

EVIDENCE FOR THE RETENTION OF KINETOPLAST
DNA IN AN ACRIFLAVINE-INDUCED
DYSKINETOPLASTIC STRAIN OF *TRYPANOSOMA*
BRUCEI WHICH REPLICATES THE
ALTERED CENTRAL ELEMENT OF THE KINETOPLAST

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ABSTRACT

A pleomorphic dyskinetoplastic strain of *Trypanosoma brucei* was produced by repeated acriflavine treatment. No kinetoplastic cells reappeared after 2 yr of maintenance in the absence of acriflavine. These dyskinetoplastic cells retained and therefore replicated the central element of the kinetoplast. This element was present in the "condensed" state typical of acriflavine-treated cells rather than the normal fibrillar state. Whole-cell DNA extracted from both normal and dyskinetoplastic strains revealed three bands upon isopycnic sedimentation, and there was no detectable alteration in buoyant density of any of these DNA components in the dyskinetoplastic strain. It seems likely that the dyskinetoplastic strain has retained its kinetoplast DNA but in an altered state.

INTRODUCTION

Flagellated protozoa belonging to the Order Kinetoplastida (4) typically contain a single tubular mitochondrion or an interconnecting tubular mitochondrial network. The kinetoplast, originally observed as a Giemsa-staining granule at the base of the flagellum, is an enlarged area of the mitochondrial tubule. This enlarged area contains DNA and fine fibrils. The fibrils, which are arranged parallel to one another, are thought to be the site of localization of kinetoplast DNA (see references 9 and 12 for review). Kinetoplast DNA in some species differs in buoyant density from nuclear DNA (3) and, like mitochondrial DNA, is circular (11), catenate (10), and is synthesized within the organelle (15). The role of kinetoplast

DNA is obscure but it may be essential for mitochondrial respiration.

Acriflavine treatment of kinetoplastid flagellates results in the production of cells in which the kinetoplast is no longer stainable with Giemsa's (18), and the fine fibrils within the kinetoplast are altered. These cells have been termed "dyskinetoplastic" (17). Kinetoplastid flagellates may be divided into two groups with reference to dyskinetoplasia. One group contains species unable to be propagated in the dyskinetoplastic condition. Such cells are cyanide sensitive (1) and probably require mitochondrial respiration. Acriflavine treatment of these cells results in the production of a high percentage of dyskinetoplastic cells but

not strains in which all cells are dyskinetoplastic. The cells may divide only a few times in the presence of the drug but then die. Studies on this group have suggested that acriflavine specifically inhibits kinetoplast DNA synthesis, resulting in cells devoid of kinetoplast DNA (14, 16). It has also been suggested that the fibrillar element of the kinetoplast became progressively smaller during drug treatment and eventually disappeared (16, 17). The second group contains species able to be propagated in the dyskinetoplastic condition. These cells are cyanide insensitive and probably do not require mitochondrial respiration (7, 8). All species in the second group belong to the subgenus *Trypanozoon*. Dyskinetoplastic strains in which all cells lack a Giemsa-stainable kinetoplast have been produced in this group by using acriflavine (7, 8). These dyskinetoplastic cells were reported to lack the central element of the kinetoplast, but no analysis of DNA composition was performed.

This paper reports the acriflavine induction of a dyskinetoplastic strain of *Trypanosoma brucei*. The cells of this strain, after propagation in the absence of acriflavine, retained the "condensed" central element of the kinetoplast, and their DNA composition did not differ detectably from that of the parental kinetoplastic strain. It seems likely that these cells retained kinetoplast DNA in an altered state.

MATERIALS AND METHODS

A pleomorphic strain of *Trypanosoma brucei* (London School of Hygiene, reference No. 14/2/164) and its derived dyskinetoplastic strain were maintained under conditions promoting chronic infection. At 4-day intervals rats (Long-Evans) were infected (all injections for infection and drug treatment were intraperitoneal) with tail blood from an infected mouse *Peromyscus leucopus* until the latter died. At weekly intervals *Peromyscus* were inoculated with blood from rats which had been infected for at least 8 days. Thus all injections for maintenance were between species, with *Peromyscus* as a reservoir.

