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Trypanosomes

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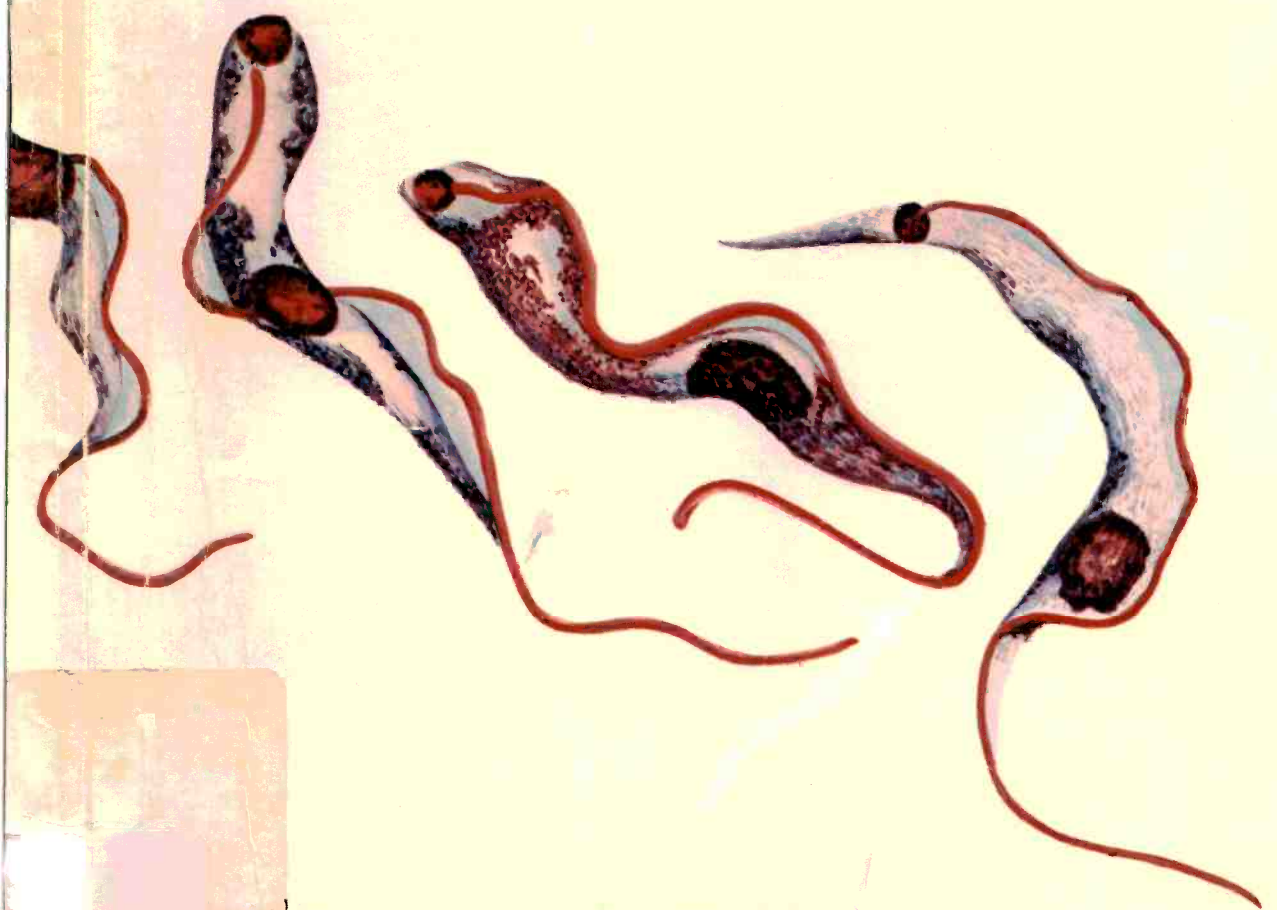
Proceedings of a workshop held at Nairobi, Kenya, 20-23 November 1978



litors: George Losos and Amy Chouinard

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Editors: **George Losos[1] and Amy Chouinard[2]**

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Biology and ultrastructure of trypanosomes in relation to pathogenesis

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Abstract. The powerful locomotory flagellum and microtubule-reinforced body of the trypanosome endow it with the ability to inflict mechanical damage on its host by penetrating through or between cells. Pinocytosis from the flagellar pocket deprives the host of plasma proteins. Destructive lysosomal enzymes and the residual bodies of autophagosomes are discharged into the flagellar pocket. Antigen–antibody complexes, which are believed to be at the root of much trypanosome pathogenesis, are produced when a host's antibody combines with either common antigens, which are released from residual bodies and from lysed trypanosomes, or variable antigens, which compose the surface coat of the trypanosome. The ability to replace one surface coat with another (antigenic variation) made of a different glycoprotein antigen enables the salivarian trypanosomes to avoid the host's immune responses.

We shall here consider the trypanosome as a destructive piece of machinery, examining the working of its parts in relation to the different ways in which it harms its host. This parasite deprives its host of essential substances while burdening it with unwanted wastes; it inflicts mechanical damage through its incessant physical activity; and it goads the host into responding to its presence and then evades the backlash. These activities will be set against the background of trypanosome biology.

