Isolation of Mitochondria and Mitochondrial RNA from *Crithidia fasciculata*

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SUMMARY

Two methods were used to isolate mitochondria from *Crithidia fasciculata*. In the first method, cells were weakened by exposure to hypotonic conditions and then disrupted by blending; mitochondria were subsequently isolated using disodium 3,5-diacetoamido-2,4,6-triiodobenzoate gradients. In the second, cells were treated with digitonin before disruption; mitochondria were purified by differential centrifugation. Both preparations were examined with the electron microscope and were also shown to possess several characteristic biochemical properties of mitochondria. Kinetoplast DNA was present in the mitochondria, uncontaminated by nuclear DNA.

Analysis by polyacrylamide gel electrophoresis showed two RNA components of molecular weights 0.47×10^6 and 0.22×10^6 , in addition to cytoplasmic RNA contamination. Four mitochondrial components with sedimentation coefficients of 14.6S, 11.4S, 10.1S and 6.9S were identified on sucrose density gradients. Ethidium bromide abolished the incorporation of [5-³H]uridine into the presumed mitochondrial RNA.

INTRODUCTION

Members of the protozoan order Kinetoplastida, which includes the genera *Crithidia*, *Leishmania* and *Trypanosoma*, are believed to contain a single mitochondrion (Paulin, 1975). If this mitochondrion is equivalent to mitochondria in other organisms, it should contain DNA coding for specific mitochondrial RNA and protein. The kinetoplast is composed of DNA (Bresslau & Scremin, 1924; Steinert, Firket & Steinert, 1958) and is a modified region of the single mitochondrion (Steinert, 1960).

The function of kinetoplast DNA (K-DNA) is unknown, although its presence or absence may be coupled to the proliferation and regression of functional mitochondria (Simpson, 1972). Verification of the presence of RNA encoded by K-DNA in the mitochondrion would give support for K-DNA functioning as mitochondrial DNA. We decided to approach the problem by isolating mitochondria from *Crithidia fasciculata*, this species being chosen because of its relative ease of culture. However, the existence of a single mitochondrion consisting of several tubular extensions makes it difficult to isolate an intact mitochondrion, especially as the kinetoplastid flagellates are relatively resistant to cell breakage by standard methods (Simpson, 1968; 1972). Several techniques have previously been used to accomplish this (Hill & White, 1968; Renger & Wolstenholme, 1970; Renger & Wolstenholme, 1972; Kusel & Storey, 1972; Toner & Weber, 1972; Braly, Simpson & Kretzer, 1974).

This paper describes the isolation of mitochondria, mitochondrial RNA and mitochondrial (kinetoplast) DNA from *C. fasciculata*.

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METHODS

Materials. Ethidium bromide was obtained from Boots Pure Drug Co., Nottingham; and actinomycin D, bovine serum albumin (fraction V), ribonuclease A, deoxyribonuclease I, MOPS [3-(*N*-morpholino)propanesulphonic acid], digitonin, and diethylpyrocarbonate from Sigma. Pronase was obtained from Calbiochem; [5-³H]uridine (5.0 Ci mmol⁻¹) from The Radiochemical Centre, Amersham; NADP from Boehringer; lithium dodecyl sulphate from Searle; Renografin (disodium 3,5-diacetoamido-2,4,6-triiodobenzoate) from Winthrop Laboratories, Surbiton-upon-Thames, Surrey; and Spurr resin from Agar-Aids, Bishop's Stortford, Hertfordshire. Other chemicals were obtained in analytical grades wherever possible.

Cultivation of organisms. *Crithidia fasciculata* was grown axenically in defined medium (Kidder & Dutta, 1958). Batch cultures (500 ml) were harvested in the mid-exponential phase (3×10^7 to 4×10^7 cells ml⁻¹) to prepare total cell RNA, and in the late-exponential phase (8×10^7 to 9×10^7 cells ml⁻¹) to prepare mitochondria.

Escherichia coli (strain MRE600) was obtained as a frozen cake from the Microbiological Research Establishment, Porton Down, Wiltshire, and was stored at -20 °C.

