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Effect of ethidium bromide on growth and morphology of *Leishmania tarentolae* promastigotes *in vitro*[1]

R. Brun, W. Leon

Summary

Incubation of *Leishmania tarentolae* promastigotes in 0.01 μg/ml ethidium bromide in Neo Ye medium for 96 h resulted in 60% inhibition of cell growth and 91% dyskinetoplasty. After 48 h incubation in ethidium bromide over 50% of the cells were scored dyskinetoplastic by light microscopy although the electron microscopical examination revealed that most promastigotes contained at least a small amount of kDNA. A few of the treated cells undergoing division contained two kinetoplasts – one devoid of kDNA and the other with a reduced amount of kDNA as seen in the electron micrographs.

Key words: Leishmania tarentolae; ethidium bromide; growth inhibition; kinetoplast DNA.

Introduction

Members of the flagellate protozoan order Kinetoplastidae (Honigberg et al., 1964) are distinguished by the possession of a structurally complex DNA within a specific region of the mitochondrion known as the kinetoplast. The kinetoplast DNA (kDNA) in *Leishmania tarentolae* consists of several molecular types held together in associations of various sizes (Renger and Wolstenholme, 1971).

Synthesis of kDNA is selectively inhibited by cationic dyes such as ethidium bromide (Riou, 1968; Morales et al., 1972; Brack et al., 1972; Ucros et al., 1977), acriflavine and by the trypanocidal drug Berenil (Leon et al., 1977). It is known that ethidium bromide preferentially inhibits the replication of extranu-

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clear circular DNA. Recent investigations with *Escherichia coli* have shown that the dye inhibits transcription by preventing the formation of a stable complex between RNA polymerase and the DNA template, particularly when the DNA is circular and supercoiled (Richardson, 1973; Richardson and Parker, 1973). It is not clear how ethidium bromide affects *L. tarentolae* kDNA morphology and cell function.

To elucidate the action of ethidium bromide on *L. tarentolae* we studied the effect of the dye upon growth, dyskinetoplasty and ultrastructure. The results of this study are the subject of the present report.

Material and methods

Cultivation. Leishmania tarentolae, strain TAR II, UCI variant (da Cruz and Krassner, 1971) was cloned and maintained in a Neopeptone yeast extract medium (Neo YE), pH 7.8, with 10 μg haemin/ml at 27° C (Leon et al., 1977). Promastigotes were grown in 50 ml Erlenmeyer flasks containing 10 ml medium, rotating at 10 rpm. Cell counts were determined in a Coulter Counter, Model ZBI with the lower threshold setting at 6 and the upper at 65.

The drug. Stock solution of ethidium bromide (EB) dissolved in Neo YE (5 mg/ml) was sterilized by filtration (Millipore GS-0.22 μM) and protected against visible light at 4° C. All experiments were done with the cell suspensions protected against visible light.

Evaluation of the percentage of dyskinetoplasty. Samples of cell cultures were mixed with an equal volume of 0.25 M sucrose – 1% (w/v) bovine serum albumin and centrifuged at 1500 *g* for 10 min at 4° C. The pellet was spread onto precleaned slides, air dried, fixed with methanol for 10 min and stained in Giemsa. Promastigotes were considered as dyskinetoplastic when their kinetoplast was not stained at all.

Electron microscopy. Promastigotes were fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer for 1½ h, washed overnight in 0.2 M cacodylate buffer with 3% (w/v) sucrose, and postfixed with 2% (w/v) OsO_4 in 0.2 M cacodylate buffer for 1½ h. The samples were dehydrated in acetone, treated with propylene oxide, and embedded in Epon. Thin sections were cut on an LKB Ultrotome III with a diamond knife. Blocks were stained during dehydration with 1% (w/v) uranyl acetate in 70% acetone and thin sections were stained with lead citrate. The sections were examined in a Zeiss EM 9S electron microscope.

Results

Cell growth and dyskinetoplasty. The optimal concentration of ethidium bromide for use in this study was determined by incubating *L. tarentolae* promastigotes for 96 h in varying concentrations of the drug. The dose response curve, plotting inhibition of cell growth and dyskinetoplasty, is shown in Fig. 1; the concentration of drug chosen for subsequent experiments was 0.01 μg/ml. This dose inhibited cell growth by 60% and induced 91% dyskinetoplastic cells. Higher drug levels resulted in morphologically altered cells containing numerous cytoplasmic granules.

