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EFFECTS OF *p*-ROSANILINE ON THE ULTRA-STRUCTURE OF THE KINETOPLAST IN *TRYPANOSOMA GAMBIENSE AND TRYPANOSOMA EVANSI*[1]

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SUMMARY When mice infected with *Trypanosoma gambiense* or *Trypanosoma evansi* were treated with a critical dose of *p*-Rosaniline, the ultra-structure of their kinetoplasts was deformed, but other cell components were unaffected. The fibrous inclusion of the kinetoplast was partially fragmented in these two species. This result was quite different from that obtained with acridine dyes. On *p*-Rosaniline treatment the envelope membrane of the kinetoplast was lost in *T. gambiense*, but not in *T. evansi* or in the *p*-Rosaniline-resistant *T. gambiense*. Akinetoplastic forms of *T. evansi* also possessed envelope membranes (Inoki and Suganuma, 1964) and akinetoplastic forms of *T. gambiense* were not viable (Inoki, 1956). These data suggest that the envelope membrane is essential for the blood-stream forms of tyrpanosomes.

INTRODUCTION

The kinetoplast of Trypanosomatidae is considered to be a self-duplicating cytoplasmic organelle. It contains DNA and is located at the base of the flagellum. It divides by binary fission prior to nuclear division of the organism (Meyer et al. 1958; Inoki and Ozeki, 1969). A number of workers examined the kinetoplast by electron microscopy and found that it consists of a dense fibrous inclusion (kinetonucleus) and a two layered envelope membrane (kinetoplast double membrane). Steinert (1960) observed that the envelope membrane is a differentiated part of the mitochondrial membrane. In some species of *Trypanosoma*, cells which have lost the kinetoplast, so-called akinetoplastic forms (AK forms), have been observed. Loss of the kinetoplast may occur spontaneously or on treatment with acriflavine or other drugs.

Inoki (1956) and Inoki et al. (1962) reported that the rate of spontaneous appearance of AK forms in about 1% or less in *T. gambiense* and about 5–6% in *T. evansi*. However, if mice infected with either *T. gambiense* or *T. evansi*

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are treated with 5–10 mg/kg of mouse body weight of *p*-Rosaniline, the rate of appearance of AK forms usually increases about 30% within 4–5 hr. In these AK forms, no kinetoplasts were found by light microscopy and the incorporation of ^{3}H-thymidine at the site of the kinetoplast was considerably reduced (Steinert and van Assel, 1967) or completely stopped (Inoki and Ono, 1969). Mühlpfordt (1963) and Trager and Rudzinska (1964) found by electron microscopy that acridine-induced AK forms lacked the fibrous inclusion, but still retained the envelope membrane. Inoki and Suganuma (1964) also demonstrated similar structures in the AK forms of *T. evansi*.

In a series of experiments to obtain information on the loss of kinetoplasts, *T. gambiense* and *T. evansi* were observed by electron-microscopy at intervals after *p*-Rosaniline treatment. The present paper describes the morphological change of the kinetoplast induced by the dye.

MATERIALS AND METHODS

Trypanosoma gambiense and *Trypanosoma evansi* were employed in this study. The Wellcome strain of *T. gambiense* was given by courtesy of Dr. Max C. McCowen, the Eli Lilly Research Laboratories, Indiana, U.S.A. *T. evansi* (Taiwan) was obtained from the National Institute of Animal Health (Tokyo). These strains were sensitive to *p*-Rosaniline. A clone of *T. gambiense* (85P) resistant to 85 mg *p*-Rosaniline per kg mouse body weight was obtained by the short passage method from the Wellcome strain by Inoki and Matsushiro (1959). An AK clone of *T. evansi* was isolated by the single cell inoculation technique (Inoki, 1960) from *p*-Rosaniline treated organisms. All these strains were maintained by subsequent passage through ddO mice.

p-Rosaniline was purchased from Chroma-Geselschaft Schmid & Co., and was dissolved in distilled water at a concentration of 1 mg/ml.

The blood-stream form of *Trypanosoma* was inoculated into normal mice. When protozoa appeared ($3 \times 10^{8-9}$ cells/ml of blood) in the blood stream of these mice, 3–4 days after inoculation, various doses of *p*-Rosaniline (2–60 mg/kg body weight) were injected into the mice by the peritoneal route. Blood containing trypanosomes was collected at intervals after the dye injection, and was fixed at 4 C for 1 hr in 0.013 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. The organisms were collected by centrifugation at 2,000 rpm for 15 min. Trypanosome cells formed a film on the top of the packed blood cells. The film was removed with a fine glass rod and washed for 1hr with phosphate buffer containing 0.25 M sucrose, changing the buffer 4 times. Then it was stored at 4 C overnight in the same isotonic buffer. The materials were post-fixed at 4 C for 1 hr with 1.5% osmium tetraoxide in isotonic buffer, and then washed once with distilled water. After dehydration with a series of increasing concentrations of alcohol, materials were embedded in Epon 812 and sections were further stained with uranyl acetate, followed by lead citrate (Reynolds, 1963). Micrographs were made in a Hitachi 11B electron microscope.