Isolation of mitochondria

Method 1: hypotonic swelling and purification by Renografin gradients. The cells were harvested by centrifuging at 4000 *g* for 5 min at 4 °C, washed twice with and resuspended (at 5×10^8 cells ml⁻¹) in 2 mM-EDTA/2 mM-Tris/HCl buffer prepared in double-distilled water and adjusted to pH 8.0 at 4 °C. After about 30 min, when the cells had swollen and assumed a spherical shape, the cells were disrupted in an MSE homogenizer for 2.5 min at full speed; 80 to 90% rupture was achieved. Mitochondria were released as swollen vesicles, some of which contained kinetoplasts. Immediately after disruption, a concentrated sucrose solution was added to the lysate to give a final concentration of 0.25 M. Swelling of the cells and the extent of breakage were routinely monitored by phase contrast microscopy.

The mitochondria were purified on Renografin gradients using the method of Braly *et al.* (1974). The gradients were centrifuged for 2 h at 77500 *g* in a Beckman SW 40 rotor at 4 °C.

Method 2: treatment with digitonin and purification by differential centrifugation. The method used was that of Kusel & Storey (1972) except that the cells were disrupted in an MSE homogenizer for 1 min at full speed. Cell rupture was always greater than 80%.

Electron microscopy. Samples were fixed in 2.5% (v/v) glutaraldehyde in 0.25 M-sucrose/0.2 M-cacodylate buffer pH 7.2 for 1 to 2 h at 4 °C, washed at 4 °C in the same buffer for 15 min and for a second time overnight. They were then embedded in 2% (w/v) Difco Bacto-agar in sucrose/cacodylate buffer and cut up into small pieces which were washed (4 × 15 min) in sucrose/cacodylate buffer. Samples were then postfixed with 2% (w/v) osmium tetroxide in 0.2 M-cacodylate for 1 h at 0 °C, dehydrated in a graded series of ethanol/water mixtures, infiltrated with Spurr resin, and finally embedded in fresh Spurr resin (1 h at 100 °C). Samples were sectioned on an LKB Ultratome III and collected on uncoated copper grids. Sections were stained for 15 min in a saturated solution of uranyl acetate in 50% ethanol and for 10 min in lead citrate before examination in a Philips EM300 electron microscope.

Extraction of RNA. RNA was extracted from cells and organelles by the method of Kirby

(1965) and Parish & Kirby (1966) as modified by Loening (1969). The RNA concentration was estimated by assuming an $E_{260}^{1\%}$ at 260 nm of 250.

Polyacrylamide gel electrophoresis of RNA. Samples of RNA (5 to 20 μ g) were analysed by polyacrylamide gel electrophoresis (Loening, 1967) using 2.6% (w/v) gels at 8 °C. rRNA of *E. coli* (mol. wts 1.07×10^6 and 0.56×10^6 ; Loening, 1967) was used as marker. The buffer (pH 7.8) contained: 40 mM-Tris; 20 mM-sodium acetate; and 1 mM-EDTA. Gels were scanned at 265 nm in a Joyce-Loebl ultraviolet scanner.

Sucrose density gradient centrifugation of RNA. RNA (20 to 60 μ g) in 0.15 M-sodium acetate, pH 6.0, containing 0.5% (w/v) lithium dodecyl sulphate, was layered on to 12 ml 15 to 30% (w/v) linear sucrose gradients prepared in 20 mM-sodium acetate/1 mM-EDTA/40 mM-Tris/HCl buffer, pH 7.8, containing 0.5% lithium dodecyl sulphate, and centrifuged at 40000 rev. min⁻¹ for 16 h at 4 °C, in the Beckman SW 40 rotor. rRNA of *E. coli* was used for calibration. The gradients were fractionated, and the absorbance was continuously measured at 254 nm using an ISCO density gradient fractionator model 183, equipped with an ISCO type 6 optical unit and model UA-5 absorbance monitor.

DNA analysis. Cells at (10^8 ml⁻¹) or organelles were lysed in 0.1 M-NaCl/0.01 M-EDTA/1 mM-Tris/HCl buffer, pH 8.0, containing 1% (w/v) sodium dodecyl sulphate and 1 mg pronase ml⁻¹ at 37 °C for 2 h. Digests were dialysed for 16 h at 4 °C against 0.1 M-NaCl/0.01 M-EDTA/1 mM-Tris/HCl buffer, pH 8.0, and the precipitated dodecyl sulphate was removed by centrifugation. The clear supernatant was stored at 4 °C until required.

