THE ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF CYTOPLASMIC AND MITOCHONDRIAL RIBOSOMES FROM A WATER-MOLD *ACHLYA*

by

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ABSTRACT

Cytoplasmic and mitochondrial ribosomes from the genus Achlya, a water-mold of the order Saprolegniales, class Oomycetes were isolated, purified, and partially characterised. The sedimentation constants of both the cytoplasmic and mitochondrial ribosomes were found to be 82S, and their subunits 53S and 38S. The stability of the ribosomes with respect to changes in Mg^{++} concentration was also studied. Cytoplasmic ribosomes dissociated readily at 0.1 mM Mg^{++} while mitochondrial ribosomes dissociated into ribosomes only in the presence of EDTA and not by lowering the Mg^{++} concentration. Cytoplasmic ribosomal RNA was isolated and found to be 25S and 18S, using E.coli 23S and 16S ribosomal RNA as standards. Mitochondrial ribosomal RNA's were observed to be very labile, and even the best preparations suffered partial degradation. But experimental evidence suggested they may have 'S' values of 25 and 18. The extreme lability of mitochondrial ribosomal RNA suggested the existence of latent nucleolytic activity in the ribosomes. Preliminary evidence points to the possible existence of a 9S ribosome-bound RNA in the mitochondrial ribosome.

ACKNOWLEDGEMENT

The author wishes to express his most sincere gratitude to Dr. H.B. LeJohn for his assistance, advice and patience throughout the course of this investigation. Sincere appreciation is also extended to Dr. P.Y. Maeba for his helpful suggestions, to Mr. F. Chu for graciously providing *E.coli* ribosomes, and to my wife for her enduring patience in the preparation of this manuscript.

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LIST OF ABBREVIATIONS

RNA	-	Ribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
tRNA	-	Transfer ribonucleic acid
DNA	-	Deoxyribonucleic acid
RNase	-	Ribonuclease
DTT	-	Dithiothreitol
EDTA-Na	-	(Ethylenedinitrilo)tetraacetate, Tetrasodium Salt
Tris	-	Tris (hydroxymethyl)aminomethane
Tris-HCl	-	Tris(hydroxymethyl)aminomethane adjusted to required pH with hydrochloric acid
SDS	-	Sodium dodecyl sulphate

INTRODUCTION

Fourteen years ago, Rendi (1959) studied the effect of chloramphenicol on protein synthesis using isolated subcellular fractions from rat liver. He discovered that chloramphenicol had no effect on the cytoplasmic protein synthesizing systems, but inhibited protein synthesis in the microsomal fraction containing mitochondria. The same phenomenon was observed independently by Mager (1960) with *Tetrahymena pyriformis*, rat and guinea pig livers. This suggested the presence of another protein-synthesizing system in eucaryotic cells apart from the cytoplasmic one.

In the years that followed, this fundamental observation was pursued with fervor. After years of hard work, a general scheme finally emerged. The presence of another protein-synthesizing system in mitochondria differing from the one in cytoplasm was established when a tRNA and aminoacyl-synthetase specific for mitochondria were isolated from *Neurospora crassa* (Barnett et al, 1967). In all eucaryotic organisms studied, the mitochondrial protein-synthesizing system is also inhibited by antibiotics that specifically interfere with bacterial protein synthesis at the ribosome level. For example, tetracycline, lincomycin, erythromycin,

spiramycin and others (Clark-Walker and Linnane, 1966; Lamb et al, 1968; Thomas and Wilkie, 1968; Linnane et al, 1968), but is insensitive to inhibitors of cell-sap protein synthesis that do not interfere with bacterial protein synthesis, like cycloheximide and emetin (Beattie et al, 1967; Perlman and Penman, 1970). Since all the antibiotics mentioned above act on ribosomes (Weisblum and Davies, 1968), the concensus was that mitochondria contain "bacterial-type" ribosomes (Huang et al, 1966).

This hypothesis was further strengthened by studies on the size and sedimentation constants of mitochondrial ribosomes and their respective rRNA's from various plants (Clark et al, 1964; Stutz and Noll, 1967; Rifkin et al, 1967; Kuntzel et al, 1967). Chloroplasts and mitochondria of plants contained polysomes consisting of 70S ribosomes, in contrast to the 80S ribosomes which make up the polysomes of the surrounding cytoplasm. They also showed that chloroplast, mitochondrial and bacterial ribosomes were indistinguishable with respect to the sedimentation behavior of the monomeric particle and the sedimentation coefficients (23S and 17S) of the RNA components corresponding to their 50S and 30S subunits. Neurospora crassa, an ascomycete, was one of the organisms used for the above studies.

Yeast, also a member of the ascomycetes, however, presented different results. Mitochondrial and cytoplasmic ribosomes from this organism have identical sedimentation coefficients (80S) and dissociate into 60S and 40S subunits (Morimoto and Halvorson, 1971). Although there exists this discrepancy in size between yeast mitochondrial ribosomes and bacterial ribosomes, yet peptide chain elongation factors and ribosomes between the two of them are interchangeable and results in active protein synthesis, whereas the cytoplasmic factors cannot be interchanged with ribosomes from mitochondria or bacteria (Richter and Lipmann, 1970). Similar results on interchangeability between mitochondrial and cytoplasmic factors and ribosomes were also obtained from Neurospora crassa (Kuntzel, 1969).

The initiation process in protein synthesis is also strikingly similar between mitochondria and bacteria. Formylmethionyl-tRNA has been found in mitochondria from yeast, Neurospora, rat liver and HeLa cells (Smith and Marcker, 1969; Epler et al, 1970); transformylase activity has been detected in Neurospora mitochondria (Epler et al, 1970). Mitochondrial ribosomes from Neurospora are able to recognise, bind and translocate E.coli formylmethionyl-tRNA in response to the codon AUG, while cytoplasmic ribosomes produced negligible translocation (Sala and Kuntzel, 1970).

A ribosome-bound, low molecular weight RNA, distinct from tRNA, was first discovered in E.coli by Rosset and Monier in 1963. This RNA, which has a sedimentation coefficient of 5S, is now considered to be a universal component of ribosomes. By reconstitution experiments, it has been found that 5S RNA plays an essential structural role in the assembly of active 50S ribosomal subunit (Erdmann et al,1971). In its absence, some functionally important proteins fail to join the reconstituting particle to form an active subunit. It has also been shown that all procaryotic 5S RNA's tested were active in reconstituted 50S subunits, whilst eucaryotic 5S RNA's were not (Wrede and Erdmann, 1973). However, the eucaryotic 5S RNA's used were from cytoplasmic ribosomes, not mitochondrial ribosomes. Isolation of 5S RNA's from mitochondria of Neurospora (Lizardi and Luck, 1971) and other organisms (Borst and Grivell, 1971) has been unsuccessful so far. The data obtained to date show some interesting and striking similarities between mitochondrial ribosomes of ascomycetes and bacterial ribosomes. It would be most interesting, therefore, to see whether an organism from Oomycetes, a group that has been suggested to have evolved independently from most of the rest of the fungi (Bartnicki-Garcia, 1970;

Lovett and Haselbey, 1971; LeJohn, 1972), would complement or contradict the results that have been accumulated so far. It is the purpose of this thesis to act as a first step in the elucidation of these problems.

MATERIALS AND METHODS:

I. Organism

The fungus used is a water-mold, Achlya sp. (1969)*, from Dr. J.S. Lovett, Purdue University.

II. Media, Buffers and Chemicals

a) G_2Y - 5 g glucose, 0.5 g yeast extract in one litre of distilled water.

b) PYG - 2 g peptone, 2 g yeast extract, 3 g glucose in one litre of distilled water.

c) AMT - 100 mM ammonium chloride, 10 mM magnesium chloride, 10 mM Tris-HCl, pH 7.5.

d) Electrophoresis Buffer (0.2% SDS-TEB) - 2.0 g sodium dodecyl sulphate, 10.8 g Tris, 0.93 g Na-EDTA, 5.5 g boric acid in 1 litre of distilled water.

e) Acrylamide - Baker grade, J.T. Baker Chem. Co.

f) Bisacrylamide (N', N'-methylene-bis-acrylamide) - Canal Industrial Corp.

g) Agarose - Biorad Lab, electrophoresis grade.

h) TEMED (tetramethylethylene diamine) - Biorad Lab, electrophoresis grade.

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* Designation given by our laboratory.

i) Sucrose - Baker analysed reagent, J.T. Baker Chem. Co. All sucrose solutions used were autoclaved for 5 min at 10 psi to destroy any contaminating ribonucleases.

III. Growth of Organism

Stock cultures were kept on slants on Cantino's PYG agar (Cantino and Lovett, 1960). Whenever needed, a few mycelial threads were transferred into 20 ml of G_2Y medium dispensed in standard size plastic petri plates, and then incubated at 22 C for 48 hr for complete growth and sporulation. Four to six cell mats were then transferred aseptically into a flask containing 100 ml of distilled water, shaken vigorously for about 1 min, the cell mat removed by filtering through sterile cotton-gauze, and the spore suspension used as inoculum for carboys. Ten Petri plates of cultures were used for each 7-litre PYG medium. The spores were allowed to germinate and grow under forced aeration for 16-18 hr (mid-exponential growth phase), the mycelial threads were then harvested by filtration on Whatman No. 1 filter paper, and washed with

0.44 M sucrose-AMT buffer. About 40 g of cells may be obtained from a single 7-l culture.

IV. Extraction of Ribosomes

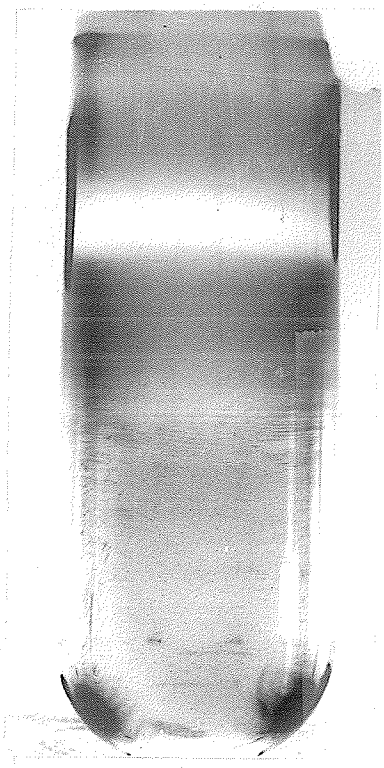
From this point onwards, all glasswares used was acid-washed with 6N hydrochloric acid, then rinsed with deionised millipored water, and heated at 500 C for three hours before use. Disposable plastic gloves were also worn during preparation of materials. Stable buffers and chemicals were autoclaved before use. The precautions taken were to minimise ribonuclease contamination and reduce heavy metal contamination and disruption of RNA.

