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ON THE SURFACE COAT AND FLAGELLAR ADHESION IN TRYPANOSOMES

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SUMMARY

Pathogenic trypanosomes in their bloodstream phase have a smooth and compact coat 12–15 nm thick enveloping the entire surface membrane of the body and flagellum. In the sleeping-sickness trypanosome *Trypanosoma rhodesiense* this coat is absent from the stages of development in the midgut of the tsetse-fly vector and from their counterparts obtained by cultivation of the trypanosome *in vitro*. In the salivary glands of the vector, however, the coat is reacquired as the trypanosomes transform from epimastigote forms into the metacyclic stage which is infective to the mammalian host. This loss and acquisition of the surface coat can be correlated with the cyclical changes in net surface charge on the trypanosome which have been observed by other workers.

The trypanosome populations of successive relapses in the blood are known to differ in their surface antigens (agglutinogens) and the loss of antigenic identity detected when any of these populations are put into culture indicates that these variable antigens are located in the surface coat. It is suggested that the coat in bloodstream trypanosomes constitutes a replaceable surface which, after being replaced, enables the trypanosome to escape the effects of host antibodies. The coat is therefore an adaptation to life in the bloodstream. Reacquisition of the surface coat by the metacyclic trypanosome after development in the vector may reflect reversion to a 'basic' antigenic type at this stage, preparatory to invading the blood of the mammalian host.

The surface coat may be removed by the wide-spectrum proteolytic enzyme pronase, and this fact together with evidence from pH/mobility relationships and chemical analysis of the variable antigens suggest that the coat is basically proteinaceous.

The coat may facilitate pinocytosis by binding proteins at sites within the pocket surrounding the base of the flagellum.

In the non-pathogenic trypanosome *T. lewisi* a more diffuse filamentous coat is present in bloodstream forms and absent from culture forms. This trypanosome is said to carry a negative charge in both bloodstream and culture phases, so it seems likely that the nature of the coat in *T. lewisi* is different from that found in the pathogenic trypanosomes.

In all these trypanosomes the flagellar membrane adheres to the surface membrane of the body throughout the life-cycle. Along the zone of adhesion lies a regular row of junctional complexes of the macula adherens type which, it is argued, serve in attachment. These attachments persist regardless of changes in the intervening cell surfaces.

INTRODUCTION

Evidence for the existence of a layer of material outside the limiting membrane of the animal cell has increased strikingly as a result of electron-microscope studies on sectioned specimens (Bennett, 1963; Fawcett, 1965; Revel & Ito, 1967). In some cases—for example, the microvillar surface of gut cells (Ito, 1965) or the plasmalemma

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of large freshwater amoebae (Pappas, 1959)—this surface coat has a recognizable filamentous form and is readily seen in electron micrographs after conventional processing without the use of specific staining techniques. For the majority of cells, however, the straightforward ultrastructural evidence that they possess a cell coat is unconvincing, and the coat can be demonstrated satisfactorily only by recourse to cytochemical staining. Thus after the thick fringe-like coat of amoebae had been shown by several workers to have a strong polysaccharide component (Bairati & Lehmann, 1953; O'Neill, 1964), a carbohydrate-rich cell coat was detected surrounding vertebrate cells by both light and electron microscopy (Gasic & Berwick, 1962; Rambourg, Neutra & Leblond, 1966; Rambourg & Leblond, 1967).

The role of the surface coat in assisting adhesion of the cell either to its substratum or to other cells has roused considerable interest as also has the part it is believed to play in the binding of particles, from ions to viruses, for subsequent pinocytosis. A third point of interest is the role of the surface coat in determining the antigenic identity of the cell, for the reaction of antibodies with the living cell is in the first place at its surface. But the possibility that surface coats may represent artifacts of fixation or other preparative procedure remains a reservation in the minds of some students of the cell surface, especially where the coat is difficult to visualize in electron micrographs and conceivably could be the result of adsorption of foreign material or the leaching out of cytoplasmic colloids (Curtis, 1967).

This paper presents electron-microscope evidence for the presence in bloodstream trypanosomes of a surface coat which is prominent in high-resolution micrographs and is unlikely to be an artifact because it is present at some stages in the life-cycle but not at others. This coat is of interest on all three counts mentioned above; first because these trypanosomes have the well-known ability to change their surface antigenic identity in order to avoid the host's immune response, secondly because there is evidence that localized pinocytosis occurs in trypanosomes, and lastly because trypanosomes exhibit adhesion of the flagellum to the body surface producing the characteristic 'undulating membrane'.

MATERIALS AND METHODS

The results described in this paper were obtained from the species and strains of mammalian trypanosomes listed below.

Bloodstream forms

Trypanosoma (*Trypanozoon*) *brucei* (3 strains); *T.* (*T.*) *rhodesiense* (10 strains); *T.* (*T.*) *evansi* (2 strains); *T.* (*T.*) *equinum*; *T. equiperdum*. (All these trypanosomes are similar in morphology and often indistinguishable from one another on morphological grounds: some of the strains of *T. brucei* and *T. rhodesiense* were pleomorphic (Vickerman, 1965) and one strain (*SAK*) of *T. evansi*, and *T. equinum*, were dyskinetoplastic. For the purpose of this paper these trypanosomes will be referred to collectively as *T. brucei*-like trypanosomes.)

Trypanosoma (*Nannomonas*) *congolense* (2 strains); *Trypanosoma* (*Duttonella*) *vivax* (2 strains); *Trypanosoma* (*Herpetosoma*) *lewisi*. All were maintained by serial syringe passage through laboratory rats or mice, except one strain of *T. vivax* which was not rodent-adapted and was maintained in sheep.

Culture forms

T. rhodesiense (3 strains) and *T. congolense* were grown on the biphasic medium of Tobie, von Brand & Mehlman (1950), subculturing at weekly intervals; *T. lewisi* was maintained on Johnson's (1947) biphasic medium, also with weekly transfers.

Vector forms

Stages in the development of 2 strains of *T. rhodesiense* in the tsetse fly were obtained from *Glossina palpalis*, *G. pallidipes* and *G. morsitans* fed on infected animals over 3 weeks previously, and subsequently shown by examination of saliva extruded during probing to be supporting salivary gland infections.

Techniques

Bloodstream forms were obtained by differential centrifugation of heparinated blood taken from infected animals at different stages in the infection. Culture forms were harvested by centrifugation of the liquid phase from 7-day cultures when the flagellates were high in the logarithmic phase of growth. Infected tsetse flies were dissected under 0·6 % saline and the midgut, cardia (proventriculus) and salivary glands removed.

Usually trypanosomes were washed in 2 × 15 min changes of Locke's solution before fixing, but some were fixed directly from their suspending plasma. Fixation was in 1 % osmium tetroxide (3 min), 1·5 % glutaraldehyde (5–10 min) or 1·5 % glutaraldehyde + 1·5 % acrolein (5–10 min), all fixatives being made up in 0·1 M phosphate buffer at pH 7·2 with 0·02 g/l. $CaCl_2$ added. For aldehyde fixation 1 % sucrose was included in the fixing fluid and after fixation the flagellates, compacted by centrifugation, were rinsed 4 times for at least 1 h in buffer with sucrose before post-fixation in the osmium tetroxide solution for 1 h. Some bloodstream trypanosomes were fixed in glutaraldehyde with ruthenium red (Kelly & Luft, 1966) added to both fixative and rinse at a concentration of 500 parts/million (w/v). Infected organs from tsetse flies were fixed in the same mixtures as the free flagellates but for longer periods (30–120 min).

Pronase digestion experiments involved incubating glutaraldehyde-fixed trypanosomes in the rinsing solution (pH 7·2) with 0·5 % (w/v) Pronase (Calbiochem Ltd., Grade B) at 37 °C for periods ranging from 10 min to 16 h: controls were incubated in the buffered sucrose alone.

Osmication was followed by rinsing 3 times in distilled water before transfer to 0·5 % aqueous uranyl acetate solution for 3–12 h. Dehydration took place via graded ethanol solutions and material was embedded directly in Araldite resin mixture. All preparative procedures from centrifugation to embedding were carried out in a cold room at 4–8 °C.

Sections were cut with a diamond knife on the LKB Ultrotome, mounted on carbon films and stained with uranyl acetate and lead citrate solutions in the usual way. Sections were examined in the EM 6B electron microscope operated at accelerating voltages of 60 and 80 kV and calibrated with a Bausch & Lomb diffraction grating replica ruled at 2160 lines/mm. Micrographs were recorded on Ilford N 60 plates.

OBSERVATIONS

Bloodstream stages of pathogenic trypanosomes

All bloodstream trypanosomes examined are bounded by a unit membrane with characteristic 3-layered structure (Fig. 2), 8–10 nm thick. This plasma membrane covers the surface of the whole body, including the pocket from which the flagellum arises (Fig. 4) and the flagellum itself (Figs. 2–4). Beneath the surface membrane of the body, but not of the flagellum or flagellar pocket, lies a row of longitudinal pellicular microtubules each of diameter 20 nm with a wall 5–6 nm thick. In transverse sections lateral connexions (Fig. 3) are sometimes seen passing from one microtubule

to the next, the spacing between the tubules being 20–30 nm. In sections taken from near the extremities of the body a second row of microtubules may be found (Fig. 2).

In *T. rhodesiense* and the other *T. brucei*-like trypanosomes, as well as in *T. congolense* and *T. vivax*, there is a continuous layer of structureless material of moderate electron density and uniform thickness, 12–15 nm, external to the entire plasma membrane (Figs. 2–5, 18–24). This surface coat is present in trypanosomes fixed by either osmium alone or aldehydes and osmium and is smoothest in the *T. brucei*-like trypanosomes. In pleomorphic members of this group it is found in all forms—stumpy, slender and intermediate. In a few trypanosomes the coat is observed to have separated from the surface membrane (Fig. 17) as though being shed: in dead trypanosomes the coat is missing, presumably lost. The coat can be removed by incubating glutaraldehyde-fixed trypanosomes in a 0·5 % solution of the wide-spectrum protease pronase for 4 h at 37 °C (Fig. 11) but not in a control solution lacking the enzyme (Fig. 12). Trypanosomes subjected to ruthenium red in the fixative showed no increase in electron density of the surface coat when compared with controls.

Where the coat lines the wall of the flagellar pocket, caveolae also bearing the surface coat (Figs. 13, 14) are found, and these are furnished with minute spines on the cytoplasmic side of the surface membrane. Spiny coated vesicles have been observed in the neighbouring cytoplasm (Fig. 15).

