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GRADUATE COLLEGE

CHARACTERIZATION OF THE MITOCHONDRIAL AND CYTOPLASMIC RIBOSOMES OF *TETRAHYMENA PYRIFORMIS*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partia. fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

DANIEL MCELHANY ZELLMER

Norman, Oklahoma

1976

CHARACTERIZATION OF THE MITOCHONDRIAL

AND CYTOPLASMIC RIBOSOMES OF

TETRAHYMENA PYRIFORMIS

APPROVED BY

DISSERTATION COMMITTEE

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TABLE OF CONTENTS

Page

LIST OF ILLUSTRATIONS v

LIST OF PLATES vii

Chapter

I. INTRODUCTION 1

II. METHODS AND MATERIALS 4

III. RESULTS 17

IV. DISCUSSION 39

SUMMARY 49

LITERATURE CITED 51

LIST OF ILLUSTRATIONS

FIGURE		Page
1.	Flow diagram of the procedures for isolation of the cytoplasmic and mitochondrial ribosomes of Tetrahymena pyriformis	6
2.	Growth of Tetrahymena pyriformis in various media	18
3.	The location of succinic dehydrogenase activity in a 30-60% linear sucrose density gradient after centrifugation for four hours in the SW27 rotor	20
4.	The location of catalase and succinic dehydrogenase activity after centrifugation for 16 hours in a step gradient in a Z-15 rotor	21
5.	The effect of 200 and 400 mM KCl on the cytoplasmic ribosome of Tetrahymena pyriformis	28
6.	The effect of 200, 300 and 400 mM KCl on the mitochondrial ribosome of Tetrahymena pyriformis	29
7.	The effect of 500 mM LiCl, 500 mM NH_4Cl and 500 mM KCl on the mitochondrial ribosome of Tetrahymena pyriformis	30
8.	The subunits of cytoplasmic and mitochondrial ribosomes after centrifugation for 11 hours 25 min in isokinetic sucrose gradients in the SW27 rotor	31
9.	Sedimentation of RNA isolated from the cytoplasmic fraction of Tetrahymena pyriformis in isokinetic sucrose density gradients	33
10.	Sedimentation of RNA isolated from the mitochondrial fraction of Tetrahymena pyriformis in isokinetic sucrose density gradients	34

FIGURE Page

11. The effect of DNase on the small subunit of the mitochondrial ribosome of Tetrahymena pyriformis 35

LIST OF PLATES

PLATE Page

1. Electron micrograph of material from pooled fractions 21-24 of an isopycnic sucrose density step gradient in the Z-15 zonal rotor 22

2. Electron micrograph of material from pooled fractions 37-39 of an isopycnic sucrose density step gradient in the Z-15 zonal rotor 23

3. Electron micrograph of material from pooled fractions 51-54 of an isopycnic sucrose density step gradient in the Z-15 zonal rotor 24

4. Electron micrograph of material from pooled fractions 66-69 of an isopycnic sucrose density step gradient in the Z-15 zonal rotor 25

5. Electron micrograph of material from pooled fractions 76-80 of an isopycnic sucrose density step gradient in the Z-15 zonal rotor 26

6. Separation of proteins extracted from ribosomal subunits of T. pyriformis by polyacrylamide gel electrophoresis 38

CHARACTERIZATION OF THE MITOCHONDRIAL AND CYTOPLASMIC RIBOSOMES OF *TETRAHYMENA PYRIFORMIS*

CHAPTER I

INTRODUCTION

The hypothesis that mitochondria are semi-autonomous has recently gained widespread acceptance. The replication of the organelles by fission is temporally independent of the replication of the cell. Protein synthesis within mitochondria may proceed during cell division--a period in which most cellular protein synthesis is inhibited. The organelle contains deoxyribonucleic acid (DNA) and a protein-synthesizing system that is apparently capable of producing some of its own constituents (Dawidowicz and Mahler, 1973; Boulter, 1972; Kuntzel, 1971). The organelle is enclosed by two membranes which separate much of its contents from the cytoplasm of the cell (Gonzalez et al., 1973).

The semi-autonomous nature of the mitochondrion has led to speculation that the mitochondrion may be derived from a symbiotic association between primitive aerobic procaryotes and primitive anaerobic eucaryotes (Boulter, 1972). The similarity between the DNA, ribosomes and mechanisms of

protein synthesis of bacteria and mitochondria is frequently cited as evidence supporting this hypothesis.

The mitochondria of plants, fungi and animals have been the subject of recent investigations concerning the structure and function of the ribosomes and their ribonucleic acids. Nevertheless, the structure and function of the ribosomes of eucaryotes are less thoroughly understood than those of procaryotes. The growth rate and simplicity of culture methods should make unicellular organisms the logical choice for the study of the structure and function of both the cytoplasmic and mitochondrial ribosomes of eucaryotic organisms.

The physiology of the ciliate, Tetrahymena pyriformis, has been extensively investigated, and the natures of the DNA (Suyama, 1966), cytoplasmic ribosome and ribosomal ribonucleic acid (Chi and Suyama, 1970; Curgy et al., 1974; Kumar, 1970; Leick and Plesner, 1968a; Leick and Plesner, 1968b; Mita, 1965; Prescott et al., 1971) have been the subject of recent investigations. The mitochondrial ribosomes have been the subject of recent investigations by Chi and Suyama (1970) and Curgy et al. (1974) who found an 80S monosome and subunits with sedimentation coefficients of 55S. No accompanying 30S-40S subunit was found. The existence of two types of 55S subunits was postulated. This hypothesis was supported by the observation that two ribosomal bands could be resolved from the 55S peak on the basis of differences in

density and that two classes of ribosomal ribonucleic acid (rRNA) could be isolated.

The report of a monosome with two subunits of identical size contrasts with the usual situation in which monosomes that contain subunits of distinctly different sizes are found. In addition, the authors of one of the reports indicated that the rRNA molecules of the mitochondrial ribosome had sedimentation coefficients of 21S and 14S (Chi and Suyama, 1970). In several cases in which the existence of similarly atypical ribosomes have been reported, subsequent investigation indicated that the ribosome had the typical structure expected and that the ribonucleic acids were larger than originally designated (Borst and Grivell, 1971).

We have undertaken to investigate the ribosomes of T. pyriformis (WH-14) with the intent to resolve the status of the mitochondrial ribosome. We have investigated the sedimentation behavior of the subunits and their ribonucleic acids. We have isolated the proteins from the subunits of the cytoplasmic and mitochondrial ribosomes for electrophoretic characterization.

CHAPTER II

METHODS AND MATERIALS

Culture of Tetrahymena pyriformis

Axenic stock cultures of Tetrahymena pyriformis WH-14 were maintained at 26°C in 5 ml screw-cap culture tubes on slant racks. The cultures were transferred to fresh media at seven-day intervals. One-liter volumes of sterile medium in Fernbach flasks were inoculated with 5 ml of stationary stock culture for procedures that required 1-4 l of culture. Eight-liter batch cultures were grown for procedures that required larger numbers of cells. The eight-liter cultures were aerated by passing air through a flask containing $CaCl_2$ and cotton and through 25 mm Millipore filters with a pore size of 45 μ.

Cells were grown in each of three media. Medium A contained 2% (w/v) proteose-peptone (Difco) and 0.1% liver infusion broth (Difco). Medium B contained 1% proteose-peptone (Difco), 0.5% liver infusion broth (Difco) and 0.5% dextrose. Medium C contained 1.25% proteose-peptone (Difco), 0.05% yeast extract (Difco), 0.25% casitone (Difco), 0.05% KH_2PO_4, 0.05% Na_2HPO_4, 0.1% NaCl, 0.0025% $MgCl_2 \cdot 6H_2O$ and 0.25% dextrose. All media were adjusted to pH 7.2. Growth

rates in each medium were determined by diluting one-milliliter aliquots of the culture in 25 ml of Isoton counting fluid and counting on a Model B Coulter Counter. Aliquots were counted 2, 30, 60, 240, 480, 720, 960, 1440 and 1920 min after inoculation.

Medium C was used for most procedures.

Preparation of Cytoplasmic Ribosomes

Cultures were harvested in late log phase (200,000 cells per ml) by centrifugation for 5 min in the GSA rotor of a Sorvall RC5 centrifuge at 4,000 xg. The cells were resuspended in 3.0 ml of Isolation Medium A (IMA) per gram wet weight of cells and homogenized in a Polytron homogenizer at a setting of 10. IMA contained 350 mM sucrose, 25 mM KCl, 10 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.8.

The homogenate was centrifuged for 10 min at 12,000 xg in the Model 870 rotor of an International B-20A centrifuge. Pellet I (Fig. 1) was retained for the isolation of mitochondria. Cytoplasmic ribosomes were isolated from Supernatant I (Fig. 1). The supernatant was layered over 1.0 ml of 1.5 M sucrose dissolved in Isolation Medium B (IMB) in 8.5-ml polycarbonate tubes and centrifuged for 2 hours at 48,000 rpm in the 50Ti rotor of a Beckman Model L2-65B ultracentrifuge. IMB contained 25 mM KCl, 10 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.8. The ribosome pellets were washed with cold distilled water and stored at -65°C.

