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Isolation of Kinetoplast-Mitochondrial Complexes from *Leishmania tarentolae**

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SYNOPSIS. Kinetoplast-mitochondrial complexes were liberated from *Leishmania tarentolae* by passing hypotonically swollen cells in dilute Tris-EDTA through a needle at 100 lbs/in^2. The complexes formed an equilibrium band by flotation in Renografin gradients at a density of 1.22 g/ml. The band was monitored by several mitochondrial and kinetoplastic markers: [^{3}H]DNA, succinate-cytochrome c reductase activity, [^{59}Fe]hemoproteins and optical density at 600 nm. Electron microscopy showed that the sole component of the 1.22 g/ml band was the kinetoplast-mitochondrial complex.

Index Key Words: *Leishmania tarentolae*; kinetoplast-mitochondrial complex; kinetoplast DNA.

THE kinetoplast is a specialized area of the mitochondrion of protozoa belonging to the order Kinetoplastida, an area which contains the mitochondrial or kinetoplast DNA (K-DNA). Due to the large amount of DNA, the kinetoplast can be seen in the light microscope as a dark granule after staining with Giemsa. Although no definitive study has yet been carried out to prove the existence of a single mitochondrion per cell, there is suggestive evidence that this is the case in all the protozoa of this order (20).

The existence of a single mitochondrion consisting of several convoluted tubular extensions attached to the disc-like region containing the kinetoplast DNA makes the isolation of the intact kinetoplast-mitochondrial complex a formidable problem. Furthermore, most kinetoplastid flagellates, especially *Crithidia* and *Leishmania*, are relatively resistant to cell breakage by standard means (19, 20), probably as a result of the structural integrity conferred upon the cells by the network of microtubules found just beneath the cell membrane (1, 20).

Simpson (19) showed that hypotonic treatment of *Leishmania tarentolae* promastigotes caused possibly the entire kinetoplast-mitochondrial complex to assume a spherical shape, and that these swollen vesicles could be released from the cells by the application of mild shearing forces in the presence of the chelating agent, EDTA. Simpson and da Silva (22) employed this rupture technic followed by differential centrifugation and DNase treatment to isolate fractions that were highly enriched for K-DNA, but which were grossly contaminated with cell ghosts, flagella, membrane fragments and some intact cells. The released kinetoplast-mitochondrial vesicles contracted upon return to isotonic sucrose, and the inner membrane was undamaged by the hypotonic exposure as evidenced by the impermeability of the structures to exogeneous DNase I.

Several other workers also have reported the use of hypotonic media in attempts to release intact mitochondria from hemoflagellates. Dubuy et al. (3) previously showed that the kinetoplast remained intact and the nucleus lysed when *L. enriettii* promastigotes were ruptured in distilled water, and Laurent & Steinert (10) used a modification of the method of Simpson (19) to release apparently intact kinetoplasts from *Trypanosoma mega*.

This report describes a procedure employing a hypotonic lysis technic for obtaining a pure kinetoplast-mitochondrial fraction from *L. tarentolae* by isopycnic flotation of the sample through a Renografin density gradient. This fraction has been partially characterized in terms of DNA content, a mitochondrial inner membrane marker enzyme, and the presence of [^{59}Fe] labeled hemoproteins, and by both light and electron microscopy.

MATERIALS AND METHODS

Cells

A clonal strain of *L. tarentolae* (Lt-C-1) was maintained by subculture in BHI medium (Brain Heart Infusion, Difco Laboratories, Detroit, Michigan) as previously described (21). Experimental cultures were grown at 27 C in 200, 400, or 1000 ml quantities in rotated bottles, or in 3.5-4.0 liter quantities in a fermentator (Fermentation Design Inc., Allentown, Pa.). Cells were harvested in late log or early stationary phase after 3-4 days growth by centrifugation at 1500 *g* for 10 min. They were washed once with cold 0.15 M NaCl, 0.02 M glucose, 0.02 M phosphate buffer, pH 7.9.