Concentrations above 0.09 μg/ml almost completely inhibited cell growth. The dyskinetoplasty curve shown in Fig. 1 drops rapidly when dye concentrations exceed 0.03 μg/ml. Above this concentration the cytoplasm became very

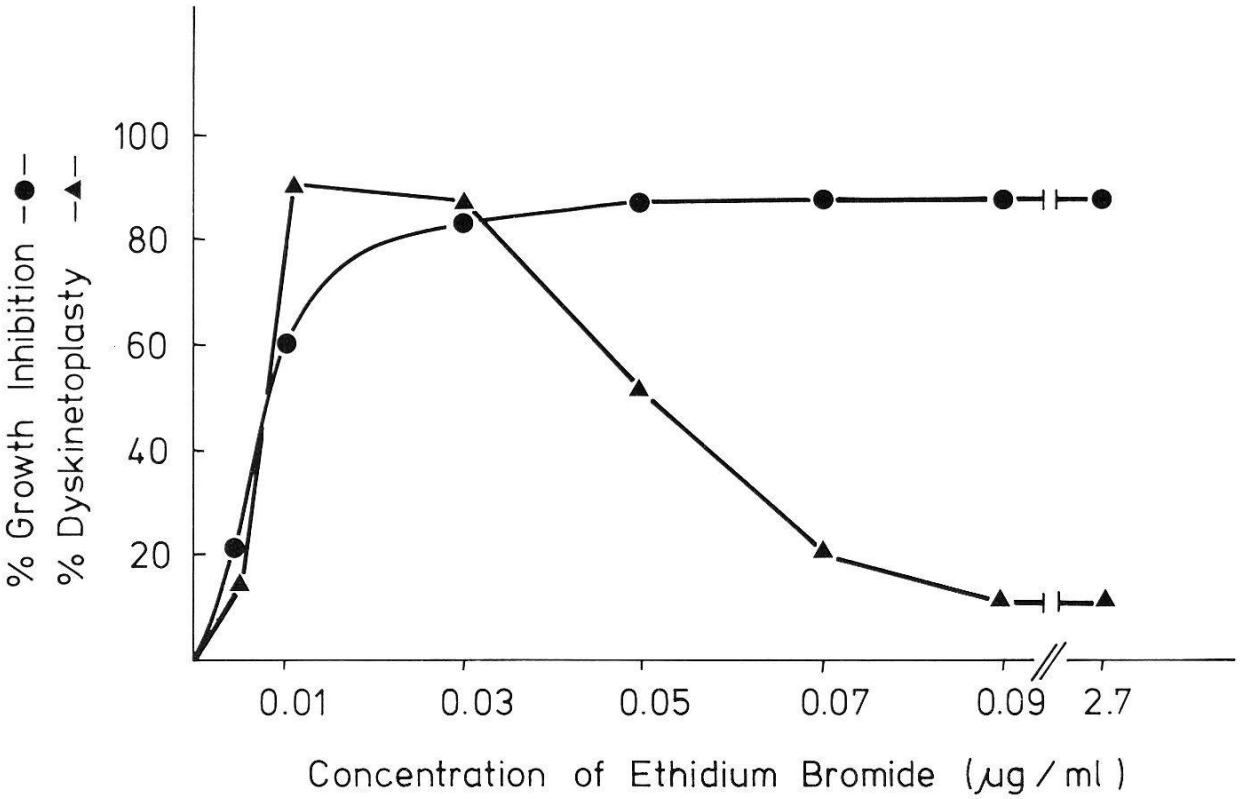


Fig. 1. Dose response of *Leishmania tarentolae* promastigotes to ethidium bromide treatment showing effect of drug concentration on growth inhibition (●) and dyskinetoplasty (▲) after 96 h. Lowest dye concentration inducing high dyskinetoplasty was 0.01 µg/ml; this concentration caused 60% inhibition of growth and 91% dyskinetoplasty. All subsequent experiments were carried out using 0.01 µg/ml. Culture conditions, cell number determination and scoring of dyskinetoplasty are described in Methods.