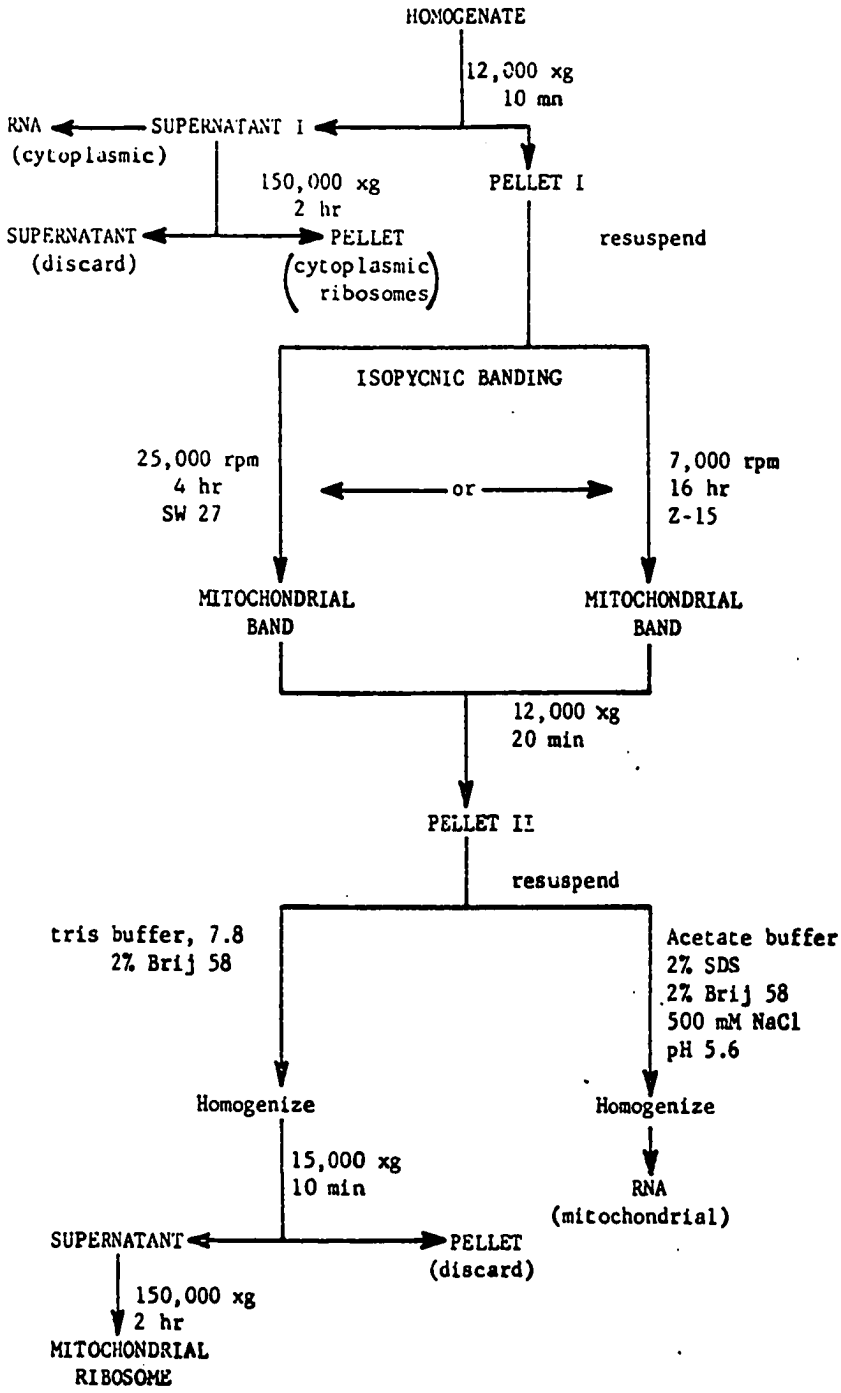


Figure 1

Flow diagram of the procedures for isolation of the cytoplasmic and mitochondrial ribosomes of Tetrahymena pyriformis.

Isolation of Mitochondria

Mitochondria were isolated from Pellet I (Fig. 1) by equilibrium centrifugation in sucrose density gradients. The procedures adopted for the isolation of mitochondria were based on the results of preliminary studies in which various procedures were analyzed for the yield and purity of mitochondria which they produced. Succinic dehydrogenase (SDH) activity was used as an indication of the presence of mitochondria in a fraction. Initial efforts to use cytochrome c oxidase activity as a mitochondrial marker were abandoned because no cytochrome c oxidase activity could be detected in Tetrahymena pyriformis (WH-14). These results agree with those reported by Ryley (1952). Catalase activity was used as an indicator of the presence of peroxisomes, considered to be the most likely contaminant of mitochondrial fractions. Selected fractions from the gradients were examined with the electron microscope to confirm the conclusions drawn from the enzyme assays.

Mitochondria were isolated from small quantities of cells (<5 grams wet weight) by isopycnic centrifugation in a Beckman SW27 rotor. Pellet I (Fig. 1) was resuspended in 3.0 ml of IMA and layered onto 37-ml linear sucrose gradients (30-60% w/w in IMB). The gradients were centrifuged in the SW27 rotor at 25,000 rpm in a Beckman L2-65B ultracentrifuge. The duration of centrifugation was varied in preliminary experiments to determine the time required to reach equilib-

rium. Fifty-one 0.72-ml fractions were collected by pumping the gradient from the bottom of the tubes with an LKB Perpex pump.

Larger quantities (>5 grams wet weight) of mitochondria were isolated from batch cultures. Ten grams of Pellet I (Fig. 1) were resuspended in 40 ml of 30% sucrose dissolved in IMB and centrifuged on a step gradient in the Z-15 rotor of an International B-20A centrifuge. A 250-300 ml overlay of 20% sucrose (w/w) dissolved in IMB was introduced at the rim, followed by the 40 ml sample, 185 ml of 42% sucrose, 200 ml of 47% sucrose and 130 ml of 60% sucrose. Excess overlay was displaced from the core as the gradient was loaded. All sucrose solutions were prepared in IMB. The Z-15 rotor was sealed, and the speed was increased from the loading speed of 2,000 rpm to the running speed of 7,000 rpm. The sample was centrifuged for 16 hours. The gradient was displaced from the rim by pumping overlay solution into the core. Ninety-six 10-ml fractions were collected and placed on ice.

SDH activity of the fractions collected from gradients in the SW27 rotor was determined by the method of Shelton and Rice (1957). An incubation medium containing 0.05 M succinate, 0.01% 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) in 0.1 M Tris-HCl buffer, pH 8.0 was incubated at 37°C for 10 min with 0.1 ml of each fraction. The reaction was terminated by adding 0.5 ml of 40% trichloroacetic acid (TCA). Three ml of ethyl acetate were added to

the reaction mixture, and the mixture was shaken vigorously. The mixture was centrifuged for 2 min at 2,000 rpm in an International Model CL centrifuge. The upper phase was decanted, and its absorbance at 490 nm was determined with a Gilford Model 2000 spectrophotometer.

SDH activity of fractions collected from the Z-15 rotor was determined by the method of Veeger, DerVartanian and Zaylemaker (1969). One-tenth ml of each fraction and 0.1 ml of 30 mM KCN were added to 2.7 ml of a reaction mixture containing 44 mM sodium succinate, 0.01% bovine serum albumin, 22 μM phenazine methosulfate, 5.6 mM 2,6-dichlorophenol-indophenol (DPIP) in 56 mM phosphate buffer, pH 7.6. The decrease in absorbance at 600 nm was recorded using a Gilford Model 2000 spectrophotometer, and SDH activity was expressed as $\Delta OD_{600} min^{-1}$.

Catalase activity was assayed by the method of Chance and Maehly (1955). One-tenth ml of each fraction was added to 3.0 ml of 1% H_2O_2 in 10 mM phosphate buffer, pH 7.0. The decrease in absorbance at 250 nm was recorded using a Gilford Model 2000 spectrophotometer. Activity was expressed as $\Delta OD_{250} min^{-1}$.

The sucrose concentration of each fraction was determined from measurements of the refractive index with a Bausch and Lomb refractometer.

Fractions 21-24, 37-39, 51-54, 66-69 and 76-80 of the zonal gradient were pooled for examination by electron microscope and centrifuged for 20 min at 12,000 xg. Each pellet

was resuspended in 1.2% glutaraldehyde and fixed for two hours at 0°C. The samples were centrifuged, and the pellet was stained with osmium tetraoxide and dehydrated with ethanol. The pellet was cleared with propylene oxide II and epon and embedded in epon. One micron sections were cut and mounted on copper grids for examination.

Preparation of Mitochondrial Ribosomes

Fractions containing mitochondria were pooled, diluted and centrifuged for 20 min at 12,000 xg. Pellet II (Fig. 1) was resuspended in 10 ml of 0.5% Brij 58 dissolved in IMA. The suspension was homogenized for 20 sec with a Polytron homogenizer at a setting of 10. The homogenate was centrifuged at 15,000 xg for 10 min in the Model 870 rotor of the International B-20A centrifuge. The supernatant was layered onto 1.0 ml of 1.0 M sucrose dissolved in IMB and centrifuged in 8.5-ml polycarbonate tubes in the 50Ti rotor for 2 hours in a Beckman L2-65B ultracentrifuge.

Preliminary Studies on the Preparation of Ribosomal Subunits

The effect of monovalent cations was determined by resuspension of the ribosomes in 50 mM Tris-HCl buffer, pH 7.8, which contained 50 μM $MgCl_2$ and the appropriate concentration of the monovalent cation to be examined. The mitochondrial ribosomes were examined in 500 mM NH_4Cl, 500 mM LiCl and concentrations of KCl from 200 mM to 500 mM. The cytoplasmic ribosomes were examined in concentrations of KCl of 200 mM and 400 mM.

The ribosomes were layered onto 5.2 ml 15-30% (w/w) linear sucrose gradients in the appropriate buffer and centrifuged for 3 h 8 min at 45,000 rpm in the SW50.1 rotor in the Beckman L2-65B ultracentrifuge.

The gradients were pumped through a flow cell in a Gilford Model 2000 spectrophotometer by introducing 60% sucrose into the bottom of the tube. Absorbance was recorded at 260 nm.

Ribosomal Subunit Preparation

Cytoplasmic ribosomes were resuspended in 50 mM Tris-HCl buffer, pH 7.8, which contained 10 μM $MgCl_2$ and 400 mM KCl and layered onto 36.6-ml isokinetic gradients made with the same buffer. The gradients were calculated for a temperature of 5°C, a particle density of 1.41 and a meniscus concentration of 10% (w/w) sucrose according to the method of McCarty, Stafford and Brown (1968). They were prepared in a gradient maker with a constant volume mixing chamber according to the method of Noll (1967). The mixing chamber contained 32.1 ml of 10% (w/w) sucrose, and the reservoir contained 26.82% (w/w) sucrose. Densities and viscosities of sucrose were calculated using the empirical equations of Barber (1966). The gradients were centrifuged for 11 h 25 min in the SW27 rotor of a Beckman L2-65B ultracentrifuge. The gradients were pumped through the flow cell of a Gilford Model 2000 spectrophotometer, and their absorbance was recorded at 260 nm. One-ml fractions were collected. The

fractions containing each subunit were pooled and centrifuged for 4 hours on the Beckman L2-65B ultracentrifuge at 48,000 rpm in a 50Ti rotor. The pellets of subunits were frozen in vials at -65°C.

The mitochondrial ribosomal subunits were prepared in the same manner, except that the buffer used in the gradients contained 500 mM KCl.

Preparation of Ribosomal RNA

Cytoplasmic rRNA was isolated from Supernatant I (Fig. 1). Supernatant I was diluted with an equal volume of 200 mM acetate buffer, pH 5.6, containing 4.0% sodium dodecyl sulfate (SDS), 0.2% Bentonite and 1 M NaCl. The mixture was placed in a glass-stoppered erlenmeyer flask with two volumes of water-saturated phenol and shaken for 10 min. Mitochondrial rRNA was isolated from Pellet II (Fig. 1). Pellet II was resuspended in 100 mM acetate buffer, pH 5.6, which contained 2% SDS, 500 mM NaCl, 2% Brij 58 and 0.1% Bentonite and homogenized for 30 sec with a Potter-Elvejehm tissue grinder. The homogenate was placed in a flask with two volumes of water-saturated phenol and shaken for 10 min.

Both the mitochondrial and cytoplasmic ribonucleic acid (RNA) preparations were centrifuged at 12,000 xg for 10 min in the Model 870 rotor of the International B-20A centrifuge. The upper phases of each preparation were removed and added to two volumes of water-saturated phenol

and shaken vigorously for 10 min at 0°C. The mixture was again centrifuged at 12,000 xg for 10 min in the Model 870 rotor of the International B-20A centrifuge. The procedure was repeated until there was no longer any protein at the interface. The upper phase was precipitated with 3 volumes of 95% ethanol which contained 3% potassium acetate for 2 h at -20°C. The preparation was centrifuged for 20 min at 15,000 xg in the Model 870 rotor of the International B-20A centrifuge. The ethanol was decanted, and the centrifuge tube was inverted and drained at -65°C.