Trypanosomes in Mammals

In cyclically transmitted trypanosomes (i.e., where the parasite has undergone multiplication and development in the vector), infection of the mammal is initiated by the metacyclic stage, the trypanosomes being injected with the saliva of the tsetse fly (Salivaria) or contaminating the ruptured epidermis from vector hindgut contents (Stercoraria). In the tsetse-borne trypanosomes, the metacyclic trypanosomes develop further in the chancre that forms in the host's skin at the site of the bite. *T. brucei* metacyclics transform into long thin slender forms that invade the local lymph vessels and then the bloodstream where they multiply by binary fission. From the vascular system the trypanosomes may invade the connective tissues. Waves of trypanosome multiplication are detectable in the host's blood, the peak and remission of each wave being characterized by an increasing percentage of nondividing short stumpy forms. Unlike the slender forms, the stumpy trypanosomes and those of intermediate morphology are poorly infective to other mammals by syringe transfer but if ingested by the tsetse fly will initiate the cycle in the vector. The differences in infectivity accompanying trypanosome pleomorphism correlate with changes in the trypanosome's single mitochondrion and correlated energy metabolism (Vickerman 1965, 1971). The stumpy forms partially activate the repressed mitochondrion and so become preadapted to life in the tsetse fly, where proline and other amino acids replace glucose as the parasite's principal source of energy.

Chronic infections in the mammal are made possible by antigenic variation. Each parasitemic wave represents a population composed of trypanosomes carrying a different surface antigen from those of the previous wave. Parasitemic remission is induced by the host's immune response (Vickerman et al. 1976) to a particular variable antigen type (VAT). The stumpy forms are more resistant to immune assault than their slender progenitors (Balber 1972), but the small minority of slender forms that bear a different surface antigen — the heterotypes — evade the immune system's attack altogether and survive to continue the infection. We do not know what halts trypanosome division and

induces genesis of the stumpy forms, but the transformation occurs in the absence of a detectable immune response (Balber 1972; Vickerman et al. 1976).

In *T. vivax* and *T. congolense*, pleomorphism and distinct dividing and nondividing stages may be present in the mammal host (Nantulya et al. 1978), but mitochondrial changes have not yet been detected. Both trypanosomes are believed to be hematic, i.e., confined to the vascular system and not prone to invade secondarily the connective tissues (Losos and Ikede 1972). *T. congolense* certainly has an extensive primary developmental phase in connective tissue of the chancre (Luckins and Gray 1978), with distinct morphological forms (reminiscent of the fly proventricular stage) (see Roberts, Gray, and Gray 1969). The bloodstream trypanosomes are attached to the endothelium of small vessels (Banks 1978; Büngener and Müller 1976) according to a circadian rhythm (Hawking 1978). *T. vivax* is not known to be localized in this way. Antigenic variation is a feature of infections with both species.

Pleomorphism is rare in the mechanically transmitted *T. evansi* and unknown in the venereally transmitted *T. equiperdum*, which is almost exclusively a parasite of the connective tissues in equines. Evidence from morphology and geographical distribution suggests that both are descendants of *T. brucei* (Hoare 1972); like the parent species both undergo antigenic variation.

In contrast, stercorarian trypanosomes illustrate the alteration of dividing and nondividing phases. *T. lewisi* undergoes multiple fission in the epimastigote form lodged in capillary vessels for 5 days following infection by metacyclics. Nondividing broad trypomastigotes are released into the blood but shortly afterwards are usually killed by a trypanolytic antibody. A few of the trypomastigotes resist the attack and survive to infect the vector, but they are later demolished by a second trypanolytic antibody. The peculiar immunoglobulin ablastin (see D'Alesandro p. 63) is responsible for checking division of *T. lewisi* in the blood, and, as in *T. brucei*, respiratory and mitochondrial changes accompany the gross morphological changes of the trypanosome (reviewed by Vickerman 1971).

Whereas *T. lewisi* is capable of limited antigenic variation (implicit in the failure of the first lytic antibody to erase all trypanosomes), *T. cruzi* does not appear to have the capability at all, having a pattern of development that renders variation unnecessary. In *T. cruzi*, division takes place in intracellular amastigotes in muscle, mononuclear phagocyte or nerve, sheltered from the host's immune assault. Nondividing trypomastigotes are released into the blood from the ruptured host cell. Although slender and broad forms have been observed among bloodstream forms of *T. cruzi*, evidence that the former may carry on the infection in the mammal and the latter serve to infect the vector (Brener 1976), paralleling the situation in *T. brucei*, is insufficient at present. Strangely, respiratory differences between the different stages in its life cycle are quantitative rather than qualitative (Gutteridge, Cover, and Gaborak 1978).

Demonstration of a common basis to the life cycles of the trypanosomes of mammals is still lacking. The claim that *T. brucei* has an "occult" dividing amastigote (or spheromastigote) stage in the mammalian host during which antigenic variation occurs (Ormerod and Venkatesan 1972) has not been confirmed. The brain-inhabiting forms of *T. brucei* that survive chemotherapy (Jennings et al. in press) may simply be slender trypanosomes located beyond the blood-brain barrier. The amastigote and multinucleate flagellates may actually have little role in the life cycle, as they are frequently the products of degeneration or aberrant morphogenesis. Ruthless selection of viable forms is probably a feature of all protozoan life cycles.

Functional Anatomy of a Trypanosome

The principal morphological features of a generalized salivarian bloodstream trypanosome are revealed by the electron microscope (Fig. 1 and 2). Cortical (pellicular) microtubules, longitudinally disposed, lie immediately beneath the plasma membrane of the flagellate's body and maintain its elongate shape. The single flagellum arises from a basal body (kinetosome) submerged in the floor of the flask-shaped flagellar pocket. After leaving the neck of the flask, the flagellum is attached to the body between two cortical microtubules to become free at the flagellate's anterior extremity; there is no projecting free flagellum in *T. congolense* nor in the advanced stumpy form of *T. brucei*. The single mitochondrion is associated with the locomotor apparatus through a capsular expansion of the former which houses the kinetoplast (Fig. 2).

