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STUDIES ON THE ULTRA-STRUCTURE OF KINETOPLASTS OF *TRYPANOSOMA CRUZI* AND *TRYPANOSOMA GAMBIENSE* BY AUTORADIOGRAPHY AND ENZYMATIC DIGESTION[1]

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SUMMARY The ultra-structure of the kinetoplasts of *Trypanosoma cruzi* and *Trypanosoma gambiense* was studied by electron microscopic autoradiography combined with enzymatic digestion with DNase, RNase or proteases. It was revealed that the kinetonucleus consists of DNA, RNA and protein.

Treatment of these trypanosomes with *p*-Rosaniline in vitro, resulted in fragmentation of the kinetonucleus and disappearance of the kinetoplastic envelope membrane with formation of a non-viable akinetoplastic form. The fragments of kinetonucleus contained RNA and protein but not DNA. These facts suggest that the envelope membrane is essential for viability and possibly also for synthesis of kinetoplastic DNA.

INTRODUCTION

Hemoflagellates of the family Trypanosomatidae all have a self-duplicating body, called the kinetoplast, which is a part of the large, single mitochondrion (Steinert, 1960). It is composed of a double layered envelope membrane containing the kinetonucleus and is located at the base of the flagellum.

The presence of DNA in the kinetonucleus has been demonstrated in the following ways. Electron microscopic autoradiography (EM-autoradiography) revealed the incorporation of radioactive thymidine into the kinetonucleus and nucleus (Burton and Dusanie, 1968; Anderson and Hill, 1969; Hill and Anderson, 1969). Anderson and Hill (1969) observed that the fibers in the kinetonucleus disappeared completely when *Crithidia fasciculata* was incubated with DNase for 4 hours. Recently, Laurent and Steinert (1970) and Ozeki et al. (1970) showed by electron microscopy that kinetoplastic DNA was released from isolated

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kinetoplasts of *Trypanosoma mega* or *Trypanosoma gambiense* on rupture by osmotic shock. The DNA of kinetoplasts may be distinguishable from nuclear DNA as satellite DNA of higher AT-content (DuBuy et al., 1965; Riou and Paoletti, 1967; Steinert and Van Assel, 1967; Simpson, 1968; Riou and Pautrizel, 1969).

However, in some species of *Trypanosoma*, cells which have lost the kinetoplast, so called dyskinetoplastic or akinetoplastic forms (AK forms), have been observed. Loss of the kinetonucleus may occur spontaneously or on treatment with acriflavine or other drugs and may be due to loss of kinetoplastic DNA (Steinert and Van Assel, 1967; Simpson, 1968). Previously, we found that fragmentation of the kinetonucleus occured on treatment of *Trypanosoma gambiense* or *Trypanosoma evansi* with *p*-Rosaniline in vivo (Inoki et al., 1969). The fragmented material was electron dense and gave a negative Feulgen reaction.

In contrast with these extensive investigations on the DNA of the kinetoplast, there has been very little work on RNA (Steinert et al., 1969) or proteins in the kinetoplast and particularly no morphological studies by electron microscope.

The present paper describes the ultra-structure of the kinetonucleus in *Trypanosoma cruzi* and *Trypanosoma gambiense* and the components of fragmented material studied by electron microscopic autoradiography and the enzymatic digestion.

MATERIALS AND METHODS

1. *Cells*

Trypanosoma gambiense (Wellcome strain) was obtained from Eli Lilly Research Laboratories, Indianapolis, Indiana, U.S.A., and *Trypanosoma cruzi* from N.I.H., Bethesda, Maryland, U.S.A.. These parasites were grown in mice as the trypanosome form. The cultured form (trypanosome form) of *T. gambiense* (Cheick strain) was given by Prof. D. Weinman, Department of Microbiology, Yale University, Connecticut, U.S.A. and cultured in Weinman's medium (1946). The cultured form (crithidia form) of the strain of *T. cruzi* mentioned above was cultured in Boné and Parent's liquid medium (1963).

2. *Materials*

^{3}H–thymidine–6–T (Spec. act. 5.0 c/mM) and ^{3}H–uridine–5–T (Spec. act. 6.0 c/mM) were purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). Sakura Autoradiography Emulsion (type NR–H_2) was obtained from Konishiroku Photo. Ind. Co. Ltd. (Tokyo, Japan). DNase (DPFF), RNase (RASE) and pepsin (PM) were obtained from Worthington Biochemical Corporation (New Jersey), DNase (DN–C) from Sigma Chemical Co. (Missouri) and pronase-p from Kaken Chemical Co. Ltd. (Tokyo, Japan). *p*-Rosaniline was purchased from Chroma Gesellschaft Schmid & Co. (Stuttgart-Unterturkheim).

3. *Enzyme treatment*

Trypanosome forms (blood stream form) of *T. gambiense* and *T. cruzi* were collected in the log-phase of growth from the blood of mice. They were fixed in 2.5% glutaraldehyde (pH 7.4) for 1 hr and washed several times with isotonic buffer solution (0.25 M sucrose in phosphate buffer, pH 7.4) and left overnight in this solution in a refrigerator at 4 C. Then they were washed with isotonic buffer solution containing 0.003 M $MgSO_4$ at pH 6.4 and treated at 37 C with the following solutions (for 5 min with pepsin and for 1 to 3 hr with other solutions).

1) For a control of DNase or RNase treatment: isotonic phosphate buffer, pH 6.4 (the optimal pH for DNase or RNase activity) containing 0.003 M Mg SO_4.

2) For DNase treatment: concentrations of 0.1% to 0.5% DNase in solution (1).

3) For RNase treatment: concentrations of 0.01% to 1% RNase in solution (1). Before use some of these solutions were heated at 80 C for 10 min to inactivate possible contaminating DNase and then acidified to pH 3 to inactivate possible contaminating protease and then adjusted to pH 6.4.

4) For pronase treatment: 10% pronase-p in isotonic solution. Before use it was heated at 80 C for 10 min to inactivate possible contaminating DNase.

5) For a control of pepsin treatment: isotonic solution containing 0.02 N HCl (the optimal strength for pepsin activity).

6) For pepsin treatment: 0.25% pepsin in solu-

tion (5).

For *p*-Rosaniline (*p*-Ros.) treatment followed by enzyme treatment, cultures of *T. gambiense* (Cheick strain) in the log-phase of growth (2×10^6 cells/ml) were treated with 5 μg/ml of *p*-Ros.. After 4 days, when the number had increased to 9×10^6 cells/ml, cells were fixed with 2.5% glutaraldehyde and divided into four parts which were used for electron microscopy, and DNase, RNase and pronase treatments as described in 2), 3) and 4), respectively.

4. *Electron microscopic autoradiography (EM-autoradiography)*

Cultures of *T. cruzi* in the log-phase of growth (1×10^7 cells/ml) were mixed with 20 μc/ml of ^{3}H-thymidine or ^{3}H-uridine. After 3, 6, 24 (1.5×10^7 cells/ml) and 48 hr (20×10^7 cells/ml), the organisms were harvested and fixed for electron microscopic observation.

