# KINETOPLAST DEOXYRIBONUCLEIC ACID OF THE HEMOFLAGELLATE *TRYPANOSOMA LEWISI*

HARTMUT C. RENGER and DAVID R. WOLSTENHOLME

From the Division of Biology, Kansas State University, Manhattan, Kansas 66502. Dr. Renger's present address is Department of Pathology, New York University School of Medicine, New York 10016. Dr. Wolstenholme's present address is Department of Biology, University of Utah, Salt Lake City Utah 84112

ABSTRACT

Cesium chloride centrifugation of DNA extracted from cells of blood strain *Trypanosoma lewisi* revealed a main band, $\rho = 1.707$, a light satellite, $\rho = 1.699$, and a heavy satellite, $\rho = 1.721$. Culture strain *T. lewisi* DNA comprised only a main band, $\rho = 1.711$, and a light satellite, $\rho = 1.699$. DNA isolated from DNase-treated kinetoplast fractions of both the blood and culture strains consisted of only the light satellite DNA. Electron microscope examination of rotary shadowed preparations of lysates revealed that DNA from kinetoplast fractions was mainly in the form of single 0.4 $\mu$ circular molecules and large masses of 0.4 $\mu$ interlocked circles with which longer, often noncircular molecules were associated. The 0.4 $\mu$ circular molecules were mainly in the covalently closed form: they showed a high degree of resistance to thermal denaturation which was lost following sonication; and they banded at a greater density than linear DNA in cesium chloride–ethidium bromide gradients. Interpretation of the large masses of DNA as comprising interlocked covalently closed 0.4 $\mu$ circles was supported by the findings that they banded with single circular molecules in cesium chloride–ethidium bromide gradients, and following breakage of some circles by mild sonication, they disappeared and were replaced by molecules made up of low numbers of apparently interlocked 0.4 $\mu$ circles. When culture strain cells were grown in the presence of either ethidium bromide or acriflavin, there was a loss of stainable kinetoplast DNA in cytological preparations. There was a parallel loss of light satellite and of circular molecules from DNA extracted from these cells.

## INTRODUCTION

Members of the protozoan order Kinetoplastida (Honigsberg et al., 1964) are characterized by the presence of a body known as the kinetoplast. The kinetoplast is a region of a mitochondrion (Meyer et al., 1958; Steinert, 1960; Clark and Wallace, 1960; Pitelka, 1961; Ris, 1962) which contains so much DNA that it can be detected by Feulgen staining (Bresslau and Scremin, 1924). DNA isolated from kinetoplast fractions of *Leishmania enriettii* by DuBuy et al. (1965) was found to have a buoyant density less than that of the cell's nuclear DNA. Riou and Paoletti (1967) and Riou and Delain (1968) separated a DNA from whole cells of *Trypanosoma cruzi* which had a lesser buoyant density than the organism's nuclear DNA and which they presumed was kinetoplast DNA. Electron microscope examination of this DNA revealed that it comprised covalently closed 0.45 $\mu$ circles, and some long noncircular molecules. They also observed that a portion of this DNA was in the form of catenanes apparently comprising many interlocked 0.45 $\mu$ circles.

The present report relates the results of experiments to determine the form and structure of the

DNA of kinetoplasts of both the rat blood strain and the culture strain of the hemoflagellate *Trypanosoma lewisi*.

## MATERIAL AND METHODS

Both the blood strain and the culture strain of *Trypanosoma lewisi* used in these experiments were obtained from D. G. Dusanic at the University of Kansas (see Dusanic, 1968, for further details of ancestry of each strain).

The blood strain of *Trypanosoma lewisi* was maintained in female albino rats (100–300 g) by syringe passage every 7 days. Trypanosomes used either for cell fractionation or for direct microscopy were harvested 5 days after infection. Infected blood was obtained by heart puncture and shaken immediately with 6 volumes of a solution containing 0.137 M sodium chloride, 0.01 M dextrose, 0.34 mM sodium citrate, and 6.6 mM sodium phosphate (pH 7.2) (Dusanic, 1968). The mixture was centrifuged at 1000 *g* for 10 min, and the trypanosomes which remained in the supernatant and concentrated above the red and white blood corpuscles were removed with a pipette. The organisms were then washed by repeated centrifugation at 3000 *g* and resuspension in the buffered dextrose saline solution, but lacking sodium citrate.

Culture strain cells of *Trypanosoma lewisi* were grown aseptically in Locke's solution over a blood-agar gel (Dusanic, 1968) in screw-cap, flat-sided bottles at 26°C. The blood-agar gel was 1 part whole rabbit blood and 7.5 parts 1.5% Difco Nutrient agar. The rabbit blood was obtained aseptically by cardiac puncture, defibrinated by shaking with glass beads, heated to 57°C for 30 min, and mixed with melted agar. Locke's solution consisted of 0.8% sodium chloride, 0.25% dextrose, 0.02% calcium chloride, 0.02% potassium chloride, 0.0089% monobasic sodium phosphate monohydrate, and 0.0038% anhydrous dibasic sodium phosphate (pH 6.5). So as to further guard against bacterial contamination, streptomycin and penicillin were present at all times in the liquid phase of the culture medium at a concentration of 50 μg/ml and 200 IU/ml, respectively. Culture strain trypanosomes were transferred every 7 days. Organisms used for cell fractionation or microscopy were harvested at 5–7 days after inoculation of the culture, by centrifuging the liquid phase at 1000 *g* for 10 min. They were washed by repeated centrifugation and resuspension in SSC (0.15 M sodium chloride, 0.015 sodium citrate, pH 7.0).

For preparing kinetoplasts, freshly harvested trypanosomes were resuspended in a solution containing 0.3 M sucrose, 1 mM EDTA (disodium ethylenediaminetetraacetate), 0.01 M Tris/HCl at pH 7.4, broken open in a Waring Blendor (15 sec at high speed), and centrifuged at 1500 *g* for 10 min. The pellet from this centrifugation contained most of the intact cells and nuclei. This step was repeated, at least twice, until all whole cells were removed from the supernatant. A pellet rich in kinetoplasts was then obtained from the supernatant by centrifugation at 8000 *g* for 10 min. This was resuspended in about 10 ml of buffer, magnesium chloride was added to 7 mM, beef pancreatic DNase I (Worthington Biochemical Corp., Freehold, N.J.) to 200 μg/ml, and the mixture was incubated at 37°C for 30 min. Digestion of DNA was monitored by adding 10 μg of $^{14}$C-labeled *Escherichia coli* DNA and measuring the loss of acid-precipitable radioactivity at the end of incubation. 96% of the free DNA was degraded consistently. The DNase digestion was stopped by adding 1 ml of 0.4 M EDTA, and the kinetoplasts were washed three times with buffer containing 0.04 M EDTA. The kinetoplasts were then suspended in 0.15 M sodium chloride, 0.1 M EDTA (pH 8.0), and either frozen or used directly.

DNA was extracted and purified by a modification of the method of Marmur (1961). Sodium dodecyl sulfate (SDS) was added to 1% to whole cells or kinetoplast fractions in 0.15 M sodium chloride and 0.1 M EDTA (pH 8.0), and the mixture was kept at 37°C for 15 min. Sodium chloride was added to a final concentration of 5 M and the solution was placed in ice. The precipitate which formed was removed by centrifugation at 10,000 *g* for 10 min, and the supernatant was diluted to twice the starting volume and gently shaken three times with an equal volume of chloroform:isoamyl alcohol (24:1, v:v) for 5 min. 400 μg/ml pancreatic ribonuclease (Sigma Chemical Co., St. Louis, previously heated to 90°C for 5 min at pH 5.0) was added to the aqueous phase which was incubated at 37°C for 30 min, and then dialyzed against sodium chloride–EDTA for 2 hr with one change. DNA was then precipitated by adding 0.55 volume of isopropyl alcohol, collected on a glass rod, and redissolved in $\frac{1}{10}$ SSC. The DNA was reprecipitated and dissolved in SSC.