Between the apposed surface membranes of flagellum and body, the surface coat forms a double layer (Fig. 3). A gap of minimum width 8 nm separates the two layers of coat in this region (Figs. 2, 3, 18–24) so that the two unit membranes are parted by a gap of 35–40 nm. In many sections of trypanosomes, however, the body and flagellum appear to have shrunk away from one another over much of their former adhesion zone (see Figs. 2, 3, 5, 18) and contact is retained only in a region of attachment by special junctional complexes. In order to describe the structure and distribution of these in further detail, some account of the structure of the trypanosome flagellum must be given.

The flagellum in trypanosomes arises from a basal body which has the characteristic 9 tubule-triplets substructure and whose open end is apposed to that capsular region of the single mitochondrion which contains the fibrous mitochondrial DNA or kinetoplast (Fig. 4). The first part of the extracellular flagellum lies in a flask-shaped pocket and the flagellum emerges from this through a short canal (Fig. 13) to lie attached to the pellicle along the length of the body. At the anterior extremity of the organism the flagellum continues as a free extension in most trypanosomes, though not in *T. congolense*. As seen in transverse section the structure of the flagellum depends upon the level at which the section is taken. The proximal extracellular flagellum (Fig. 4) has peripheral tubule doublets replacing the triplets of the basal body around an electron-transparent core. The main shaft has the typical axoneme (axial filament complex) arrangement of tubules characteristic of the majority of cilia and flagella, i.e. two central tubules linked radially via secondary fibres to 9 peripheral doublets which have clockwise-pointing arms as seen from the flagellum base looking towards its tip (see Fig. 2). A feature of the trypanosomatid flagellates is the

internal partition on the non-arm-bearing subtubule of each axonemal doublet. As the flagellum emerges from the canal portion of the flagellar pocket, the axoneme acquires a partner structure—the paraxial rod (Figs. 2, 3). The infrastructure of the paraxial rod remains to be resolved in detail, but as seen in sections it appears as a three-dimensional lattice lying parallel to the two central tubules of the axoneme. Using the axoneme doublet terminology of Afzelius (1959), subtubule *B* on doublets 5 and 7 (see Figs. 2, 3, 5) shows a bifurcated process linking the axoneme to the paraxial rod.

The junctional complexes between body and flagellum are seen as a series of apposed macular densities (each 25 nm in diameter) on the cytoplasmic side of each surface membrane. These complexes, which fall within the spectrum of the macula adherens of Farquhar & Palade (1963), are spaced at an interval of 95 nm centre-to-centre along a line of attachment which parallels adjacent microtubules and bears a constant relationship to these surface structures. If in a transverse section, such as Fig. 5, we are looking along the flagellum from its basal end with the flagellum lying on top of the body surface, the macula is interposed in a gap in the pellicular microtubules of about 70 nm and the microtubules immediately to the left of the gap are embraced by a diverticulum of the granular reticulum. Within the microtubule gap between the macula and this flagellum-associated reticulum, a microtubule of considerably diminished diameter can usually be seen (Figs. 3, 5, 18, 21–23): more rarely a similar reduced microtubule is found in the gap on the opposite side of the macula (Fig. 20). The dense and finely fibrous material of the macula is more abundant on the body side and fine strands can be seen radiating from it into the cytoplasm beneath. High-resolution micrographs show cortical filaments about 5 nm across running parallel to the surface of the flagellate in this region and the macular strands terminating among them (Fig. 19). On the flagellar side the macular material shows fine strands linking it to the junction between the paraxial rod and the process from axonemal doublet no. 7 (Figs. 5, 18, 23). No penetration of the surface membranes and their intervening material by macular filaments has been observed (Fig. 21).

Although the maculae might appear to depend upon the paraxial rod for anchorage inside the flagellum, they are to be found along the wall of the flagellar canal (Fig. 16) apposing a region of the flagellum that lacks the paraxial rod. Here their anchoring link is with doublet 7 and possibly dense material in the flagellar sheath (Fig. 22). The same pellicular modifications are present along the canal region (Fig. 16) as are found along the main shaft of the flagellum. The flagellum-associated reticulum first becomes evident pressed against its 4 microtubules as the flagellum emerges from the canal; towards the anterior end of the body this reticulum is confined to less than 4 microtubules and at the very tip it is missing from the attachment region. The microtubules of the flagellum-associated reticulum are unique in that they alone arise from the foot of the flagellum's basal body and pass along the wall of the flagellar pocket: they border the line of maculae while executing a half-turn around the flagellar canal and from then on lie along the body surface paralleling the line of flagellar adhesion.

Culture forms of pathogenic trypanosomes

Culture forms of *T. rhodesiense* have the surface membrane and pellicular microtubules described for bloodstream forms, but are not enveloped by a prominent surface coat. Examination of the region of apposition of body and pellicle shows that there may be some traces of material between the 2 unit membranes but the width of the intermembranous space is less than in bloodstream forms and in the region of 20 nm. Although some fine irregular deposit may be visible on the surface membrane, a comparison of micrographs of bloodstream and cultured trypanosomes fixed at the same time and later subjected to the same treatment (Figs. 5, 6) shows that there can be no confusion between the two.

The details of flagellar adhesion apart from the absence of surface coat are very much the same for cultured as for bloodstream *T. rhodesiense*, though the reduced pellicular microtubule seems to be a less constant feature and flagellum-associated reticulum is less prominent than in bloodstream forms. Cultured *T. congolense* also lacks a surface coat.

Vector stages of Trypanosoma rhodesiense

T. rhodesiense undergoing multiplication and development in the midgut and cardia of the tsetse fly is known to resemble morphologically the forms obtained in culture, in that the kinetoplast occupies an almost post-nuclear position in contrast to the bloodstream forms where the kinetoplast lies close to the posterior end of the body (see Fig. 1). The pellicular structure of these gut forms (Fig. 7) is likewise identical with that of culture forms in that here too a surface coat is lacking, and details of flagellar adhesion are the same as in cultured trypanosomes.

Two distinct morphological stages in the trypanosome life-cycle occur in the salivary gland of the tsetse vector (see Fig. 1). The epimastigote (crithidial) forms (terminology of Hoare & Wallace, 1966) have the kinetoplast in front of the nucleus (see Fig. 1) and undergo division while attached to the microvillar luminal border of the salivary gland secretory cells. The metacyclic trypanosomes are, like the midgut and bloodstream flagellates, trypomastigote forms; that is, have a post-nuclear kinetoplast, in this case close to the posterior extremity of the body. The metacyclic trypanosomes do not divide and are found free in the lumen of the gland along with stages in the transformation of epimastigote to metacyclic trypanosomes. In sections the two stages can be distinguished, therefore, by their position in the gland as well as by their morphology. Under the electron microscope other differences are evident. The epimastigote forms (like the midgut stages) have a network of mitochondrial canals while the metacyclic forms (like the mammalian bloodstream stages) have just a single longitudinal mitochondrial canal (Vickerman, 1966). A definite assessment of the developmental stage of a particular trypanosome can therefore be made from the number of mitochondrial profiles to be seen in the section, and then the surface characters of the two forms can be compared. The epimastigote forms resemble midgut and culture forms in having a naked plasma membrane (Fig. 8) whereas the metacyclics have reacquired a surface coat (Fig. 10) of similar dimensions and character

to that seen in the bloodstream trypanosomes. Sections of epimastigotes in the process of transforming into metacyclics exhibit a somewhat thinner surface coat (Fig. 11) but one which is uniform in its distribution over the whole flagellate.

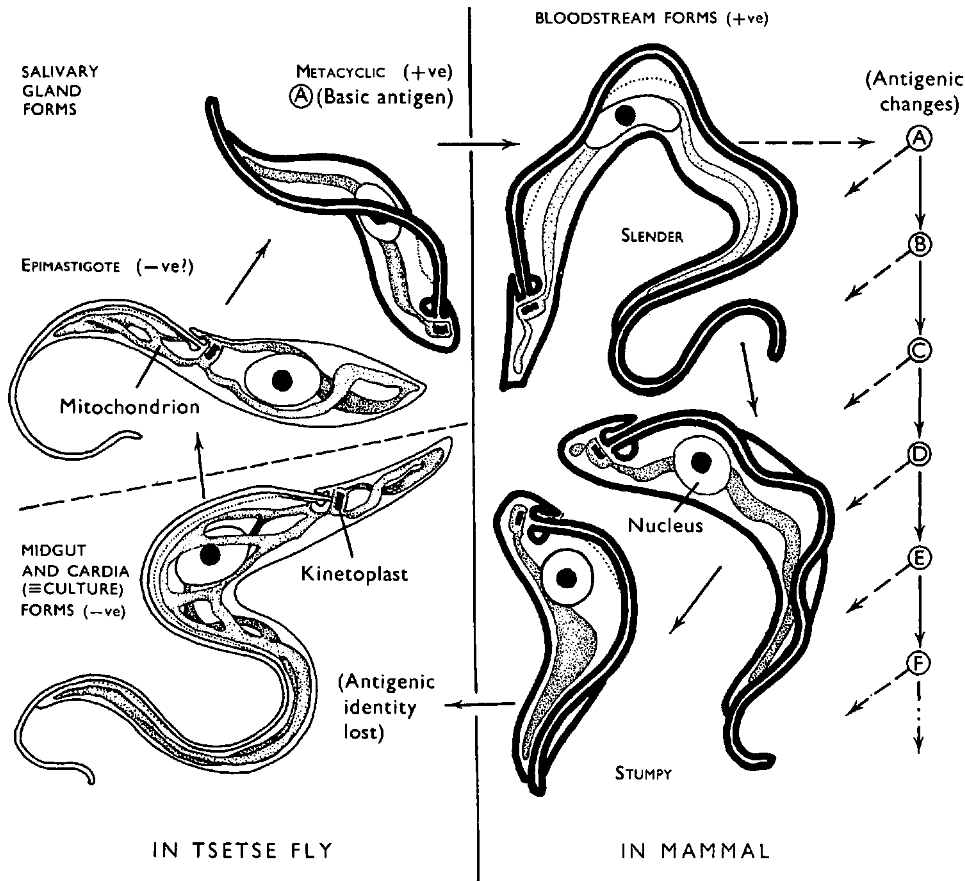

Fig. 1. Diagram of stages in the life-cycle of *Trypanosoma rhodesiense* to show cyclical changes in surface coat in relation to net surface charge, antigenic identity, and gross morphological characters (position of nucleus and kinetoplast, form of mitochondrion). Forms with a surface coat are drawn with a thick outline. The surface charge is indicated crudely, positive and negative signs reflecting relative electrophoretic mobilities rather than actual net charge. The letters A, B, C, D etc. refer to the sequence of antigenic variants characterizing successive relapse populations in the blood. The transformation from slender to stumpy trypanosomes takes place within each relapse population. On entering the fly all variants lose their antigenic identity and eventually revert to the basic antigen A as they reacquire the surface coat in becoming metacyclic forms.

