the stimulation of a macrophage plasma membrane-bound NADPH-oxidase. Failure to establish a Leishmania infection in a normal macrophage (non-activated) correlates well with the degree of stimulation of the RB (Murray, 1981) and successful invasion of the macrophage indicates little or no triggering of the RB following attachment of the flagellate: activated macrophages appear to exaggerate these responses.

Promastigotes of Leishmania species appear to stimulate the RB and are more sensitive to H_2O_2 than amastigotes, which induce a reduced response (Murray, 1982; Channon et al, 1984). This could explain the differential survival of amastigotes and promastigotes when infecting macrophages. The promastigote plasma membrane-bound acid phosphatase described by Gottleib and Dwyer (1981), however, has been shown to supress oxygen radical production in human neutrophils (Remaley, 1984) and one function of this enzyme might be to exert some control over the RB activity. Nevertheless, promastigotes have a lower survival rate and this may be largely explained by the differences in type and distribution of their surface ligands compared with those on amastigotes which permit binding to the macrophage membrane receptors.

During interiorization of the leishmanias the microbicidal activity can proceed within the newly formed parasitophorus

vacuole and may result in early parasite destruction. A more rapid transformation from promastigote to amastigote after entry into the host cell should thus favour survival. Neutralization of the toxic oxygen metabolites of the RB may be a defence mechanism and catalase and glutathione-peroxidase - scavengers of H_2O_2 - appear to be important in this respect. Leishmania promastigotes display a low level of these enzymes and this may explain their poor survival (Murray, 1981).

Having overcome the RB the flagellates must resist intracellular destruction by surviving incorporation into host cell secondary lysosomes. Since Leishmania and T. cruzi do not adopt the strategy of preventing phago-lysosome fusion (compare Toxoplasma gondii, where a parasite mechanism seems to prevent this (Sethi et al, 1981)), some means of countering the hostile environment must have evolved. One adaptation might be resistance to lysosomal enzymes either imparted by surface properties of the transformed parasite or by a means of altering host enzyme activity.

A carbohydrate containing 'excretion factor' has been identified and as this polyaniomic substance has anti-β-galactosidase activity it is thought that it might be a broad spectrum inhibitor of lysosomal enzymes (El-On et al, 1980). Changing the intra-lysosomal pH by release of ammonia from the parasite might interfere with hydrolase activity. It has been suggested by Coombs (1982) that ammonium compound release might be a consequence of proteinase activity. This activity is detectable in the soluble fraction of Leishmania mexicana mexicana amastigotes at a level which is considerably higher than that found in macrophages or promastigotes. If secreted by the amastigote these proteinases might inactivate host

enzymes. Inhibitors of such parasite proteinases are being investigated as potential antileishmanial drugs. Previously described host lysosomal enzyme activity may now prove to be, in part, parasite derived.

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Section 2.

The preparation or freeze-fracture replicas using a cold-stage module system.

SUMMARY

Modification to the construction and operation of a basic Nanotech/Polaron type freeze-fracture module have been made to examine stages in the trypanosome life-cycle in insect tissue samples using the knife-fracture method for freeze-fracture replication. Construction and installation of a complementary replica device further improved the specimen handling capabilities to permit the production of complementary fracture faces of tissue and suspension samples. Details of vacuum system, cold stage and evaporation source improvements are described which result in more reliable production of freeze-fracture replicas. The reduction in size of the specimen support (increasing the cooling rate of the sample) and adoption of liquid propane/ isopentane as coolant allowed the examination of non-cryoprotected suspension samples.

Introduction

Since the introduction of freeze-fracturing/etching by Steere (1957), Moor et al. (1961) and Bullivant and Ames (1966), the use of this ultrastructural technique has become very widespread, both as an adjunct to conventional transmission electron microscopy of sections and, independently, as a unique method for revealing membrane architecture.

Biological specimens rapidly cooled below -100°C may be fractured, either under vacuum or at atmospheric pressure, and the exposed cold fracture-face metal-shadowed and carbon-replicated under vacuum (freeze-fracture). Alternatively, the fracture face may be left for some time to allow the frozen matrix to sublime before the evaporation step (freeze-etching).

Several devices have been developed to perform the above procedure, the most popular being those which employ a cooled microtome to cleave the specimen *in vacuo*. Such devices based on the original design of Moor *et al* (1961) are commercially available from Balzers Union (Liechtenstein). Freeze-cleaving systems not employing microtomes such as those using hinged devices (Sleytr and Umrath, 1976) or those cleaving the specimen under liquid nitrogen (Bullivant and Ames, 1966) work equally well and are preferred by some authors. The merits and disadvantages of the various systems have been the subject of several reviews (Benedetti and Favard, 1973; Sleytr and Robards, 1977; Willison and Rowe, 1980; Sleytr and Robards, 1982).

The conditions necessary for the production of a high resolution replica of a metal-shadowed fracture face using any system, includes

(1) the elimination of condensable gases as far as possible from the specimen vicinity; (2) the use of evaporation sources which exert the minimum heat load on the exposed specimen; the sources should be outgassed before evaporation to avoid generation of condensable gases; (3) rapid cooling of the specimen to prevent ice-crystal damage and maintenance of the specimen at a suitable low temperature throughout the fracture (± etching) and replication procedures; (4) the successful cleaning and retrieval of the replica for electron microscopical observation. Thus, in freeze-etching, examination and recording of details from the replica are the last steps in a multi-event process. Attention should therefore be paid to all aspects of equipment design and operation to minimise the formation of artefacts which may influence the final interpretation.

A freeze-etch module is an inexpensive 'add-on' system which accompanies a high vacuum coating unit - common to most electron microscope laboratories - providing a microtome-based freeze-etching facility for about one tenth of the price of the dedicated system. While freeze-fracturing tsetse fly organs for stages in the developmental cycles of trypanosomes several modifications to the module system proved necessary to permit the production of good quality replicas. These modifications enabled capabilities of the system to be extended to complementary replicas of both tissue and single cell suspension samples.

The Nanotech/Polaron module system: The basic top plate design comprises seven ports, six at the corners of a hexagon and one in the centre of this arrangement. Four of the outer seven ports take evaporation sources and blanking or vacuum feedthrough plates for electrical and thermocouple connections. The remaining two ports house the liquid nitrogen integral dewars - the cooled microtome arm

and the low temperature specimen stage (see fig. 1). The entire assembly rests on a glass-walled cylinder which forms the directly pumped vacuum chamber (fig. 2).

Design modifications

Vacuum system The Nanotech 300S coating unit which evacuates the module, comprises a 12m^3/hr. two-stage rotary pump backing a 650 litre per second oil diffusion pump. The vacuum fluid in both pumps was replaced by Invoil 30 (Inland Vacuum Industries, U.S.A.) a low vapour pressure (5 x 10^{-8} torr., trapped) hydrocarbon pumping fluid incorporating an antioxidant. This eliminated contamination of the diffusion pump oil with low grade mineral oil backstreaming from the rotary pump, a serious limiting factor in vacuum system performance. Additionally, a liquid nitrogen cold trap was incorporated between the diffusion pump and chamber which, along with the cryotrapping capacity of the integral liquid nitrogen dewar arms of the module, resulted in a large cryopumping surface area yielding an ultimate chamber vacuum better than 10^{-6} torr. The system was brought to atmospheric pressure by venting with dry nitrogen gas (oxygen-free) passed over a type 5A molecular sieve column at 5 p.s.i. (see figs. 1 and 2).

Specimen handling and fracture methods In order to freeze-fracture tsetse fly salivary glands successfully several modifications to accommodate the very small tissue pieces were adopted. Firstly, the 8mm diameter thick copper supports (fig. 4) supplied were replaced with 3mm diameter gold planchettes (Balzers Union) and the original specimen sub-stage substituted by a triple specimen holder (Nickel et al. 1978) constructed from brass and stainless steel (fig. 3b). This was adapted to the 2BA screw insert method for installation onto the

cold stage (figs. 3b and 4).

For 'planing down' to the level of the specimen using the cold microtome, extra knife length was required as the ice quickly blunted the blade. Removal of the microtome upper cold shroud facilitated the use of the full 4 cm length of the Schick injector blade in the knife holder and through careful manipulation of specimen and microtome arms the level of the specimen in its frozen matrix could be reached by repeated pre-fracturing and selecting a fresh, clean region of the knife edge for the next pass. Previously, after the final pass, the knife had to be retracted across the freshly exposed surface, dragging with it ice chips which cause contamination, this artefact being exacerbated by the delay incurred between freeze-cleavage and replication steps. A cold shield was fitted in place of the original shroud which did not compromise microtome function. This was a flat polished brass plate which can be brought to within a few hundred microns above the fracture face (for example during etching experiments) (fig. 3a).

To assess the proximity of specimens to the surface of the frozen matrix during pre-fracturing, a light source and stereo-binocular microscope system (Bausch and Lomb, x 45, zoom) was installed in front of the vacuum chamber. The arcs swept by microtome and specimen arms coincide just inside the chamber wall (fig. 3b) and the progress of fracturing may be followed in detail by observation at 30-40x magnification.

The requirement to examine both fracture faces led to the construction of a complementary replica device (Sleytr and Umrath 1974) adapted to use two sets of copper plates (Balzers Union BUO 12 056T). These specimen supports (fig. 4) are thin enough - 100 µm - to permit two plates, with sample between, to be plunge-cooled in liquid propane/isopentane around its solidifying point (-190°C) (Jehl

et al. 1981). Further, thin layer suspension samples may also be frozen without recourse to cryoprotectant addition such as in surface etching experiments (Costello and Corless, 1978).

The complementary replica device was operated by a rotating arm moving a lever to release a spring-loaded mechanism pulling apart the two halves of the double support plate assembly. A vacuum rotation leadthrough was used to acheive this, the shaft being turned manually from outside the vacuum chamber (figs.2 and 3a).

Substage temperature The attachment of either the triple specimen holder or the complementary replica device to the cold stage placed an additional load on the temperature controller. The ability of the controller to stabilise at the preset temperature by heating the cold (-180°C) stage up via a heating element inserted in its base, was additionally monitored with copper/constantan (type T) thermocouples held firmly by the specimen retainers. Temperature was registered on a previously calibrated Comark 5000 or similar multichannel digital thermometer and was compared to readings indicated on the cold stage controller. In practice even after ensuring good contact between all interfaces (by polishing all metal surfaces), a drop of 10°C was observed at the specimen position relative to that recorded at the base of the cold stage. Since this temperature is sensed by the controller it is necessary to compensate settings on the controller by 10°C (i.e. for -100°C set at -110°C).

Evaporation sources The resistance evaporation sources prior to modification proved to give irreproducible and frequently heavy depositions of platinum/carbon or carbon. The use of expensive electron beam evaporators has been advocated for overcoming such problems in reliably producing freeze-fracture replicas (Willison and

Rowe 1980). Other workers have illustrated (Steere et al. 1977; Steere 1982) however, that equally good results can be obtained if details of evaporator construction and operation are critically evaluated.

Modifications based on Steere's work and introduced here included the machining of evaporators to accept 6.05mm diameter 'Specpure' compacted graphite rods (Johnson Matthey) and the use of carbon rod sharpeners (Balzers Union KS 600 and KS 610) to produce spigots 1mm diameter and 1.5mm long which acted as the carbon source and supported the 0.1mm diameter platinum wire coil of the shadowing source. The coil was produced using a mandrel and loaded onto the spigot, assembled against a flattened graphite rod and premelted in vacuo to form a platinum bead. The bead was rotated to the correct position to exit through the evaporator shield via the 45° window (fig. 4) before re-installation of the source for an actual freeze-fracture run. The production and orientation of the platinum bead in this way serve to outgas the small graphite spigots and both carbon and platinum/carbon sources were similarly pre-fired. Spigot and platinum wire length were arrived at empirically for the fixed source to specimen distance (10cm) and were considerably more economical in use of platinum than the source originally supplied by the manufacturers. A reproducible current density was a feature of the spigot and bead sources avoiding thermal damage to both specimen and evaporators.

Results and Conclusions

Typical replicas obtained from the triple specimen holder/knife fracture method and the complementary replica device are shown in figures 5 and 6, respectively. In figure 5, fine grain and good contrast of the replica, turgidity of the specimen cellular structure

and even distribution of intra-membranous particles indicate a good preparation (Staehelin, 1979). The sample in fig. 6 obtained using the complementary device illustrates a lipid vesicle suspension (Baillie et al. 1985) frozen directly from distilled water showing a matrix of low ice crystal size and no obvious contamination. The complementary device has also been used on fixed, cryoprotected tissue as illustrated throughout this thesis and, specifically, to record fracture face complementarity of junctional complexes (section). It is concluded that the modified freeze-etch module performs well and forms a basis for adaptation to other cryotechniques such as deep-etch rotary shadowing (Heuser and Saltpeter 1979) for which the equipment is presently being further modified.

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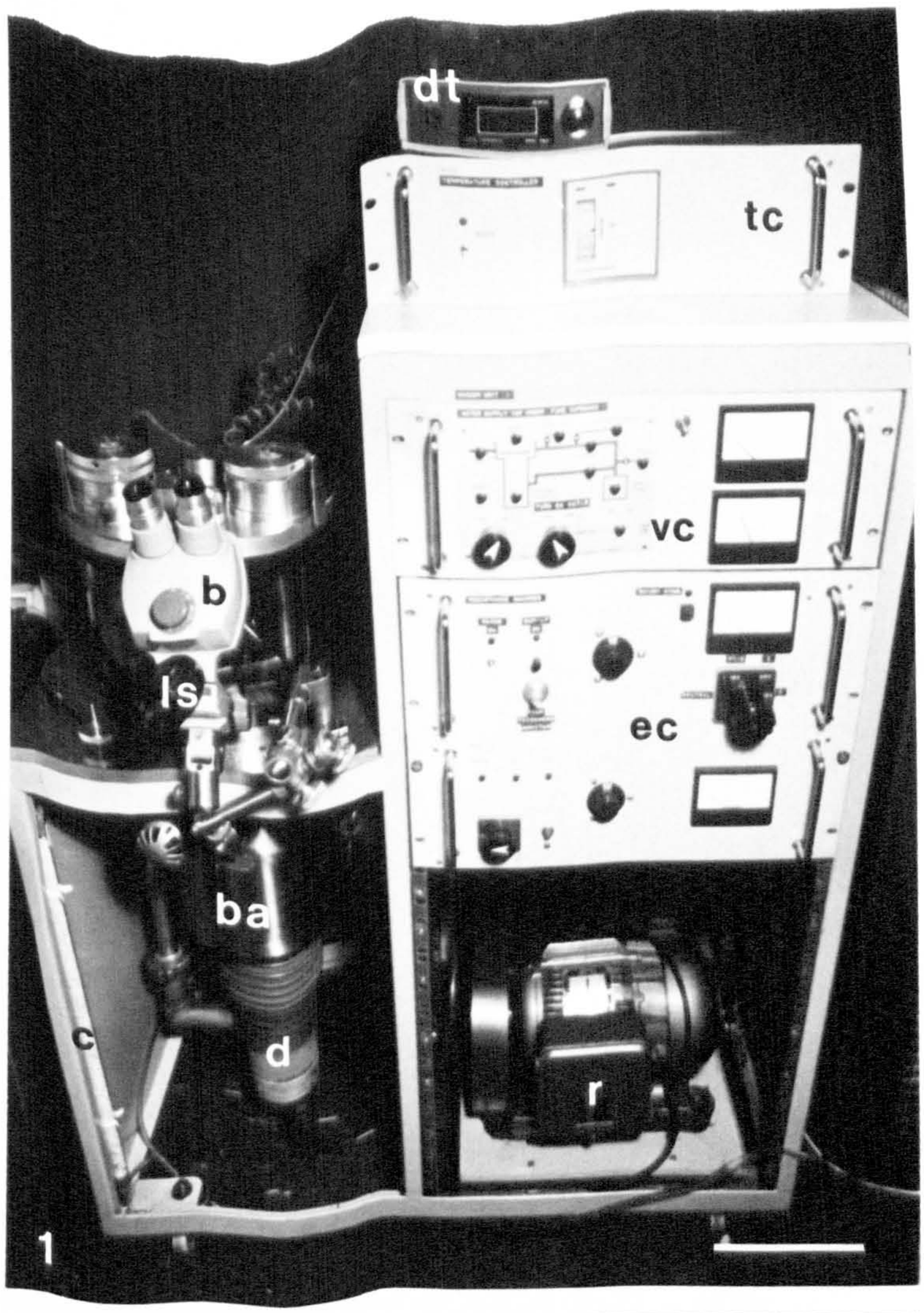
Figures 1 and 2

Fig. 1. Front view of the complete vacuum plant and installed cold-stage module.

The stereo-binocular (b) microscope system and light source (ls) are sited in front of the cold stage module vacuum chamber. Beneath the chamber the liquid nitrogen baffle(ba) is shown atop the oil diffusion pump (d) beside the molecular sieve gas drying column (c). A two-stage rotary pump (r) sits beneath the vacuum control (vc) and evaporation supply control (ec) panels: temperature controller (tc) and digital thermometer (dt) are located, for best visibility and access, on top of the main unit. Scale bar = 20cms

Fig. 2. View of vacuum chamber showing ports in the top plate and position of liquid nitrogen dewar arms.

The specimen stage dewar arm (sa) and knife arm (ka) have vapour exhaust pipes (p) (for evolving cold nitrogen) loosely installed to direct condensing vapour away from the access port when opening the cooled module under a backflow of dry nitrogen gas. In the front is the carbon source (cs) and in the centre of the top plate is the platinum/carbon source (ps) behind which are the three ports for electrical and vacuum leadthroughs. Outside the vacuum chamber is the knob (k) for rotation of the arm (a) which moves the lever (l) to open the complementary replica device. Scale bar = 10cms



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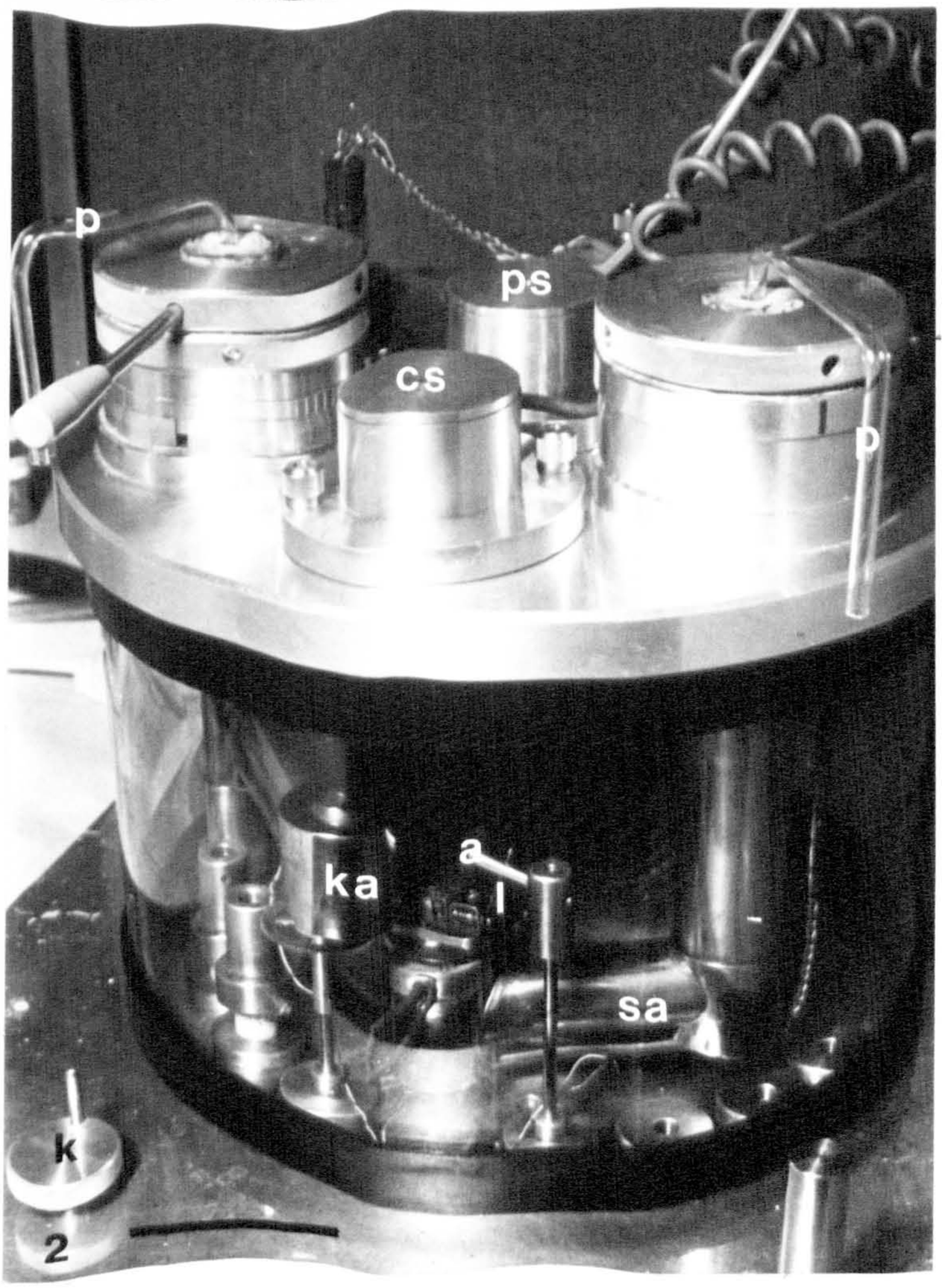

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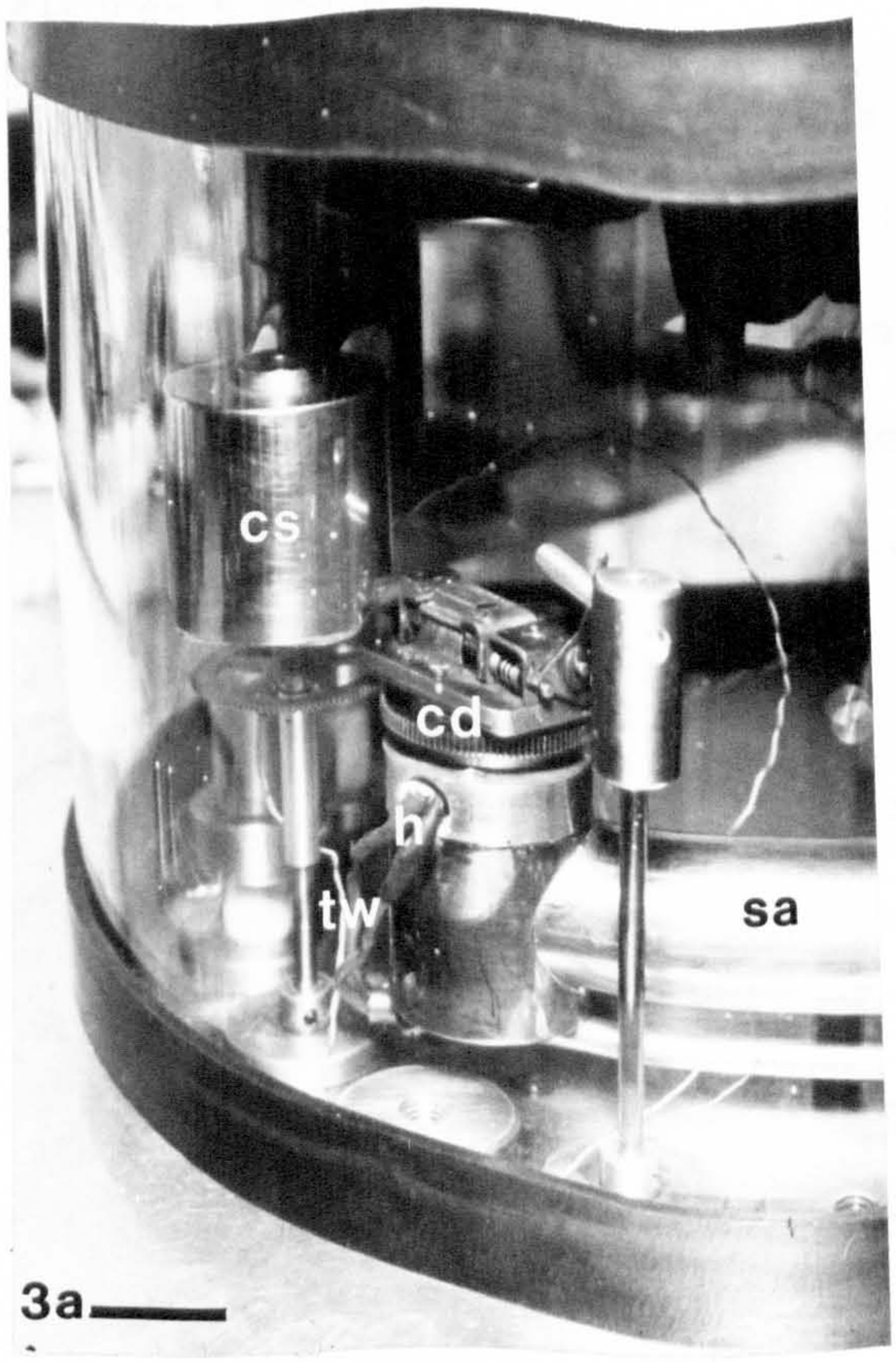
Figures 3 and 4

Fig. 3(a). Close up of installed complementary replica device (cd) in the closed position with the cold shield (cs) swung away. The device accepts two sandwiched specimens, a total of four replicas being produced per run. The cold stage heater (h) and thermocouple sensor wire (tw) are seen inserted in the base of the specimen arm (sa). Scale bar = 2cms

Fig. 3(b). Close up of the triple specimen holder (th) and knife blade (b) combination. The knife arm (ka) can be raised or lowered externally to plane the specimens by pre-fracturing before the final knife pass produces the desired fracture face: knife height adjustment is calibrated in 10 μm steps. Scale bar = 2cms

Fig. 4. Layout showing specimen cooling station, substages, various supports and insertion tools. A platinum/carbon evaporator is also shown. Seen inside the polystyrene container (p) for liquid nitrogen, is the brass coolant cup (cc) containing propane/isopentane near its solidifying point (-190°C) into which specimens are plunged. The original supports (os), the gold planchettes (gs) for use in the triple device, and the copper plates (cs) are shown in front of the brass cup. The triple specimen holder is mounted in a polypropylene base (b) to facilitate loading under liquid nitrogen and unloading after retrieval from the cold stage: the demountable arm (a) opens the sprung steel retaining clip of the holder. The complementary replica device is displayed in the open position to show the two halves of one pair of copper support plates (arrow heads) as they would appear after fracture. The insertion tool for the substages (ts) and the knife holder (tk) are in the foreground. The pre-fired platinum/carbon source (ps) is positioned to reveal the platinum bead (arrowed) mounted on its carbon spigot. Scale bar = 4cms



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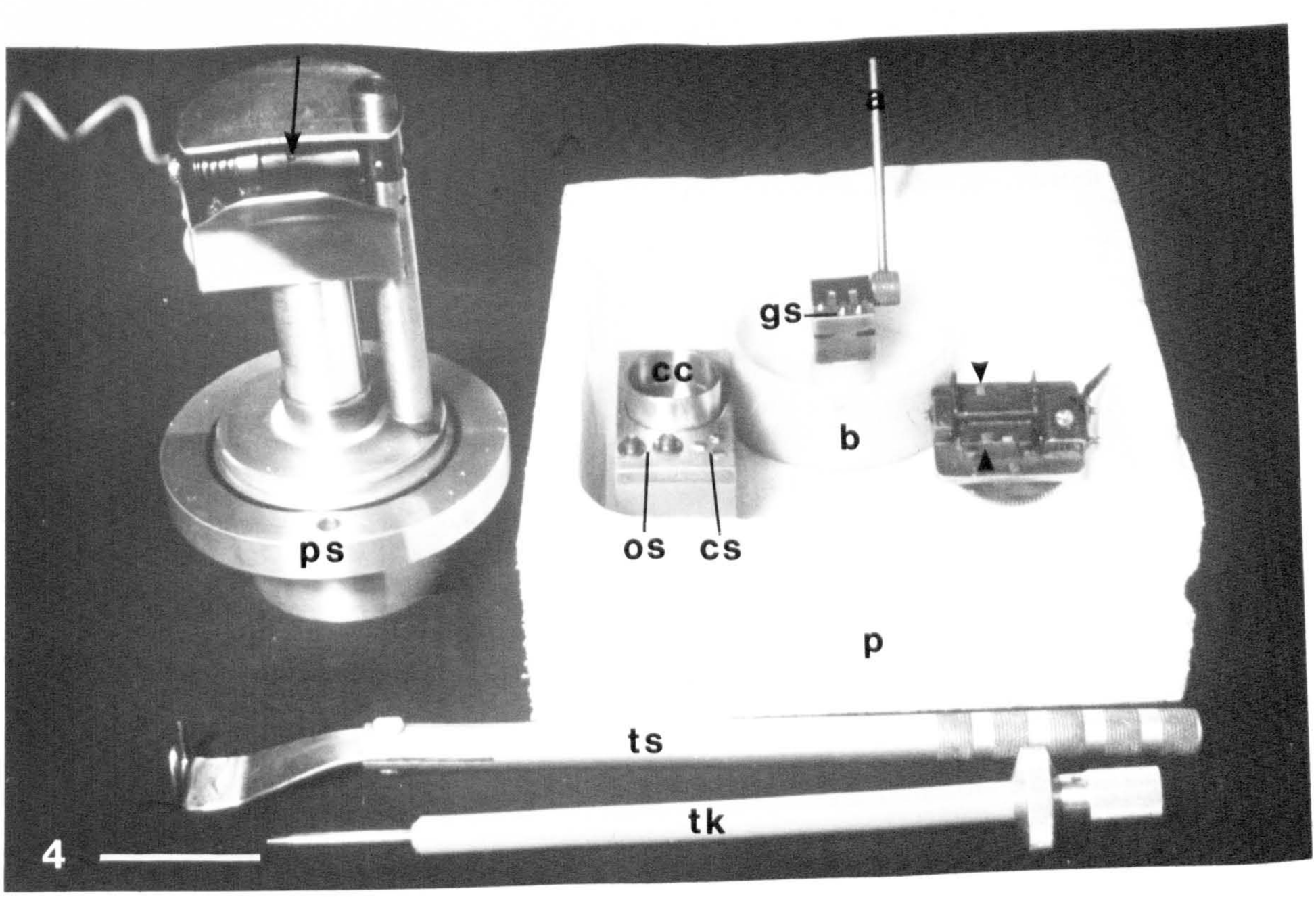

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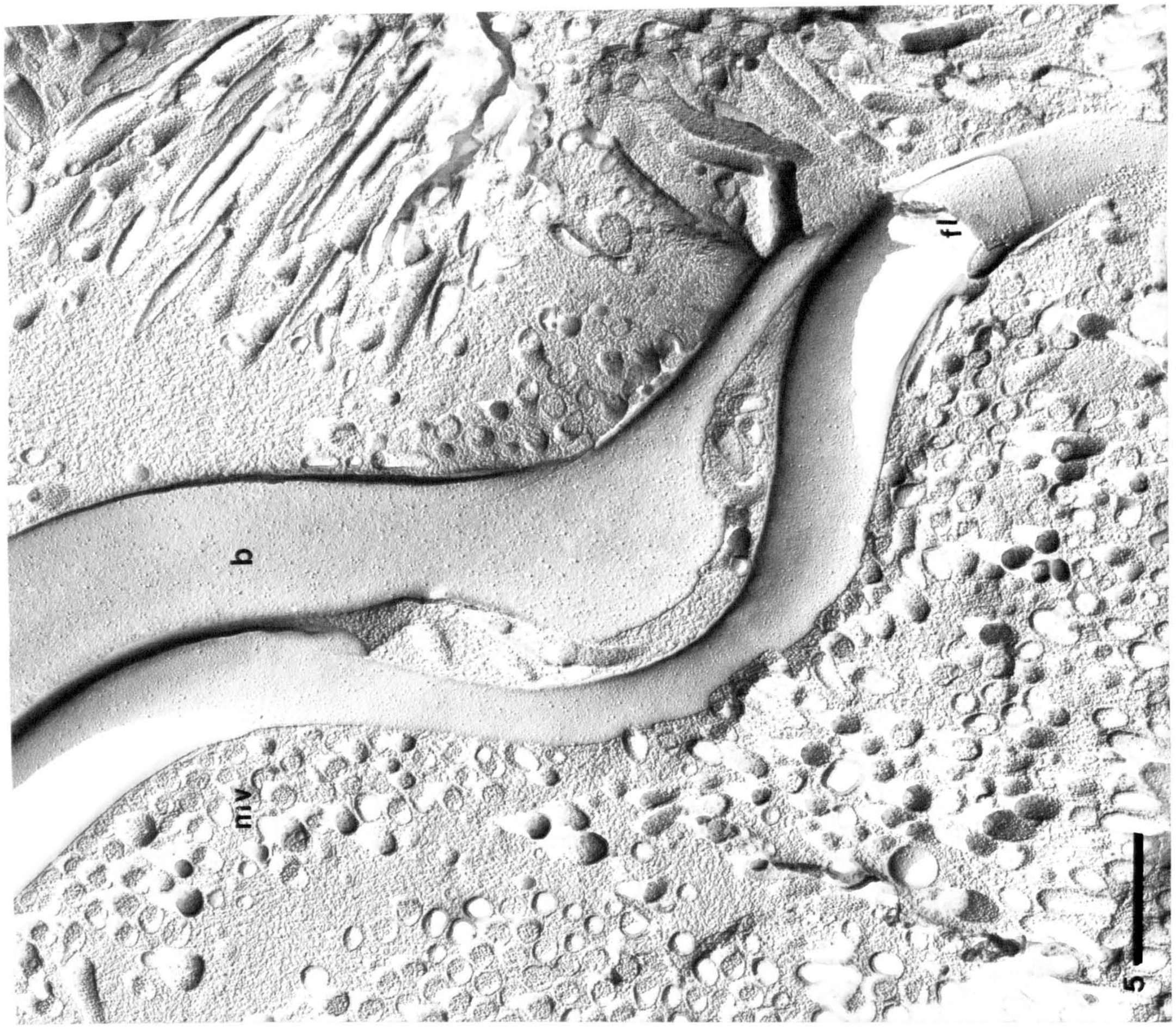
Figures 5 and 6

Fig. 5. Replica produced by knife fracturing at -100°C, 10^{-6} torr. of glutaraldehyde fixed, glycerol (25%) cryoprotected tsetse fly mid gut. The profiles in the centre of the field are body (b) and flagellum (fl) membrane fracture faces of Trypanosoma brucei adjacent to the midgut epithelial cell microvilli (mv). Scale bar = 0.5µm

Fig. 6. Replica produced using the complementary replica device of a fractured suspension of non-ionic surfactant 'niosomes' frozen from the distilled water in which they were prepared. The vesicle fracture faces and background are essentially smooth illustrating a low ice crystal size and negligible contamination rate: fracture temperature -100°C, vacuum better than 10^{-6} torr., replicated within 3 seconds of fracture. Scale bar = 0.5µm



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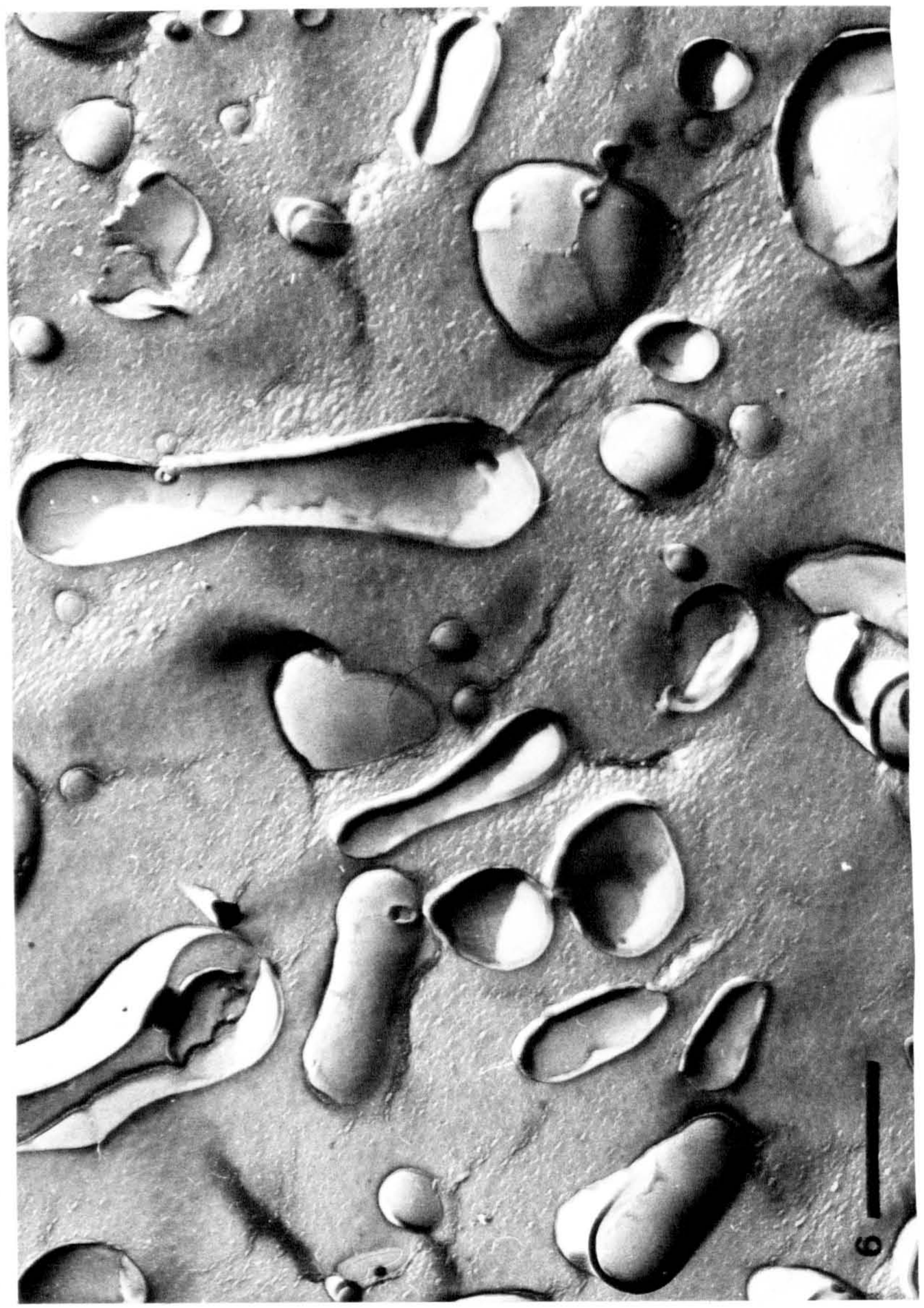

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Section 3.

