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A quantitative ultrastructural study on the transformation of *Trypanosoma brucei brucei* metacyclic to bloodstream forms in vitro

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Summary

The transformation of metacyclic to bloodstream forms of *Trypanosoma brucei brucei* was studied in vitro using light and electron microscopy. The ultrastructural composition was investigated with stereological methods and the mean cell volume determined in a Coulter Channelyzer. The mitochondrion showed the most significant changes during transformation with a reduction in volume as well as in the membrane areas. The glycosomes remained unchanged whereas the lipid inclusions increased over the 24 h incubation period. The metacyclic forms contained many vesicles in the reservoir vicinity with surface coat-like material on the inner side of the membrane. Metacyclic forms also contained a previously undescribed structure, termed «inclusion body». These large, polymorphic structures whose function and origin are unknown disappeared during transformation. The mean cell volume for metacyclic forms was 15–16 μm^3. During transformation the values first dropped and then increased to about 20 μm^3 after 24 h.

Key words: *Trypanosoma brucei brucei;* in vitro transformation; metacyclic forms; mean cell volume; morphometry.

Introduction

Transformation from the metacyclic to the bloodstream form is an important part of the life cycle of *Trypanosoma (T.) brucei*. After the infectious tsetse bite the trypanosomes become established and multiply in the skin prior to dissemination to the bloodstream via the lymphatic system (Emery and Moloo, 1980). When they appear in the blood as slender forms they have already com-

pleted this transformation. Gordon and Willett (1958) described in detail the transformation as seen on stained smears during the first 24 h in vitro.

A new culture system has been developed to grow pleomorphic bloodstream forms of *Trypanosoma (T.) brucei* (Brun et al., 1981). In this system metacyclic forms transformed within 8 h to slender bloodstream forms and could continuously be cultivated as infective mammalian forms (Jenni and Brun, 1981). In vitro cultivated bloodstream forms were found to be morphometrically similar to lymph and bloodstream forms from laboratory animals (Hecker and Brun, 1982). The present study was aimed to examine the morphological changes of the parasites during transformation with respect to their mean cell volume and cellular organelles.

Materials and Methods

Trypanosoma brucei brucei STIB 247, a pleomorphic stock, is readily transmissible by the tsetse fly. It was isolated in 1971 in the Serengeti National Park from a hartebeest *(Alcelaphus buselaphus)* and cryopreserved in liquid N_2 after one rat passage. ILRAD 923 used is a derivative of STIB 247.

Glossina morsitans morsitans used were from the ILRAD R^6 colony (Moloo, 1979). The infected flies were kept at 25°C and 70% relative humidity.

Three East African adult Galla crossbred goats were injected intramuscularly on different days with *T. brucei brucei* ILRAD 923. To determine infection, the goats were bled from the ear daily except Sundays, and the parasitaemia was determined by haematocrit centrifuge technique and by wet blood film examination. When the infection became patent, teneral (young/unfed) tsetse were allowed to feed on the clipped and cleaned flanks of the goats every day except Sundays. On day 35 post-emergence, the tsetse were allowed to probe singly on slides at 38°C and those which showed metacyclics in saliva were used for the present study. Altogether 920 tsetse were used and the infection rate was 14.1%.

Harvest and incubation of metacyclic forms

Infected tsetse were coaxed to salivate singly into drops of culture medium, each of 50 μl at 37°C. Metacyclics were harvested for 60 min from 100 to 150 tsetse. The drops were pooled into a vial on ice. The pooled metacyclics were then counted in a Neubauer haemocytometer and the remaining metacyclics were centrifuged at 1000 g at 4°C for 10 min. The pellet was resuspended in the medium to a final density of 5×10^5/ml and the tube incubated at 36.5°C in 4% CO_2/96% air for 0–24 h. After the respective incubation period 300,000 cells were taken and used as follows: approx. 75,000 cells for Coulter Channelyzer analysis, approx. 5,000 cells for infectivity tests in 2 mice, approx. 20,000 cells for smears for light microscopy, approx. 200,000 cells for electron microscopy.

Culture medium

The medium used for the transformation experiments was Minimum Essential Medium (MEM) with Earle's salts, 25 mM HEPES buffer, 1% MEM nonessential amino acids (100×) and additional 2 g/l glucose. The medium was supplemented with 15% heat-inactivated rabbit serum and 20 μg/ml gentamycin. MEM and the nonessential amino acids were from GIBCO Bio-Cult Ltd. The rabbit serum was prepared from blood of adult New Zealand White rabbits. It was filter sterilized, heat-inactivated and stored at −20°C.

Infectivity test

Infectivity of the metacyclic forms after the in vitro incubation was tested in adult A/J mice. Two mice were injected intraperitoneally with 2,500 trypanosomes each and the mice were examined daily.

Size distribution analysis

Size distribution and determination of the mean cell volume were carried out with a Coulter Counter ZBI (with a 70 μm orifice and the settings A = 2, I = ¼) in combination with a Coulter Channelyzer C-1000, setting the base channel threshold to 6 and the window width to 50 with EDIT on. For each analysis 75,000 trypanosomes (= 150 μl incubation fluid) were put in 8 ml Isoton II electrolyte solution (Coulter Electronics) and immediately analyzed. The counted number of trypanosomes per channel was multiplied by the given volume of the respective channel. The mean cell volume (Vc) of all trypanosomes classified in the channels 6 to 61 (2–3×10^4) was determined.