Isolation of kinetoplast fraction

The cells were resuspended in 2 mM Tris-HCl, 2 mM EDTA, pH 7.9 at 4 C at a concentration of 1.2×10^9 cells per ml. This cell suspension was passed through a #26 G syringe needle at 100 psi. The procedure ruptured close to 100% of the cells and freed the swollen kinetoplast-mitochondrial complexes from the cells, possibly as intact structures. A concentrated sucrose solution was immediately added to the lysate to a final concentration of 0.25 M to minimize the osmotic damage caused by the hypotonic solution. The swelling of the cells and the extent of rupture were monitored routinely by phase contrast microscopy. The lysate was centrifuged at 16,000 × *g* for 10 min at 4 C and the pellet resuspended in 0.25 M sucrose, 0.02 M Tris, pH 7.9, 3 mM $MgSO_4$ and treated with DNase I (10 μg/ml) for 1 hr at 0-4 C. The treatment was terminated by the addition of 2 to 3 volumes of cold 0.25 M sucrose, 0.02 M Tris, HCl, 2 mM EDTA, pH 7.9 (STE) and the lysate was centrifuged at 16,000 *g* for 10 min. The pellet was then washed once with STE and resuspended by vortexing in cold 60% (w/v) Renografin (Reno-M 60, Meglumine Diatrizoate, Squibb, New York) containing 0.25 M sucrose and 0.1 mM EDTA. Four ml of the suspension were placed at the bottom of a 34 ml linear 20-40% Renografin gradient that also contained 0.25 M sucrose, 0.02 M Tris HCl, 0.1 mM EDTA, pH 7.9. The dilution of the pellet with 60% Renografin must be sufficient to allow the suspension to sink beneath the 40% Renografin-sucrose. We have found recently that a linear 20-35% Renografin gradient yields a cleaner separation of the

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bands and is more convenient for introducing the homogenate in 60% Renografin underneath the gradient due to the larger density difference.

The gradients were centrifuged for 2 hr at 82,500 g_{av} in a Beckman SW27 rotor at 4 C and were fractionated either from the top with an Isco Model D density gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebraska) by pumping 60% Renografin through the bottom, or from the bottom by puncturing the tube and collecting drops.

The distribution pattern of light scattering material within the gradient was monitored by following the optical density at 600 nm using a flow cell with an optical path of 0.5 cm.

Enzyme assays

The succinate-cytochrome c reductase activity of the fractions was measured spectrophotometrically by following the reduction of cytochrome c at 550 nm described by Sottocasa et al. (23).

Catalase was measured with a Clark oxygen electrode according to the method of Ganschow and Schimke (6) using bovine liver catalase (Cal Biochem., 47,000 IU/mg) as a standard. This assay was run on *L. tarentolae* cell homogenates (up to 1 mg protein/ml) obtained from sonication of cells in phosphate buffer or from hypotonic syringe lysis as described above.

Oxidase activities with several substrates were determined polarographically using the reaction mixture described by Evans & Brown (4) and a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Protein was determined by the method of Lowry et al. (11) using bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Mo.) as a standard.

Isolation of DNA

Kinetoplast-mitochondrial complexes were recovered for DNA extraction by pooling the 1.22 g/ml bands from several Renografin gradients, diluting with 2-3 volumes of STE and centrifuging at 16,000 *g* for 10 min. The pellet was then washed twice with STE and resuspended in 0.15 M NaCl, 0.1 M EDTA, pH 8.0. Pronase (Grade B, Cal Biochem., pre-digested for 30 min at 37 C) was added to a final concentration of 2.5 mg/ml and Na sarkosinate (Geigy Chemical Co., Ardsley, New York) to a final concentration of 3% (w/v). The sample was left at 60 C for 4-5 hr, after which is was dialyzed against 0.15 M NaCl, 0.015 M Na citrate, pH 7.0 (SSC). The solution was deproteinized by shaking with chloroform-isoamyl alcohol (25:1, v/v) and then was redialyzed against SSC.

Analytical CsCl equilibrium centrifugation was carried out in a Spinco model E ultracentrifuge essentially as described by Meselson et al. (13). Tracings of the films were made with a Joyce-Lobel densitometer.