Freeze-Fracture studies on the surface membranes of pleomorphic bloodstream and in vitro transformed procyclic Trypanosoma brucei.

Summary

The surface membranes of bloodstream long slender, short stumpy and culture procyclic stages of Trypanosoma brucei brucei were compared with respect to freeze-fracture electron microscopy, intramembrane particle (IMP) distribution and B-hydroxysterol content as shown by the characteristic intramembrane lesions induced by the polyene antibiotic filipin. Little difference was observed between IMP density of long slender and short stumpy form body membranes: IMP's were more abundant on the protoplasmic face (PF) than on the exoplasmic face (EF). The procyclic culture form body membrane showed an increased density of PF IMPs and a decreased density of EF IMPs over their bloodstream short stumpy form predecessors. Flagellar membrane fracture faces displayed higher IMP densities than body membrane fracture faces of the same trypanosome. The numbers of filipin-induced lesions (FIL) indicated an increased level of B-hydroxysterols in the short stumpy forms relative to the level in the long slender bloodforms. FIL density was further increased in the body membrane of the procyclic culture form. FIL density was higher in the flagellar membrane than in the corresponding body membrane and FIL were excluded from flagellum to body attachment zones of the flagellar membrane of all stages. The polarity of the FIL in the surface membranes was reversed on transforming from bloodstream to culture procyclic stages. These observations indicate qualitative differences between the surface membranes of the three stages, independent of the presence or absence of the surface coat.

Key Words

Freeze-fracture electron microscopy, surface membranes, sterol cytochemistry, Trypanosoma brucei

INTRODUCTION

Changes in the character of the surface during the life cycle of the African (salivarian) trypanosomes have been revealed by lectin binding, complement activation and measurements of surface charge, as well as by conventional ultrastructural studies on sections of trypanosomes (Vickerman, 1985). The most striking changes accompany loss and reacquisition of the surface or variable antigen coat (Vickerman, 1969; Steiger, 1973). This coat consists of a monomolecular layer of glycoprotein; sequential replacement of one glycoprotein with others of different antigenic specificity is responsible for antigenic variation and thus evasion of the host's immune response by the parasite (reviewed Vickerman, 1978; Borst and Cross 1982; Boothroyd 1985). In Trypanosoma brucei the coat is present in all stages in the mammalian host, but is lost on entering the tsetse fly vector, and reacquired during differentiation to the metacyclic stage.

The focus of attention in recent years has been on molecular characterisation of the variable antigen coat; (reviewed Cross 1984). Less attention has been paid to the structure of the membrane itself; some information on its composition is available for the slender (monomorphic) bloodstream form only (Rovis and Baekkeskov, 1980; Voorheis et al 1979). The freeze-fracture replica technique allows comparison of the internal structure of membranes. A detailed study of the membrane changes occurring during acquisition of the coat as seen in freeze-fracture replicas has already been published (Tetley and Vickerman, 1985). The present observations are of the intra-membrane differences between multiplicative long slender trypomastigotes and non-dividing short stumpy trypomastigotes from the mammalian host, and between these stages and the uncoated procyclic trypomastigotes which arise from them when they are cultivated in vitro at 26°C.

Materials and Methods

Parasites. Slender and stumpy form bloodstream Trypanosoma brucei brucei of the AnTaR1 serodeme (AnTat 1.8 and 1.9 cloned stock EATRO 1125) were grown

in female CFLP mice previously given 600 rads' whole body irradiation to increase trypanosome yields (Luckins 1972). All bloodstream forms were separated from blood by anion exchange chromatography on DEAE cellulose columns (Lanham 1968) and harvested by centrifugation at 1000g for 10 minutes in phosphate saline glucose.

Slender blood forms were obtained from ether-anaesthetised mice in blood withdrawn by cardiac puncture just prior to peak parasitaemia (10^8/ml). Thirty six hours later when > 90% of parasites in the remaining mice were of stumpy morphology, trypanosomes were recovered after similarly sacrificing the animals.

Culture T.brucei procyclics derived from AnTat 1.8 stumpy bloodforms were transformed in vitro and maintained by serial culture in Cunningham's medium (Cunningham, 1977) at 26°C then harvested in log phase by centrifugation for 10 mins at 1000g. Completed transformation was confirmed by transmission electron microscopy of embedded pellets of trypanosomes.

Filipin treatments were performed on established culture procyclics which had been twice-weekly sub-cultured in Cunningham's medium for at least one month after initial transformation.

Filipin treatments Filipin solution was prepared by dissolving the powder in DMSO (dimethylsulphoxide) and adding the mixture to 0.1M cacodylate buffer, pH 7.4 to give a final filipin concentration of 50 µg/ml (final DMSO concentration 0.5% $^{v}/_{v}$. Fixed parasites were pelleted then resuspended in filipin solution to a concentration of 10^6/ml and incubated in the dark at 20°C for 4-6 hours. Parasites were then washed in 0.1M cacodylate buffer, infiltrated overnight at 4°C with 25% glycerol in the same buffer and freeze-fractured.

Electron microscopy. Pelleted parasites were fixed in 2.5% glutaraldehyde (Sigma) in 0.1M cacodylate buffer, pH 7.4 at 20°C. Half the pellet was taken for freeze-fracture electron microscopy and the remainder processed essentially as detailed previously (Brown et al., 1973) for embedding and sectioning.

Freeze-fracture electron microscopy. Fixed, centrifuged parasites in glycerol buffer were sandwiched between two Balzer's copper support plates and rapidly cooled to -180°C by plunging into liquified propane. The specimens were transferred to a complementary replica device under liquid nitrogen and subsequently fractured in a vacuum of 2×10^{-6} torr. at -100°C. Replicas were obtained of the exposed fracture faces immediately by unidirectional platinum-carbon shadowing at 45° followed by carbon evaporation at 90°. The replicas were then retrieved by thawing into glycerol buffer, washing in distilled water and cleaning with 40% chromic acid overnight at 20°C. Further repeated washings in distilled water preceded collection of replicas on 300 mesh grids and observation in an AEI 801 transmission electron microscope operating at 60Kv. Images were recorded on Ilford EM4 cut film.

Counting of intramembrane particles (IMPs) and filipin-induced lesions. IMPs and lesions were counted from prints at a final magnification of 100,000x and using a Mk I Counting Calculator (Agar Aids) which registers points manually by a pen as counting procedes. Counts made on micrographs were of regions evenly shadowed and a graphics digitising tablet and microcomputer were employed to obtain areas under irregular perimeter profiles. Filipin-induced lesions were identified and counted as 25-30 nm pits or protrusions.

Results

Intramembrane Particles. Examination of the inner, protoplasmic face (PF) and outer, exoplasmic face (EF) fractures of the surface membranes of freeze-fractured trypanosomes showed a greater density of IMPs on the PF compared with the EF in all forms studied. Figures for the IMP densities in the body membranes of the three forms studied are given in Table I.

There was little difference between the IMP densities on corresponding halves of the plasma membrane of slender and stumpy parasites. However, in culture forms a marked change in IMP distribution between the two membrane halves became apparent; a decrease in EF IMP numbers was accompanied by an

increase in PF IMP numbers (figs. 1 and 2). This was paralleled by a loss of surface coat revealed by transmission electron microscopy of sectioned pellets (not illustrated).

A constant feature in all forms where a trypanosome's flagellum and body membrane fractured together was the higher IMP density on the body membrane than on the flagellar fracture faces (see figs. 3-6). Clusters of IMPs corresponding to the macular attachment sites of the trypanosome's flagellum/body junction (figs 3 and 6) were seen on the PF of the flagellar membrane in all forms.

The number of filipin-induced lesions per square micron observed in the body and flagellar membranes of long slender blood forms was markedly fewer than in corresponding surface membrane regions of short stumpy forms (figs 3 and 4, and Table I). An increase in lesion density was also seen after transformation of short stumpy forms into procyclic culture forms (Table I).

Filipin-induced lesions appeared as pits on the PF and protuberances on the EF of both the body and flagellar surface membranes of short stumpy and long slender blood forms (figs 3 and 4). However, the reverse was seen for the corresponding procyclic surface membrane fracture faces: protuberances occurred on the PF and pits were found on the EF (figs. 5 and 6).

Filipin-induced lesions were always at a higher density on the flagellar membrane fracture face compared with the adjacent body membrane fracture of the same trypanosome in all forms. The lesions were excluded from the IMP clusters of the flagellum to body macular attachment zones in the flagellar membrane PF (figs. 3 and 6).

Discussion

In freeze fracture replicas the short stumpy form body membrane appears little different from that of the long slender form with respect to IMP numbers. This could be explained on the basis that coated forms have similar surface membrane enzymes and transport sites necessary for physiological function in the blood environment and that alteration of the surface membrane protein composition may be unnecessary. In addition the

architecture of the surface membrane may be constrained by the need to support the glycoprotein coat.

Changes in body membrane ultrastructure accompanying transformation may reflect the differing requirements of respiratory substrate of bloodstream and procyclic forms. The necessity for replacement of transport sites and receptors during loss of the surface coat glycoprotein seems likely. Interestingly, as seen from Table I, the PF IMP densities increase during progression from stumpy to procyclic form, whereas the EF IMP densities decrease.

There is however, no evidence for EF IMPs being quantitatively related to the variant surface glycoprotein (VSG) molecules comprising the surface coat. The latter have a far higher packing density on the surface than is represented by particles in the membrane (600 IMPs/μm^2 compared with 60,750 VSG molecules/μm^2); Jackson et al 1984). Few eukaryote cell plasma membrane IMPs are well characterised in biochemical and physico-chemical terms. Only in the relatively simple systems such as the erythrocyte or bacterial membranes has analysis of specific IMP function been possible (Pinto da Silva and Nicolson 1976; Verkleij and Vervegaert 1978). It appears likely that IMPs are the morphological representations of intercalated globular proteins (possibly associated with lipids) within the membrane. IMPs have been produced by, for example, the introduction of rhodopsin molecules into artificial lipid bilayers (Hong and Hubbell, 1972). Further structural analyses including complementarity studies of both membrane halves (Verkleij and Vervegaert, 1978) and the use of labelling techniques with freeze-fracture (Pinto da Silva et al 1981; Pinto da Silva and Kan, 1984) may help to clarify the nature of IMPs.

Freeze-fracture cytochemistry using the polyene antibiotic filipin shows the distribution of β-hydroxy sterols through the morphologically identifiable 25 nm lesions produced where filipin and sterols interact within the membrane. The specificity, mode of action and use of filipin as a tool in determining membrane cholesterol have been evaluated (Norman et al

1976, Robinson and Karnowsky, 1980; Friend and Bearer, 1981) and recently reviewed (Severs and Robenek, 1983; Miller 1984). The marked increase in filipin-induced lesions during the slender-stumpy transformation contrasts with the lack of change in IMP density and indicates a marked increase in cholesterol content.

The general effect of increasing cholesterol in membranes is to decrease the fluidity and permeability thus resulting in a more rigid membrane structure. A cholesterol:phospholipid ratio of around 0.5 has been shown for monomorphic (long slender) T.brucei (Rovis and Baekkeskov 1980). This ratio falls within the lowest end of the range observed for mammalian cells (0.49-1.04, Emmelot, 1977). Rovis and Baekkeskov (1980) predicted that stumpy forms might need a higher cholesterol:phospholipid ratio for entering the invertebrate host in order to prevent the phospholipid gel to liquid-crystal transition effect at the lower temperature encountered in the fly.

The increase in cholesterol content of stumpy T. brucei surface membranes may explain the ability of this stage to resist lysis for longer than long slender trypanosomes during the Blood Incubation Infectivity Test (BIIT). This test, developed by Rickman and Robson (1970), attempts to discriminate between non human-infective T. brucei brucei and the human infective sub-species (T.b.rhodesiense and T.b.gambiense) by incubation of trypanosomes in human serum in vitro; only the former are lysed.

These authors and Rifkin (1978) noted the relative resistance to lysis of stumpy forms exposed to normal (non-immune) human serum. The active constituent of human serum is supposedly high density lipoprotein (HDL). Rifkin (1978) postulated that HDL removes surface membrane cholesterol. Colloid osmotic swelling results from the increase in permeability and eventually produces lysis.

A possible consequence of the higher surface membrane cholesterol levels in stumpy forms is reduced motility as this depends upon deformation of the body membrane by the beating flagellum to form the undulating

membrane. Movement is more rapid in long slender forms perhaps because they have a less rigid body membrane due to decreased cholesterol content.

The increased density of filipin-induced lesions in the body membrane of culture procyclics over their stumpy progenitors may reflect a change in the sterol present or a change in permeability to filipin. Procyclic trypanosomes are likely to contain ergosterol rather than cholesterol in their membranes (Dixon et al 1972), but since both are β-hydroxysterols, both react with filipin. Ergosterol is synthesized only by culture forms when cholesterol is limited and may be required for specialised functions for which cholesterol cannot substitute. Mammalian blood forms have no synthetic capacity for sterols, and rely on uptake of cholesterol from the abundant supply in the host circulation (Dixon et al 1972; Venkatesan and Ormerod 1976).

There is no potentially restrictive barrier to filipin penetration at the surface membranes of the procyclic as exists for the blood stages in the form of the 15nm glycoprotein layer (fig. 4) cross-linked by glutaraldehyde prior to incubation. This barrier might influence lesion density through steric hindrance of filipin-sterol interaction by limiting diffusion of the antibiotic across the fixed, coated surface of the blood forms. Rifkin (1983) noted a three fold increase in binding of radio-iodinated HDL to the surface of procyclics over that bound to long slender blood forms and indicated that the presence of the surface coat could influence the binding of HDL to the membrane to a degree probably dependent on the molecular packing density of the coat molecules. No figures are available, as yet, for the relative densities of VSG molecules on slender and stumpy forms, but the low molecular weight of filipin (566 daltons) suggests that its penetration of the surface coat should not present a problem.

The increase in numbers of filipin-induced lesions in the flagellum over that in the body membrane of the trypanosome (Table I) indicates a functionally less fluid membrane region which may be significant in the action of the beating flagellum. Flagellum to body attachments which appear

as clusters of IMPs on the flagellar PF (Smith et al 1974; Hogan and Patton 1976; Vickerman and Tetley 1979) (fig. 6) are apparently insensitive to filipin in common with other intramembrane particle arrays studied so far (Severs and Robenek, 1983). During the attached stages of development of T.brucei in the salivary glands of the tsetse fly flagellar attachment plaques to the host microvilli have also been shown to be devoid of filipin induced lesions (Tetley and Vickerman 1985), so it appears that sterols are excluded from these regions also.

The tendency for lesions to occur mainly as pits and correspondingly as protuberances in the PF and EF respectively in blood forms (and conversely in procyclics) argues for an unequal partitioning of sterol according to current interpretations of the molecular interaction of filipin with membranes (Severs and Robenek 1983; Miller 1984). Sterol-filipin binding initiates deformation in one half of the bilayer due to re-orientation of sterol from a horizontal to a vertical position in the plane of the membrane (Severs et al., 1981). Consequent increase in surface pressure results in a bulge (lesion) towards the monolayer of origin of the filipin-sterol complex. The monolayer exhibiting most pits would thus seem to represent the membrane half with the highest sterol content. This model is complicated by the possibility of 'flip-flop' of sterol from one membrane half to the other (Miller 1984) and also by the effects of fixation on filipin-sterol complex symmetry: it can reverse the polarity seen in unfixed preparations (Robinson and Karnowsky 1980). No influence of the sub-surface cytoskeletal elements on lesion partition between EF and PF was seen. The alignment of filipin-induced lesions with underlying microtubules in the PF of some T.cruzi body membranes (Souto-Padron and De Souza 1983) has not been observed in this study and there was no indication of restriction to lateral mobility of sterols by sub-membrane connections such as are sometimes seen emanating from the sub-pellicular microtubule array.

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Table 1. Intramembrane particle (IMP) and filipin-induced lesion counts (FIL) on T.brucei fracture faces represented as the mean number per square micron ± 2 S.E., taken from the number of measurements given in parenthesis.

T.brucei stage	IMP/μm^2		FIL/μm^2	
	Body membrane PF	Body membrane EF	Body membrane	Flagellar membrane
Bloodstream Long Slender	2353± 150 (6)	600± 57 (6)	5± 2 (12)	36± 13 (10)
Bloodstream Short Stumpy	2314± 184 (6)	536± 52 (5)	104± 22 (17)	184± 39 (13)
Culture Procyclic	3332± 213 (4)	241± 12 (4)	162± 52 (13)	258± 46 (14)

Figure legends

Pt/C shadow direction is from the bottom to the top of all micrographs.

Abbreviations: PF_{fl} = flagellar membrane PF.

EF_{fl} = flagellar membrane EF.

PF_{bm} = body membrane PF.

EF_{bm} = body membrane EF.

Arrows = filipin-induced lesion (protuberance)

Arrowheads = filipin-induced lesion (pit)

IMP = intramembrane particle

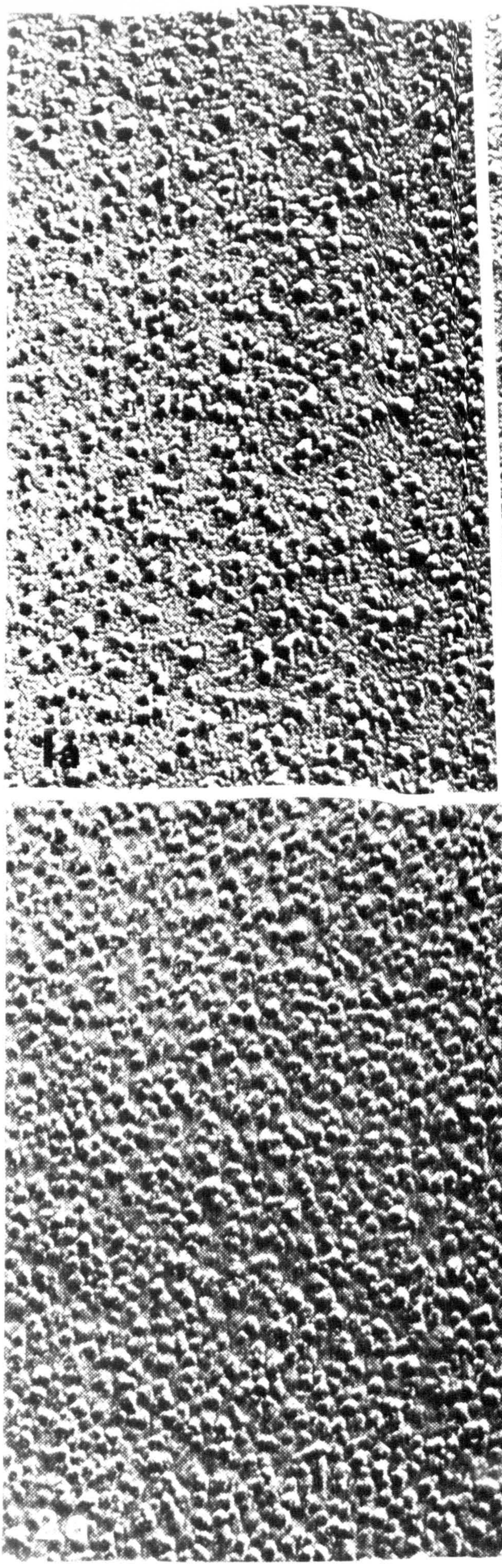
fl = flagellum fp = flagellar pocket

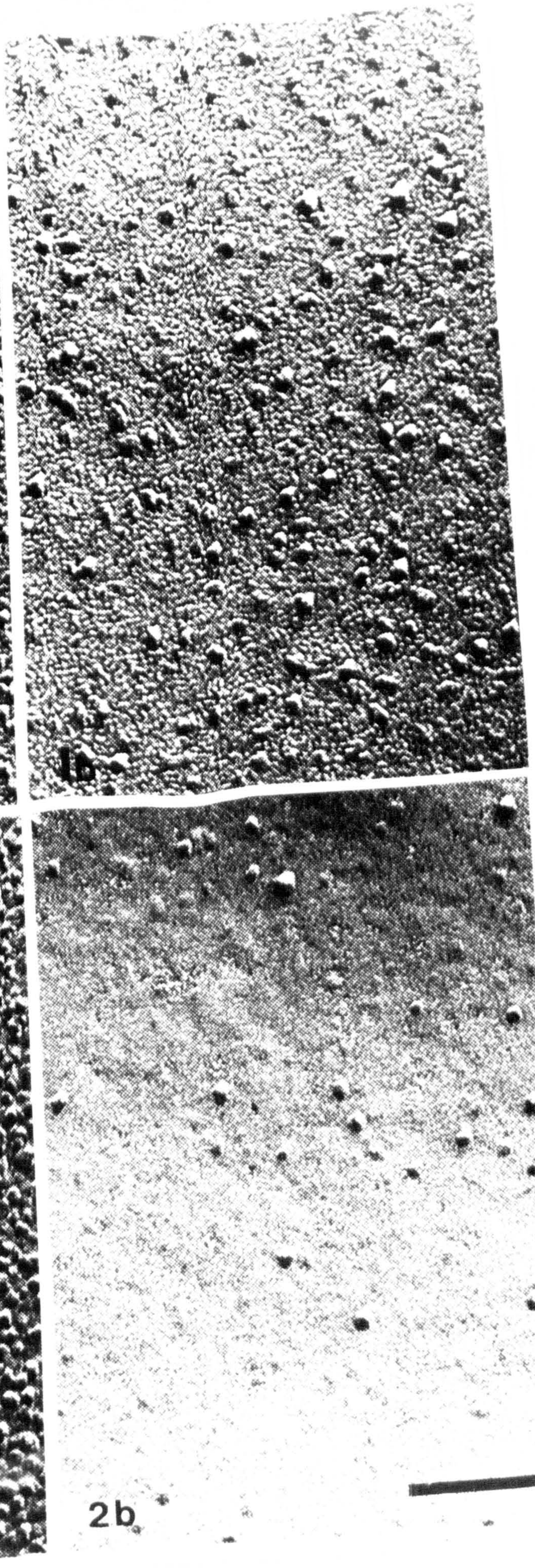
SC = surface coat

Figs. 1 and 2 Fracture faces of the body membrane of T.brucei stages to show IMP distributions. Scale bar = 100 nm

Fig. 1. Short stumpy blood form. (a) PF, (b) EF

Fig. 2. Procyclic culture form. (a) PF, (b) EF


1a

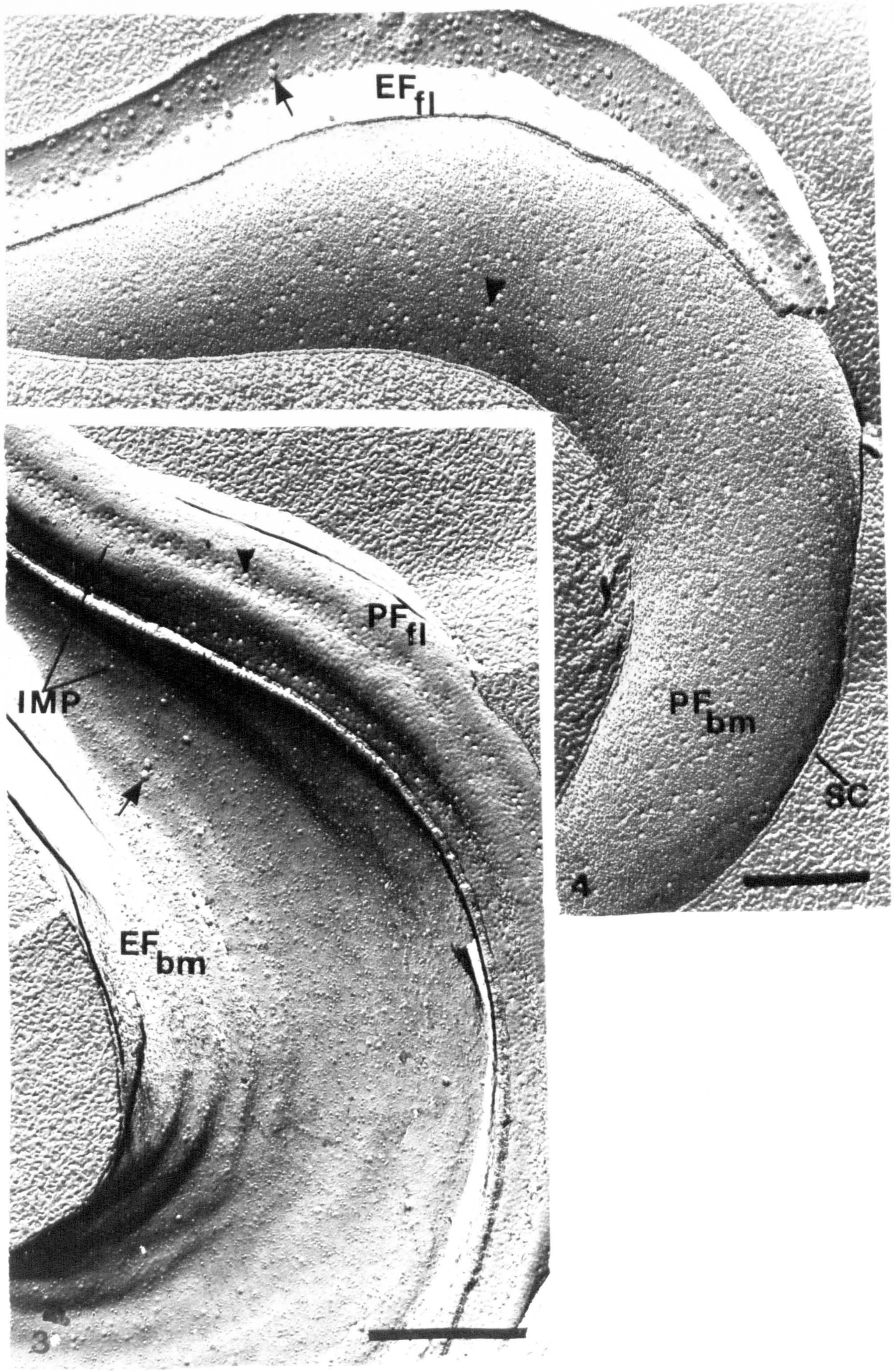

1b

2b

Figs. 3 and 4 Body and flagellar membrane fracture faces of blood forms of T.brucei showing filipin-induced lesions. Scale bar = 0.5 μm.

Fig. 3. Long slender form.

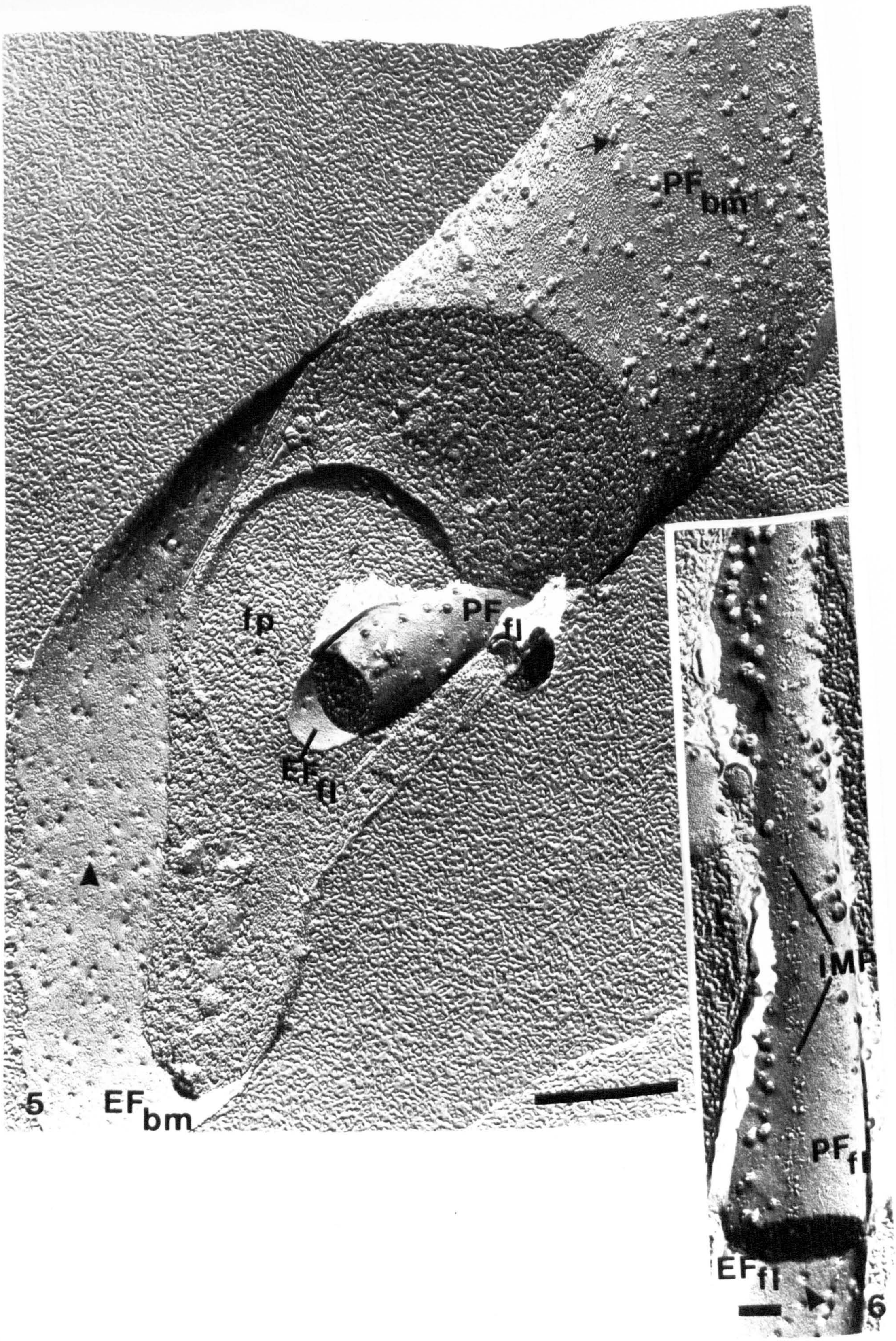
Fig. 4. Short stumpy form.


EF fl
PF fl
IMP
EF bm
PF bm
SC
4
3

Figs. 5 and 6 Body and flagellar fracture faces of culture procyclic T. brucei showing filipin-induced lesions.

Fig. 5. PF and EF of surface membranes. Scale bar = 0.5 μm

Fig. 6. Flagellar membrane showing absence of filipin-induced lesions from PF IMP clusters at flagellar attachment zone. Scale bar = 100 nm.


PF bm
fp
PF fl
EF fl
5
EF bm
IMP
PF fl
EF fl
6

Section 4.

Differentiation in Trypanosoma brucei: Host-parasite cell junctions and their persistence during acquisition of the variable antigen coat.

SUMMARY

Acquisition of the variable antigen-containing surface coat of Trypanosoma brucei occurs at the metacyclic stage in the salivary glands of the tsetse fly vector. The differentiation of the metacyclic trypanosome in the gland has been studied by scanning electron microscopy and by transmission electron microscopy of thin sections and freeze-fracture replicas. The uncoated epimastigote trypanosomes (with a pre-nuclear kinetoplast) divide while attached to the salivary gland epithelium brush border by elaborate branched flagellar outgrowths which ramify between the host cell microvilli and form punctate hemidesmosome-like attachment plaques where they are indented by the microvilli. These outgrowths become reduced as the epimastigotes transform to uncoated trypomastigotes (with post-nuclear kinetoplast) which remain attached and capable of binary fission. The flagellar outgrowths disappear but the attachment plaques persist as the uncoated trypomastigotes (premetacyclics) stop dividing and acquire the surface coat to become "nascent metacyclics". Coat acquisition therefore occurs in the attached trypanosome and not, as previously believed, after detachment. Coating is accompanied by morphological changes in the glycosomes and mitochondrion of the parasite. Freeze-fracture replicas of the host-parasite junctional complexes show membrane particle aggregates on the host-membrane but not on the parasite membrane. It is suggested that disruption of the complex occurs when maximum packing of the glycoprotein molecules has been achieved in the trypanosome surface coat, releasing the metacyclic trypanosome into the lumen of the gland.