Bloodstream and culture forms of Trypanosoma lewisi

The non-pathogenic *T. lewisi* differs appreciably from the pathogenic trypanosomes described above in its surface features and mode of flagellar adhesion. A surface coat is present in bloodstream forms, but this consists of a fuzz of fine filaments attached to the plasma membrane (Figs. 25–27). In transverse sections of the flagellar adhesion

zone, a dense line is often seen lying in the space between the 2 bounding membranes (Fig. 27) and this may represent a line of overlap between filaments of the 2 surface coats. Macular attachments between body and flagellum are essentially similar to those described for the pathogenic trypanosomes. Reduced microtubules and flagellum-associated reticulum are not a feature of the *T. lewisi* pellicle attachment zone. A single sub-pellicular cisterna which lies down one side of the trypanosome (Fig. 25) stops short of the macula adherens but is not closely applied to any of the pellicular microtubules. The fuzzy surface coat is absent from culture forms.

DISCUSSION

The surface coat

Electron microscopy reveals a coat of moderately electron-dense material about 15 nm thick covering the plasma membrane in the bloodstream phase of the *Trypanosoma brucei*-like trypanosomes, *T. congolense* and *T. vivax*, i.e. in the pathogenic trypanosomes which are transmitted through the biting mouth parts of insects. A coat of a different nature, filamentous and rough as opposed to amorphous and smooth, has been found in the non-pathogenic *T. lewisi* which belongs to the other division of mammalian trypanosomes which are transmitted by contamination through the faeces of biting insects.

In *T. rhodesiense* the surface coat would seem to disappear when the flagellate enters the tsetse-fly vector, as midgut and cardial stages in development of the trypanosome, and their counterparts obtained by cultivation *in vitro*, lack this coating layer. In the salivary glands of the fly the multiplicative epimastigote trypanosomes also lack a coat, but their metacyclic descendants, which initiate infection when they are inoculated into a fresh mammalian host, develop a new one so that they are already coated when they subsequently become bloodstream flagellates. The significance of the surface coat will be discussed in relation to the life-cycle of *T. rhodesiense* and the other *T. brucei*-like trypanosomes: how far this discussion is relevant to *T. congolense*, *T. vivax*, and *T. lewisi* will be dealt with briefly as a postscript.

The surface coat and antigenic change in trypanosomes. In chronic infections with the pathogenic trypanosomes, the number of flagellates in the blood fluctuates considerably from day to day. Each remission of parasitaemia is due to the destruction of the majority of trypanosomes by host antibodies. The recrudescences are thought to be due to proliferation of trypanosomes which have a different antigenic constitution from their predecessors and which have managed to escape the host's immune onslaught (Levaditi & Mutermilch, 1909). This succession of antigenic types (A → B → C..., etc) occurs in clone strains and is to some extent reversible (e.g. A → B → C → A; Lourie & O'Connor, 1937). Gray (1962) found a sequential pattern of antigenic variation in cyclically transmitted *T. brucei* infections, starting from a 'basic antigen' to which the trypanosomes reverted each time they passed through the tsetse fly. He concluded (Gray, 1967) that antigenic change is the outcome of selection acting upon individual adaptation rather than upon random mutations, so that the situation may be compared to antigenic variation in *Paramecium aurelia* (Beale, 1954),

where different genes express themselves as different antigens at different temperatures. The variable antigens of trypanosomes have been identified as unconjugated proteins (Brown & Williamson, 1962; Seed & Weinman, 1963; Williamson & Brown, 1964). Le Page (1968) could not recognize common peptides among the major variant antigens after trypsin digestion and concluded that the antigens were the products of different genes. Although the writer favours the adaptation theory of antigenic variation, it should be mentioned that some investigators still explain their results on the basis of selection of genetic mutants (Watkins, 1964; Seed & Gam, 1966*b*).

The variable antigens include agglutinogens and the variant succession has been investigated largely using the agglutination reaction (Inoki, Kitaura, Makaboyosi & Kurogochi, 1952; Inoki, Osaki, & Nakabayashi, 1956; Soltys, 1957; Cunningham & Vickerman, 1962; Gray, 1962, 1965*a*, *b*, 1966; Seed, 1963; Seed & Gam, 1966*a*). Seed (1963) showed that the agglutinogens of bloodstream forms disappear entirely in culture and therefore presumably on entering the fly. He found later (Seed, 1964) that culture trypanosomes from several strains of *T. brucei* and *T. rhodesiense* have common surface antigens: antigenic variation is not a property of the cultured flagellates and these forms are poorly immunogenic.

These antigenic changes during the life-cycle of the trypanosome are intelligible in terms of what is happening to the surface coat, which no doubt contains the surface agglutinogens. The loss of surface antigenic character which the trypanosomes undergo in culture, or in the tsetse midgut, parallels loss of the surface coat. Reversion to the basic antigenic type probably occurs when metacyclic trypanosomes are produced in the antibody-free environment of the fly salivary gland: Cunningham (1966) reported that the metacyclics were of the same antigenic type as the first population to appear in the blood following infection, as shown by neutralization tests. Antigenic changes in the bloodstream population take place adaptively as the host produces the corresponding antibodies (Gray, 1962, 1965*a*). Gray (1965*b*) noted that in the antibody-free surroundings of the non-immune host the basic antigen tended to replace the variant antigens.

It is suggested, then, that antigenic change might involve changes in the surface coat of the trypanosome. As the coat is shed on transfer to the vector, or in culture, the coat appears to be an adaptation to life in the bloodstream and may constitute a surface which the parasite can replace by another, which being antigenically different is less vulnerable when the trypanosome is assailed by antibodies. The mechanism of replacement will be discussed later. The reacquisition of the coat in the metacyclic trypanosome pre-adapts it to its bloodstream existence.

The surface coat is probably an important component of the 'exo-antigen' of Weitz (1960). This soluble variable antigen found in the sera of infected rats induced the formation of antibodies which agglutinated members of the trypanosome population from which the exo-antigen had been derived. The loss of viability and infectivity of saline-washed trypanosomes, which Weitz put down to the loss of protective exo-antigen by the flagellates, can be interpreted as being due to the loss of surface coat which was noted in the moribund trypanosome.

It is significant that a surface coat is found in all forms of the pleomorphic strains

studied here, in that the host's immune response has been suspected of inducing the transformation from long slender to short stumpy trypanosomes. The case for a relationship between pleomorphism and antigenic variation has been argued elsewhere (Vickerman, 1965), but still rests *sub judice*. The present study can add nothing to the controversy over which particular form if any is responsible for antigenic change.

Surface charge during the life-cycle. There have been many attempts to determine the net surface charge on bloodstream trypanosomes, and the earlier literature was summarized by Broom, Brown & Hoare (1936). Extensive studies were undertaken by Broom & Brown (1937, 1939) on the surface charge of *T. gambiense* (*T. brucei*-like) throughout its life-cycle. They utilized the erythrocyte's permanent negative charge in a simple cell adhesion test, suspending the trypanosomes and red cells in an isotonic medium. Under these conditions adhesion of red cells to trypanosomes indicated a positive charge on the latter. They claimed that whereas trypanosomes of the first parasitaemia in the blood were invariably positively charged, those of the relapse (recrudescence) could be negative. On entering the tsetse fly, or being inoculated into culture medium, however, the onset of development of the vector forms was accompanied by the assumption of a negative charge by all trypanosomes. In the salivary glands of the tsetse-fly vector reversion to a positively charged surface took place.

More recently, Hollingshead, Pethica & Ryley (1963) have studied the surface characteristics of bloodstream and culture *T. rhodesiense* and bloodstream forms of some other species. These authors found that for the culture (fly midgut) forms the isoelectric point was about pH 3 while for the bloodstream form it was about pH 7, so that the circulating trypanosomes probably carry no net surface charge, though some may be slightly positive or slightly negative depending upon the population studied—a fact that may reconcile the conflicting results of earlier workers. Electrophoresis, unlike the red-cell adhesion test, revealed no appreciable charge difference between relapse populations of the same strain.

When the trypanosome enters the fly or culture tube it sheds its surface coat to expose the plasma membrane beneath: this coincides with a shift towards negative mobility. The reacquisition of the surface coat in the salivary glands of the fly might be expected to result in a reverse mobility shift, and the findings of Broom & Brown (1939) are significant here, for although their method of charge determination was a crude one (they used a medium of rather low ionic strength for the erythrocyte to behave reliably as a polyanion), they detected charge reversal at this stage (see Fig. 1).

It is suggested therefore that cyclical loss and acquisition of the surface coat can account for changes in electrophoretic behaviour of pathogenic trypanosomes at different stages in their life-histories.

Origin and nature of the surface coat in trypanosomes. At the moment little can be said under this heading. In blood flukes there is evidence (Smithers, Terry & Hockley, 1968) that host serum proteins can become adsorbed on to the surface so that the parasite can mimic its host antigenically and so avoid rejection as foreign tissue. The possibility that the coat of trypanosomes is an extraneous accretion of host serum proteins can be dismissed on the grounds that an antigenically and morphologically

similar coat occurs on the trypanosome before it enters the mammalian bloodstream. The coat *must* be endogenous, but whether it is formed as an integral part of the cell membrane or represents a secretion plastered all over it, cannot yet be decided. An important consequence of the mode of origin of the coat is the way in which it is replaced. Does the trypanosome actually shed its coat in the face of antibody attack? It is tempting to draw an analogy with the behaviour of ciliates such as *Tetrahymena* when these are placed in antiserum (Harrison, 1955): the surface antigens combine with their homologous antibodies to form an envelope from which the organism can escape dramatically. Coat replacement and antigenic change in trypanosomes could be as simple as this. We have evidence that the trypanosome can shed its coat (see Fig. 17), but whether, having done so, it can produce another, we do not know.

In *T. rhodesiense* transforming to the metacyclic stage the coat seems to appear uniformly over the whole surface rather than in patches, as might be expected if the coat were an integral part of the membrane and therefore formed only when membrane was being manufactured. The presence of all the trappings of a secretory cell (Golgi with subtending granular reticulum, vesicles with secretion—see Fig. 13) close to the flagellar pocket in *T. congolense* suggested to the writer (Vickerman, 1969) that the surface material might be secreted just as the surface scales of phytoflagellates are secreted around the flagella bases (see e.g. Manton, 1967, for references).