Cultures of *T. cruzi* which had been incubated with ^{3}H-thymidine or ^{3}H-uridine for 48 hr were fixed with 2.5% glutaraldehyde with or without previous incubation with 2.5%μg/ml of *p*-Ros. for 4 days. Then the cells were treated with DNase or RNase or pronase solution as described in (2), (3) and (4) above.

After enzyme treatment all these specimens were double fixed with 1.5% osmium tetraoxide in isotonic buffer and mounted in Epon 812 in the usual way for electron microscopic observation.

For EM-autoradiography, ultra-thin sections of specimens on grids were mounted with Sakura Emulsion. After about 3 weeks exposure they were developed by "Sakura" Konidol-X and fixed with Kodak Fixer. The gelatin layer was removed by washing with 0.02 N NaOH and preparations were stained with uranylacetate and lead citrate. Micrographs were made using a Hitachi 11 B electron microscope.

RESULTS

1. *Presence and localization of nucleic acid and protein in kinetoplasts of crithidia form of T. cruzi*

On incorporation of ^{3}H-thymidine into the crithidia form of *T. cruzi*, silver grains of ^{3}H-thymidine were mainly seen in the nucleus and kinetonucleus, especially at their peripheral membranes (Fig. 1 to 4). During synthesis of the kinetonucleus many grains were seen (Fig. 3). Grains were also seen in the blepharoplast (Fig. 4). Fewer grains were seen after incubation for only 3 or 6 hr than after incubation for 48 hr.

On EM-autoradiography followed by DNase treatment of *T. cruzi* which had been labelled with ^{3}H-thymidine for 48 hr various extents of digestion of the kinetonucleus and nucleus were observed. On partial digestion, the fibrillar structure of the kinetonucleus and nucleus were lost and electron-dense material with no grains was seen (Fig. 5). Treatment with DNase at high concentration or for a long time resulted in complete loss of the fibrillar structure of both the kinetonucleus and nucleus with no appearance of grains (Fig. 6).

On digestion of *T. cruzi* with RNase after ^{3}H-thymidine labelling, there was extensive digestion of the cytoplasm but not the kinetonucleus or nucleus where many grains remained (Fig. 7). When unlabelled *T. cruzi* was treated with RNase similar digestion was observed (Fig. 8). In contrast treatment of *T. gambiense* (Wellcome Strain) with RNase resulted in extensive digestion of the kinetonucleus (cf. Fig. 28, 29 and 30). Use of an RNase preparation which had been heated and acidified to a low pH as described in the Materials and Methods, gave similar results. Thus the RNase preparation (RASE-Worthington) was not contaminated with DNase or protease.

On EM-autoradiography of *T. cruzi* after labelling with ^{3}H-uridine, silver grains were observed in the cytoplasm, nucleolus, nucleus and kinetonucleus (Fig. 9, 10 and 11). There was no consistent difference in the distributions of grains in the cytoplasm of cells harvested after 3, 6, 24 and 48 hr. However, fewer grains were seen in the nucleus and kinetonucleus after 3 and 6 hr, than after 24 and 48 hr.

On digestion of *T. cruzi* labelled with ^{3}H-uridine with DNase, the fibrillar structures in the kinetonucleus and nucleus were completely lost while grains of ^{3}H-uridine still remained in the kinetonucleus, nucleus and cytoplasm (Fig. 11). Thus the grains seem to be entirely due

to the presence of RNA. However, grains were still observed in the kinetonucleus and nucleus after their complete loss from the cytoplasm by digestion with RNase. Thus if the kinetonucleus contains RNA, it must be in a form which is resistant to RNase.

2. *Effects of p-Rosaniline on T. cruzi and T. gambiense in vitro*

It has been shown that *p*-Ros. effects the kinetoplast of both *T. gambiense* (Cheick Strain) and *T. cruzi* in vitro. When *p*-Ros. (2.5 μg/ml to 5 μg/ml) was added to cultures in the log-phase of growth, the cell number continued to increase and the akinetoplastic form (AK form) also increased in number. Comparison with control cells by electron microscopy (Fig. 12) showed that fragmentation of the kinetonucleus and disappearance of the kinetoplast envelope membrane occurred under these conditions (Fig. 13 to 19). The fragmented material had no fibrillar structure but was electron-dense. The AK form induced could only duplicate for a few generation, so no AK strain developed. These observations are the same as those obtained previously on the effect of *p*-Ros. on the kinetoplast of *T. gambiense* (Wellcome strain) but not of *T. evansi*. In the latter, fragmentation of the kinetonucleus occurred but the kinetoplast envelope membranes remained intact and cells could survive, multiply and form an AK strain (Inoki et al., 1969).

On *p*-Ros. treatment in vitro followed by DNase treatment, the fibrillar structure of the kinetonucleus and nucleus were lost but the electron density in the fragmented material did not change (Fig. 15). However, on treatment with RNase, the electron density of the fragmented material decreased (Fig. 16). On pronase treatment also, the kinetonucleus, nucleus and fragmented material decreased in electron density as shown in Fig. 17.

On EM-autoradiography of ^{3}H-thymidine labelled *T. cruzi* and then *p*-Ros. treatment, no grains were seen in fragmented material while many were found in the kinetonucleus and nucleus (Fig. 18 and 19). In contrast, on similar treatment of cells labelled with ^{3}H-uridine grains were seen in fragmented material as well as in the normal kinetonucleus, nucleus, nucleolus and microsomes (Fig. 20 and 21).

3. *Kinetoplasts of trypanosome forms of T. gambiense and T. cruzi*

Fig. 22 showed the blood stream form (trypanosome form) of *T. cruzi* as control of RNase treatment. In this form, the kinetonucleus appeared to have an uncoiled fibrillar structure (Inoki et al., 1971). On RNase treatment, these fibrillar structures in the kinetonucleus and nucleus were not affected but the cytoplasm was digested extensively (Fig. 23). This was similar to the case with the cultured form (crithidia form) of *T. cruzi* (see Fig. 7 and 8) but different from that with the trypanosome form of the Wellcome strain of *T. gambiense* (cf. Fig. 28, 29 and 30).

In the trypanosome form of *T. gambiense* (Wellcome strain), the control for DNase or RNase treatment appeared similar to untreated material (Fig. 24 and 25). On DNase treatment, the kinetonucleus and nucleus were extensively digested (Fig. 26 and 27). After digestion the kinetonucleus showed a matrix-like, rather than fibrillar structure. On RNase treatment, the kinetonucleus and cytoplasm were digested extensively (Fig. 28, 29 and 30) and the nucleus very little. Digestion of kinetonucleus was much greater than in *T. cruzi* (cf. Fig. 7, 8 and 23). RNase treatment of preparations of *T. cruzi* and *T. gambiense* was repeated more than 10 times with similar results. Fig. 29 and 30 show the effects of partial digestion of *T. gambiense* (Wellcome strain) with RNase, indicating that the electron density of the kinetonucleus gradually became less.