INTRODUCTION

One of the most crucial stages in the transmission of trypanosome infections is the development of the metacyclic trypanosome in the vector. In Trypanosoma brucei, the species responsible for human sleeping sickness as well as disease in cattle, this development takes place in the salivary gland of the tsetse fly (Glossina spp.). Here epimastigote trypanosomes (with a prenuclear kinetoplast), attached to the host's glandular epithelium, transform to trypomastigote metacyclic forms (with a postnuclear kinetoplast); the mature metacyclic trypomastigotes lie free in the gland lumen, ready to be discharged with the saliva when the fly bites a mammal. The discharged metacyclic trypanosome resembles all the mammal stages of the parasite in that it possesses a 12-15 nm thick surface coat on both body and flagellum (Vickerman, 1969). This coat is made up of a monomolecular layer of glycoprotein which represents the variable antigen of the trypanosome (Vickerman and Luckins, 1970; Cross 1975; Fruit et al. 1977). By expressing different variable antigen genes the trypanosome can change the antigenic character of its surface and so evade the host's immune response (Vickerman 1978, Borst & Cross, 1982). The coat is absent from the epimastigote predecessors of the metacyclic: uncoated trypanosomes are non-infective as they readily fall prey to the mammalian host's non-specific defense mechanisms (Mosser & Roberts, 1982; Ferrante & Allison, 1983). As the metacyclic trypanosomes of a clone infection are heterogeneous with respect to variable antigen type (Le Ray, Barry and Vickerman, 1978), the generation of variable antigen type diversity must occur during differentation of the metacyclic forms from the epimastigotes.

Although there has been some chronicling of the ultrastructural events accompanying acquisition of the surface coat and infectivity during metacyclic differentiation (Vickerman, 1969; Steiger, 1973), detailed study has been retarded by the difficulties inherent in securing the tsetse salivary gland stages (as fly infection rates in the laboratory are notoriously low) and in obtaining satisfactory fixation of this material. These difficulties have now been largely surmounted in this laboratory and we present here an account of metacyclic differentiation with emphasis on host parasite attachment in relation to acquisition of the surface coat.

MATERIALS AND METHODS

Infection of tsetse flies with trypanosomes

Trypanosomes of the AnTAR 1 and ETAR 1 serodemes were used to infect tsetse flies, Glossina morsitans morsitans, as described previously (Hajduk, Cameron, Barry & Vickerman, 1981). Flies were monitored for infection by allowing them to probe saliva onto warm glass slides and recording the presence of mature metacyclic tryapnosomes. Salivary glands from positive flies were removed into Cunningham's medium (Cunningham 1977) supplemented with 10% heat-inactivated foetal calf serum and examined by phase contrast microscopy to ascertain the extent of infection.

Electron Microscopy

For transmission microscopy, heavily-infected glands were transferred into fixative at room temperature (20°C) and immediately cut into 4 or 5 segments to facilitate access of fixative to the attached trypanosomes. Egress of saliva from the gland segments allowed

collection of unattached forms in fixative and small pellets of these flagellates were detained by centrifugation (1000g for 10 min) for further processing and ultrastructural comparison with attached stages.

For scanning electron microscopy an infected gland was pinned onto 'Sylgard' silicon rubber (Dow Chemicals Ltd.) in a pool of Cunningham's medium and slit along its length from the blind end to the salivary duct using a micro-knife fitted with a fractured razor blade; saliva was flushed gently from the exposed infected epithelium with fresh medium before fixation of the gland in glutaraldehyde.

Primary fixation of gland pieces was in 2.5% glutaraldehyde in 0.1M phosphate buffer containing 10 mM $CaCl_2$ at pH 7.4 for 1-2 h at 20°C. Overnight storage in 0.1M phosphate buffer containing 2% w/v sucrose was followed by several rapid changes of this solution and then gradual introduction of 4% w/v aqueous osmium tetroxide solution to give a final OsO_4 concentration of 2% in the buffered sucrose solution.

After 1-2 h post fixation and several changes of distilled water to remove residual osmium (3 x 5 min), specimens for TEM were stained 'en bloc' with 0.5% aqueous uranyl acetate for 30 min and routinely dehydrated in an alcohol series followed by propylene oxide infiltration and embedding in Araldite.

Blocks polymerized in silicon rubber moulds for 48 h at 50°C were cut on an LKB MkI Ultratome to give sections of 60-80 nm thickness range (as judged by interference colours). These were either mounted on 300 mesh copper grids or Formvar coated 3 x 1 mm slot grids for serial reconstructions. All sections were contrasted with uranyl acetate and lead citrate and images recorded on Ilford EM4 cut film using an AEI 801

transmission electron microscope operating at 60 Kv.

For SEM, infected glands were transferred after postosmication into a retaining chamber comprising 2 stainless steel meshes separated by a Teflon spacer and housed in a brass fitting to facilitate dehydration in acetone and critical point drying from CO_2. Dried glands were attached to stubs with double-sided Sellotape and sputter coated with gold to 20-40 nm thickness. The specimens were examined in a Philips 500 SEM operating at 12 or 25 Kv.

Freeze-fracture procedures

After primary glutaraldehyde fixation, salivary gland pieces were rinsed in 0.1M phosphate buffer and gradually infiltrated over 2-3 h with 25% glycerol in the same buffer, at 20°C. Individual pieces mounted in 25% glycerol on Balzers' copper support plates (flat) were sandwiched in a thin liquid layer by placing a second support plate over the first and then the assembly was rapidly cooled in liquid propane held at near -180°C Such preparations were stored under liquid nitrogen until required.

Fracturing was accomplished at -100°C and 2×10^{-6} torr with a complementary replica device (Sleytr & Umrath, 1974) installed in a diffusion pumped vacuum system fitted with a liquid nitrogen baffle and a temperature controlled specimen table, also liquid nitrogen cooled. Conventional 45° Platinum/Carbon and 90° Carbon evaporation from resistance sources gave replicas which were cleaned with 40% chromic acid and picked up from distilled water, after several changes, on 200-mesh copper grids. The matching of complementary replicas was facilitated by retrieving each on individual referenced grids (Polaron, G200FZ).

In order to compare distribution of β-hydroxy sterols in the

membranes of different developmental stages, gluteraldehyde-fixed salivary gland pieces were incubated in 50 μg ml^{-1} filipin (UpJohn, a gift from Dr. T.E. Grady) dissolved in DMSO and phosphate buffer (0.5% DMSO final concn) for 4-8 h at 20°C. in the dark. After several buffer washes over 30 min the specimens were infiltrated with 25% glycerol in phosphate buffer and freeze-fractured as detailed above.

RESULTS

Light microscopy, scanning electron microscopy, and transmission electron microscopy of thin sections have enabled us to distinguish four different stages in the development of Trypanosoma brucei in the salivary gland of Glossina morsitans. These are (1) the epimastigote, (2) the premetacyclic tryponastigote, (3) the nascent metacyclic and (4) the mature metacyclic tryponastigote which is discharged by the vector with its saliva on feeding. Stages 1 to 3 are attached to the salivary gland epithelium; stage 4 lies free in the gland lumen.

No obvious differences were discerned using any technique between the ultrastructure of AnTAR 1 (non infective to man - T.brucei brucei) and ETAR 1 (infective to man - T.brucei rhodesiense) trypanosomes.

Transmission electron microscopy of thin sections

(1) Epimastigotes. In the epimastigote form of the trypanosome the kinetoplast lies anterior to the nucleus and the flagellate multiplies by binary fission while attached to the salivary gland epithelium. The luminal border of the epithelium is uneven and covered with tangled microvilli, each approximately 1-1.5 μm long. It is to these that the

trypanosomes attach.

A detailed description of the ultrastructural features of the epimastigote stage of the African trypanosomes has been given for T.vivax (Vickerman, 1973) and the T.brucei epimastigote differs most strikingly from this in the nature of its attachment to the vector, as T.vivax epimastigotes attach to the chitinous lining of the tsetse food canal. As in T.vivax the body and flagellar membranes are uncoated and attachment to the host is by the flagellum: in T.brucei, however, the flagellar membrane and underlying sheath are greatly expanded to form arborescent outgrowths which penetrate between the supporting host microvilli and embrace them, bringing the parasite into intimate contact with the apical surface of the host epithelial cell (Figs.1,2,18). Where microvilli indent the branched flagellar outgrowths, local attachment plaques c.100-130 nm in diameter are visible; fibrillar electron dense material lines each cup-like plaque only on the flagellar membrane side of the junction; there is no plaque on the confronting host cell membrane. A gap of c.20 nm separates the host and flagellar membranes (Fig. 3). Where the parasite membrane is in sustained contact with host cell apical membrane, focal plaques punctuate the flagellar membrane at regular intervals (c.40-50 nm); there is no continuous attachment "hemidesmosome" as there is in the junction between the T.vivax flagellum and the chitinous lining of the tsetse fly proboscis. The body membrane of the epimastigote does not appear to attach to host surfaces. The tentacular outgrowths with their attachment plaques are absent from the recently emerged flagellum and appear to be predominantly a feature of its distal end, though less marked on its "free" (non body-attached) terminal

portion; they arise largely from the peri-axonemal sheath (Fig.2) but not infrequently from that half of the flagellar sheath which surrounds the paraxial rod; the membrane of the "free" portion of the flagellum may form junctional complexes around its entire perimeter.

Other ultrastructural features of the epimastigote include the branched mitochondrion (usually two strands are visible in the pre-kinetoplastic portion of the body and at least two in the post-kinetoplastic portion alongside the nucleus) with its crowded tubular cristae (Fig.2), and the dense bacilliform glycosomes (Fig.4).

(2) Premetacyclic trypomastigotes. Scattered among the attached epimastigotes are flagellates which, because they have a postnuclear kinetoplast, and flagellar origin, can be identified as trypomastigotes, but they differ from the mature metacyclic trypomastigotes and resemble epimastigotes in that they are uncoated, in that they are patently capable of division (Fig.6), and in that they are attached. Flagellar outgrowths are again present, but noticeably shorter and not so well developed as in the epimastigote. Attachment plaques are abundant but mainly along the membrane of the flagellar shaft (Fig.3). The mitochondrion is still branched in the pre-kinetoplastic part of the body and the glycosomes are bacilliform.

(3) Nascent metacyclics. Profiles of coated trypomastigotes whose flagella lack tentacular outgrowths but retain the attachment plaque junctional complexes with the microvillar membrane are frequently encountered in sections (Fig.7). In their cytoplasmic organization, these flagellates resemble mature metacyclics in that the pre-kinetoplastic mitochondrion is a single strand with sparser ampulliform cristae and the

glycosomes are spherical (Fig.5), less electron dense, and not bacilliform. Dividing stages of these coated attached trypomastigotes have not been observed. In all but their attached flagella then, these flagellates are metacyclic trypanosomes and are here interpreted as "nascent metacyclics", i.e. trypanosomes that have recently acquired the coat but have not yet been released.

A notable feature of these nascent metacyclics is the persistence of an extension of the flagellum beyond the anterior tip of the body - the so called "free flagellum" which is reputedly absent or greatly truncated in the mature metacyclic (Hoare, 1972). In the nascent metacyclic this flagellar tip either lies attached by focal junctions among the host microvilli or it is inserted into the apical cytoplasm of the host cell (Fig 8). Whether embedding of the flagellar tip occurs before or after coating is unknown but inserted uncoated flagella have not been observed.

(4) Mature metacyclics. These lie free in the lumen of the salivary gland. They have never been observed in division. Their flagella bear no traces of junctional complexes other than the desmosome-like junctions between trypanosome body and flagellum. The mitochondrion and glycosomes are essentially similar to those of the nascent metacyclic.

Scanning electron microscope observations.

Owing to dense crowding of the flagellates in the carpet of parasites lining the salivary gland, and too deep penetration of the microvillar layer by flagella, details of attachment (including the arborisation of the epimastigote flagellum) cannot be visualized with the SEM. The different stages in development of the metacyclic, however, can

be distinguished by their posterior ends (Fig.9). During the transition from epimastigote to metacyclic progressive shortening of the distance from flagellar base to posterior end occurs and the acute angle formed by the posterior extremity in profile increases. In the nascent and mature metacyclic the posterior extremity is blunt and not acute.

Freeze-fracture observations.

Survey views of replicas with the transmission electron microscope at low magnification confirm the branched nature of epimastigote flagellar outgrowths and show them penetrating between irregularly-shaped host cell microvilli (Figs. 10, 13). Attachment plaque sites with interposed plaque-free areas are seen as conspicuous concavities (PF) and convexities (EF) in cleaved flagellar membranes and occur in groups along the length of attached flagella. Trypanosome body membranes and the flagellar membranes of luminal flagellates lack these plaques.

Fracture faces of the surface membranes of attached epimastigotes differ strikingly in their intramembranous particle (IMP) distribution from those of mature metacyclic trypanosomes found free in the lumen of the gland. During the transformation from epimastigote to metacyclic trypomastigote the density of IMPs in the PF becomes reduced while there is a slight reduction in IMP density in the EF (Figs.11,12). The cleaved flagellar membranes of both forms show a reduced IMP distribution compared with their respective trypanosome body membranes (Figs. 13, 14). Trypanosome attachment plaques are of uniform size (100nm diameter) as seen in replicas of cleaved flagellar membrane and largely devoid of IMPs on both fracture faces.

The cleaved host cell membrane, however, shows patches with regimented linear arrays of IMPs on the PF (Figs.14,15); these arrays correspond in size to the concavities/convexities of the flagellar membrane attachment plaques and in some replicas (Fig.14) can be seen to be apposed to them. PFs of the basal region of the host cell microvillar border, may show several IMP arrays, confirming that indentation of the flagellar membrane by microvilli is not necessary for the generation of attachment plaques. Such PFs show that the regular IMP arrays are composed of particles of a particular size class (10nm) and clearly distinct from larger (16nm) unaggregated particles also present in the host membrane PF (Fig.15). Particle aggregates are not found in the freeze-fractured microvillar membrane of uninfected salivary glands. Complementary replicas of the flagellar membrane attachment plaques (Fig.17) confirm that the particle arrays are entirely absent from the cleaved parasite membrane. Filipin treatment of attached trypanosomes results in characteristic lesions of the flagellar membrane (Fig.16) except at sites of flagellar attachment plaques suggesting that cholesterol is largely excluded from these regions.

DISCUSSION

Eukaryote flagella and cilia are primarily contractile organelles involved in cell locomotion or in the propulsion of fluid over cells, but in protists the flagellar or ciliary membrane may play a part in recognition processes, especially in mating reactions as is now well established for Chlamydomonas spp. (Goodenough and Thorner, 1983) and the ciliate Paramecium (Miyake, 1981). The all-parasitic

trypanosomatid flagellates appear to be unique in that they have evolved the ability to modify the flagellum as an attachment organ and, in at least one phase of the life cycle, they multiply while attached to a host surface. This surface may be the intert chitinous lining of the hindgut or proboscis of the arthropod host or the living microvillar border of its midgut, malpighian tubule or salivary gland epithelium. Although the host in question is usually an arthropod, Trypanosoma congolense has the ability to attach to capillary vessel endothelia in its mammalian host (Banks, 1978). Sometimes these attachments occur between the flagella of adjacent parasites so that attachment to the host surface is indirect (Vickerman, 1973). Flagellar beating continues unimpaired in such attached forms and may help to circulate the surrounding medium about the multiplying parasites, or, in some cases drive this medium through the flagellar pocket from which pinocytosis occurs (Vickerman and Preston, 1976).

How specific are these trypanosomatid flagellar attachments is uncertain. Thus Crithidia fasciculata in vivo will adhere to the chitinous intima of the mosquito hindgut (Brooker, 1971a), but in vitro it will attach to Millipore filters (Brooker, 1971b). The epimastigote stage of T.congolense which multiplies while clinging to the chitinous wall of the food canal in the tsetse proboscis will, in vitro, attach to the wall of plastic culture flasks (Gray et al. 1981). On the other hand, the specificity shown by attachment of the flagella of certain species of Leptomonas for the water and salt-transporting rectal ampullae as opposed to other chitinized regions of the hindgut of their insect hosts is quite remarkable (Lauge & Nishioka, 1977).

Those flagellates that attach to living host cells, however, have not as yet been cultivated on artificial substrates and it is possible that either growth of the attached phase in the life cycle or maturation of the free-swimming phase that develops from it is dependent upon exchange between parasite and host cell.

The ultrastructure of the hemidesmosome-like attachments formed where the flagellar membrane contacts an inert substratum has been described for several trypanosomatid species by numerous investigators (reviewed Vickerman and Preston, 1976; Molyneux, 1977), but attachments to living host microvilli have been poorly characterized. No hemidesmosomes were discerned with microvilli for Leishmania mexicana in Lutzomya longipalpis (Killick-Kendrick, et al, 1974) or Trypanosoma melophagium in Melophagus ovinus (Molyneux 1975). Steiger (1973) briefly described the punctate attachment plaques of T.brucei epimastigote flagella with their insect host's microvilli, but not the remarkable dendroid flagellar outgrowths which also play a part in attachment. He envisaged coating taking place after release of the trypanosome from its mooring.

We report here that the acquisition of the variable antigen-containing surface coat occurs while the trypanosome is still attached and that this non-dividing nascent metacyclic stage is preceded by a dividing uncoated trypomastigote (the premetacyclic) which arises from the main multiplicative stage in the salivary gland (the epimastigote). The recognition of these different stages in metacyclic differentiation (Fig.18) is crucial to the problem of the generation of trypanosome variable antigen diversity in the vector's salivary glands and to the interpretation of data on this population heterogeneity (Le Ray, et

al. 1978).

In our experience, mature (coated) metacyclics only are discharged from the fly salivary gland; it is possible, however, that the practice in some laboratories of squeezing the abdomen of the fly to force it to discharge more trypanosomes will result in dislodgements of attached uncoated trypanosomes. This practice should therefore be avoided as it unnecessarily complicates the picture of metacyclic variable antigen type (VAT) heterogeneity as studied using the immuno-fluorescence reaction. The ability to recognize nascent metacyclics in sections of the vector salivary gland will be useful in answering the question "is VAT heterogeneity present ab initio or do all nascent metacyclics initially acquire a coat of the same antigenic type so that heterogeneity arises as a consequence of antigenic change in the mature metacyclic population?" Electron immunocytochemical studies on nascent metacyclics in situ are in progress towards this end.

The host-parasite junctional complex.

Junctional complexes described to date between cells of the same organism may be classified as adhering (desmosome, hemidesmosome), impermeable (tight, septate) and communicating (gap, synapse) junctions. The punctate junctions described here are most probably of the adhering type, performing an anchoring function and distributing any shearing forces through the salivary gland epithelium. Each time the tsetse fly feeds, muscular contraction of the wall of the salivary glands to expel saliva will create a shearing force along the microvillar border threatening to dislodge the trypanosome.

The flagellar attachment plaques differ from hemidesmosomes in that there is as yet little evidence for a tonofibril-like filament system connecting the plaques to one another or to the main cytoskeletal elements - in this case the axoneme-paraxial rod complex. This absence may reflect lack of preservation. In T.vivax (Vickerman, 1973) and T.congolense (Evans, Ellis and Stamford, 1979; Thevenaz and Hecker, 1980) the belt-like hemidesmosome which attaches the flagellum to the chitinous lining of the tsetse proboscis does appear to show such connections to particular doublets of the axoneme (6,7,8,9 and 1). Similarly the spot desmosome-like junctions that bind the flagellum to the body in all trypanosomes show a filamentous "Y" connection between the flagellar plaque and axonemal doublet and the paraxial rod (Vickerman, 1969). Comparable connectives between the axoneme or paraxial rod and surface mastigonemes of the flagella of euglenid flagellates have been demonstrated (Melkonian et al. 1982).

In T.brucei the efficiency of anchoring is increased by the great expansion of the flagellar membrane so that many more attachment plaques can be accommodated on the dendroid outgrowths and attachment can be effected to a large number of microvilli in the plane of shear. Although limited flowing of the flagellar cortex into branches can be observed in T.vivax and T.congolense attached to inert substrata (cf.Thevanez & Hecker, 1980; Gray et al. 1981), the extraordinary scale on which branching occurs in T.brucei has no known parallel among other trypanosomatids. In the related biflagellate kinetoplastid family, Bodonidae, however, comparable branching of the anterior flagellum for the purpose of attachment has been described for Cryptobia helicis in the

spermatheca of pulmonate snails, though no differentiated junctional complexes were seen in these attachments (Current, 1980).

It is interesting to note that these junctional complexes persist in the trypanosome flagellum during the acquisition of the surface coat, so that the forces that bind host and parasite membranes together must endure, at least initially, the intervention of the parasite glycoprotein coat. Barry (1978) has presented evidence that the variable antigen glycoprotein is freely diffusible in the plane of the surface membrane of the trypanosome and that during coat acquisition the density of packing of the glycoprotein molecules increases over the entire surface of the flagellate. We postulate here that the achievement of maximum glycoprotein packing disrupts the bond between host and parasite. It is equally possible, however, that metabolic changes taking place in the trypanosome during metacyclic maturation result in products which disrupt the unusual junctional complex, liberating the mature metacyclic into the lumen of the gland.

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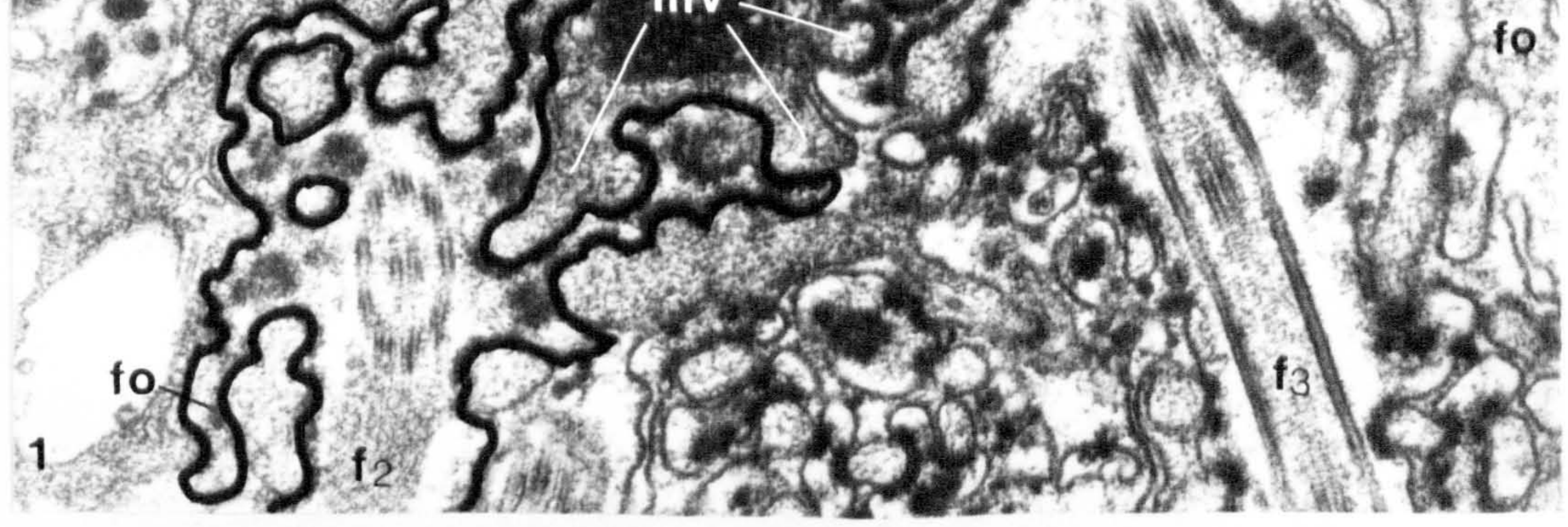
Legends to Figures

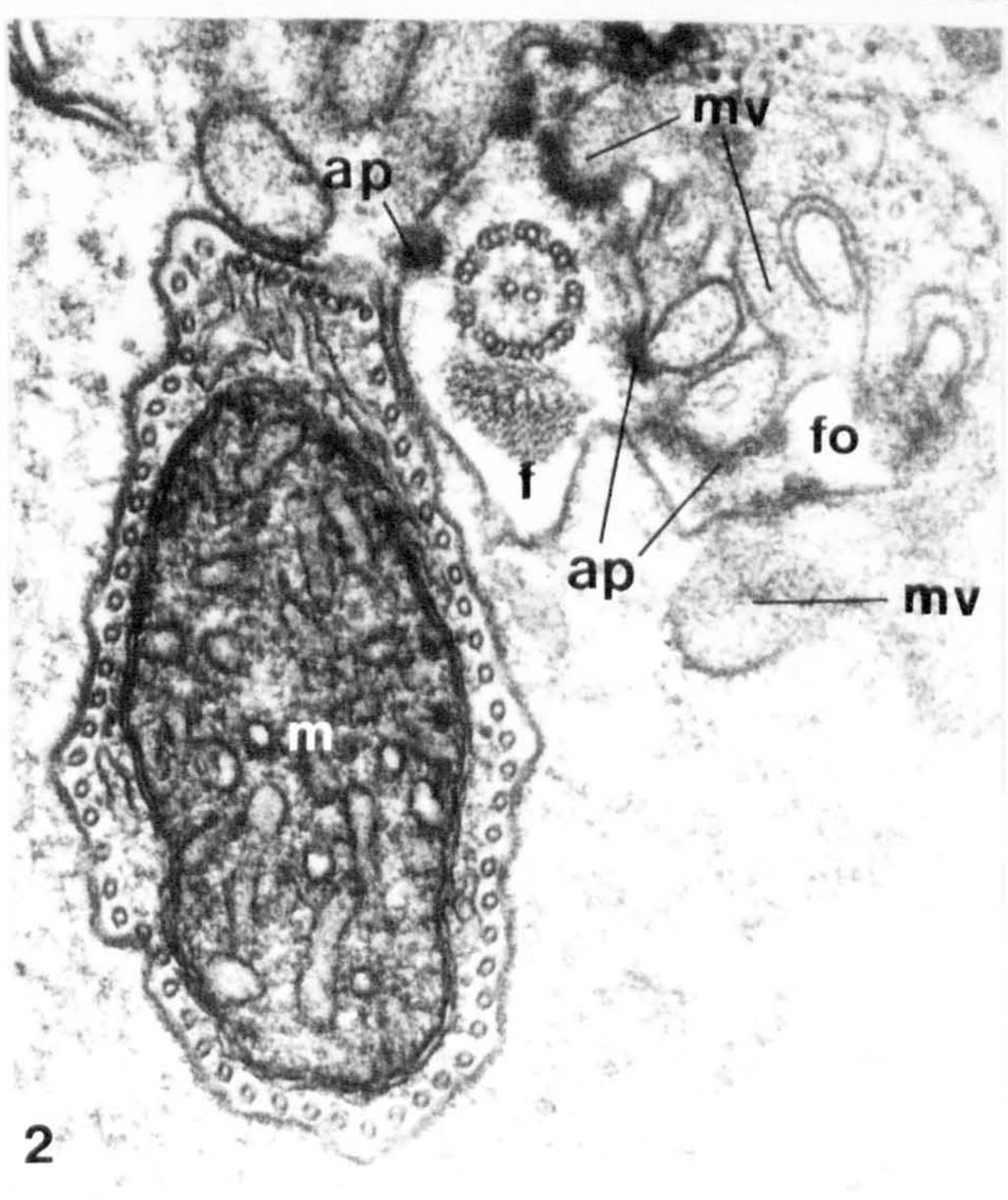
Figs.1-10,11 & 12 are of AnTAR 1; Figs.9 and 13-17 of ETAR 1 trypanosomes.

Fig. 1. Tangential section through apical region of microvillar border of tsetse salivary gland cell with attached flagella of Trypanosoma brucei epimastigotes. Three profiles of trypanosome flagellar shafts (f_1, f_2, f_3) are visible, each showing extensive branched outgrowths (fo) of the flagellar sheath. The outgrowths penetrate between the microvilli (mv) and focal attachment plaques (ap) are visible where the microvilli indent the flagellar outgrowths. Electron dense material lines each cup-like plaque only on the flagellar membrane side of the junction. The flagellar membrane of f_2 has been traced in black to indicate more clearly the extent of branching in this section. X42,000.

Fig. 2. Transverse section of epimastigote showing flagellum with outgrowth (fo) and several attachment plaque junctions (ap) with adjacent host microvilli (mv). Both flagellar and body surface membranes of the epimastigote lack a surface coat, though a thin uneven precipitate of salivary protein covers all visible surface membranes. The trypanosome mitochondrion (m) shows the tubular cristae characteristic of the epimastigote stage. X48,800.

Fig. 3. Detail of junctions between epimastigote flagellum (f) and host cell (hc). Note the fibrillar plaque regions (ap) on the flagellar membrane where it is indented by host cell microvilli (mv). The gap between confronting parasite and host membranes is considerably wider between plaques (large arrowheads) than in the plaque region (small arrowheads). X150,000.


f1
ap
fo
ap
mv
fo
fo
f2
1
f3


mv
ap
f
fo
ap
mv
m
2

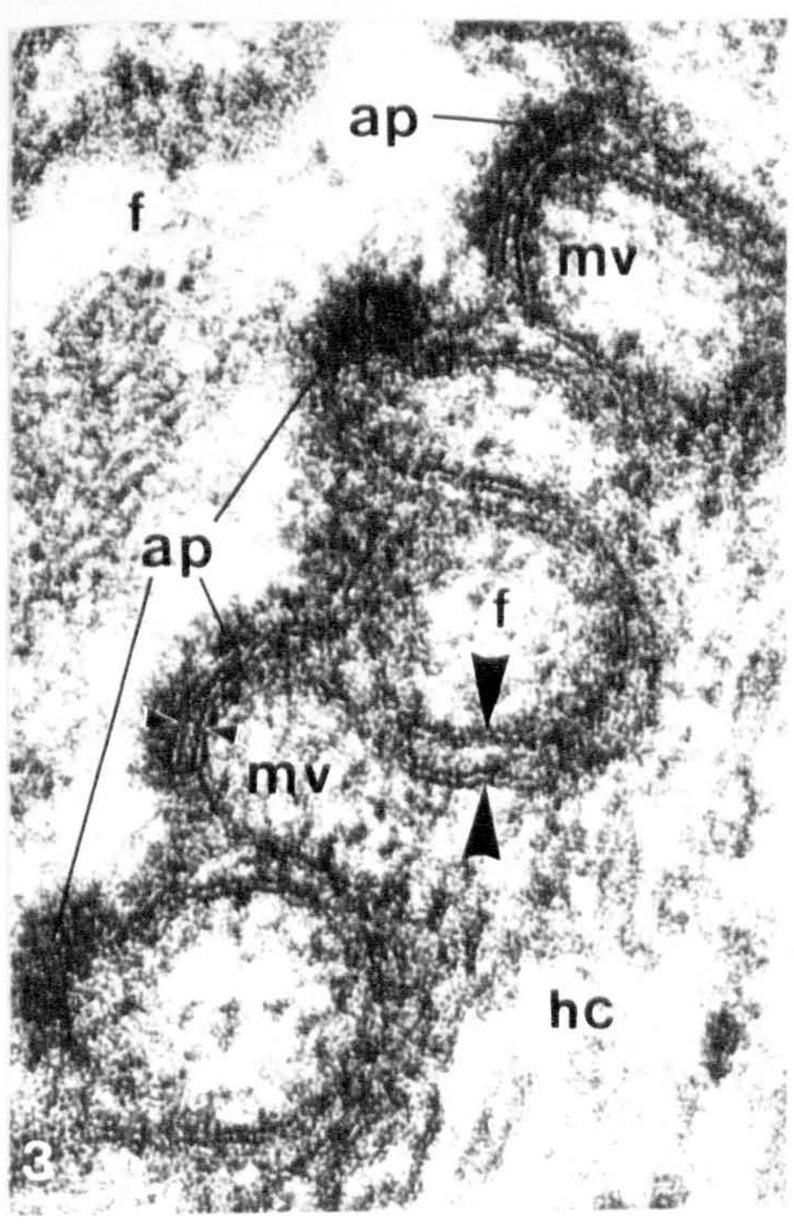
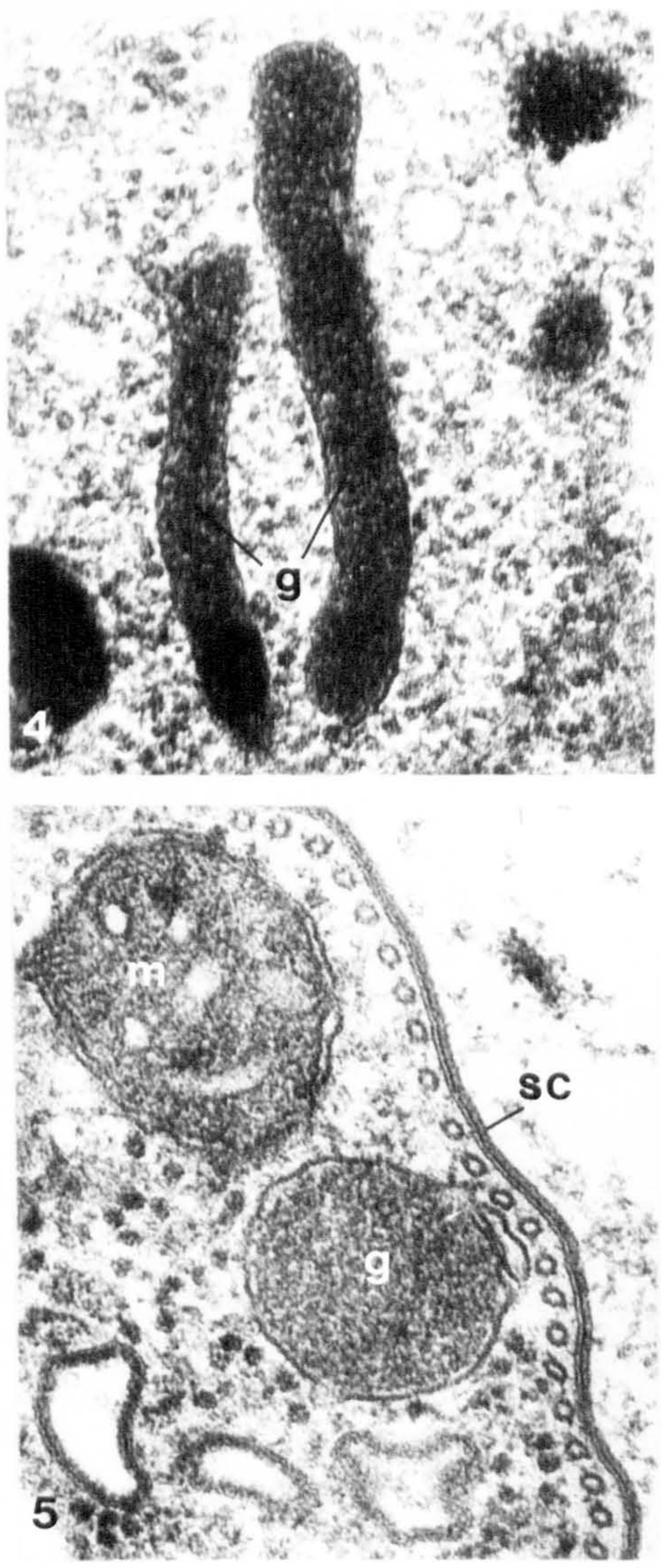
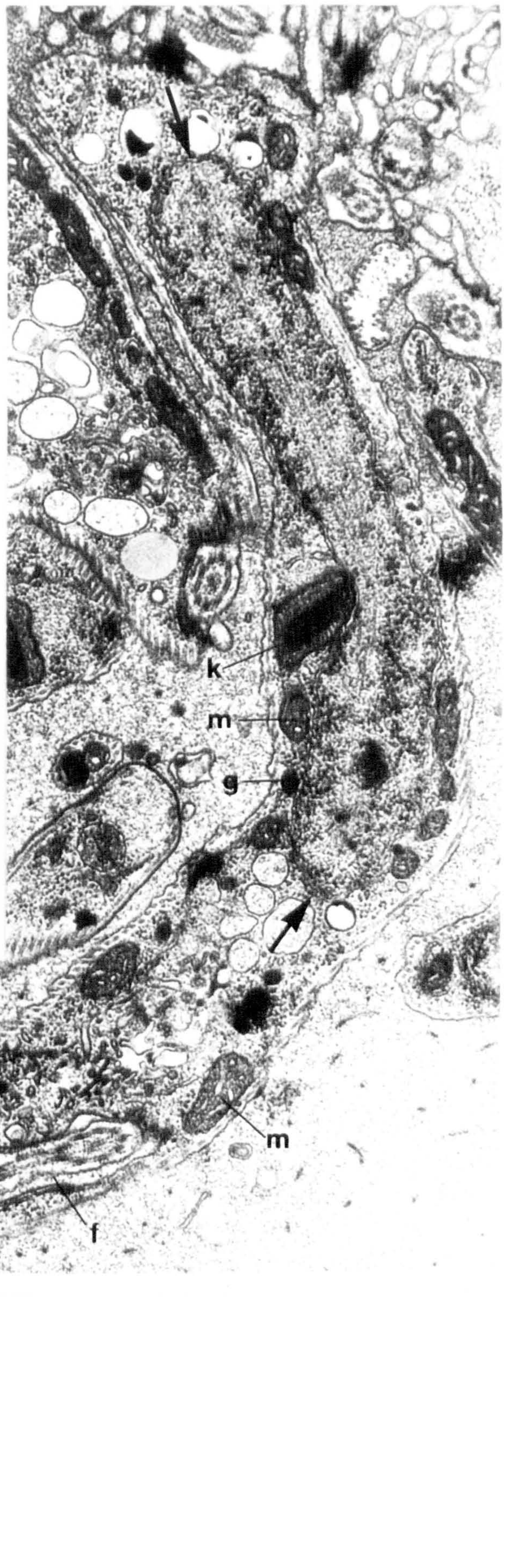

ap
f
mv
ap
f
mv
hc
3

Fig. 4. Bacilliform glycosomes (g) of epimastigote stage showing dense homogeneous contents. X78,000.

