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PHYSICO-CHEMICAL PROPERTIES OF RIBOSOMAL RNA FROM THERMOPHILIC FUNGI

by

ERIC R. HALL, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

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AUTHOR: Eric R. Hall, B.Sc. (McMaster University)

SUPERVISOR: Dr. K.B. Freeman, Professor and Chairman, Department of Biochemistry

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SCOPE AND CONTENTS: Thermophilic fungi have the highest maximum growth temperature (60°C) of any eukaryotic organisms. It might, therefore, be expected that its cellular components would have unusual physico-chemical properties resulting in increased thermostability. To investigate this, ribosomes and ribosomal RNA from Chaetomium thermophile var. coprophile were compared to those of rat liver. Chaetomium cytosol ribosomes were observed to be more thermostable than rat liver ribosomes. However, the opposite relationship was found for their component ribosomal RNAs. These findings suggest that the thermostability of the Chaetomium ribosome results from unique RNA-protein interactions rather than from novel characteristics of its ribosomal RNAs.

An examination of Chaetomium mitochondrial ribosomal RNA revealed that in terms of sedimentation properties and electrophoretic mobilities, the two high molecular weight species resembled their cytosol counterparts. However, their G+C contents were intermediate between those of the cytosol RNAs and those of previously studied mitochondrial RNAs from

aerophilic fungi. These observations represent the first demonstration in ascomycetes and animals of mitochondrial ribosomal RNA with properties similar to those of its homologous cytosol component.

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LIST OF ABBREVIATIONS

EDTA	=	ethylene diaminetetraacetc
LCS	=	liquid chromatography system
MDH	=	malate dehydrogenase
rRNA	=	ribosomal ribonucleic acid
SDS	=	sodium dodecyl sulphate
STINS	=	sodium tri-isopropylnapthalene sulphonate
T_m	=	thermal transition midpoint
Tris	=	tris (hydroxymethyl) aminomethane

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I. INTRODUCTION

Mitochondria of eukaryotic organisms have been shown to contain a protein synthesizing system that is distinct from the corresponding cytosol* system. To date, a large number of criteria are available by which this distinction may be made. Mitochondrial protein synthesis shows a pattern of antibiotic sensitivity that is very different from that seen in the cytosol. Mitochondria are also known to contain unique species of messenger RNA, transfer RNA, ribosomal and ribosomal RNA. Reviews by Ashwell and Work (1970), Küntzel (1971), Borst (1972) and Kuhler (1973) cover these areas and others, in adequate detail. It is the unusual properties of mitochondrial ribosomes and their [illegible] RNA molecules which led to the investigations reported in this thesis.

Mitochondrial Ribosomes

Early reports on the existence of a protein synthetic apparatus in mitochondria met with a great deal of resistance. Many attributed the observations to contamination of the mitochondria with bacteria or with cytosol ribosomes. But improvements in technique have allowed the isolation of very pure mitochondria, free from either of these contaminants, and it has been shown conclusively that mitochondria do contain unique species of ribosomes that are active in protein synthesis. When classified on the basis of physico-chemical properties, mitochondrial

* This term is used to indicate extra-organellar cytoplasm.

ribosomes can be identified with one of three general categories (Borst & Grivell, 1971).

Mitochondria from animal tissues contain the "mini-ribosome" of [illegible] and Kalf (1967), so-called because its sedimentation coefficient is unusually low (55-60 S). The subunits of particles seen with these preparations have low S-values also, averaging 40 S and [illegible] for the large and small subunits respectively. However, [illegible] the validity of the "mini-ribosome" as the [illegible] protein synthesis [illegible] from reports of [illegible] particles in the lysates of rodent mitochondria (Borst & Grivell, 1971). Only recently has the argument abated. Green et al. (1974) have isolated 55 S and 39 S particles from rat liver mitochondria which [illegible] able to carry out protein synthesis which is [illegible] presence of (a specific inhibitor of [illegible] and [illegible] protein synthesis). They have further shown that the [illegible] S particles [illegible] probably due to both contamination with cytosol [illegible] of the 55 S particles.

The suggested peculiar size of the "mini-ribosome" [illegible] on its S-value and those of its subunits seemed to be confirmed when these particles were found to contain RNA molecules whose sedimentation coefficients, 16 S and 12 S (Borst, 1972), were also low in comparison to values of 28 S and 18 S for the corresponding cytosol species. However, later reports indicate that the mitochondrial ribosome is not at all "mini" in terms of molecular weight or volume, but that its slow sedimentation behavior is due to a high ratio of protein to RNA (O'Brien et al., 1974; De Vries & Van der Koogh-Schuuring, 1973). [illegible] ribosome

of such unusual composition can maintain functional similarities to other ribosomes is intriguing, and, to date, ribosomes from animal mitochondria remain the most studied and the least understood in the field of mitochondrial biogenesis.

At present, the second category of mitochondrial ribosomes is limited to those found in the protozoan Tetrahymena pyriformis. Ott & Suyama (1970) originally characterized these ribosomes, finding their sedimentation coefficient (80 S) to be identical to that of the cytosol ribosomes. The two types could be distinguished, however, by their differing buoyant densities in CsCl and by the fact that in EDTA, the 80 S mitochondrial ribosome is transformed to a species sedimenting at a peak at 55 S. They also found that whereas cytosol ribosomes contained 26 S and 17 S ribosomal RNA, mitochondrial ribosomes and their subunits yielded RNA sedimenting at 21 S and 14 S. Recently, Stevens et al. (1974) further authenticated the 80 S mitochondrial particle by demonstrating that its unique properties are shown by polyacrylamide gel electrophoresis and electron microscopy.

The final category encompasses those ribosomes isolated from the mitochondria of the Ascomycetes. Most authors have shown that the mitochondrial ribosomes of yeast and fungi have sedimentation coefficients intermediate between those of their cytosol counterparts and those of E. coli. Morimoto & Halvorson (1971) were a notable exception. They maintained that for yeast, 80 S was the correct sedimentation coefficient. In addition, Datema et al. (1974) have isolated 80 S particles from the mitochondria of the fungus Neurospora crassa, using heparin as a ribonuclease inhibitor. They extended this finding to show

that only 80 S monosomes are found in mitochondrial polysomes (Grivell-Labbe et al., 1974), a fact which may force revision of the previously accepted value of 73 S.

Although controversy now surrounds the S-value of mitochondrial ribosomes of ascomycetes, there is agreement on the S-value of the subribosomal particles. Until their cytosolic counterparts, small mitochondrial ribosomes readily dissociate into two subunits (55 S and 37 S) when the magnesium concentration is less than 1 mM (Lamb).

The ascomycete mitochondrion is also unique in the size of mitochondrial ribosomal RNA (rRNA). Fungi have the largest sedimentation coefficients of any mitochondrial rRNAs (21 S and 16 S), differing very little from their corresponding cytosol species (25 S and 17 S).

Mitochondrial Ribosomal RNA

In a reference to prokaryotic and eukaryotic ribosomes, Lamb et al. (1972) observed that the basic structure of rRNA has been conserved through evolution; perhaps because a working alternative could not be found. In their study, mitochondrial rRNA was not included. It may be unjustified to say that mitochondrial ribosomes are in fact the "working alternative", but certainly the unique physical and chemical properties of mitochondrial rRNA present quite interesting questions in terms of ribosome structure and function.

In all species studied so far, mitochondrial ribosomes have been shown to contain two high molecular weight RNA molecules. The existence of a low molecular weight component is uncertain at this point. Dubin, Jones & Cleaves (1974) have found an unmethylated, ethidium bromide

sensitive species of RNA in hamster cell mitochondria, which sediments at 5 S. However, until this component can be isolated from purified ribosomes, its validity as the mitochondrial equivalent to 5 S RNA in conventional ribosomes is questionable.

