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Isolation and Characterization of Kinetoplast DNA Networks and Minicircles from *Crithidia fasciculata**

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SYNOPSIS. Covalently closed kinetoplast DNA networks have been isolated from stationary phase *Crithidia fasciculata* cells by a technic involving selective pelleting of the networks at a low centrifugal field. Approximately 62% of the kinetoplast DNA of the cell was recovered free of nuclear DNA by simple differential centrifugation.

Purified kinetoplast DNA networks were visualized both in the electron microscope and in the light microscope.

Closed networks sedimented as a homogeneous band both in neutral and alkaline sucrose, with an $s_{20,w}$ in neutral sucrose of approximately 5×10^3.

Closed monomeric minicircles were isolated from purified networks by mild sonication and band sedimentation in alkaline sucrose. Several physical properties of closed monomeric minicircles were measured. These included molecular weight, buoyant density in CsCl, superhelix density and sedimentation coefficient.

Index Key Words: *Crithidia fasciculata*; kinetoplast DNA; network, minicircles, isolation and characterization of.

THE K-DNA of several species of hemoflagellates has been isolated in the form of high molecular weight masses of DNA termed "networks" (9). Laurent *et al.* (5) used a 2 step isolation procedure consisting of sedimentation on a sucrose pad and neutral CsCl equilibrium centrifugation. They obtained homogeneous particles from *Crithidia luciliae* that were visible in the fluorescent microscope after staining with ethidium bromide. Renger and Wolstenholme (7) isolated equivalent structures from a kinetoplast-enriched fraction of *C. acanthocephali* and successfully observed the organization of molecules within the structure in the electron microscope. They found that the large masses of DNA were composed of approximately 800 rosettes, each of which consisted of approximately 33 minicircles catenated together. The rosettes were interconnected laterally by the catenation of one or more component minicircles. Simpson and Berliner (9) isolated K-DNA networks from *Leishmania tarentolae* by a technic similar to that described in this report, and performed several physical measurements on these structures.

We have isolated covalently closed K-DNA networks from *C. fasciculata* and have studied some physical properties of these networks and the component minicircles.

* Supported in part by NIH Research Grant No. AI09012 to L. Simpson, and by a UCLA Biomedical Science Support Grant.

MATERIALS AND METHODS

Culture and Labeling of Cells

The cells represent a clone of a *C. fasciculata* culture originally obtained from Dr. Stuart Krassner. This clonal cell line has been growing in our laboratory for 3 years as previously described (10). The experimental cultures were grown in either modified (9) Trager's (13) defined medium CA for *L. tarentolae,* or in Kidder and Dutta's (4) defined medium for *Crithidia.* Cells were grown for at least 3 subcultures in the appropriate defined medium prior to use in labeling experiments.

[Methyl-^{3}H] thymidine (18 Ci/mM) was purchased from Schwarz/Mann Co. Experimental cultures were grown in bottles with rotation at 12 rpm (27 C).

Cell counts were performed by mixing samples of culture 1:1 (v/v) with 3% (w/v) formalin and counting 200-500 cells in a hemocytometer.

Isolation of K-DNA Networks

The isolation procedure was carried out at 0-4 C unless specified. Cells were harvested in early stationary phase ($\sim 2 \times 10^8$ cells/ml). Centrifugation was at 4,000 rpm for 10 min in the Sorvall HB-4 rotor and the pellet obtained was washed once with SBG (0.15 M NaCl, 0.02 M phosphate buffer pH 7.9, 0.02, M glucose). This final pellet was resuspended in 0.5 M EDTA-0.15 M NaCl pH 8.0 to a 5% (w/v) final concentration. Sarkosyl (Sarkosyl NL 97, Geigy Industrial Chemicals, Ardsley, N. Y.) was added to 1% (w/v) final concentration, and pronase B or pronase^{CB}, B grade, (Calbiochem) predigested for 30 min at 37 C, was added to a concentration of 2 mg/ml or 0.5 mg/ml respectively. The solution was incubated at 56 C for 3 to 5 hr with occasional agitation. The lysate was then passed through a #20 or #18 needle at 25 lbs/in^2 and centrifuged at 20,000 rpm in the SW39 rotor for 20 min. The supernatant was poured off carefully and the pellet was resuspended either in 1/10 SSC or SSC and centrifuged again under the same conditions to eliminate nuclear DNA contamination. The final pellet was resuspended in either SSC or 1/10 SSC and was extracted with chloroform-isoamyl alcohol (24:1 v/v). The interface was reextracted to avoid loss. In some experiments the K-DNA was also treated with RNase A + RNase T_1 (20 μg/ml and 20 units/ml respectively) for 30 min at 37 C. Then pronase^{CB} was added to a final concentration of 100 μg/ml and the solution was reincubated at 37 C for 30 min. Deproteinization was then performed with chloroform-isoamyl alcohol. The solution was dialyzed overnight against SSC at 4 C. When necessary the K-DNA solution was concentrated by evaporation in dialysis tubing and redialyzed against SSC.

If complete recovery of K-DNA was desired along with some nuclear DNA contamination, the syringed lysate was centrifuged in the SW27 rotor for 2 hr at 25,000 rpm at 5 C. The pellet was resuspended in SSC and treated as above with RNase A+T_1 and pronase. The DNA was then subjected to EthBr-CsCl equilibrium centrifugation as described below, and the lower fluorescent band of K-DNA was collected. The dye was removed as described below.