The freshly collected cell mat was broken up into small pieces, put into a pre-cooled Waring blender, and 4 vol of cold 0.44M sucrose-AMT buffer added. Cells were then homogenized at high speed with 30 sec bursts, 5 times, with 30 sec cooling intervals in between bursts. The homogenate was then filtered through 4 double layers of cotton gauze, and the extract was centrifuged at 2 C at 5,900 X g for 10 min in a Sorvall RC2-B centrifuge to remove cell debris. The supernatant was then

centrifuged at 27,000 X g for 20 min to sediment mitochondria. The post-mitochondrial supernatant was saved for isolation of cytoplasmic ribosomes. The crude mitochondrial pellet was then suspended in 0.44M sucrose-AMT (1 ml per 20 gm wet weight of cells). Two millilitre portions of this suspension was layered on top of 60 ml linear (40% - 60% w/v) sucrose-AMT gradients with 10 ml of 60% sucrose as a cushion. Centrifugation was carried out with a Beckman SW25.2 rotor for 2½ hr at 106,000 X g at 4 C in a Spinco ultracentrifuge. A yellowish mitochondrial band was formed about half way down the gradient and this collected by aspiration from the top using a Pasteur pipette (Fig. 1). To prepare mitochondrial ribosomes, the mitochondrial fraction was diluted with 1½ vol of AMT buffer, containing 5% (v/v) Triton X-100 and mixed thoroughly to lyse the mitochondria. Treatment with Triton X-100 does not lyse any possible contaminating bacteria (Parenti and Margulies, 1967). The lysate was then subjected to centrifugation at 65,000 X g for 20 min, using a Beckman Type 60 Ti rotor, the pellet discarded, and supernatant again centrifuged at 100,000 X g for 1 hr. The translucent pellet (mitochondrial ribosomes) was dissolved in a very small volume of

Figure 1

PURIFICATION OF MITOCHONDRIAL FRACTION BY ISOPYCNIC CENTRIFUGATION IN LINEAR SUCROSE GRADIENT. PROCEDURES AS DESCRIBED IN MATERIALS AND METHODS.


MITOCHONDRIAL
BAND

AMT buffer and kept at -70 C until used.

To prepare cytoplasmic ribosomes, the post-mitochondrial supernatant was centrifuged in a Sorvall RC2-B at 48,200 X g for 20 min, pellet discarded and the supernatant recentrifuged at 100,000 X g for 1 hr in a Spinco ultracentrifuge. The translucent yellow pellet was rinsed with AMT buffer and dissolved in same. After a clarifying spin at 48,200 X g for 20 min, the turbid supernatant containing the cytoplasmic ribosomes was stored at -70 C until used.

V. Biological Activity of Mitochondria

To ensure that the 'mitochondrial' preparations obtained from the sucrose gradients were indeed mitochondrial, their ability to oxidize metabolizable substrates such as glutamate, etc., was monitored with a Gilson's oxygraph. The reaction mixture consisted of 0.3 ml 0.1M Tris-acetate buffer pH 7.0, 0.5 ml distilled water, and 10-20 µl of mitochondrial preparation (depending on the concentration). After endogenous substrates were used up, then the following substrates were added in a total volume of 1 ml: 100 µl of each either 50 mM malate or 50 mM glutamate,

and 10 mM $CaCl_2$. The rate of oxygen uptake was then followed.

VI. Sedimentation Analysis

Determination of the sedimentation values (S) of the two types of ribosomes was achieved using a Beckman Model E analytical ultracentrifuge. Before the analysis, the two types of ribosomes (in AMT buffer) were dialysed against a buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT) for 8 hr at 5 C. To determine the sedimentation values of the subunits of the ribosomes, they were dialysed instead in another buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, 1 mM EDTA, 1 mM DTT. Samples were run at 130,000 X g at 20 C, and after attaining the pre-set speed, Schlieren photographs were taken at 4-min intervals. Approximately 50 O.D. units of ribosomal material (0.5 ml) were used in each run.

VII. Zone Sedimentation Analysis

The effect of Mg^{++} ion concentration on the dissociation and re-association of the subunits of the ribosomes were also studied in zone velocity

centrifugation in isokinetic sucrose gradients (Noll, 1967). Two O.D. units of ribosomes in 20 µl buffer was layered onto 5 ml sucrose density gradients (5 - 20% w/v) containing 10 mM Tris-Cl pH 7.5, 50 mM NH_4Cl and varying Mg^{++} concentrations or EDTA. Centrifugation was carried out with an SW 50.1 rotor in a Spinco L2B-65 ultracentrifuge at 125,000 X g for 1 hr. The absorption spectrum of the gradient was determined at A_{260} in a Gilford model 2400 recording spectrophotometer with a flow cell attachment. For preparation of subunits, 60-ml 5% - 20% linear sucrose gradients containing the same buffer but with 1 mM EDTA added were used. Centrifugation was for 11½ hr at 100,000 X g using an SW 25.2 rotor at 4 C. One and a half ml fractions were collected using an ISCO fraction collector Model 272, and analysed spectrophotometrically at A_{260}. Fractions that belong to the same peak were pooled, adjusted to 10 mM with Mg^{++}, and subunits precipitated by addition of 0.7 vol of -20 C absolute ethanol, and stored at -20 C overnight. The precipitate were then collected by centrifugation, redissolved in a buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, and 5 mM $MgCl_2$, dialysed against the same buffer with

DTT added in the cold for 4 hr. Samples not used immediately were stored at -70 C.

VIII. Extraction of rRNA

Cytoplasmic rRNA were extracted using the method of Lovett and Leaver (1969). But instead of using whole cell extracts, purified cytoplasmic ribosomes were used. This is to eliminate any possible contamination by mitochondrial rRNA. Ribosomal suspensions were added to 5 mls of cold PAS-TNS (6% p-aminosalicylic acid and 1% triisopropylnaphthalene sulfonic acid in 0.01 M Tris-HCl buffer at pH 7.5), 5 ml of buffer saturated redistilled phenol, and mixed rapidly for 10 secs with a vortex mixer. The phases were then separated by centrifugation at 770 X g for 5 min at 0 C, and the upper, clear, aqueous phase re-extracted two more times with phenol by the same procedure. The RNA was precipitated from the aqueous layer with 0.1 volume of 20% potassium acetate (pH 5.0) and 2 volumes of cold absolute ethanol at -15 C, and stored as precipitate at -20 C until used. When necessary, the precipitate was centrifuged at 2,000 X g for 5 min, washed, and 'redissolved' in electrophoresis buffer.

IX. Gel Electrophoresis

RNA or ribosome samples were analysed by method of Peacock and Dingman (1967, 1968). Acrylamide and bisacrylamide were recrystallized before use. The final gel concentration in all cases were 0.5% agarose and 2.5% polyacrylamide unless otherwise specified. Gels were cast in 10 cm X 0.6 cm plexiglass tubes. Between 0.5 and 1.0 O.D. units of either RNA or ribosomes dissolved in 45 µl electrophoresis buffer and 5 µl glycerol were applied to each gel, and electrophoresed at 1 mA/tube for 10 min, then at 5 mA/tube for 70 min at 8 C. At the end of the electrophoretic run, gels were removed and soaked in 1% acetic acid for 1 hr. Absorption profiles of the gels were then obtained by scanning them at 258 nm using either a Joyce-Loebl Chromoscan or a Shimadzu Model MPS-50L Multipurpose Recording Spectrophotometer.

RESULTS AND DISCUSSIONS

I. Purity of Mitochondrial Preparations

To eliminate significant contamination of mitochondrial preparations by cytoplasmic ribosomes we have isolated mitochondria by isopycnic centrifugation in sucrose gradients according to the method by Luck (1963). Preparations of mitochondria thus obtained are essentially pure and free from contamination. The biological activity of the 'mitochondrial' preparations were assessed by measuring their ability to oxidize either glutamate or malate in a Gilson's oxygraph.

II. Physical Properties of Cytoplasmic and Mitochondrial Ribosomes

To determine whether cytoplasmic and mitochondrial ribosomes are different, the sedimentation behavior of ribosome preparations from the two sources were examined using zone velocity centrifugation in isokinetic sucrose gradients (Noll, 1967). Figures 2 and 3 show the sedimentation patterns of mitochondrial and cytoplasmic ribosomes. Both preparations sedimented as strong monomeric peaks. The distance travelled by the monomeric peak down the gradient was the same

Figure 2

SEDIMENTATION PATTERN OF MITOCHONDRIAL RIBOSOMES (MONOSOMES) ON SUCROSE GRADIENTS

One A_{260} O.D. unit of mitochondrial ribosome in 20 µl AMT buffer was layered on a 5 ml 5 - 20% (w/v) linear sucrose gradient (in AMT buffer) and centrifuged at 125,000 X g for 1¼ hr in a Spinco SW50.1 rotor at 4 C. The absorption spectrum of the gradient was determined at A_{260} in a Gilford Model 2400 recording sepctrophotometer with a flow cell attachment.