Crude lysates were used to make cesium chloride buoyant density gradients and rotary shadowed preparations for electron microscopy. These were prepared by suspending whole cells or kinetoplast fractions in a solution containing 0.015 M sodium chloride, 0.01 M EDTA (pH 8.0), 0.2% SDS, and 1 mg/ml papain [Difco Laboratories, Detroit, Mich.: recrystallized and dissolved in 0.02 M cysteine (Kimmel and Smith, 1954)] and incubating them at 37°C for 30 min. Crude lysates for making rotary shadowed preparations were also obtained by incubating at 37°C for 4–8 hr with 1 mg/ml of DNase-freed pronase (Calbiochem, Los Angeles, Calif.) (Wolstenholme and Gross, 1968).

Cesium chloride analytical ultracentrifugations were carried out according to Meselson et al. (1957).

The solutions were centrifuged in a Beckman Spinco model E at 42,040 rpm, for at least 20 hr in an An-D rotor. Microdensitometer tracings of ultraviolet photographs were made with a Joyce-Loebl densitometer. The proportion of DNA in each satellite was estimated by weighing pieces of the graph paper delineated by the densitometry tracings.

Cesium chloride–ethidium bromide preparative ultracentrifugations (Radloff et al., 1967) were made in a Beckman Spinco model L2 65B using polyallomer tubes and a SW-65 titanium rotor. Centrifugation was done at 40,000 rpm for 48 hr. The cesium chloride density was adjusted to 1.55 g/ml and the ethidium bromide (a gift of Boots Pure Drug Co., Nottingham, England) was used at a final concentration of 150 $\mu$g/ml. Fractions of approximately 75 $\mu$l were collected by piercing a small hole in the bottom of the tube. Ethidium bromide was removed from the DNA suspension by a single passage through Dowex 50W resin (Radloff et al., 1967).

Thermal denaturation of DNA was followed in a Gilford 240 spectrophotometer equipped with dual thermoplates connected to a Haake heating unit. The DNA at a concentration of 10–15 $\mu$g/ml in SSC was overlayed with mineral oil and continuously heated at the rate of 0.5°C/min in 1.4 ml or 0.7 ml quartz cuvettes. The temperature was measured with a thermocouple placed in the same compartment as the sample.

DNA solutions cooled with water from an ice bath were sonicated at 1 amp for either 1 or 3 × 1 min using a 10 KC Raytheon sonic oscillator.

Whole cells or pellets of kinetoplast fractions were fixed in Kellenberger's 1% osmium tetroxide for 12 hr, treated with uranyl acetate (Ryter et al., 1958), dehydrated in a graded series of ethanols, and embedded in Epon (Luft, 1961). Thin sections were cut on an LKB Ultrotome III microtome with a Dupont diamond knife and stained with aqueous uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965). For the preparation of protein monolayers, 10 $\mu$l of a crude lysate or of a purified DNA solution was picked up with a wide-bore (at least 2 mm) pipette and added to 0.1 ml of a 1 M ammonium acetate solution containing 0.05% cytochrome *c*, 0.5% formaldehyde (Freifelder and Kleinschmidt, 1965). The DNA was then spread, picked up on copper grids, and shadowed as described previously (Wolstenholme and Gross, 1968). Electron micrographs were made with an Hitachi HU-11B electron microscope. The shadowed molecules were photographed (using projector pole piece 2) at an original magnification of 11,000 (calibrated with a diffraction grating replica [2,160 lines/mm]). Measurements of molecules were made on positive prints at magnifications of either 50,000 or 160,000.

Smears of trypanosomes were made on gelatinized slides, dried, fixed in acetic acid:ethanol (1:3), stained with Giemsa's, or by the Feulgen procedure, mounted in immersion oil, and examined by bright-field illumination in a Zeiss Photomicroscope with a 100 X apochromatic oil immersion objective. Micrographs were made on Adox KB14 film.

## RESULTS

### *Kinetoplast Fractions*

The kinetoplast fractions were monitored by electron microscopy. In thin sections of whole cells of *Trypanosoma lewisi*, the kinetoplast appeared as a mass of DNA-containing fibrils, about 25 A in diameter (Ris, 1962; Mühlpfordt, 1963), lying in parallel array and situated within an enlarged portion of the mitochondrion (Fig. 1). The DNase-treated kinetoplast fractions included an abundance of intact, membrane-bounded structures having the morphological characteristics of kinetoplasts and mitochondria (Figs. 2–4). The DNA-containing fibrils and their arrangement in parallel array within the kinetoplasts were clearly preserved (Fig. 4). Fragments of other cell components, particularly basal bodies, flagella, and microtubule-bearing cell walls, were also present. Nuclei were virtually absent. As intact membranes were rarely present around the basal bodies, it is unlikely that any DNA which might be associated with them (Randall and Disbrey, 1965) would survive the DNase treatment.

### *Buoyant Densities*

The cesium chloride density gradient equilibrium band positions of DNA from whole cells and from DNase-treated kinetoplast fractions of both the blood and culture strains are shown in Figs. 5 and 6. Most of the DNA extracted from blood strain cells banded at a density of 1.707, but there were two distinct satellites, one lighter, $\rho$ = 1.699, and one heavier, $\rho$ = 1.721, than the main band. The light and heavy satellites each accounted for approximately 9% of the total DNA. That all these bands did, in fact, represent DNA was indicated by their absence when DNase treatment preceded centrifugation (Fig. 5). If the absence of unusual bases is assumed, the buoyant density values obtained for the light satellite, main band, and heavy satellite DNA's correspond, respectively, to base compositions of 39.8%, 48.0%, and 62.3% GC (guanylic plus cytidylic acid) (Schildkraut et al., 1962).

Most of the DNA extracted from cells of the

culture strain of *T. lewisi* (Fig. 6) had a buoyant density of 1.711 which is greater than the main band DNA of cells of the blood strain, and corresponds to a base composition of 52.0% GC. Approximately 19% of the DNA was a light satellite with a buoyant density similar to that of the blood strain DNA. The heavy satellite DNA isolated from cells of the blood strain was not apparent in the DNA from cells of the culture strain.

DNA from DNase-treated kinetoplast fractions of cells of both the blood strain and the culture strain banded exclusively at the same density as the light satellite.

### *Form and Size of DNA Molecules*

In rotary shadowed electron microscope preparations, DNA from whole cells of both strains consisted mainly of long linear molecules (for one sample of whole cell culture strain DNA carefully prepared from a crude lysate, the mean length of 58 linear molecules was 38.4 $\mu$; standard deviation $\pm$ 27.0, range 1–105 $\mu$). Circular DNA molecules having a mean contour length of 0.4 $\mu$ were also found (Figs. 7–14). There was no evidence of a length difference between circular molecules derived from cells of the blood strain and cells of the culture strain (Fig. 14). A number of large masses were also seen (Figs. 12 and 13), the appearance of which was consistent with their comprising interlocked 0.4 $\mu$ circles. Evidence supporting this interpretation is presented below, and the large masses are, therefore, referred to as catenanes.

