EFFECTS OF ACRIFLAVINE ON THE MITOCHONDRIA AND KINETOPLAST OF *CRITHIDIA FASCICULATA*

Correlation of Fine Structure Changes with Decreased Mitochondrial Enzyme Activity

GEORGE C. HILL and WINSTON A. ANDERSON

From the Department of Biochemistry, University of Kentucky Medical School, Lexington, Kentucky 40506, and the Laboratoire de Biologie Cellulaire 4, Université de Paris, Faculté des Sciences, 91-Orsay, France. Dr. Anderson's present address is the Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

The effects of acriflavine on the fine structure and function of the mitochondria and the kinetoplast in *Crithidia fasciculata* have been investigated. A mitochondrial fraction was prepared by differential centrifugation of cells broken by grinding with neutral alumina. Isolated mitochondria or intact cells revealed by spectrophotometric measurements the presence of cytochromes $a + a_3$, b, c_{555}, and o. After cells were grown in acriflavine for 3–4 days, the fine structure of the mitochondria and their cytochrome content were affected. Cells grown in 5.0 μM acriflavine had a threefold decrease in cytochrome $a + a_3$ and decreased respiratory activity. The mitochondrial preparation from these cells had a fivefold decrease in cytochrome $a + a_3$ and a less but significant decrease of other cytochromes present. There was also a decrease in the mitochondrial enzyme activities of NADH, succinic and L-α-glycerophosphate oxidases, and succinic and L-α-glycerophosphate dehydrogenases. Dyskinetoplastic cells could be demonstrated after growth in 1.0 μM acriflavine. At 5 μM, 80–90% of the cells were dyskinetoplastic. The kinetoplastic DNA was condensed, nonfibrillar, and did not incorporate thymidine-^{3}H. The mitochondria in these cells had few cristae and were shorter and more swollen than the controls. Acriflavine may induce the fine structure effects we have observed and may affect the formation of the mitochondria in *C. fasciculata*.

INTRODUCTION

The biogenesis of mitochondria in eukaryotic cells remains an intriguing problem. This is especially true in blood trypanosomes and in insect trypanosomatids because of the large amount of cytoplasmic DNA they contain (15–20% of the total cellular DNA) (49). This extranuclear DNA is chiefly localized in a specific region of the mitochondrion, the kinetoplast. The continuity of the kinetoplast and mitochondrial envelope is well established (39). The kinetoplast-mitochondrion complex has been isolated in mitochondrial preparations which have electron-transport activity (16–18, 24).

Acriflavine, an antitrypanocidal drug, has

marked effect on the structure of the kinetoplast and mitochondria in members of the family Trypanosomatidae. As first observed at the ultrastructural level by Trager and Rudzinska (54) and Mühlpfordt (31, 32), this dye induces the loss of the DNA-containing material in the kinetoplast; however, the kinetoplast envelope remains. In the presence of acriflavine, the mitochondrial cristae degenerate or become vacuolated, while other organelles appear not to be affected. According to Trager and Rudzinska (54), these organisms are dyskinetoplastic.

Although acriflavine significantly affects the structure of the mitochondria in trypanosomes, there has been no correlation between the structural and the biochemical changes which occur when dyskinetoplastic organisms are produced. Acriflavine could be affecting the formation and function of mitochondria in trypanosomes, as it does in yeast (8, 9, 50).

Crithidia fasciculata is an insect trypanosomatid isolated from the gut and rectum of *Anopheles quadrimaculatus*. It grows in vitro in quantities sufficient for biochemical studies, in contrast to blood trypanosomes which cannot be maintained outside of a vertebrate in significant quantities without reverting to the vector form. The cytochrome system in *C. fasciculata* has been characterized as containing cytochromes $a + a_3$, b, c_{555}, and o (18). The present study reports various changes in the activity of mitochondrial enzymes and in the fine structure of the mitochondria and kinetoplast after the growth of *C. fasciculata* in the presence of acriflavine.

MATERIALS AND METHODS

Materials

Neutral acriflavine was obtained from Eli Lilly and Company, Indianapolis, Indiana. Just prior to use, acriflavine was dissolved in distilled water and sterilized by filtration. Protoporphyrin IX was obtained from Calbiochem (Los Angeles, Calif.). The stock solution of protoporphyrin was dissolved in a small amount of pyridine and diluted with 0.5 M NH_4OH to a final concentration of 3.0 mM. The solution was kept in the dark at −16°C. Tritiated thymidine (specific activity 6.7 c/mmole) was purchased from the New England Nuclear Corp. (Boston, Mass.).

Growth

Crithidia fasciculata (ATCC No. 11745) was grown in a medium containing 0.5% Trypticase (Baltimore Biological Laboratories, Baltimore, Md.), 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), 0.01% liver extract (Nutritional Biochemicals Corporation, Cleveland, Ohio), and 0.05 M D-xylose (Sigma Chemical Co., St. Louis, Mo.). The pH was adjusted to 8.0 with KOH. After the medium was autoclaved for 20 min at 121°C, it was cooled, and protoporphyrin was added at a final concentration of 0.75 μM unless otherwise indicated. The organisms were shaken in baffled sidearm flasks for 3–4 days, as described by Hill and White (18).

Preparation of the Mitochondrial Pellet

The mitochondrial pellet was prepared by grinding the cells with alumina and by differential centrifugation of the homogenate as described (18).

Respirometric and Spectrophotometric Procedures

Difference spectra and absolute spectra were performed as described by Hill and White (18). Oxygen uptake of mitochondria or cells was measured polarographically (58). Succinate and L-α-glycerol phosphate dehydrogenase activity were measured spectrophotometrically with 2, 6-dichlorophenol-indophenol as electron acceptor (29).

Electron Microscopy

Cells were fixed for 45–60 min in 2% OsO_4 in Na cacodylate buffer, pH 6.1 (44). The cells were then dehydrated, embedded, and stained as described (16). Thin sections were observed in a Siemens Elmiskop 1A electron microscope.

Thymidine-^3H Incorporation

Control and acriflavine-treated cells were centrifuged to a pellet and washed once with 0.05 M sodium phosphate buffer (pH 7.5). The cells were given a 30 min pulse of 50 μc/ml of thymidine-^3H as described (52). This incubation was stopped by mixing the cells with nine volumes of ice-cold buffered isotonic sucrose solution, containing 1 mg/ml carrier thymidine. The cells were centrifuged and then treated as previously described in this study for electron microscopic examination.