The nature of the surface coat in trypanosomes must also await further work, for few relevant pieces of information are available as yet. Hollingshead and co-workers (1963) presented the following evidence from pH/mobility relationships and charge reversal spectra as to the nature of the surface in bloodstream and culture forms of *T. rhodesiense*. Culture forms of this trypanosome show a similar pH/mobility relationship to most other cells and to phosphatide sols: the charge reversal spectrum with respect to uranyl ions is also characteristic of phosphatide and phosphate surfaces (i.e. surfaces in which the ionized phosphate groups of phospholipids are to the outside), that is the concentration of uranyl ions required to bring about charge reversal is low. As phospholipids figure in models of membrane structure, this speculation would be in keeping with the presence of 'exposed' unit membrane in culture forms. The low mobility of bloodstream forms at pH 7·0 could indicate a polysaccharide surface but the dependence of mobility upon pH suggests the presence of charged groups, both acidic and basic, on the trypanosome surface. The *p*K values for these groups in *T. rhodesiense* suggest carboxyl and amine groups and therefore a protein nature for the surface layer.

The fact that the wide-spectrum protease of *Streptomyces griseus* (pronase) can remove the surface coat indicates that at least its structural matrix is protein. The time taken for digestion of the fixed coat is rather long, but persistence of the coat in controls incubated without the enzyme suggests that digestion is specific. Ruthenium-red staining, believed by Kelly & Luft (1966) to reveal mucopolysaccharides in surface coats, gives negative results with *T. rhodesiense* and so fails to convince us that these substances are present in any quantity.

In considering the nature of the surface coat it is interesting to recall that the variable agglutinogens have been identified as unconjugated proteins. Williamson & Desowitz

(1961) found that the only carbohydrates (apart from nucleotide pentose) detectable in hydrolysates of *T. rhodesiense* were traces of glucosamine. There is the possibility that these were derived from surface glycoprotein. The evidence that glycoproteins play an important role in surface membrane structure is accumulating rapidly (see Cook, 1968). Studies in cell electrophoresis combined with specific enzyme treatments now point to sialic acid-containing glycoproteins rather than ionizable phosphate groups as being responsible for the net negative charge on intact cells. Glycoproteins have been amply demonstrated in coats outside the cell membrane (see Introduction) but there is much to suggest that glycoproteins are also an integral part of the membrane itself (Benedetti & Emmelot, 1967), moreover that they constitute important surface agglutinogens. In the absence of any indication that carbohydrates are present in the surface antigens of trypanosomes, and bearing in mind the inability of the surface coat to bind ruthenium red, it is suggested that the glucosamine might be a component of the surface membrane beneath the trypanosome coat and that the surface coat of pathogenic trypanosomes is therefore very different in its composition from that of other cells.

The surface coat and pinocytosis. The most familiar example of a surface coat in protozoa is that of the large amoebae such as *Amoeba proteus* and *Chaos chaos* (*Pelomyxa carolinensis*). Although pinocytosis has not been observed under natural conditions, in the laboratory this coat can be shown to provide binding sites for particulate matter which is later taken into the amoeba by membrane flow into pinocytosis channels and vesicles (reviewed by Marshall & Nachmias, 1965). In metazoan cells localized coated membranes have been shown to act as binding sites for proteins which are pinocytosed by formation of first a coated caveola and then a coated vesicle. Such binding sites can be recognized in electron micrographs by the spinous processes on the cytoplasmic side of the membrane (see e.g. Roth & Porter, 1964; Fawcett, 1965; Friend & Farquhar, 1967).

In culture forms of trypanosomes from poikilotherms a differentiated cytostome has been shown to be functional in the ingestion of ferritin and possibly other proteins (Steinert & Novikoff, 1960): such a structure does not seem to be present in the pathogenic trypanosomes of mammals. In *T. rhodesiense*, however, Brown, Armstrong & Valentine (1965) have shown by electron microscopy that ferritin can traverse the wall of the flagellar pocket and find its way into nearby vesicles, presumably by pinocytosis though they did not demonstrate the ultrastructural details of this process. In the present study both caveolae and vesicles with spiny processes comparable to those recorded for pinocytotic cells of metazoa have been seen frequently in sections of *T. rhodesiense*, *T. congolense* and *T. vivax* at the site predicted by Brown and co-workers along the wall of the flagellar pocket. Although the surface coat is found over the whole trypanosome, pinocytosis appears to be associated only with sites in the flagellar pocket which are backed by the spiny fringe on the cytoplasmic side.

The surface coat of lower trypanosomes. The above discussion is based almost entirely on results obtained with the *T. brucei*-like trypanosomes, but a similar story of antigenic variation is emerging for *T. congolense* (Wilson, 1968) and *T. vivax* (Clarkson & Awan, 1968). These trypanosomes appear to carry a slightly negative charge in the

bloodstream phase (Hollingshead *et al.* 1963; Lanham, 1968). Despite the presence of a coat in the bloodstream but not the culture forms of *T. lewisi*, the trypanosome is negatively charged in both phases (Hollingshead *et al.* 1963). The very limited ability of *T. lewisi* to escape the trypanocidal antibodies of its rat host (Taliaferro, 1932) might prove eventually to be related to the fact that the surface coat of this flagellate has an entirely different nature from that of the pathogenic trypanosomes.

Flagellar adhesion

The undulating membrane formed by the flagellum is the most noticeable feature of the living trypanosome but the structural basis of this supposed adaptation to movement in a viscous medium is still open to dispute. In those trypanosomes that have a very prominent undulating membrane, e.g. *T. avium* (Baker & Bird, 1968), this structure seems to be formed by the moving flagellum distorting the pellicle and drawing it out into a series of fins or folds, rather than by distortion of the membrane bounding the flagellum itself. The undulating membrane region of the mammalian trypanosomes studied here, however, is not so telling when seen in section, so this discussion will be confined to the mode of adhesion of flagellum to pellicle.

The extent of adhesion of the flagellum to the body has been found to vary in transverse section but, minimally, adhesion is confined to a narrow zone which corresponds to a row of junctional complexes. These can be classified in the macula adherens (Farquhar & Palade, 1963) or desmosome region of the spectrum of junctional complexes drawn up by Kelly & Luft (1966), in that they have macular densities on either side of apposed membranes with fine filaments converging on the dense regions from the cytoplasmic side of each membrane. Anderson & Ellis (1965) described a desmosome-like attachment between flagellum and pellicle in *T. lewisi*, and Boisson, Mattei & Boisson (1965) named the junctional complexes of *T. gambiense* 'rivets lipoprotéiques'. The present paper characterizes these junctional complexes in considerably more detail than previous ones as regards form, distribution and structural connexions: the discussion of their function will be brief.

T. lewisi alone has the median dense line between apposed membranes which is characteristic of desmosomes in higher organisms, and in this trypanosome the line seems to be formed by overlap of the filaments of two surface coats, though this overlap is by no means a constant feature. Because of the median line's inconstant presence in *T. lewisi* and its apparent absence in the flagellar junctional complex of the pathogenic trypanosomes, the generic term 'macula adherens' is preferred to 'desmosome' here. As there is no evidence for lipoprotein material penetrating the apposed membranes at the maculae, the term 'lipoprotein rivet' seems inappropriate.

The adherent maculae of the trypanosome flagellum have a very small diameter (25 nm) compared to the desmosomes of higher organisms and also differ in that they occur between adjacent parts of the same cell rather than between different cells. A further interesting point—and the sole excuse for including flagellar adhesion in this paper—is that these highly specific attachments persist regardless of the nature of the apposed surfaces; that is, whether the surface coat is present or not.

Because doubt has been cast upon the desmosome type of junction in the attach-

ment of cell surfaces (e.g. see Curtis, 1967, pp. 89–90) the observation that adhesion is always maintained in the region of the macula when it has been disrupted over the wider zone of flagellum/pellicle apposition is highly significant. It suggests that two mechanisms of binding may operate. The first is weak (in that it is easily disrupted by the preparative procedures for electron microscopy) and exists over the zone of adhesion as a whole, though the extent of this zone may vary with the position of the flagellum in its stroke so that this binding is temporary. The second mechanism of binding is much stronger and operates at the macula only. The minimum gap (10–20 nm) between the apposed surfaces in the zone of adhesion is characteristic of adhesions between cells of higher organisms, but the strength of adhesion at this distance (the 'secondary minimum' of Curtis, 1967) is weak in comparison with that between surfaces in direct contact (0·5 nm apart—the 'primary minimum' of Curtis). To account for adhesion in the face of flagellar movement, some sort of bridging mechanism (Pethica, 1961; Steinberg, 1962) must be postulated to provide a strengthening bond, at least in the region of the macular attachment. A calcium bridge wherein calcium ions link surface cationic groups to an intervening molecule of cementing substance is plausible. Dividing trypanosomes in citrated blood produce a completely free 'daughter flagellum' while the 'parent flagellum' remains attached (Vickerman, unpublished observations), suggesting that calcium ions chelated by the citrate are necessary for adhesion of the developing flagellum, but not for maintenance of attachment.

The development of relationships between the flagellum, flagellum-associated reticulum and surface microtubules during morphogenesis remains to be explored, but it is interesting to note, meanwhile, that even when the flagellum differentiates intracellularly (Vickerman, 1969), flagellum-associated reticulum is still a feature of the adjacent pellicle in the absence of maculae. It is hoped that experimental treatment of the morphogenesis of flagellar attachment will be the subject of another paper.

