

# REPLICATION OF KINETOPLAST DNA OF *CRITHIDIA ACANTHOCEPHALI*

## I. Density Shift Experiments Using Deuterium Oxide

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### ABSTRACT

The protozoan *Crithidia acanthocephali* contains, within a modified region of a mitochondrion, a mass of DNA known as kinetoplast DNA (kDNA). This DNA consists mainly of an association of approximately 27,000 covalently closed 0.8- $\mu\text{m}$  circular molecules which are apparently held together in a definite ordered manner by topological interlocking. After culturing of *C. acanthocephali* cells for 25 generations in medium containing 75% deuterium oxide, both nuclear DNA ( $\rho^{\text{native, nondeuterated}} = 1.717 \text{ g/cm}^3$ ) and kDNA ( $\rho^{\text{native, nondeuterated}} = 1.702 \text{ g/cm}^3$ ) increased in buoyant density by  $0.012 \text{ g/cm}^3$ . The replication of the two DNAs was studied by cesium chloride buoyant density analysis of DNAs from exponentially growing cells taken at 1.0, 1.4, 2.0, 3.0, and 4.0 cell doublings after transfer of cells from  $\text{D}_2\text{O}$ -containing medium into medium containing only normal water. The results obtained from analysis of both native and denatured nuclear DNAs indicate that this DNA replicates semiconservatively. From an analysis of intact associations of kDNA, it appears that this DNA doubles once per generation and that the newly synthesized DNA does not segregate from parental DNA. Fractions of covalently closed single circular molecules and of open circular and unit length linear molecules were obtained from associations of kDNA by sonication, sucrose sedimentation, and cesium chloride-ethidium bromide equilibrium gradient centrifugation. Buoyant density profiles obtained from these fractions indicate that: (a) doubling of the kDNA results from the replication of each circular molecule rather than from repeated replication of a small fraction of the circular molecules; (b) replication of kDNA is semiconservative rather than conservative, but there is recombination between the circles at an undefined time during the cell cycle.

The protozoan flagellate *Crithidia acanthocephali* belongs to the order Kinetoplastida (10), members of which are characterized by the possession of a structurally highly complex DNA situated within a mitochondrion, and known as kinetoplast

DNA (kDNA). In the case of *C. acanthocephali*, this DNA comprises on the average 27,000 covalently closed circular molecules, each 0.8  $\mu\text{m}$  in contour length (mol wt  $1.5 \times 10^6$  daltons), which are held together, apparently by topological inter-

locking, in a definite ordered manner to form a structure termed an association (17, 22). Some long linear molecules are found associated with some associations. The results of a number of physicochemical studies on the kDNA of *C. acanthocephali* are consistent with the interpretation that most, if not all, of the circular molecules are homogeneous in regard to their nucleotide sequences (7).

We found that *C. acanthocephali*, like *Euglena gracilis* (11), will grow in the presence of high concentrations of deuterium oxide ( $D_2O$ ) and that such growth results in an increase in the buoyant densities of both nuclear DNA and kDNA. Making use of this observation, we have conducted density shift experiments to try to elucidate the mode by which an association of circular molecules of kDNA is duplicated. The results of these experiments and the conclusions we have drawn from them are the subject of this report.

#### MATERIALS AND METHODS

All experiments involved cells of the same strain of *Crithidia acanthocephali* as that used by Renger and Wolstenholme (11) and originally obtained from Dr. Helene Guttman at the University of Illinois, Chicago Circle, Ill. Stocks of organisms were maintained by growth without agitation in 20-ml culture tubes at 27°C under sterile conditions in the trypticase broth medium of Guttman and Eisenman (9) prepared as given previously (17). Transfers of cells were made every 20 days. Organisms were also grown without agitation in a similar trypticase broth medium but containing 50% deuterium oxide ( $D_2O$ ) (Bio-Rad Laboratories, Richmond, Calif.) by weight for a total of 25 generations. These organisms were then transferred to trypticase broth medium containing 75%  $D_2O$  and cultured for a further 25 generations before being used for experiments.

Organisms used for the density shift experiments resulted from culturing in the presence of 75%  $D_2O$  for 35 generations or more. For each experiment, one liter of trypticase medium containing 75%  $D_2O$  was inoculated at an initial cell concentration of  $2.1 \times 10^5$  cells/ml. The cells were grown with agitation to a concentration of  $1.6 \times 10^7$  cells/ml and the culture was divided into a 600-ml sample and a 400-ml sample. The cells in the 400-ml sample were collected by centrifugation at 10,000 g for 10 min at 4°C, washed once with 0.15 M sodium chloride, 0.015 M sodium citrate (SSC), pH 7.5, at 4°C, and stored at -20°C. The cells in the 600-ml sample were collected by centrifugation at 10,000 g for 10 min at 25°C, washed in fresh trypticase broth medium not containing  $D_2O$  (i.e. containing only normal water) and collected by centrifugation. These cells were then suspended in 3,500 ml of trypticase medium containing only normal water and cultured from an initial cell concentration of  $1.5 \times 10^6$  cells/ml. Samples were taken at cell

concentrations of  $3.1 \times 10^6$ ,  $4.0 \times 10^6$ ,  $6.1 \times 10^6$ ,  $1.2 \times 10^7$ , and  $2.4 \times 10^7$  cells/ml (Fig. 2) by centrifugation at 10,000 g at 4°C, washed once in SSC at 4°C, and stored at -20°C. All cell counts were made in a Spencer Bright Line Hemocytometer (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.), with 1.0-ml culture samples to which 10  $\mu$ l of 37% formaldehyde had been added.