Isolation of K-DNA Minicircles

Covalently closed K-DNA networks were isolated as described previously and the purified networks were subjected to sonication in a Biosonic III sonicator. For maximum yield the DNA was sonicated either in 0.2 ml or 3 ml volumes for 5 sec, small probe-power of "zero," or for 7 sec, medium probe-power of 20 respectively. The solutions were bubbled with argon gas (Matheson) prior to and after each sonication. The sample was then layered onto an alkaline band velocity sucrose gradient for recovery of the closed monomeric minicircle peak. The denatured closed monomers were renatured by the following technic which was developed by Strider (12). The monomers were dialyzed versus 2 mM EDTA pH 8.0 and then two volumes of monomers (20-60 μg/ml) were combined with 1 volume of fresh "annealing solution" at pH 10.7 (9.0 ml of 4 M NaCl, 0.24 ml of 0.5 M EDTA pH 8, 1.2 ml of 1 M K_3HPO_4 and 1.56 ml of 1 M K_2HPO_4). This was brought to 70 C for 5 min and then neutralized with 2 volumes of 2 M Tris-HCl pH 7.0 and cooled.

[^{32}P]-labeled closed monomeric and dimeric minicircles were isolated from *L. tarentolae,* for use as s-value markers by the technic of Wesley and Simpson (15), involving alkaline band sedimentation of sonicated networks. The initial specific activity of the purified closed monomers was 246,689 cpm/μg (9).

Isolation of N-DNA

The supernatant from the first centrifugation of the cell lysate was saved and the N-DNA was isolated according to the method of Marmur (6). Further purification was performed by running the DNA in a CsCl equilibrium gradient ($\rho_0 = 1.700$ g/ml) for 56 hr at 40,000 rpm in a #50 rotor, 20 C. The fractions were collected by drops from the bottom of the tube and the A_{260} nm was measured in a Gilford spectrophotometer. The N-DNA peak was collected and dialyzed against SSC.

Isolation of Tetrahymena pyriformis *DNA*

Tetrahymena pyriformis strain HSM-3 was a gift from Dr. C. Brunk. The cells were grown in 3½ liters of 1% (w/v) Bacto-Peptone (Difco) with 0.1% (w/v) fraction L (Difco) in a fermentator with 2 l of air/min, 175 rpm, 26 C. After 3 days of growth the cells were centrifuged in a Sorval centrifuge at 4,000 rpm for 15 min in a GSA rotor. The pellet was washed in 0.5 M NaCl, 0.05 M EDTA, 0.05 M Tris pH 8.5 (1). This final pellet was resuspended at 8% (w/v) in the above solution and SDS (Sodium Dodecyl Sulphate) was added to 2% (w/v) final concentration. The solution was incubated at 60 C for 15 min and then cooled to room temperature. Sodium perchlorate was added to give a 1M final concentration. The mixture was extracted twice with an equal volume of chloroform-isoamyl alcohol (25:1 v/v) for 30 min in the cold, and the DNA was precipitated with ethanol and redissolved in SSC. This was repeated twice. The DNA was then treated with RNase A (20 μg/ml) and RNase T1 (20 U/ml) for 30 min at 37 C, reextracted with chloroform, reprecipitated with ethanol and dissolved in SSC.

Preparative EthBr-CsCl Equilibrium Centrifugation

This was carried out in a Spinco L2 ultracentrifuge in a #50 fixed angle rotor at 40,000 rpm for 48 hr at 20 C. The initial refractive index at 25 C was usually 1.3875, and the tube contained 6.5 ml of CsCl solution with 200-300 μg/ml of EthBr. Four or 5 drop fractions were collected from the bottom onto Whatman 3MM filter discs or into test tubes. If the radioactive profile was desired, then 5 μl samples of the fractions were spotted onto discs, which were processed and counted. In some cases the K-DNA band was directly visualized by UV illumination of the tube and the band was collected by eye.

The filters were processed through cold 5% (w/v) trichloroacetic acid, ethanol and ether, and counted in Omnifluor (New England Nuclear) in toluene.

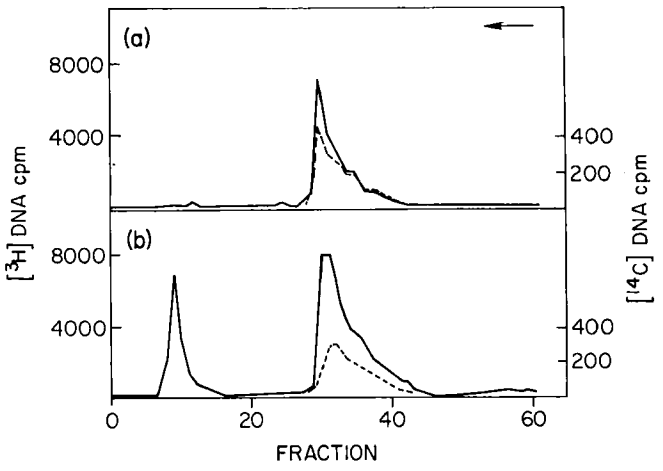

Fig. 1. EthBr-CsCl equilibrium gradients of [^{3}H]thymidine-labeled *C. fasciculata* total cell lysates. Cells were lysed by the sarkosyl pronase method under different conditions in an attempt to optimize the yield of K-DNA. (a) Cells were resuspended at a concentration of 5% (w/v) in 0.5 M EDTA - 0.15 M NaCl (pH 8), and sarkosinate and pronase^{CB} were added to 1% (w/v) and 0.5 mg/ml respectively. As soon as the solution was mixed, an aliquot was taken and put into an EthBr-CsCl solution. (b) The lysis procedure was as in (a) but the solution was placed at 60 C for 3 hr before adding to the EthBr-CsCl solution. Centrifugation conditions: 39,000 rpm, #50 rotor, 60 hr, 20 C, n_D^{25} = 1.3878, 200 μg/ml EthBr. *C. fasciculata* [^{14}C]-labeled nuclear DNA was added as an internal upper band marker to each gradient (-----). Three drop fractions were collected onto Whatman 3MM filter discs and processed as described in Materials and Methods.