Preparation for microscopy

After incubation approximately 220,000 cells (= 440 μl) were transferred to a 3 ml conical centrifuge tube and 2 ml of a 2% bovine serum albumin solution in medium was added. The trypanosomes were centrifuged for 10 min at 1000 g at 4° C and the supernate removed leaving about 30 μl. After thorough mixing a small amount of 2–3 μl was removed and two smears prepared with an Eppendorf tip. They were fixed with menthanol and stained with Giemsa. The rest of the concentrated trypanosome suspension was transferred into a capillary tube. At one end fresh mouse blood was added to a length of about 1 mm, this end was then sealed with plasticine. During a 10 min centrifugation at 1000 g the trypanosomes were pelleted on top of the mouse erythrocytes. The pellets were fixed and processed for electron microscopy (Hecker and Brun, 1982).

Stereology

Trypanosome populations were examined quantitative-morphologically using stereological principles (Weibel, 1979; Burri and Hecker, 1979). For each population 100–120 micrographs were used at a final magnification of 58,000× corresponding to about 420 μm² of randomly sectioned trypanosomes. The volume of the flagellum was added to the cytoplasmic volume since the Coulter Counter measures the cell volume inclusive the flagellum. Flagellar parameters were somewhat underestimated due to the omission of some of the free flagellar profiles. Stereological parameters as presented in Tables 1 and 2 were investigated as described previously (Hecker and Brun, 1982).

Results

Light microscopy

The metacyclic forms harvested from the tsetse flies were short trypomastigotes with the kinetoplast in a terminal position and with a very short free flagellum. Dividing metacyclic forms could not be observed. In some of the metacyclic forms a stained body located between kinetoplast and nucleus was present (Fig. 1). After 2 h (Fig. 2) the kinetoplast had moved towards the nucleus and the free flagellum as well as the cell itself was longer as compared to 0 h. These changes were more evident after 8 h. At that time, some of the trypanosomes could already be considered as long slender bloodstream forms (Fig. 3). After 24 h all cells had completed transformation and revealed the typical features of long slender bloodstream forms, i.e. with the kinetoplast in a subterminal position, with an elongated body and with a long free flagellum (Fig. 4). Cells in division could first be seen after 12–15 h.

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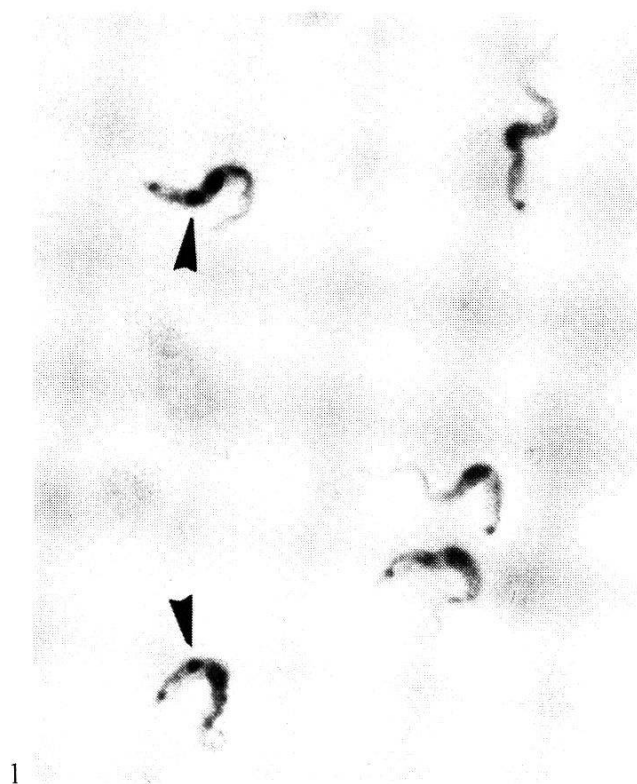
Figs. 1–4. Light micrographs. *Trypanosoma brucei brucei* STIB 247 metacyclic forms during transformation from metacyclic to bloodstream stage in vitro. Fig. 1, metacyclic forms at 0 h. Note the inclusion body (◄) located between nucleus and kinetoplast. Fig. 2, after 2 h incubation the cells start to elongate and the free flagellum becomes visible. Fig. 3, after 8 h incubation there is further elongation of the cell body and outgrowing of the flagellum; most cells seem to have completed transformation. Fig. 4, after 24 h the population consists of fully transformed long slender bloodstream forms. Giemsa stained smears, magnification 1280×.