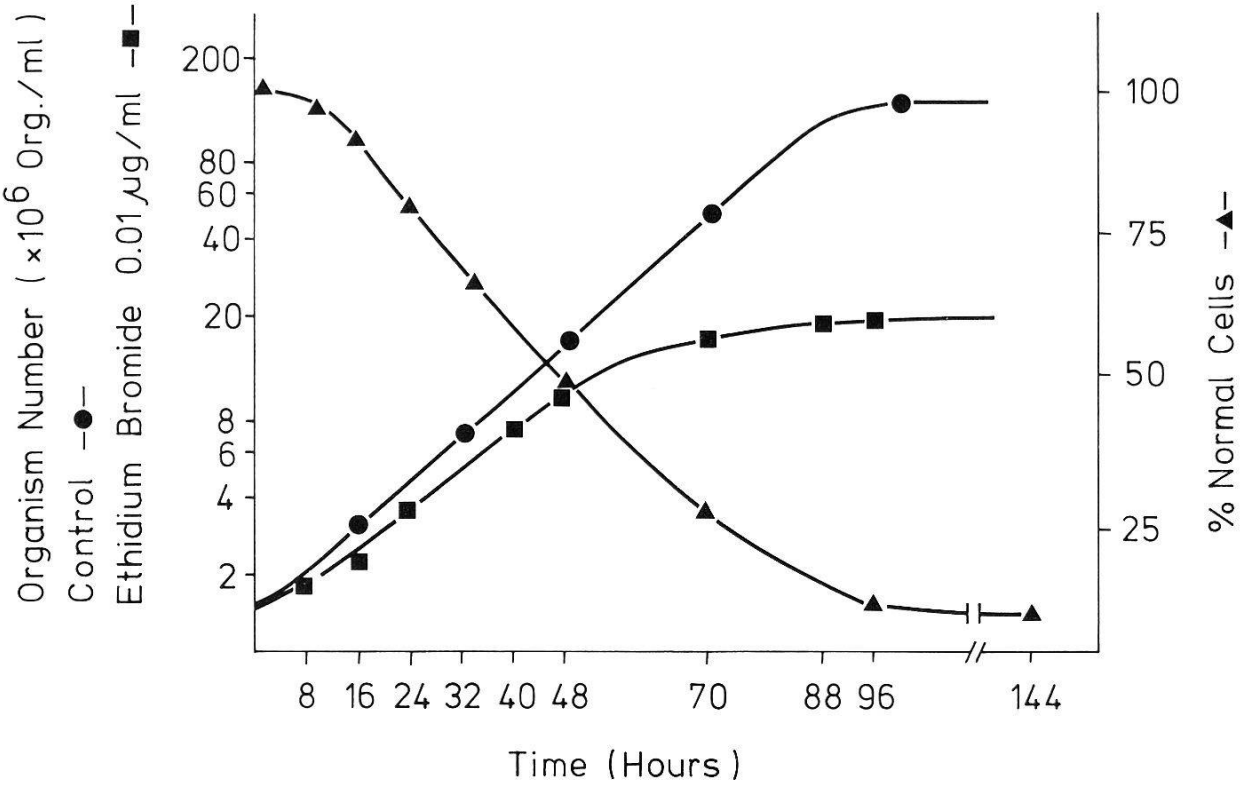


Fig. 2. Kinetics of the effect of ethidium bromide (0.01 µg/ml) on *Leishmania tarentolae*. Comparison of growth curves of cells with (■) and without (●) dye and the kinetics of loss of normal (kinetoplastic) cells (▲). Culture conditions and cell number determination described in Methods.

granular and made scoring of dyskinetoplasty very difficult. The effect of 0.01 μg/ml ethidium bromide upon dyskinetoplasty and cell growth is shown in Fig. 2.