The trypanosome's ability to move and its ability to alter its shape go hand in hand. Beating of the flagellum, and hence propulsion of the trypanosome, may proceed in either direction but usually the waves pass from tip to base of the flagellum; hence the flagellar tip marks the organism's anterior end. A series of junctional complexes of the desmosome type (see Brooks 1978) attach the flagellum to the body and the body is deformed by the beating flagellum to give the characteristic undulating membrane appearance. Powerful swimming movements combined with the lon-

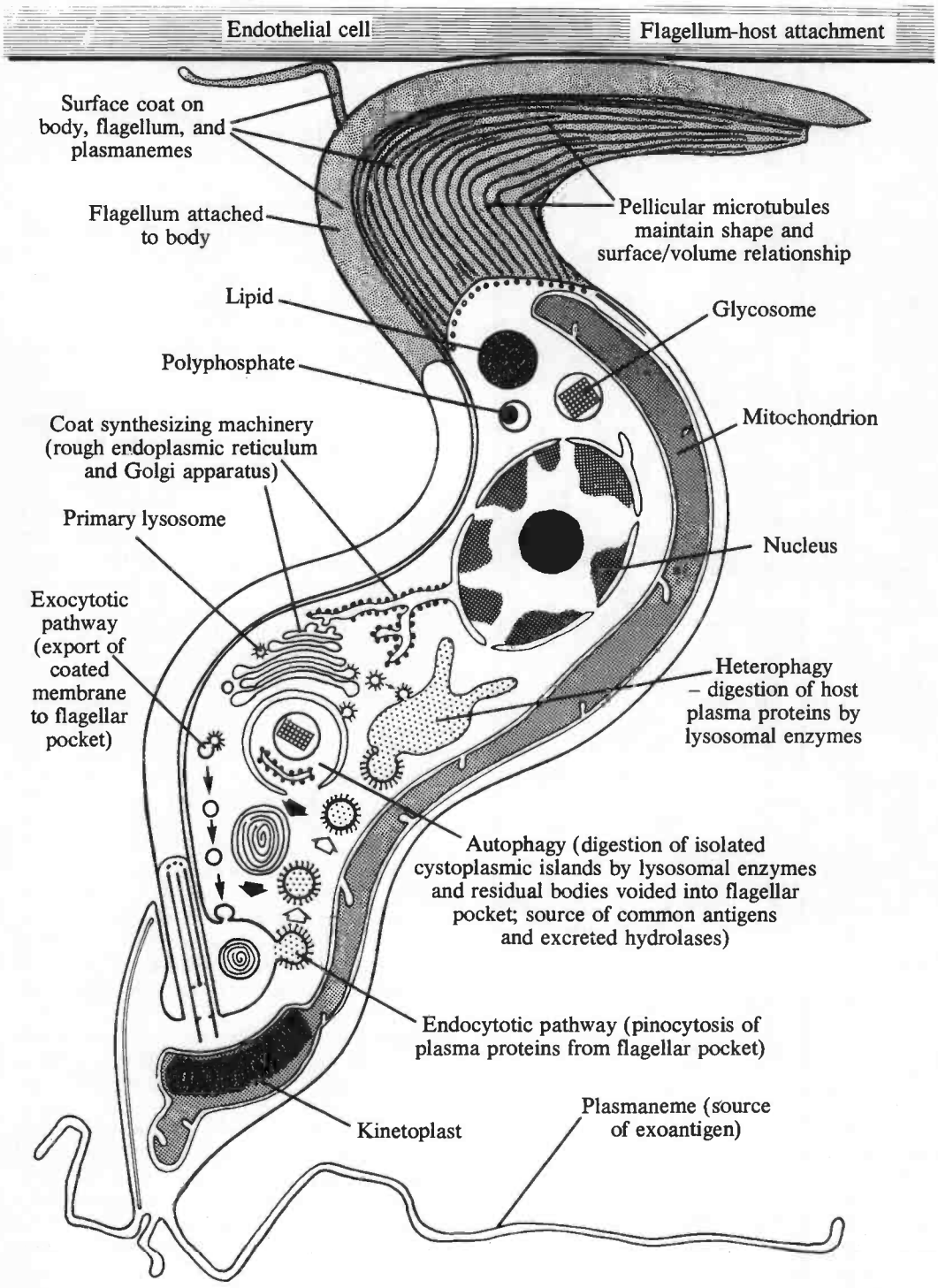

Fig. 1. *Principal structures of a salivarian trypanosome and their functions in relation to pathogenesis.*

