PHYSICOCHEMICAL PROPERTIES OF KINETOPLAST DNA FROM *CRITHIDIA ACANTHOCEPHALI,* *CRITHIDIA LUCILIAE,* AND *TRYPANOSOMA LEWISI*

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ABSTRACT

The protozoa *Crithidia* and *Trypanosoma* contain within a mitochondrion a mass of DNA known as kinetoplast DNA (kDNA) which consists mainly of an association of thousands of small circular molecules of similar size held together by topological interlocking. Using kDNA from *Crithidia acanthocephali, Crithidia luciliae,* and *Trypanosoma lewisi,* physicochemical studies have been carried out with intact associations and with fractions of covalently closed single circular molecules, and of open single circular and unit length linear molecules obtained from kDNA associations by sonication, sucrose sedimentation, and cesium chloride-ethidium bromide equilibrium centrifugation. Buoyant density analyses failed to provide evidence for base composition heterogeneity among kDNA molecules within a species. The complementary nucleotide strands of kDNA molecules of all three species had distinct buoyant densities in both alkaline and neutral cesium chloride. For *C. acanthocephali* kDNA, these buoyant density differences were shown to be a reflection of differences in base composition between the complementary nucleotide strands. The molar ratios of adenine: thymine:guanine:cytosine, obtained from deoxyribonucleotide analyses were 16.8:41.0:28.1:14.1 for the heavy strand and 41.6:16.6:12.8:29.0 for the light strand. Covalently closed single circular molecules of *C. acanthocephali* (as well as intact kDNA associations of *C. acanthocephali* and *T. lewisi*) formed a single band in alkaline cesium chloride gradients, indicating their component nucleotide strands to be alkaline insensitive. Data from buoyant density, base composition, and thermal melting analyses suggested that minor bases are either rare or absent in *Crithidia* kDNA. The kinetics of renaturation of ^{32}P labeled *C. acanthocephali* kDNA measured using hydroxyapatite chromatography were consistent with at least 70% of the circular molecules of this DNA having the same nucleotide sequence. Evidence for sequence homologies among the kDNAs of all three species was obtained from buoyant density analyses of DNA in annealed mixtures containing one component kDNA strand from each of two species.

Members of an order of protozoa, the kinetoplas-
tida (Honigberg et al., 1964), are distinguished by the presence of a DNA of high structural complexity known as kinetoplast DNA (kDNA) which is

situated in an enlarged region of a mitochondrion associated with the basal body of the organism's flagellum. We have previously reported the results of physicochemical and electron microscope studies of the kDNA of *Crithidia acanthocephali* which led us to propose (Renger and Wolstenholme, 1972; Wolstenholme et al., 1974) a model for the structure of this DNA involving a hierarchy consisting of: (a) circular molecules each 0.8 μm in contour length (mol. wt. 1.54×10^6 daltons), as the basic unit of organization, (b) groups of such circles as the second level of organization, each group containing an average of 33 circles held together by topological interlocking of each circle with a large number of other circles in the group, and (c) a collection of, on the average, 804 such groups to form a structure termed an association, the final level of organization. Each group within the association is attached to several adjacent groups. The attachment between two groups is effected by one or more circles shared between them; each such circle is never shared by more than two groups. The groups of circles are arranged in such a way that the association (comprising about 27,000 circles with a total mol. wt. of about 41×10^9 daltons) has a topologically two-dimensional form. Associations of circles with some species-specific variation in contour length (0.3 μm to 0.8 μm) of the component circular molecules, in the arrangement of the individual circular molecules, and in the degree to which linear DNA is associated with the associations have been described for kDNA from *Trypanosoma cruzi* (Riou and Delain, 1968), *Trypanosoma lewisi* (Renger and Wolstenholme, 1970), *Trypanosoma mega* (Laurent and Steinert, 1970), *Trypanosoma congolense* and *Trypanosoma equiperdum* (Renger and Wolstenholme, 1971), *Leishmania tarentolae* (Simpson and Da Silva, 1971), and *Crithidia fasciculata*, *Crithidia luciliae*, and *Crithidia rileyi* (Renger and Wolstenholme, 1972).

In order to learn more about the structure of kDNAs of *Crithidia* and *Trypanosoma*, particularly in regard to the amount of genetic information they could carry, we have conducted a number of further physicochemical studies. The results of these studies are the subject of this report.

MATERIALS AND METHODS

Culturing of Organisms

Cells of *C. acanthocephali*, *C. luciliae*, and *T. lewisi* (blood strain) used in these studies were of the same strains as those used by Renger and Wolstenholme (1972

and 1970). Stocks of *C. acanthocephali* and *C. luciliae* were maintained by growing without agitation in 20-ml culture tubes at 27°C under sterile conditions in the trypticase broth medium of Guttman and Eisenman (1965) prepared as described by Renger and Wolstenholme (1972). Transfers of cells were made every 20 days. Cells used for DNA extraction were grown in a similar way except that 250-ml or 4-liter glass flasks were used and the cultures were placed on a gyratory shaker (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) and agitated at approximately 200 rpm. Upon reaching a titer of approximately 2×10^7 cells/ml, the cells were harvested by centrifugation at 6,000 g for 10 min, washed once with a solution containing 0.15 M sodium chloride, 0.1 M EDTA, 0.05 M sodium phosphate (pH 8.5) and either used immediately for DNA extraction or frozen.

Cells of *T. lewisi* were grown in the blood of albino rats and maintained by syringe passage every 7 days. Whole rat blood was obtained by cardiac puncture 7 days after infection.

Isolation of DNAs

DNA was isolated from whole cells of *Crithidia* or from *T. lewisi* infected rat whole blood by a method modified from that of Marmur (1961). Cells suspended in 0.05 M sodium phosphate, 0.15 M sodium chloride, 0.1 M EDTA (pH 8.5) were lysed by addition of 2% sodium dodecyl sulfate and 1 mg/ml Pronase (grade B, Calbiochem, San Diego, Calif., freed from DNase activity by the method of Hotta and Bassel [1965]) and stirring for 30 min at 25°C. An equal volume of phenol saturated with the above mentioned isolation buffer was added and the mixture was gently shaken at 5-min intervals over a 30-min period. The mixture was then centrifuged at 7,700 g for 10 min at 4°C and the aqueous phase was removed. The phenol phase was further extracted with ½ vol of fresh isolation buffer. The two aqueous fractions were then pooled, 5 M sodium perchlorate was added to a final concentration of 1.0 M, and an equal volume of 24:1 mixture of chloroform:isoamyl alcohol was added. The mixture was shaken at 5-min intervals over a 30-min period and centrifuged at 7,700 g for 10 min at 4°C. The aqueous phase was removed and the DNA was precipitated by addition of 2 vol of cold (-20°C) 95% ethanol, collected by spooling on a glass rod and suspended in 0.15 M sodium chloride, 0.015 M sodium citrate (SSC). Pancreatic ribonuclease A (freed from DNase activity by heating at 80°C for 10 min at 20 mg/ml in 0.1 M sodium acetate, pH 5.0; Worthington Biochemical Corp., Freehold, N. J.) was added to a final concentration of 200 μg/ml and the mixture was incubated for 30 min at 37°C. The RNase was removed by extraction with an equal volume of chloroform:isoamyl alcohol and the DNA was precipitated from the resulting aqueous phase by addition of 2 vol of cold ethanol, collected on a glass rod and suspended in SSC and stored at -20°C.

Associations of kDNA were separated from nuclear DNA (and in the case of *T. lewisi*, also from rat

leukocyte DNA) by a modification of the procedure of Laurent et al. (1971). 3 ml of solution containing approximately 1.0 mg of whole cell DNA was layered on 10 ml of 20% sucrose in SSC and centrifuged for 1 h at 21,000 rpm at 4°C, using an SW 41 rotor in a Beckman Spinco L2 65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pellet was suspended in SSC and clarified by centrifugation at 4°C for 10 min at 2,000 g. The supernate containing the DNA was again layered on 20% sucrose in SSC and the whole of the above procedure was repeated six times. The final pellet of kDNA was suspended in SSC and stored at −20°C.

Nuclear DNA of *C. acanthocephali* and *C. luciliae* was obtained from the supernate of the first sucrose sedimentation of the respective whole cell DNA after removal of the pelleted kDNA. The DNA was precipitated from the 20% sucrose solution by addition of cold 95% ethanol and redissolved in 0.1 SSC. This suspension of DNA was relayered on 10 ml of 20% sucrose, and the sedimentation, precipitation, and redissolving in 0.1 SSC were repeated. The DNA was then centrifuged to equilibrium in a preparative neutral cesium chloride buoyant density gradient. Fractions were collected and the distribution of DNA was determined by examination, using 260 nm illumination in a Gilford 2400-S spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The fractions containing the dense half of the major band of DNA were pooled and dialyzed against four changes of 500 ml SSC at 4°C for a total of 16 h. The resultant DNA suspension was stored at −20°C. The purity of kDNA samples and of nuclear DNA samples was monitored by analytical cesium chloride equilibrium buoyant density centrifugation. Only DNAs with a purity greater than 98% were used (see, for example, Fig. 1).

Escherichia coli bromouracil-labeled hybrid DNA was prepared as described by Richards et al. (1971). Bacteriophage T4 DNA was a gift of Dr. O. C. Richards (Dept. of Biochemistry, University of Utah) and bacteriophage SPO1 DNA was a gift of Dr. D. Wilson (Dept. of Biophysics, University of Chicago). *Micrococcus lysodeikticus* DNA and *Clostridium perfringens* DNA were obtained from the Sigma Chemical Co. (St. Louis, Mo.).

Preparation of Single kDNA Molecules

In order to obtain fractions containing collections of single circular and linear molecules of kDNA, 4-ml suspensions of kDNA associations (100 μg/ml) in SSC were first sonicated for 10 s at 4°C, using a Branson Sonifier Model LS-75 (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) equipped with a microprobe at a power setting of 1. 1-ml samples of sonicated kDNA suspensions were then layered on 5% to 20% linear sucrose gradients in SSC and centrifuged for 14 h at 35,000 rpm at 4°C in an SW 41 rotor in a Spinco L2 65B. After centrifugation the tube was punctured at the bottom with

an 18 gauge hypodermic needle and 15 drop fractions were collected. The flow rate was regulated to approximately 1 ml/min by use of a Buchler polystaltic pump (Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N. J.). The fractions were analyzed by absorbance at 260 nm, and the occurrence of different molecular forms in the various fractions was determined by electron miroscopy.