Electron microscopy

The appropriate band from the Renografin gradient was diluted with 3-4 volumes of cold STE and the suspension centrifuged at 16,000 *g* for 10 min. The pellet was washed once with STE and was then scooped up with a weighing spatula and thoroughly resuspended with the round end of a glass rod in a drop of buffer solution at 0-4 C [0.135 M Na phosphate buffer, pH 7.4, 0.04 M sucrose, 0.015 M NaCl (9)]. All subsequent fixations and washes were made in this buffer. The resuspended pellet was transferred as a small puddle to a glass slide at 30 C and mixed with a drop of 3% (w/v) agar made with the above buffer solution. After cooling the agar-pellet suspension, it was sliced with a razor blade into 0.5 mm square pieces. Twenty such pieces were treated in sequence as follows: (a) Two hr fixation in 100 ml of 2% (v/v) glutaraldehyde, which was prepared from ampules of glutaraldehyde sealed over inert gas (Ladd Research Industries, Inc., Burlington, Vermont); (b) Two hr rinse in 0 C buffer; (c) Post fixation for 2 hr in 100 ml of 1% (w/v) OsO_4 (Ventron Alfa Products, Beverly, Massachusetts); (d) Two hr rinse in 0 C buffer; (e) Post-staining for 2 hr at 24 C in 200 ml of 0.5% (w/v) uranyl acetate (J. T. Baker Chem. Corp., Phillipsburg, New Jersey) made with distilled water 12 hr before use and filtered through a fine glass filter just prior to use; (f) Dehydration along an acetone gradient at 25 C consisting of 30 min in each 30, 50, 70, 95% acetone, and 1 hr with 4 changes in 100% acetone; (g) Infiltration with Vestopal (17) for 12 hr in each of the following mixtures: 1:3 (v/v), Vestopal-acetone; 1:1 (v/v), Vestopal-acetone; 3:1 (v/v), Vestopal-acetone, and 2 days in pure Vestopal. All Vestopal infiltrations occurred while the tissue was being tumbled in closed 8 ml vials placed on a rotating wheel. The infiltrated agar pieces were transferred to gelatin capsules and polymerization allowed to proceed for 7 days at 60 C. The blocks were sectioned on an LKB 4800 Ultratome with glass knives. Sections were stained for 1 hr in a 60 C saturated solution of uranyl acetate (8 *g*/100 ml triple distilled H_2O) prepared 24 hr before use and filtered through fine glass filters immediately before use, and then with lead citrate (16). Sections were picked up on one-hole slot grids covered with Formvar (Monsanto, Springfield, Massachusetts), carbon coated, and examined in a Hitachi HU 11E-1 at 80 KV accelerating voltage with a 200 μm condenser aperture and a 50 μm objective aperture.

In certain experiments, continuous ribbons, comprised of 20-30 attached serial sections each about 50 nm thick, were cut and collected intact on one-hole slot grids according to the method of Kretzer (9).

Stereology of electron micrographs

A grid of 17 equidistant parallel lines (1 cm apart, 13 cm long) was produced on a piece of clear cellulose acetate. This grid was randomly placed over randomly chosen micrographs. The number of intersections of the lines with the kinetoplast-mitochondrial complex peripheral membrane and the number of intersections with the arc of the K-DNA networks projected onto the periphery of the complex were recorded (Table 5). The data were used for a stereological analysis (27) of the average relative surface area of the kinetoplast-mitochondrial complex contiguous to K-DNA.

Distribution of [^3H] DNA and [^{59}Fe] hemoproteins in the Renografin gradient

This was measured by spotting samples onto Whatman #3 MM filter discs, which were processed through cold 5% (w/v) trichloroacetic acid (TCA), 70% ethanol, 95% ethanol and absolute ether, and counted in a toluene-based scintillation fluid (Omnifluor, New England Nuclear Co.) in a Beckman Scintillation Counter. [^{59}Fe] hemin was prepared chemically from $^{59}FeCl_2$ (ICN) and protophorphyrin IX according to the method described by Falk (5). The initial specific activity of the final product was 7×10^3 cpm/μg. Purity was verified by thin layer chromatography as described by Falk (5).

RESULTS

Cell rupture

Cells were made more susceptible to shearing forces by exposure to hypotonic medium. The tonicity of the medium was