Details of the following techniques employed were as described previously (7): (a) isolation of whole cell DNA; (b) separation and isolation of purified kDNA associations and main band (nuclear) DNA. Kinetoplast DNA associations used directly for buoyant density analyses were obtained from whole cell DNA by a single sucrose sedimentation. Kinetoplast DNA associations used for the preparation of fractions of single circular molecules were purified by six successive sucrose sedimentations. Nuclear DNAs used for buoyant density analyses were prepared from the first supernate of whole cell DNA by performing six sucrose sedimentations to remove kDNA; (c) preparation of fractions consisting of covalently closed single circular molecules and of fractions consisting of a mixture of open circular molecules and linear molecules of single circle length (unit length linear molecules). From  $OD_{260}$  measurements, it was estimated for six preparations that between 20% and 40% of the kDNA in associations was recovered after sonication and sucrose sedimentation, as single circular and unit length linear molecules. The ratio of covalently closed circular molecules to open circular molecules and unit length linear molecules after separation by cesium chloride-ethidium bromide equilibrium centrifugation was between 1:4 and 1:3; (d) preparative neutral cesium chloride and cesium chloride-ethidium bromide equilibrium density gradient centrifugation; (e) analytical neutral and alkaline cesium chloride equilibrium density gradient centrifugation; (f) electron microscopy, except that measurements of molecules were made with a Graphics calculator (Numonics Corp., North Wales, Pa.).

*Escherichia coli* bromouracil-labeled hybrid DNA was prepared as described by Richards et al. (18). *Clostridium perfringens* DNA was a gift of Dr. Robert S. Ryan of the University of Chicago, Chicago, Ill.

Analysis of single-stranded DNA was performed by the method of Manning and Richards (12). DNA was denatured in SSC (0.15 M sodium chloride, 0.015 M sodium citrate) containing 0.2 N sodium hydroxide and 2% formaldehyde for 20 min at room temperature and then reneutralized with 1 M monobasic potassium phosphate. Analytical neutral cesium chloride equilibrium density gradient centrifugation was then carried out as for double-stranded DNA (7).

#### RESULTS

The buoyant densities of kinetoplast DNA (kDNA) and nuclear DNA of *C. acanthocephali* cultured in medium which contained only normal

water (nondeuterated DNA) were determined to be 1.702 g/cm<sup>3</sup> and 1.717 g/cm<sup>3</sup>, respectively (Figs. 1 and 3), in agreement with our previous observations (17, 7). After growth of cells of *C. acanthocephali* in medium containing 75% deuterium oxide (D<sub>2</sub>O) for 25 generations, both kDNA and nuclear DNA had a buoyant density 0.012 g/cm<sup>3</sup> greater than the buoyant density of the respective native DNAs (Fig. 1). Further cell doublings in this medium did not result in a further increase in the buoyant density of either DNA.

Cells growing exponentially in medium containing 75% D<sub>2</sub>O were pelleted, a sample was removed (zero cell doublings), and the remainder were transferred into medium containing only normal water (Fig. 2). Further samples were taken at 1.0, 1.4, 2.0, 3.0, and 4.0 cell doublings after the transfer into normal water medium (Fig. 2). DNA was extracted from cells of each sample and the nuclear DNA and kDNA associations were separated and analyzed by cesium chloride equilibrium density gradient centrifugation.

### Replication of Nuclear DNA

The buoyant densities of double-stranded nuclear DNAs from the various samples are given in Fig. 3. DNA from cells taken at zero cell doublings had the heavy buoyant density (1.729 g/cm<sup>3</sup>) expected. DNA from cells which had undergone 1.0 cell doubling after transfer into nor-

mal water medium had a buoyant density (1.722 g/cm<sup>3</sup>) intermediate between that of nondeuterated nuclear DNA (1.717 g/cm<sup>3</sup>) and that of nuclear DNA taken at zero cell doublings (1.729 g/cm<sup>3</sup>). The band of DNA from cells which had undergone 1.4 cell doublings exhibited a mode at the intermediate density of 1.722 g/cm<sup>3</sup>, but there was also a distinct shoulder on the less dense side of the band. Two distinct modes were visible in the banded DNA from cells which had undergone 2.0 cell doublings, one at the intermediate density and one at a buoyant density slightly greater than that of native nuclear DNA. These results are similar to those obtained by Messelson and Stahl (13) for *E. coli* DNA after transfer of bacterial cells from an N<sup>15</sup>- into an N<sup>14</sup>-containing medium, and are what would be expected if the nuclear DNA of *C. acanthocephali* replicated semiconservatively (5). By 1.0 cell doubling, all of the double-stranded molecules would contain one parental (heavy) nucleotide strand and one newly replicated (light) nucleotide strand. By 1.4 cell doublings, replication of the DNA of a proportion of the cells would result in approximately 25% of this DNA comprising two light strands which would account for the shoulder on the less dense side of the band of nuclear DNA from cells taken at this time. By 2.0 cell doublings, all of the DNA would have undergone two cycles of replication resulting in a population of DNA molecules, of which one-half comprise one heavy and one light strand, and the other half comprise only light strands.

To test this interpretation further, nuclear DNA from each sample was denatured and analyzed by neutral cesium chloride equilibrium centrifugation. The results are shown in Fig. 4. Denatured nondeuterated nuclear DNA and denatured DNA from cells taken at zero cell doublings each formed a single band at buoyant densities of 1.728 g/cm<sup>3</sup> and 1.740 g/cm<sup>3</sup>, respectively. Two bands comprising approximately equal amounts of DNA were formed by denatured nuclear DNA from cells which had undergone 1.0 cell doubling after transfer from D<sub>2</sub>O medium to normal water medium, one at the density of denatured DNA from cells taken at zero cell doublings (1.740 g/cm<sup>3</sup>) and one at the density of denatured nondeuterated DNA (1.728 g/cm<sup>3</sup>). Two bands at similar positions were formed by denatured DNAs from 1.4 and 2.0 cell doublings after transfer to normal water medium; however, the relative amount of DNA in

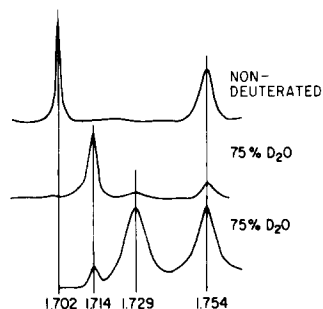


FIGURE 1 Photoelectric scans, using 260 nm illumination, of cesium chloride buoyant density gradients of DNAs from exponentially growing cells of *C. acanthocephali*. The upper tracing (nondeuterated) represents kDNA associations isolated from cells cultured only in normal water-containing medium. The center and lower tracings represent kDNA associations and whole cell DNA, respectively, isolated from cells cultured in medium containing 75% D<sub>2</sub>O for more than 25 generations. The reference bands ( $\rho = 1.754$  g/cm<sup>3</sup>) to the right are *E. coli* bromouracil-labeled hybrid DNA.