Preparation of Radioautographs

Specimens were prepared for radioautography according to Granboulan (11) and Droz (6). Ilford L4 emulsion was used to detect the radioactivity in the cells. After 4–5 wk of exposure, the radioautographs were developed for 4 min in Microdol (Eastman Kodak Co., Rochester, New York) and fixed for 5 min in sodium thiosulfate solutions.

TABLE I

Effects of Acriflavine on the Total Growth and Respiratory Activity of Crithidia fasciculata

Acriflavine concentration	Growth (absorbancy at 750 mμ)		Respiratory activity	
	D-Xylose	Glucose	D-Xylose	Glucose
μM			mμ moles O_2/min/mg protein	
0	0.80	1.00	38.2	18.6
1	0.71	0.90	36.0	16.2
5	0.31	0.28	33.6	11.6
10	0.11	0.12	14.8	6.8

The total growth of cells, grown on 0.05 M glucose or D-xylose, was measured as described in Materials and Methods. The cells were centrifuged, and an aliquot was used to measure initial endogenous respiratory rate. See Fig. 3 for the growth rate of the cells.

RESULTS

Growth of Cells

Sucrose or glucose has usually been used as carbon source in nutritional studies with insect trypanosomatids. In order to induce as high a respiratory activity as possible in *C. fasciculata*, a comparison was made of growth of cells on D-xylose and glucose. The respiratory activity of cells grown on xylose was twice that of cells grown on glucose (Table I). The generation time of cells growing on D-xylose was 7 hr, in contrast to 5 hr for cells growing on glucose (Fig. 1). The total cell yield was usually the same. The acid production decreased markedly in cells grown with xylose.

Properties of Isolated Mitochondria

The isolated mitochondria could be completely reduced in a few minutes in the presence of 10.0 mM succinate. Succinate oxidase activity was 40.2 mμmoles O_2/min/mg protein as compared to an endogenous respiratory rate of 38.2 mμmoles O_2/min/mg protein for the intact cells.

Respiratory pigments, measured spectrophotometrically, of intact cells or of isolated mitochondria were essentially the same. Fig. 2 illustrates a succinate reduced minus oxidized difference spectrum and a carbon monoxide difference spectrum of the mitochondrial preparation. The spectrum of mitochondria reduced with succinate minus mitochondria oxidized in the presence of air suggests the presence of cytochrome *a* + a_3 (605 mμ), cytochrome *b* (559 mμ), and cytochrome *c* (553 mμ). In the CO difference spectrum, the following two CO-binding pigments were evident: cytochrome a_3 with an α peak at 590 mμ and a Soret at 432 mμ, and cytochrome oxidase *o* with peaks at 570 mμ, 540 mμ, and 410 mμ.

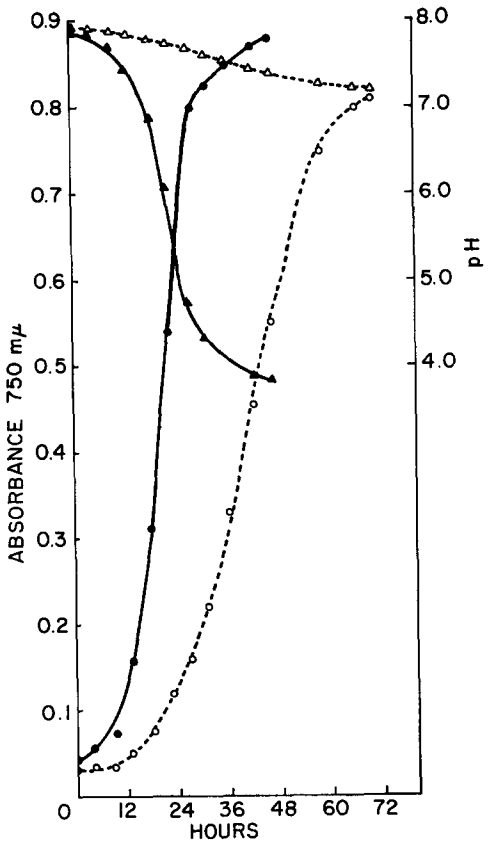


FIGURE 1 Growth of cells on glucose (●—●) is compared to growth of cells on xylose (○—○). The pH in cells grown on glucose (▲—▲) dropped, while the pH of cells grown on xylose remained above pH 7 (△—△). Cells were grown at 1.5 μM protoheme.

EFFECTS OF ACRIFLAVINE

Growth of Cells

The growth of *C. fasciculata* was markedly affected by the presence of acriflavine (Fig. 3). In the presence of 5.0 μM acriflavine, there is a 50–60% decrease in total growth of the cells and an increased generation time of the cells.

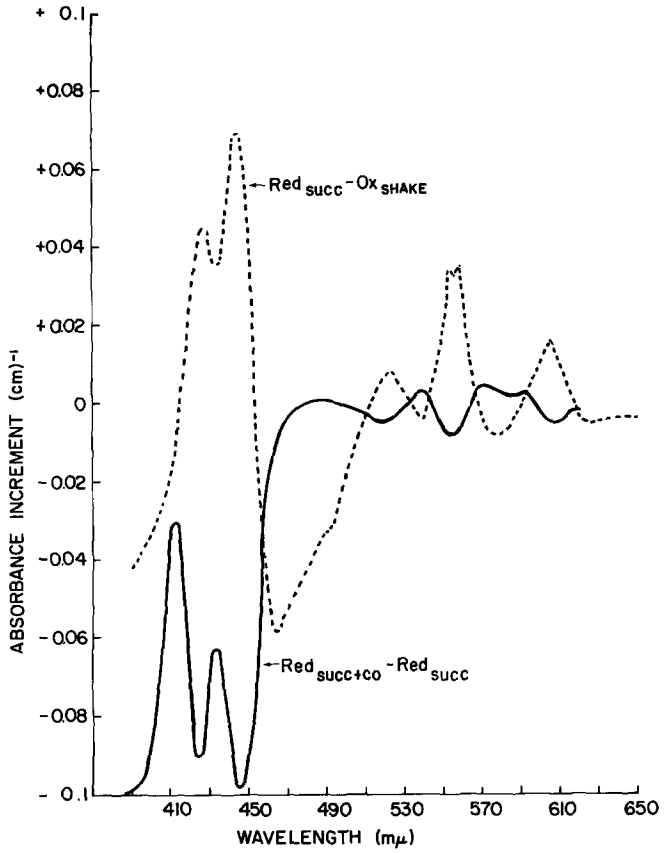


FIGURE 2 Difference spectra of mitochondria isolated from *C. fasciculata* grown with 1.5 μM protoporphyrin. Dashed line represents the difference spectrum of mitochondria with pigments reduced in the presence of 10 mM succinate, as compared with mitochondria with the pigments oxidized by shaking in air. Reduction of pigments was complete 2–3 min after addition of succinate. Solid line represents the difference spectrum of mitochondria reduced in the presence of succinate and saturated with carbon monoxide for 10 min, as compared with mitochondria reduced in the presence of succinate. The protein concentration was 10.0 mg of mitochondrial protein/ml. All other procedures are described in Materials and Methods.