Removal of the dye was accomplished either by shaking the DNA dye solution with isoamyl alcohol or by dialysis against Dowex 50w resin in 1.5 M NaCl at 27 C.

Analytical CsCl Centrifugation

This was performed in a Spinco Model E ultracentrifuge at 44,770 rpm for 20 hr at 25 C. Tracings of the UV films were made with a Joyce-Loebel densitometer. The buoyant densities were calculated as described by Vinograd and Hearst (14) using two markers: *Tetrahymena pyriformis* (1.685 g/ml), and *Micrococcus lysodeiticus* (1.731 g/ml) DNA.

Electron Microscopy

DNA samples were prepared for electron microscopy as described previously (8). Photographs were taken either in a Philips 200 or a Jeolco JEM-T7 electron microscope.

øX-RF-II DNA molecules were used as internal length standards. The DNA was a gift from Dr. Ronald Wesley.

Light Microscope Autoradiography

The microscope slides were presubbed with 0.1% (w/v) gelatin-0.01% (w/v) chrome alum. The K-DNA was smeared onto the slide with a platinum loop and air-dried quickly, fixed in methanol, and extracted with cold 5% trichloroacetic acid, 70% ethanol and 95% ethanol. The Ilford L-4 emulsion diluted 1:1 with water was applied by dipping the slides into the melted emulsion. The slides were kept in a box at 4 C until they were developed for 4 min in Kodak D-19 developer at 20 C. The slides were stained through the emulsion with Giemsa stain diluted 1 : 40 with 0.02 M phosphate buffer, pH 6.8.

TABLE 1. *Percentage of K-DNA in total cell DNA of* C. fasciculata *measured by three independent methods.*

Autoradiography					
N-grains	K-grains	cells	% K-DNA*	% K-DNA calculated from analytical CsCl tracings	% K-DNA calculated from EthBr-CsCl equilibrium gradient of [^{3}H] thymidine-labeled total cell lysate*
787	344	100	24	23 ± 0.6 (n = 4)†	22 ± 1.7 (n = 4)†

* Corrected for thymine content of 21.5% in K-DNA versus 28% in nuclear DNA.
† Average ± standard deviation (n = number of runs).

Sucrose Gradient Sedimentation

Low speed neutral and alkaline gradients.—Neutral sucrose gradients were used to calculate the sedimentation coefficients of closed networks. The gradients were prepared with Ultra Pure sucrose (Schwarz/Mann) in SSC. Centrifugation conditions were 11,500 rpm for 12 min at 4 C in the SW39 rotor or SW65 rotor. The gradients were fractionated from the top in an Isco Model D fractionator.

High speed alkaline gradients.—These gradients were used to separate closed minicircles from nicked minicircles or other molecules. Sucrose was dissolved in a solution consisting of 0.3 M NaOH, 1 M NaCl, 0.01 M Tris, 1 mM EDTA. The final pH of the 5 and 20% sucrose solutions was adjusted to 12.5. Centrifugation conditions were 2.5 hr at 65,000 rpm at 4 C in the SW65 rotor, or 15 hr in the SW27 rotor.

RESULTS

Isolation of Covalently Closed Kinetoplast DNA Networks From Stationary Phase Cells

The use of a high concentration of EDTA (0.5 M) in the lysis medium allowed complete recovery of K-DNA networks as covalently closed structures in EthBr-CsCl equilibrium gradients. The use of the lysis medium for *L. tarentolae* (9), which contains 0.025 M EDTA, consistently gave open or partially open networks.

Attempts were made to optimize the conditions for cell lysis in terms of the release of lower band material in EthBr-CsCl gradients. A brief lysis at room temperature did not release any lower band material (Fig. 1a). Incubation of the lysate at 60 C for 5 min gave a profile in which 20% of the total DNA was in the lower band, and incubation of the lysate at 60 C for 3 hr gave the profile shown in Fig. 1b in which 32% of the total DNA was in the lower band. Passage of the lysate through a #20 syringe needle at 25 lbs/in^2 to lower the viscosity did not affect the banding pattern in EthBr-CsCl. A 3 hr treatment at 56 C was selected as the standard lysis procedure.

In three separate total cell lysate preparations an average of 24% of the DNA was released as lower band material by the standard lysis procedure: 0.5 M EDTA-0.15 M NaCl (pH 8.0) 1% Na sarkosinate, 0.5 mg/ml pronase^{CB}, 3 hr at 56 C. This value is equivalent to the relative amount of K-DNA in the cell as measured by 2 independent methods (Table 1): light microscope autoradiography of long-term [^{3}H] thymidine-labeled cells, and measurement of the areas under the peaks in analytical CsCl equilibrium gradients (Fig. 2). Therefore, it is likely that essentially all of the K-DNA of stationary phase cells is present in a covalently closed circular configuration.