A dyskinetoplastic strain was produced by repeated acriflavine treatment while the trypanosomes were passaged through Swiss mice. Mice were injected with a sterile solution of acriflavine (Nutritional Biochemicals Corporation, Cleveland, Ohio) in Locke's solution each time the infection became fairly heavy, about 10^5 cells/ml, and subinoculations were made daily after acriflavine treatment. Initially about 0.005 ml was transferred at each subinoculation, but later in the experiment about 0.3 ml was transferred. The

level of the infection was estimated by observing wet mounts under phase contrast, and the per cent of dyskinetoplastic cells was determined by scoring 200 cells stained with Giemsa's stain after methanol fixation and 5 min hydrolysis in *N* HCl at 60°C. This procedure was continued until no kinetoplastic cells reappeared in mice infected with the dyskinetoplastic strain but not treated with acriflavine.

The isolation of trypanosomes for electron microscopy and DNA extraction was by the method of Lanham (6) which utilized diethylaminoethyl-ion-exchange cellulose. The trypanosome layer, recovered after centrifugation of infected blood, was diluted with buffer 1:3 and applied to the column. The eluted trypanosomes, thus freed from rat blood cells which remained in the cellulose column, were then washed twice by centrifugation with buffer.

The isolated, washed cells were fixed for 30 min in an ice-cold, freshly prepared mixture of 1.0 ml, 0.2 M, pH 7.4 phosphate buffer, 0.5 ml 5% osmium tetroxide, and 1.5 ml 2.4% glutaraldehyde. After fixation the cells were washed in buffer, suspended in 30% ethanol, and embedded in 2% agar. The embedded cells were then dehydrated, embedded in Epon, sectioned, and stained for 2 min each in uranyl acetate and lead citrate. The sectioned cells were examined in a RCA EMV 3b electron microscope.

DNA was isolated after lysing about 1.5 ml packed cells with 12 ml 0.1% sodium dodecyl sulphate (SDS) containing 500 µg/ml pronase, and incubating for 1 hr in an ice bath. SDS (1.33%) was added to a final volume of 45 ml, and the sample was gently homogenized with a Teflon homogenizer. The nucleic acids were sedimented into a 1 ml 20% sucrose cushion containing 1.33% SDS at a speed of 49,000 rpm for 18 hr in a SW-50 rotor. The nucleic acids were dispersed in 40 ml Tris (1 mM, pH 8.0), exhaustively dialyzed against Tris, and centrifuged at 10,000 rpm for 20 min to remove the resultant precipitate. After treatment with RNase (3 mg total for 30 min at 37°C), SDS was added to a concentration of 0.02% and the DNA was sedimented into a 1.5 ml sucrose cushion (without SDS) by 23 hr centrifugation at 49,000 rpm. The resultant gelatinous pellet was rinsed carefully with Tris without being dislodged after the supernatant and sucrose cushion had been removed. The pellet was dispersed in 16 ml Tris, dialyzed overnight, and centrifuged at 10,000 rpm for 20 min.

Approximately 3 µg of this DNA was dissolved in cesium chloride solution ($\rho = 1700$ g/cc) and centrifuged in a Spinco Model E analytical centrifuge at 44,000 rpm for 20 hr. Ultraviolet absorption photographs were scanned with a Joyce-Loebl microdensitometer (Joyce, Loebl and Co., Inc., Burlington, Mass.) and buoyant densities were calculated according to Schildkraut et al. (13) with *Micrococcus lysodeikticus* DNA ($\rho = 1731$ g/cc) as a density marker.