Electron microscopy

Metacyclic forms freshly harvested from tsetse flies showed the well known ultrastructure of *Trypanosoma brucei brucei* (Fig. 5). However, some of the structures differed from those found in bloodstream forms or have not been described previously. In the area around the reservoir many vesicles could be found. Some contained material which resembled in thickness and density surface coat material (Fig. 6). Besides these vesicles also Golgi zones and multivesiculated bodies were present between nucleus and kinetoplast (Fig. 7). In the same region and often close to the nucleus a structure which has not yet been described was observed. This structure, we termed it "inclusion body", is of a similar size as the nucleus. About every other cell seemed to contain one of these bodies. They did not seem to be surrounded by a continuous membrane, exhibited a polymorphic shape, and contained heterogenous contents, e.g. light flocculant material (Fig. 8) or dense material (Fig. 9).

After 12 h in vitro incubation the number and type of vesicles in the reservoir vicinity seemed to be unchanged. On the other hand, the number of inclusion bodies was drastically reduced. Also the shape of these structures was altered. They had transformed into dense fragments (Fig. 10). This process continued, and after 24 h only very few and small inclusion bodies could be found.

Mean cell volume

The mean cell colume (Vc) of trypanosome populations during transformation is presented in Fig. 11. Although the values were taken from several experiments a clear tendency became evident. During the first 2 h of transformation the mean cell colume dropped by approximately 1 μm^3 from values between 15 and 16 μm^3. After 4 h the volume began to increase and by 6 h it reached the initial value of that of metacyclic forms. While transformation continued, the cell volume further increased until it finally reached the values of the bloodstream forms (Table 2). This may take several days, which is longer than the morphological changes observed in the light microscope, which seemed to be completed after 8 to 10 h.

Stereology

Morphometric parameters of *T. brucei brucei* STIB 247 transforming from metacyclic to bloodstream forms during the first 24 h in vitro are presented in Tables 1 and 2. Previous data calculated for a slender blood population from mouse and of a lymph node population from rat (Hecker and Brun, 1982) are included for comparison.