It was found that K-DNA networks would selectively pellet

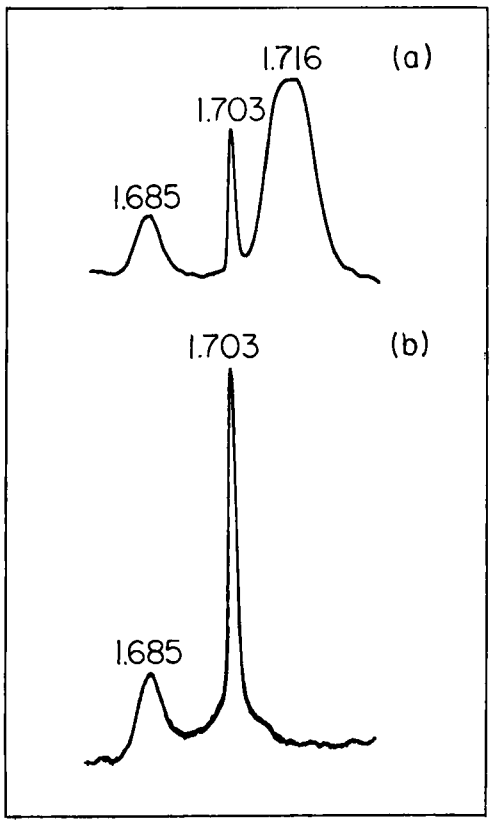

Fig. 2. Densitometer tracings of UV films of analytical CsCl equilibrium centrifugations of *C. fasciculata* total cell lysate and of purified K-DNA networks. The centrifugation was carried out in a Spinco model E ultracentrifuge at 44,770 rpm for 20 hr at, 20 C. *Tetrahymena pyriformis* DNA (1.685 g/ml) was used as a density marker. Traces of the films were made with a Joyce-Loebel densitometer. (a) *C. fasciculata* total cell lysate. (b) *C. fasciculata* purified K-DNA networks.

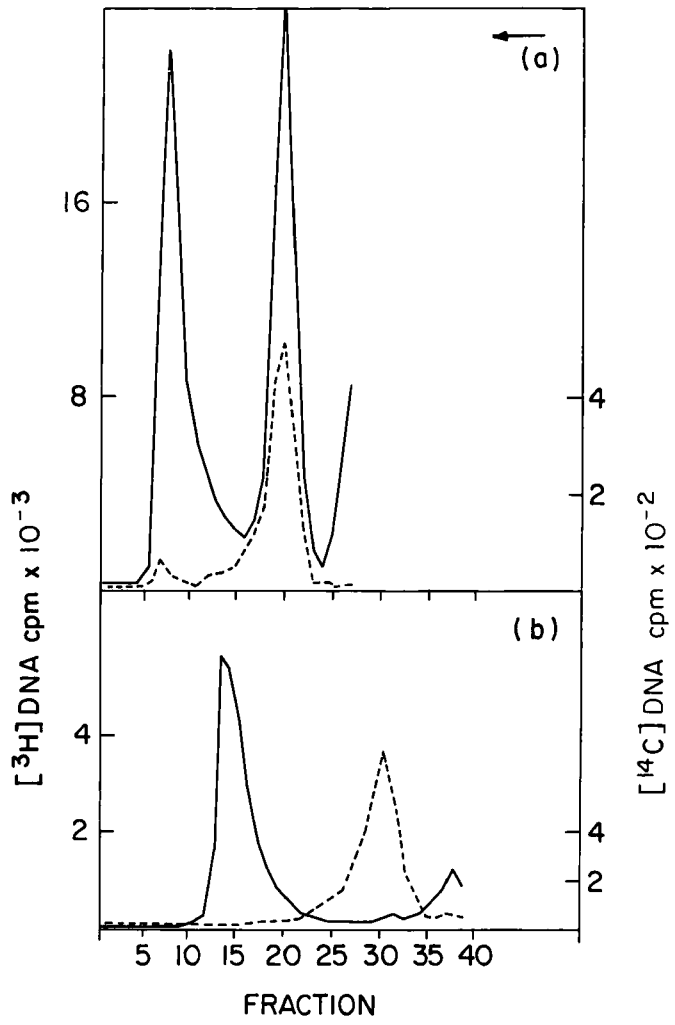

Fig. 3. EthBr-CsCl equilibrium gradients of *C. fasciculata* [^{3}H]-labeled K-DNA networks. Centrifugation conditions: 40,000 rpm, 48 hr, #50 rotor, 20 C, $n_D^{25} = 1.3880$. *C. fasciculata* [^{14}C]-DNA was added as an upper band market (------). (a) gradient of a K-DNA pellet isolated according to the hot-sarkosyl pronase method but not sheared prior to the differential centrifugation. (b) Sheared by passage through a #20 needle at 25 lbs/in^2.

at fairly low centrifugal forces. In order to obtain pure K-DNA uncontaminated with nuclear DNA in the case of a reasonably concentrated lysate, it was found necessary to lower the viscosity of the lysate and wash the pellet by a single resuspension and recentrifugation. The former was accomplished by passage of the lysate through a syringe needle at 25 lbs/in^2. As shown by the EthBr-CsCl profiles in Fig. 3, the pellet obtained at a centrifugal force of 652,880 $g_{av} \times$ min from an unsyringed lysate was contaminated with nuclear DNA (Fig. 4a), whereas the pellet from a syringed lysate (Fig. 3b) consisted entirely of lower band material. There was no effect of the syringing on the position of the lower band material in the EthBr-CsCl gradient.

The position and shape of the K-DNA lower band was not affected by removal of the dye and rebanding the DNA in another EthBr-CsCl gradient by phenol extraction, or by sequential treatment with RNase A + T_1, pronase and phenol.

The standard differential centrifugation isolation technic resulted in the recovery of 15 ± 1.4% (n = 4)† of the total cell DNA as pure K-DNA. This represents a recovery of 62% of the K-DNA of the cell, assuming 24% of the cell DNA is K-DNA.

† The standard deviation and the number of experiments are given.

Light and Electron Microscopy of Isolated K-DNA Networks

Covalently closed K-DNA networks spread on a water hypophase by the method of Freifelder and Kleinschmidt (3) appeared as compact circular structures surrounded with a fringe of minicircle-sized loops. The interior of the structures appeared condensed (Fig. 4). The average area of such freshly isolated networks was 20 μm^2. After further treatment with chloroform-isoamyl alcohol, the networks spread over a large area (63 ± 13 μm^2 [n = 17]) and possessed less condensed material (Fig. 5). These networks, however, still had a ring of highly condensed material near the periphery. It was impossible to visualize the orientation of individual molecules within the network sheet.