Analytical CsCl centrifugation was performed in a Beckman model E ultracentrifuge at 140700 g (44000 rev. min⁻¹) at 20 °C for 16 to 18 h (Schildkraut, Marmur & Doty, 1962). Ultraviolet absorption photographs of the DNA distribution at equilibrium were scanned using a Joyce-Loebl recording microdensitometer. Buoyant densities were calculated using *Micrococcus luteus* DNA (1.731 g ml⁻¹) as marker (Szybalski, 1968).

Enzyme assays. Succinate dehydrogenase (succinate:(acceptor) oxidoreductase; EC 1.3.99.1) activity was assayed by the method of King (1967). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was assayed by the method of Langdon (1966).

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Equivalent amounts of buffer were included in the blank when necessary.

RESULTS

Properties of the mitochondrial preparations

After centrifugation in Renografin gradients (method 1), two bands could usually be seen by eye, although the size of the upper band, containing membrane fragments and flagella in addition to mitochondria, was very variable. The lower band, at a density of 1.18 g ml⁻¹, contained mitochondria with little cell debris and no whole cells (Fig. 1*a*). Smaller mitochondria, of 0.5 μ m diameter, had a condensed matrix and large intracristal spaces, whereas larger mitochondria, some having a diameter of over 1.0 μ m, had a less electron-dense matrix and smaller intracristal spaces. Some mitochondria contained a section through a kinetoplast. The existence of various densities in the matrices could represent various degrees of leakiness of the mitochondria. Absorbance at 600 nm was used to follow the distribution of light-scattering material in the gradients; the distribution of protein and succinate dehydrogenase activity coincided with the material at 1.18 g ml⁻¹, with the peak activity being 57 nmol succinate oxidized min⁻¹ (mg protein)⁻¹. Analysis by CsCl equilibrium centrifugation showed a fast-banding DNA component in the mitochondrial lysates, which is a characteristic of kinetoplast DNA (Dubuy, Mattern & Riley, 1965). At equilibrium

Fig. 1. Electron micrographs of mitochondria isolated from *C. fasciculata*. (a) Mitochondria obtained by hypotonic swelling and disruption in an MSE homogenizer. Purification was by flotation on a Renografin gradient. (b) Mitochondria obtained by treatment with digitonin and disruption in an MSE homogenizer. Purification was by differential centrifugation. (c) Mitochondria obtained as for (b). Purification was by flotation on a Renografin gradient. K, Kinetoplast DNA; F, flagella; CM, cell membrane; UC, unidentified component. Bar markers represent 1 μ m.

Fig. 2. Ultraviolet scans of RNA extracted from whole cells and mitochondria and analysed by polyacrylamide gel electrophoresis. Electrophoresis was performed in acetate buffer at 8 °C for 2.5 h. RNA molecular weights were determined relative to *E. coli* rRNA (mol. wts 1.07×10^6 and 0.56×10^6) run as an internal marker in duplicate gels. —, Total rRNA extracted from *C. fasciculata* (load 10 μ g); ---, RNA extracted from mitochondria.

(18 h) there was only one component of buoyant density 1.703 g ml^{-1} , which was identified as kinetoplast DNA. Nuclear DNA (1.717 g ml^{-1}) was absent from all such mitochondrial preparations.

The final mitochondrial pellet prepared by method 2 (Fig. 1b) was less pure than preparations obtained by Renografin centrifugation. Many flagella and cell membranes were present. The mitochondria were of a more uniform size than Renografin preparations and were all of approximately $0.5 \mu\text{m}$ diameter. The matrix was electron dense with large intracristal spaces. The smaller mitochondria obtained by this method may be due to the isolation medium being isotonic at all stages of the preparation, whereas in those preparations involving hypotonic swelling, the mitochondria may have been damaged and become swollen. Succinate dehydrogenase activity was determined during the purification of mitochondria and in two different preparations the specific activity of the final mitochondrial pellet had increased to 2.5 times and 6 times that of the broken-cell homogenate. Glucose-6-phosphate dehydrogenase is an enzyme of the pentose phosphate pathway which is generally considered to be cytoplasmic and occurs in trypanosomes (Mancilla & Náquira, 1964). Glucose-6-phosphate dehydrogenase activity was detected in extracts of sonicated *C. fasciculata* and its specific activity was monitored during the purification of mitochondria. Only 1 to 5 % of the activity of the initial homogenate was found in the final mitochondrial preparations.

The best mitochondrial preparations (Fig. 1c) were obtained by a combination of digitonin treatment and Renografin centrifugation.