Fig. 5. Part of section of metacyclic trypanosome showing spherical glycosome (g) with less dense contents characteristic of trypomastigote stages which bear a surface coat (sc). m, mitochondrion. X78,000.

Fig. 6. Longitudinal section of uncoated dividing trypomastigote (pre-metacyclic). Note elongate nucleus (n) with spindle microtubules, passing from pole to pole (poles arrowed), two kinetoplasts (k) and profiles of branched mitochondrion (m). The morphology of the glycosomes (g) suggest that these organelles resemble those of the uncoated epimastigote rather than those of the coated (metacyclic) trypomastigotes. f, flagellum. X26,250.


g
4

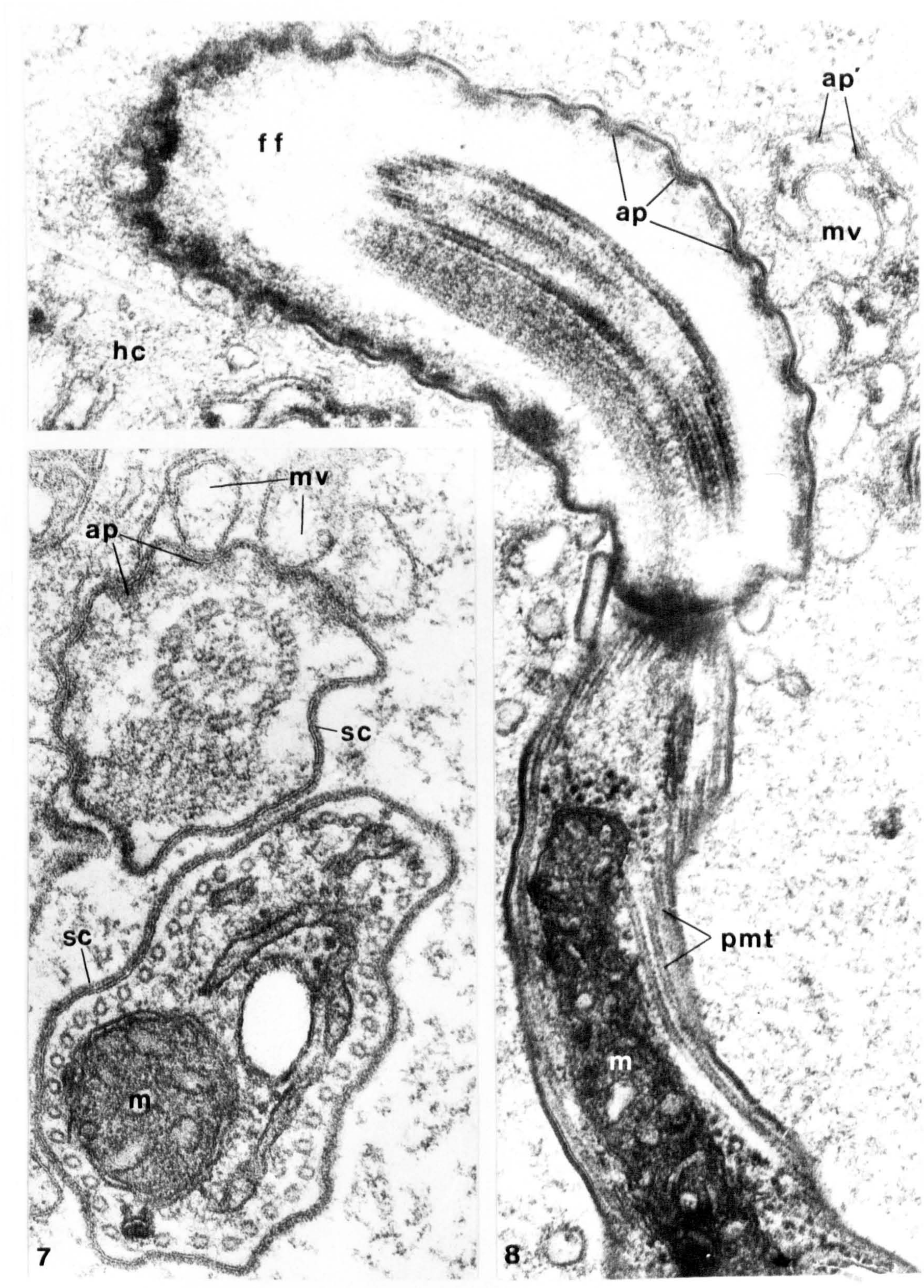

k
m
g

m
sc
g
5

k
m
f

Fig. 7. Transverse section of anterior extremity of attached coated trypomastigote ("nascent" metacyclic). Attachment plaques (ap) are still present where host cell microvilli (mv) indent the flagellar membrane but outgrowths are not present on the metacyclic flagellum (f). sc, surface coat; m, mitochondrion. X92,000.

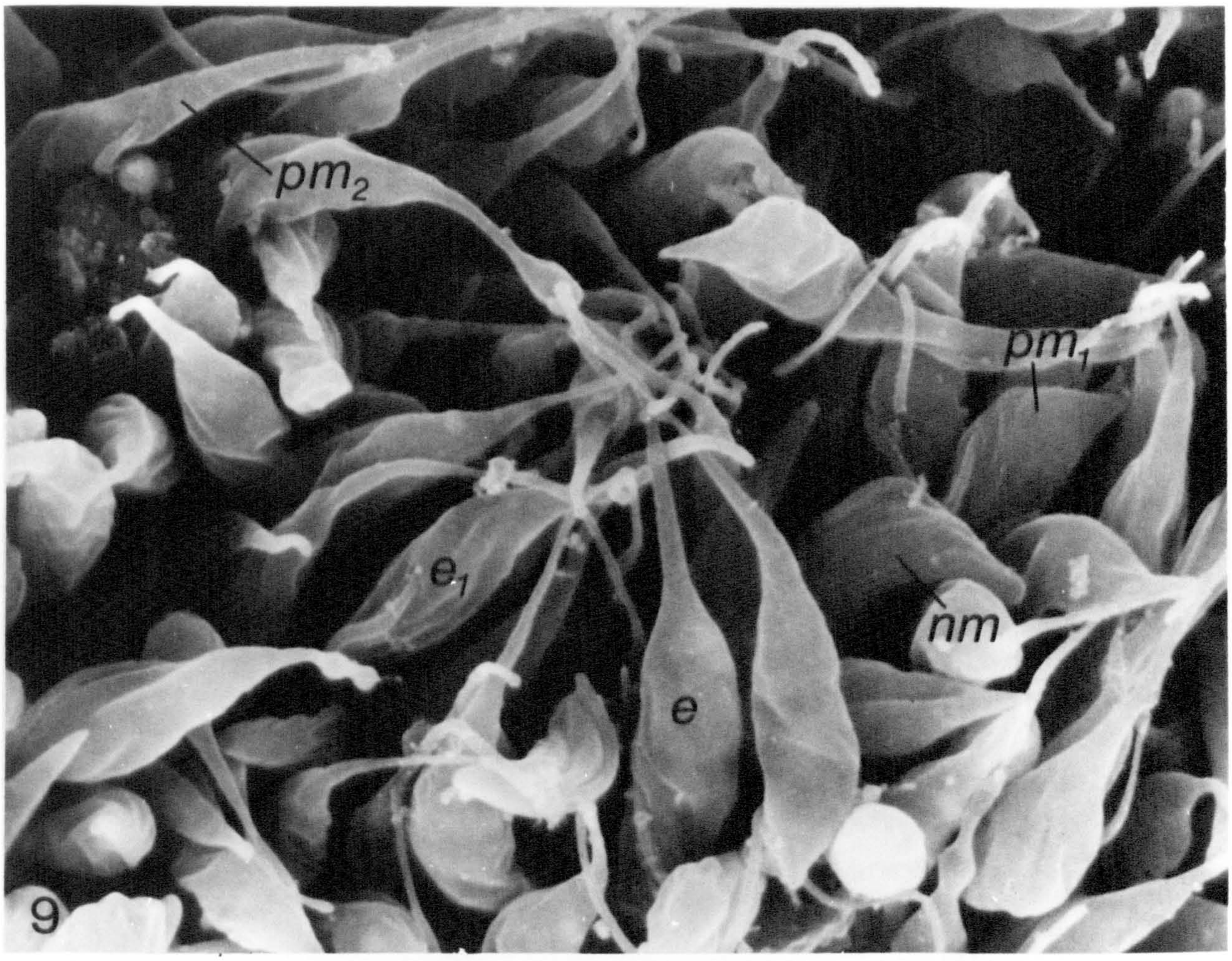
Fig. 8. Obligue longitudinal section through "free flagellum" (ff) and part of body of nascent metacyclic trypanosome. The flagellum is inserted beyond the microvillar (mv) border into the cytoplasm of a host epithelial cell (hc). Evenly-spaced attachment plaques (ap) occur throughout the region of parasite-host cell membrane contact (compare with attachment plaques (ap′) of adjacent epimastigote flagellar outgrowths). m, mitochondrion; pmt, pellicular micro tubules of trypanosome body. X66,000.



7

8

Fig. 9. Scanning electron micrograph of carpet of trypanosomes attached to salivary gland epithelium. Epimastigotes (e) have anterior insertion of the flagella (e_1) and long drawn out posterior extremities. Premetacyclic trypomastigotes show posteriorly inserted flagella and retain the pointed extremity which may be short (pm_1) or long(pm_2), depending on the stage in development. Nascent metacyclic trypanosomes (nm) have a blunt posterior end. X6,700.


pm_2
pm_1
e_1
nm
e
9

Figs. 10-16 are of freeze fracture replicas. The direction of shadowing is from bottom to top of each micrograph.

Fig. 10. Survey view of microvillar border of infected tsetse salivary gland as seen in freeze-fracture replica preparation. Fracture faces of numerous flagella and host cell microvilli (mv) pointing in different directions, are visible. Attachment plaques are evident as circular concavities (at arrowheads) in the flagellar membrane PF (PFf) and as convexities (arrowed) in the EF (EFf). hc, cytoplasm of host cell; sgl, salivary gland lumen. X30,000.

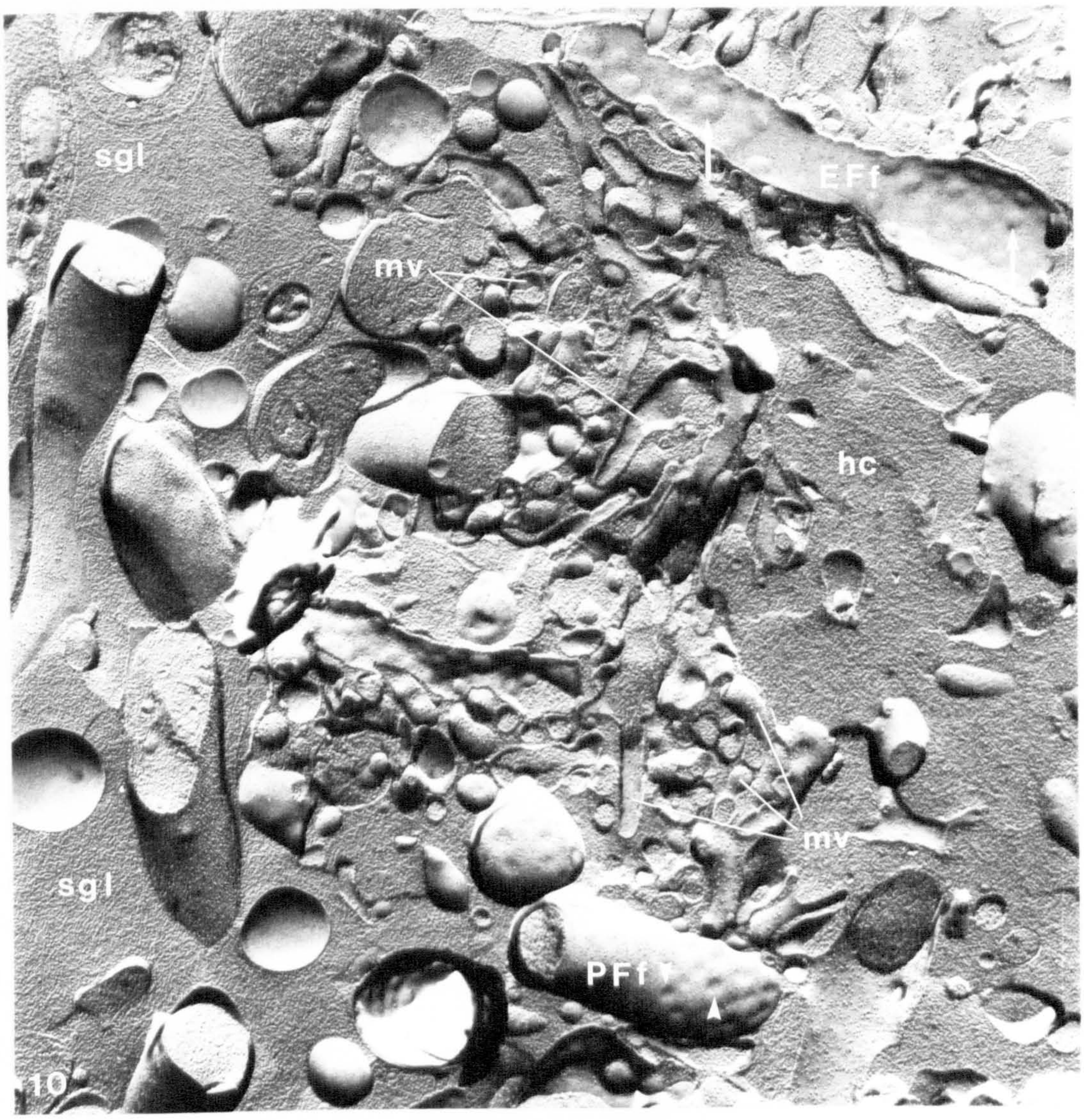
Figs. 11 and 12. Fracture faces of surface membranes of bodies of mature metacyclic (coated trypomastigote; fig. 11) and epimastigote (uncoated; Fig.12) to show differences in intramembranous particle distribution. X100,000.

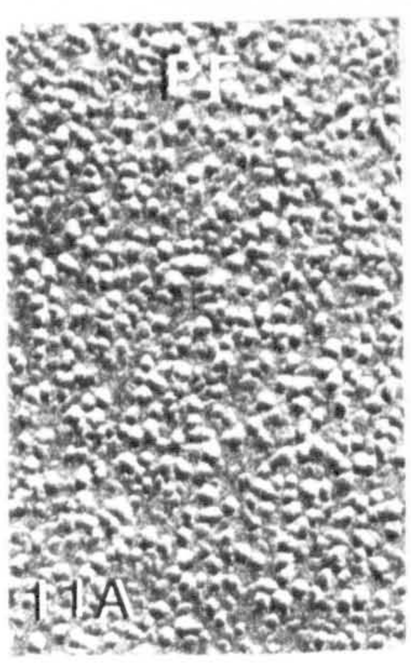
Particle counts with SDs: coated, EF 1096 $\pm$ 150μm^{-2} (n = 10)

PF 4872 $\pm$ 397μm^{-2} (n = 10)

uncoated, EF 1697 $\pm$ 159μm^{-2} (n = 14)

PF 7814 $\pm$ 873μm^{-2} (n = 7)


sgl
EFf
mv
hc
mv
sgl
PFf
10


PF
11A


EF
11B


PF
12A

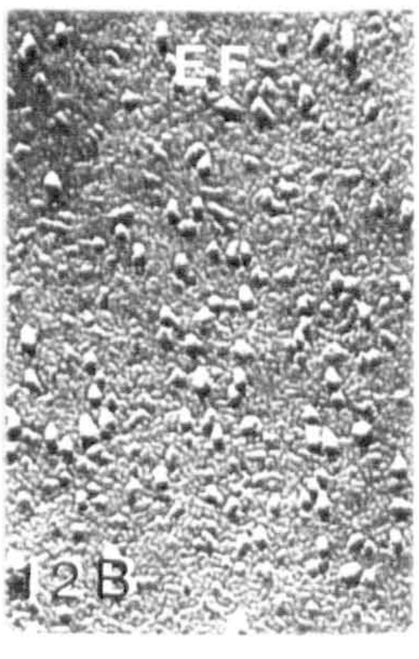
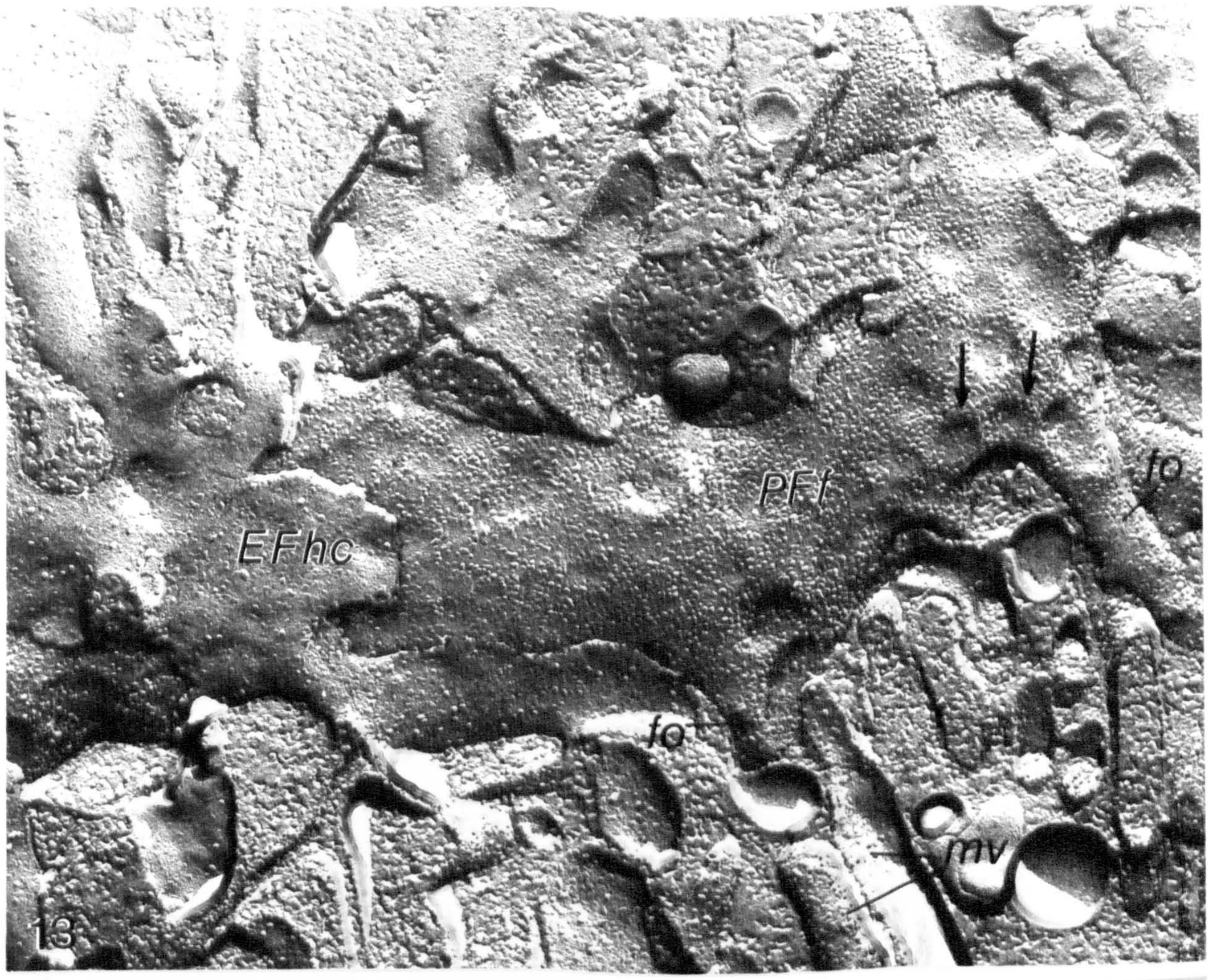

EF
12B

Fig. 13. Fractured junction of epimastigote flagellum and microvillar border. The fracture plane exposes the EF of part of the host cell membrane (EFhc) and the PF of part of the apposed flagellar shaft (PFf) and some of its outgrowths (fo) between host microvilli (mv). The indentations (arrowed) which mark the sites of junctional complexes are relatively free of intramembranous particles. X56,000.

Fig. 14. Fractured body (tb) and (unbranched) flagellum (f_1) of nascent metacyclic; the flagellum shows indented (attachment plaque) and non-indented regions. The line of extracellular particles (at arrows) is believed to represent the surface coat. Note difference in IMP density between PFs of body (PFtb) and flagellum (PFf) (suggesting differences in composition although both bear the glycoprotein coat) and relative paucity of IMPs on metacyclic flagellar PF compared with that of epimastigote (Fig. 13). In lower right-hand corner note array of IMPs (arrowhead) on host microvillus PF at site of indentation of adjacent flagellum (f_2) (attachment plaque). mv, host cell microvillus. X75,000.


EFhc
PFf
fo
fo
mv
13

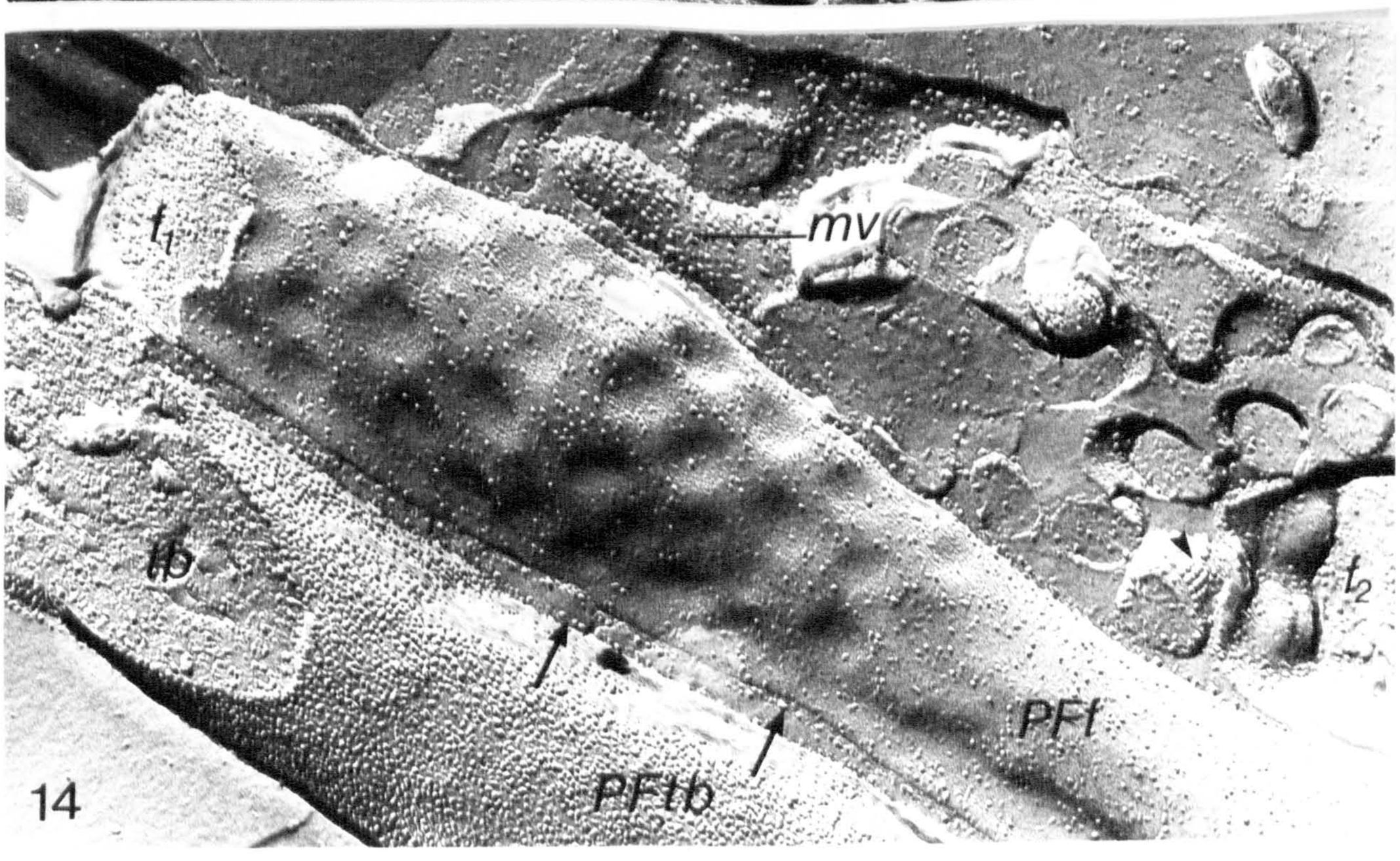
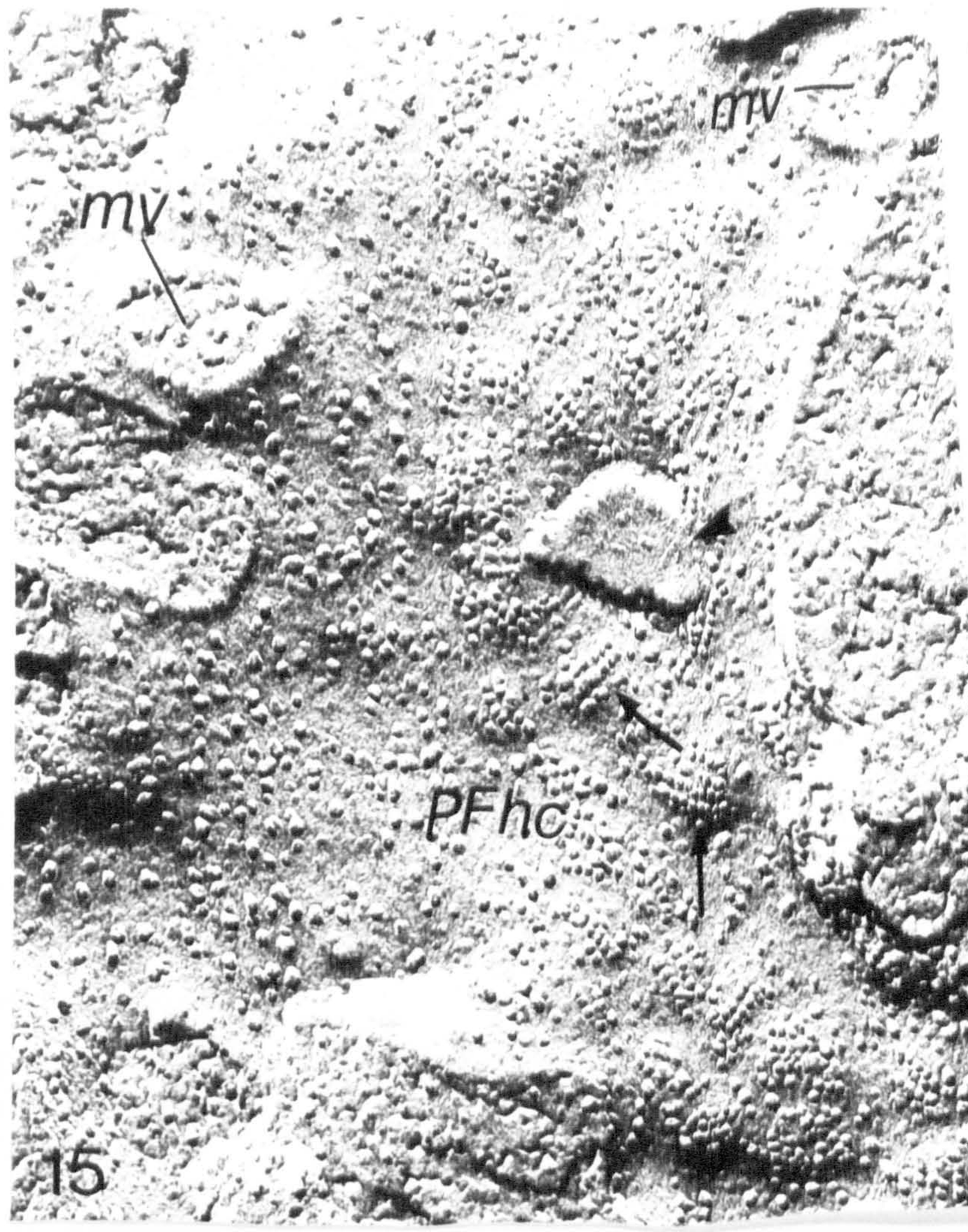

t_1
mv
fb
t_2
PFf
PFfb
14

Fig. 15. Fractured basal region of microvillar border of host epithelial cell to which flagellates are attached. The exposed PF of the intermicrovillar host membrane (PFhc) shows clustered regular aggregates of intramembranous particles (arrowed) at sites of host-parasite attachment plaques. A residual fragment of adhering parasite membrane (large arrowhead) shows no corresponding aggregates in its EF. Stumps of microvilli are visible at mv. X72,000.

Fig. 16. Fractured flagellum of metacyclic trypanosome after filipin treatment. Characteristic filipin-induced lesions (arrowed) indicating β-hydroxysterol-rich areas of the flagellar membrane are visible on the PF. Such lesions are lacking on the EF convexities which correspond to parasite attachment plaques suggesting that the membrane of the plaque region is less rigid than that of the rest of the flagellum. X37,000.

Fig. 17. Complementary replicas of the PF and EF of part of a trypanosome flagellum attached to salivary gland microvilli (mv). Note the absence of particle arrays (such as are found in host cell membrane PF) at sites of parasite attachment (arrowed) in complementary PF and EF. X67,000.



15

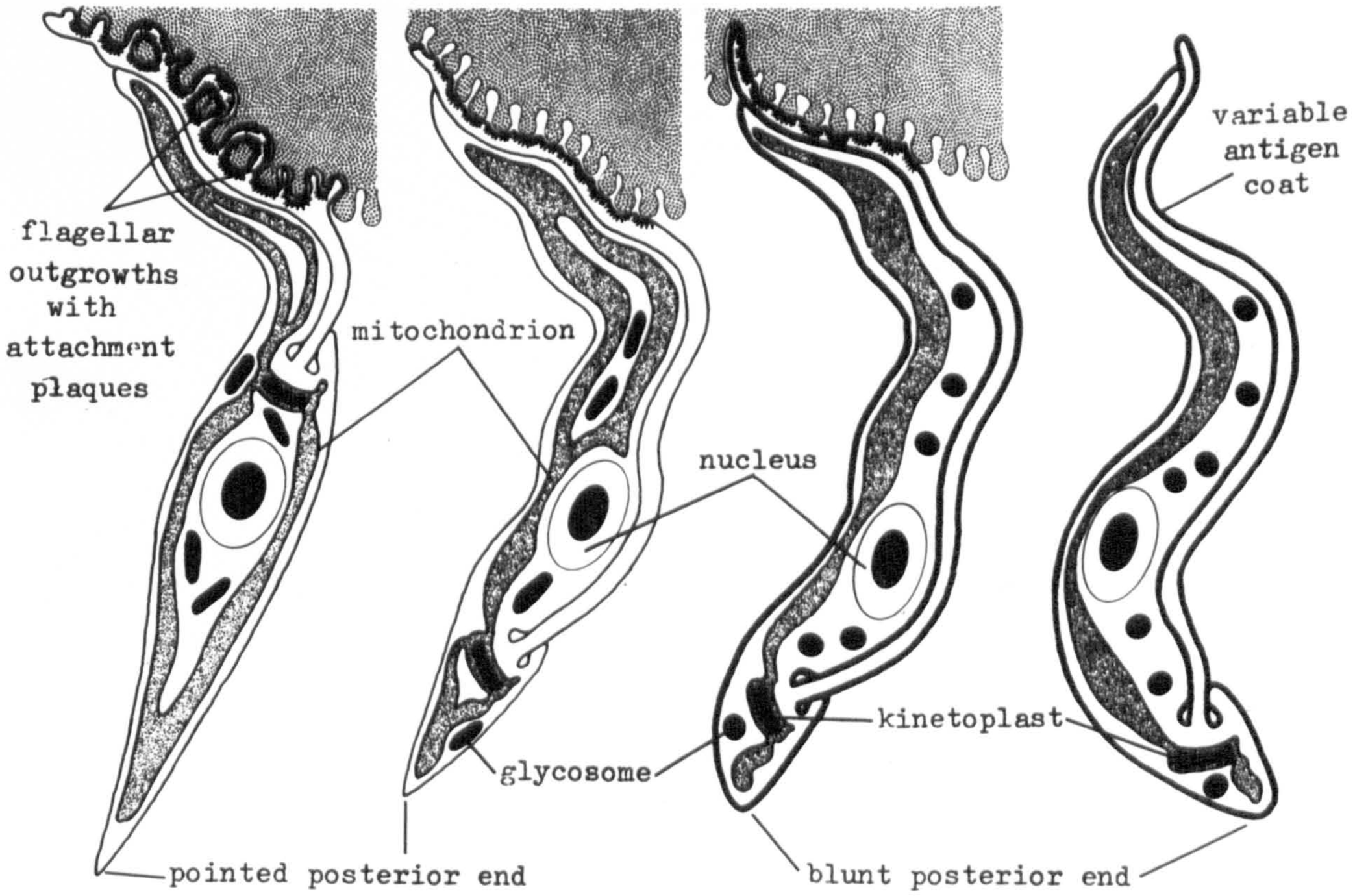
16

17A

17B

Fig. 18. Schematic diagrams of stages in development of mature metacyclic from epimastigote via premetacyclic and nascent metacyclic stages showing principal morphological changes taking place during differentiation.

attached to microvilli of salivary gland epithelium — free in lumen



EPIMASTIGOTE	PREMETACYCLIC	NASCENT METACYCLIC	METACYCLIC
prenuclear kinetoplast	postnuclear kinetoplast		
uncoated, dividing		coated, non-dividing	
branched mitochondrion		unbranched mitochondrion	
bacilliform glycosomes		spherical glycosomes	

Section 5.

The surface membrane of Leishmania mexicana mexicana: comparison of amastigote and promastigote using freeze-fracture cytochemistry.