DNA from kinetoplast fractions of cells of both strains was found to consist of the 0.4 $\mu$ circular molecules and catenanes. Long, often noncircular molecules (up to at least 7 $\mu$ in length) were also found associated with the catenanes (Fig. 13) and were also occasionally found lying free. Very rare circular molecules were found which were two, three, and four times larger than the 0.4 $\mu$ circle (Fig. 10). Also molecules apparently consisting of two or three 0.4 $\mu$ interlocked circles were seen.

### *Structure of the Circular DNA Molecules*

The DNA molecules of a number of viruses (Vinograd and Lebowitz, 1966), bacterial plasmids and sex factors (Roth and Helinski, 1967; Hickson et al., 1967), and mitochondria from metazoan animals (Hudson and Vinograd, 1967; Dawid and Wolstenholme, 1967) have also been shown to be circular. In each case, the circles are mainly covalently closed; that is, all of the phosphodiester bonds in each of the two polynucleotide chains of each molecule are intact (Vinograd and Lebowitz, 1966). We have made studies to determine whether the circular kinetoplast DNA molecules of *T. lewisi* are also covalently closed.

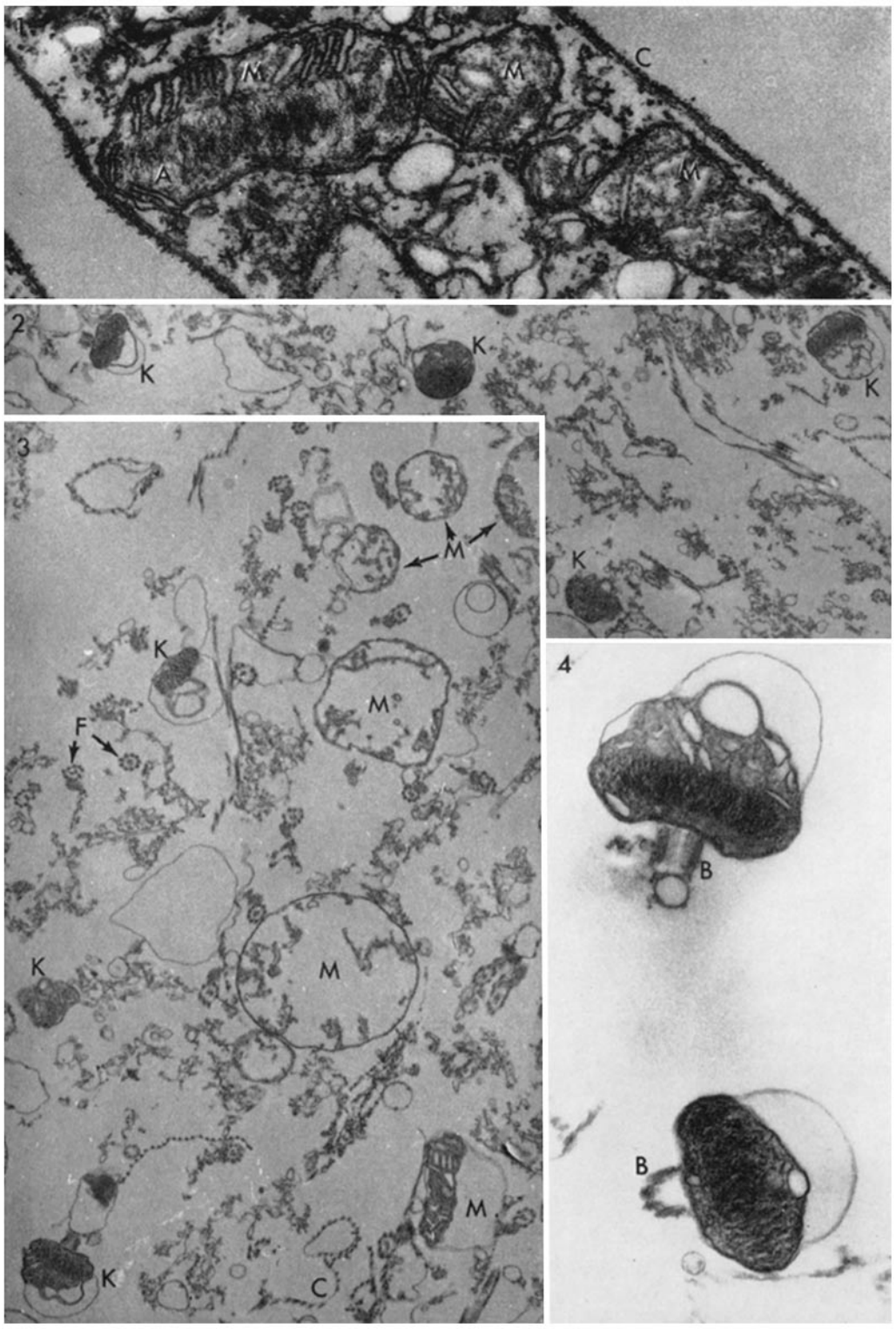
When whole-cell DNA was centrifuged to equilibrium in a cesium chloride–ethidium bromide gradient, two distinct bands were formed. Rotary shadowed DNA from each band was examined in the electron microscope. The denser band comprised circular molecules mainly 0.4 $\mu$ in contour length and large catenanes with a few associated linear molecules, whilst the lighter band consisted almost entirely of long linear molecules. Only a few circles were found in the lighter band. Since covalently closed circles bind less dye than circles containing at least one phosphodiester bond break (open circles) or linear molecules, and, therefore, band at a greater density (Radloff et al., 1967; Bauer and Vinograd, 1968), our observa-

FIGURE 1 An electron micrograph of a longitudinal section through a blood strain *Trypanosoma lewisi* showing the kinetoplast consisting of a mass of DNA-containing fibrils (*A*) within an enlarged portion of a mitochondrion (*M*). The fibrils lie in parallel array and are transversely oriented with respect to the mitochondrial portion of the kinetoplast. *C*, cell wall. × 50,000.

FIGURES 2–4 Electron micrographs of sections of a pellet of a DNase-treated kinetoplast fraction prepared from blood strain *Trypanosoma lewisi*.

FIGURES 2 and 3 The fraction contains intact membrane-bounded kinetoplasts (*K*), mitochondria (*M*). Fragments of other cell components are also present, particularly flagella (*F*) and microtubule-bearing cell walls (*C*). × 15,000.

FIGURE 4 Two kinetoplasts with attached basal bodies (*B*). The DNA-containing fibrils and their arrangement in parallel array are clearly preserved. × 35,000.