Preparative Cesium Chloride Equilibrium Density Gradient Centrifugations

Preparative neutral cesium chloride equilibrium density gradient centrifugations were performed in a Beckman Spinco Model L2 65B, using pollyallomer tubes and a fixed angle 65 rotor. The initial density of cesium chloride (analytical grade, Harshaw Chemical Co., Kewanee Oil Co., Solon, Ohio) was adjusted to 1.700 g/cm³. After centrifugation for 48 h at 42,000 rpm at 20°C, five drop fractions (approximately 75 μl) were collected, 0.5 ml of SSC was added to each fraction, and the absorbance of each fraction was determined using 260 nm illumination.

Preparative alkaline cesium chloride equilibrium density gradient centrifugations of 40–100 μg DNA were performed in a similar way except that the initial cesium chloride density was adjusted to 1.760 g/cm³, the solution contained 0.04 M tribasic potassium phosphate (pH 12.5), and centrifugation was carried out for 72 h.

Preparative cesium chloride-ethidium bromide equilibrium density gradient centrifugations (Radloff et al., 1967) were performed using the same tubes, rotor, and centrifuge mentioned above. The initial cesium chloride density was 1.550 g/cm³ and the ethidium bromide (a gift of Boots Pure Drug Co., Ltd., Nottingham, England) was used at a final concentration of 300 μg/ml. After centrifugation at 42,000 rpm for 48 h at 20°C, gradients were examined and photographed as described by Renger and Wolstenholme, 1972. The fluorescent bands were removed in series from the top of the tube using a Pasteur pipette. The cesium chloride and ethidium bromide were removed from each of these fractions by dialysis in the dark against four changes of 500 ml of a solution containing 0.05 M Tris, 0.005 M EDTA (pH 7.5), and 20 ml of Dowex 50 (Dow Chemical Co., Midland, Mich.) over a total period of 16 h. Before use, the Dowex 50 was washed serially with 2 liters of 0.5 N hydrochloric acid, 2 liters of 0.5 N sodium hydroxide, and 1.5 liters of 0.5 M Tris, 0.05 M EDTA (pH 7.5).

Analytical Cesium Chloride Equilibrium Density Gradient Centrifugation

Analytical neutral cesium chloride equilibrium density gradient centrifugations (Meselson et al., 1957) were performed with a Beckman Spinco Model E and a titanium An-F rotor. Each gradient contained between 2 and 5 μg of test DNA except when the purpose of the

analysis was to monitor purity, in which case 20 μg of DNA was used. The initial density of cesium chloride (analytical grade) was adjusted to 1.700 g/cm³ and centrifugation was carried out for at least 18 h at 20°C. Ultraviolet absorbance tracings were made directly with a photoelectric scanner. The density of each DNA was determined by its position in the gradient relative to a reference DNA by the method of Schildkraut et al. (1962). The buoyant densities of the DNAs used for reference were determined from their positions at equilibrium in analytical neutral gradients relative to the position of *M. lysodeikticus* native DNA. Taking the buoyant density of *M. lysodeikticus* DNA to be 1.731 g/cm³ (Schildkraut et al., 1962), the following values were obtained: bacteriophage SPOl native DNA, ρ = 1.742 g/cm³; *C. perfringens* native DNA, ρ = 1.691 g/cm³; and the single preparation of *E. coli* bromouracil-labeled hybrid DNA used in these experiments, ρ = 1.754 g/cm³.

Analytical alkaline cesium chloride equilibrium buoyant density centrifugations were performed under conditions similar to those described for neutral gradients except that the initial cesium chloride density was adjusted to 1.760 g/cm³; the solutions contained 6–10 μg DNA, and 0.04 M tribasic potassium phosphate (pH 12.5), and centrifugation time was 36–48 h. The buoyant densities of bacteriophage T4 DNA and *M. lysodeikticus* DNA used for reference in analytical alkaline cesium chloride gradients were taken as 1.756 g/cm³ and 1.788 g/cm³ (Vinograd et al., 1963).

Thermal Denaturation

Thermal denaturation of nuclear or kDNA in 0.1 SSC was followed in a Gilford 2400-S spectrophotometer equipped with dual thermal plates connected to a Haake heating unit (Haake Inc., Saddle Brook, N. J.). The DNA solutions at a concentration of 15–20 μg/ml were continuously heated at a rate of 0.5°C/min in 1.0-ml stoppered cuvettes. Changes in absorbance at 260 nm and temperature were automatically recorded.

Annealing of Separated Single Strands of kDNA

After optical density analysis of a kDNA-containing alkaline cesium chloride gradient, a sample containing heavy strand kDNA was obtained by pooling fractions containing the denser portion (approximately half) of the denser DNA band, and a sample containing light strand kDNA was obtained by pooling fractions containing the less dense portion of the less dense band (see Fig. 13). Each sample was dialyzed against four changes of 500 ml of 0.1 SSC over a total period of 48 h. Each sample was then concentrated ten-fold, using a Buchler flash evaporator. For coannealing experiments, samples (0.05–0.2 ml) each containing approximately 2 μg of a selected type of single-stranded kDNA were mixed, solid cesium

chloride was added to 5.8 M and the mixture was placed at 60°C for 24 h. Self-annealing of single-stranded kDNA was similarly performed, but with 4 μg of one type of single-stranded kDNA. Buoyant density analysis was performed after cooling of the specimens to 20°C and, where appropriate, addition of marker DNA.

Denaturation and Reannealing of Individual kDNAs

100 μg of kDNA associations contained in 1 ml SSC at 4°C were sonicated in a Branson sonifier, using a microprobe at a power setting of 1 for a total of 180 s in 60-s pulses with intervening 120-s rest periods. The absence of circular molecules following shearing of each kDNA was ascertained by electron microscope examination. The sonicated DNA was denatured by adding 1 N sodium hydroxide to a final concentration of 0.2 N. After 15 min, the solution was neutralized by adding 1.0 M monobasic potassium phosphate until a pH of 7.1 was achieved. kDNAs were reannealed and subjected to buoyant density analysis as described for coannealing of single strands.

Base Composition Analysis

C. acanthocephali was grown to a titer of 2 × 10⁷ cells/ml in medium containing 12.5 mC/l of ³²P (New England Nuclear, Boston, Mass.). Whole cell DNA was isolated and kDNA associations, fractions comprising covalently closed single circular molecules and fractions comprising open single circular and unit length linear molecules, were prepared. From a portion of the latter fraction, heavy and light complementary single strands were obtained by preparative alkaline cesium chloride equilibrium centrifugation. Deoxyribonucleotide analyses (following the method of Richardson, 1966) of each kDNA fraction and of nuclear DNA were then carried out. At the beginning of the experiments, the specific activities of the different kDNA samples used varied from 3,200 to 11,400 cpm/μg and the specific activity of nuclear DNA was 4,000 cpm/μg. Variations in the specific activity of different kDNA fractions derived from cells of a single culture were due to differences in time after labeling at which the different fractions were analyzed, as a function of ³²P decay. To a sample of DNA in 0.1 SSC with a total radioactivity of approximately 20,000 cpm, 25 μg of unlabeled, sonicated (as described above but for 3 min) denatured calf thymus DNA (produced by boiling in 0.1 SSC for 10 min and quenching in ice water for 5 min) was added and the total DNA was then precipitated by addition of 2 vol of cold 95% ethanol and storage at −20°C for 48 h. The precipitated DNA was recovered by centrifugation at 7,700 g for 20 min at 4°C. The supernate was discarded and 1 ml of cold 95% ethanol was added to the precipitated DNA. This mixture was placed at −20°C for 20 min and the centrifugation repeated. The whole

washing procedure with 95% ethanol was repeated three times. The DNA was dried *in vacuo* and then suspended in 0.05 ml of a solution containing 0.05 M Tris (pH 7.6) and 0.01 M magnesium chloride. To obtain 5'-deoxyribonucleotides, 20 μl of pancreatic DNase I (1 mg/ml, Worthington Biochemical Corp., Freehold, N. J.) was added and the mixture was incubated at 37°C for 1 h. 5 μl of 1 M glycine (pH 9.2) was added, followed by 2 μl of snake venom phosphodiesterase I (1 mg/ml, Worthington Biochemical Corp., and freed from protease activity by passage through a Dowex 50 column [Keller, 1964]) and incubation was continued for 1 h at 37°C. 20 μl of a solution containing 0.5 mg/ml of each of the 5'-deoxyribonucleotides of adenine, cytosine, guanine, and thymine (Sigma Chemical Co.) was added to the hydrolysate. The mixture was spotted onto a 30-inch strip of Whatman 3MM chromatography paper. Descending chromatography was carried out for 26 h, using a solution containing saturated ammonium sulfate, 1 M sodium acetate, and isopropanol in the proportions 80:18:2 as the solvent system. Nucleotides were visualized in the chromatograph using a UV S-11 mineral light (Ultra-Violet Products, Inc., San Gabriel, Calif.) and their positions outlined with a pencil. The chromatograph was cut into 1-cm strips and the radioactivity in each strip was determined using 10 ml of 2,5-bis-2-(5-tert-butyl-benzoxazolyl) thiophene (scintillation grade, Packard Instrument Co., Inc., Downers Grove, Ill.) (4 gm/1 toluene) as a fluor in a Packard Model 3320 liquid scintillation system. Identification of the four nucleotides on the chromatograph was accomplished by cochromatography of individual unlabeled nucleotides. Determination of the distribution of radioactivity in three entire chromatographs revealed that 96.9–99% of the ^{32}P was in the area of the four nucleotide spots, 0.1–2.5% was in the solvent front, and 0.2–0.3% remained at the origin of the chromatograph.