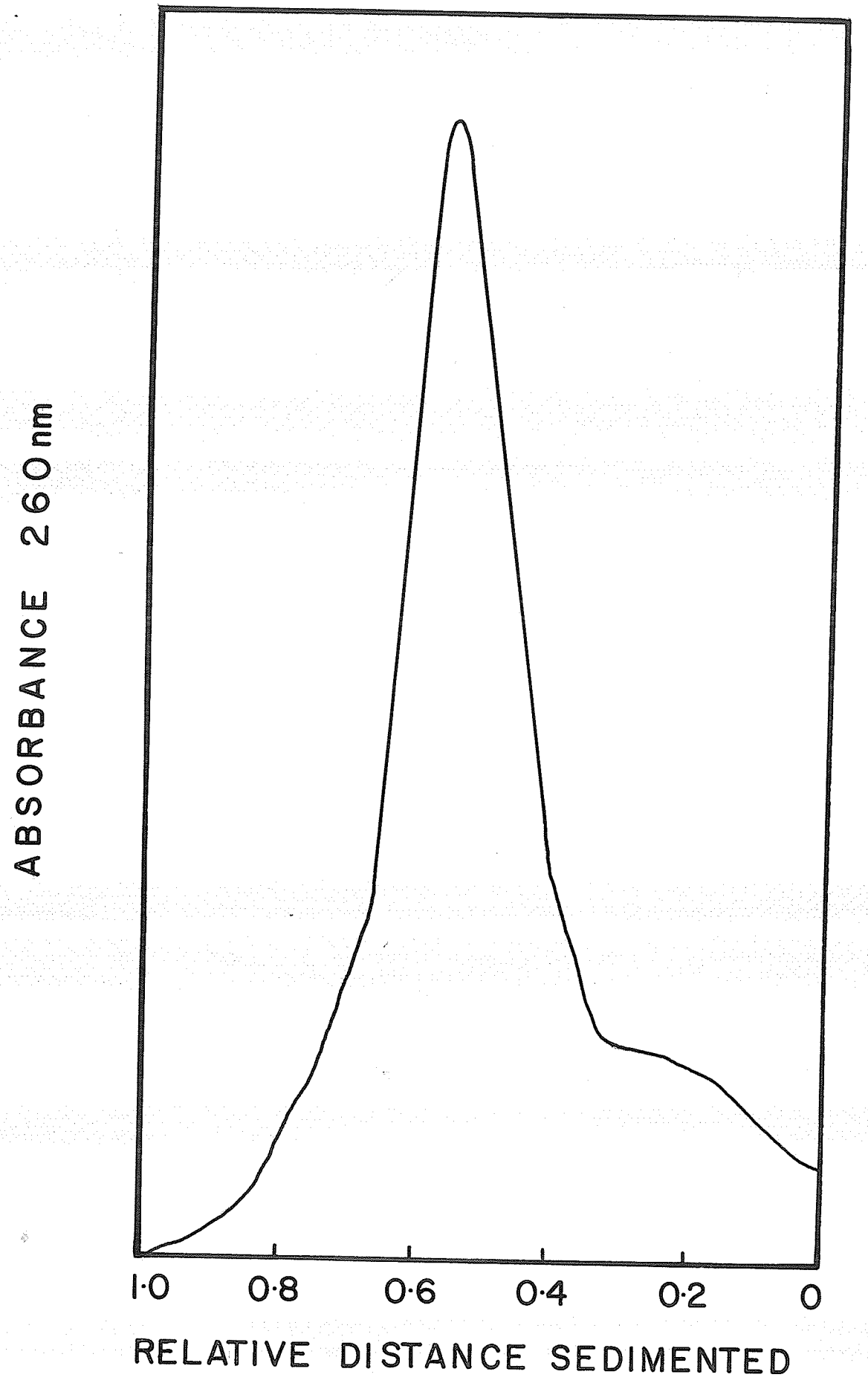
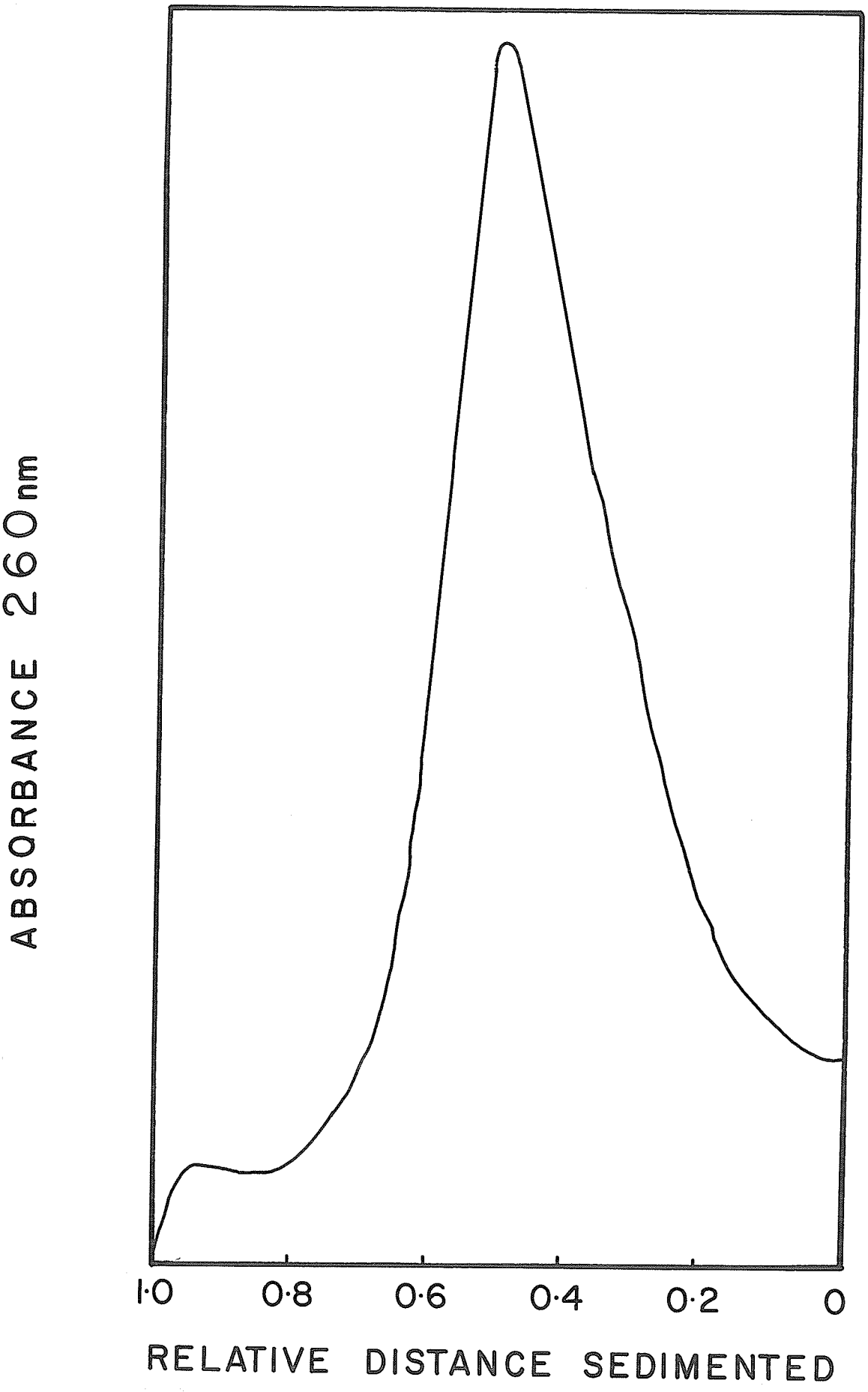

ABSORBANCE 260nm
1·0
0·8
0·6
0·4
0·2
0
RELATIVE DISTANCE SEDIMENTED

Figure 3

SEDIMENTATION PATTERN OF CYTOPLASMIC RIBOSOMES (MONOSOMES) ON SUCROSE GRADIENTS.

Procedures same as in legend of Figure 2.


ABSORBANCE 260nm
1·0
0·8
0·6
0·4
0·2
0
RELATIVE DISTANCE SEDIMENTED

in both. Cosedimentation experiments of cytoplasmic and mitochondrial ribosomes yielded only one single peak. This seemed to suggest that the two types of ribosomes have the same, or very close sedimentation constants. The sensitivities of both types of ribosomes to Mg^{++} ions was also investigated. In Neurospora crassa, mitochondrial ribosomal monomers dissociated into subunits at 2 mM Mg^{++} while cytoplasmic monomers dissociated only when the Mg^{++} concentration in the preparative medium was lowered to 0.1 mM (Rifkin et al, 1967; Kuntzel, 1969). It has also been reported that cytoplasmic ribosomes from Neurospora must be dialysed for at least 18 hr against 0.04 mM Mg^{++} to achieve dissociation (Alberghina and Suskind, 1967). It turned out that the dialysis step is unnecessary for Achlya. Complete dissociation into subunits was achieved by layering CYTOPLASMIC RIBOSOMES suspended in AMT buffer on sucrose gradients containing 0.1 mM Mg^{++} and centrifuging for 1 hr at 125,000 X g in Spinco SW50.1 rotor (Fig. 5). But to our surprise, the MITOCHONDRIAL RIBOSOMES did not dissociate into its subunits under the same conditions. They remained as monomers even when the gradient contained no Mg^{++}. Only when the

Figure 4

SEDIMENTATION PATTERN OF MITOCHONDRIAL RIBOSOMAL SUBUNITS ON SUCROSE GRADIENTS

1.5 A_{260} O.D. units of ribosomes suspended in 20 µl of 0.1 mM Mg - AT buffer was layered on a 5 ml 5 - 20% (w/v) linear sucrose gradient (in buffer containing 0.1 mM Mg^{++} - AT and 1 mM EDTA), and centrifuged at 125,000 X g for 1¾ hr in a Spinco SW50.1 rotor at 4 C. The absorption spectrum of the gradient was analysed at A_{260} in a Gilford Model 2400 recording spectrophotometer.

