THE FORM AND STRUCTURE OF KINETOPLAST DNA OF *CRITHIDIA*

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ABSTRACT

Cesium chloride centrifugation of each of the DNAs extracted from eight strains of *Crithidia* revealed a main band at $\rho = 1.717$ g/cm^3 and a satellite band varying from $\rho = 1.701$ to 1.705 g/cm^3 for the different strains By electron microscopy each DNA was shown to include circular molecules, 0.69–0.80 μ in mean contour length, and large, topologically two-dimensional masses of DNA in which the molecules appeared in the form of rosettes. DNA isolated from kinetoplast fractions of *Crithidia acanthocephali* was shown to consist of light satellite DNA and to be mainly in the form of large masses, 0.8 μ (mol wt $= 1.54 \times 10^6$ daltons) circular molecules, and a few long, linear molecules. The results of experiments involving ultracentrifugation, heating, and quenching, sonication, and endodeoxyribonuclease digestion, combined with electron microscopy, are consistent with the following hypothesis. The large DNA masses are associations of 0.8 μ circles which are mainly covalently closed. The circles are held together in groups (the rosettes) of up to 46 by the topological interlocking of each circle with many other circles in the group. A group of circles is attached to an adjacent group by one or more circles, each interlocking with many circles of both groups. Each of the associations comprises, on the average, about 27,000 circles (total mol wt $\simeq 41 \times 10^9$ daltons). A model is proposed for the *in situ* arrangement of the associations which takes into consideration their form and structure, and appearance in thin sections

INTRODUCTION

Members of the protozoan order kinetoplastida (Honigberg et al., 1964) which includes the hemoflagellate genera *Trypanosoma* and *Leishmania*, and the insect parasites *Crithidia*, are characterized by the presence of a body known as the kinetoplast. This organelle is a modified region of a mitochondrion (Meyer et al, 1958; Steinert, 1960; Clark and Wallace, 1960, Pitelka, 1961; Ris, 1962), which contains DNA detectable by Feulgen staining (Bresslau and Scremin, 1924). DNAs isolated from kinetoplast fractions of a number of species of hemoflagellates have been shown in each case to have a buoyant density less than that of the respective organism's nuclear DNA (DuBuy et al, 1965, Renger and Wolstenholme, 1970, 1971; Simpson and da Silva, 1971) In electron microscope preparations kinetoplast DNA was found to include circular molecules ranging in contour length from about 0.3 μ in *Trypanosoma congolense* and *Trypanosoma equiperdum* (Renger and Wolstenholme, 1971) and *Leishmania tarentolae* (Simpson and da Silva, 1971) to 0 74 μ in *Trypanosoma mega* (Laurent and Steinert, 1970) Kinetoplast DNA from each species also included large masses. For *Trypanosoma lewisi* (Renger and Wolstenholme, 1970) and *L. tarentolae* (Simpson and da Silva, 1971) evidence was presented that these masses consisted of interlocked small circular

molecules which were covalently closed Some linear or long molecules were found in the DNA from all kinetoplast fractions examined up to the present time A DNA separated from whole cells of *Trypanosoma cruzi* and presumed to be kinetoplast DNA, had similar properties (Riou and Paoletti, 1967; Riou and Delain, 1968). The contour length of circular molecules from this species was 0.45 μ. Simpson and da Silva (1971) have argued that in the case of *L. tarentolae*, long kinetoplast DNA, which accounts for 33% of this DNA, holds together associations of circles by threading through the small circles and catenanes.

The present report describes the results of experiments designed to determine the form, structure, and arrangement of kinetoplast DNA molecules of members of the genus *Crithidia* whose natural life cycle involves only an insect host.

MATERIAL AND METHODS

Cultures of eight strains of *Crithidia* were obtained from Dr. Helene Guttman at the University of Illinois, Chicago Circle. These were: *Crithidia acanthocephali*; *Crithidia fasciculata* (the *Culex pipiens* strain of Nöller, American Type Culture Collection #12858), *Crithidia fasciculata* (the *Culex pipiens* strain of Wallace, ATCC #12857); *Crithidia luciliae* (ATCC #14765); *Crithidia rileyi*; *Crithidia* sp. from *Arilus*, *Crithidia* sp. from *Euryophthalmus davisi*; *Crithidia* sp. from a Syrphid.

The organisms were grown without agitation in 250-ml flasks at 27°C under sterile conditions in a broth containing 0.5% trypticase, 0.01% liver fraction "L" (Nutritional Biochemicals Corporation, Cleveland, Ohio), 0.5% yeast extract, 0.0025% hemin, 0.25% triethanolamine, and 0.5% sucrose, and adjusted to pH 7.9 (Guttman and Eisenman, 1965 *a*). Preparation of the broth involved first making a solution of 50% triethanolamine containing 0.4% hemin. This was then added to the other ingredients in solution to give the final concentrations reported above, and the whole broth was autoclaved. Transfer of cells was made every 10 days Cells were harvested by centrifugation at 1000 *g* for 10 min and washed three times with 0.15 M sodium chloride, 0.015 M sodium citrate (SSC), pH 7.5.

A kinetoplast-enriched fraction was isolated from cells of *C. acanthocephali* by a method similar to that used previously for the isolation of kinetoplasts from *T. lewisi* (Renger and Wolstenholme, 1970). Freshly harvested organisms were resuspended in a solution containing 0.3 M sucrose, 1 mM disodium ethylenediaminetetraacetate (EDTA), 0.01 M Tris/HCl at pH 7.4, broken open in a Waring Blender (15 sec at high speed), and centrifuged at 700 *g* for 10 min This centrifugation 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 the aforementioned buffer and incubated with 200 μg/ml beef pancreatic DNase I (Worthington Biochemical Corp., Freehold, N.J.) at 37°C for 30 min in the presence of 7 mM magnesium chloride (Rabinowitz et al., 1965; Renger and Wolstenholme, 1970). The DNase was removed by washing three times with buffer containing 0.04 M EDTA. A portion of the kinetoplast-enriched fraction was then fixed for electron microscopy and the remainder was suspended in 0.15 M sodium chloride, 0.1 M EDTA, and 0.05 M sodium phosphate (pH 8.0) and either frozen or used directly.

DNA was extracted from whole cells after lysis with 2% sodium dodecyl sulfate by modification of the method described by Kirschner et al. (1968). The lysate was shaken three times for 60 min at room temperature with phenol equilibrated with the same buffer as that in which the cells were suspended. Phenol was removed from the aqueous phase by shaking with ether which was finally evaporated away by blowing air through the solution Pancreatic ribonuclease-A (Sigma Chemical Co, St. Louis, Mo., previously heated to 90°C for 5 min at pH 5 0) was added to 200 μg/ml, and the mixture incubated at 37°C for 30 min. The solution was dialized against 20 vol of 0 35 M NaCl, 2 mM EDTA, and 0.05 M sodium phosphate at pH 6.7 for 2 hr with two changes DNA was then precipitated by adding 0.55 vol of isopropyl alcohol, collected on a glass rod, and redissolved in $\frac{1}{10}$ SSC The DNA was reprecipitated and dissolved in SSC.

Cesium chloride analytical ultracentrifugations were carried out according to Meselson et al. (1957), using an An-D rotor in a Beckman Spinco Model E analytical ultracentrifuge at 42,040 rpm for at least 20 hr at 20°C. Microdensitometer tracings were made either of ultraviolet photographs using a Joyce Loebl densitometer, or directly using a photoelectric scanner.

Preparative cesium chloride–ethidium bromide density gradient centrifugations (Radloff et al, 1967) were performed in a Beckman Spinco Model, L2-65B using polyallomer tubes and an SW-65 titanium rotor. The initial cesium chloride density was adjusted to 1.55 g/ml and the ethidium bromide (a gift of Boots Pure Drug Co., Ltd., Nottingham, England) was used at a concentration of 300 μg/ml. After centrifugation at 47,000 rpm for 44 hr, gradients were irradiated with light from a high pressure HBO 200 mercury lamp, transmitted by Zeiss UG1 and BG38 exciter filters. The bands of fluorescence, resulting from ethidium bromide–DNA complexes, were observed using a Kodak Wratten number 16 filter. Photographs were made using the same

illumination and filter on Kodak Panatomic-X film. The distances between bands were estimated by making measurements on photographs representing a gradient magnification of 4. Fractions were collected and the ethidium bromide was removed from the DNA suspension by a single passage through Dowex 50W resin (Radloff et al., 1967).