Abstract. The freeze fracture replica technique has been used to compare the plasma membranes of amastigote and promastigote stages of Leishmania mexicana mexicana with respect to intramembranous particle (integral protein) distribution and to β-hydroxysterol content as revealed by the distribution of lesions induced by the polyene antibiotic filipin. Intramembranous particle (IMP) density was greater in promastigote than in amastigote plasma membranes. IMPs were more abundant in the PF (protoplasmic face) than in the EF (exoplasmic face) of promastigotes, but this situation was found to be reversed in amastigotes. Filipin-induced lesions in glutaraldehyde-fixed parasites indicated higher levels of β-hydroxysterols in the amastigote than in the promastigote plasma membrane, and in the promastigote flagellar membrane than in the body membrane. Amphotericin B (a related polyene antibiotic used in chemotherapy of leishmaniasis) induced IMP aggregation in the PF of unfixed amastigotes but did not appear to influence sterol distribution as demonstrated by freeze-fracture of subsequently-fixed and filipin-treated organisms.

INTRODUCTION

The surface membranes of protozoan parasites often show adaptations which aid survival in the host (Vickerman, 1982). Leishmania parasites occur in two main morphological forms. They grow *in vitro* at 25°C as the promastigote - a form with a locomotory flagellum - that is considered equivalent to the stage that occurs in the midgut of the sandfly vector. In the mammalian host, leishmanias multiply within mononuclear phagocytes as small amastigotes. These lie within a parasitophorous vacuole, which is apparently equivalent to a secondary lysosome, and are potentially exposed to the microbicidal attack of the host cell. The mechanisms whereby the parasite survives this attack are at present not fully understood, but in part may involve an adaptation of the parasite surface membrane to withstand the degradative activities of the host cell. Studies of the leishmania surface membrane have so far been concerned mainly with the promastigote as it is readily cultivated *in vitro*. In neither amastigote nor promastigote is the plasma membrane protected by a surface coat comparable to that in related African trypanosome mammalian stages (Vickerman & Preston, 1976). The promastigote surface membrane contains various sugar residues and, unusually, a 5'-nucleotidase and a 3'-nucleotidase, although the significance of these enzymes has not been determined (Dwyer, 1977; Dwyer & Gottlieb, 1984).

In an attempt to gain more information on the adaptations of the leishmanial plasma membrane to life in the parasitophorous vacuole, and on the extent of the changes that occur upon transformation of the promastigote to the amastigote, we have studied the plasma membranes of both stages in the life cycle of *Leishmania mexicana mexicana* using freeze-fracture cytochemistry. In particular we have used the polyene antibiotic filipin, which binds specifically and stoichiometrically to β-hydroxysterols (Kinsky, 1970; Hamilton-Miller, 1973) to form easily identified complexes in replicas of freeze-fractured membranes, to compare surface membrane sterol distribution in the two stages.

MATERIALS AND METHODS

Parasites. Leishmania mexicana mexicana amastigotes were isolated from firm unruptured lesions in female NIH mice (Hacking and Churchill, Huntingdon, Cambridge) as described previously (Hart et al., 1981). Parasites were liberated from host cells by gently grinding the excised lesion in a phosphate albumin (PSGEMKA) buffer (Hart et al., 1981) between stainless steel gauzes and large host cell debris was removed by filtration through No.42 Whatman filter paper. Clumps of amastigotes in the crude, filtered preparation were dispersed by 2 strokes of a Potter homogenizer operating at the lowest speed and the parasites were harvested by centrifugation at 2100 g for 10 min at 4°C. Promastigotes were derived from amastigotes as described by North & Coombs (1981) and maintained in HOMEM medium (Berens et al., 1976) with 10% heat-inactivated foetal calf serum (FCS) and air as the gas phase; they were harvested during the logarithmic phase of growth by centrifugation at 2100 g for 10 min at 20°C.

Fixation. Parasites were fixed in 2.5% (v/v) glutaraldehyde (Sigma, EM grade) in 0.1M cacodylate buffer, pH 7.4, for 30 min at 20°C, pelleted by centrifugation as before and rinsed by immersion in buffer plus 2% (w/v) sucrose for 1-2 h.

Polyene treatments.

a) Filipin: Filipin solution was prepared by dissolving powder in dimethyl sulphoxide and adding to 0.1 M cacodylate buffer, pH 7.4, to give a final filipin concentration of 50 µg/ml [DMSO final concentration 0.5% (v/v)]. Fixed, pelleted parasites were resuspended in filipin solution to a concentration of 10^6/ml and incubated in the dark at room temperature (~ 20°C) for 4 - 6 h. After this, the parasites were washed in 0.1M cacodylate buffer, pH 7.4, infiltrated overnight with 25% glycerol in the same buffer and processed for electron microscopy, half the pellet for freeze-fracture and half for embedding and sectioning.

b) Amphotericin B: Treatments with this antibiotic were performed on living, freshly isolated amastigotes. Parasites were incubated for 1 h at 25°C in PSGEMKA buffer containing 13 µg/ml amphotericin B (Fungizone, Flow Laboratories, Irvine, Scotland). At the end of the incubation, approximately 20% of the amastigotes were dead as assessed by their non-refractile appearance using phase contrast microscopy. In the absence of drug, all parasites survived. Treated parasites and controls were glutaraldehyde-fixed and either freeze-fractured as described below or incubated for 4 - 6 h at 20°C in 50 µg/ml filipin and subsequently processed as described above.

Freeze fracture replicas. Parasites pelleted in glycerol buffer were sandwiched between two Balzer's specimen plates, frozen by immersion in liquid propane at -180°C, transferred under liquid nitrogen into a complementary replica device and fractured under a vacuum of 2×10^{-6} Torr at -100°C. Replicas of the cleaved surface were obtained by conventional platinum-carbon shadowing at 45°, backed with carbon, removed from the vacuum, then floated onto distilled water and cleaned with 40% (v/v) chromic acid overnight. After further washing in distilled water, replicas were collected on 300 mesh copper grids.

Electron microscopy. Pelleted parasites were processed for embedding and sectioning as described by Alexander & Vickerman (1975). Replicas and sections were examined in an AEI EM 801 transmission microscope operating at 60 KV and photographed on Ilford EM4 cut film.

Counting of intramembranous particles (IMPs) and lesions. IMPs and filipin-induced lesions were counted from prints at a final magnification of 75,000X under a 2X illuminated magnifier and using a MkI Counting Calculator (Agar Aids) which registers points marked manually by a pen as counting

proceeds. Areas on micrographs were obtained by placing 1μm^2 quadrats at random over regions evenly shadowed or, in the case of flagellar profiles especially, by digitizing smaller areas using a graphic digitizing tablet and microprocessor. Filipin-induced lesions were identified and counted as 25-30nm pits or protrusions.

RESULTS

Intramembranous particles.

The distribution and sizes of intramembranous particles (IMPs) in the protoplasmic face (PF) and exoplasmic face (EF) of the surface membrane of amastigote and promastigote forms of L. m. mexicana are shown in Figs 1 and 2 and Table 1. IMPs in the plasma membrane of the amastigote were unusually distributed between the two membrane halves; more particles were found on the EF than on the PF, although particle densities were not high on either fracture face. The promastigote plasma membrane fracture faces exhibited more conventional particle partitioning in that the PF had more densely packed smaller IMPs than the complementary EF (Fig. 2). The promastigote flagellar membrane (Fig. 3) had few particles on both EF and PF; there were clusters of particles in both fracture faces at the region of flagellar emergence (see Benchimol and De Souza 1980, Fig. 10).

Polyene treatments.

Filipin treatment resulted in characteristic doughnut-shaped complexes in the surface membranes of both forms of the parasite; the complexes appeared as 20-30 nm protuberances or pits in freeze-fracture replicas. These complexes represent intramembranous aggregates of sterol plus filipin molecules. The amastigote forms exhibited dense packing of complexes over the entire plasma membrane (Fig. 4). Filipin-treated amastigotes revealed bulges at sites of complex formation (Fig. 5) in vertical section; in tangential section the stained complexes appeared as annuli. An occasional region of reduced lesion density was seen in some amastigotes (Fig. 6) and other profiles showed apparent alignment of lesions in tracks across the

membrane PF (Fig. 6). Amastigote PF fractures invariably revealed protuberances whereas EF fractures exhibited corresponding pits (not illustrated).

The promastigote plasma membrane PF had relatively few intramembranous lesions compared with the amastigotes after incubation with the same concentration of filipin (Fig. 7). Additional differences were also recognised in the appearance of lesions in this fracture face; in contrast to the amastigote, protuberances and pits were found together in the same fracture face (Fig. 7 inset). This configuration was not observed in the flagellar membrane fracture faces which displayed only protrusions on the PF and pits on the EF. The density of filipin/sterol complexes in the flagellar membrane was consistently higher than in the corresponding plasma membrane fracture faces for any given cell (Table 1).

Amphotericin B/filipin treatments.

After amphotericin B treatment of living amastigotes, segregation of PF particles into islands surrounded by particle-free areas was common (Fig. 8) though extensive particle rearrangements were not seen in the EF (Fig. 9). The drug-induced depressions (Fig. 9) - evident as swellings on the true outer surface of the parasite - may have been related to particle-free areas in the complementary PF. Few EF fractures displayed this feature, however, and it could be an indication of incipient lysis.

When similar amphotericin B-treated, fixed parasites were incubated with filipin, no change in overall filipin/sterol complex arrangement was seen when compared with controls which had not received amphotericin B treatment. An amphotericin B/filipin-treated amastigote is shown in Fig. 10; note the similarity to the lesion density in Fig. 4 which shows material treated with filipin only.

DISCUSSION

Freeze-fracture techniques together with the cytochemical marker filipin have been used to investigate the structure of a variety of cell

membranes in recent years (Feltkamp & Van Der Waerden, 1982; Robenek et al., 1982; Bridgman & Nakajima, 1983; Gotow & Hashimoto, 1983; Risinger & Larsen, 1983; Tamm & Tamm, 1983). Application of these methods to the human parasite Leishmania mexicana mexicana has revealed marked differences between the surface membranes of amastigotes and promastigotes. Analysis of the IMP numbers in the fracture faces of the amastigote plasma membrane has shown a generally low density and since IMP density is thought to reflect integral protein distribution and functional complexity (Zingsheim & Plattner, 1976) it could be argued that the low IMP density of the amastigote plasma membrane reflects a relatively low membrane enzyme content. Another unusual feature of the membrane is the inversion of the usual particle partition between PF and EF. This has been reported also for Leptomonas samueli promastigote plasma membrane (Souto-Padron et al., 1980) and roughly equal distribution of IMPs between PF and EF has been found in studies of several trypanosomatid plasma membranes (reviewed in Benchimol & De Souza, 1980). The most common particle partition state in eukaryote plasma membranes - that of highest IMP density on the PF (Zingsheim & Plattner, 1976) - was observed for the L. mexicana mexicana promastigote plasma membrane in the present study. The IMP density reported recently for the same fracture face in L.m. amazonensis (Benchimol & De Souza, 1980) was considerably lower (2,040/μm^2) than in the present study whereas EF IMP density was almost identical (890/μm^2). The significance of these differences is unclear but could relate to conditions of growth.

The relative abundance of filipin/sterol complexes - reflecting β-hydroxysterol distribution specifically (Kinsky, 1970; Hamilton-Miller, 1973; Norman et al., 1976) - suggests that there is a greater concentration of these lipids in the surface membrane of leishmanial amastigotes than in promastigotes. The high sterol content of the amastigote surface membrane could be related to the resistance of the intracellular form to the microbicidal activity of the host cell. This could be either a direct effect, the sterols rendering the membrane insusceptible to hydrolytic

attack, or an indirect effect, the high sterol content being a corollary of a low protein content and the absence of the components involved in stimulating the microbicidal activity of macrophages. Recent work on Leishmania promastigotes (Goad et al., 1984) has identified the major sterol present as ergosterol. This β-hydroxysterol is also the form present in the culture forms (corresponding to insect gut stages) of the trypanosomatids Trypanosoma brucei, Trypanosoma cruzi and Crithidia fasciculata (Korn et al., 1969; Dixon & Williamson, 1970). As yet, however, no information on the sterol composition of leishmania amastigotes is available. The mammalian host environment of the amastigote will be rich in cholesterol which may be incorporated by the parasite. It is possible that ergosterol also is present in the amastigote membrane. Ergosterol biosynthesis continues even during the incorporation of exogenous cholesterol by Leishmania major promastigotes (Goad, et al., 1984) and ergosterol may be required for specialized functions for which cholesterol cannot substitute.

The effectiveness of amphotericin B, a polyene antibiotic related to filipin, in the treatment of leishmaniasis (Kern, 1981) may be due to the drug's stronger affinity for ergosterol than for cholesterol (Archer & Gale, 1975) and its selective attack on parasite membranes containing ergosterol. The plasma membranes of promastigotes of Leishmania compare with yeast and fungal plasma membranes in possessing ergosterol as the main sterol (Goad et al., 1984) and amphotericin B has potent anti-fungal activity. Freeze-fracture examination of the effect of amphotericin B on the plasma membrane of the human pathogenic fungus Epidermophyton floccosum revealed a similar particle aggregation pattern on the PF (Kitajima, et al., 1976) to that found with Leishmania amastigotes.

The similarity of the PF of amphotericin B-treated fixed amastigotes (Fig. 10) after filipin treatment to the PF in amastigotes treated only with filipin (Fig. 4 and 5) suggests that the sterol distribution was apparently unmodified by the amphotericin B incubation. It is possible, however, that the high sterol concentration in the membrane could account for the lack of

obvious amphotericin B effects after subsequent filipin exposure. Close packing of the filipin/sterol complexes prevents visualization of the particle distribution in those regions of fracture face between complexes.

The striated appearance of some amastigote PF structures (Fig. 6) probably reflects the underlying pellicular microtubule distribution (Fig 5); such appearances are absent from EF fractures. It is possible that the striations are the result of volume shrinkage of the parasite rather than a structural association of the plasma membrane with the underlying cytoskeleton though such an association has been reported (Linder & Staehelin, 1977). The interaction between membranes and cytoskeletal elements has been considered restrictive to sterol-filipin complex formation (Severs & Simons, 1983) but clearly no such effect was apparent in the present study.

The differences in density of both IMP and filipin/sterol complexes on the flagellum and plasma membranes of promastigotes of L. mexicana mexicana suggests that these membranes represent distinct domains. Indeed, the presence of a collar of IMPs at the neck of the emergent flagellum observed in some trypanosomatids (Linder & Staehelin, 1977; De Souza et al., 1978) would seem to form a delineation of the two regions of otherwise continuous membrane. Since several kinetoplastid flagellates, including Leishmania spp., attach to a substratum by their flagellum (but not by the body) at certain stages in their life cycles (reviewed Molyneux, 1983; Tetley & Vickerman, 1985), it is possible that the flagellar membrane is adapted for attachment. However, this difference may also relate to the greater requirement for fluidity of the actively motile, locomotory flagellum in the free-swimming stages.

ACKNOWLEDGEMENTS

We thank Helen Hendry and Maureen Gardner for photographic assistance.

Filipin was a gift from Dr J.E. Grady, Upjohn Co., Kalamazoo, Michigan, USA.

TABLE 1. Intramembranous particle (IMP) and filipin-sterol complex (FC) counts on amastigote and promastigote fracture faces, represented as the mean number per square micron taken from the number of measurements given in parenthesis.

Leishmania mexicana mexicana	IMP/μm^2				FC/μm^2	
	Plasma membrane		Flagellar membrane		Plasma membrane	Flagellar membrane
	PF	EF	PF	EF		
Amastigote	761(7)	1338(6)	-	-	461(10)	-
Promastigote	3641(6)	896(6)	650(5)	543(3)	146(10)	704(4)

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Figures 1-3 Fracture faces of surface membranes of Leishmania mexicana mexicana showing intramembranous particle distributions.

Scale bar = 100 nm.

Fig. 1a PF (protoplasmic face) of amastigote plasma membrane.

1b EF (exoplasmic face) of amastigote plasma membrane.

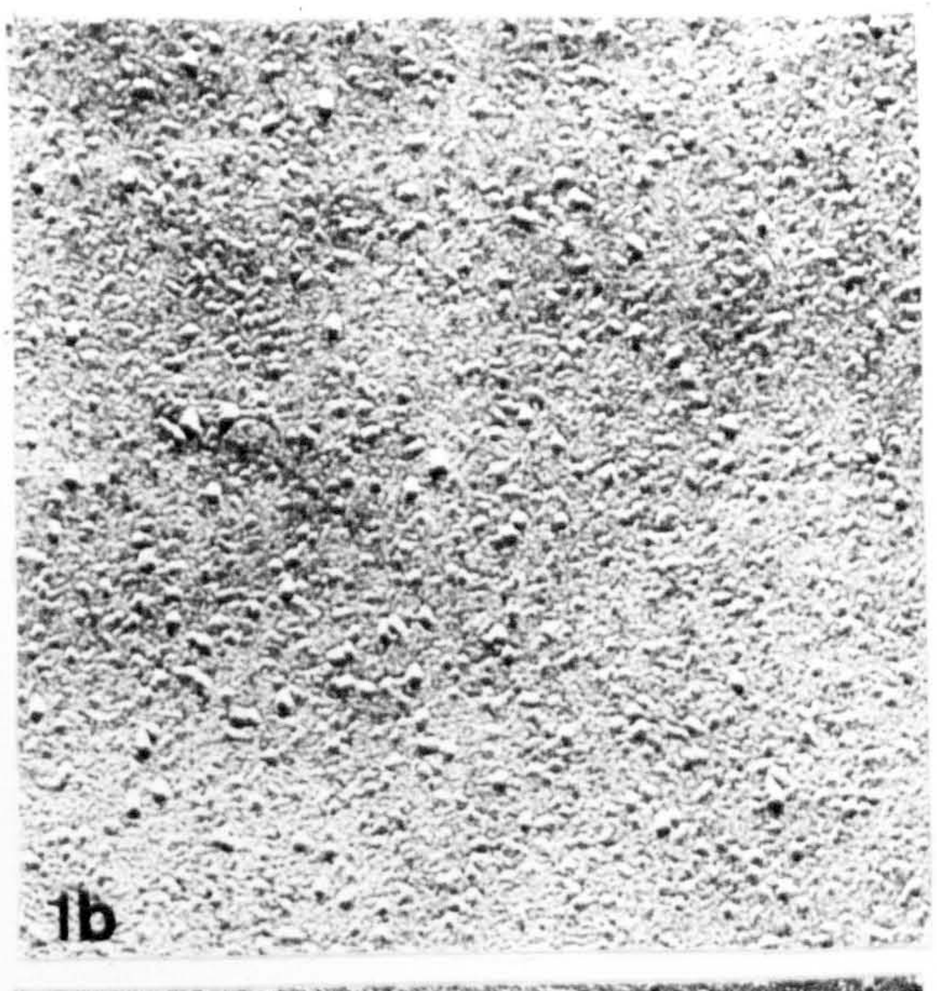
Fig. 2a PF promastigote plasma membrane.

2b EF promastigote plasma membrane.

Fig. 3a PF promastigote flagellar membrane.

EF promastigote flagellar membrane.


1a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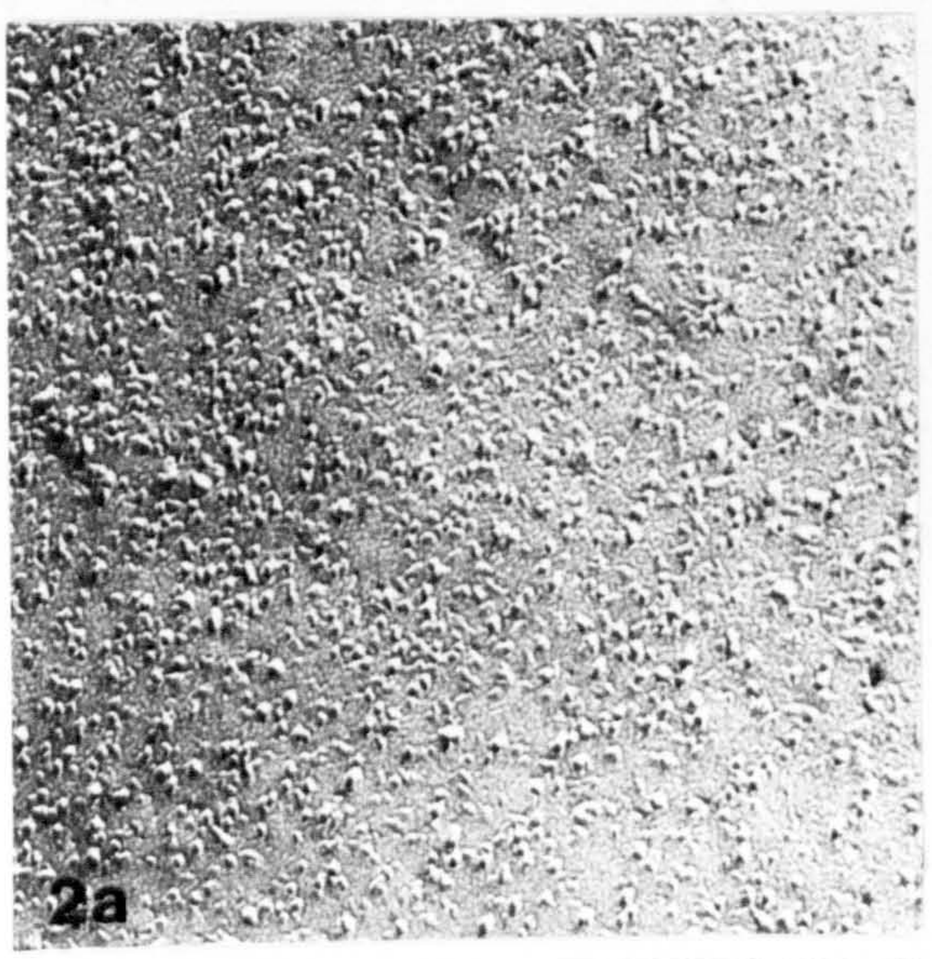
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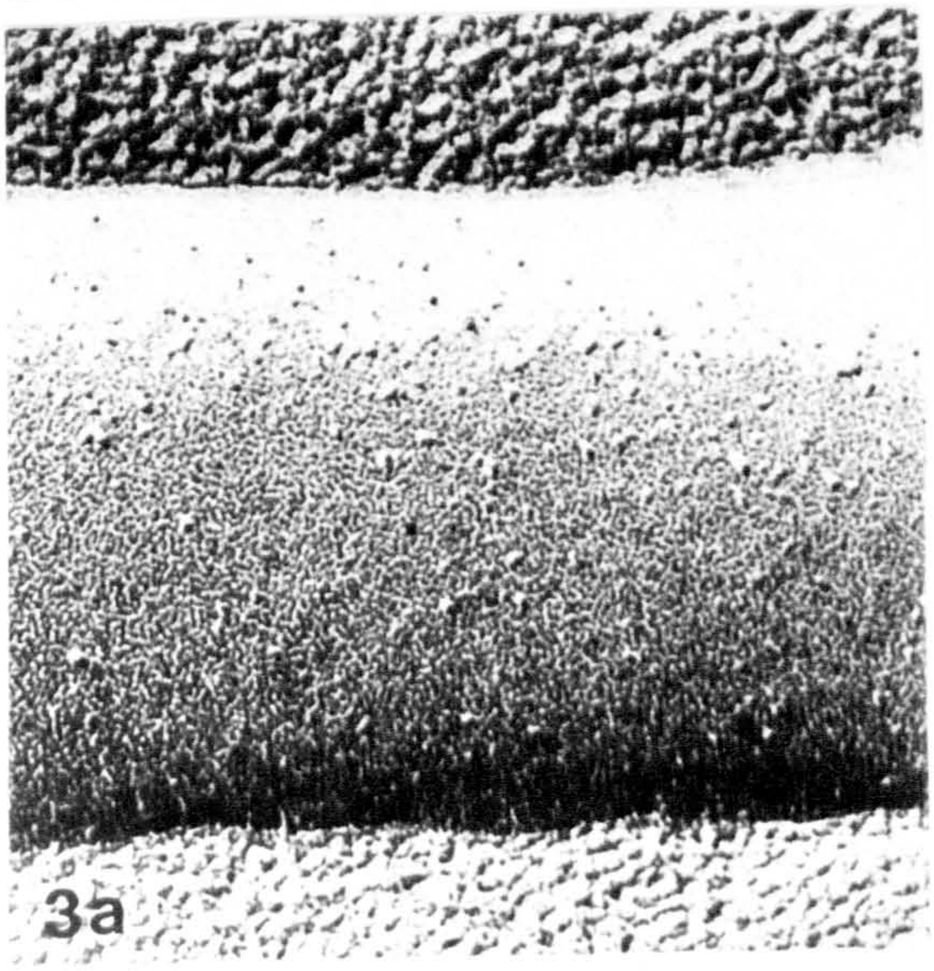
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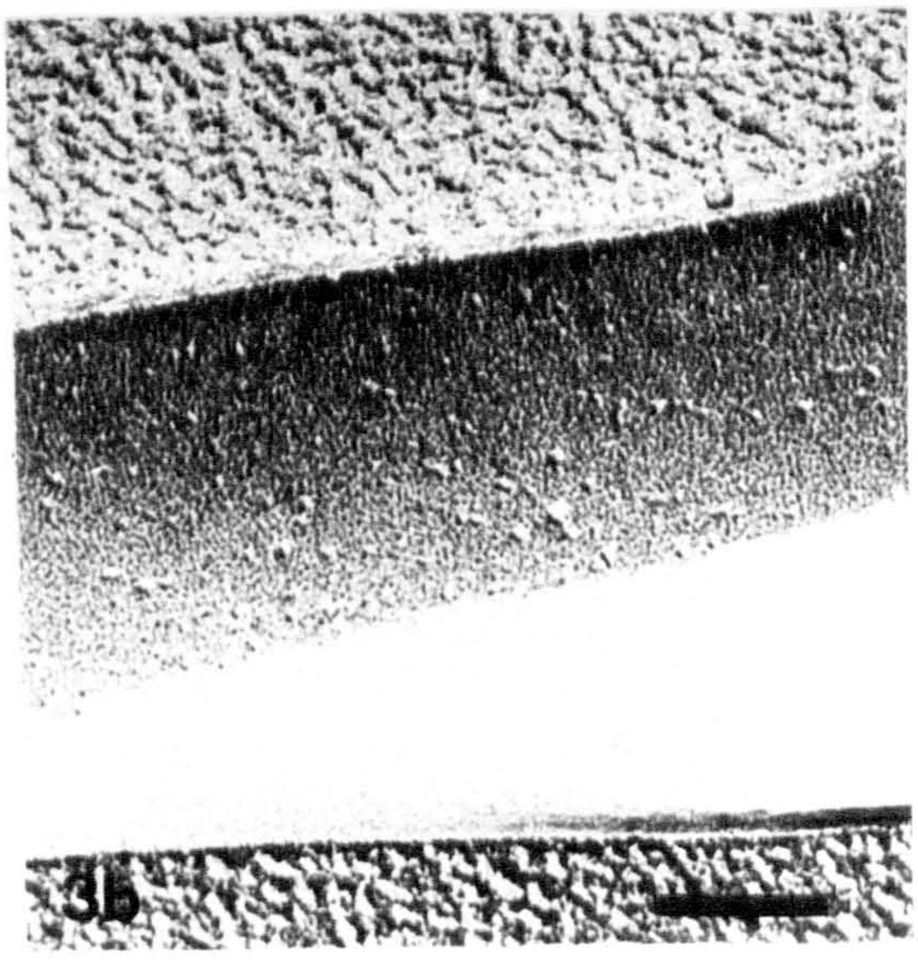
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Figures 4-7 Filipin treated Leishmania mexicana mexicana stages

Fig. 4 PF fracture face of filipin-treated amastigote.
fp = flagellar pocket opening. Scale bar = 0.5 μm.

Fig. 5 Section through surface of filipin-treated amastigote showing circular profiles of filipin/sterol complexes in glancing section (arrows) represented as bulges (arrowheads), produced by the intramembrane complexes, on the cell surface.
pmt = pellicular microtubules
pm = plasma membrane Scale bar = 200 nm.

Fig. 6 Filipin-treated amastigotes showing regions of reduced lesion density (star) and striated appearance (arrows) found in some PF fracture faces.
fp = flagellar pocket opening. Scale bar = 1.0 μm.

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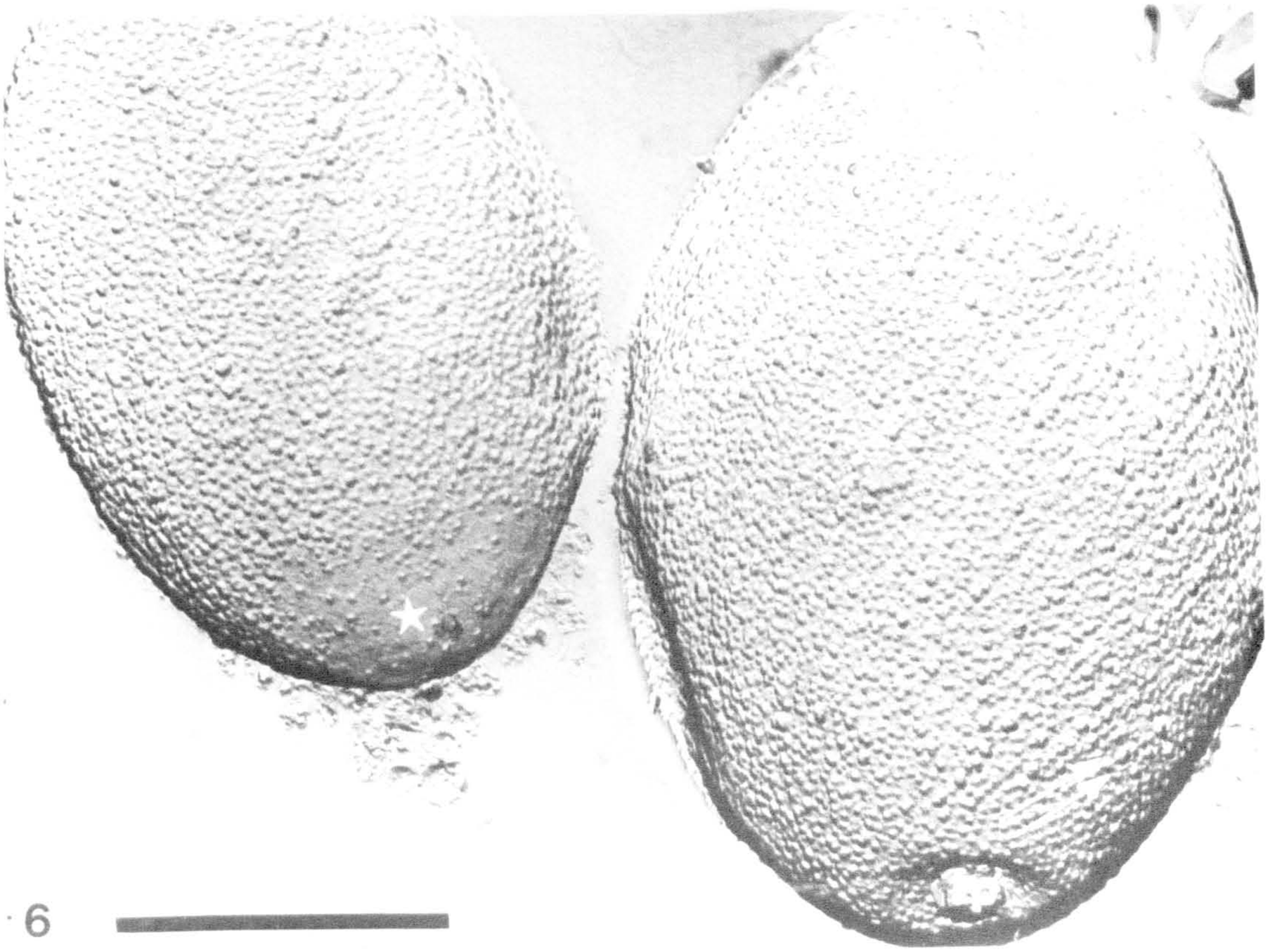

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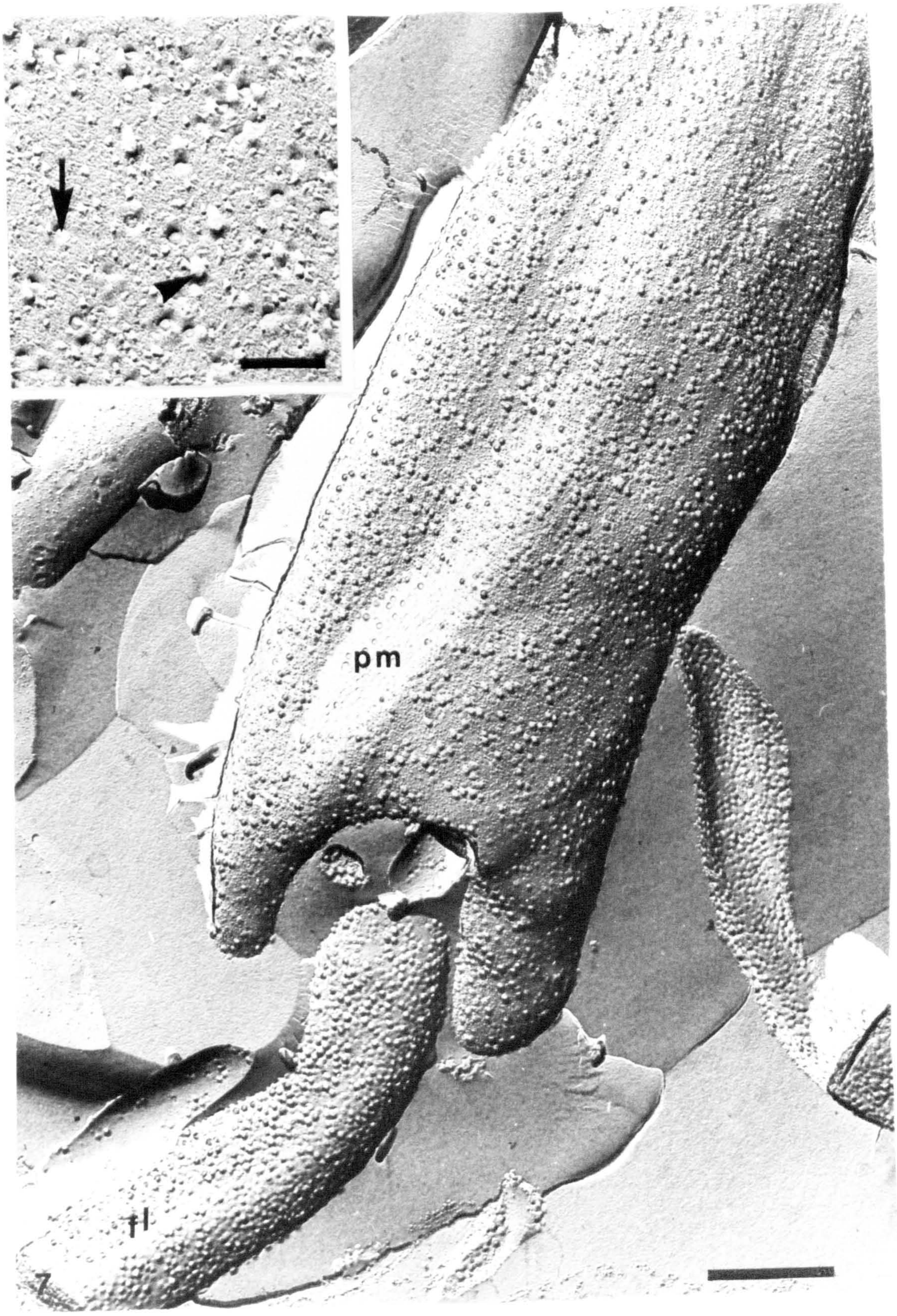
Fig. 7 PF fracture faces of promastigote body and flagellum to show relative distribution of filipin-induced lesions.

fl = flagellar PF.

pm = plasma membrane PF Scale bar = 0.5 μm.