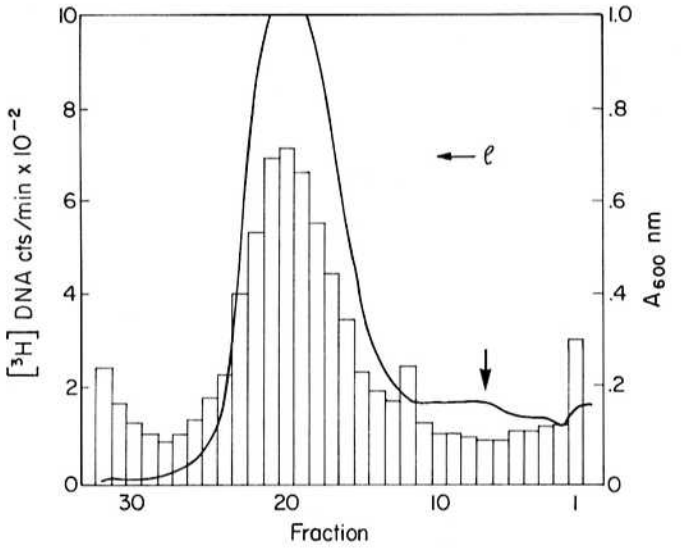

Fig. 1. Isopycnic distribution of a hypotonic lysate of *L. tarentolae* in terms of [^{3}H] DNA and optical density at 600 nm in a linear 20-40% Renografin gradient. The sample, after treatment with DNase I, was resuspended in 60% Renografin-sucrose and placed beneath the gradient. Centrifugation was for 2 hr at 82,500 g_{av} in the SW 27 rotor at 5 C. The gradient was fractionated from the top. The solid line represents the $A_{600_{nm}}$ and the bars represent the [^{3}H] DNA cpm. The position of the lighter band is indicated by an arrow.

critical and probably differs for each of the different hemoflagellate species. In the case of *L. tarentolae*, a lysis medium of 0.02 M Tris HCl, 0.002 M EDTA, pH 7.9 at 4 C allowed 75% of the cells to rupture on a single passage through a #26 needle at 100 psi, but the results were variable, and it was necessary to wash the cells with the medium several times before syringing. A tenfold decrease in the concentration of Tris HCl caused virtually all of the cells to be ruptured by a single passage through the needle without any prior washing. This method, as described in Methods, was used in all of the following experiments.

Nuclei released from ruptured cells lysed rapidly, as seen by phase contrast microscopy, and hence were not recovered intact in any gradient separation.

Comparison of sucrose and Renografin separations

Renografin was chosen as the density gradient material after testing sucrose isopycnic gradient separations. The kinetoplast band from sucrose gradients, which banded at a density of 1.22 g/ml, was extensively contaminated with cell membrane ghosts, flagella, and other material, whereas the kinetoplast band from Renografin gradients (Fig. 1) had little contamination, as shown below.

Flotation was far superior to sedimentation in achieving isopycnic separation in Renografin gradients. Sedimentation consistantly resulted in the trapping of a large percentage of kinetoplasts in the upper band and a corresponding reduction in recovery of kinetoplasts in the lower band.

The lighter band was quite variable in relative amounts in different preparations, possibly due to uncontrolled physiological variations in the cells or to variations in the extent of cell rupture. The extent of variability in the lighter band is best illustrated by comparing the experiments shown in Figs. 1 and 2(b).