On pronase treatment, the electron densities of both the nucleus and kinetonucleus decreased (Fig. 31). Even in the control for pepsin (incubated in 0.02 N HCl isotonic solution) the whole cell was deformed (Fig. 32 and 33), the fibrillar structure of the kinetonucleus was lost and the outline of the cell became irregular. Pepsin treatment resulted in exten-

sive digestion of the whole cell, especially the microtubules (Fig. 34).

DISCUSSION

In this work, we confirmed the incorporation of ^{3}H-thymidine into the kinetonucleus by EM-autoradiography (Fig. 1 to 4). On digestion of ^{3}H-thymidine labelled cells with DNase the fibrillar structure of the kinetonucleus was lost (Fig. 5 and 6). This confirms that the fibrils of the kinetonucleus are composed of DNA.

We also observed grains of ^{3}H-thymidine in the blepharoplasts (Fig. 4), showing that blepharoplasts in these organisms contain DNA.

In 1969 Kallinikova reported that the kinetoplast contains RNA from cytochemical studies by light microscope while Steinert et al. (1969) observed the incorporation of ^{3}H-uridine into the kinetoplast by EM-autoradiography. Our experiments confirm the incorporation of ^{3}H-uridine into the kinetonucleus as well as into the nucleus and cytoplasm (Fig. 9 and 10). However, on digestion of ^{3}H-uridine labelled cells of *T. cruzi* with RNase, no morphological deformation of the kinetonucleus or nucleus was observed although the cytoplasm was extensively digested (Fig. 7 and 8). Thus, as mentioned above, both the kinetonucleus and nucleus seem to contain RNA which is resistant to RNase, like chromosomal RNA or nuclear RNA in higher organisms (Bonner et al., 1965 and 1967; Benjamin et al., 1966; Shearer et al., 1967; John et all, 1969).

In contrast, similar treatment of the Wellcome strain of *T. gambiense* with RNase caused severe deformation of the kinetonucleus (Fig. 27, 28 and 29), while with the Cheick strain of *T. gambiense* moderate deformation was observe ed. In the Wellcome strain of *T. gambiense*, it appeares that RNA is somehow involved in maintaining the structure of the kinetonucleus. No further experiments were made on this, since unfortunately under our culture conditions this strain could not be labelled.

Both pronase and pepsin caused morphological alteration of the kinetonucleus (Fig. 17, 31 and 34) indicating that in latter also contains protein. However, according to Steinert (1965), the kinetonucleus does not contain any histone. As shown in Fig. 17, after pronase treatment less electron-dense lines or tiny dots were seen along the fibril threads after pronase treatment, suggesting that DNA and protein together formed a chromonema-like structure. Thus, electron microscopic studies on labelled material and material after enzyme treatment showed that the kinetonucleus of Trypanosomatidae contains not only DNA but also RNA and proteins.

These techniques were also applied to study the fragmented kinetonucleus induced by *p*-Ros.. In *T. cruzi* and *T. gambiense* in vitro, the fragmented material was labelled with ^{3}H-uridine but not ^{3}H-thymidine and was digested by pronase. This indicates that this fragmented material contains RNA and protein but not DNA. *p*-Ros. treatment also resulted in disappearance of the envelope membrane in these organisms in vitro, so that they could not survive or maintain an AK strain. In contrast on treatment of *T. evansi* with *p*-Ros. in vivo, the envelope membrane was not affected and an AK strain was formed. Electron-dense material, like the fragmented kinetonucleus induced by *p*-Ros., was seen inside the envelope membrane in this AK strain (Inoki et al, 1969) and AK strain cells had kinetoplastic DNA (Ono et al. in press). These facts reconfirm that the kinetoplastic envelope membrane of these flagellates has some essential function related to viability, and this may be why *T. gambiense* (Wellcome strain and Cheick strain) and *T. cruzi* cannot survive as the AK form.

p-Ros. seems to inhibit synthesis of kinetoplastic DNA when the envelope membrane has disappeared so there may be some ralationship between the synthesis of kinetoplastic DNA and the existence of the kinetoplastic envelope membrane.

ACKNOWLEDGEMENT

We are grateful to Assistant Professor S. Okubo of

this department for providing materials and for his helpful discussion throughout this study. We also wish to thank Miss Y. Tanaka for assistance in preparing this paper.

Cultured T. cruzi (crithidia form) incubated with ^{3}H-thymidine (20μc/ml) for 48 hr.

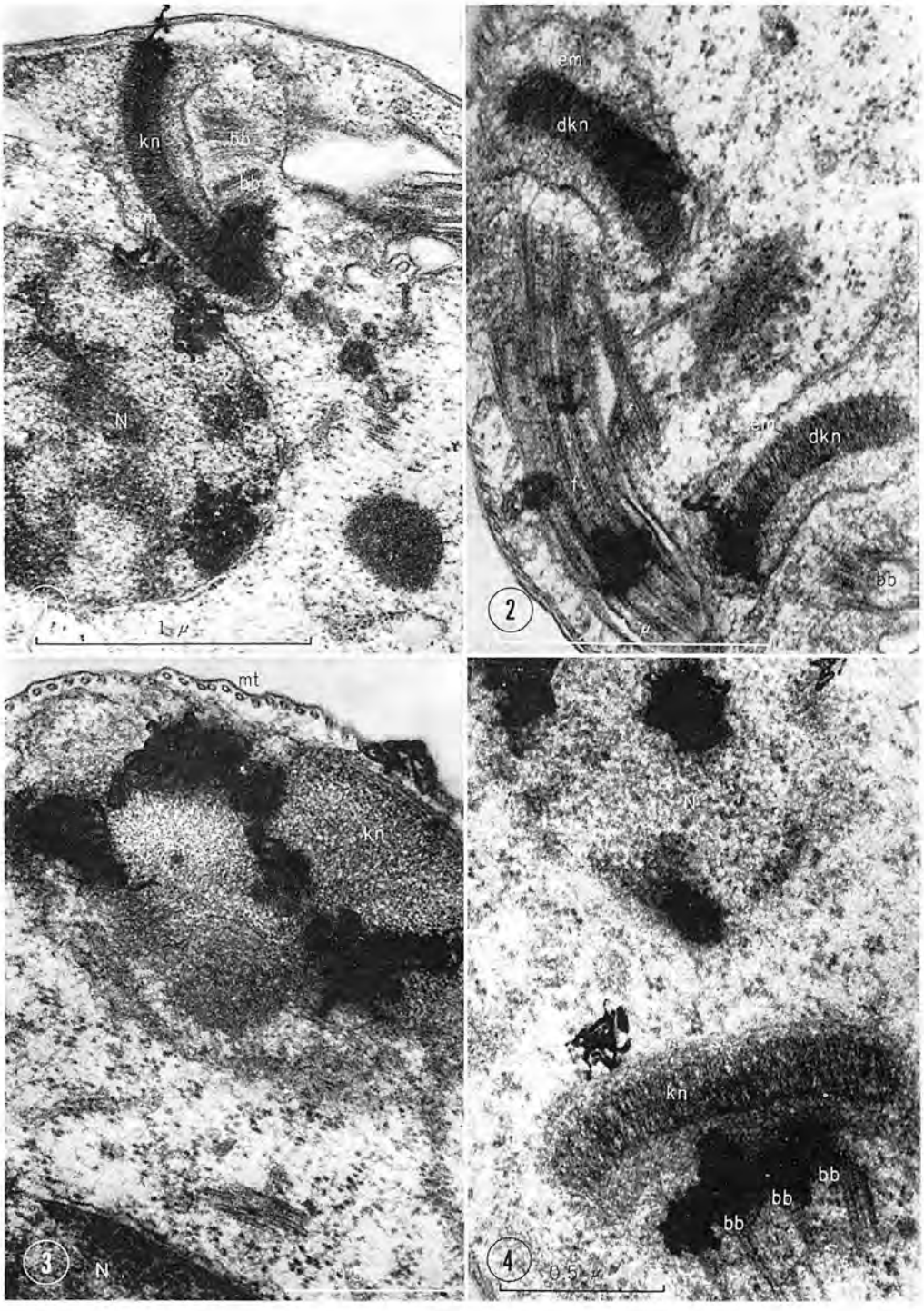
FIGURE 1. *Resting stage of kinetonucleus and nucleus. Silver grains are observed in the kinetonucleus and at the periphery of the nucleus.*