Isolated networks could be spread onto glass slides, air-dried, fixed in methanol and stained with Giemsa, and then visualized in the light microscope. As shown in Fig. 6, the networks appeared as sheets of DNA which possessed lips as a result of the two-dimensional constraint imposed on the originally cup-shaped structure. The lips were more darkly

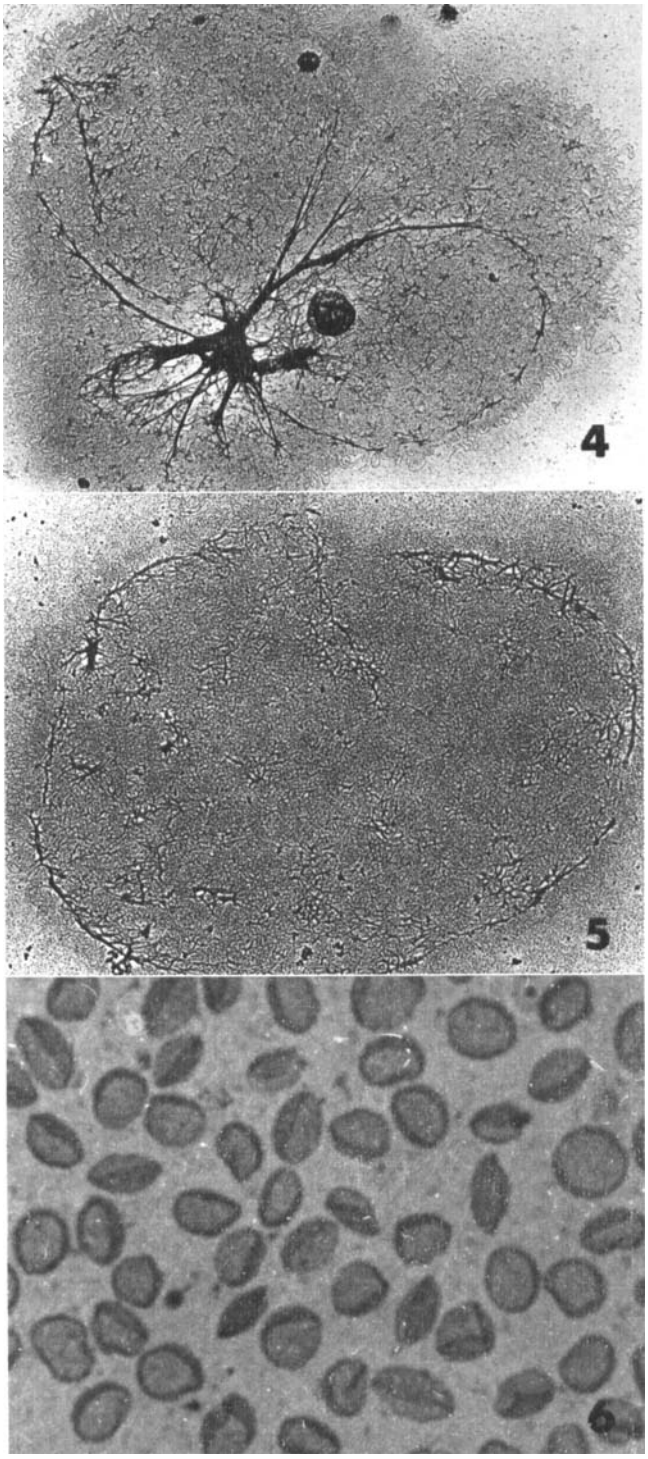

Fig. 4. Electron micrograph of a freshly pelleted K-DNA network spread on a water hypophase. K-DNA networks were obtained as described in Materials and Methods but not subjected to further chloroform-isoamyl alcohol extraction. × 9,700.

Fig. 5. Electron micrograph of *C. fasciculata* K-DNA network after extraction with choloroform-isoamyl alcohol as described in Materials and Methods. × 6,000.

Fig. 6. Light micrographs of Giemsa-stained K-DNA networks. The networks were prepared for light microscopy as described in Materials and Methods. × 1,100.

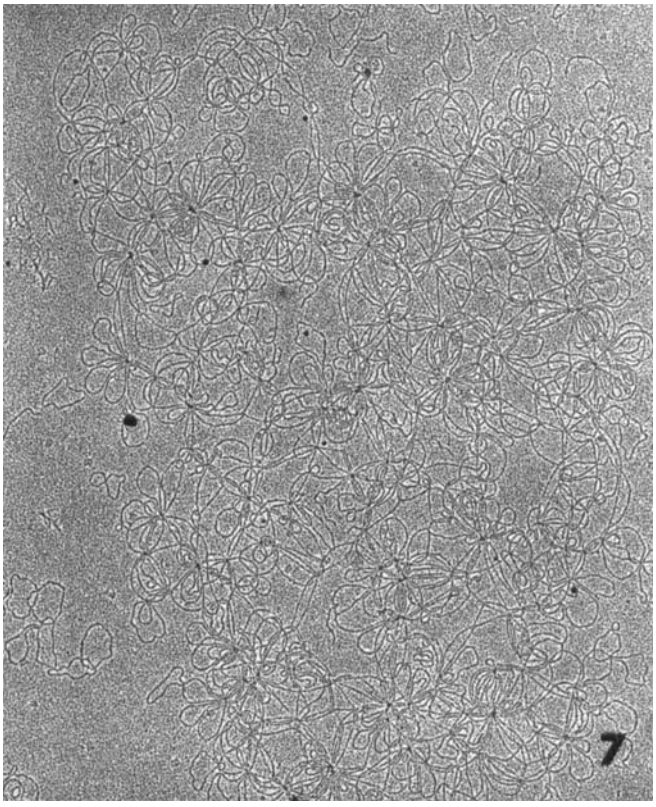

Fig. 7. Electron micrographs of mildly sonicated *C. fasciculata* networks. Note the free minicircles as well as the rosettes made by the interlocked molecules inside of the association. × 20,000.

stained than the interior but this is probably due merely to the double thickness of DNA at that point.