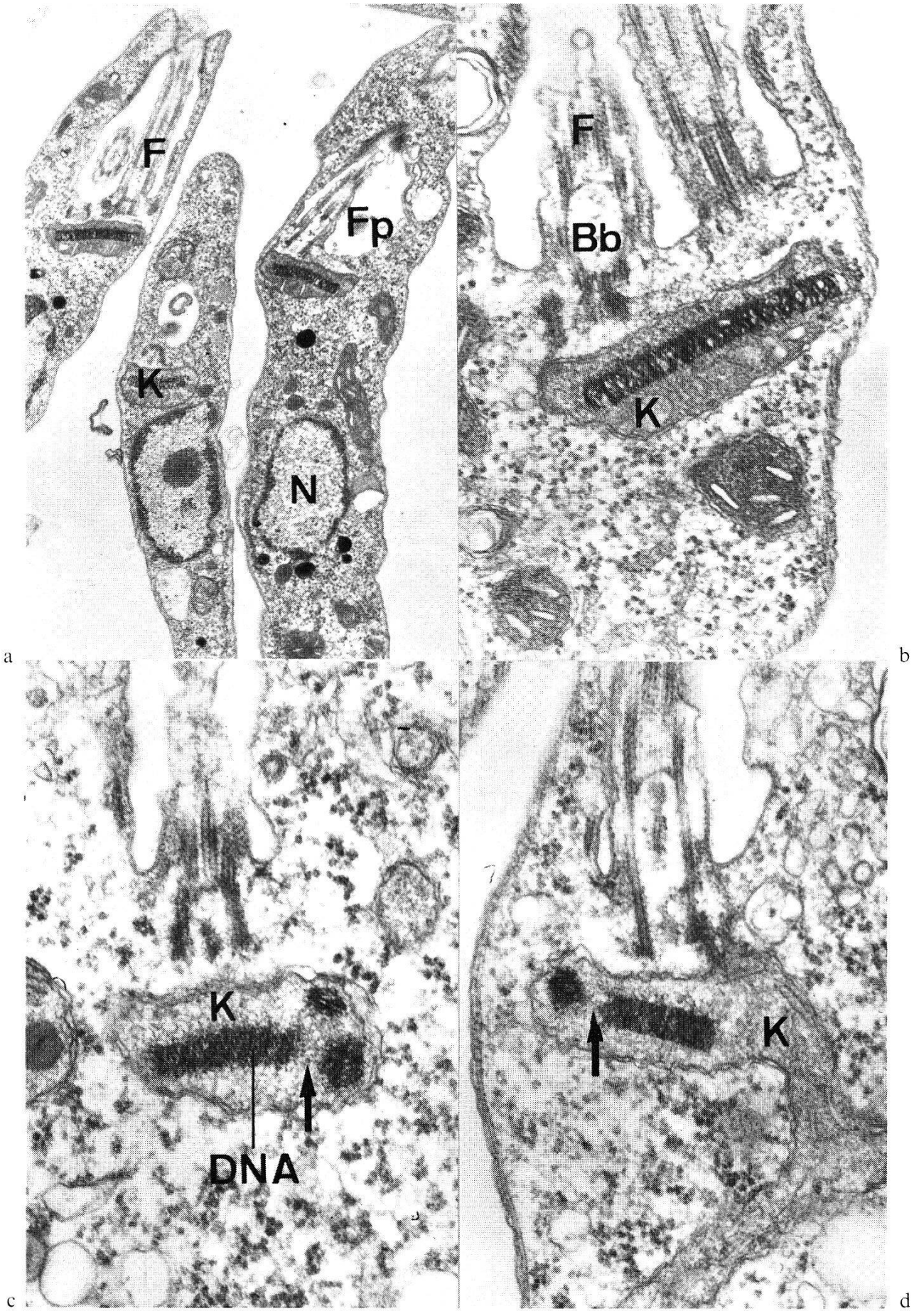
Ultrastructure. The general morphology on the ultrastructural level was unchanged after an incubation in 0.01 μg/ml EB compared to control cells, with the exception of the kinetoplast. Cells exposed to EB for 12 h showed structural changes in the k-DNA (Fig. 3c, d) when compared with kDNA of non-treated cells (Fig. 3a, b). After 48 h exposure, the fibrillar band consisted of a broken dense material which had lost the normal comb-like parallel arrangement of fibres (Fig. 4a, b) and some cells appeared completely devoid of kDNA (Fig. 4d). Most of the cells, however, in disagreement with the light microscopical observations, contained an altered and reduced amount of kDNA (Fig. 4a, b). Some dividing promastigotes contained two kinetoplasts: one devoid of kDNA and the other with a small amount of kDNA (Fig. 4c). After 96 h about half of the cells showed a greatly reduced amount of kDNA which was completely altered. The fibrous structure was lost and replaced by amorphous material (Fig. 5a). The other half of the cells appeared completely devoid of kDNA in the portion of the mitochondrion closest to the basal body (Fig. 5b).