It has been difficult to define the size relationship between the high molecular weight rRNAs of mitochondria and those of the cytosol. Table I gives a summary of reported sedimentation coefficients and apparent molecular weights for most of the organisms studied to date. A general conclusion is that, in terms of S-value, the mitochondrial species are smaller. However, large discrepancies are seen between the S-values and the apparent molecular weights determined by polyacrylamide gel electrophoresis. This is most pronounced among the ascomycetes, where in contrast to their sedimentation coefficients, the mitochondrial rRNAs have apparent molecular weights which are equal to, or greater than those of their cytosol counterparts. It has been generally accepted (Loening, 1967, 1969) that in neutral salt buffers, the rate of migration of RNAs through polyacrylamide gels is linearly related to the logarithm of their molecular weights. But the appearance of the anomalies listed in Table I prompted further investigations into the use of gel electrophoresis to determine molecular weights of mitochondrial rRNA.

Groot et al. (1970) first showed that the apparent molecular weight of rat liver mitochondrial RNA decreases drastically relative to cytosol RNA as the temperature of electrophoresis is raised from 2 to 28°C. In addition to this temperature effect, other authors (Forrester et al., 1970; Grivell et al., 1971; Herzog et al., 1970; Edelman et al.

TABLE I

Organism	Heavy Component		Light Component		Reference
	Sedimentation coefficient (S)	Apparent mol. weight ($\times 10^{-6}$)	Sedimentation coefficient (S)	Apparent mol. weight ($\times 10^{-6}$)	
Yeast	21-22 (25)*	1.73 (1.25)	14 (17)	1.19 (0.82)	Grivell et al. (1971)
	21 (26)		15 (17)		Forrester et al. (1970)
Neurospora crassa		1.28 (1.28)		0.72 (0.67	Neupert et al. (1969)
	24.5 (28)		19 (18)		Rifkin et al. (1967)
	20.5 (25)		16.4 (17.4)		Küntzel & Noll (1967)
Aspergillus nidulans	23.5 (26.5)	1.29 (1.29)	15.5 (17)	0.72 (0.72)	Edelman et al. (1970)
Tetrahymena pyriformis	21 (26)	0.82 (1.41)	14 (17)	0.52 (0.66)	Borst & Grivell (1971)
Xenopus laevis	18 (28)	0.95 (1.54)	13 (18)	0.4 (0.69)	Borst & Grivell (1971)
House (L-cells)	16 (28)	0.68 (1.71)	13 (18)	0.43 (0.70)	Mitra et al. (1972)
Rat liver	16 (28)	0.95 (1.75)	13 (18)	0.50 (0.70)	Groot et al. (1970)
Hamster (BHK-21 cells)	17 (28)	0.73 (1.72)	13 (18)	0.41 (0.70)	Dubin & Montenecourt (1970) Montencourt & Dubin (1970)
Human (HeLa cells)	16 (28)	0.7 (1.65)	12 (18)	0.4 (0.65)	Attardi & Attardi (1971)

* figures in brackets indicate values for cytoplasmic components

1971) found that the mobilities of mitochondrial rRNAs are retarded in buffers of low ionic strength. Because the data given in Table 1 were obtained at various temperatures and in buffers of varying composition, it is possible that the molecular weights may be in error. It may also explain the large differences in size reported for mitochondrial rRNAs of related species.

These large variations in electrophoretic mobility have been attributed to an exceptional sensitivity of the secondary structure of mitochondrial rRNA to changes in temperature and ionic strength. Because mitochondrial rRNAs have a very low G+C content (Table II), this property was originally thought to be the entire basis for their unusual electrophoretic behaviour. But, although it likely does play some role, it cannot be the sole explanation of the phenomenon. Edelman et al. (1971) compared the electrophoretic behaviour of mitochondrial rRNAs from three fungi under a variety of conditions and found that no simple relationship exists between the degree of retardation on gels and the G+C content of the rRNAs. A more likely explanation is given by Grivell et al. (1971). They found that the electrophoretic mobility of yeast mitochondrial rRNA decreased greatly between 7 and 9°C, whereas the melting curve showed almost no change in this temperature range. This may suggest that the melting of a small number of critical base-paired regions may lead to a large change in conformation and a corresponding decrease in electrophoretic mobility.

Because Loening's method of gel electrophoresis is so sensitive to changes in secondary structure, Reijnders et al. (1973a) and Reijnders et al. (1973b) have determined the molecular weights of yeast

mitochondrial rRNA by methods which are unaffected by the secondary structure of the rRNA. The basis of their method is to examine the molecules by electrophoresis on polyacrylamide gels under highly denaturing conditions: either 88% urea at 60°C, or 98% formamide at 50°C. The molecular weights determined by these methods were found to compare very well with values obtained from sedimentation equilibrium centrifugation studies and from electron microscopy of the rRNA denatured in dimethylsulfoxide. The averages of the four methods (1.30×10^6 and 0.70×10^6) were very similar to the molecular weight reported for other fungi (Table 1).

Although the unusually low G+C content of mitochondrial rRNA may not entirely explain their behaviour on polyacrylamide gels, it seems to be the basis of their distinctive optical absorbance properties. Ferna, Kay & Littauer (1971) have determined that like cytoplasmic rRNA, mitochondrial rRNA is stabilized by both hydrogen bonding and base stacking in helical segments of the molecules. The absorbance changes induced when these molecules are heated are reflections of breakdown in this secondary structure. The thermal denaturation patterns of mitochondrial and cytosol rRNA have been examined by Edelman et al. (1971) and two observations are consistent. The first is that mitochondrial rRNAs have much lower thermal-denaturation midpoints (T_m) than their corresponding cytosol-rRNAs. The second is that inferred from the ratios of the amount of hyperchromicity measured at 240 nm, to that measured at 260 nm (Fresco, Klotz & Richards, 1963; Cox, 1966); mitochondrial rRNAs have smaller percentages of GC pairs in the helical regions of their molecules than do the cytosol species. Indeed, this

unusual characteristic is not only associated with the ordered regions of the molecules, but is also reflected in the total base composition of mitochondrial rRNA.

The previous discussion has suggested that a low G+C content is at least partly the basis for the unusual physico-chemical properties of mitochondrial rRNA. From Table II it is apparent that mitochondrial rRNA from every source examined so far has a lower G+C content than the homologous cytosol rRNA (Freeman, Mitra & Bartoov, 1973). But is this a universal characteristic in eukaryotes? Although the number of organisms studied to date is limited, the results do indicate that the low G+C content of mitochondrial rRNA compared to cytosol rRNA has been conserved through evolution and, therefore, this property would seem to be of fundamental importance to the mitochondrial ribosome. By extending the survey of the structure of mitochondrial rRNA, we may be able to decide whether a low G+C content is indeed of fundamental importance, or whether the ribosome functions in spite of it. If the universality of a low G+C content among mitochondrial rRNAs is further supported, this would strongly suggest that it is of great importance and has therefore been conserved. However, if it is demonstrated that there exists a species of mitochondrial rRNA whose G+C content resembles that in its cytosol rRNA, we would conclude that mitochondrial ribosomes can dispense with this property and still remain functional.