This effect of acriflavine was observed whether the cells were growing with xylose or glucose.

Respiratory and Enzyme Activity

The endogenous respiration of the cells was decreased by growth in acriflavine (Table I). The respiration of the control and drug-treated cells was inhibited 70–80% by 0.01 M sodium azide.

The relative amounts of cytochromes $a + a_3$, b, and c in cells grown at 5.0 μM acriflavine were measured from the absolute spectrum of the cells (Table II). Nearly three times more cytochrome $a + a_3$ was present in the control as compared to that present in acriflavine-treated cells. The respiratory activity of the cells was inhibited 40%.

Mitochondria were isolated from these cells grown in 5.0 μM acriflavine. Enzyme activities were decreased to varying degrees (Table III). From the difference spectrum, the marked decrease of functional cytochrome $a + a_3$ in these isolated mitochondria can be seen (Fig. 4). Control mitochondria had 10-fold higher cytochrome $a + a_3$ than mitochondria isolated from acriflavine-treated cells. There was ⅓ more cytochrome b and cytochrome c in control mitochondria. No cytochrome a_3 was suggested either at 590 mμ or at 432 mμ (Fig. 5).

The turnover number for the cytochrome oxidase activity (micromoles O_2 per minute per absorbancy increment × 1000) was 2.58 for control mitochondria and 1.08 for mitochondria from acriflavine-treated cells.

Ultrastructural Observations

Dyskinetoplastic cells could be detected after *C. fasciculata* was grown in 1.0–10.0 μM acriflavine. The increase in dyskinetoplasty was proportional to the concentration of acriflavine present between 1.0 and 5.0 μM (Fig. 6). The fine structure of the kinetoplast of most cells treated with 1.0 μM acriflavine was not greatly affected (Figs. 7 and 14). On the basis of counts of 250–300 cells, 75% of the kinetoplasts and mitochondria were found

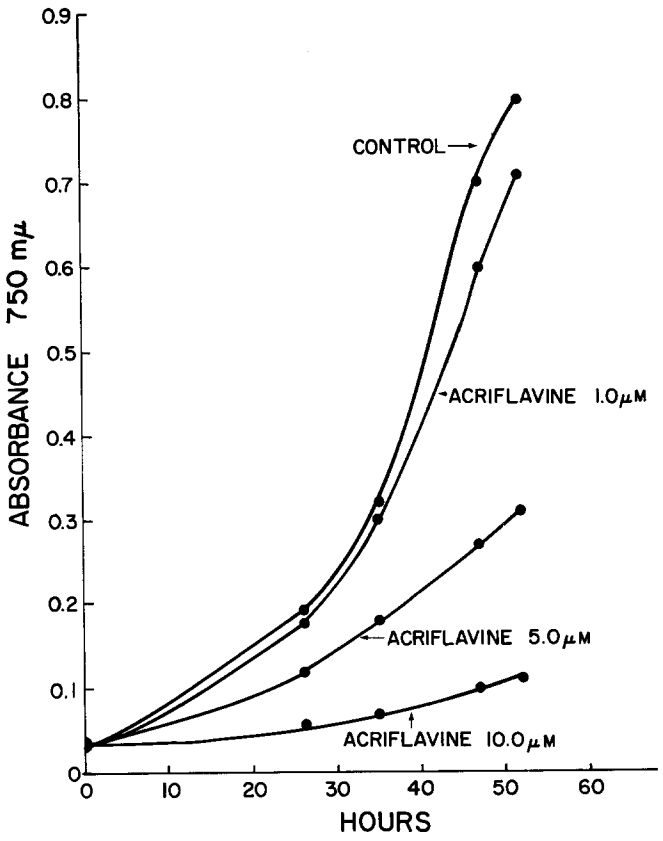


FIGURE 3 Effect of acriflavine on the growth rate of *C. fasciculata*. Cells were inoculated into medium containing designated amounts of acriflavine. 0.05 M D-xylose and 0.75 μM protoporphyrin were present. Growth was measured as described in Materials and Methods.

to be structurally unchanged. Mitochondria extending from the kinetoplast or free in the cytoplasm were unaltered and displayed the presence of well-formed cristae. In most cases, cell division was normal, and each cell received a normal kinetoplast. In some cases, however, the division of the kinetoplast resulted in one organism becoming bikinetoplastic (Fig. 8). The fine structure of the nucleus was not affected by any concentration of acriflavine.

Cells treated with 5.0 μM acriflavine were 85% dyskinetoplastic. The DNA-containing fibers of the kinetoplast were markedly affected; they appeared thicker and more electron opaque (Figs. 15 and 16) and were retracted from the envelope of the mitochondrion. The fibers were reduced to an amorphous, granular or fibrous mass within the mitochondrion (Figs. 9, 10, and 16). In comparison to controls, the mitochondria attached to the kinetoplast or free in the cytoplasm lacked well-developed cristae; the free mitochondria were swollen with enlarged cristae that sometimes appeared as vesicles (Figs. 9 and 10).

With an increase in concentration of acriflavine (10.0 μM), only 2% of the cells showed unmodified kinetoplasts. The kinetoplast and mitochondria appeared similar to those in cells treated with 5.0 μM acriflavine. After cell division, the kinetoplast was reduced in size and lost its original relationship to the basal body of the flagellum. Binucleate cells were common.