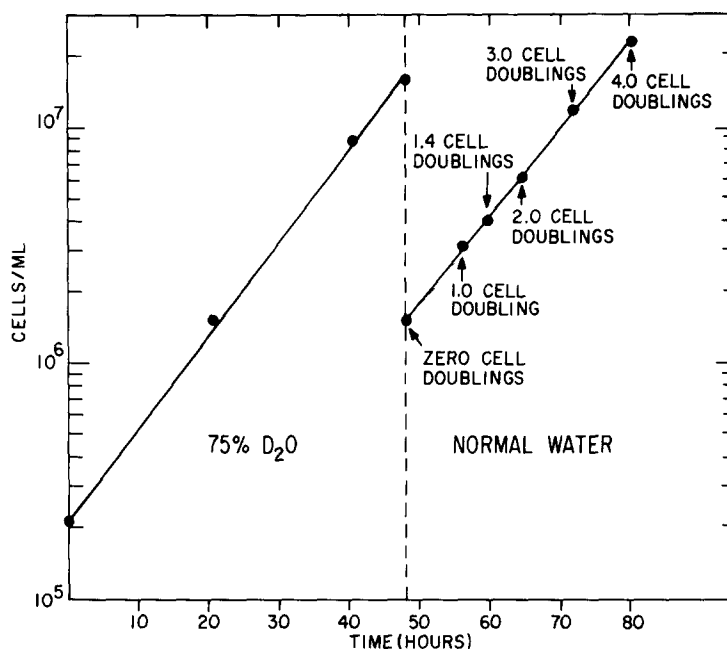


FIGURE 2 Cell multiplication in the culture of *C. acanthocephali* used in the density shift experiment. The cells (previously grown in 75% D<sub>2</sub>O-containing medium for approximately 35 generations) were first cultured in a medium containing 75% D<sub>2</sub>O. At the time indicated by the vertical broken line, the cells were transferred to a medium containing only normal water. The doubling times indicated are the times at which cell samples were taken for DNA isolations. The solid circles indicate the actual times at which cell concentrations were determined. The mean cell doubling time in 75% D<sub>2</sub>O medium was 7.68 hours and in normal water medium was 8.10 h.

the band at 1.728 g/cm<sup>3</sup> increased and the relative amount of DNA in the band at 1.740 g/cm<sup>3</sup> decreased with increased doubling time. In no case was denatured DNA of intermediate buoyant density detected. These results are, therefore, in complete agreement with the interpretation that nuclear DNA of *C. acanthocephali* replicates semiconservatively.

#### Replication of Kinetoplast DNA

The results of buoyant density analyses of kDNA associations are given in Figs. 5 and 6. Kinetoplast DNA taken from cells which had undergone 1.0 cell doubling after transfer from D<sub>2</sub>O medium to normal water medium formed a single sharp band with a buoyant density of 1.708 g/cm<sup>3</sup>, midway between that of kDNA taken from cells at zero cell doublings (1.714 g/cm<sup>3</sup>) and that of nondeuterated kDNA (1.702 g/cm<sup>3</sup>). Kinetoplast DNA from cells at 2.0 cell doublings formed a single band at  $\rho = 1.705$  g/cm<sup>3</sup>. A single band with two modes at  $\rho = 1.708$  g/cm<sup>3</sup> and  $\rho = 1.705$  g/cm<sup>3</sup> was found for kDNA of

cells taken at 1.4 cell doublings. This latter observation is consistent with the buoyant density values found for kDNA associations taken from cells at 1.0 and 2.0 cell doublings, as it is expected that approximately one-half of the cells taken from the culture at 1.4 cell doublings are the product of a single cell doubling and that the other half are the product of two cell doublings after transfer into normal water medium. Kinetoplast DNA from cells at 3.0 and 4.0 cell doublings each formed a single band at  $\rho = 1.703$  g/cm<sup>3</sup> and  $\rho = 1.702$  g/cm<sup>3</sup>, respectively.

The single bands observed for kDNA from cells taken at 1.0, 2.0, 3.0, and 4.0 cell doublings could in each case result from all of the individual associations having similar mean buoyant densities or from aggregation in the gradient of associations with different mean buoyant densities. To test the validity of this latter explanation, nondeuterated kDNA associations and kDNA associations from cells taken at different cell doubling times were mixed in pairs, and cesium chloride buoyant density analyses were performed. The

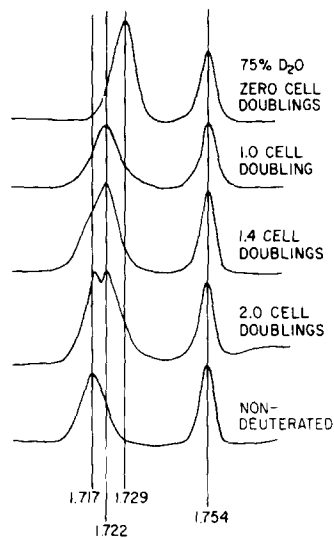


FIGURE 3 Photoelectric scans, using 260 nm illumination, of cesium chloride buoyant density gradients of double-stranded nuclear DNAs from exponentially growing cells of *C. acanthocephali*. The top tracing represents DNA isolated from cells cultured in medium containing 75% D<sub>2</sub>O, and the following three tracings represent DNA isolated from cells from the same culture taken at the given intervals of cell doubling after transfer into medium containing only normal water (Fig. 2). The bottom tracing (nondeuterated) represents DNA isolated from cells cultured only in normal water-containing medium. The reference band ( $\rho = 1.754 \text{ g/cm}^3$ ) to the right is *E. coli* bromouracil-labeled hybrid DNA.

results (Fig. 6) do not provide evidence that aggregation takes place in the cesium chloride gradients: in each gradient the absorbance profile indicates separate banding of the two component DNAs at buoyant density values similar to those obtained when the DNAs were centrifuged separately to equilibrium (Fig. 5).