A related question arising at this point is how the mitochondrial ribosomes studied to date can remain functional over the same temperature range as cytosol ribosomes. It has been established that at physiological temperatures in neutral salt solutions, mitochondrial rRNA assumes a

TABLE II. Base composition of mitochondrial and cytoplasmic rRNA*

Organism	Mitochondrial rRNA: Light component (or total)† (Moles %) A	U	G	C	GC%	Mitochondrial rRNA: Heavy component (Moles %) A	U	G	C	GC%	Cytoplasmic rRNA: GC%	Reference
Yeast	38.4	34.5	16.1	11.0	27.1	40.3	34.6	14.0	11.0	25.2	49.0	Faiman et al. (1968)
	33.3	35.4	17.4	12.9	30.3	32.6	34.0	17.2	16.1	33.3		Morimoto et al. (1971)
	38.3	39.6	14.0	8.7	22.7							Kellndera et al. (1972)
Neurospora crassa	31.8	31.7	20.8	16.6	36.4	33.9	31.9	19.1	15.0	34.1	51.0	Rifkin et al. (1967)
	27.2	29.8	22.9	14.8	37.7							Küntzel & Noll (1967)
Aspergillus nidulans	34.5	34.0	18.0	13.5	31.5	36.0	33.5	18.0	12.5	34.1	53.7	Verma et al. (1971)
Trichoderma viride	31.5	33.0	22.5	13.0	35.5	35.5	29.0	19.5	12.0	31.5	49.7	Edelman et al. (1971)
Tetrahymena pyriformis	33.8	35.7	16.8	11.8	30.6	36.9	35.2	16.2	11.7	27.9	45.2	Chi & Suyama (1970)
Euglena gracilis	39.5	33.2	14.9	12.5	27.4						54.4	Krawiec et al. (1970)
Xenopus laevis	34	23	21	22	43	36	24	20	20	40	62	David et al. (1972)
Mouse (L cells)	34.1	26.3	17.4	22.2	39.6						63.4	Barroov et al. (1970)
	38.1	26.1	15.2	20.3	35.5	37.7	23.8	16.0	17.3	33.3		Montenecourt et al. (1970)
Rat (liver)	30.5	22.9	21.6	24.9	46.5						64.4	Barroov et al. (1970)
(hepatoma cells)	31.5	27.5	26.4	24.5	50.9							Barroov (1971)
Hamster (BHK cells)	33.2	27.4	18.4	20.8	39.2	33.0	27.0	17.7	22.3	40.0		Montenecourt et al. (1970)
Human (HeLa cells)	33.3	23.4	18.4	24.9	43.3	33.9	24.4	18.1	23.6	41.7	65.2	Vesco & Penman (1969)
	32.7	22.6	18.3	26.3	44.6	32.0	23.4	18.9	25.7	44.6		Attardi & Attardi (1971)

* after Freeman, Mitra and Barroov (1973)

† when values are given for only one component, data is for total rRNA

less compact structure than its cytosol counterpart. Whether the rRNA in an intact ribosome can undergo the same changes that it demonstrates in solution, is not known. To partially understand the functioning of mitochondrial ribosomes, we must ask how significant the contribution made by the rRNA to the overall thermal stability of the ribosome is. Does mitochondrial rRNA from an organism living at high temperature - an environment of thermal stress - have a low G+C content? If the answer is yes, then we may speculate that mitochondrial rRNA plays only a minor role, if any, in the resistance of the ribosome to thermal denaturation. Therefore, in organisms living at more moderate temperatures, an rRNA with a low G+C content would not be expected to affect the functioning of the mitochondrial ribosome. However, if a higher than usual G+C content is found in the thermophile, it may suggest that there exists a lower limit in the relationship between rRNA stability and ribosome function at a particular temperature and that in the organisms studied previously, this limit has not been reached.

In an attempt to answer these kinds of questions, one objective of this investigation is to compare the physico-chemical properties of mitochondrial and cytosol rRNA from a thermophilic fungus. Thermophilic fungi will grow at temperatures up to 60°C, the highest for any eukaryotic organism. It might be expected that its cellular components would exhibit unusual properties resulting in increased thermostability.

Studies on Thermophile Ribosomes and rRNAs

There are many studies which indicate that at least moderate increases in G+C content of rRNAs are seen with increasing maximum

growth temperature of the organism. A review by Friedman (1968) provides a concise summary of studies on the RNA, ribosomes and ribosomal proteins from thermophiles. Bacteria, because of their ability to thrive at more extreme temperatures, than eucaryotic organisms have been more thoroughly investigated than the eucaryotic ones. As mentioned previously, the upper temperature limit for the growth of eucaryotic organisms is near 60°C, while prokaryotic organisms have been found to temperatures over 90°C, and even boiling water (Brock, 1967).

A large number of reports have confirmed that the protein synthetic machinery of thermophiles has a greater capacity to resist thermal denaturation. Ribosomes isolated from thermophilic bacteria, Bacillus stearothermophilus (Tsuji et al., 1968), Thermus aquaticus (Oshima, Taylor & Brock, 1970) and thermophilic Clostridium (Irvin, Akiba & Hines, 1973) all showed significantly higher thermal denaturation midpoints (T_m) than the ribosomes of related mesophiles, or those of E. coli. Imahori, Kano & Campbell (1967) found a positive correlation between the maximum growth temperature of 19 psychrophilic, mesophilic and thermophilic bacteria, and the T_ms of their ribosomes. These physical studies, however, do not necessarily relate to the biological function of the ribosomes. With this in mind, Friedman, Axel & Weinstein (1969) and Hoshino et al. (1971) preincubated ribosomes at high temperatures, removed aliquots at various times, and assayed their ability to support poly U-directed phenylalanine incorporation. In both cases, after preincubation for 30 minutes, thermophile ribosomes lost no more than 20% of their activity.

On the other hand, ribosomes from the mesophiles had lost virtually all activity after 15 minutes.

In an attempt to explain this extra thermostability, various aspects of ribosomal structure have been explored, including ribosomal proteins, ribosomal RNA and the effect of ions and polyamines. Because the characteristics of thermophile RNA are of primary importance to this thesis, further discussion will be limited to properties of this component. Reports by Mangiantini et al. (1965) on B. stearothermophilus, and by Zeikus et al. (1970) on T. aquaticus are typical of the findings of other authors.

In both cases, rRNA isolated from the thermophiles was more thermostable than that of E. coli. However, this extra thermostability was only about half of the amount seen for the intact ribosomes; a fact which suggests that the contribution of the rRNA alone cannot account for this variance. Of interest also, was the nucleotide composition of the thermophile rRNA. The G+C content of B. stearothermophilus rRNA was 7 mole % higher than that of E. coli rRNA. The difference between T. aquaticus and E. coli was only 1.0 mole %. The authors agree that the higher G+C content in the thermophiles would imply higher melting points, but not high enough to explain the observed T_ms.

Since the differences in the G+C content between the thermophiles and E. coli are small and variable (7% & 1%), it is reasonable to assume that they may be due only to normal species diversity rather than to any special thermophilic character. To rule out this possibility, Stenesh & Holazo (1967) determined the base compositions of rRNA isolated from mesophilic and thermophilic strains of the same genus.

Bacillus. The average G+C content for the thermophiles was 60%, a value statistically distinct from that of the mesophiles, [illegible]. However, when the same study was done with related species of *Clostridium*, no difference in G+C content could be detected ([illegible] et al., [illegible]).