Discussion

Selective inhibition of hemoflagellate nucleic acid synthesis by the intercalating dye, ethidium bromide, was first observed by Newton (1957). Cationic dyes such as ethidium bromide selectively inhibit kDNA replication for several reasons: their adenine and thymine binding affinity, the relatively high AT base composition of kDNA and the absence of histone proteins in kDNA which might otherwise hinder access of cationic drugs and dyes (Simpson, 1972).

Growth inhibition increased very rapidly from 21% to 83% between EB concentrations of 0.005 μg/ml and 0.03 μg/ml. With higher concentrations growth was almost completely inhibited. The percentage of dyskinetoplasty showed a similar increase up to the concentration of 0.03 μg/ml, followed by a decrease to 11% dyskinetoplasty at 0.09 μg/ml. This pattern confirms the findings of Simpson (1968) and Steinert (1969) who postulated that the loss of kDNA depends on cell divisions. At lower EB concentrations, the cell and the kinetoplast still divide but the kDNA no longer replicates, leading to a dilution of the kDNA. The final disappearance of the kDNA may occur in a "all-and-

Fig. 3. *Leishmania tarentolae* promastigotes grown in Neo YE medium at 27° C. a) Control cells in log phase with nucleus (N), kinetoplast (K) with kDNA, flagellum (F) and flagellar pocket (Fp). 12,800×. b) Higher magnification of control cell showing the enlarged kinetoplast (K) with the kDNA disc prior to division and two flagella (F). Basal body (Bb) 42,500×. c, d) Cells exposed 12 h to ethidium bromide (0.01 μg/ml). The kDNA has lost its normal structure revealing disturbances in the parallel arrangement of the minicircles. Fragments are separated from the main kDNA body (→). c = 47,300×. d = 44,200×.