The buoyant density values obtained for kDNA associations from cells taken at progressive cell doubling times indicate that the kDNA of an association doubles once per cell doubling, that upon segregation of the daughter associations newly synthesized DNA remains attached to parental DNA, and that newly synthesized and parental DNA are on the average equally divided between daughter associations. These results are also what would be expected if each component circular molecule of the kDNA association was replicated semiconservatively with molecules containing one-generation and two-generation old parental strands segregating at random between new associations. However, similar results

would also have been expected if doubling of kDNA in an association were achieved by repeated replication beginning with a few parental circles followed by equal division of parental and newly synthesized circular molecules in the daughter associations in each generation.

To elucidate the replication of kDNA further, we conducted buoyant density analyses of fractions consisting of covalently closed single circular molecules and fractions consisting of a mixture of open circular and linear molecules of single circle length (unit length linear molecules) obtained from nondeuterated kDNA associations and from kDNA associations from cells taken at the different cell doubling times after transfer from 75% D<sub>2</sub>O-containing medium to normal water medium. Kinetoplast DNA associations from each cell sample were sonicated and the product was subjected to neutral sucrose sedimentation. Fractions were examined in the elec-

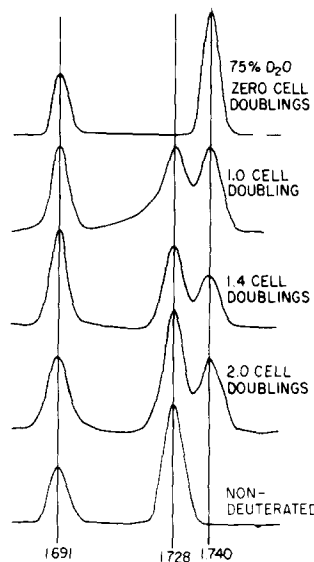


FIGURE 4 Photoelectric scans, using 260 nm illumination, of cesium chloride buoyant density gradients of denatured (single-stranded) nuclear DNAs from exponentially growing cells of *C. acanthocephali*. The top tracing represents DNA isolated from cells cultured in medium containing 75% D<sub>2</sub>O, and the following three tracings represent DNAs isolated from cells from the same culture taken at the given intervals of cell doubling after transfer into medium containing only normal water (Fig. 2). The bottom tracing (nondeuterated) represents DNA isolated from cells cultured only in normal water-containing medium. The reference band ( $\rho = 1.691 \text{ g/cm}^3$ ) to the left is *C. perfringens* native (double-stranded) DNA.

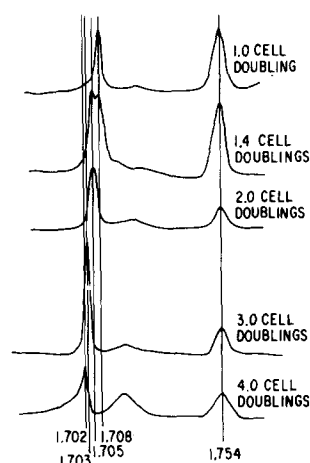


FIGURE 5 Photoelectric scans, using 260 nm illumination, of cesium chloride buoyant density gradients of kDNA associations from exponentially growing cells of *C. acanthocephali*. The DNAs were isolated from cells cultured in medium containing 75% D<sub>2</sub>O and taken at the given intervals of cell doubling after transfer into medium containing only normal water (Fig. 2). Varying amounts of nuclear DNA are apparent in each preparation as each of the kDNA associations used were separated from whole cell DNA by only a single sucrose sedimentation. The reference band ( $\rho = 1.754 \text{ g/cm}^3$ ) to the right is *E. coli* bromouracil-labeled hybrid DNA.

tron microscope, and those consisting mainly of single circular and unit length linear DNA molecules were pooled and the DNA was centrifuged to equilibrium in a cesium chloride-ethidium bromide gradient. The form, frequency, and size of the DNA molecules in each of the two fluorescent bands which resulted (Fig. 7) for each kDNA sample were determined by electron microscope analysis. The results are summarized in Table I. For each kDNA sample, greater than 86% of the DNA in the lower band (Fig. 9) was in the form of single circular molecules 0.78–0.80  $\mu\text{m}$  in contour length. The remainder in each case comprised various proportions of apparently catenated circular oligomers and linear molecules. In view of this and the relative position of these molecules in the gradient (15, 1, 7), these fractions will be referred to as fractions of covalently closed single circular molecules. For each kDNA sample, between 64% and 84% of the DNA in the upper band (Fig. 8) was in the form of circular molecules 0.77–0.80  $\mu\text{m}$  in contour length and unit length linear molecules. The remainder of each consisted mainly of linear molecules which were either less or greater than unit

length (Table I). Due to their relative position in the gradient, the circular molecules in these fractions are interpreted as open circles (circular molecules containing at least one broken phosphodiester bond). The upper band fractions will be referred to as fractions of open single circular molecules and unit length linear molecules.

The results of buoyant density analyses of fractions of covalently closed single circular molecules are shown in Fig. 10. A single band was observed for each sample centrifuged. The buoyant density values obtained were identical to the buoyant density values obtained for the corresponding kDNA associations (Fig. 5).