Mitochondrion. The volume density of the mitochondrion ($V_{Vmi,cy}$, Table 1) was significantly reduced during transformation. The absolute volume of the mitochondrion (Vmi,c) remained unchanged (Table 2) due to the increase of the absolute cytoplasmic volume (Vcy,c). A pronounced reduction of the surface
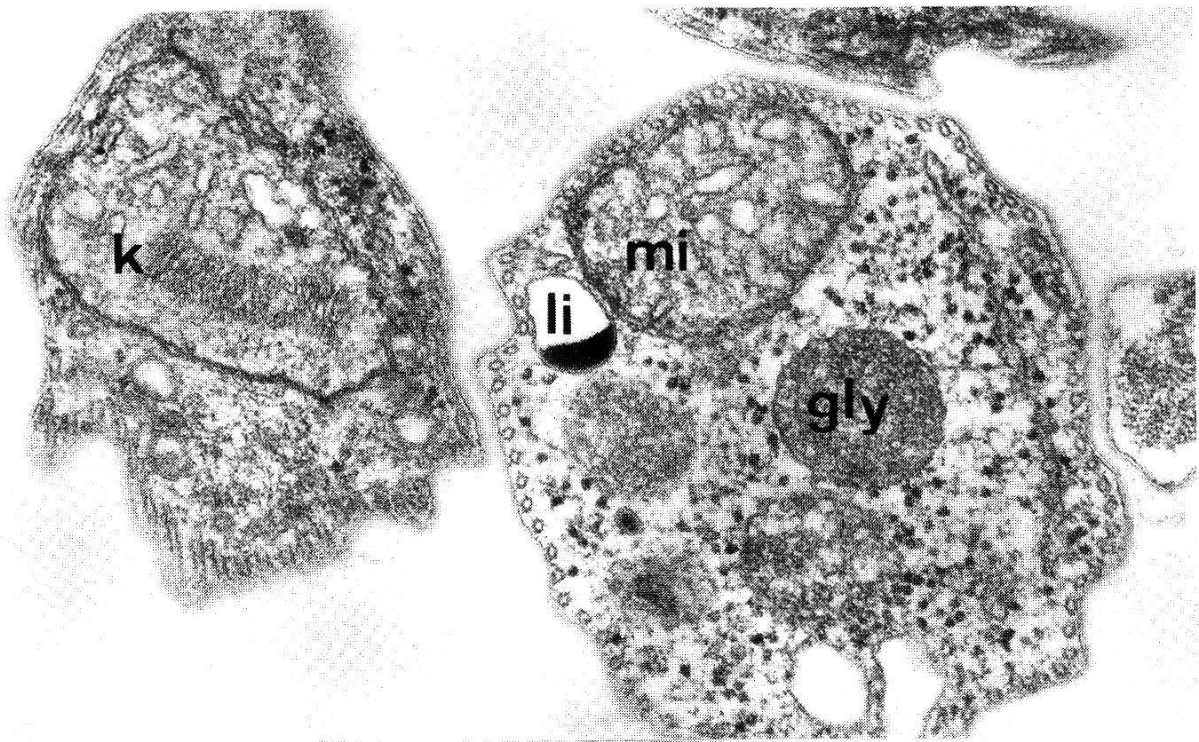
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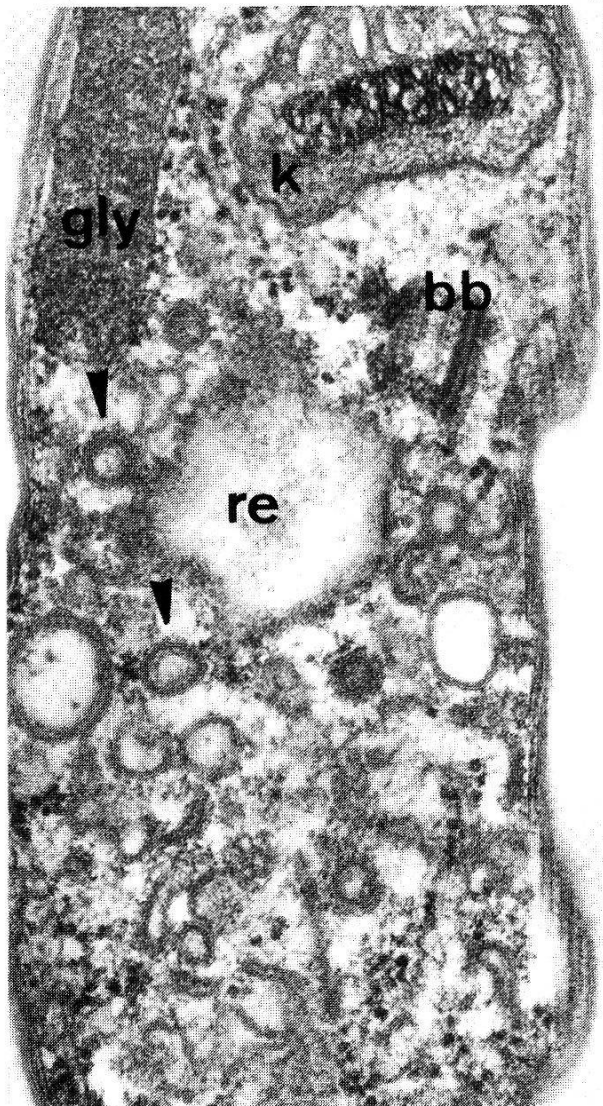
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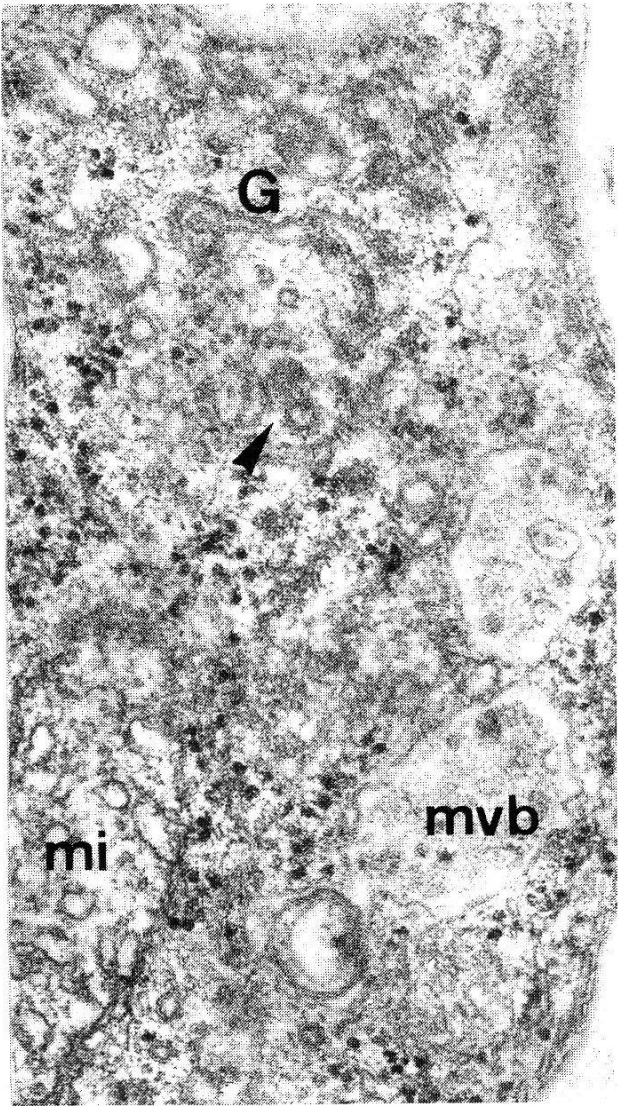density of the inner mitochondrial membrane ($S_{Vmii,cy}$) was measured. Also the absolute surface area of the inner membrane (Smii,c) tended to decrease. The reduction of the inner membrane area, however, was more distinct than the one of the mitochondrial volume. – The surface density of the mitochondrial outer membrane ($S_{Vmio,cy}$) remained constant during transformation. The ratio of the outer membrane surface to the mitochondrial volume, (S/V) mio, increased demonstrating a reduction of the mean diameter of the chondriome.

Glycosomes. The volume density of the glycosomes ($V_{Vgly,cy}$) remained unchanged and their absolute volume (Vgly,c) increased to the same extent as the cells enlarged. This finding indicates that the metacyclic forms are preadapted for transformation to a form requiring extramitochondrial respiration.