DNA Reassociation Kinetics

The kinetics of reassociation of kDNA was studied using the hydroxyapatite procedure described by Britten and Kohne (1968). Kinetoplast DNA associations labeled with ^{32}P were prepared as described above. The DNA was sheared to yield pieces about 450 nucleotides in length by forcing it three consecutive times through a needle valve using a pressure of 15,000 psi in a French pressure cell at 4°C in 0.12 M sodium phosphate, pH 6.8. The absence of circular molecules after shearing was ascertained by electron microscope examination. The weight average fragment length of the sheared kDNA was determined from the results of alkaline sucrose gradient analysis, using either ^{3}H-labeled bacteriophage ϕX 174 DNA or ^{3}H-labeled bacteriophage λ b_2b_5 DNA as internal markers and the weight to sedimentation distance relationship derived by Studier (1965). Reassociation experiments were carried out using approximately 1.5 μg of ^{32}P-labeled kDNA (sp act 20,000 cpm/μg)

mixed with approximately 120 μg of ^{3}H-labeled *Bacillus subtilis* DNA (sp act ~250 cpm/μg) sheared to the same size as the kDNA. The DNA samples were denatured in alkali as described by Kram et al. (1972). To 4.0 ml of the DNA mixture in 0.05 M sodium phosphate (pH 6.8) was added 0.5 ml of 1.0 *N* sodium hydroxide. After 10 min at 25°C, 5.0 ml of distilled water at 60°C was added and the solution was equilibrated at 60°C. The solution was then neutralized with 0.5 ml of 2 M monobasic sodium phosphate (at 60°C) to give a final concentration of 0.12 M phosphate (pH 6.8). The DNA was then allowed to reassociate at 60°C and aliquots were removed at the appropriate Cot (moles·seconds·liters^{-1}) (Britten and Kohne, 1967) for fractionation on hydroxyapatite. The reassociated DNA was poured onto a 1-ml hydroxyapatite column which was equilibrated with 0.12 M sodium phosphate and maintained at 60°C in a water-jacketed column. The single-stranded DNA was eluted by washing the column four times with 2 ml of 0.12 M sodium phosphate. The reassociated DNA was then eluted by washing three times with 2 ml of 0.3 M sodium phosphate. The percentage of DNA which had reassociated at each Cot value was determined by measuring the radioactivity eluted in the 0.12 M and 0.3 M sodium phosphate fractions. The elution procedure removed greater than 98% of all radioactivity from the column.

Electron Microscopy

DNAs were prepared for electron microscopy by the aqueous protein monolayer technique of Freifelder and Kleinschmidt (1965) and the grids were rotary shadowed with platinum-palladium exactly as described by Wolstenholme and Gross (1968). Grids were examined in the Siemens Elmiskop 101 electron microscope at approximately 8,000 × and shadowed molecules were photographed (using projector pole piece 1) at either 8,000 × or 10,000 ×. Exact calibrations were obtained by use of a diffraction grating replica (2160 lines/mm, Ernest F. Fullam, Inc. Schenectady, N. Y.). Measurements of molecules were made on positive prints, using a map measure at a magnification of 160,000 ×.

RESULTS

Studies Concerning the Base Composition of kDNAs

BUOYANT DENSITY ANALYSES OF FRAC-TIONATED ASSOCIATIONS: The neutral cesium chloride density gradient equilibrium band positions of whole cell DNA and of purified kDNA associations of *C. luciliae* are shown in Fig. 1. The values for kDNA (ρ = 1.705 g/cm^3) and for main band nuclear DNA (ρ = 1.717 g/cm^3) correspond to those reported previously (Renger and Wolstenholme, 1972) for this species. The buoyant density
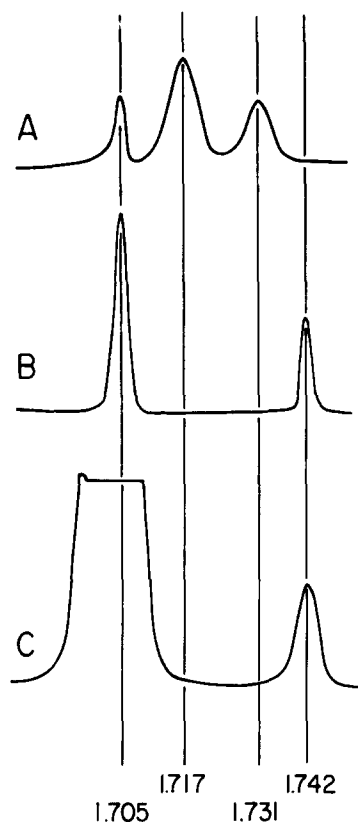
FIGURE 1 Photoelectric scans, using 260 nm illumination, of neutral cesium chloride equilibrium buoyant density gradients of DNAs from *C. luciliae*. A, whole cell DNA; B, kDNA associations (2 μg) purified by six sucrose velocity gradient sedimentations; C, as B but the gradient contained 20 μg of kDNA associations. The reference band (ρ = 1.731 g/cm³) in A is native DNA of *M. lysodeikticus*, and the reference band (ρ = 1.742 g/cm³) in B and C is native DNA of bacteriophage SPO1.

profile for a gradient overloaded with kDNA indicated that nuclear DNA contamination in this preparation of kDNA was less than 1% (Fig. 1C). In rotary shadowed protein monolayer preparations, this kDNA comprised large associations (Fig. 2), single circular molecules with a mean contour length of 0.77 μm (n = 30; SD = ± 0.027 μm), and some linear molecules up to 15 μm in length. These molecular forms are the same as those described by Renger and Wolstenholme (1972) for kDNA from four *Crithidia* species including *C. luciliae* and *C. acanthocephali*. The same authors provided evidence that the associations of *C. acanthocephali* kDNA comprised mainly, if not completely, covalently closed topo-

logically interlocked 0.8 μm circular molecules. The molecular forms of *T. lewisi* kDNA were found to be similar to those described for kDNAs of *Crithidia* species but the component circular molecules were 0.4 μm in contour length (Renger and Wolstenholme, 1970).

An attempt was made to detect variation in base composition among the circular molecules in kDNA associations of *C. luciliae*. Suspensions of kDNA associations were sonicated and the sonicated suspensions were subjected to centrifugation in a linear 5%–20% sucrose gradient. Fractions from the resultant DNA distribution (Fig. 3) were examined in the electron microscope. Most of the DNA in fractions 8–12 was in the form of single circular molecules and linear molecules of approximately single circle length (unit length linear molecules). These fractions were pooled. A second sample of kDNA was constructed by mixing the pellet from the bottom of the tube, fractions 1–7 and fractions 13–30 from the sucrose gradient. The kDNA in the pellet accounted for about 65% of the DNA in the mixture and comprised associations of up to 100 circular molecules. The kDNA in fractions 1–7 comprised molecules containing two or three apparently interlocked circles and the kDNA in fractions 13–30 comprised linear molecules of less than single circle length. A portion of this sample and of the sample comprising single circular molecules and unit length linear molecules was subjected to analytical cesium chloride buoyant density centrifugation. Only one band with a mean position equal to that of the unsonicated kDNA associations (ρ = 1.705 g/cm³) was evident in each sample.

The pooled fractions containing single circular molecules and unit length linear molecules (fractions 8–12, Fig. 3) were centrifuged to equilibrium in a cesium chloride-ethidium bromide density gradient. Two fluorescent bands resulted (Fig. 4). The bands were removed separately from the tube and, after removal of cesium chloride and ethidium bromide, a sample of each band was examined in the electron microscope. Of a sample of DNA totaling 130.6 μm from the upper band (Fig. 5), 46.6% was circular molecules, 49.7% was linear molecules and 3.7% was in the form of a single (0.8 μm) circular molecule with a linear molecule up to 0.8 μm associated with it. Of the circular DNA in this band, 92.2% was single circles (mean length = 0.78 μm ± 0.004 μm [SE], n = 72) and 7.8% was catenated dimers. Of the linear DNA, 78.4% was
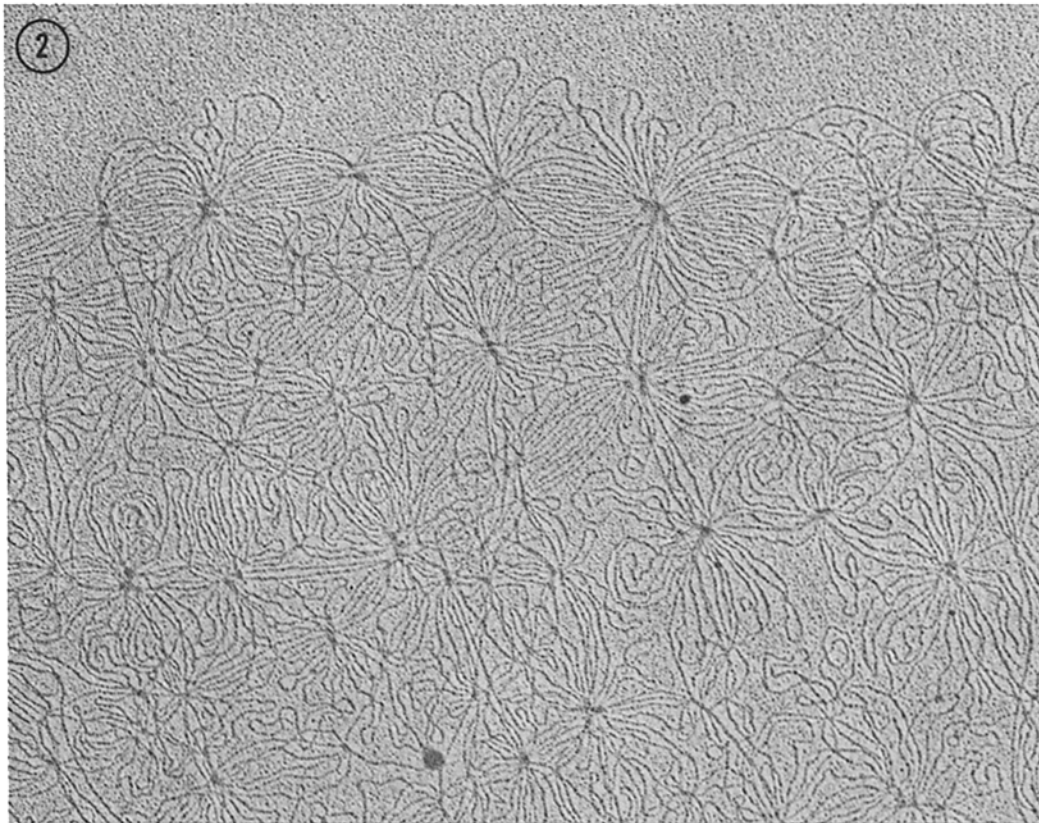
FIGURE 2   Electron micrograph of a rotary shadowed association of kDNA from a preparation of kDNA of *C. luciliae* separated from whole cell DNA by successive sedimentation through sucrose. × 53,200.
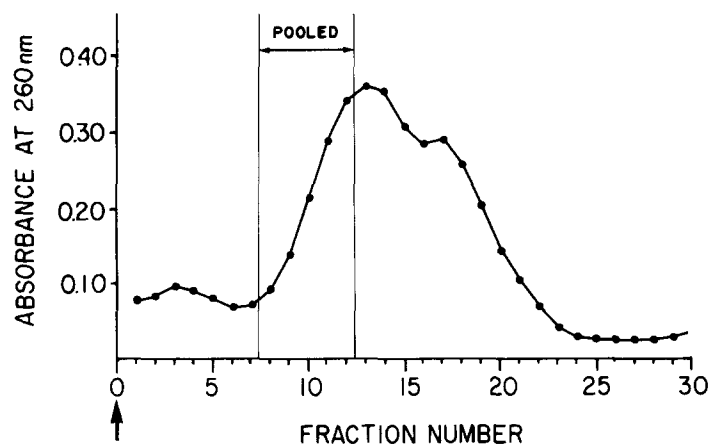