RESULTS

The production of a strain of *Trypanosoma brucei* in which all cells were dyskinetoplastic required 102 days, 22 acriflavine injections, and 14 subinoculations (Figs. 1 and 2). In the first five mice there was great fluctuation in population size and per cent dyskinetoplastic cells. These mice had been inoculated with about 0.005 ml blood. The last 10 mice were inoculated with about 0.3 ml blood, and the numbers of trypanosomes and the per cent dyskinetoplastic cells increased steadily in these animals. The trypanosomes in animals 10–14 were not all dyskinetoplastic. Kinetoplastic cells were seen in the blood of mice subinoculated from these animals but not treated with acriflavine. However, no kinetoplastic cells appeared in such a line derived from animal 15. This line was then maintained in *Peromyscus* and rats and has remained free of kinetoplastic cells for 2 yr. Like the parental strain, the dyskinetoplastic strain remained pleomorphic, but fewer intermediate and stumpy cells were seen at any given time. Up to 84% intermediate and stumpy forms were seen in kinetoplastic populations, while no more than 12% were seen in the dyskinetoplastic strain.

An attempt was made to grow both the kinetoplastic and dyskinetoplastic strains in Pittams culture medium. Although neither strain became established in culture, the kinetoplastic cells remained motile for 2 wk, whereas the dyskinetoplastic cells stopped moving within hours.

The ultrastructure of the kinetoplast in the dyskinetoplastic cells differed from that in normal cells. This organelle in the kinetoplastic cells (Fig. 3) was bound by a double membrane, continuous with the rest of the tubular mitochondrion, and both structures contained a matrix that was generally denser than the surrounding cytoplasm. The normal kinetoplast contained the typical central element (11) composed of darkly stained fibrils aligned parallel to one another. These fibrils were embedded in an electron-transparent matrix. Both the kinetoplast and the mitochondrion contained occasional membranous profiles reminiscent of cristae. The kinetoplast in dyskinetoplastic cells (Fig. 4) did not contain the typical fibrillar element but instead contained a darkly stained dense body. The occasional membranous profiles appeared swollen in the dyskinetoplastic cells. Other than these two features, the two strains were identical in ultrastructure.