RESULTS

1. *T. gambiense* (*p-Rosaniline sensitive clone*)

The general structures of the kinetoplast in

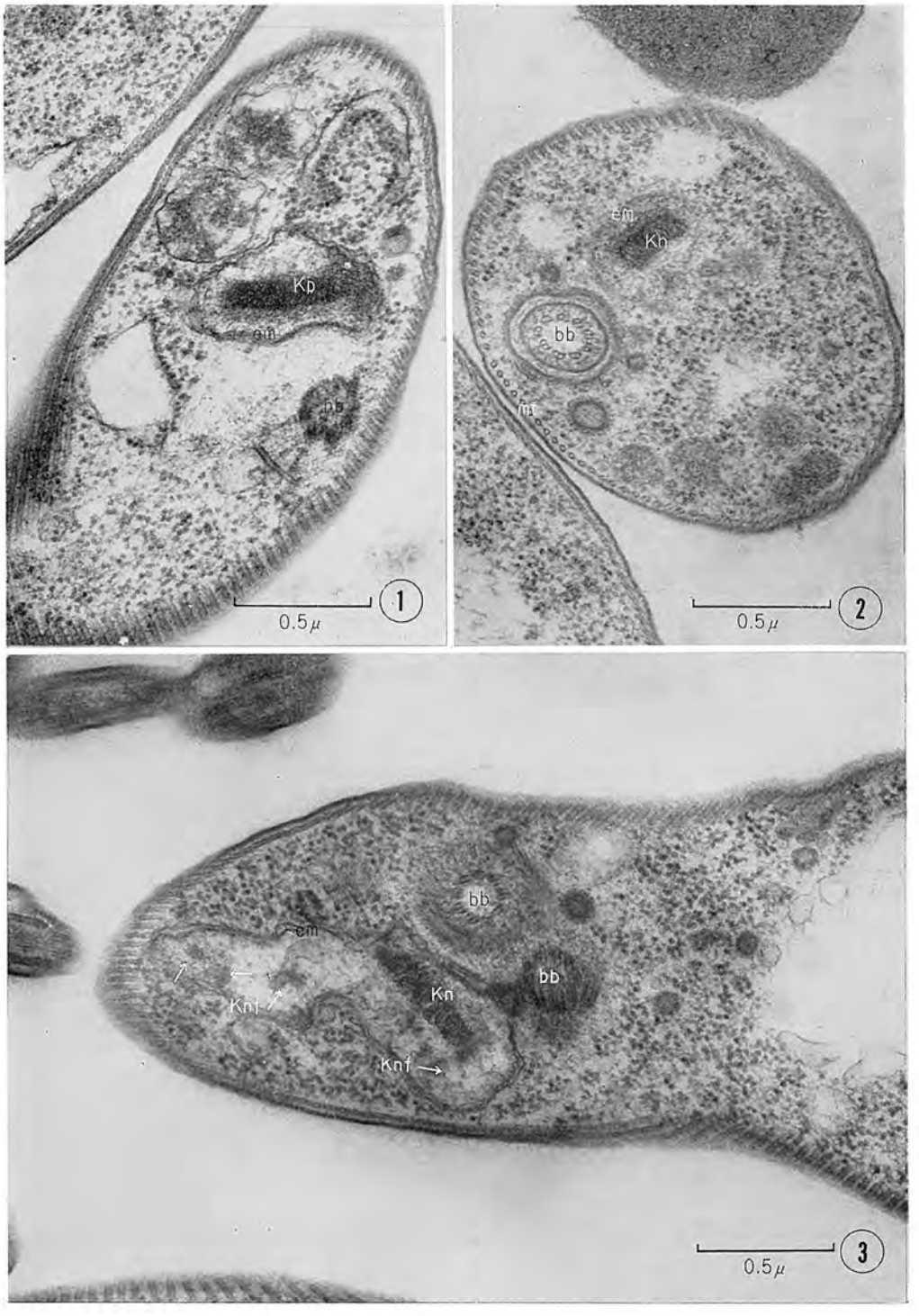
T. gambiense (*p-Rosaniline sensitive clone*)

FIGURE 1. *Untreated cell at the late log-phase of growth. The envelope membrane is clearly seen and the kinetonucleus shows the typical fibrous structure.*

FIGURE 2. *Untreated cell at the middle log-phase of growth. The envelope membrane is not so clear as that shown in Fig.* 1.