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ABBREVIATIONS ON PLATES

ac	acanthosomes	*mif*	membranous inclusion of flagellum
ar	agranular membranes	*mit*	profiles of the single mitochondrion
ax	axoneme	*mvb*	multivesicular bodies
bb	basal body	*n*	nucleus
cv	coated vesicle	*ne*	nuclear envelope
deb	debris	*nuc*	nucleolus
f	flagellum	*p*	peroxisome-like organelle
far	flagellum-associated reticulum	*pm*	pellicular microtubule
ff	free portion of flagellum	*pl*	plate-like termination
fp	flagellar pocket	*pr*	paraxial rod
fs	flagellar sheath	*rib*	ribosome
g	Golgi apparatus	*rm*	reduced microtubule
gr	granular reticulum	*sar*	sacs of agranular membranes
k	kinetoplast (mitochondrial DNA)	*sm*	surface (plasma) membrane
ma	macula adherens		

Fig. 2. Transverse section of anterior tip of bloodstream *T. evansi*, to show the smooth continuous dense coat overlying the surface unit membrane (*sm*) of the body (below) and its attached flagellum (above). The body shows the characteristic corselet of pellicular microtubules (*pm* 1), some of which appear to have been relegated to the underlying cytoplasm to form a second row (*pm* 2) as they approach the apex of the body. Within the flagellum the axoneme (*ax*) and paraxial rod (*pr*) complexes are readily distinguishable, the latter showing little detail in this micrograph. The peripheral doublets of the axoneme have been numbered in clockwise rotation after Afzelius (1959). Subtubule *A* of each doublet shows the characteristic 'arms', and subtubule *B* the internal partition which is a feature of the trypanosomatid axoneme. Subtubule *A* on doublet 2 shows part of a radial link to the ring of secondary fibres which are seen in shadowy outline (small arrowheads) surrounding the two central tubules. On subtubule *B* of doublets 5 and 7 connectives (arrowed) to the paraxial rod are visible. The attachment zone of the flagellum to the body in life probably extended over the distance between the two large arrowheads but preparative procedures have resulted in a certain amount of mutual repulsion of body and flagellum. Details of the macula adherens are not apparent here owing to the oblique plane of section of the flagellum in this region, but the anterior extremity of the flagellum-associated reticulum (*far*) can be made out applied to one peripheral microtubule. Glutaraldehyde fixation. × 250000.

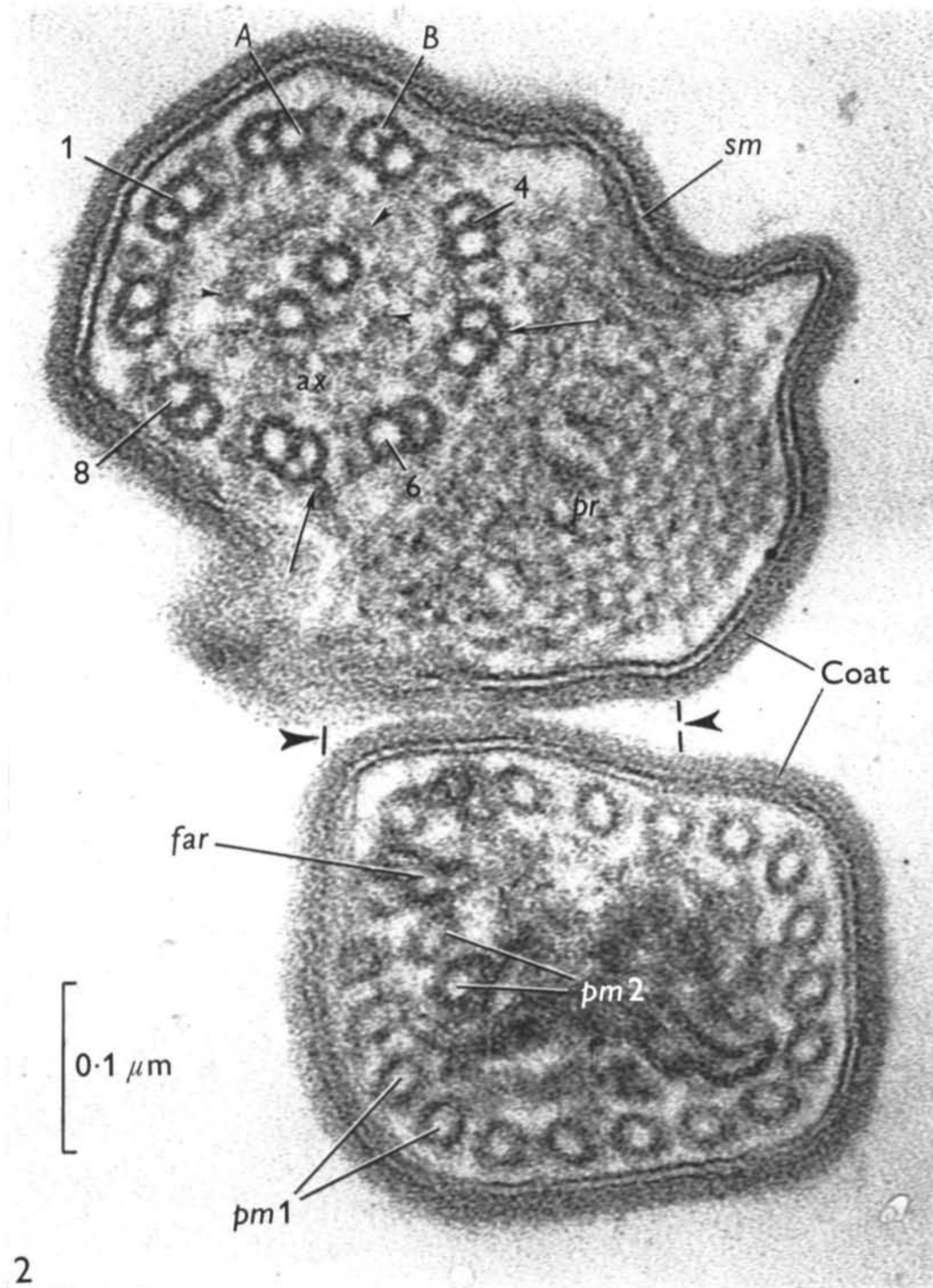
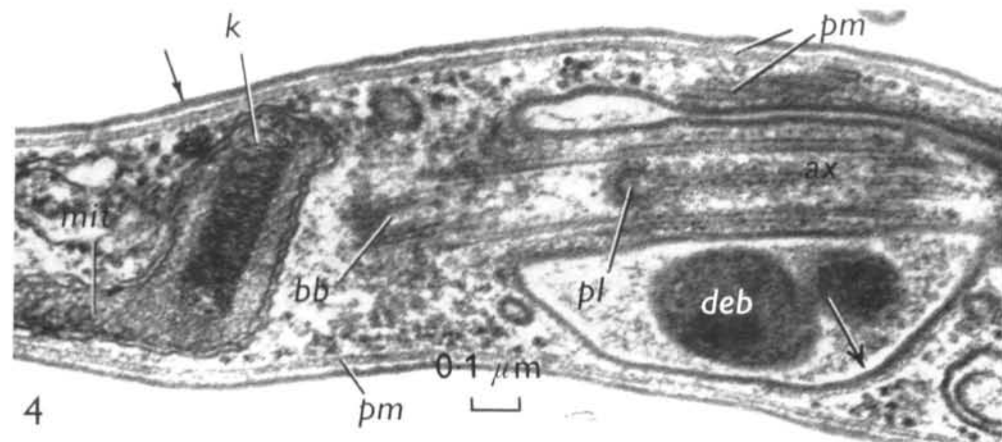

A
B
1
sm
4
ax
8
6
pr
Coat
far
pm2
0·1 μm
pm1
2

Fig. 3. *T. evansi* (from bloodstream). Transverse section of a dividing flagellate towards the anterior end showing the uniform thickness of the surface coat around the body, and both old (*f* 1), and new (*f* 2) flagella. Close to the attachment zone of the old flagellum, a diverticulum of the granular endoplasmic reticulum (*gr*) the flagellum-associated reticulum (*far* 1), is evident: flagellum-associated reticulum is as yet poorly developed (*far* 2) alongside the new flagellum. Note the broad attachment zone of each flagellum; some slight separation of the old flagellum has occurred, but this flagellum, unlike the other, shows some details of the macula adherens (*ma*). Within the cytoplasm dense spherical bodies (*p*) are prominent; these are of uncertain function but may be peroxisome-like organelles which house the para-mitochondrial enzyme systems of terminal respiration in bloodstream trypanosomes. Possible connexions between the pellicular microtubules are seen at the small arrows. Glutaraldehyde fixation. × 78000.

Fig. 4. *T. rhodesiense* bloodstream form. Longitudinal section of posterior end in region of base of flagellum, to show surface coat (arrowed) overlying the membrane covering the body and lining the flagellar pocket. Pellicular microtubules (*pm*) are seen cut along their length: the microtubule running alongside the flagellum is probably one of the 4 which wind from the basal body of the flagellum towards the mouth of the flagellar canal to become embraced by the flagellum-associated reticulum along the entire length of the body. The plate-like termination (*pl*) of the central tubules of the axoneme (*ax*) is seen inside the flagellum whose basal body (*bb*) is apposed to the kinetoplast (*k*) region of the mitochondrion (*mit*). Dense globular particles of debris (*deb*) lie in the cavity of the flagellar pocket. Glutaraldehyde fixation. × 62000.

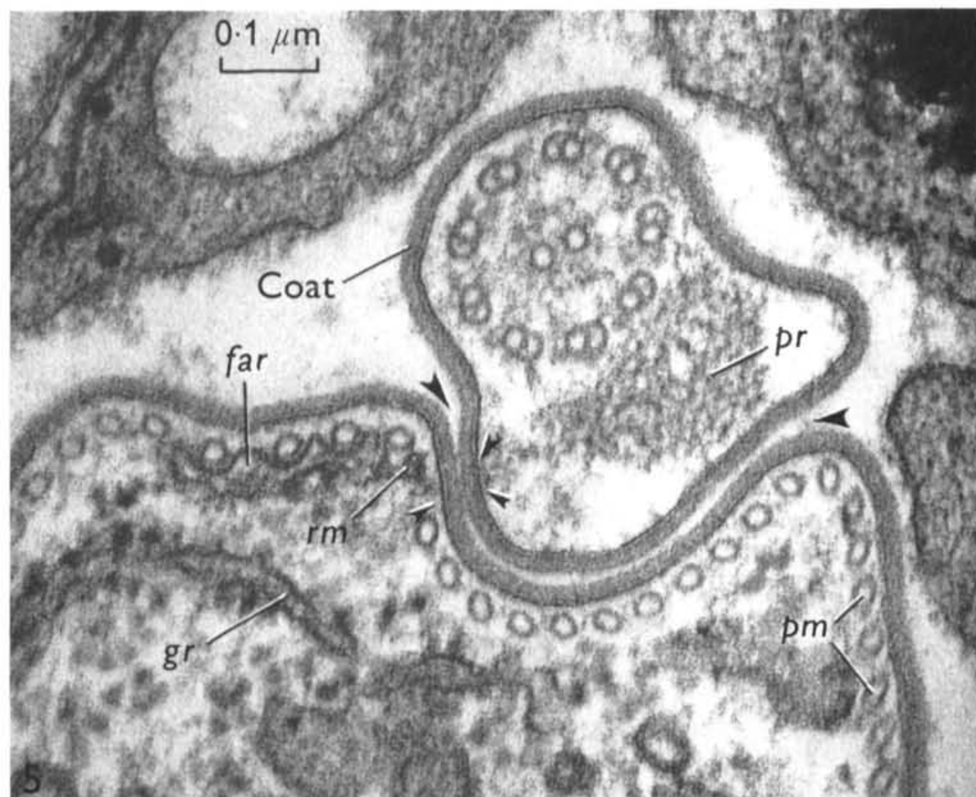
ax
f2
far 2
f1
far 1
ma
p
p
gr
pr
mit
rib
Coat
sm
pm
0·1 μm
3