1
M
M
C
A
M
2
K
K
K
K
3
M
K
F
M
K
M
M
K
C
4
B
B

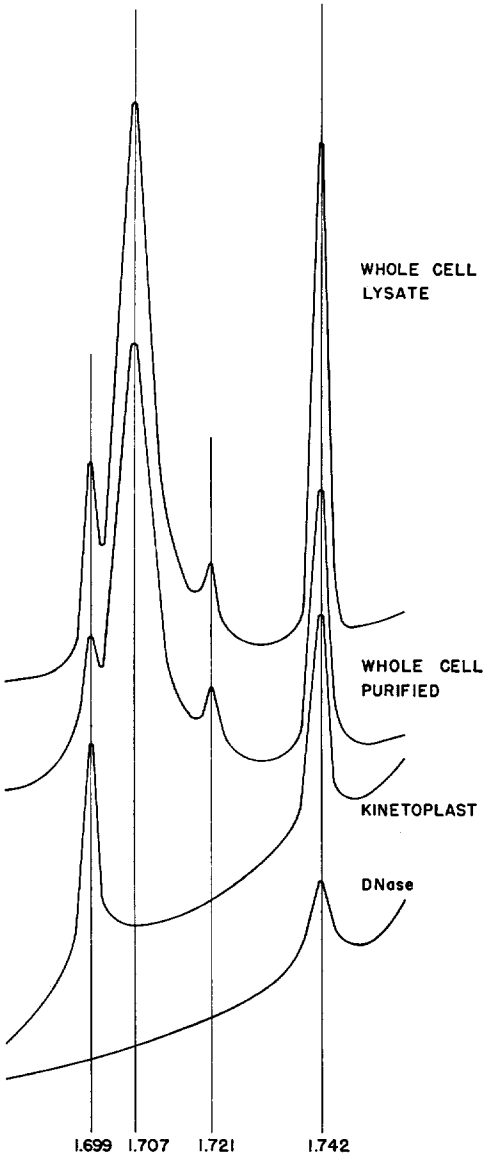



FIGURE 5 Microdensitometer tracings of ultraviolet photographs of cesium chloride buoyant density gradients of DNA from whole cells, and from a kinetoplast fractioe of blood strain *Trypanosoma lewisi*. The reference band (1.742) to the right is native DNA of SPO1.

tion indicates that the 0.4 $\mu$ circles are covalently closed. Also, the finding that the catenanes were confined to the denser band indicates that the molecules of which these forms are made up are mainly covalently closed circles.

Covalently closed circles show resistance to denaturation upon heating (Vinograd and Lebowitz, 1966; Nass, 1969). Even if hydrogen bond separation occurs, the two polynucleotide strands cannot separate from each other owing to their topological bonding, and "snap back" into their native configuration upon cooling (Dawid and Wolstenholme, 1967). The equilibrium band positions in cesium chloride of DNA from culture strain cells which was heated at 100°C and quenched in ice are shown in Fig. 15. The buoyant density of the light satellite DNA was increased by only 4 mg/ml compared to an increase of 18 mg/ml for the main band DNA, indicating that much less of the light satellite than of the main band DNA denatured. Confirmation of this was obtained by examining the heated and quenched DNA in the electron microscope. Many apparently double-stranded 0.4 $\mu$ circular molecules were seen which were indistinguishable from native circular molecules. (Under the conditions used to make these preparations, single-stranded DNA either collapses or appears as kinky threads, poor in contrast and, therefore, easily distinguished from double-stranded DNA. For further discussion, see Dawid and Wolstenholme, 1968.)

Thermal denaturation curves for whole cell *T. lewisi* culture strain DNA are given in Fig. 16. The melting temperature ($T_m$) for unsonicated

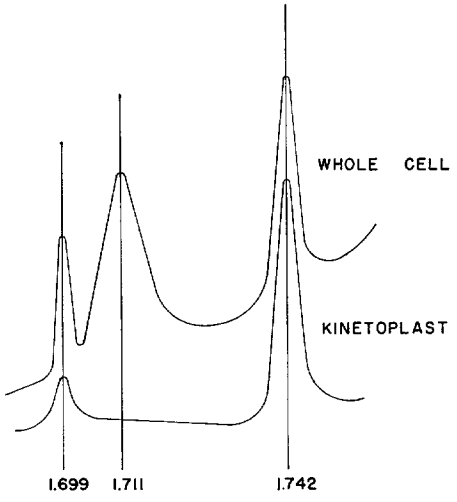
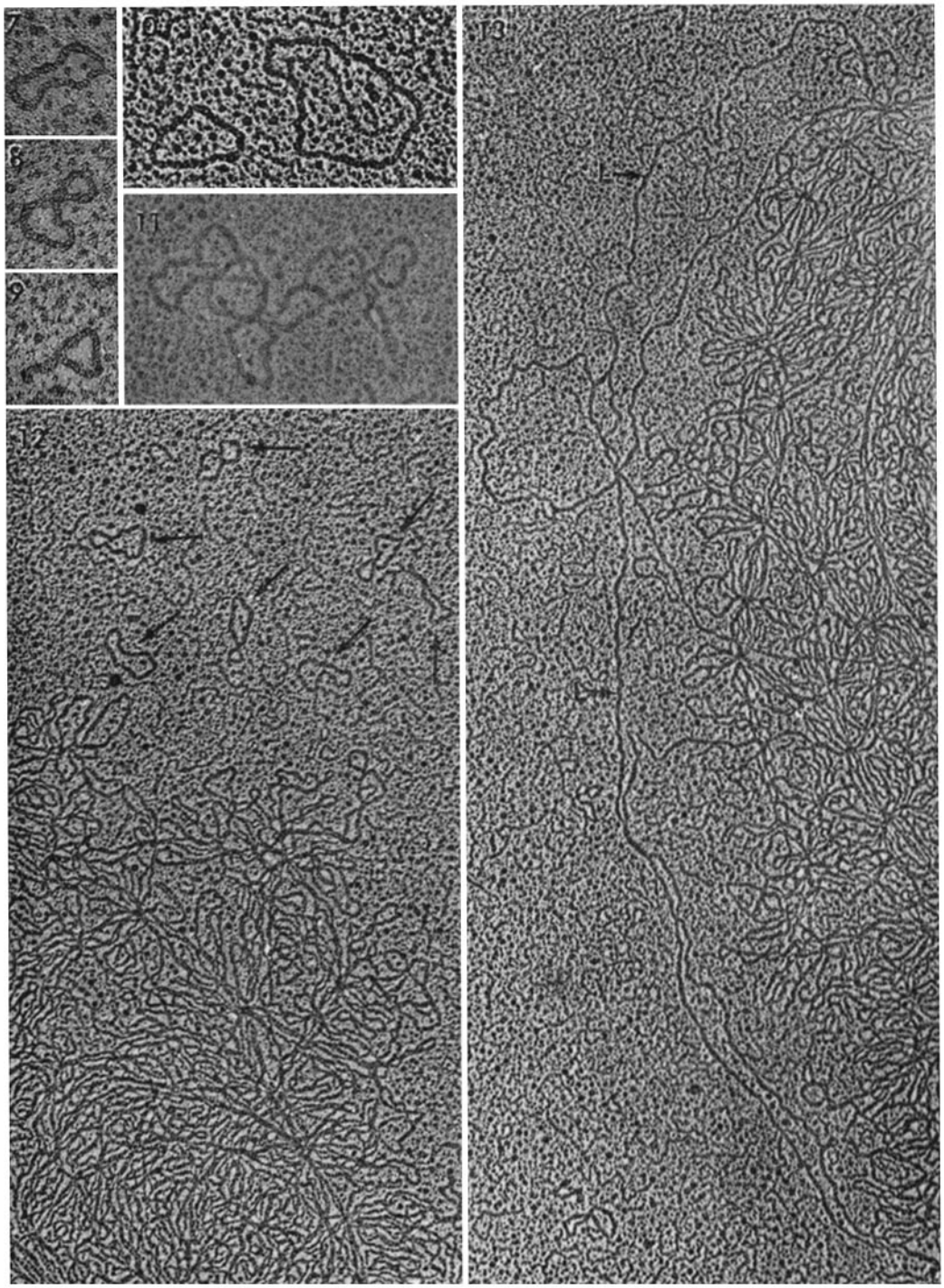



FIGURE 6 Microdensitometer tracings of ultraviolet photographs of cesium chloride buoyant density gradients of DNA from whole cells, and from a kinetoplast fraction of culture strain *Trypanosoma lewisi*. The reference band (1.742) to the right is native DNA of SPO1.