FIGURE 3. *p-Rosaniline treated cell at the late log-phase of growth* (1.5 *hr after treatment with* 10 *mg p-Ros./kg mouse body weight). The kinetonucleus consists of both fibrous and fragmented regions. Arrows show the fragmented regions. The envelope membrane is still observed.*

bb, basal body; kn, kinetonucleus (fibrous inclusion); em, envelope membrane (kinetoplast envelope); knf, kinetonuclear fragment; mt, microtubules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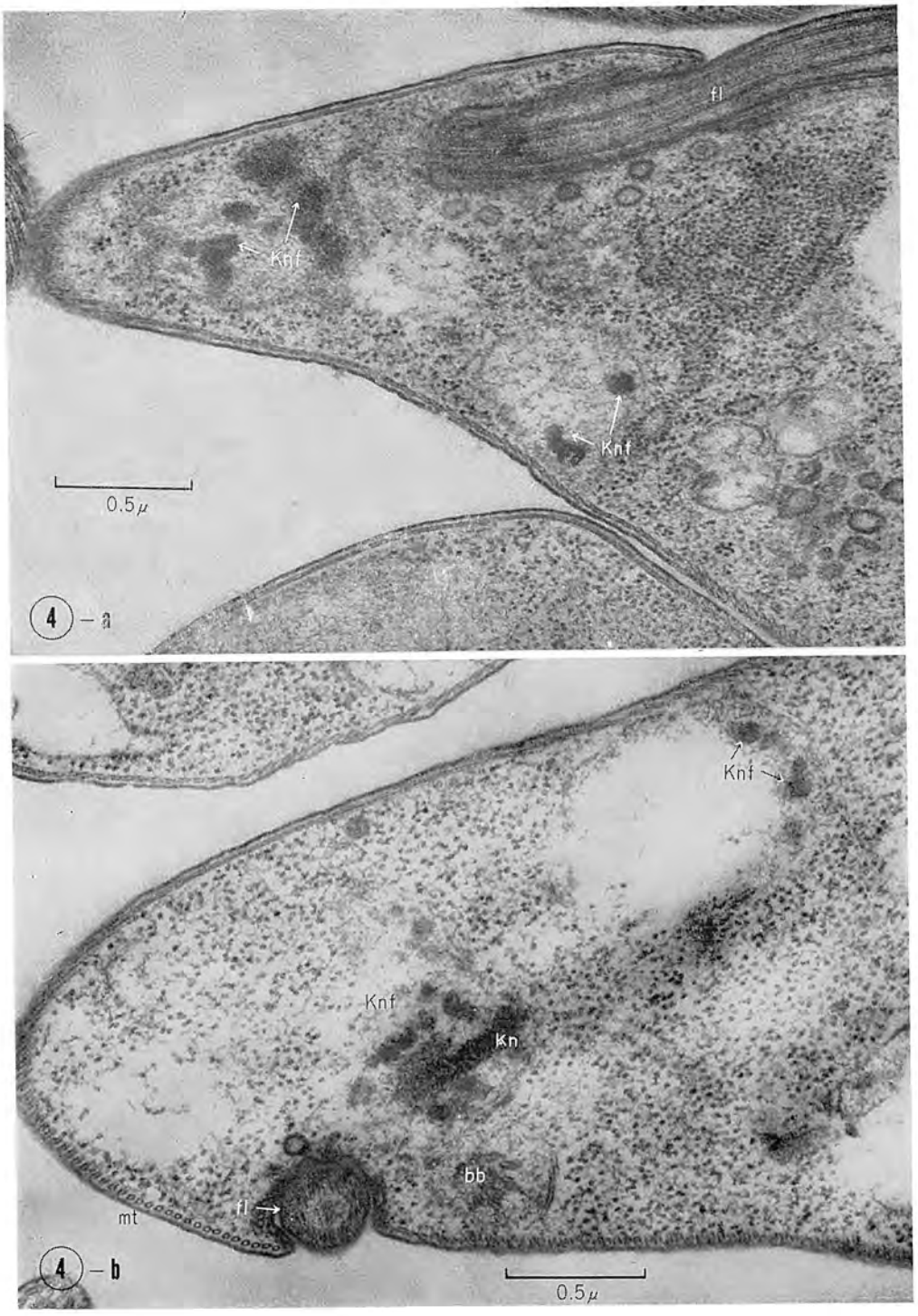
T. gambiense are shown in Fig. 1. In the non-dividing cell, the kinetoplast usually appears elongated or rod-shaped in longitudinal section. The dense inclusion (kinetonucleus) of a kinetoplast is filamentous, and is present within a two layered envelope membrane. The envelope membrane is more clearly seen in cells (Fig. 1) in the late log-phase of growth (ca 10^9 cells/ml of blood) than in those in the middle log-phase (ca $1–3 \times 10^8$ cells/ml Fig. 2) with this fixative. Moreover, the kinetoplast at the late log-phase is more resistant than that at the middle log-phase; namely, the abnormality was first observed in the former cells at a dosage of 9 mg *p*-Rosaniline/kg body weight, while with the latter it was detected with 5 mg/kg. With the dose range of *p*-Rosaniline used in these experiments, observable effects appeared only in the kinetoplasts, while the nucleus and other cytoplasmic structures appeared unaffected morphologically. Fig. 3 shows the effect on the kinetoplast of *p*-Rosaniline treatment at the late log-phase with a dosage of 10 mg/kg for 1.5 hr. More prolonged treatment causes the disappearance of the envelope membrane. For example, Fig. 4a and 4b show the typical appearance of kinetoplasts obtained 5 hr after *p*-Rosaniline (10 mg/kg) injection into infected mice at the late log-phase. As shown in Fig. 4a, the fibrous inclusion was extensively fragmented and the envelope membrane disappeared. However, some cells retained fibrous structures beside materials being fragmented (Fig. 4b). If this type of affected cell divides, one of the two daughter cells may receive only fragments of material and cells with only fragments may appear in stained preparations under the light microscope just like AK forms. When the same treatment was prolonged from 4–5 hr to 15–18 hr, a few cells containing a rod-shaped kinetoplast without an envelope membrane were observed (Fig. 5). These observations could be explained by supposing that fragmentation of the fibrous inclusion takes place only on synthesis of kinetonuclear materials after *p*-Rosaniline treatment and not in resting cells.