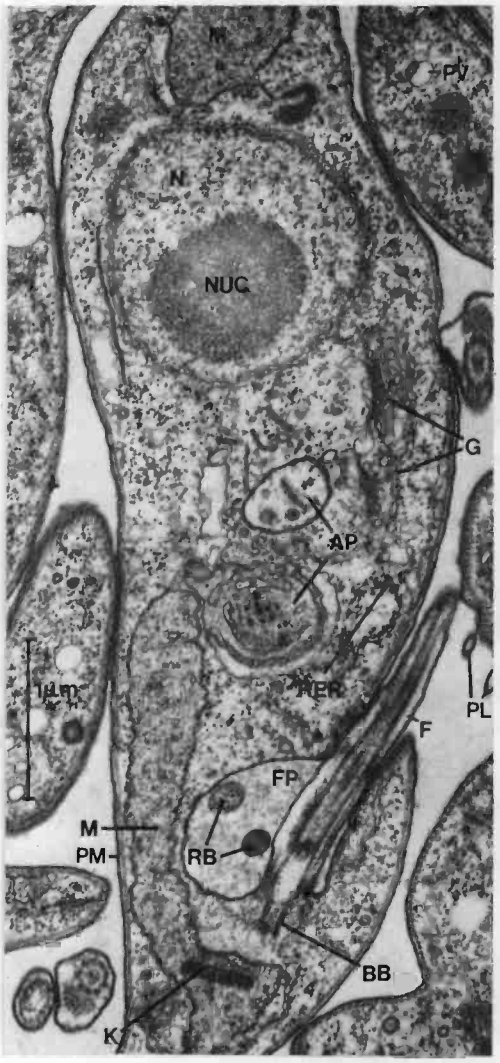

Fig. 2. Electron micrograph of longitudinal section of posterior half of stumpy bloodstream form of T. brucei: *the flagellum (F) arising from the flagellar pocket (FP), its basal body (BB) apposed to the kinetoplast (K)-bearing region of the single mitochondrion (M), the nucleus (N) with its large nucleolus (NUC), rough endoplasmic reticulum (RER), the Golgi apparatus (G), and partially formed autophagosomes (AP). The body is supported by pellicular microtubules (PM), the flagellar pocket contains exocytosed residual bodies (RB). Adjacent trypanosome profiles show a polyphosphate vacuole (PV) and plasmanemes (PL). Fixation in glutaraldehyde, followed by osmium tetroxide, staining with uranyl and lead.*

gitudinal reinforcement of the slim body by pellicular microtubules and lateral deformability enable the trypanosome to penetrate between cells and into cells. Details of how penetration is effected, however, are surprisingly sparse. Whether in their migratory activities in the mammalian body trypanosomes can split apart junctional complexes binding endothelial cells, actively penetrate the endothelial cell membrane, or induce the cell to phagocytose and then exocytose them, is not known. *T. brucei* procyclics appear to invade *Glossina* midgut epithelial cells (Evans and Ellis 1978) by active penetration. *T. cruzi* blood trypomastigotes induce even "nonprofessional" phagocytes to engulf them (Nogueira and Cohn 1976), and the ability of *T. cruzi* actually to penetrate cell membranes (e.g., that of the parasitophorous vacuole) is well established (Kipnis, Calich, and DaSilva 1979).

One unique feature of the trypanosomatid flagellum is its ability to form attachments to host surfaces. The ultrastructural details of the attachments to the chitin-lined regions of the vector's gut are now well known (reviewed by Molyneux 1977). Not so well characterized is the attachment of *T. congolense* to the walls of small (10–30 µm diameter) blood vessels (Banks 1978). Electron micrographs of the attachment (Büngener and Müller 1976) have not as yet revealed the characteristic hemidesmosome-like arrangement of filaments (Vickerman 1973) converging on the flagellate's membrane in the attachment region, but interdigitations of the membranes of parasite and host have been reported. Attachment of trypanosomes to phagocytic cells also seems to be mediated initially by the flagellum (Stevens and Moulton 1978; Takayanagi, Nakatake, and Enriquez 1974). Macrophage cytophilic antibody induces attachment in vitro, but opsonizing antibody is necessary for engulfment of the parasite (Vasquez, Cattan, and Herbert 1975).

When attached to host surfaces, the trypanosome flagellum continues to beat, and its movement may prevent stagnation of the medium surrounding the parasite in the vector. Its role in the free-swimming trypanosome is less clear. One likely possibility is that it circulates the contents of the flagellar pocket (Vickerman and Preston 1976). The pocket is an important site of entry and exit of materials in bulk (Fig. 1), as the pellicular microtubules preclude these activities elsewhere on the flagellate's surface.

Pinocytosis of proteins by trypanosomes has been observed by investigators using ferritin and other electron-dense tracers that can be located by electron microscopy. Details of the relationships between pinocytosis vesicles and lysosomes containing digestive enzymes (recognized by their positive reaction in Gomori staining for acid phosphatase) have been elucidated by Langreth and Balber (1975). Ferritin is taken up in spiny vesicles (Fig. 1) pinched off from the flagellar pocket

lining. These vesicles fuse with an elaborate network of smooth-membraned cisternae that appear to store the protein. Primary lysosomes from the Golgi complex fuse with parts of this network presumably digesting its contents. In some stages of the life cycles of certain trypanosomes, e.g., the intracellular amastigote and culture epimastigote forms of *T. cruzi*, but never Salivaria, a specialized cytostome and cytopharynx are present to mediate such "heterophagy." The protein sequestering system of *T. brucei* is so well developed that the uptake of host serum protein might be envisaged as taking place on a massive scale in relation to the size of the organism. Serum albumin has long been recognized as a prolonger of the active life of bloodstream trypanosomes in vitro and might be expected to be consumed avidly in vivo. Quantitative estimates of its uptake, however, have proved surprisingly low — 700 ng/h/mg trypanosome protein for monomorphic (slender) bloodstream *T. rhodesiense* though this figure was double for a pleomorphic line (A.H. Fairlamb personal communication), implying greater activity on the part of the stumpy trypanosomes.

Autophagy — the digestion of isolated islands of cytoplasm containing unwanted organelles — also appears to be more active in the stumpy forms (Langreth and Balber 1975). The cytoplasm to be destroyed is first walled off by membrane (probably supplied by the adjacent Golgi complex) to form an autophagic vacuole (Fig. 2) with which primary lysosomes fuse. Amino acids released from autophagic vacuoles may be utilized as an energy source in the stumpy form, which is activating its proline oxidase system.