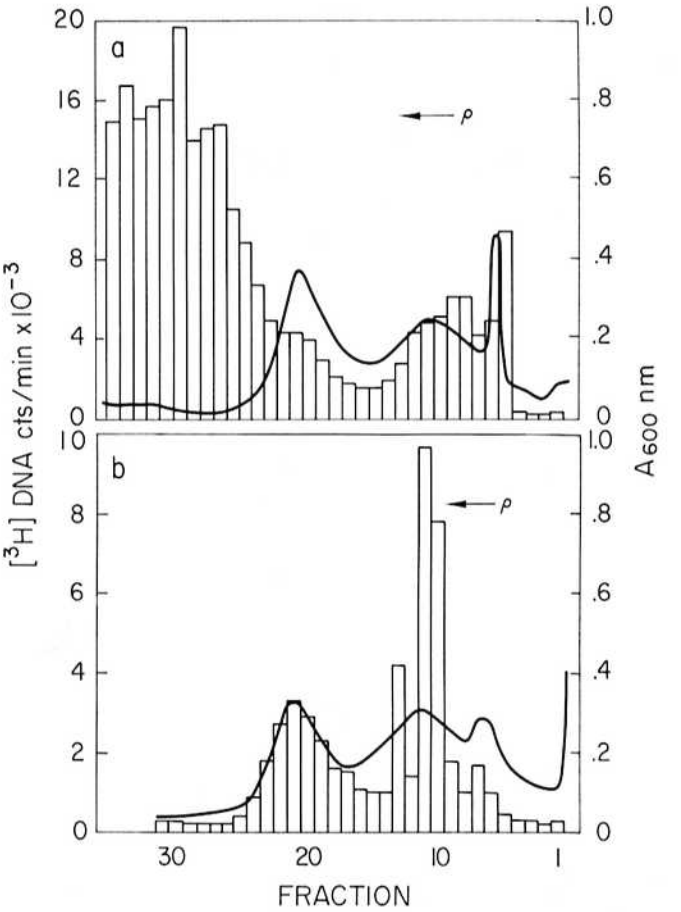

Fig. 2. Comparison of the isopycnic distribution of a hypotonic lysate that was treated with DNase I prior to running, with a lysate that was not treated. The cell lysate sample was divided into two portions, one of which (b) was treated with DNase I as described in Methods and the other of which (a) was not treated. Centrifugation conditions were as in Fig. 1. (a) Untreated sample; (b) DNase-treated sample.

DNase treatment

Elimination of nuclear DNA by pretreatment of the cell lysate with DNase I prior to isopycnic separation in Renografin was essential to prevent agglutination artifacts and to achieve optimal purity of the kinetoplast band. A comparison of isopycnic Renografin separations of untreated and DNase-treated samples of the same cell lysate is shown in Figs. 2(a) and (b). Again, the [^{3}H] DNA and the optical density at 600 nm were monitored throughout the gradients. Notwithstanding the similar optical density profiles of the two gradients, the lower band of the untreated sample in Fig. 2(a) was grossly contaminated with intact cells as shown by phase contrast microscopy. This contamination was apparently due to the large amount of DNA in the lower part of the gradient, which was not present in the gradient of Fig. 2(b).

Evidence for the nuclear origin of the DNA present in the lower portion of Renografin gradients of non-DNase pre-treated cell lysates was obtained by extracting the DNA and performing analytical CsCl equilibrium centrifugation. As shown in the densitometer tracings of Fig. 3(a) and 3(b), nuclear DNA at 1.716 g/ml (22) was the major DNA component of the 1.22 g/ml band from the gradient in Fig. 2(a), whereas the characteristically narrow band of high molecular weight K-DNA net-

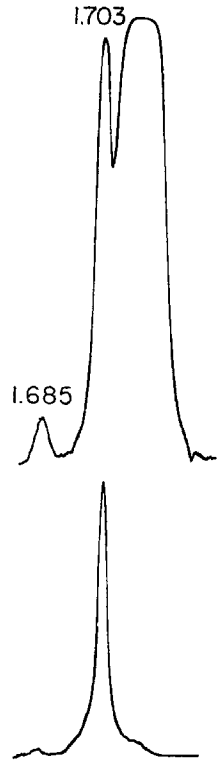

Fig. 3. Densitometer tracings of UV films of analytical CsCl equilibrium gradients of DNA isolated from the 1.22 g/ml bands of the Renografin gradients shown in Fig. 2. (a) DNA isolated from the 1.22 g/ml band of the gradient in Fig. 2(a). The gradient is overloaded with nuclear DNA in order to visualize the K-DNA present. The reference DNA is *Tetrahymena pyriformis* (1.685 g/ml, [2]); (b) DNA isolated from the 1.22 g/ml band of the gradient in Fig. 2(b).

works was the sole component of the 1.22 g/ml band from the gradient in Fig. 2(b).

Use of [^{59}Fe] hemoprotein, succinate-cytochrome c reductase, and [^{3}H] DNA as markers in the same Renografin gradient

Several markers were followed in the same gradient in order to correlate the distribution of K-DNA and mitochondrial membranes (Fig. 4). [^{59}Fe] hemoprotein and succinate-cytochrome c reductase were used as markers for the mitochondrial inner membrane, and [^{3}H] DNA was used as a marker for K-DNA. The optical density at 600 nm was used to follow the total distribution of light-scattering material.

The upper band in this experiment (Fig. 4) was relatively large and consisted of small vesicles, membrane fragments and trapped kinetoplast-mitochondrial complexes. The lower band, however, was composed almost exclusively of kinetoplast-mitochondrial complexes, as monitored by phase contrast microscopy. The complexes formed an equilibrium band at 1.22 g/ml in this Renografin gradient, a density identical to that previously observed in sucrose gradients.