FIGURE 3   The distribution of DNA (measured as OD_{260}) which resulted from sonication (10 s) of kDNA associations followed by centrifugation in a 5% to 20% linear sucrose gradient. It was determined by electron microscopy that the DNA in the fractions (8–12) within the two vertical lines comprised single circular molecules and linear molecules of approximately single circle length (unit length linear molecules). The arrow below the abscissa indicates the bottom of the gradient.

in lengths lying within the range of lengths of single circular molecules in this sample, 15.9% was in lengths less than, and 5.7% (three molecules with lengths of 0.88 μm, 1.08 μm, and 1.75 μm) was in lengths greater than, the range of lengths of circular molecules.

Of a sample of DNA totaling 227.5 μm from the lower band (Fig. 6), 98.5% was circular molecules, 1.1% was linear, and 0.4% comprised a single 0.8 μm circular molecule with a 0.4 μm linear molecule associated with it. Of the circular DNA, 83.6% was in the form of single circles (mean length = 0.78 μm ± 0.004 μm [SE], n = 50) and the remaining 16.4% of the DNA was catenated dimers.

These findings are consistent with the circles from the lower (denser) band being covalently closed: such circles bind less ethidium bromide than circles containing a broken phosphodiester bond (open circles) or linear molecules and therefore band at a greater density (Radloff et al., 1967; Bauer and Vinograd, 1968). The less dense fraction will be referred to as comprising open single circular and unit length linear molecules and the denser fraction as comprising covalently closed single circular molecules.

After analytical neutral cesium chloride buoyant density centrifugation, both the covalently closed circular molecules and the mixture of open circular and unit length linear molecules formed single bands at ρ = 1.705 g/cm³. The possibility that the single band observed for the covalently closed circles represented a mixture of molecules with different buoyant densities was tested. 20 μg of the covalently closed single circular DNA molecules were centrifuged to equilibrium in a neutral cesium chloride gradient. The gradient was fractionated and the distribution of DNA determined (Fig. 7). Fractions were then pooled so as to provide samples containing the DNA from the more dense, the center, and the less dense parts of the DNA band as indicated in the upper part of Fig. 7. Each of these samples was subjected to analytical cesium chloride equilibrium density centrifugation. The results (Fig. 7) did not provide evidence for heterogeneity. The DNA in each sample again formed a single band at ρ = 1.705 g/cm³.

Buoyant density analyses were carried out on whole cell DNA, kDNA associations, and fractions comprising covalently closed single circular molecules and open single circular and unit length linear molecules of kDNA from C. acanthocephali

and from T. lewisi. The results are shown in Table I. The buoyant density values for whole cell DNA and for kDNA from both species are similar to those reported previously (Renger and Wolstenholme, 1972, 1970). As was found for C. luciliae, fractions comprising essentially single molecules in all cases formed a single band with a buoyant density characteristic of the kDNA associations of the respective species.

THERMAL DENATURATION STUDIES: Data concerning thermal denaturation of various fractions of C. luciliae kDNA are given in Fig. 8 and Table I. The DNAs in the fraction comprising sonicated associations and in the fraction comprising open single circular and linear molecules both exhibited a clear hyperchromic shift with melting temperatures (T_m) of 72.7°C and 73.4°C, respectively. Covalently closed single circular molecules showed a resistance to denaturation, noted previously for covalently closed circular viral DNAs (Vinograd and Lebowitz, 1966; Follett and Crawford, 1967), metazoan mitochondrial DNAs (Dawid and Wolstenholme, 1967; Borst et al., 1967; Nass, 1969; Leffler et al., 1970; Wolstenholme et al., 1972), and kDNAs of T. lewisi (Renger and Wolstenholme, 1970) and L. tarentolae (Wesley and Simpson, 1973 a). In the present case, a definite but relatively small hyperchromic shift was observed with a T_m of 74.0°C, suggesting the presence of some open circular molecules in the fraction. When another sample of this fraction was heated after sonication (which broke all the circles as determined by electron microscope examination), a greatly increased hyperchromic shift was observed with a T_m (72.7°C) identical to that observed for kDNA of sonicated associations. This result is again in agreement with earlier findings for T. lewisi kDNA (Renger and Wolstenholme, 1970) and L. tarentolae kDNA (Wesley and Simpson, 1973 a). The guanine plus cytosine (GC) content of C. luciliae kDNA calculated from the mean T_m (Mandel and Marmur, 1968) (Table I) of the various kDNA fractions is 46.3%, which is very close to the GC content (46.0%) of this DNA calculated from buoyant density values (Table I).

Thermal denaturation data for C. acanthocephali and T. lewisi kDNAs are given in Table I. As was found for C. luciliae kDNA, the GC content of C. acanthocephali kDNA calculated from T_m values (42.9%) is in good agreement with the GC content calculated from the results of buoyant
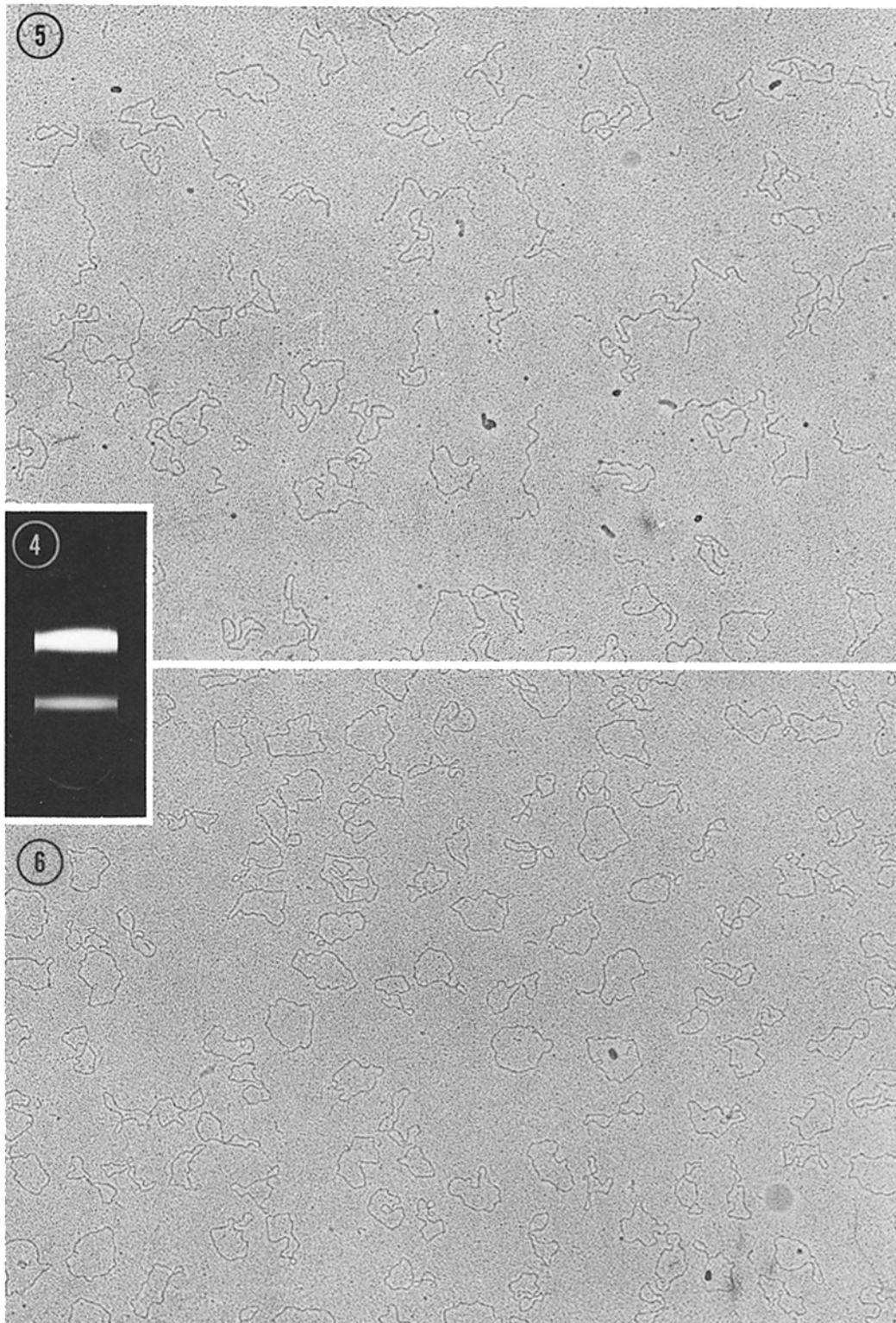
FIGURE 4   Fluorescence micrograph of the cesium chloride-ethidium bromide gradient resulting from centrifugation to equilibrium of approximately 300 μg of kDNA comprising a mixture of single circular molecules and unit length linear molecules obtained by sucrose sedimentation (fractions 8-12, Fig. 3) of sonicated kDNA associations of *C. luciliae.* × 0.8.