FIGURE 2. *Daughter kinetonuclei in the process of division. A silver grain is observed in each.*

FIGURE 3. *Stage of kinetonucleus synthesis. Silver grains seem to be arranged in a line.*

FIGURE 4. *Resting stage. ^{3}H-thymidine is incorporated into basal bodies (blepharoplasts), and at the periphery of the kinetonucleus and in the nucleus.*

bb, basal body; em, envelope membrane; f, flagellum; kn, kinetonucleus; dkn, daughter kinetonucleus; mt, microtubules; n, nucleus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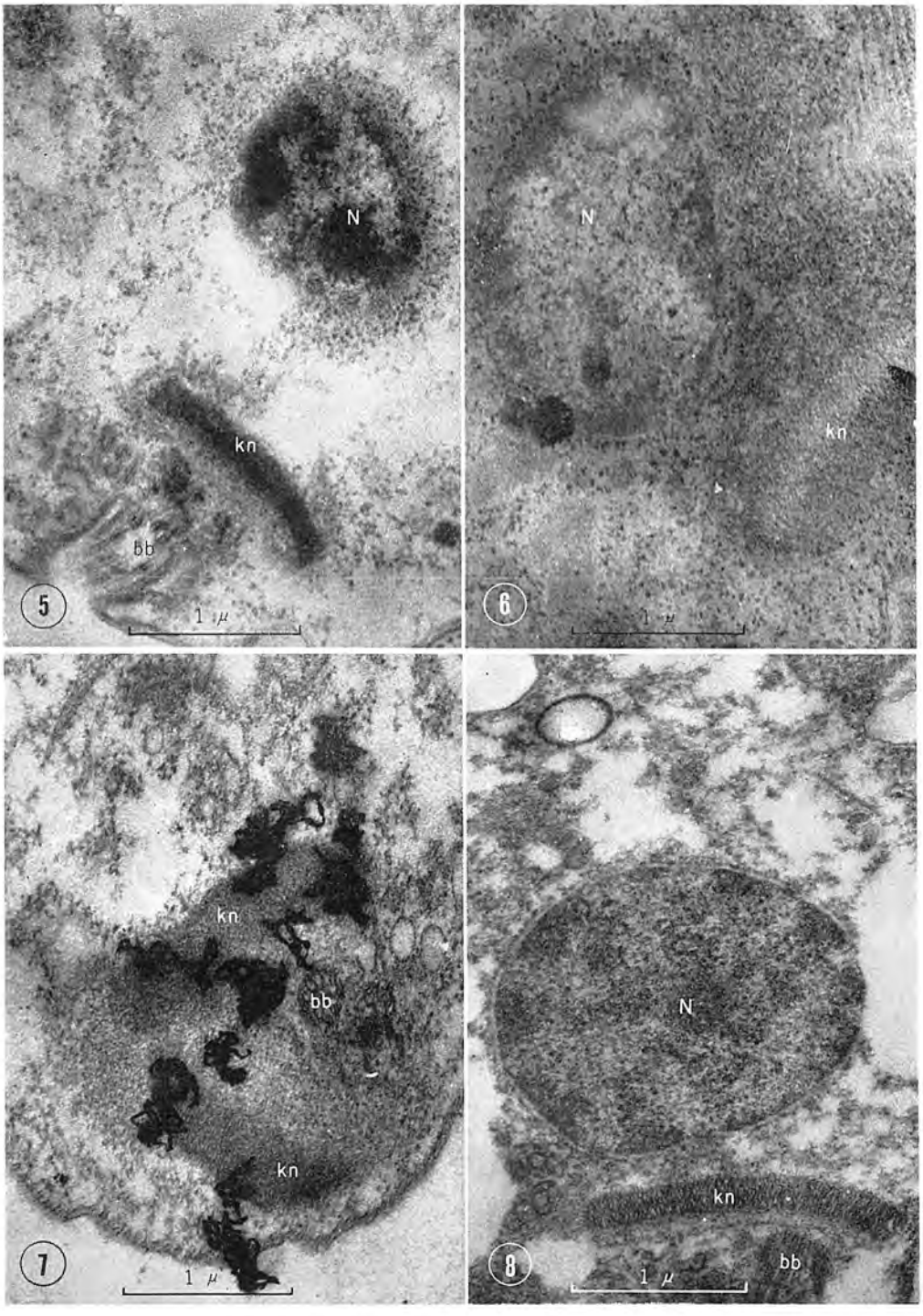
Cultured T. cruzi (crithidia form)

FIGURE 5 and 6. *Cells were incubated with ^{3}H-thymidine (20μc/ml) for 48 hr and then fixed and treated with DNase (Worthington DPFF). Different extents of digestion are seen. No grains or fibrillar structure is visible.*

FIGURE 7. *Cells were incubated with ^{3}H-thymidine(20μc/ml) for 48 hr and then fixed and treated with RNase. The cytoplasm is extensively digested. The kinetonucleus is not affected and is in the process of division. Many grains are seen.*