Determination of Sedimentation Coefficients

Sedimentation coefficients were determined by analytical boundary sedimentation, sucrose density gradient sedimentation with isokinetic gradients and sucrose density gradients with an internal marker.

Eight mg of each monosome were resuspended in 0.7 ml of 10 mM Tris-HCl buffer, pH 7.2, which contained 100 mM NaCl and 1.0 mM $MgCl_2$. Sedimentation rates were determined by centrifugation at 30,000 rpm and 20°C in a 12 mm, 4°Epon cell in an AnD rotor modified for use in a Beckman L2-65B ultracentrifuge. The position of the schlieren boundaries were determined by Polaroid photographs taken at intervals during the run. The subunits of mitochondrial ribosomes were disassembled under the conditions used for boundary sedimentation. Consequently, they were fixed in 6% formaldehyde for 2 hours at 0°C prior to analysis.

The sedimentation coefficients of the ribosomal subunits were determined by sucrose density gradient sedimentation in isokinetic gradients. Five-hundred µg of cytoplasmic ribosomes were resuspended in 50 mM Tris-HCl buffer, pH 7.8, which contained 10 µM $MgCl_2$ and 400 mM KCl and layered onto 36.6-ml isokinetic gradients made with the same buffer. The gradients were calculated for a temperature of 5°C, a particle density of 1.41 and a meniscus concentration of 10% (w/w) sucrose according to the method of McCarty, Stafford and Brown (1968). They were prepared in a gradient maker with a constant volume mixing chamber according to the method of Noll (1967). The mixing chamber contained 32.1 ml of 10% (w/w) sucrose, and the reservoir contained 26.82% (w/w) sucrose. Densities and viscosities of sucrose were calculated using the empirical equations of Barber (1966). The gradients were centrifuged for 11 h 25 min in the SW27 rotor of a Beckman L2-65B ultracentrifuge. The gradients were pumped through the flow cell of a Gilford Model 2000 spectrophotometer, and their absorbance was recorded at 260 nm.

The sedimentation coefficients of the mitochondrial ribosomal subunits were determined in the same manner, except that the buffer used in the gradients contained 500 mM KCl.

The pellet of RNA was resuspended in 100 mM acetate buffer, pH 5.6, containing 10 mM $MgCl_2$ and 0.5% SDS. One-hundred µg of cytoplasmic rRNA or mitochondrial rRNA and 10 µg of tritium-labelled Escherichia coli rRNA were layered

onto 4.8 ml isokinetic gradients made with 5.1 ml of 5% (w/w) sucrose in the above buffer in the mixing chamber and 26.22% (w/w) sucrose in the reservoir of an isokinetic gradient maker. The gradients were calculated for a meniscus concentration of 5% (w/w) sucrose, a temperature of 20°C and a particle density of 1.89. The gradients were centrifuged for 2 h 27 min in the SW50.1 rotor at 45,000 rpm in a Beckman L2-65B ultracentrifuge.

Two-tenths-ml fractions were collected by puncturing the bottom of the tube and displacing the gradients from the bottom of the tube with mineral oil delivered from a Hamilton metering syringe. One-half ml of water was added to each fraction, and absorbance at 260 nm was measured with a Gilford Model 2000 spectrophotometer. One-half ml of each fraction was counted in 5 ml of Beckman Biosolve IA Scintillation Cocktail in a Beckman LS-133 liquid scintillation spectrometer.

Sedimentation coefficients were calculated by the method of McCarty, Stafford and Brown (1968). These values were compared to those calculated by the method of Martin and Ames (1961). Sedimentation coefficients for the labelled Escherichia coli rRNA used as an internal standard were assumed to be 23S and 16S.

Incubation with Deoxyribonuclease

Mitochondrial 31.4S subunits were treated with 10 µg/ml deoxyribonuclease (DNase) for 10 min at 0°C. Ten µg of the

DNase-treated 31.4S subunits were layered onto 5.2-ml 15-30% (w/w) linear sucrose gradients prepared in IMB and centrifuged for 3 h 47 min at 45,000 rpm in the SW50.1 rotor in the Beckman L2-65B ultracentrifuge. Ten µg of untreated 31.4S subunits were centrifuged at the same time on a second gradient. The sedimentation of the DNase-treated sample was compared to that of the untreated control.

The gradients were pumped through a flow cell of a Gilford Model 2000 spectrophotometer, and the absorbance was recorded at 260 nm.

Electrophoresis of Ribosomal Proteins

Subunits were resuspended in a solution which contained 2.0 M LiCl, 0.1 M mercaptoethanol and 4.0 M urea. Ribosomal proteins were extracted overnight at 4°C (Spitnik-Elson, 1965). The samples were centrifuged at 15,000 xg for 20 min in the Model 870 rotor of the International B-20A centrifuge. The proteins were precipitated with 1/10 volume of 40% TCA for 12 h at 4°C and centrifuged at 15,000 xg in the Model 870 rotor of the International B-20A centrifuge for 20 min. The proteins were resuspended in a solution which contained 8.0 M deionized urea, 2 mg/ml dithiothreitol and 0.2 M sucrose for electrophoresis. Electrophoresis was performed by the Gesteland and Staehelin (1967) modification of the Reisfeld, Lewis and Williams (1962) method. The gels were stained for 15 min in Amido-Schwartz in 7% acetic acid, stabilized by treating for 10 min in 6% formaldehyde and destained electrophoretically in 7% acetic acid.

CHAPTER III

RESULTS

Growth of Tetrahymena Pyriformis

Strain WH-14 grows most rapidly in Medium B. The generation times in the three media, A, B and C are 3.63, 2.81 and 3.87 hours, respectively (Fig. 2). Stationary phase cultures contained about 350,000 cells/ml of medium in all media. Medium C was used for most procedures.

A gradual increase in the generation times was observed during prolonged culture in any of the three media. This effect was less pronounced in Medium C. Rapid growth could be restored by transferring the cultures to an alternate medium. We have subsequently discovered (Fisher, 1976), that the reduction in growth rate was caused by light-induced degradation of thiamine and riboflavin in the media after prolonged exposure to light. Fisher's results are in agreement with the studies of Lee (1969) and Phelps (1959). This phenomenon could be eliminated by storing the media in the dark prior to use.

Studies on the Isolation of a Pure Mitochondrial Fraction

The mitochondrial band in 30-60% (w/w) sucrose gradients was routinely collected after four hours of centrifugation at

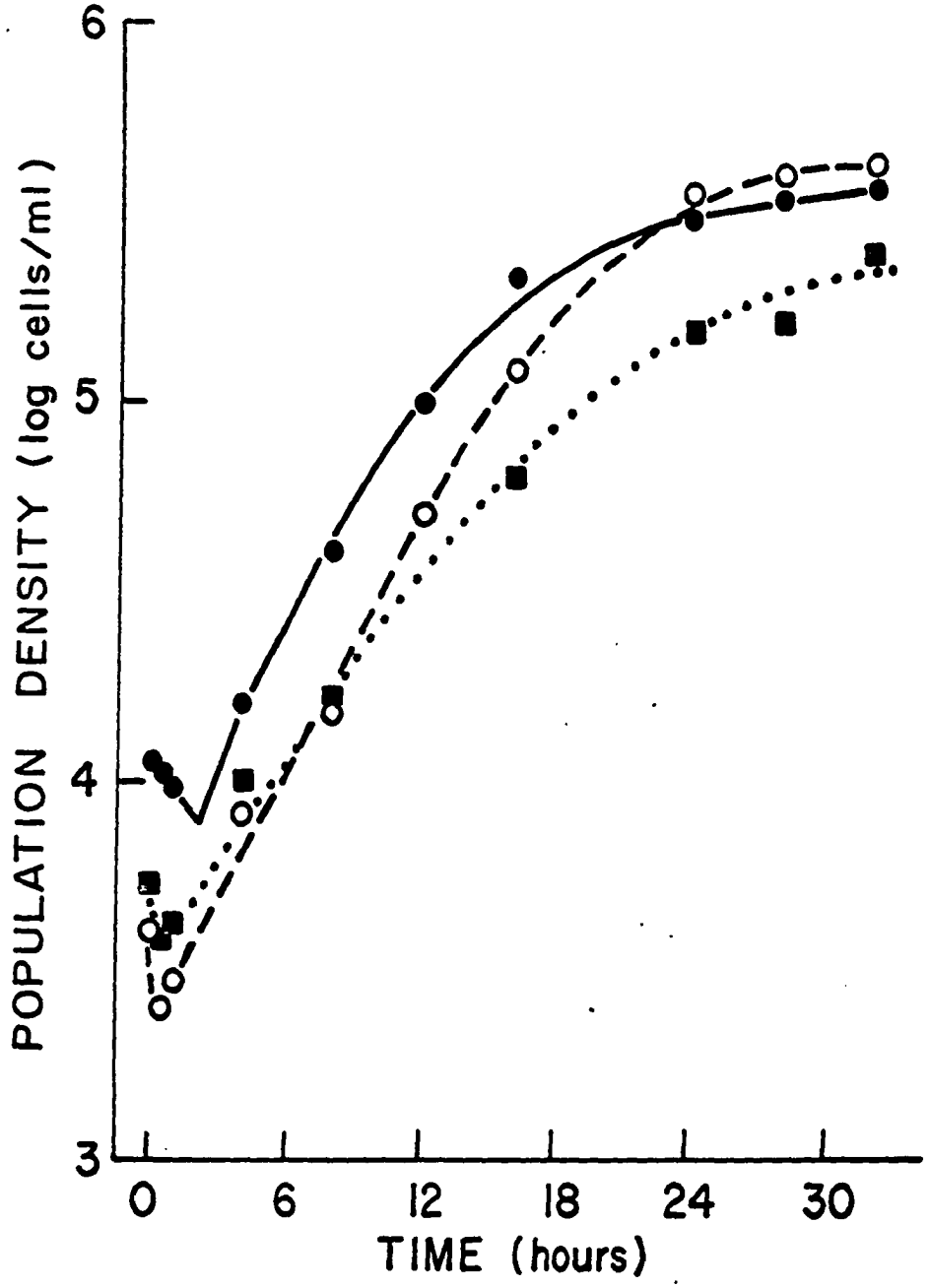


Figure 2

Growth of Tetrahymena pyriformis in various media.
● Medium A, O Medium B, ■ Medium C

25,000 rpm. Observations indicated that the band was stationary after two hours. The SDH activity peak was found to be isopycnic in 48% sucrose (Fig. 3). This corresponds to a density of 1.22. Brightwell et al. (1968) found a density of 1.21 for mitochondria.

SDH activity was associated primarily with fractions 21-32 in the zonal gradient with smaller amounts in fractions 42 and 54 (Fig. 4). There was considerable activity on the meniscus. Electron micrographs indicated that fractions 21-24 and 37-39 (Plates 1 & 2) contained large quantities of mitochondria, but other organelles were absent. We isolated mitochondrial ribosomes from fractions 24-33 which corresponded to the upper part of the major peak of SDH activity.