Fig. 7(inset) Enlargement of PF of filipin treated promastigote plasma membrane to illustrate existence of protuberances (arrowhead) and pits (arrow) on the same fracture face.

Scale bar = 100 nm.

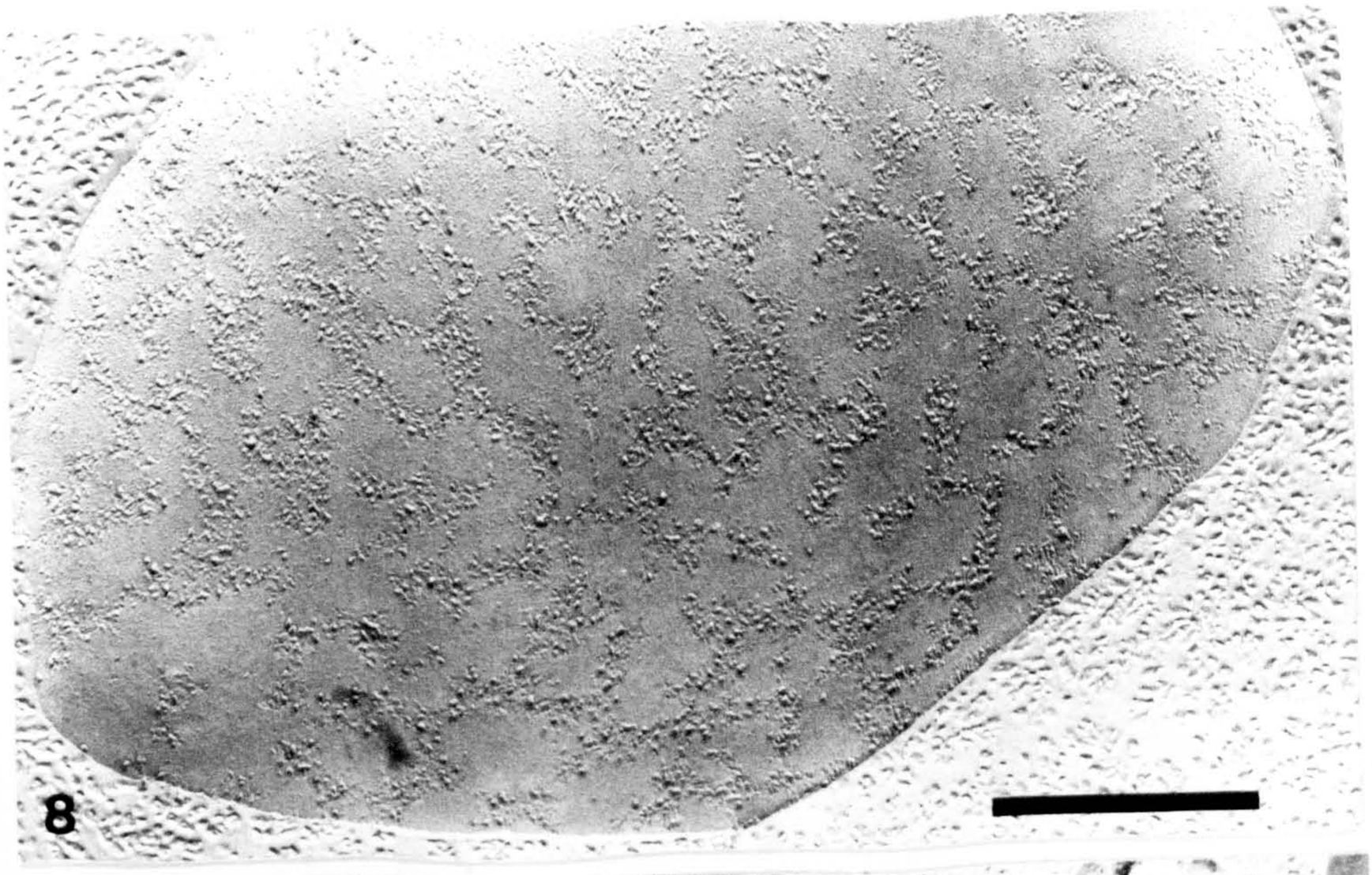

pm

Figures 8 & 9 Amphotericin B treated Leishmania mexicana mexicana amastigotes

Fig. 8 Particle aggregation pattern seen on PF of amastigote plasma membrane after amphotericin B treatment. Scale bar = 0.5 μm.

Fig. 9 Numerous depressions (d) on EF of amastigote plasma membrane after amphotericin B treatment. Particle aggregation is not apparent. Scale bar = 0.5 μm.

Fig. 10 Amphotericin B/filipin treated amastigote PF of plasma membrane showing filipin sterol complexes distributed similarly to that found in filipin-only treated amastigotes. (Compare Fig. 4). Scale bar = 0.5 μm.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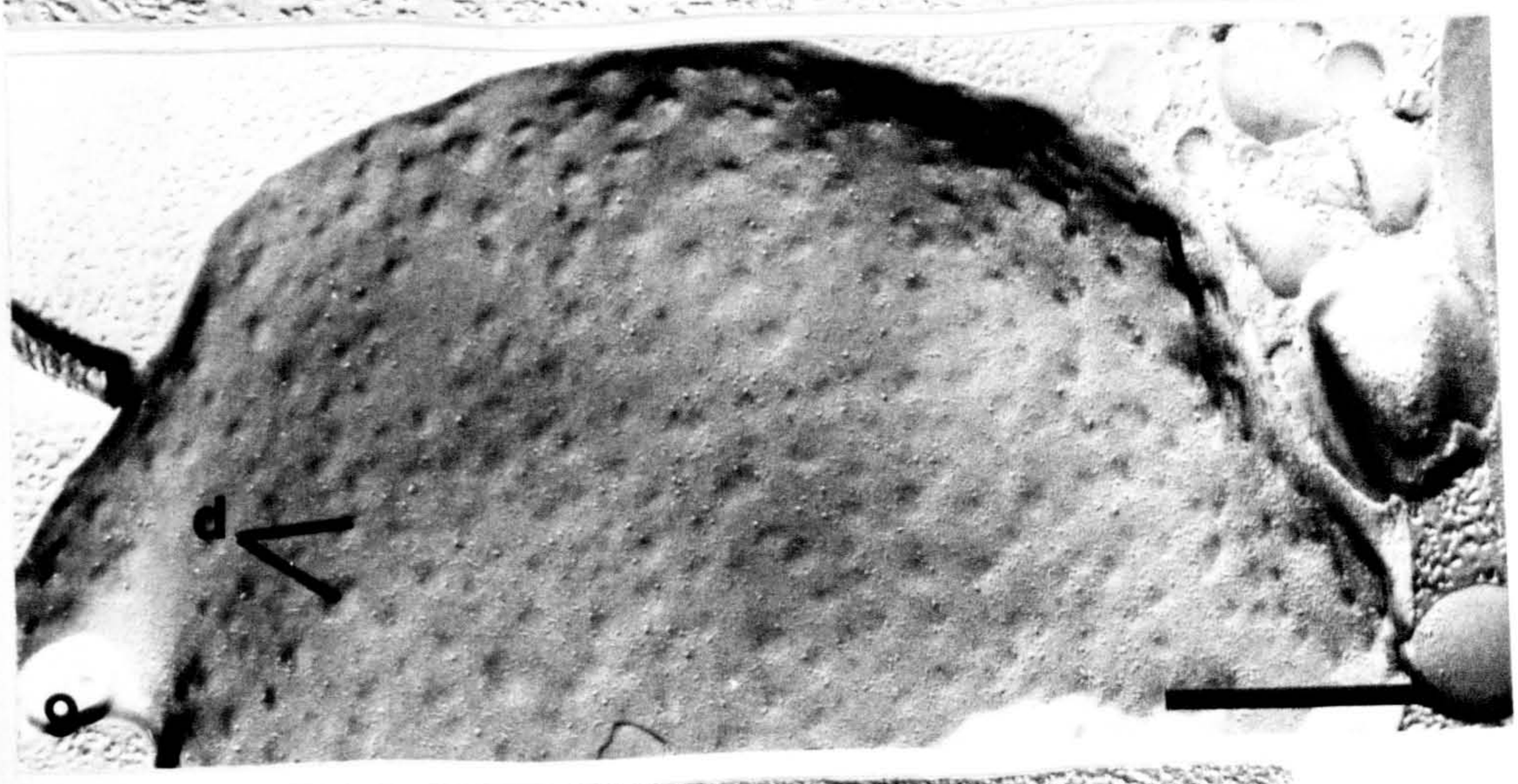
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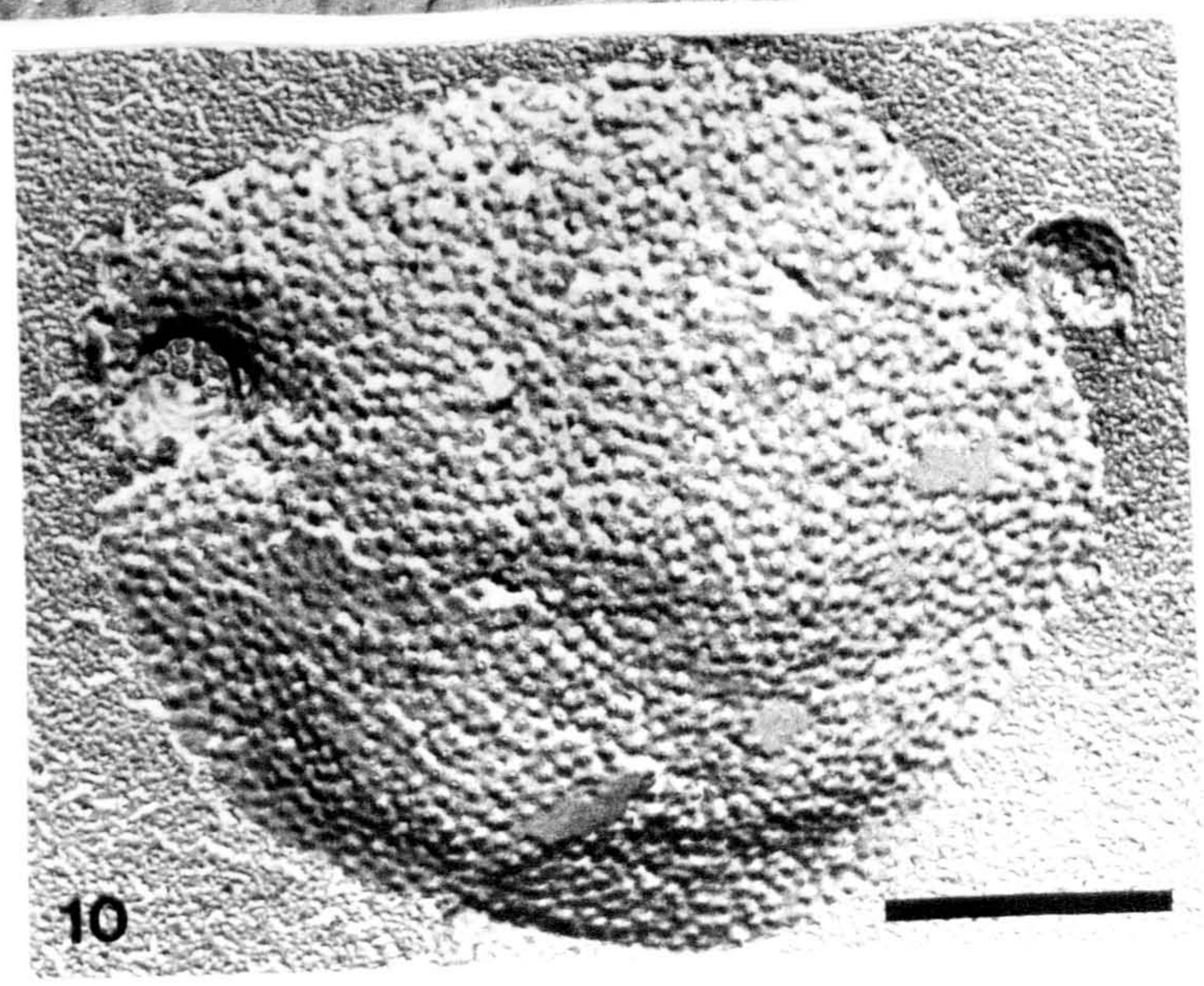
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Section 6.

Three dimensional structure of the <u>leishmania</u> amastigote as revealed by computer-aided reconstruction from serial sections.

SUMMARY

Computer-aided reconstruction from serial sections has been used to analyse the three dimensional structure of entire amastigotes of Leishmania mexicana mexicana and determine the number, arrangement and volume of each organelle. In two reconstructions,the lysosome-like "megasomes" were the most numerous organelle, there being 34 in one amastigote, and they comprised as much as 15% of the total cell volume. In contrast, as few as nine glycosomes were present accounting for 1% of the cell volume. The unitary nature of the mitochondrion was confirmed and its complex basket-like structure was revealed. The spatial arrangement of the cell organelles is here displayed in stereo pairs

INTRODUCTION

The trypanosomes and leishmanias appear to combine simplicity of cell structure with striking morphological and physiological adaptability in the face of environmental change. Transformation from one stage in the life-cycle to the next is accompanied by changes in the structure, size, number and distribution of certain organelles. Although these changes have been to some extent quantified for Trypanosoma brucei using morphometric methods (Hecker, Burri, Steiger and Geigy 1972; Bohringer and Hecker 1974, 1975; Opperdoes, Baudhuin, Coppens, De Roe, Edwards, Weijers & Misset, 1984), for other trypanosomatids they have almost invariably been surmised from individual, randomly cut sections or from a limited number of serial sections of the two stages in question. Studies of whole cells from serial sections are few and have been concerned mainly with demonstration of the unitary nature of the mitochondrion (Paulin 1975, 1977, 1983) and not with its relationships with other organelles, though recently reconstructions of the mitotic apparatus of trypanosomatids have been undertaken (Solari, 1980, 1983). The only published set of serial sections of a trypanosomatid is that of a stumpy bloodstream form of Trypanosoma brucei by Bird, Molloy and Ormerod (1966). There have been no reports describing three-dimensional reconstructions of the entire flagellates, yet studies of this type would do much to improve our understanding of the relationship between form and function in these parasites.

The development of computer programmes designed to collect information on organelle distribution from serial sections and then to display it in three-dimensional form has greatly facilitated the preparation of such reconstructions. It has removed the need to prepare and photograph contour models of clay or fretted plastic sheets, because graphic plots of the reconstruction from different viewpoints can be obtained and viewed by stereoscopy.

In this paper, we present the three-dimensional arrangement of

organelles in the amastigote of Leishmania mexicana mexicana isolated from its macrophage host cell, together with data on the number and volume of the organelles. We are particularly concerned with the mitchondrion and its relationships with the glycosomes and with lysosomal organelles which we provisionally termed "megasomes" (Alexander and Vickerman, 1975). Megasomes are abundant in L. m.mexicana amastigotes, but apparently absent from or present only in low number in culture promastigotes and not yet reported from amastigotes of other Leishmania species.

MATERIALS AND METHODS

Parasites

Leishmania mexicana mexicana amastigotes were grown in NIH mice and partially purified according to previously described methods (Hart, Vickerman and Coombs, 1981). Briefly, amastigotes were liberated from excised lesions in PSGEMKA buffer by gentle grinding between stainless steel plates. Large debris was removed by filtration (Whatman No. 42 paper) and the resultant preparation dispersed by two strokes of a Potter homogeniser operating at minimum speed. The liberated amastigotes were concentrated by centrifugation at 2,100 g for 10min. at 18°-C and processed for electron microscopy.

Electron Microscopy

Pelleted amastigotes were fixed for 30-40 min. at 18° C in 2.5% (v/v) glutaraldehyde (Sigma) in 0.1M phosphate buffer, pH 7.4, and subsequently rinsed as small fragments of the original pellet in a 0.1M phosphate buffer, pH 7.4, containing 2% (w/v) sucrose. After several washes in this solution, specimens were post-osmicated by the gradual addition of 4% (v/v) osmium

tetroxide in buffer to give a final 2% (v/v) OsO_4 concentration. The parasites were incubated in this for 1 hr. at 18°C. Removal of the osmium solution by washing in distilled water was followed by a 30 min. 'en bloc' treatment in 0.5% (w/v) aqueous uranyl acetate, dehydration in an alcohol series, and embedding in Araldite using propylene oxide as transition solvent. Polymerization of the resin was for 48 hrs at 60°C.

Serial sections were cut on an LKB Mk1 Ultratome to give ribbons with an interference colour silver (i.e. about 80 nm thick) at constant thickness using a diamond knife (Diatome Ltd, Switzerland). Ribbons were collected sequentially on Formvar coated 2 x 1 mm slot grids and the sections contrasted with uranyl acetate and lead citrate before viewing in an AEI 801 transmission electron microscope operating at 60Kv.

Image Recording

Ilford EM 4 cut film negatives were exposed at 15,550 times calibrated instrument magnification and 2.5 times photographic enlargements were printed. Profiles of organelles were traced from the photographs onto thin acetate sheets using a colour coding system to distinguish the different structures. Individual organelles of the same type were further identified numerically.

Serial Section Reconstruction

The coordinates of the boundary of each cell component were recorded using a magneto-strictive digitising tablet (Summagraphics ID model) linked to a mini-computer (Digital PDP-11 minc). These values were stored on a magnetic disc and recalled for the serial section reconstruction which was plotted on a storage oscilloscope (Tetronix T4010) or digital plotter (Tetronix T4662)

The micrographs were digitised by taking up to 500 (x,y) coordinates at approximately equal distances (ca. 1mm) around the profile of the cell or cell component. These values, together with the object reference number,

section thickness, magnification and some derived values from these coordinates (e.g. area and centre of gravity) were saved on disc. Each of the profiles on that section was then digitised, before changing to the next serial section. The relative orientation of the successive sections was decided by eye, taking into account the position of all the structures present. The various profiles of a single organelle were identified by the same reference number on each section on which they appeared.

The serial section reconstruction was made by selecting data from these files and calculating the corresponding coordinates for an isometric projection with the data transformed according to the rotation and tilt of the view. By indicating the object reference number, selected features could be plotted. The masking of hidden features was achieved by plotting the sections from the top layer and noting the lowest y-value plotted for each x-value in a data array (1024 elements). Where a calculated y-value was lower than this lowest value from the previous layers it could be plotted, whereas if higher it was masked. This simple algorithm was adequate for the reconstruction and avoided mapping the complete display to detect hidden features. The method of masking did not slow the plotting and did not require any extra computer memory for the mapping. Visible features were shown as lines, or dotted, and masked features as dots or not plotted. These dots represented the points originally digitised from the micrographs. For a stereo pair two plots were made with 5 degree difference in rotation.

Object volumes were calculated in the following way from the areas on each profile. Area (A) = $\sum x \quad \delta y$, where the first and last point for a closed profile must be the same. Object Volume = $\sum z \ (A_i + \sqrt{A_i A_{i+1}} + A_{i+1})$ /3, assuming each layer (thickness z) to be a truncated cone. A zero value for the area was assumed for the sections above the top layer and below the lowest layer on which the object was observed.

The method has been published in brief (Moss, 1985) and full details of the programme will be published elsewhere.

RESULTS

The appearance and distinguishing features of the different organelles of the Leishmania amastigote are shown in Figs 1-3. Reconstructions of two amastigotes were undertaken and the results are shown in Figs 4-12 and Table 1. The table gives the number, total volumes and relative volumes of the various organelles in each amastigote calculated as described above.

Both amastigotes were non-dividing cells in so far as each possessed a single nucleus and kinetoplast, but the volume of one amastigote was almost twice that of the other. As one might expect, the individual organelles were generally smaller in the smaller amastigote. They composed, however, a similar percentage of the total cell volume in the two cells. In both cases, the organelles recorded occupied 40% of the total cell volume. The nuclei of the two amastigotes showed a bigger difference in relative volumes than was noted for any of the other organelles. This could suggest that the two cells were at different stages in the cell cycle.

The mitochondrion of both amastigotes was a unitary structure, basket-like in form, but not composed of strands of uniform diameter. One of the most striking revelations of the reconstruction was the broad flattened expanses of mitochondrion which are joined by narrower strands, most components lying close to the surface of the cell (Figs 10-12), the entire organelle occupying approximately one-tenth of the cell's volume. The fibrillar kinetoplast formed a disc in the mitochrondrial matrix apposed to the flagellar base (Fig. 1); the matrix also contained flattened (plate-like) cristae (Fig. 1).

Glycosomes were identified by their lack of inclusions and relatively thin (6 nm) bounding membrane (Fig. 3). The amastigotes appeared to have nine and ten of these organelles which showed no clear-cut pattern of distribution in the cell (Figs 5,8). They were not noticeably associated with any other organelle or with one another. They were heterogeneous in shape (spherical to elongate) and size, the largest being several times the

volume of the smallest. Together they contributed only 1% of the total cell volume, approximately one-tenth of the volume of the mitochondrion.

Storage substances included lipid globules and electron-dense inclusion vacuoles (Fig. 3) identical in appearance to the polyphosphate vacuoles of trypanosomes (Vickerman and Tetley, 1977). The few (3 or 6) lipid globules lacked a bounding membrane, were spheroidal, variable in size and markedly anterior in their distribution in the cell (Figs 4,8). The polyphosphate vacuoles were also variable in size, 11 or 12 per cell and apparently randomly distributed in the amastigote. Their bounding membrane was comparable in thickness to that of the plasma membrane (10 nm).

The most abundant organelle in the amastigotes was the megasome with 34 and 18 of these structures present in the two cells. These thick membrane (10 nm)-bound organelles (Fig. 3) were spherical to elongate or dumb-bell shaped, variable in size (almost one-hundred fold in volume, 0.0064 to 0.44 um^3) and had markedly heterogeneous contents (Figs 1,2) with electron-dense spherules, vesicles and occasionally elongate crystalloids (Fig. 2) present in a matrix of moderate electron density. The megasomes were closely packed together in the posterior part of the cell, although extending forward or as far as the flagellar pocket (Figs 1,6,9) and occupied 13-15% of its volume.

DISCUSSION

Computer-aided reconstruction from serial sections has already been used by several groups of workers to study the three dimensional architecture of cell organelles and elaborations of the cell surface (for references see Nirzwicki-Bauer, Balkwill and Stevens, 1983). Few of these studies, however, have been concerned with the interrelationship of the major organelles within a single cell, largely because the programmes available did not have the capacity to handle sufficient data to allow meaningful analysis of an entire eukaryotic cell. The present study was facilitated by the development of a suitable programme, by use of a computer

with a large memory, and by the minute size of the Leishmania amastigote compared with other eukaryotic cells. This has allowed for the first time the accurate analysis of the three dimensional structure of an entire eukaryotic cell including all of the major organelles.

Now that the methods have been developed, it is perfectly feasible for other eukaryotic cells to be studied in a similar way. For practical reasons, however, such as the difficulty in obtaining serial sections throughout the cell and the problems in handling all the data produced, it seems probable that such analyses will be restricted to small cells for the time being.

The present study involved only two amastigotes. Nevertheless, the overall structure of the two was found to be very similar which suggests that the amastigotes analysed were not abnormal.

Perhaps the most remarkable finding of this investigation was the large number and total volume of the megasomes found in a single amastigote, together with the great diversity in their size. As there was no clear correlation between size and location within the cell and neither were associations with other cells detected, it is not possible to make firm judgements on the origin of the organelles or their function. They have similarities in structure and enzyme content (Pupkis, Tetley and Coombs, 1985) to lysosomes but their abundance clearly suggests that they are a rather specialised type of organelle which possibly play a part in the survival of the Leishmania m. mexicana amastigote in the macrophage host cell. Interestingly, they appear to be absent from amastigotes of L. major or L. donovani donovani but are present in L. m. amazonensis (Pupkis et al., 1985).

The small number of glycosomes found in L. m. mexicana amastigotes and their rather variable size is in contrast with Trypanosoma brucei bloodstream forms which are said to contain 200-300 glycosomes of very similar size (Opperdoes et al., 1984). Fractionation experiments with L. m. mexicana promastigotes, however, have shown that the glycosomes differ in

size more than those of T. brucei bloodstream forms (Hart and Opperdoes, 1984, Mottram and Coombs, 1985). It has been stated that the cultured promatigotes of L. major may contain as few as 50-100 glycosomes (Hart, D.T., unpublished observations). As culture promastigotes are approximately 5-10 x larger than amastigotes, it would appear that glycosomes may be equally abundant in the two forms. The lower density of glycosomes in leishmanias compared with bloodstream T. brucei may be, in part, a reflection of the latter relying more on glycolysis for energy production. There was no clear cut association of glycosomes with any other organelle in L. mexicana amastigotes nor with each other, suggesting that there is unlikely to be any direct metabolic coupling of the organelles with, for instance, the mitochondrion.

This study has revealed the complex structure of the single mitochondrion which appears to differ considerably from the relatively simple one reported for some trypanosomes (Paulin, 1977, 1983). The overall similarity of the structure of the mitochondrion in the two amastigotes, however, gives credence to the results and its relatively large volume confirms that it has an important role in amastigote metabolism.

Many questions concerning the functional reationships of the various amastigote organelles remain unanswered. The present study, however, has given for the first time an accurate insight into the three dimensional organisation of the leishmanial amastigote and so provides a framework on which other investigations can be built. In addition, it has emphasised the great possibilities offered by computer graphics in the study of the spatial arrangement of cells and cell components.

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TABLE 1 The number and volume of the organelles of Leishmania mexicana mexicana amastigotes.

Organelle	AMASTIGOTE 1				AMASTIGOTE 2			
	Number	Total Volume (um^3)	% of Total Cell Volume	Volume Range (um^3)	Number	Total Volume (um^3)	% of Total Cell Value	Volume Range (um^3)
1. Whole cell+	1	11.0	100	-	1	5.4	100	-
2. Nucleus	1	1.06	9.6	-	1	0.77	14.2	-
3. Mitochondrion	1	1.17	10.7	-	1	0.52	9.5	-
4. Kinetoplast	1	ND	ND	-	1	0.031	0.57	-
5. Glycosome	10	0.090	0.82	0.0039-0.015	9	0.035	0.64	0.00092-0.0056
6. Lipid globules	6	0.30	2.7	0.019-0.081	3	0.085	1.6	0.0065-0.054
7. Polyphosphate	11	0.060	0.54	0.0013-0.0096	12	0.025	0.45	0.00057-0.0053
8. Megasome	34	1.71	15.5	0.0064-0.44	18	0.69	12.6	0.0088-0.14
9. All organelles (2-8)	-	-	40	-	-	-	40	-

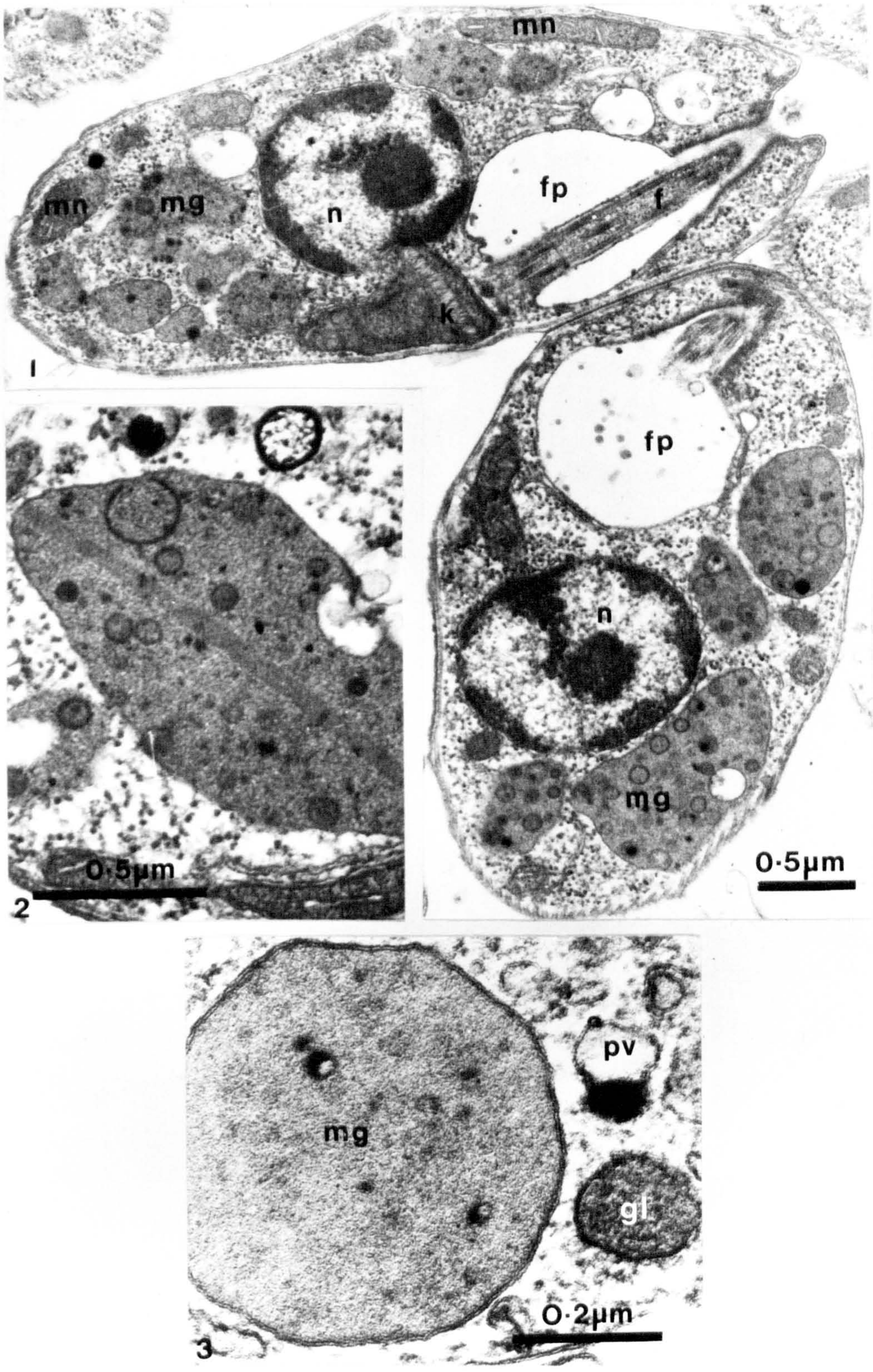
+ Volume of whole cell is after subtraction of the flagellar pocket volume.

Figs 1-3 Transmission electron micrographs of sections of amastigotes of Leishmania mexicana mexicana.

Fig. 1. Longitudinal sections of entire amastigotes showing nucleus (n), flagellum (f), flagellar pocket (fp), kinetoplast (k), profiles of mitochondrial network (mn) and megasomes (mg).

Fig. 2 Detail of megasome with heterogeneous contents including vesicles and an elongate crystalloid.

Fig. 3. Detail of megasome (mg), glycosome (gl) and polyphosphate vacuole (pv). Note thick (10nm) and thin (6nm) bounding membranes of megasome and glycosome respectively.

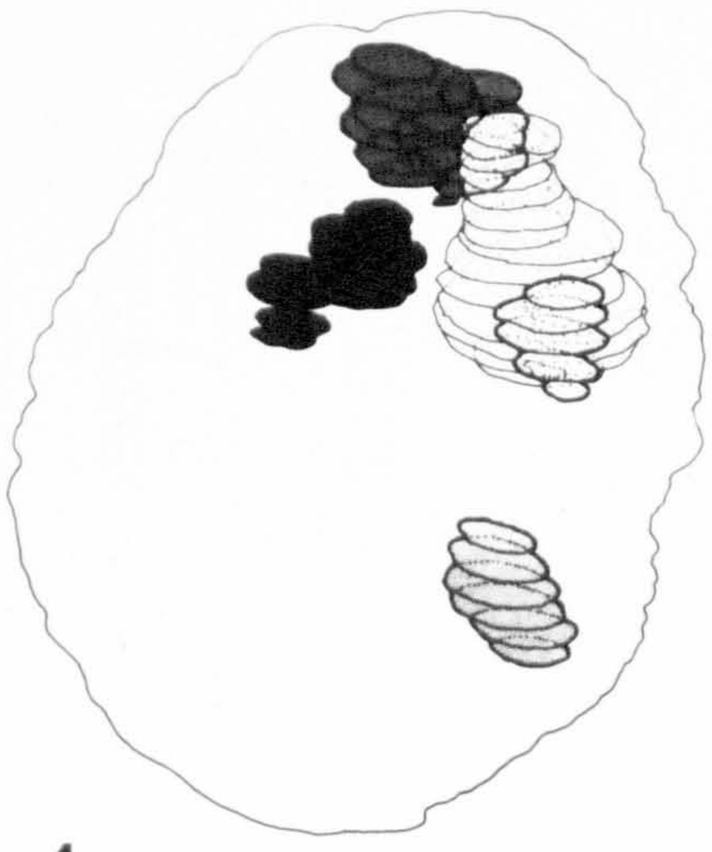

mn
fp
f
mn
mg
n
k
1
fp
n
mg
0·5µm
0·5µm
2
pv
mg
gl
0·2µm
3

Figs. 4-6 Reconstruction of amastigote organelles showing serial sections of individual organelles, stippled to aid identification; lighter stippled organelles are nearer to the observer than darker shaded ones. Only unmasked parts of organelles are stippled. The amastigotes surface outline and flagellar pocket (unshaded) are included for reference.