DNA solutions cooled with water from an ice bath were sonicated at 1 amp for 35 sec using a 10 KC Ratheon oscillator.

Crude SDS-papain lysates of kinetoplast-enriched fractions were prepared as described by Renger and Wolstenholme (1970) and used to make cesium chloride buoyant density gradients and rotary shadowed preparations for electron microscopy.

Whole cells or pellets of kinetoplast-enriched 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 uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965) Protein monolayers of DNA in crude lysates and of purified DNA were prepared, picked up on copper grids, and shadowed as described previously (Renger and Wolstenholme, 1970). Electron micrographs were made with an Hitachi HU-11B electron microscope. The shadowed molecules were photographed (using projector pole piece 2) at original magnifications of 11,000 (calibrated with a diffraction grating replica [Fullam, 2160 lines/mm]). Measurements of molecules were made on positive prints at a magnification of 150,000 using a map measure.

Crithidia were prepared for light microscopy, examined, and photographed as described for *T. lewisi* (Renger and Wolstenholme, 1970).

RESULTS

Kinetoplasts In Situ and Kinetoplast-Enriched Fractions

Light microscopy of Giemsa-stained organisms confirmed that cells of all eight strains contained a kinetoplast.

In thin longitudinal sections of whole cells of *Crithidia*, the kinetoplast appeared as an oblong mass of DNA-containing fibrils, about 25 A in diameter (Ris, 1962, Mühlpfordt, 1963), situated in an enlarged portion of a mitochondrion (Fig. 1). In some longitudinal sections, the DNA-containing fibrils were seen to be in parallel array (Fig. 2) The dimension of the mass in the direction of the parallel array was always about 0.5 μ. In transverse sections the over-all shape of the kinetoplast DNA was that of a disc measuring up to 1.9 μ in diameter (Fig. 3), and comprising short fibrils showing no particular orientation, and dots. The latter suggest an orientation of some fibrils perpendicular to the transverse section, which is in agreement with the observations of parallel arrays of fibrils in longitudinal sections.

The DNase-treated kinetoplast-enriched fractions included an abundance of intact, membrane-bounded structures having the morphological characteristics of kinetoplasts and mitochondria (Figs. 4–7). The DNA-containing fibrils and their arrangement in parallel array within the kinetoplasts were clearly preserved (Figs. 6 and 7). Fragments of flagella and basal bodies, sometimes seen in association with kinetoplasts, and membrane fragments were also present. Nuclei

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FIGURES 1–3 Electron micrographs of sections through the kinetoplast of whole cells of *C. acanthocephali*. *C*, cell wall. All micrographs, × 22,000.

FIGURE 1 A longitudinal section through a kinetoplast. The DNA (*K*) is situated within an enlarged portion of a mitochondrion (*M*).

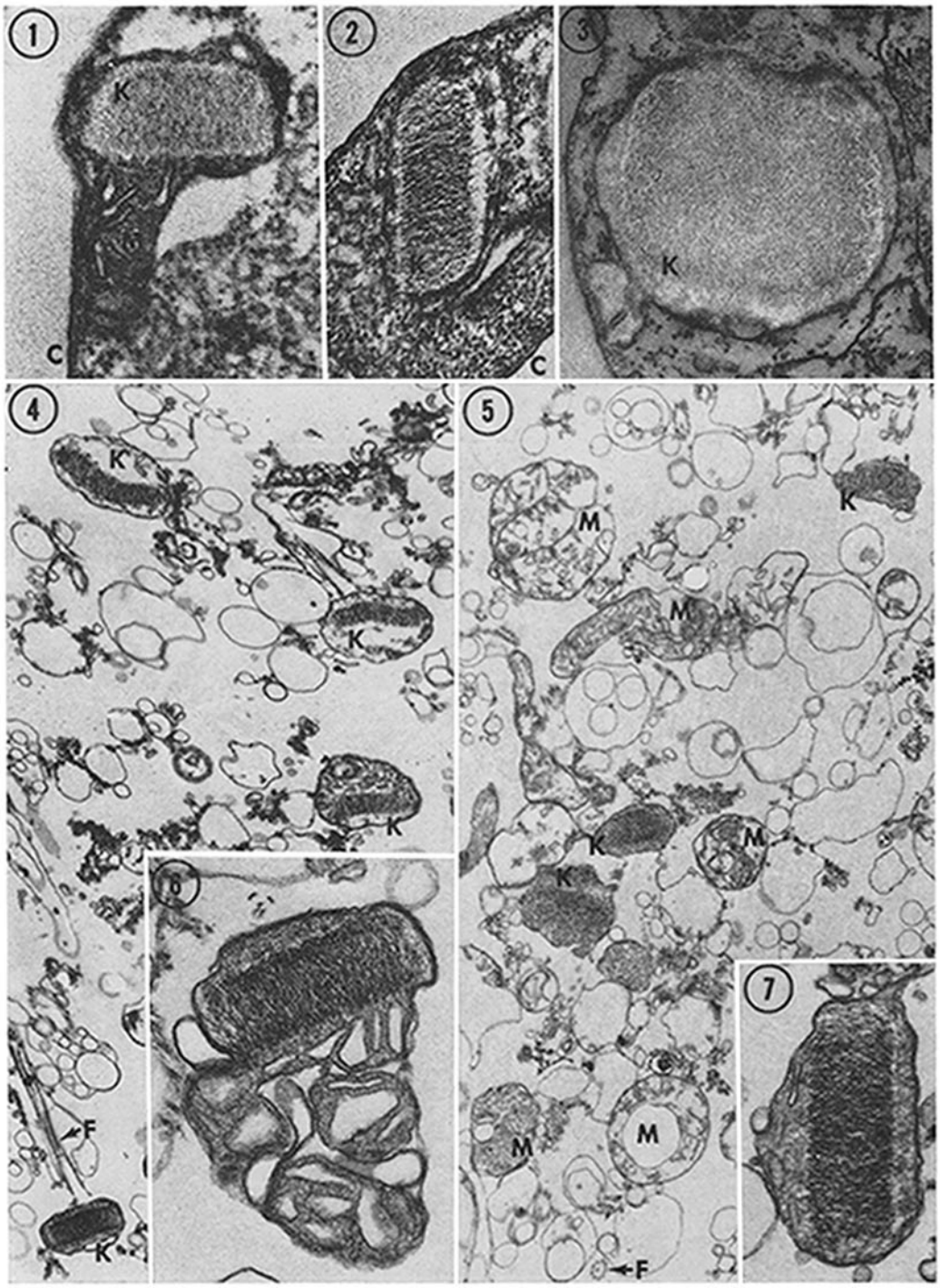
FIGURE 2 A longitudinal section through a kinetoplast. The DNA fibrils (*K*) lie in parallel array.

FIGURE 3 A transverse section through a kinetoplast (*K*). *N*, nucleus

FIGURES 4–7 Electron micrographs of sections of a pellet of a DNase-treated, kinetoplast-enriched fraction prepared from *C. acanthocephali*

FIGURES 4 and 5 The fraction contains intact, membrane-bounded kinetoplasts (*K*) and mitochondria (*M*). Flagella (*F*) sometimes seen in association with kinetoplasts, and membrane fragments are also present. Fig. 4, × 7000; Fig. 5, × 10,000.

FIGURES 6 and 7 Two kinetoplasts. The DNA-containing fibrils and their arrangement in parallel array are clearly preserved. Both micrographs, × 25,000.


1
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C
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F
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C

TABLE I

Buoyant Densities of Main Band and Satellite DNAs and Contour Lengths of Circular DNA Molecules of Eight Strains of Crithidia

For each of the buoyant density values the standard error and the number of runs are given. For each of the mean contour lengths, the standard error and the number of molecules examined are given The insect of origin is shown in parentheses for some of the strains

Strain	Buoyant density (g/cm³)		Mean contour length in microns
	Main band	Satellite band	
C. acanthocephali	1.717 ± 0.0001 (5)	1.702 ± 0.0001 (8)	0.80 ± 0.002 (95)
C. fasciculata (*Culex pipiens*) (Nöller strain)	1.717 ± 0.0001 (2)	1.701 ± 0.0001 (2)	0.73 ± 0.007 (20)
C. fasciculata (*Culex pipiens*)	1.717 ± 0.0002 (2)	1.701 ± 0.0002 (2)	0.72 ± 0.013 (20)
C. luciliae	1.717 ± 0.0001 (3)	1.705 ± 0.0002 (3)	0.75 ± 0.011 (20)
C. rileyi	1.717 ± 0.0002 (2)	1.703 ± 0.0001 (2)	0.72 ± 0.016 (20)
Crithidia sp. (*Arilus*)	1.717 ± 0.0002 (2)	1.702 ± 0.0004 (2)	0.80 ± 0.004 (20)
Crithidia sp. (*E. davisi*)	1.717 ± 0.0002 (2)	1.701 ± 0.0003 (2)	0 73 ± 0.006 (20)
Crithidia sp. (Syrphid)	1.717 ± 0.0002 (2)	1.701 ± 0.0003 (2)	0.69 ± 0 012 (20)

were virtually absent. Membranes surrounding basal bodies were rarely intact, or were absent. It seems unlikely, therefore, that any DNA which might be associated with them (Randall and Disbrey, 1965) would survive the DNase treatment.

