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Swiss Tropical Institute, Basle

On the Ultrastructure
of *Trypanosoma (Trypanozoon) brucei* in the Course
of its Life Cycle and Some Related Aspects

ROLF FRANKLIN STEIGER

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Abstract

Based on previous investigations by other authors on the ultrastructure of bloodstream and culture forms of *brucei*-subgroup trypanosomes, the present publication gives a detailed account of the structural transformations undergone by *T. brucei* in the course of its developmental cycle in the blood and in the vector. The most striking structural alterations concern the surface coat, the mitochondrion, the cytoplasmic membrane systems and the lipid droplets. The present study also deals with the cytochemical characterization of organelles and inclusions, and with the composition of the peritrophic "membrane". The penetration of the latter by special ectoperitrophic midgut forms is described.

The results are discussed with reference to the latest findings in the fields of trypanosome ultrastructure, immunology and biochemistry. The ultrastructural modifications during the life cycle of *T. brucei* reflect physiological alterations, such as mitochondrial activity, loss and reacquisition of surface antigens and lipid composition. Special emphasis is put on the formation of the surface coat in metacyclic salivary gland forms, and it is hoped that tracer experiments will elucidate the role of endogenous and exogenous factors.

I. Introduction

The systematics of mammalian trypanosomes have undergone considerable changes in the course of time and were finally reviewed by HOARE (1964), who worked out the valid modern classification based on morphological and physiological grounds (1966).

The former *T. brucei*-subgroup belongs to the section Salivaria, which is characterized by the development of the infective metacyclic (metatrypomastigote¹)

¹ For the characterization of the different developmental stages we follow the terminology by HOARE & WALLACE (1966).

forms in the "anterior station". The pleomorphic *T. brucei*, *T. gambiense* and *T. rhodesiense* as well as trypanosomes of the former *T. evansi*-subgroup range in the subgenus *Trypanozoon*. *Trypanosoma (Trypanozoon) brucei*, *T. (T.) gambiense* and *T. (T.) rhodesiense* are morphologically indistinguishable both in the mammalian bloodstream and in the vector *Glossina* (HOARE, 1949). Their main differences deal with a few physiological peculiarities, such as pathogenicity for mammalian hosts. *T. (T.) brucei* is the causative agent of Nagana in certain domestic ungulates; *T. (T.) gambiense* causes classical chronic human Sleeping Sickness and *T. (T.) rhodesiense* the acute Rhodesian type.

The cyclic development of the *brucei*-subgroup trypanosomes in the tsetse fly has extensively been studied with the aid of light microscopy for more than half a century. It has been summarized and revised by several authors (e.g. WENYON, 1926; BUXTON, 1955; GEIGY & HERBIG, 1955; NOBLE, 1955; MULLIGAN, 1970).

The existence of a developmental cycle of *T. brucei* in *G. palpalis* was first presumed by KLEINE (1909). About at the same time BRUCE et al. (1909) noted that trypanosomes taken up with a bloodmeal reacquire their infectivity for a monkey after some period (14th-18th day after the infecting bloodmeal).

BRUCE et al. (1911) observed that *T. gambiense* consecutively invades different fly organs at different periods. They identified several trypanosome forms and noticed that certain salivary gland forms resembled bloodforms. ROBERTSON (1913) gave a full account of the successive stages of *T. gambiense* in *G. palpalis*. She noted that the tsetse fly was capable of transmission some two to five days after invasion of the salivary glands, whereas proventricular forms failed to cause new infections in the mammalian host.

ROBERTSON (1912), ASHCROFT (1957) and WIJERS & WILLETT (1960) concluded from their experiments that the "stumpy" pleomorphic bloodstream forms are the stages most infecting to the tsetse fly and thus prerequisite to establish infections in the vector. This view has slightly been modified recently by BALBER (1970), who demonstrated that a few "slender" bloodstream forms retain a limited capability of transforming *in vitro*.

It was stated quite early (THOMSON & SINTON, 1912, quoted by VICKERMAN, 1962) that cultured *T. rhodesiense* are morphologically very much similar to midgut forms in the tsetse intestinal tract; a statement supported by recent ultra-structural and biochemical studies.

With the bloodmeal the trypanosomes enter the crop and the endoperitrophic space of the fly's midgut. They transform to elongated slender midgutforms by the 4th day and reach the ectoperitrophic space, in migrating down the intestinal tract, subsequently round the free end of the peritrophic "membrane" in the hindgut region and finally forward along the midgut (TAYLOR, 1932; YORKE et al., 1933). They multiply intensively and are concentrated in the foremost part of the midgut in the proventricular region. The flagellates cross the peritrophic "membrane" barrier, which is in secretion and therefore fluid-like, and reach the proventricular lumen around the 10th day (TAYLOR, 1932; VAN HOOFF & HENRARD, 1934; GEIGY et al., 1971). Opinions differ on the mode of development in the proventriculus (cardia). LEWIS & LANGRIDGE (1947) studied the development of *T. brucei* in *G. pallidipes* and *G. austeni*. They assumed a transformation of elongated trypomastigote proventricular forms to "post-proventricular" stages, which are supposed to give rise by unequal fission to long and short "crithidial" forms (epimastigotes); the latter are considered responsible for reaching the salivary glands. According to TAYLOR (1932) "filamentar crithidia" besides a lower amount of trypomastigotes are regarded as being migrating forms invading the salivary glands. BRUMPT (1927) stated that the invasion is "crithidial", whereas ROBERTSON (1913) and WENYON (1926) took it for being effected by trypanosomes

of the trypomastigote proventricular type. LEWIS & LANGRIDGE (1947) supposed that changes in the basic pattern of development might possibly be affected by the different *Glossina* species.

From the proventriculus the trypanosomes move forward down the foregut to the labrum and thence via the hypopharynx upwards to the salivary glands. An infection of the proventriculus usually precedes a gland infection (MURGATROYD & YORKE, 1937). The "crithidias" attach themselves to the epithelial lining in the middle part of the salivary glands adjacent to the salivary duct (ROBERTSON, 1913). There intensive multiplication occurs (TAYLOR, 1932). The epimastigotes attached to the epithelium transform to metacyclic forms, which are infective for the mammal. The process of invasion into the salivary glands is regarded as being continuous and thought to be effected by proventricular trypomastigotes (FAIRBAIRN & BURTT, 1946) or proventricular and subsequently "post-proventricular" forms (LEWIS & LANGRIDGE, 1947).

A salivary gland infection once established persists for the whole life of the tsetse fly (BURTT, 1953). Slender bloodforms develop from the metacyclic forms injected by the fly (GORDON & WILLETT, 1958). In contrast to HOARE (1945) some investigators suggested that salivary gland infections of *T. brucei*, *T. gambiense* and *T. rhodesiense* might be discernible on the basis of the flagellates' density and localization (VAN HOOF et al., 1944; LLOYD & JOHNSON, 1924).

Although flies proved to transmit an infection, trypanosomes could not always be found by dissection. These "cryptic" infections were first recorded by DUKE (1921). WARD & BELL (1971) even assumed infective proventricular forms in these cases; an observation which has not been confirmed in the course of this study.

The longevity of the flies is not influenced by infections with trypanosomes (DUKE, 1928 a). It is well known, however, that many factors affect the development of *brucei*-subgroup trypanosomes in the vector. Strain specificity is one factor (DUKE, 1928 b; TAYLOR, 1932; GEIGY et al., 1971). Strains with a low virulence, i.e. with a pronounced pleomorphism, induce higher infection rates possibly also concomitantly with a different speed of development (VAN HOOF et al., 1938), whereas monomorphic syringe-passaged laboratory strains of *brucei*-subgroup do not readily develop; their infections never reach the salivary glands (ROUBAUD & COLAS-BELCOUR, 1936; MURGATROYD & YORKE, 1937; reviewed by BUXTON, 1955).

There are evident indications for the temperature being one of the main factors with respect to infection rates and speed of development. Infected tsetse flies kept at higher temperatures (TAYLOR, 1932; DUKE, 1933) or the pupae of which have been incubated at raised temperatures (BURTT, 1946; FAIRBAIRN & BURTT, 1946), yield higher infection rates. Also the species of *Glossina* (e.g. GEIGY et al., 1971) and the fly's individuality (MURGATROYD & YORKE, 1937) might affect the ratio. BUXTON (1955) speaks in favour of a genetical explanation.

Some evidence exists that the mammalian species which the infecting meal has been derived from (CORSON, 1938; VAN HOOF et al., 1938; FAIRBAIRN & BURTT, 1946; VAN HOOF, 1947) or on which the flies are maintained after the infecting feed may affect the development of trypanosomes, as shown by ROUBAUD (1911) with *T. vivax* in *G. palpalis*.

This matter was followed up with cultured *T. brucei* (AMREIN et al., 1965; AMREIN & HANNEMAN, 1969), where it could be shown that the restitution of infectivity for the mammalian host partly depends on the individual donor of the blood added to the haemocultures. Parallel experiments with the fly material proved, however, that the infection rates of *T. brucei* in *Glossina morsitans* and *G. fuscipes* are not dependent on the individual blood quality offered to the flies (GEIGY et al., 1971).

The first electron microscopic observations of trypanosomes date back about 20 years, when whole mounts of *T. lewisi* (KLEINSCHMIDT & KINDER, 1950), *T. brucei* (KLEINSCHMIDT & SCHLEICH, 1951) and *T. evansi* (KRANEVELD et al., 1951) were studied. MUEHLPFORDT & BAYER (1961) examined the fine structural organization of monomorphic *T. gambiense* bloodforms. These authors were the first to describe the mitochondrion as a single tubular canal devoid of cristae. The connection between the kinetoplast and the mitochondrion was first observed in *T. cruzi* (MEYER et al., 1958), *T. mega* (STEINERT, 1960) and in other Trypanosomatids (CLARK & WALLACE, 1960). The structure and possible function of the kinetoplast was discussed and reviewed by MUEHLPFORDT (1963; 1964). Exact data on the comparative ultrastructure of bloodstream and culture (midgut) forms of *T. brucei* were provided by VICKERMAN (1962), who showed that the chondriome of the latter appears better developed and constitutes an elaborate network lined with numerous cristae. The author related the mitochondrial proliferation to changes in the pattern of respiration. Moreover, he suggested that the kinetoplast's shift to a more anterior position is achieved by the outgrowing posterior part of the mitochondrion. The switch in mitochondrial activity was the subject of further publications (VICKERMAN, 1965 a; b), where biochemical data were referred to. The same investigator studied the replication of the mitochondrion and the genetic systems (VICKERMAN, 1966 a; b). In addition, preliminary reports on the tsetse salivary gland forms (VICKERMAN, 1966 c), the description of the structure and function of the surface coat (VICKERMAN, 1968; 1969 a; b) and of the unique mode of nuclear division (VICKERMAN & PRESTON, 1970) as well as the *in vitro* transformation of bloodforms (VICKERMAN, 1971 a) were provided. The results obtained from these studies have recently been reviewed and discussed with respect to immunological and biochemical data (VICKERMAN, 1971 b).

The fine structure of the developmental forms of the same and allied trypanosome species have also been studied by other research workers. Briefly summarized it concerns the following trypanosome species: *T. avium* (BAKER & BIRD, 1968), *T. brucei* (WRIGHT & LUMSDEN, 1964; KUBO, 1968; RUDZINSKA & VICKERMAN, 1968), *T. conorhini* (MILDER & DEANE, 1967; MUSE & ROBERTS, 1969), *T. cruzi* (SCHULZ & MACCLURE, 1961; SANABRIA, 1963; 1964; 1966; 1968; 1969; WÉRY & DE GROODT-LASSEEL, 1966; MEYER, 1968; 1969; BRACK, 1968; SANABRIA & ARISTIMUÑO, 1970), *T. congolense* (VICKERMAN, 1969 b), *T. lewisi* (JUDGE & ANDERSON, 1964; ANDERSON & ELLIS, 1965; MOLYNEUX, 1969 a; b), *T. raiiae* (BOISSON et al., 1967 a), *T. rotatorium* (CREEMERS & JADIN, 1966), and akinetoplastic (dyskinetoplastic) trypanosomes (VICKERMAN, 1963; MILDER & DEANE, 1969).

It is the aim of the present study to give a comprehensive description of *T. brucei* in the course of its developmental cycle in the bloodstream and vector; pure ultrastructural analysis is supplemented by some cytochemical tests.

It concerns the subcellular modifications undergone during transformation to the different stages. Furthermore, related aspects, such as the nature and significance of the peritrophic "membrane", are taken into account.

The present publication is a thesis submitted in partial fulfillment of the requirements for the degree of Ph.D. at the University of Basle.

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II. Material and Methods

A. Basic ultra-cytology

1. Bloodstream forms of *T. brucei*

The pleomorphic bloodforms of the strain STIB 33 were processed as previously described (HECKER et al., 1972). The trypanosomes were separated from the blood of the rats by means of the DEAE-cellulose anion-exchanger method according to LANHAM & GODFREY (1970). The eluated flagellates were spun down at 1700 x g and thereafter fixed in 0.1 M cacodylate-buffered 2.5% glutaraldehyde (pH 7.3) for 1½ hour at 4°C, washed in cold 0.2 M cacodylate buffer with 5% saccharose and encapsulated in a drop of warm agar 2% (DIFCO Bacto) dissolved in the wash buffer. Prior to the solidification of the agar the trypanosomes were centrifuged down at 3000 x g. Small cubes of agar containing the bloodstream forms were cut with a razor blade, post-fixed and further processed as small tissue samples according to the method described below.

2. Vector stages

The fly maintenance, in the E.A.T.R.O. insectary at Tororo (Uganda), experimental procedures, light microscopical results and routine techniques needed for the light microscopical examination were essentially described elsewhere (GEIGY et al., 1971). Crops, midguts, proventriculi and salivary glands of *Glossina morsitans*, *G. fuscipes* and *G. pallidipes* heavily infected with *T. brucei* were collected at different intervals after the infecting bloodmeal (day 1 until day 30) in the course of infection experiments, the results of which were summarized in the publication cited (GEIGY et al., 1971). In consequence of its size the midgut was cut into three portions. The same was true of some infected salivary glands. This procedure helped to define the exact localization of the flagellates.

The specimens were fixed for electron microscopy in 0.1 M cacodylate-buffered glutaraldehyde (pH 7.3), at concentrations ranging from 1.5 to 5%, for 1–2 hours at 4°C.

Washing was carried out overnight in several changes of 0.2 M cacodylate buffer (pH 7.3) with 5% saccharose.

The organs were then post-fixed in 0.2 M cacodylate-buffered 2% osmium tetroxide (pH 7.2) for 1–2 hours at 4°C and at room temperature, dehydrated in acetone and propyleneoxide, and penetrated in mixtures of propyleneoxide/embedding medium. They were then embedded in Epon according to LUFT (1960), in freshly prepared Epon or Durcupan (Fluka).

Ultrathin sections (400–800 Å) were cut on Reichert OmU₂ and LKB Ultratome III microtomes with glass and diamond knives, respectively. The sections were mounted on parlodion-carbon-covered copper grids. Block-staining was performed during dehydration with 1% uranyl acetate in 70% acetone for 1 hour, and post-staining of the thin sections with 5% aqueous uranyl acetate for 10 minutes followed by REYNOLDS' (1963) lead citrate.

Finally, the sections were examined with Philips EM 300 or Zeiss EM 9 electron microscopes. Micrographs were taken on 70 mm roll films or plates, respectively, at magnifications ranging from 1100× to 220,000×. Semithin sections (1–2 μ) were prepared with the Reichert OmU₂ for checking the flagellates' density in the different organs concerned. They were examined light microscopically either by phase contrast or after staining with azur-II-methylene blue (Siegfried).

B. Cytochemical experiments on thin sections

1. Extraction of lipids

Epon-embedded sections from bloodstream and midgut forms of *T. brucei* as well as from the midgut epithelium were mounted on copper grids with or without a supporting film.

The method described by EURENIUS & JARSKAER (1970) used for the demonstration of lipids in crab oocytes was employed. It constitutes a modification of the original method recommended for the extraction of epoxy resin from light microscope sections (MAYOR et al., 1961). The grids with the sections were dipped into the lipid-specific epoxy solvent, made up by three parts stock solution and one part methanol-benzene mixture (1 : 1), for 10–40 seconds. The concentrated stock solution was sodium-methoxide dissolved in methanol-benzene.

Subsequent rinsing was performed in the methanol-benzene mixture for 1 minute, in two changes of 100% acetone for 30 seconds each and finally in redistilled water for 5 minutes.

If necessary the sections were contrasted. In the control experiment sodium-methoxide was omitted.

2. Demonstration of polysaccharides

Epon-embedded ultrathin sections of *T. brucei* bloodforms, midgut and foregut epithelia, and the peritrophic "membrane" in the proventriculus and the midgut region were mounted on filmless 300 mesh gold grids. The PA-TCH-silver albumose technique by THIÉRY (1967; 1969) was applied.

The sections were preoxidized with 1% periodic acid (PA) for 30 minutes, subsequently quickly washed in three changes of redistilled water and then kept floating on 0.2% thiocarbohydrazid (TCH) in 20% acetic acid for varying periods (1, 12, 16, 24, 36, 48 and 72 hours). Afterwards they were washed in 10 and 5% acetic acid for 15 minutes each and finally in three changes of redistilled water.

The grids containing the sections were then transferred onto a drop of aqueous 1% silver albumose solution; they were exposed in total darkness for 30–40 minutes, and finally rinsed again in redistilled water. The control sections were either preoxidized with 1% H₂O₂ or processed omitting TCH incubation. All reaction steps were carried out at room temperature; the sections were not post-stained.

3. Digestion with proteases

Slightly modified methods originally described by several authors were used (LEDUC & BERNHARD, 1961; ANDERSON & ANDRÉ, 1968; GEYER, 1969; WOLFF & SCHREINER, 1971).

Some of these investigators used water-soluble embedding media, which permitted a better enzyme penetration and action. For this study only Epon-embedded sections were available, which are known to reduce markedly the rate of penetration and hydrolysis. This might explain the limited reproducibility encountered.

Thin sections from newly synthesized and fully formed peritrophic "membranes" mounted on gold grids were preoxidized in 2–5% H_2O_2 or PA for 10–60 minutes at room temperature. Subsequent incubation was performed in a wide variety of enzyme solutions:

a) 0.01–0.1% pronase (Calbiochem) in redistilled water, adjusted to pH 7.4 with 0.01 N NaOH, for 20–40 minutes at 37 °C.

b) 0.05–0.1% pronase in 0.01 M phosphate buffer (pH 7.4) from 15 minutes up to 3 hours at 37 °C.

c) 0.1 and 0.2% pepsin (Schuchardt) dissolved in 0.1 N HCl for 10 and 20 minutes at 37 °C.

d) 0.2–0.3% trypsin (Schuchardt) dissolved in 0.1 M barbital-acetate buffer adjusted to pH 8.0, for 10–40 minutes at 37 °C.

The control sections were incubated in the enzyme solvents and without previous oxidation. Before use, the sections were rinsed with redistilled water and regularly post-stained with uranyl acetate and lead citrate. In order to investigate the chemical composition of the surface coat, similar procedures were conferred to ultrathin sections of bloodstream forms of *T. brucei*. These experiments resulted in an extreme decomposition of all cellular components thus impeding a reasonable interpretation.

C. Localization of enzymes by incubation

1. Peroxidase/catalase

Bloodforms of *T. brucei* were likewise used to demonstrate an eventual localization of peroxidase or catalase activity in the peroxisome-like organelles.

Slightly modified methods previously worked out by KARNOVSKY (1965) and GRAHAM & KARNOVSKY (1966) for tracing protein transport and used for the demonstration of endogenous peroxidases or catalases (MAGALHÃES & MAGALHÃES, 1971) were applied.

Fixation of the trypanosomes was carried out in 0.1 M cacodylate-buffered 2.5% glutaraldehyde (pH 7.3) for 20 minutes at 4 °C, or in 0.1 M phosphate-buffered 0.75% freshly prepared formaldehyde (pH 7.2) for 1 hour at 4 °C. The flagellates were then transferred to the incubation medium and left there for 10–30 minutes at room temperature. The standard medium was composed of 5 mg 3'3-DABTH (diaminobenzidine tetrahydrochloride, Sigma) dissolved in 10 ml 0.05 M tris-HCl buffer (pH 7.6), and 0.1 ml 1% H_2O_2 (freshly prepared from 30% Perohydrol, Merck).

The trypanosomes were then washed in three changes of cold tris-HCl-buffer for 5 minutes each; a quick rinse in saline followed the first buffer wash. Further processing was the same as for normal bloodforms. The incubation media of the control reactions either lacked H_2O_2 or DABTH.

2. Acid phosphatase

Tests for acid phosphatase with bloodforms of *T. brucei* were carried out using the widely applied lead-salt technique (GOMORI, 1956), modified by many research workers (e.g. ERICSSON & TRUMP, 1964-65) and recently employed for *Crithidia fasciculata* (BROOKER, 1971 a). After fixation in 0.1 M cacodylate-buffered 2.5% glutaraldehyde (pH 7.3) for 30-60 minutes at 4 °C, the flagellates were washed in two changes of 0.2 M NaCl in 0.1 M cacodylate buffer (pH 7.3) for 30 minutes each at 4 °C. They were then placed in the Gomori medium for 1 and 2 hours at room temperature.

Three different types of media were employed:

a) a standard Gomori medium, made up by 5 ml 3% Na- β -glycerophosphate and 60 mg Pb(NO₃)₂ in 50 ml 0.05 M acetate buffer (pH 5.0), incubated for 24 hours at 37 °C and filtered before use;

b) a freshly prepared filtrated medium of the same composition and application;

c) *do.* with 10% DMSO (dimethyl sulphoxide) added; according to GANDER & MOPPERT (1969) DMSO enhances the enzyme activity in the rat liver lysosomes, as revealed by both histochemical staining and quantitative evaluation of tissue homogenates. DMSO possibly induces reversible changes of the membrane proteins' configuration and transport selectivity (GERHARDS & GIBIAN, 1968).

Control specimens were incubated in media devoid of either the substrate or the acceptor.

After incubation the flagellates were extensively washed alternatively in two changes of the fixative and 2% acetic acid for 15 minutes each at 4 °C. Further processing was like with normal bloodforms.

The incubation methods for the eventual demonstration of the enzymes acid phosphatase and peroxidase/catalase required the centrifugation of the trypanosomes between the single steps of preparation before they were embedded in agar as mentioned before.

III. Results

A. Some light microscopical observations and annotations

Our results (GEIGY et al., 1971) obtained by the dissection of 1,500 infected flies principally corroborate what has comprehensively been said on the cyclic development of *T. brucei* in *Glossina* (see introduction). Variations within the same fly species and fly batch with respect to the speed and mode of development, besides infection rates, were evident. Following an infecting bloodmeal the trypanosomes are first found in the crop and then in the endoperitrophic space of the midgut, being slightly concentrated in the posterior part. They persist in the crop until the 6th day without establishing themselves; this was shown before by DUKE & MELLANBY (1936) with *T. rhodesiense* in *G. palpalis*. An "adjustment period" for trypanosomes in this organ of young flies is strongly suggested by HARMSSEN (1972), who presumes an enzymatic transformation of the trypanosomes just after the infecting feed. It would mean therefore that the destruction of non-transforming trypanosomes, derived from the crop, in transitory midgut infections, consti-

tutes an important establishment barrier. The flagellates failing to establish themselves are lysed and digested. The view of the author aforementioned is endorsed by the fact that occurrence of viable trypanosomes in the endoperitrophic space and in the crop parallels late established ectoperitrophic, midgut, proventricular und salivary gland infections.

It can be concluded that several factors must be prerequisite to the establishment of trypanosomes in their vector. The low infection rates (reviewed by BUXTON, 1955) normally encountered would imply the absence of at least one of these essential factors.

Only the interaction of all these prerequisites, e.g. enzymatic pre-adaptation of *T. brucei* in the bloodstream (formation of stumpy forms) and the vector ("adjustment period" in the crop), the age of the fly on the day of the infecting feed (length of the peritrophic "membrane"), nutritional requirements (COSGROVE, 1963), etc., would warrant the successful development in *Glossina*.

Normally by the 4th day after the bloodmeal the first ectoperitrophic midgutforms are located in the posterior segment of the midgut. The density of the flagellates varies considerably. Cases were observed, where on the 3rd day abundant trypanosomes, some of them still in transition, were encountered in the anterior part.

Yet, it seemed that the high division rate was confined to the posterior part only; from there daughter flagellates would move forward in the ectoperitrophic space. Around the 7th day the trypanosomes become concentrated in the foremost part of the midgut immediately posterior to the proventriculus and exhibit an enormous rate of division. In most cases the infection then spreads equally throughout the whole intestinal tract. The foremost part of the midgut remains a zone of intensive multiplication during the whole life of the fly.

Around the 10th day the proventriculus is invaded. At times, however, flies showing heavy midgut infections around the 15th day were devoid of a proventriculus infection. As to the development in this organ, pronounced variations were common. Either only elongated trypomastigote proventricular forms or epimastigotes mixed with the former stages were observed. This proved true for all three species of *Glossina* used. It must be admitted, however, that the ages of infection were not always the same; a different speed of development in the three fly species cannot be excluded. In *G. morsitans* the epimastigote phase in the proventriculus was practically always present and it is assumed therefore that the stages involved in the migration to the salivary glands are, in fact, epimastigote forms. It has to be mentioned that unequal binary fission of the "post-proventricular" forms giving rise to two types of epimastigotes (LEWIS & LANGRIDGE, 1947) was never observed with certainty. In one case a similar pattern appeared to be present.

This variation may also be attributed to degenerating developmental forms.

When the salivary glands became infected from the 18th day onwards epimastigotes could at first be seen attached to the epithelial lining. Most of the forms were concentrated in the middle part of the salivary glands nearest to the duct. Infections limited to one gland only (LLOYD & JOHNSON, 1924) were not observed with our material. The production of metacyclic (metatrypomastigote) forms from epimastigotes commences and these infective forms are located free in the glands' lumen. Some time (1-2 days) passed before they could be ascertained, an observation analogous to that by ROBERTSON (1913). It seems likely that this period is needed for the transitional development. It was a striking fact that the density of the flagellates in the salivary glands differed very much within the three fly species, irrespective of the age of infection. *G. morsitans* possessed the heaviest gland infections, whereas *G. fuscipes* always had the lowest ones.

Several mature metacyclic infections were indirectly traced by feeding the flies on clean rats. In some of the flies, which proved to transmit, hardly any trypanosomes could be found by dissection. Maybe the trypanosome reserve was exhausted. In these "cryptic" infections (DUKE, 1921; BURTT, 1946) a few flagellates appeared in the salivary duct only. This point indicates that periodic fluctuations in the flagellates' density do occur; this was previously shown by means of the saliva probing technique (FAIRBAIRN & BURTT, 1946), where the total number of metacyclic stages and other developmental forms ejected varied considerably from day to day. This can be explained in terms of different rates of division and the fortified attachment (p. 87) of the epimastigotes, and different rates of invasion of the salivary glands. Therefore also the production of metacyclics may vary. Moreover, one can presume that the dose of infective trypanosomes injected by the fly into the mammal is decisive (FAIRBAIRN & BURTT, 1946).

B. Electron microscopical observations

1. Normal ultrastructure (Table 1, p. 96)

1.1. Bloodstream forms (Figs. 1-18)

1.1.1. Non-dividing slender bloodform

Accounts of the fine structure of *T. brucei* bloodforms were provided by MUEHLFORDT & BAYER (1961), RUDZINSKA & VICKERMAN (1968) and KUBO (1968). The whole trypanosomal cell, flagellum and reservoir (flagellar pocket) included, is limited by a trilaminar unit membrane (pellicula, pellicle), measuring 60-80 Å in thickness (Figs. 1, 2).

The outer layer of the pellicula appears somewhat thicker and more electron-dense. Additionally, the pellicula is uniformly covered with a surface coat 120–150 Å thick of moderate electron-density (VICKERMAN, 1969a) (Figs. 2, 10). This coat appears homogenous and amorphous, but at higher magnifications rather fine-flocculent to filamentous (Fig. 10). Regularly arranged subpellicular microtubules (ANDERSON & ELLIS, 1965; “subpellicular striations”: MEYER & PORTER, 1954; “pellicular fibrils”: VICKERMAN, 1962), run beneath the pellicula and form the periplast layer (Figs. 1 inset, 2, 10). These microtubules (SCHULZ & MACCLURE, 1961) measure about 180–250 Å in diameter. The structure and arrangement of the pellicular tubules in Trypanosomatids was investigated by ANGELOPOULOS (1970). The microtubular walls are clearly made up by subunits as shown by osmiophilic globular particles (Fig. 10). The distance between the subpellicular microtubules and the pellicula measures some 100 Å, whereas single microtubules are separated from each other by a rather wide gap of 250–300 Å. Fine-filamentous intertubular connections (FUGE, 1968) between subpellicular microtubules can be seen (Fig. 10). Microtubules are absent from the invaginated cell membrane portion of the flagellar pocket, except for three to four tubules, which lie much closer together than those aforementioned and perpendicular to them. They are located a short distance (80 Å) from the reservoir membrane (Fig. 1, inset).

The unique subpellicular organelle, originally described by FUGE (1968) and TAYLOR & GODFREY (1969), is frequently encountered. It is a structure lying near the point of flagellar attachment, made up by four subpellicular microtubules and a vesicular membrane, which is continuous with the rer and the perinuclear cisterna (Fig. 10, inset). The subpellicular microtubules show wider gaps at flagellar attachment zones (ANDERSON & ELLIS, 1965; VICKERMAN, 1969a). At these points, desmosome-like plaques (macula adhaerens type: FAWCETT, 1966), 65–70 m μ wide, can be seen at regular intervals (20 m μ) (Fig. 10).

The nucleus lies in the middle of the cell and is slightly rounded to elliptical, the long diameter being 1.8–2.5 μ , the shorter one 1.1–1.5 μ . It is surrounded by two nuclear membranes (nuclear envelope), which are separated from each other by a space of 150–200 Å (perinuclear cisterna) (Figs. 1, 5). More or less regularly arranged nuclear pores in the nuclear envelope are visible. The outer nuclear membrane is studded with ribosomes (Fig. 5). The nucleoplasm is fine-granular to filamentous and of low electron-density. It is interspersed with coarsely granular chromatin-like masses (“chromatin-like material”: VICKERMAN & PRESTON, 1970), which are very often concentrated on the inner nuclear membrane (Fig. 5). The round endosome representing the structure analogous to the nucleolus of eukaryotic cells lies in the middle of the nucleus or slightly eccentric (Figs. 1, 5). It consists

of fine-granular homogenous material and measures $0.5-0.8 \mu$ in diameter.

The characteristic mitochondrion (chondriome) originates in the subterminal kinetoplast (Fig. 2). The kinetoplast has a capsular shape and appears rod-like in transverse sections (Figs. 2, 3). It can be regarded as a specialized part of the chondriome, where mitochondrial DNA is concentrated (STEINERT, 1964); reviews on this subject were given by MUEHLPFORDT (1963, 1964) and VICKERMAN (1970a). The kinetoplast is bounded by the two mitochondrial membranes (Figs. 2, 3). The concentrated mitochondrial DNA, often referred to as "kinetonucleus" (INOKI et al., 1971), appears regularly coiled comprising closely packed figure-8 circles of high electron-density (Fig. 3). The arrangement of the kinetoplast DNA (K-DNA) in *T. cruzi* was thoroughly studied and described by BRACK (1968). The kinetoplast of *T. cruzi* is larger than that of *T. brucei*.

The kinetoplast measures $0.6-0.65 \mu$ in length and $0.2-0.3 \mu$ in width, whereas the coiled DNA structures measures $0.45-0.50 \mu$ and $0.10-0.15 \mu$, respectively. Moreover, the kinetonucleus is embedded in a matrix of moderate electron-density, which conforms to the normal mitochondrial matrix (Fig. 2). The kinetoplast is slightly curved; on the convex side some tubular cristae may be seen (Fig. 2). On the concave side facing the basal body a more-electron-dense, plate-like structure lining the kinetoplast envelope is sometimes found (Fig. 2). This structure has recently been described and discussed as "basement plate" (INOKI et al., 1971).

A single large mitochondrial tube (MUEHLPFORDT & BAYER, 1961; VICKERMAN, 1962) originating in the kinetoplast is composed of a long anterior and a very short posterior part; it extends along the cell more or less parallel to and underneath the pellicular membrane (Figs. 1, 2). The matrix is moderately electron-dense. Mitochondrial tubules are rare or absent (Fig. 1). The mitochondrial membranes appear somewhat undulated, the space between the two membranes being about 100 \AA wide. Occasionally, in the post-nuclear region, the chondriome proliferates and forms a labyrinth-like structure lacking any cristae (BROOKER, 1971a) (Fig. 4).

The Golgi apparatus ("parabasal body": GRIMSTONE, 1959; GRASSÉ, 1966) lies near the reservoir between kinetoplast and nucleus. It comprises four to six parallel cisternae and many Golgi vesicles budding off laterally (VICKERMAN, 1969b) (Figs. 1, 11). At times, large cisternae giving rise to a great number of vesicles can be seen, which give this area a fenestrated aspect (Fig. 11). Golgi vesicles may also be seen around the flagellar pocket (see cytochemical experiments) and near multivesiculate bodies (Fig. 13). The Golgi complex is closely associated with the membrane systems of the smooth and the rough endo-

plasmic reticulum, as well as lysosomal structures, a feature described as GERL (NOVIKOFF et al., 1971) (Fig. 12).

The rer is rather inconspicuous and is made up by but a few profiles regularly lined with ribosomes (Fig. 5). These small bits of membrane are continuous with the membranes of the nuclear envelope (perinuclear cisterna). The smooth endoplasmic reticulum (ser), however, is very well developed and composed of many tubes of different size and shape not showing any evident orientation (Fig. 12). They can be found in all parts of the cell, but occur concentrated in the GERL area. Segments of ser tubes appear quite often swollen and filled with material of moderate electron-density much alike the matrix of peroxisome-like organelles (Fig. 7). Sometimes, the smooth endoplasmic reticulum forms a vesicular network reminding of the labyrinth-like structure described above for mitochondrial extensions (Figs. 6, 49).

Peroxisome-like organelles (VICKERMAN, 1969a), synonymous with the "Type I granules" (BIRD et al., 1966), "the dense bodies" (VICKERMAN, 1971b) and the "GPO bodies" (BAYNE et al., 1969b) are scattered throughout the cell but seem to be somewhat more numerous in the GERL area between nucleus and reservoir. They are spherical (0.25 to 0.50 μ in diameter) and membrane-bounded (Figs. 1, 7). The limiting membrane, quite similar to the mitochondrial membranes, appears undulated. The matrix is flocculent to fine-granular sometimes equipped with small membraneous inclusions (Fig. 7, inset). The peroxisome-like organelles appear closely associated with the smooth endoplasmic reticulum. They seem to lead, by transition, to the formation of multivesiculate bodies (Fig. 13). The matrix becomes heterogenous and many vesicular structures emerge. These vesicles seem to be contributed by the Golgi zone. In addition, cytoplasmic "exocytotic" processes into the mvb's lumen are observed.

Besides multivesiculate bodies, a variety of other lysosomal organelles can be found (see also cytochemical experiments). The Golgi vesicles around the base of the flagellum probably represent primary and/or secondary lysosomes (DE DUVE & WATTIAUX, 1966), a fact which is strongly supported by cytochemical staining (p. 95). Autolyosomal structures formed by concentric growth of ser tubes are frequently seen (Fig. 12). Even dense-body-like organelles containing membraneous material are found. The multivesiculate bodies (mvb) are identical with the "Type III granules" (BIRD et al., 1966); they are found in the GERL zone, too.

The reservoir or flagellar pocket constitutes an invaginated portion of the pellicular membrane. This structure is considered by many authors to represent the site of food storage (especially proteins), and the reservoir membrane is attributed pinocytotic activity (ARMSTRONG et al., 1964; BROOKER & VICKERMAN, 1964; BROWN et al., 1965;

BROOKER & PRESTON, 1967; SEED et al., 1967; JADIN & CREEMERS, 1968; VICKERMAN, 1969a, b; GEIGY et al., 1970). The lumen of the flagellar pocket is mostly filled with flocculent material and possibly exocytosed particles ("débris": VICKERMAN, 1969b) (Fig. 8). The sequence of the formation of pinocytotic vesicles can be traced: first, invaginations of the reservoir membrane into the cytoplasm are visible; then, vesicles are formed at the tips of these membrane invaginations (Fig. 9), which, finally, are released into the trypanosome's cytoplasm. The perfected vesicles are similar to the "coated" vesicles, previously described as pinocytotic vesicles in insect cells (ANDERSON, 1969; ROTH & PORTER, 1964), and have already been reported from trypanosomes (VICKERMAN, 1969a). They are relatively small (0.1μ in diameter). In contrast to the "coated" vesicles of insect oocytes, they do not lose their proteinaceous flocculent covering on the surface of their limiting membrane upon entry into the cytoplasm.

The basal body of the flagellum lies anterior to the kinetoplast (Fig. 1, inset). Its fine structural organization has extensively been studied in *T. cruzi* by BRACK (1968) among other investigators (GIBBONS & GRIMSTONE, 1960; VICKERMAN, 1970a). This cylindrical centriole-like organelle is limited laterally by the nine peripheral flagellar fibrils. At the basis these fibrils, consisting of two connected subfibrils, divide into three subfibrils, which exhibit the typical triplet structure viewed in transverse sections. The triplets appear slightly twisted. Moreover, two septa arising from the basal body's wall project towards the basal body's centre. The two central fibrils of the flagellar axoneme pass the distal septum and end in the basal plate. The flagellum, which originates in the basal body, possesses the two central fibrils and nine peripheral fibril doublets composed of two subfibrils A and B (Fig. 10). Subfibril A has a circular profile, while subfibril B appears crescentic and sharing a sector of the wall of subfibril A. Arm-like appendages of the subfibrils A face the subfibrils B of adjacent doublets (VICKERMAN, 1969a). The flagellar fine structure was investigated by many authors (VICKERMAN, 1962; ANDERSON & ELLIS, 1965; BOISSON et al., 1965; GRIMSTONE & KLUG, 1966; FUGE, 1968). The fibrils and subfibrils, in fact, are microtubular structures and coincide in dimension and morphology with the subpellicular microtubules. Again, at higher magnifications, subunits in the microtubular walls represented by globular osmiophilic particles can be recognized. Fine-filamentous connections between the central tubules and the peripheral tubules, as well as between the peripheral tubules and the flagellar membrane are common (Fig. 10).