Sedimentation of Covalently Closed Networks

Closed networks sedimented mainly as a single homogeneous peak in both neutral (Fig. 8a) and alkaline (Fig. 8b) sucrose. The $s_{20,w}$ in neutral sucrose, calculated by the equation of Burgi and Hershey (2), was $5.0 \pm 0.5 \times 10^3$ ($n = 7$), whereas the $s_{apparent}$ in alkaline sucrose was 7.4×10^3. The sedimentation coefficient in neutral sucrose was unaffected by extraction of the networks with chloroform-isoamyl alcohol, or by variation in the ionic strength of NaCl in the sucrose gradient from 0.05 to 1.0.

Isolation of Minicircles from Sonicated Networks

As is the case with *L. tarentolae*, mild sonication of isolated closed K-DNA networks broke down the compact network structure and caused a release of minicircles, linear fragments, and small associations of minicircles in which the "rosette" type of organization reported by Renger and Wolstenholme (7) could be seen in (Fig. 7).

The kinetics of the release of closed minicircles from networks by sonication was investigated. Pooled network DNA from several preparations was centrifuged to equilibrium in EthBr-CsCl and the lower band selected. This DNA was originally completely closed circular, but had suffered some breakdown during storage at −20 C for several months. Due to the presence of some intermediate band networks in the selected fractions, there was a breakdown of 47% of the DNA

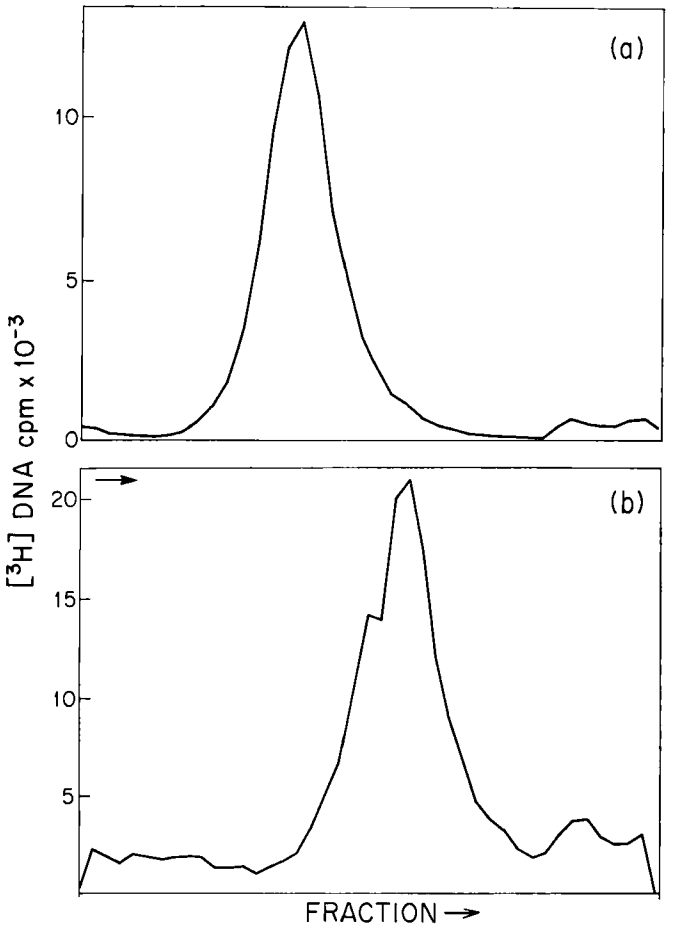

Fig. 8. Band velocity sedimentation of covalently closed *C. fasciculata* [³H]-labeled K-DNA networks. Networks were isolated by the hot-sarkosyl pronase method and subjected to an EthBr-CsCl equilibrium centrifugation. The lower band was collected and the dye removed by dialysis against Dowex 50W resin. Centrifugation conditions: 12,500 rpm SW39 rotor, 10 min, 4C. Three-drop fractions were collected onto Whatman 3MM filter discs which were processed as described in Materials and Methods. (a) 5-20% neutral band velocity sucrose gradient. (b) 5-20% alkaline band velocity sucrose gradient (pH 12.5). The apparent shoulder on the trailing edge of the band in the alkaline gradient was not reproducible and probably is artificial.

in alkaline sucrose. This DNA preparation was sonicated for various times and assayed for the presence of closed monomers by band velocity sedimentation in alkaline sucrose, which separated closed monomeric minicircles from single-stranded linear fragments. The values for the percentage of closed monomers released from closed network DNA are shown in Fig. 9. A maximum of 16% of the closed network DNA could be released in the form of closed monomers. This represents a minimal value for the percentage of K-DNA in the form of minicircles due to the destructive nature of the process.

For the preparative isolation of closed monomers, 5 sec was chosen as the optimal time of sonication. Monomers obtained by this technic were "irreversibly denatured" by exposure to the high pH, as shown by electron microscopy. They could be renatured to typical form I closed circular molecules by the technic of Strider and Warner (12) as described in Materials and Methods.