Buoyant Densities

The results of analytical cesium chloride buoyant density centrifugation of DNA from whole cells of each of the eight *Crithidia* strains are given in Table I and Fig. 8. Most of the DNA of each strain banded at a density of 1.717 g/cm³, but as has been found for all kinetoplastida examined to date (see Renger and Wolstenholme, 1970 and 1971 for references), in each case a light satellite was present. The satellite DNA varied in density from 1.701 g/cm³ to 1.705 g/cm³.

DNA from lysates of DNase-treated, kinetoplast-enriched fractions of cells of *C acanthocephali* banded exclusively at the same density as light satellite DNA, 1.702 g/cm³, of this species (Fig. 8).

Forms of Kinetoplast DNA

In rotary shadowed electron microscope preparations, DNA from whole cells of each strain was mainly in the form of linear molecules. However, each whole cell DNA included circular molecules which appeared to be of one size class for an individual strain, but to vary in mean contour length from 0.69 to 0.80 μ for the different strains (Table I, Figs. 9–12, and 15). As some variation has been found in mean contour length of circular DNA molecules from a single DNA sample in different protein monolayer preparations (Wolstenholme and Dawid, 1968), it remains possible that the lengths of the circles of the different strains are in fact the same. In each DNA preparation large masses of DNA were also seen (Fig. 13). These masses had an over-all circular or oval shape and covered areas up to 73 μ^2. Evidence will be presented below that they comprise circular molecules, and they are, therefore, referred to as associations of circles.

DNA from kinetoplast-enriched fractions of *C. acanthocephali* was found to consist of associations of circles and single 0.8 μ circles. Some linear molecules reaching measured lengths of 12 μ were occasionally found in contact with the edges of the associations, or lying free. Molecules apparently consisting of two to four interlocked single length (0 8 μ) circles were also found.

It is known that the mass per unit length of DNA prepared by the protein monolayer technique is influenced by the salt concentration of both

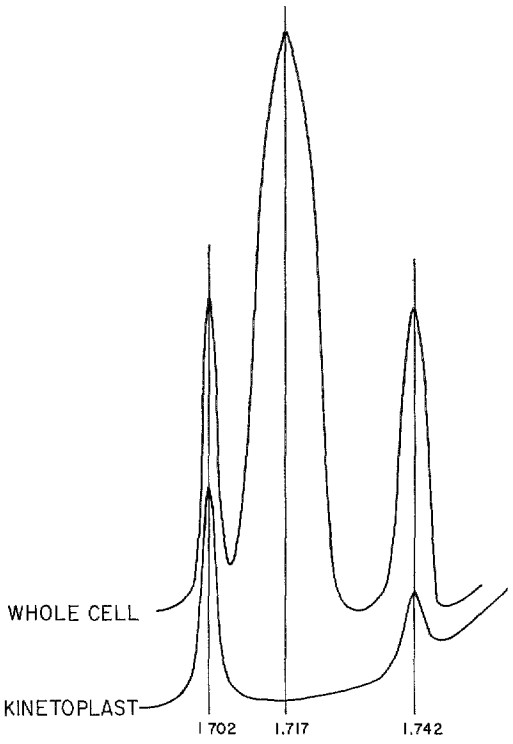


FIGURE 8 Microdensitometer tracings of ultraviolet photographs of cesium chloride buoyant density gradients of DNA from whole cells, and from a lysate of a DNase-treated kinetoplast-enriched fraction of *C. acanthocephali*. The reference band (ρ = 1.742 g/cm^3) to the right is native DNA of bacteriophage SPO1.

the hypophase and the spreading solution (Inman, 1967, Lang, 1970). In order, therefore, to make an estimate of the molecular weight of a molecule from its length, it is necessary to compare it with that of a molecule whose molecular weight has been determined by independent techniques This was done for circular kinetoplast DNA molecules by mixing *C. acanthocephali* DNA with bacteriophage ϕX174 double-stranded replicative form DNA, preparing for electron microscopy, and comparing the lengths of the two classes of molecule found on the same grid square (Fig. 15). A ratio of lengths of 0.42:1.00 was found for *C. acanthocephali* circles and ϕX174 RF circles, respectively. Taking the mol wt of ϕX174 RF to be 3.4×10^6 daltons (which is twice the molecular weight reported for the single-stranded form of this DNA [Sinsheimer, 1959]), a mol wt of 1.54×10^6 daltons (SE = $\pm$ 0 008 $\times 10^6$ daltons) is indicated for the *C. acanthocephali* circular kinetoplast DNA molecule.

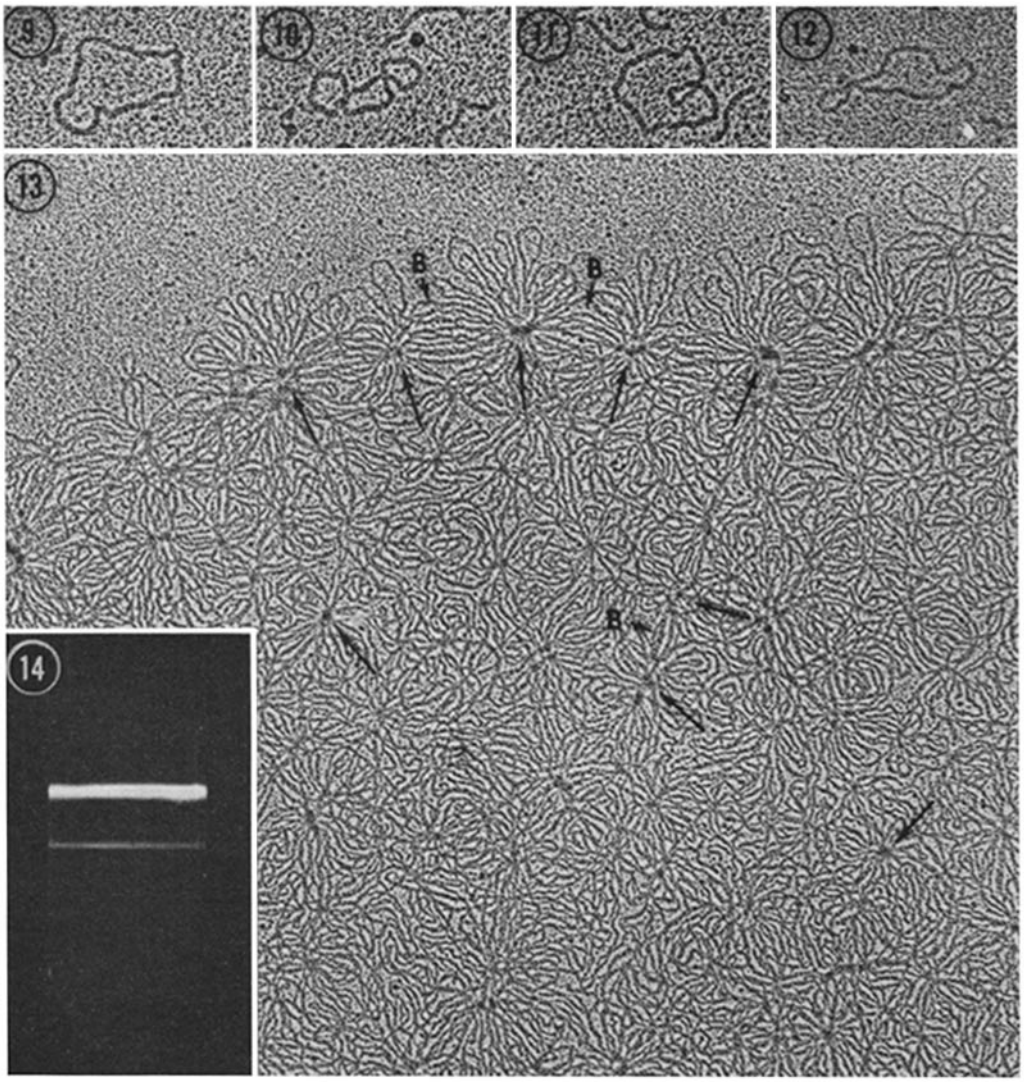
Structure of the Kinetoplast DNA

Most of the kinetoplast DNA of *T. lewisi* (Renger and Wolstenholme, 1970), *T cruzi* (Riou and Delain, 1968), and *L. tarentolae* (Simpson and da Silva, 1971) was shown to be in the form of circular molecules which are interlocked into large masses. The circular molecules were found in each case to be mainly covalently closed structures in the sense defined by Vinograd and Lebowitz (1966), that is, all of the phosphodiester bonds in each of the two polynucleotide chains of a molecule were intact.