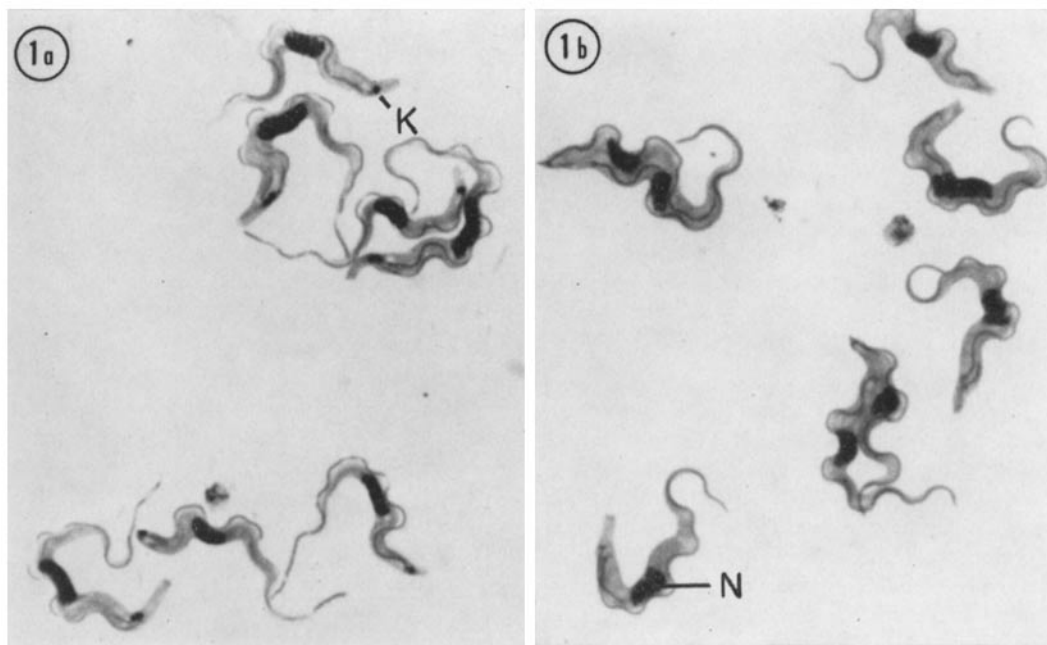


FIGURE 1 Giemsa-stained kinetoplastic (A) and dyskinetoplastic (B) cells showing kinetoplast (K) and nucleus (N). $\times 1500$.

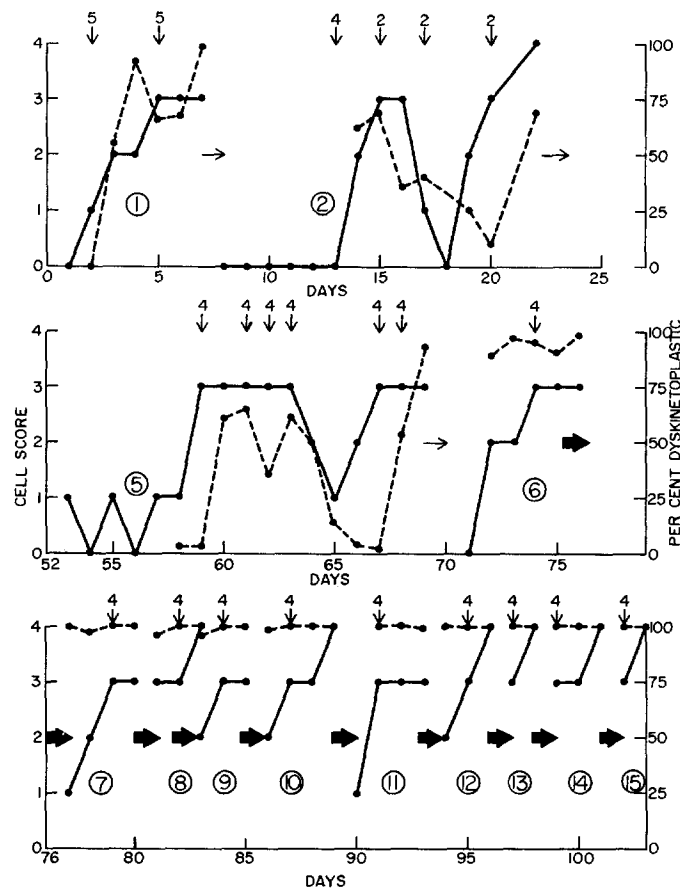


FIGURE 2 Results of an experiment whereby a totally dyskinetoplastic strain was produced. Animals are indicated by the circled numbers. The numbers associated with the vertical arrows indicate the milligram per kilogram dose of acriflavine administered on the day indicated by the arrow. The horizontal arrows indicate a subinoculation, the slender arrows indicating a transfer of about 0.005 ml of infected blood and the thick arrows indicating a transfer of about 0.3 ml. The per cent of dyskinetoplastic cells is indicated by the dashed line, while the cell score is indicated by the solid line. One or less trypanosomes per field of a wet mount viewed at $40\times$ was scored 1; 2–20 cells per field was scored 2; more than 20 per field but less than one trypanosome per red blood cell was scored 3; more than one trypanosome per red blood cell was scored 4; and no detectable trypanosomes was scored 0.

No differences were detected in the DNA composition of extracts from whole kinetoplastic and dyskinetoplastic cells (Fig. 5). Both strains contained three density DNA species in isopycnic cesium chloride gradients. In each case there was a major band with a density of 1.707 g/cc, a larger satellite with a density of 1.702 g/cc, and a small satellite with a density of 1.692 g/cc the kinetoplast DNA.¹ There was no detectable dif-

ference in buoyant density or relative concentration of any of the DNA species from the dyskinetoplastic cells or the total DNA content (about 1.0 mg DNA/ml packed cells).