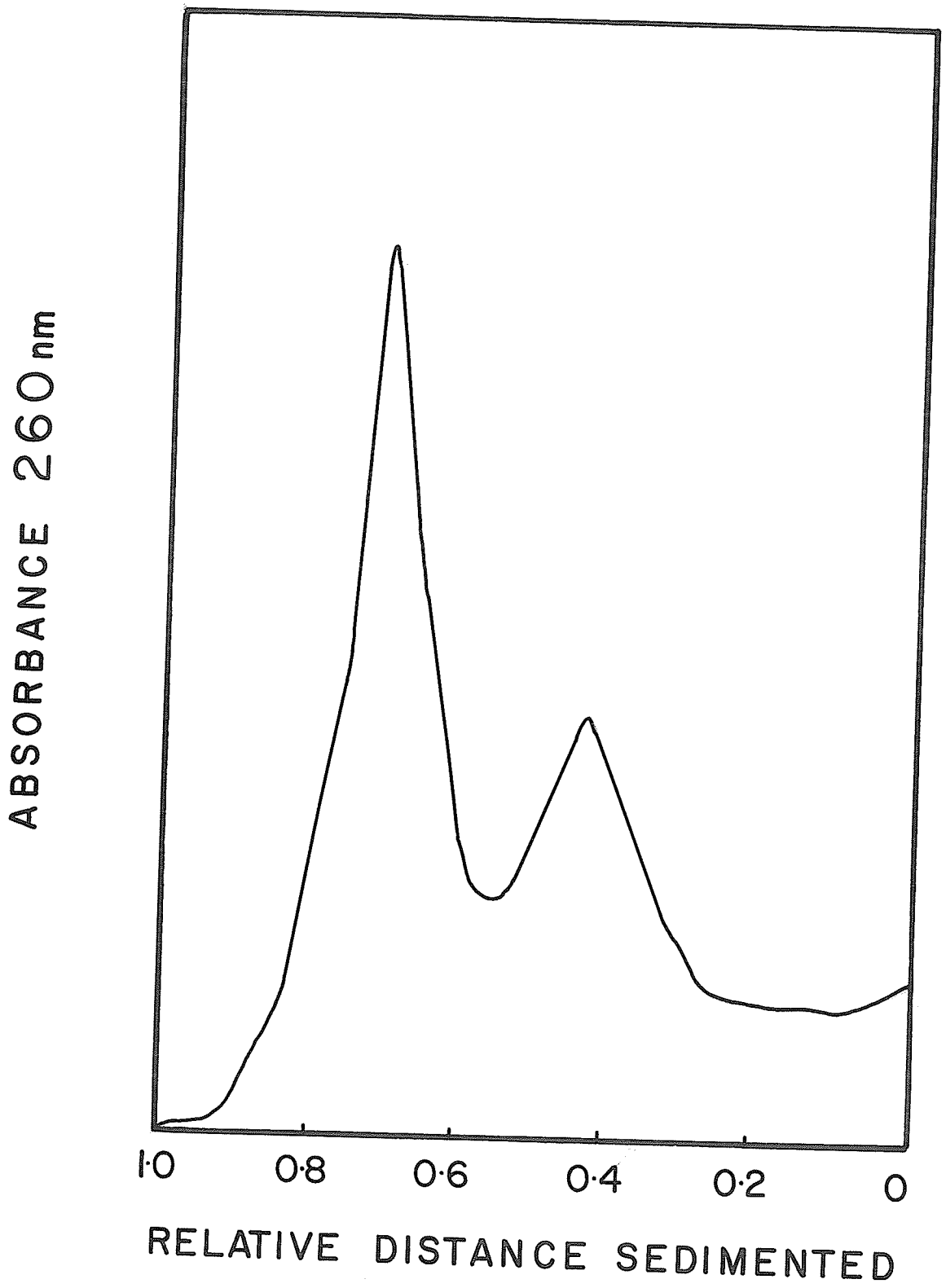
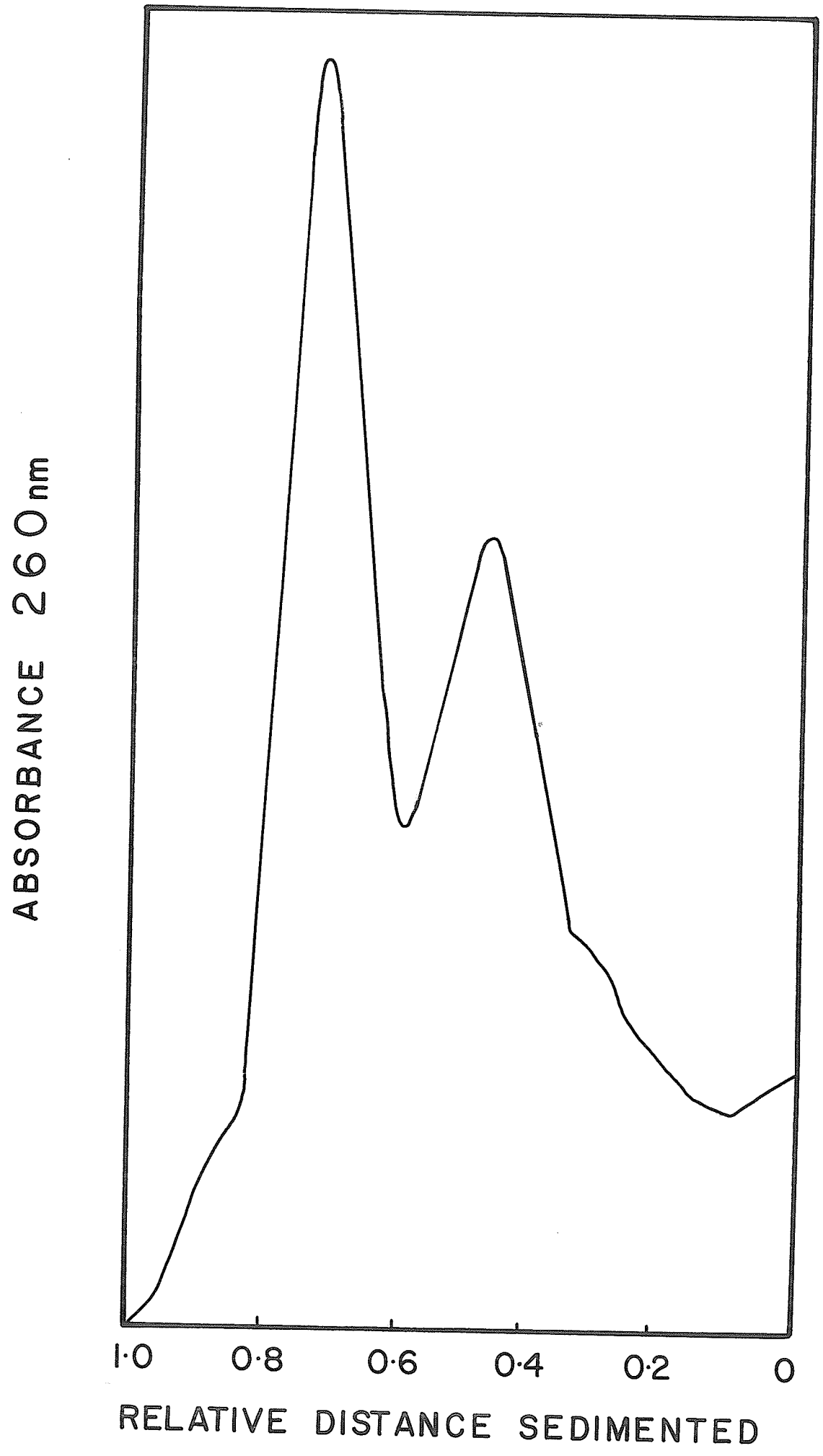

ABSORBANCE 260nm
1·0
0·8
0·6
0·4
0·2
0
RELATIVE DISTANCE SEDIMENTED

Figure 5

SEDIMENTATION PATTERN OF CYTOPLASMIC RIBOSOMAL SUBUNITS ON SUCROSE GRADIENTS.

Procedures same as in legend of Figure 4 except the gradient was made up in 0.1 mM Mg - AT buffer and no EDTA was present.


ABSORBANCE 260nm
1·0
0·8
0·6
0·4
0·2
0
RELATIVE DISTANCE SEDIMENTED

mitochondrial ribosomes were suspended in 0.1 mM Mg^{++}-AT buffer and centrifuged into a gradient containing 1 mM EDTA did it dissociate into their subunits (Fig. 4). Our results complement that found in Tetrahymena pyriformis. Chi and Suyama have shown that in Tetrahymena pyriformis, cytoplasmic ribosomes dissociate at 0.1 mM Mg^{++}, but dissociation of mitochondrial ribosomes required the presence of EDTA (Chi and Suyama, 1970). It therefore shows that in Achlya, the mitochondrial ribosomes are much more stable than cytoplasmic ribosomes with respect to Mg^{++} concentrations. This is contrary to the general belief that mitochondrial ribosomes, like bacterial ribosomes, are more sensitive to Mg^{++} concentration than their cytoplasmic counterparts (Boardman et al, 1966; Rifkin et al, 1967; Kuntzel 1969; Lizardi and Luck, 1971).

The purity of the two types of ribosomes were checked by measuring the ratio of RNA to protein. The UV absorption profiles for purified mitochondrial ribosome A_{260}/A_{280} = 1.96, while that of purified cytoplasmic ribosomes = 1.98. The high A_{260}/A_{280} ratio indicates ribonucleoprotein particles with little extraneous protein contamination (Petermann, 1964)

In Saccharomyces cerevisiae, it was found that in order to get mitochondrial ribosomes with A_{260}/A_{280} close to 1.9, a sucrose gradient centrifugation step is necessary for purification (Morimoto and Halvorson, 1971; Yu et al, 1972). This was found to be unnecessary in Achlya because even after the sucrose gradient purification step, A_{260}/A_{280} remained the same. UV absorption maxima and minima are 258 nm and 235 nm respectively for both cytoplasmic and mitochondrial ribosomes.

A precise determination of the sedimentation coefficients ($S_{20,\,w}$) of both cytoplasmic and mitochondrial ribosomes and their subunits was achieved using the Model E analytical ultracentrifuge equipped with Schleiren optics (Figures 6 and 7). Interestingly enough, both mitochondrial and cytoplasmic ribosomes have a sedimentation value of 82S, and moreover, their subunits have the same sedimentation values of 53S and 38S.

III. RNA From Cytoplasmic and Mitochondrial Ribosomes

An attempt was made to isolate RNA from both cytoplasmic and mitochondrial ribosomes for further

characterization. For isolation of rRNA from cytoplasmic ribosomes, the method of Lovett and Leaver (Loeining, 1968; Lovett and Leaver, 1969) was followed. But for mitochondrial rRNA, this method was unsuccessful as the RNA extracted were all degraded regardless whether whole mitochondria or purified mitochondrial ribosomes were used.