Whole cell DNA of *C. acanthocephali* was centrifuged to equilibrium in a cesium chloride-ethidium bromide gradient. Two distinct bands were formed at a mean distance of 4.51 mm apart (SE = $\pm$ 0.30 mm; No. = 3) (Fig. 14). The contents of each band were examined in rotary shadowed preparations in the electron microscope. The denser band comprised associations of circles, a few single 0.8 μ circular molecules, and a few linear molecules The linear molecules (1 $-$ 6 μ length) were always lying free and never found in contact with the associations (50 associations were examined). The less dense band consisted almost entirely of long linear molecules. Since covalently closed circular molecules bind less dye than circular molecules containing at least one phosphodiester bond break, or linear molecules, and therefore band at a greater density (Radloff et al, 1967; Bauer and Vinograd, 1968), the present observations indicate that the associations of *C. acanthocephali* comprise covalently closed circular molecules.

To further test this interpretation, the effect of heating kinetoplast DNA in 1/10 SSC at 100°C for 5 min and quenching in ice was examined. Covalently closed circles have been shown to be more resistant to denaturation by heating than noncovalently closed DNA (Vinograd and Lebowitz, 1966; Nass, 1969; Renger and Wolstenholme, 1970, Wolstenholme et al., 1972) and to appear in their native configuration upon cooling (Renger and Wolstenholme, 1970, Wolstenholme et al, 1972). The equilibrium band positions in cesium chloride of heated and quenched whole cell DNA of *C. acanthocephali* are shown in Fig. 16. The buoyant density of the light satellite DNA was increased by only 2 mg/cm^3 compared to 14 mg/cm^3 for the main band DNA, indicating that most of the

kinetoplast DNA did not denature. The small band at 1.717 g/cm³ could be either denatured light satellite DNA or undenatured main band DNA. When heated and quenched whole cell DNA was examined in the electron microscope, double-stranded 0.8 μ circular molecules and associations apparently comprising double-stranded circular molecules undistinguishable



FIGURES 9–13 Electron micrographs of rotary shadowed molecules from whole cell DNA of *C. acanthocephali.*

FIGURES 9–12 Circular molecules approximately 0.8 μ in contour length. All micrographs, × 68,000.

FIGURE 13 An association of DNA apparently comprising rosettes. The centers of some of the rosettes are indicated by arrows, and in some places appear to be connected by bundles of filaments, *B*. The loops of the rosettes can be clearly seen at the edge (top) of the association. × 44,000.

FIGURE 14 Fluorescence photograph of a cesium chloride–ethidium bromide gradient resulting from centrifuging 150 μg whole cell DNA of *C. acanthocephali* at 47,000 rpm for 44 hr in a Beckman SW65 rotor. The photograph was taken using illumination from a high pressure HBO 200 mercury lamp, Zeiss UG1 and BG38 exciter filters, and a Kodak Wratten number 16 filter as a barrier filter. Two fluorescence bands, the center-to-center distance of which is 45 mm, are clearly visible. Photograph, × 1.65.

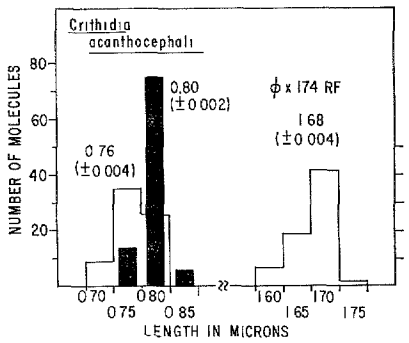


FIGURE 15 Frequency distributions of lengths of circular molecules of *C. acanthocephali* (in whole cell DNA preparations) spread separately (black areas) and of *C. acanthocephali* DNA and bacteriophage φX174 replicative form DNA spread together (open areas). The mean and standard error of each sample are given.

from native associations (see below) were found. (Under the conditions used to make these preparations, single-stranded DNA either collapses or appears as kinky threads poor in contrast and is, therefore, easily distinguished from double-stranded DNA; see Dawid and Wolstenholme, 1968)

In the electron microscope the edges of the associations from preparations of whole cell DNA, from lysates of kinetoplast-enriched fractions, and from the lower band of cesium chloride–ethidium bromide gradients of whole cell DNA always appeared to be made up of "rosettes" (Figs. 13 and 17). Each rosette had a center from which loops radiated. The centers of adjacent rosettes were usually less than 0 4 μ apart. Where they could be clearly traced, the loops were found never to exceed 0.85 μ in total length and rarely to have more than two points of crossover. Rosette centers were visible in the inner areas of some associations (Fig. 13), especially when the association was well spread (Fig. 17). These observations, together with the results cited above, suggested that the associations are made up of groups of 0.8 μ circular molecules. Using kinetoplast DNA isolated from whole cell DNA by cesium chloride–ethidium bromide centrifugation experiments were conducted to further test this hypothesis and to determine in more detail the structure of the associations

Kinetoplast DNA was sonicated and examined in the electron microscope. Single rosettes were observed The number of loops in a rosette was estimated by counting the number of strands radiating from the center and dividing by two. Rosettes comprising up to approximately 46 loops were found (Figs. 19–25) A single loop never exceeded 0 85 μ in length When the number of loops was small, their lengths could be accurately measured and were always found to be in the range 0.73 – 0 85 μ (see, for example, Figs 21–24) This supports the view that the rosettes comprise 0 8 μ circles. The circles could be held together by topological interlocking. The rosette

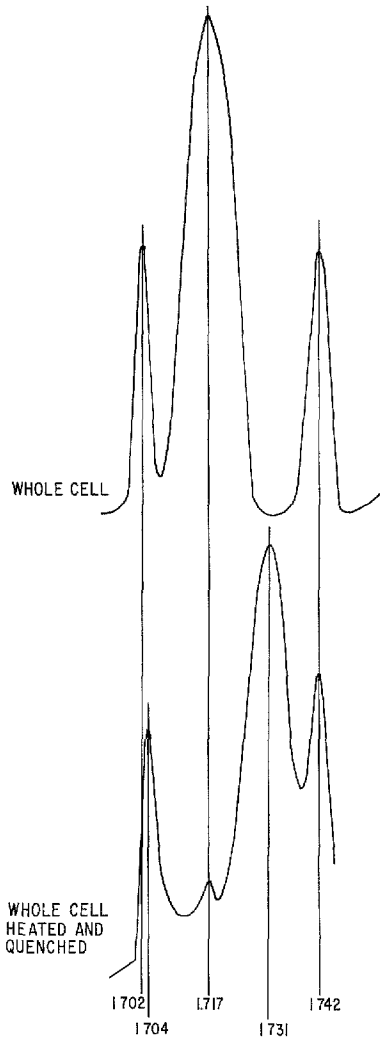


FIGURE 16 Microdensitometer tracings of ultraviolet photographs of cesium chloride buoyant density gradients of DNA isolated from whole cells of *C. acanthocephali*. The upper tracing is of native DNA; the lower one is of DNA which was heated at 100°C for 5 min in $\frac{1}{10}$ SSC and then quenched in ice. The reference band (ρ = 1.742 g/cm^3) to the right is native DNA of bacteriophage SPO1.