Lipid inclusions. Relative ($V_{Vli,cy}$) and absolute volume (Vli,c) of lipid inclusions increased significantly during transformation. The values obtained after 24 h were about 2-times the values previously measured for bloodstream forms from the mouse (Hecker and Brun, 1982).

"Inclusion bodies". Polymorphic structures with a heterogenous content were very conspicuous in metacyclic forms (Figs. 8, 9). Their volume density ($V_{Vin,cy}$) amounted to 9% of the cytoplasmic volume. During transformation a drastic reduction of these structures took place. After 24 h in vitro these structures could hardly be found any longer.

Flagellum. Despite an underestimation of flagellar parameters, an increase of the absolute volume of the flagellum (Vfl,c) was found indicating elongation and formation of a free flagellum during transformation.

Infectivity for mice

Metacyclic trypanosomes, transforming stages and transformed forms always proved to be infective for mice with a prepatent period of 3 days.

Discussion

The transformation of metacyclic to bloodstream forms as seen in vitro was described in detail for the first 24 h by Gorden and Willett (1958). Our findings confirm their excellent observations made with light microscopy. The ultrastructure of the metacyclic form has been investigated by Vickerman (1966),

Figs. 5–10. Electron micrographs. *Trypanosoma brucei brucei* STIB 247 metacyclic forms.

Fig. 5. Cross section showing the following organelles: mi = mitochondrion; k = kinetoplast; gly = glycosome; li = lipid inclusions. 48,500×.

Fig. 6. Longitudinal section through reservoir area. Note the coated vesicles (◄) located between nucleus and kinetoplast. re = reservoir; bb = basal body of the flagellum; k = kinetoplast; gly = glycosome. 54,000×.