The well known intra-flagellar structure (IFS: ANDERSON & ELLIS, 1965; FUGE, 1969) runs alongside the tubular elements (axoneme) of the flagellum, which exhibit the characteristic 9 + 2 arrangement

(Fig. 10). The IFS is often referred to by other authors as “honeycomb” (MILDER & DEANE, 1967), “paraxial rod” (VICKERMAN, 1969a; b) and “bande striée du flagelle” (BOISSON et al., 1965). It is composed of filaments, approximately 40–60 Å thick, running in different planes giving this structure a lattice-like feature. The detailed ultrastructure was studied in *T. brucei* by FUGE (1969). The IFS shows fine-filamentous connections with the peripheral doublets and is attached to the flagellar membrane (Fig. 10). This membrane is continuous with the pellicula in the region of the reservoir and is formed when a new flagellum grows out from the basal body into the flagellar pocket, and from there alongside the cell body (Fig. 1). Then, evident attachment zones between the pellicular and the flagellar membrane are formed (p. 121). It must be added that the basal body and the flagellum do not undergo basic changes in the ultrastructural organization in the course of the whole developmental cycle of *T. brucei* in the bloodstream and the vector. Sometimes, small vesicles often next to or continuous with the flagellar membrane can be seen at the flagellar periphery (Fig. 8). Similar vesicles are also found in *Herpetomonas muscarum* (BRUN, personal communication).

Lipid inclusions (“Type II granules”, “endoplasmic reticular granules” according to BIRD et al., 1966, and MOLLOY & ORMEROD, 1971, respectively) are quite abundant in the whole cytoplasm. Their lipoid composition was strongly suggested by BIRD et al. (1970) and by VENKATESAN (1972), and will be confirmed by a cytochemical test (p. 91).

These lipid droplets are small (0.15–0.20 μ in diameter) and spherical in shape, never bounded by a true unit membrane, and appear dark electron-dense and osmiophilic (Figs. 1, 13). They do not show any structural connections with other organelles.

Masses of free ribosomes are often knitted together to form polysome-like complexes; they are visible in the whole cytoplasm and appear embedded in a rather flocculent ground cytoplasm (Fig. 1, inset).

1.1.2. Dividing slender bloodform

Many dividing trypanosomes are observed in the bloodstream. Their division mechanism involves some cytoplasmic organelles. The division cycle in trypanosomes with respect to the behaviour of the kinetoplast and the basal body (BURTON & DUSANIC, 1968; INOKI & OZEKI, 1969), nucleus and endosome (VICKERMAN & PRESTON, 1970), as well as the mitochondrion (VICKERMAN, 1966a) has extensively been studied.

First, the basal body replicates, the daughter basal body ("prokinetosome": PAULIN, 1969) lying next to the flagellum-bearing old one. The daughter basal body represents a newly synthesized centriole-like structure, which the new flagellum is growing out from.

At the same time, the kinetoplast and the kinetoplast DNA (kinetoplast nucleus) increase in length until reaching the double size needed for equal division of the kinetoplast (kinetoplast approx. $1.0-1.2\ \mu$ in length; K-DNA $0.8\ \mu$ in length). Then the elongated kinetoplast bends in the middle and cytoplasmic inpocketing (RUDZINSKA & VICKERMAN, 1968) accompanied by membrane incision separates the kinetoplast.

DNA replication shows the following features: during the kinetoplast's bending the fibrillar aspect of the DNA disappears to some extent being replaced by an amorphous structure (INOKI & OZEKI, 1969), which might represent the site of active DNA synthesis. As division proceeds, the amorphous mass gets lost and fibres become visible again. Finally, the normal shape and configuration is re-acquired. Amorphous structures after the complete separation of kinetoplasts have never been observed. The outgrowing new flagellum seems to be bounded by a separate flagellar sheath of its own. However, modifications in this context are possible (p. 86).

The nuclear division is effected by endonuclear spindle microtubules, an event fully described in *T. rhodesiense* and *T. raiae* by VICKERMAN & PRESTON (1970). Similar observations were made by BIANCHI et al. (1969) in the leptomonads of *Leishmania tropica* and by INOKI & OZEKI (1969) in *T. gambiense*.

In consecutive steps the nucleus becomes elongated, whilst the nuclear membranes persist, and the two halves of the nucleus are pushed apart by the outgrowing spindle microtubules (Figs. 14, 15). Thereby the endosome is also pulled apart in the spindle axis and forms the "laminated plaques" (VICKERMAN & PRESTON, 1970) (Fig. 16). Condensed chromatin-like masses appear sometimes attached to the inner membrane of the nuclear envelope. A gradually increased constriction in the middle part of the nucleus leads to the complete separation of the two daughter nuclei.

During division, the mitochondrion of *T. brucei* is supposed to split (VICKERMAN, 1966a). However, it is assumed that splitting is accompanied by partial outgrowth of a new mitochondrion following cell division.

The remaining cell organelles seem to be distributed in more or less equal numbers when the cell body divides, at last. Cell divisions of *T. brucei* proceed principally in the same way, with a few exceptions, in all developmental forms during the whole life-cycle. Equatorial divisions characteristic of epimastigotes of *T. cruzi* have not been observed in bloodforms.

1.1.3. Stumpy bloodform

Apart from physiological differences the stumpy forms of *T. brucei* exhibit evident structural modifications as compared to the slender form.

The nucleus is more rounded, and the nucleoplasm contains only inconspicuous chromatin-like masses (Fig. 17). By analogy with eukaryotic cells its state could be circumscribed as "euchromatic". The membrane systems (Golgi, rer, ser and lysosomes = GERL) show more elaborate features (Figs. 17, 18). Especially the ser-mediated formation of peroxisome-like organelles is intensified. The reservoir or flagellar pocket appears somewhat larger.

Intrinsic modifications concern the morphology of the mitochondrion. The chondriome is considerably enlarged (VICKERMAN, 1965a; b), due to the outgrowth of the anterior part of the mitochondrial tube; the mitochondrion begins to form typical branchings, which are to become even more elaborate during the transformation to midgutforms in the vector *Glossina*. Many mitochondrial tubules (tubular cristae) are present (Figs. 17, 18). They are not parallelly aligned and project into a fine-flocculent little electron-dense matrix.

The mitochondrial proliferation and the acquisition of cristae can clearly be correlated with differences in enzymatic activities (VICKERMAN, 1965a, b; 1970a; 1971a).

Biochemical data in this context and especially concerning the comparison between bloodstream and culture/midgut forms were provided by several authors (FULTON & SPOONER, 1957; VON BRAND, 1960; GRANT & SARGENT, 1960; GRANT & SARGENT, 1961b; RYLEY, 1962, 1966; RAUSCHENBACH, 1967; BOWMAN & FLYNN, 1968; FLYNN & BOWMAN, 1970; reviewed by HILL & ANDERSON, 1970).

1.1.4. Intermediate bloodform

The stage of the stumpy form is reached by the slender form through intermediate stages. The latter are similar to the stumpy forms from the structural and physiological point of view (VICKERMAN, 1965a).

The mitochondrion is about to proliferate and quite a number of tubular cristae are already present. Morphometrical data on the transition of the three pleomorphic types of *T. brucei* bloodforms, with respect to the different cell organelles, have recently been contributed by HECKER et al. (1972).

1.2. Midgut forms (Figs. 19–29)

1.2.1. Deteriorated transition form

This term comprises bloodforms in the endoperitrophic space, which, upon entry into the vector, are not able to transform to viable midgut stages and to establish themselves.

They are found in early infections (day 1-4) and are structurally comparable to lysed flagellates found also in advanced established midgut infections.

Their morphology is characterized by a gradual desintegration of the whole trypanosomal cell, akin to the structure of *T. cruzi* in aged cultures (WÉRY & DE GROODT-LASSEEL, 1966).

The nucleus becomes more and more pycnotic, while vacuoles of different shape and size accumulate (Fig. 19). These increase in number. Finally, a lytic aspect of the whole cell, as revealed by large mvb complexes, is given (Fig. 20). Moreover, the ground cytoplasm appears structureless, and the cell contours shrunk with irregular features. Defined membranes disappear, the kinetoplast DNA remaining practically unaffected (Fig. 19). Total vacuolization and the loss of any cell differentiation account for the last stage of the deterioration processes.

The surface coat is immediately lost and the periplast microtubules display total desorientation.

1.2.2. Surviving transition form

The transition of bloodstream to culture forms was studied by BROWN & EVANS (1971) and by VICKERMAN (1971b). The surface coat is lost (VICKERMAN, 1964a), though it can persist, at times, until the 4th day after the ingestion of an infecting bloodmeal (Fig. 21, inset). The kinetoplast migrates forward (Fig. 21). The number of rer profiles decreases paralleled by an apparent increase of free ribosomes or polyosomes (Fig. 21). The Golgi complex is large being composed of extended cisternae. The mitochondrion is markedly proliferating (VICKERMAN, 1962), the posterior part being expanding to a great extent. This event is closely connected with the kinetoplast's displacement (Fig. 21).

The tubular cristae (mixed type) are enlarged, in comparison with the ones occurring in the stumpy bloodform (Fig. 21). The peroxisome-like organelles sometimes tend to be somewhat oblong and are therefore comparable to the "bacilliform bodies" in *Crithidia* (BROOKER, 1971a). The kinetoplast retains its normal ultrastructure described before for pleomorphic bloodforms. Multivesiculate bodies are present, whereas lipid inclusions are rare. The ser is inconspicuous or absent. Pinocytosis is not observed, whereas exocytosed material can frequently be found in the flagellar pocket.

Many kinetoplast divisions are seen. The divisions either proceed conformably to the mode described before in bloodforms or they are of the equatorial type (BRACK, 1968) (Fig. 22). The surviving transition forms occur in the endoperitrophic space only; never have parasites actively crossing the peritrophic "membrane" been observed.

1.2.3. Established midgutform

These developmental stages occur in the ectoperitrophic space from day 4 on. However, it must be pointed out that, in late heavy midgut infections, they are never absent from the endoperitrophic space, where they appear viable and unaffected. The bulk of the ectoperitrophic midgutforms is concentrated in the forepart of the midgut. They are regarded as being different, in terms of structural organization, from the ectoperitrophic forms in the foremost part of the midgut (proventricular region). The latter are described later.

The former resemble in fine structure trypomastigote culture forms (VICKERMAN, 1962) and exhibit the following morphological peculiarities: the surface coat is absent (Fig. 25). The nucleus is oval in shape, measuring 2.0–2.5 μ in length (Fig. 23). The structure of the kinetoplast tallies with the general organization mentioned for the precedent stages. It is situated at half-distance between the nucleus and the posterior end. The mitochondrial network is even more extended and possesses a great number of tubular cristae (Fig. 26); these are not parallelly arranged and do not show any evident orientation. The mitochondrial matrix remains moderately electron-dense. The Golgi apparatus seems to be well developed (Fig. 25). It has a vesicular appearance produced by many vesicles of different size and electron-density. Some of these vesicles may represent primary lysosomes or contribute to the formation of mvb, because they are often found close to the multivesiculate bodies and correspond in shape and size to the mvb vesicles. Additionally, larger Golgi vesicles (0.15 μ in diameter) display an association with some er profiles (Fig. 25).

The rer, made up by but a few tubes, seems to be in formation (Fig. 27). The ribosomal lining is irregular and not very dense like in bloodforms. Also the ser system is clearly reduced. The basal body and the flagellum appear unchanged. Many desmosome-like junctions between the flagellum and the pellicle are detected (Fig. 25).

Lysosomal structures increase in number. Golgi-derived vesicles are frequent near the reservoir membrane. Multivesiculate bodies are abundant (Figs. 24, 25, 27), while dense and residual bodies are missing.

Lipid inclusions quite different from those found in bloodforms are numerous. They are significantly larger (0.3–0.5 μ in diameter) and much less electron-dense (Figs. 23, 24, 27). These droplets are evenly distributed in the cytoplasm. Due to slightly inadequate fixation they often appear washed out or wrinkled (for identification of the lipid nature see cytochemical experiments).

Peroxisome-like organelles remain unchanged as regards distribution and morphology. Even more free ribosomes give the ground cytoplasm a homogeneously granular aspect (Fig. 2).

Many dividing midgutforms are found; the division cycle follows the regular pattern formerly described for bloodforms. Equatorial division was observed in one case.

Most frequently, trypanosomes in the deep folds of the peritrophic "membrane" and sometimes also embedded in the "PM" are seen (Fig. 24). Embedded trypanosomes appear between layer I and layer II (MOLOO et al., 1970) of the peritrophic "membrane". The two layers are pulled apart leaving a cavity (up to 2.5μ in width) with a fine flocculent content enfolding midgutforms of *T. brucei* (Fig. 24, inset).

For the cytochemical staining of the "PM" look up cytochemical experiments (p. 93).

1.2.4. Ectoperitrophic midgutform in the proventricular region

This stage of development of *T. brucei* is found in the foremost part of the midgut, located in the space limited by the midgut epithelium and the peritrophic "membrane" in front of the "press" (WIGGLESWORTH, 1929), which is formed by the fore-gut invagination (cardial valve) and the proximal midgut epithelium (Fig. 28). There the flagellates are closely packed and crowded together (Figs. 28, 29). Generally, these forms resemble the established midgutforms just described. Yet, some structural modifications can be recognized.

The nucleus is longer (up to 3.5μ), and the endosome indistinct and less contrasted. The nucleus/cytoplasm ratio shifts in favour of the nucleus.

An increased number of large multivesiculate bodies, 1μ in diameter, can be found (Fig. 29). They seem to be built up, in part, by the transformation of peroxisome-like organelles, since the latter show a definite decrease in quantity.

The small osmiophilic lipid inclusions reported from bloodforms and absent from the preceding midgut stage, emerge again besides the larger lipid droplet type cited before (Fig. 29). The latter, however, is less numerous.

The ectoperitrophic midgutforms of the proventricular region are considered transition stages giving rise to the trypomastigote proventricular form. They are responsible, with certainty, for crossing the peritrophic "membrane" barrier.

1.2.5. Penetration of the peritrophic "membrane"

The pervasion of the "PM" is effected by the transition forms mentioned above. It is achieved in the region of the Type III midgut epithelial cells (MOLOO et al., 1970), which synthesize the second, thicker layer of the peritrophic "membrane". It has not been observed in the

region of the Type II cells, as predicted by the same authors. The penetration area lies ahead of the "press" (WIGGLESWORTH, 1929), where the thickness of the "PM" varies considerably (0.5–3.5 μ). There the peritrophic laminae seem not to be fully polymerized yet (YORKE et al., 1933; FAIRBAIRN, 1958). Different steps of penetration are distinguished:

a) the trypanosomes are embedded in the "PM" (Fig. 29, inset);

b) later on, they appear at the periphery (layer I) and lie there in a stratum-like distribution (Fig. 29). Layer I still persists and borders the trypanosomes towards the proventricular lumen;

c) the third and final step is specified by the rupture of layer I and the subsequent release of the trypanosomes into the lumen of the proventriculus (cardia), where they transform to trypomastigote proventricular forms.

An interesting observation concerns the direction of penetration, never were trypanosomes lying perpendicular to the "PM" found, but only flagellates in oblique or nearly horizontal positions.

1.3. Trypanosomes in the proventriculus (cardia) (Figs. 30–35)

Most of the forms occurring in the proventriculus are trypomastigotes; however, epimastigotes and transition forms, constituting the intermediate stage, are always present.

1.3.1. *Trypomastigote proventricular form*

This stage is detected from day 9 on. These forms are structurally akin to the ectoperitrophic midgutforms in the foremost part of the midgut involved in the penetration of the "PM". The nucleus is slightly longer (up to 4 μ). The endosome remains small (0.3–0.4 μ diameter), and its position is strictly central.

The kinetoplast, again, appears practically unchanged being somewhat more spherical, at best (Figs. 31, 33). A few large tubular cristae line its posterior side (Figs. 32, 33). Also the dimensions correspond to those calculated for the kinetoplast of blood and midgutforms. Large and wide mitochondrial tubes arise from the kinetoplast and form an elaborate network (Figs. 31, 32), comparable to that observed in midgut or culture forms (VICKERMAN, 1962). The shorter posterior part of the chondriome is even larger, possibly accounting for the kinetoplast's moving forward, whereby the outgrowing mitochondrion acts as a transport system.

The Golgi apparatus reveals only insignificant features; cisternae and vesicles are only rarely seen. Again, like in the midgutform, the rer is only developed to a reduced extent.

Some few membrane-bound ribosomes are visible. The rer tubes

are fairly short, but seem to be in a phase of formation and outgrowth (Fig. 32, inset). A similar statement applies to the smooth endoplasmic reticulum (ser).

The most drastic and impressive modification is the enormous increase of the lysosomal organelles. More mvb than in the developmental stages aforementioned occur. Their size has altered, too. They have definitely widened and become more heterogenous as to their shape and vesicular contents (Figs. 31, 32). In addition, membrane-bounded bodies containing membranous residues ("myelin" figures) are often visible. They are regarded as dense bodies and/or residual bodies (DE DUVE & WATTIAUX, 1966; NOVIKOFF, 1967). The peroxisome-like organelles seem to contribute, by transition, to the formation of multivesiculate bodies. The small, osmiophilic lipid inclusions encountered in bloodforms have become more frequent (Fig. 31). Free ribosomes have decreased in number, as compared to the midgutform, and are possibly mobilized for a limited rer formation (Fig. 32, inset).

Many dividing forms are present; they behave in conformity with the schematic mode of division explained before. An interesting modification concerning the dividing flagellum supervenes. Cases have been observed where the new daughter flagellum grows out into the old flagellar sheath, which contains then two axonemes either equipped with the intra-flagellar structure (Figs. 34a, b). Similar observations are known from promastigotes of *Leishmania donovani* (CHATTERJEE & SEN GUPTA, 1970). The actual division of the flagellum is completed by membrane incision and longitudinal splitting.

1.3.2. Proventricular transition form

This stage is regularly found in infections from day 10 on. It has never been found in division and is characterized by a juxta-nuclear position of the kinetoplast (Fig. 35). The most striking difference concerns the ultrastructure of the kinetoplast. During the kinetoplast's migration the DNA becomes larger, looser and more or less uncoiled. The typical figure-8 configuration is replaced by a more filamentous network (Fig. 35). Similar observations are reported from transforming kinetoplasts in developmental forms of *Herpetomonas muscarum* (BRUN, personal communication). The DNA fills nearly the entire kinetoplast. It measures 0.45–0.55 μ in length and 0.3–0.35 μ in width. The larger DNA fibrils are interconnected by small filaments running in oblique directions. The fibrils lie likewise on a "basement plate" (INOKI et al., 1971) made up by an amorphous mass of relatively high electron-density.

The kinetonucleus regains its coiled, figure-8 configuration upon transformation to the epimastigote form.

1.3.3. Epimastigote proventricular form

The morphology of this stage is characterized by the anterior position of the kinetoplast (Fig. 35, inset). They are never found in division. The kinetoplast with its typical coiled structure lies in an electron-dense matrix. The kinetoplast appears a bit smaller (0.35 to 0.40 μ in length; 0.15–0.20 μ in width). For the detailed description note the epimastigotes in the salivary glands.

In heavy proventricular infections trypanosomes of the trypomastigote as well as the epimastigote type are also present in the crop duct and the crop lumen. Yet, fixation has proved to be unsatisfactory.

1.4. Salivary gland forms (Figs. 36–50)

Infections of *T. brucei* in *G. morsitans* were much heavier than in *G. fuscipes* and *G. pallidipes*. Difficulties with the fixation and localization of the flagellates had to be overcome. Sometimes, with the concentrations of the fixatives used, the epithelium showed a good preservation, whereas the parasites were poorly fixed, and vice versa.

The parasites' density was optimal in the middle parts of the salivary glands immediately ahead of the salivary duct (Fig. 37). Trypanosomes were only occasionally seen in the distal part of the glands and the salivary duct.

48 EM specimens could be evaluated, but only 3 turned out to be ideal with respect to the number of parasites and fixation. These were all derived from *G. morsitans*. A schematic description of the salivary gland cycle is given in Fig. 36.

1.4.1. Epimastigote form

This stage was always much more frequent than the metacyclic form. The parasites are strictly concentrated at the epithelial lining, at places being closely attached to the epithelium by the flagellum, which project deeply into the microvillar zone, functioning as an anchor (Fig. 38). In addition, hemidesmosomal plaques sustain the attachment. These are akin to the maculae adhaerentes involved in the flagellum's adhesion to the pellicle.

The nucleus is rounded to oval (1.5–2.0 μ in diameter) (Figs. 37, 40). The endosome is very distinct and lies slightly eccentric (0.4–0.6 μ in diameter) (Fig. 40). The nucleoplasm appears filamentous to fine-granular interspersed with heterochromatin-like masses, the bulk of which is concentrated at the periphery (Figs. 40, 41). The pellicula, periplast, basal body, flagellum, nuclear membranes and basic struc-

ture of the kinetoplast do not show alterations compared with the vector stages already described (Figs. 40, 41).

The kinetoplast lies anterior to the nucleus, and so does the basal body ("epimastigote": HOARE & WALLACE, 1966).

A mitochondrial network arises from the kinetoplast (Fig. 41). It has expanded much more in the anterior part of the cell. Two bifurcate branches of the anterior and the posterior part of the mitochondrion converge on the kinetoplast.

The mitochondrion possesses a great number of tubular cristae, which are hardly arranged in a parallel fashion and sometimes even reveal a position parallel to the mitochondrial wall, as already observed by BROOKER (1971a) in *Crithidia fasciculata*. The mitochondrial cristae are rather dilated and bulbous, comparable to the ones of the metacyclic form (Fig. 41). Another indication for the final transformation to the metatrypomastigote (metacyclic) form is given by the mitochondrion, which appears as a single reduced tube on a fairly long distance lacking any ramifications.

The Golgi complex is well developed and of vesicular appearance (Fig. 40). Parallel stacks of membrane are not frequent. Many Golgi vesicles may indicate that the Golgi apparatus is more active than in proventricular and midgut forms. Golgi vesicles can clearly be distinguished from pinocytotic vesicles, located in the vicinity of the flagellar pocket and the GERL area. The latter resemble "coated" vesicles described in insect cells (ROTH & PORTER, 1964; FRIEND & FARQUHAR, 1967; ANDERSON, 1969; JENNI, 1971) and are covered with a flocculent proteinaceous coat, 270-320 Å thick (Fig. 40, inset). The formation of these pinocytotic vesicles by invagination of the reservoir membrane can be followed up; they do not lose their spiny coat upon release into the cytoplasm. Their diameters range from 0.07-0.20 μ .

It is evident that the pinocytotic activity is augmented in epimastigotes compared with the stages aforementioned. On the other hand, exocytosis is remarkably diminished.

Peroxisome-like organelles have again increased in number, and their mobilization for the formation of multivesiculate bodies seems to be scanty. The former are probably newly synthesized as indicated by a definite change in the consistence of their matrix, which becomes more homogenous, electron-dense and amorphous. They correspond to the ones previously depicted in bloodforms.

Lipid inclusions of both types are not numerous. The quantity of free ribosomes has considerably decreased (Figs. 40, 41). Divisions in epimastigotes are extremely abundant. More than half of all forms displayed two basal bodies and kinetoplasts in duplication (Fig. 40). The divisions undergo the same processes as in slender bloodforms and the other vector stages. Endonuclear spindles (p. 101) and the presence of

condensed "heterochromatic" masses located at the nuclear periphery and/or running cross-wise through the nucleoplasm, typify the well known pattern of division.

The daughter kinetoplast, nevertheless, is migrating towards the posterior end of the cell body and contributes to the formation of transition forms (see below).

1.4.2. Transition forms

1.4.2.1. Juxta-nuclear form

This form occurs quite frequently. It is defined by the position of the kinetoplast during its mitochondrion-dependent migration from the ante-nuclear to the post-nuclear location following a division of an epimastigote form. The kinetoplast lies by the side of the nucleus (Fig. 43). As to size and morphology it appears unchanged compared with the former stages. This is also in keeping with the structural organization of the mitochondrion. The kinetoplast is derived from a dividing epimastigote, and its migration towards the posterior end of the cell seems to be mediated by the outgrowing anterior part of a new mitochondrion, as estimated in relation to the mitochondrial profiles sectioned (Fig. 44). The chondriome, on the whole, is markedly reduced and hardly any branchings are recognized (Fig. 43).

The surface coat is still absent. The Golgi zone appears somewhat more extended than in the epimastigote and is composed of vesicles and tubules with electron-dense contents (Fig. 44). The rer and ser seem to be in formation, whereas only a few lysosomal organelles occur.

1.4.2.2. Immature metatrypomastigote form (Fig. 45)

This stage is reached by advanced migration of the kinetoplast. On morphological grounds it differs very much from proventricular trypomastigotes.

The nucleus is rather rounded, and the mitochondrion even more reduced, above all the anterior part.

The number and size of the mitochondrial tubules have decreased. Moreover, the mitochondrial tubules are smaller and vesicular-shaped. The Golgi complex contains many cisternae with electron-dense contents and a great number of vesicles in the GERL zone and around the reservoir.

The surface coat, having been absent from the precedent vector stages, is about to reappear. However, it is not entirely homogenous yet, but clearly thinner and rather flocculent; it measures some 90 Å in thickness (Fig. 46).

Sporadic lipid inclusions of the small osmiophilic type and free ribosomes (Fig. 45) occur. Pinocytosis and exocytosis are insignificant. Immature metatrypomastigotes are able to divide (Fig. 45).

1.4.3. Mature metatrypomastigote (metacyclic) form

Forms of this kind occur free in the lumen of the salivary glands and are not attached to the epithelium (Fig. 39). The nucleus is smaller and more rounded, situated in the middle of the cell. Again, chromatin-like masses are fixed to the inner nuclear membrane and running cross-wise through the nucleoplasm (Fig. 39).

The chondriome is strictly reduced to one mitochondrial tube (VICKERMAN, 1966c; STEIGER, 1971), made up by a long anterior and very short posterior part (Fig. 47). Mitochondrial tubules of a rounded, bulbous type are less numerous and hardly parallelly aligned (Figs. 47, 49). Sometimes, labyrinth-like mitochondrial extensions are observed; they are comparable to those found in slender bloodforms and in *Crithidia fasciculata* (BROOKER, 1971a) (Fig. 49).

The pellicula is uniformly covered with a surface coat, 110–130 Å thick (VICKERMAN, 1966c; STEIGER, 1971) which corresponds in morphology to the surface coat of bloodstream forms (VICKERMAN, 1969a, and Fig. 47).

The most drastic alterations concern the state of development of the Golgi zone, which is extremely enlarged and composed of 3–4 parallel cisternae and many vesicles of different size (0.06–0.25 μ in diameter) (Figs. 47, 48). The smaller vesicles containing electron-dense material arise from the Golgi membranes and seem to fuse to larger vesicles (STEIGER, 1971). These are strictly concentrated close to the reservoir and can be distinguished from pinocytotic vesicles in lacking an external “coat” (STEIGER, 1971). The larger Golgi-derived vesicles are lined on the inside of the limiting membrane with a layer of amorphous, moderately electron-dense material, 100–130 Å thick (Fig. 48). This intravesicular lining coincides with the surface coat material on the pellicle of the trypanosome. The mode of formation of these Golgi-derived vesicles can be traced step by step (STEIGER, 1971). First, the electron-dense material is evenly dispersed in the small vesicles (Fig. 48). Then, as the vesicles transform possibly by fusion, to larger vesicles, this material becomes condensed at the intravesicular periphery (Fig. 48).

This type of Golgi-derived vesicle has never been observed neither in epimastigotes nor in transition forms.

The rer system is like in bloodforms (Fig. 47), whilst the smooth endoplasmic reticulum is very inconspicuous and made up by but a few small pieces. The number of lysosomal structures has much been

reduced. Some vesicles near the flagellar pocket could be considered primary lysosomes. A few multivesiculate bodies persist, being sometimes quite large ($1\text{--}1.5\ \mu$ in diameter), but mostly small ($0.3\text{--}0.4\ \mu$ in diameter), possibly representing the derivatives of peroxisomes-like organelles. The intrusion of Golgi vesicles into the former can be affirmed. Lipid-inclusions of the small, osmiophilic type are rare or absent; larger lipid inclusions like the ones found in midgutforms have never been observed in metacyclic forms. A lot of peroxisome-like organelles are scattered throughout the cytoplasm (Fig. 47, comp. Fig. 1). Their matrix looks similar to that of the chondriome (Fig. 47). A limited number of free ribosomes are visible (Figs. 47–49); they are considerably less abundant than in the precedent vector stages.

A remarkable observation has been made: quite frequently mature metacyclic forms are encountered in division (Figs. 49, 50). This is in opposition to the general view expressed by many earlier investigators (e.g. BUXTON, 1955) that mature metacyclics are not able to multiply. Also in this case, the division is initiated by the duplication of the basal body and by a clear enlargement and subsequent replication of the kinetoplast and its DNA. It has to be added that the division proceeds with accordance to the general mode described in bloodforms. The duplication and consequent splitting of the mitochondrion is possibly effected by means of the labyrinth-like extensions (BROOKER, 1971a) (Fig. 49).

2. Cytochemical experiments

2.1. Lipid extraction (Figs. 51–54)

This method was not totally specific and reproduceable. Reactions on thin sections mounted on grids without a supporting parlodion film were more precise, but the Epon medium is dissolved faster. The optimal incubation time was about 20–30 seconds, depending on the section thickness and the concentration of the solvent used. The Epon is completely removed with prolonged incubation periods. The control reactions with the solvent devoid of Na-methoxide, not showing affinity to the supposed lipid droplets proved the specificity of the method.

As regards the reactions procedures on thin sections of midgutforms the results were more accurate due to the fact that the midgut epithelium contributed to a certain consistence despite the action of the lipid-Epon solvent.

2.1.1. Bloodstream forms

No difference in reactivity was found between slender and stumpy forms.

After 10–20 seconds the small osmiophilic inclusions ($0.15\text{--}0.20\ \mu$ in diameter) lose their spherical shape and appear somewhat faded,

more granular and less contrasted (Fig. 51a, b). They seem to be "digested" to some extent. A "lytic" zone surrounds these granules ("Type II granules": BIRD et al., 1966; MOLLOY & ORMEROD, 1971).

After an exposure time of 30 seconds or longer many of these inclusions disappear leaving a white hole representative of the former site of the lipid material (Fig. 51). It has to be mentioned that other cell structures are sensitive to this treatment. Membranes become vague, above all those of the mitochondrion. The ground cytoplasm gets loose and gives the cell a spotty aspect. With prolonged incubation periods the Epon appears perforated.

2.1.2. Midgut forms

To test the reaction in midgutforms for specificity, a parallel experiment was carried out with the thin sections of the midgut epithelium, the cells of which contain huge droplets, probably lipids, in the apical part of the cytoplasm.

These droplets are related to blood uptake and digestion. After an incubation time of 30-40 seconds the moderately electron-dense droplets of the epithelial cells get shrunk and washed out. Finally, a white spot remains indicating the former presence of lipids (Fig. 52).

Relatively large droplets (0.3-0.5 μ in diameter) of the midgutforms of *T. brucei* are thought to represent lipid-like material. They are apparently affected by the incubation in the lipid-specific Epon solvent. The optimal length of incubation ranged from 10-40 seconds (with or without a parlodion film), whereby the lipid material is gradually "digested" and leaves at first small white spots, and finally large light zones (Figs. 53, 54). The bulk of the cell organelles appears only slightly affected.

At least the mitochondrial membranes are completely decomposed, and remnants are found in loose agglomerations (Fig. 53).

2.2. Demonstration of polysaccharides (Figs. 55-63)

The reactions carried out on thin sections of pleomorphic blood-forms, midgut epithelium and peritrophic "membrane" were highly specific; the controls either without TCH-incubation or with pre-oxidation in H_2O_2 were always negative (Fig. 58). Membranes stained very readily in all experiments. Prolonged incubation periods (48 and 72 hours) in TCH produced a lot of unspecific silver deposits. The optimal, most precise localization of the reaction product was obtained after 24 hours' incubation.

2.2.1. Bloodstream forms

No differences between the three pleomorphic types with respect to localization and intensity of the reaction product were noticed. One-hour treatment with TCH causes the membranes to be slightly contrasted. Single Golgi vesicles and pieces of ser stain positive. After 12 and 17 hours the reaction appears considerably intensified as revealed by the Golgi complex, including vesicles in the reservoir area, which show more granular deposits (Figs. 55, 57). However, not all Golgi cisternae and vesicles are stained (Figs. 55, 57). The marked vesicles are mainly lateral. Fine granular precipitates are also found in the reservoir and very distinctly in exocytosed particles. Presumable primary lysosomes next to the reservoir membrane and osmiophilic lipid inclusions are very markedly stained (Fig. 57). In this respect they can be distinguished from pinocytotic vesicles, which lack any specific granulation.

Marked ser tubes run throughout the cell as far as the reservoir zone and have the appearance of a coherent canal system (Fig. 57). They seem to be involved in the formation of primary lysosomes; a suggestion, which is supported by a further cytochemical experiment (p. 97). Yet, many vesicles in the GERL zone stain negative; the same applies to the rer system. Mostly, only pieces of the Golgi tubes are marked. The most compact reaction product is found in fenestrated Golgi saccules (Fig. 55).

The pellicula (surface membrane) is darkly contrasted, whereas the surface coat material is always devoid of any granulation (Fig. 56).

After 24 hours' incubation the distribution of the reaction product is much alike the situation after 17 hours. However, the localization appears somewhat more accurate and distinct. Above all, the concave side of the lamellar Golgi apparatus and the lipid inclusions as well as the cytoplasmic membranes are even more electron-opaque (Fig. 55), whilst the convex side of the Golgi stacks of membrane and the surface coat remain unstained (Figs. 55, 56).

2.2.2. Peritrophic "membrane" in the midgut

The bilaminar structure of the peritrophic "membrane" is known to be made up by two layers of different type and thickness (MOLOO et al., 1970). Layer I of the condensed "PM" in the midgut measures 15–25 m μ , layer II 0.30–0.35 μ .

The site of polysaccharide compounds can unobjectionably be demonstrated. Using different incubation periods (optimal: 12 hours in TCH) dark relatively large silver granules are seen restricted to layer I. Layer II stained only faintly indicated by very small indistinct grains homogenously spread in this lamella of the "PM" (Fig. 59).

Both control experiments were negative. To test specificity, rosettes of α -glycogen in the fore-gut epithelium were marked (Fig. 62).

2.2.3. *Epithelia and peritrophic "membrane" in the proventriculus*

The "PM" in this region exhibits fine-granular homogenous granulations both in layer I and layer II (Fig. 60). This is in opposition to what has been described for the peritrophic "membrane" in the mid-gut. The density of the reaction product in the two layers does not differ.

Additionally, the chitinous intima of the fore-gut invagination (cardial valve) stains much more intensively. The positive zone is about 0.25–0.40 μ thick (Fig. 60). As mentioned before, glycogen in the fore-gut epithelium is clearly marked (Fig. 62).

The proximal midgut epithelium in the proventricular region known to contribute to the formation of the "PM" shows specific silver precipitates (Fig. 61). These are visible in the glycocalyx and in vesicles of different size and possibly different type in the apical zone of the cells (Fig. 61).

The control experiments all proved specific in lacking any deposits of reduced silver (Fig. 60, inset).

Yet, the control experiment after PA oxidation, but without subsequent incubation in TCH, combined with post-staining of the thin sections in lead citrate, showed extraction zones in the apical part of the cardinal valve revealed by a thick white line (Fig. 63). These zones correspond topographically with the chitinous intima, and contain a homogeneously dispersed fine granulation. PA treatment together with post-staining was used to demonstrate polysaccharides in the sperm of a cephalopod (LONGO & ANDERSON, 1970).

2.3. Protease digestion (Fig. 64 + inset)

Digestion experiments with several proteases (pronase, trypsin and pepsin), at different concentrations and incubation periods, were performed on thin sections to demonstrate proteinaceous components in the peritrophic "membrane". Sections floated on 0.05–0.10% pronase for 20–60 minutes after preoxidation with 5% PA or H_2O_2 (40 to 60 minutes) yielded the best results. Controls without previous incubation in the oxidatives did not show enzymatic digestion effects. Yet, strictly speaking, the digestion experiments were not incontestably reproducible. It is not clear as to whether this fact should be imputed, apart from the partly unsuitable embedding medium, to variations in section thickness or in activities of the enzymes concerned. The fine structure of the "PM" in the control experiments

bears analogy to the normal ultrastructure already described (MOLOO et al., 1970) (Fig. 64, inset).

After enzyme digestion following preoxidation visible changes occur only in the peritrophic "membrane" of the proventricular region. These are marked by an initial decrease in electron-density caused by pre-oxidation of bound osmium fixative. Subsequently, the thick layer II of the "PM" becomes looser, rather fine-filamentous, whilst layer I is practically left unchanged (Fig. 64). Then, lighter zones are found which are thought to be induced by the digestive activity of the proteases.

In the peritrophic "membrane" of the distal parts of the midgut, where the "PM" is a compressed structure after having been drawn through the cleft between the proventricular wall and the invaginated fore-gut, hardly any structural modifications are observed.

2.4. Localization of peroxidase/catalase (Figs. 65, 66)

Contrary to expectation, the presence of peroxidase activity in the peroxisome-like organelles of blood trypanosomes could not readily be demonstrated, neither following formaldehyde nor glutaraldehyde fixation.

Precipitates of oxidized benzidine (DABTH) are confined to places outside the trypanosomal cell, indicating contamination with peroxidase from lysed blood cells, and to but a few intracellular vesicles (Figs. 65, 66). The marked vesicles correspond in size to the peroxisome-like organelles or "GPO" bodies. The boundary membranes of the stained bodies are only vague (Fig. 66, inset). The term "peroxisome" seems to be justified.

In the control experiments no positive staining is seen, neither extracellularly nor intracellularly (Fig. 65, inset). To test the incubation media, pieces of rat liver were treated and processed in the same way. Rat liver microbodies were specifically contrasted by oxidized DABTH deposits.

2.5. Tests for acid phosphatase (Figs. 67–70)

The cytochemical tests for acid phosphatase in bloodforms of *T. brucei* turned out to be specific. Control reactions in incubation media devoid of the substrate (glycerophosphate) or the acceptor (Pb^{2+} cations) did not lead to reaction products (Fig. 67, inset).

As to the normal experiments all procedures either in freshly prepared media with or without DMSO or in the filtrated standard Gomori medium yielded lead salt precipitates of comparable quality. The cytochemical staining after incubation in the DMSO-containing medium was markedly augmented, whereas incubation in the standard medium produced the most accurate localization of the reaction product.