T. gambiense (*p-Rosaniline sensitive clone*)

FIGURE 4a. and 4b. *p-Rosaniline treated cell at the late log-phase of growth (5 hr after treatment with 10 mg/kg). Greater fragmentation is observed (arrows). Fragmented regions have no fibrous structure. Note the disappearance of the envelope membrane.*

bb, basal body; fl, flagellum; kn, kinetonucleus (fibrous inclusion); knf, kinetonuclear fragment; mt, microtubules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2. *T. gambiense* (85p) (*p-Rosaniline resistant clone*)

The general structures of the kinetoplast in this resistant clone are indistinguishable from those in the original sensitive one (as Fig. 1). The appearance of the kinetoplast after *p*-Rosaniline treatment (10 mg/kg) is shown in Fig. 6. Fragmentation of the fibrous inclusion occurs just as in the sensitive clone, but no disappearance of the envelope membrane was observed, even when high doses of dye (20 mg/kg, 40 mg/kg and 60 mg/kg) were administered. Fig. 7 shows that after treating the kinetoplast with 60 mg/kg dye for 4 hr it still has an envelope membrane.

3. *T. evansi* (*kinetoplastic form clone*)

For comparison of *T. evansi* and *T. gambiense*, *T. evansi* in mice were also treated with *p*-Rosaniline (10 mg/kg) for 4–5 hr. Fragmentation of the fibrous inclusion of *T. evansi* also occured but not disappearance of the membrane (Fig. 8). When the cells were exposed to *p*-Rosaniline for 15–18 hr, the envelope membrane of the kinetoplast persisted as a " ghost " and the fibrous inclusion was replaced by fragmented material, as shown in Fig. 9. Similar observations have been made by many investigators with acriflavine instead of *p*-Rosaniline employing other species of trypanosomes (Mühlpfordt, 1963; Vickerman, 1963; Trager and Rudzinska, 1964; Steinert and van Assel, 1967; Simpson, 1968).

4. *T. evansi* (*akinetoplastic form clone*)

In the electron micrograph (Fig. 10), the fibrous inclusion appears to be replaced by fragmented material, just as in the kinetoplastic form of *T. evansi* treated with *p*-Rosaniline for 15–18 hr (cf. Fig. 9). Similar observations have already been reported by Inoki and Suganuma (1964).

T. gambiense (*p-Rosaniline sensitive clone*)

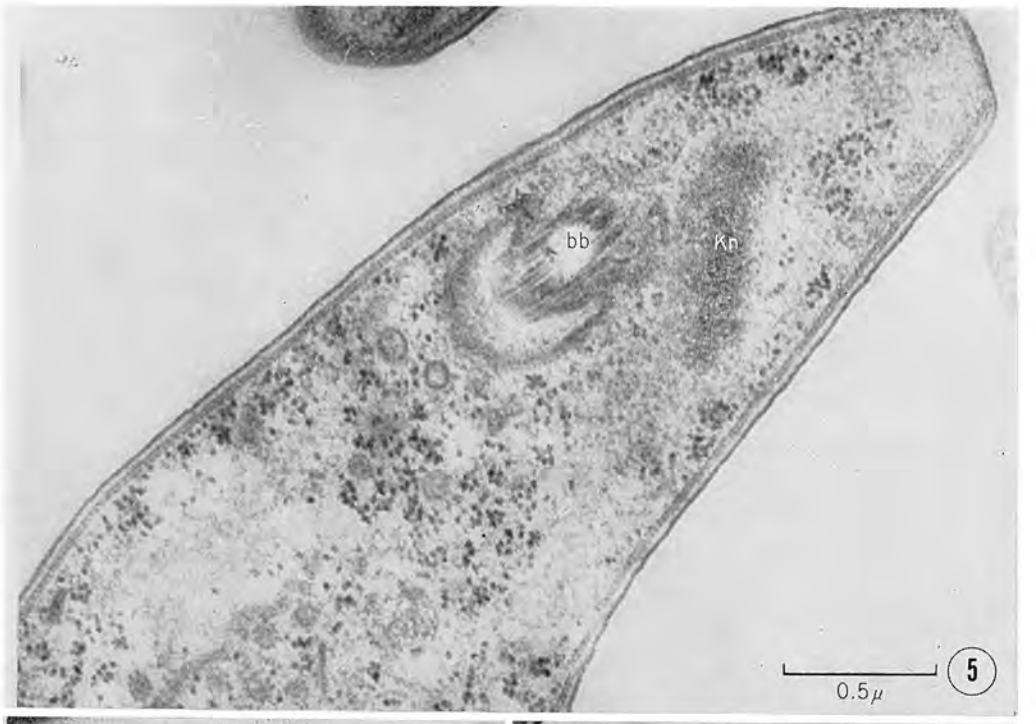
FIGURE 5. *p-Rosaniline treated cell at the late log-phase of growth* (15 *hr after treatment with* 10 *mg/kg*). *In this preparation, cells were fixed in hypertonic fluid. This cell may be dead, since no plasmolysis takes place.*