Thymidine-^3H Observations

Normal cells pulsed for 30 min demonstrated radioactive kinetoplasts (Figs. 11 and 12). Silver grains were restricted to the nucleoid of the kinetoplast, with some label over the basal body. Acriflavine-treated cells rarely incorporated thymidine-^3H into the kinetoplast; however, the nucleus continued to synthesize DNA in cells treated with 1.0 and 5.0 μM acriflavine. In cells cultured in 1.0 μM acriflavine, silver grains were observed over the centriole and the nucleus but not over

TABLE II

Effect of Acriflavine on the Cytochrome Content and Respiratory Activity of Crithidia fasciculata

Cells	Absorbance increment × 1000/mg protein			Respiratory activity
	605 mμ	558 mμ	552 mμ	
				mμmoles O_2/min/mg protein
Control	0.3048	1.3008	1.5040	40.2
Acriflavine 5.0 μM	0.1159	0.8115	0.8695	24.0
Relative amounts				
Control cells:Acriflavine-treated cells	2.6:1.0	1.6:1.0	1.7:1.0	

Cells were grown in 5.0 μM acriflavine for 3 days. Absolute spectra and O_2 uptake were measured as described in Materials and Methods.

the partially modified nucleoid of the kinetoplast (Fig. 13).

DISCUSSION

The cytochrome system of *C. fasciculata* contains cytochromes $a + a_3$, *b*, *c*, and *o*. While cytochrome *c* has been suggested to be absent in this organism (24), it is readily detected in succinate-reduced minus oxidized difference spectra of isolated mitochondria from cells grown with limiting concentrations of protoporphyrin or protoheme. Cytochrome c_{555} has been extracted from cells and isolated mitochondria and partially purified (18). At high concentrations of protoheme or protoporphyrin in the medium, the increased formation of functional cytochrome *b* obscures the detection of cytochrome *c* in room temperature difference spectra.

The studies reported on cytochromes in insect trypanosomatids and blood trypanosomes support the probable presence of a similar cytochrome system in all cyanide-sensitive members of the family Trypanosomatidae (1, 10, 22, 27, 40–43). The stumpy blood trypanosomes appear to possess a cytochrome system (57), in contrast to the slender bloodstream forms which lack detectable cytochrome pigments and possess an L-α-glycerol phosphate oxidase–L-α-glycerol phosphate dehydrogenase cycle (12–14). The development of a cytochrome system during the transition from slender to stumpy trypanosomes in the vertebrate's bloodstream is essential if the pathogen is to return to the vector. If the formation of the cytochrome system in the stumpy trypanosomes could be prevented, perhaps the life cycle of pathogenic trypanosomes could be stopped.

TABLE III

Effects of Acriflavine on Mitochondrial Enzyme Activity in Crithidia fasciculata

Enzyme	Mitochondria from control cells	Mitochondria from acriflavine-treated cells
Succinic oxidase	35.9	7.2
NADH oxidase	7.0	1.4
L-α-glycerol phosphate oxidase	7.5	3.8
Succinic dehydrogenase	0.0890	0.0225
L-α-glycerol phosphate dehydrogenase	0.0771	0.0154

Mitochondria were isolated from control cells and cells grown in 5.0 μM acriflavine. All enzyme activities were measured as described in Materials and Methods. The specific activities of the oxidase enzymes is expressed as mμmoles O_2/min/mg protein. The specific activity of dehydrogenases measured is expressed as Δ OD at 600 mμ/min/mg protein.

The effects of acriflavine on the fine structure of members of the family Trypanosomatidae have been reported by numerous investigators (23, 31, 32, 53, 54, 56). Dyskinetoplastic organisms have also been observed in our studies. We have observed these changes when *C. fasciculata* was grown in low concentrations of acriflavine (e.g. 2.59 μg/ml). Mitochondria of acriflavine-treated cells were consistently swollen and displayed vesicular

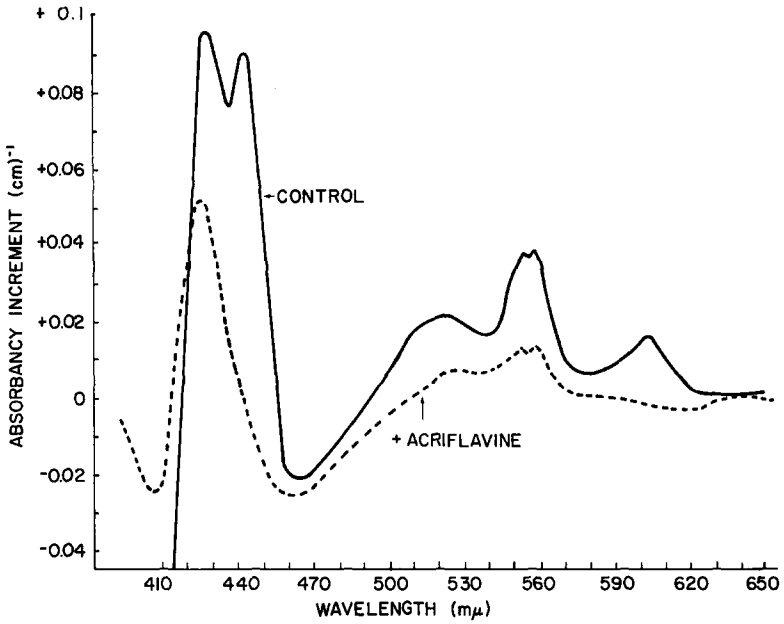


FIGURE 4 Difference spectra of isolated mitochondria from *C. fasciculata* with pigments reduced in the presence of 10 mM succinate compared with mitochondria with the pigments oxidized by shaking in air. The solid line represents mitochondria isolated from control cells. The dashed line represents mitochondria isolated from cells grown in 5.0 μM acriflavine. The protein concentration was 10.0 mg of mitochondrial protein/ml.

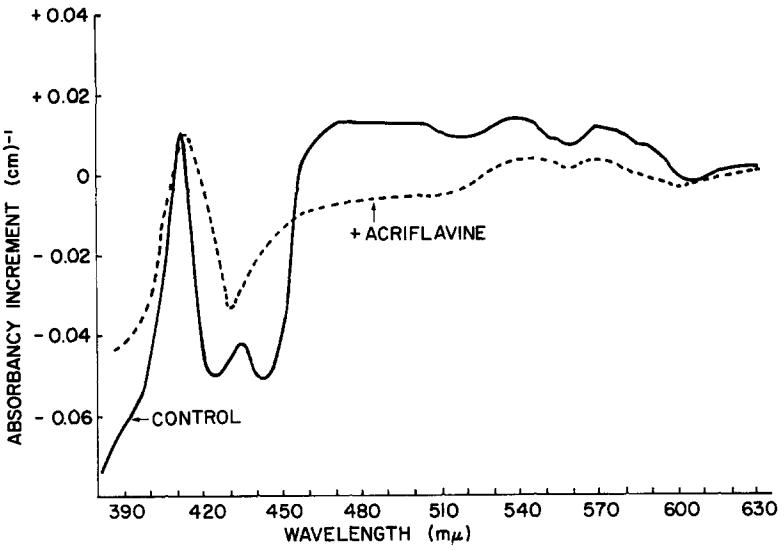


FIGURE 5 Difference spectra of isolated mitochondria from *C. fasciculata* reduced in the presence of succinate and saturated with carbon monoxide compared with mitochondria reduced in the presence of succinate. The solid line represents mitochondria isolated from control cells. The dashed line represents mitochondria isolated from cells grown in 5.0 μM acriflavine. The protein concentration was 10.0 mg of mitochondrial protein/ml.