Although these experiments do not present a conclusive picture, most of the evidence suggests that organisms with higher growth temperatures tend to contain rRNAs which have higher [illegible] and higher percentages of guanosine and cytosine. As a basis for making predictions [illegible] the results we might expect with thermophilic fungi, the work of Pace and Campbell ([illegible]) is particularly interesting. In this study, the maximum growth temperature of 19 different organisms, covering a range of [illegible] ([illegible]°C), was correlated with the G+C content of their rRNA. With one or two exceptions, the G+C content tends to increase with increasing growth temperature. If a linear relationship between the two is assumed (Figure 1), we can calculate a slope of approximately 0.18 mole % G+C/°C. Over the temperature range examined by Pace and Campbell, this results in an increase in G+C content of over [illegible] mole %. However, the difference in rRNA G+C content between thermophilic and mesophilic fungi could very likely be smaller. The highest growth temperature for thermophilic fungi is about 60°C; for mesophiles of the type studied in Table II, the maximum growth temperature is 35-40°C ([illegible], [illegible]). In applying this temperature difference of 20-25°C to the relationship of Pace & Campbell, we might expect an increase in the G+C content of rRNA from thermophilic fungi of from 3.6-4.5 mole %. Although this increase is small, it should still be detectable, and it would probably make the G+C content significantly different from the average value obtained for

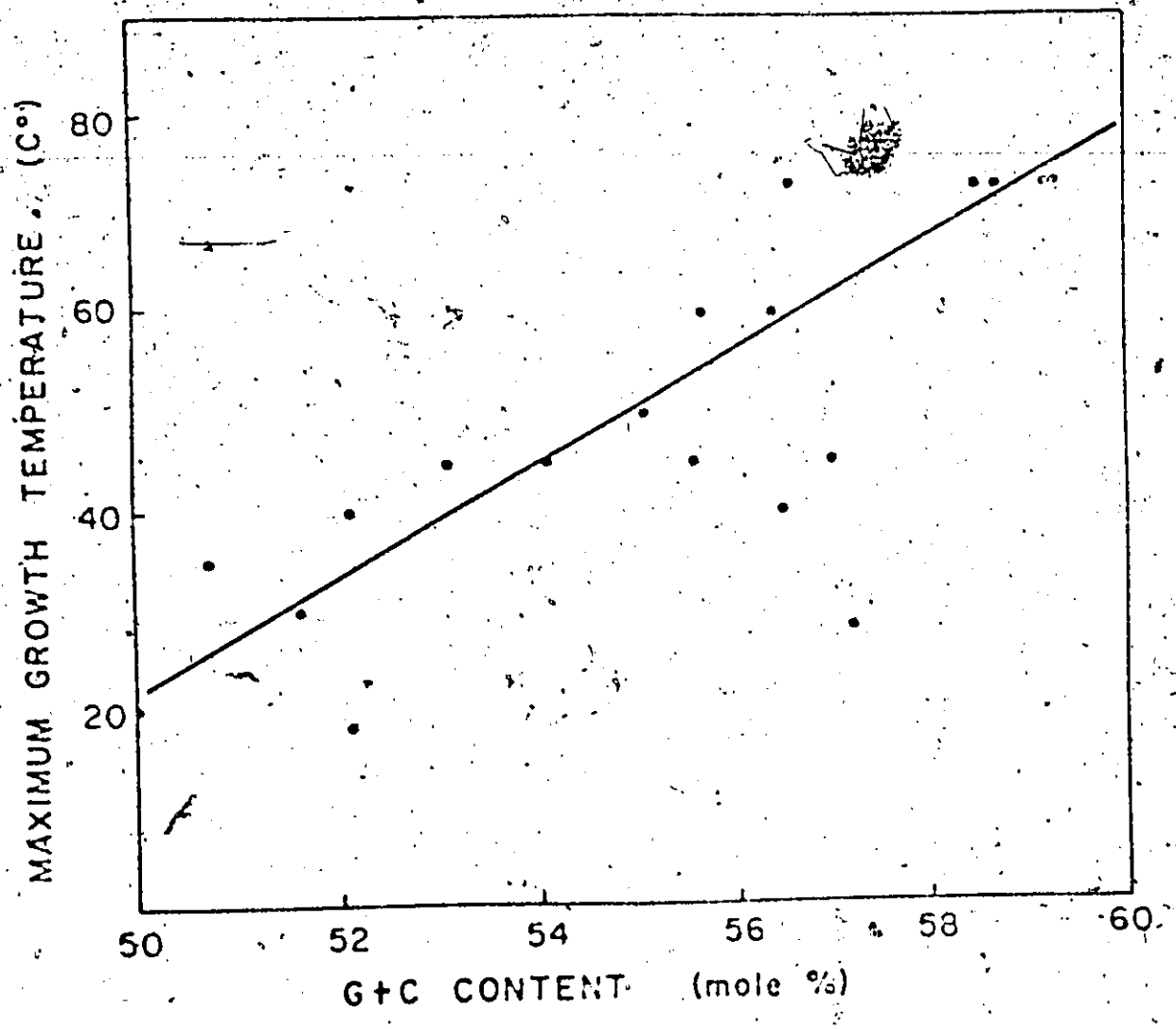

Figure 1. Relationship between G+C content of rRNA and maximum growth temperature for a variety of organisms. Data from Pace & Campbell (1967).

mitochondrial rRNA from the three mesophiles of Table II.

Keeping the results of Pace & Campbell in mind, it may be reasonable, on other grounds, to predict more than a 3.6-4.5 mole % increase in the G+C content of thermophilic mitochondrial rRNA. As suggested previously, unlike other types of RNA, mitochondrial rRNA may be at, or close to the limit of G+C content, below which ribosomes cannot remain functional. If this is true, then perhaps a 20-25% increase in maximum growth temperature would apply enough thermal stress to require a larger increase in G+C content than would normally be predicted from the results of Pace & Campbell.

Molecular Weights of rRNA and Evolution

On the basis of a fairly extensive survey of prokaryotic and eukaryotic cytosol sources, Loening (1968) hypothesized a pattern of evolution based upon the molecular weights of rRNAs. Loening's data, plus some values published after 1968, appear summarized in Table III.

Prokaryotic organisms were regarded as a distinct phylogenetic class, whose rRNAs have molecular weights averaging 1.09×10^6 and 0.56×10^6. Even those organisms which are similar to eukaryotes show rRNA molecular weights comparable to those of bacteria. Actinomycetes which bud like yeast, and blue-green algae which in the past were regarded as plants, had respective molecular weights (1.11×10^6 & 0.56×10^6; 1.07×10^6 & 0.56×10^6) coinciding well with those of E. coli (1.07×10^6 & 0.56×10^6).

In the eukaryotic class, both rRNAs were larger than the corresponding prokaryotic species. However, some interesting patterns

Table III

Molecular Weights of Ribosomal RNA

Species	Molecular Weights ($\times 10^6$)		
ANIMALS			
Human	1.75	0.70	Loening (1968)
Rat (liver)	1.75	0.70	"
Mouse (liver)	1.71	0.70	"
Rabbit	1.72	0.70	"
Chick (liver)	1.58	0.70	"
Xenopus (liver)	1.54	0.69	"
Drosophila	1.40	0.73	"
PLANTS & PROTOZOA			
Protozoa			
Amoeba	1.53	0.89	"
Euglena	1.3	0.85	"
Tetrahymena	1.30	0.65	"
Paramecium	1.31	0.69	"
Higher plants			
Pea, corn, bean	1.29	0.70	"
Algae			
Chlorella	1.28	0.69	"
Chlamydomonas	1.30	0.69	"
Fern			
Dryopterus	1.34	0.72	"

Fungi			
Myxomycetes	1.45	0.75	Jewett & Haseloy (1971)
Oomycetes	1.42	0.73	"
Hyphochytridiomycetes	1.36	0.73	"
Chytridiomycetes	1.34	0.73	"
Zygomycetes	1.34	0.72	"
Ascomycetes	1.30	0.72	"
Basidiomycetes	1.31	0.73	"
PROKARYOTIC CELLS			
Bacteria			
E. coli	1.07	0.56	Loenin (1963)
Rhodopseudomonas	1.08	0.59	"
Actinomycetes			
Streptomyces	1.11	0.56	"
Others	1.13	0.56	"
Blue-green Algae			
Anabaena	1.07	0.55	"
Nostoc	1.07	0.56	"
Oscillatoria	1.07	0.56	"

were apparent. All the eukaryotic organisms from the lower plants to the mammals, seem to share the smaller rRNA component of molecular weight 0.70×10^6. Conversely, the larger component shows a range of molecular weights. Among the animals, the large subunit has increased in size in the more recently evolved organisms from 1.40×10^6 in the sea urchin to 1.75×10^6 in human cells. Among the plants and protozoa, the size of the large subunit remained constant at 1.30×10^6. Loening sampled only five fungi, primarily ascomycetes, and included them in the category of lower plants. A later paper by Lovett and Haselby (1971) reported that a broader survey of fungi showed a range of molecular weights ($1.30-1.45 \times 10^6$) for the large component and that this was consistent with the concept of Fungi as a separate Kingdom, in addition to Plantae and Animalia.