DISCUSSION

All dyskinetoplastic cells so far studied have three features in common: the kinetoplast does not stain with Giemsa's, the fibrillar element of the kinetoplast is altered, and dyskinetoplastic cells are unable to be propagated under conditions believed

¹ D. H. Williamson, K. D. Stuart, W. E. Gutteridge, and I. Burdett. In preparation.

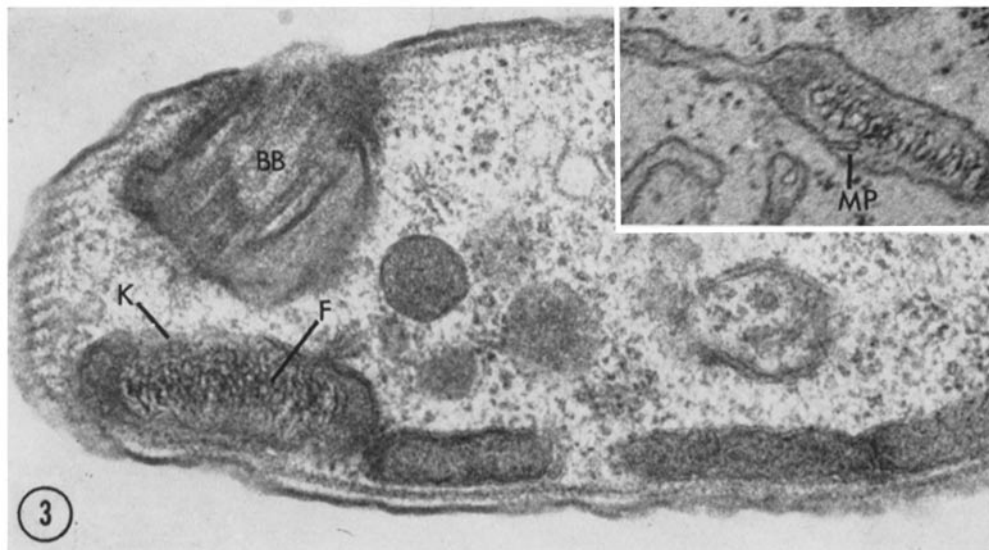


FIGURE 3 Electron micrograph of kinetoplastic cell. The kinetoplast (*K*) lying near the basal body (*BB*) contains the typical fibrillar central element (*F*). The *inset* shows a kinetoplast containing a crista-like membranous profile (*MP*). Fig. 3, $\times 56,400$; *inset*, $\times 79,000$.