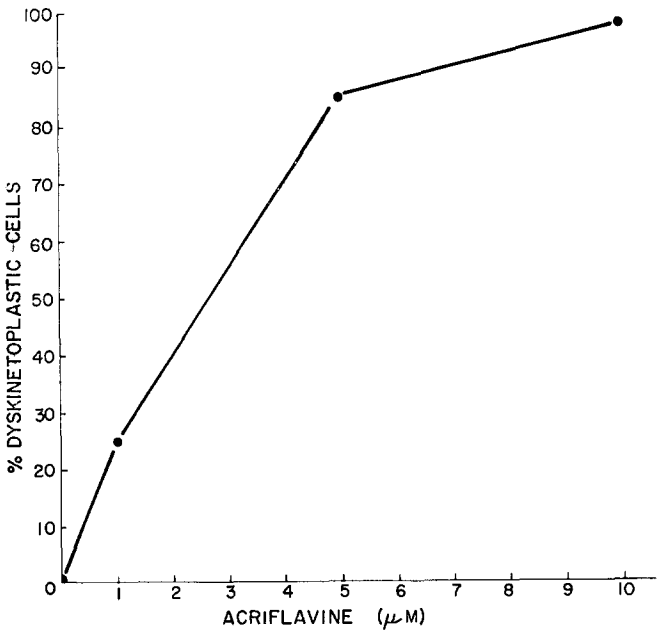


FIGURE 6 The effect of acriflavine on the formation of dyskinetoplastic cells in *C. fasciculata*. The percentage of dyskinetoplastic cells was determined by examination of 250–300 cells with the electron microscope. Cells in which the DNA fibers of the kinetoplast were thicker, more electron opaque, amorphous or atypical in comparison to the controls were regarded as dyskinetoplastic.

cristae. Previous investigators using *C. fasciculata* have reported the production of dyskinetoplastic organisms, but 10–20 times more acriflavine was required to produce their results (2, 3, 15, 23). However, Simpson (49) has also observed that very low levels of acriflavine (e.g. 136 mμg/ml) produced 62% dyskinetoplasty in *Leishmania tarentolae* after 3–4 days of growth. He also observed that hemin and protoporphyrin formed an intracellular complex with acriflavine in *L. tarentolae*. This could affect the formation of hemoproteins in heme-requiring trypanosomatids.

The effects of acriflavine on the formation of the mitochondria result in decreased respiration of the cells, decreased activities for mitochondrial enzymes, and a significant decrease in the level of functional cytochrome $a + a_3$. Numerous investigators have reported effects of acriflavine on mitochondrial formation. In yeast, acriflavine induces the formation of "petites," cytoplasmic respiratory deficient mutants which have been phenotypically characterized by a lack of the mitochondrial enzymes, cytochromes $a + a_3$ and b (4, 5, 8, 9, 33, 47, 50). The effect of the cytoplasmic mutation in yeast appears to be confined to the insoluble enzymes of the mitochondrial cristae, while cytochrome c and a number of readily solubilized citric acid cycle enzymes of the mitochondria are still formed in appreciable amounts by the organism (28, 29, 48).

The effects of acriflavine in *C. fasciculata* suggest the interference by this drug in the formation of mitochondria. Strong evidence exists for the intercalation of the acridines between two otherwise sequential DNA base pairs (25, 26, 35). Tubbs et al. (55) have shown that the binding of acriflavine to DNA is particularly strong in the case of DNA with a high adenine-thymine base pair composition. Guttman and Eisenman (15) suggested that the degree of dyskinetoplasty produced in *C. fasciculata* is related to the increase in adenine-thymine: guanine-cytosine base pair ratio in the kinetoplast DNA. This formation of a DNA-acriflavine complex may explain the absence of extractable mitochondrial or satellite DNA in DNA preparations from dyskinetoplastic cells (30, 52).

Acriflavine also inhibits the action of purified RNA polymerase, preventing the formation of mRNA from DNA templates (19, 20, 34, 45, 46, 51, 59). This could also be the result of intercalation of acriflavine with DNA. Richardson (36) has shown that acriflavine prevents the binding of RNA polymerase along specific sites of the DNA molecule, thus preventing transcription from occurring at well-defined regions. It seems possible that the acriflavine prevents the transcription of template mitochondrial DNA into mRNA, thus preventing the translation of mRNA into a specific protein. If this is so, there would be a decrease in