Fig. 4. Lipid globules (6)

Fig. 5. Glycosomes (10)

Fig. 6. Megasomes (34)



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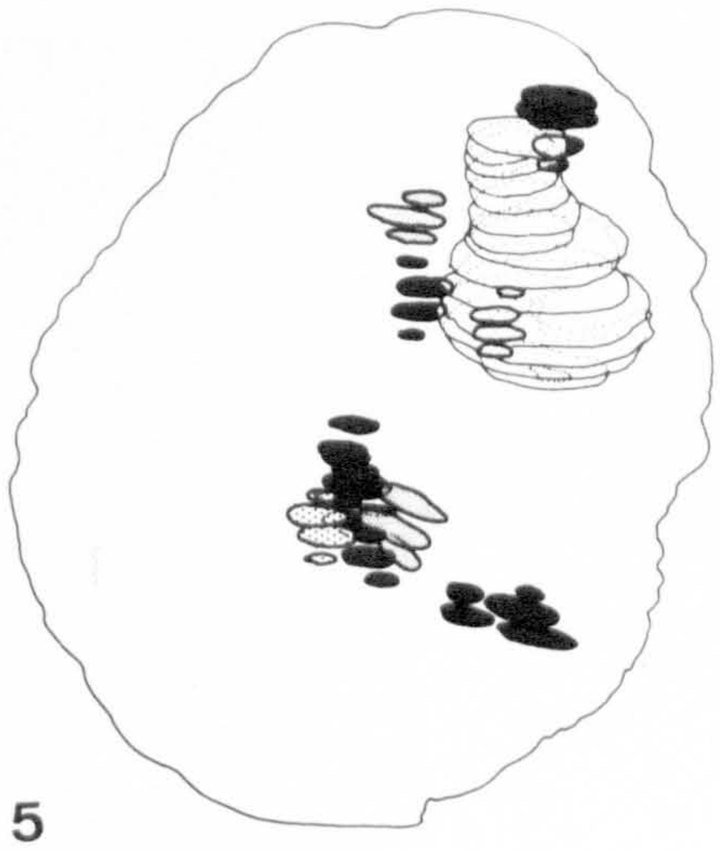

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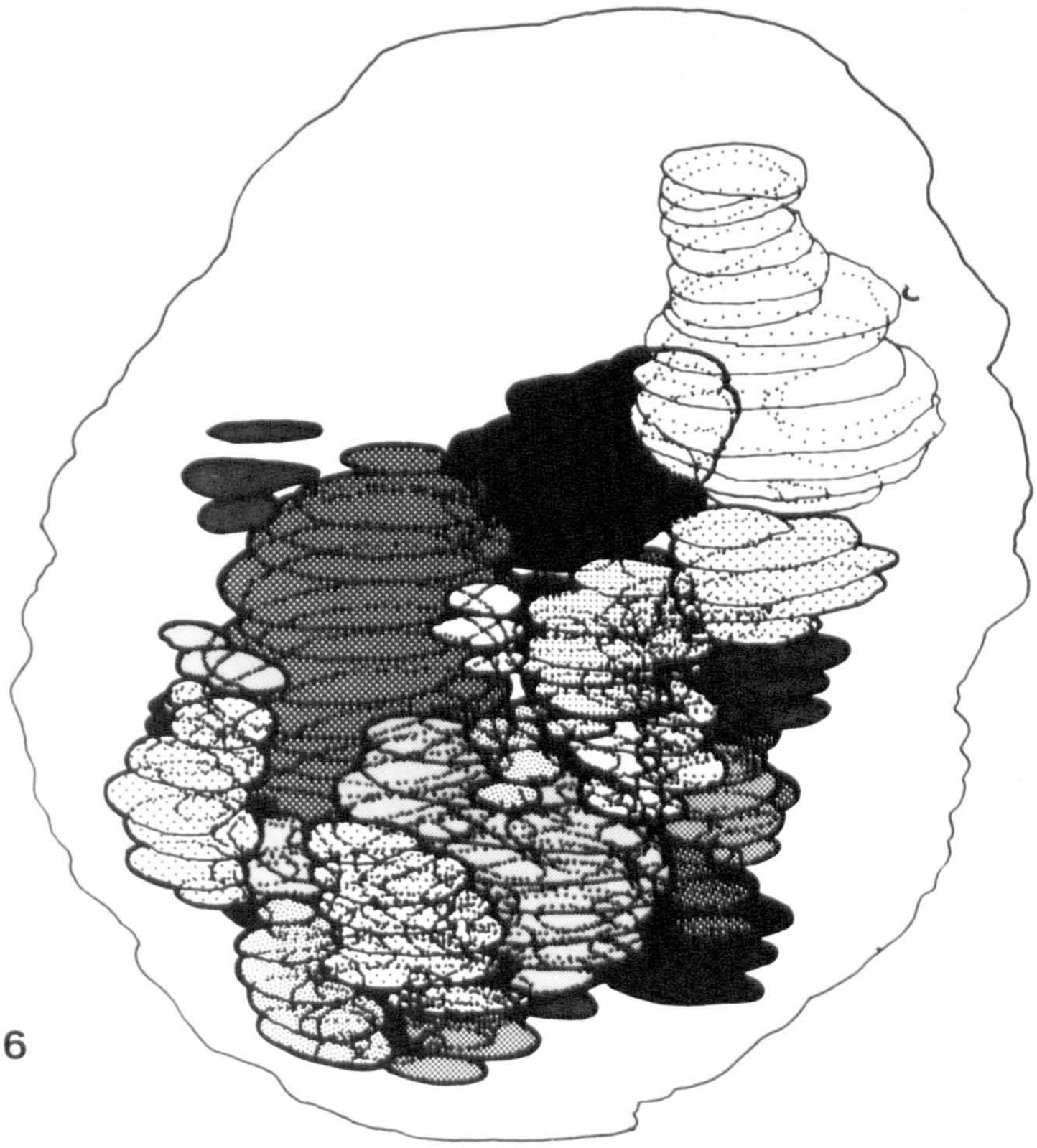
1 µm



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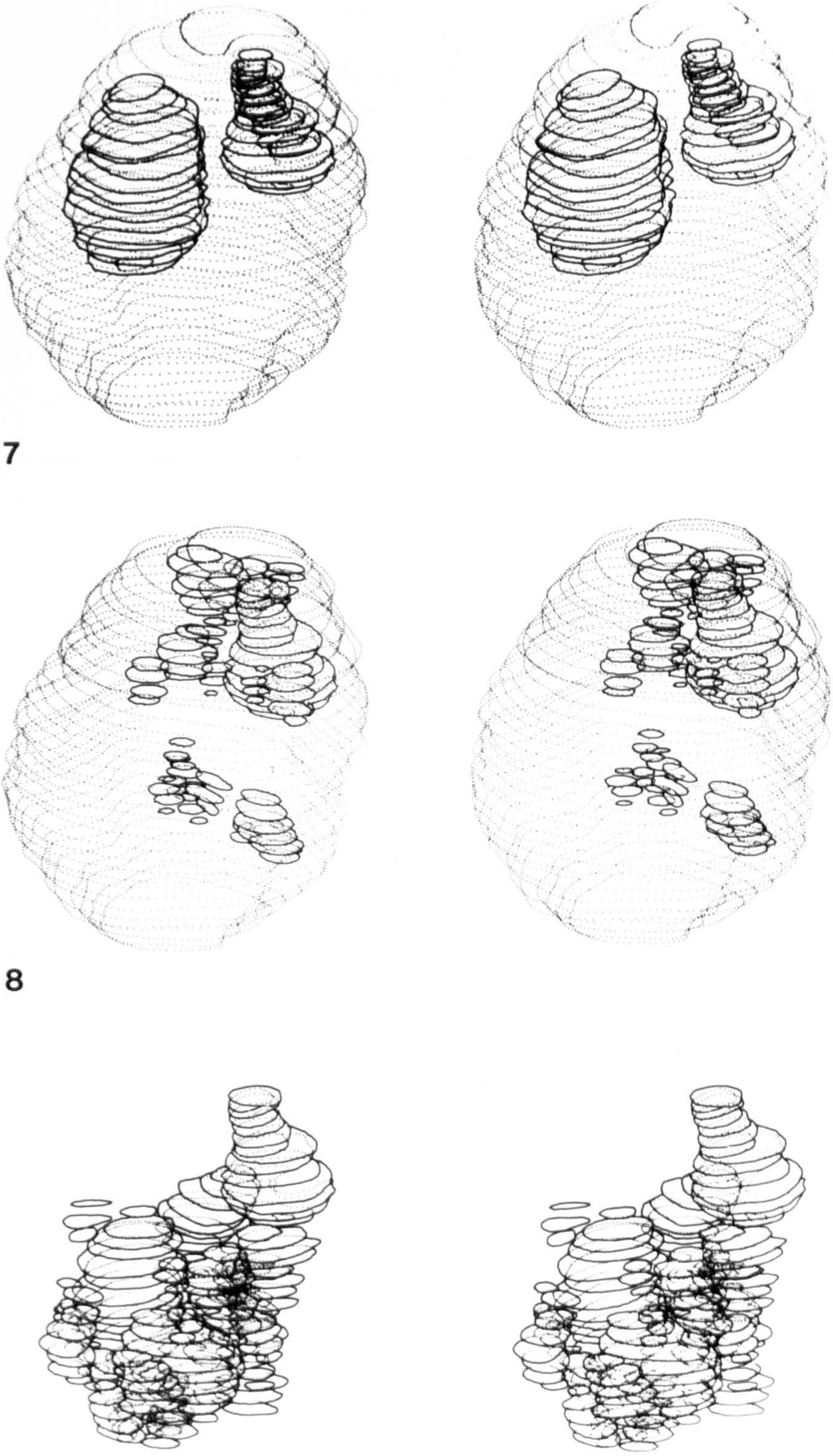

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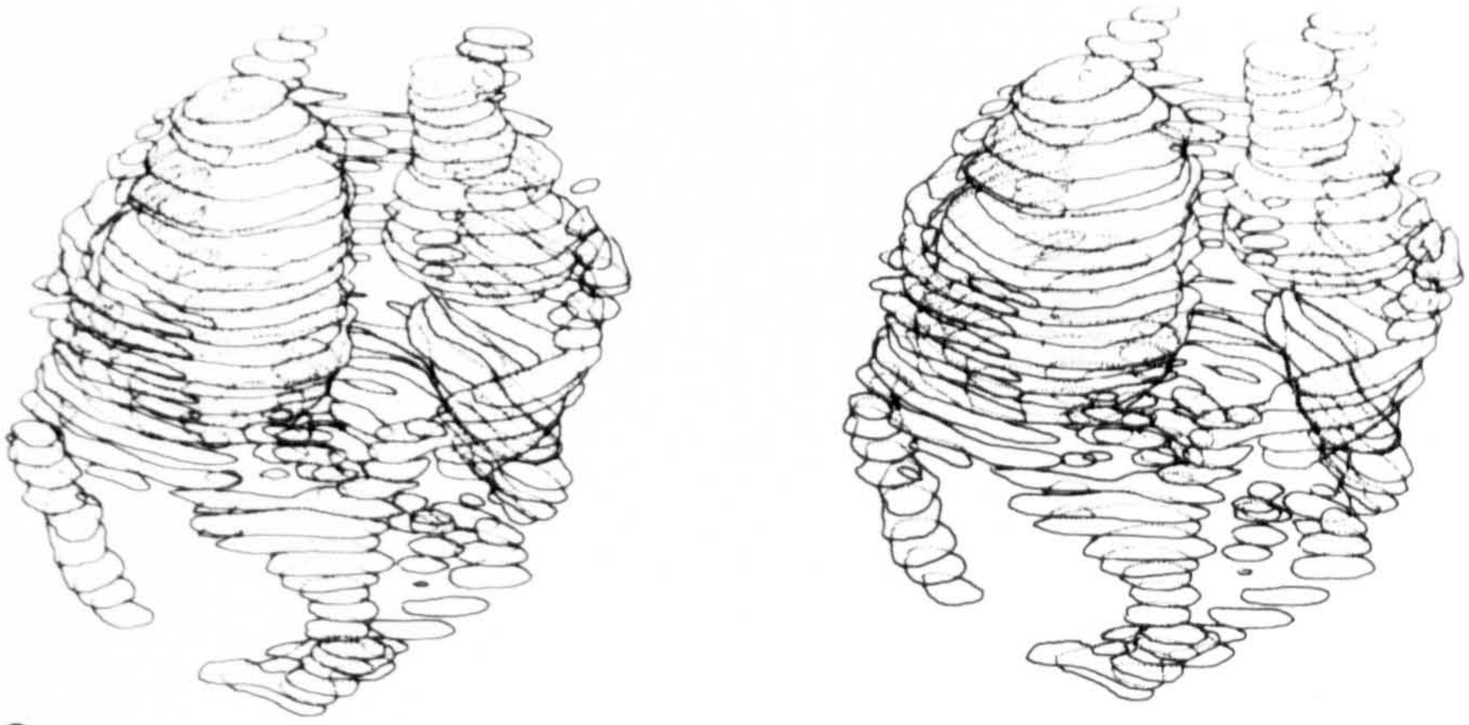
Fig. 10. Amastigote 1 showing relationships between mitochondrial network, nucleus, glycosomes and flagellar pocket.

Fig. 11. Amastigote 1 showing mitochondrial network alone. The kinetoplast was located within the flattened portion to the right of the network and adjacent to the flagellar pocket.

Fig. 12. Amastigote 2 showing the mitochondrial network within the parasite's surface membrane and also the flagellar pocket. Profiles of the kinetoplast nucleoid are shown (shaded in) within profiles of the inward-orientated part of the mitochondrion (kinetoplast capsule) adjacent to the base of the flagellar pocket; the pocket opens to the surface of the amastigote on the observer's right. Note that whereas amastigote 1 was sectioned transversely with respect to the longitudinal axis of the cell, amastigote 2 was sectioned slightly oblique to the longitudinal axis, hence the different appearance of the organelles, particularly the flagellar pocket, in the two amastigotes.



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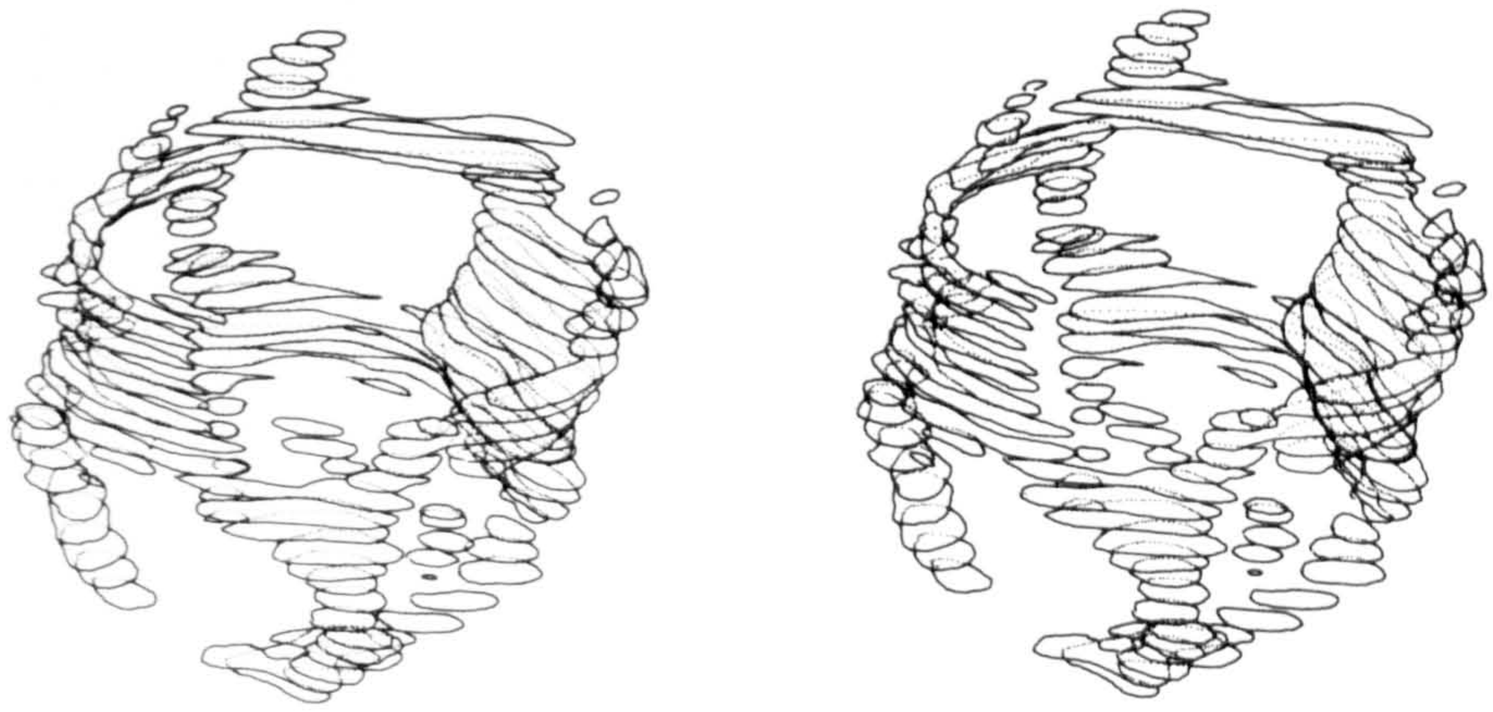

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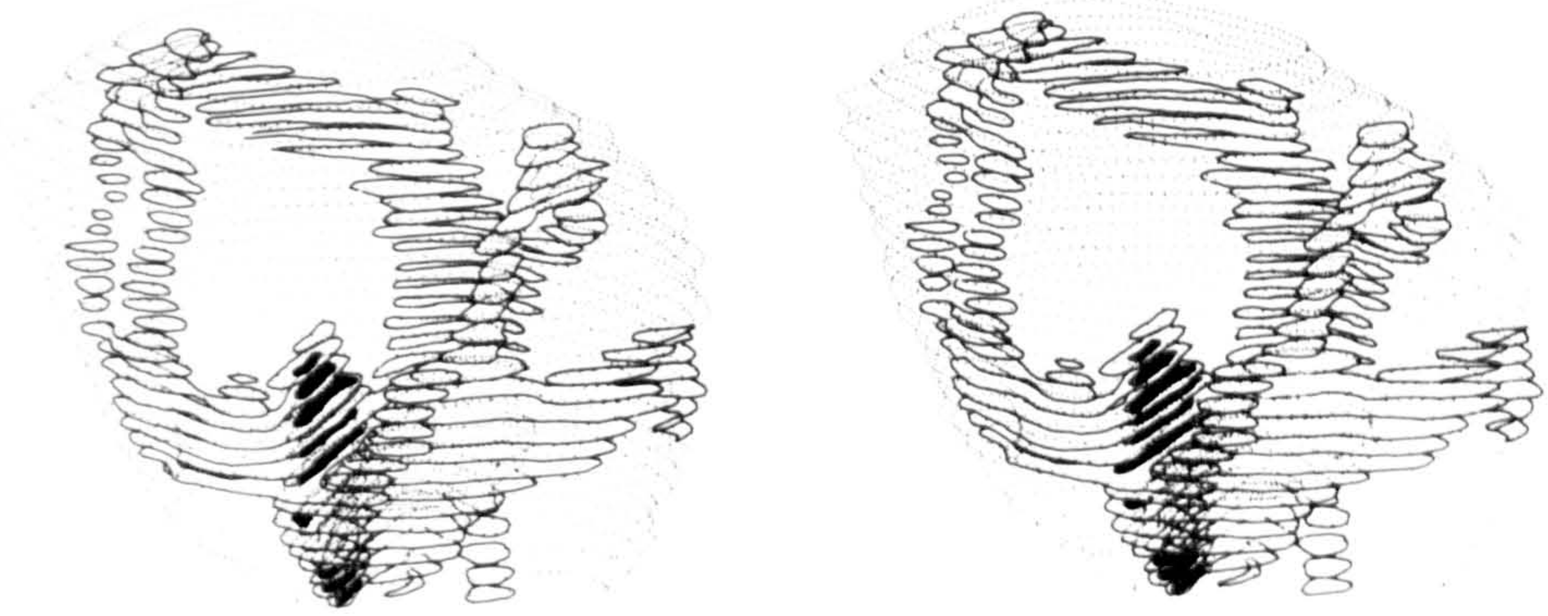

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Section 7.

Leishmania mexicana : Amastigote hydrolases in unusual lysosomes.

ABSTRACT

Leishmania mexicana mexicana (M379) amastigotes were found to contain much higher activities than cultured promastigotes of five putative lysosomal enzymes: cysteine proteinase; arylsulphatase (EC 3.1.6.1); β -glucuronidase (EC 3.2.1.31); DNase (EC 3.1.22.1) and RNase (EC 3.1.27.1). The release profiles of the first three of these enzymes from digitonin-permeabilized amastigotes suggests that they are located within organelles. Cytochemical staining for cysteine proteinase, using gold labelled antibodies, and arylsulphatase showed that both were present in large organelles previously named 'megasomes'. Comparative studies with L. m. amazonensis (LV78), L. d. donovani (LV9) and L. major (LV39) revealed that L. m. amazonensis was similar to L. m. mexicana in possessing both high amastigote cysteine proteinase activity and large numbers of megasome organelles in amastigotes, whereas the other two species lacked both these features. The results suggest that the presence of numerous lysosome-like organelles in the amastigote is a characteristic of the L. mexicana group of parasites.

INDEX DESCRIPTORS: Leishmania mexicana mexicana; Leishmania mexicana amazonensis; Leishmania donovani donovani; Leishmania major; amastigotes: promastigotes: lysosome.

INTRODUCTION

Leishmania mexicana mexicana amastigotes have been shown to possess much higher cysteine proteinase activity than cultured promastigotes of the same species (Coombs 1982). This observed difference is apparently due to an amastigote-specific enzyme which is believed to play a crucial role in the survival of the parasite within the host cell macrophage and so provides a useful target for chemotherapeutic attack (Pupkis and Coombs 1984). In an attempt to understand better the involvement of the cysteine proteinase in L. m. mexicana amastigotes, we have investigated the location of the proteinase within the cell, and similarly studied various other putative lysosomal enzymes in L. m. mexicana, as well as carrying out comparative studies with L.m. amazonensis, L. donovani donovani and L. major.

MATERIALS AND METHODS

Triton X-100, ∝- cellulose, sigmacell (type 50), azocasein, digitonin, methylumbelliferyl sulphate, 4-nitrocatechol sulphate, gluteraldehyde, .p-nitrophenylglucuronide, p-nitrophenylglucoside, p-nitrophenol, dimethylformamide, L-proline-L-phenylalanine-L-arginine, L-phenylalanine-L-proline, L-leucine-L-alanine, L-proline-L-leucine, L-proline-glycine, glycine-glycine, glycine-L-leucine, L-leucine-L-leucine-L-leucine, glycine-glycine-glycine, L-leucine-glycine-glycine, RNA, DNA, L-amino acid oxidase (from Crotalus atrox), horse radish peroxidase and o-dianisidine hydrochloride were obtained from Sigma London Chemical Company (Poole, Dorset, England, U.K.). Panasorbin was obtained from Calbiochem (C.P. Laboratories Ltd., Bishops Stortford, Herts, U.K.). All other reagents were of analar grade and obtained from BDH Laboratory Chemicals Division (Poole, Dorset, England, U.K.).

Leishmania m. mexicana (M379) amastigotes were grown in female CBA mice (Department of Zoology, University of Glasgow, Glasgow, U.K.) and isolated as described previously (Hart et al. 1981). The purified amastigotes were viable and essentially free from contamination by intact host cells (less than 0.2% cell/cell) and cellular debris. Promastigotes of L. m. mexicana were grown as described previously (North and Coombs 1981) and only cells which had been subpassaged not less than ten times were used in these studies. Crude homogenates of washed cells in 0.25M sucrose were obtained by the addition of Triton X-100 to 0.1% (v/v). Leishmania m. amazonensis (LV78) and L. major (LV39) were grown in male Balb/c mice and L. donovani donovani (LV9) was grown in male cotton rats. The infected cotton rats were kindly supplied by Dr R. Stokes (Liverpool School of Tropical Medicine). The amastigotes were isolated from the infected animals as for L. m. mexicana with the number of purified amastigotes and % cell/cell contamination being, respectively: 2 x 10^9, 0.2%; 0.8 x 10^9 0.2%; 0.5 x 10^9, 0.4%. Promastigotes of each species were obtained by transformation of the amastigotes and growth in HOMEM medium as for L. m. mexicana.

To study the release of enzymes from amastigotes permeabilized with digitonin, purified L. m. mexicana amastigotes (10^9 cells per sample) were suspended in buffer (0.25M sucrose; 25 mM Tris HCl, pH 7.3; 1 mM EDTA) and incubated at 27°C with digitonin (previously dissolved in dimethylformamide) at a concentration of 75 µg/mg protein in a final volume of 1 ml. Samples were removed at appropriate time intervals during the 1 hr incubation. After centrifugation at 10,000 g for 15 min at 18°C, the supernatant and pellet fractions (the latter lysed by 0.1% (v/v) Triton X-100) were assayed for the following enzymes using methods described previously: azocaseinase (AZCase) (Coombs 1982) BZ-proline-phenylalanine-arginine

peptidase (PPAase) (Pupkis and Coombs 1984) arylsulphatase (EC 3.1.6.1., Sherman and Stanfield 1967); glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucose phosphate isomerase (EC 5.3.1.9.) and glutamate dehydrogenase (EC 1.4.1.3) (Mottram and Coombs 1985). Protein concentrations were estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Leishmanial amastigotes and promastigotes were lysed using 0.1% (v/v) Triton X-100 and assayed for the following enzymes using the methods indicated: β-D-glucuronidase (EC 3.2.1.31), β-D-glucosidase (EC 3.2.1.21), deoxyribonuclease II (EC 3.1.22.1) and ribonuclease (EC 3.1.27.1) (Barrett 1972); acid phosphatase (EC 3.1.3.2) (Trouet 1974); peptidases and amino acid oxidase (EC 1.4.3.2) (Coombs et al. 1982); mono-, di- and polyamine oxidases (EC 1.4.3.4.) (Tabor et al. 1954); phospholipase C (EC 3.1.4.3) (Kurioka, S. and Matsuda, M. 1976); acid lipase using p-nitrophenyl derivatives of fatty acids (Mahaderan and Tappel 1968); catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7) (Bergmeyer 1974).

Pellets of isolated amastigotes of the four species and blocks of excised infected tissue were fixed for electron microscopy by incubation in 2.5% glutaraldehyde in phosphate buffer (0.1M, pH 7.4) at 20^{0}C for 30 min. Subsequent washing in phosphate buffer (0.1M, pH 7.4) containing 2% (w/v) sucrose was followed by 1 hr in phosphate-buffered (0.1M, pH 7.4) OsO_4 (2% w/v), 30 min in uranyl acetate (0.5% aqu) and dehydration in an alcohol series. The material was embedded (via propylene oxide) in Araldite which was polymerised at 60^{0}C for 2 days. Sections (60nm) were contrasted with uranyl acetate and lead citrate after collection on 300 mesh uncoated grids and examined in an AEI 801 TEM at 60Kv.

The method of Hopsu-Hasvu and Helminen (1974) which employed 4-

nitrocatechol sulphate as substrate was used to demonstrate the location of arylsulphatase A or B in amastigotes. Bariumchloride was used as capture agent, forming electron dense barium deposits at reaction sites. Freshly obtained small pieces of amastigote-rich lesion were immediately placed in 0.5% glutaraldehyde in cold (4^{0}C) cacodylate buffer (0.1M, pH 7.4) for 30 min and cut into blocks (< 1 mm^3). Excess, cold cacodylate buffer containing 2% (w/v) sucrose was added to terminate the reaction and the solution changed several times to remove unreacted fixative. The specimen buffer was changed to 0.1M acetate, pH 5.2, by gradually increasing the concentration of acetate through several changes and with a final overnight wash at 4^{0}C. Substrate incubation was performed at 37^{0}C for 1 hr at pH 5.2 in the dark and terminated by the addition of cold acetate buffer. The specimens were subsequently prepared as described previously, except that embedding was achieved by overnight infiltration in Spurr's resin. Polymerisation in fresh resin was carried out at 60^{0}C for 24 hr. All processing except during the resin infiltration and enzyme incubation was at 0-4^{0}C. Sections (60 nm) were examined unstained or after 5 min in Reynold's lead citrate to improve general contrast.

A rabbit (NZW 1/2 LOP, 5 months old, female) was challenged on three occasions with 200 µg L. m. mexicana amastigote cysteine proteinase (specific activity 3.0 units/mg protein) purified as described previously (Pupkis and Coombs 1984). One week after the final challenge the serum IgG fraction was purified by an affinity method using Staphylococcus aureus (Johnstone and Thorpe 1982). The nuclear-specific antibodies in the preparation were absorbed out as follows. An enriched nuclear fraction was prepared from L. m. mexicana promastigotes (4 x 10^9 cells) by the method of Coombs et al. (1982) and incubated with the pure IgG fraction (10 mg protein) at

22°C for 30 min in phosphate buffer (0.15 M, pH 7.3). The supernatant resulting from centrifugation (2100 g for 10 min at 22°C) was incubated in Tris HCl buffer (0.15 M, pH 8.6) for 1 hr at 4°C with Staphylococcus aureus fixed cells to absorb the immunoglobulins. The complex was obtained by centrifugation (10,000 g for 10 min at 20°C) and the immunoglobulins were subsequently eluted using phosphate buffer (0.5 M, pH 6.0).

Colloidal gold (5-10 nm diameter) was produced as detailed elsewhere (de Mey 1983). The gold particles were complexed at pH 9.0 at the stabilizing concentration of the purified IgG protein (de Mey 1983). The resultant IgG/gold probe was washed 3 times by resuspension in Tris buffer (20 mM, pH 7.8; 1% w/v BSA) and centrifugation at 60,000 g for 1 hr at 4°C to remove unbound antibody. The probe was resuspended in Tris buffer to a dark red colour and unwanted aggregates were removed by centrifugation at 2,000 g for 15 min at 4°C. Studies on the localisation of the cysteine proteinase using the IgG/gold probe were carried out with amastigotes and promastigotes fixed in 0.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for 30 min and dehydrated rapidly in the cold through an alcohol series and via brief, cold propylene oxide exchange. The material was infiltrated with Araldite overnight at 20°C and polymerised at 60°C for 2 days. On-section marking using the gold probe was performed essentially as detailed by Roth (1983) using Araldite sections (80 nm) mounted on nickel grids and etched for 10 min in H_2O_2(10% v/v). Controls included section incubations in 5% normal rabbit serum for 30 min prior to gold probe exposure. Irrelevant antibody/gold probe substitution and blocking with antibody alone (at 1 in 100 dilution) were carried out in Tris HCl buffer (20 mM, pH 7.8) containing 0.1% (w/v) BSA.

RESULTS

Five of the seven putative lysosomal enzymes detected in L. m. mexicana were present at much higher activity in amastigotes than promastigotes (Table 1). The exceptions were β-glucosidase, which was present at very low levels in promastigotes and was undetectable in amastigotes, and acid phosphatase, which has been shown previously to be present in a variety of cell locations and may represent the activities of several enzymes (Coombs et al. 1982; Gottlieb and Dwyer 1983).

The profiles of the release of two of the soluble enzymes from amastigotes treated with digitonin are shown in Fig. 1, together with the profiles of soluble enzymes used as markers for the cytosol (glucose-6-phosphate dehydrogenase), mitochondrion (glutamate dehydrogenase) and glycosome (glucose phosphate isomerase). These last three enzymes were fully released sequentially and relatively rapidly. In contrast, proteinase (AZCase and PPAase) and arylsulphatase were released only slowly from the amastigotes such that by 30 min approximately 50% of the activity was recovered in the supernatant. β-Glucuronidase was similarly released slowly, and extending the incubation period to 60 min resulted in approximately 80% of each of the three activities being recovered in the supernatant fraction (data not shown).

The use of cytochemical staining methods and electron microscopy revealed (Fig. 2) that arylsulphatase was detectable only in the organelles characteristically abundant in L. m. mexicana amastigotes that have previously been referred to as 'megasomes' (Alexander and Vickerman 1975). IgG antibody purified from a rabbit challenged with highly purified L. m. mexicana amastigote cysteine proteinase was found to stain not only megasomes but also, and more abundantly, the nucleus and kinetoplast of the parasite (Fig. 3a) as well as the

macrophage nucleus. This was visualised by 'on-section' marking using a gold/antibody probe. After absorption of antibodies by the nuclear-enriched fraction of L. m. mexicana promastigotes, however, gold staining occurred only in megasomes, albeit at a rather low level (Fig 3b).

None of the peptidases investigated were found to be present at higher activity in amastigotes of L. m. mexicana than promastigotes (Table 2) and various enzymes characteristic of mammalian peroxisomes (catalase, various peroxidases and amino acid oxidase) and lysosomes (phospholipase C and acid lipase) were undetectable in amastigotes and promastigotes, as were mono-, di- and polyamine oxidases. The limits of detection for these enzymes in the amastigote, expressed as munits/mg protein, were as follows: catalase, 0.2; general peroxidase, 2.0; cytochrome C peroxidase, 4.0; amino acid oxidase, 3.0; phospholipase C, 25; general acid lipase, 3.0; mono-, di- and polyamine oxidases, 3.0.

A high activity of cysteine proteinase was detected with amastigotes of L. m. amazonensis as well as L. m. mexicana, but not with L. d. donovani and L. major (Table 3). Transmission electron micrographs of the amastigotes of these four parasites revealed a clear correlation between the presence of high cysteine proteinase activity and the presence of megasomes; the organelles were abundant in both L. m. mexicana and L. m. amazonensis, but not seen in L. d. donovana or L. major (Fig. 4).

DISCUSSION

We have been unsuccessful so far in our efforts to fractionate amastigotes of L. m. mexicana by the classical methods used for promastigotes (Coombs et al. 1982; Mottram and Coombs 1985) and so prevented from characterising isolated organelles. The use of

digitonin to release soluble enzymes from amastigotes, however, has demonstrated that the cysteine proteinase, arylsulphatase and -glucuronidase are present within an organelle, but not the glycosome or the mitochondrion. The similarity of the release profiles of the three enzymes suggests that they may be located together.

The histochemical staining technique employed for arylsulphatase provides strong evidence that this enzyme is present within the structures previously called megasomes (Alexander and Vickerman 1975). In addition, the use of gold-labelled anti-cysteine proteinase antibody showed this enzyme also is present within these organelles. The gold labelling found associated with the nucleus and kinetoplast of the amastigote and also the nucleus of host cell macrophage could have been due to an impurity in the purified proteinase used to elicit the rabbit antibody production. It is feasible that if the impurity was much more immunogenic than the amastigote cysteine proteinase then there would be more antibodies produced against the impurity even though it comprised less than 1% of the total protein in the purified proteinase preparation (Pupkis and Coombs 1984). A second possibility is that the antibodies that reacted with the leishmanial nuclear and kinetoplast material were present in the rabbit serum for reasons other than the challenge with the proteinase preparation. The target of the antibodies is not known, but it is interesting that there appears to be an antigenic component common to the kinetoplast and the nucleus of the parasite and also the nucleus of host macrophages. The successful removal of the contaminating antibodies by using a promastigote nuclear-enriched fraction, whilst the megasome-labelling antibodies remained (albeit at a low level), revealed both that the kinetoplast/nuclear staining was not due to the anti-proteinase antibodies and that the proteinase was not present in the promastigote material used. The low level of

gold marking over the megasomes was possibly a consequence of the relatively low titre of anti-proteinase antibodies in the rabbit serum, reflecting the low immunogenicity of the cysteine proteinase, and the removal of low avidity antibodies during the numerous washing procedures.

The conclusion that the cysteine proteinase is located within megasomes is supported by the finding that L. m. amazonensis is similar to L. m. mexicana in possessing both high cysteine proteinase activity and large numbers of these organelles in the amastigote, whereas L. d. donovani and L. major amastigotes contained very low proteinase activities and few if any structures equivalent to megasomes. It is tempting to speculate that these characteristics of L. mexicana are related to the production of the large parasitophorous vacuoles in which the amastigotes of the species typically grow, unlike L. d. donovani and L. major which reside in much smaller vacuoles. Unquestionably, however, the similarities of L. m. mexicana and L. m. amazonensis are supportive of their being closely related organisms.

The similarities between arylsulphatase, β-glucuronidase, DNase, RNase and the cysteine proteinase with respect to their amastigote/promastigote activity ratios and their release from digitonin-treated amastigotes suggests that all may be located within megasomes in the amastigote. The apparent absence of catalase, peroxidases and amino acid oxidase from L. m. mexicana suggests that megasomes are not peroxisome-like. Ultrastructurally they resemble lysosomes in that they possess a thick (10nm) surrounding membrane (Coombs et al. 1985) and, as shown in this study, contain several acid hydrolases commonly associated with lysosomes. They are very abundant in L. m. mexicana amastigotes, however, with up to 34 of the

organelles being present comprising approximately 15% of the total cell volume (Coombs et al. 1985), and in this respect they differ considerably from most trypanosomatid lysosomes. In order to simplify discussion of the organelles and avoid confusion between these structures and the lysosomes of other trypanosomatids, they should be given different names. Megasome is not very appropriate for the L. mexicana amastigote structures, as they vary considerably in both size (0.0064-0.44 μm^3) and shape (Coombs et al. 1985). Nevertheless, we propose that its use should continue until we have a better understanding of the roles of the organelles and so are able to devise a more suitable name.

Further studies are required to elucidate the part megasomes play in the survival of L. mexicana in the host macrophage. We have been unable to detect cysteine proteinase secretion from L. m. mexicana amastigotes even using a highly sensitive proteinase assay involving (^3H)-acetylcasein, nor could we detect the uptake of gold-labelled BSA, low density lipoprotein or cationised ferritin into megasomes. It is possible, however, that steric interference by the 20 nm gold particles used prevented access of these proteins into the flagellar pocket. There is very little known concerning the occurrence of pinocytosis in leishmanial amastigotes and further studies are necessary to establish the extent and route of uptake of exogenous material into megasomes.