From Table III, the most striking observation is that no intermediate sizes of rRNA are found between the prokaryotic and eukaryotic classes, a fact which presents an interesting possibility for investigation with thermophilic fungi.

Theories regarding the origin or evolutionary position of thermophiles have proceeded along two lines: either mesophiles developed from thermophiles or vice versa. Allen (1953) has presented some very good arguments for the mesophilic origin of thermophiles via either adaptation or mutation. However, if evolution proceeded from a hot to a temperate environment, it may be more sensible to consider thermophiles as the forerunners of mesophiles.

Therefore, the second objective of this investigation is to examine the molecular weights of cytosol rRNAs from thermophilic fungi

and to determine the evolutionary implications of the results according to Loening (1968) and Lovett and Haselby (1971). If thermophilic fungi do precede mesophilic fungi in the evolutionary scheme, their positions may be reflected in the relative molecular weights of their rRNAs. Perhaps the rRNA of thermophilic fungi will be of the intermediate category between the prokaryotic and eukaryotic classes.

II METHOD AND MATERIALS

1. Growth of Fungi

Humicola lanuginosa (Griffon and Maublanc) Bunce (1961) and Chaetomium thermophile var. coprophile (Cooney and Emerson, 1964) were obtained from Dr. M.R. Tansey, Dept. of Microbiology, Indiana University. Mycelia were grown in liquid cultures of Emerson YpSs medium (1.5% soluble starch, 0.4% Bacto-Yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4$), with moderate shaking (70-80 RPM) in a New Brunswick controlled environment incubator shaker. Normally, each 2800 ml Fernbach flask containing 1000 ml of medium was seeded with 0.2 g of spores and grown for 30-40 hr at 45°C.

2. Growth Experiments

(a) Determination of wet and dry weights

A stock culture of mycelia was grown into late log phase at 45°C. The suspension was then diluted to about 10 mg fungal mycelia (wet weight) per ml, and 0.5 ml aliquots (5 mg) were added to 250 ml Erlenmeyer flasks containing 100 ml of growth medium. At various times, flasks, in triplicate, were filtered under vacuum onto Whatman no. 1 filter paper. The wet weight of the filter plus fungus was determined and after drying for 48 hr at 60°C, the combined dry weight was measured. Individual filters were pretreated in the same manner to calculate tare weights.

(b) Determination of RNA content

RNA was estimated by a modified Schmidt-Thannhauser method

(Munro and Fleck, 1966). One gram of filtered fungus was ground at 0°C with 0.5 g of glass powder (Fisher, 200 mesh), and was then suspended in ice-cold SET medium (0.44 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5), to a final volume of 20 ml. To 5 ml aliquots, 2.5 ml of ice-cold 0.6 N $HClO_4$ was added, and after 15 minutes at 0°C, the precipitate was recovered by centrifugation. The precipitate was washed twice with ice-cold 0.2 N $HClO_4$. RNA was hydrolysed by incubation in 4 ml of 0.3 N KOH at 37°C for 2 hr. After cooling in ice, protein and DNA were precipitated by adding 2.5 ml of 1.2 N $HClO_4$. After 10 minutes at 0°C, the precipitate was removed by centrifugation, the supernatant was recovered and the pellet washed twice with 5 ml of ice-cold 0.2 N $HClO_4$. The RNA content of the combined supernatant solutions was then measured using either ultraviolet absorption at 260 nm (ϵ_M = 7,600), or the orcinol method of Mejbaum (1939), with adenosine as the standard.

3. Electron Microscopy of Fungal Hyphae

Fungus was prepared for electron microscopy in two ways. In the first method, osmium tetroxide was added directly to the growth medium to a final concentration of 2%. After fixation at 0°C for 15 minutes, the samples were washed, dehydrated with ethanol, and embedded in Epon 18. Sections were then cut with an LKB ultramicrotome, and specimens were examined in a Philips Electron Microscope, Model 300.

In the second method, growth medium was made 1% in glutaraldehyde. After 15 minutes at 0°C, osmium tetroxide was added, and the preparation continued as described above.

4. Isolation of Fungal Mitochondria

A mitochondrial fraction was prepared by a modification of the method of Edelman, Verma, & Littauer (1969). The outer membrane was removed by a digitonin wash as described by De Vries & Van der Kooij-Schuuring (1973). In order to minimize bacterial contamination and ribonuclease activity, all steps in the procedure were carried out at 4°C in sterile solutions, using baked glassware. Mycelia were recovered by vacuum filtration and washed well with ice-cold, glass-distilled water. They were then transferred to a mortar and pestle, mixed with half their wet weight of glass powder (Fisher, 200 mesh), and ground vigorously with slow addition of 2.5 volumes of SET medium (0.44 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5). The homogenate was centrifuged at 500 x g for 15 minutes. The supernatant was saved, and the pellet was rehomed and recentrifuged as described above. The combined supernatant solutions were centrifuged at 26,000 x g for 20 minutes to obtain a crude mitochondrial pellet. The mitochondria were washed by resuspension in SE(10)T medium (SET medium, containing 10 mM EDTA) with the aid of a teflon homogenizer, and 20 ml portions were layered over a sucrose cushion of 5 ml of 2.0 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. The tubes were centrifuged for 15 minutes at 10,000 x g and 3°C in the Hb-4 rotor of a Sorvall RC2-B centrifuge. The upper layer and the band at the interface were recovered, combined and centrifuged 20 minutes at 26,000 x g. The pellet was resuspended in 15 ml of SET medium and the mitochondria were further purified by layering 5 ml portions over discontinuous sucrose gradients, containing 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, and the following sucrose concentrations: 1.75 M (7 ml), 1.35 M (5 ml), 1.20 M (5 ml), 0.90 M (4 ml). Following centrifugation at 50,000 x g and 3°C

for 1 hr in the SW25.1 rotor in a Spinco preparative ultracentrifuge, the upper layers of the gradient were aspirated, and the band at the 1.15-1.75 M sucrose interface was recovered directly with a Pasteur pipette. Similar mitochondrial fractions were combined and diluted with 2.5 volumes of 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, and centrifuged at 24,000 x g for 10 minutes. The purified mitochondria were then re-suspended, with homogenization, in 5 ml of ST medium (0.44 M sucrose, 10 mM Tris-HCl, pH 7.5), the concentration of mitochondrial protein was estimated, and 5 ml of ST medium containing digitonin was added to give a ratio of 0.12 mg digitonin/mg protein. After 2 minutes at 0°C, the suspension was diluted with 15 ml of ST medium, and mitochondria were pelleted at 39,000 x g for 20 minutes.