Catalase activity was present in fractions 48-60 and the meniscus. Electron micrographs indicated that fractions 51-54 (Plate 3) contained mitochondria and peroxisomes, with no other organelles.

Electron micrographs indicated that fractions 66-69 and 76-80 (Plates 4 & 5) contained mitochondrial fragments, peroxisomes, other organelles and fragments of membranes.

Studies on Subunit Preparation

Preliminary attempts to dissociate the monosomes by reducting Mg^{++} concentration failed to yield subunits. Increasing the monovalent cation resulted in the breakdown of polysomes into monosomes and, subsequently, into the subunits. The extent of breakdown into subunits is proportional to the

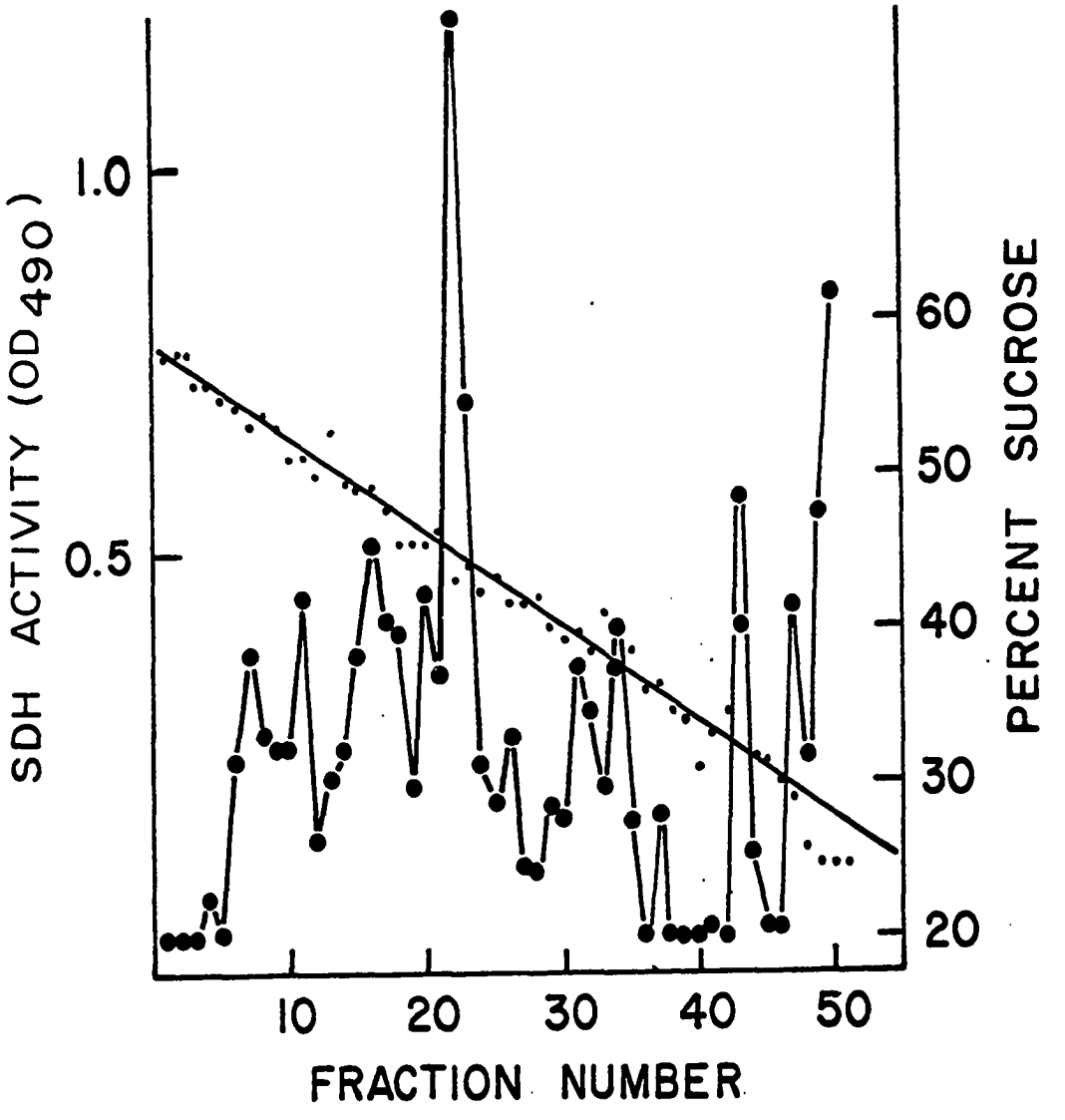


Figure 3

The location of succinic dehydrogenase activity in a 30-60% linear sucrose density gradient after centrifugation for four hours in the SW27 rotor. The optimal density readings indicate the concentration of INT formazan formed in 10 min at 37°C in the presence of 50 mM succinate and INT.

• % sucrose ● SDH activity

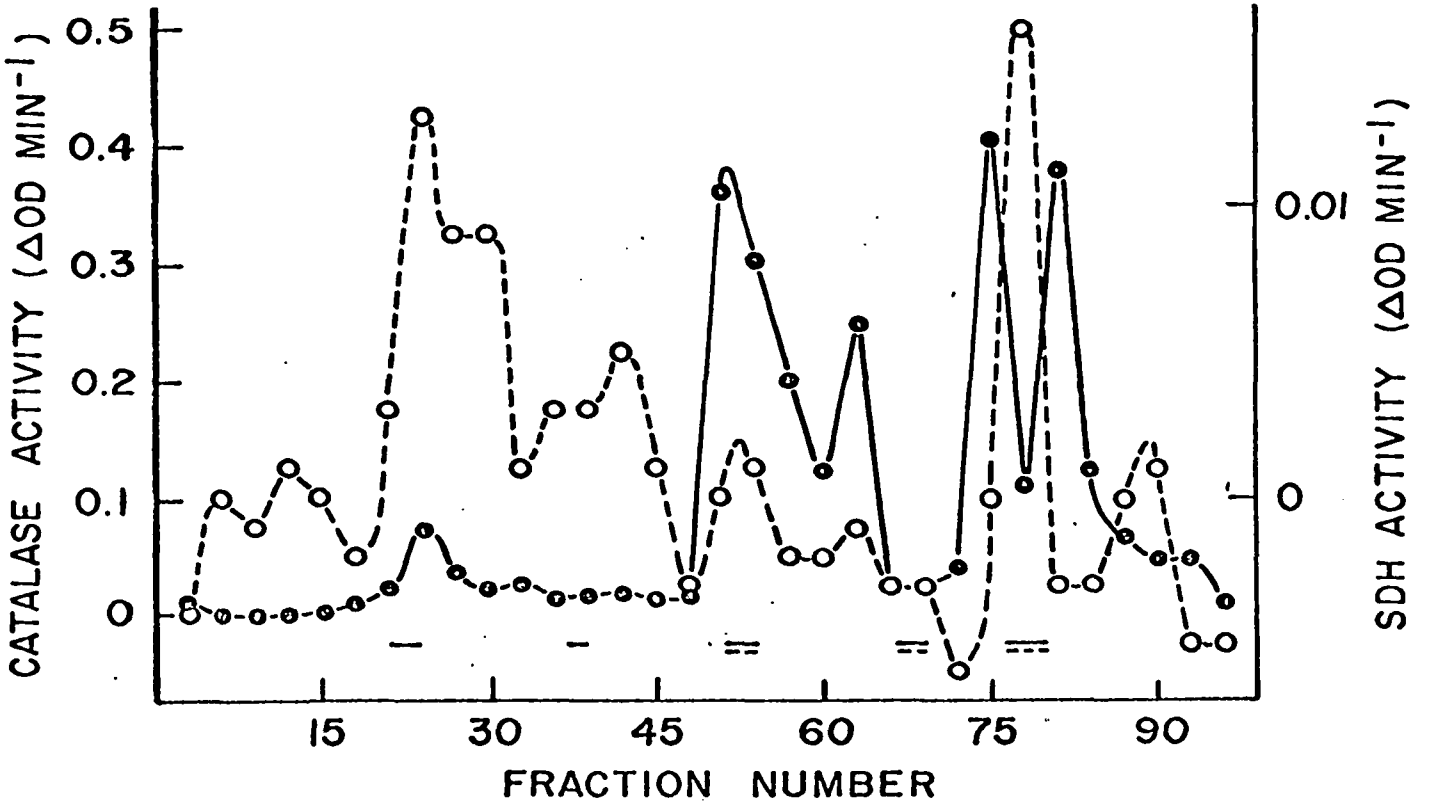


Figure 4

The location of catalase and succinic dehydrogenase activity after centrifugation for 16 hours in a step gradient in a Z-15 rotor.
—●— Catalase activity, --O-- SDH activity, —— fractions containing mitochondria, — — — fractions containing peroxisomes.



Plate 1

Electron micrograph of material from pooled fractions 21-24 (Fig. 4) of an isopycnic sucrose density step gradient in the Z-15 zonal rotor. The mitochondria (m) of T. pyriformis have tubular cristae. X 41,000.



Plate 2

Electron micrograph of material from pooled fractions 37-39 (Fig. 4) of an isopycnic sucrose density step gradient in the Z-15 zonal rotor. The mitochondria (m) lost most of their cristae during preparation. X 31,000.



Plate 3

Electron micrograph of material from pooled fractions 51-54 (Fig. 4) of an isopycnic sucrose density step gradient in the Z-15 zonal rotor. The mitochondria (m) lost many of their cristae during preparation. The peroxisome (p) has a dense nucleoid. X 41,000.



Plate 4

Electron micrograph of material from pooled fractions 66-69 (Fig. 4) of an isopycnic sucrose density step gradient in the Z-15 zonal rotor. Membrane fragments containing basal bodies (b) and a few mitochondrial fragments (mf) are seen on the meniscus. X 20,000.



Plate 5

Electron micrograph of material from pooled fractions 76-80 (Fig. 4) of an isopycnic sucrose density step gradient in the Z-15 zonal rotor. A few mitochondrial fragments (mf) are seen on the meniscus. X 20,000.

amount of monovalent cation present (Figs. 5 & 6). Each of the three monovalent cations examined can produce the same effect. Very little difference in dissociation behavior was seen when the dissociation medium contained the same concentrations of Li^+, NH_4^+ or K^+ (Fig. 7). Five-hundred mM monovalent cation was required to consistently induce dissociation of the mitochondrial ribosomes into subunits. Four-hundred mM KCl would consistently produce subunits in the cytoplasmic ribosomes.

Sedimentation Coefficients of the Ribosomal Particles

The sedimentation coefficient of the cytoplasmic monosome was 82.0S as determined by analytical boundary sedimentation. This value is in essential agreement with those of Whitson, Padilla and Fisher (1966) who reported a value of 82S, Leick and Plesner (1968a) who reported 80S and Chi and Suyama (1970) who reported 80S. The mitochondrial monosome has a sedimentation coefficient of 72.3S. This differs substantially from the values of 80S reported by Chi and Suyama (1970) and Curgy et al. (1974).