Buoyant density analyses were performed on fractions of open single circular and unit length linear molecules obtained from nondeuterated kDNA and kDNA from cells taken at zero, 1.0, and 2.0 cell doublings. Again, a single band was observed for each sample examined; however, the mode of each band was at a buoyant density 1–3  $\text{mg/cm}^3$  greater than the buoyant density found for the mode of the corresponding covalently

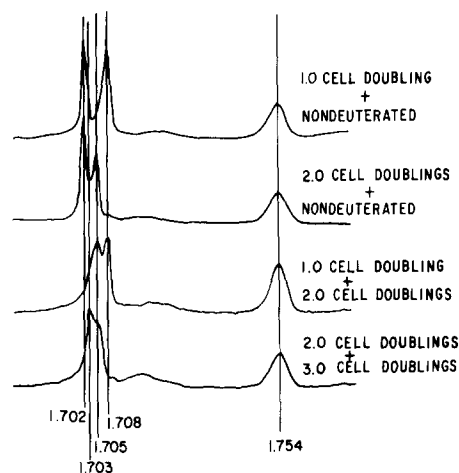


FIGURE 6 Photoelectric scans, using 260 nm illumination, of cesium chloride buoyant density gradients of kDNA associations from exponentially growing cells of *C. acanthocephali*. Each tracing represents an approximately equimolar mixture of two of the DNAs represented in Figs. 1 and 5. These are kDNA associations isolated from cells cultured only in normal water-containing medium (nondeuterated), and from cells cultured in medium containing 75% D<sub>2</sub>O and taken at the given intervals of cell doubling after transfer into medium containing only normal water (Fig. 2). The reference band ( $\rho = 1.754 \text{ g/cm}^3$ ) to the right is *E. coli* bromouracil-labeled hybrid DNA.

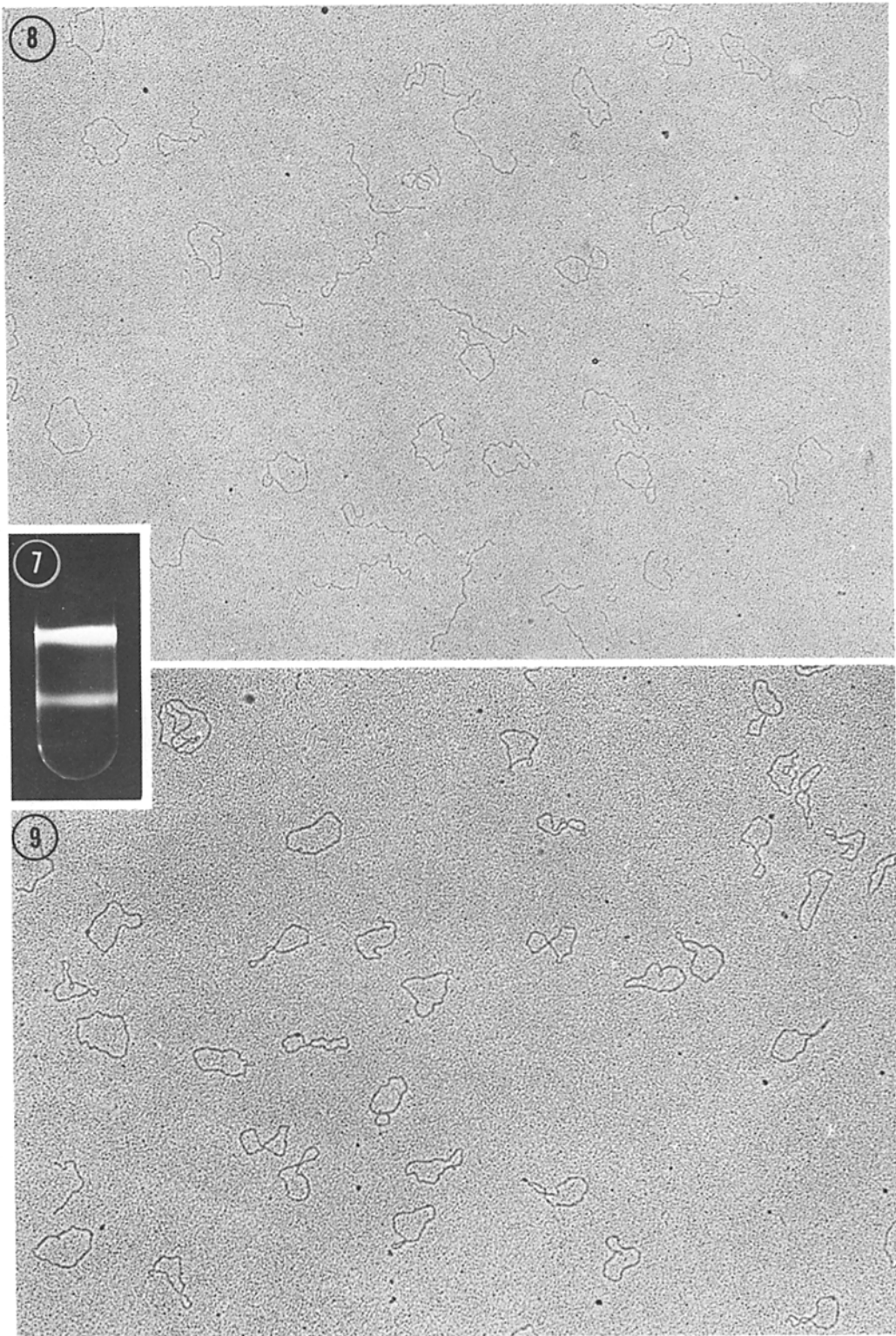


FIGURE 7 Fluorescence photograph of a cesium chloride-ethidium bromide gradient resulting from centrifugation to equilibrium of approximately  $100 \mu\text{g}$  of kDNA consisting mainly of single circular molecules and unit length linear molecules obtained by sucrose sedimentation of sonicated kDNA associations. The kDNA associations were isolated from cells taken at zero cell doublings. The photograph was taken using illumination from a high-pressure HBO 200 mercury lamp, Zeiss UGI and BG38 exciter filters, and a Kodak Wratten no. 16 filter as a barrier filter. Two fluorescence bands are clearly visible. Photograph,  $\times 0.8$ .

FIGURES 8 and 9 Electron micrographs of rotary shadowed molecules in protein monolayer preparations of kDNA in the upper (Fig. 8) and lower (Fig. 9) bands of the cesium chloride-ethidium bromide density gradient shown in Fig. 7. Both micrographs,  $\times 31,200$ .