Table 1. Ultrastructural features of developmental stages of *T. brucei*

Location	Stage	Pellicula	Chondriome	GERL system	free Ribosomes	GPO bodies	Lipids
<i>Blood</i>	slender	with coat	cristaeless tube	few rer, Golgi +	many	numerous,	spherical
	intermediate	with coat	cristae forming enlarged	ser well developed	many	seem to give rise to mvb	osmiophilic, abundant
	stumpy	with coat	begins to ramify	more developed	many		
<i>Midgut</i>	deteriorated	coat absent	decomposed	lysis and vacuolization	increase	decrease bacilliform	only few
	transition	persists up to day 4	proliferation	reduced rer	increase	decrease bacilliform	large type
	established	coat absent	elaborate network, shift of the kinetoplast	no ser, numerous lysosomes	abundant	decrease bacilliform	large type
	ectoperi. in the prov. region	coat absent		increase of large mvb	abundant	decrease, formation of mvb	both types 2nd type rare
<i>Cardia</i>	trypomastigote	coat absent	unchanged, shorter cristae	Golgi insignificant rer in formation more lysosomes	decrease	some persist	osmiophilic type, larger type rare
	epimastigote	like in the salivary glands
<i>Salivary gland</i>	epimastigote	coat absent	unchanged, cristae dilated	Golgi active, more pinocytosis	evident decrease	increase, mvb formation rare	both types rare
	transition	coat precursor	reduction, cristae less numerous and smaller	Golgi extended er formation	evident decrease	increase, mvb formation rare	sporadic, only small type
	metacyclic	perfected coat like in blood-forms	single tube, few cristae of a vesicular type	Golgi extremely enlarged and active presence of Golgi-derived vesicles	evident decrease	like in bloodforms	sporadic, only small type

Homogenously dispersed fine lead salt deposits are seen in the flagellar pocket indicating the presence of acid phosphatase in this extracellular space (Fig. 67). This observation accords with results of other authors (JADIN & CREEMERS, 1970a; b). Many marked vesicles are situated around the reservoir membrane (BROOKER & VICKERMAN, 1964; SEED et al., 1967; Fig. 67). They are likely to represent primary and secondary lysosomes, and may be distinguished from marked pinocytotic vesicles by a higher contrast. Only a few Golgi cisternae stain positive, a single cisterna being sometimes only partially marked (Fig. 68). Lateral Golgi vesicles are marked, too (Fig. 68). Extended cisternae, possibly ser saccules, contain, at times, a widely distributed reaction product (Fig. 70, inset). Large tubules of ser run throughout the cell and reach the reservoir area. They are often filled up with lead salt deposits and have the appearance of a coherent canal system (Fig. 69). They seem also to be concerned with the formation of primary lysosomes (Fig. 70), which, upon extrusion into the flagellar pocket, would make up the extracellular phosphatase-positive material. However, a great number of vesicles located in the GERL zone are clearly negative (Figs. 68, 69). The rer system stains only faintly, the lead phosphate precipitates being restricted to parts of the perinuclear space. Furthermore, structures certainly identical with multivesiculate bodies stain phosphatase-positive (Fig. 68, inset), whereas the remainder of the organelles, such as "Type I granules", interpreted as lysosomes (BIRD et al., 1966; MOLLOY & ORMEROD, 1971) always lack any specific staining for acid phosphatase.

IV. Discussion

This study shows that the transformation of *T. brucei* during its cycle in the bloodstream and vector is characterized by drastic modifications of its organelles. However, some cell components remain practically unchanged, such as the subpellicular microtubules, the subpellicular organelle, the desmosome-like attachment zones, the kinetoplast (+DNA) and the basal body with the flagellum.

A. Microtubular elements

Microtubules are found in the periplast (subpellicular microtubules), in the subpellicular organelle, in the vicinity of the reservoir, during the division of the nucleus and as components of the flagellar axoneme and the basal body.

Microtubular structures are attributed cytoskeletal functions in a wide variety of cells (reviewed by PORTER, 1966), concerned in maintaining cell shape and being responsible for giving rigidity to certain areas of the cell (FAWCETT, 1966).

On the other hand, they seem to be implicated in internal and external cell movements as well as in alterations of the cell shape (TILNEY & PORTER, 1965; FAWCETT, 1966; MACGREGOR & STEBBINGS, 1970). Microtubular walls are known to be composed of protein subunits, some 40–45 Å across (OLSON & HEATH, 1971), of various types respecting their configuration and number per tubule (LEDBETTER & PORTER, 1964). Their proteinaceous nature was proved in whole mounts of trypanosomes by protease digestion (MEYER & PORTER, 1954). Yet, the tubules are resistant to treatments known to affect the microtubular structures in other cells (colchicine); after cell death, however, they are completely digested (MESSIER, 1971), possibly caused by proteases released from ruptured lysosomes. It was shown for *Tetrahymena* that all microtubules within a cell include common protein subunits (TAMURA, 1971). The globular units of the microtubular wall have an amino acid composition comparable to actin, which is probably bound to a myosin component showing ATPase activity to form an actino-myosin complex (AMBROSE & EASTY, 1970). Analogously thrombosthenins were isolated from the microtubular apparatus of human blood platelets (BETTEX-GALLAND et al., 1969).

As a matter of fact, an ATPase system attached to the peripheral tubules of *Tetrahymena* cilia was previously found by GIBBONS (1967). ATPase could be demonstrated, likewise, in the flagellum of the *Helix* spermatozoa (ANDERSON et al., 1968).

The intertubular filamentous connections (actin-like) of *T. brucei* could house a similar ATPase system enabling microtubular motility, but they could at the same time act as a sustaining structure, which warrants the regular spatial arrangement of the microtubules. Similar mechanisms are assumed in conjunction with transformations in *Crithidia fasciculata* and trypanosomes (BROOKER, 1971a). Investigations on the array of pellicular microtubules in different Trypanosomatids by means of a modified surface tension spreading method yielded interesting results (ANGELOPOULOS, 1970). The majority of the tubules was seen to follow a regular spiral course from the flagellar end to the apical area and are thought to terminate near the apex. In *T. lewisi*, representative of the genus *Trypanosoma*, the tubules do not end at the posterior apex but appear to be bent backward to continue their course towards the base of the flagellum. The observations made by MEYER et al. (1958), VICKERMAN (1962) and SANABRIA (1963) that the periplast tubuli follow a continuous parallel course around the cell body without free ends, as well as the model of an increase in number

by branching (MEYER & PORTER, 1954), are not consistent with the impressive results obtained by ANGELOPOULOS (1970). Moreover, we tend to think that transformation of the different developmental stages of *T. brucei*, in terms of synthesis and partial motility, can be best explained using the interpretation by the author aforementioned. Partial motility ("limited flexibility") of the microtubules might provoke winding and unwinding of the cell body. The "intertubular substance" reported by the same investigator is perhaps identical with the intertubular connections discussed above.

The same speculations hold for the tubular elements of the flagellum. The peripheral tubular doublets are likely to represent partially contractile structures with an associated ATPase system, which would provide energy for a muscle-like contraction/relaxation mechanism manifesting as flagellar movement.

In this case the two mechanism brought about by the contractile proteins of the subpellicular microtubules and the axoneme would have all the makings of a synchronized interdependent system.

It is conceivable that the interdependence is favoured by the desmosome-like attachment zones between the pellicle and the flagellar membrane. These elements, i.e. limited contractibility of the flagellar axoneme and the subpellicular microtubules, accompanied by pellicular extension and distortion, as well as the fortified flagellum/pellicula cohesion can give the impression of an "undulating membrane", as it is seen in the light microscope. An extension of the pellicular membrane could be demonstrated in the "undulating membrane" of a reptile trypanosome (RANQUE et al., 1970).

Partial flexibility of the microtubules concerned, however, cannot account alone for transformations, growth and divisions in trypanosomes. It must be exceeded by a new synthesis of microtubular proteins. The flexibility, after all, is remarkably restricted; this was shown before (ANGELOPOULOS, 1970) with the aid of a great number of uneven breaks occurring on spread preparations of pellicular tubules ("the concept of substructural rigidity").

Microtubules of the flagellum arise from the basal body (blepharoplast, kinetosome), the peripheral doublets being continuous with those of the basal body in the transitional zone (VICKERMAN, 1970a), whereas the central tubules originate in the basal plate situated in the centre slightly farther along the shaft.

Replication of the basal body takes place in anticipation to the division of the kinetoplast, and it is generally accepted that the cell division cycle is initiated by the basal body's replication (MEYER et al., 1958; BAKER & BIRD, 1968; BURTON & DUSANIC, 1968; INOKI & OZEKI, 1969). The newly synthesized free basal body lies at first perpendicular to the old flagellum-bearing one (INOKI & OZEKI, 1969; PAULIN, 1969).

Later on, however, it comes to lie more or less parallel to the old basal body (BURTON & DUSANIC, 1968; PAULIN, 1969) and gives rise to the daughter flagellum.

The basal body seems to constitute a *de novo* synthesized structure. Precursor material, referred to as "pericorpuscular satellites" (SCHULZ & MACCLURE, 1961), "procentriolar masses" (BURTON & DUSANIC, 1968) or "accumulations of dense material" (PAULIN, 1969), is visible in the vicinity of mature basal bodies (kinetosomes) during or shortly after the shift to the parallel position. A prokinetosome is formed which grows out building up a new flagellum.

As to the actual mode of synthesis only little information is available. Basal bodies, like centrioles, do incorporate H_3 -thymidine (BURTON & DUSANIC, 1968; OZEKI et al., 1971) indicating active DNA synthesis typical of "self-replicating" organelles. The presence of DNA at the level of the kinetosomes was demonstrated in ciliates (RANDALL & DISBREY, 1965; SMITH-SONNEBORN & PLAUT, 1967).

Only suggestions exist so far how tubular subunits of the basal body are synthesized and built in. There is some evidence for different organelles (Golgi complex, nuclear envelope) being involved in the synthesis of tubule subunits in a wide variety of cells (reviewed by HEATH & GREENWOOD, 1971). It is noteworthy that in *Acanthamoeba* a distinct association between the Golgi zone and the microtubules was observed reflecting a possible role of the Golgi apparatus in the formation of microtubular material (BOWERS & KORN, 1968). However, such a pattern of association has never been seen in *T. brucei*.

SEAMAN (1962) proved that kinetosomes isolated from *Tetrahymena* are capable of protein synthesis. On the other hand, it was put forward that the tubular organization of the centriolar structure might act as a "template", which initiates polymerization of already synthesized subunits (6 S protein: RENAUD et al., 1968).

We tend to think that actual synthesis may be perfected by a combined system of protein synthesis, which nuclear components, numerous polyribosomes and the basal body itself are involved in. A modified model would apply, without the participation of the basal body, to the formation and outgrowth of subpellicular microtubules.

The unique subpellicular organelle described by FUGE (1968) and TAYLOR & GODFREY (1969) represents a structural complex never found in other types of cells. The apparent association with the flagellar attachment zones might imply a role in controlling movement (TAYLOR & GODFREY, 1969). However, linkage with the perinuclear cisterna, which is part of the rer system, seems to favour the suggestion that newly synthesized proteins are transported through this membranous canal in order to contribute to the formation of microtubules (TAYLOR & GODFREY, 1969) or to provide the supposed actinomyosin complex

of the pellicular microtubules with sufficient amounts of ATPase. It is conceivable that pellicular microtubules in the area of flagellar attachment would have to exhibit a higher degree of motility.

Spindle microtubules are known to occur in the dividing nuclei of trypanosomes (VICKERMAN & PRESTON, 1970). They have been reported from *Leishmania tropica* (BIANCHI et al., 1969) and have also been observed by INOKI & OZEKI (1969) in bloodforms of *T. gambiense* and in the present study. They represent structures requisite for the nuclear division process. Again, they display involvement in cell movements, namely the separation of the nuclear halves and the equal distribution of endosomal and chromatin-like material. In these respects they exert a role analogous to the function of mitotic spindles in higher eucaryotic cells. However, pronounced modifications occur. The nuclear envelope persists during division. The endonuclear spindle in *T. brucei* is devoid of any centriolar apparatus and connections to basal bodies. The spindle microtubules in trypanosomes are insensitive to colchicine treatment (LUCKINS et al., 1967), which is in contrast to mitotic spindles at metaphase. They resemble *Euglena gracilis* (LEEDALE, 1968), but are unlike other zooflagellates, which possess condensed chromosomes, kinetochores and a centriolar system (GRIMSTONE & GIBBONS, 1966). Chromosomes in trypanosomes have never been seen with certainty (ROBERTSON, 1927; WALKER, 1964).

B. Surface coat

The loss and reacquisition of the external surface coat (VICKERMAN, 1969a) during the life-cycle of *T. brucei* is one of the most spectacular changes. This structure is found in the bloodstream phase of *brucei*-subgroup trypanosomes, *T. congolense*, *T. vivax* and *T. lewisi*. It is lost when the flagellates enter the vector to complete their cycle, as well as in the culture medium.

Midgutforms, like culture forms, the developmental forms in the proventriculus and the epimastigotes of the salivary glands do not have a surface coat (VICKERMAN, 1969a; 1971a). As soon as the transformation to the metatrypomastigote stage is being completed, the formation of the coat sets in (STEIGER, 1971). The juxta-nuclear transition form is still devoid of any external covering; the first coat precursor material on the cell surface appears when the immature metatrypomastigote stage is reached. Then, a thinner coat (90 Å across) becomes visible.

It was suggested that the Golgi apparatus in metacyclic forms contributes to the synthesis and release of coat material, which is thought

to be transported by means of “Golgi-derived” vesicles to the reservoir to be liberated there by exocytosis onto the pellicular membrane (STEIGER, 1971). This view is endorsed by the state of development of the Golgi complex, which, during this phase, appears large and active, rather vesicular; this is in contrast with the Golgi complex of epimastigotes, where it looks reduced. Vesicles are rarer and lack the intravesicular lining.

It was previously mentioned (STEIGER, 1971) that the “cytoplasmic membrane system” (VICKERMAN, 1969b) and the “sacs of secretion” in *T. congolense* ought to be interpreted rather in terms of the formation of cell organelles (peroxisome-like organelles) and lipid storage droplets, since they do not resemble the “Golgi-derived” vesicles reported here. Similar objections may apply to the possible function of the distended rer tubules and the “zymogen” granules (ORMEROD, 1966), as well as to the “appareil élaborateur singulier” in *T. congolense* (BOISSON et al., 1967b).

“Golgi-derived” vesicles of metacyclic forms possibly involved in the coat formation, and pinocytotic vesicles known to occur quite frequently are clearly discernible. The former do not possess the external flocculent coat typical of “coated” vesicles and “acanthosomes” (VICKERMAN, 1969a; b).

It must be added that “Golgi-derived” vesicles are absent from bloodstream forms. It is obvious that the presence or absence of the surface coat parallels differences in the physiological state of the trypanosome. The coat is basically composed of protein substances, as revealed by pronase digestion (VICKERMAN, 1969a). WRIGHT & HALES (1970), using different fixation methods, demonstrated the presence of carbohydrates in the surface coat and they assumed a stratified distribution. Their results are not consistent with the fact that ruthenium red does not show any affinity (VICKERMAN, 1969a). Moreover, with our material (bloodforms of *T. brucei*), using the TCH-silver-albumose technique by THIÉRY (1967; 1969), the presence of polysaccharides in the coat could never be detected. Yet, the pellicula stains very apparently as shown by an electron-opaque granulation, a feature true of all other cytoplasmic membranes. The positive reaction is possibly due to membrane-associated compounds, such as phosphatidyl choline – ethanolamine – inositol (MEYER & HOLZ, 1966) known to show affinity to periodic acid oxidation.

It was clearly shown by means of ferritin-labelled antibodies that the surface coat represents the site of surface antigens (BOISSON et al., 1968; VICKERMAN & LUCKINS, 1969). The ferritin particles appeared adhering to the surface coat of the cell body and the flagellum. In addition, it could be demonstrated that these marked antigens are, in fact, variable antigens (VICKERMAN & LUCKINS, 1969).

Antigens of trypanosomes have been characterized as being predominantly unconjugated proteins (4S and 1S proteins: BROWN & WILLIAMSON, 1962, 1964; WILLIAMSON & BROWN, 1964). Agglutinogens belong to the group of the variable antigens (INOKI et al., 1956; CUNNINGHAM & VICKERMAN, 1962; GRAY, 1965a, b; 1966). They disappear entirely when the trypanosomes enter the culture medium concomitantly with the loss of the surface coat. GRAY (1962) found that cyclically transmitted *T. brucei* follow a sequence of different antigenic types, starting from a basic antigen, which is reacquired upon transformation to the metacyclic stage when the coat is built up anew (VICKERMAN, 1969a). Antigenic variants in clone strains follow a similar sequence, which is partly reversible (LOURIE & O'CONNOR, 1937).

The results cited support the view that the variable antigens are located in the surface coat, which might represent a component of the "exo-antigen" (WEIZ, 1960).

WRIGHT, LUMSDEN & HALES (1970) observed the formation of filopodium-like processes by *T. brucei*. These filamentous protrusions of the pellicle consist of a cytoplasmic core covered likewise with surface coat material, which is shed off into the plasma with the aid of these processes ("fibrils": MOLLOY & ORMEROD, 1964).

Recent studies on the composition of the surface coat, following earlier investigations (HUMPHRYES, 1970; NJOGU & HUMPHRYES, 1971; NJOGU & NGULI, 1971), revealed the identity of the 4S antigens and the exoantigens (ALLSOPP et al., 1971); according to these authors, protein-carbohydrate complexes are present in the surface coat. Four neutral sugars were detected; D-galactose and D-mannose could be identified.

The negative cytochemical staining of the surface coat in blood-forms of *T. brucei* might support the suggestion that the sugar components do not occur stratified as anticipated by WRIGHT & HALES (1970), but they are likely to constitute determinant end groups concealed by extreme coiling of the glycoproteins. It could also be that the sugars are camouflaged by host antibodies, and that the cytochemical staining is strain-specific (CROSS, personal communication). Preliminary cytochemical studies on the surface coat in metacyclic salivary gland forms yielded interesting results (JENNI, in preparation). With the same TCH-silver-albumose technique a definitely enhanced contrast on the pellicula was obtained. The immature metatrypomastigotes and the epimastigotes stain negative. Micrographs at higher magnifications demonstrate that the granular reaction product is clearly located in the coat lining of the outer leaflet of the pellicular unit membrane. The positive granulation is not homogeneously spread, but consists of small agglomerations of electron-opaque deposits. These observations may indicate that we deal in fact with stages of the coat formation. It is possible that the coiled configuration of the glycopro-

tein chains is not marked yet, and that the absence of host antibodies favours the genuine localization of the carbohydrates.

The darker appearance of the outer leaflet of the pellicular membrane, as seen in the normal cytological picture of bloodstream *T. brucei*, might be induced by a high amount of linkage groups between the membrane and the glycoprotein chains of the surface coat; these could be of lipoid nature, a presumption which gains support from the results obtained by GODFREY & TAYLOR (1969). These authors showed that cobra venom, containing phospholipase A and a direct lytic factor, is able to remove the surface coat in washed *T. brucei*.

Recapitulating the possible vesicle-mediated formation of the surface coat in the metacyclic forms of the salivary glands we think that we deal with a partly endogenous mechanism. An analogous contribution of the Golgi complex to the synthesis of cell coat material could be demonstrated in *Amoebae* (WISE & FLICKINGER, 1970) and in a variety of intestinal cells (ITO & REVEL, 1966; WETZEL et al., 1966; BONNEVILLE & WEINSTOCK, 1970).

It has to be emphasized, however, that the vesicle-mediated formation of the surface coat in trypanosomes could concern predominantly the construction of a homogenous ground substance composed of glycoproteins. The hypothesis is conceivable that determinant sugar end groups are incorporated in a different way. In the bloodstream forms different carbohydrates and proteins (amino acids) could consecutively be built in, bringing about antigenic variants in response to antibodies.

The variant determinant sugar end groups would then be much alike the well known bloodgroups specific carbohydrate chains, which contain likewise D-galactose (quoted by KARLSON, 1967).

As a matter of fact, a specific hexose transport system, as demonstrated by competitive interaction of several carbohydrates, was reported from *T. lewisi* (SANCHEZ & READ, 1969). Specific membrane binding sites for glucose and glucosamine could thus be identified.

For the uptake of pteridines (essential growth factors) into *Crithidia fasciculata* a carrier-linked energy-dependent transport mechanism was found (REMBOLD & VAUBEL, 1970). The view was adopted that an analogous carrier-mediated amino acid transport exists in bloodforms of *T. brucei* (VOORHEIS, 1971) and culture forms of *T. cruzi* (HAMPTON, 1971). The involvement of membrane-bound carriers in the bidirectional transport flux of amino acids and sugars through membranes of erythrocytes and kidney cells has extensively been studied (e.g. LEVINE et al., 1965; MAWE & HEMPLING, 1965; SCRIVER & WILSON, 1967).

These facts raise the question as to whether the salivary glands contribute to the formation of the surface coat in metacyclic forms, thus providing exogenous factors. Amino acids and sugars were ex-

tracted from the salivary glands and characterized (WILLIAMSON, 1956). Mucoprotein proved to be absent. 12 amino acids, but only the sugar alcohol inositol and possibly arabinose were found. The common hexoses were shown to be absent. Besides, GEIGY et al. (1959) found trace amounts of trehalose and glucose in extracts from salivary glands.

AMREIN et al. (1965) succeeded in growing virulent culture forms of *Trypanosoma brucei* in haemocultures, though direct evidence for the presence of real culture metacyclics has not been produced yet. They found that the reacquisition of virulence depends on three factors, namely age of the culture medium, the human blood donor and the length of storage of the media before inoculation. By addition of substances known to occur in the salivary glands of *Glossina*, however, no significant influence on the virulence of cultured *T. brucei* was obtained (GEIGY & KAUFFMANN, 1964).

In this case a contribution of blood sugar and galactose (Agar) from the medium cannot be excluded. The same statement holds for amino acids, which could also be provided by the salivary glands or the culture medium, respectively. Be that as it may, it is to be hoped that tracer experiments with a mixture of radioactive amino acids and sugars as well as with the electron-dense marker iron-dextrane can elucidate the roles of endogenous and exogenous factors in the formation of the surface coat (JENNI, in preparation).

Reviewing these aspects we could support the idea that the coat formation in the metatrypomastigotes of *T. brucei* represents an interaction of exogenous and endogenous factors. A higher degree of amino acid and sugar uptake by the flagellates may initiate protein synthesis (reviewed by MANCILLA, 1968) and assembly of glycoproteins in the Golgi apparatus, which are then released via "Golgi-derived" vesicles onto the trypanosome's pellicle.

It is well established that bloodstream forms of *T. brucei* subgroup trypanosomes exhibit a surface charge (BROOM et al., 1936). Using erythrocytes, which are known to have a permanent negative charge, as a reference cell system in an adhesion test, *T. gambiense* in the bloodstream could be ascribed a positive charge. On the other hand, it could be shown by electrophoretic behaviour that bloodstream *T. rhodesiense* were unlikely to have a definite surface charge, whereas in culture forms devoid of the coat a clear negative charge was apparent (HOLLINGSHEAD et al., 1963). Therefore VICKERMAN (1969a) concluded that the loss and reacquisition of the surface coat could indeed reflect "changes in electrophoretic behaviour". Moreover, the same author pointed out that low mobility of bloodforms at pH 7.0 could indicate the presence of polysaccharides; in addition, pH-dependent mobility and the pK values speak in favour of proteins (carboxyl and amine groups). LANHAM & GODFREY (1970) made use of the facts cited in

separating bloodstreams trypanosomes of different species at optimal ionic strength from erythrocytes by means of a DEAE-cellulose anion exchanger. As regards the "coatless" forms in fly and culture, their negative surface charge, comparable to erythrocytes, may be due to sialic acid residues of the pellicular membrane, which give cell surfaces a polyanionic character (carboxyl groups) (COOK et al., 1961; COOK, 1962; EYLAR et al., 1962). The more or less immediate loss of the surface coat upon entry of the bloodforms into the vector could partly be caused by the action of proteolytic enzymes, known to reside in the middle segment of the midgut of *Glossina* (WIGGLESWORTH, 1929). In dying bloodforms where the coat is lost, too, the rupture of lysosomes and the action of released proteases could elicit a similar effect. The fact, however, that the bloodforms transforming to culture or midgut-forms also lose their coat, possibly without the presence of proteases, offers scope for a more speculative interpretation. The loss of the surface coat could be related to the "respiratory switch" undergone during transformation, which might bring about differences in the formation of linkage groups between the pellicula and the coat-borne glycoproteins.

For the possible function of the coat as a specific protein binding site in the course of pinocytosis we refer to VICKERMAN (1969a) and GEIGY et al. (1970).

C. Mitochondrion and kinetoplast

It was previously shown that the cyclic development of *T. brucei* trypanosomes involves changes in the mitochondrion's size and shape (VICKERMAN, 1962; 1965a, b).

The mitochondrion of the slender bloodstream forms consists of but one single tube extending forward from the kinetoplast; in addition, a short post-kinetoplastic part is present. At the slender stage mitochondrial tubules (cristae) are scarce or absent, but they become more numerous as the slender form transforms to the stumpy form through intermediate stages, concomitantly with the mitochondrion's increase in width (VICKERMAN, 1962).

The transformation of pleomorphic bloodforms with respect to the changes in mitochondrial volume densities was elucidated with the aid of stereological methods (HECKER et al., 1972). The volume density increases significantly from 0.022 in slender forms to 0.085 in "extrapolated" stumpy forms.

Following the transformation to culture (VICKERMAN, 1962) or midgut-forms the chondriome appears even more elaborate and ramified

lined with abundant cristae. This mitochondrial network is retained in trypomastigote proventricular and epimastigote forms (VICKERMAN, 1966a; b; c). In culture and midgut forms the kinetoplast moves to a more anterior position lying then at half-distance between the nucleus and the posterior end of the cell. This migration proceeds farther in this direction until the kinetoplast comes to lie anterior to the nucleus deemed as "epimastigote". It is thought that the kinetoplast's migrations (VICKERMAN, 1962; 1971b) is effected by the mitochondrial proliferation.

Similar events were reported from the leishmania-leptomonad transformation of *Leishmania donovani* (RUDZINSKA et al., 1964). There the kinetoplast enlarges and develops mitochondrial branches. Moreover, SIMPSON (1968a) observed that the transformation process of *Leishmania donovani* is dependent on the available nutrients (amino acids and glucose) and concomitant with changes in the soluble antigens.

Reduction of the mitochondrion in anticipation to the formation of metacyclic forms already starts when the epimastigote stage is reached. This is incompatible with VICKERMAN's (1966c; 1969a; 1970a) observations that the reduction sets in when the metatrypomastigote stage is reached. It results from our observations that the movement of the kinetoplast from the ante-nuclear to the post-nuclear position during the transformation in the salivary glands is likely to be mediated by a new outgrowing mitochondrial tube following the division of an epimastigote form. This would mean that the mitochondrion of an infective metacyclic form constitutes rather a newly synthesized than a converted structure. The reduced mitochondrial tube of the metacyclic form is comparable to that of the slender bloodstream form (VICKERMAN, 1969a; 1970a; STEIGER, 1971). However, internal cristae are still relatively numerous, the matrix being more electron-dense than in the blood stages.

In the present study several transition forms have been identified. On the basis of structural organization, especially of the mitochondrion, they can clearly be interpreted as leading to mature stages. Attention was paid to morphological changes of the mitochondrial cristae in the course of the life-cycle (VICKERMAN, 1971a). However, a strict differentiation into definite types of cristae cannot unobjectionably be confirmed. Tubular cristae in stumpy bloodforms seem to give rise to rather a mixed type of cristae encountered in midgut and proventricular forms. "Closely packed tubular cristae" could be ascertained in epimastigotes, while those in the metacyclics are fairly short and bulbous.

The crithidial (epimastigote) forms in the salivary glands were found attached to the epithelium, as reported elsewhere (VICKERMAN, 1966c). They could be regarded as a specialized division phase re-

sponsible for the proliferation of many daughter flagellates for the purpose of production of metacyclic forms. As intimated, this process involves an augmented synthesis of mitochondrial membranes, rather than a simple splitting of the existing mitochondrial network (VICKERMAN, 1966a). However, partial splitting is probable, possibly supported by "labyrinth-like extensions" (BROOKER, 1971a).

It is widely accepted that morphological differences of the mitochondrion in *brucei* subgroup trypanosomes are to be correlated with differences in enzymatic activities (VICKERMAN, 1962; 1963; 1965a; 1971a). This is in keeping with the fact that the cristaeless mitochondrion in slender bloodstream forms of *T. brucei* is inactive as revealed by the absence of NAD-diaphorase activity (VICKERMAN, 1965a). Slender (monomorphic) bloodforms contain an aerobic glycolytic pathway respiring glucose as far as pyruvate (VON BRAND et al., 1955; VON BRAND, 1960). This system accounts alone for the production of energy-rich ATP (VICKERMAN, 1970a). Moreover, these forms are CN-insensitive indicating the absence of a functional cytochrome chain. A functional Krebs cycle is not detectable (RYLEY, 1962). It was shown that the particular terminal respiration is effected by a unique L- α -glycerophosphate oxidase system substituting for the cytochrome chain (FULTON & SPOONER, 1959; GRANT & SARGENT, 1960; 1961a, b).

NAD-diaphorase was identified as a lipoyl dehydrogenase, which can be engaged in catalyzing the reversible oxidation of NADH and reduction of NAD (MASSEY, 1960). NADH formed in bloodforms is thought to be reoxidized by the paramitochondrial L- α -glycerophosphate oxidase system (HILL & ANDERSON, 1970). RYLEY (1966) and VICKERMAN (1965a), demonstrated that the L- α -GPO and the lipoyl dehydrogenase activity, respectively, are found in "discrete" extramitochondrial bodies. These organelles are most probably identical with the "peroxisome-like" organelles (VICKERMAN, 1969a), the "Type I granules" (BIRD et al., 1966; MOLLOY & ORMEROD, 1971), the "dense bodies" (VICKERMAN, 1971a) and the "GPO bodies" (BAYNE et al., 1969b). The latter were isolated from a subcellular fraction of *T. equiperdum* homogenates. These spherical bodies stained indeed positive for L- α -GPO not inhibited by cyanide added.

The appearance of mitochondrial cristae and the proliferation of the chondriome in intermediate and stumpy forms parallel the detection of mitochondrial enzymes rendering the chondriome "active" (NAD-diaphorase positive: VICKERMAN, 1965a). This proves that the "respiratory switch" takes place in the bloodstream to get stumpy forms preadapted to the development in the vector or culture.

BOWMAN & FLYNN (1968) produced evidence, however, that the Krebs cycle and the cytochrome system in intermediate and stumpy bloodforms are still not functional yet. It was suggested that a fully

developed cytochrome chain is acquired when the stumpy form has reached the vector (HILL & ANDERSON, 1970). Established culture forms are known to possess a cyanide-sensitive terminal respiration and a fully functional Krebs cycle both residing in the well developed elaborate mitochondrial network (RYLEY, 1962). Just recently EVANS & BROWN (1971) have pointed out that "immature" culture forms transforming from the bloodstream to the established midgut stage are considerably different from either of these stages. They showed that the oxidation of succinate, NADH and L- α -glycerophosphate by trypanosomes of cultures up to 4 days old was completely insensitive to azide, antimycin A and cyanide, and they suggested that terminal respiration, again, is linked to the L- α -GPO mechanism. Additionally, these authors found that the respiratory switch is reversible if trypanosomes are transferred to a culture medium containing a higher amount of blood lysate (BROWN & EVANS, 1971). The physiologically "immature" period (up to 4 days) is in line with the morphological situation. "Surviving transition forms" in the midgut are found up to the 4th day after the infective bloodmeal. It signifies that the morphological changes during this period, namely the outgrowth of the mitochondrion and the final differentiation of the cristae, imply the final differentiation of the fully active Krebs cycle and the cytochrome system.

It can be assumed that the active chondriome is retained in the midgut, proventricular and epimastigote forms, but that terminal respiration is reverted back to the extramitochondrial L- α -GPO pathway when the metacyclic stage is reached, concomitantly with the repression of the chondriome and the reduction of the cristae. This repression may proceed stepwise or only partially, since NAD-diaphorase activity was reported from the mitochondrion of the metacyclic form (VICKERMAN, 1970c).

The presence of different mitochondrial enzymes in several species of trypanosomes was ascertained, confirmed and reviewed by RAUSCHENBACH (1967), whereas the electron transport systems in Trypanosomatids were revised by BAERNSTEIN (1963) and HILL & ANDERSON (1970).

There is some indication that the dyskinetoplasmic *T. evansi* and *T. equiperdum* resemble biochemically and ultrastructurally the slender (monomorphic) bloodforms of *T. brucei* (VICKERMAN, 1970a). The monomorphic forms of *T. congolense* and *T. vivax* do have an active mitochondrion with many cristae (RUDZINSKA & VICKERMAN, 1968; VICKERMAN, 1966b; 1969b). The repression of the chondriome seems to be much restricted.

Glucose in bloodstream forms of *T. congolense* is metabolized more completely, since carbon dioxide, acetic and succinic acid can be traced (AGOSIN & VON BRAND, 1954; RYLEY, 1955). However, some doubt exists whether the tricarboxylic acid cycle in *T. congolense* is fully

functional. RAUSCHENBACH (1967) stated that possibly only the blood-forms of *T. cruzi* and *T. lewisi* dispose of a quantitatively significant citric acid cycle. Again, extramitochondrial terminal respiration could be functioning (VICKERMAN, 1969b).

As regards the culture forms of *T. congolense* the respiration is sensitive to cyanide and cycle inhibitors indicating the presence of a tri-carboxylic acid cycle linked to a cytochrome system (VON BRAND & TOBIE, 1959). This accords with VICKERMAN's description (unpublished observations) of an elaborate mitochondrial network in epimastigotes of *T. congolense* in the proboscis of *Glossina* reflecting the active state of this organelle. A similar respiratory switch, although not so accentuated, can be assumed therefore for *T. congolense*. KALLINIKOVA (1968; 1969) found that cytochrome oxidase and NAD-diaphorase are present in all developmental stages of *T. cruzi*, quantitatively varying during the development *in vitro*. The epimastigotes reveal peroxidase activity, which is wanting in other stages. The results obtained by the author aforementioned are consistent with the presence of a functional Krebs cycle (RAUSCHENBACH, 1967), as indicated by several other Krebs cycle enzymes (AGOSIN & VON BRAND, 1955; AGOSIN & WEINBACH, 1956), and the presence of a well developed mitochondrion during the whole life-cycle (BRACK, 1968).

It can be deduced from the results cited in the present study that the structural organization of the kinetoplast in the course of the developmental cycle of *T. brucei* remains practically unchanged. This conflicts, apart from the division cycle, with the descriptions of different types of kinetoplasts during the development within the same species (*T. cruzi*: BRACK, 1968; *Herpetomonas muscarum*: BRUN, in preparation). *T. cruzi* and *H. muscarum* are more archaic flagellate types.

Kinetoplasts are confined to the Trypanosomatids (reviewed by MUEHLFORDT, 1963; 1964) and Bodonids (PITELKA, 1963).

A wide variety of kinetoplasts are found in Trypanosomatids (CLARK & WALLACE, 1960). Their continuity with the mitochondrial system was reported from all trypanosomes (MUEHLFORDT, 1964).

The morphological organization is characterized by the two limiting membranes (mitochondrial membranes) and a fine flocculent matrix containing electron-dense fibrillar structures representative of the kinetoplast's DNA (MUEHLFORDT, 1963; 1964; MUEHLFORDT & BAYER, 1961). Earlier investigators confirmed the presence of DNA by Feulgen staining and by incorporation of H_3 -thymidine (BRESSLAU & SCREMIN, 1924; STEINER et al., 1958), as well as by DNase digestion (COSGROVE & ANDERSON, 1954; OZEKI et al., 1971). The kinetoplast is self-duplicating and is considered a specialized part of the mitochondrion, where DNA is concentrated (STEINERT, 1960; ANDERSON & ELLIS, 1965). It is known that K-DNA differs from nuclear DNA in some

physical properties, such as buoyant density in CsCl, base composition and "melting" temperature (DU BUY et al., 1965; 1966; RIOU et al., 1966; RIOU & PAOLETTI, 1967; STEINERT & VAN ASSEL, 1967; SIMPSON, 1968). Electron microscope studies showed that K-DNA is circular, however, accompanied by long linear DNA molecules (RIOU & DELAIN, 1969). Loss of kinetoplast DNA ("dyskinetoplastic": TRAGER & RUDZINSKA, 1964) is known to occur in bloodstream forms of trypanosomes; it can arise spontaneously or be induced. Dyskinetoplastic trypanosomes can survive and divide in the bloodstream, but they are not able to be transmitted cyclically or to grow in culture media (TOBIE, 1951; HOARE, 1954).

The loss of K-DNA can be effected by trypanocidal drugs, such as acriflavines (KUSEL et al., 1967; STEINERT & VAN ASSEL, 1967; HILL & ANDERSON, 1969), ethidium bromide (DELAIN & RIOU, 1969; STEINERT, 1969; STEINERT et al., 1969), berenil (NEWTON & LE PAGE, 1968; MACADAM & WILLIAMSON, 1969; RIOU et al., 1970) and stilbamidines (DELAIN et al., 1970; DELAIN et al., 1971). A similar affinity to K-DNA is exerted by p-rosaniline (INOKI et al., 1969). It was supposed that accessibility to these drugs is largely due to the absence of DNA-associated histones (STEINERT, 1965). It resulted that the kinetoplast's envelope in dyskinetoplastic forms is retained lacking, however, the fibrous DNA inclusion (MUEHLFORDT, 1963; INOKI & SUGANUMA, 1964; TRAGER & RUDZINSKA, 1964). This statement also applies to trypanosomes treated with drugs, of which p-rosaniline is the exception in affecting irreversibly the mitochondrial membranes, too (INOKI et al., 1969). Yet, a dense body of unknown nature always remains in the kinetoplast (VICKERMAN, 1966b; MILDER & DEANE, 1969). GUTTERIDGE et al. (1971) found that the kinetoplast of dyskinetoplastic trypanosomes species still contains K-DNA.

DNA-interacting drugs can be distinguished with respect to their mode of action (DELAIN et al., 1971). One deals with intercalating dyes (acridines and ethidium bromide) and drugs which preferentially bind to AT-rich DNA (hydroxystilbamidine and berenil). P-rosaniline is supposed to act "on the synthesis of kinetonuclear materials, but not directly on the kinetonucleus in the resting stage" (INOKI et al., 1969).

The loss of K-DNA can clearly be correlated with respiratory deficiencies, which would mean that the kinetoplast is of some importance for regulating the mitochondrial activity.

STEINERT & STEINERT (1962) postulated that the kinetoplast DNA presumably controls the formation of a fully active mitochondrion. Intercalating ethidium bromide selectively inhibits the replication of K-DNA, which is diluted out with divisions (STEINERT, 1969). Moreover, it was suggested that kinetoplast DNA is engaged in regulating the respiratory switch in *T. brucei* (BAYNE et al., 1969a). Artificial

drug-induced dyskinetoplasty is concomitant with the death of the culture forms (TRAGER & RUDZINSKA, 1964; STEINERT & VAN ASSEL, 1967; STUART & HANSON, 1967), and with the inability of trypanosomes to establish themselves in the vector (REICHENOW, 1940). Furthermore, it was shown that treatment with acriflavine or ethidium bromide induces a decrease of mitochondrial respiratory enzymes, accompanied by an increase of glycolytic enzymes (KUSEL et al., 1967; HILL & ANDERSON, 1969; 1970). HILL & HUTNER (1968) noted that succinic dehydrogenase represents the main target of various trypanocides.

Important conclusions can be drawn from results obtained with other cells. Mitochondrial DNA can code for only a small fraction of all mitochondrial components (ROODYN & WILKIE, 1968; BORST & KROON, 1969), which is consistent with the results of BAYNE et al. (1969a), who pointed out that only membrane proteins are coded for in mitochondrial fractions from *T. conorhini*. It was demonstrated that in yeast the information for the synthesis of mitochondrial ribosomal proteins is provided by the mitochondrial DNA (LINNANE et al., 1968). Ethidium bromide and acridine dyes do, in fact, inhibit mitochondrial DNA polymerase and interfere with mitochondrial RNA and protein synthesis (reviewed by DELAIN et al., 1971). Most of the proteins synthesized in mitochondria are generally thought to be membrane components (BEATTIE et al., 1967); moreover, cytochromes in yeast are produced in the mitochondria (LINNANE et al., 1968).