FIGURE 8. *RNase treatment. The cytoplasm is digested extensively, while the kinetonucleus and nucleus are not affected.*

bb, basal body; kn, kinetonucleus; n, nucleus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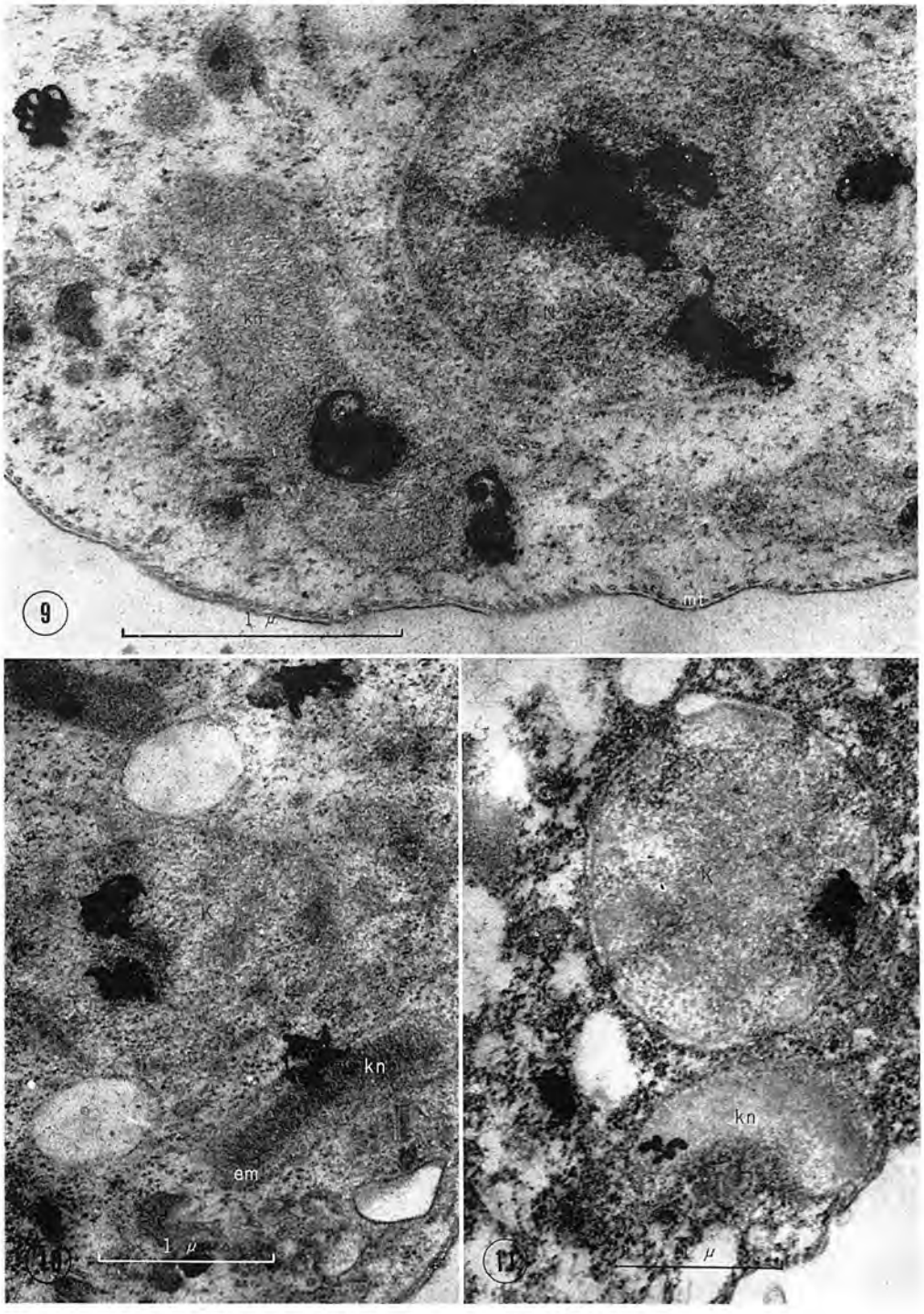
Cultured T. cruzi (crithidia form) incubated with ^{3}Huridine (20μc/ml) for 48 hr
FIGURE 9 and 10. *Grains are seen in the nucleolus, nucleus, kinetonucleus and cytoplasm.*

FIGURE 11. *Cell treated with DNase after incorporation of ^{3}H-uridine. The kinetonucleus and nucleus have been almost completely digested, grains are still seen in the kinetonucleus, nucleus and cytoplasm.*
em, envelope membrane; kn, kinetonucleus; n, nucleus; mt, microtubules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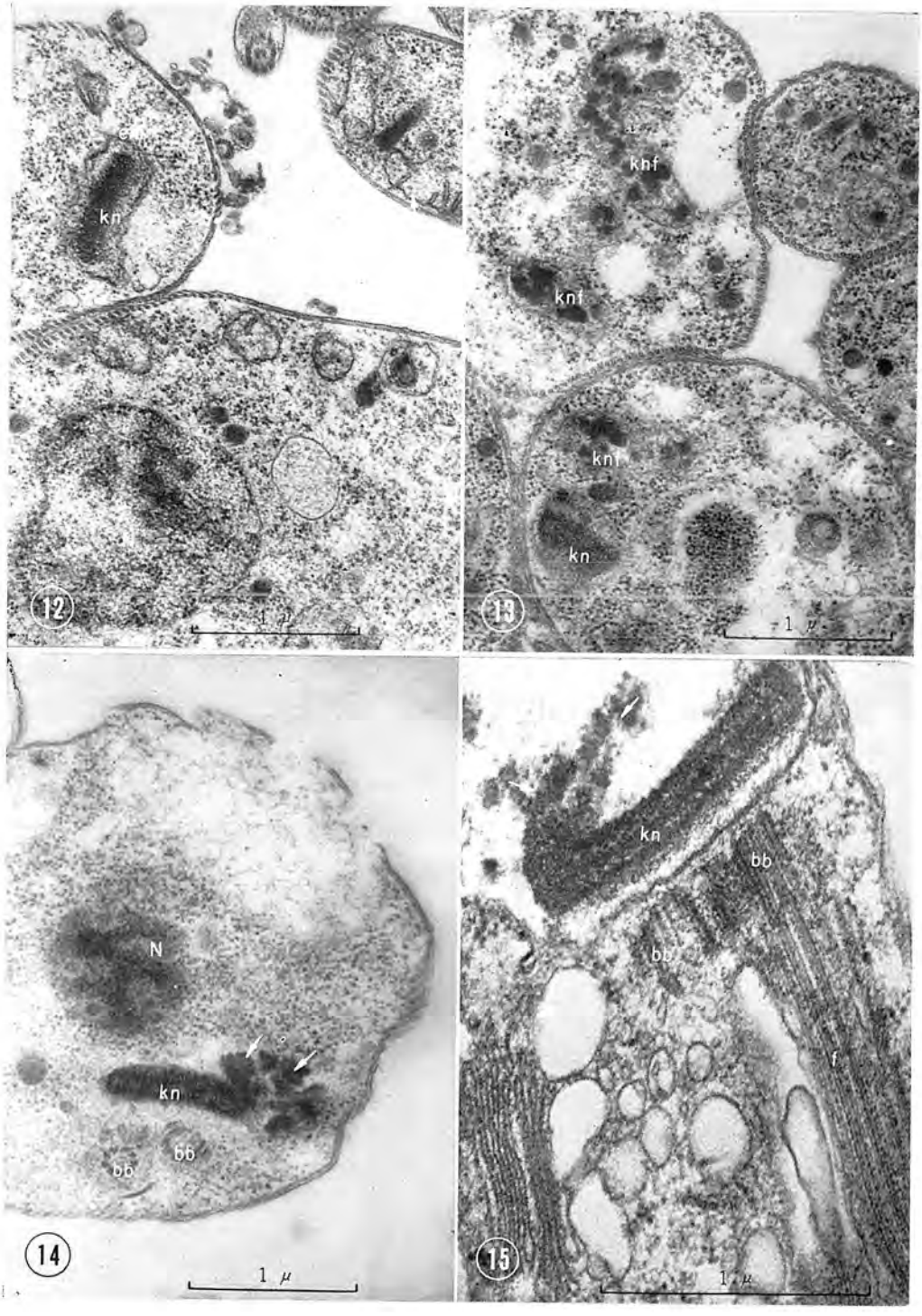
p-Rosaniline treatment