5. Isolation of Ribosomes

(a) Preparation of rat liver cytoplasmic-ribosomal fraction

Rat liver ribosomes were prepared by a method similar to that described by Kohlhaw-Tredahl and Arnstein (1972). Livers were homogenized with a Potter teflon homogenizer in 10 volumes of ice-cold STKM medium (0.25 M sucrose, 10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 7.6). The homogenate was centrifuged at 12,000 x g for 15 minutes at 3°C and the post-mitochondrial supernatant was filtered through gauze to remove excess fat. A solution of 10% Triton X-100 in STKM was added to the supernatant to a final concentration of 1%, and the mixture was kept at 0°C for 15 minutes. The lysate was then centrifuged at 100,000 x g for 1 hr in the 60Ti rotor in a Beckman Model L2 ultracentrifuge. The microsomal pellets were rinsed with TKM buffer (STKM medium minus sucrose) and then suspended in TKM with the aid of a teflon homogenizer. Following a clarifying spin

(20 minutes, 26,000 x g), the ribosomal suspension was layered over 9 ml of 1.5 M sucrose in TKM and centrifuged for 4 hr at 150,000 x g in the 60Ti rotor. The ribosomal pellets thus obtained were either covered with STKM medium, or suspended in TKM at concentrations greater than 10 mg/ml. In both cases, ribosomes were stored at -70°C.

(b) Preparation of fungal cytoplasmic ribosomal fraction

Fungal cytoplasmic ribosomes were obtained from a post-mitochondrial supernatant, prepared as described in the section on the isolation of mitochondria. A 10% Triton X-100 solution in isolation medium was added directly to the supernatant to a final concentration of 1%. All further steps were identical to those given above for rat liver cytoplasmic ribosomes.

(c) Preparation of fungal mitochondrial-ribosomal fraction

Mitochondria, purified as described above, were suspended in 8 ml of ice-cold TKMD (10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 50 mg dextran sulphate/ml, pH 7.6), and lysed by the addition of 20% Triton X-100 in TKMD to a final concentration of 2%. After 30 minutes at 0°C, the lysate was layered over 3 ml of 1.5 M sucrose in TKM and spun for 4 hr at 150,000 x g in a type 65 rotor in a Beckman Model L3 ultracentrifuge, at 3°C. The pellets were rinsed, resuspended in TKM buffer and stored at -70°C.

6. Isolation of Ribonucleic Acids

(a) Fungal cytoplasmic ribosomal RNA

Fungal cytoplasmic ribosomal RNA was isolated by two methods.

Method I.- Cytoplasmic ribosomes were suspended in 7.0 ml of medium D [2% (w/v) SDS, 0.1% (w/v) Macaloid, 1% (w/v) NaCl, 10 mM $MgCl_2$,

and 25 mM-sodium acetate, pH 4.8, Bartoov, 1971). After 1 minute, 7.0 ml of phenol-cresol mixture (300 g distilled phenol, 42 ml distilled m-cresol, 33 ml water and 0.3 g 8-hydroxyquinoline), made 5% in STISS (sodium tri-isopropylnaphthalene-sulphonate), was added and the mixture stirred for 15 minutes at room temperature. The aqueous layer was re-extracted once with an equal volume of phenol-cresol, and the RNA was precipitated by the addition of 3% (w/v) NaCl, 20% (v/v) sodium benzoate and 10% (w/v) m-cresol. After 1 hr at 0°C, the RNA was pelleted by centrifugation at 10,000 x g for 10 minutes. The RNA was washed twice with ice-cold 70% ethanol, 20% sodium benzoate, 10% m-cresol, once with 1 M NaCl in 75% ethanol, once with 75% ethanol, twice with absolute ethanol, dried overnight in vacuo and stored desiccated, at -20°C.

Method II - In a modification of the method of Loening and Ingle (1971), 20-30 g of filtered fungus was carefully frozen in a mortar and pestle containing liquid nitrogen. The mycelia were then ground to a fine powder and when the nitrogen was completely evaporated the ruptured cells were suspended in 50 ml of medium D. The suspension was extracted with phenol-cresol and the ribosomal RNA was precipitated as described in method I.

(b) Fungal mitochondrial ribosomal RNA

Mitochondrial pellets were suspended in 2 ml of medium D, frozen immediately in liquid nitrogen and stored at -70°C until needed. From three to five mitochondrial preparations treated in this way were thawed, pooled, and added to 1 volume of 4% (w/v) amino-salicylic acid, 2% (w/v) SDS, 0.1% (w/v) Macaloid, 25 mM sodium acetate, pH 4.8 and two volumes of phenol-cresol made 5% in STISS. The mixture was stirred for 1 hr at 4°C and the aqueous phase was recovered and re-extracted with 1 volume of

phenol-cresol. RNA was precipitated by the addition of 0.2 volumes of 5 M NaCl and 2.5 volumes of cold 96% ethanol and was kept overnight at -20°C. The RNA was washed twice with 1% NaCl in 75% ethanol, once with 75% ethanol, twice with absolute ethanol and dried under vacuum overnight.

(c) Rat liver cytoplasmic ribosomal RNA

Ribosomal RNA was extracted from rat liver by method I of Kirby (1965), dried under vacuum and stored desiccated at -20°C.

(d) E. coli ribosomal RNA

Ribosomes, prepared by the method of Nirenberg and Matthaei (1960), were suspended in medium B, and their RNA was extracted as described above for fungal cytoplasmic ribosomal RNA.

7. Radioactive Labelling of Ribosomes and RNA

(a) Ribosomes

Cytoplasmic ribosomes of rat liver were labelled as described by Bartley (1971).

(b) L-cell cytoplasmic RNA

L cells were grown and their RNA extracted as described by Bantle, Mitra and Freeman (1970). Suspension cultures of 250-350 ml were labelled for 48 hr with 10 uCi of 2-^{14}C-uridine.

8. Sucrose Density Gradient Centrifugation

(a) Analysis of ribosomes

Isokinetic sucrose gradients from 15-33% sucrose in TKM buffer were prepared as described by Noll (1967). The mixing chamber contained 10 ml of 15% (w/v) sucrose solution and 12 ml of 40% (w/v) sucrose was added dropwise at a rate of 1 ml/minute, with rapid mixing. From 2-4 mg

of ribosomes in 0.5 ml of RSB buffer were layered on top of the gradient and centrifuged for 4 hr at 3°C at 195,000 x g in the SW41 rotor in a Beckman Model L2-65B ultracentrifuge. The gradients were fractionated in two ways.

In the first method, the tubes were punctured from the bottom and 0.2 ml fractions were collected dropwise. The optical density at 260 nm of each fraction was read in a microcuvette holding 0.4 ml and then the solution was transferred to scintillation vials for measurement of radioactivity.

In the second method, tubes were punctured from the bottom and the gradients were monitored continuously at 254 nm with a Chromatronix Model 200 UV photometer. The gradients were forced through the photometer by pumping water onto the top of the gradients at a rate of 0.7 ml/minute, with an ISCO Model 185 density gradient fractionator.

(b) Analysis of RNA

Isokinetic sucrose gradients from 15-30% sucrose were prepared according to Noll (1967), using either of two buffer systems. In the first system, the sucrose solutions were buffered with 0.1 M sodium acetate, pH 6.0. Approximately 260 µg of RNA in 0.5 ml of 0.1 M sodium acetate, pH 6.0 was layered on top of the gradient and centrifuged for 15 hr. at 3°C and 142,000 x g in the SW41 rotor.

In the second system, the sucrose solutions were in 0.1 M NaCl, 1 mM EDTA, 0.5% (w/v) SDS, 10 mM Tris-HCl, pH 7.0 (Storrie and Attardi, 1971). Centrifugation was for 16 hr at 25°C at 71,000 x g in a Spinco SW41 rotor.