Fig. 7. Longitudinal section between reservoir and nucleus containing Golgi apparaturs (G), coated vesicles (◄) and multivesiculated bodies (mvb). The mitochondrion (mi) contains many cristae. 54,000×.
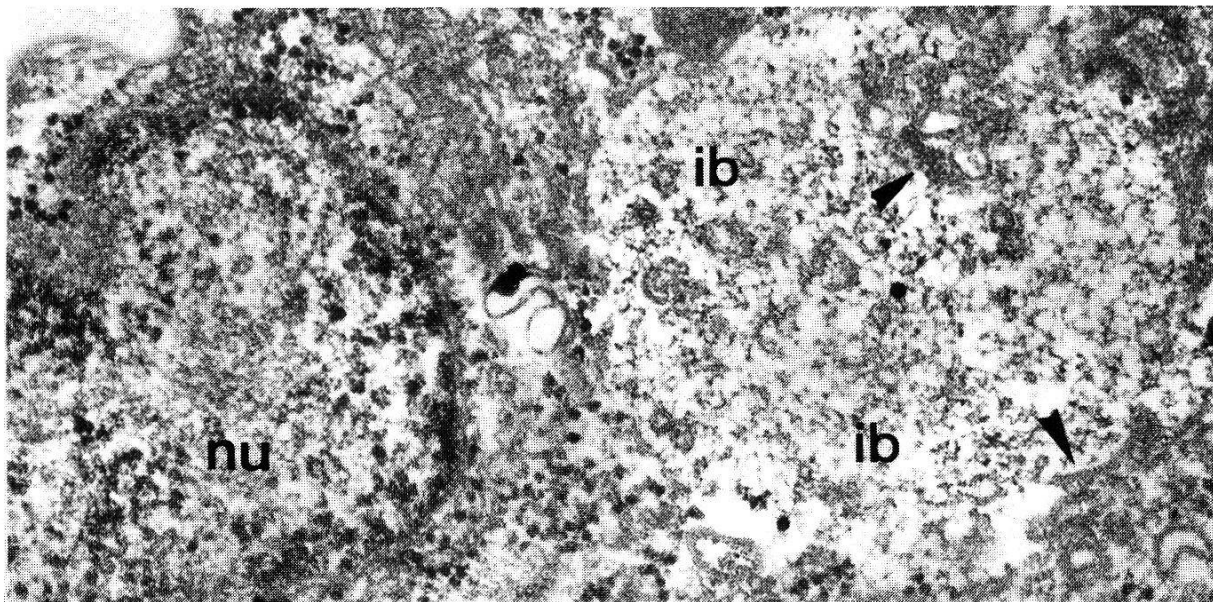
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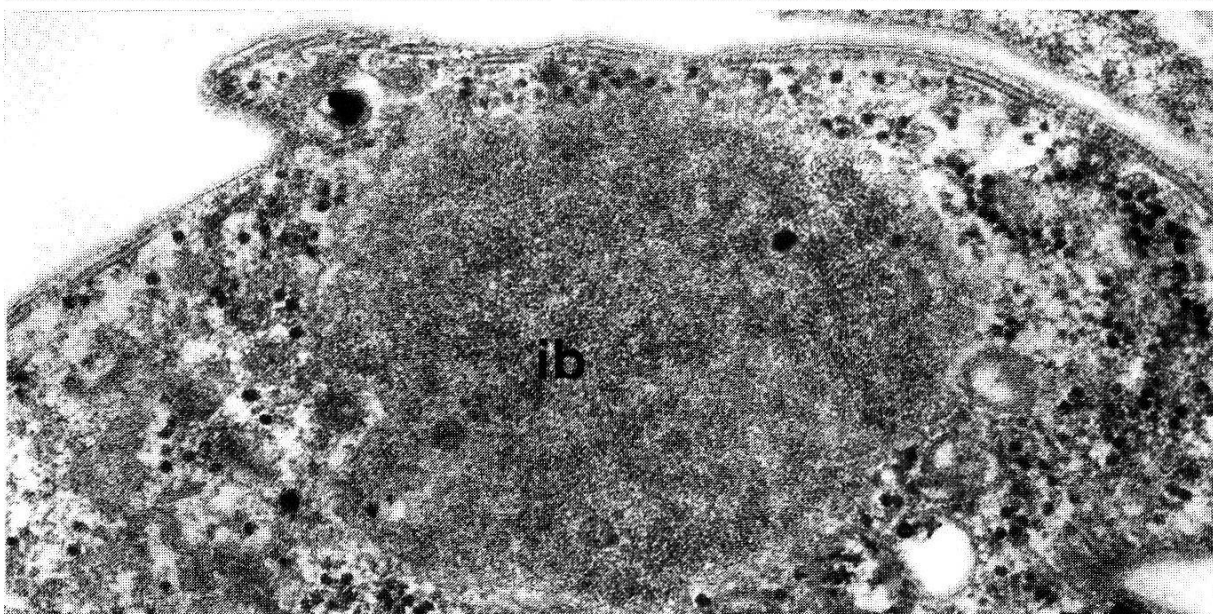
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Fig. 11. Mean cell volume of *Trypanosoma brucei brucei* STIB 247 metacyclic forms during transformation to bloodstream forms in vitro. (●) values obtained from one experiment. (○) values from several different experiments. The mean volumes were determined with a Coulter Channelyzer C-1000 as described in Materials and Methods.
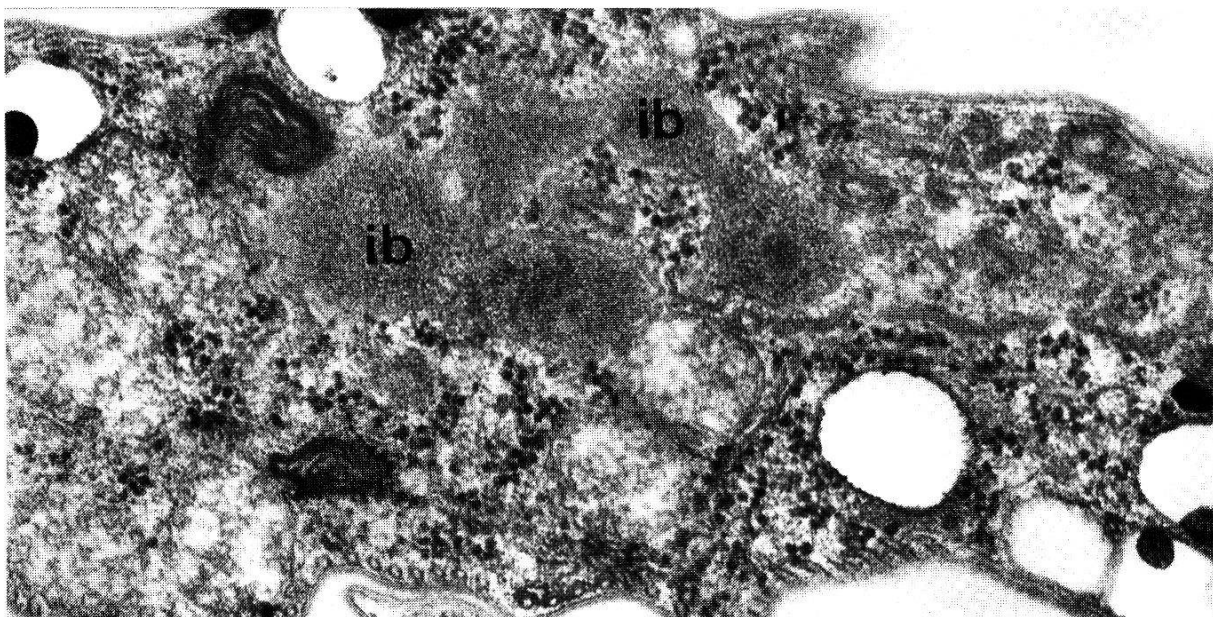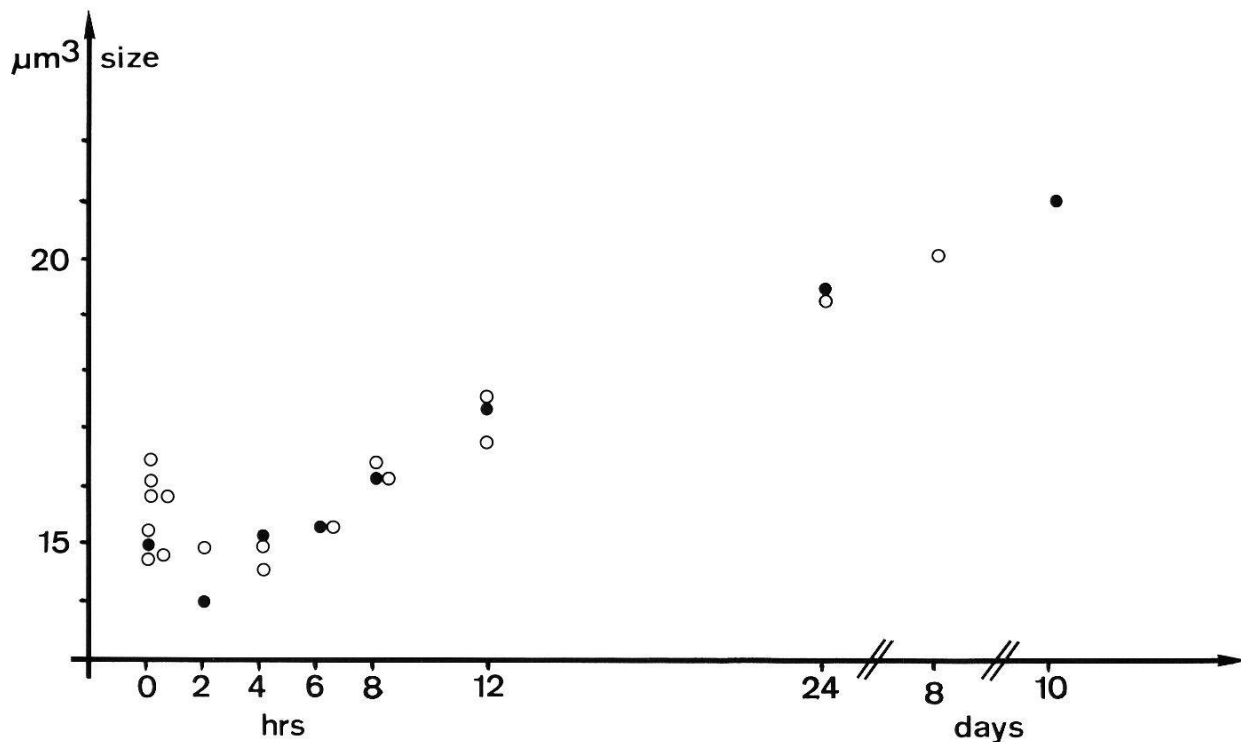
Steiger (1973) and by Böhringer and Hecker (1975). These authors as well as others also examined the ultrastructure of bloodstream forms on a qualitative or a quantitative basis.