FIGURES 7–13 Electron micrographs of rotary shadowed molecules of DNA from kinetoplast fractions of *Trypanosoma lewisi*.

FIGURES 7–9 Single circular molecules of contour lengths approximately 0.4 $\mu$. × 92,700.

FIGURE 10 Two circular molecules, of contour lengths 0.4 $\mu$ and 0.8 $\mu$. × 87,500.

FIGURE 11 A molecule apparently comprising eight interlocked 0.4 $\mu$ circles. From a preparation of kinetoplast DNA sonicated for 1 min. × 75,000.

FIGURE 12 A mass of DNA apparently made up of interlocking circles of approximate contour length 0.4 $\mu$. Seven 0.4 $\mu$ circles (arrows) are visible lying free at the edge of the mass. × 56,000.

FIGURE 13 A mass of DNA apparently made up of interlocking 0.4 $\mu$ circles with which much longer molecules (*L*) are associated. × 51,000.

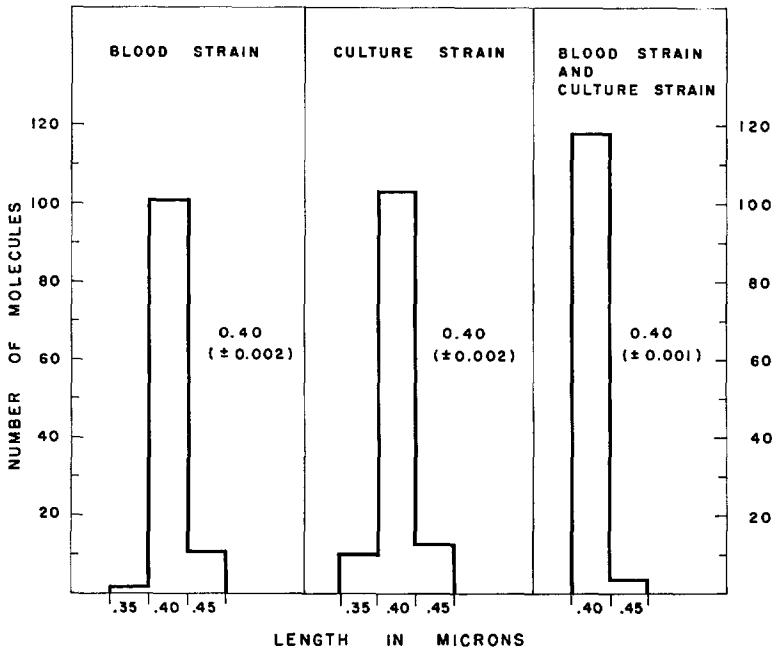



FIGURE 14 Frequency distribution of lengths of circular molecules of blood strain DNA (n=114), culture strain DNA (n=125), and an approximately equal mixture of blood strain and culture strain DNA (n=122). The mean and standard error of each sample are given.

DNA was 91.1°C which corresponds to a GC content of 53.2% (Marmur and Doty, 1962). This value is higher than, but in good agreement with that calculated from the buoyant density of main band DNA. There was no evidence that the light satellite DNA was denatured. Sonication of whole cell DNA, which broke all the circles as confirmed by electron microscopy, resulted in a lowering of the $T_m$ to 88.9°C corresponding to a GC content of 47.8%. A $T_m$ of 89.7°C would be expected for a mixture of light satellite and main band DNAs in the average proportion (19:81) in which they were found in isolated DNA, calculated from the respective buoyant densities. Resistance to denaturation of circular kinetoplast DNA was confirmed by melting experiments with a pure sample of circular DNA obtained by centrifugation of whole culture cell DNA in cesium chloride–ethidium bromide gradients. The melting profiles are shown in Fig. 17. The circular DNA showed a very small increase in absorbancy. Sonicated DNA (again shown by electron microscopy not to contain circles), on the other hand, exhibited a clear thermal transition with a $T_m$ of 86.5°C. This corresponds to a GC content of 42.0% which compares to the 39.8% calculated from the buoyant density of the light satellite DNA.

One sample of kinetoplast DNA was sonicated for 1 min. Upon continuous heating, this DNA exhibited a rise in absorbancy to only about one-half that shown by kinetoplast DNA sonicated for 3 min. When this DNA was examined in the electron microscope, the large catenanes which were frequently found in unsonicated DNA were not seen. Instead, an abundance of molecules comprising up to 10 apparently interlocked 0.4 $\mu$ circles was found (Fig. 11). These observations are consistent with the interpretation of the large masses of DNA as comprising mainly 0.4 $\mu$ circles which are held together by the interlocking of each circle with one or more other circles.

### *Effect of Culturing in the Presence of Ethidium Bromide and Acriflavin*

Cells of the culture strain were grown in the presence of 2 $\mu$g/ml ethidium bromide for 10 days or in the presence of 2 $\mu$g/ml acriflavin for 12 days. A kinetoplast could not be discerned in 95.5% of Giemsa-stained and 97% of Feulgen-

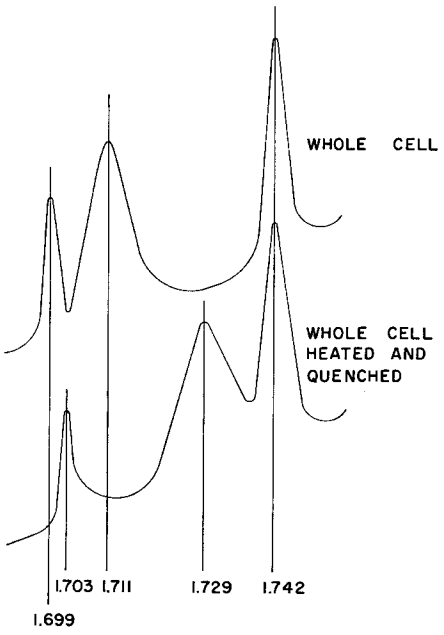



FIGURE 15 Microdensitometer tracings of ultraviolet photographs of cesium chloride buoyant density gradients of DNA isolated from whole cells of culture strain *Trypanosoma lewisi*. The upper tracing is of native DNA, the lower one of DNA which was heated at 100°C for 5 min and then quenched in ice. The reference band (1.742) to the right is native DNA of SPO1.

stained cells cultured in the presence of ethidium bromide (Figs. 18–21). In the remaining 4.5% and 3% of the cells, a stained body, smaller than but in the same position as the kinetroplast in normal cells, was visible (Fig. 20). Profiles of mitochondria were seen in thin sections of each organism examined in the electron microscope. However, the usual parallel array of fibrils which represents the kinetoplast DNA either was not found or was replaced by a dense body. The ultrastructure of this body suggested that it was formed by condensation of the DNA fibrils (Figs. 22 and 23) (see also Trager and Rudzinska, 1964; and Steinert and Van Assel, 1967). No light-satellite DNA band could be detected in DNA extracted from cells grown in the presence of ethidium bromide (Fig. 24). A lysate was prepared from the ethidium bromide culture and from a culture grown in the absence of the dye, and rotary shadowed preparations were made. For each sample, grid squares were methodically searched from one side to the other and the first 22–58 linear molecules seen, which did not run out of view off the grid square, or were not excessively tangled so as to make measuring meaningless, were photographed and measured. The mean length was calculated. The number of single circles per linear molecule was then obtained by scanning the same grid squares. From these data, the approximate percentage of DNA in the form of free 0.4 $\mu$ circles in each of the preparations was estimated. No catenanes were found in the DNA from cells grown in the presence of ethidium bromide, and single circles were estimated to account

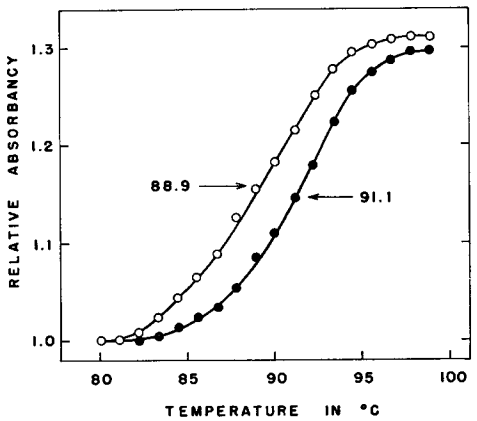



FIGURE 16 The effect on absorbancy at 260 m$\mu$ of heating unsonicated (—●—●—) and sonicated (—○—○—) whole cell culture strain DNA in SSC. The $T_m$ values are given.

