

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/in². 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 kinetoplasmic markers: [³H]DNA, succinate-cytochrome c reductase activity, [⁵⁹Fe]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 exogenous 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 [⁵⁹Fe] 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 $16,000 \times g$ for 10 min at 4 C and the pellet resuspended in 0.25 M sucrose, 0.02 M Tris, pH 7.9, 3 mM MgSO₄ 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 it 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) OsO₄ (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 H₂O) 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 μ m condenser aperture and a 50 μ m 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 [⁵⁹Fe] 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. [⁵⁹Fe] hemin was prepared chemically from ⁵⁹FeCl₂ (ICN) and protophorphyrin IX according to the method described by Falk (5). The initial specific activity of the final product was 7 × 10³ cpm/ μ g. 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

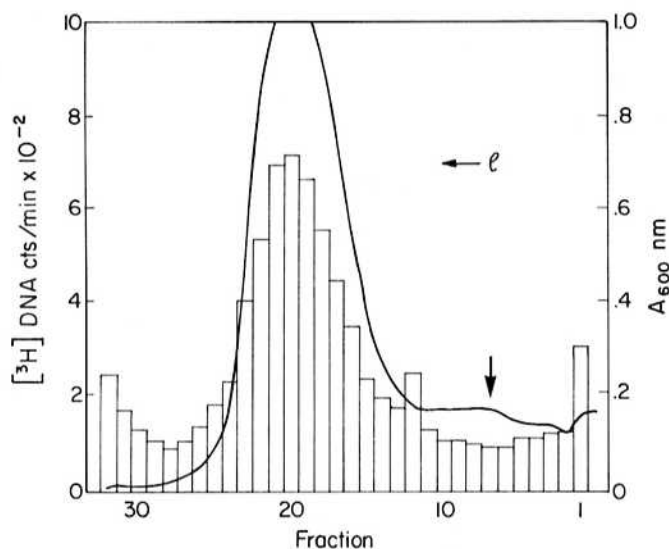


Fig. 1. Isopycnic distribution of a hypotonic lysate of *L. tarentolae* in terms of [³H] DNA and optical density at 600 nm in a linear 20-40% Renografin gradient. The sample, after treatment with DNase I, was resuspended in 60% Renografin-sucrose and placed beneath the gradient. Centrifugation was for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

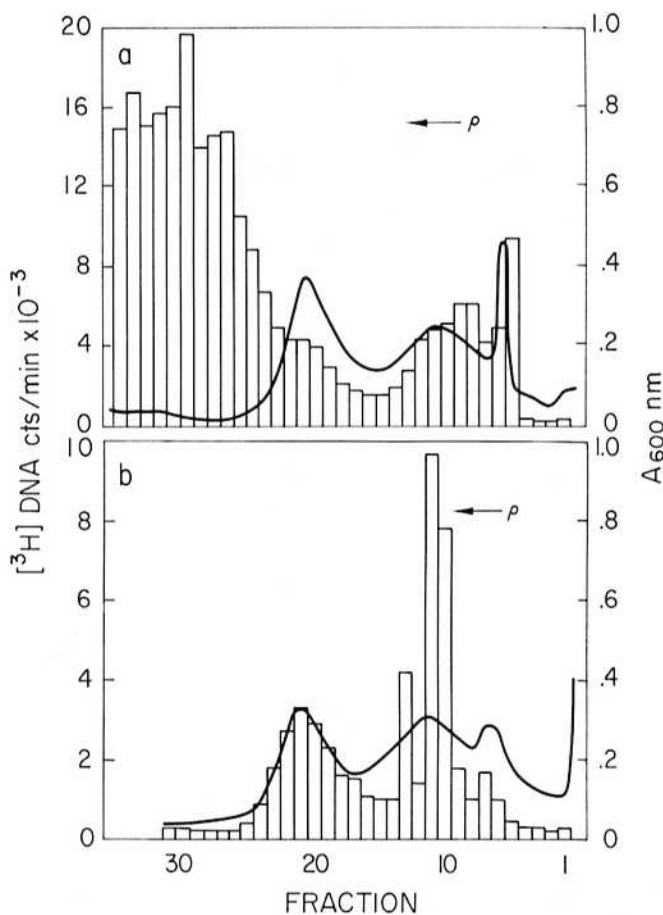


Fig. 2. Comparison of the isopycnic distribution of a hypotonic lysate that was treated with DNase I prior to running, with a lysate that was not treated. The cell lysate sample was divided into two portions, one of which (b) was treated with DNase I as described in Methods and the other of which (a) 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 [³H] 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-