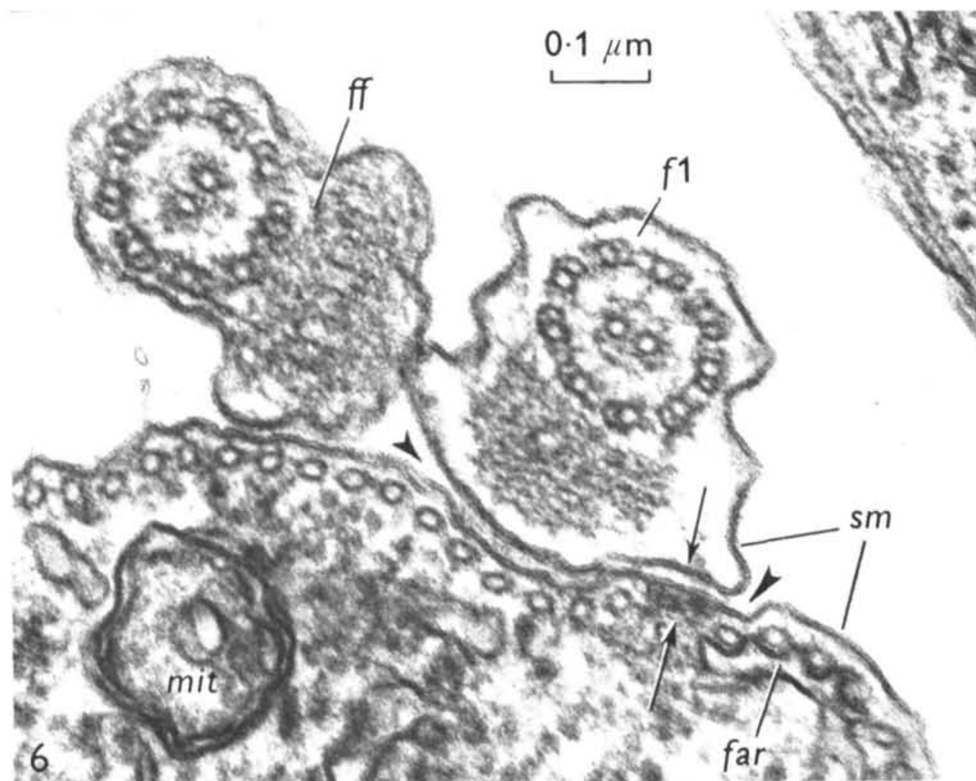

k
pm
ax
mit
bb
pl
deb
0·1 μm
pm
4

Figs. 5, 6. *T. rhodesiense* fixed in glutaraldehyde to show difference in cell surface between bloodstream and culture forms. × 130000.

Fig. 5. Bloodstream form. Transverse section of flagellum (viewed from its base) and adjacent pellicle to show adhesion zone (between large arrowheads). The surface coat of the flagellum is separated from that of the body by a gap of more or less uniform width except in the region of the macula (small arrows) indicating that the gap may be a contraction artifact. The fibrous material of the macula adherens is poorly represented in this figure but other features of the macular region—the reduced pellicular microtubule (*rm*) and nearby flagellum-associated reticulum (*far*)—are evident. The axoneme shows many of the structures seen in Fig. 1, including the connective from doublet 7 to the paraxial rod.

Fig. 6. Culture form. A section comparable to that shown in Fig. 4 but the attached flagellum (*f* 1) is viewed from the tip (as shown by the counter-clockwise direction of arms on the axoneme doublets and the position of the flagellum-associated reticulum (*far*)). The surface coat is not present, though some diffuse material is visible in the gap between membranes in the adhesion zone (between large arrows). The gap between the flagellar and body membranes in the region of the macula is less than the similar gap in the bloodstream form. The flagellum to the left represents the free flagellum (*ff*) of another trypanosome and is not a daughter flagellum as the axonemal doublets point in the opposite direction.


0·1 μm
Coat
far
pr
rm
gr
pm
5


0·1 μm
ff
f1
sm
sm
mit
far
6

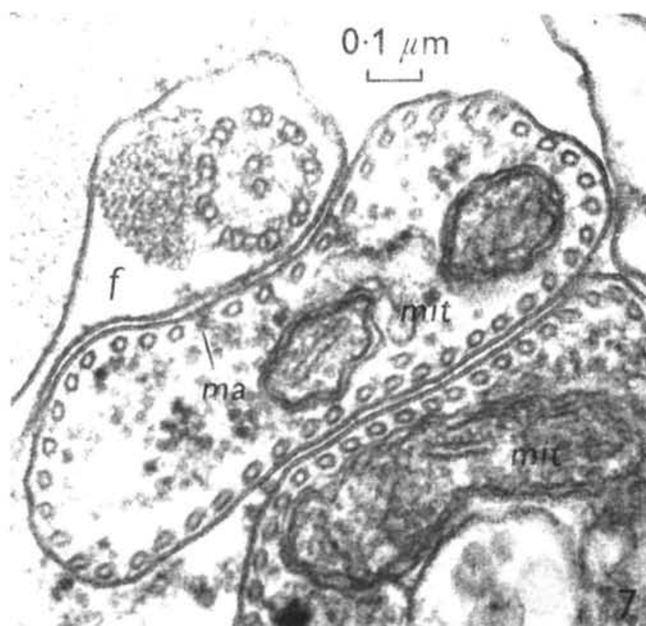
Figs. 7–10. Sections of various stages in development of *T. rhodesiense* in the vector, *G. pallidipes*, to show changes taking place at the cell surface. All were taken from the same infected fly. OsO4 fixation.

Fig. 7. Transverse section of anterior end of trypomastigote form and part of another flagellate from the midgut cardia (proventriculus); the surface coat is clearly missing, The membrane surrounding the flagellum (*f*) is very stretched and applied to the adjacent trypanosome body over a broad adhesion zone. The gap between flagellum and body is approximately the same as that between the bodies of the two adjacent flagellates. The macula adherens is not flanked by a reduced microtubule in this particular case. × 72000.

Figs. 8–10. Details from one micrograph of adjacent flagellates in a section of the salivary gland. Fig. 8. Attached epimastigote form; the coat is still not present. Fig. 9. Detached epimastigote form believed to be in process of transforming into metacyclic: a thin surface coat is visible. Fig. 10. Mature metacyclic trypanosome: the surface coat is thicker than that shown in Fig. 9. × 90000.

Fig. 11. *T. rhodesiense* bloodstream form. Transverse section of trypanosome after glutaraldehyde fixation and incubation for 4 h at 37 °C in phosphate-buffered 0·5 % pronase with 1 % sucrose: the surface coat has been removed from both body and flagellum. × 80000.

Fig. 12. Section of similar trypanosome incubated at the same temperature for the period in buffered 1 % sucrose only: the dense surface coat is still present. × 80000.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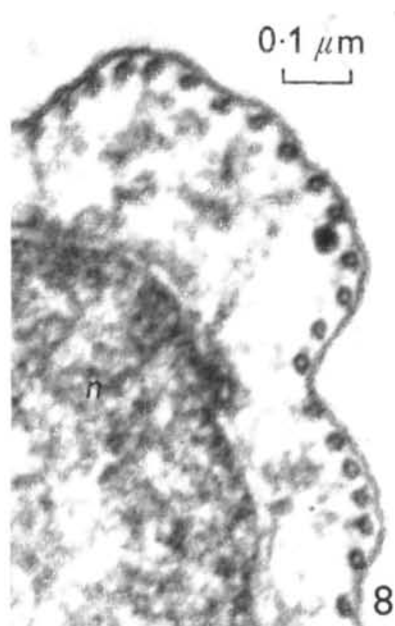
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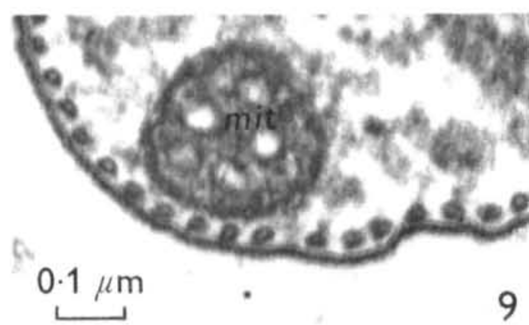
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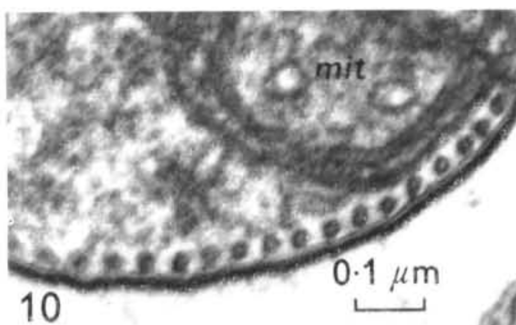
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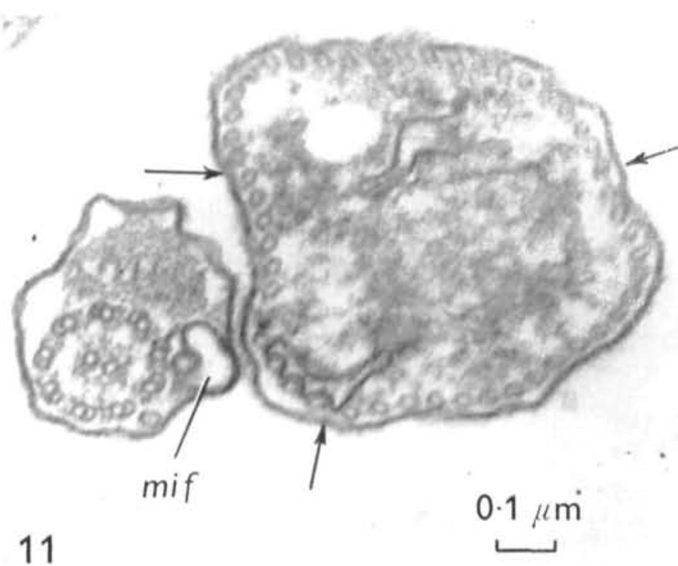
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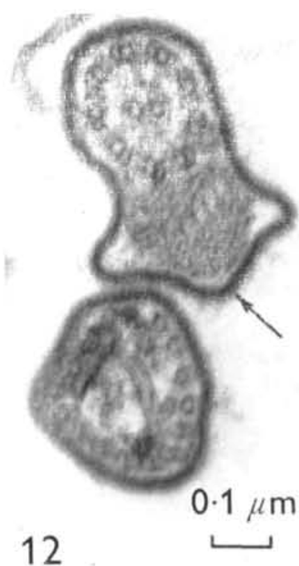
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12