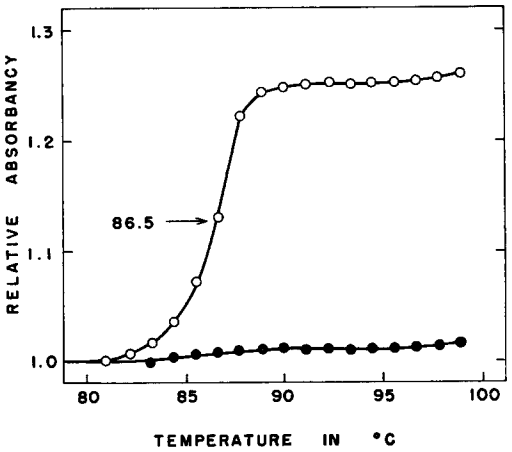



FIGURE 17 The effect on absorbancy at 260 m$\mu$ of heating unsonicated (—●—●—) and sonicated (—○—○—) circular kinetoplast DNA in SSC. The $T_m$ values are given.

for only 0.06% of the DNA compared to 6.4% of the the DNA from cells grown under normal conditions (Table I).

Similar results were obtained when cells of the culture strain were grown in the presence of acriflavin (Figs. 21 and 24, Table I). In this case, 93.8% of Giemsa-stained cells and 95.8% of Feulgen-stained cells appeared to lack a kinetoplast, and in electron microscope preparations circles were estimated to represent only 0.07% of the DNA.

## DISCUSSION

The data presented clearly indicate that the DNA of kinetoplasts of *Trypanosoma lewisi* has a lesser guanylic plus cytidylic acid content than the nuclear DNA and comprises mainly circular molecules with a contour length of about 0.4 $\mu$. The 0.4 $\mu$ circular molecules are mainly in the covalently closed form: They show a high degree of resistance to thermal denaturation which is lost following sonication, and they band at a greater density than linear DNA in cesium chloride-ethidium bromide gradients.

It was found that intact circular *T. lewisi* DNA does not continue to increase in absorbancy upon continuous heating in SSC. A similar observation has been made on covalently closed circular guinea-pig mitochondrial DNA (Wolstenholme, unpublished). It has been previously reported, however, that covalently closed circular polyoma DNA (Vinograd and Lebowitz, 1966) and mouse L-cell mitochondrial DNA (Nass, 1969) show a much lower but constant rate of increase in absorbancy than open DNA upon continuous heating in SSC.

The interpretation of the masses of DNA from kinetoplasts as comprising interlocked covalently closed circular 0.4 $\mu$ molecules is supported by the findings that they band with single circular molecules at a greater density than linear molecules in cesium chloride–ethidium bromide gradients, and that following breakage of some circles by mild sonication they disappear and are replaced by molecules made up of low numbers of apparently interlocked 0.4 $\mu$ circles.

Our findings concerning the forms and structure of DNA of kinetoplasts of *T. lewisi* are similar to those of Riou and Delain (1968) for the light-satellite DNA of *T. cruzi*, and, therefore, support their suggestion that the latter DNA is also from kinetoplasts.

It is not known what genetic information is carried by kinetoplast DNA. A single molecule of DNA with a contour length of 0.4 $\mu$, molecular weight $7.7 \times 10^5$ (MacHattie and Thomas, 1964), could contain the information for determining the sequences of amino acids in only two proteins with an average molecular weight of 20,000. The circles are homogeneous with respect to size, but there is no indication from our data as to whether they all have identical nucleotide sequences and

FIGURES 18–21 Bright-field light micrographs of Giemsa-stained cells of culture strain *Trypanosoma lewisi*. × 3,000.

FIGURE 18 Cells cultured under normal conditions. The kinetoplasts (*K*) and nuclei (*N*) are clearly visible.

FIGURES 19 and 20 Cells cultured in the presence of ethidium bromide.

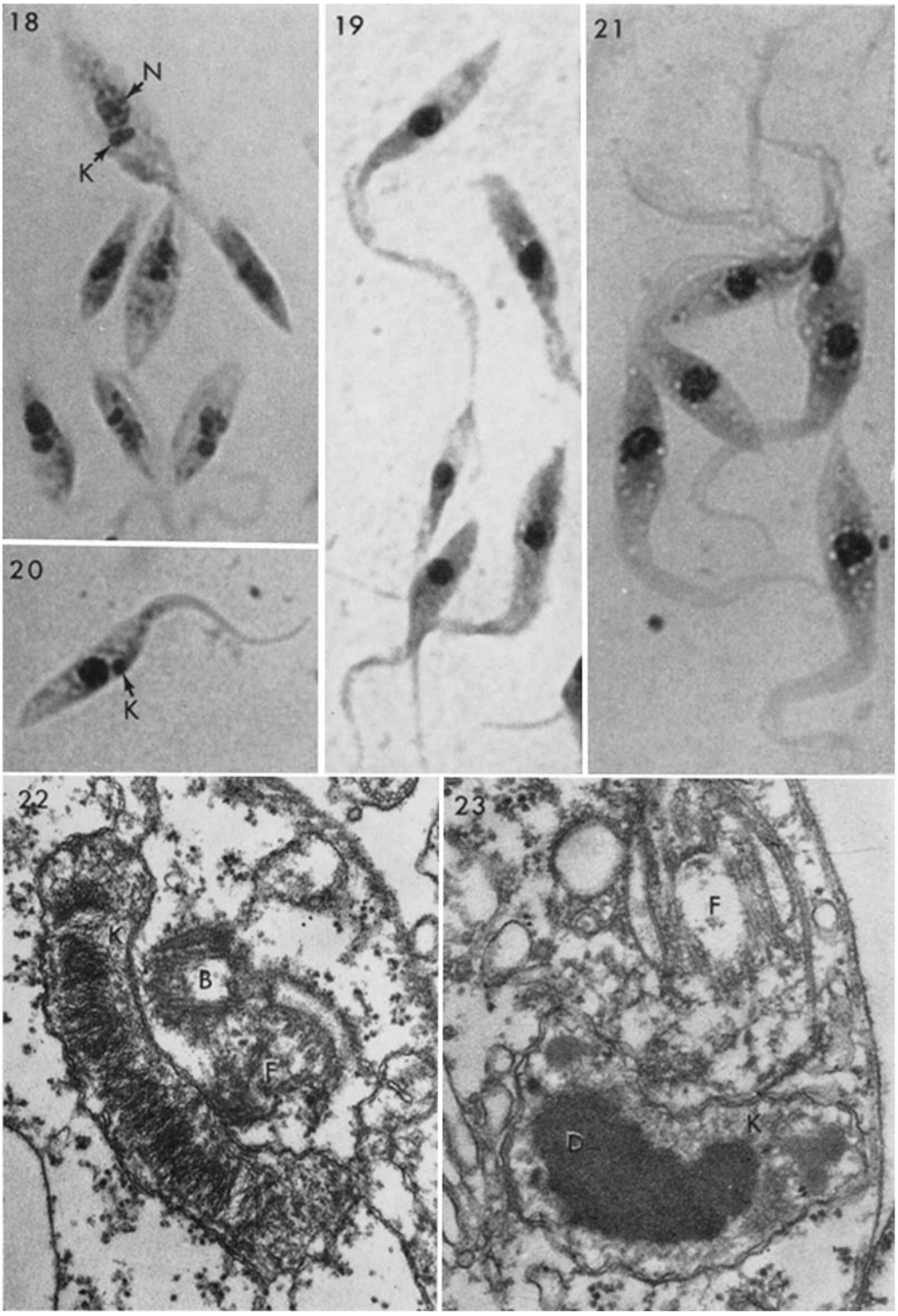
FIGURE 19 Cells lacking a kinetoplast.