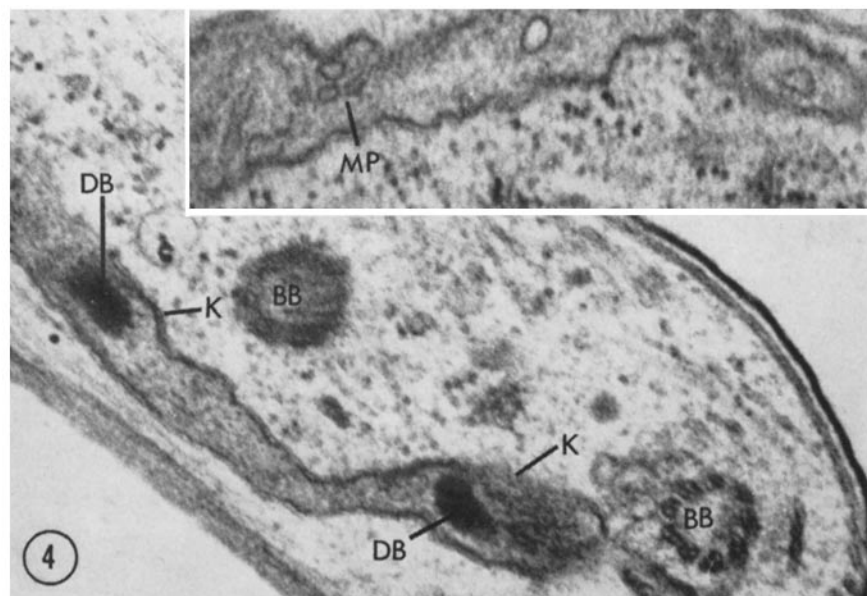


FIGURE 4 Electron micrograph of a dyskinetoplastic cell. In this cell which was in the process of division, the kinetoplast (*K*) contains two dense bodies (*DB*) each near a basal body (*BB*). The *inset* shows a section of the mitochondrion containing membranous profiles (*MP*). Fig. 4, $\times 79,000$; *inset*, $\times 79,000$.

to require mitochondrial respiration. The acriflavine-induced dyskinetoplastic strain of *Trypanosoma brucei* produced in this study fulfills these criteria. Its kinetoplast does not stain with

Giemsa's and the fibrillar element is altered. Although neither the dyskinetoplastic nor the parental normal strain could be cultivated after laboratory maintenance, kinetoplastic cells survived 2

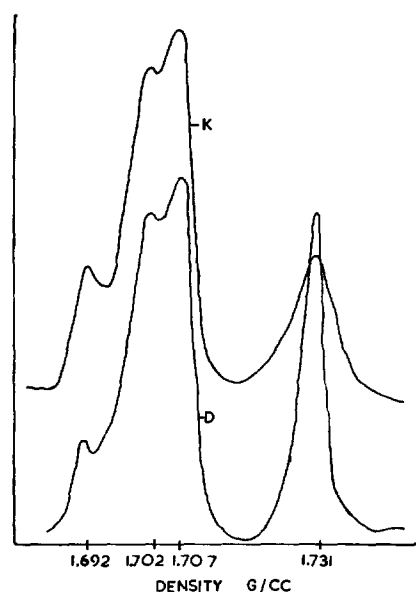


FIGURE 5 Equilibrium sedimentation profiles of whole-cell DNA, extracted from kinetoplastic (K) and dyskinetoplastic (D) cells. The peak at 1.731 g/cc is *Micrococcus lysodeikticus* DNA which was used as a density marker.

wk in culture while the dyskinetoplastic cells died within hours.

This dyskinetoplastic strain retained the central element of the kinetoplast in the condensed state typical of acriflavine-treated cells and contained all three DNA components upon isopycnic sedimentation of whole-cell DNA. These results differ from the findings that acriflavine treatment results in the condensation and then loss of the central element of the kinetoplast (17), and the absence of a DNA component as a result of specific inhibition of kinetoplast DNA synthesis (14, 16). However, studies leading to these latter findings were done with species which, unlike *T. brucei*, are unable to grow in the dyskinetoplastic condition, and the analyses were done shortly after drug treatment. Previous studies with species able to grow in the dyskinetoplastic condition also reported the absence of the central element of the kinetoplast in dyskinetoplastic cells (7, 8). However, these observations may have been due to a difference in fixation or to strain differences. Recent studies employing naturally occurring dyskinetoplastic strains indicate the retention of the condensed central element of the kinetoplast (12,¹). These

strains also contain the typical three DNA components.¹ The retention of the condensed central element of the kinetoplast is, therefore, not peculiar to acriflavine-induced dyskinetoplastic cells. Hence dyskinetoplastic cells may retain and replicate the condensed central element of the kinetoplast as well as kinetoplast DNA, at least in species able to be propagated in the dyskinetoplastic condition.