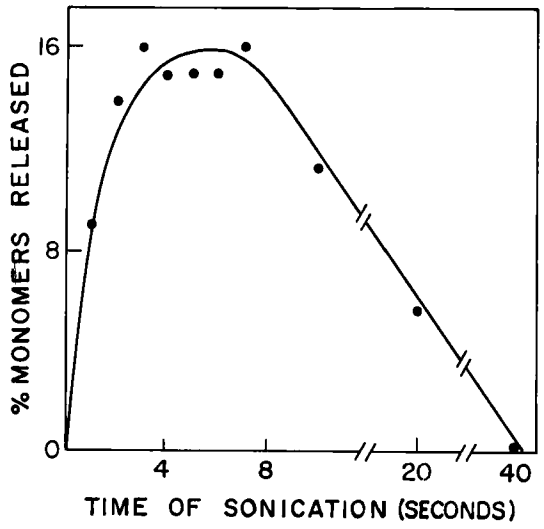

Fig. 9. Percentage of covalently closed minicircles released from closed networks as a function of time of sonication. K-DNA networks labeled with [³H] were subjected to sonication in 0.24 ml quantities as described in Materials and Methods. The % values were corrected for the original % of alkaline-unstable K-DNA in the particular network preparation used. Centrifugation conditions: 60,000 rpm, SW 65 rotor, 3 hr, 4 C. Three drop fractions were collected from the top onto Whatman 3MM filter discs which were processed as described in Materials and Methods.

Buoyant Properties of Minicircles in CsCl

Covalently closed monomeric minicircles, after renaturation by the technique of Strider & Warner (12), had the same buoyant density in one analytical CsCl equilibrium gradient as intact networks, 1.703 g/cc. Networks gave an average value of 1.703 ± 0.001 (n = 69) g/cc. Nuclear DNA from *C. fasciculata* had a buoyant density of 1.716 ± 0.001 (n = 15) g/cc.

Contour Length and Molecular Weight of Minicircles

The average contour length of *C. fasciculata* minicircles co-spread with øX RF II molecules was measured. As shown in Table 2, the ratio between the weight average contour lengths of *Crithidia* minicircles and øX RF II molecules was 0.44, yielding a molecular weight for the minicircles of 1.50 × 10^6 daltons. The weight average contour length of *Crithidia* minicircles was 0.79 μm. This value is 2.72 × that reported for *L. tarentolae* K-DNA minicircles by Simpson and Da Silva (8).

TABLE 2. *Measurement of* C. fasciculata *K-DNA minicircles co-spread with øX174 RF II molecules.*

Experiment	L_w (cm)*	Ratio Cf/øX	Calculated molecular weight	Equivalent length (μm)	Actual length (μm)†
C. fasciculata‡	5.6 ± 0.4 (n = 34)	0.44	1.49 × 10^6	0.79	0.88
øX RF II‡	12.6 ± 0.9 (n = 27)				

* Weight average contour length (μm).
† Compared to carbon replica of a diffraction grating.
‡ Hypophase was water.

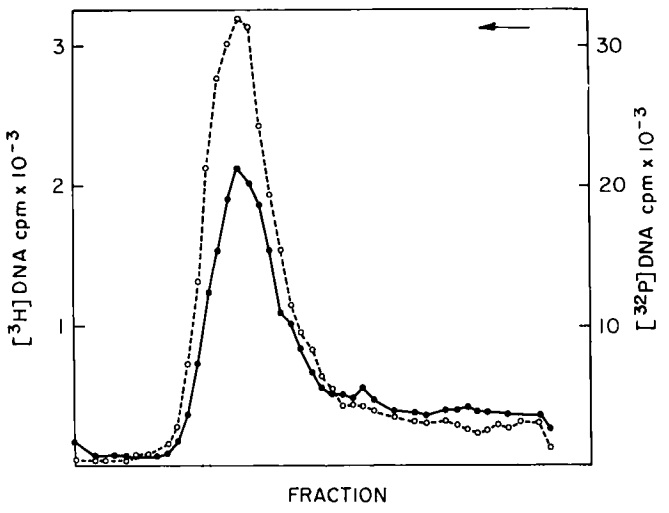

Fig. **10**. EthBr-CsCl equilibrium centrifugation of *C. fasciculata* [^{3}H]-labeled covalently closed monomers. Three-drop fractions were collected from the bottom onto Whatman 3MM filter discs and processed as described in Materials and Methods. *L. tarentolae* [^{32}P]-labeled, covalently closed minicircles (o-o-o-o-) were added as an internal marker in the gradient to calculate the superhelix density. Centrifugation conditions: 40,000 rpm, #50 rotor, 72 hr, 20 C, 200 μg/ml EthBr.