Mitochondrial and kinetoplast DNA are unlikely to contain enough information for all products needed; BAYNE et al. (1969a) assume therefore a "necessary nucleo-cytoplasmic regulation".

The synthesis of special membrane components could, for instance, be regulated by K-DNA, whereas mitochondrial enzymes and electron-carriers would be synthesized in the cytoplasm, regulated by nuclear DNA, which, on the other hand, could at the same time be responsible for the respiratory switch to the cyanide-insensitive para-mitochondrial terminal respiration in trypanosomes (BAYNE et al., 1969a).

As a matter of fact, RNA and proteins could be detected in kinetoplasts of *T. cruzi* and *T. gambiense* (OZEKI et al., 1971).

We tend to think that the synthesis of mitochondrial membranes, especially in midgutforms of *T. brucei*, could be supplied with structural proteins synthesized on the large number of free cytoplasmic polysomes.

At times, nucleo-kinetoplastic interaction was explained on the basis of a structural connection between the nucleus and the kinetoplast (MUEHLFORDT, 1963; 1964). Such structural links have never been observed with our material.

From the facts cited follows that the kinetoplast DNA may partly be responsible for the activation of the mitochondrion. It may be in-

volved in the synthesis of specific membrane proteins and possibly also of a few enzymes. The kinetoplast seems to represent a semi-autonomous organelle, being part of a nucleo-kinetoplasmic interaction mechanism and being subject to nuclear control.

D. Cytoplasmic membrane systems

The close association of the cellular membrane systems coincides with the feature termed as "GERL" reported from neurons (NOVIKOFF, 1967; NOVIKOFF et al., 1971). The cytochemical tests applied in the present study endorse the idea that we deal with a coherent canal system. The Golgi apparatus, Golgi vesicles and vesicles around the reservoir stained positive for polysaccharides, which proves that the vesicles of the latter type, in fact, represent descendants from the GERL zone. Also a high number of ser tubes, multivesiculate bodies and pieces of rer are clearly marked. The heavy staining of the Golgi complex with the TCH-silver-albumose technique conforms to a high activity of this organelle, which is involved in the synthesis of polysaccharides and glycoproteins (THIÉRY, 1967; 1969). The marked rer tubes could provide proteins to be incorporated in the Golgi area into glycoproteins; the carbohydrate-positive ser canal system is likely to give rise to vesicles located close to the flagellar pocket.

It is known that the cytoplasmic membrane systems are to be reduced upon transformation to culture forms (VICKERMAN, 1971a). Our results make it evident that in all vector stages, except "immature" and "mature" metatrypomastigotes in the salivary glands, the bulk of the membrane canal systems is in retrogression. The smooth endoplasmic reticulum is practically lacking, the rer is absent and the diminished Golgi apparatus appears inactive. The number of free ribosomes has strongly increased. This ought to be correlated with the intensified synthesis of structural proteins needed for the proliferation of the mitochondrion and the cell growth. We endorse the hypothesis that the GERL membrane system is decomposed by the action of lysosomal organelles, namely autophagosomes (autophagic vacuoles), in order to lay out a membrane pool, from which membrane subunits for the formation of an extended mitochondrion can be derived. Similar observations with regard to modifications of the Golgi apparatus and the endoplasmic reticular system were made in other trypanosome species. In epimastigotes of *T. cruzi* (BRACK, 1968) only smooth membranes are visible; free ribosomes are abundant, whereas in trypomastigote bloodforms the er is made up by long flat saccules densely lined with ribosomes. In opposition to that, SANABRIA (1966) found rer

in either of these stages. In epimastigotes of *T. conorhini* the ser is only little developed composed predominantly of bits of smooth membranes (MILDER & DEANE, 1967). The statement by HERBERT (1965) that growing culture forms of *T. theileri* and *T. melophagium* possess a wide, active Golgi zone deserves special interest.

It is thinkable that the well developed active GERL zone, e.g. in bloodforms of *T. brucei*, is absolutely necessary to cope with the high division rate of the flagellates; however, the non-dividing stumpy bloodforms have got a well developed GERL area.

It is to be hoped that exact morphometrical studies would help to clarify the ins and outs of this matter. We do not think that the ser is responsible for the formation of "sacs of secretion", as suggested by VICKERMAN (1969b). Likewise, the "local dilatations" of the "agranular (secretory) reticulum" are not thought to be involved in the process of secretion. It was pointed out before (STEIGER, 1971) that the swellings of the ser tubes might rather lead to the stepwise formation of organelles, such as peroxisome-like organelles and lysosomes, a view endorsed by the results obtained in this study. At the same time, we tentatively rule out the possibility that secretion products of the "membrane systems" (VICKERMAN, 1971a) should result in coat formation. The formation of basic surface coat material in the metacyclics appears more plausible.

If we were dealing with a true Golgi-derived secretion product, the PA-silver method would surely reveal the presence of carbohydrates. However, these seem to be absent, which speaks in favour of newly formed organelles. The distribution of the lead phosphate deposits in the tests for acid phosphatase fits well to his general picture. Acid Pase activity in bloodforms of *T. brucei* is confined to parts of the Golgi complex, to the lumen of the reservoir, to vesicles near the reservoir membrane, to multivesiculate bodies, very distinctly to the smooth endoplasmic reticulum (ser) and to parts of the perinuclear cisterna.

Endocytosis in *brucei* trypanosomes is believed to take place in the reservoir substituting for a cytopharynx/cytostome complex as revealed by protein tracer experiments (ARMSTRONG et al., 1964; BROWN et al., 1965; GEIGY et al., 1970). Pinocytosis is in conjunction with the catabolic activity of lysosomal structures. In these respects acid phosphatase as a marker enzyme could be demonstrated several times in trypanosomes (BROOKER & VICKERMAN, 1964; WÉRY & DE GROODT-LASSEEL, 1966; HARMSSEN, 1967; SEED et al., 1967; SANABRIA, 1969). The lysosomes appeared thereby concentrated around the flagellar pocket. Our results confirm the extracellular localization of acid phosphatase in the flagellar pocket recently reported (JADIN & CREEMERS, 1970b).

Extrusion of acid Pase-positive vesicles derived from the ser into the reservoir was described by JADIN & CREEMERS (1970b).

Glucose-6-Pase was localized in the flagellar pocket, too (SEED et al., 1967). PRESTON (1969) and the authors aforementioned attribute to the reservoir an important role in extracellular digestion (pre-digestion), which would favour the phagocytotic uptake of proteins. The localization of acid Pase and of the peroxidase tracer (GEIGY et al., 1970) in the flagellar pocket revealed a definite condensation of the reaction products, which would signify that besides extracellular digestion a distinct concentration and storage of proteins take place. These events justify the term "reservoir". The abundance of acid Pase-positive pieces of ser in the vicinity of the reservoir membrane may indeed contribute to the formation of vesicles destined to discharge their enzymatic contents into the extracellular space. Other vesicles or tubules, however, seem to represent primary lysosomes ready to fuse with phagosomes budding off from the reservoir membrane. These phagosomes ("pinosomes": VICKERMAN, 1969b) can be marked, too, when they are concerned in the uptake of "predigested" stained material. Primary lysosomes, which are supposed to be represented by the Golgi-derived "acanthosomes" (VICKERMAN, 1969a; b), would account, upon fusion with the phagosomes, for the presence of secondary lysosomes. "Acanthosomes" with the morphology described by the above author for *T. congolense* could never be found in *T. brucei*. We tended to suppose that these specialized vesicles are identical with the pinocytotic "coated" vesicles (STEIGER, 1971). The secondary lysosomes, in turn, might end up, by coalescence, in the formation of multivesiculate bodies.

The peroxisome-like organelles, once described as "Type I granules" and deemed as "lysosomes" (BIRD et al., 1966; MOLLOY & ORMEROD, 1971) do never stain for acid phosphatase. At several stages in the bloodstream and the vector, however, they seem to be in transition contributing to the formation of multivesiculate bodies and they could then exhibit lysosomal character. Sequestration of exogenous proteins by multivesiculate bodies is well known from the rat's vas deferens (FRIEND & FARQUHAR, 1967) and from the fat body of an insect (LOCKE & COLLINS, 1968). The presence of lead salt deposits in the nuclear envelope, which is continuous with the protein-synthesizing rer system, may reflect an important role of this organelle in the synthesis of the actual Pase enzyme. This enzymatic moiety might be transported to the Golgi complex and packed there for the lysosomes and the direct delivery via the ser system to the reservoir. The heterogenous origin of the multivesiculate bodies cannot always be followed up with certainty. They could be built up by fusing secondary lysosomes, by the transformation of peroxisome-like organelles, as intimated before, or they could represent *de novo* synthesized structures derived from the GERL's membrane pool (ser) or the fenestrated Golgi saccules. The

swellings of ser tubes, possibly concerned in the formation of peroxisome-like organelles, could also account for the formation of mvb. The supply with the enzymatic outfit would be facilitated in both cases.

In transitional deteriorating midgutforms the quantity of lysosomal structures, especially multivesiculate bodies, increases significantly, comparable to the situation in aged culture forms of *T. cruzi* (WÉRY & DE GROODT-LASSEEL, 1966). It can be concluded that these structures might be involved in the digestion of cytoplasmic material. With increased age of the infection in *Glossina* (e.g. trypomastigote proventricular stage) even dense and residual body-like structures (DE DUVE & WATTIAUX, 1966) accumulate.

The release of undigestible material into the reservoir was suggested by BROOKER (1971a). This material is likely to be identical with the "débris" (CREEMERS & JADIN, 1967). "Dense globular particles of débris" (VICKERMAN, 1969a; b) are found at several stages during the life-cycle of *T. brucei*. In bloodforms they stain plainly positive for carbohydrates; this probably confirms that they are Golgi derivatives. They are membrane-bounded and are possibly exocytosed, as proved by the peripheral localization of the surface coat. Since lysosomes do occasionally contain silver grains indicative of polysaccharides, this "débris" material could just as well be of lysosomal nature (= discharged residual bodies?). "L'appareil élaborateur" (BOISSON et al., 1967b), which was thought to take part in the production of nutritives or hormones, could be an autophagosome. This suggestion is supported by the presence of material accessible to digestion with DNase and oxy-RNase.

E. Peroxisome-like organelles

As intimated before, the term "peroxisome-like organelle" originally used by VICKERMAN (1969a) is synonymous with the expressions "Type I granule" or "lysosome" (BIRD et al., 1966), "dense body" (VICKERMAN, 1971a) and "GPO body" (BAYNE et al., 1969a; b). The term "dense body" is misleading, because this name is to be reserved to a particular post-lysosomal organelle (DE DUVE & WATTIAUX, 1966).

It was mentioned and discussed before that the formation of these spherical peroxisome-like organelles may be ascribed to the ser system. In the course of the life-cycle these bodies undergo differences as to their number and shape. The ectoperitrophic midgutforms in the proventricular region contain a definitely reduced set of GPO bodies, possibly due to the fact that these organelles give rise to the formation of digestive multivesiculate bodies. The same statement holds for the trypomastigote proventricular forms. The number of GPO bodies increases again in the epimastigotes of the salivary glands until reaching

its normal density in metacyclic forms. It was noted before that the GPO bodies lodge the extramitochondrial terminal respiration (L- α -glycerophosphate cycle), which is fully active in bloodforms and early culture forms (up to day 4) of *T. brucei*. It can be assumed that they remain inactive in the vector stages till they are activated again when the metacyclic forms are extruded into the bloodstream to cause a new infection.

These events parallel the different metabolic states of the mitochondrion. There is some evidence that, when the mitochondrion is active, the peroxisome-like organelles can partly be mobilized for the formation of multivesiculate bodies. They seem to have got the capability, however, of being reactivated when the culture forms are put into media with a higher blood content (EVANS & BROWN, 1971).

On morphological grounds they resemble peroxisomes or microbodies of other protozoa (MUELLER, 1969). In fact, at times, they contain membranous inclusions embedded in the matrix typical of microbodies (HRUBAN & RECHCIGL, 1969). Yet, marker enzymes, such as catalase or peroxidase, could not readily be demonstrated with our material.

It is well known that peroxisomes in *Tetrahymena* contain glyoxysomal components (HOGG, 1969, quoted by MUELLER, 1969). They are likely to be concerned with glyconeogenesis, too (HOGG & KORNBERG, 1963). This could also be the case in *T. brucei* in relation to a possibly augmented synthesis of glucose from amino acids to satisfy the great demand of the aerobic glycolytic pathway in bloodforms of this species. In turn, these organelles could also be involved in the production of oxaloacetate used for the Krebs cycle when the mitochondrion is active in established vector or culture stages. This would mean, however, that the peroxisome-like organelles must retain at least a limited activity throughout all vector stages.

Yet, exact data on the activity of the GPO bodies during the life-cycle are not available. A high peroxidatic activity was reported from culture epimastigotes of *T. cruzi* (KALLINIKOVA, 1968), which could indicate an activity of the organelles concerned; identical bodies are, in fact, present in this trypanosome species (SANABRIA, 1966; BRACK, 1968). Despite limited staining for peroxidase in our material, the presence of this enzyme in *T. brucei* seems probable in view of the fact that hydrogen peroxide would inhibit the activity of L- α -glycerophosphate dehydrogenase (GRANT & SARGENT, 1961a). The action of a substrate-specific peroxidase is likely to intervene in the two-step reaction of the L- α -glycerophosphate oxidase system (GRANT & BOWMAN, 1963). These findings are supported by the results obtained by MICHEL (1964), who found naphtholperoxidase-positive granules in the cytoplasm of *T. gambiense*. Since peroxidase was also localized in lysosomes in conjunction

with the digestion of pinocytosed material (JADIN & CREEMERS, 1970a), it could well be that some of the peroxidase-positive reflex-microscopical granules aforementioned represent lysosomal organelles. Earlier investigators revealed the presence of at least two different types of granules in the cytoplasm of *T. brucei* trypanosomes (MICHEL, 1964; 1966; BIRD et al., 1966; MOLLOY & ORMEROD, 1971). The term "volutin granule" was applied to the Giemsa-positive "Type I granule" (BIRD et al., 1966) and to the "Methylenblaugranula" (MICHEL, 1964; 1966), as well as to the "Type II granule" (ORMEROD, 1958; BIRD et al., 1966).

The nature and function of the latter type shall be discussed later. The suggestion by HERBERT (1965) that inclusions considered "volutin granules" might be made up by several types of inclusions of different function extricates from the confusion existing in this context. Morphometrical data (HECKER et al., 1972) cannot directly be related to the observations by ORMEROD (1961) that "volutin granules" are more frequent in stumpy than in slender bloodstream forms of *T. rhodesiense*. ORMEROD worked with absolute numbers, whereas the morphometrical data represent relative numbers of vesicles, which are more representative of a functional interpretation. Strain-specific differences of the trypanosomes used in both studies, however, cannot be excluded yet.

On the other hand, the frequency of light microscopical granules can clearly be correlated with the course of infection of monomorphic *T. gambiense* in several laboratory animals (MICHEL, 1966). The same author demonstrated "Azur-I-Granula", which proved to be identical with the granules exhibiting an unspecific tetrazolium reaction. This type of granule, in turn, is identical with the extramitochondrial respiratory or GPO body. Thiol groups proved to be responsible for the affinity to the Azur-I-dye. These SH-groups were supposed to be related directly to the essential SH-groups of the L- α -glycerophosphate oxidase enzyme. This suggestion is strongly supported by the fact that this enzyme is indeed inhibited by thiol reagents (GRANT & SARGENT, 1960). The methylene blue-positive "RNS-Granula" (Volutingranula) (MICHEL, 1964; 1966) might represent pieces of reticulating their affinity to this stain to the membrane-associated ribosomes.

F. Lipid inclusions

Our observations showed that two types of lipid inclusions occur in *T. brucei* in the course of its developmental cycle.

In bloodforms only the small osmiophilic type exists, whereas in midgutforms only the larger less electron-dense type can be found. In the ectoperitrophic midgutform of the proventricular region the first

type reappears accompanied by the second type. Both inclusion types are rare in epimastigotes. When the metatrypomastigote stage is reached, only the small droplet may occasionally be encountered.

It is most probable that changes in type and frequency may imply physiological modifications, as suggested by DIXON & WILLIAMSON (1970).

The lipid nature of either of these inclusion types has been proved by the application of the lipid extraction method carried out on thin sections.

The small osmiophilic lipid droplet of bloodforms seems to be identical with the "Type II granule" (BIRD et al., 1966; MOLLOY & ORMEROD, 1971) and the "endoplasmic reticular granule" (MOLLOY & ORMEROD, 1971). However, connections with the endoplasmic reticulum have never been observed with our material. The view expressed by the latter authors that the "Type II (endoplasmic reticular) granules are concerned with storage and secretion of lipoprotein material..." is rather inexact. Yet, some indications for their lipid nature existed before; they stain with fat soluble dyes and are removed upon fixation in methanol (ORMEROD & PAGE, 1967). Evidence has recently been produced (VENKATESAN, 1972) that the "Type II granules" (= osmiophilic type) contain large amounts of lipids. This author compared two strains of *T. brucei* containing a different number of these granules at the peak of infection. He assumed that differences in lipid content should rather be attributed to differences in membrane synthesis or uptake than to strain-specificity. Moreover, the presence of esterified sterols in these granules could be ascertained. It has been demonstrated in this paper that the small osmiophilic lipid droplet type stains very distinctly for carbohydrates, whereas the reaction in the large less electron-dense lipid droplet type is lowered (unpublished observation).

The degree of osmiophily in the two types differs significantly. This might be due to a high or low amount, respectively, of unsaturated fatty acids and sterols. This view is endorsed by the fact that the osmiophilic type is not so readily extracted as the less electron-dense type. Analogous to the situation in the pellicle, with respect to the positive staining for carbohydrates, similar phosphatidyl compounds might be present in the small osmiophilic lipid inclusion type. This would sustain the suggestion that the small droplet type would contribute to the formation of subcellular membranes, especially since this inclusion type is absent from midgutforms where the proliferation of the chondriome has already ceased.

In view of these points we think that the larger lipid droplet type common in midgutforms represents a storage product. It was suggested before (DIXON & WILLIAMSON, 1970) that total lipid composition may

parallel the proliferation or regression of the lipid-rich mitochondrion. As a matter of fact, the same authors found specific differences in the amount of triglycerides detected. Cultured *T. rhodesiense* contain more than bloodforms. The triglyceride pool is considered a fatty acid reserve for culture or midgutforms. The phospholipids of blood *T. rhodesiense* include a high sphingomyelin content accounting for the plasma membrane (pellicle), since the mitochondrion is only poorly developed.

GODFREY (1967) noted that *T. lewisi*, known to possess an elaborate mitochondrial complex, exhibits a relatively high content of phosphatidyl choline in bloodforms; this compound is also typical of mitochondria in mammalian cells. It is thought that the high amount of "lipoprotein/lipopolysaccharide" material in culture forms of *T. rhodesiense* (WILLIAMSON & GINGER, 1965) may be correlated with the well known mitochondrial outgrowth.

DIXON & WILLIAMSON (1970) found that polyunsaturated fatty acids are much more frequent in bloodforms than in culture forms of *T. rhodesiense*. This fact was discussed by these investigators with respect to the loss of ability to synthesize these compounds, and hence to the adaptation to the insect host. The incorporation of such acids from the host environment would warrant successful survival and development in the invertebrate vector. The same authors pointed out, on the other hand, that the fatty acid pattern of the blood trypanosomes need not be a copy of the lipid composition of the host's plasma. Trypanosomes are therefore attributed a definite control function over the composition and distribution of their lipids. It was furthermore suggested by these authors that essential exogenous lipids are required.

It can be concluded that the osmiophilic lipid droplets in bloodforms containing considerable amounts of polyunsaturated fatty acids may mainly be concerned in providing material needed for the proliferation of mitochondrial membranes; in turn, the larger less electron-dense lipid droplets in midgutforms may constitute rather a triglyceride pool destined to be incorporated, in case of need, into the metabolic pathways. The reappearance of the first type concomitantly with the gradual disappearance of the second type in the course of the life-cycle would signify that the ability to synthesize membrane-lipid precursors for the next mitochondrial proliferation (stumpy form/midgutform) is reacquired.

G. Attachment zones

"Intracellular" attachment zones between the pellicula and the flagellar membrane were previously described in *T. lewisi* (ANDERSON & ELLIS, 1965), *T. gambiense* (BOISSON et al., 1965) and *T. rhodesiense*

(VICKERMAN, 1969a). These complexes constitute junctions of the "macula adhaerens" type (FAWCETT, 1966). These adhesion zones persist in all developmental forms, but seem to vary, at times, in number; this could be explained in terms of the state of development of an "undulating membrane". VICKERMAN (1969a) reviewing these aspects assumes two mechanisms of binding. The first one is supposed to be temporary and varying with the flagellum's stroke; the second one operates at the macula junction and seems to be stronger. It was suggested by the same research worker that calcium ions are needed for adhesion, "but not for maintenance of attachment zones".

It has been demonstrated in the present study that attachment of epimastigotes to the epithelium of the salivary gland is achieved by deep insertion of the flagella into the epithelial lining, thus confirming preliminary results (VICKERMAN, 1966c). In addition, junctional complexes between apposed pellicular (flagellar) and epithelial membranes (microvillar border) have been found. They seem to warrant the close attachment preventing the epimastigotes from being ejected with the tsetse fly's saliva. Quite analogous reports in this context stem from *T. lewisi* in its natural host *Nosopsyllus fasciatus* (MOLYNEUX, 1969a) and from other Stercorarian trypanosomes, namely *T. grayi* in *Glossina palpalis* (HOARE, 1931a) and *T. avium* in *Ornithomyia avicularia* (BAKER, 1956).

According to the former author the epimastigotes of *T. lewisi* get attached to the wall of the flea's hindgut. Moreover, they appear concentrated there. Electron micrographs elucidated the situation very accurately. In one of two types of epimastigotes an "increased amount of intraflagellar material" could be observed; this feature allows a multiple mode of division. The "intraflagellar area" is then sometimes equipped with up to four axonemes. It is closely associated with the rectal wall forming a zonula adhaerens junction. The desmosome-like condensation appears on the inside of the flagellar membrane only. The term "hemidesmosome" would be appropriate, since the cuticular lining of the flea's rectal wall is devoid of any electron-dense plaque material. BRUN (in preparation), working with *Herpetomonas mirabilis* in *Calliphoridae*, found a similar situation. Hemidesmosomes are seen in the intraflagellar area; however, only one axoneme per flagellar sheath is found. In addition, true "maculae adhaerentes" between adjacent pellicles are seen. These may bring about a stronger adhesion between single flagellates. In *H. muscarum* these junctional complexes are formed by the giant cercoplasmatic promastigotes. Similar observations were made in *Crithidia fasciculata*, which develop "adhesion plaques" associated with the flagellar membrane when it comes into contact with the matrix of a millipore filtre (BROOKER, 1971b). The author called them "hemidesmosomes"; these seem to be confined to

the apex of a flagellar membrane's evagination. The plaques appear continuous with filaments converging from the cytoplasm on these structures. BROOKER (1971c) also described hemidesmosome-like complexes in haptomonads of *C. fasciculata* attached to the cuticular lining of the hindgut in *Anopheles gambiae*. They are comparable to the adhesion plaques in rosettes of *C. fasciculata* (BROOKER, 1970), which seem to be responsible for maintaining the integrity of these rosettes. The fact that de-adhesion can readily be induced (quoted by BROOKER, 1971b) leads to the suggestion that junctional complexes in Trypanosomatids can spontaneously be formed and decomposed.

Hemidesmosomes were also reported from phytoflagellates, where they have a role in maintaining the close contact between the protoplast and the theca (SCHNEPF & MAIWALD, 1970).

H. Peritrophic "membrane" and its penetration

The fine structure of the peritrophic "membrane" ("PM") in *Glossina* and its relation to the migration of the trypanosomes was described and discussed by MOLOO et al. (1970). Three cell types in the annular pad of the proximal midgut epithelium take part in the synthesis of material needed for the formation of the "PM". Type I and II cells seem to be involved in providing polysaccharides for the electron-dense first layer, whilst Type III cells contribute proteins for the thicker second layer. The "PM" is regarded as a bilaminate structure and does not represent a true unit membrane; as put forward by GANDER (1968) the name "peritrophic lamina" is more appropriate, though this author worked with mosquitoes, where the mode of formation is quite different. The presence of proteins and hexose substances was demonstrated histochemically (MOLOO et al., 1970). These results confirm those obtained by WIGGLESWORTH (1929) and WATERHOUSE (1957), who assumed chitinous ground substances containing proteins.

Our ultrahistochemical experiments shed some light on the localization of the polysaccharides and the proteins. In the proventricular region ahead of the "press" (WIGGLESWORTH, 1929), the "PM" is quite thick (3-4 μ) and possibly constitutes the "fluid secretion" described before (YORKE et al., 1933). In this area the staining for carbohydrates in both layers of the peritrophic "membrane" is made up by homogeneously spread fine granulations. It seems that here the carbohydrates of the thin first layer are not polymerized yet.

After the "PM" has been drawn through the "press", it represents a condensed structure only 0.35 μ thick. This portion is the fully formed "membrane" extending along the midgut. The first layer reveals then an increased concentration of silver granules, which appear diminished

in the second layer. Moreover, digestion with proteases have shown that the second layer contains a high amount of proteins. This lamina of the peritrophic "membrane", however, is only in the proventriculus accessible to enzymatic digestion.

It follows from these facts that the first layer contains probably chitinous substances, which get polymerized concomitantly with the condensation of the "PM". The second layer is supposed to be predominantly made up by glycoproteins and is remarkably compressed, possibly also accompanied by a polymerization of the protein units. The ultrahistological tests accord with the mode of formation anticipated by MOLOO et al. (1970). VICKERMAN & PERRY (1968) assumed a condensation of the peritrophic "membrane" in the absence of a "press" effect, especially in proventriculi heavily infected with trypanosomes.

It is widely accepted that the "PM" is closely related to the migration of trypanosomes in their vector. YORKE et al. (1933) emphasized that trypanosomes pass round the posterior free end of the peritrophic "membrane" and that they must subsequently cross this barrier in order to reach the proventricular lumen on their way to the "anterior station". They assumed that the fluid-like consistence of the "PM" favours penetration by the flagellates. On the other hand, FAIRBAIRN (1958) thought that the trypanosomes would pervade the "membrane" after it has been drawn through the "press", where it is considered to be still soft enough.

It is supposed that, unless "ectoperitrophic midgutforms in the foremost part of the midgut" are formed, the crossing cannot be perfected. These developmental forms seem to be prerequisite to transverse the "membrane" barrier. Their ultrastructural peculiarities, especially the type of lipid droplet involved, might imply physiological changes. Reconsidering this context, we think that penetration is effected mechanically, which conflicts with the "necessary enzymatic means" anticipated (MOLOO et al., 1970). It seems probable that the "PM" is really semi-liquid in the proventricular region, and that therefore the passage is possible.

The fully formed, condensed "PM" in the midgut cannot be penetrated by trypanosomes (HOARE, 1931b). However, a great number of flagellates are visible in the "membrane" folds and occasionally even embedded in the peritrophic "membrane". Yet, actual crossing, as postulated by FREEMAN (1970), has never been observed. This author also found trypanosomes embedded in the "PM".

The significance of the peritrophic "membranes" of blood-sucking Diptera for the development of blood parasites, with respect to the different modes of formation, was extensively discussed by STOHLER (1961).

WIJERS (1958) and WARD & BELL (1971), in opposition to HARLEY (1971), stated that the age of the tsetse fly has an influence on the infection rates with *T. brucei*. Young flies taking their infective blood-meal on the day of emergence develop higher infection rates than older flies. This fact was correlated with the outgrowth of the "PM", which constitutes a hindrance to the trypanosome migration. The outgrowth is stimulated by bloodmeals (WIGGLESWORTH, 1929); it is considerably slower in fasting or teneral flies (HOARE, 1931; WILLETT, 1966). We tend to think therefore that an interaction of some factors could enable to actual penetration in the proventriculus: e.g. first, the rate of secretion and the consistence of the "PM", secondly, the formation of the particular trypanosome stage mentioned before.

A mode of formation similar to that in *Glossina* exists in the blow-fly *Calliphora* (SMITH, 1968). Yet, the "PM" in this fly species is more complex, being composed of three laminar components. These are supplied likewise by Golgi derived secretion products and proteins from epithelial cells of the midgut, by chitinous material contributed by ectodermal and/or midgut cells, and finally by a third component shed off from the epithelial brush border.

In *Chironomus* the "PM" is formed analogously by the specialized columnar cells in the proventricular area (PLATZER-SCHULTZ & WELSCH, 1969). However, distinct modifications occur in this fly species: endodermal epithelial cells undergo classical apocrine secretion. The "PM" is a bilaminate structure derived from secretion granules and from the "pinched off upper half of the cell". Two types of cells are probably concerned in the formation of the peritrophic "membrane": the "ER-cells" and the "M-cells". Glycogen is provided additionally by the former ones. As put forward by STOHLER (1961) three different basic types of peritrophic "membranes" are found in insects. The "membranes" reviewed here belong to the "Type II", where the constituent parts are continuously secreted by a ring of specialized cells in the foremost part of the midgut.

It follows from the facts cited that genus-specific or even species-specific modifications are conceivable.

This is in keeping with the observations that in mosquitoes ("Type III") differences in the secretion mechanism and in the composition of the "PM" between *Aedes* and *Anopheles* exist (FREYVOGEL & STAEUBLI, 1965). In this context a particular publication deserves special notice: RICHARDS & RICHARDS (1971) demonstrated that differences in the formation of the "PM" prevail even between the imagines and larvae of *Aedes aegypti*. In contrast to the adult mosquito, the larva secretes "PM" material with the aid of specialized "microvillate midgut cells" in the anterior half of the proventricular pouch, which is much alike the annular pad in *Glossina*. The peritrophic "membrane"

becomes thicker with the age of the larva, and the fully formed "PM" (4th instar larva) is then composed of four layers of similar composition as in the tsetse fly ("chitin-protein units"). An analogous post-secretional, polymerization process ("post-secretion aggregation") sets in, however, in the absence of a "press" effect. This mode of formation conflicts with the statement that different developmental stages within the same insect species possess the same "PM" type (PETERS, 1969).

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Zusammenfassung

In der vorliegenden Arbeit wurden die ultrastrukturellen Veränderungen von *T. brucei* im Verlauf des Entwicklungszyklus im Blut und im Überträger *Glossina* untersucht. Zudem wurden einige zytochemische Experimente durchgeführt, welche die Funktion von Organellen, Vesikeln und Lipid-Einschlüssen sowie den Aufbau der peritrophischen Membran zu klären hatten.

Es konnte gezeigt werden, daß sich das Mitochondrium vom einfachen cristae-losen Zustand in schlanken Blutformen zu einem weitverzweigten Netzwerk mit vielen mitochondrialen Tubuli in Mitteldarm- und Proventrikelformen entwickelt. In epimastigoten Speicheldrüsenformen (Crithidia) setzt die Reduktion des Mitochondriums wieder ein, so daß schließlich in metazyklischen Formen der Speicheldrüse der einfache Zustand wieder erreicht wird; allerdings sind Cristae noch vorhanden.

Der Oberflächenschicht (surface coat) der Blutformen, der Sitz der Oberflächenantigene, geht im Vektor verloren und wird erst wieder in den trypomastigoten Speicheldrüsenformen sichtbar. Dabei zeigen unreife metatrypomastigote Stadien schon Vorläufer des Coats auf der Pellicula-Membran; die reifen metatrypomastigoten (metazyklischen) Formen besitzen den fertig ausgebildeten Oberflächencoat, wie er auch für die Blutformen charakteristisch ist. Es wird vermutet, daß die Grundsubstanz des Coats im Golgi-Apparat gebildet und mittels Vesikeln («Golgi derived vesicles») auf die Oberfläche der Trypanosomen abgegeben wird.

Die zytoplasmatischen Membransysteme (ER, Golgi, Lysosomen) verändern sich ebenfalls im Verlauf des Zyklus.

In den Blutformen ist das glatte und rauhe Endoplasmatische Reticulum gut ausgebildet. In den stumpfen Blutformen scheinen sie aktiver, und die Bildung von Peroxisomen (peroxisome-like organelles) durch das glatte ER ist intensiviert. Die Mitteldarmformen besitzen praktisch kein ER mehr, an seiner Stelle jedoch vermehrt freie Ribosomen. In den trypomastigoten Proventrikelformen scheint die Bildung von rauhem Endoplasmatischen Reticulum wieder einzusetzen. Die Lysosomen erscheinen in den Mitteldarm- und Proventrikelformen häufiger als in den Blutstadien. Die Peroxisomen tragen zur Bildung von multivesikulären Körpern (multivesiculate bodies) bei, sowohl in Blut- als auch in Vektorformen.

Hinsichtlich des Entwicklungszustandes der cytoplasmatischen Membransysteme sind die metacyclischen Formen weitgehend den Blutformen ähnlich, allerdings fehlt den ersteren das glatte ER vollständig.

Alle diese morphologischen Veränderungen stehen in engem Zusammenhang mit physiologischen und biochemischen Umwandlungen, wie sie von andern Autoren beschrieben wurden.

Die Durchdringung der peritrophischen Membran auf der Höhe des Proventrikels konnte verfolgt werden. Spezielle ektoperitrophische Mitteldarmformen durchdringen dort die noch nicht auspolymerisierte Struktur wohl rein mechanisch.

Die cytochemischen Experimente charakterisierten zwei Typen von Lipideinschlüssen, die einerseits in den Blutformen, andererseits in den Mitteldarmformen vorkommen. Die Darstellung von Polysacchariden erlaubte es, auf den Aktivitätszustand des Golgi-Apparates in Blutformen, den Ursprung gewisser Vesikel im Cytoplasma und die Zusammensetzung des Coats zu schließen. Außerdem konnte, verbunden mit Proteasen-Verdauungen, der Aufbau der peritrophischen Membran (Glycoproteine + Polysaccharide) abgeklärt werden.

Peroxidase/Katalase wurde in den «Typ I-Vesikeln» nachgewiesen, die damit als Peroxisomen charakterisiert sind. Bei diesen Vesikeln handelt es sich im übrigen um diejenigen Organellen, welche auch die Enzyme für die extramitochondriale terminale Respiration ($L\text{-}\alpha$ -Glycerophosphat-Zyklus) enthalten.

Mit der Lokalisation von saurer Phosphatase im Reservoir, im ER (RER und GER), im Golgi-Apparat und in verschiedenen Typen von Lysosomen in Blutformen von *T. brucei* konnte die Bedeutung gewisser Zellkompartimente und vor allem deren unmittelbare Verbindung (GERL-System) erkannt werden. Die Bedeutung der Mikrotubuli für das Wachstum, die Teilung, die Bewegung und die Stützung der Trypanosomenzelle sowie die Funktion der desmosomenartigen «Zellverbindungen» wurden diskutiert.

Résumé

Dans la présente publication sont décrites les altérations ultrastructurales de *Trypanosoma brucei* pendant son cycle évolutif dans le sang de l'hôte et dans le vecteur *Glossina*. De plus, quelques expériences cytochimiques ont été accomplies dans le but de comprendre la fonction des organelles, des vésicules et des lipides ainsi que la constitution de la « membrane » péritrophique.

On a pu démontrer que la mitochondrie se développe, dans les formes sanguicoles élançées, d'un état simple, sans cristae, à un système réticuliforme pourvu de beaucoup de tubules mitochondriaux. Ce stade se trouve dans les formes de l'intestin moyen et du proventricule. Dans les formes épimastigotes (crithidia) de la glande salivaire, on enregistre une réduction de la mitochondrie, sorte de retour à l'état simple, visible dans les formes métacycliques; cependant, les mitochondries régressées possèdent encore des cristae.

La couche de surface (surface coat) des formes sanguicoles, le siège des

antigènes agglutinants, est perdue dans le vecteur et elle redevient visible dans les formes trypomastigotes de la glande salivaire. Les formes métatrypomastigotes immatures contiennent déjà un matériel, précurseur du « coat », sur la membrane pelliculaire; les métatrypomastigotes (métacycliques) possèdent la couche définitive caractéristique des formes sanguicoles. On suppose que la substance fondamentale du « coat » est formée par l'appareil de Golgi et qu'elle est libérée par des vésicules (« Golgi derived vesicles ») à la surface des trypanosomes.

Les systèmes des membranes cytoplasmiques (ER, Golgi, Lysosomes) changent également pendant le cycle évolutif. Le réticulum endoplasmique ribosomal et le réticulum lisse sont bien développés dans les formes sanguicoles. Dans les formes sanguicoles courtes, ils apparaissent plus développés et la formation de péroxisomes (peroxisome-like organelles) à partir du réticulum endoplasmique lisse est augmentée. Les formes de l'intestin moyen ne possèdent que peu d'ER, mais par contre un plus grand nombre de ribosomes libres (free ribosomes). La formation d'ER semble recommencer dans les formes trypomastigotes du proventricule. Les lysosomes sont plus fréquents dans les formes de l'intestin et du proventricule que dans les formes sanguicoles.

Les péroxisomes contribuent à la formation des corps multivésiculaires (multivesiculate bodies) et cela dans toutes les formes. Quant à l'état de développement des systèmes de membranes cytoplasmiques, les formes métacycliques ressemblent fortement aux formes sanguicoles. On note cependant une absence totale de réticulum endoplasmique lisse dans les formes métacycliques. Toutes ces altérations morphologiques sont en rapport avec les changements physiologiques et biochimiques décrits par d'autres auteurs.

La pénétration de la « membrane » pérityrophique dans la région du proventricule a pu être étudiée. Des formes ectopérityrophiques spéciales y pénètrent mécaniquement, alors que la structure de la membrane n'est pas encore polymérisée.

Les expériences cytochimiques ont dénoncé deux types d'inclusions lipidiques. Elles se trouvent, d'une part dans les formes sanguicoles, et, d'autre part, dans les formes de l'intestin moyen. Dans les formes sanguicoles, la localisation des polysaccharides permet de préciser l'état de développement de l'appareil de Golgi, l'origine de certaines vésicules dans le cytoplasme et la composition du « coat ». En combinaison avec des digestions de protéases, la nature de la membrane pérityrophique (glycoprotéines et polysaccharides) a pu être démontrée.

La peroxydase/catalase a été localisée dans les « vésicules de type I », qui sont donc de vrais péroxisomes. Ceux-ci sont identiques aux organelles responsables de la respiration terminale extramitochondriale (cycle du L- α -glycérophosphate).

La localisation de la phosphatase acide dans le réservoir, dans les systèmes de réticulum endoplasmique, dans l'appareil de Golgi et dans plusieurs types de lysosomes des formes sanguicoles démontrent la fonction de certains compartiments cellulaires et leurs communications directes entre eux. La signification des microtubules pour la croissance, la division, le mouvement et le soutien du trypanosome, de même que la fonction des jonctions cellulaires de type desmosome sont également discutées.