T. gambiense (85 *p*) (*p-Rosaniline resistant clone*)

FIGURE 6. *p-Rosaniline treated cell at the late log-phase of growth* (4 *hr after treatment with* 10 *mg/kg*). *Fragmentation of the kinetonucleus occurs, but the envelope membrane still remains* (*cf. Fig.* 4).

FIGURE 7. *p-Rosaniline treated cell at the late log-phase of growth* (4 *hr after treatment with* 60 *mg/kg*). *The envelope membrane still remains.*

bb, basal body; kn, kinetonucleus (*fibrous inclusion*); *em, envelope membrane* (*kinetoplast envelope*); *knf, kinetonuclear fragment*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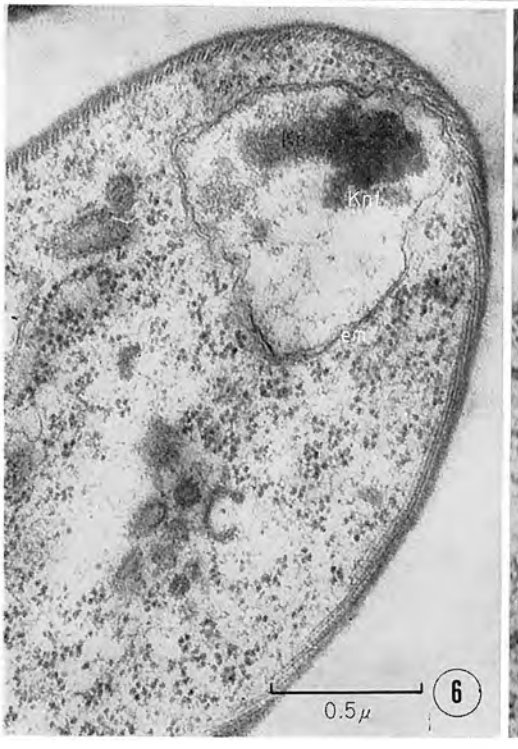
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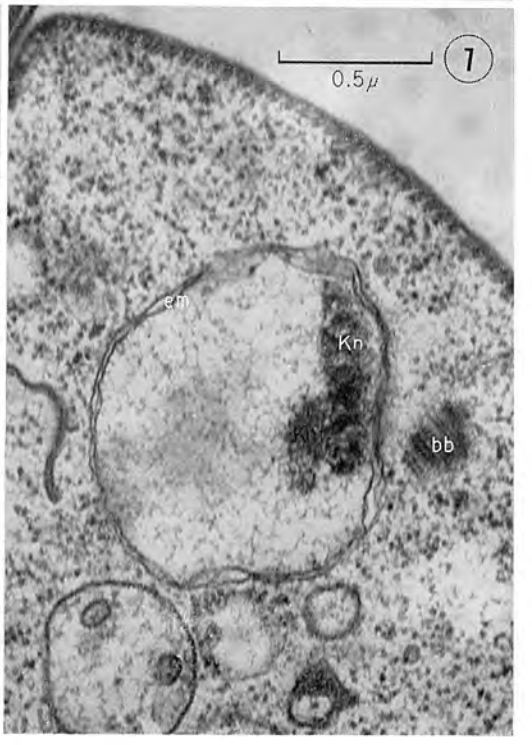
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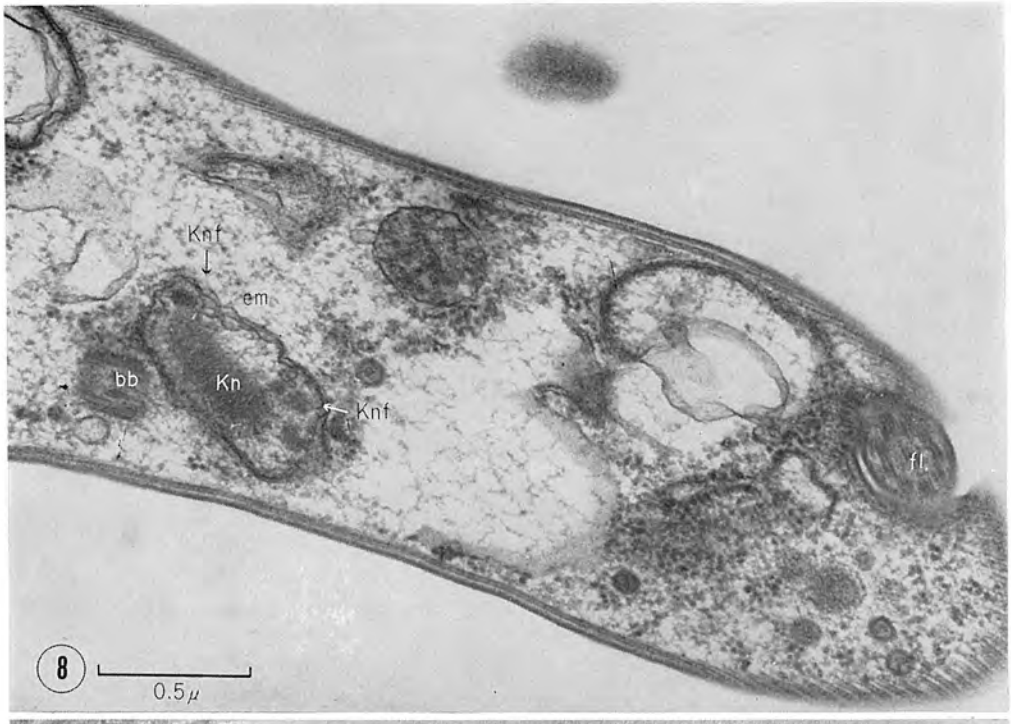
T. evansi (*a strain of kinetoplastic form*)