TABLE I  
Analysis of Form, Frequency, and Size of kDNA Molecules

Kinetoplast DNA preparation	Denser band				Less dense band				Length of single circular molecules	
	Percentage of DNA as		Mean length $\pm$ standard deviation (number of molecules)	Total length in micrometers of DNA examined	Percentage of DNA as		Single circular and unit length linear molecules¶	Linear molecules less than unit length**		Circular and linear molecules greater than unit length**
	Single circular molecules	Catenated circular oligomers‡			Linear molecules	Linear molecules less than unit length				
Nondeuterated	91.6	2.8	5.6	191.6	83.9	5.8	10.3	0.77 $\pm$ 0.038 (20)	0.70-0.84	
75% D <sub>2</sub> O (zero doublings)	86.9	8.5	4.6	117.5	71.9	9.4	7.6	0.80 $\pm$ 0.031 (20)	0.73-0.86	
One cell doublings	86.3	6.7	7.0	¶	¶	¶	¶	0.78 $\pm$ 0.031 (20)	¶	
Two cell doublings	90.1	2.7	7.2	141.3	68.4	10.2	21.4	0.78 $\pm$ 0.028 (20)	0.73-0.83	
Three cell doublings	90.9	5.5	3.6	129.8	64.5	8.8	26.7	0.80 $\pm$ 0.030 (20)	0.74-0.86	

Data obtained by electron microscope analysis concerning the form, frequency, and size of DNA molecules in the denser and less dense fluorescence bands resulting from cesium chloride-ethidium bromide equilibrium density gradient centrifugation of kDNA fractions, each consisting of a collection of mainly single molecules. The latter fractions were produced by sucrose sedimentation of sonicated nondeuterated kDNA associations and kDNA associations obtained from cells taken at the various doubling times indicated after transfer from D<sub>2</sub>O medium into normal water medium.

\* The sum of the measured lengths of all of the linear molecules and single circular molecules with attached linear molecules observed, plus the product of the number of single circular molecule equivalents observed and the calculated mean length of the single circular molecules.

† This category comprises catenated circular dimers and trimers (never accounting for more than 0.5% of the total DNA examined in any one fraction), and single circular molecules with an attached linear molecule of a length less than that of the circle (never accounting for more than 1.0% of the total DNA examined in any one fraction).

‡ Range of lengths, 0.1-1.6  $\mu$ m.

§ The sum of the measured lengths of all of the linear molecules, single circular molecules with attached linear molecules, and forked linear molecules observed, plus the product of the number of single circular molecule equivalents observed and the calculated mean length of the single circular molecules.

¶ Unit length linear molecules were defined as linear molecules with a measured length within the range of lengths of single circular molecules in the same preparation. Single circular molecules accounted for between 31% and 62% of the molecules of this class in the different preparations.

\*\* This category comprises catenated circular dimers, linear molecules with measured lengths greater than the range of lengths of single circular molecules, but no greater than twice the maximum measured length of single circular molecules in the same preparation, catenated circular trimers, single circular molecules with an attached linear molecule, catenated circular dimers with a linear molecule attached to one of the component circles, and forked linear molecules (with lengths of individual component segments never greater than the length of a single circular molecule).

‡‡ Upper band of this kDNA sample not examined.



lently closed single circular molecule fraction. These higher values could have resulted from the presence of single-strand sections produced during the preparative sonication step (14) on the ends of the component linear molecules of these fractions.

The buoyant density of 1.708 g/cm<sup>3</sup> found for the single band of covalently closed single circular molecules taken from cells at 1.0 cell doubling is consistent with each circular molecule having replicated once semiconservatively after transfer from D<sub>2</sub>O medium to normal water medium. Each molecule would comprise one parental (heavy) nucleotide strand and one newly replicated (light) nucleotide strand. An alternative explanation, that the single band observed at  $\rho = 1.708$  g/cm<sup>3</sup> comprises an equal mixture of circular molecules of the buoyant densities of 1.714 g/cm<sup>3</sup> and 1.702 g/cm<sup>3</sup>, seems unlikely: when a mixture of single circular molecules from cells grown in normal water ( $\rho = 1.702$  g/cm<sup>3</sup>) and from cells taken at 1.0 cell doubling ( $\rho = 1.708$  g/cm<sup>3</sup>) were centrifuged to equilibrium, the resulting absorbance profile was a single band with two modes at approximately the values found for the component DNAs when they were centrifuged to equilibrium separately (Fig. 10).

Determinations of the buoyant densities of the separated nucleotide strands of single molecules from kDNA of cells at 1.0 cell doubling could provide a direct indication as to whether a parental strand is conserved during replication of each molecule. Such an analysis was attempted (Fig. 11) on denatured DNA from fractions comprising open single circular and unit length linear molecules. Denatured nondeuterated kDNA formed two bands at equilibrium in cesium chloride, each with a single mode at  $\rho = 1.706$  g/cm<sup>3</sup> and  $\rho = 1.731$  g/cm<sup>3</sup>. It has been previously shown that these bands represent the complementary nucleotide strands of kDNA molecules (7). (We determined that the lower buoyant density reported here for the heavy strand, 1.731 g/cm<sup>3</sup> compared to 1.739 g/cm<sup>3</sup> reported by Fouts et al. [7], resulted from the use of formaldehyde to prevent reassociation). Denatured kDNA from cells taken at zero cell doublings also formed two bands at equilibrium at  $\rho = 1.713$  g/cm<sup>3</sup> and  $\rho = 1.737$  g/cm<sup>3</sup>. Denatured kDNA from cells taken at 1.0 cell doubling formed two broad bands with modes at approximately  $\rho = 1.710$  g/cm<sup>3</sup> and  $\rho = 1.734$  g/cm<sup>3</sup>. Two bands,

similar in shape and position to those found for 1.0 cell doubling kDNA, were formed when an approximately equimolar mixture of denatured nondeuterated kDNA and zero cell doubling kDNA was centrifuged to equilibrium. While these results are again consistent with a semiconservative mode of replication for kDNA, failure to demonstrate four buoyant density classes for kDNA from cells taken at 1.0 cell doubling prevents confirmation of this conclusion.