FIGURE 12. *Cheick strain of T. gambiense (trypanosome form) as a control for p-Ros. treatment. The fibrillar structure of the kinetonucleus is clearly seen and the arrow shows mitochondrial cristae which appear in the cultured form.*

FIGURE 13. *Cheick strain of T. gambiense (trypanosome form) treated with 5 μg/ml of p-Ros. for 4 days. Fragmentation of the kinetonucleus and disappearance of the envelope membrane are seen. The fragmented material shows no fibrillar structure.*

FIGURE 14 and 15. *Cultured T. cruzi (crithidia form) treated with 2.5 μg/ml of p-Ros. for 4 days. Fragmentation and disappearance of the envelope membrane are apparent. The specimens were then treated with DNase (Sigma DN-C) as shown in Fig. 15 resulting in digestion of the fibrillar structure of the kinetonucleus. However, the electron density of fragmented material (arrow) did not decrease.*
bb, basal body; em, envelope membrane; f, flagellum;
kn, kinetonucleus; knf, kinetonuclear fragment;
n, nucleus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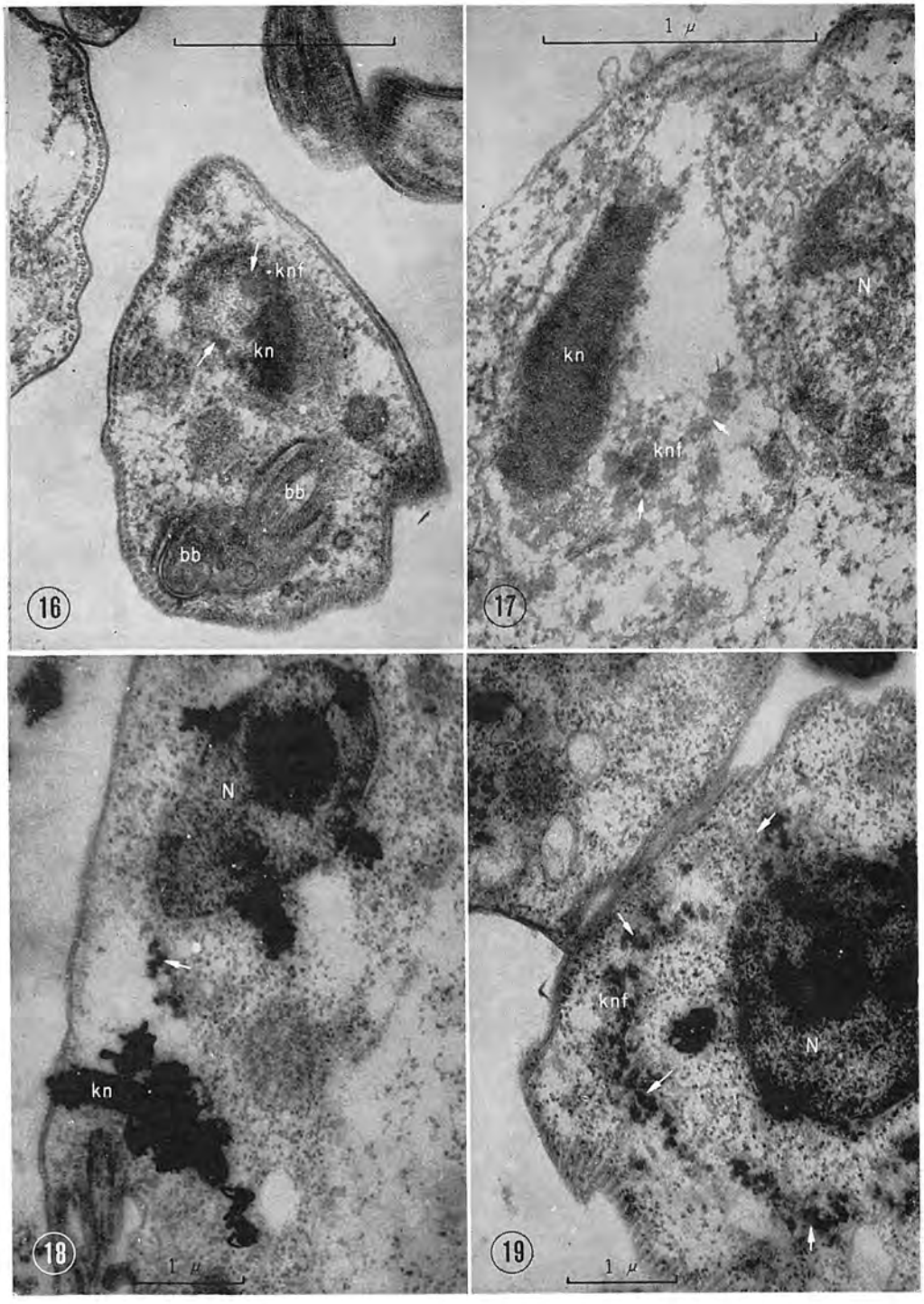
p-Rosaniline treatment

FIGURE 16. *Wellcome strain of T. gambiense (blood form) treated with 10 mg p-Ros./kg mouse body weight for 5 days and with RNase after fixation. The kinetonucleus and fragmented materials (arrows) are less electron dense than in the control.*