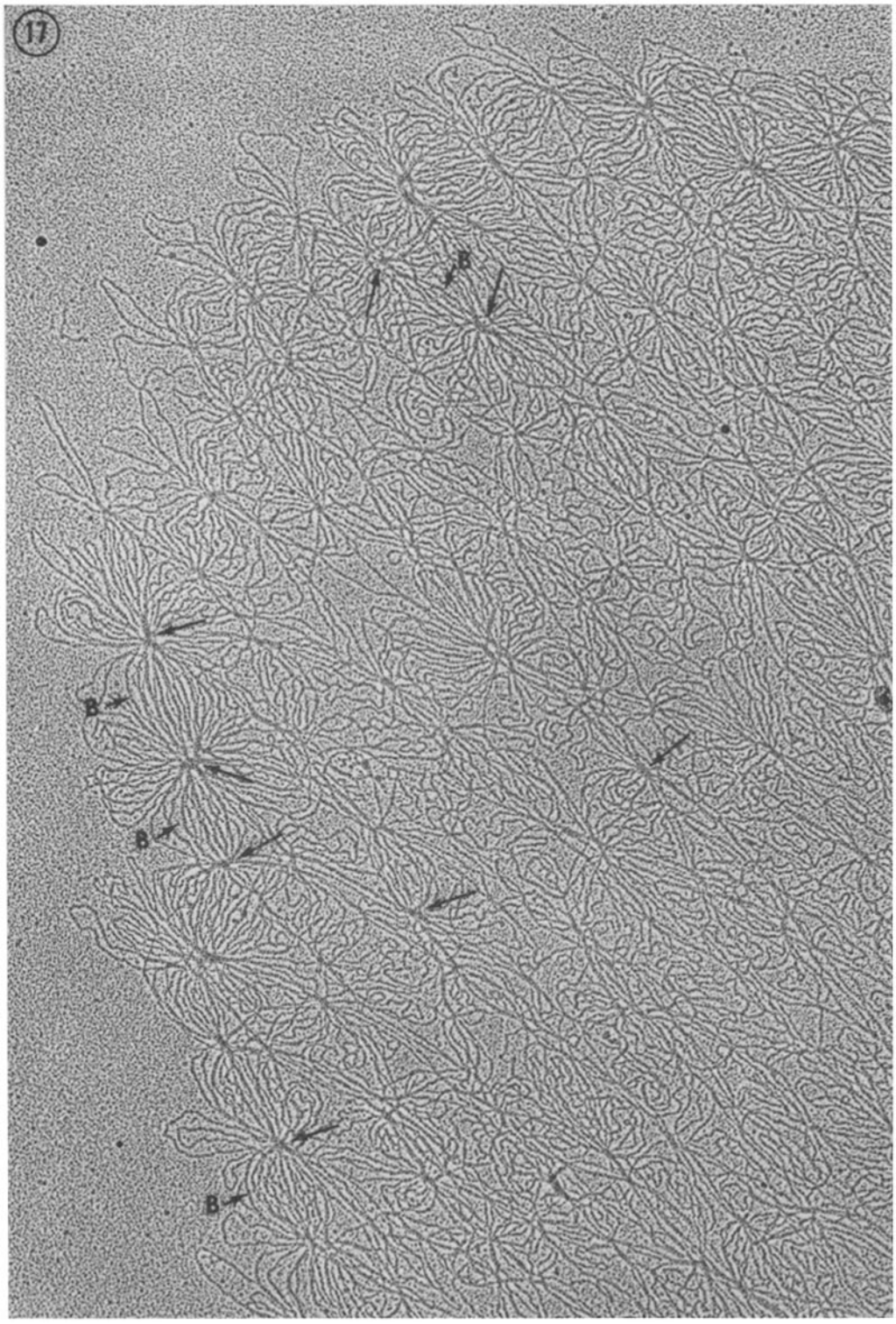


FIGURE 17 Electron micrograph of rotary shadowed kinetoplast DNA of *C acanthocephali* This association from a preparation of whole cell DNA is well spread out and the rosette forms are apparent. The centers of the rosettes (some of which are indicated by arrows) appear in some places to be connected by bundles of fibers (*B*). × 50,000.

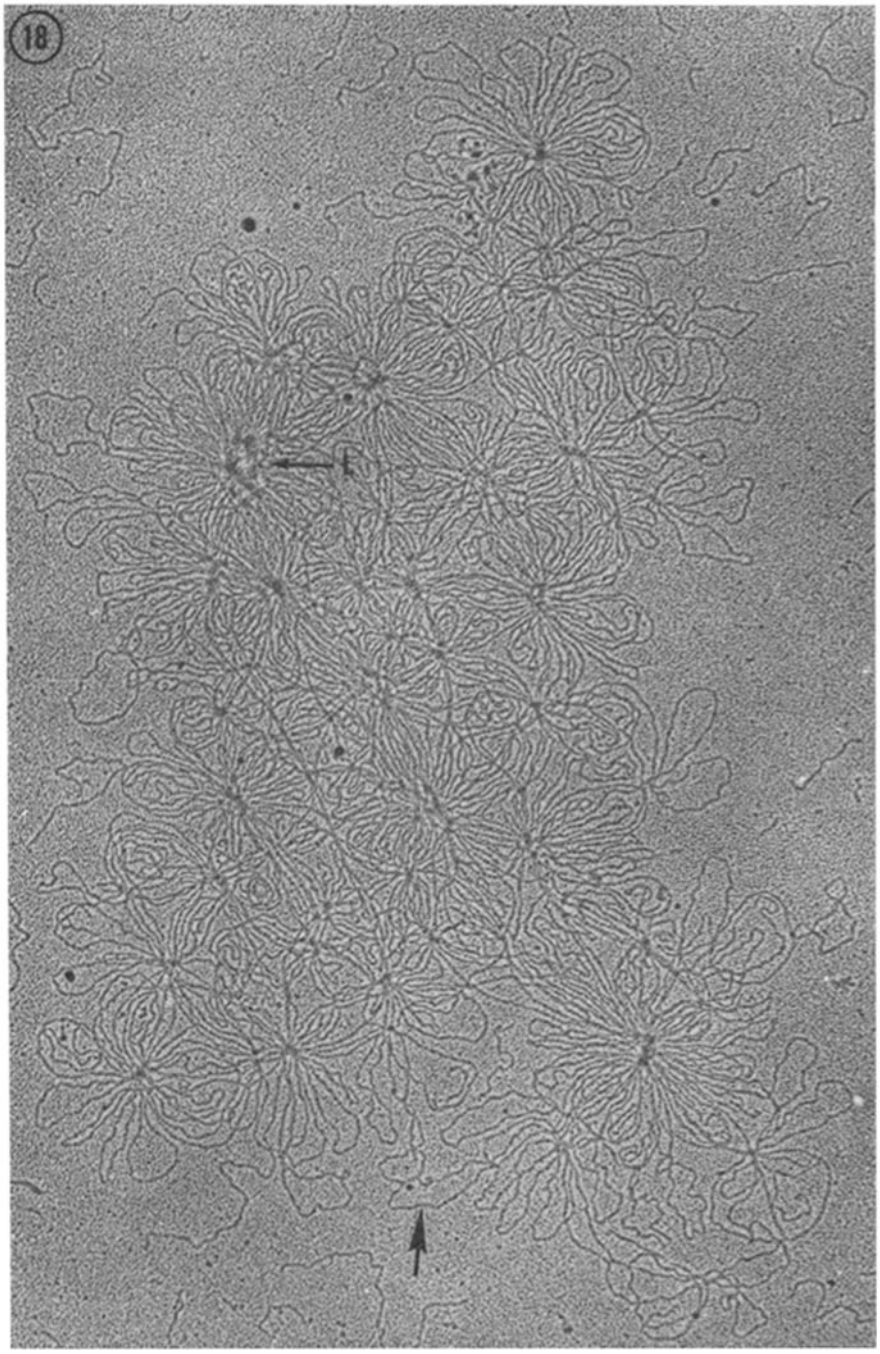


FIGURE 18 Electron micrograph of a rotary shadowed fragment of an association of kinetoplast DNA of *C. acanthocephali* produced by sonication for 35 sec. Rosettes can be clearly distinguished. The arrow indicates two filaments apparently comprising a chain of four 0.8 μ circles connecting the centers of two rosettes. One rosette (*L*) has a large, more diffuse center from which an apparently larger number of loops radiate. × 50,000.

forms could be produced if each circle were interlocked with all or many of the other circles in a group and, upon spreading, the circles were restrained from a tendency to separate from each other. In support of this latter interpretation was the finding that chains of more than three circles were rare. Structures made up of varying numbers of rosettes were also observed (Figs. 18 and 25). A few rosettes were seen which had a larger, more diffuse center, from which an apparently larger (but uncountable) number of loops radiated (Fig. 18). Whether these were in fact double or multiple forms of single rosettes was not clear. In some cases, as shown in Fig. 25, the center of a rosette appeared to be joined to that of an adjacent rosette by even numbers of strands which never exceeded 0 42 μ in length. This suggests that groups of circles are held together by one or more circles, each interlocking with many of the circles of adjacent groups. The orientation and length of bundles of fibers running between rosette centers in intact associations (Figs. 13 and 17) are also in agreement with this interpretation. In some cases the center of one rosette was connected to that of an adjacent rosette by a chain apparently comprising two, three, or four circles (Fig. 18, arrow) Neither longer chains of circles nor long linear molecules connecting rosettes or groups of rosettes were ever found Linear molecules and single circles were abundant in this preparation. However, more than 99% of the linear molecules had lengths equal to, or less than, that of a single circle.