Abbreviations used in the figures

- a = anterior portion of the mitochondrion
- ax = axoneme (tubular elements of the flagellum)
- bb = basal body (base of the flagellum)
- cm = cytoplasmic membrane systems
- cr = cristae (mitochondrial tubules)

- cv = "coated" pinocytotic vesicles
- d = desmosomes (maculae adhaerentes)
- e = endosome ("nucleolus")
- ec = ectoperitrophic space of the midgut
- en = endoperitrophic space
- es = endonuclear spindle microtubules
- ex = exocytosed material
- F = flagellum
- fep = fore-gut epithelium
- fl = flocculent material in the reservoir
- fr = free rebosomes or polysomes
- G = Golgi apparatus
- g = glycogen
- hc = heterochromatin-like masses
- hd = hemidesmosomes
- i = chitinous intima of the fore-gut epithelium
- ifs = intra-flagellar structure
- K = kinetoplast
- kn = kinetonucleus (K-DNA)
- L = lumen (proventriculus, midgut, salivary glands)
- le = labyrinth-like extensions (mitochondrion)
- li = lipid inclusions
- ly = lysosomal structures
- m = membranes
- ma = mitochondrial (kinetoplastic) matrix
- mep = midgut epithelium
- Mi = mitochondrion (chondriome)
- mv = microvilli
- mvb = multivesiculate bodies
- N = nucleus
- ne = nuclear envelope (nuclear membranes)
- P = pellicula, pellicle (limiting membrane)
- p = posterior portion of the mitochondrion
- pm = peritrophic "membrane" ("PM")
- I, II = first and second layer of the "PM"
- po = peroxisome-like organelles (GPO bodies)
- R = reservoir (flagellar pocket)
- rer = rough-surfaced endoplasmic reticulum
- sep = salivary gland epithelium
- ser = smooth-surfaced endoplasmic reticulum
- sm = subpellicular microtubules
- so = subpellicular organelle
- T = trypanosomes
- v = vacuoles
- ve = vesicles (Golgi-derived)

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Fig. 1. General view of a slender bloodstream form. Note the single cristaeless mitochondrial tube originating in the subterminal kinetoplast. The nucleus with the endosome lies in the middle of the cell body. The Golgi zone, pieces of rER (\rightarrow), peroxisome-like organelles and small spherical lipid inclusions are seen. 17,500 \times .

Inset: details of the kinetoplast-reservoir region. The pellicula is covered with the surface coat (\rightarrow); the outer leaflet of the unit membrane is more electron-dense. Subpellicular microtubules run underneath the pellicula (\rightarrow), four special microtubules accompanying the reservoir membrane (\rightarrow). The reservoir (flagellar pocket) is filled with flocculent material. The base of the flagellum and free ribosomes are visible. 44,000 \times .

Fig. 2. Details from a kinetoplast. The posterior portion of the mitochondrion is only small. The kinetoplast is bounded by the two mitochondrial membranes and contains the coiled DNA structure (kinetoplast) embedded in the mitochondrial matrix. A few small mitochondrial tubules are common (\rightarrow). The "basement plate" is the electron-dense material between the K-DNA and the anterior part of the kinetoplast's envelope (\approx). 69,000 \times .

Fig. 3. Longitudinal section through a kinetoplast. The DNA is composed of closely packed figure-8 coils. 69,000 \times .

Fig. 4. The mitochondrion often shows labyrinth-like extensions. The cristae are then inconspicuous. 44,000 \times .

Fig. 5. Details from the nucleus. The two nuclear membranes (= nuclear envelope) are continuous with rER cisternae. The outer one is likewise studded with ribosomes. Nuclear pores (\rightarrow) are frequent. Heterochromatin-like masses are attached to the inner nuclear membrane; the endosome consists of homogenous fine-granular material. 44,000 \times .

Fig. 6. Tubes of ser forming a vesicular network. 44,000 \times .

Fig. 7. GERL zone. Ser tubes seem to contribute to the formation of peroxisome-like organelles. They appear swollen and filled with material similar to the PO's matrix. 86,000 \times .

Inset: membranous inclusions in the matrix of the peroxisome-like organelles (\times). 34,500 \times .

Fig. 8. Exocytosed vesicles and flocculent material in the flagellar pocket. Intraflagellar vesicles are common (\rightarrow). 51,500 \times .

Fig. 9. Formation of a pinocytotic vesicle at the reservoir membrane (\rightarrow). Note the flocculent external coat (\rightarrow). 172,000 \times .

Fig. 10. Transverse section of a slender bloodform through the pellicula and the flagellum. The subpellicular microtubules are regularly arranged and connected by fine-filamentous processes (\rightarrow). Flagellar attachment is reinforced by desmosome-like plaques (maculae adhaerentes) inserted in a gap between subpellicular microtubules and at the flagellar membrane, respectively. The flagellum exhibits the typical 9+2 arrangement of the tubular elements, of which the peripheral ones consist of two subtubules (A+B) and arm-like appendages (\rightarrow). The axoneme is accompanied by the intraflagellar structure, which shows filamentous connections with the flagellar membrane and the axoneme (\bullet). All microtubular walls

demonstrated here are made up by globular osmiophilic subunits (\rightarrow). The outer leaflet of the pellicular unit membrane is again more electron-dense. The surface coat appears rather flocculent to filamentous (\blacktriangleright). 220,000 \times .

Inset: vesicular structure of the subpellicular organelle near the flagellar attachment zone comprising 4 subpellicular microtubules and a rer-derived membrane (\rightarrow). 86,000 \times .

Fig. 11. Active large Golgi apparatus (fenestrated) with many vesicles budding off laterally. 69,000 \times .

Fig. 12. Close association of the various membrane systems (GERL): Golgi apparatus, rer, ser and a lysosomal organelle. 44,000 \times .

Fig. 13. Formation of multivesiculate bodies from peroxisome-like organelles in the GERL zone. Cytoplasmic intrusions (\rightarrow) and small vesicles (\blacktriangleright), possibly contributed by the Golgi complex and ser, are visible. 54,000 \times .

Inset: mature mvb containing vesicles of uniform size. 86,000 \times .

Fig. 14. Nuclear division by means of endonuclear spindle microtubules. The nucleus becomes elongated and constricted and the endosome fragmented. 22,000 \times .

Fig. 15. Details: the nuclear membranes persist during the division. The endosome material is stretched along the tubule axis. 44,000 \times .

Fig. 16. The fragmentation of the endosome results in the formation of "laminated plaques" (\blacktriangleright). 86,000 \times .

Fig. 17. General view of a stumpy bloodstream form. The mitochondrion is distinctly enlarged lined with many tubular cristae (\rightarrow). The cytoplasmic membrane systems appear well developed. 17,000 \times .

Fig. 18. Details: the enlarged mitochondrion is rich in tubular cristae (\rightarrow). The formation of autolysosomal structures is effected by concentric growth of ser tubes. Many free ribosomes are present. The surface coat is present (\blacktriangleright). 44,000 \times .

Fig. 19. Deterioration of transitional forms in the vector *Glossina* (day 1 after the infecting feed). The nucleus becomes pycnotic, extreme vacuolization sets in and defined cellular membranes disappear (\blacktriangleright). The surface coat is lost (\rightarrow). The K-DNA remains unaffected, whereas cell shrinkage is evident. 18,500 \times .

Fig. 20. Formation of large mvb complexes. The surface coat is absent (\rightarrow). 27,000 \times .

Fig. 21. Surviving transition form (day 3). The mitochondrion grows out combined with the kinetoplast's shift to a more anterior position. The mitochondrial tubules are enlarged (\rightarrow). Masses of free ribosomes emerge, whereas the rer becomes insignificant. The Golgi apparatus is rather extended. In most cases the surface coat is immediately lost (\rightarrow). 22,000 \times .

Inset: sometimes, the coat persists up to day 4, both on the flagellum and the cell body (\rightarrow). 85,000 \times .

Fig. 22. Equatorial mode of kinetoplast division. The daughter kinetoplast lies above the original one and is displaced then sideways and comes to lie by the side of the old DNA structure (\rightarrow). 96,500 \times .

Fig. 23. General view of established midgutforms (day 16) in the ectoperitrophic space close to the midgut epithelium. Different cell organelles, such as the nucleus, mitochondrion, peroxisome-like organelles, Golgi complex and lipid inclusions can be recognized. The coat is absent (\blacktriangleright). 84,000 \times .

Fig. 24. Ectoperitrophic midgutform in the folds of the peritrophic "membrane" (day 20). Note the free ribosomes (\bullet), multivesiculate bodies (\blacktriangleright) and the lipid inclusions. 17,500 \times .

Inset: transverse section through trypanosomes embedded in the peritrophic "membrane" (day 12). The first and second layer of the "PM" are pulled apart leaving a cavity containing the flagellates. 17,500 \times .

Fig. 25. Details from an established midgutform (day 30). Pairs of desmosome-like plaques (\blacktriangleright) line the flagellar attachment zone. The surface coat is absent (\rightarrow). Many free ribosomes and an extended Golgi zone with many vesicles, as well as a typical large multivesiculate body are visible. 34,500 \times .

Fig. 26. Parts of the characteristic mitochondrial network with elongated tubular (mixed type) cristae (day 30). 44,000 \times .

Fig. 27. Details: large little electron-dense lipid droplets. Cisternae of rer seem to be in formation. A multivesiculate body with heterogenous vesicular contents can be seen. 44,000 \times .

Fig. 28. Transverse section through the proventricular region (day 16). The invaginated fore-gut portion (cardial valve) with its typical chitinous lining (\rightarrow) is separated from the foremost part of the midgut epithelium (type III cells) by a rather wide space. The "PM" seems not to be fully polymerized yet. Trypanosomes in the ectoperitrophic space (\rightarrow) and penetrating the peritrophic "membrane" (\rightarrow) are visible. 6,900 \times .

Inset: details from the ectoperitrophic space in the proventricular region. The trypanosomes appear closely packed in the zone between the microvilli of the type III cells and the "PM". 14,800 \times .

Fig. 29. Zone of the "PM"'s penetration in the region of the type III epithelial cells (day 17). Different steps of pervasion are identified. First in the ectoperitrophic space, the trypanosomes are then found embedded in the second layer of the "PM" (inset) and finally in a stratum-like distribution beneath the first layer (\blacktriangleright), which, upon rupture, releases the flagellates into the proventricular lumen. 14,800 \times .

Fig. 30. Proventricular forms concentrated in the lumen of the proventriculus. 18,500 \times .

Fig. 31. Details from a trypomastigote proventricular form (day 17). A large mitochondrial tube with many long tubular cristae originates in the capsule-shaped kinetoplast. Two basal bodies indicate the first step of cell division. Large mvb complexes (\blacktriangle), and lipid inclusions of both types (\blackstar) are seen. 22,000 \times .

Fig. 32. Details from the wide mitochondrial tube, kinetoplast and a mvb with heterogenous contents (day 17). 54,000 \times .

Inset: pieces of rer in formation (day 18); a few irregularly arranged ribosomes are visible (\rightarrow). 86,000 \times .

Fig. 33. Details from *Fig. 31*. Duplication of the basal body and replication of the K-DNA (doubling in length) precede cell division. The basal portion of the "daughter" basal body (prokinetosome) exhibits the typical peripheral microtubular triplets (\rightarrow). 69,000 \times .

Figs. 34 a/b. Special mode of flagellar division. a: two axonemes in one flagellar sheath. b: subsequent splitting effected by membrane in-pocketing (\bullet). 130,000 \times .

Fig. 35. Transition form (day 12) showing the juxta-nuclear position of the kinetoplast. The K-DNA appears somewhat coarser fibrillar to spongy, the matrix more electron-dense. 68,000 \times .

Inset: details from a proventricular epimastigote form (day 12). The kinetoplast lies anterior to the nucleus. 54,000 \times .

Fig. 36. Schematic drawing of the development of *T. brucei* in the middle part of the salivary glands. a: Epimastigote form attached by flagellar insertion and hemidesmosomes to the microvillar border of the salivary gland epithelium. A large mitochondrial network and pinocytotic "coated" vesicles are found; b: dividing epimastigote still attached to the epithelium showing kinetoplast replication and endonuclear spindle microtubules; c: detached transition form arising from a dividing epimastigote; combined with the outgrowth of the anterior part of the mitochondrion the kinetoplast moves to the posterior position; d: immature metatrypomastigote form with a reduced mitochondrion, an activated Golgi zone and coat precursor material on its surface (\rightarrow); e: mature metatrypomastigote (metacyclic) form showing many Golgi-derived vesicles and a fully formed surface coat (\rightarrow).

Fig. 37. General view of trypanosomes in the middle part of a salivary gland (day 19). 6,800 \times .

Fig. 38. Epimastigote forms (day 28) attached by means of flagellar insertion and hemidesmosomes (\rightarrow) to the microvilli of the salivary gland's epithelium. 44,000 \times . Inset: details from a hemidesmosomal plaque (\rightarrow). 172,000 \times .

Fig. 39. Metacyclic forms free in the lumen of the gland (day 28). Note the postero-nuclear position of the kinetoplast. 14,800 \times .

Fig. 40. Dividing epimastigote form (day 22) showing two parallelly arranged basal bodies. The nucleus contains an eccentric endosome and peripheral chromatin-like masses (\rightarrow). The Golgi zone is rather vesicular. Free ribosomes occur. 54,000 \times . Inset: pinocytotic vesicles are quite different from Golgi vesicles in having an external flocculent covering ("coated" vesicles, \rightarrow). 44,000 \times .

Fig. 41. Dividing epimastigote form (day 19). A large chondriome originates in

- the kinetoplast and possesses a great number of enlarged mitochondrial tubules (\rightarrow). The surface coat is still absent (\rightarrow). Free ribosomes are present. 34,500 \times .
- Fig. 42.* Endonuclear spindle microtubules involved in nuclear division. 54,000 \times .
- Fig. 43.* Transition form with the kinetoplast in the juxta-nuclear position (day 20). The mitochondrion appears reduced and less ramified. 27,000 \times .
- Fig. 44.* Advanced migration of the kinetoplast to a nearly posteronuclear position (day 22). The reduced mitochondrion still contains numerous tubular cristae (\rightarrow). Bits of rer can be seen. The surface coat is still absent. 27,000 \times .
- Fig. 45.* Immature metatrypomastigote form (day 22). The kinetoplast and the basal body have reached the final position. This form is dividing. The Golgi apparatus has enlarged comprising many vesicles close to the reservoir (\bullet). The reduced mitochondrion possesses fewer cristae. 27,000 \times .
- Fig. 46.* Details from *Fig. 45* showing the pellicula, subpellicular microtubules, base of the flagellum and the reservoir. The pellicular unit membrane is covered with flocculent coat precursor material, and so is the reservoir membrane (\blacktriangleright), 110,000 \times .
- Fig. 47.* Mature metatrypomastigote (metacyclic) form (day 22). The pellicula is uniformly covered with a homogenous fully formed surface coat (\rightarrow). The single mitochondrial tube consists of a long anterior and a small posterior part; cristae are less abundant and rather vesicular to bulbous (\bullet). The Golgi zone is markedly enlarged containing many vesicles of different size. Free ribosomes have decreased. The peroxisome-like organelles resemble those in bloodforms. 34,500 \times .
- Fig. 48.* Details from a mature metacyclic form (day 25). The Golgi complex gives rise to small electron-dense vesicles (\rightarrow), which, in turn, may lead to the formation of the larger Golgi-derived vesicles with their typical coat-like intravesicular lining (\rightarrow). 86,000 \times .
- Fig. 49.* Dividing metacyclic form (day 26). The mitochondrion forms a concentric labyrinth-like structure. 44,000 \times .
- Fig. 50.* Dividing metacyclic form (day 26), characterized by the surface coat (\rightarrow), two basal bodies and an elongated kinetoplast. 69,000 \times .
- Fig. 51.* Lipid extraction in bloodforms (30 sec). The small osmiophilic lipid inclusions have completely been extracted leaving white spots indicating their former presence (\blacktriangle). Also the rest of the cell is affected. 22,000 \times .
- Insets: steps of lipid extraction. a: After 20 sec the inclusions lose their strictly spherical shape; "lytic" zones appear (\rightarrow). 44,000 \times . b: After 25 sec the lipid droplets become smaller and somewhat faded; a decrease of electron-density and distinct "lytic" zones (\rightarrow) are typical. 44,000 \times .
- Fig. 52.* Parallel experiment (25 sec). The huge lipid droplets of the midgut epithelium (day 1 after a bloodmeal) are affected and consecutively extracted. 44,000 \times .
- Figs. 53, 54.* Lipid extractions in midgut forms (30 and 40 sec, respectively). The lipid material is partly affected and gives the inclusion a spotty aspect. Mitochondrial membranes are decomposed by the treatment (\blacktriangle). Finally, the whole lipid material is extracted leaving a large white hole (\bullet). 44,000 \times .
- Fig. 55.* Localization of polysaccharides in a bloodform by means of the PA-TCH-silver albumose technique. After 24 hours' incubation in TCH the Golgi cisternae and most of the vesicles (\rightarrow) stain positive. Membranes are contrasted, too. 110 000 \times .
- Fig. 56.* After the same incubation period, rer, all lipid inclusions and the pellicular membrane (\rightarrow) stain positive, whereas the external surface coat appears unstained (\blacktriangleright). 130,000 \times .
- Fig. 57.* After the same incubation period, also ser tubes and vesicles around the reservoir membrane (\rightarrow) show a positive reaction for carbohydrates. 110,000 \times .
- Fig. 58.* Control experiment with preoxidation in H_2O_2 . No positive staining in any parts of the trypanosomal cell. 93,000 \times .
- Fig. 59.* Localization of carbohydrates in the peritrophic "membrane" of the midgut by means of the PA-TCH-silver albumose technique. After 12 hours' incubation in TCH the first layer (\rightarrow) shows a distinct coarse granulation. The second layer has only fine-granular silver deposits (*). 44,000 \times .
- Inset: details from the "PM" of the midgut. Coarse granules are restricted to the

first layer (\rightarrow), whereas fine-granular reaction product is found in the second layer (*) 86,000 \times .

Fig. 60. After 24 hours' incubation, the "PM" in the proventricular region shows an equally dispersed fine-granular reaction product in both layers and the glyco-calyx (\rightarrow). The chitinous intima of the cardiac valve stains very markedly for carbohydrates, too. 44,000 \times .

Inset: no positive staining in the control experiment, where incubation in TCH has been omitted. 44,000 \times .

Fig. 61. Parallel experiment with the same histochemical method. After 12 hours' incubation in TCH marked vesicles (\rightarrow) in the apical zone of the midgut epithelium are observed. 22,000 \times .

Fig. 62. Parallel experiment. α -Glycogen particles in the fore-gut epithelium stain positive. 44,000 \times .

Fig. 63. Further experiment with PA oxidation and subsequent post-staining with lead citrate. The chitinous lining of the fore-gut invagination shows a light extraction zone containing a fine granulation. 44,000 \times .

Fig. 64. Protease digestion with 0.05% pronase in 0.01 M phosphate buffer (pH 7.4) for 40 min at 37 °C. The peritrophic "membrane" in the proventriculus is affected showing a definite decrease in consistence and electron-density of the "PM"'s second layer. 87,000 \times .

Inset: control experiment without previous oxidation with H₂O₂. The "PM" remains unaffected and appears compact. 87,000 \times .

Fig. 65. Localization of peroxidase/catalase in bloodforms. Reaction product is regularly found extracellularly (\rightarrow), possibly due to contaminations from lysed blood cells (pseudoperoxidase). Fix.: formaldehyde. 44,000 \times .

Inset: control experiment with the medium devoid of H₂O₂. Reaction products are absent both intracellularly and extracellularly. Fix.: formaldehyde. 44,000 \times .

Fig. 66. Localization of peroxidase/catalase. Sometimes peroxisome-like organelles in the cytoplasm stain positive indicated by a homogenous black precipitate. Fix.: formaldehyde. 44,000 \times .

Inset: details from a positively stained PO. The limiting membrane is indistinct (\rightarrow). Fix.: formaldehyde. 110,000 \times .

Fig. 67. Localization of acid phosphatase in bloodforms: standard Gomori medium. Homogenously distributed reaction product in the flagellar pocket. Additionally, vesicles in close proximity stain positive (\rightarrow). 69,000 \times .

Inset: control experiment with the medium lacking the substrate. The reservoir remains unstained. 69,000 \times .

Fig. 68. Standard Gomori medium. Only one cisterna of the Golgi apparatus and some peripheral vesicles (\rightarrow) stain positive for acid phosphatase. 130,000 \times .

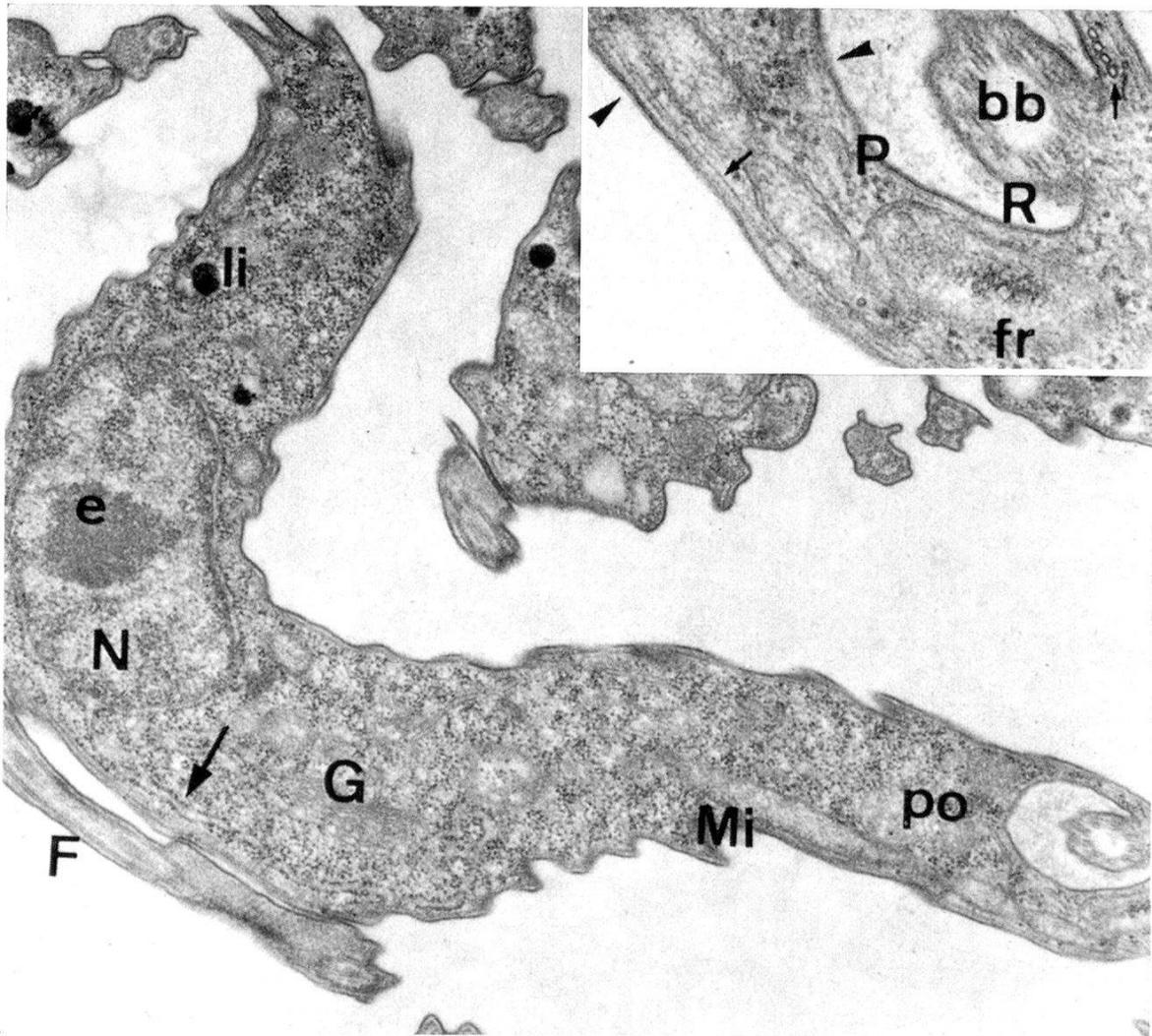
Inset: multivesiculate body containing lead salt precipitates. 130,000 \times .

Fig. 69. Standard Gomori medium. The coherent ser system is very heavily marked. 69,000 \times .

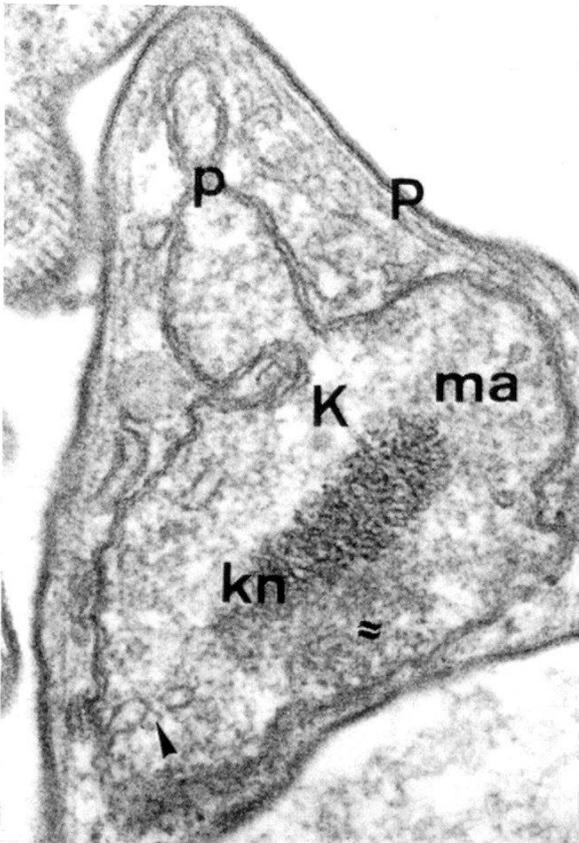
Inset: profiles of ser tubes (\rightarrow) containing lead phosphate deposits. 130,000 \times .

Fig. 70. Freshly prepared medium without DMSO. Presumable primary lysosomes (\rightarrow) close by a pinocytotic reservoir membrane invagination (\rightarrow) stain positive for acid phosphatase. 110,000 \times .

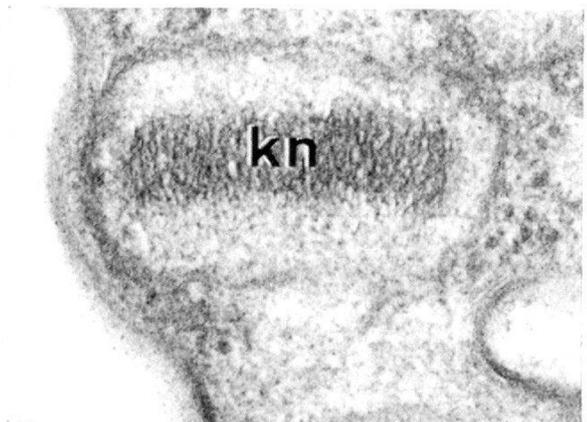
Inset: widened ser cisterna containing reaction product. 54,000 \times .