Fig. 3. Incorporation of [5-³H]uridine into RNA extracted from mitochondria of *C. fasciculata* grown for 1.5 generations in the presence of [5-³H]uridine (2 μ M, 500 mCi mmol⁻¹). Electrophoresis was performed in acetate buffer at 8 °C for 2.75 h. rRNA (2 μ g) of *E. coli* was included as marker. The gels were cut into 1 mm slices with a Mickle gel slicer and, after drying and digestion with H₂O₂, the radioactivity in the slices was determined in Triton/toluene scintillant. —, Absorbance at 265 nm; ---, radioactivity.

Analysis of mitochondrial RNA by polyacrylamide gel electrophoresis

RNA species present in mitochondria, especially rRNA, are distinct from their cytoplasmic counterparts (Borst, 1972), and it was hoped that this would also apply in the case of mitochondrial RNA from *C. fasciculata*. However, it was first necessary to identify cytoplasmic RNA.

rRNA extracted from *C. fasciculata* and analysed by polyacrylamide gel electrophoresis consisted of two major components with molecular weights (relative to *E. coli* rRNA) of 1.28×10^6 and 0.82×10^6 (Fig. 2). Two minor components with molecular weights of 0.71×10^6 and 0.56×10^6 are believed to be breakdown products of the larger rRNA species, due to a 'hidden' break in the molecule (Pace, 1973) as in *Crithidia oncopelti* (Spencer & Cross, 1976) and *Crithidia luciliae* (Reijnders *et al.*, 1973). The small high molecular weight peak in the position expected for DNA (Loening, 1967) was absent after DNAase treatment of the sample.

RNA extracted from mitochondria prepared by digitonin treatment and differential centrifugation had two additional components (Fig. 2); their average molecular weights were calculated to be 0.47×10^6 and 0.22×10^6 (corresponding to sedimentation coefficients of 14.5S and 9.5S). The amount of contamination by cytoplasmic RNA varied from 45 to 63%. Treatment of samples with DNAase or RNAase confirmed that all components were RNA except for that of the highest molecular weight band which was DNA.

Incorporation of [5-³H]uridine into growing cells [for 1.5 generations in the presence of 1 μ Ci ml⁻¹ [5-³H]uridine (2 μ M, 500 mCi mmol⁻¹)] gave mitochondrial RNA components of

Fig. 4. Sucrose density gradient analysis of (a) total rRNA (20 μ g) from *C. fasciculata* and (b) RNA isolated from mitochondria. The RNA was analysed at 4 °C for 16 h in 12 ml 15 to 30 % sucrose gradients in 20 mM-sodium acetate/1 mM-EDTA/40 mM-Tris/HCl buffer, pH 7.8, containing 0.5 % (w/v) lithium dodecyl sulphate. RNA sedimentation coefficients were determined relative to *E. coli* rRNA run in duplicate gradients.

the same specific activity as the cytoplasmic components (Fig. 3). Additional small radioactive peaks were detected which had not previously been detected by absorbance alone. These were not investigated further. Only 0.07 % of incorporated [5-³H]uridine was recovered in the final mitochondrial preparation. Allowing for the estimated recovery of mitochondria this suggests that mitochondrial RNA represents about 1 % of total cellular RNA.

RNA could not be detected in mitochondria purified on Renografin gradients. Addition of diethylpyrocarbonate, an RNAase inhibitor, was without effect. This result was disappointing since these mitochondrial preparations were of greater purity.

Selective inhibition of cytoplasmic mitochondrial RNA synthesis

The effects of two RNA inhibitors were investigated: actinomycin D, which preferentially inhibits cytoplasmic RNA synthesis (Vesco & Penman, 1969); and ethidium bromide, an inhibitor of mitochondrial RNA synthesis (Attardi *et al.*, 1970).

Actinomycin D was required at 50 μ g ml⁻¹ to inhibit the incorporation of [5-³H]uridine into the total cell RNA of *C. fasciculata* by 97 %. This is much greater than the concentration required to inhibit mammalian RNA synthesis (approximately 0.04 μ g ml⁻¹) suggesting that *C. fasciculata* may be relatively impermeable to the drug. Ethidium bromide at 2 μ g ml⁻¹ gave no detectable inhibition of total RNA synthesis, as would be expected if it were only inhibiting mitochondrial RNA synthesis. Higher concentrations of drug (20 μ g ml⁻¹ and 40 μ g ml⁻¹) inhibited the incorporation of [5-³H]uridine into RNA by 12 % and 45 %, respectively.