FIGURE 17, 18 and 19. *Cultured T. cruzi (crithidia form) treated with 2.5 μg/ml of p-Ros. for 4 days. Fig. 17. Cells treated with pronase. The kinetonucleus and fragmented material (arrow) have become less electron dense.*
The cells in Fig. 18 and 19 were incubated with ^{3}H-thymidine (20 μc/ml) for 48 hr before treatment with p-Ros.. Many grains are seen in the normal kinetonucleus and nucleus but not in fragmented material (arrows).
bb, basal body; kn, kinetonucleus; knf, kinetonuclear fragment; n, nucleus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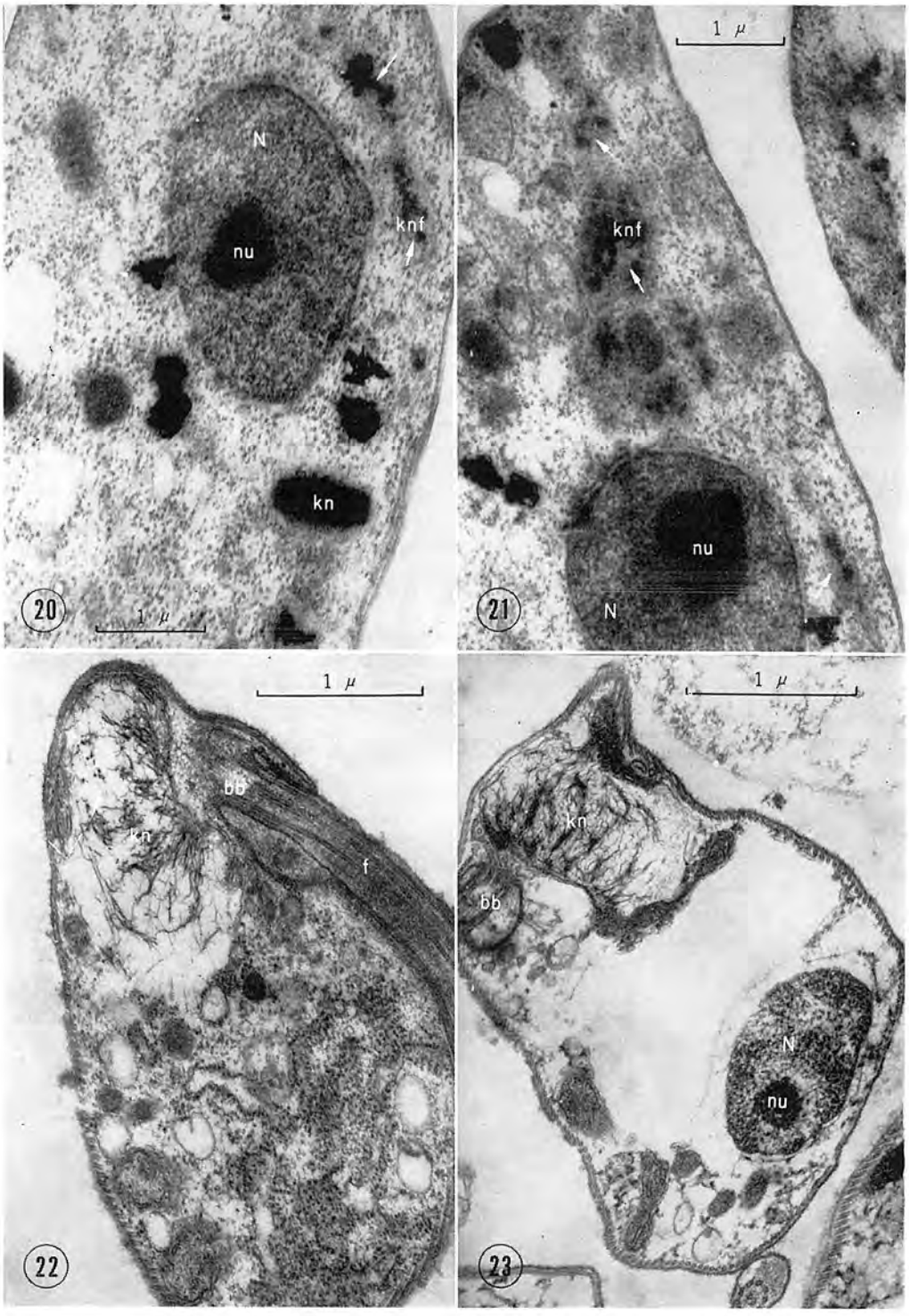
T. cruzi (crithidia and trypanosome forms)

FIGURE 20 and 21. *Cultured T. cruzi treated with ^{3}H-uridine (20 μc/ml) for 48 hr and then with 2.5 μg/ml of p-Ros. for 4 days. ^{3}H-uridine is incorporated into fragmented material (arrows) and the nucleolus, kinetonucleus and cytoplasm.*

FIGURE 22 and 23. *Blood form of T. cruzi. Fig. 22, control for RNase treatment. The kinetonucleus has an uncoiled fibrillar structure (cf. Inoki et al., 1971). Fig. 23. Cells after RNase treatment. The cytoplasm but not the kinetonucleus or nucleus is digested (cf. Wellcome strain of T. gambiense Fig. 28, 29 and 30). bb, basal body; f, flagellum; kn, kinetonucleus; knf, kinetonuclear fragment; n, nucleus; nu, nucleolus*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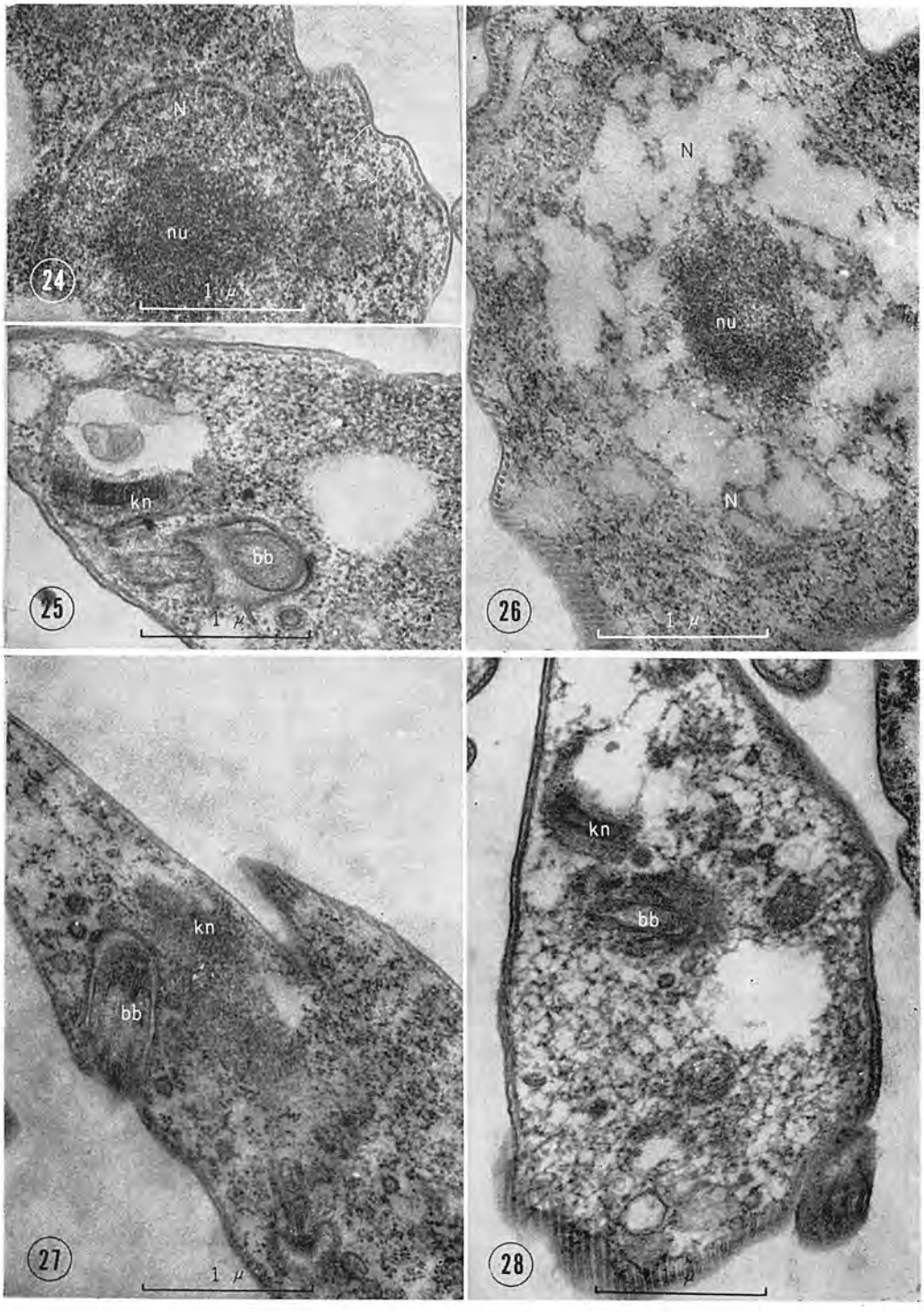
Enzyme treatment of the Wellcome strain of T. gambiense (trypanosome form)