These findings, together with the observation of groups of 20–30 rosettes in the form exemplified in Fig. 18, clearly indicate that the rosette form is a uniform component throughout the associations

If, in fact, associations are made up of groups of circular DNA molecules held together by topological interlocking, then, upon limited digestion with an endodeoxyribonuclease, only circular molecules and linear pieces no longer than the circular molecules should be generated. Kinetoplast DNA prepared by cesium chloride–ethidium bromide centrifugation was, therefore, digested with 1 5 $\times$ 10^{-6} μg/ml of pancreatic DNase for 5 min. This nuclease breaks only one polynucleotide chain at a time, but eventually results in double-strand breakage of the molecule. When this DNA preparation was examined in the electron microscope before digestion, it was found that in an area containing 50 associations there was present an average of 26 free circles and a total of 15 μ of linear DNA per association As this DNA represents only 0.17% of the weight of DNA in the average association (see next section), it was not taken into consideration.

After the DNase digestion, the DNA appeared as in Fig. 28. Of the total DNA (molecules measuring a total length of 652 μ were examined), 2%

FIGURES 19–25 Electron micrographs of rotary shadowed kinetoplast DNA after sonication for 35 sec.

Figs. 19 and 25, $\times$ 68,000; Figs. 20–25, $\times$ 78,000

FIGURES 19 and 20 Single rosettes comprising approximately 30 and 40 loops, respectively.

FIGURE 21 A single rosette comprising 10 loops. The loops are 0.74–0.82 μ in contour length.

FIGURE 22 A single rosette comprising six loops. The loops are 0.74–0.82 μ in contour length

FIGURE 23 A structure comprising three loops, 0.74–0.82 μ in contour length.

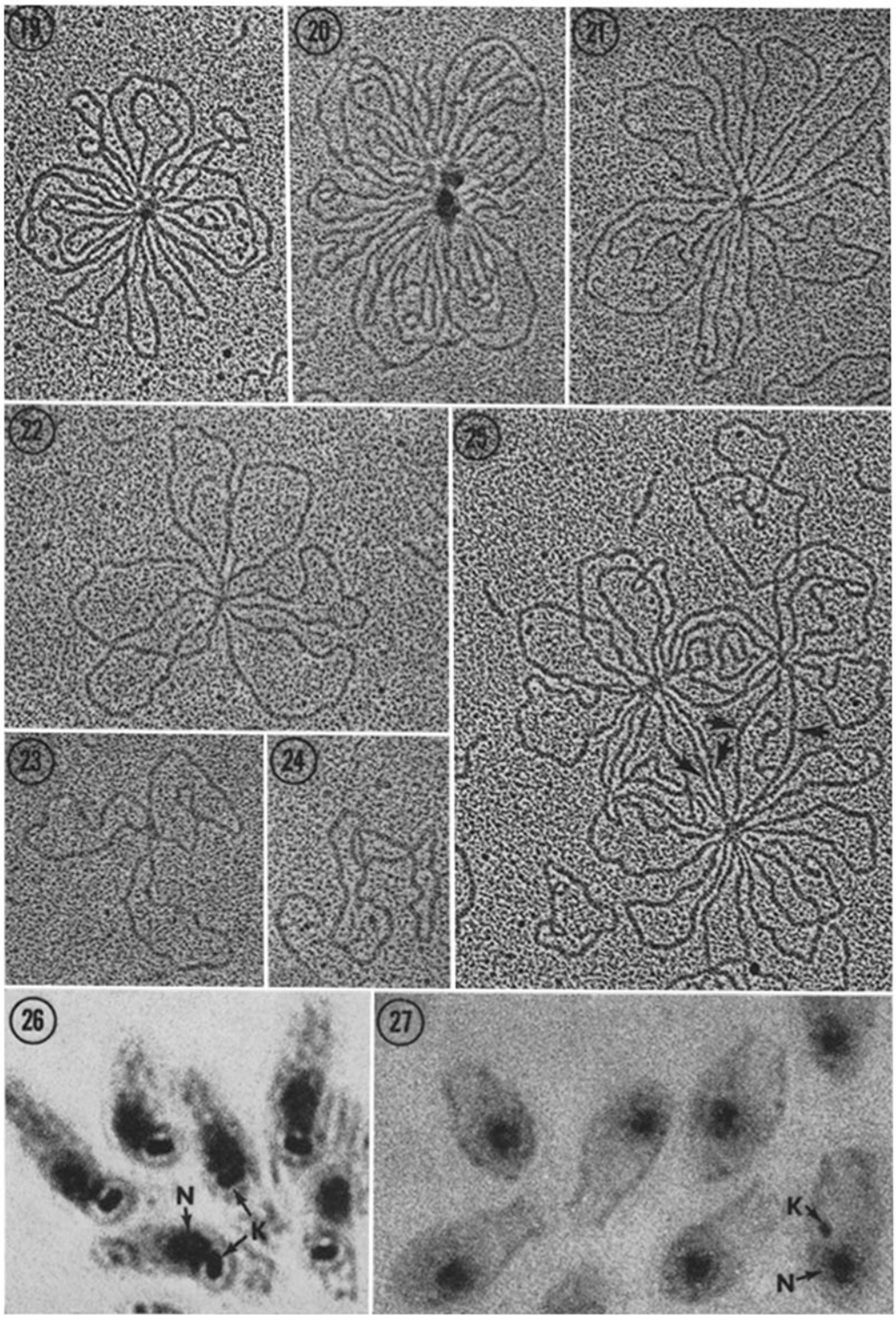
FIGURE 24 Two apparently connected 0.8 μ circles

FIGURE 25 Three rosettes apparently held together by molecules approximately 0.4 μ in length joining their centers (arrows)

FIGURES 26 and 27 Bright field micrographs of Giemsa-stained cells of *C. acanthocephali*. Both micrographs, $\times$ 3000.

FIGURE 26 Cells cultured under normal conditions. The kinetoplasts (*K*) and nuclei (*N*) are clearly visible.

FIGURE 27 Cells cultured in the presence of acriflavin. A kinetoplast (*K*) is visible in only one of the cells and is reduced in size. *N*, nucleus.