The undigested remains of heterophagic and autophagic vacuoles are cast out into the flagellar pocket along with their accompanying lysosomal enzymes (Fig. 1, 2, and 3). This exocytosis of residual bodies may be of considerable significance in relation to pathogenesis. Trypanosome lysosomes contain acid proteases, leucine aminopeptidase, phosphatases, phosphodiesterases, deoxyribonucleases, α-mannosidase, β-hexosaminidase, and lipase (Venkatesan, Bird, and Ormerod 1977; Steiger 1975) as well as phospholipases (Tizard et al. 1977). Their destructive effects have not been fully investigated, but Tizard et al.p.103 have explored phospholipase and its role in the generation of lytic fatty acids.

The residual bodies exocytosed by *T. brucei* may be quite large in relation to the size of the trypanosome (Fig. 3). They may embody a mechanism for release of common (i.e., nonvari-

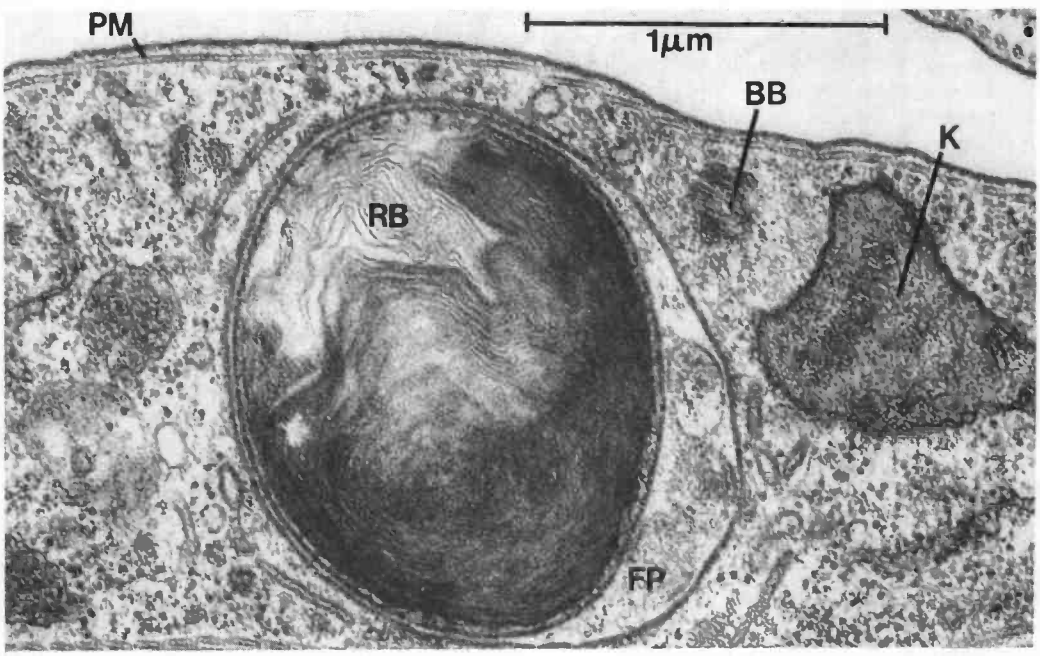

Fig. 3. *Electron micrograph of longitudinal section of the distended flagellar pocket (FP) of* T. brucei *(bloodstream stumpy form) containing large residual body (RB), which has the structure of a liposome, i.e., several concentric membrane layers. Surface coat on the membrane lining the pocket and covering the residual body as well as the trypanosome surface increases the apparent thickness of the membrane at this low resolution. Other labeled structures are the basal body (BB), pellicular microtubules (PM), and the kinetoplast (K).*

able) antigens before the wholesale liberation of these agents, which occurs when trypanosomes are destroyed by lytic antibody at a crisis. Discharge of these bodies as thick streamers from the flagellar canal has been described by Ellis, Ormerod, and Lumsden (1976).

The functional significance of many trypanosome cytoplasmic inclusions still eludes us. Trypanosomes have no means of storing energy as carbohydrate. These flagellates sequester triglyceride (Dixon, Ginger, and Williamson 1971), but it seems quite probable that, in the absence of an exogenous supply of respirable substrate, protein (of either internal or external origin) provides the only source of energy. Our recent X-ray microanalysis studies (Vickerman and Tetley 1977) of dense inclusions that lie to one side of a thick-membraned vacuole (Fig. 2 and 3) in trypanosome cytoplasm suggest that these are polyphosphate, but their role in trypanosome physiology is uncertain. Although they may function as phosphagens, i.e., to store phosphate bond energy when ATP production exceeds demand, they probably serve as phosphate reserves. The calcium and zinc associated with these granules suggest that they play a part in regulation of divalent cation concentrations in the cytoplasm — a function that can be vitally important in unicellular organisms. The mitochondrial cycle of the trypanosomes may be related to sequestration and release of divalent cations, as the ability of mitochondria to perform this function is well known. The pathological significance of polyphosphates discharged from the trypanosome on lysis deserves investigation, as these polyanions are utilized commercially as detergents.

One cytoplasmic organelle whose function has recently come to light is the glycosome (previously referred to as a microbody or peroxisome-like organelle by Vickerman and Preston 1976). Opperdoes and Borst (1978) have shown that in bloodstream *T. brucei*, it houses enzymes for the breakdown of glucose to glycerol-3-phosphate (G-3-P) and 3-phospho-glycerate (3-PG), which pass out into the cytoplasm. G-3-P is oxidized by molecular oxygen in the outer membrane of the mitochondrion and the dihydroxyacetone phosphate produced is shipped back to the glycosome for recycling. The 3-PG is converted to pyruvate in the cytoplasmic matrix, with linked phosphorylation of ADP, and the pyruvate is excreted by the trypanosome. The contribution of excreted pyruvate to pathogenesis is discussed in Newton p.17.