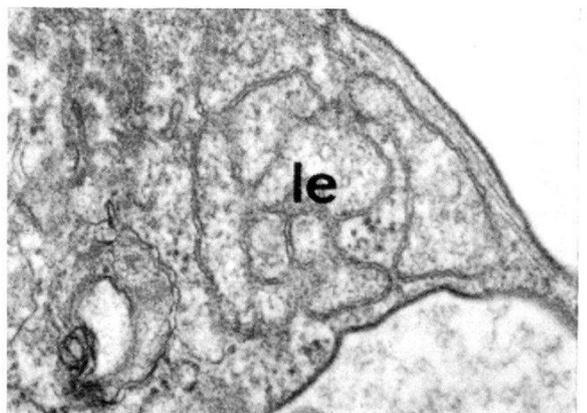
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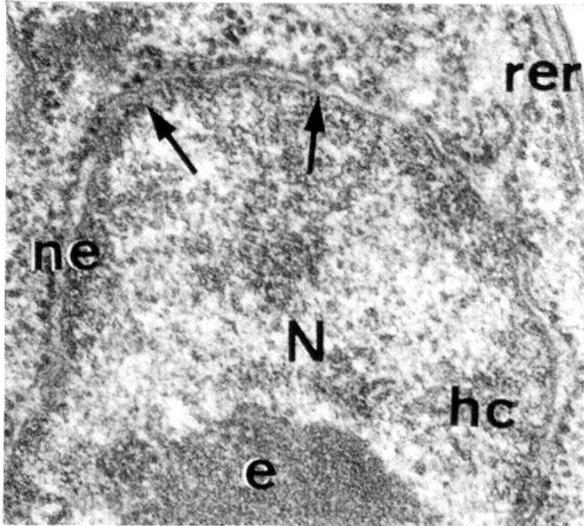
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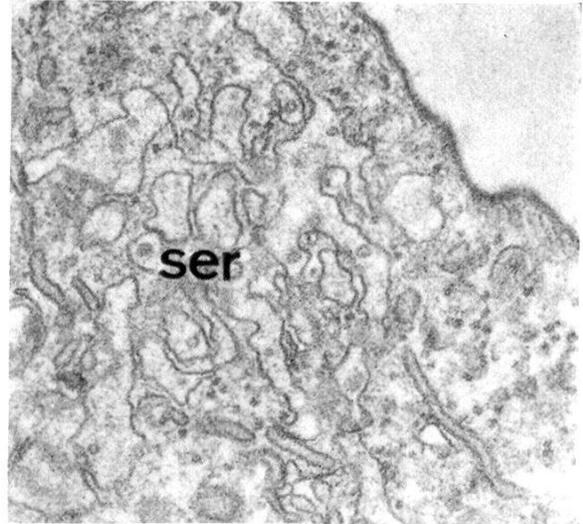
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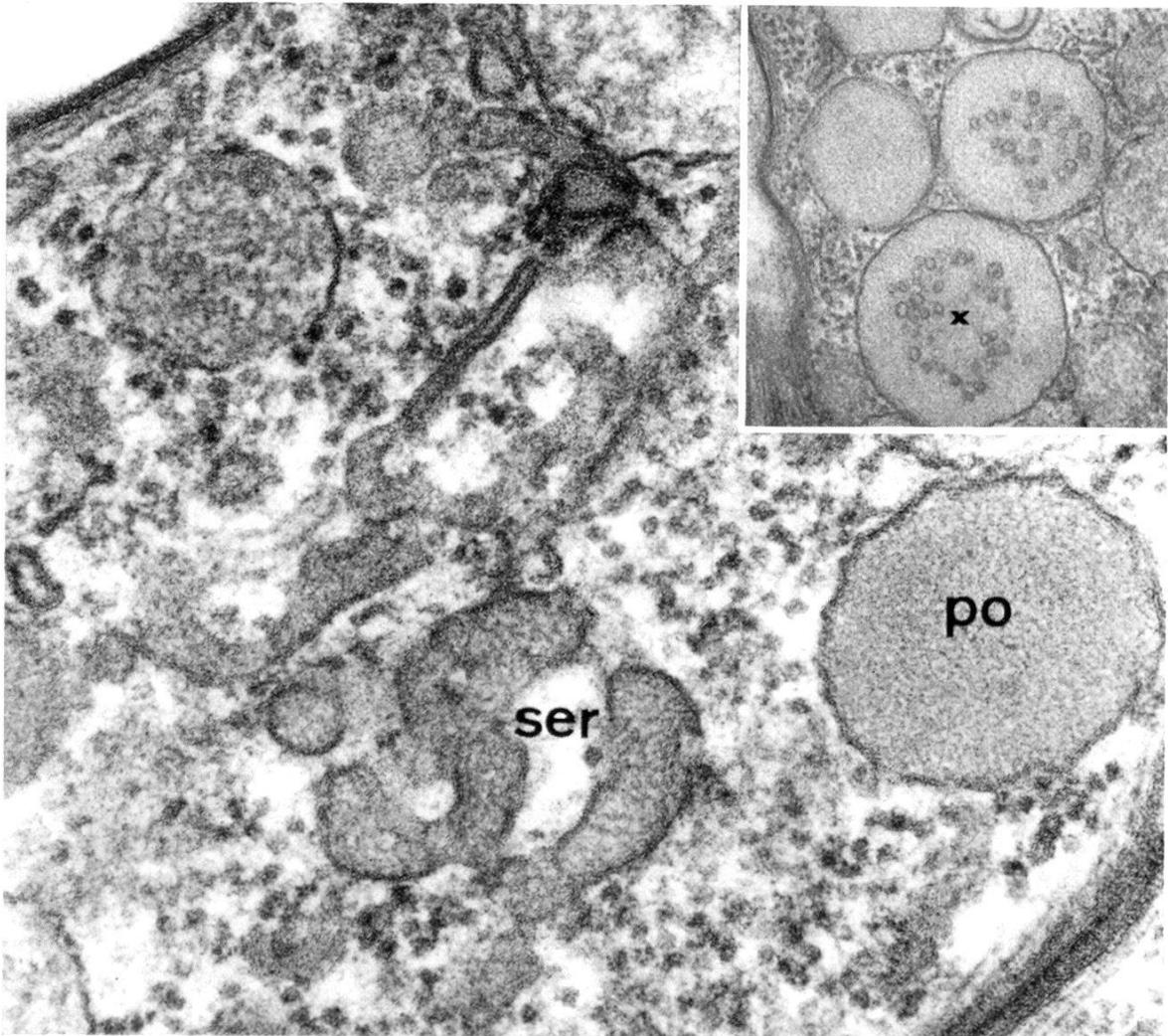
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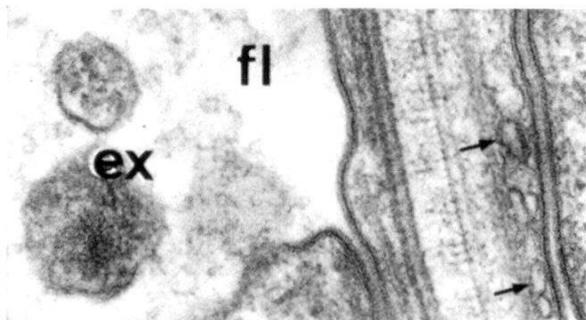
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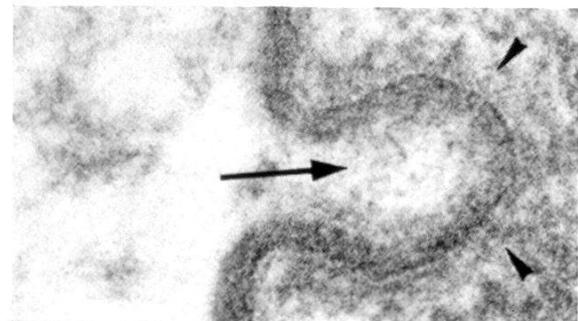
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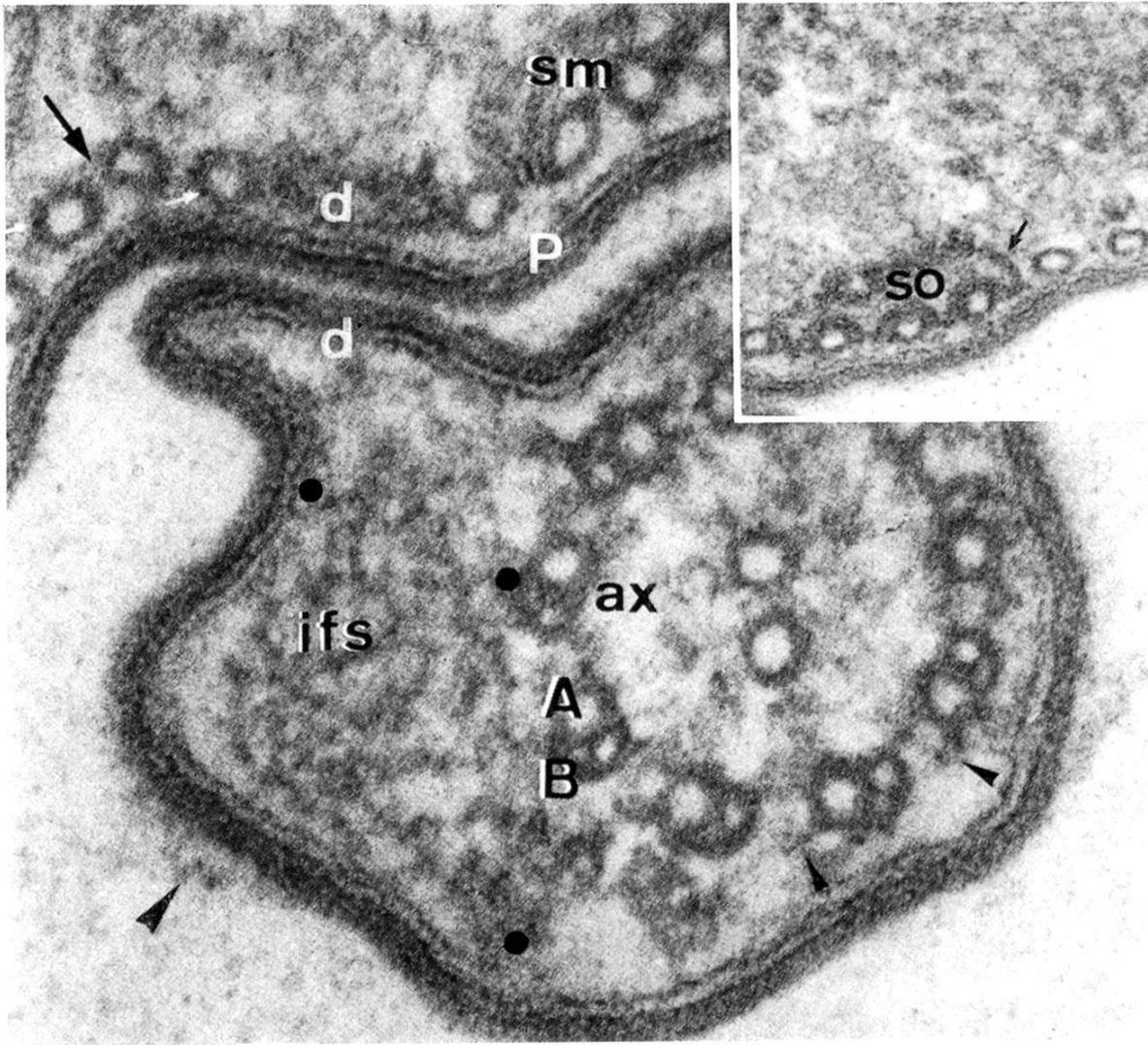
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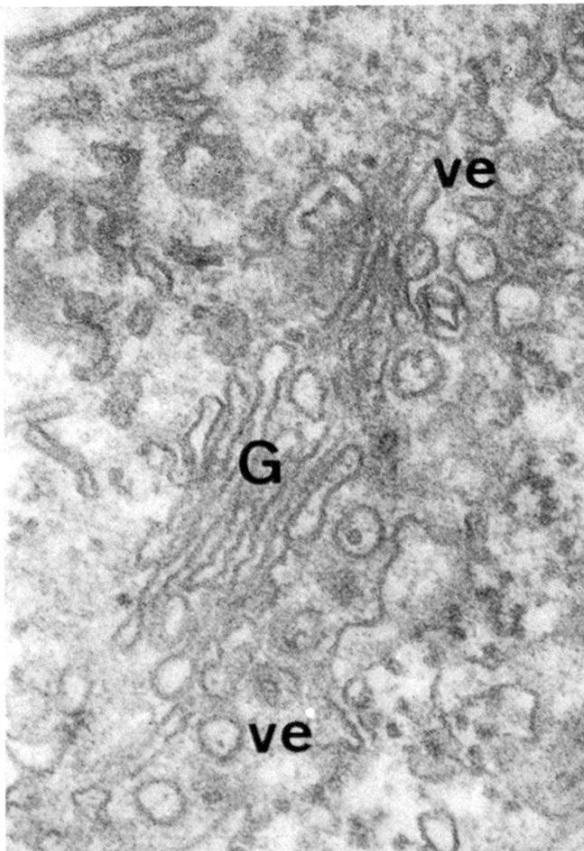
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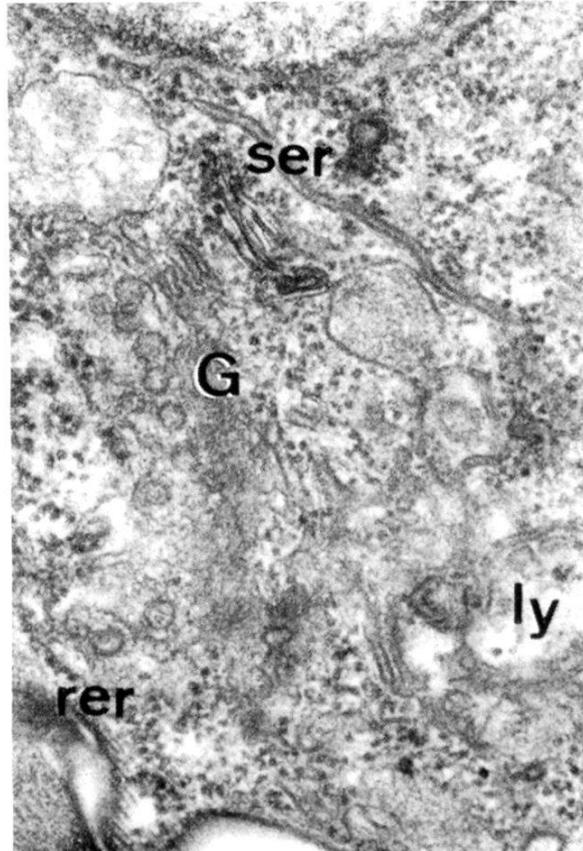
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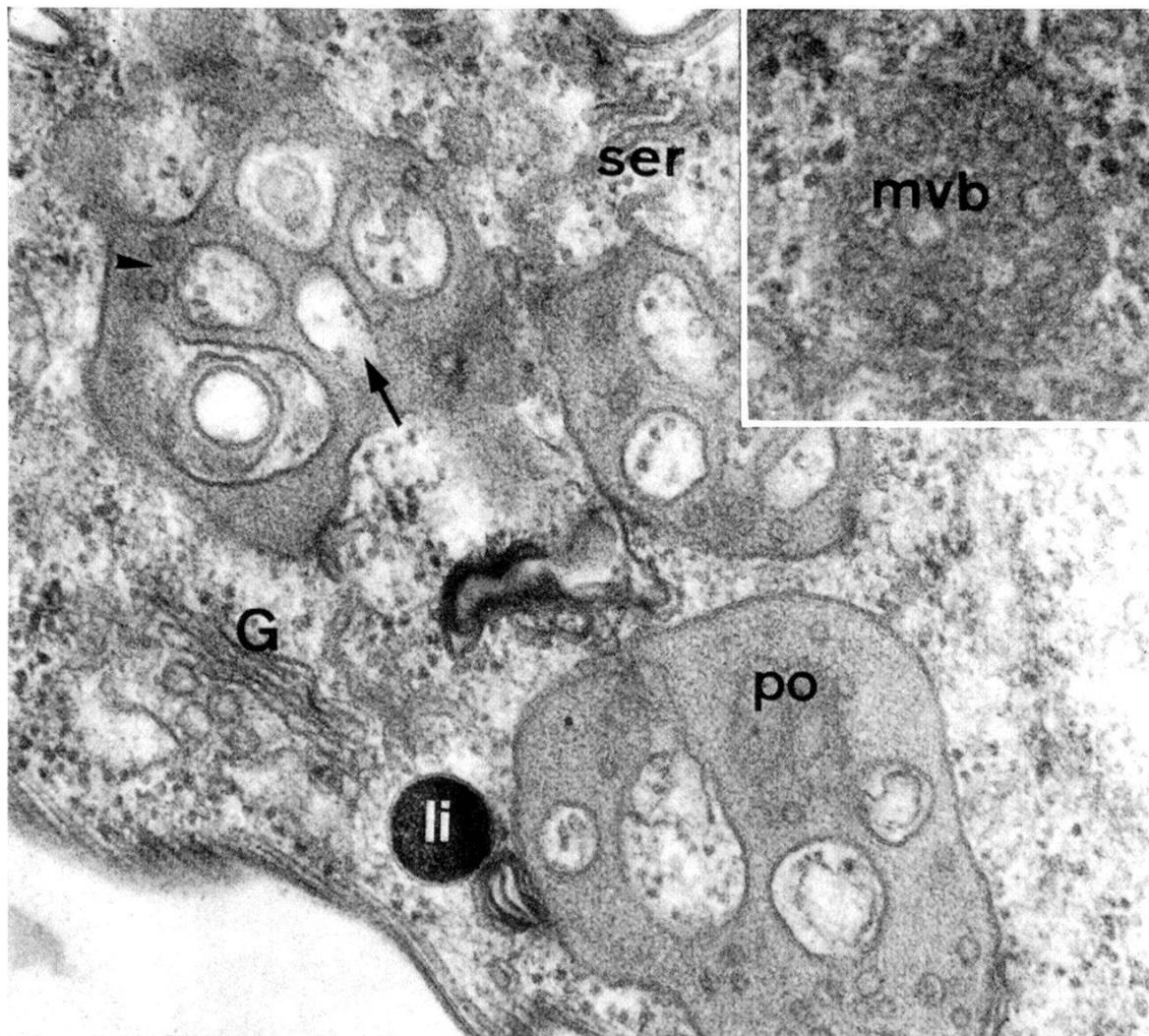
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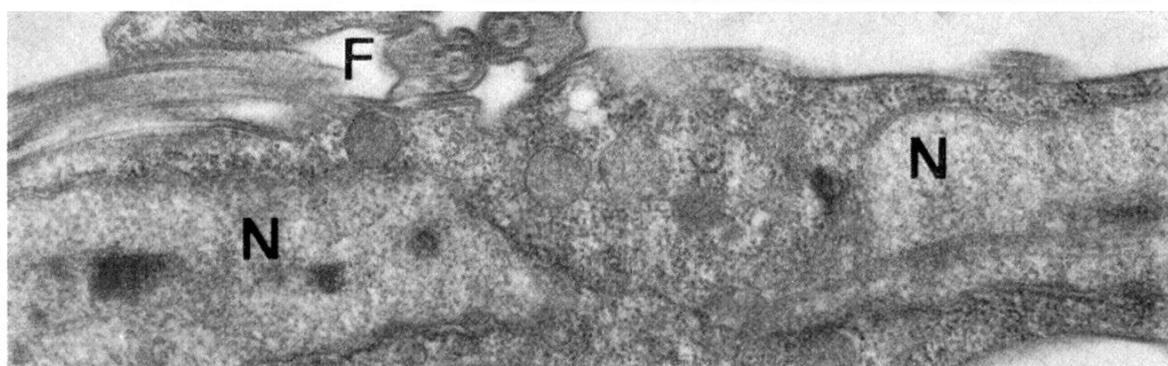
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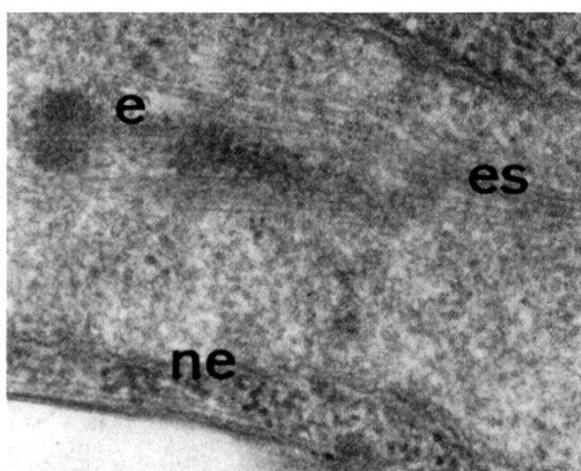
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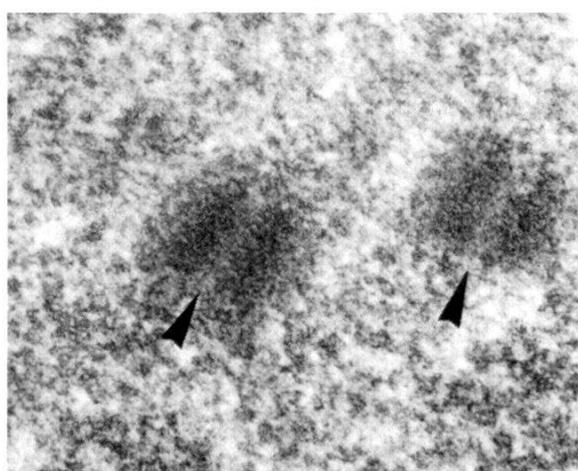
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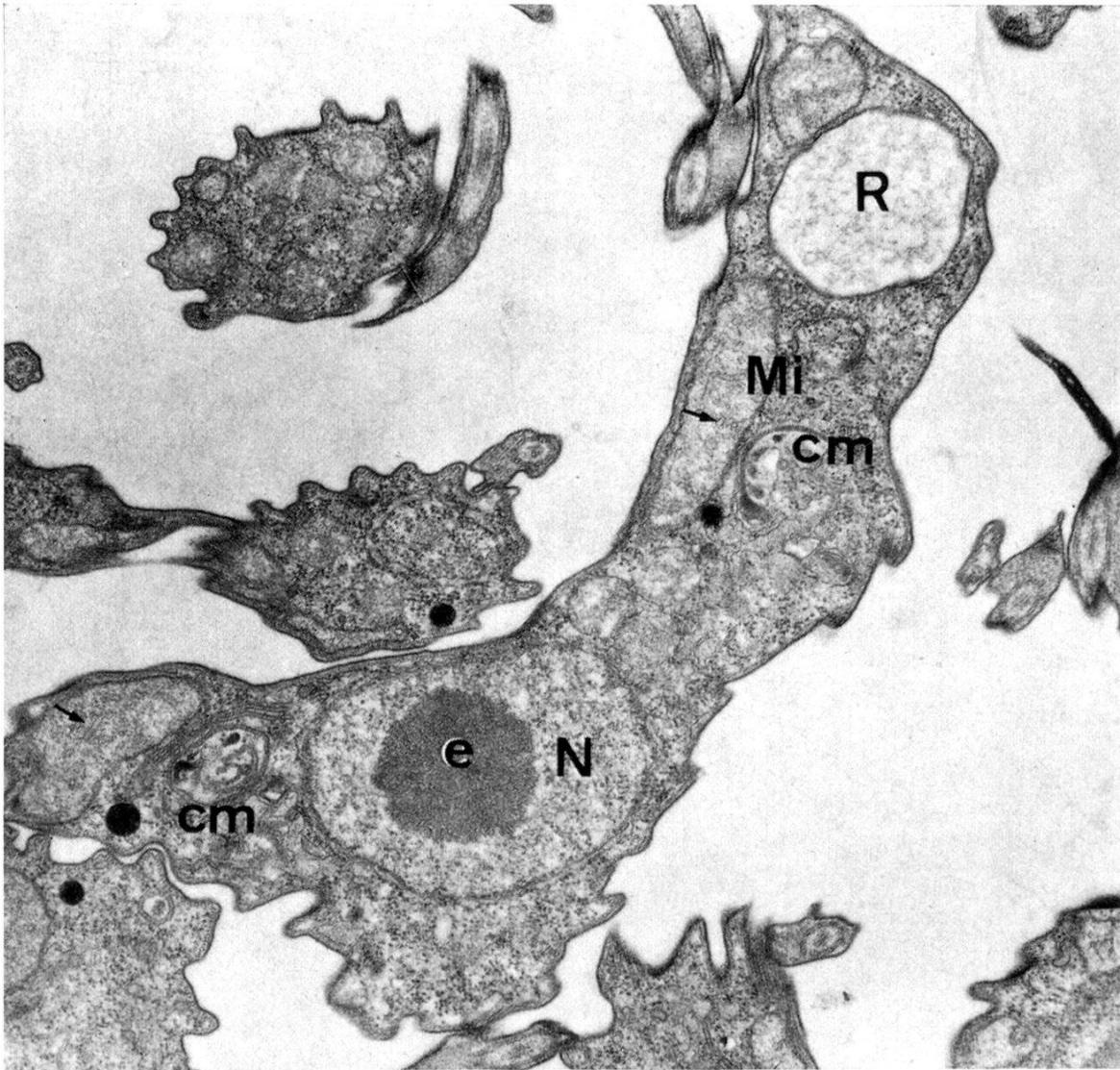
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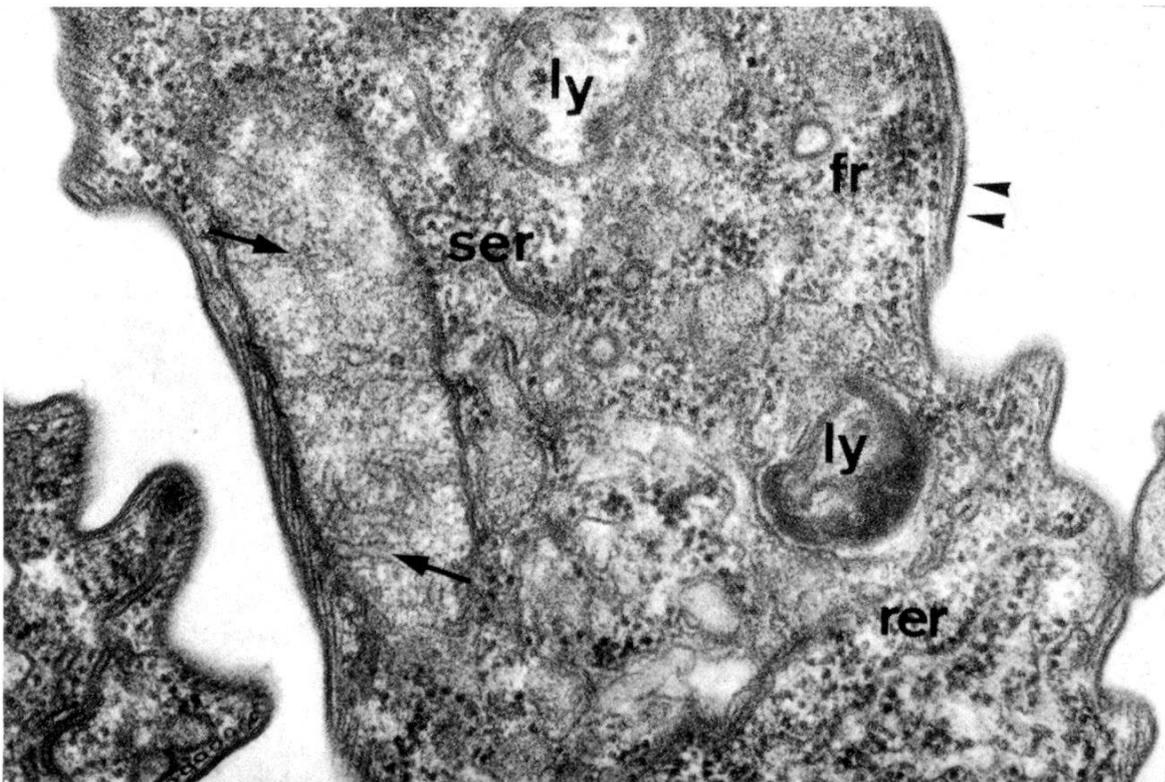
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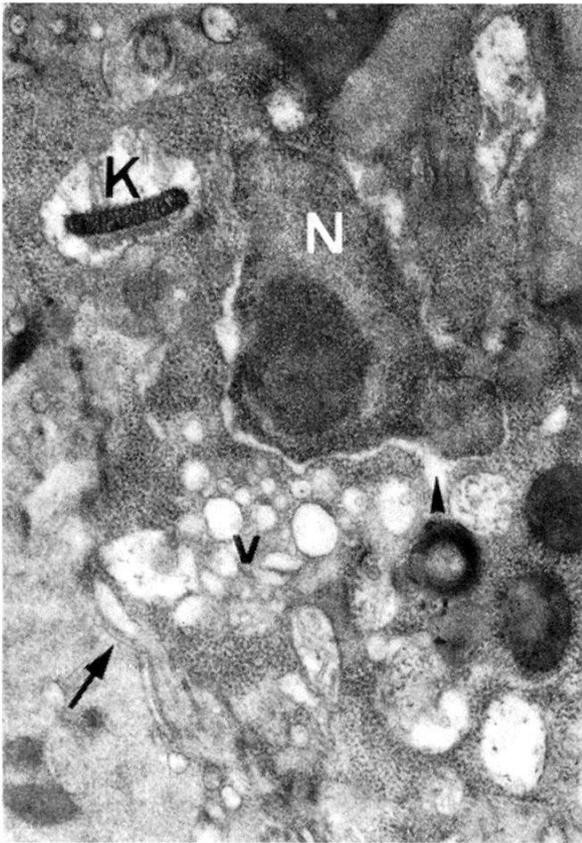
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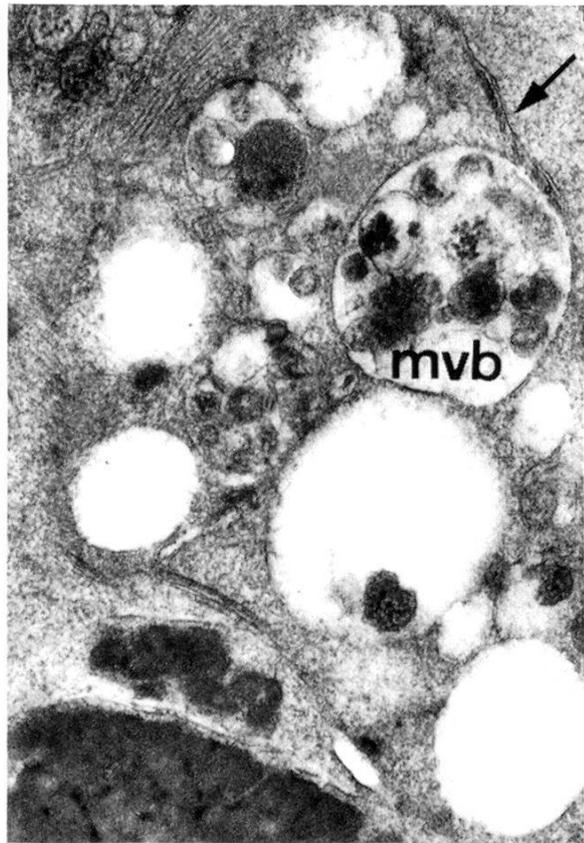
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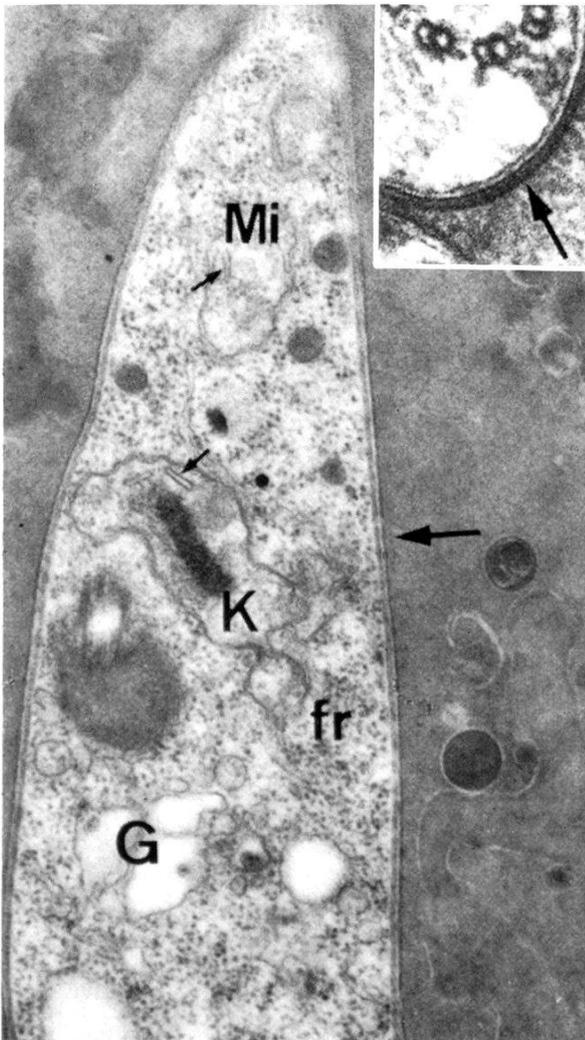
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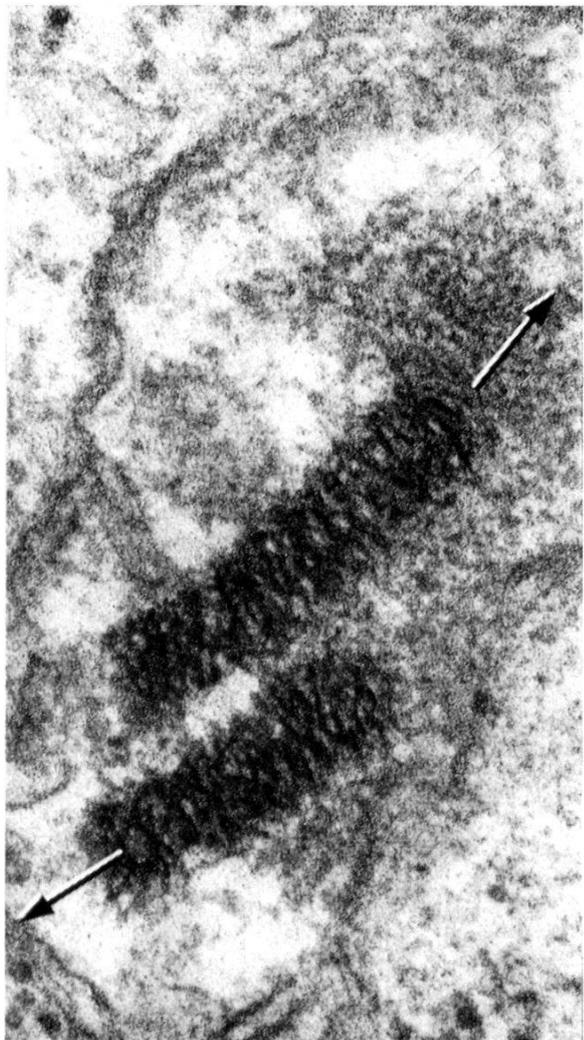
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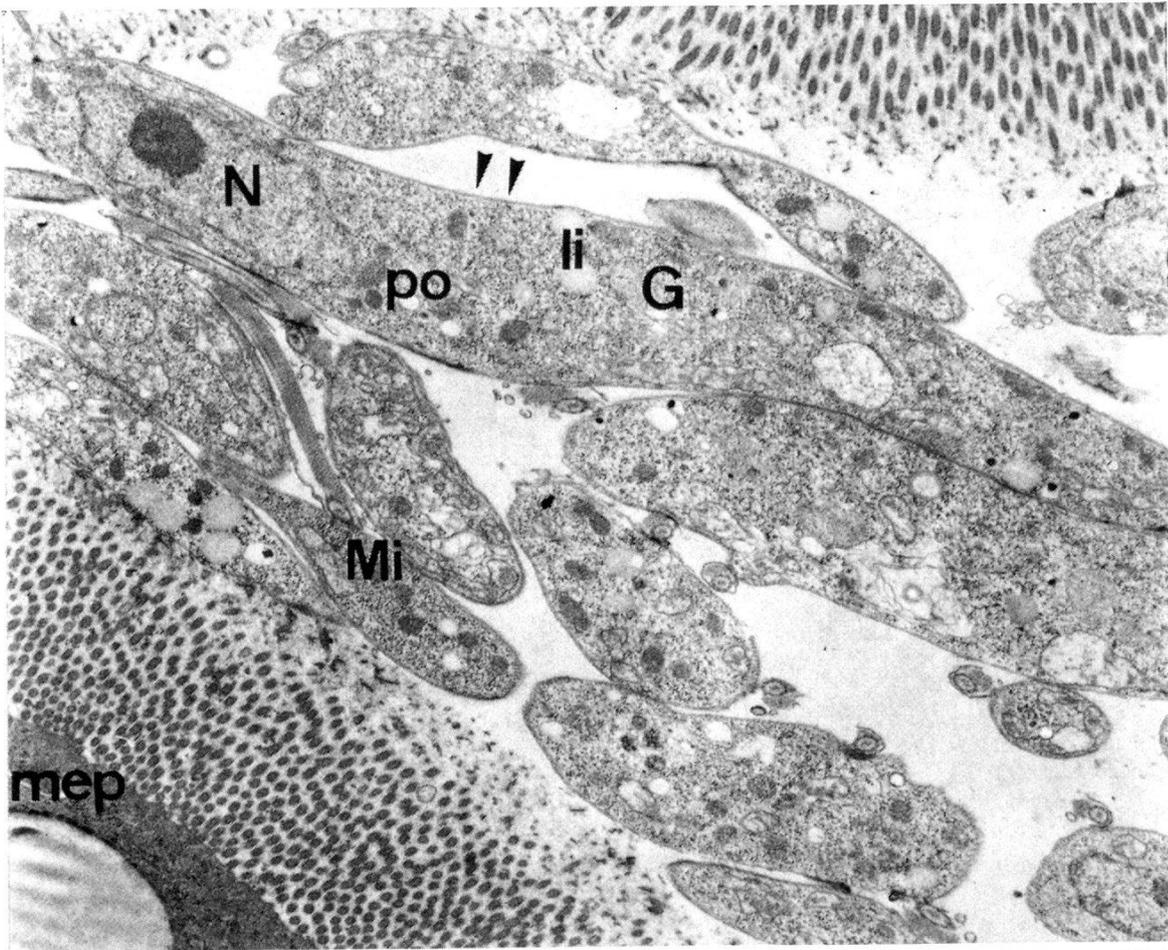
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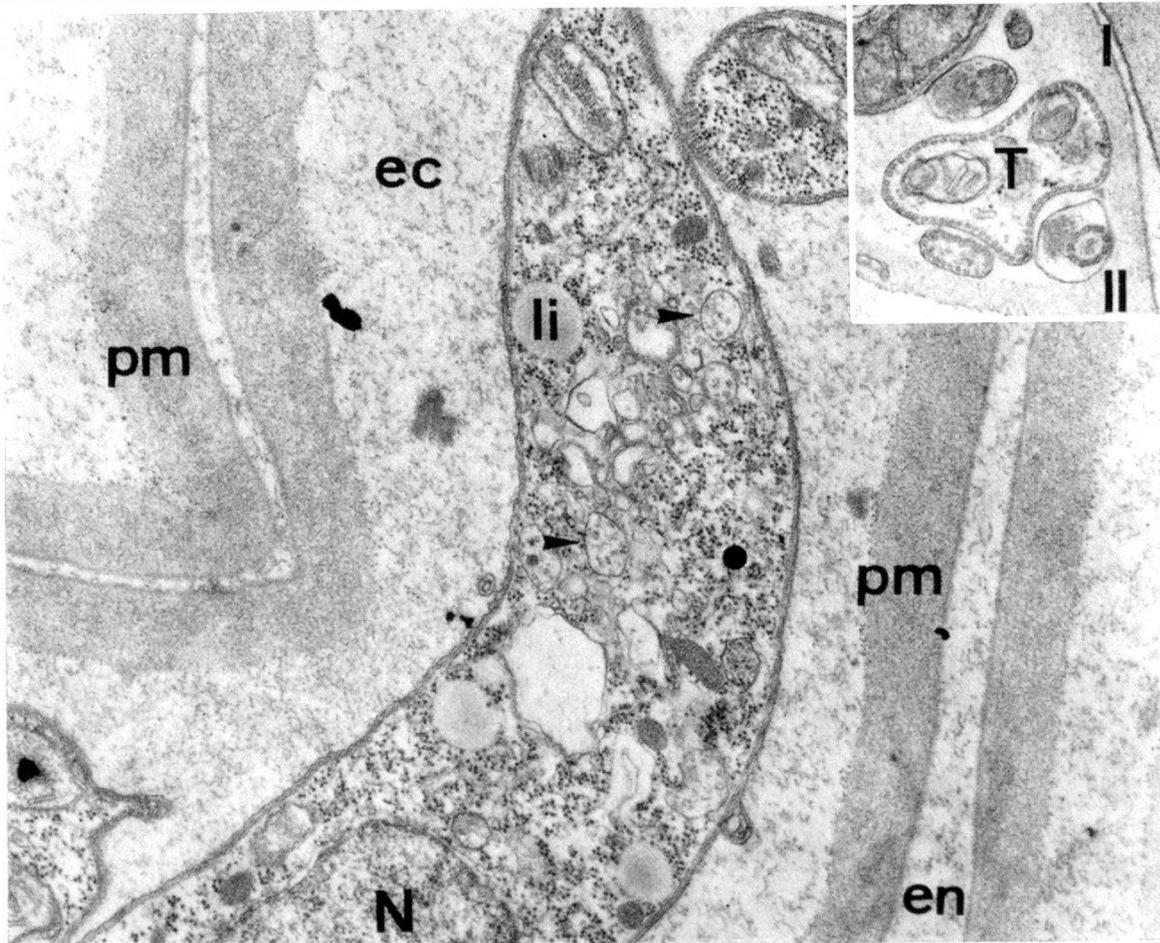
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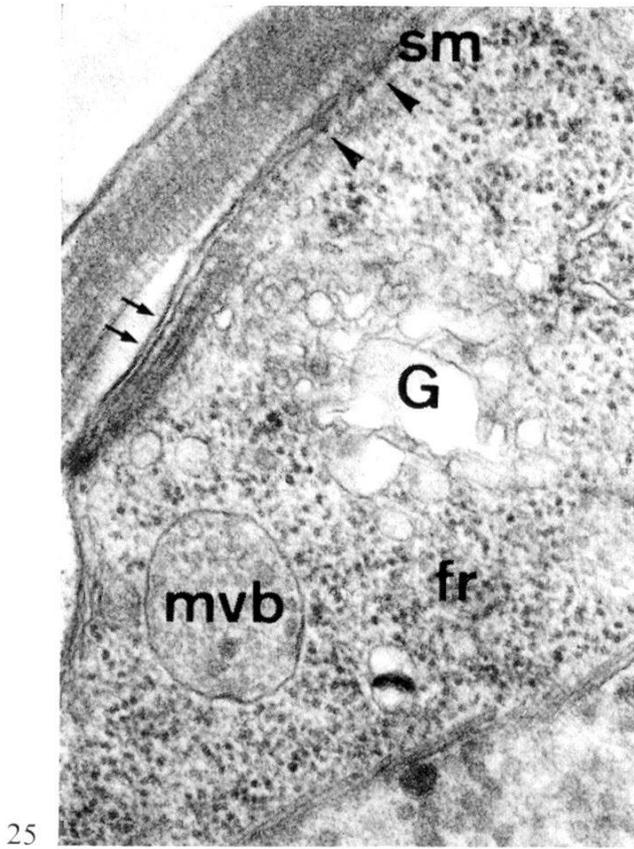
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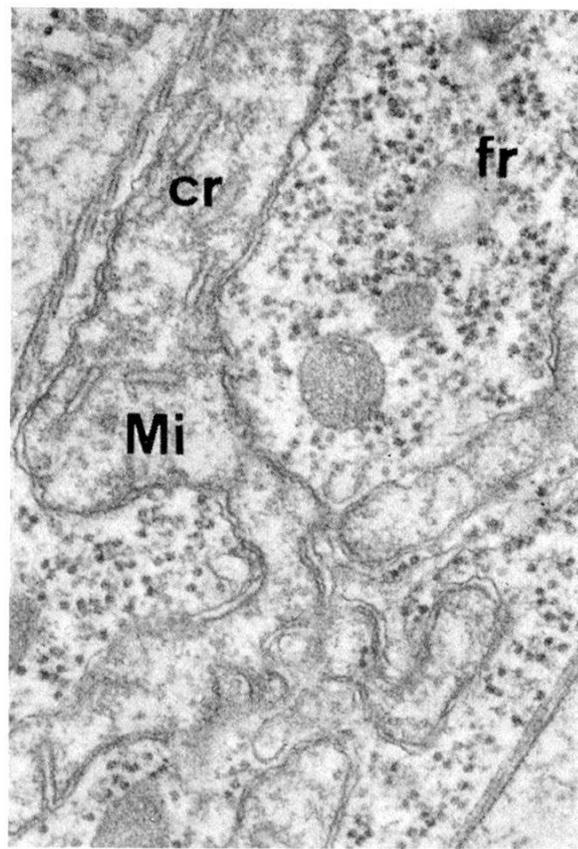
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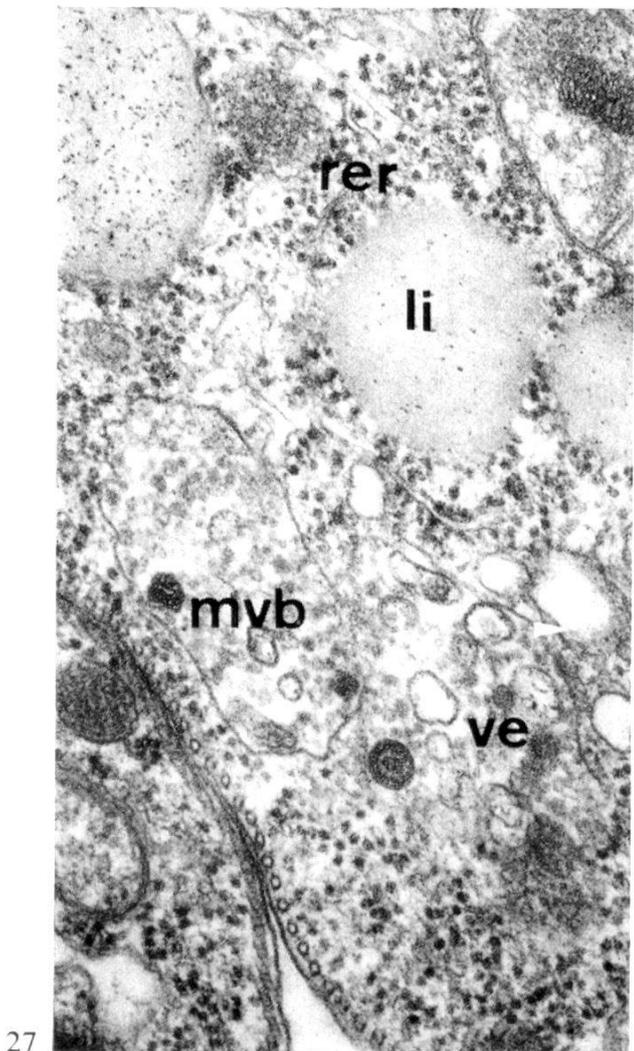
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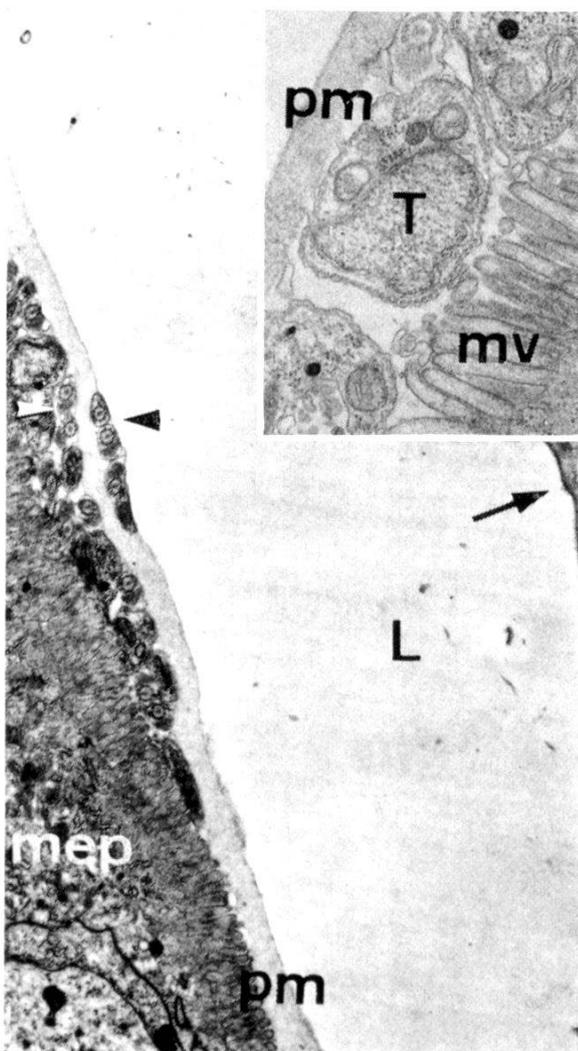
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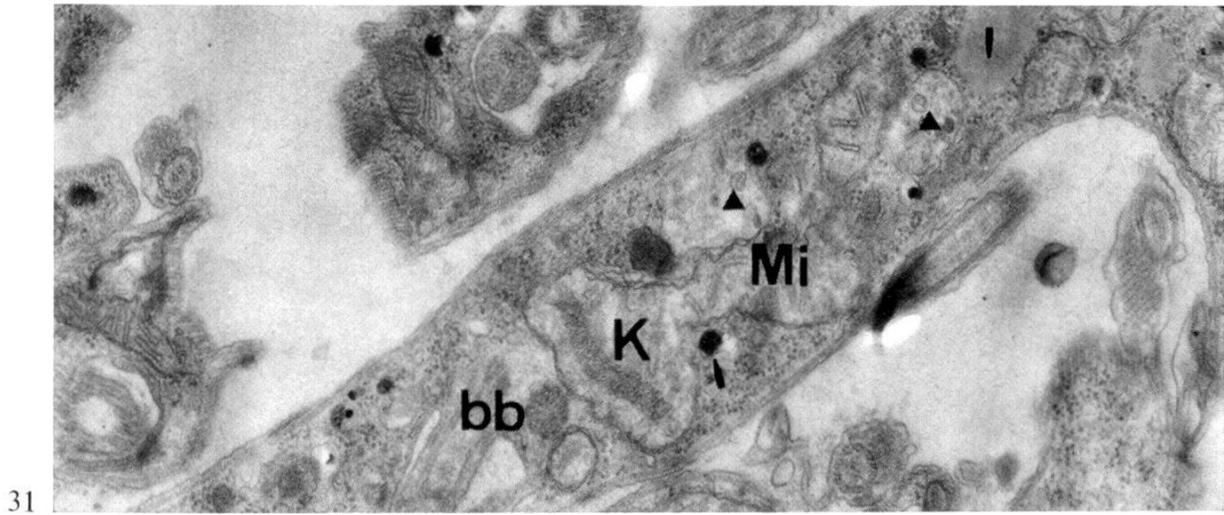
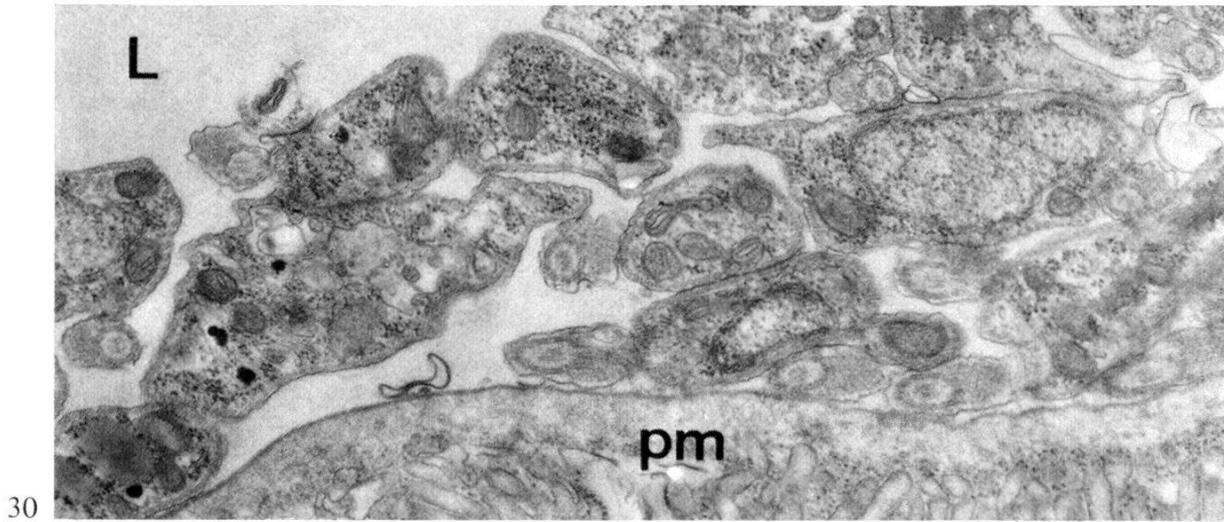
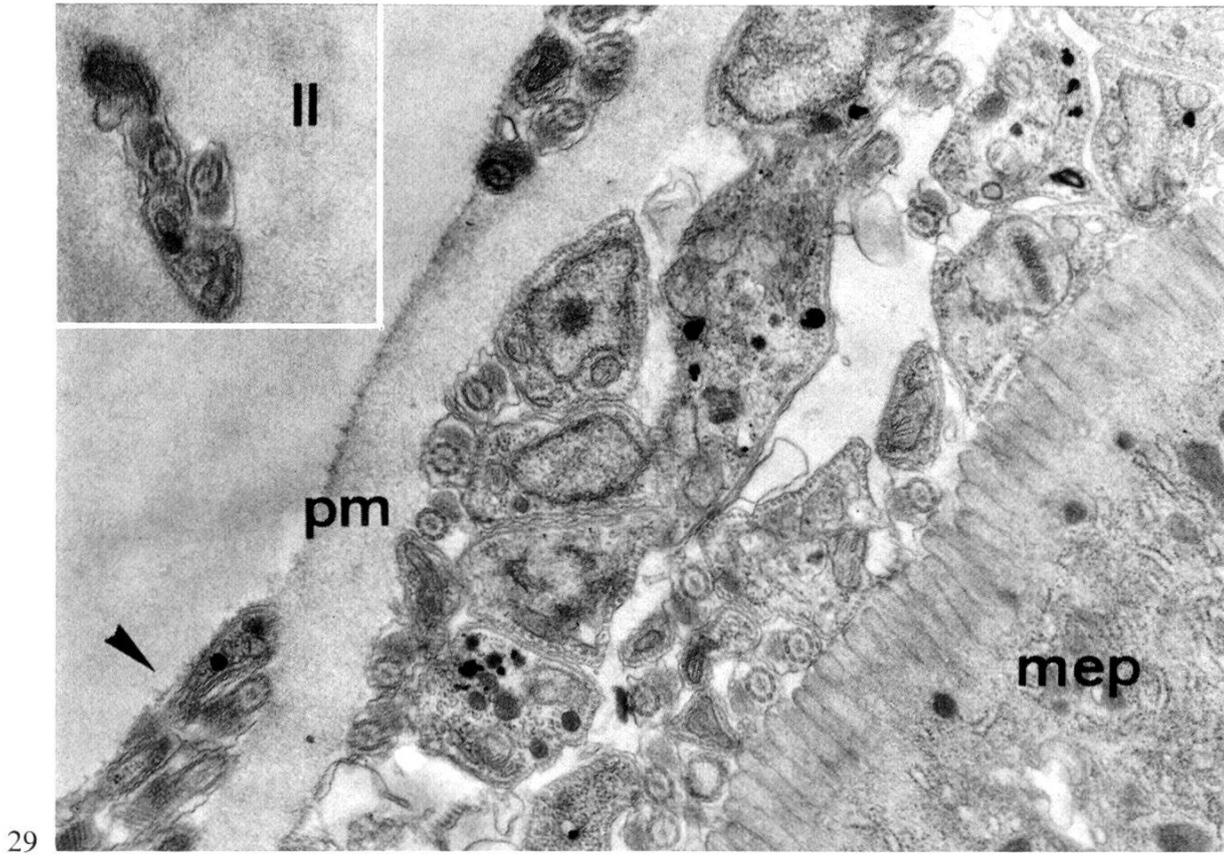
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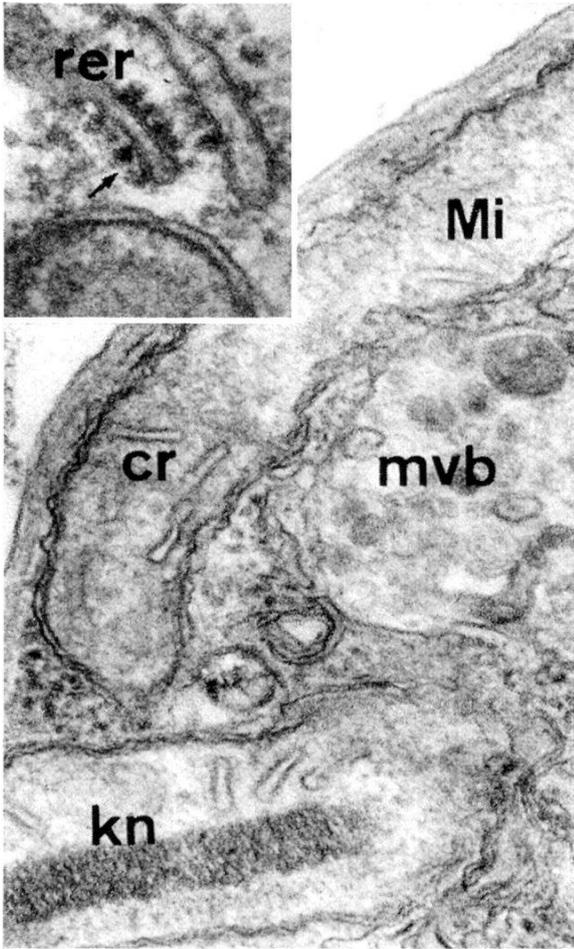