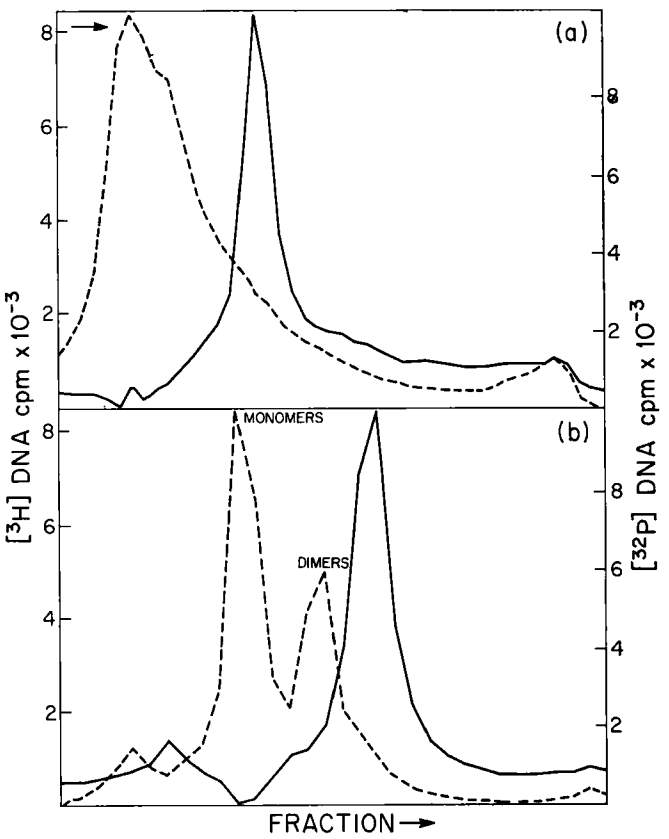

Fig. **11**. Band velocity sedimentation of [^{3}H]-labeled *C. fasciculata* covalently closed monomers in 5-20% linear sucrose gradients. (a) Neutral band velocity gradient. (b) Alkaline band velocity gradient. [^{32}P]-labeled *L. tarentolae* covalently closed monomers and closed dimers were added to both gradients as markers (----). There was no resolution of monomers and dimers in the neutral gradient. Four-drop fractions were collected from the top onto Whatman 3MM filter discs which were processed and counted as described in Materials and Methods. Centrifugation conditions: 25,000 rpm, SW65 rotor, 4 C, 16 hr.

Superhelix Density of Covalently Closed Minicircles

Covalently closed [^{3}H]-labeled minicircles isolated from sonicated networks as described above were co-run with [^{32}P]-labeled *L. tarentolae* closed minicircles in an EthBr-CsCl equilibrium gradient. As shown in Fig. 10, the *Crithidia* minicircle band was coincident with the *Leishmania* minicircle band, implying an identical superhelix density in view of the previously demonstrated equality of the buoyant densities in neutral CsCl.

Wesley and Simpson (16) previously calculated the superhelix density of *L. tarentolae* minicircles to be 0.011 ± 0.004 turns per 10 base pairs.

Sedimentation Properties of Minicircles

The banding patterns of covalently closed monomeric *C. fasciculata* minicircles in neutral and alkaline sucrose gradients are presented in Figs. 11a and 11b. In both cases internal markers of [^{32}P]-labeled *L. tarentolae* closed monomeric and dimeric minicircles were used. The $s_{20,w}$ of *C. fasciculata* closed monomers in neutral sucrose was 27, whereas the s_{obs} in alkaline sucrose was 33.

DISCUSSION

The network isolation procedure described in this paper has certain advantages over that of Laurent et al. (5). The differential centrifugation step is rapid and provides K-DNA uncontaminated with nuclear DNA with a yield of approximately 62% of the total cell K-DNA. If an EthBr-CsCl equilibrium gradient step is added, then one can extend the time of differential centrifugation and recover all of the K-DNA of the cell. Furthermore, the use of 0.5 M EDTA allowed the recovery of covalently closed network DNA. Similar procedures were found to work for *L. tarentolae* (9) and it is probable that this method can be applied to all the hemoflagellate species. The isolation technic has been successfully used in a study of the replication of K-DNA of *Crithidia* and *Leishmania* (11).

The selective recovery of K-DNA networks at low centrifugal fields was found to be due to the extremely large sedimentation coefficients of these structures. Another feature of *C. fasciculata* K-DNA that proved useful in developing an isolation technic was its relative stability to shear forces due to the compactness of packing of the DNA and the many topological links holding together the structure. This property permitted a selective fragmentation of nuclear DNA in the lysate by controlled shearing.

Both Laurent et al. (5) and Renger and Wolstenholme (7) have shown that the K-DNA networks of *C. luciliae* and *C. acanthocephali* were unaffected by treatment with RNase and pronase at least in terms of morphology in the light and electron microscopes. In the case of *C. fasciculata* we have shown that the covalently closed circular nature of the networks from nondividing cells is also unaffected by RNase, pronase and alkaline pH. This implies that the closed network of *C. fasciculata* is not held together by RNA or protein linkers, and therefore can be distinguished from the "folded chromosome" of *Escherichia coli* (18), which is sensitive to RNase, ionic detergents and alkaline pH.

When we examined closed networks from *C. fasciculata* in the electron microscope we were not able to see the rosette type of organization reported by Renger and Wolstenholme (7) in the K-DNA of *C. acanthocephali*. This difference may either be due to the fact that the *C. fasciculata* networks were definitely covalently closed or to variations in spreading tech-

nics. We in fact noticed a progressive change in the compactness of the networks after additional deproteinization with chloroform, but we only consistently saw the rosette formation after partial disruption of the network by sonication.

The recovery of covalently closed networks from *C. fasciculata* allowed the development of a technic to isolate closed minicircles. This technic was based on that of Wesley and Simpson (15) and involved the disruption of networks by sonication and the recovery of closed monomeric minicircles by alkaline band velocity sedimentation. In the case of *Crithidia*, however, the denatured minicircles did not "snap back" on lowering the pH but remained as "irreversibly" denatured molecules. These were renatured to form I circles by using Strider and Warner's technic (12).

All attempts to recover a class of "free" or loosely bound minicircles by the "Alkaline lysis method II" of Wesley and Simpson (15) failed. This negative result may imply an absence of this class of minicircles in *C. fasciculata* K-DNA.

The size of the K-DNA minicircle seems to be species-specific. For example, minicircles from the monogenetic parasite, *C. fasciculata*, are 2.7 × the contour length of minicircles from the digenetic parasite, *L. tarentolae*. *C. fasciculata* minicircles are identical to those isolated from another species in the same genus, *C. acanthocephali*, and are 2.0× and 1.8× those isolated from the stecorarian trypanosomes, *T. lewisi* and *T. cruzi* respectively. In general, there seems to be a restriction in the amount of genetic information in the minicircle component of the K-DNA [assuming that the genetic complexity is equal to the contour length as is the case with *L. tarentolae* (17)], as the hemoflagellate species evolved into highly biphasic parasites which underwent a cyclical development in 2 hosts. More information on the K-DNA of other genera will be necessary to make evolutionary sense out of this trend.

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