Different types of ribonuclease inhibitors were then employed in attempts to overcome this problem (Dingman and Sporn, 1962); Frankel-Conrat's phenol-SDS-bentonite method (Frankel-Conrat et al, 1961; Singer and Frankel-Conrat, 1961); Griffin and Breuker's phenol-SDS-bentonite-polyvinyl sulphate method (Clark et al, 1964; Griffin and Breuker, 1969) and Solymosy's diethyl pyrocarbonate method (Solymosy et al, 1968). All of these approaches were unsuccessful. Gel-electrophoretic analysis showed that the mitochondrial rRNA's were degraded. Urea-LiCl extraction of mitochondrial rRNA (Traub, Nomura and Tu, 1966) was also tried with no avail. The failure of all these methods to isolate mitochondrial rRNA seemed to suggest either the rRNA is very labile, or that one of the ribosomal proteins is an active ribonuclease. But since gel

Figure 6

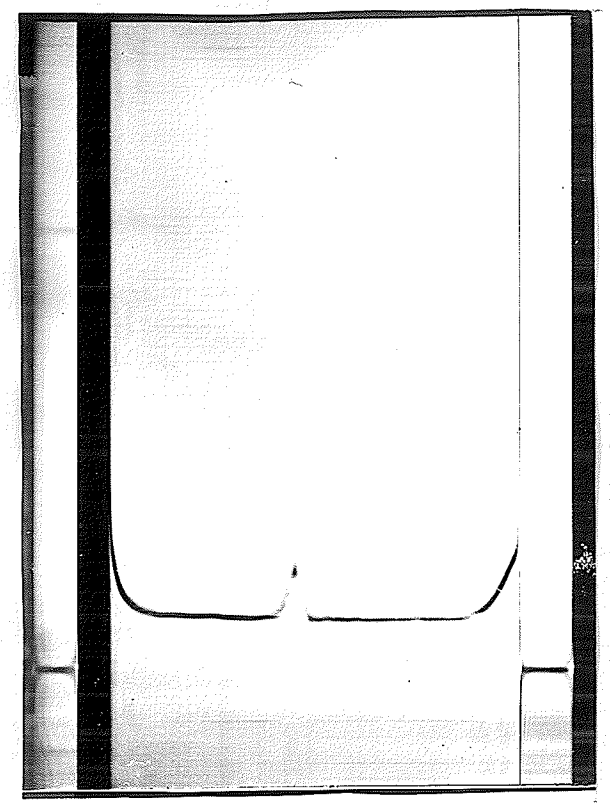
DETERMINATIONS OF THE SEDIMENTATION CONSTANTS ($S_{20, w}$) OF MITOCHONDRIAL AND CYTOPLASMIC RIBOSOMES USING THE MODEL E ANALYTICAL ULTRACENTRIFUGE

Schlieren photographs showing the monosome peaks

a) mitochondrial ribosomes. Sedimentation was from left to right.

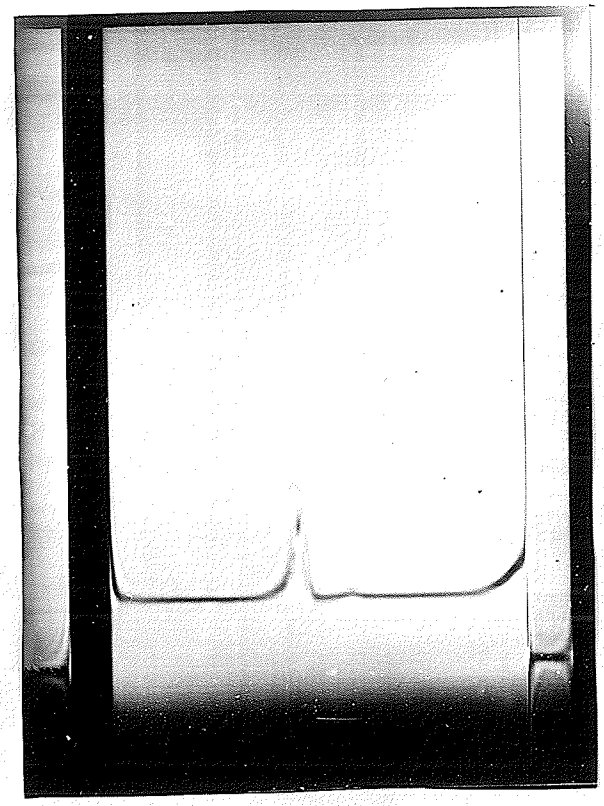
b) cytoplasmic ribosomes. Sedimentation was from left to right.

Procedures as outlined in Materials and Methods.



a

SEDIMENTATION



b

SEDIMENTATION

Figure 7

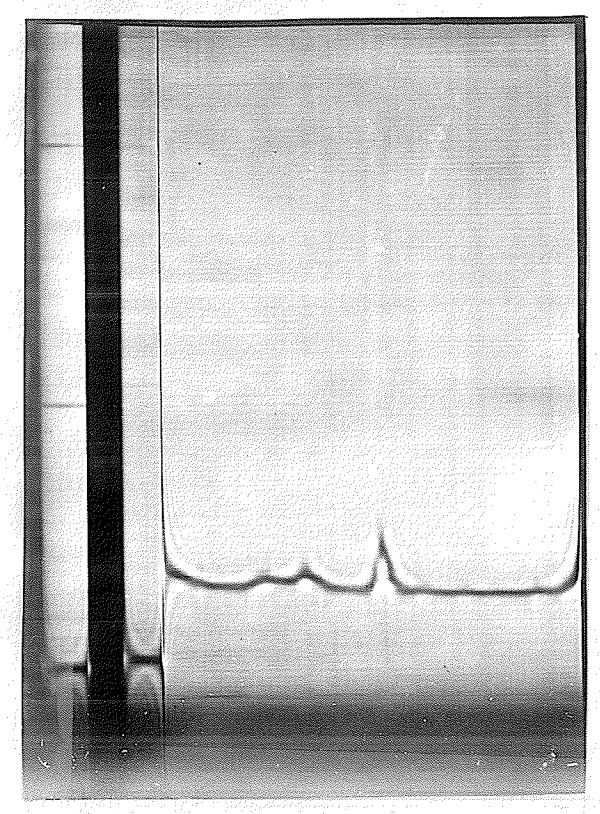
DETERMINATIONS OF THE SEDIMENTATION CONSTANTS ($S_{20, w}$) OF MITOCHONDRIAL AND CYTOPLASMIC RIBOSOMAL SUBUNITS USING THE MODEL E ANALYTICAL ULTRACENTRIFUGE

Schlieren photographs showing the monosome and subunit peaks:

a) mitochondrial ribosomes. Sedimentation was from left to right.

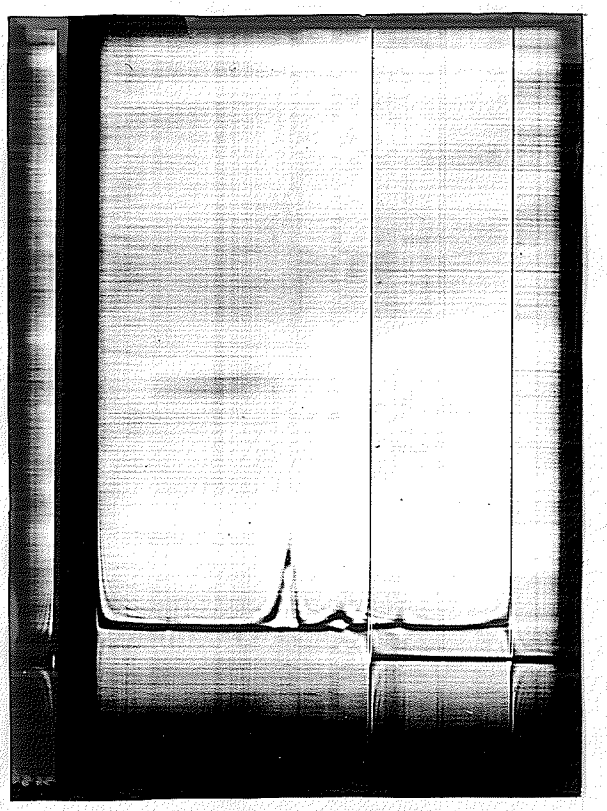
b) cytoplasmic ribosomes. Sedimentation was from right to left.

Procedures as outlined in Materials and Methods.



a

SEDIMENTATION →



b

SEDIMENTATION ←

patterns of the break-down products were consistently showing relatively large fragments of RNA, it seems to suggest the presence of an endonuclease. This degradation problem was also encountered in Neurospora though not to such a great extent (Kuntzel and Noll, 1967). Using Kuntzel's method (Kuntzel and Noll, 1967), in which the ribosomes were suspended in 2% SDS-TEB, applied to gels, and electrophoresed immediately, only 4 major peaks appeared in the gel profile (Fig. 9a).

In order to ensure that this method is satisfactory for studying sedimentation values in gel electrophoresis, the same procedure was used with cytoplasmic ribosomes and E.coli ribosomes. Ribosomal RNA's were also extracted from cytoplasmic ribosomes by Lovett's method (Lovett and Leaver, 1969), and from E.coli ribosomes using urea-LiCl extraction (Traub, Nomura and Tu, 1966). Electrophoresis was then carried out on the RNA's and ribosomes. Migration of the RNA's down the gels were compared. The distances travelled by extracted RNA's were the same as those when whole ribosomes were used. All subsequent gel analyses of ribosomal RNA's were then done by Kuntzel's method, with the assumption that SDS

would strip-off ribosomal proteins from mitochondrial rRNA as effectively as it does in E.coli and cytoplasmic ribosomes.