The effect of these drugs on individual RNA components was investigated, the RNA being analysed by sucrose density gradient centrifugation since it was occasionally found that RNA isolated from mitochondrial preparations would not enter polyacrylamide gels

Fig. 5. Sucrose density gradient analysis of RNA isolated from mitochondria of *C. fasciculata* labelled with [5-³H]uridine in the presence and absence of (a) ethidium bromide and (b) actinomycin D. *Crithidia fasciculata* was grown to a density of 5×10^7 to 6×10^7 cells ml⁻¹ before adding ethidium bromide ($10 \mu\text{g ml}^{-1}$) or actinomycin D ($25 \mu\text{g ml}^{-1}$) and, 30 min later, [5-³H]uridine ($2 \mu\text{M}$, 500 mCi mmol⁻¹). The cells were harvested 3 h later. Conditions for centrifugation were as for Fig. 4. Arrows indicate species of RNA believed to be of mitochondrial origin. —, Control; ---, in the presence of (a) ethidium bromide or (b) actinomycin D.

(possibly due to the presence of large amounts of K-DNA or protein). RNA isolated from mitochondria (Fig. 4b) contained four components of 14.6S, 11.4S, 10.1S and 6.9S, in addition to cytoplasmic contaminants. Ethidium bromide ($10 \mu\text{g ml}^{-1}$) altered the radioactive profile of RNA isolated from mitochondria from cells labelled with [5-³H]uridine (Fig. 5a). Synthesis of the presumptive mitochondrial components was abolished, whereas incorporation of [5-³H]uridine into the cytoplasmic contaminants was unaffected. Although the total incorporation of [5-³H]uridine into the cytoplasmic components was not reduced substantially by actinomycin D, the large rRNA component was very much decreased compared with the control (i.e. the ratio of large rRNA to small rRNA decreased) and there was a concomitant increase in the breakdown product at 13.9S (Fig. 5b). There was no definitive effect upon the synthesis of the mitochondrial RNA components.

DISCUSSION

Previous attempts to isolate mitochondria from kinetoplastid flagellates usually involved vigorous disruption which probably caused extensive fragmentation of mitochondria. Two methods are described here. Preparations obtained by method 1 were morphologically pure; intact cells, flagella and cell membranes were absent. Mitochondria prepared by method 2 were accompanied by flagella and cell membranes.

There is no definitive evidence that these mitochondrial preparations represent whole mitochondria. If they do, each mitochondrion should contain a kinetoplast, but this would not be observed in a thin section. Braly *et al.* (1974) calculated that in any one thin section of *Leishmania tarentolae* mitochondria, the probability of sectioning the K-DNA of any kinetoplast-mitochondrion complex was 24%. In this work 19% of the mitochondria in the

electron micrographs contained a section through K-DNA. Therefore, it seems likely that each mitochondrial profile represents a whole mitochondrion.

There is some earlier evidence that kinetoplasts contain RNA. In 1969, Kallnikova stated that the kinetoplast of *Trypanosoma cruzi* contained RNA, although no experimental evidence was given. Steinert *et al.* (1969) showed by autoradiography and pulse labelling with [³H]uridine that the kinetoplast was a site of RNA synthesis. However, our studies (Fig. 3) suggest that K-DNA may be labelled by [³H]uridine. Autoradiography, coupled with enzymic digestion was used by Ozeki *et al.* (1971) to show that the kinetoplasts of *T. cruzi* and *T. gambiense* contained DNA, RNA and protein. More recently, Simpson (1973) demonstrated two RNA species, isolated from mitochondria of *L. tarentolae*, with sedimentation coefficients of 9S and 12S. The synthesis of these RNA molecules was inhibited by ethidium bromide. These RNA components accounted for less than 2% of the total cell RNA and Simpson & Lasky (1975) proposed that they represent stable mitochondrial mRNAs. We found RNA in mitochondria represented approximately 1% of total cell RNA. The sizes of these components are similar to those obtained by Simpson (1973).

The presence of RNA in the mitochondria supports the hypothesis that kinetoplast DNA may be transcribed, but hybridization studies are required to confirm the relationship between the numerous molecular species that have been identified in K-DNA (see, for example, Simpson, 1972; Renger & Wolstenholme, 1970, 1972; Steinert & Van Assel, 1975) and the isolated RNA. This should also determine which molecular species represents the true mitochondrial DNA.

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