FIGURES 5 and 6   Electron micrographs of rotary shadowed molecules in protein monolayer preparations of kDNA from the upper (Fig. 5) and lower (Fig. 6) bands of the cesium chloride-ethidium bromide density gradient shown in Fig. 4. Both × 33,100.

FIGURE 7 The upper diagram illustrates the distribution (measured as OD_{260}) of 20 μg of covalently closed single circular molecules of kDNA of *C. luciliae* (lower band, Fig. 4) after centrifugation to equilibrium in a neutral cesium chloride density gradient. Fractions were then pooled so as to provide three samples; A, B, and C, containing respectively, DNA from the more dense, center, and less dense parts of the kDNA band. The lower diagram comprises photoelectric scans using 260 nm illumination of analytical neutral cesium chloride equilibrium buoyant density gradients of the kDNA in the three samples, A, B, and C. The reference band to the right (ρ = 1.731 g/cm³) is native DNA of *M. lysodeikticus*.

density analysis (42.7%). The GC content of *T. lewisi* kDNA calculated from thermal melting data is considerably lower than the GC content calculated from buoyant density data. A similar discrepancy was noted previously for this kDNA (Renger and Wolstenholme, 1970) and may indicate the presence of minor bases (bases in addition to guanine, cytosine, thymine, and adenine) (Marmur and Cordes, 1963).

BUOYANT DENSITY STUDIES ON SEPARATED NUCLEOTIDE STRANDS: The complementary nucleotide strands of mitochondrial

DNA from a number of metazoans can be separated by centrifugation to equilibrium in alkaline cesium chloride (Nass and Buck, 1970; Attardi et al., 1970; Dawid, 1972). To test whether this was the case for kDNA, fractions comprising open single circular and unit length linear molecules of kDNA of *C. luciliae, C. acanthocephali,* and *T. lewisi* were centrifuged to equilibrium in alkaline cesium chloride. For the kDNA of each species, two bands were observed (Fig. 9). The mean buoyant density of the less dense band (the light fraction) was similar for all three species (1.726-1.727 g/cm³). The buoyant density of the denser band (heavy fraction) varied among the three species: 1.775 g/cm³ for *T. lewisi*; 1.787 g/cm³ for *C. acanthocephali;* and 1.790 g/cm³ for *C. luciliae.* After centrifugation of nuclear DNA of *C. luciliae* to equilibrium in alkaline cesium chloride, only one band, at ρ = 1.771 g/cm³, was observed.

Heavy and light fractions of kDNA of each of the three species were obtained by preparative alkaline cesium chloride equilibrium centrifugation as indicated in Fig. 10. These fractions were neutralized and analyzed by cesium chloride equilibrium centrifugation. The results are shown in Fig. 11. The mean buoyant density of the light fraction was similar for each species (1.706-1.707 g/cm³). The mean buoyant density of the heavy fraction of the kDNA of *C. luciliae* was 1.741 g/cm³, of *C. acanthocephali* 1.739 g/cm³, and of *T. lewisi* 1.724 g/cm³. Experiments were next conducted to test whether the heavy and light fractions represent the complementary nucleotide strands of kDNA molecules of each of the species. Each separated fraction of kDNA was self-annealed, and for each species approximately equimolar quantities of heavy and light kDNAs were coannealed. The results of neutral cesium chloride buoyant density analysis of the products are shown in Fig. 12. Self-annealing did not result in a change in the buoyant density of any of the light fractions or of any of the heavy DNA fractions tested. This confirms that each fraction comprises single-stranded molecules of like polarity. On the other hand, the products of co-annealing of the heavy and light fractions of kDNA of each species formed a single band narrower than that formed by each of the component heavy and light fractions and with a mean buoyant density 0.002-0.009 g/cm³ denser than the buoyant densities of the respective native kDNAs. In protein monolayer preparations of separately annealed light and heavy fractions of kDNA, only small

TABLE I

A Summary of Data Concerning Base Composition of Various Fractions of kDNA from C. luciliae, C. acantho-cephali, and T. lewisi

Form of kinetoplast DNA	Buoyant density	n	Guanine + cytosine*	Tm	ΔTm	n	Guanine + cytosine‡	Guanine + cytosine determined by chromatography of 5'-deoxy-ribonucleotides	n
	$g/cm^3 \pm SE$		%	$°C \pm SE$	$°C \pm SE$		%	%	
C. luciliae									
Native associations	1.7048 ± 0.0001§	11	45.7	72.5 ± 0.27||	18.8 ± 0.42	5	45.4	—	
Covalently closed single circular molecules	1.7050 ± 0.0002	5	45.9	72.7||	11.9	1	45.9	—	
Open single circular and unit length linear molecules	1.7054 ± 0.0003	8	46.3	73.4	18.0	1	47.6	—	
Mean¶	1.705		46.0	72.9	16.2		46.3		
C. acanthocephali									
Native associations	1.7017 ± 0.0001**	12	42.6	71.6 ± 0.13||	18.5 ± 1.04	3	43.1	44.0 ± 0.22	7
Covalently closed single circular molecules	1.7020 ± 0.0005	3	42.9	—		—	—	43.2 ± 0.16	8
Open single circular and unit length linear molecules	1.7018 ± 0.0003	4	42.7	71.4	18.4	1	42.7	42.0 ± 0.25‡‡	5
Mean¶	1.702		42.7	71.5	18.5		42.9	43.1	
T. lewisi									
Native associations	1.6979 ± 0.0003§§	7	38.7	66.3 ± 0.43||	20.65 ± 1.28	4	30.15		
Covalently closed single circular molecules	1.6978 ± 0.0002	2	38.6	—		—				
Open single circular and unit length linear molecules	1.6981 ± 0.0003	2	38.9	68.4	18.9	1	35.4	—	
Mean¶	1.698		38.7	67.6	19.8		32.8		

n = the number of observations. Tm = melting temperature (temperature at one-half hyperchromic transition, Doty et al. 1959). Δ Tm = temperature range of hyperchromic transition.

* Calculated from the mean buoyant density values by the method of Schildkraut et al. (1962).

‡ Calculated from the mean melting temperature values in 0.1 SSC by the method given by Mandel and Marmur (1968).

§ The 11 observations include nine for isolated native kDNA associations and two for the light satellite in whole cell DNA.

|| Fractions of DNA which had been sonicated and determined by electron microscopy not to contain circular molecules.

¶ This value is in each case the mean of the means for each kDNA fraction.

** The 12 observations include 10 for isolated native kDNA associations and two for the light satellite in whole cell DNA.

‡‡ Calculated from the combined data for separated heavy and light complementary strands (Table II).

§§ The seven observations include six for isolated native kDNA associations and one for the light satellite in whole cell DNA.

molecules having the characteristic form of single-stranded DNA were observed. In contrast, examination of coannealed light and heavy kDNA fractions in the electron microscope revealed apparently double-stranded single circular and linear molecules and large complex masses, the component molecules of which also had the characteristic form of double-stranded DNA. The latter forms were similar to the concatenanes of renatured mitochondrial DNA reported by Dawid and Wolstenholme (1968). These results confirm that the heavy and light fractions represent complementary strands of kDNA. Doubling the time of coanneal-

ing (48 h) of each kDNA did not result in further decrease in buoyant density (toward native density) of any of the products. Also, similar buoyant densities were obtained for the product of renaturation of alkali- or heat-denatured sonicated kDNA associations. This contrasts with the finding of Simpson and DaSilva (1971) that heat-denatured sonicated kDNA of *L. tarentolae* did return to native buoyant density upon reannealing. It has also been found for mitochondrial DNA from some organisms, though not for others, that reannealing of alkali- or heat-denatured DNA results in a product with a buoyant density greater
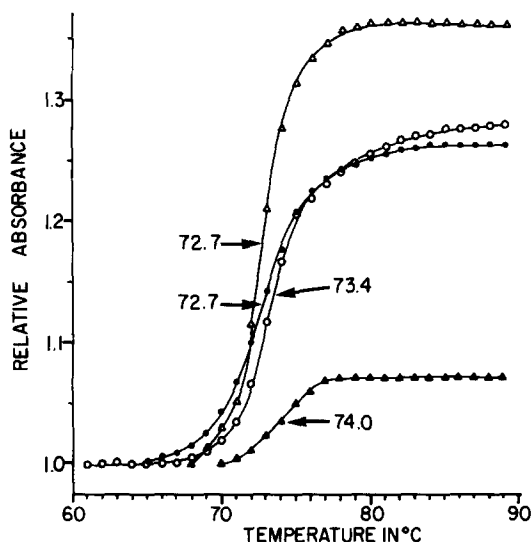
FIGURE 8 The effect on absorbancy at 260 nm of continuously heating kDNA of *C. luciliae* in 0.1 SSC. The solid circles represent sonicated associations. The open circles represent a mixture of open single circular and unit length linear molecules, the solid triangles represent covalently closed single circular molecules, and the open triangles sonicated covalently closed single circular molecules. The T_m values are given.

than that of the native mitochondrial DNA (Borst and Ruttenberg, 1966; Corneo et al., 1966, 1968 *b*; Sinclair et al., 1967; Dawid and Wolstenholme, 1968; Wolstenholme and Gross, 1968; Polan et al., 1973). While failure to achieve native buoyant density upon reannealing could indicate nucleotide sequence heterogeneity between molecules, it is also possible that this results from incomplete base pairing due to the formation of complex molecular configurations.