19
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K
27
K
N

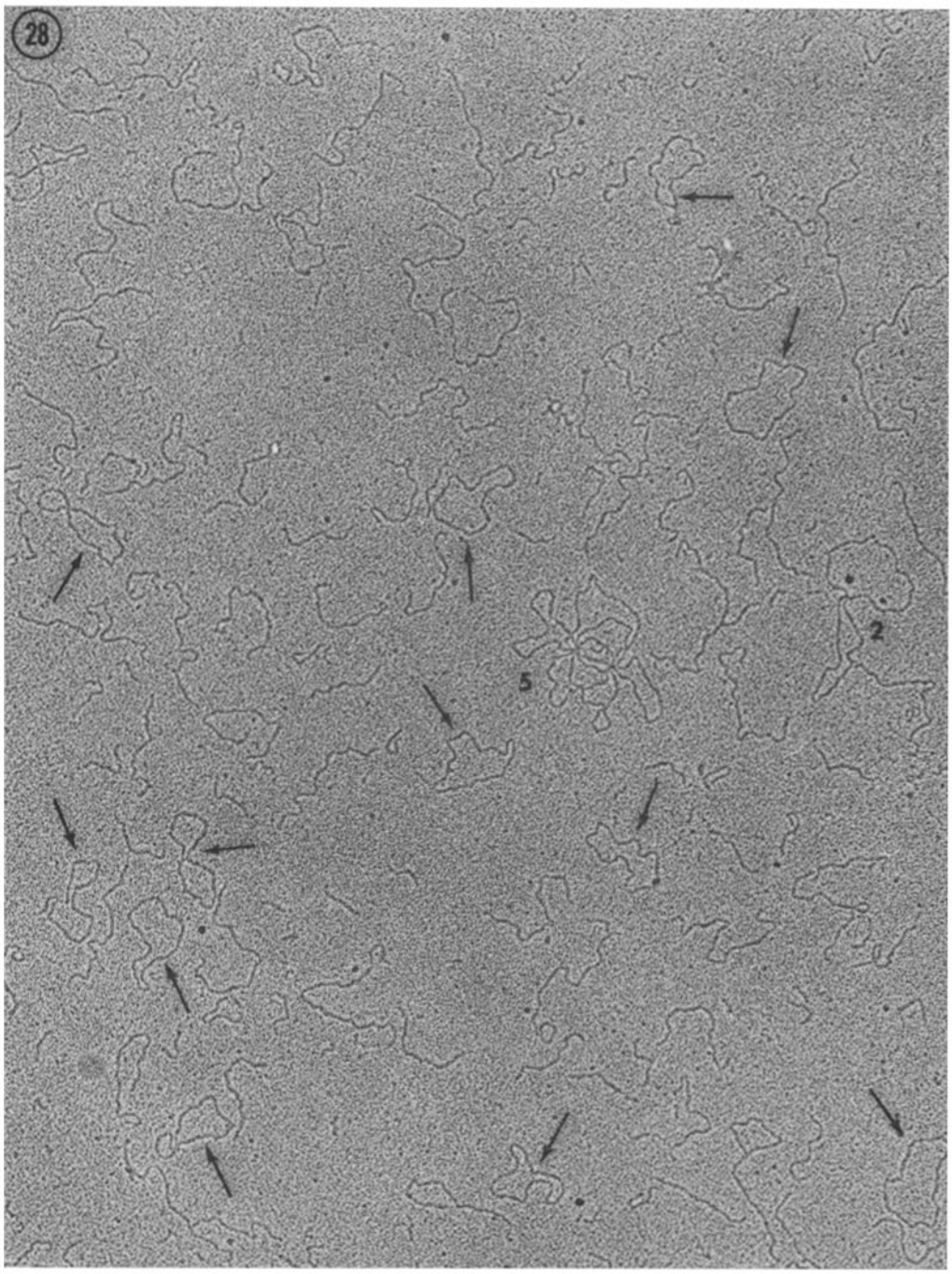

FIGURE 28 Electron micrograph of rotary shadowed kinetoplast DNA of *C ancanthocephali* digested with pancreatic DNase (1.5×10^{-6} μg/ml in the presence of 40 μg/ml serum albumin, 0 05 M Tris buffer, pH 7.4, and 5 mM $MgCl_2$ at 23°C for 5 min). Circular molecules, approximately 0 8 μ in contour length, are indicated by the arrows. Forms apparently consisting of two (*2*) and five (*5*) interlocked circles are also visible. None of the linear molecules are more than 0 82 μ in length. × 50,000.

appeared as two interlocked circles, and 4% as three to seven interlocked circles. The remaining 94% was in the form of single molecules. 7% was single circles with a mean contour length of 0 8 μ (SE = $\pm$ 0 003 μ, No = 58) and 87% was linear molecules This result indicates that on the average not more than 12 circles need to be broken in order to release a single circle. Only 0.8% of the linear DNA was represented by molecules longer than the length of a single circle. 11 single circles, each with an apparently attached linear piece measuring from 0.3 to 0.8 μ, were found, suggesting the possibility that some of the circular molecules of the associations are in the form of "fused dimers" (Clayton et al , 1970), referred to as "figure eights" by Simpson and da Silva (1971).

These results are clearly consistent with the suggestion that the major basic unit of the association is a 0.8 μ circular DNA molecule.

When a sample of this same kinetoplast preparation was similarly digested with DNase, but for approximately one-half the time taken to reduce the associations to 94% single molecules, molecular forms identical in appearance to those described for the sonicated sample were found.

Molecular Weights of Associations

In order to make estimates of the total molecular weights of the associations, five complete associations from whole cell DNA were photographed in the electron microscope. For each association a montage was constructed and the area covered by the association was determined The mean distance between the centers of adjacent rosettes was next determined by making 40 measurements on areas of each association where the centers were most clearly visible. These data were used to calculate the mean area taken up by a rosette by assuming that the rosettes were packed in a hexagonal array, from this value and the total association area the number of rosettes per association was calculated The mean number of circles per rosette was estimated by counting the filaments visible in 90° sectors of rosettes at the edges of the association and multiplying by 2 Multiplication of this value by the total number of rosettes gave the total number of circular DNA molecules in an association. This estimate is based upon the assumption that the average number of circles in a group at the periphery of the association is the same as that in a more centrally located group From these values and the molecular weight of a circle (1 54 $\times$ 10^6 daltons), the molecular weight of each association was derived

The results are summarized in Table II. Associations were estimated to comprise between 21,000 and 31,000 circular molecules, and to have molecular weights of between 32 $\times$ 10^9 and 47 $\times$ 10^9 daltons

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

Cells of *C. acanthocephali* were grown in the presence of 4 μg/ml ethidium bromide for 8 days, or in the presence of 4 μg/ml acriflavin for 6 days. A kinetoplast could not be discerned in 93% of Giemsa-stained cells cultured in the presence of ethidium bromide In the remaining 7% of the cells, a stained body smaller than, but in the same position as, the kinetoplast in normal cells was visible. No light satellite DNA band could be detected in DNA extracted from whole cells grown in the presence of ethidium bromide (Fig. 29) When this whole cell DNA was examined in the electron microscope, no associations were seen Single circles were rare. They had a mean contour length 0 75 μ (SE = $\pm$ 0 008 μ, No. = 35), similar to that of circles grown under normal conditions.

After growth in the presence of acriflavin, 81%

TABLE II

Estimates of the Number of Rosettes, Circular DNA Molecules, and Molecular Weights for Five Associations of Kinetoplast DNA of Crithidia acanthocephali

	Total rosettes	Mean number of circles per rosette (No = 20)	Total circles	Molecular weight $\times$ 10^9
Mean	804	33.2	26,693	41 12
Range	734–1095	28–36	21,046–30,660	32.41–47 22

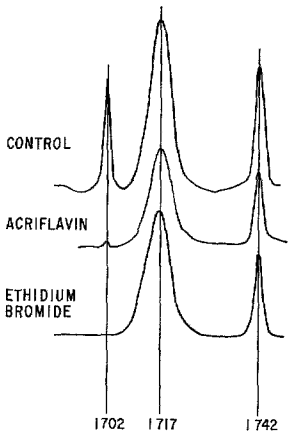

FIGURE 29 Photoelectric scans using 260 mμ illumination of cesium chloride buoyant density gradients of DNA from whole cells of *C. acanthocephali* grown under normal conditions and in the presence of acriflavin and ethidium bromide. The reference band (ρ = 1.742 g/cm^3) to the right is native DNA of bacteriophage SPO1.

of the cells had no discernible kinetoplast and, again, the kinetoplasts in the remaining 19% of the cells were reduced (Figs. 26 and 27). A light satellite band was still detectable in DNA isolated from these cells grown in acriflavin, but accounted for only about 2% of the total DNA compared to 15% of the total DNA from normal cells (Fig. 29). Again associations were not detected in electron microscope preparations. Single circles were present but rare, and had a mean contour length of 0.78 μ (SE = ± 0 005 μ; No. = 64).

DISCUSSION

The data presented are clearly consistent with the interpretation that kinetoplast DNA of *C acanthocephali* is in the form of circular molecules approximately 0.8 μ in contour length, and that these circles are topologically interlocked into groups of up to 46 circles. The rosette forms of the DNA which were observed would only result upon spreading if one circle was interlocked with many others in the group. As, however, the mean number of circles in a rosette was indicated to be about 33, but a single circle was released by breaking on the average no more than 12 other circles, each circle cannot be topologically interlocked to all the other circles in its group. A group of circles appears to be attached to an adjacent group by one or more circles, each interlocking with many circles of both groups. It is also possible that some groups of circles are joined either by the interlocking of a single circle from each of two groups, or by a third circle interlocking with a single circle from two groups.

The possibility that the rosette forms result from a spreading artifact seems very unlikely: such a structure is peculiar to DNA of kinetoplast origin and to DNA from the lower band of cesium chloride–ethidium bromide gradients of whole cell DNA The rosette form is maintained in this DNA after a variety of treatments including mild DNase digestion and sonication. Also, wherever clearly measurable, the loops of the rosettes have a constant length.