FIGURE 24 and 25. *Controls for DNase or RNase treatment (incubated at 37 C for 1 hr in isotonic buffer solution).*
The nucleus (Fig. 24) and kinetonucleus (Fig. 25) are similar to those of untreated cells.

FIGURE 26 and 27. *DNase (Worthington DPFF) treatment results in extensive digestion of the nucleus (Fig. 26) but not the nucleolus. The kinetonucleus (Fig. 27) is digested but still retains a matrix-like structure.*

FIGURE 28. *RNase treatment causes digestion of the kinetonucleus (cf. Fig. 7, 8 and 23) and cytoplasm.*
bb, basal body; kn, kinetonucleus; n, nucleus;
nu, nucleolus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Enzyme treatment of T. gambiense (trypanosome form)

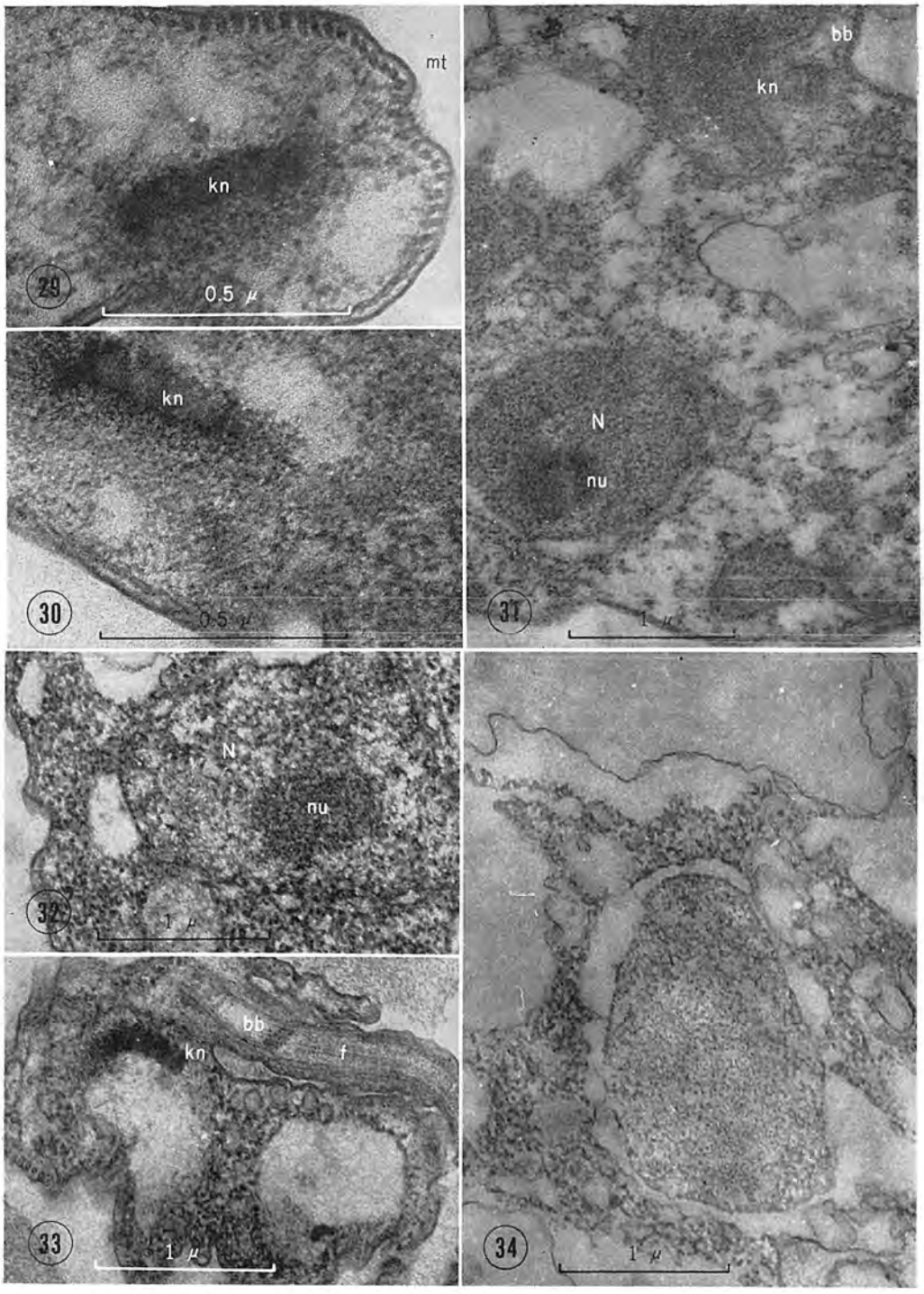
FIGURE 29 and 30. *Different extents of RNase digestion of the kinetonucleus in the Wellcome strain of T. gambiense. RNase digestion causes gradual reduction in the electron density of the kinetonucleus.*

FIGURE 31. *Pronase treatment of the Cheick strain of T. gambiense, results in reduction in electron density of all cell components.*

FIGURE 32 and 33. *Control for pepsin treatment (incubated at 37 C for 30 min in isotonic 0.02 N HCl solution). The whole cell is deformed. No fibrillar structure is seen in the kinetonucleus and the outline of the cell is irregular.*

FIGURE 34. *Pepsin treatment. The whole cell is extensively digested within 5 min on incubation at 37 C. No microtubles are seen.*

bb, basal body; f, flagellum; kn, kinetonucleus;
mt, microtubules; n, nucleus; nu, nucleolus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