F
Fp
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DNA
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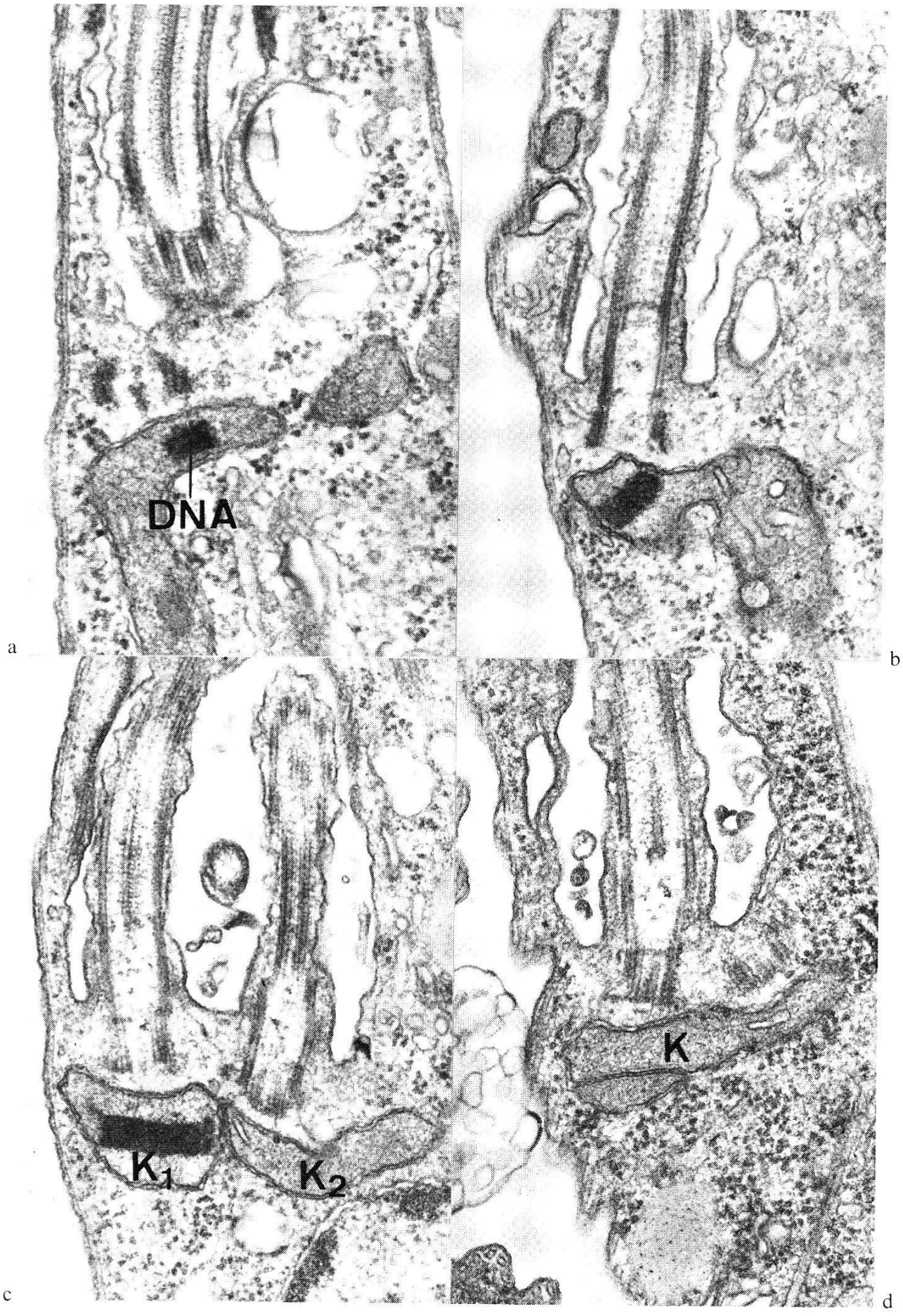
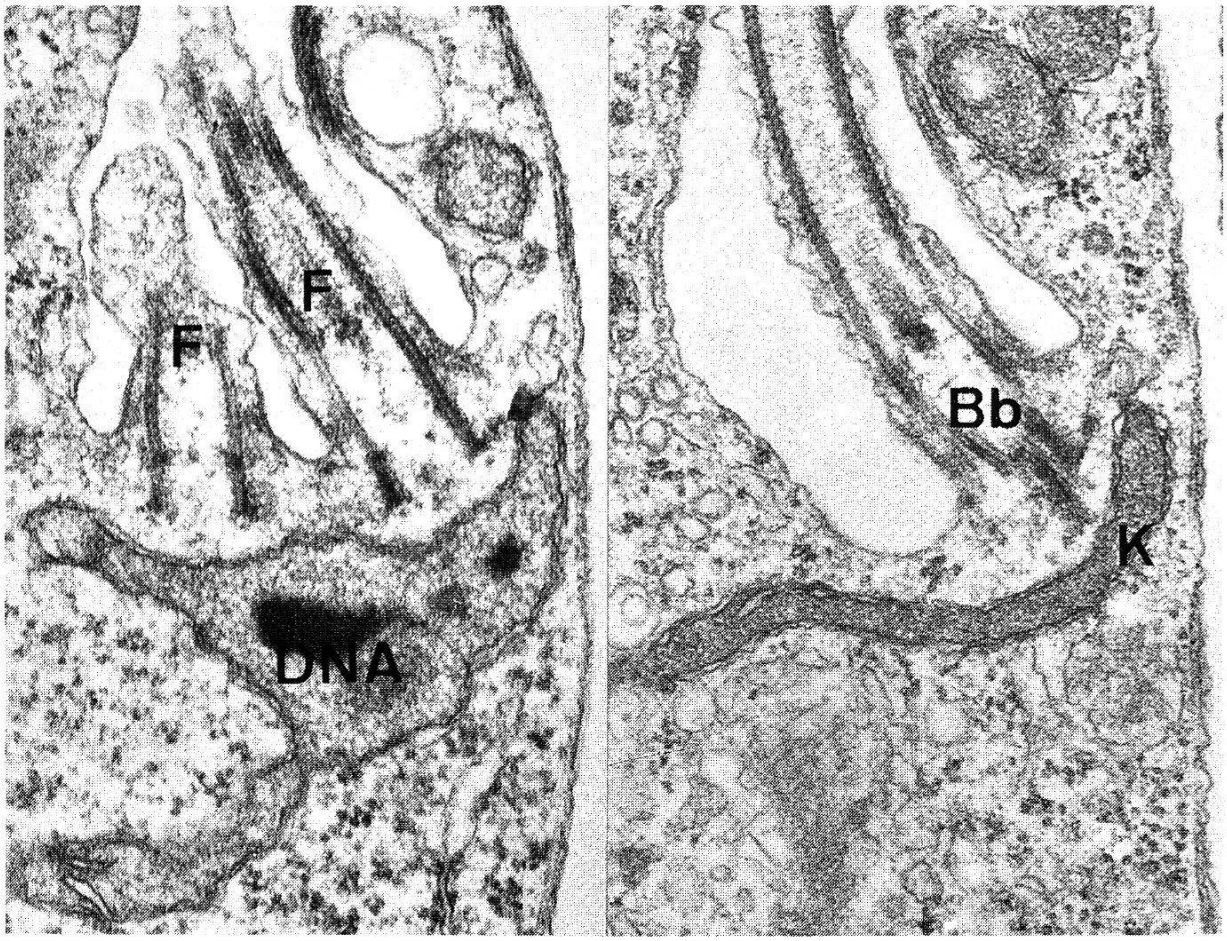