Subunits of the cytoplasmic ribosomes have sedimentation coefficients (Fig. 8) of 56.8 ± 1.3 ($\bar{x} \pm$ 1 SD, n=19) and 34.0 ± 1.7 ($\bar{x} \pm$ 1 SD, n=23), which is significantly different from the values of 50S and 30S reported by Leick and Plesner (1968a) and Leick and Plesner (1968b), and the 60S and 40S reported by Chi and Suyama (1970). Subunits of the mitochondrial ribosomes have sedimentation coefficients (Fig. 8)

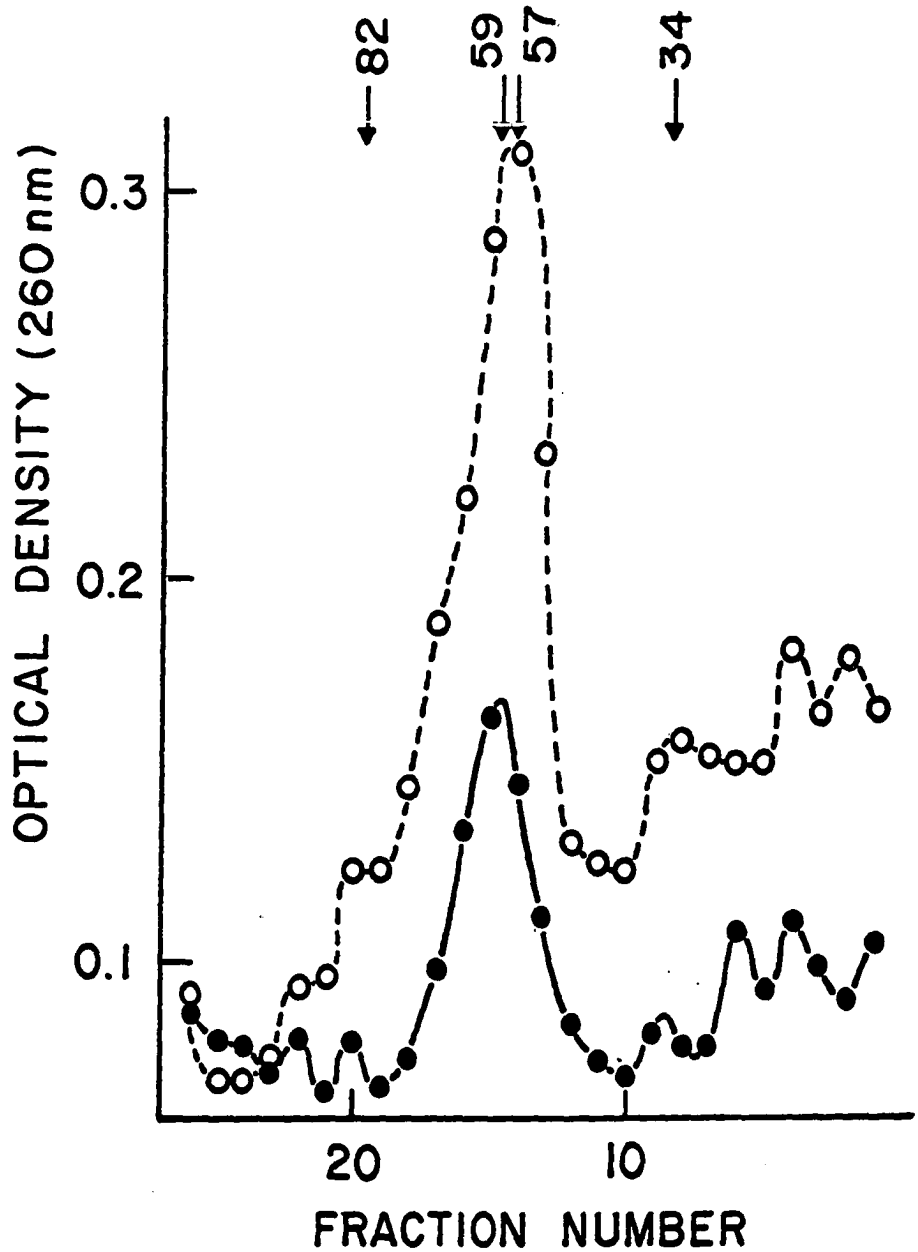


Figure 5

The effect of 200 and 400 mM KCl on the cytoplasmic ribosome of Tetrahymena pyriformis.
● 200 mM KCL, ○ 400 mM KCL

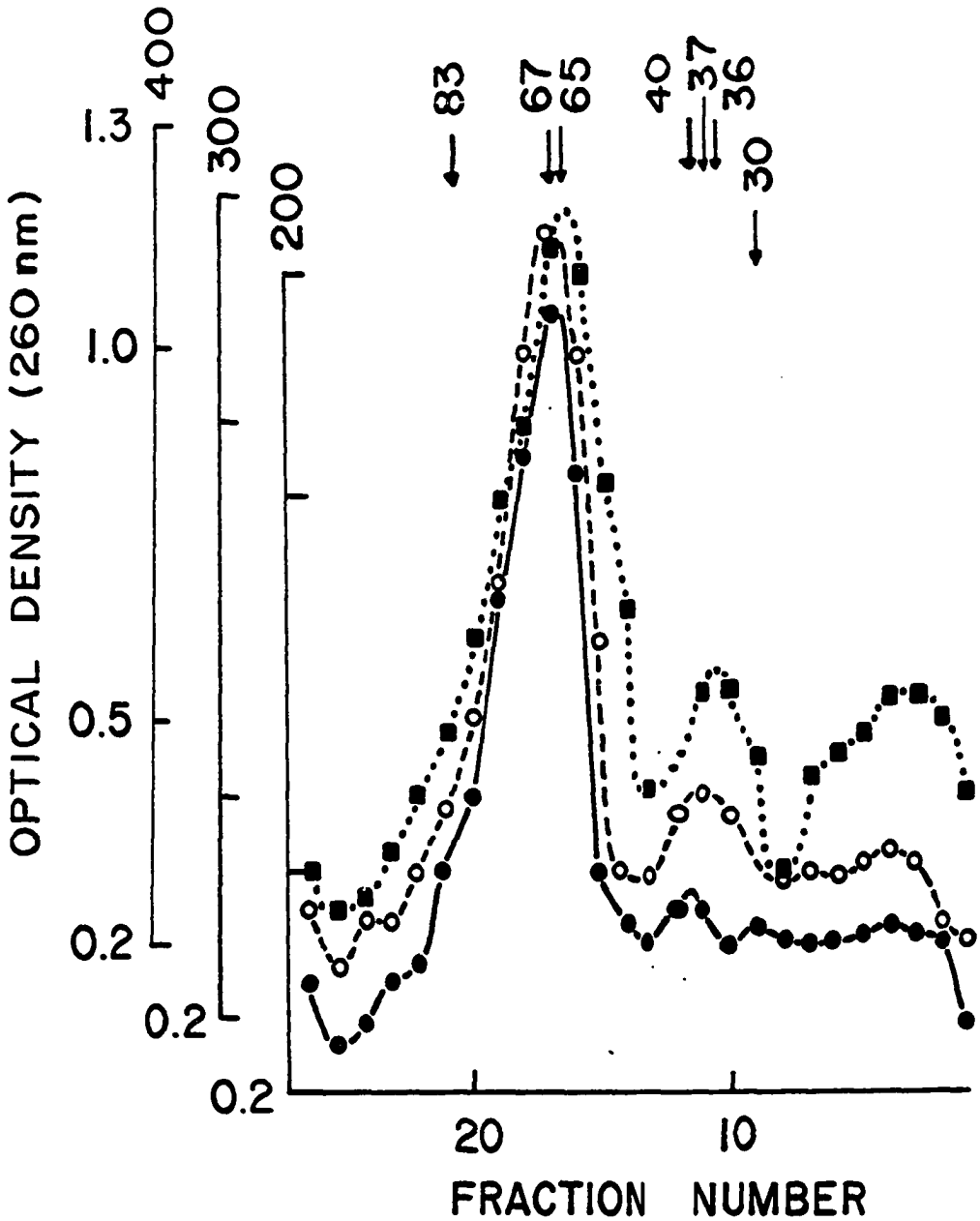


Figure 6

The effect of 200, 300 and 400 mM KCl on the mitochondrial ribosome of Tetrahymena pyriformis.
● 200 mM KCl, ○300 mM KCl, ■400 mM KCl

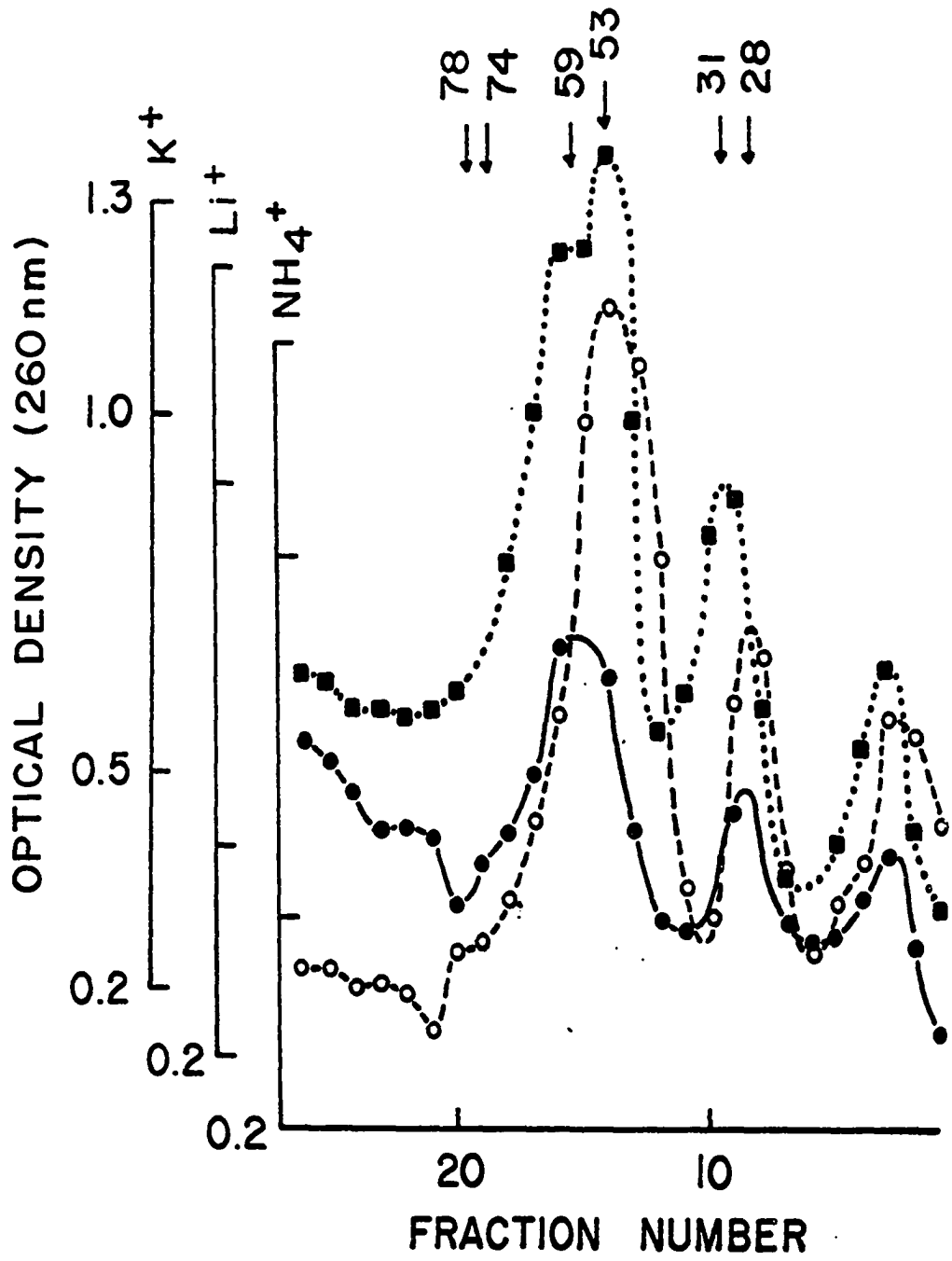


Figure 7

The effect of 500 mM LiCl, 500 mM NH_4Cl and 500 mM KCl on the mitochondrial ribosome of Tetrahymena pyriformis.
○ 500 mM LiCl, ● 500 mM NH_4Cl, ■ 500 mM KCl