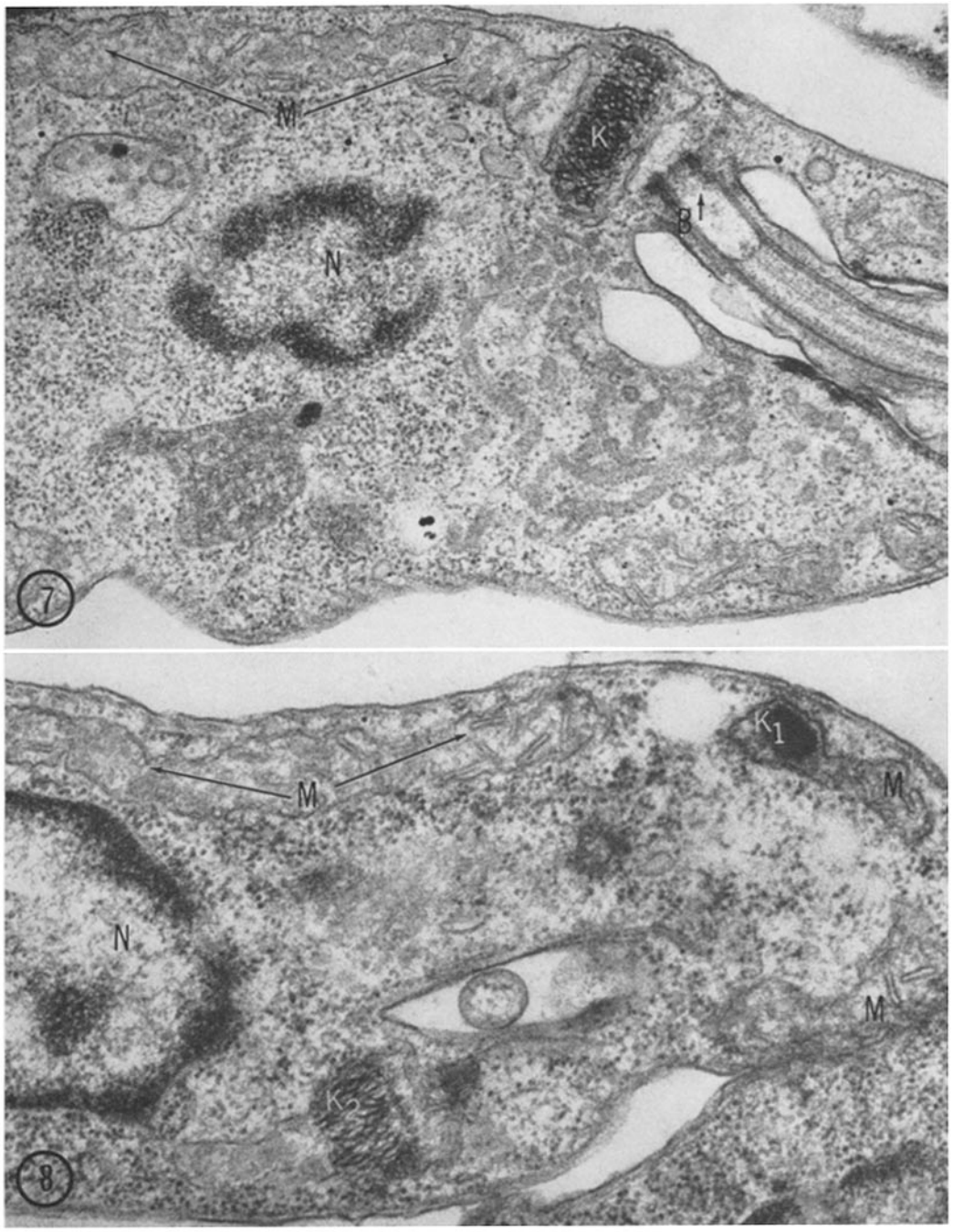
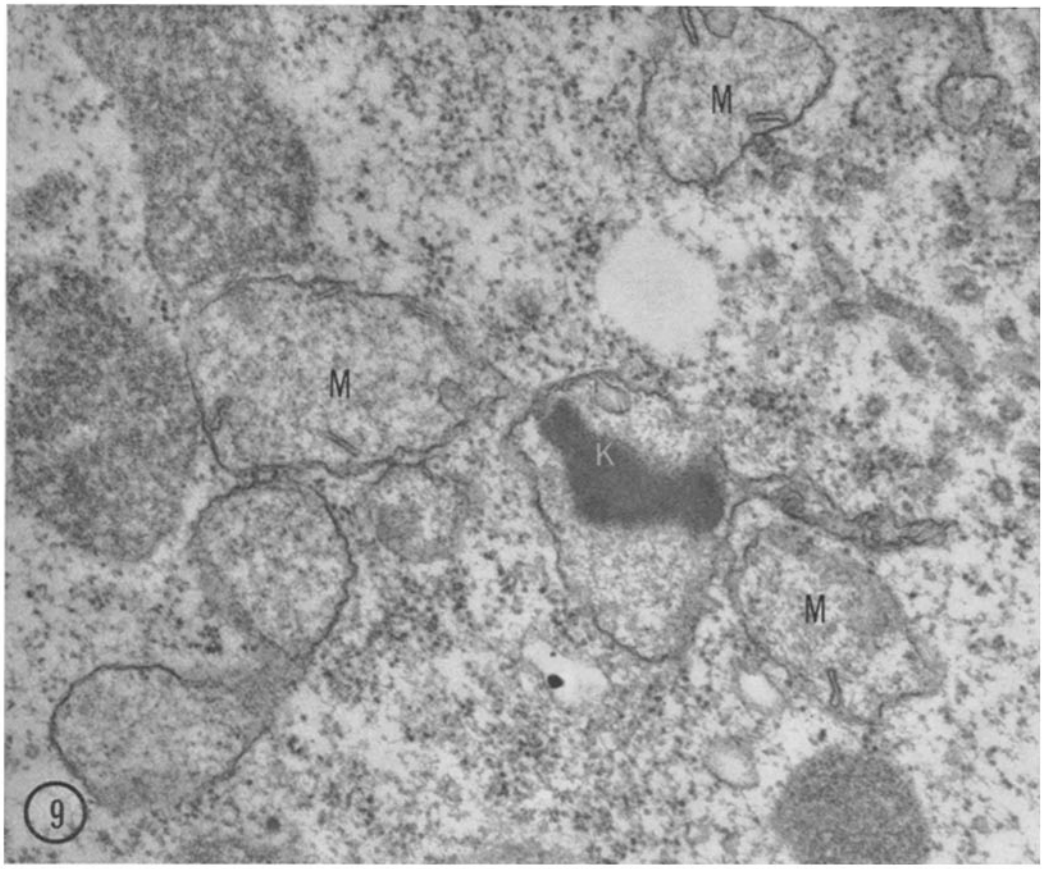


FIGURE 7 *Crithidia fasciculata*, after growth for 3 days in 1.0 μM acriflavine. The kinetoplast (*K*) and mitochondria (*M*) are unaffected. The DNA fibers of the kinetoplast appear normal and the associated mitochondrion has well-developed cristae. *B*, basal body; *N*, nucleus. ×36,000.

FIGURE 8 *Crithidia fasciculata*, after growth for 3 days in 1.0 μM acriflavine. The cell has two kinetoplasts, one normal (K_2) and one dyskinetoplastic (K_1). The latter kinetoplast shows the condensed state of the DNA and its detachment from the envelope. The mitochondria (*M*) are typical. *N*, nucleus. ×36,000.