DNA
a
b
K
K1
K2
c
d



a b

Fig. 5. *Leishmania tarentolae* promastigotes grown in the presence of ethidium bromide (0.01 μg/ml) in Neo YE medium at 27° C. After 96 h about half of the cells contained a reduced and altered amount of kDNA although 91% of the cells were scored dyskinetoplastic by light microscopy. a) Longitudinal section of a cell containing two flagella (F). The kDNA is severely disordered and present as amorphous material. 40,500×. b) In this longitudinal section no kDNA is visible in the kinetoplast (K) region of the mitochondrion closest to the basal body (Bb). 40,500×.

none" division in which one daughter cell receives all the remaining kDNA, as described for e.g. *Crithidia fasciculata* (Cosgrove, 1966) and *Leishmania tarentolae* (Simpson, 1968) using acriflavine treated culture forms.

Electron microscopical examination of thin sections showed that EB treatment greatly reduced the amount of kDNA in the cells, although only a few cells possessed no detectable kDNA after 48 h. Non-network DNA may still be

Fig. 4. *Leishmania tarentolae* promastigotes grown in the presence of ethidium bromide (0.01 μg/ml) in Neo YE medium at 27° C. a) After 36 h in EB the cells show a shortened kDNA complex which has lost the normal parallel arrangement of the minicircles. 49,900×. b) After 48 h in EB about 50% of the cells were scored dyskinetoplastic by light microscopy. Electron microscopical examination revealed that most kinetoplasts still contained a reduced amount of kDNA. 40,100×. c) Dividing cell after 48 h in EB. "All-and-none" division with one daughter kinetoplast receiving a small, abnormal kDNA disc (K1) and the other daughter kinetoplast devoid of kDNA (K2). 40,200×. d) Rare case of a cell after 48 h in EB totally devoid of visible kDNA in the kinetoplast (K). 47,100×.

present in the mitochondrion of dyskinetoplastic forms, as this would probably be undetectable by EM (Hadjuk, 1976). The kDNA remaining after EB treatment was abnormal in its structure, exhibiting disturbances in the parallel alignments of the fibers and the formation of compact globular masses. After 96 h in the presence of EB only about half of the cells contained no kDNA at all although 91% of the same cells were scored dyskinetoplastic by light microscopy. The described alterations of the kDNA and its continuous loss are similar to those of Berenil treated *L. tarentolae* promastigotes (Leon et al., 1977) and of other EB and acriflavine treated flagellates (reviewed by Simpson, 1972).

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