Although the central element of the kinetoplast has been retained in dyskinetoplastic cells, it has clearly been altered. Lowered viability and protein alterations in the dyskinetoplastic strain² suggest that the alteration has physiological significance. The alteration may be in the nucleotide sequence of kinetoplast DNA. This suggestion is attractive in view of the heritable, stable nature of the alteration. The fact that there is no buoyant density alteration in any of the DNA components in the dyskinetoplastic strain does not preclude such an alteration. For example, certain cytoplasmic petite mutants of yeast have no detectable alteration of the buoyant density of mitochondrial DNA, yet this DNA has an altered base composition and the cells are respiratory deficient (2). On the other hand, the primary structure of kinetoplast DNA may not be altered but there may be a conformational alteration or altered binding of the DNA to other molecules. The loss of Giesma- and acridine orange-stainability¹ while kinetoplast DNA is probably present is consistent with such a suggestion. Although the possibility that the condensed central element is not kinetoplast DNA has yet to be excluded, but this suggestion seems unlikely. The absence of Feulgen stainability of plant nuclei which contained reasonable amounts of DNA provides a precedent for the presence of DNA that is cytologically undetectable (5). Many factors including the presence of certain proteins were found to affect Feulgen stainability in these studies.

The role of kinetoplast DNA is obscure. Most kinetoplastid species have cyanide- and azide-sensitive respiration and cannot grow in the dyskinetoplastic conditions. Members of the subgenus *Trypanozoon* have life cycle stages in which respiration is cyanide and azide insensitive. Dyskinetoplastic strains of this subgenus have been propagated under conditions where respiration is cyanide and azide insensitive but not under conditions where respiration is sensitive to these in-

² K. D. Stuart. In preparation.

hibitors (see references 1 and 12 for review). These findings have led to the suggestion that kinetoplast DNA is necessary for mitochondrial respiration (12). The similarities between dyskinetoplastic trypanosomes and cytoplasmic petite mutants of yeast as well as the similarities between kinetoplast and mitochondrial DNA have made attractive the suggestion that these two DNA's have a similar function. However, the absence of sexuality in kinetoplastid flagellates prevents discrimination between nuclear and cytoplasmic mutants and, since dyskinetoplastic strains are not generally cloned, they are likely to be genetically heterogeneous.

It has been suggested that a manifestation of the role of kinetoplast DNA in mitochondrial activity is the observation that, in pleomorphic populations of trypanosomes growing in the vertebrate host, stumpy cells have mitochondrial activity while slender cells do not. Since dyskinetoplastic strains are reported to contain only slender cells, it was suggested that restriction of the activity of kinetoplast DNA in these cells resulted in the restriction of mitochondrial activity (12). The findings that the dyskinetoplastic strain produced in this study was pleomorphic and that the SAK dyskinetoplastic strain of *Trypanosoma evansi* isolated by Dr. Hoare was originally pleomorphic³ place this suggestion in doubt.

The assistance of Ken Moore and Ian Burdet with the electron microscopy is gratefully acknowledged as is the assistance of Dr. Roger Chalkley in the preparation of DNA. I am specially indebted to Dr. Don Williamson for assistance with the Model E analyses and assistance in preparation of the manuscript and thank Dr. Joseph Frankel for his interest and encouragement during the course of this study.

This work was supported in part by United States Public Health Service Predoctoral Fellowship F1 GM-32, 677 and was done in partial fulfillment of the degree of doctor of philosophy at the University of Iowa.

Received for publication 6 July 1970, and in revised form 23 October 1970.

³ K. Vickerman. Personal communication.

Note Added in Proof: Recently, in collaboration with Drs. W. E. Gutteridge and D. H. Williamson, detergent extracts of whole-cell DNA were analyzed by analytical isopycnic centrifugation in cesium chloride. In these crude extracts from the kinetoplastic strain the band with a density of 1.692 g/cc, the density of kinetoplast DNA was very sharp as has been reported by other authors (see references 3 and 16). This band, however, was more broad in extracts from the dyskinetoplastic strain as in Fig. 5.

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