Our previous suggestion (Coombs 1982) that the amastigote cysteine proteinase, now shown to be present in megasomes, may be involved in the secretion of amines into the parasitophorous vacuole with the consequent antagonism of the microbicidal activity of the macrophage is still consistent with the available data. The much greater activities of arylsulphatase, DNase and RNase detected in L. m. mexicana amastigotes compared with cultured promastigotes suggests

that these enzymes may also play important parts in the survival and growth of L. m. mexicana in the mammal. The restriction of megasomes to amastigotes of L. mexicana suggests that these parasites are adapted for life in macrophages in ways different, at least in part, from other leishmanias including L. d. donovani and L. major. A similar conclusion has been reached from studies concerning the polypeptide composition of the amastigotes of these parasites (Pupkis and Coombs 1985).

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Fig. 1. Release of soluble enzymes from L. m. mexicana amastigotes permeabilized with digitonin at a concentration of 75 µg/mg protein.

Key: ■ , glutamate dehydrogenase (NAD-specific)(EC 1.4.1.3); □ , glucose -6-phosphate dehydrogenase (EC 1.1.1.49); ● , glucose phosphate isomerase (EC 5.3.1.9); ○ , azocaseinase; ▲ , arylsulphatase (EC 3.1.6.1); △ , proline-phenylalanine-arginine peptidase; ◆ , protein.

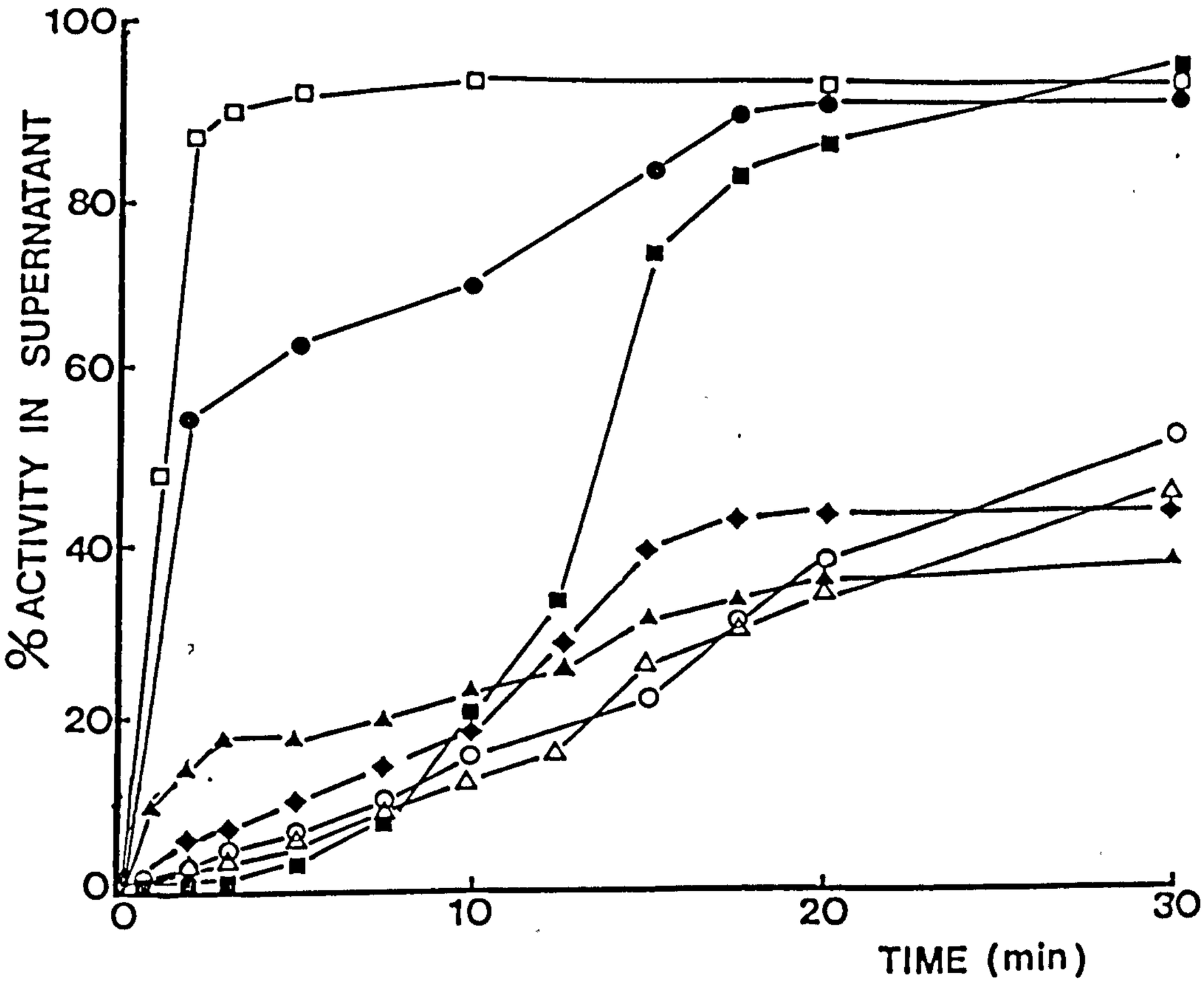

100
80
60
40
20
0
% ACTIVITY IN SUPERNATANT
0
10
20
30
TIME (min)

Table 1. Activity of putative lysosomal enzymes in L. m. mexicana[a].

Enzyme	Amastigote	Promastigote	Ratio
Cysteine proteinase	153	4	38
Arylsulphatase	187	5	37
β-Glucuronidase	1.3	0.04	33
Deoxyribonuclease	2	0.2	10
Ribonuclease	9	0.3	30
β-Glucosidase	0.01	0.03	0.3
Acid phosphatase	43	60	0.7

a. The results given are expressed as munits per mg protein and are th
means from replicate experiments.

Table 2. Peptidase activites of L. m. mexicana[a].

Substrate	Amastigote	Promastigote
L-Phe-L-Pro	15.9	31.1
L-Leu-L-Ala	8.8	20.6
L-Pro-L-Leu	1.2	28.9
L-Pro-Gly	10.6	21.1
Gly-Gly	0.9	0.2
Gly-L-Leu	1.9	1.2
L-Leu-L-Leu-L-Leu	35.2	76.5
Gly-Gly-Gly	1.9	0.7
L-Leu-Gly-Gly	3.0	7.9

a. The results given are expressed as munits per mg protein and are the means from replicate determinations.

Table 3. Proteinase activities of L. m. mexicana, L. m. amazonensis, L. d. donovani and L. major.

Activity	Conditions	L. m. mexicana		L. m. amazonensis		L. d. donovani		L. major	
		Amast[d]	Prom[d]	Amast	Prom	Amast	Prom	Amast	Prom
Azocaseinase[a]	-DTT	74	6	28	7	10	6	9	7
	+DTT(1 mM)	153	4	36	5	8	5	7	6
	+Leupeptin[c]	12	86	10	90	20	100	100	91
PPAase[b]	-DTT	143	23	166	0.5	6	0.8	6	ND[e]
	+DTT(1 mM)	240	27	190	0.5	20	0.5	7	ND[e]

a. Azocaseinase activity expressed as munits per mg protein and given as the means from replicate experiments.

b. L-Pro-L-Phe-L-Arg peptidase (PPAase) activity expressed as munits per mg protein and given as the means from replicate experiments. All of these activities were totally inhibited by leupeptin (33 ug/ml).

c. The effect of leupeptin is expressed as the activity in the presence of 33 ug/ml leupeptin as a percentage of the control activity in the presence of DTT.

d. Abbreviations: Amast, amastigotes; Prom, promastigotes; DTT, dithiothreitol.

e. ND, not determined.

Fig. 2. Demonstration of arylsulphatase in the megasomes of Leishmania mexicana mexicana.

Fig. 2a. Specific reaction product (arrowed) can be seen in the megasomes (m) only.

Fig. 2b. Control omitting substrate, nitrocatechol sulphate. No barium deposits can be observed.

Sections were counterstained with uranyl acetate and lead citrate. Scale bar represents 0.5 μm.

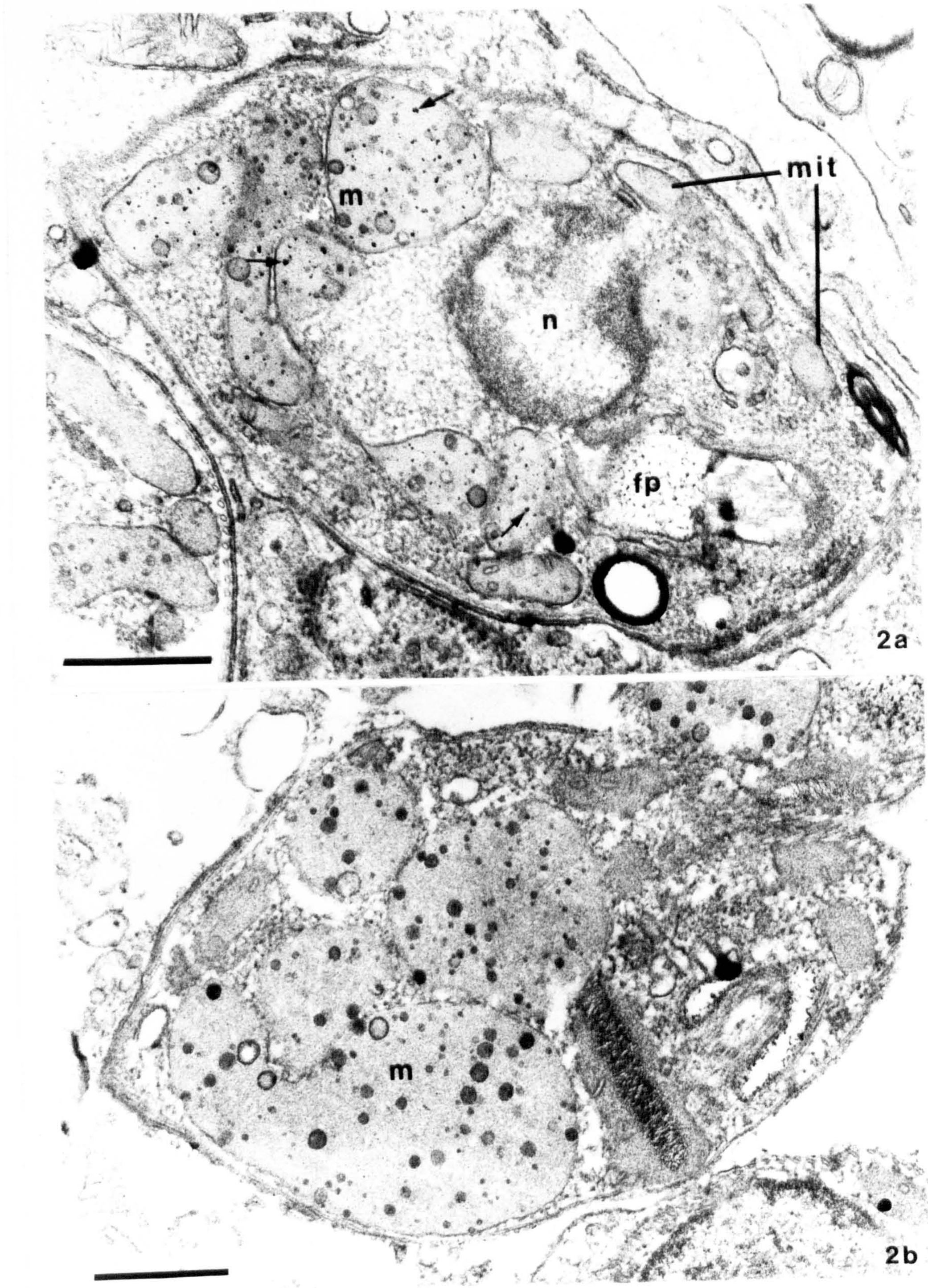
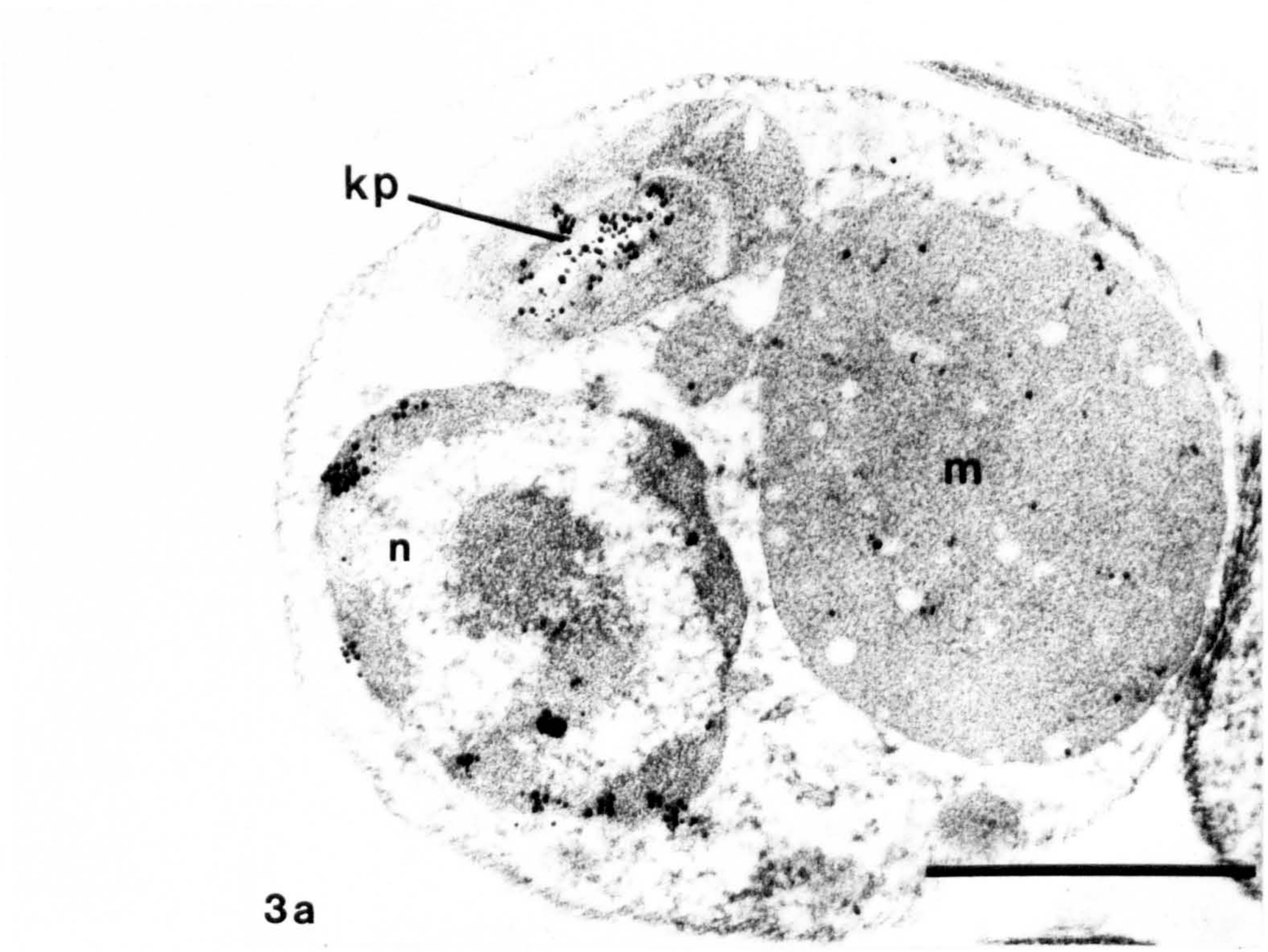

m
mit
n
fp
2a
m
2b

Fig. 3. Localization of cysteine proteinase in Leishmania mexicana mexicana amastigotes using immuno-gold staining.

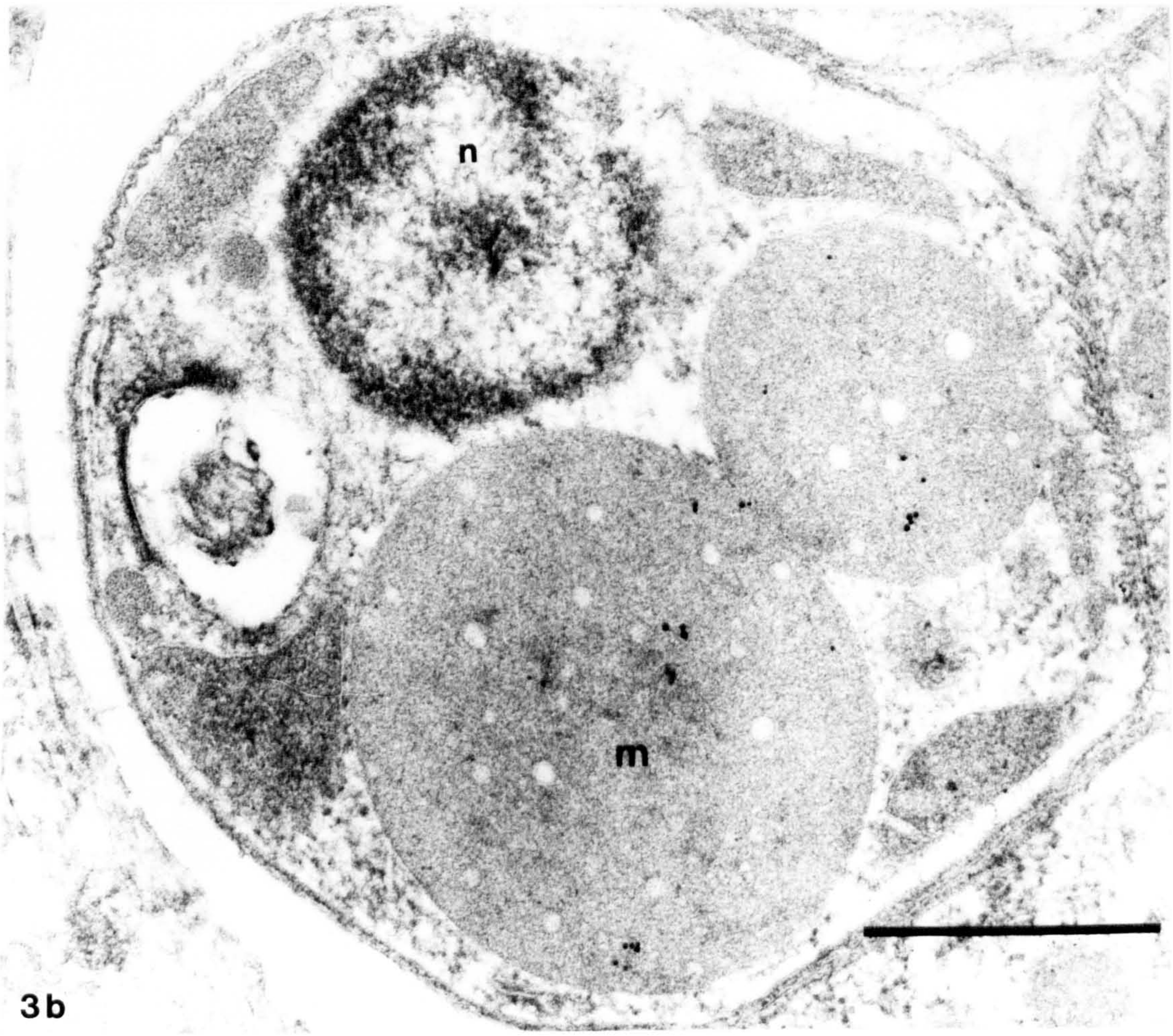
Fig. 3a. Staining pattern obtained using original IgG preparation. Note that the gold probe is present over DNA-containing structures as well as megasomes (m). Key: kp, kinetoplast; n, nucleus.

Fig. 3b. Staining pattern after absorption of irrelevant IgG. Probe is now found associated with megasomes only.

The sections were not counterstained with lead or uranyl salts in this procedure. Scale bar represents 0.5 μm.



3a

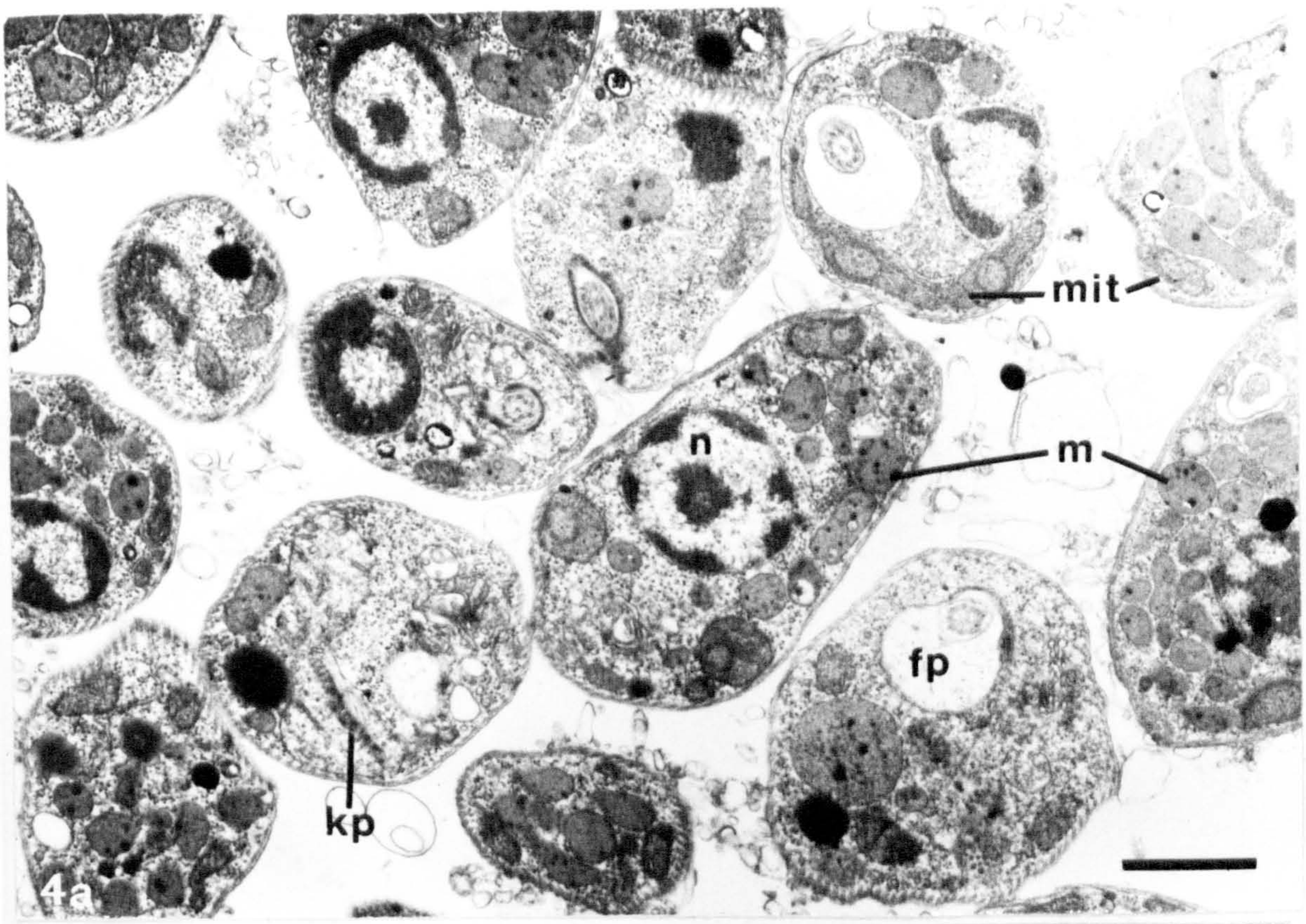


3b

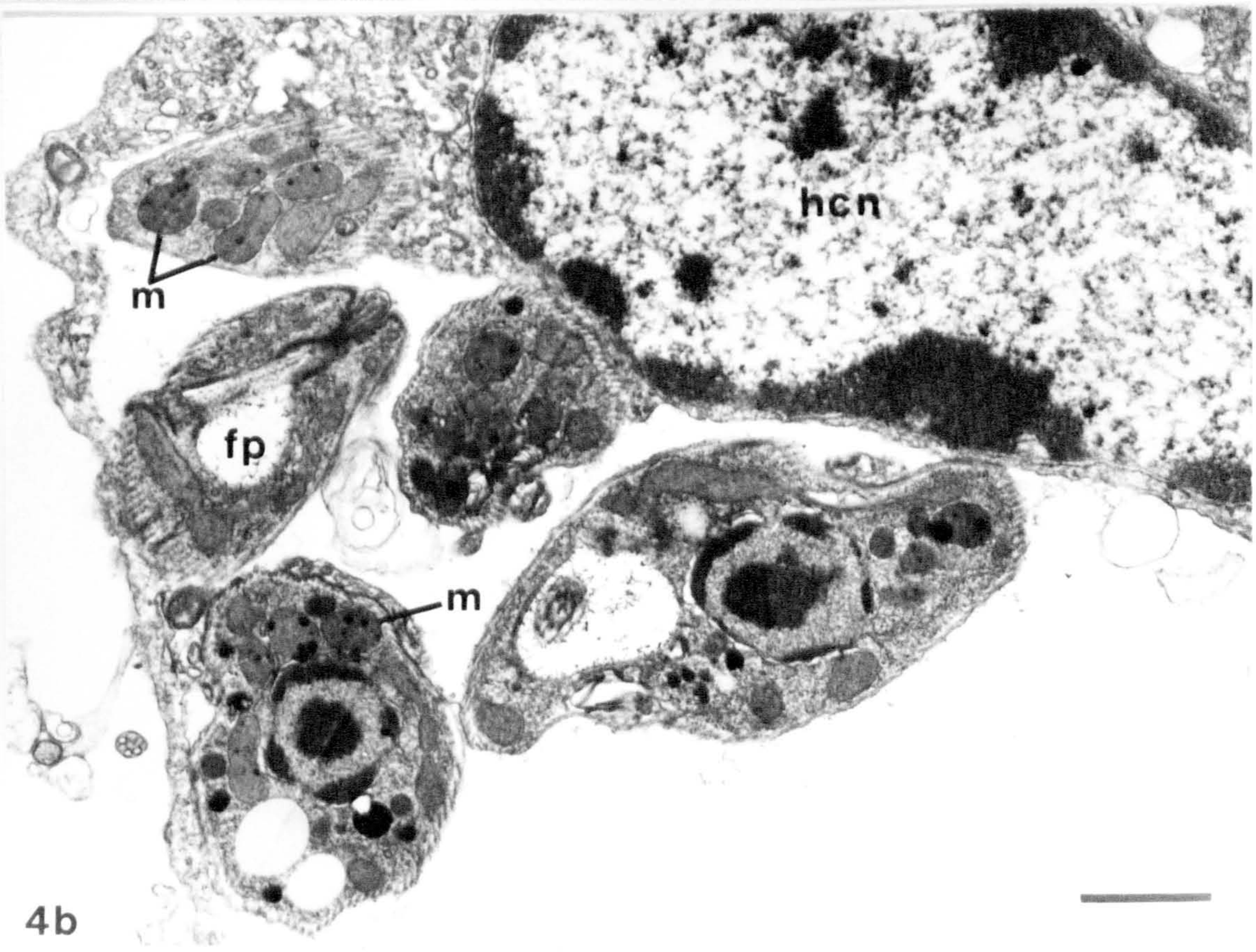
Fig. 4. Transmission electron micrographs of amastigotes of Leishmania species. Abbreviations used: mit, mitochondrion; m, megasome; kp, kinetoplast; fp, flagellar pocket; n, nucleus; hcn, host cell nucleus; l, lysosome. Scale bar represents 1.0 μm.

Fig. 4a. Leishmania mexicana mexicana amastigotes showing abundant megasomes.

Fig. 4b. Leishmania mexicana amazonensis amastigotes, in host cell parasitophorous vacuole, with many megasomes.



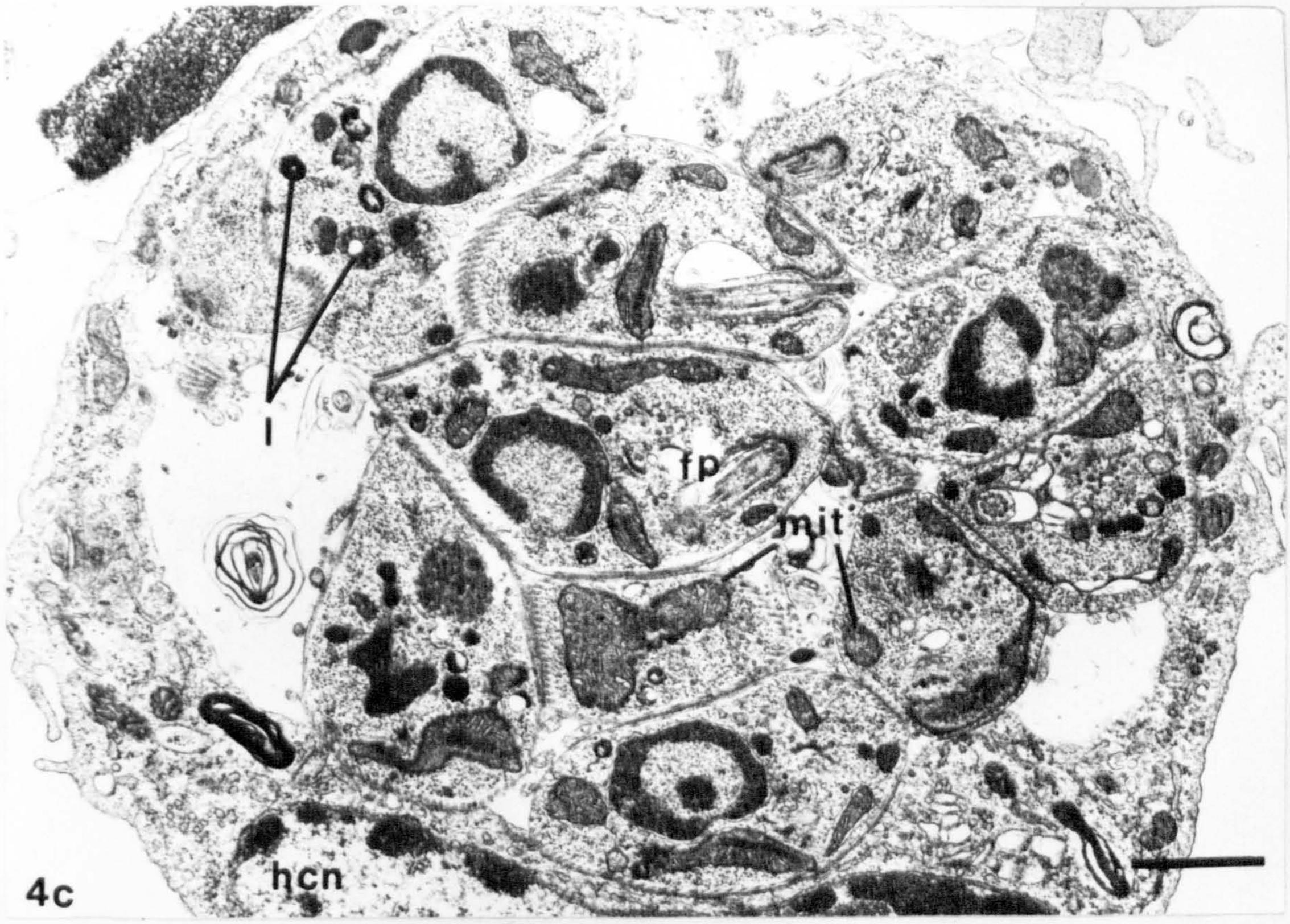
4a



4b

Fig. 4c. Leishmania major. A cluster of parasites within a host cell. Megasomes are absent although a few lysosome-like organelles (l) can be observed.

Fig. 4d. Leishmania donovani. Partially-purified amastigotes lacking demonstrable megasomes but with occasional lysosomes.



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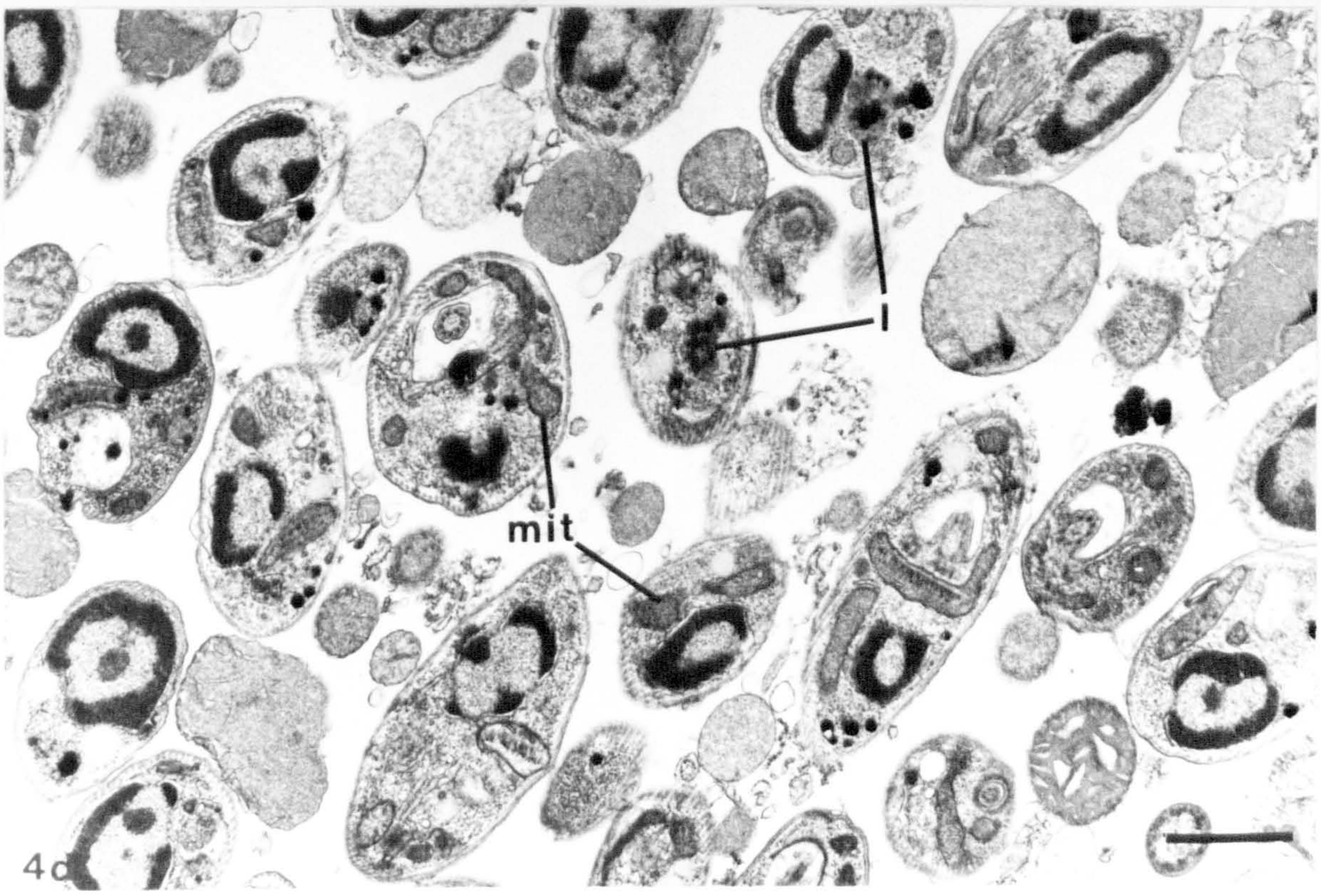

4d