Figure 8 shows polyacrylamide gel electrophoresis of cytoplasmic ribosomes and its subunits. Subunits were prepared as in Materials and Methods. When whole ribosomes were applied to gels, two distinct peaks were observed, with a shoulder in front and another shoulder behind the faster moving peak. Using E.coli 23S and 16S RNA as standards, these two peaks were found to be 25S and 18S. When the 53S cytoplasmic ribosomal subunit was electrophoresed alone, only the 25S peak was present. A small shoulder, however, was also present, and this coincided with the slow moving shoulder of the 18S peak (compare Fig. 8a and Fig. 8b). For the cytoplasmic 38S ribosomal subunit, however, there was extensive degradation of the RNA as shown in Fig. 8c. The 18S RNA was degraded almost completely. A small peak was observed which coincided with the fast moving shoulder of the 18S peak in Fig. 8a. There were also a build up of small molecular weight RNA's at the end of the gel. The peak at the top of the

Figure 8

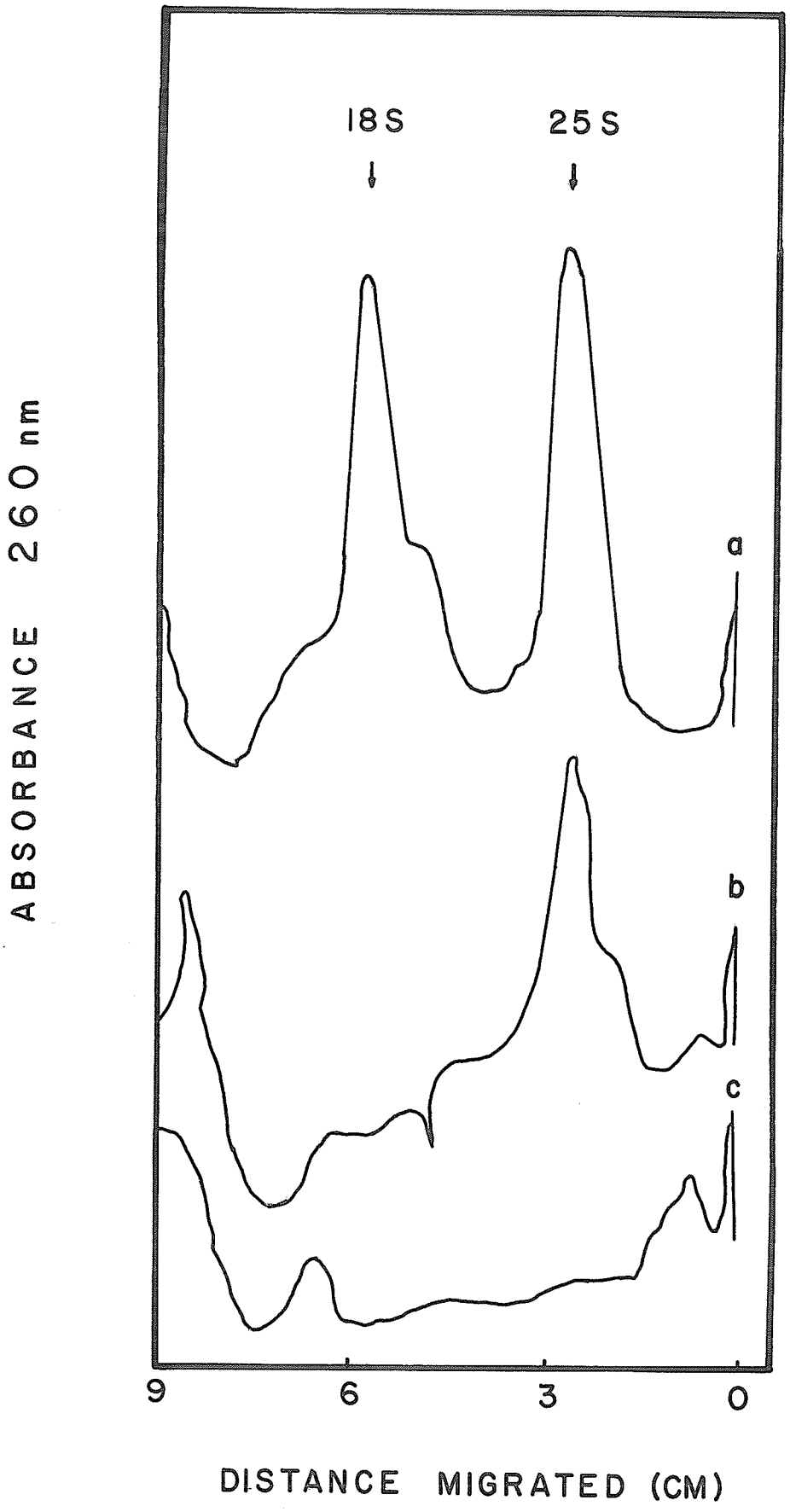
POLYACRYLAMIDE GEL ELECTROPHORESIS OF CYTOPLASMIC rRNA'S

1 A_{260} unit of ribosomes or subunits were suspended in 2% SDS-TEB, incubated at 37 C for 10 secs, chilled, and mixture layered immediately on gels.

a = rRNA's (25S and 18S) from 82S ribosome

b = rRNA (25S) from 53S subunit

c = rRNA (18S) from 38S subunit


18S
25S
a
b
c
9
6
3
0
ABSORBANCE 260 nm
DISTANCE MIGRATED (CM)

gel might represent an aggregate of RNA's complexed with each other because of base homology. From the above results, we can postulate that the 53S cytoplasmic ribosomal subunit is more stable than the 38S subunit, and that when the ribosome is present in its 82S state, the 18S RNA is somehow stabilized. The 2 shoulders shown in Fig. 8a represent partial breakdown products of the 25S and 18S RNA's.

Profiles of gel electrophoresis of the mitochondrial ribosomes and its subunits are shown in Fig. 9. Four peaks were observed. Using E.coli ribosomal RNA as standards, they were found to be 25S, 23S, 13S and 9S respectively (Fig. 10). When the mitochondrial 53S subunit was electrophoresed, a 25S RNA was observed, and not a 23S RNA. This suggested that the 23S RNA was only a breakdown product from the 25S RNA. For the 38S mitochondrial ribosomal subunit, extensive breakdown of the RNA occurred as in its cytoplasmic counterpart. Three small peaks were present: 20S, 18S and 13S. It would seem most likely that the undegraded RNA would be 18S, but because of its instability, degradation gave rise to a 13S fragment and smaller

Figure 9

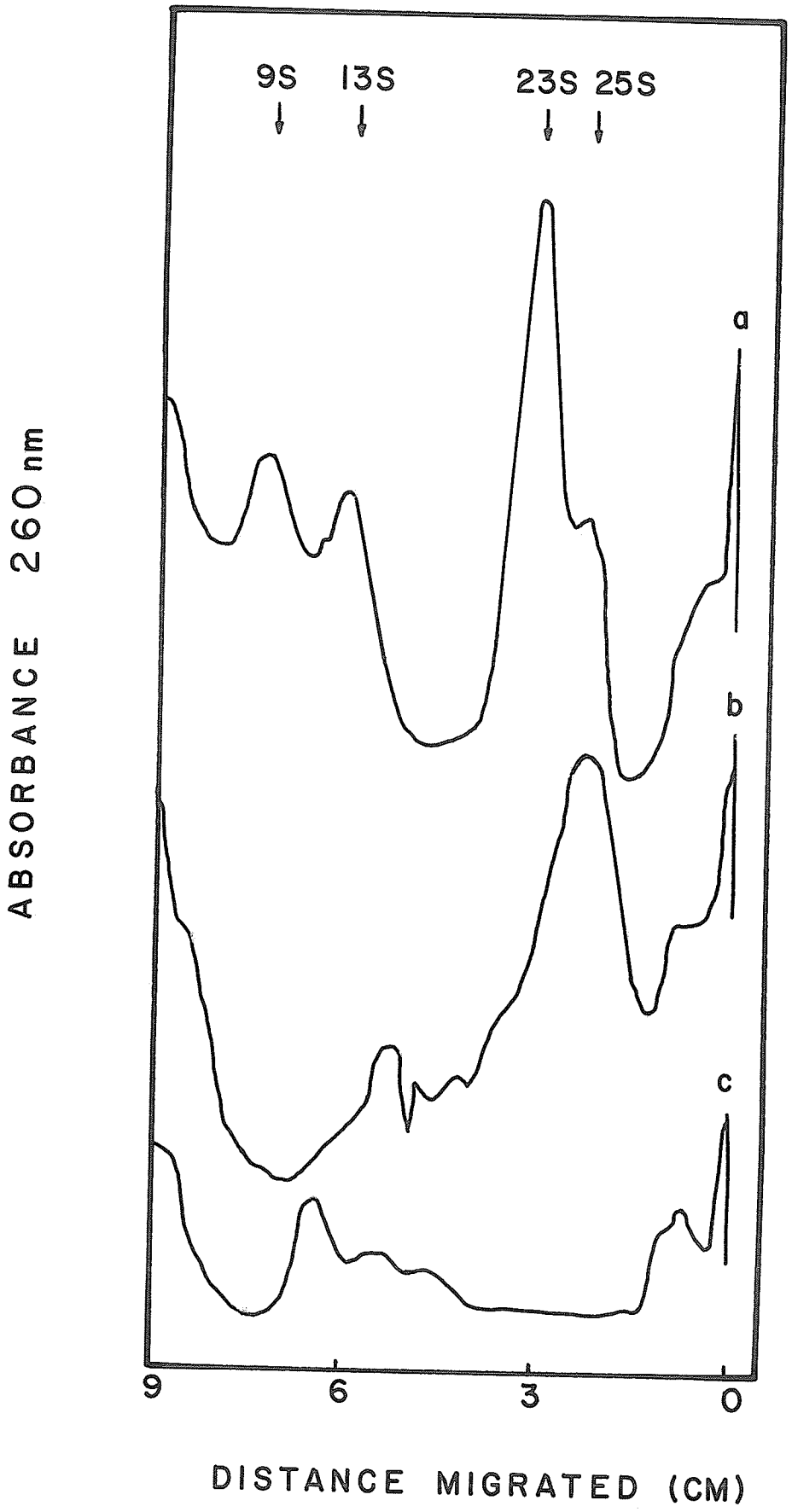
POLYACRYLAMIDE GEL ELECTROPHORESIS OF MITOCHONDRIAL RIBOSOMES AND THEIR SUBUNITS

Procedure as outlined in legends in Figure 8.