FIGURE 20 A cell in which the kinetoplast (*K*) is greatly reduced in size.

FIGURE 21 Cells cultured in the presence of acriflavin and lacking a kinetoplast.

FIGURE 22 An electron micrograph of a section through the kinetoplast (*K*) of a cell of *Trypanosoma lewisi* grown in culture under normal conditions. The parallel array of DNA-containing fibrils of the kinetoplast is clearly visible. *B*, basal body, *F*, flagellum. × 45,000.

FIGURE 23 An electron micrograph of a section through the kinetoplast (*K*) of a cell of *Trypanosoma lewisi* grown in culture in the presence of ethidium bromide. In place of the parallel array of DNA-containing fibrils seen in kinetoplasts of cells grown under normal conditions is a dense body (*D*), apparently formed by condensation of the fibrils. *F*, flagellum. × 45,000.


18
N
K
19
21
20
K
22
K
B
F
23
F
D
K

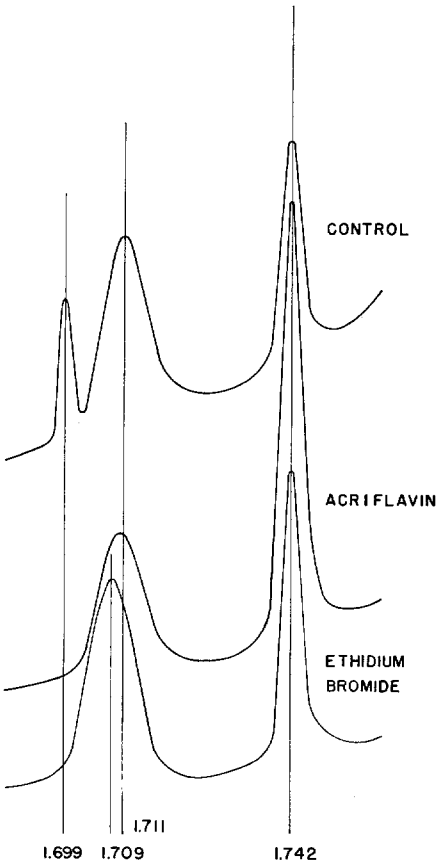



FIGURE 24 Microdensitometer tracings of ultraviolet photographs of cesium chloride buoyant density gradients of DNA from whole cells of culture strain *Trypanosoma lewisi* grown under normal conditions and in the presence of acriflavin and ethidium bromide. The reference band (1.742) to the right is native DNA of SPO1.

therefore, carry the same genetic information. The relatively long linear molecules which were always found in preparations of kinetoplast DNA might carry considerably more information than the circles. It is also possible, however, that the linear molecules comprise tandem repeats of a single 0.4 $\mu$ nucleotide sequence length.

Our finding that kinetoplast DNA is greatly reduced in *T. lewisi* cultured in the presence of acriflavin is in agreement with the observations of Trager and Rudzinska (1964), and Simpson (1968) on *Leishmania tarentolae*, Guttman and Eisenman (1965) on *Crithidia fasciculata*, and Steinert and Van Assel (1967) on *Trypanosoma mega* and *Crithidia luciliae* (for a review of earlier reports of the elimination of kinetoplast DNA by acridine dyes, see Mühlpfordt, 1959 and 1963). Reduction of kinetoplast DNA in cells of *Trypanosoma cruzi* cultured in the presence of ethidium bromide has been reported by Riou (1968) and Riou and Delain (1969). However, the light satellite DNA was reduced by only 30%, and multiple-length 0.45 $\mu$ circles, rarely found in DNA from untreated cells, accounted for more than 30% of this DNA. These observations clearly differ from the present findings that culturing *T. lewisi* cells in the presence of ethidium bromide resulted in disappearance of the light-satellite DNA and reduction of the kinetoplast circles by more than 99%. It is plausible that in both species the ethidium bromide, which intercalates between DNA base pairs (Crawford and Waring, 1967), is interfering with replication of the circular molecules. The extreme effect in *T. lewisi* might be the result of more of the ethidium bromide penetrating the kinetoplast membrane in this species, even though a similar concentration of the drug was present in the culture medium in the two experiments.

In agreement with previous reports, we found

TABLE I

*A Comparison of the Proportion of DNA as Linear and as Circular Molecules Isolated from Whole Cells of Trypanosoma lewisi Grown under Normal Conditions of Culture, and in the Presence of Either Ethidium Bromide or Acriflavin*

Catenanes, apparently comprising many interlocked 0.4 $\mu$ circles, were not found in the DNA of cells grown in the presence of either ethidium bromide or acriflavin and were not included in the estimates of circular DNA from cells grown under normal culture conditions.

| | Control | Ethidium bromide | Acriflavin |
|---|---|---|---|
| Mean length of linear molecules in microns (n) | 38.4 (58) | 22.8 (22) | 33.0 (25) |
| Ratio of molecules observed, linear: circles | 58:372 | 84:3 | 153:9 |
| % DNA as 0.4 $\mu$ circles | 6.409 | 0.064 | 0.073 |

that cells of *T. lewisi* lacking a kinetoplast could not be cultured indefinitely.

In hemoflagellates, including *T. lewisi* it is, likely that the entire mitochondrial complement of the cell, which is highly extended and convoluted, is connected to the kinetoplast portion (see Simpson, 1968). At present, however, there is no evidence as to whether or not regions of this organelle other than the kinetoplast portion contain DNA.

We wish to thank Dr. D. G. Dusanic for providing stocks of both the blood strain and the culture strain of *Trypanosoma lewisi*, Dr. K. G. Lark for the *Escherichia coli* $^{14}$C-labeled DNA, Dr. D. Wilson for the SP01 DNA, and Misses Kay Curry, Patricia Meinhardt, Cheryl Hetherington, and Claudia Garrett for technical assistance. We are also indebted to Dr. K. G. Lark for constructive criticism of the text.

This study was supported by National Institutes of Health grant No. GM-16636 and American Cancer Society grant No. E-531.

*Received for publication 30 March 1970, and in revised form 22 July 1970.*

## REFERENCES

BAUER, W., and J. VINOGRAD. 1968. The intercalation of closed circular DNA with intercalative dyes. I. The superhelix density of SV40 DNA in the presence and absence of dye. *J. Mol. Biol.* **33**:141.

BRESSLAU, E., and L. SCREMIN. 1924. Die Kerne der Tryapnosomen und ihr Verhalten zur Nuklealreaktion. *Arch. Protistenk.* **55**:509.