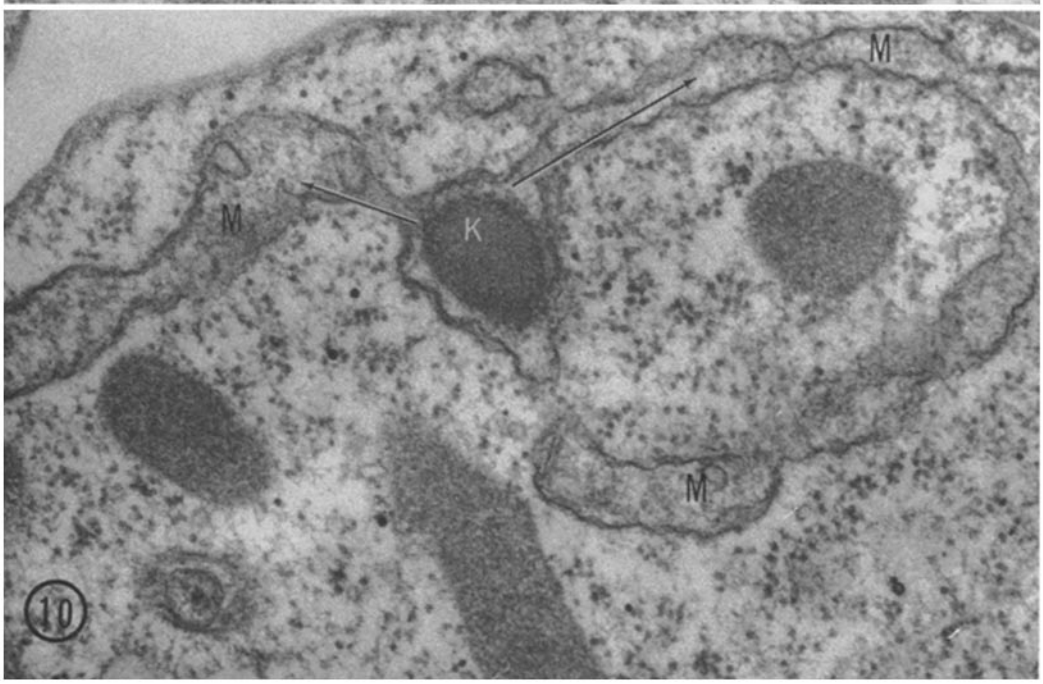
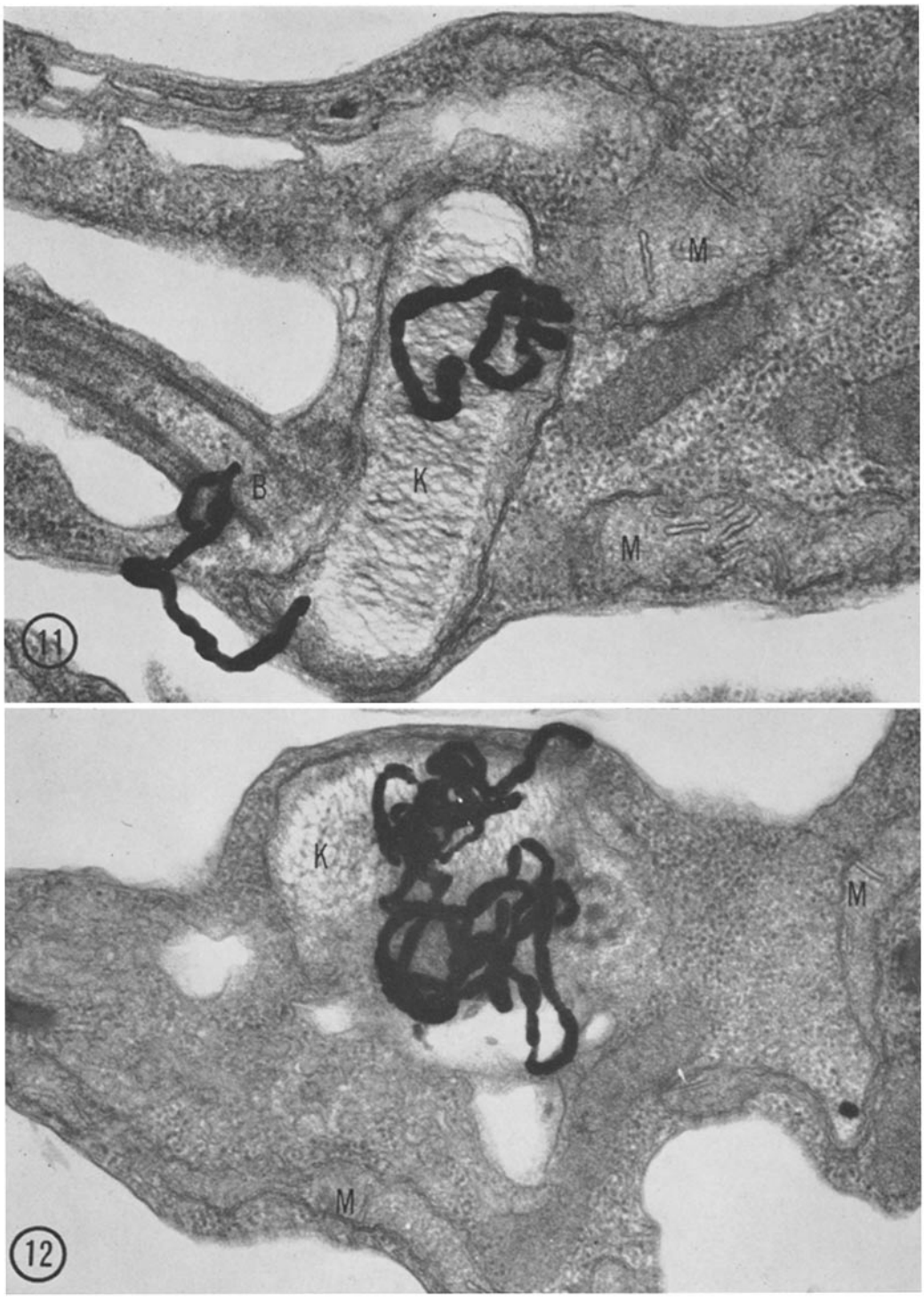


FIGURE 9 *Crithidia fasciculata*, treated for 3 days with 5.0 μM acriflavine. Observe the condensed nature of the kinetoplast DNA (*K*). The mitochondria (*M*) are swollen, with few if any cristae. ×50,000.

FIGURE 10 *Crithidia fasciculata*, treated for 3 days with 5.0 μM acriflavine. The mitochondria (*M*, arrows) extending from the kinetoplast (*K*) have few vesicular cristae. ×40,000.