a = rRNA's from 82S ribosome

b = rRNA from 53S subunit

c = rRNA from 38S subunit


9S
13S
23S
25S
a
b
c
ABSORBANCE 260 nm
9
6
3
0
DISTANCE MIGRATED (CM)

RNA's that accumulate at the end of the gel. The 20S peak was probably due to aggregate formation between homologous fragments of the 13S and smaller RNA fragments. There are 2 interesting aspects when we compare the 3 gel scans in Fig. 9. First, the 9S RNA peak which was consistently present when whole ribosomes were used was absent in both cases when subunits were electrophoresed. If the 9S was a breakdown product, then it should show up in either Fig. 9b or Fig. 9c. But it did not. If it was not a degradation product, then what could it be? A 5S, ribosome-bound RNA component was found in E.coli (Rosset and Monier, 1963), and is considered to be a universal component of all ribosomes. Then it was shown to be absent from mitochondrial ribosomes of Neurospora crassa (Lizardi and Luck, 1971). Could this 9S RNA, then, be the same type of low molecular weight ribosome-bound RNA's as the 5S RNA in E.coli, or could it be the 7S RNA that was found in cytoplasmic ribosomal preparations in Neurospora crassa (Lizardi and Luck, 1971)? If it were indeed bound to cytoplasmic ribosomes, then it should have been detected in those gel scans. But no such RNA peak was found in cytoplasmic ribosomal gel scans. But whether the 9S

Figure 10

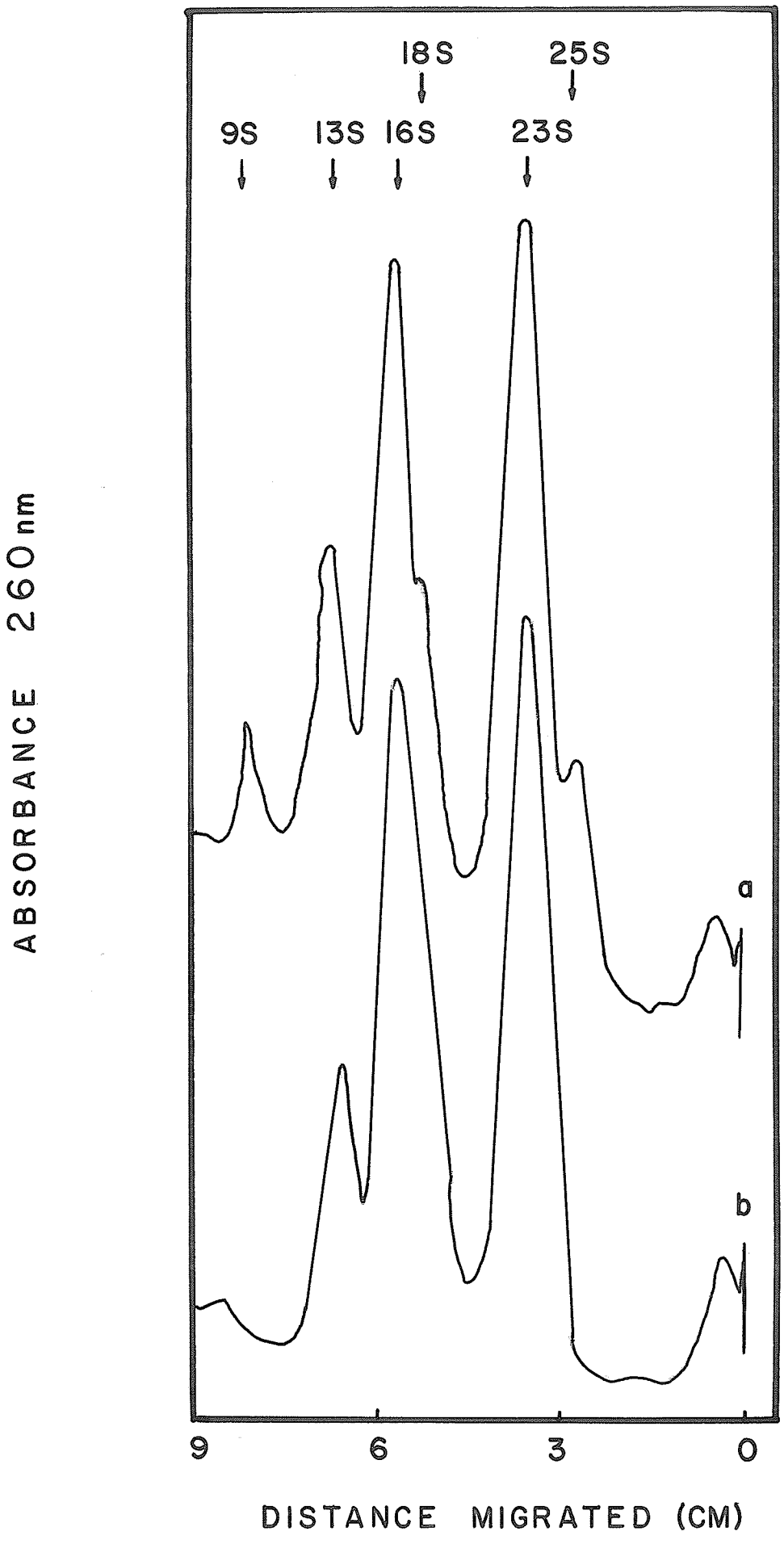
CO-ELECTROPHORESIS OF MITOCHONDRIAL AND

E.COLI RIBOSOMES

Procedure as in legend in Figure 8.

a = rRNA's from E.coli ribosomes (1.2 A_{260} units used) and from mitochondrial ribosomes (0.9 A_{260} units used)

b = rRNA's (23S and 16S) from E.coli ribosomes (1.2 A_{260} units of ribosomes were used).


18S
25S
9S
13S
16S
23S
a
b
9
6
3
0
ABSORBANCE 260nm
DISTANCE MIGRATED (CM)

RNA is really the low molecular weight mitochondrial ribosome-bound RNA cannot be decided on the evidence presented so far, but it can be determined through base composition studies of RNA's of whole and sub-units of mitochondrial ribosomes. Second, the mitochondrial 25S RNA seemed to be stable by itself (Fig. 9b), but when whole ribosomes were electrophoresed, it was degraded down into a 23S RNA. This seemed to suggest again the presence of an endonuclease in the 38S subunit, and that this endonuclease could degrade partially the 25S RNA.

In order to confirm that the absorption peaks in the gels were really RNA's, ribosomes were treated with either ribonuclease or deoxyribonuclease before electrophoresis. For those treated with ribonuclease, the gel scans registered no peaks at all while the isolates treated with deoxyribonuclease produced the same gel scans as controls. This showed that the peaks registered on the scans were truly RNA.

An attempt was then made to inhibit the endonuclease activity of the mitochondrial 38S subunit. Ribonuclease inhibitors: diethyl pyrocarbonate (Solymosy et al, 1968), polyvinyl sulphate (Clark

et al, 1964) Hg^{++} (Cantoni and Davies), Zn^{++} (Barker and Hollinshead, 1967) were added directly to the ribosome-2% SDS-TEB mixture, mixed and then electrophoresis performed. Results are shown in Figs. 11 and 12. The diethyl pyrocarbonate did not seem to have any effect, while Hg^{++} and polyvinyl sulphate seemed to aggravate the situation. Zn^{++} had the same effect as Hg^{++}. So all attempts to isolate an intact mitochondrial 18S RNA have been unsuccessful, and is probably due to the presence of a highly active endonuclease which might be present as a ribosomal protein in the 38S subunit itself.

From the above experimental evidence, we can see that both cytoplasmic and mitochondrial ribosomes have the same sedimentation constants of 82S, and 53S and 38S for their subunits. Their ribosomal RNA's too, are the same size: 25S and 18S. But this similarity in size does not necessarily mean that they are identical. This is suggested by their difference in response towards Mg^{++} concentration; the different degrees of stability of their respective ribosomal RNA's; and the possible presence of an extra low molecular weight ribosome-bound RNA on the mitochondrial ribosome, which was absent in

Figure 11

POLYACRYLAMIDE GEL ELECTROPHORESIS OF:

a = rRNA profile from mitochondrial ribosome

b = rRNA profile from mitochondrial ribosome treated with diethyl pyrocarbonate (5 µl/50 µl)

Procedure as in legend in Fig. 8.