TEST FOR ALKALI SENSITIVITY OF NU-
CLEOTIDE STRANDS: The nucleotide strands of circular mitochondrial DNA molecules of a number of metazoan species have been found to be sensitive to alkali (Dawid and Wolsten-holme, 1967; Borst et al., 1967; Pikó et al., 1968; Robberson et al., 1971), apparently due to the presence of a small number of ribonu-cleotides (Miyaki et al., 1973; Wong-Staal et al, 1973; Grossman et al., 1973). To test whether this was the case for kDNA of *C. acanthocephali*, native intact kDNA associations from stationary phase cells of *C. acanthocephali* and a fraction of kDNA of the same species comprising covalently closed single circular mole-

cules were centrifuged to equlibrium in alkaline cesium chloride. (kDNA associations from stationary phase cells of *C. acanthocephali* were used, as such associations appear to comprise mostly, if not entirely, covalently closed circular molecules. In contrast, about one-third of the associations isolated from exponentially growing cells have associated with them long, noncircular molecules which appear to comprise tandem arrangements of nucleotide sequences identical to those of circular kDNA molecules [Manning and Wolstenholme, unpublished observations].) The results are shown in Fig. 13. Instead of the two bands which were observed when open circular and linear kDNAs were similarly treated, a single band was observed for each kDNA fraction. The mean buoyant densities of the associations and the covalently closed single circles were 1.780 g/cm^3 and 1.779 g/cm^3, respectively. The sharp band formed by the kDNA associations, characteristic of native asso-ciations and due to their high molecular weight, is a further indication that exposure to alkali did not result in degradation of the kDNA molecules. When intact kDNA associations of *T. lewisi* were centrifuged to equilibrium in an alkaline cesium chloride gradient, again a single sharp band was observed, at ρ = 1.772 g/cm^3 (taking the buoyant density of *M. lysodeikticus* DNA in the same
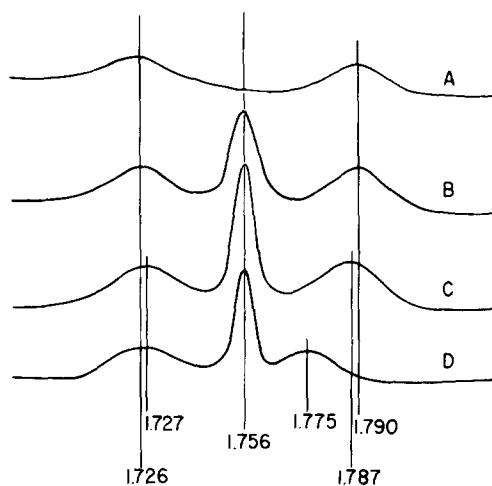

FIGURE 9 Photoelectric scans using 260 nm illumination of alkaline cesium chloride equilibrium buoyant density gradients of samples of open single circular and unit length linear kDNA molecules from *C. luciliae* (A and B), *C. acanthocephali* (C), and *T. lewisi* (D). The reference band in the center is DNA of bacteriophage T4 (ρ = 1.756 g/cm^3, Vinograd et al., 1963).
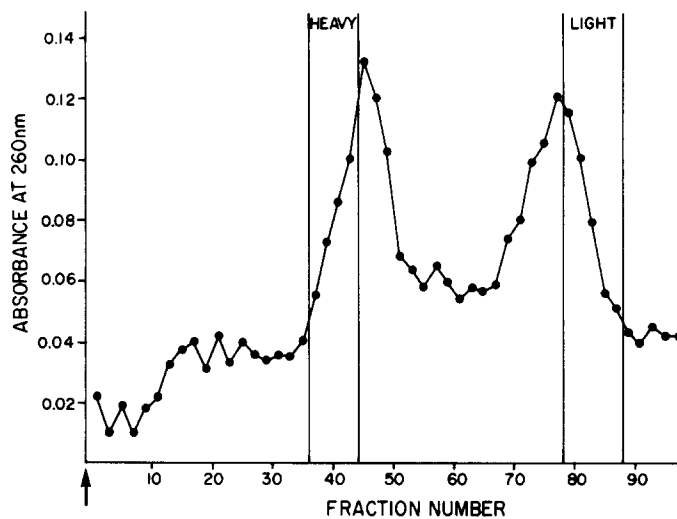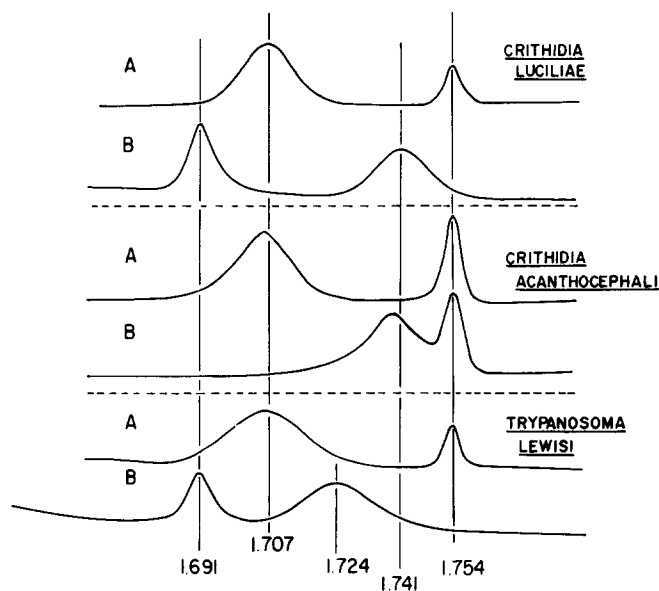
FIGURE 10   The distribution (measured as OD_{260}) of kDNA in a preparative alkaline cesium chloride gradient. The DNA used was a fraction comprising open single circular and unit length linear molecules prepared from *C. acanthocephali* kDNA associations. The DNA in the fractions between the two vertical lines to the left and the two vertical lines to the right were separately pooled and used in further experiments as the heavy and light fractions, respectively. The arrow below the abscissa indicates the bottom of the gradient.

FIGURE 11   Photoelectric scans, using 260 nm illumination, of neutral cesium chloride equilibrium buoyant density gradients of light (A) and heavy (B) fractions of kDNA of the species indicated. The light and heavy fractions were separated by preparative alkaline cesium chloride equilibrium buoyant density gradient centrifugation of fractions comprising open single circular and unit length linear molecules. The reference band to the left (ρ = 1.691 g/cm³) is native DNA of *C. perfringens*. The reference band to the right (ρ = 1.754 g/cm³) is *E. coli* bromouracil-labeled hybrid DNA.
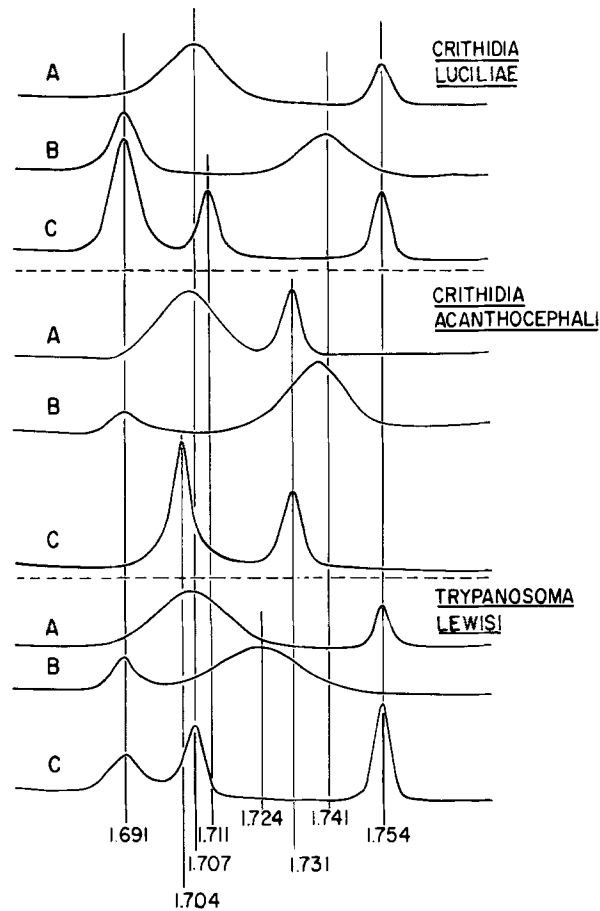
FIGURE 12 Photoelectric scans, using 260 nm illumination, of neutral cesium chloride equilibrium buoyant density gradients of self-annealed light (A), self-annealed heavy (B) and coannealed light and heavy (C) fractions of kDNA of the species indicated. The light and heavy fractions were separated in each case by preparative alkaline cesium chloride equilibrium buoyant density gradient centrifugation of fractions comprising open single circular and unit length linear molecules. The reference bands are ρ = 1.691 g/cm^3, native DNA of *C. perfringens*; ρ = 1.731 g/cm^3, native DNA of *M. lysodeikticus*; ρ = 1.754 g/cm^3, *E. coli* bromouracil-labeled hybrid DNA.

gradient to be 1.788 g/cm^3). These results clearly indicate that neither ribonucleotides nor any form of alkaline-sensitive bonds are involved in the continuity of either of the nucleotide strands of circular kDNA molecules of *C. acanthocephali* or *T. lewisi*.