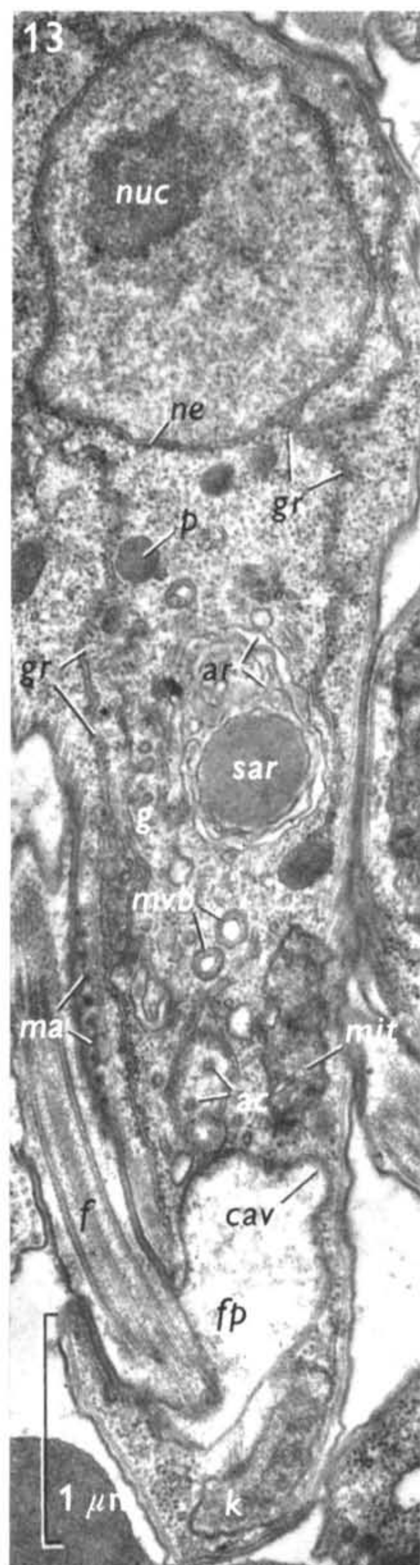
Fig. 13. *T. congolense* bloodstream form. Longitudinal section of posterior end of flagellate, showing nucleus, kinetoplast (*k*), flagellar pocket (*fp*) with emerging flagellum (*f*) and some of the cytoplasmic membrane systems. The basal body of the flagellum is not represented in this section but part of the row of maculae adherentes (*ma*) attaching the flagellum to the body of the trypanosome are visible. The flagellar pocket shows a single caveola which may indicate incipient pinocytosis. One of the many profiles of granular reticulum passes from the nucleus to the flagellar pocket subtending the Golgi apparatus (*g*) of which only the peripheral part is visible here. Close to the Golgi lies a system of smooth (agranular) membranes (*ar*) arranged in concentric fashion and dilated locally to form sacs (*sar*) filled with a substance of medium density—probably a secretion. The granular reticulum–Golgi–smooth cisternae may form a system for synthesis and transport of the secretion to the flagellar pocket. The multivesicular bodies (*mvb*) and acanthosomes (small spiny vesicles—*ac*) may represent activating and transporting components of this system. Glutaraldehyde fixation. × 30000.

Fig. 14. *T. congolense* bloodstream form. Detail of part of flagellar pocket showing 2 caveolae, one with spinous coat (arrowed). Glutaraldehyde fixation. × 38000.

Fig. 15. *T. rhodesiense* bloodstream form. Transverse section just in front of flagellar pocket showing profiles of spinous coated vesicles (*cv*). Flagellum shows connexion between macula adherens and paraxial rod (arrowhead). Glutaraldehyde fixation. × 45000.

Fig. 16. *T. congolense* bloodstream form. Thick vertical section through base of flagellum to show row of maculae adherentes winding around the wall of the flagellar canal. Glutaraldehyde fixation. × 48000.

Fig. 17. *T. congolense* bloodstream form. Transverse section showing shedding of surface coat (at arrows). Glutaraldehyde fixation. × 48000.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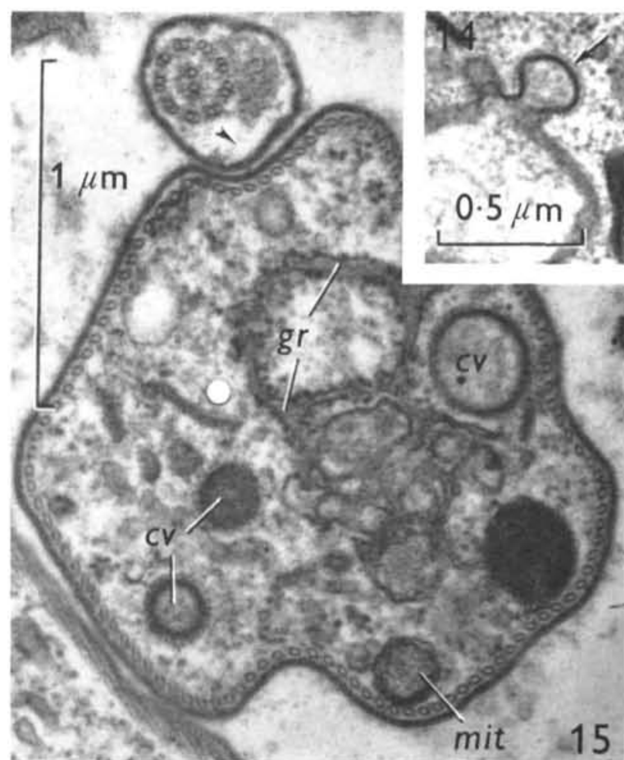
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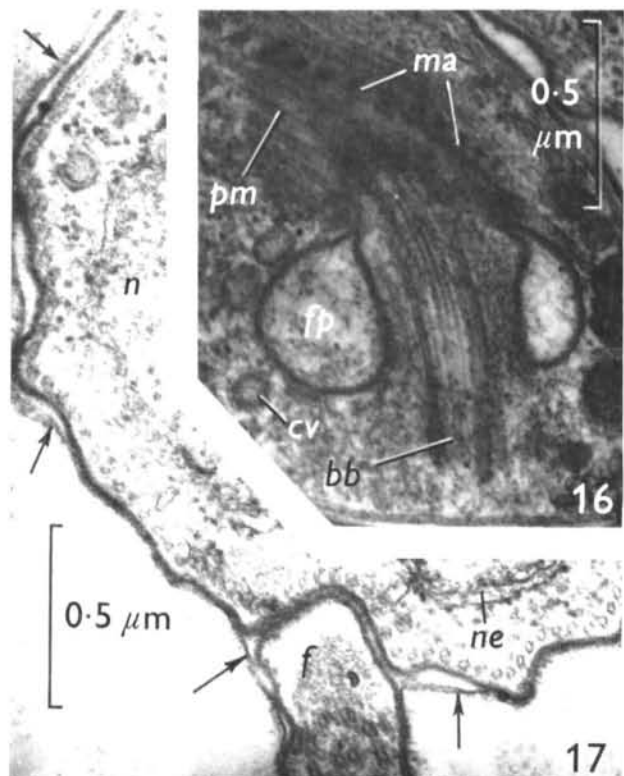
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Figs. 18–23. *T. congolense* bloodstream forms in section to show features of flagellar adhesion to pellicle. Glutaraldehyde fixation.

Fig. 18. Transverse section to show surface coat, and fibrous material of macula adherens (*ma*) in relation to other surface structures. Fine strands radiate from the macula towards underlying granular reticulum elements (*gr*) through a region of point densities (between small arrowheads) which are possibly fine filaments seen in cross-section. Between the macula and the flagellum-associated reticulum (*far*) lie 2 microtubules, one of normal dimensions, the other (*rm*) considerably reduced in diameter. On the flagellar side of the macula, a loose connexion to the paraxial rod (arrowed) can be seen. Some separation of flagellum and body has occurred in the adhesion zone (between large arrowheads). × 130000.

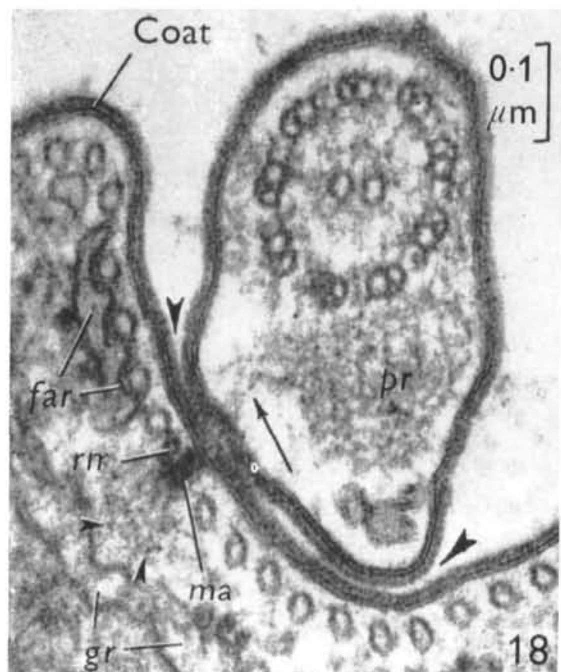
Fig. 19. Longitudinal section showing a series of maculae adherentes with their anchoring strands passing to longitudinally oriented filaments (arrowed) lying above the reticulum cisterna (*gr*). Note the gap between apposed surface coats. × 94000.

Fig. 20. Detail of transverse section of attachment, passing through space between two maculae, to show (in this case) two reduced microtubules (*rm*) one on either side of the row of maculae. × 125000.