CLARK, T. B., and F. G. WALLACE. 1960. A comparative study of kinetoplast ultrastructure in the Trypanosomatidae. *J. Protozool.* **7**:115.

CRAWFORD, L. V., and M. J. WARING. 1967. Supercoiling of polyoma virus DNA measured by intercalation with ethidium bromide. *J. Mol. Biol.* **25**:23.

DAWID, I. B., and D. R. WOLSTENHOLME. 1967. Ultracentrifuge and electron microscope studies on the structure of mitochondrial DNA. *J. Mol. Biol.* **28**:233.

DAWID, I. B., and D. R. WOLSTENHOLME. 1968. Renaturation and hybridation studies of mitochondrial DNA. *Biophys. J.* **8**:65.

DUBUY, H. G., C. F. T. MATTERN, and F. L. RILEY. 1965. Isolation and characterization of DNA from kinetoplasts of *Leishmania enriettii*. *Science* (*Washington*). **147**:754.

DUSANIC, D. G. 1968. Growth and immunological studies on the culture forms of *Trypanosoma lewisi*. *J. Protozool.* **15**:328.

FREIFELDER, D., and A. K. KLEINSCHMIDT. 1965. Single strand breaks in duplex DNA of coliphage T7 as demonstrated by electron microscopy. *J. Mol. Biol.* **14**:271.

GUTTMAN, H. M., and R. N. EISENMAN. 1965. Acriflavin-induced loss of kinetoplast deoxyribocleic acid in *Crithidia fasciculata* (*Culex pipiens* strain). *Nature* (*London*). **207**:1280.

HICKSON, F. T., T. F. ROTH, and D. R. HELINSKI. 1967. Circular DNA form of a bacterial sex factor. *Proc. Nat. Acad. Sci. U. S. A.* **58**:1731.

HONIGBERG, B. M., W. BALAMUTH, E. C. BOVEE, J. O. CORLISS, M. GOJDICS, R. P. HALL, R. R. KUDO, N. D. LEVINE, A. R. LOEBLICH, J. WEISER, and D. H. WENRICH. 1964. A revised classification of the phylum Protozoa. *J. Protozool.* **11**:7.

HUDSON, B., and J. VINOGRAD. 1967. Catenated circular DNA molecules in HeLa cells. *Nature* (*London*). **216**:647.

KIMMEL, J. R., and E. L. SMITH. 1954. Crystalline papain. I. Preparation, specificity, and activation. *J. Biol. Chem.* **207**:515.

LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.

MACHATTIE, L. A., and C. A. THOMAS. 1964. DNA from bacteriophage Lambda: Molecular length and conformation. *Science* (*Washington*). **144**:1142.

MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic-acid from micro-organisms. *J. Mol. Biol.* **3**:208.

MARMUR, J., and P. DOTY. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* **5**:119.

MESELSON, M., F. W. STAHL, and J. VINOGRAD. 1957. Equilibrium sedimentation of macromolecules in density gradients. *Proc. Nat. Acad. Sci. U. S. A.* **43**:581.

MEYER, H., M. DE OLIVEIRA MUSACCHIO, and I. DE ANDRADE MENDONÇA. 1958. Electron microscopic study of *Trypanosoma cruzi* in thin sections of infected tissue cultures and of blood-agar forms. *Parasitology*. **48**:1.

MÜHLPFORDT, H. 1959. Vergleichende Untersuchung über die Wirkung des Trypaflavins auf den Blepharoplast verschiedener Trypanosomenarten. *J. Trop. Parasitol.* **10**:19.

MÜHLPFORDT, H. 1963. Über die Bedeutung und Feinstruktur des Blepharoplasten bei parasitischen Flagellaten. I. Teil. *Z. Tropenmed. Parasitol.* **14**:357.

MÜHLPFORDT, H. 1963. Über die Bedeutung und Feinstruktur des Blepharoplasten bei Parasitischen Flagellaten. II. Teil. *Z. Tropenmed. Parasitol.* **14**:475.

NASS, M. M. K. 1969. Mitochondrial DNA. II. Structure and physicochemical properties of isolated DNA. *J. Mol. Biol.* **42**:529.

PITELKA, D. R. 1961. Observations on the kinetoplast mitochondrion and cytostome of *Bodo*. *Exp. Cell Res.* **25**:87.

RADLOFF, R., W. BAUER, and J. VINOGRAD. 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA. *Proc. Nat. Acad. Sci. U. S. A.* **57**:1514.

RANDALL, J., and C. DISBREY. 1965. Evidence for the presence of DNA at basal body sites in *Tetrahymena pyriformis*. *Proc. Roy. Soc. London Ser. B.* **162**:473.

RIOU, G. 1968. Disparition de l'ADN du kinetoplaste de *Trypanosoma cruzi* cultive au presence de bromure d'ethidium. *Compt. Rend.* **266**:250.

RIOU, G., and E. DELAIN. 1968. Electron microscopy of the circular kinetoplastic DNA from *Trypanosoma cruzi*: occurrence of catenated forms. *Proc. Nat. Acad. Sci. U. S. A.* **62**:210.

RIOU, G., and E. DELAIN. 1969. Abnormal circular DNA molecules induced by ethidium bromide in the kinetoplast of *Trypanosoma cruzi*. *Proc. Nat. Acad. Sci. U. S. A.* **64**:618.

RIOU, G., and C. PAOLETTI. 1967. Preparation and properties of nuclear and satellite deoxyribonucleic acid of *Trypanosoma cruzi*. *J. Mol. Biol.* **28**:377.

RIS, H. 1962. Ultrastructure of certain self-dependent cytoplasmic organelles. Proceedings 5th International Congress of Electron Microscopy. Philadelphia. **2**:XX-1.

ROTH, T. F., and D. R. HELINSKI. 1967. Evidence for circular DNA forms of bacterial plasmid. *Proc. Nat. Acad. Sci. U. S. A.* **58**:650.

RYTER, A., E. KELLENBERGER, A. BIRCH-ANDERSON, and O. MAALØE. 1958. Étude au microscope électronique de plasmas contenant de l'acide désoxyribonucléique. I. des nucléoides des bactéries en croissance active. *Z. Naturforsch.* **13b**: 597.

SCHILDKRAUT, C. L., J. MARMUR, and P. DOTY. 1962. Determination of the base composition of deoxy ribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* **4**:430.

SIMPSON, L. 1968. Effect of acriflavin on the kinetoplast of *Leishmania tarentolae*. Mode of action and physiological correlates of the loss of kinetoplast DNA. *J. Cell Biol.* **37**:660.

STEINERT, M. 1960. Mitochondria associated with the kinetonucleus of *Trypanosoma mega*. *J. Biophys. Biochem. Cytol.* **8**:542.

STEINERT, M., and S. VAN ASSEL. 1967. The loss of kinetoplastic DNA in two species of Trypanosomatidae treated with acriflavin. *J. Cell. Biol.* **34**:489.

TRAGER, W., and RUDZINSKA. 1964. The riboflavin requirement and the effects of acriflavin on the fine structure of the kinetoplast of *Leishmania tarentolae*. *J. Protozool.* **11**:133.

VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.

VINOGRAD, J., and J. LEBOWITZ. 1966. Physical and topological properties of circular DNA. *J. Gen. Physiol.* **49**:103.

WATSON, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.* **4**:475.

WOLSTENHOLME, D. R., and N. J. GROSS. 1968. The form and size of mitochondria DNA of the red bean, *Phaseolus vulgaris*. *Proc. Nat. Acad. Sci. U. S. A.* **61**:245.