NUCLEOTIDE COMPOSITION ANALYSIS: Buoyant density bias of complementary nucleotide strands has been demonstrated by neural and alkaline cesium chloride density gradient analysis for a number of viral DNAs (Marmur and Cordes, 1963; Guild and Robison, 1963; Sheldrick and

Szybalski, 1967; Doerfler and Hogness, 1968; Riva et al., 1968, 1969), metazoan mitochondrial DNAs (Corneo et al., 1968 *b*; Borst and Aaij, 1969; Attardi et al., 1970), chloroplast DNA (Stutz and Rawson, 1970), and eukaryote nuclear satellite DNA (Flamm et al., 1967; Corneo et al., 1968 *a*; Schildkraut and Maio, 1969). Separation of strands in neutral gradients is due to differences in base composition (Marmur and Cordes, 1963; Riva et al., 1969). Some differences are exaggerated in alkaline gradients as thymine and guanine residues are deprotonated and cesium ions are
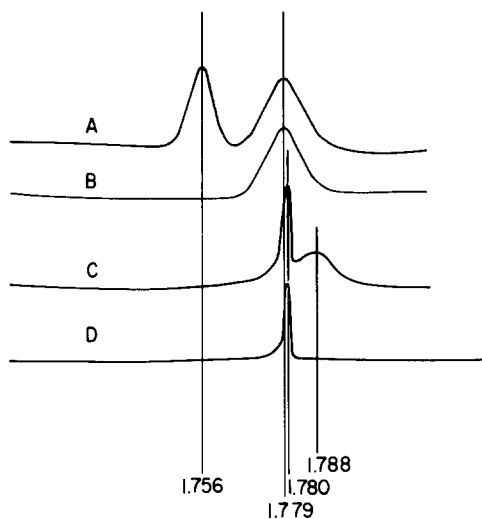
FIGURE 13 Photoelectric scans, using 260 nm illumination, of alkaline cesium chloride equilibrium buoyant density gradients of kDNA of *C. acanthocephali* with and without added density-standard DNAs; A and B, a kDNA fraction comprising covalently closed single circles. C and D, a fraction comprising intact associations of circles. The reference band in A (ρ = 1.756 g/cm³) is bacteriophage T4 DNA. The reference band in C (ρ = 1.788 g/cm³) is *M. lysodeikticus* DNA. The buoyant densities in alkaline cesium chloride for T4 DNA and *M. lysodeikticus* DNA are taken from Vinograd et al., (1963).

selectively bound, resulting in increased buoyant density (Vinograd et al., 1963; Szybalski et al., 1971).

In order to elucidate the basis of the observed very large buoyant density bias of the nucleotide strands of kDNA molecules, a base composition analysis was conducted using paper chromatography of enzyme-digested ³²P-labeled kDNA from *C. acanthocephali*. The results are summarized in Table II. In all of the chromatographs, the radioactivity was distributed into four clearly defined areas corresponding to the positions of the co-chromatographed unlabeled 5'-deoxyribonucleotides of adenine, guanine, thymine, and cytosine. The GC contents of native associations and of fractions comprising covalently closed single circular molecules were 44.0% and 43.2%, respectively. These values compare favorably with the values of 42.7% and 42.9% GC for these DNAs calculated from the results of buoyant density and thermal melting analyses, respectively (Table I). These results, together with the observed ratios of adenine:thymine and guanine:cytosine (Table II), do not provide support for the presence of minor bases in the kDNA of *C. acanthocephali*. The fractional values for the individual bases show a high degree of complementarity between the separately analyzed heavy and light strands. The GC contents of the heavy and light strands were found

TABLE II

Base Composition of Various Fractions of C. acanthocephali kDNA and Nuclear DNA as Determined by Paper Chromatography of ³²P-labeled 5'-deoxyribonucleotides Produced by Consecutive Pancreatic DNase and Snake Venom Phosphodiesterase Digestion

	Kinetoplast DNA				
	Native associations	Covalently closed single circular molecules	Heavy strand	Light strand	Nuclear DNA
Number of chromatographs	7	8	5	5	5
Adenine	28.7 ± 0.28	29.2 ± 0.11	16.8 ± 0.11	41.6 ± 0.24	21.6 ± 0.10
Thymine	27.4 ± 0.18	27.6 ± 0.11	41.0 ± 0.24	16.6 ± 0.40	21.2 ± 0.31
Guanine	21.9 ± 0.15	21.9 ± 0.04	28.1 ± 0.25	12.8 ± 0.45	28.5 ± 0.17
Cytosine	22.1 ± 0.29	21.3 ± 0.17	14.1 ± 0.28	29.0 ± 0.63	28.7 ± 0.35
Guanine + cytosine	44.0 ± 0.22	43.2 ± 0.16	42.2 ± 0.17	41.8 ± 0.32	57.2 ± 0.34
Guanine + thymine	49.7 ± 0.19	49.3 ± 0.12	69.1 ± 0.23	29.4 ± 0.43	49.7 ± 0.05
Ratio, adenine:thymine	1.05	1.06	—	—	1.02
Ratio, guanine:cytosine	0.99	1.03	—	—	0.99

to be 42.2% and 41.8%, respectively. These values are in good agreement with each other and with the values observed for double-stranded kDNAs. However, there was a distinct base bias between the complementary strands. The heavy strands contained 41.0% thymine and 28.1% guanine, compared to 16.6% thymine and 12.8% guanine in the light strands.

The results of a similar base composition analysis of purified nuclear DNA are also given in Table II. The adenine:thymine and guanine:cytosine ratios are again close to unity and the GC content was 57.2%. The latter value compares well with the GC content of 58.2% calculated from the buoyant density of 1.717 g/cm³ for this DNA (Schildkraut et al., 1962).

Sequence Complexity of C. acanthocephali kDNA

The data from the various analyses presented above fail to provide evidence for heterogeneity in base composition between circular molecules of kDNA of a single species. In an attempt to elucidate whether kDNA comprises more than one kind of circular molecule with respect to nucleotide sequences, we have studied the kinetics of renaturation of C. acanthocephali kDNA. It is possible to determine the sequence complexity of a given DNA by comparing the kinetics of renaturation of that DNA with those of a DNA of known sequence complexity (under standard conditions of cation concentration, temperature, and DNA fragment length, and after making appropriate corrections for nucleotide composition [Britten and Kohne, 1967; Wetmur and Davidson, 1968]). A measure of the rate of renaturation used is Cot ½; that is, the product of the DNA concentration (moles nucleotide/liter) and time (sec), required for one-half of the DNA to renature (Britten and Kohne, 1967). The results of measuring the kinetics of renaturation of kDNA from C. acanthocephali are given in Fig. 14. Curves were calculated for the data points by least squares analysis, using both one and two second order components (Manning et al., 1975). The best fitting curve (SD = ± 0.0027) shown in Fig. 14 was obtained using two second order components. The faster component accounted for 70% of the kDNA and had a Cot ½ of 0.0021. The slower component accounted for 15% of the kDNA and had a Cot ½ of 0.015. Renaturation of 12% of the DNA was too fast to measure (zero time) and the remaining 3% failed to renature. The

Cot ½ obtained for B. subtilis DNA contained in the same reaction mixture (Fig. 14) was 2.0. As the average GC content of B. subtilis DNA (43.1%, calculated from values given by MacDonald and MacDonald, 1962; Dubnau et al., 1965; and Welker and Campbell, 1967) is similar to that (42.9%) for C. acanthocephali kDNA (Table I), correction for base composition is unnecessary. Taking the molecular weight of the B. subtilis DNA to be 2.4 × 10⁹ daltons (Gillis et al., 1970) and assuming it to be nonrepetitious, the sequence complexity of the faster component of C. acanthocephali kDNA is indicated to be equivalent to that of a nonrepetitious molecule of mol wt 2.5 × 10⁶ daltons. This is 1.64 times the mol wt of a single circular molecule of this species (1.54 × 10⁶ daltons, Renger and Wolstenholme, 1972). By a similar consideration, the Cot ½ of the slower component is what would be expected for a nonrepetitious molecule of mol wt 18.0 × 10⁶ daltons, which is 11.7 times the sequence complexity of a single circular molecule of C. acanthocephali kDNA.