Fig. 21. Similar detail to Fig. 20, but passing through a macula, showing that the unit membranes of flagellum and pellicle are not interrupted. Note the space between the two surface coats (small arrowheads) in the region of adhesion. × 168000.

Fig. 22. Transverse section of flagellum at point of emergence from the flagellar pocket, and just before the paraxial rod appears as a component of the flagellum: dense amorphous masses are present in the flagellar sheath (*fs*). The pellicular microtubules show the characteristics of the macular zone (*rm, far*) despite the absence of a paraxial rod. × 84000.

Fig. 23. Transverse section showing region of adhesion apposed to arc of axonemal portion of the flagellum. × 84000.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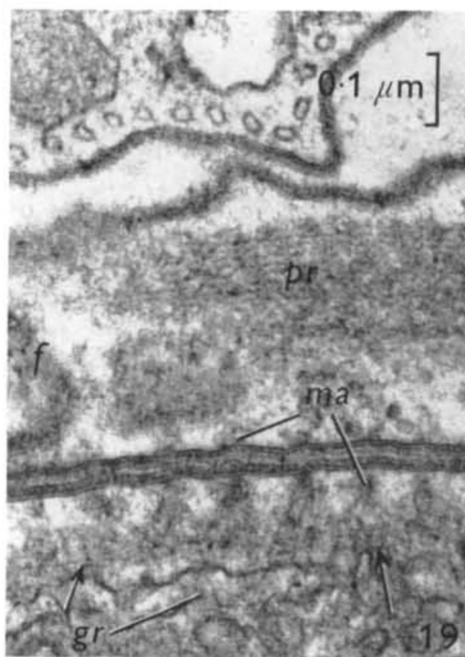
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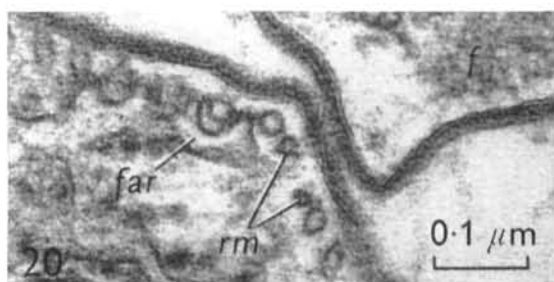
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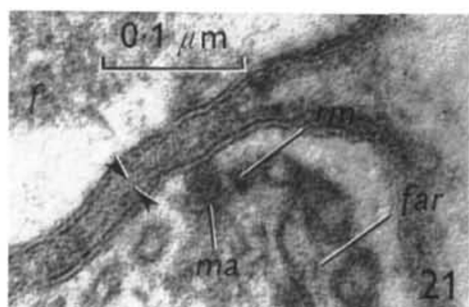
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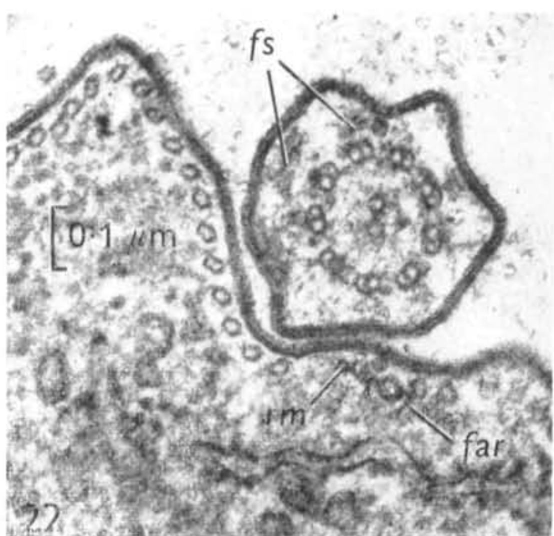
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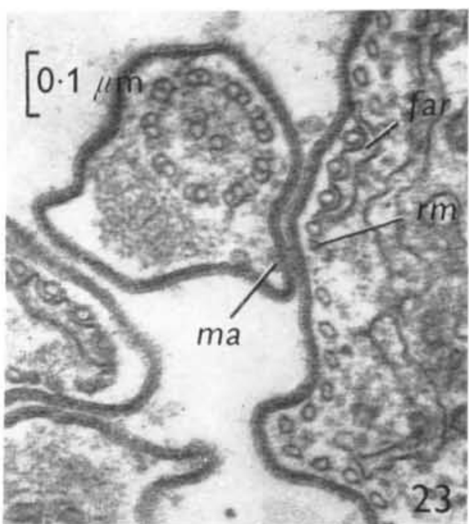
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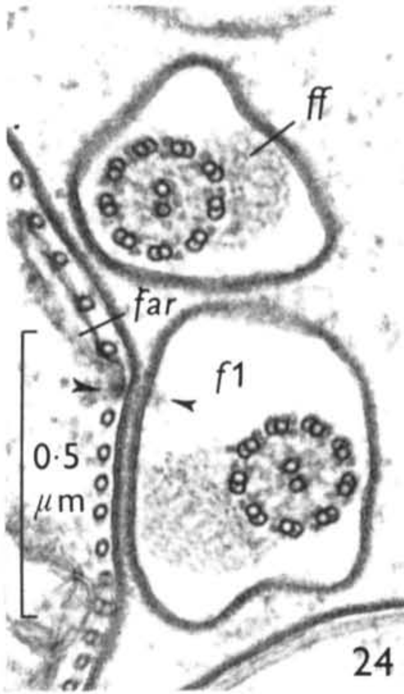
Fig. 24. *T. vivax* bloodstream form. Transverse section of flagellum (*f* 1) and adjacent surface with section of free flagellum (*ff*) of another trypanosome: the flagellar matrix is very swollen. Details of the surface coat and adhesion are very similar to those found in *T. rhodesiense* (and other *T. brucei*-like flagellates) and *T. congolense*. The macular attachment is indicated by arrowheads. OsO_4 fixation. × 75 000.

Fig. 25. *T. lewisi* late bloodstream form. Transverse section of pre-nuclear part of trypanosome showing fuzzy coat at low magnification. The macula adherens binds the flagellum to the body in the narrow adhesion zone. The subpellicular cisterna of granular reticulum to the right abuts on the macular region but is not in intimate contact with nearby microtubules as in the other species of trypanosome investigated. Glutaraldehyde fixation. × 62 000.

Fig. 26. *T. lewisi* late bloodstream form. Anterior tip of body with attached flagellum, showing surface coat at higher resolution. The coat is composed of fine filaments. The macular region shows no reduced microtubules or flagellum-associated reticulum. Glutaraldehyde fixation. × 84 000.

Fig. 27. *T. lewisi* late bloodstream form. Detail of flagellar attachment in mid-body. Note the inter-membranous line where the coats of flagellum and body meet (arrowed) in the adhesion zone which is partly apposed to the axoneme. Glutaraldehyde fixation. × 102 000.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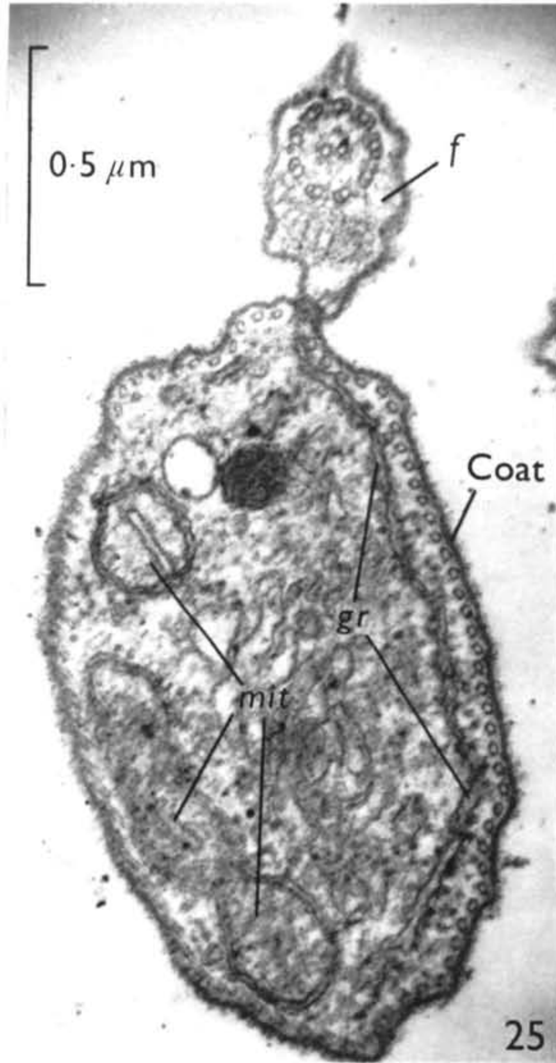
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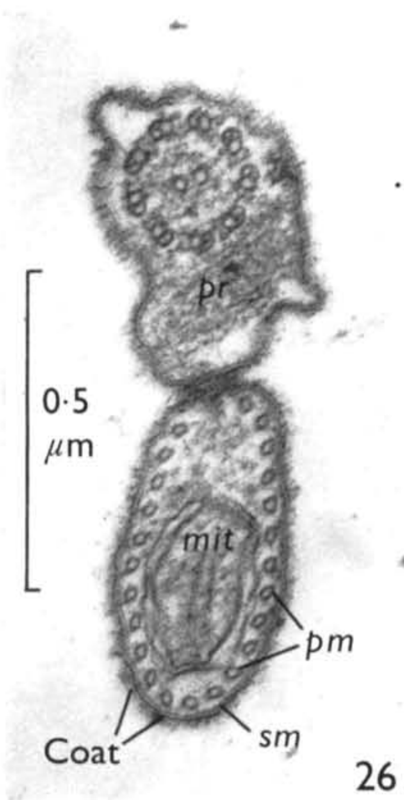
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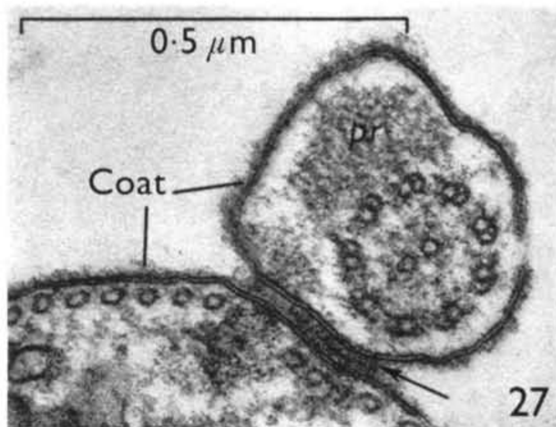
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