The gradients were fractionated by puncturing the tubes from the bottom and collecting 0.2 ml fractions dropwise. After measuring its

optical density at 260 mμ, each fraction was counted for radioactivity.

7. Agarose-Polyacrylamide Gel [illegible]

Electrophoresis of RNA was carried out by a modification of the methods of [illegible], [illegible] and [illegible] (19[illegible]) and [illegible] and Peng[illegible] (19[illegible]). A solution containing [illegible] (w/v) acrylamide, [illegible] (w/v) methylenebisacrylamide, [illegible] (w/v) N,N,N',N'-tetramethylethylenediamine and 0.4% (w/v) [illegible] in [illegible] concentrated [illegible] buffer, was kept at 0°C for [illegible] minutes. Ammonium persulfate was added to 0.1[illegible] (w/v) and the solution was mixed with an equal volume of a boiling solution of 0.6% (w/v) agarose in distilled water. The mixture was immediately poured into plugged electrophoresis tubes [illegible] x 65 mm, [illegible] [illegible] and allowed to solidify for 2 hr [illegible]. The plug was removed to form a well at the top of the tube and the sample (30 [illegible] of RNA in 0.1 M sodium acetate, pH [illegible]) was applied to the surface of the gel.

Two buffer systems were used and both were [illegible] 0.2[illegible] in SDS. The first system was E-buffer (Loening, 196[illegible]) containing [illegible] mM Tris-HCl, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.7. In this system, electrophoresis was carried out at 25°C and 7 volts/cm or 5 mA/gel.

The second buffer was low-salt buffer (Loening, 196[illegible]) containing 10 mM Tris, 16 mM HCl, 0.1 mM EDTA, pH [illegible]. Gels in low-salt buffer were run at 25°C and 7 volts/cm or [illegible] mA/gel.

In all cases, gels were pre-run for at least [illegible] minutes and following electrophoresis of the sample, the gels were scanned at 260 nm in a Gilford Model 2400 recording spectrophotometer equipped with a linear transport accessory. Because the duration of electrophoresis was varied,

times for individual runs are given in the figures.

10. Base Composition Analysis

RNA base composition was [illegible] by the method of [illegible] (1974). Approximately [illegible] of RNA [illegible] nucleotides [illegible] with [illegible] of [illegible] [illegible] in 0.3 M sodium [illegible], 0.07 M [illegible] for [illegible] to 18 hr.

Chromatographic analysis was carried out [illegible] 1000 [illegible] Aminex A-7 column. An aliquot of [illegible] units was applied to the column and the [illegible] with 0.2 M ammonium formate (pH [illegible]), at [illegible] [illegible]. The effluent was monitored at 254 nm and the elution profile was recorded on a Varian [illegible] chart recorder. [illegible] at the [illegible], the elution profile [illegible] and weighed and the weights were divided by the [illegible] of the appropriate nucleoside in the elution buffer at 254 nm. [illegible] coefficients were determined using known [illegible] of nucleosides. They were standardized using a Zeiss PMQ II spectrophotometer and the [illegible] coefficients reported by Dunn and Hall (1970).

11. Thermal Denaturation Analysis

(a) Ribosomes

Ribosomes purified on sucrose gradients were either dialyzed overnight against thermal denaturation buffer (TD buffer), or diluted by

direct addition of Tm-buffer (10 mM Tris-HCl, 95 mM KCl, 1 mM $MgCl_2$, pH 7.0). Thermal denaturation profiles were then obtained with a Zeiss PMQ II spectrophotometer equipped with a water jacketed cuvette holder connected to a circulating water bath. The temperature was monitored by entering the sensor of a Mettler TH-10 digital thermometer covered with paraffin oil. Solutions were degassed before use, either under vacuum or by bubbling helium through them. Optical density measurements were made at 260 nm, at intervals of 0.5 °C, as the temperature was raised at a rate of 0.25 °C/minute. Measurements were corrected for solvent expansion (Mandel and Marmur, 1963).

(b) DNA

Thermal denaturation profiles of DNA were obtained using essentially the same method as that described for ribosomes. For DNA, the Tm buffer was 50 mM sodium chloride, 50 mM sodium acetate, pH 6.7 (Edelman, Verma and Littauer, 1970), and absorbancies were monitored at both 260 and 250 nm.

12. Measurement of Radioactivity

Fractions from sucrose gradients containing ^{14}C- or ^{3}H-labelled samples were transferred to 4 ml glass shell vials and diluted with 0.2 ml of water. A 3 ml portion of PCS solubilizer (Amersham/Searle) was added and the samples were counted at 10 °C in a Nuclear Chicago Mark I scintillation counter. Counting efficiencies for ^{3}H were about 20%, and for ^{14}C, about 85-90%.

11. Chemical and Enzymatic Measurements

(a) Chemical methods

(i) Protein concentrations were measured by the method of Lowry et al. (1951). A solution of bovine serum albumin at 1 mg/ml served as the standard.

(ii) RNA was measured by the orcinol hot acid test of Mejbaum (1939), with calf thymus as the standard. The heating time was 45 minutes (Albaum and Umbreit, 1947).

(iii) DNA was estimated by the diphenylamine method of Dische (1955).

(b) Enzymatic methods

(i) Malate dehydrogenase and cytochrome oxidase activities were assayed as described by Freeman (1963). In both cases, one unit of activity was defined as that amount of enzyme giving a change in optical density of 1.0 per minute, in a cuvette of 1.0 cm path length.

(ii) Microsomal ribonuclease activity was assayed by a modification of the method of Argyris and Wenneyanz (1963). The reaction mixture contained: 8.8 ml of the appropriate buffer, 0.2 ml of a solution of 5 mg fungal cytoplasmic ribosomal RNA per ml of 0.1 M Tris-HCl, pH 7.0, and 0.66 ml of a microsomal fraction in 0.1 M Tris-HCl, containing 1.0 mg microsomal protein. After incubation at 37°C for the required time, 1.5 ml aliquots were withdrawn and added directly to 1.5 ml of ice-cold 7% (v/v) $HClO_4$. The mixtures were kept at 0°C for 30 minutes and then filtered through glass fibre filters. The acid soluble optical density of the filtrate at 260 nm was read against a zero time blank, in a Zeiss PMQ II spectrophotometer.

4. Composition of buffers and solutions

SET medium: 0.44 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5.

SE(10)T medium: 0.44 M sucrose, 10 mM EDTA, 10 mM Tris-HCl, pH 7.5.

ST medium: 0.44 M sucrose, 10 mM Tris-HCl, pH 7.5.

TKM medium: 10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 7.6.

Medium D: 2% (w/v) SDS, 0.1% (w/v) Macaloid, 1% (w/v) NaCl, 10 mM MgCl and 25 mM sodium acetate, pH 4.8.

Te buffers:

(i) Ribonuclease: 10 mM Tris-HCl, 95 mM KCl, 3 mM $MgCl_2$, pH 7.6.

(ii) RNA: 50 mM NaCl, 50 mM sodium acetate, pH 5.7.

5. Materials

All chemicals and reagents used in this study were of the highest grade and the water used for all solutions was deionized and glass distilled. Diphtonin (A grade) was obtained from CalBiochem. Macaloid, a gift of the Imperial Co., Las Vegas, was prepared as described by Barrow (1971). PCS solubilizer and ^{32}P- and ^{14}C-labelled compounds were obtained from Amer sham/Searle, Toronto. Acrylamide, N,N,N',N',-tetramethylethylenediamine and N,N',-methylenebisacrylamide (purified as described by Loening, 1967) were obtained from the Eastman Kodak Co., Rochester, N.Y. Deoxyribonuclease (electrophoretically purified from ribonuclease), alkaline phosphatase (E. coli, chromatographically purified) and venom phosphodiesterase I were obtained from Worthington Biochemical Corp., Freehold, N.J.