In the present study we investigated the changes of ultrastructural composition of *T. brucei brucei* during the course of transformation from metacyclic to bloodstream forms in vitro. The ultrastructure of metacyclic forms is different from that of midgut forms and that of bloodstream stages (Böhringer and Hecker, 1975).

Structures typical for metacyclic and transforming forms were the vesicles found in the reservoir vicinity. These vesicles contained a coat-like intravesicular lining (Steiger, 1973). It can be speculated that they transport new coat material to the outside of the cell possibly via the reservoir. After 4 h of transformation there is an increase of the cell volume and of the cell surface due to elongation of the trypanosome body and the flagellum. Therefore, the cells have

Fig. 8. Inclusion body (ib) with light flocculant content located near nucleus (nu). Membranous structures (◀) are associated with and located within the inclusion body. 54,000×.

Fig. 9. Inclusion body (ib) containing dense amorphous material. 52,500×.

Fig. 10. Transformed cell after 12 h in vitro incubation. Inclusion body (ib) in a state of fragmentation and/or condensation. 52,500×.

125

Table 1. Morphometric parameters (means ± standard errors) of *T. brucei brucei* STIB 247 metacyclic forms during transformation to bloodstream forms in vitro

	$V_{Vmii.cy}$	$S_{Vmito.cy}$	$S_{Vmii.cy}$	$(S/V)mio$	$(S/V)mii$	$Smii/Smio$	$V_{Vgly.cy}$	$V_{Vli.cy}$	$V_{Vmi.cy}$	$V_{Vfl.cy}$
Metacyclic forms	0.091	1.056	2.171	11.9	24.1	2.06	0.046	0.015	0.089	0.144
	±0.008	0.083	0.190	0.5	0.5	0.08	0.004	0.001	0.012	0.010
12 h in culture ...	0.083	1.128	1.577	13.6	19.0	1.40	0.045	0.042	0.018	0.135
	±0.005	0.067	0.093	0.4	0.5	0.02	0.002	0.003	0.005	0.009
24 h in culture ...	0.063	0.939	1.302	15.5	21.1	1.38	0.047	0.053	0.012	0.135
	±0.007	0.080	0.122	0.9	0.7	0.03	0.004	0.006	0.003	0.009
Blood mouse*	0.077	1.048	1.464	14.0	19.4	1.40	0.051	0.028	–	0.145
	±0.006	0.062	0.090	0.6	0.7	0.03	0.004	0.002	–	0.006
Lymph rat* ...	0.046	0.839	0.898	18.9	20.0	1.07	0.067	0.010	–	0.166
	±0.004	0.041	0.052	1.0	0.8	0.03	0.005	0.001	–	0.008