FIGURE 8. *p-Rosaniline treated cell at the late log-phase of growth* (1.5 *hr after treatment with* 10 *mg/kg*). *Fragmentation of the kinetonucleus* (*arrows*) *and the remaining envelope membrane are observed.*

FIGURE 9. *p-Rosaniline treated cell at the late log-phase of growth* (4 *hr after treatment with* 10 *mg/kg*). *Greater fragmentation is observed, but the envelope membrane does not disappear* (*cf. Fig.* 4).

bb, basal body; fl, flagellum; kn, kinetonucleus (*fibrous inclusion*); *em, envelope membrane* (*kinetoplast envelope*); *knf, kinetonuclear fragment*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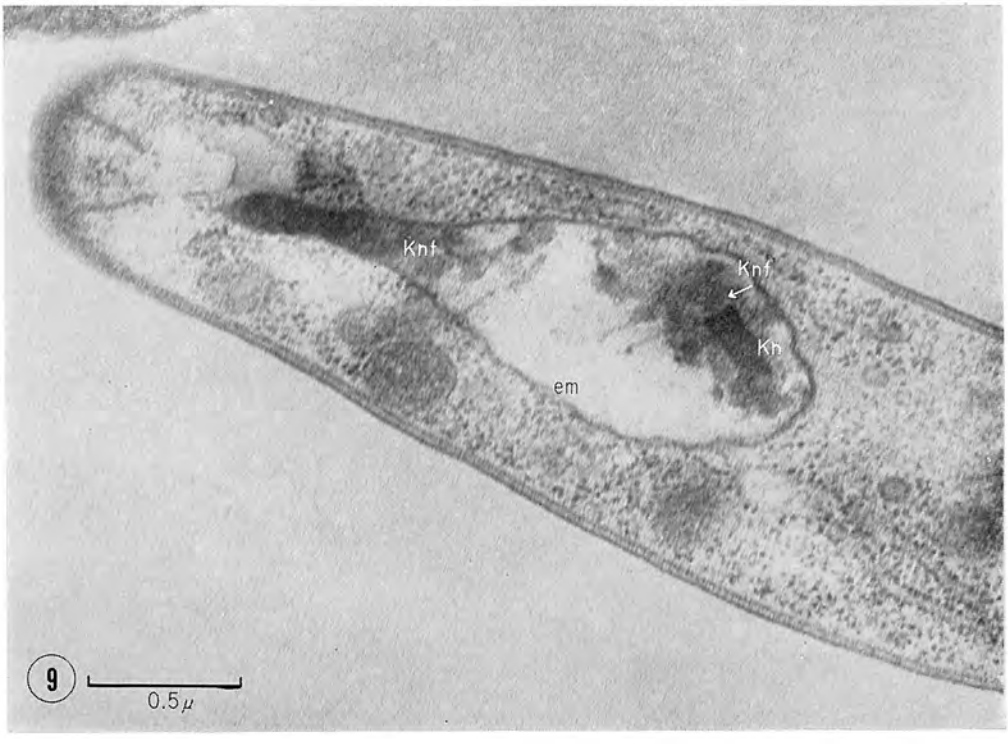
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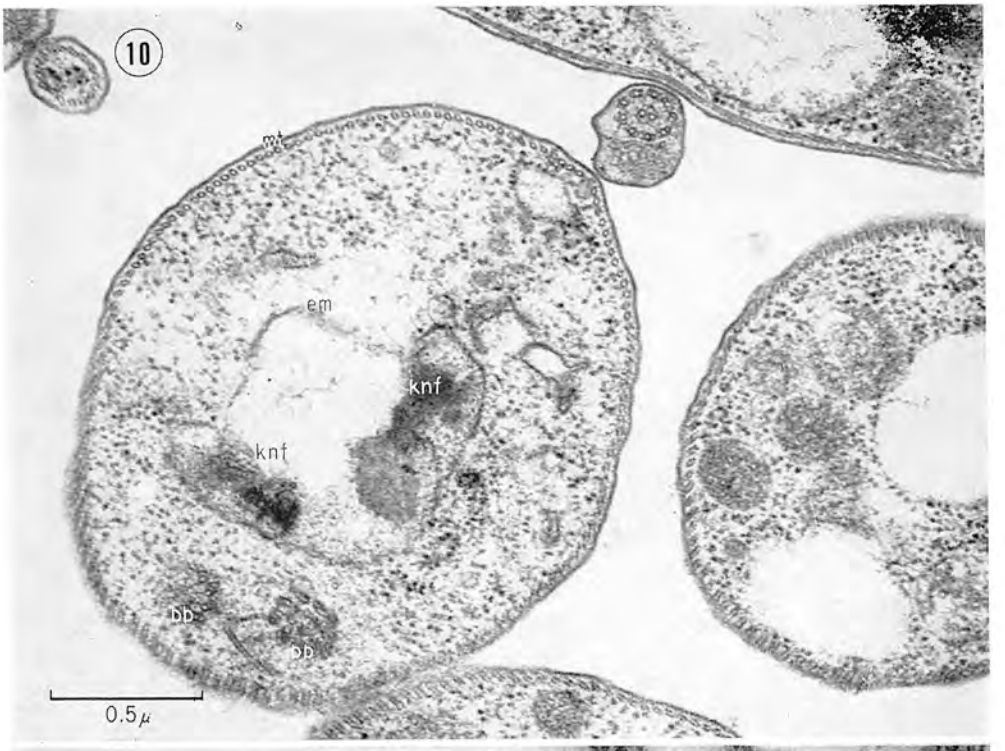
T. evansi (*a strain of kinetoplastic form*)