Base Sequence Homologies of kDNAs

To test whether the kDNAs of C. acanthocephali, C. luciliae, and T. lewisi have sequence homologies, separated strands from the three species were coannealed in various pairs. The products resulting from coannealing heavy strands of C. luciliae and heavy strands of either C. acanthocephali or T. lewisi kDNA and from coannealing light strands of C. luciliae and light strands of either C. acanthocephali or T. lewisi kDNA each formed a single diffuse band in cesium chloride gradients (Fig. 15). In each case, the midpoint of the band was at a buoyant density which could represent a simple mixture of the respective pairs of strands. These results are therefore consistent with there having been little or no hybrid formation between the heavy or light kDNA strands of the different species. In contrast to the above findings, the two products resulting from coannealing heavy strands of one of the Crithidia species and light strands of the other species both formed a single narrow band in cesium chloride density gradients, indicating that in each case the component strands had hybridized into a high molecular weight structure. Further, the mean buoyant density (1.706 g/cm³) of the product formed by coannealing C. luciliae light strands and C. acanthocephali heavy strands was between the
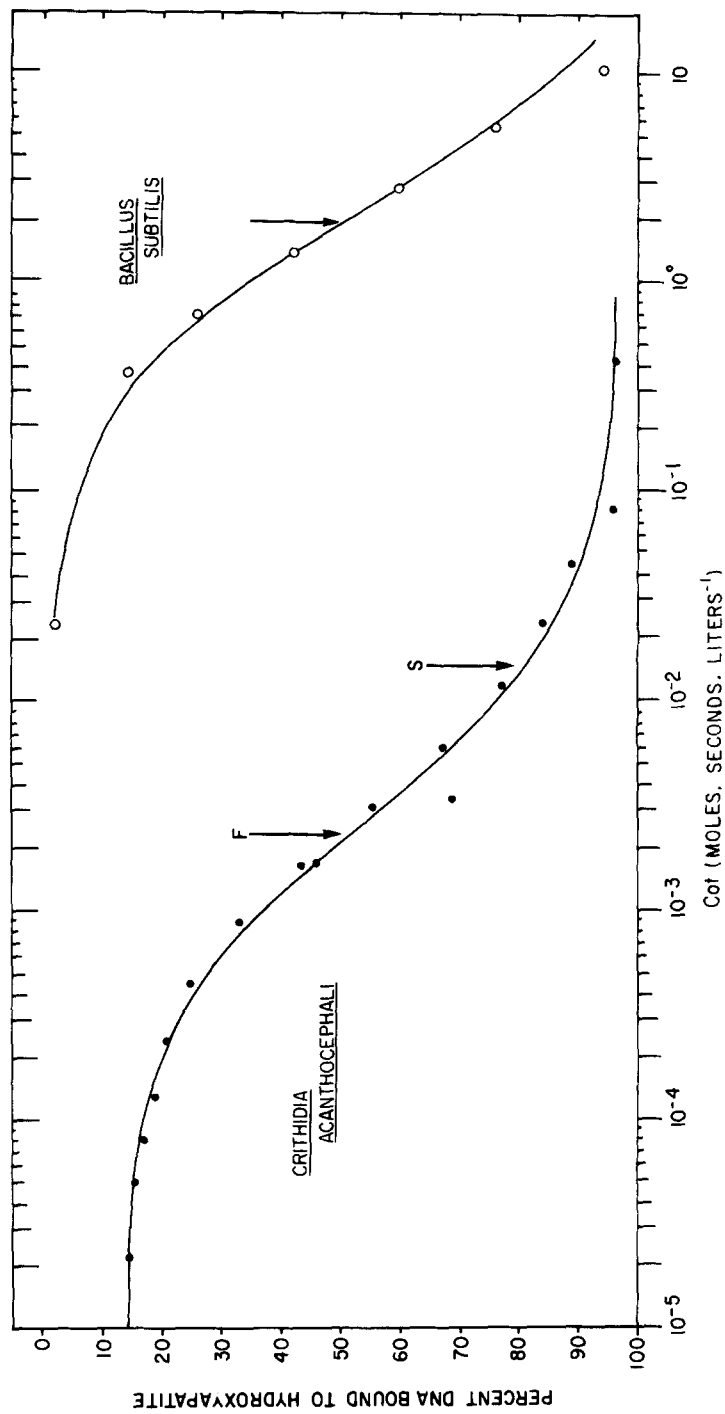
FIGURE 14 Renaturation kinetics of kDNA of *C. acanthocephali* and of *B. subtilis* DNA in the same reaction mixture. The curve for the *C. acanthocephali* kDNA (SD = ± 0.0270) was fitted to the data points (solid circles) by least squares analysis using two second order components. *F* indicates the Cot $\frac{1}{2}$ (0.0021) for the faster component which accounted for 70% of the kDNA in the reaction and *S* indicates the Cot $\frac{1}{2}$ (0.015) for the slower component which accounted for 15% of the kDNA; 12% of the kDNA renatured at zero time and 3% did not renature. The curve for *B. subtilis* DNA (SD = ± 0.0017) was also fitted to the data points (open circles) by least squares analysis, but using a single second order component. The arrow indicates the Cot $\frac{1}{2}$ of 2.0.
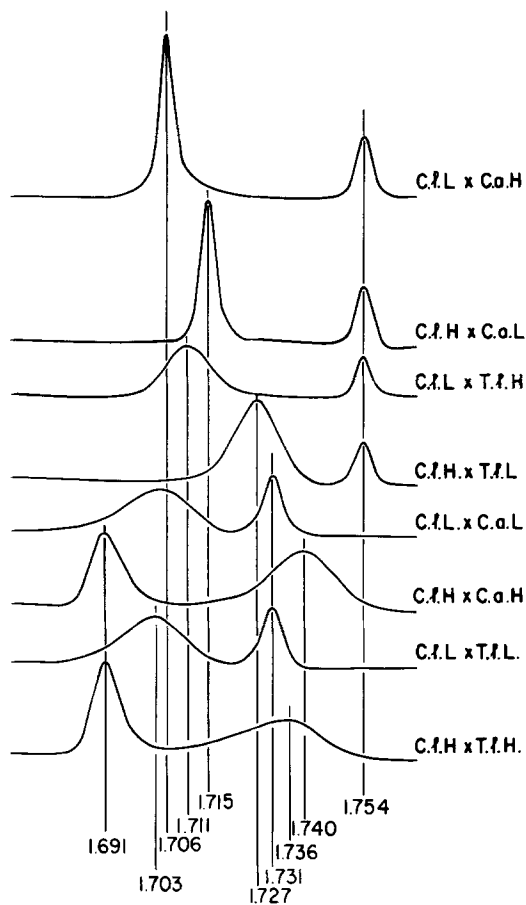
FIGURE 15 Photoelectric scans, using 260 nm illuminations, of neutral cesium chloride equilibrium buoyant density gradients of light (*L*) and heavy (*H*) strands of kDNA from *C. luciliae* (*C.l.*), *C. acanthocephali* (*C.a.*), and *T. lewisi* (*T.l.*) co-annealed in the combinations indicated. For each species, the heavy and light strands were obtained by preparative alkaline cesium chloride equilibrium centrifugation of fractions of kDNA comprising open single circular and unit length linear molecules (see Fig. 13). The reference bands are ρ = 1.691 g/cm³, native DNA of *C. perfringens*; ρ = 1.731 g/cm³, native DNA of *M. lysodeikticus*; ρ = 1.754 g/cm³, *E. coli* bromouracil labeled hybrid DNA.

buoyant density values of coannealed heavy and light strands of *C. acanthocephali* (1.704 g/cm³) and coannealed heavy and light strands of *C. luciliae* (1.711 g/cm³) (Fig. 15), suggesting a high degree of homology between the kDNAs of the two species. However, the mean buoyant density of the product of coannealing *C. luciliae* heavy strands and *C. acanthocephali* light strands was

1.715 g/cm³. Co-annealing of reciprocal complementary strands of *C. acanthocephali* and *C. luciliae* was repeated twice, and in each case buoyant density values for the respective products were similar to those given above. These differences in buoyant density values could result from there being differences in base composition between the unpaired regions of nucleotide strands in the reciprocal renaturation products.

The products of coannealing *C. luciliae* heavy strands and *T. lewisi* light strands and of *C. luciliae* light strands and *T. lewisi* heavy strands both formed a single band in cesium chloride, indicating that in both cases hybridization had occurred and therefore that the kDNAs of the two species have sequence homologies. The degree of sequence homology between *C. luciliae* and *T. lewisi* kDNAs relative to that between *C. luciliae* and *C. acanthocephali* kDNAs cannot be estimated from the buoyant densities of the coannealed kDNAs due to the fact that the effect of the differences in size between *C. luciliae* and *T. lewisi* kDNAs on the buoyant densities of the products of coannealing is unknown. Evidence for sequence homology between kDNAs of *L. tarentolae* and *T. cruzi* was obtained by Simpson and DaSilva (1971) from buoyant density analysis of coannealed sonicated whole kDNAs of these two species.

DISCUSSION

The data presented from both neutral and alkaline cesium chloride buoyant density analyses and from base composition analyses are consistent with the view that all of the circular molecules of kDNA of a given species are similar in base composition. Further, it appears from the kinetics of renaturation of kDNA of *C. acanthocephali* that at least 70% of this DNA could comprise only one kind of molecule in regard to nucleotide sequence. A similar conclusion concerning the circular molecules of kDNA of *L. tarentolae* has been reached by Wesley and Simpson (1973 *a* and *b*) from studies of renaturation kinetics and other physicochemical properties of this DNA.

The fraction (12%) of kDNA of *C. acanthocephali* which reassociated too fast to measure could represent tandemly arranged inverted repeat sequences. Such sequences, which renature essentially at zero time, have recently been demonstrated in mitochondrial DNA from certain petite yeast cells (Locker et al., 1974). The fraction (15%) of *C. acanthocephali* kDNA which

renatured with a Cot ½ of 0.015 may represent molecules with a sequence complexity about 12 times that of a single circular molecule. This observation might, however, also result from renaturation of the faster component not following second order kinetics after a certain point in the reaction is reached, possibly because of concatenate formation. The finding that reassociated kDNA does not return to native density is also consistent with this explanation. Wesley and Simpson (1973 *b*) also found components of whole kDNA of *L. tarentolae* which renatured faster and slower than the majority of this DNA. They considered the possibility that the slower component represented the long noncircular (linear) molecules found associated with the associations of circles of *L. tarentolae* kDNA. This explanation is unlikely in the case of *C. acanthocephali*, as we have recently found evidence that the long linear molecules associated with kDNA associations of this species are, in fact, tandem repetitions of the kDNA unit circle sequence (Manning and Wolstenholme, unpublished observations).

The agreement between base composition calculated from thermal melting data and that calculated from buoyant densities, as well as the direct base composition analysis of *C. acanthocephali* kDNA, makes it seem unlikely that minor bases (bases other than guanine, cytosine, thymine, and adenine) are present in significant amounts in kDNA of *Crithidia*. Whether or not such minor bases constitute a significant fraction of *T. lewisi* kDNA remains an open question. A direct base composition analysis as performed for *C. acanthocephali* would at present be prohibitively difficult in the case of *T. lewisi* because an in vitro culture system (permitting easy preparation of the required ^{32}P-labeled DNA) has not yet been developed for the blood form strain of *T. lewisi* we are studying. A form of *T. lewisi* have been cultured in vitro (Dusanic, 1968) but we have found that the buoyant density of the nuclear DNA of these organisms differs from that of the blood form strain of *T. lewisi* (Renger and Wolstenholme, 1970).

The observed difference in molar content of guanine plus thymine in the heavy and light strands of kDNA of *C. acanthocephali* offers an explanation in agreement with the finding of others concerning a variety of DNAs (see for example, Sheldrick and Szybalski, 1967; Doerfler and Hogness, 1968; Corneo et al, 1968a; and Flamm et al., 1967) for the very large difference in buoyant

density in alkaline cesium chloride of kDNAs of the three species studied. Also, the differences in base composition between the heavy and light strands of kDNA of *C. acanthocephali* appear to be adequate to explain the large observed differences in buoyant density in neutral cesium chloride gradients between the two strands. The buoyant densities of the light and heavy strands calculated from the observed base compositions according to the formula given by Riva et al. (1969) ($\rho^{single\ strand\ DNA} = 1.62669$ A + 1.75804 G + 1.76809 C + 1.74241 T g/cm^3; where A, G, C, and T are the molar fractions of adenine, guanine, cytosine, and thymine) are 1.704 and 1.731 g/cm^3, respectively, compared to the observed values of buoyant densities of $\rho^{light\ strand} = 1.706$ g/cm^3 and $\rho^{heavy\ strand} = 1.739$ g/cm^3.

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