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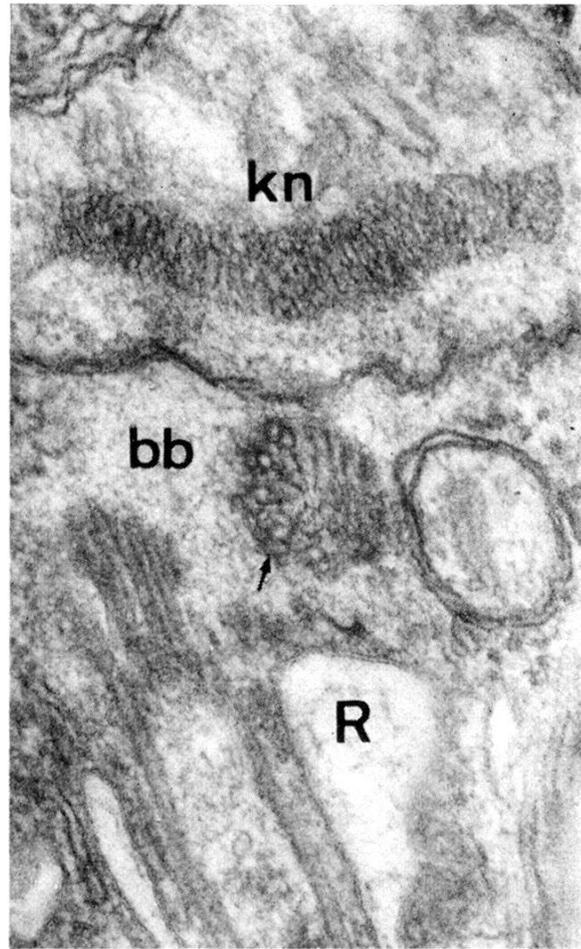


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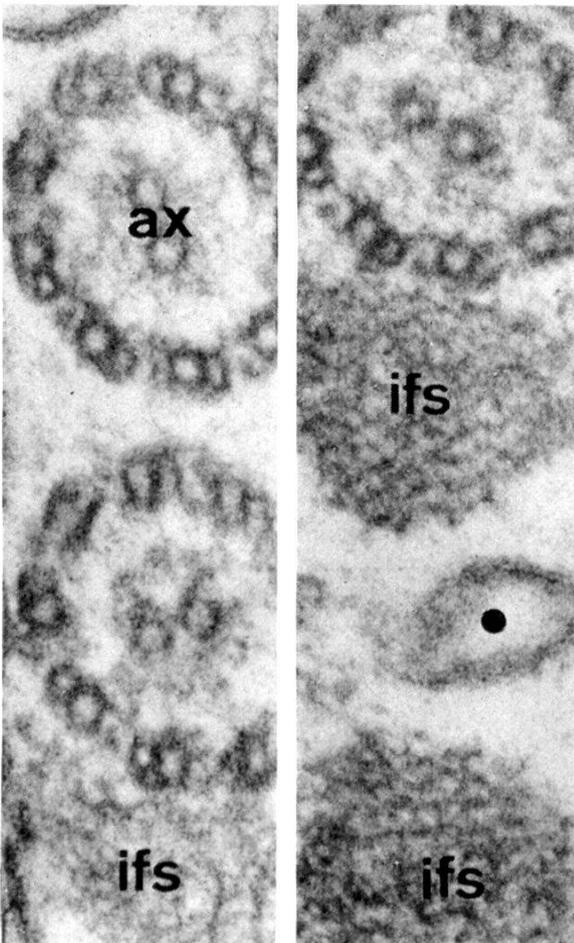




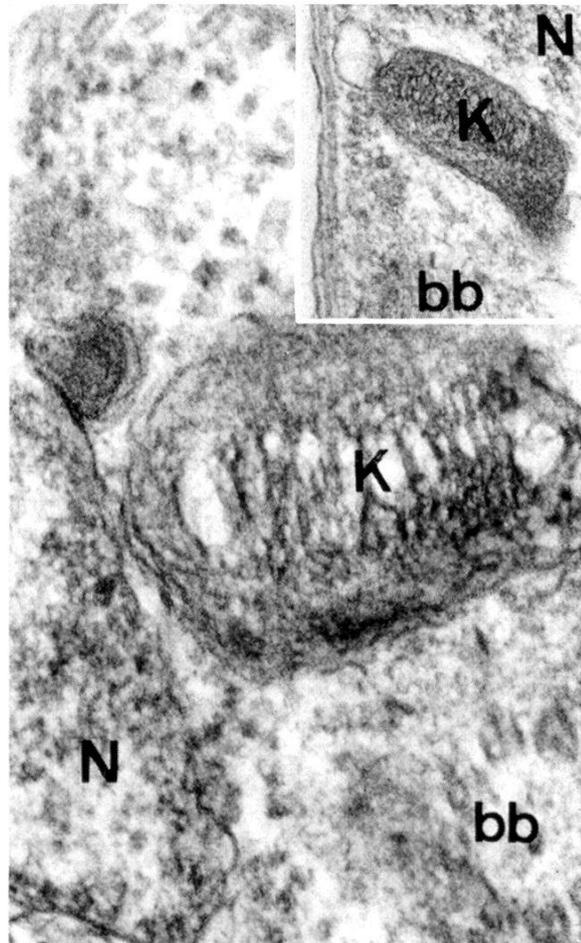
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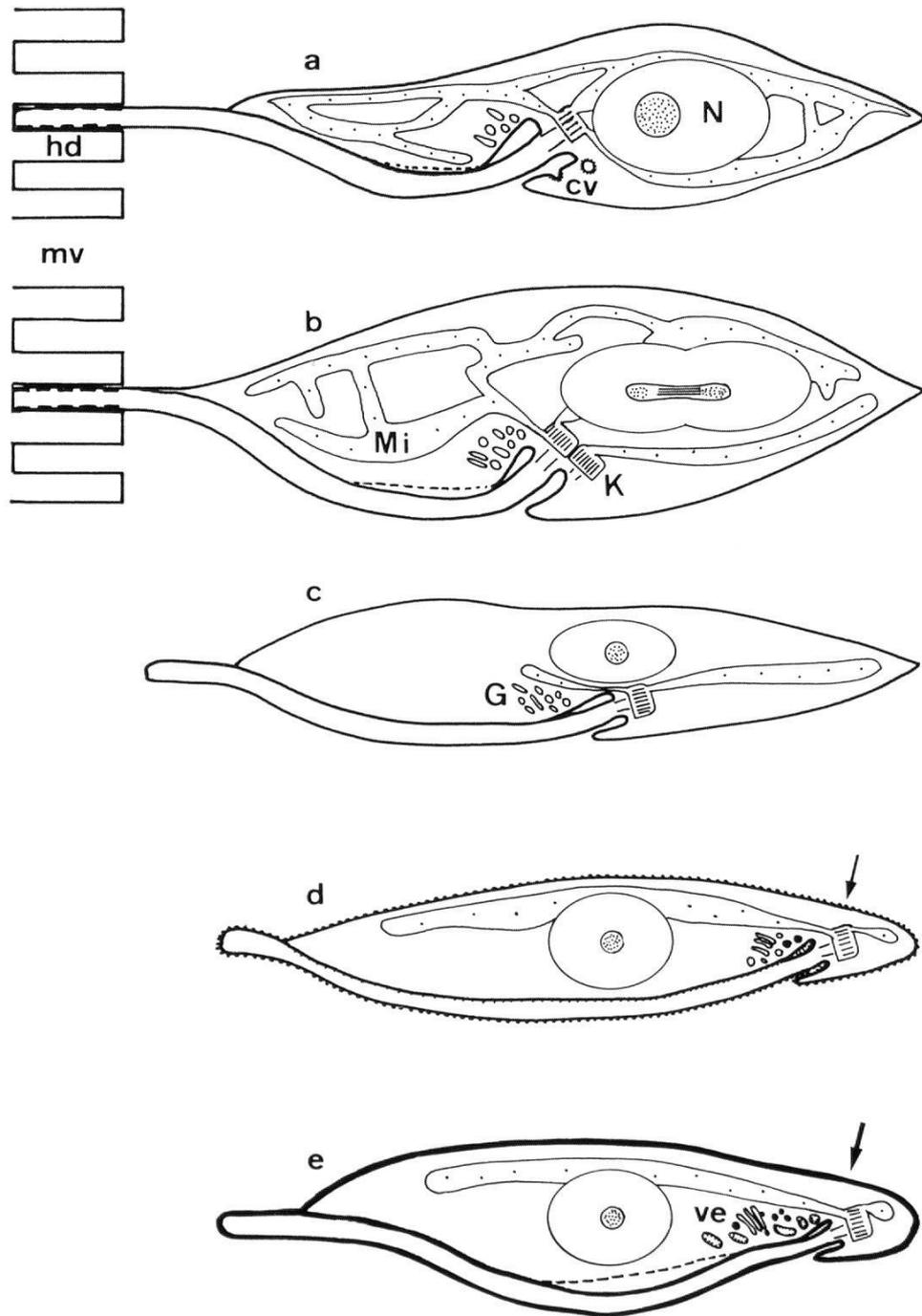
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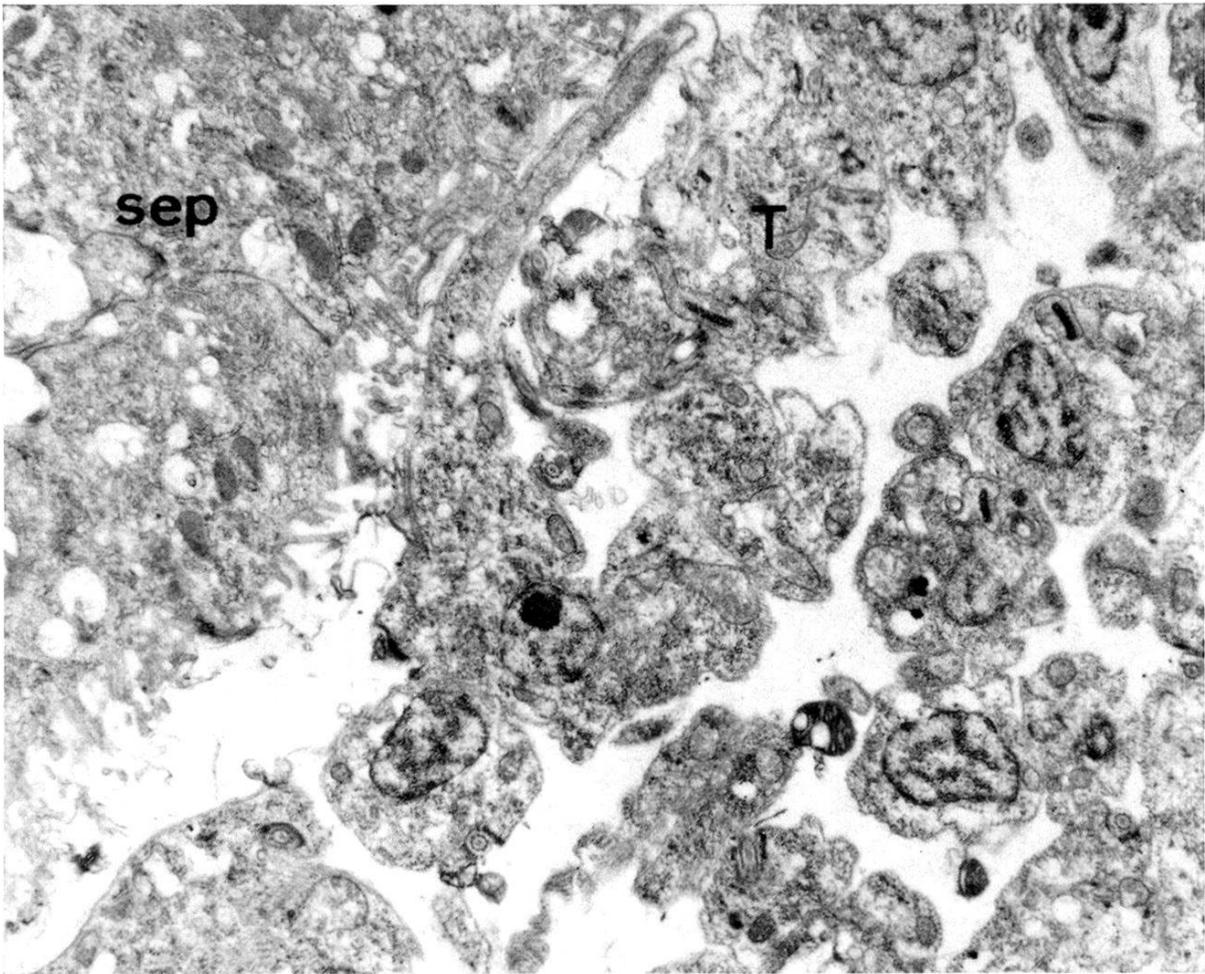


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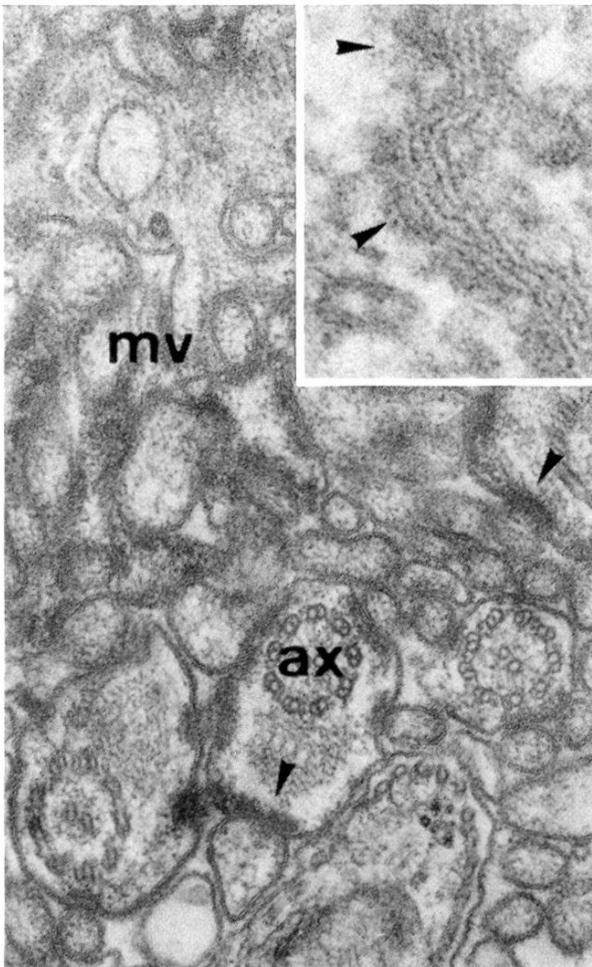


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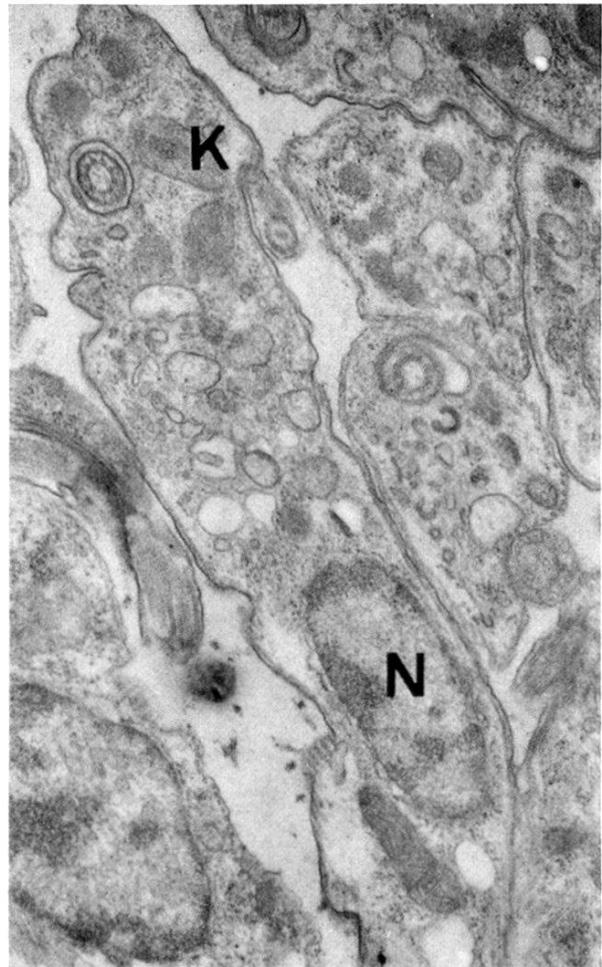