The data presented do not rule out the possibility that the circular DNA molecules are in fact held together by a specialized structural joint. However, such a joint would have to involve bonding sensitive to pancreatic DNase in order to be consistent with the molecular forms observed after digestion of the associations with this nuclease. Also, as linear forms were only very rarely seen attached to rosette centers after either DNase digestion or sonication, the joint would, for each circle, have to be broken before the circularity of the molecule was destroyed.

As has been found for the circular molecules of hemoflagellate kinetoplast DNA (Riou and Delain, 1968; Renger and Wolstenholme, 1970 and 1971, Simpson and da Silva, 1971), the circular molecules of *C. acanthocephali* are apparently covalently closed.

Linear or long molecules have always been found in DNA isolated from kinetoplast fractions. The possibility that at least some of these molecules represent contamination by relatively small amounts of nuclear DNA has not been ruled out

Simpson and da Silva (1971) have interpreted their observations concerning *L. tarentolae* as indicating that the associations of the kinetoplast DNA of this organism are made up by long molecules holding together small circular molecules and groups of circular molecules by threading through them. These authors presented micrographs showing long chains of "figure eight" molecules joining together groups of circles.

From the present results there is no evidence to suggest that long or linear DNA molecules play a major role in holding together the groups of circular kinetoplast DNA molecules of *C acanthocephali*. The observation that after cesium chloride–ethidium bromide centrifugation linear DNA

molecules were no longer in contact with the edges of the associations suggests the possibility that linear DNA is in no way involved in the structure of the associations of this organism.

Laurent and Steinert (1970) observed rosette patterns in DNA masses which they obtained from kinetoplast fractions of *T. mega*. However, they reported that the loops of the rosettes were of variable size, and suggested that the rosettes resulted from an artifactual folding of a long molecule upon spreading The present results are clearly at variance with this interpretation

A molecule of mol wt 1.54×10^6 daltons could contain the information for determining the amino acid sequences of only four polypeptides with an average mol wt of 20,000 daltons (assuming an average mol wt of an amino acid of 135 daltons). The circles appear to be of one size class, but there is no indication from our data as to whether they all have the same nucleotide sequences and, therefore, carry the same genetic information The circle size of kinetoplast DNA of hemoflagellates examined up to the present time, with the exception of *T. mega* (Laurent and Steinert, 1970), is smaller than that of *C acanthocephali* (Riou and Delain, 1968; Renger and Wolstenholme, 1970, 1971; Simpson and da Silva, 1971), raising the possibility that they carry less information. If in *C acanthocephali* all the circles code for the same information, then our data indicate that each organism carries on the average at least 27,000 copies

There is no evidence from the present data as to whether each kinetoplast contains one or more associations Upon examination of light satellite DNA from *C lucıliae* by fluoresce microscopy, Laurent et al. (1971) found it to comprise large masses. They presented evidence that the masses had about the same molecular weight as *in situ* kinetoplast DNA of this organism, and concluded, therefore, that a single mass represented the total kinetoplast DNA compliment of a single cell. The mean mol wt they obtained for the masses was 22×10^9 daltons Considering the difference in the two techniques employed, this value is in good agreement with the present estimate of a mean value of 41×10^9 daltons for a kinetoplast association of *C. acanthocephali*. There may, of course, be a species difference. Also, as in both cases estimates were made on kinetoplast DNA of unsynchronized cells, the observed variation in values for molecular weights of associations from a single species was not unexpected. Delain and Riou (1969) and Riou and Delain (1969) estimated a total of about 24,000 circular DNA molecules per kinetoplast of *T cruzi* from a consideration of the total weight of DNA in a single kinetoplast and the contour length of a circular molecule of this organisms. Similar estimates, made by Simpson and da Silva (1971) for *L tarentolae*, yielded a mean value of 10,000 circles per kinetoplast

The fact that in protein monolayer preparations the DNA molecules of the associations can be clearly visualized indicates that *in situ* the groups of circles must be held together in a form which is topologically two dimensional. That is, a single group of circles is bound to groups of circles all around it, but not to groups of circles at some distance away If the latter were true, then upon spreading the DNA would retain a three-dimensional arrangement and shadowing would not result in the individual filaments being discernible

From examinations of thin sections, it is clear that the kinetoplast DNA of *Crithidia* is in the shape of a disc, *in situ* (see also Kussel et al , 1967; Clark and Wallace, 1960) The diameter of the disc is about 1 9 μ and the height, 0.5 μ. Also, the DNA filaments appear to be preferentially oriented to the height of the disc. This latter observation would be explained if most of the circular molecules of an individual group were fully open (i. e., formed perfect circles), and the plane of each circle was oriented parallel to the height of the disc. The maximum dimension of such an arrangement, which would be equal to two 0.8 μ circles arranged tangentially, would be 0.5 μ: the correlation is clear The circular molecules binding together individual adjacent groups of circles might be oriented in any plane but, owing to their relatively small number, would not interfere with the general appearance of the kinetoplast DNA in parallel arrays in longitudinal sections

The structures seen in protein monolayer preparations are compatible with this model They would result simply from an over-all centrifugal expansion of the associations upon spreading. This would result in elongation of the circular configuration of the circular molecules holding together the groups of circules. The rosette configurations would result from the circles of a group being restrained from a tendency to leave each other upon spreading It is interesting to note that, in *T. lewisi*, where the contour length of the kineto-

plast circular DNA molecules is 0 4 μ, the height of the kinetoplast mass seen in longitudinal thin sections in which parallel arrays of DNA filaments are evident is only 0.25 μ (see Renger and Wolstenholme, 1970) This is what would be expected if the kinetoplast DNA of this organism has the same structure and is arranged *in situ* in the manner proposed for *C acanthocephali*. In protein monolayer preparation of DNA associations from *T. lewisi* kinetoplasts, the arrangement of the component molecules was not clear, probably due to the small size of the circles However, in sonicated preparations of this DNA, rosette formations have been observed (Renger and Wolstenholme, unpublished)

Delain and Riou (1969) have proposed that in the kinetoplast of *T cruzi* the circular molecules each contain a single twist and are stacked side by side to produce the parallel configuration of DNA filaments and dimensions of the DNA mass they observed in thin longitudinal sections Simpson and da Silva (1971) have reported that in *L tarentolae* the width (height) of the disc of kinetoplasts DNA is equal to the deameter of a *single* fully opened circular molecule of this species, and suggest that the circles or loops of long molecules they observe are "aligned in pancake fashion" to form the structures seen in thin sections of kinetoplasts. They have also given a similar explanation for the arrangement of *T. cruzi* kinetoplast DNA. Neither of these models take into consideration what is known of the quaternary structure of the respective DNAs

The finding that kinetoplast DNA is greatly reduced in *C. acanthocephali* grown in the presence of acriflavin, is in agreement with previous observations concerning *C fasciculata* (Guttman and Eisenman, 1965 *b*), *T mega* and *C luciliae* (Steinert and Van Assel, 1967), *L. tarentolae* (Trager and Rudzinska, 1964, Simpson, 1968), and *T. lewisi* (Renger and Wolstenholme, 1970). (Earlier reports of elimination of kinetoplasts by acridine dyes have been reviewed by Mühlpfordt, 1959, 1963) Recently, Stuart (1971) reported that treatment of *Trypanosoma brucei* with acriflavin eliminated kinetoplasts detectable by light microscopy of stained cells However, he interpreted a buoyant density analysis of whole cell DNA from acriflavin-treated organisms to indicate that the kinetoplast DNA had not been reduced. This author did not, however, take into consideration the possibility of a nonkinetoplastic satellite DNA in these cells which has a buoyant density identical to that of kinetoplast DNA. This is important because such a satellite DNA has been reported in DNA of cells of the related *T. equiperdum* and *T equinum*, and also in *T. congolense* (Renger and Wolstenholme, 1971).

Reduction of kinetoplast DNA in cells of *T. lewisi* (Renger and Wolstenholme, 1970) and *T cruzi* (Riou, 1968; Riou and Delain, 1969) grown in the presence of ethidium bromide has been reported In the latter case, the light satellite DNA was reduced by only 30%, and 30% of the remaining DNA was in the form of multiple length circles. Possible explanations for the different effects of ethidium bromide have been discussed by us previously (Renger and Wolstenholme, 1970).

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