One of the most important trypanosome components in any discussion of pathogenesis is the surface membrane — the interface between parasite and host. In bloodstream African trypanosomes, the plasma membrane proper is overlaid by a surface coat of glycoprotein, 12–15 nm thick as seen in electron micrographs of sections (Vickerman 1969). Compact and dense in *T. brucei* and *T. congolense*, this coat is somewhat diffuse in *T. vivax*. An even more diffuse coat is present in the stercorarian trypanosomes such as *T. lewisi* and *T. cruzi* bloodstream forms. The coat appears to be an adaptation to life in the mammalian host, for it is discarded in the vector until it is reacquired at the metacyclic stage of development. In the Salivaria, it contains the variable antigen (VA) of the trypanosomes (Vickerman 1969b; Vickerman and Luckins 1969; Cross 1975; Fruit et al. 1977), and antigenic variation occurs when one coat is replaced with another of different antigenic type (reviewed by Cross 1978a; Vickerman 1978). The way in which replacement is instigated eludes us, but we believe that the change is phenotypic (i.e., genetic mutation is not required), that it occurs in only a small number of individuals (heterotypes) in a given population (approximately 1 in 10 000), and that antibody is not necessary for induction of the switch to expressing a new VA glycoprotein.

Cytochemical, especially lectin-binding, techniques indicate that in *T. brucei* the carbohydrate moieties attached to the single polypeptide chain of the variable antigen glycoprotein are located close to the membrane proper (Wright and Hales 1970; Steiger 1975; Cross and Johnson 1976; Seed, Seed, and Brindley 1976; Renwrantz and Schottelius 1977) and may even play a part in attachment of the molecule to the membrane. Whether in all variable antigen types of the subgenus Trypanozoon it is unexposed and plays no part in host–parasite interactions remains controversial (Baltz, Baltz, and Pautrizel 1977). In *T. congolense* (Rovis, Barbet, and Williams 1978; Jackson, Honigberg, and Holt 1978), *T. vivax* (Tetley unpublished observations), and the stercorarian trypanosomes (Dwyer 1976; Dwyer and D'Alesandro 1976), carbohydrate groups appear to be exposed on the surface coat rather than concealed.

The ease with which variable antigen is dissociated from the surface of the trypanosome in vitro suggests that it is a peripheral protein, i.e., has weak attachment to the membrane. In addition, the VA molecules appear to be mobile over the surface of the trypanosome in that they can be capped by homologous antibody in the indirect immunofluorescence reaction (Barry in press) and are lost uniformly from the surface as shown by electron microscopy and immunofluorescence studies on the trypanosomes transforming to culture forms in vitro (Barry and Vickerman in press). The