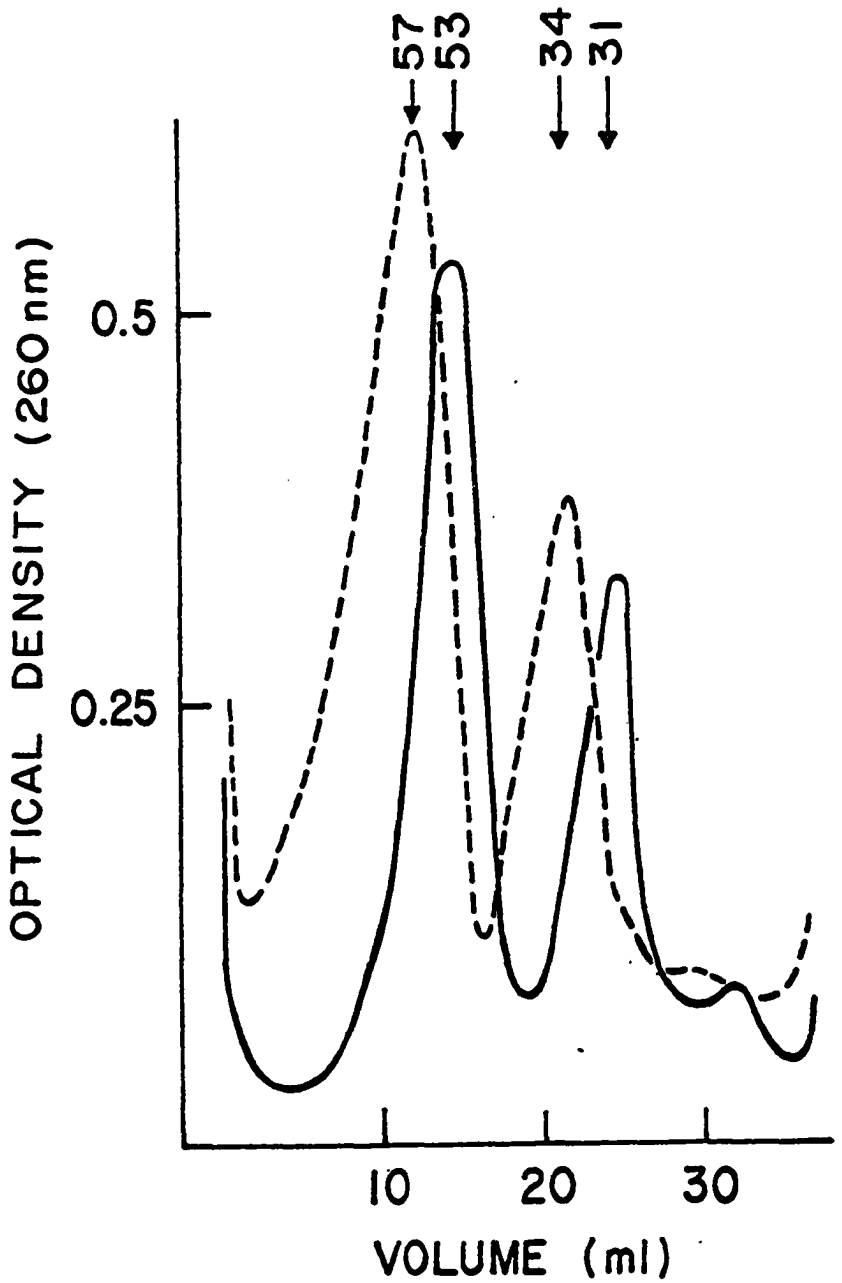


Figure 8

The subunits of cytoplasmic and mitochondrial ribosomes after centrifugation for 11 hours 25 min in isokinetic sucrose gradients in the SW27 rotor.
—— mitochondrial subunits in 500 mM KCl,
---- cytoplasmic subunits in 400 mM KCl.

of 53.3 ± 2.6 ($\bar{x} \pm$ 1 SD, n=38) and 31.4 ± 2.5 ($\bar{x} \pm$ 1 SD, n=39). Chi and Suyama (1970) found one peak at 55S which contained two particles of different densities. The two particles were presumed to contain one rRNA molecule each.

Sedimentation Coefficients of the Ribosomal Ribonucleic Acids

Sedimentation coefficients of 26.0 and 16.4 were obtained for RNA isolated from the cytoplasmic supernatant (Fig. 9). Leick and Plesner (1968a) and Leick and Plesner (1968b) found values of 25S and 17S. Sedimentation coefficients of 26.1 and 15.4 were obtained from RNA isolated from mitochondria (Fig. 10). This differs significantly from the 21S and 14S particles found by Chi and Suyama (1970).

Incubation of the 31.4S Particle with DNase

Incubation of the 31.4S particle with DNase resulted in the disappearance of a shoulder of the 31.4S peak (Fig. 11). Most of the peak was insensitive to DNase degradation. These results agree with the conclusions of Chi and Suyama (1970) that a DNA that sediments at 30S is produced under certain conditions of high ionic concentration. However, they contradict their conclusion that there is no 30S mitochondrial subunit.

Ribosomal Proteins of the Subunits

The small subunit of the cytoplasmic ribosome contains 22 proteins, and the large subunit contains 21 proteins. Ten of the proteins of the small subunit have Rf values similar

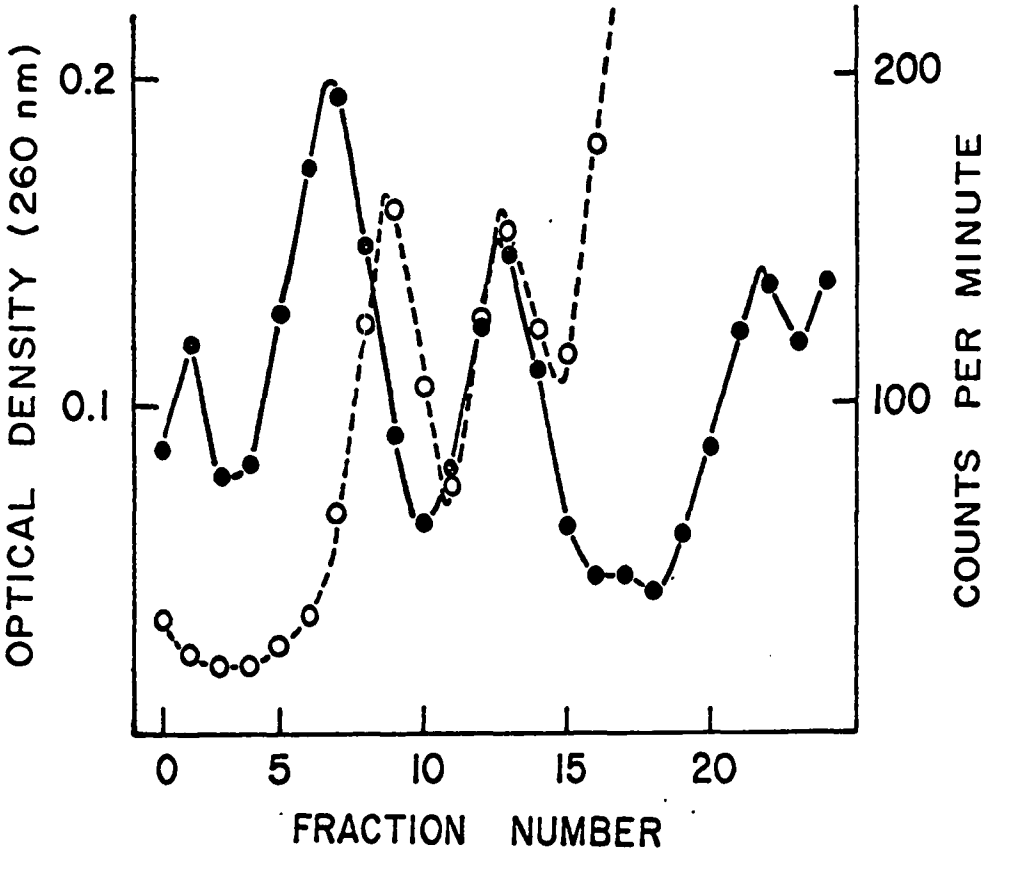


Figure 9

Sedimentation of RNA isolated from the cytoplasmic fraction of Tetrahymena pyriformis in isokinetic sucrose density gradients. The radioactivity is from Escherichia coli ribosomal RNA that had been labelled with ^{3}H-uridine.
—— OD_{260}; – – CPM

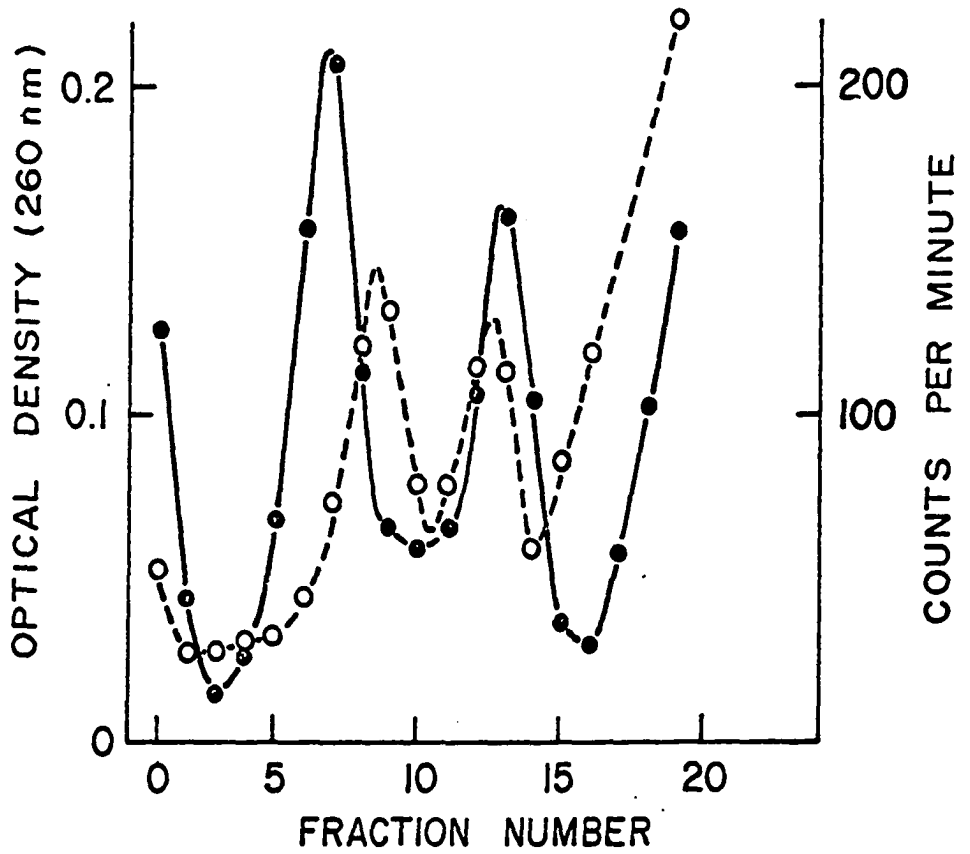


Figure 10

Sedimentation of RNA isolated from the mitochondrial fraction of Tetrahymena pyriformis in isokinetic sucrose density gradients. The radioactivity is from Escherichia coli ribosomal RNA that had been labelled with ^{3}H-urudine.
—— OD_{260}; – – CPM