FIGURE 10. *p-Rosaniline treated cell at the late log-phase of growth* (16 *hr after treatment with* 10 *mg/kg*). *The envelope membrane still persists. The kinetonucleus is replaced with fragmented material* (*no-fibrous structures. cf. Fig.* 5).

T. evansi (*an established AK form clone*)

FIGURE 11. *Untreated cell at the late log-phase of growth. The envelope membrane is clearly observed. An electron-dense mass but no normal kinetonucleus is seen* (*arrow*).

bb, basal body; em, envelope membrane (*kinetoplast envelope*); *knf, kinetonuclear fragment; mt, microtubules*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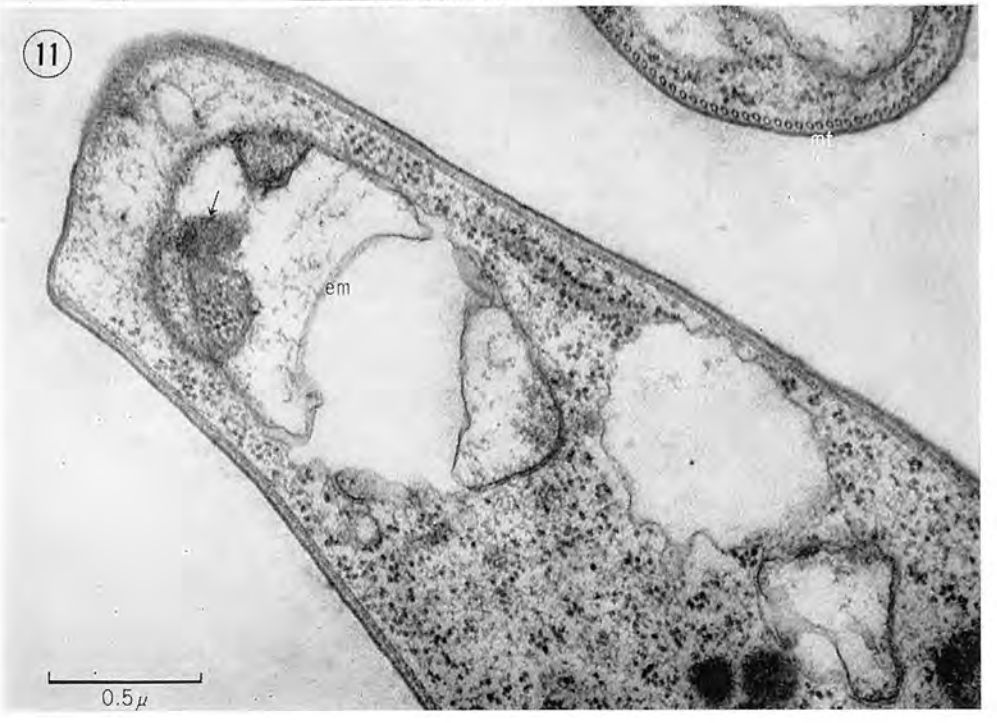
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DISCUSSION