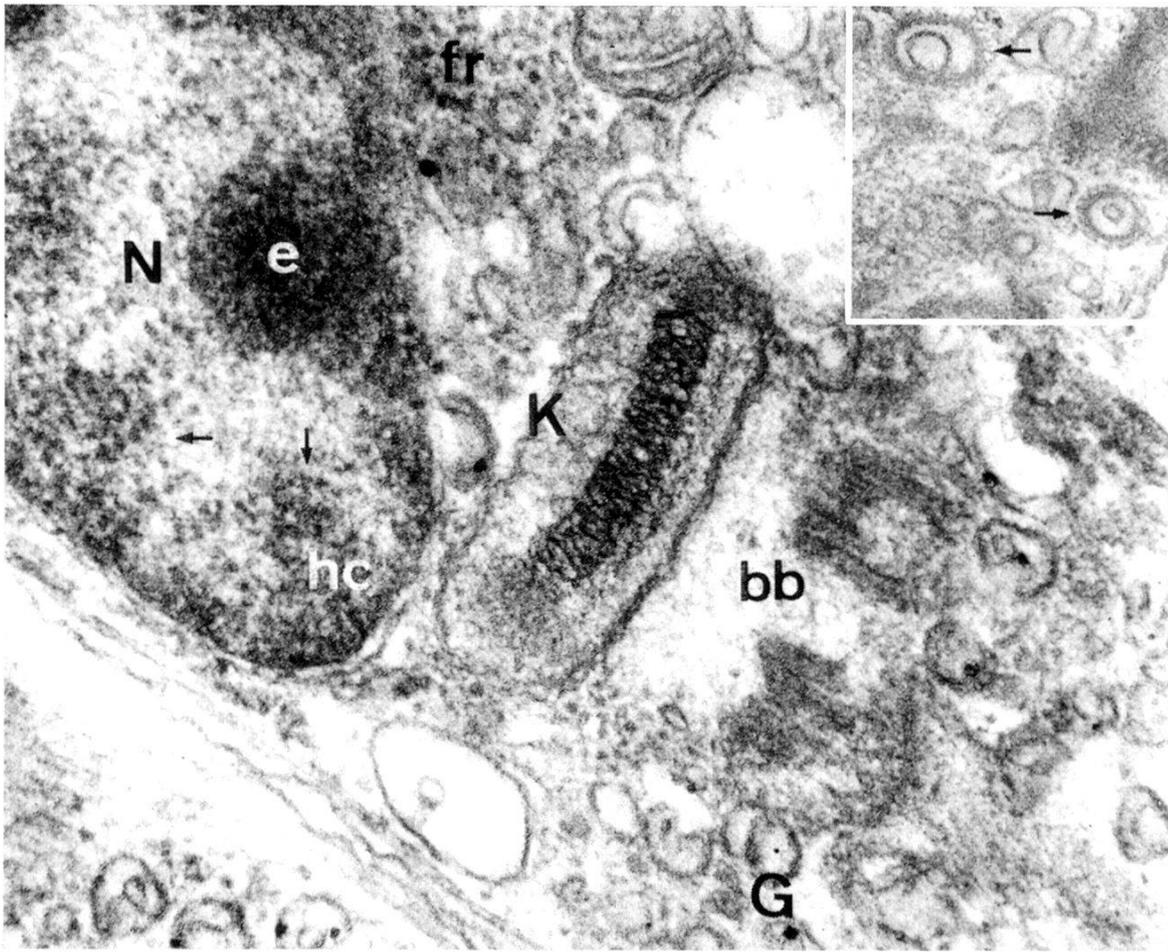
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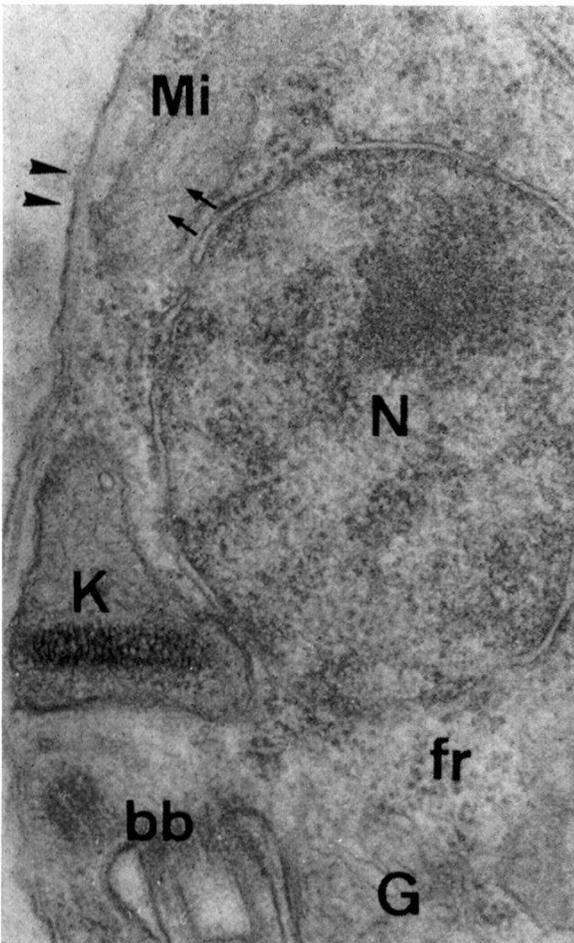
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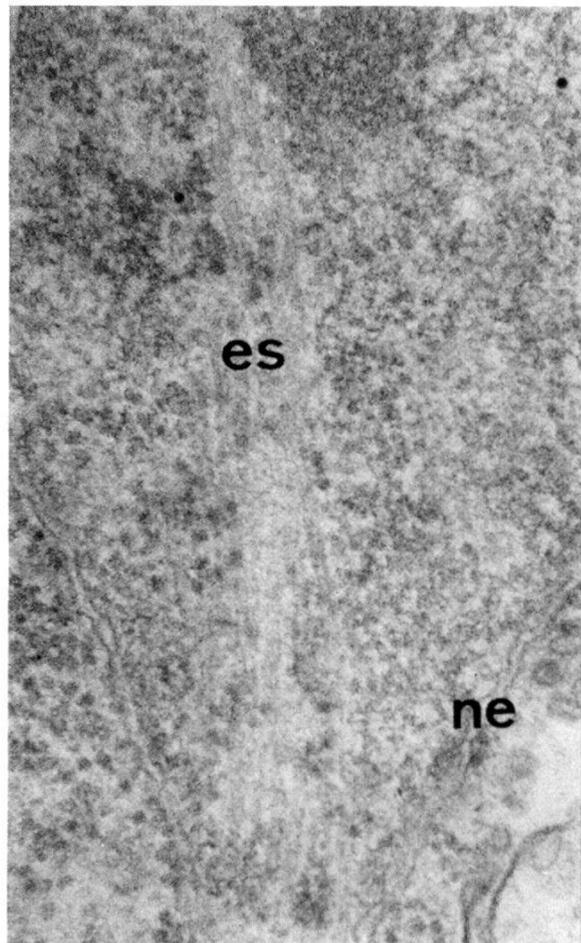
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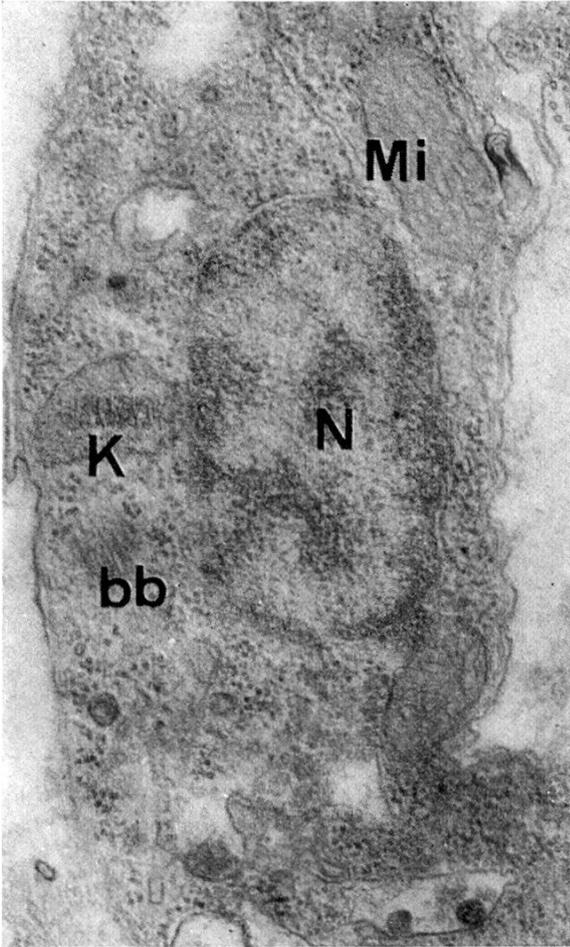
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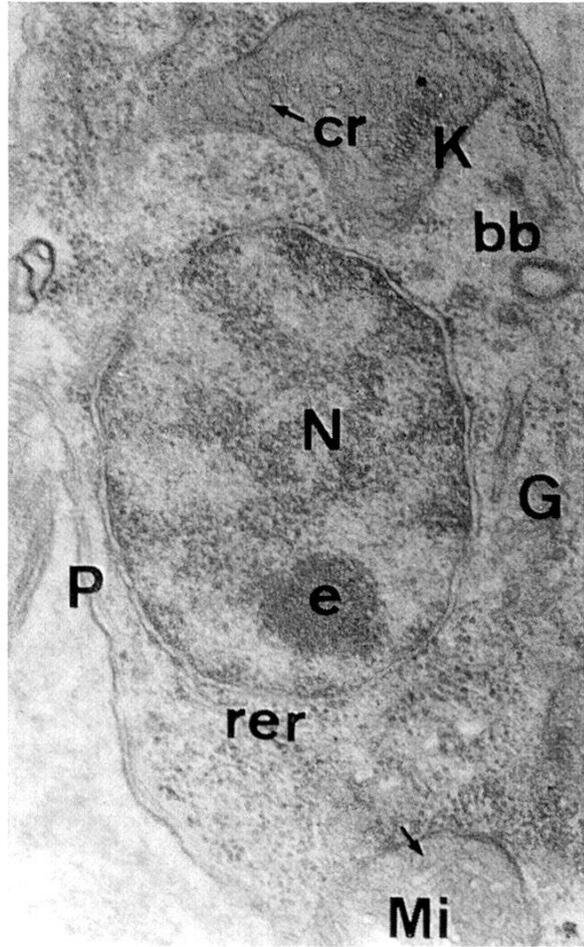
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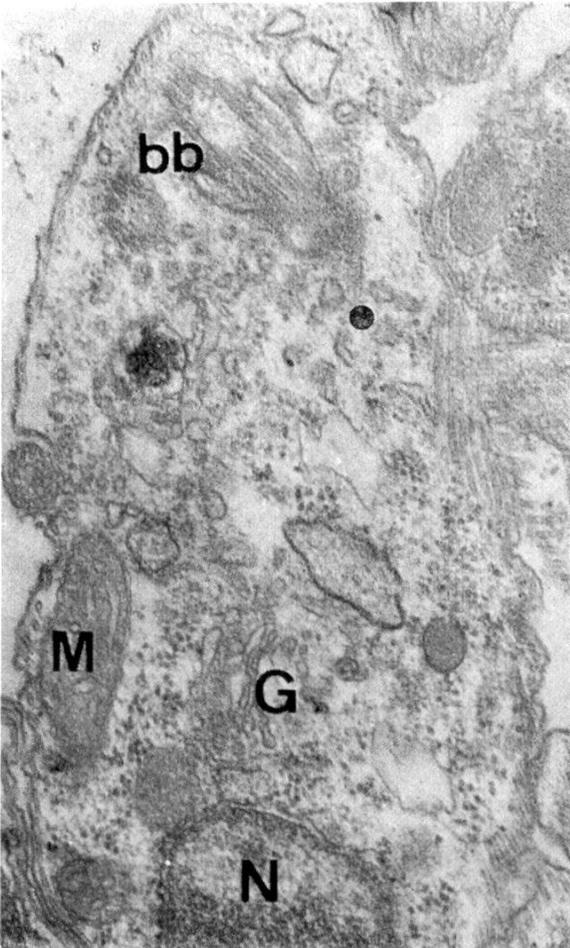
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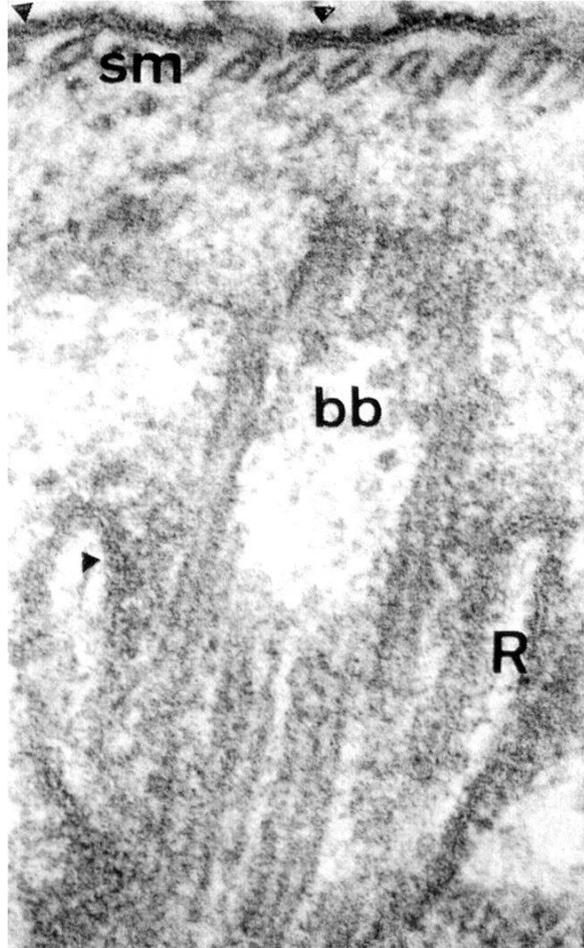
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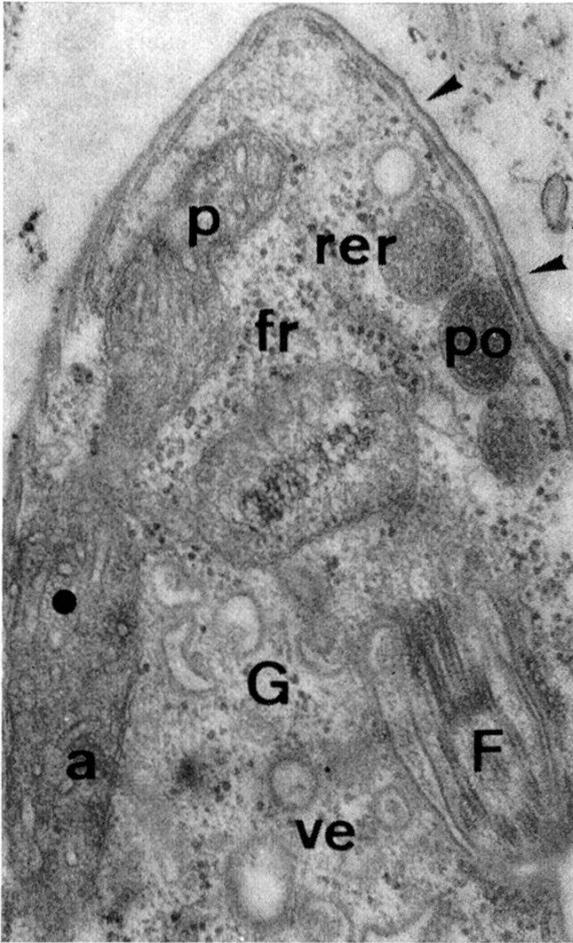
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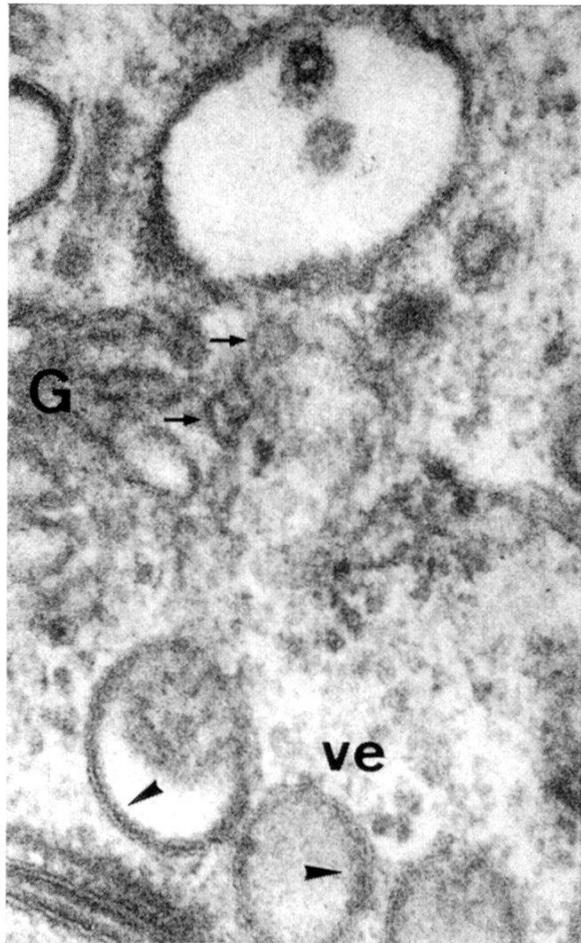
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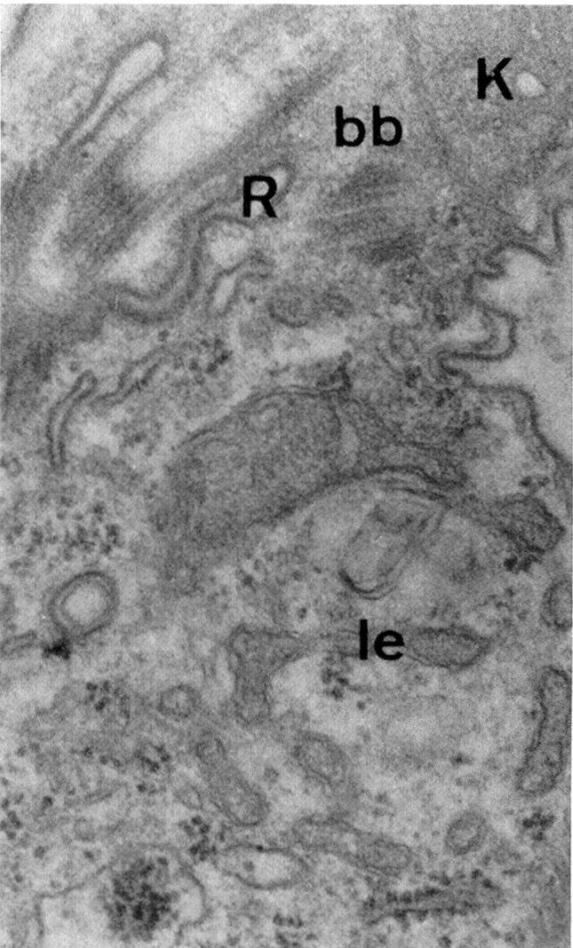
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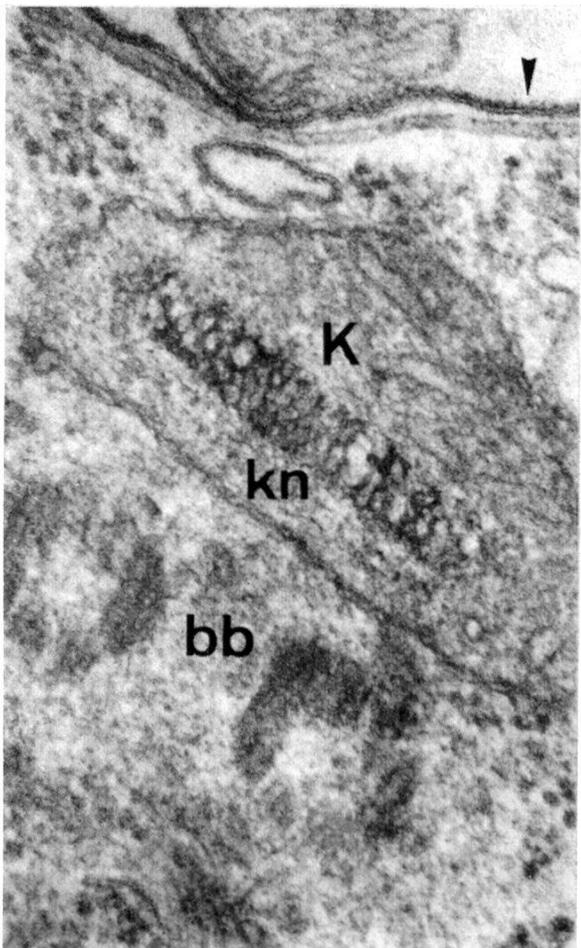
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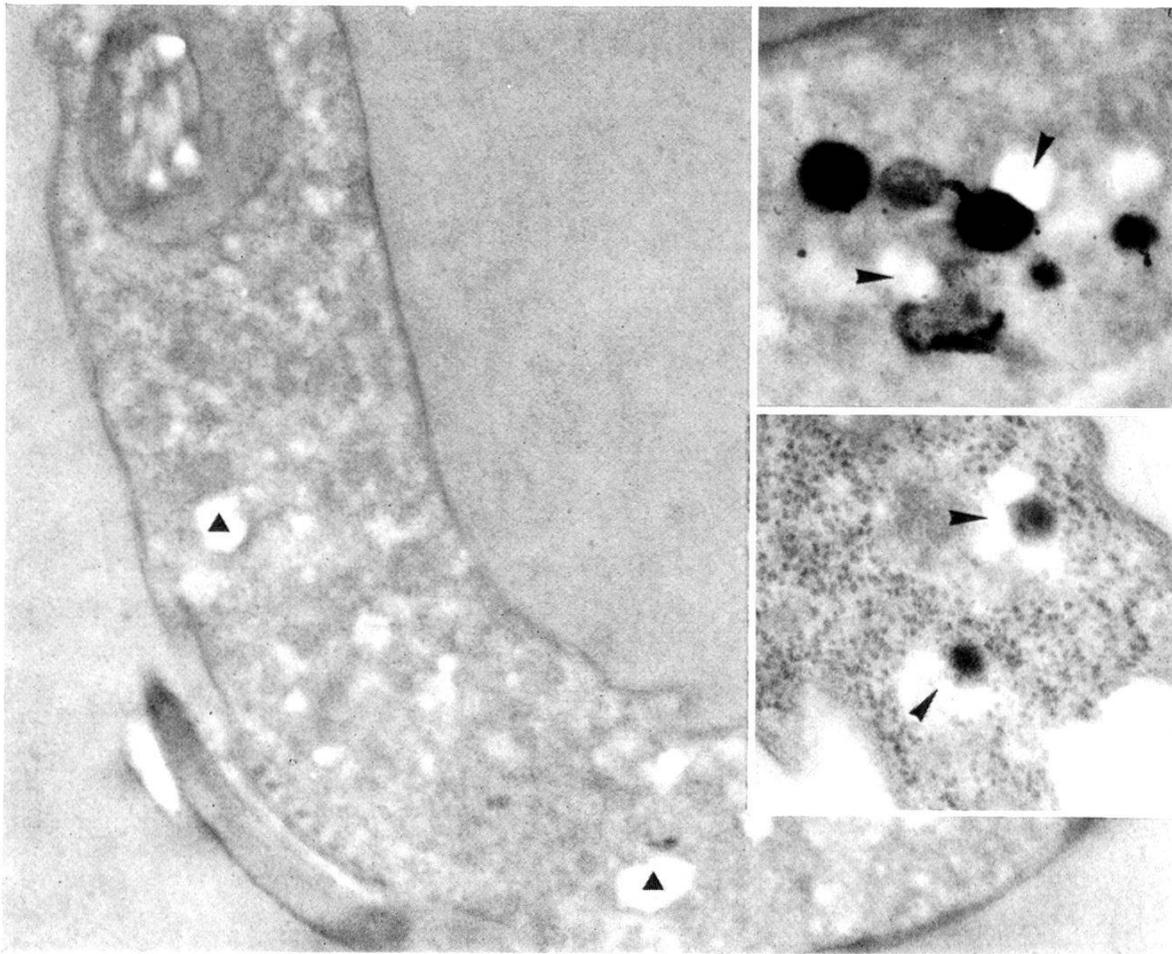
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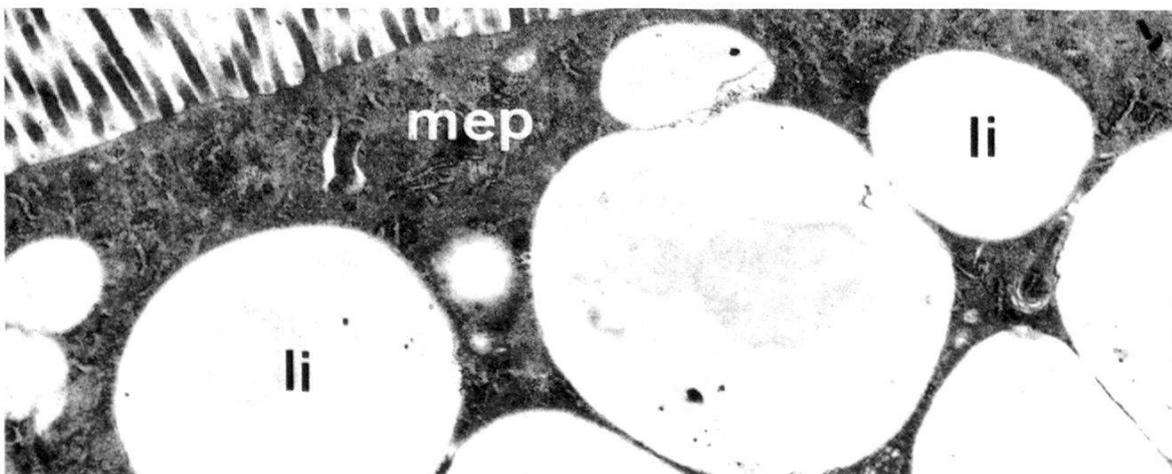
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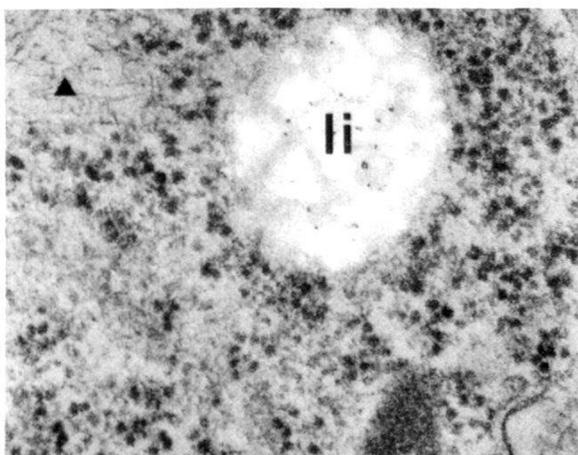
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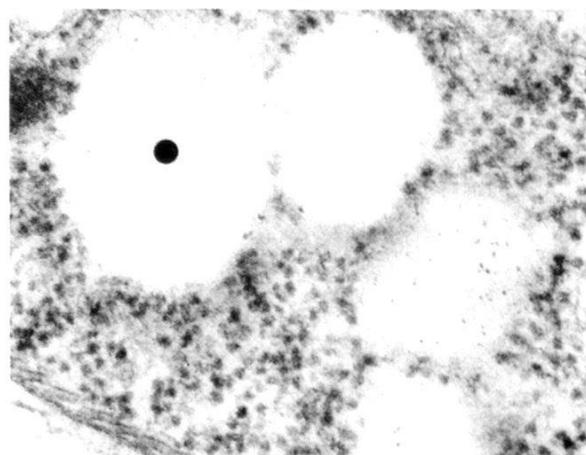
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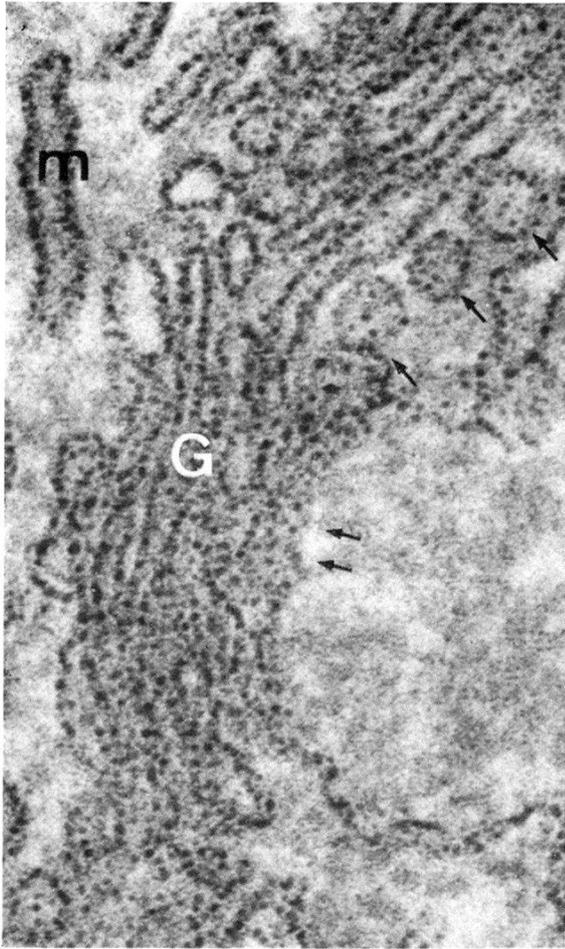
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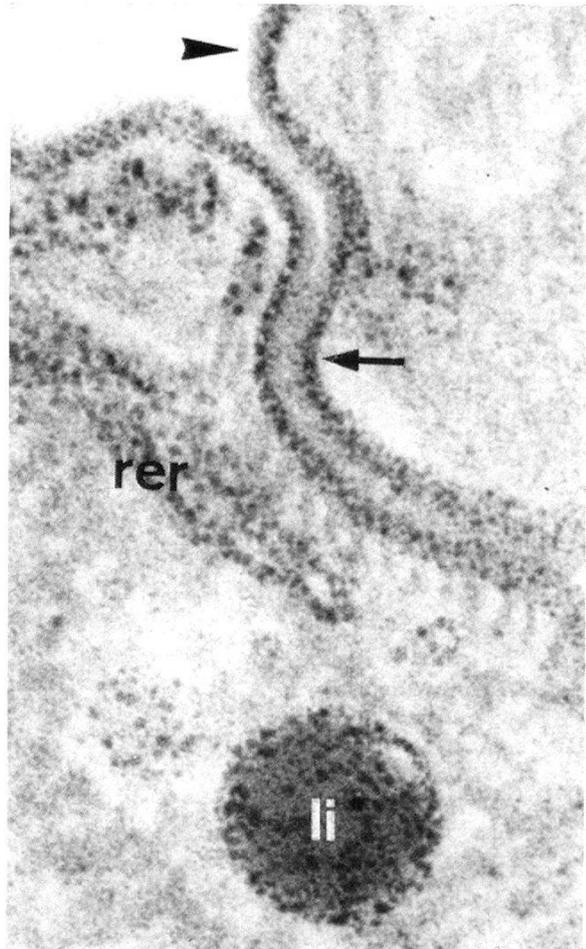
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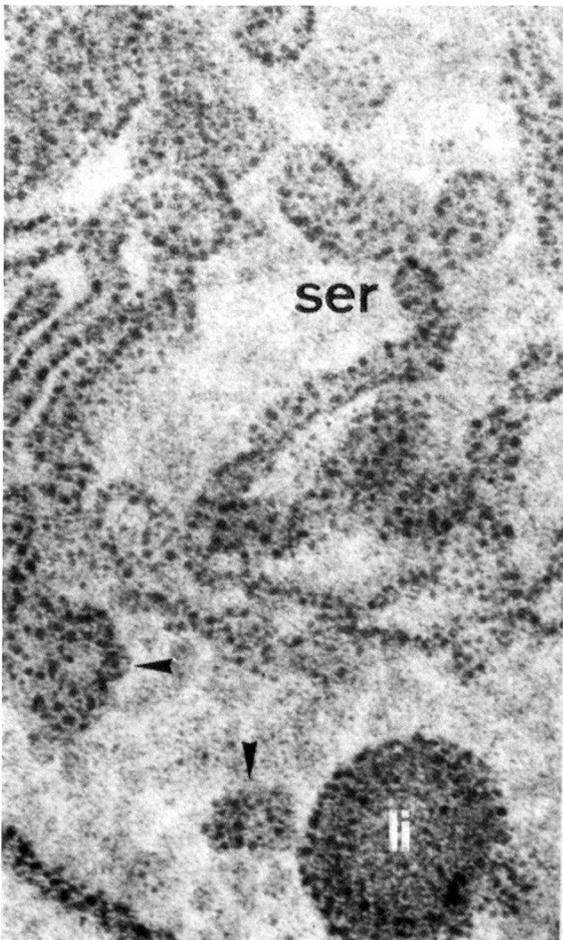
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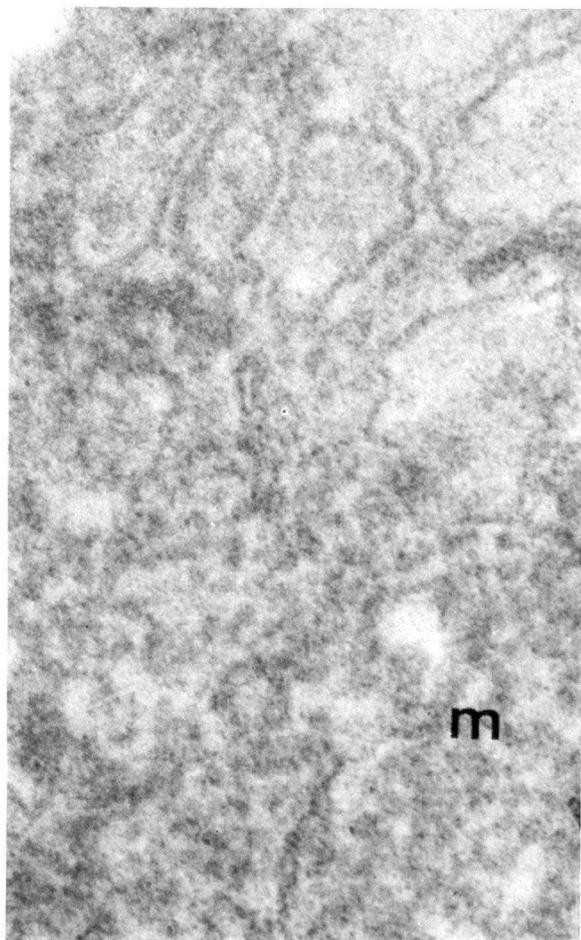
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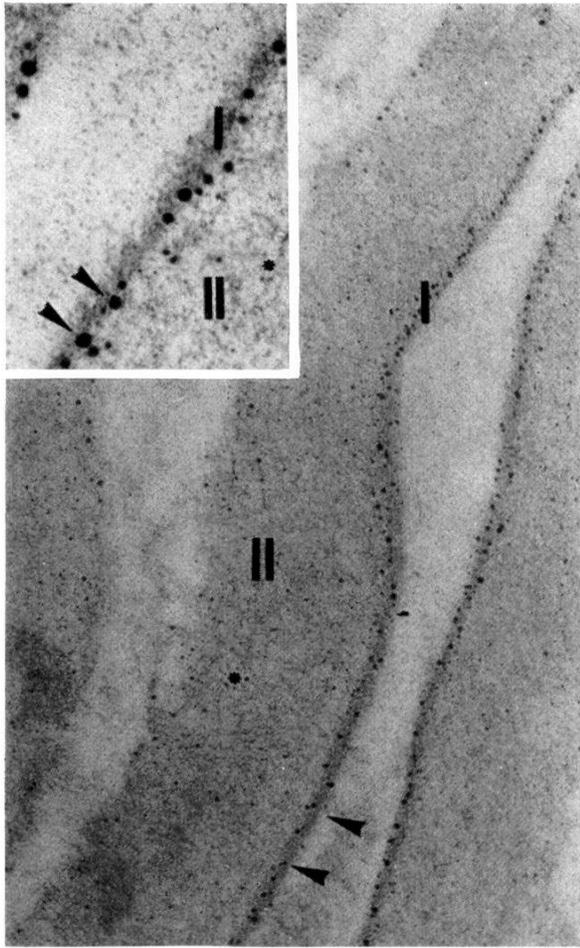
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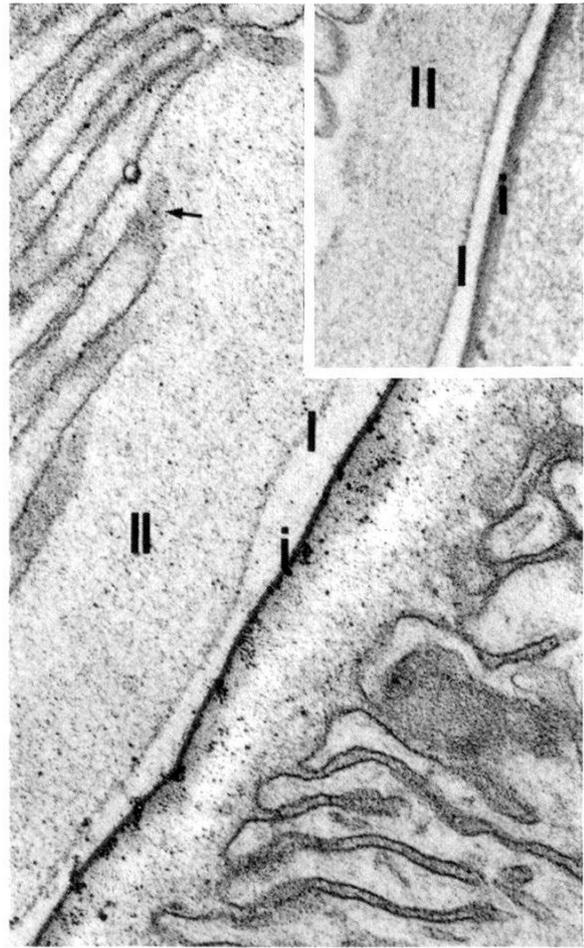
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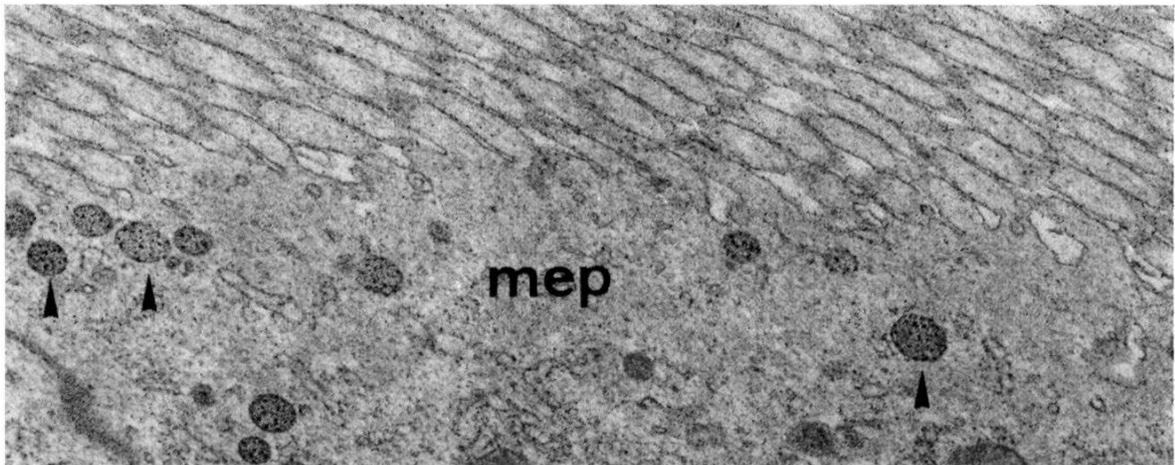
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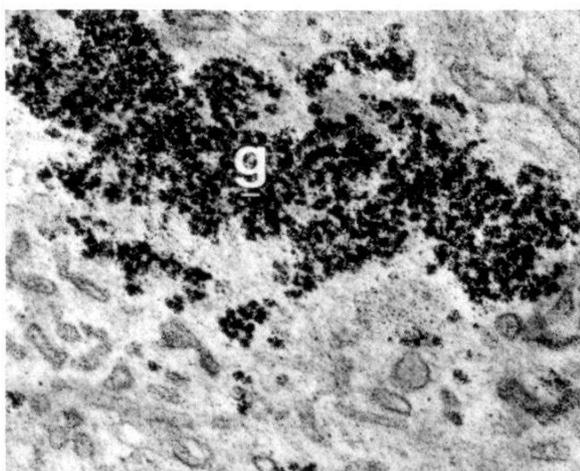
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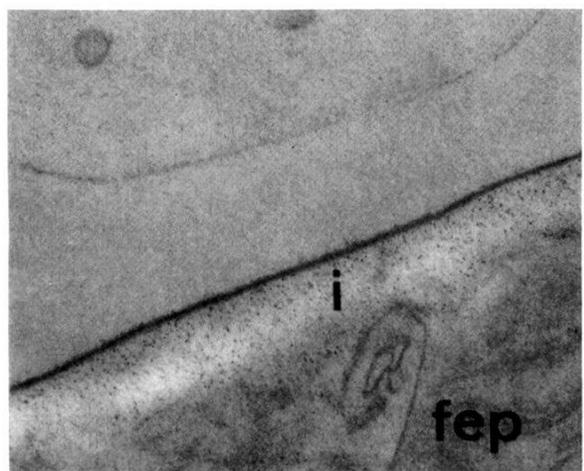
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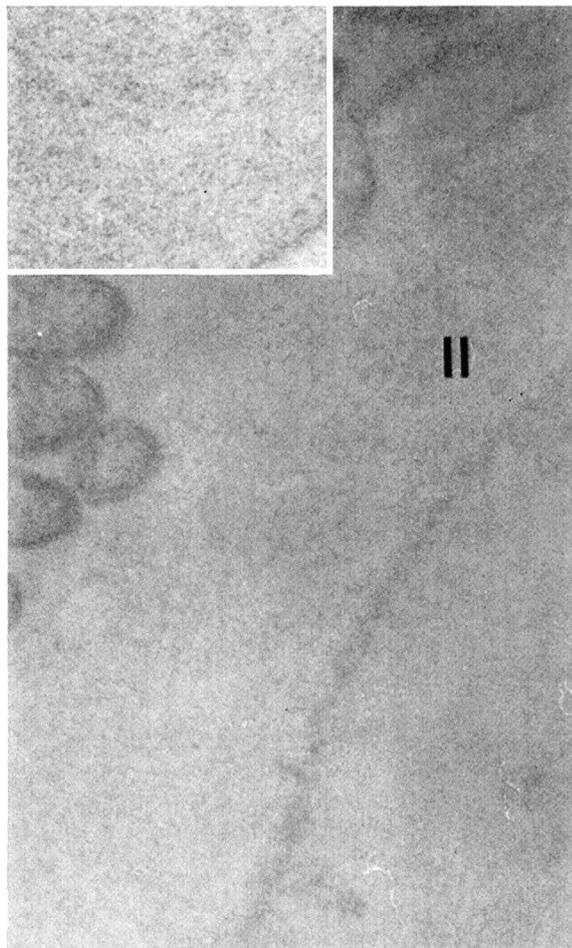
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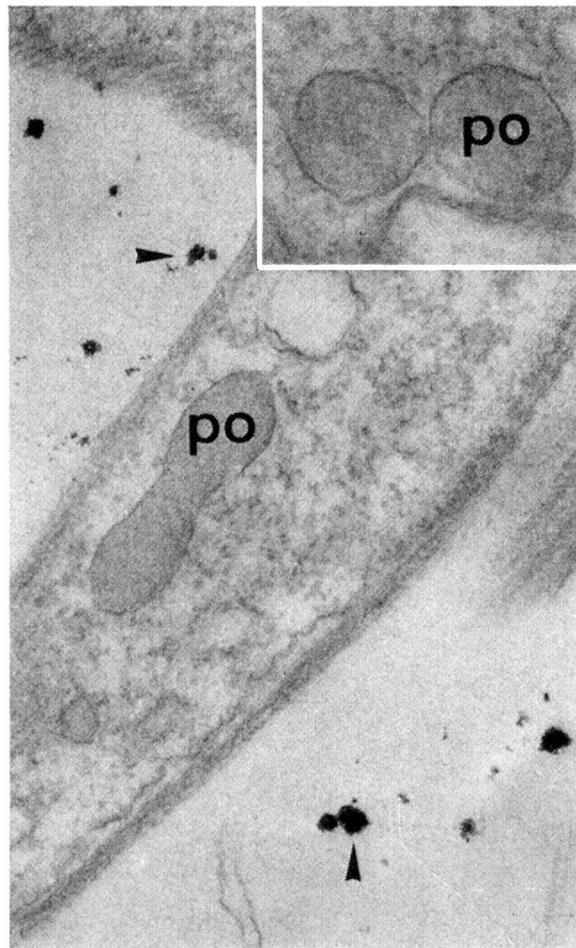
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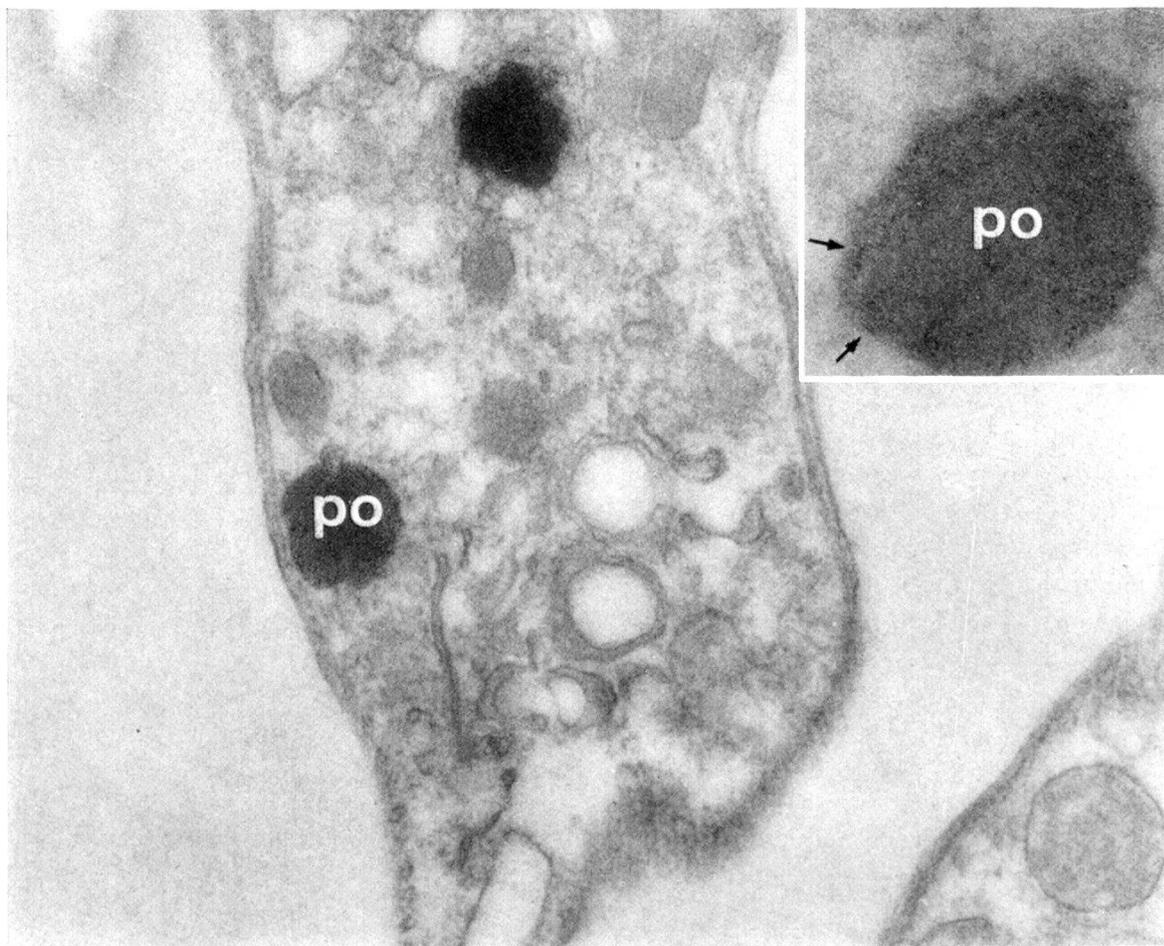
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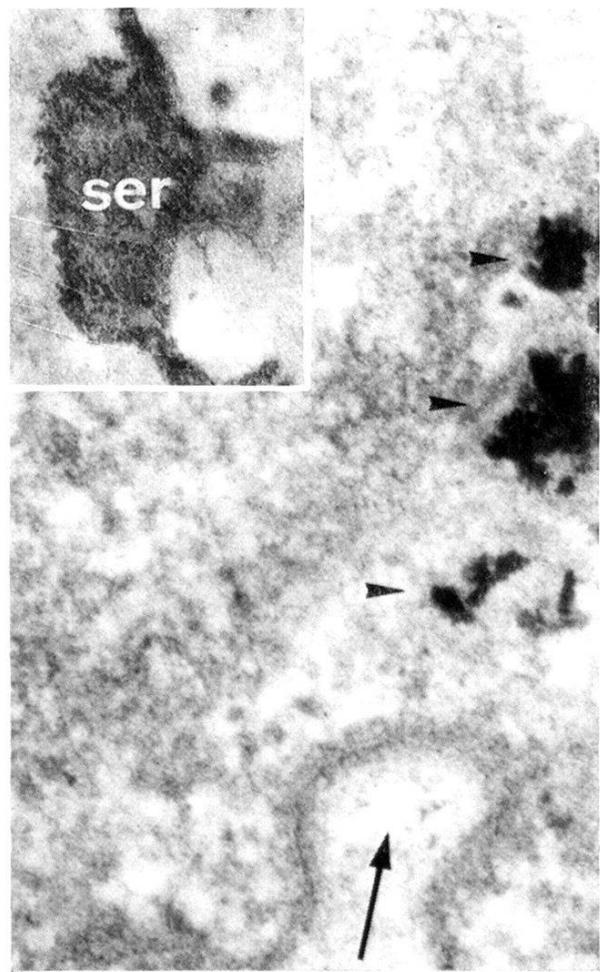
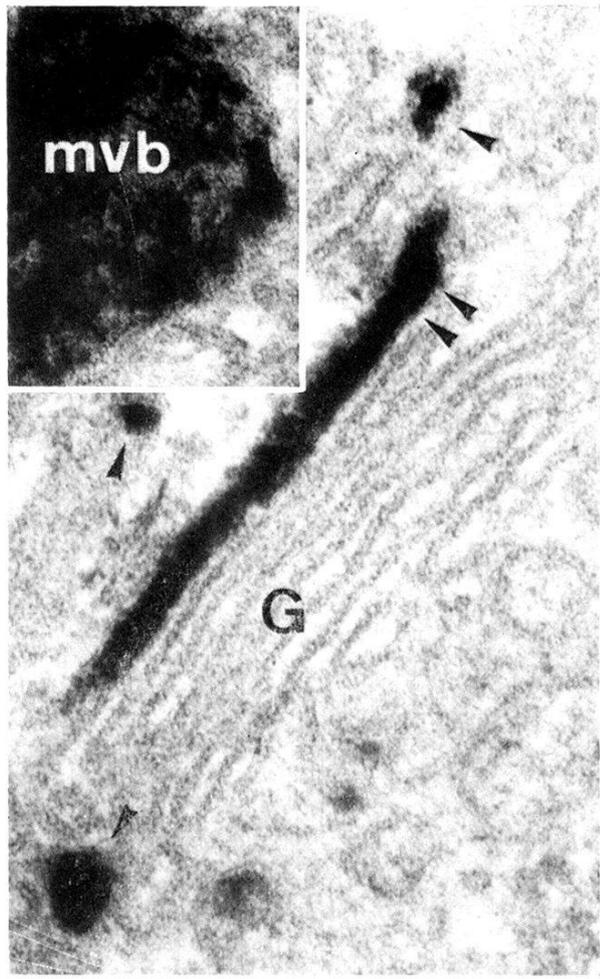
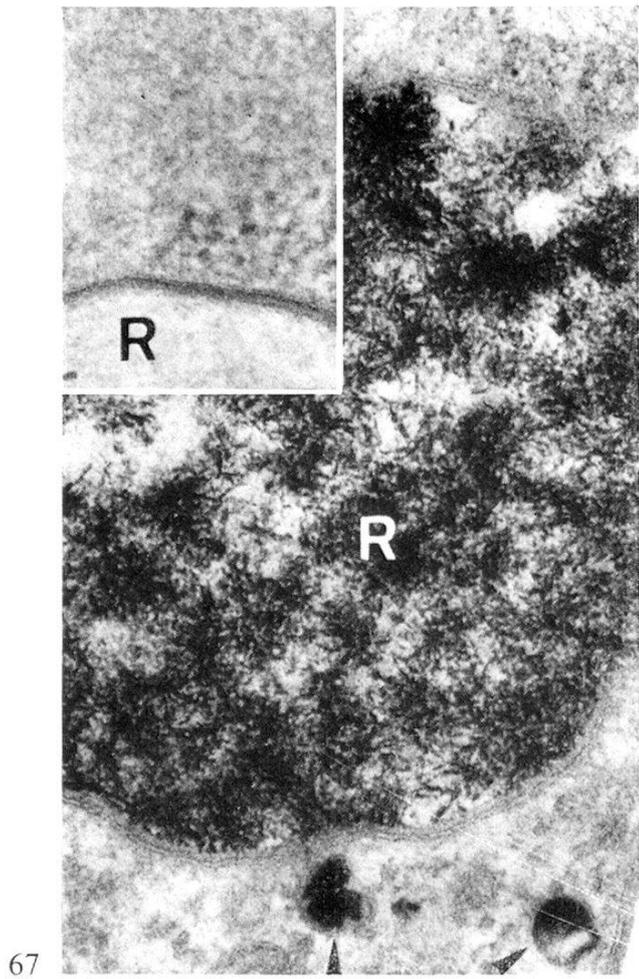
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