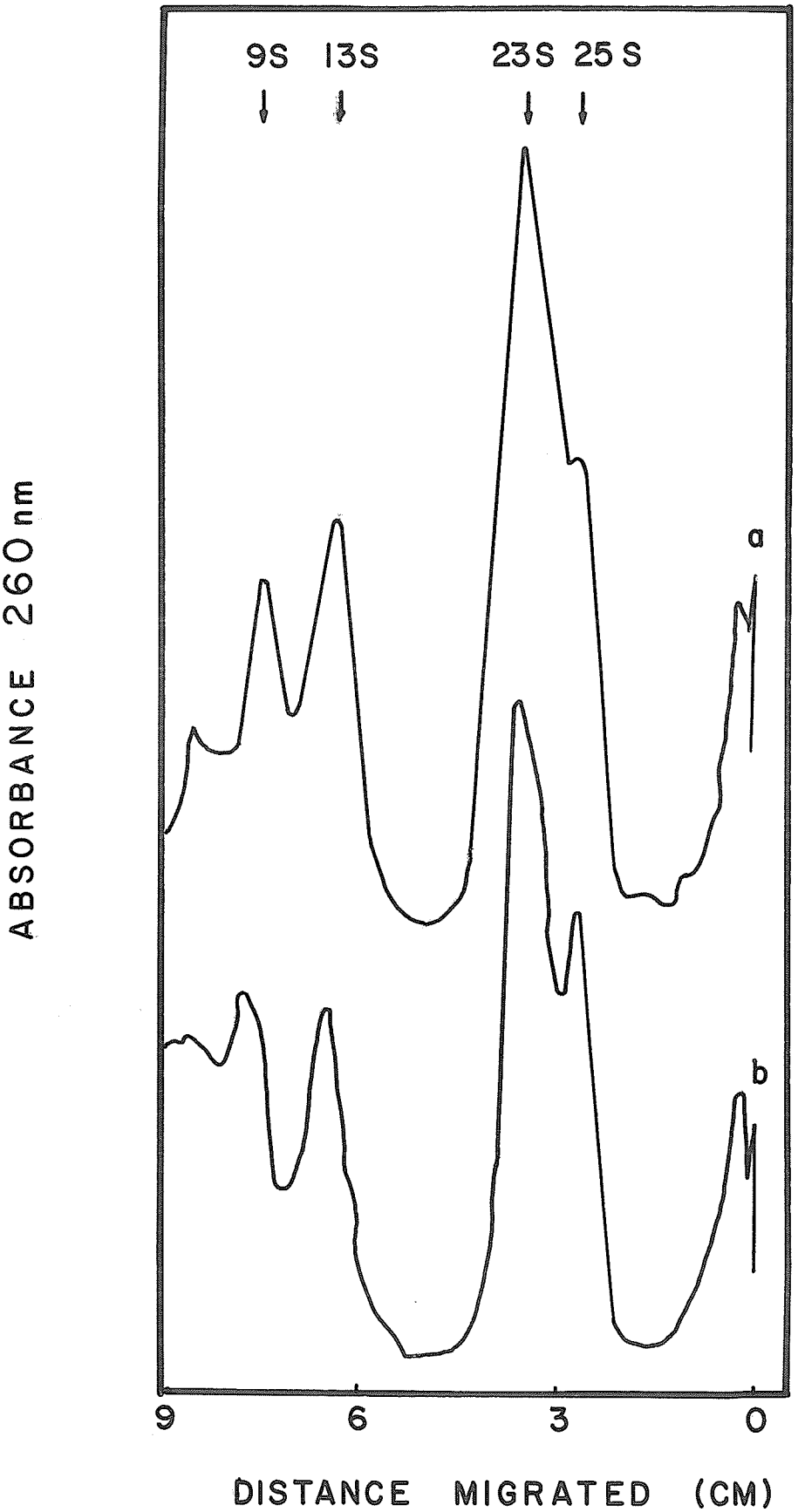

9S
13S
23S
25S
a
b
ABSORBANCE 260nm
9
6
3
0
DISTANCE MIGRATED (CM)

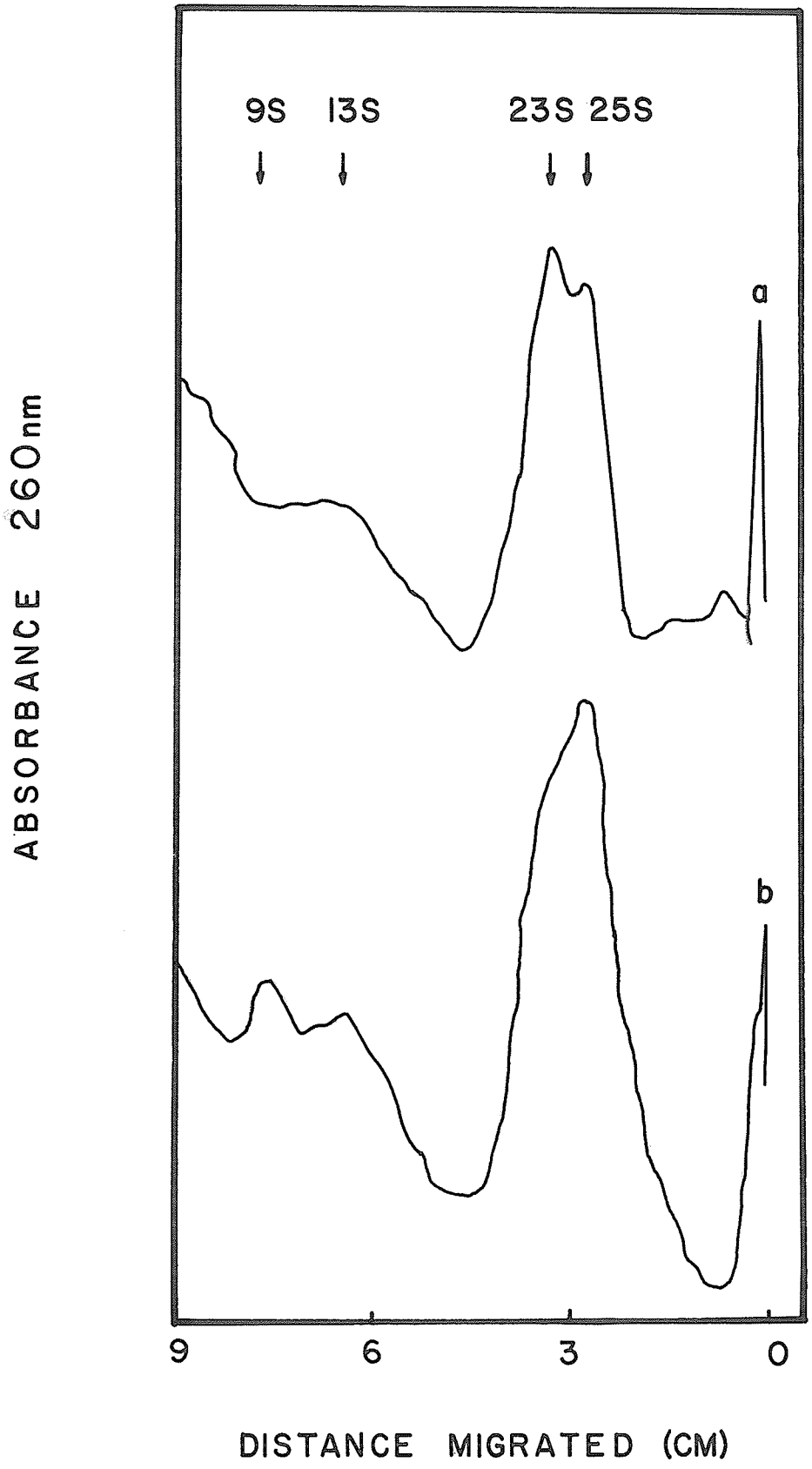
Figure 12

POLYACRYLAMIDE GEL ELECTROPHORESIS OF:

a = rRNA profile from mitochondrial ribosomes treated with Hg^{++} (10mM)

b = rRNA profile from mitochondrial ribosomes treated with polyvinylsulphate (PVS: 5 µgm/50 µl)

Procedure as in legend in Fig. 8.


9S
13S
23S
25S
a
b
ABSORBANCE 260nm
9
6
3
0
DISTANCE MIGRATED (CM)

the cytoplasmic ribosomes. More differences between ribosomes from mitochondria and cytoplasm are known in other organisms. In Neurospora (Kuntzel, 1969), Yeast (Morimoto and Halvorson, 1971), and Aspergillus nidulans (Edelman et al, 1970; Verma et al, 1970) mitochondrial and cytoplasmic ribosomes have different patterns of ribosomal proteins. Also, in all cases, mitochondrial ribosomal RNA has a lower 'G + C' content than does cytoplasmic ribosomal RNA. The melting profiles of the two ribosome preparations are different in yeast (Morimoto and Halvorson, 1971; Yu et al, 1972). The conformational changes accompanying thermal denaturation of ribosomes reflect both protein-RNA interactions and RNA composition (Saunders and Campbell, 1966).

Sensitivities of mitochondrial and cytoplasmic ribosomes towards different antibiotics are different also. In Neurospora (Kuntzel, 1969), yeast (Morimoto, 1971) and HeLa cells (Linnane, 1968; Giuseppe and Ojala, 1971) cytoplasmic protein-synthesizing systems are inhibited by cycloheximide, whereas the mitochondrial systems are sensitive to chloramphenicol, similar to behavior of bacterial ribosomes (Kroon & De Vries, 1971). Differences are also seen in peptide initiation and elongation factors.

Formylmethionyl-tRNA has been found in mitochondria from yeast (Smith and Marcker, 1969), Neurospora (Elper et al, 1970), rat liver (Smith and Marcker, 1969), and HeLa cells (Galper and Darnell, 1969) but is absent from the cytoplasm. Chain elongation factors are also different between cytoplasmic and ribosomal protein synthesis in Neurospora (Kuntzel, 1969), and yeast (Borst and Grivell, 1971). Neurospora, yeast and Aspergillus all belong to Ascomycetes while Achlya belongs to Oomycetes. It has been suggested that Oomycetes represent a group that evolved independently from most of the rest of the fungi (Bartnicki-Garcia, 1970; Lovett and Haselbey, 1971; LeJohn, 1972). A study and comparison between mitochondrial and cytoplasmic ribosomal RNA's between Oomycetes and Ascomycetes on the above parameters should then be most interesting, and should throw more light on the question of divergent evolution.

CONCLUSIONS

The cytoplasmic and mitochondrial ribosomes of Achlya have been isolated, purified, and partially characterized. It was found that both types of ribosomes have a sedimentation constant of 82S, with values of 53S and 38S for their ribosomal subunits. The cytoplasmic ribosomal RNA's were isolated and found to be 25S and 18S by gel electrophoresis. The mitochondrial ribosomal RNA's were extremely labile, but experimental evidence points towards the fact that they were also 25S and 18S. Identical sedimentation values for both cytoplasmic and mitochondrial ribosomes and their respective RNA's do not, however, mean they are identical. This is shown by (1) differences in their stability towards low Mg^{++} concentrations; cytoplasmic ribosomes dissociating at 0.1 mM Mg^{++} whereas dissociation of mitochondrial ribosomes required the additional presence of EDTA. (2) The different degrees of stability of their RNA's during isolation. The cytoplasmic rRNA showing a much higher degree of stability than the mitochondrial rRNA. (3) The possible presence of an extra low molecular weight ribosome-bound RNA on the mitochondrial ribosome. (4) The possible presence of a nuclease as one of the mitochondrial ribosomal proteins

of the 38S subunit. Further studies of the base-compositions of the two types of rRNA's; of the thermal transitions of the RNA's as a measure of their differences; the degree of methylations of the RNA's; comparisons between the ribosomal proteins; sensitivities towards different types of antibiotics in protein synthesis; and comparison of the different factors required in peptide initiation and elongation would certainly provide a deeper insight into the relationships between the cytoplasmic and mitochondrial ribosomes.

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