KINETOPLAST DEOXYRIBONUCLEIC ACID
OF THE HEMOFLAGELLATE TRYPANOSOMA LEWISI

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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 µ circular molecules and large masses of 0.4 µ interlocked circles with which longer, often noncircular molecules were associated. The 0.4 µ 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 µ 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 µ 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; Clerk and Wallace, 1960; Fitelka, 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 µ 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 µ 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 mM sodium chloride, 0.01 mM 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-sodium citrate. The red and white blood corpuscles were removed by repeated centrifugation at 3000 g and resuspension 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.

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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 µg/ml. Fractions of approximately 75 µl 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 µg/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 KG 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 ethanol, and embedded in Epon (Luft, 1961). Thin sections were cut on an LKB Ulrotome 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 µl 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, 0.3% formaldehyde (FreiJeder 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; Mühlfordt, 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, ρ = 1.699, and one heavier, ρ = 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 (guanilyc 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 μ; standard deviation ± 27.0, range 1–105 μ). Circular DNA molecules having a mean contour length of 0.4 μ 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 μ 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 μ circular molecules and catenanes. Long, often noncircular molecules (up to at least 7 μ 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 μ circle (Fig. 10). Also molecules apparently consisting of two or three 0.4 μ 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 μ 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-
The reference band (1.742) to the right is native DNA of SPOT. 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 µ 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 (Tm) for unsonicated DNA from both the whole cell and kinetoplast fraction of T. lewisi trypanosomes is shown in Fig. 17. The Tm for the kinetoplast fraction is slightly lower than for the whole cell fraction, but both show a single, sharp transition. The buoyant density of the DNA from the kinetoplast fraction is shown in Fig. 18. The density of the main band is approximately 1.742, while the density of the light satellite band is approximately 1.711. The differences in density between the main band and light satellite are consistent with the differences in melting temperature between the two fractions. The equilibrium band positions in cesium chloride of DNA from whole cells, and from a kinetoplast fraction of blood strain Trypanosoma lewisi. The reference band (1.742) to the right is native DNA of SPOT.
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 µ. × 92,700.

**Figure 10**  Two circular molecules, of contour lengths 0.4 µ and 0.8 µ. × 87,500.

**Figure 11**  A molecule apparently comprising eight interlocked 0.4 µ 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 µ. Seven 0.4 µ 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 µ circles with which much longer molecules (*L*) are associated. × 51,000.
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 86.3°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 µ circles was found (Fig. 11). These observations are consistent with the interpretation of the large masses of DNA as comprising mainly 0.4 µ 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 µg/ml ethidium bromide for 10 days or in the presence of 2 µg/ml acriflavin for 12 days. A kinetoplast could not be discerned in 95.5% of Giemsa-stained and 97% of Feulgen-
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 kinetoplast 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, and single circles were estimated to account

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 µ 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

for the remaining 96.5% of the DNA. No light-satellite DNA band could be detected in DNA extracted from cells grown in the presence of ethidium bromide, and single circles were estimated to account
for only 0.06% of the DNA compared to 6.4% of 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 kine-
toplast, 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 μ. The 0.4 μ 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 μ 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 μ 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 μ, molecular weight 7.7 × 10^6 (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 kineto-

plast 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-con-
taining 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.
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 SPOT.

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 µ 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 µ 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 |

|                     | 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 µ 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.

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