FIGURES 11 and 12 *Crithidia fasciculata*, control cells pulsed with thymidine-^{3}H for 30 min. Silver grains are located over the DNA-containing fibers of the kinetoplast (*K*). Adjacent mitochondria (*M*) are not radioactive. *B*, basal body. Fig. 11, ×80,000; Fig. 12, ×50,000.

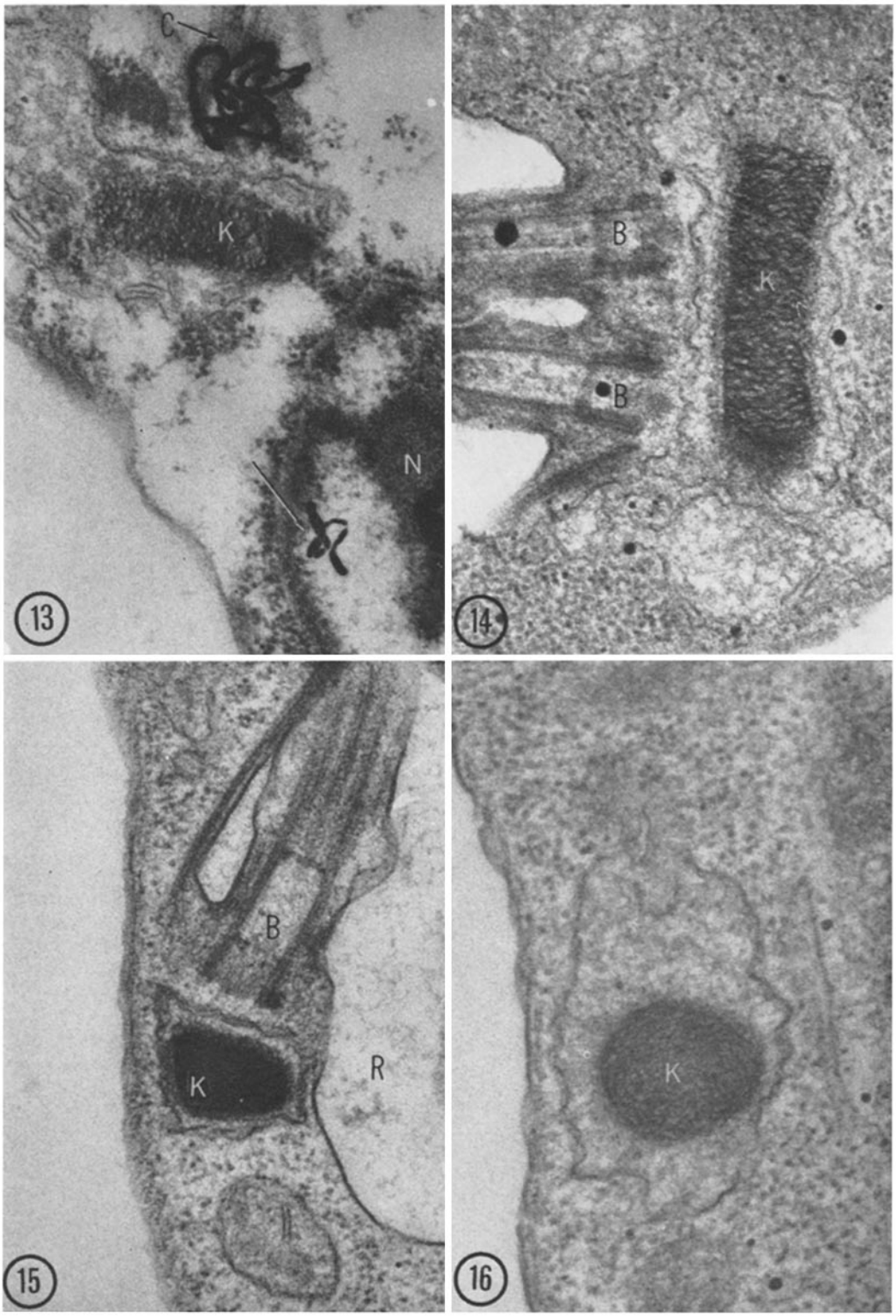


FIGURE 13 *Crithidia fasciculata*, cultured for 3 days in 1.0 μM acriflavine and pulsed for 30 min with thymidine-^{3}H. In this radioautograph, silver grains are located over the nucleus (*N*, arrow) and the centriole (*C*). The kinetoplast (*K*) is not labeled. ×36,000.

FIGURE 14 *Crithidia fasciculata*, cultured in 1.0 μM acriflavine for 3 days. The kinetoplast (*K*) DNA fibers are slightly modified, and no mitochondrial cristae are present. The kinetoplast maintains its original relationship to the basal bodies (*B*), in comparison to Figs. 9 and 10. ×40,000.

FIGURES 15 and 16 *Crithidia fasciculata*, after growth in 5.0 μM acriflavine. The highly condensed DNA fibers of the kinetoplast (*K*) are retracted from the envelope of the kinetoplast. *B*, basal body; *R*, reservoir. Fig. 15, ×40,000; Fig. 16, ×50,000.

proteins synthesized by the mitochondria; and we have observed a decrease in particulate mitochondrial enzyme activities. However, these enzymes may not be synthesized in the mitochondria. Kadenbach (21) has provided evidence that isolated rat liver mitochondria incorporate labeled amino acids preferentially into the structural protein. Only 22% of incorporated activity was found in other structurally bound proteins, and no activity was observed in soluble protein (enzyme). It seems clear that cytochrome *c* is not synthesized in the mitochondria (37).

There is little doubt that mitochondrial DNA exists in a double-stranded form. Its rapid rate of renaturation after thermal denaturation has been observed in various organisms and indicates that mitochondrial DNA may be more homogeneous in its base composition than nuclear DNA. Presumably, as discussed by Roodyn and Wilkie (38), a DNA that codes for the synthesis of one or two proteins would be more homogeneous in base composition than a DNA coding for hundreds of proteins. DuBuy et al. (7) found that the kinetoplast DNA isolated from *Leishmania enrietti* renatured 80–90% and did so more rapidly than the nuclear DNA. Acriflavine could prevent the formation of structural protein, which could explain the decrease in the number of cristae in mitochondria of the dyskinetoplastic organisms. Studies on the observation of the effects of acriflavine on the synthesis of mitochondrial proteins in isolated mitochondrial preparations from trypanosomatids should help to shed light on these hypotheses.

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