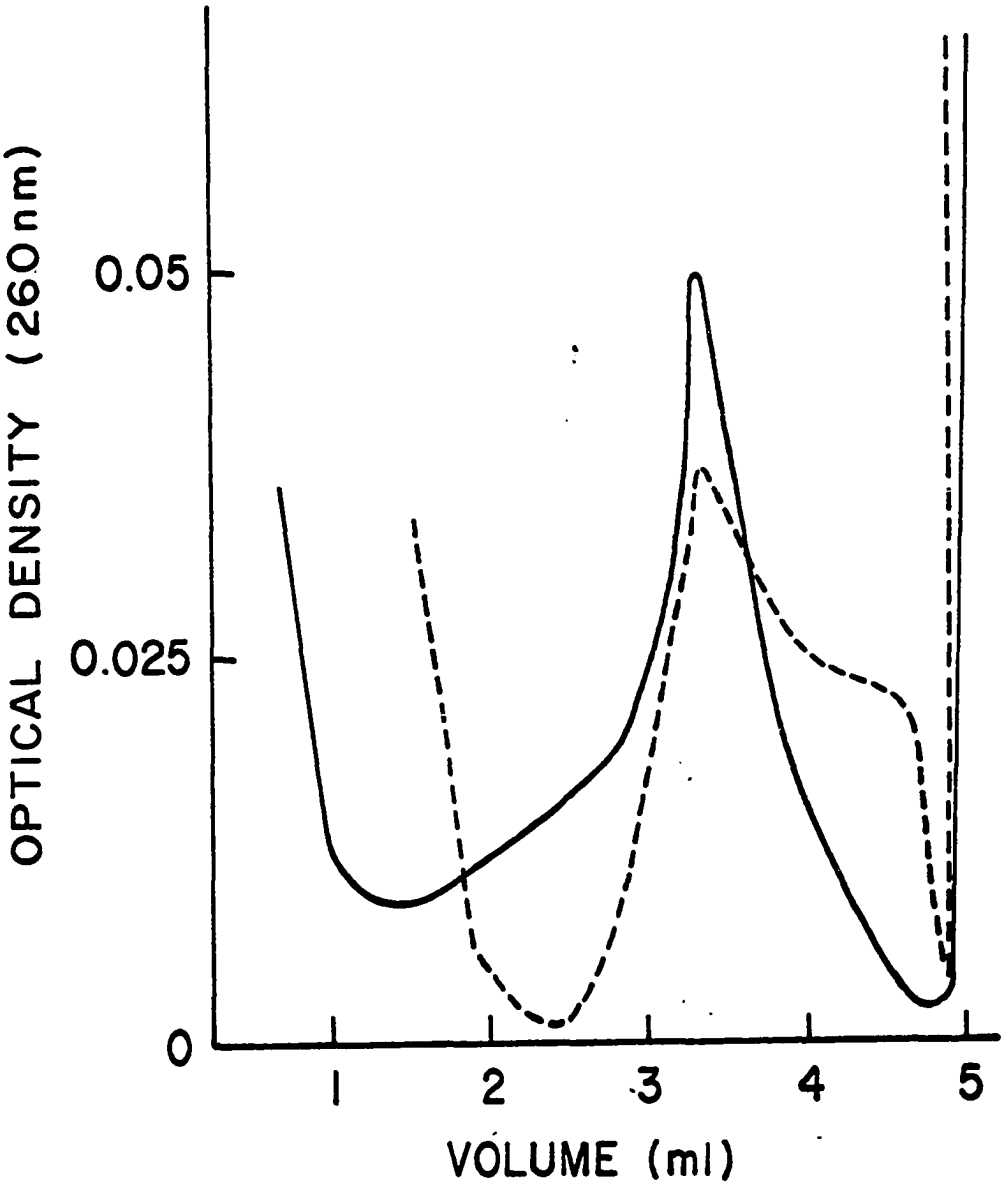


Figure 11

The effect of Dnase on the small subunit of the mitochondrial ribosome of Tetrahymena pyriformis.
– – control, —— DNase-treated

to those of the large subunit, but the subunits were extracted from the gradients in a manner that would preclude any contamination of the proteins of one class of subunits with those of another (Plate 6). Chi and Suyama (1970) found 27 proteins in the cytoplasmic monosome.

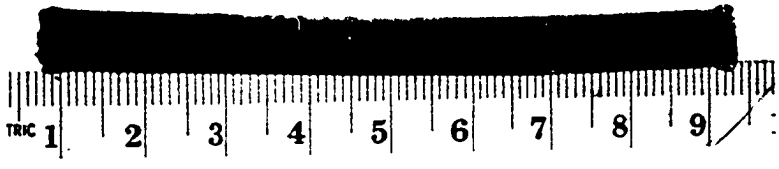
Twenty-three proteins were isolated from the large subunit of the mitochondrial ribosome (Plate 6). Nineteen proteins were isolated from the small subunit. Chi and Suyama (1970) found 28 proteins in the mitochondrial monosome, of which only 22 were consistently extracted.

Plate 6

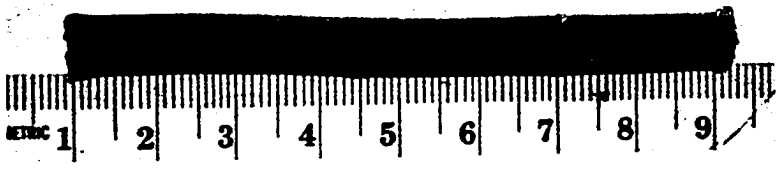
Separation of proteins extracted from ribosomal subunits of T. pyriformis by polyacrylamide gel electrophoresis. Proteins were extracted into 4.0 M urea, 2.0 M LiCl and precipitated with 4% TCA. 50 µg of protein were applied to 10% gels and run in B-alanine buffer, pH 4.5, at 4°C for 5 hours. A current of 2.5 ma/gel was applied. (a) proteins of the 56.8S cytoplasmic subunit, (b) proteins of the 34.0S cytoplasmic subunit, (c) proteins of the 53.3S mitochondrial subunit, (d) proteins of the 31.4S mitochondrial subunit.


1 2 3 4 5 6 7 8 9 10

a


1 2 3 4 5 6 7 8 9

b


METRIC 1 2 3 4 5 6 7 8 9

c


METRIC 1 2 3 4 5 6 7 8 9

d

CHAPTER IV

DISCUSSION

The ribosomes of procaryotes and the mitochondrial ribosomes of some eucaryotes are similar in size, mechanisms of initiation, processing of rRNA precursors and responses to antibiotics. The similarity in structure and function lends support to the hypothesis that mitochondria originated as aerobic procaryots which became symbiotic with an anaerobic eucaryote.

The sedimentation coefficients of mitochondrial ribosomes of plants and fungi more closely approximate the 70S sedimentation coefficients of procaryotic ribosomes than the 80S sedimentation coefficients of the cytoplasmic ribosomes of eucaryotes. The sedimentation coefficients of the mitochondrial ribosomes of many higher animals have been reported to be in the 55S-60S range. These ribosomes dissociate into subunits with sedimentation coefficients of 35S-25S and contain 12S and 20S rRNA which are considerably smaller than those from larger ribosomes. This has resulted in their assignment to a "mini-ribosome" class.

Although many reports of the occurrence of miniribosomes and mini-ribonucleic acids in mitochondria have been

made, reinvestigation of some of them indicated that a larger ribosome could be found which contained higher molecular weight rRNAs. In one such study, the 55S ribosome was reported to contain only degraded rRNA (Borst and Grivell, 1971). O'Brien and Kalf reported in 1967 that rat liver mitochondria contained 55S ribosomes. O'Brien subsequently reported (1969) that the ribosome was 70S. Hamster mitochondrial rRNAs have been reported to have sedimentation coefficients of 17S and 13S (Borst and Grivell, 1971). Larger hamster mitochondrial rRNAs which have sedimentation coefficients of 27S and 17S (Dubin and Brown, 1967) and 28S and 18S (Dubin and Montenecourt, 1970) have been reported.

Sacchi et al. (1973) and de Vries and van der Koogh-Schuuring (1973) reported that the 55S miniribosome has a density in CsCl gradients (1.42-1.45) which is less than that of other ribosomes measured by the same method (1.5-1.6). Chua and Luck (1974) calculated from these densities that the miniribosome would have 70% protein. They were able to calculate from the molecular weight of the RNA and the estimate of protein content that the molecular mass of the miniribosomes of Xenopus laevis would be greater than that of the ribosomes of E. coli.

The rRNA processing of mitochondria proceeds in a manner more characteristic of procaryotic rRNA processing than cytoplasmic rRNA processing in eucaryotes. A 32S precursor to both 25S and 19S rRNA was reported by Kuriyama and Luck (1973). This closely resembles the situation in E. coli

in which a 30S precursor contains the 23S and 16S rRNA (Schlessinger, 1974). The processing of cytoplasmic rRNA in eucaryotes, on the other hand, involves larger precursor molecules. A larger fraction of the precursor is turned over within the nucleus and is not incorporated into the mature rRNA.

The rRNA of mitochondria is less methylated than that of procaryotes and the cytoplasmic rRNA of eucaryotes. The methylation that exists is predominantly base methylation rather than ribose methylation (Klootwijk, Klein and Grivell, 1975; Borst and Grivell, 1971). Recently, Klootwijk, Klein and Grivell (1975) found a yeast mutant in which mitochondrial protein synthesis is not susceptible to inhibition by chloramphenicol is also characterized by lack of methylation. The mitochondrial ribosome is not as sensitive to chloramphenicol as is the ribosome of procaryotes (Allen and Suyama, 1972). The reduced sensitivity may be due to the reduced methylation.