cy = with respect to cytoplasmic volume. flagellum included. $V_{Vmii.cy}$ = *volume density* of mitochondrion ($\mu m^3/\mu m^3$). $V_{Vgly.cy}$ = of glycosomes. $V_{Vli.cy}$ = of lipid inclusions. $V_{Vmi.cy}$ = of "inclusion bodies". $V_{Vfl.cy}$ = of flagellum. $S_{Vmio.cy}$ = *surface density* of mitochondrial outer membrane ($\mu m^2/\mu m^3$). $S_{Vmii.cy}$ = of mitochondrial inner membrane. $(S/V)mio$ = *ratio* of surface area of outer membrane to mitochondrial volume ($\mu m^2/\mu m^3$), $(S/V)mii$ = of surface area of inner membrane to mitochondrial volume, $Smii/Smio$ = of surface area of inner to outer mitochondrial membrane ($\mu m^2/\mu m^2$)

* from Hecker and Brun (1982)

Table 2. Morphometric parameters (means ± standard errors) of *T. brucei brucei* STIB 247 metacyclic forms during transformation to blood-stream forms in vitro

	Vc	$V_{Vnu.c}$	Vnu.c	Vcy.c	Vmi.c	Smio.c	Smii.c	Vgly.c	Vli.c	Vin.c	Vfl.c
Metacyclic forms .... ±	14.92 0.04	0.140 0.019	2.1 0.3	12.8 0.3	1.17 0.11	13.6 1.1	27.9 2.5	0.59 0.05	0.19 0.01	1.14 0.16	1.8 0.1
12 h in culture .... ±	16.85 0.03	0.152 0.012	2.6 0.2	14.3 0.2	1.19 0.07	16.1 1.0	22.5 1.4	0.64 0.03	0.60 0.04	0.26 0.07	1.9 0.1
24 h in culture .... ±	19.35 0.04	0.130 0.010	2.5 0.2	16.8 0.2	1.06 0.12	15.8 1.4	21.9 2.1	0.79 0.07	0.89 0.10	0.20 0.05	2.3 0.2
Blood mouse* ... ±	20.40 0.02	0.111 0.010	2.3 0.2	18.1 0.2	1.43 0.11	19.4 1.2	27.1 1.7	0.94 0.08	0.52 0.04	– –	2.7 0.1
Lymph rat* .... ±	19.60 0.02	0.145 0.014	2.8 0.3	16.8 0.3	0.77 0.07	14.1 0.7	15.1 0.9	1.13 0.09	0.17 0.02	– –	2.8 0.1

c = with respect to cell volume, flagellum included. Vc = *absolute volume* of cell (μm^3), Vnu.c = of nucleus. Vcy.c = of cytoplasm. Vmi.c = of mitochondrion, Vgly.c = of glycosomes. Vli.c = of lipid inclusions, Vin.c = of "inclusion bodies", Vfl.c = of flagellum. $V_{Vnu.c}$ = *volume density* of nucleus ($\mu m^3/\mu m^3$). Smio.c = *absolute surface area* of mitochondrial outer membrane (μm^2), Smii.c = of mitochondrial inner membrane

* from Hecker and Brun (1982)

to synthesize more surface glycoproteins and transport them to the surface by exocytosis. These vesicles did not disappear during the course of our experiment (24 h).

The metacyclic forms also contained an unknown structure which we termed "inclusion body". This large, polymorphic body contained flocculant, sometimes very heterogenous material with dense inclusions. As far as we are aware, this structure has not been described before. During transformation the inclusion bodies disappeared. The mode of degradation is unclear, however, they seem to condense to structures resembling lysosomes. The origin and function of these structures are unknown. They cannot be an artefact caused by the in vitro incubation since they were already present in the metacyclic population extruded by the flies. Metacyclics of the same stock produced at the Swiss Tropical Institute in Basel did not contain "inclusion bodies" (unpublished observation). These flies were fed on reconstituted lyophylised pig's blood by membrane feeding while in the present study tsetse were fed on goats infected with *T. brucei brucei* and those with mature infections were maintained on rabbits. It would be of interest to further study the effects on trypanosomes in terms of these inclusion bodies when infected tsetse are maintained on different diets using different hosts.

Based on the values of the morphometric parameters, metacyclic forms have to be placed between midgut forms (Böhringer and Hecker, 1975) or procyclic trypomastigotes from culture (Ghiotto et al., 1979) and bloodstream forms (Böhringer and Hecker, 1974; Ghiotto et al., 1979). After 24 h in vitro the transformed metacyclics approached a fine structure similar to that of bloodstream forms found in the mammalian host.

During transformation from the metacyclic to the bloodstream form the mitochondrion was reduced to a simple tube containing only few cristae. The morphometrically determined distinct decrease of all mitochondrial parameters, volume as well as surface area parameters, was the most striking quantitative change observed in the present study. These changes are to be considered as the morphological expression of the switch from mitochondrial to extramitochondrial respiration, the latter taking place in the glycosomes of bloodstream forms (Opperdoes and Borst, 1977).

128

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