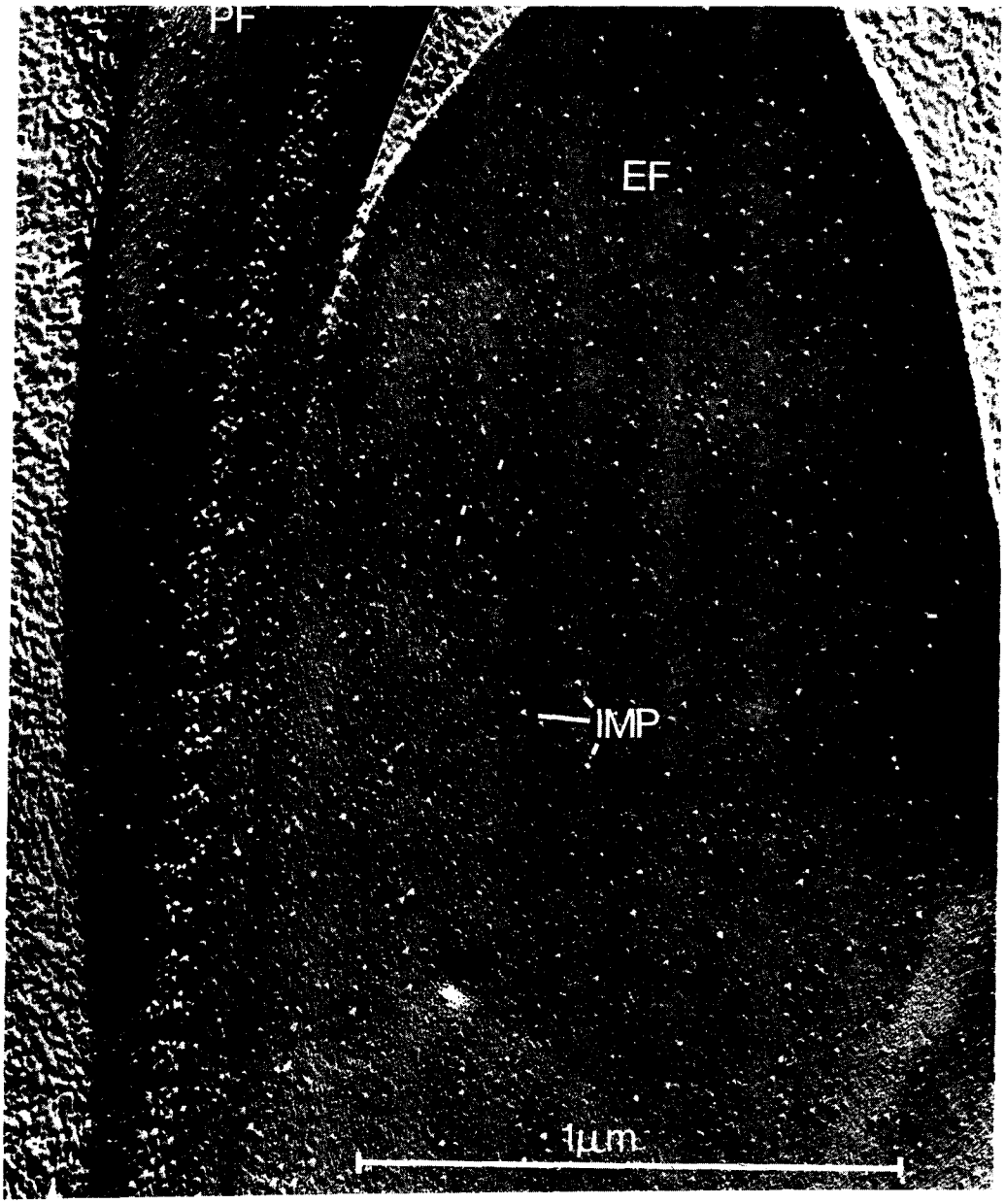

Fig. 4. *Electron micrograph of replica of freeze-fractured* T. brucei *(cultured procyclic form). The fracture plane has passed along the centre of the plasma membrane of the body (right) and the attached flagellum (left), revealing intramembranous particles (IMPs, integral proteins or lipoprotein complexes) that are embedded in the lipid bilayer. The extracellular face (EF) of the body membrane is shown and the protoplasmic face (PF) of the flagellar membrane. The clustering IMPs (a band running the length of the PF) represent the desmosome-like attachments of the flagellum to the body. Arrow indicates direction of shadowing of the replica.*

strong antigenic character of the VA may be due to its release from the trypanosome either on its entire surface, perhaps after combination with antibody, or on those enigmatic structures the plasmaneines or "filopodium-like appendages" (Wright, Lumsden, and Hales 1970; Vickerman and Luckins 1969). These fine extensions of the body and flagellum (Fig. 1) are readily observed on trypano-