The distribution of [^{3}H] DNA in the lower band paralleled that of succinate-cytochrome c reductase activity and [^{59}Fe] hemoprotein, confirming that kinetoplast-mitochondrial complexes containing both K-DNA and mitochondrial enzymes were localized in this position.

Because of the contamination of the upper band with kinetoplast-mitochondrial complexes in this experiment, no evidence could be obtained by the use of markers regarding the presence

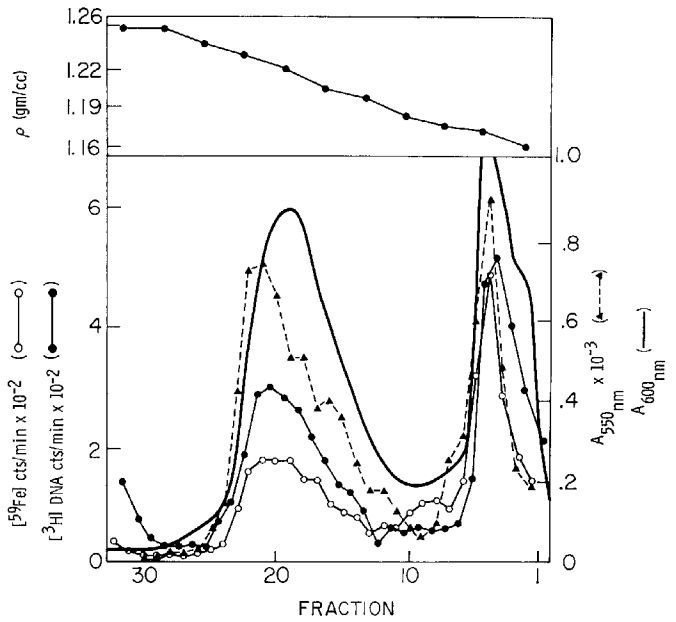

Fig. 4. Isopycnic distribution of a hypotonic lysate of *L. tarentolae* in terms of [^{3}H] DNA (●——●), [^{59}Fe] hemoprotein (○——○), optical density at 600 nm (——), and succinate-cytochrome c reductase activity (▲---▲) in a 20-40% linear Renografin gradient. The Renografin density distribution as measured with a pycnometer is also included. The sample was pretreated with DNase I as in Methods, and was resuspended in 60% Renografin sucrose and placed beneath the gradient. Centrifugation conditions were as in Fig. 1.

or absence of a light density band containing mitochondrial fragments devoid of kinetoplast DNA.

The use of [^{59}Fe] hemoprotein as a marker for the mitochondrial inner membrane was justified by negative results for the presence of catalase in these cells. No measurable catalase activity was observed with *L. tarentolae* cell homogenates even when the homogenate was present at a concentration of 1 mg protein/ml.

Oxidase activity of kinetoplast-mitochondrial complex fraction

The kinetoplast-mitochondrial complex fraction isolated as in Fig. 1 was assayed for oxidase activity using succinate, DL-α-glycerophosphate and NADH as substrates. The succinoxidase specific activity was increased fourfold over that of the total cell homogenate (Table 1). The activity observed with NADH as substrate indicated that a small percentage of the kinetoplast-mitochondrial complexes had damaged inner membranes that were permeable to NADH.

TABLE 1. *Oxidase activity of 1.22 g/ml Renografin band and unfractionated cell homogenate.*

Substrate	Oxidase activity* 1.22 g/ml Renografin band	Cell homogenate
Succinate (10 mM)	349	85
DL-α-glycerophosphate (10 mM)	116	—
NADH (2 mM)	70	—

* nmol oxygen/hour/mg protein.

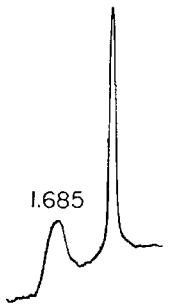

Fig. **5**. Densitometer tracing of UV film of analytical CsCl equilibrium gradient of DNA isolated from a 1.22 g/ml Renografin band which had been DNase-treated after removal from the Renografin gradient. The washed kinetoplast-mitochondrial complex fraction was treated with DNase I (100 μg/ml, 30 min, 4 C) prior to DNA isolation. The reference DNA is *Tetrahymena pyriformis* (1.685 g/ml).