It has been shown that acriflavine-induced AK forms lack a kinetoplast DNA structure but retain the kinetoplast membrane (Mühlpfordt, 1963; Trager and Rudzinska, 1964; Steinert and van Assel, 1967). In the process of loss of the kinetoplast DNA structure, the ultra-structure becomes extremely abnormal; DNA fibrils are condensed forming a homogeneous mass separated from the membrane by a space of low electron-opacity (Steinert and van Assel, 1967). These observations have already been confirmed with the Wellcome strain of *T. gambiense* (Inoki, Ozeki and Ono, Unpublished). It was found that loss of kinetoplast DNA occurs gradually generation by generation, reducing the amount of DNA/kinetoplast (Simpson, 1968).

In contrast, *p*-Rosaniline treatment had a very different affect on the kinetoplasts in *T. gambiense* and *T. evansi*, as is reported here. In this case, the abnormality results in fragmentation of kinetonuclear materials. In many cells, these fragments coexisted with a normal kinetonucleus (Fig. 3, 4, 6, 7, 8 and 9). The generation time of the blood stream form of *T. gambiense* or *T. evansi* was estimated as about 4–6 hr in mice. After injecting *p*-Rosaniline into mice (10 mg *p*-Ros./kg body weight), fragmentation of the kinetonuclei (fibrous inclusions) was observed in a few cells after 1.5 hr., and in many cells after 4–5 hr; but even after 15–18 hr, a few kinetonuclei still remained (Fig. 5). These facts may be explained by supposing that *p*-Rosaniline dye acts only on the synthesis of kinetonuclear materials, but not directly on the kinetonucleus in the resting stage. Kinetonuclear materials which fragment may contain, some other components of kinetonuclear materials, such as protein or/and RNA, rather than kinetoplast DNA since no DNA fibril structure was demonstrated (Fig. 4, 6, 10 and 11). The following observations also support this possibility. In the AK forms of *T. evansi*, the fragments observed inside the envelope membrane were Feulgen negative. Moreover, on treatment of *T. gambiense* with DNase similar fragments were not dissolved, while the fibrous structure persisting in the kinetonucleus (normal appearance) was completely destroyed (to be published). These observations imply that *p*-Rosaniline acts specifically on the synthesis of kinetoplast DNA.

If cells containing fragments and part of the normal kinetonucleus (such as the cell shown in Fig. 4b) divide, one of the daughter cells may receive only fragments. Such a cell will probably appear as an AK form by light microscopy. This may explain the previous data by Inoki (1956) that the rate of appearance of the AK forms was about 30% 4 hr after *p*-Rosaniline treatment.

In this study, we found that the kinetoplast envelope membrane had disappeared in most cells of the sensitive clone of *T. gambiense* at the late log-phase of growth by 4–5 hr after treatment with *p*-Rosaniline (Fig. 4a, 4b and 5). The AK forms of this species are known to be nonviable (Inoki, 1956). On the other hand, in the *p*-Rosaniline resistant clone of *T. gambiense* (Fig. 6 and 7) and *T. evansi* (Fig. 9 and 10) the envelope membrane did not disappear. Likewise in the AK forms of *T. evansi*, *p*-Rosaniline-induced AK forms and an established clone of the AK form, the envelope membrane did not disappear, and these AK forms were all viable.

These facts suggest that the kinetoplast envelope membrane of these flagellates has some essential function related to their viability. Since Steinert (1960) revealed that the kinetoplast of Trypanosomatidae is a differentiated part of the large, single mitochondrion, the kinetoplast membrane might have some of the same functions as the mitochondrial membrane. In the blood stream form of the trypanosomes employed in this experiment, a simple tubular mitochondrion with scarcely any cristae is found extending from the kinetoplast to the posterior end of the flagellate. Schnaitman et al. (1967) showed that monoamine oxidase is located on the outer membrane of the mitochondria in rat liver, while cytochrome oxidase

is located on the cristae and inner membrane of the mitochondria. This suggests that the blood stream forms of *T. gambiense* and *T. evansi* do not have so much cytochrome oxidase in their kinetoplast. If monoamine oxidase is located on the kinetoplast outer membrane, its disappearance might cause respiratory defects. Simpson (1968) considered that when *Leishmania tarentolae* is treated with acriflavine, loss of kinetoplast DNA leads to a respiratory defect. This study, however, suggests that disappearance of the envelope membrane is more directly related to death of trypanosomes than loss of kinetoplast DNA.

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