Initiation of protein synthesis by mitochondria and procaryotes requires formyl-methionyl transfer RNA (Smith and Marcker, 1968; Epler, Shugart and Barnett, 1970; Galper and Darnell, 1969). The cytoplasmic ribosome requires methionyl transfer RNA for initiation. Initiation factors 1 and 2 and elongation factors Tu and G isolated from mitochondria of Neurospora are active in the polyuridine-directed synthesis of polyphenylalanine by E. coli ribosomes (Ciferri and Parisi, 1970; Grandi and Kuntzel, 1970; Borst and Grivell, 1971). Elongation factor Ts was inactive in this capacity,

possibly due to lability (Grandi and Kuntzel, 1970). Interchangeability of mitochondrial ribosomal proteins with those of procaryotes has not been demonstrated. Mitochondrial ribosomes of T. pyriformis are sensitive to inhibition of protein synthesis by chloramphenicol (Allen and Suyama, 1972).

The sedimentation coefficients of the mitochondrial rRNA of T. pyriformis that have been reported resemble those that would be expected from miniribosomes, although the mitochondrial ribosome was reported to be larger. Our investigations indicate that there is little difference in the sedimentation coefficients of the mitochondrial rRNA and cytoplasmic rRNA. A sedimentation coefficient of 80.0S was obtained for the cytoplasmic ribosome which is in essential agreement with the reports of 80S by Chi and Suyama (1970) and Leick and Plesner (1968a) and 82S by Whitson, Padilla and Fisher (1966). The sedimentation coefficients of the subunits were 56.8S and 34.0S. Chi and Suyama (1970) found sedimentation coefficients of 60S and 40S, but Leick and Plesner (1968a) and Leick and Plesner (1968b) found sedimentation coefficients of 50S and 30S. However, Leick and Plesner (1968a) and Leick and Plesner (1968b) indicated the presence of 60S and 40S subunits which were considered to be precursor to the 50S and 30S subunits.

The 26.0S and 16.4S sedimentation coefficients of the cytoplasmic rRNA which we found were in essential agreement with the reports of Mita (1965), Leick and Plesner (1968a),

Leick and Plesner (1968b), and Chi and Suyama (1970). However, Chi and Suyama reported a 1:10 ratio of 25S to 17S. We were able to obtain a 2:1 ratio of 26S to 16.4S in the presence of 500 mM NaCl. This is consistent with the report by Bostock, Prescott and Lauth (1971) that 500 mM NaCl was required to isolate undegraded rRNA due to the extreme lability of the 26S rRNA.

The sedimentation coefficient of the mitochondrial ribosome is 72.3S, which is significantly different from the 80S value reported by Chi and Suyama (1970) and Curgy et al. (1974). Curgy et al. (1974) reported that the mitochondrial ribosome was larger than the cytoplasmic ribosome of T. pyriformis and had a more elongate shape. In another report, the mitochondrial ribosome has been reported to be smaller than the cytoplasmic ribosome in electron micrographs (Swift, Adams and Larsen, 1964). Borst and Grivell (1971) predicted that a sedimentation coefficient of about 70S would eventually be found for the mitochondrial ribosome. This prediction was based on the observation that the mitochondrial ribosomes appear smaller than the cytoplasmic ribosomes in electron micrographs.

We were able to resolve the mitochondrial ribosome into 53.8S and 31.4S subunits. Chi and Suyama (1970) found only a 55S peak which contained two particles which were distinguishable by their different densities in CsCl. Curgy et al. (1974) reported that the 55S components migrated coincidentally on polyacrylamide gel electrophoresis. In

both reports, EDTA was used to dissociate the monosome. We were able to produce similar results by treating the monosome with 100 μM EDTA or by reducing the Mg^{++} concentration to 50 μM. However, we have concluded that these results do not represent dissociation of the monosome into subunits.

Chi and Suyama (1970) reported a peak of 35S to 45S which appeared in the presence of 1 M KCl, which was thought to be DNA on the basis of its density in CsCl. The appearance of this peak was irregular. Curgy et al. (1974) reported the presence of a DNA peak in polyacrylamide gel electrophoresis of RNA extracted from whole mitochondria which migrated more slowly than the large mitochondrial rRNA. The 31.4S peak which we found appeared consistently in isokinetic gradients that contained 500 mM KCl. The 31.4S peak remained after the treatment with DNase. The disappearance of a shoulder which existed in the control indicates the presence of heterogeneous DNA which is isolated by our procedure and which sediments coincident with the small subunit.

The sedimentation coefficients of 26.1S and 15.4S which we obtained for the mitochondrial rRNA were significantly different from the values reported by Chi and Suyama (1970). The values were sufficiently similar to the values for cytoplasmic rRNA to raise the question of contamination. We feel that we have demonstrated that we extracted ribosomes from mitochondrial fractions that were free from contaminants likely to contain cytoplasmic ribosomes. Curgy et al. (1974)

indicated that the small rRNA of the mitochondrial ribosomes and the cytoplasmic ribosomes migrated coincidentally in polyacrylamide gel electrophoresis. We have found that the small rRNAs of both ribosomes are similar, but not identical, in size. Curgy et al. (1974) reported that the large cytoplasmic rRNA is larger than the large mitochondrial rRNA. We found no significant difference in size. This would suggest that: (1) their mitochondrial rRNA may be slightly degraded, (2) our cytoplasmic rRNA may be slightly degraded or (3) this represents a difference in strains of T. pyriformis. Both the rRNA that Curgy et al. (1974) obtained and that we obtained manifested 2:1 ratios. This would indicate minimal degradation. The rRNA sizes reported by Curgy et al. (1974) are more similar to our results than to the results of Chi and Suyama (1970).

Chi and Suyama (1970) extracted mitochondrial and cytoplasmic rRNA in the presence of 300 mM NaCl. We were unable to isolate undegraded rRNA from either ribosome in salt concentrations less than 500 mM NaCl. Bostock, Prescott and Lauth (1971) reported similar results. The mitochondrial rRNA obtained by Chi and Suyama (1970) had a ratio of the 21S to 14S (0.927 and 0.431 X 10^6 daltons, respectively) of 4:9 rather than the usually obtained ratio of 2:1 of the larger to the smaller. A ratio of 2.15:1 would be predicted by the relative size of the RNA molecules that they reported if it is assumed that each subunit contains one RNA molecule. Ratios near 2:1 are generally taken as evidence of undegraded

preparations. They concluded that the rRNA was undegraded since: (1) the isolated mitochondria were capable of incorporation of amino acids into interpolypeptide linkages of mitochondrial proteins, (2) the extracted mitochondrial ribosomes were active in polyuridine-directed phenylalanine synthesis and (3) mitochondrial rRNA exhibited distinct bands in polyacrylamide gel columns.

Our procedure for the isolation of mitochondria is such that several hours elapse between the homogenization of the cells and the preparation of mitochondrial rRNA. Nevertheless, we were able to isolate rRNA from the mitochondria which had a 2:1 ratio characteristic of undegraded rRNA. This suggests that the rRNA remains undegraded while the mitochondria are intact. Our experience indicates that the mitochondrial rRNA is most likely to be degraded during the course of its isolation and purification.

Chua and Luck (1974) suggest that the ratio of proteins to RNA in mitochondrial ribosomes may be higher than in other ribosomes. We were unable to obtain as many proteins as are found in bacterial ribosomes or the cytoplasmic ribosomes of eucaryotes. This would suggest that either the proteins are larger or that we failed to extract all the proteins of the ribosome. The cytoplasmic ribosome of eucaryots usually has 36-50 proteins associated with the larger subunit and 27-33 proteins associated with the small subunit. The procaryotic ribosome usually has 26-31 proteins associated with the small subunit and 37-41 proteins associated with the large subunit.

Leister and Dawid (1975) reported that the mitochondrial ribosome of Xenopus laevis contain about 80 proteins. There appear to be 23 distinct spots associated with two-dimensional gel electrophoresis of the large subunit proteins (Leister and Dawid, 1975).

The possibility that the high salt concentrations used to dissociate the monosomes into subunits may have resulted in the removal of some of the proteins cannot be excluded. Chi and Suyama (1970) suggest that the Spitnik-Elson (1965) technique may not isolate acidic proteins. We were unable to detect acidic proteins in preparations extracted from cytoplasmic or mitochondrial monosomes by the techniques of Berger (1974) or Spitnik-Elson (1965). We were able to obtain proteins from E. coli which migrated in an anionic polyacrylamide gel electrophoresis system by this method. Thus, the absence of acidic proteins cannot be explained by their possible loss during dissociation procedures.

The cytoplasmic ribosomes of eucaryotes are larger than those of procaryotes. They have more and larger proteins (Wool and Stoffler, 1974). They have larger rRNAs. The mitochondrial ribosomes of eucaryotes are usually described as being like those of procaryotes. This relationship is more distinct in plants. The miniribosomes of higher animals differ from procaryotic ribosomes in the physical properties of their components, but such criteria as methylation of rRNA and sensitivity to antibiotics suggest they are basically similar.

The cytoplasmic ribosomes of Tetrahymena pyriformis manifest properties similar to those of other eucaryotes. The mitochondrial ribosome of T. pyriformis has rRNAs which resemble those found in fungi and plants. Mitochondrial rRNAs of some higher animals have been reported to be in this size range (Borst and Grivell, 1971: Dubin and Brown, 1967; Dubin and Montenecourt, 1970).

The number of species, especially in higher animals, in which the mitochondrial ribosome has been characterized is surprisingly small. Speculation about relationships at this time is probably premature.

SUMMARY

The cytoplasmic and mitochondrial ribosomes of Tetrahymena pyriformis (WH-14) have been characterized.

The mitochondrial ribosomes were isolated from purified mitochondrial fractions. The mitochondria were isolated by isopycnic banding in sucrose density gradients. Enzyme assays and electron micrographs confirmed their purity. The mitochondrial monosome has a sedimentation coefficient of 72.3S. The subunits have sedimentation coefficients of 53.3 $\pm$ 2.6 ($\bar{x} \pm 1$ SD, n=38) and 31.4 + 2.5 ($\bar{x} \pm 1$ SD, n=39). The 31.4S subunit has been treated with deoxyribonuclease. This treatment has shown that a portion of the 31.4S peak is DNase sensitive, but most of the peak is DNase resistant. The mitochondrial ribosomal ribonucleic acids have sedimentation coefficients of 26.1S and 15.4S. Nineteen proteins of the small mitochondrial subunit and 23 proteins of the large mitochondrial subunit were extracted and characterized by polyacrylamide gel electrophoresis.

The sedimentation coefficient of the cytoplasmic monosome is 80.0S. The subunits of the cytoplasmic ribosome have sedimentation coefficients of 56.8 $\pm$ 1.3 ($\bar{x} \pm 1$ SD, n=19) and 34.0 $\pm$ 1.7 ($\bar{x} \pm 1$ SD, n=23). The ribosomal ribonucleic acids

of the cytoplasmic ribosome have sedimentation coefficients of 26.0S and 16.4S. Twenty-two proteins of the small cytoplasmic subunit and 21 proteins of the large cytoplasmic subunit were extracted and characterized by polyacrylamide gel electrophoresis.

No acidic proteins were found in either monosome.

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