Effect of DNase treatment on kinetoplast-mitochondrial complex fraction

DNase I treatment of the 1.22 g/ml band (100 μg/ml, 30 min, 4 C) caused a decrease of 20-25% in the amount of K-DNA recoverable from the fraction, but had no effect on the narrow CsCl equilibrium profile of the remaining K-DNA (Fig. 5). This implied that 75-80% of the complexes isolated in the Renografin gradient possessed intact inner membranes that were impermeable to exogenous DNase.

Electron microscopy of kinetoplast-mitochondrial complex fraction

Approximately 300 overlapping pictures forming 16 montages were taken to analyze the purity of the 1.22 g/ml Renografin fraction. Two ribbons at right angles to each other were always cut from the same block when testing for homogeneity.

TABLE 2. *Proportion of kinetoplast-mitochondrial complexes in the 1.22 g/ml Renografin fraction that contain K-DNA in electron micrographs of thin sections.*

Negative number	Number of kinetoplast-mitochondrial complexes	Number of complexes containing K-DNA	Percentage of complexes with K-DNA
3416	48	12	25
3413	44	12	27
3412	40	10	25
3417	52	14	27
3534	46	11	24
3554	44	11	25
3577	42	11	26
3582	51	14	27
3552	53	14	26
3539	46	12	26
3420	37	9	24
TOTAL	503	130	
AVERAGE ..... [SD = ± 1%, n (No. of trials) = 11]			

TABLE 3. *Proportion of kinetoplast-mitochondrial complexes in the lighter Renografin fraction that contain K-DNA in electron micrographs of thin sections.*

Negative number	Number of kinetoplast-mitochondrial complexes	Number of complexes containing K-DNA	Percentage of complexes with K-DNA
3444	79	12	15
3447	98	16	16
3463	62	10	16
3449	62	9	15
3452	65	10	15
3455	78	12	15
3457	56	9	16
TOTAL	500	78	
AVERAGE ..... [SD = ± 1%, n (No. of trials) =7]			

The resuspended pellet of the 1.22 g/ml fraction proved completely homogeneous with regard to orientation of the sections within the block (Figs. 6 & 7).

Furthermore, the homogeneous character of the pellet was quantitatively analyzed by weighing an 8-picture montage taken at 3,500 × electron magnification and photographically enlarged 2.5 ×; this montage weighed 25.1 g. All non-mitochondrial structures were cut out and weighed. The weight of such contamination was 0.31 g, or 1.2% of the overall montage weight. This represents the maximal contamination of the pellet with non-mitochondrial structures.

The proportion of kinetoplast-mitochondrial complexes in randomly chosen thin sections of the 1.22 g/ml Renografin band that contained K-DNA was measured from 11 randomly chosen micrographs which were taken at 13,000 × magnification and enlarged 3 × photographically, two of which are shown in Figs. 6, 7. The data (Table 2) indicate that an average of 26%, [SD = ± 1%, n (no. of trials) = 11], of the complexes in any given thin section possess K-DNA. This is in contrast to the values obtained from seven micrographs of thin sections of the upper Renografin band, in which an average of 16% ± 1% (n = 7) of the complexes had K-DNA in any given thin section (Table 3).

In an attempt to prove unequivocally that all of the kinetoplast-mitochondrial complexes in the 1.22 g/ml Renografin band contain K-DNA, a preliminary analysis of serial sections was performed. Twenty kinetoplast-mitochondrial complexes were chosen at random and the organelles were followed in successive sections as far as possible. In each case, the K-DNA was apparent in a total span of 3-5 successive sections (Table 4), implying a height of the K-DNA network of 4 × 50 nm or 200 nm. In 2 cases (Table 4, F, J), kinetoplast-mitochondrial complexes were completely traversed in serial sections. In both cases 17 sections were required, implying a thickness of 1,000 nm for the entire spherical-shaped complex. Difficulties were encountered in traversing more complexes completely due to the dense packing of structures in the pellet employed for serial sections. However, the serial section data (Table 4) is suggestive that most, if not all, of the complexes in the 1.22 g/ml band contain K-DNA, but that the probability of sectioning the K-DNA of any one complex

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Fig. **6**. Electron micrograph of a thin-section from a resuspended pellet of the 1.22 g/ml band from a Renografin gradient. Note its homogeneous nature, the K-DNA (→), the various matrix densities from dense (D) to empty (E), and the vesiculate, bloated cristae (V). × 23,000.