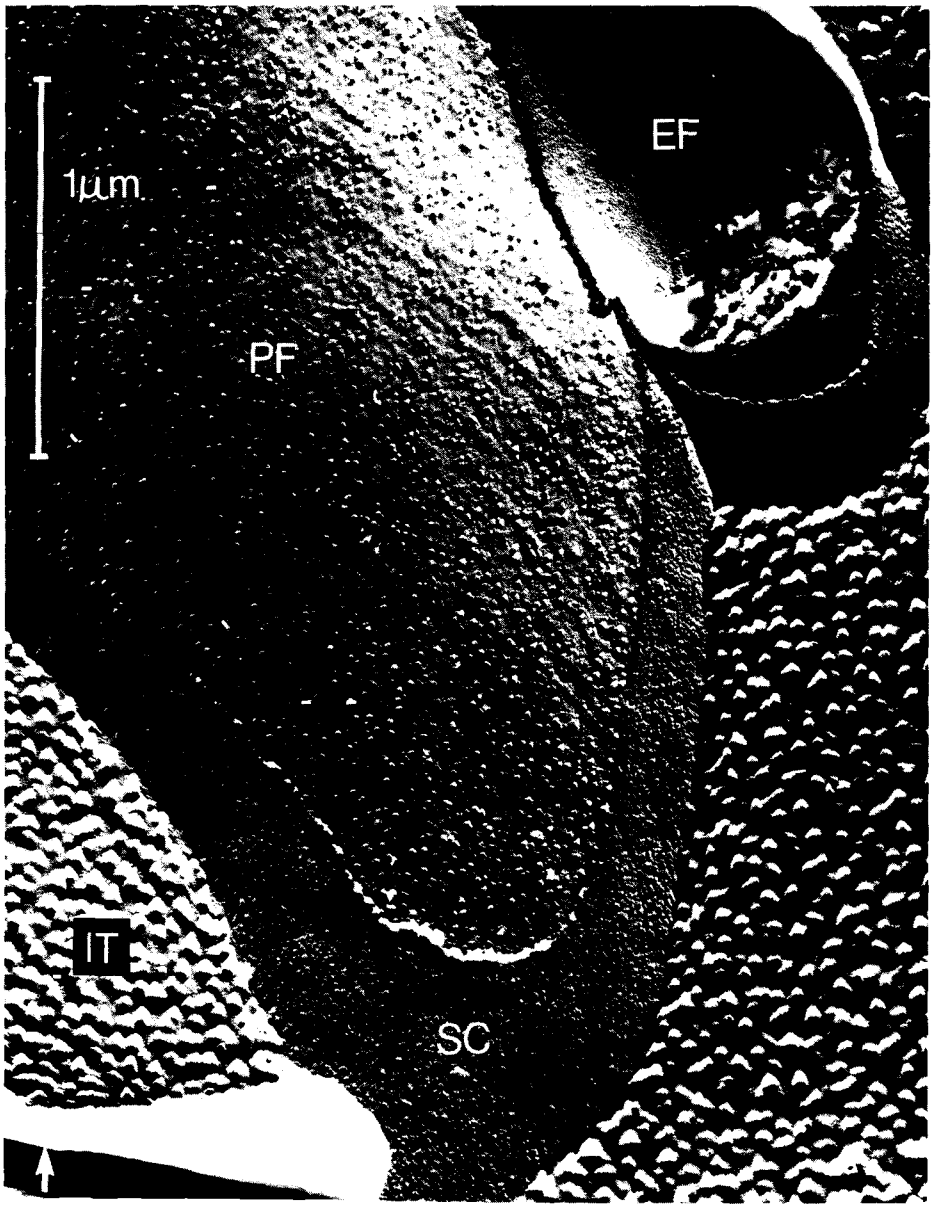

Fig. 5. *Electron micrograph of a replica of a freeze-fractured* T. vivax *(bloodstream form), which has been subjected to etching before shadowing. The protoplasmic face (PF) of the trypanosome body and the extracellular face (EF) of the flagellum plasma membrane are visible with their scattered intramembranous particles. In the etching process some of the covering ice is sublimed away to disclose the true surface of the membrane adjacent to the internal faces exposed by fracturing. The rough surface coat (SC) is visible because of the retreat of the ice table (IT).*

somes in vitro by light and electron microscopy, but definite proof of their formation in circulating blood is still lacking. Plasmanemes themselves are highly immunogenic (Herbert and MacAdam 1971), and their attachment to endothelial or red blood cells would provide a major source of antigen–antibody complexes to account for vascular damage.

Organized surface glycoproteins on flagellates are usually secreted by an active rough endoplasmic

reticulum-Golgi complex system, with externalization of new surface membrane bearing the secretion close to the flagellar bases. The existence of such a well developed secretory apparatus between the nucleus and flagellar pocket (Fig. 1 and 2) strongly suggests that a similar pathway exists in trypanosomes (Vickerman 1969). Owing to presence of the pellicular corselet of microtubules, it is difficult to see where new coated membrane could be inserted into the surface other than in the flagellar pocket. The possibility that secretion is controlled by lysosomal enzymes (as in the coccolithophorid flagellates)(Pienaar 1971) also exists. Secretion of a mitogen by salivarian trypanosomes has been suggested (Esuruoso 1976; Mansfield, Craig, and Stelzer 1976) and that variant antigen itself has a mitogenic effect has been postulated.

There is now convincing evidence that the soluble "exo-antigen" believed by Weitz (1960) to be a secretion is surface coat material liberated from the trypanosomes into the surrounding medium (Allsopp, Njogu, and Humphreys 1971). Whether the material is also liberated in vivo from viable trypanosomes is a matter of considerable interest to pathologists who are investigating complement activation by antigen–antibody complexes and its role in inflammatory disease (see Boreham p.114). Musoke and Barbet (1977) found that purified (soluble) variable antigen of *T. brucei* activated complement via the classical pathway, but a particulate fraction from the same clone of trypanosomes activated the alternate pathway of complement fixation. This fraction probably included the surface membrane proper of the trypanosome. Uncoated trypanosomes, as represented by culture forms, are susceptible to lysis by normal serum. In *T. cruzi* lysis appears to be due to the activation of complement via the alternate pathway (Nogueira, Bianco, and Cohn 1975). It seems likely that the trypanosome membrane proper has complement-activating components that in the intact flagellate are inaccessible owing to the barrier presented by the surface coat (see Nielsen, Tizard, and Sheppard p.94).

In the short term, the glycoprotein coat protects vulnerable membrane proteins (such as substrate transport sites) from complement components and in the long term the coat provides a replaceable surface enabling the trypanosome to evade the host's immune response. But that the variable antigens are the only proteins exposed on the surface of the bloodstream trypanosomes is unlikely. De Souza and colleagues (1978) have demonstrated differentiated regions on the surface of *T. cruzi* using cytochemical staining at the electron microscope level and the freeze-fracture replica technique. Differentiated regions of the surface of salivarian trypanosomes may prove to be discernible using similar methods.

In the freeze-fracture technique, frozen cells are cleaved; their membranes tend to split along the middle of the lipid bilayer, the fracture plane passing over and under integral proteins. The proteins then stand out as "intramembranous particles" (IMPs) when metal-shadowed replicas are made of the exposed fracture faces (Fig. 4 and 5). The true surface of the membrane — for example the surface coat of bloodstream trypanosomes — can be revealed by freeze-etching, i.e, subliming away surface ice adjacent to the fractured region of membrane (Fig. 5). No regular packing of coat glycoprotein units has been observed. The density of intramembranous particles visible (summing counts made from both faces of the split membrane) is $\sim 3 \times 10^3/\mu m^2$ for bloodstream *T. brucei* (Vickerman and Tetley 1977), whereas the density of coat glycoprotein molecules was calculated by Cross (1975) to be $\sim 10^5/\mu m^2$ so that coat molecules outnumber IMPs 20 to 1.

The proteins (or lipoprotein complexes) represented by IMPs are probably heterogeneous in function as well as size. Most noticeable of the IMPs are those that lie in clusters along the flagellum-body junction (Fig. 4) the clusters presumably corresponding with the desmosomal attachments of the flagellum (for further details see Smith et al. 1974; Hogan and Patton 1976; Vickerman and Tetley 1977) and the particles with the intramembranous portions of molecules that bridge the gap, i.e., protrude beyond the surface coat. There may be similar membrane differentiations, for example, where *T. congolense* attaches to host endothelium or where non VAT-specific cytophilic antibody binds the trypanosome to a macrophage. We are confident that in the not too distant future the combination of freeze-etching with techniques for the labeling of specific membrane components will result in significant information on the organization of the trypanosome surface and, indirectly, lead to a deeper understanding of the intricate relationship between parasite and host.

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