If kDNA molecules replicate semiconservatively, it would be expected that single circular molecules obtained from cells at 2.0 cell doublings would show two modes at equilibrium in buoyant density gradients representing equal amounts of DNA at buoyant density values of 1.702 g/cm<sup>3</sup> (each molecule comprising two light strands) and 1.708 g/cm<sup>3</sup> (each molecule comprising one heavy and one light strand). This was not observed. Covalently closed single circular molecules obtained from cells at 2.0 cell doublings formed a single band in cesium chloride gradients. However, the modal density (1.705 g/cm<sup>3</sup>) was equal to the mean of the two modal values expected.

To elucidate the buoyant densities of the component circular molecules of kDNA at 2.0 cell doublings, the following experiment was conducted. A density shift experiment identical to that summarized in Fig. 2 was performed except that cells were taken only at 2.0 cell doublings after transfer from D<sub>2</sub>O medium into normal water medium. Covalently closed single circular kDNA molecules were prepared as before, and buoyant density analyses again revealed a single band with a mean value of  $\rho = 1.705$  g/cm<sup>3</sup> as was found in the original experiment. Approximately 20  $\mu$ g of the covalently closed single circular molecules were then centrifuged to equilibrium in a preparative neutral cesium chloride gradient. The gradient was fractionated and the distribution of DNA determined (Fig. 12). Fractions were then pooled so as to provide samples containing DNA from the more dense, the center, and the less dense portions of the DNA band as indicated in the upper portion of Fig. 12. Each of these samples was subjected to analytical cesium chloride equilibrium density centrifugation. The results are shown in the lower portion of Fig. 12. The DNA of each of the three fractions formed a single band; however, the mean buoyant density of the DNA from the more dense,

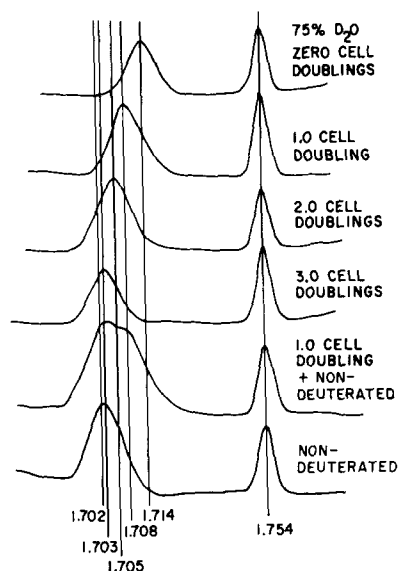


FIGURE 10 Photoelectric scans, using 260 nm illumination, of cesium chloride buoyant density gradients of covalently closed single circular DNA molecules obtained from kDNA associations of exponentially growing cells of *C. acanthocephali*. The top tracing represents DNA isolated from cells cultured in medium containing 75% D<sub>2</sub>O, and the following three tracings represent DNAs isolated from cells from the same culture taken at the given intervals of cell doubling after transfer into medium containing only normal water (Fig. 2). The bottom tracing (nondeuterated) represents DNA isolated from cells cultured only in normal water-containing medium. The next to bottom tracing represents an approximately equimolar mixture of the DNAs indicated. The reference band ( $\rho = 1.754 \text{ g/cm}^3$ ) to the right is *E. coli* bromouracil-labeled hybrid DNA.

center, and less dense portions of the preparative gradient were  $1.708 \text{ g/cm}^3$ ,  $1.705 \text{ g/cm}^3$ , and  $1.703 \text{ g/cm}^3$ , respectively.

These results clearly indicate that circular molecules of kDNA from cells taken at 2.0 cell doublings comprise more than one kind in regard to buoyant density. The buoyant density values found for the kDNA in the more dense portion ( $1.708 \text{ g/cm}^3$ ) and less dense portion ( $1.703 \text{ g/cm}^3$ ) of the preparative gradient are consistent with there being present hybrid molecules (comprising one heavy and one light strand) and molecules comprising only light strands as would be expected at 2.0 cell doublings if the mode of replication is semiconservative. However, the finding of a single band with a modal buoyant density of  $1.705 \text{ g/cm}^3$  for the fraction compris-

ing kDNA from the central portion of the preparative gradient suggests the presence of circular molecules with buoyant densities intermediate between that of hybrid DNA molecules ( $1.708 \text{ g/cm}^3$ ) and that of DNA molecules comprising only light strands ( $1.702 \text{ g/cm}^3$ ).

## DISCUSSION

From the results presented it is clear that nuclear DNA of *C. acanthocephali* replicates semiconservatively. Data consistent with this mode of replication have been obtained for the nuclear DNA of a number of organisms (13, 21, 19, 4, 6, 16), for chloroplast DNA (3, 12), and for mitochondrial DNA from *Neurospora crassa* (16) and rat liver (8) and from HeLa cells (6a).

The data presented concerning kDNA of *C. acanthocephali* clearly indicate that duplication of the DNA of an association during each gener-

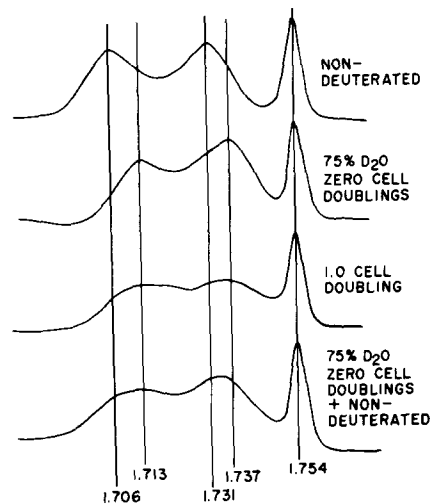


FIGURE 11 Photoelectric scans, using 260 nm illumination, of cesium chloride buoyant density gradients of denatured (single-stranded) open single circular and unit length linear DNA molecules obtained from kDNA associations of exponentially growing cells of *C. acanthocephali*. The top tracing (nondeuterated) represents DNA isolated from cells cultured only in normal water-containing medium. The next two tracings represent, respectively, DNA isolated from cells cultured in medium containing 75% D<sub>2</sub>O and DNA molecules from cells of the same culture taken at one cell doubling after transfer into medium containing only normal water. The bottom tracing represents an approximately equimolar mixture of the DNAs indicated. The reference band ( $\rho = 1.754 \text{ g/cm}^3$ ) to the right is *E. coli* bromouracil-labeled hybrid DNA.

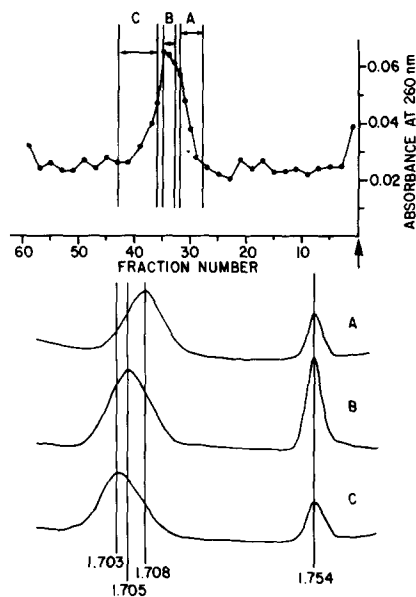


FIGURE 12 The upper diagram indicates the distribution of approximately 20  $\mu\text{g}$  of covalently closed single circular molecules of kDNA after centrifugation to equilibrium in a preparative neutral cesium chloride gradient as indicated by absorbance at 260 nm. The covalently closed single circular molecules were obtained from kDNA associations of cells of *C. acanthocephali* cultured in medium containing 75%  $\text{D}_2\text{O}$  and taken at 2.0 cell doublings after transfer into medium containing only normal water. 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 portions of the kDNA band. The arrow below the abscissa indicates the bottom of the cesium chloride gradient. The lower diagram comprises photoelectric scans, using 260 nm illumination, of analytical neutral cesium chloride buoyant density gradients of the kDNA in each of the three samples A, B, and C. The reference band ( $\rho = 1.754 \text{ g/cm}^3$ ) to the right is *E. coli* bromouracil-labeled hybrid DNA.

ation results from the replication of the DNA of each circular molecule. The buoyant density values obtained for covalently closed single circular molecules taken from cells at 1.0 cell doubling after transfer from  $\text{D}_2\text{O}$  medium to normal water medium exclude the possibility that duplication of an association results from repeated replication of a small fraction of the circular molecules in an association.

The buoyant density values obtained from covalently closed single circular molecules of kDNA taken from cells at 1.0 cell doubling are

consistent with a semiconservative mode of replication. The finding of circular molecules with buoyant densities intermediate between the hybrid value ( $\rho = 1.708 \text{ g/cm}^3$ ) and the nondeuterated value ( $\rho = 1.702 \text{ g/cm}^3$ ) in kDNA from 2.0 cell doublings is still consistent with a semiconservative mode of replication if it is assumed that after replication of the nucleotide strands at least some of the circular molecules engage in one or more recombination events. Recombination of the circular molecules could involve the exchange of a homologous double-stranded segment of two circular molecules brought about by crossing over of the nucleotide strands at two locations. Or recombination might involve exchange of homologous single-stranded segments between molecules as occurs, for example, in the mechanism proposed by Benbow et al. (2) for recombination between circular RF DNA molecules of bacteriophage  $\phi\text{X-174}$ . Recombination of either sort between some of the molecules predicted as the product of semiconservative replication in cells having undergone 2.0 cell doublings could result in circular molecules with buoyant densities between  $1.702 \text{ g/cm}^3$  and  $1.708 \text{ g/cm}^3$  as observed. Further testing of this hypothesis by analysis of single strands of molecules of kDNA from cells taken at 1.0 and 2.0 cell doublings is not possible with the present procedures due to the limited resolution obtainable for the buoyant densities of single strands as discussed above.

The high degree of recombination among circular molecules during replication, which must be postulated to render the results of our density shift experiments consistent with semiconservative replication, may be related to the amount of circle breakage and rejoining that can be expected to accompany replication of the kDNA associations given their topological structure. Evidence has been obtained with light microscope autoradiography (20) and electron microscope autoradiography (Manning and Wolstenholme, unpublished observations) that synthesis of kDNA of *Crithidia* is limited to the peripheral regions of the associations. Breakage and rejoining of the circular molecules must occur for these observations to be consistent with our conclusion that duplication of kDNA involves semiconservative replication of each circular molecule. Breakage and rejoining of circular molecules must also occur to separate the products of replicating topologically interlocked circles in a way that preserves the highly ordered arrangement of molecules in the association (17,

22) and provides for segregation of molecules into two daughter associations.

It should be mentioned that Simpson et al. (20) analyzed single circular kDNA molecules and nuclear DNA from cells of *Leishmania tarentolae* grown continuously in [<sup>3</sup>H]thymidine and then shifted into a medium containing bromodeoxyuridine and <sup>32</sup>P. At 1.0 cell doubling they found that more than half of both newly labeled kDNA and newly labeled nuclear DNA had the buoyant density expected for DNA in which both nucleotide strands contained the density label. The remaining newly labeled DNA in each case was of hybrid density. By 1.9 cell doublings, more of the <sup>32</sup>P label was found associated with both kDNA and nuclear DNA density labeled in both strands. The similar density patterns found for kDNA and nuclear DNA suggest the possibility that the result may be explained by there being more than one round of DNA replication per cell doubling after transfer of cells into bromodeoxyuridine-containing medium. However, in the absence of further analysis of DNA replication within the 1.0 cell doubling period, the data of Simpson et al. (20) do not establish that either nuclear DNA or kDNA circular molecules replicate semiconservatively, and leave open the possibility that in *L. tarentolae* duplication of some of the kDNA results from repeated replication of a portion of the molecules.

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