III RESULTS

1. Growth Experiments

Three species of thermophilic fungi were obtained for this investigation: Humicola lanuginosa (Griffon & Maublanc) Bunce (1961), Chaetomium thermophile var. dissitum (Cooney & Emerson, 1964) and Chaetomium thermophile var. coprophile (Cooney & Emerson, 1964). These three are particularly suited for our study because, of the approximately twenty thermophilic fungi known at that time, Humicola and Chaetomium have the highest maximum growth temperature (58-60°C).

It was first necessary to determine the optimum time of growth and conditions of growth of the fungi, for extraction of rRNA. Growth experiments were undertaken to determine which of the fungi had the highest growth rate in liquid culture and also which fungus produced the largest standing crop. Presumably, a higher growth rate indicates a more active protein synthetic apparatus and perhaps a higher rRNA content. The necessity of these preliminary experiments was due to the fact that as a colony of fungus grows, either from spores or from an initial inoculum of mycelia, it sends out thick-walled, cylindrical hyphae in all directions. As these hyphae increase in length, cytoplasm is maintained primarily in their growing tips and, therefore, only a minor percentage of the mass of the organism is synthetically active cytoplasm. By maximizing grow rates and crop yield, it was hoped that the problem of a paucity of ribosomes and rRNA could be overcome.

The growth of Chaetomium thermophile var. coprophile was examined

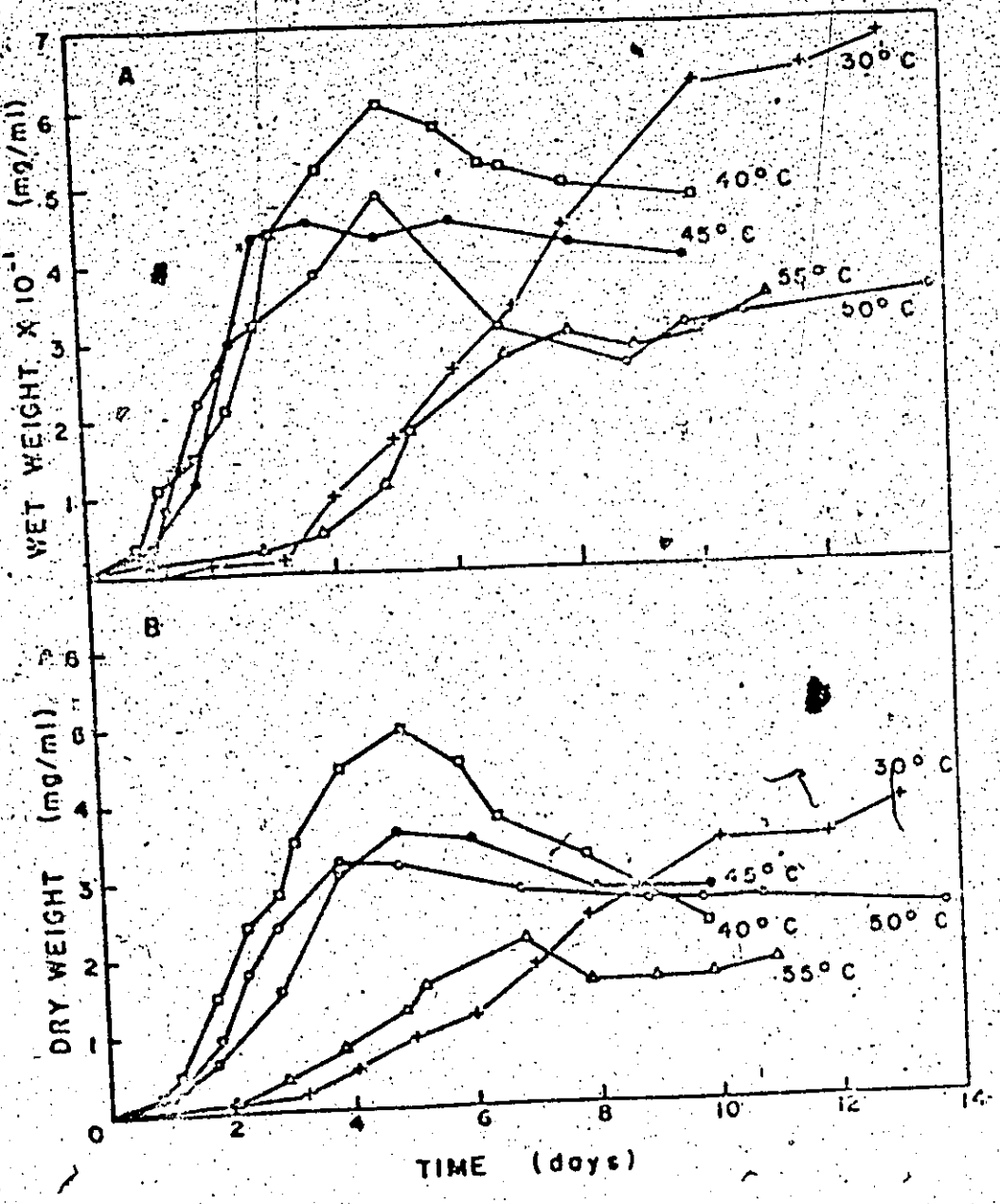

Figure 2. The effect of temperature on the growth of Chaetomium thermophile. (A) Wet weight of cell mass. (B) Dry weight of cell mass. Representative standard deviations are shown in Figure 4.

first. Figure 2 shows growth curves obtained over almost the entire temperature range at which this fungus will grow (Cooney & Emerson, 1964). At 20° and 55°, the fungus appears to be growing at or close to its temperature minimum and maximum respectively. At these temperatures there is a very long lag phase of about 3 days, followed by an exponential phase of relatively slow growth. The maximum standing crop is also less than that for the 40-50°C cultures and is reached only after a lengthy growth period of 7-10 days.

From the growth curves, the temperature optimum of this fungus appears to be 40 to 45°C. At these temperatures the lag phase is short, followed by an exponential phase of comparatively rapid growth and reaching a maximum crop after 4-5 days. The characteristics of the Chaetomium growth curves are summarized in Table IV. At 45°C, the fungus exhibits its highest maximum growth rate, although it is probably not significantly different from that at 40°C. Both at 30 and 40°C, the fungus produces the largest standing crop, but at 30°C, this is not reached until after 10 days of growth. In terms of RNA content, the highest values are found at 45 and 50°C. However, samples were taken for RNA determinations at arbitrary times approximating mid log phase and, as shown below, RNA content is extremely variable over this section of the growth curve. On the basis of growth rate and maximum standing crop, the temperature optimum of Chaetomium appears to be 40-45°C. For this study, 45°C was chosen to select for thermophilic characteristics and to reduce the frequency of mesophilic contamination.

Figure 3 shows two growth curves for Humicola lanuginosa. Because the published temperature optimum for Humicola is the same as that for

Table IV

Growth Experiments

Temp.	Maximum Growth rate (g/day)		Maximum Standing crop (g/100 ml)		RNA content
°C	wet* weight	dry* weight	wet* weight	dry* weight	% dry weight*
Chaetomium thermophile var. *coprophile*:					
30°	0.95	0.052	6.80	0.38	7.32
40°	2.5	0.15	5.97	0.49	8.28
45°	2.8	0.18	4.80	0.37	9.19
50°	1.1	0.12	4.83	0.32	11.8
55°	0.56	0.07	3.44	0.29	6.09
Humicola lanuginosa:					
45°	1.1	0.13	3.79	0.41	7.33
55°	0.66	0.08	2.55	0.32	7.40

*standard deviations were from 5-15% of the indicated values.

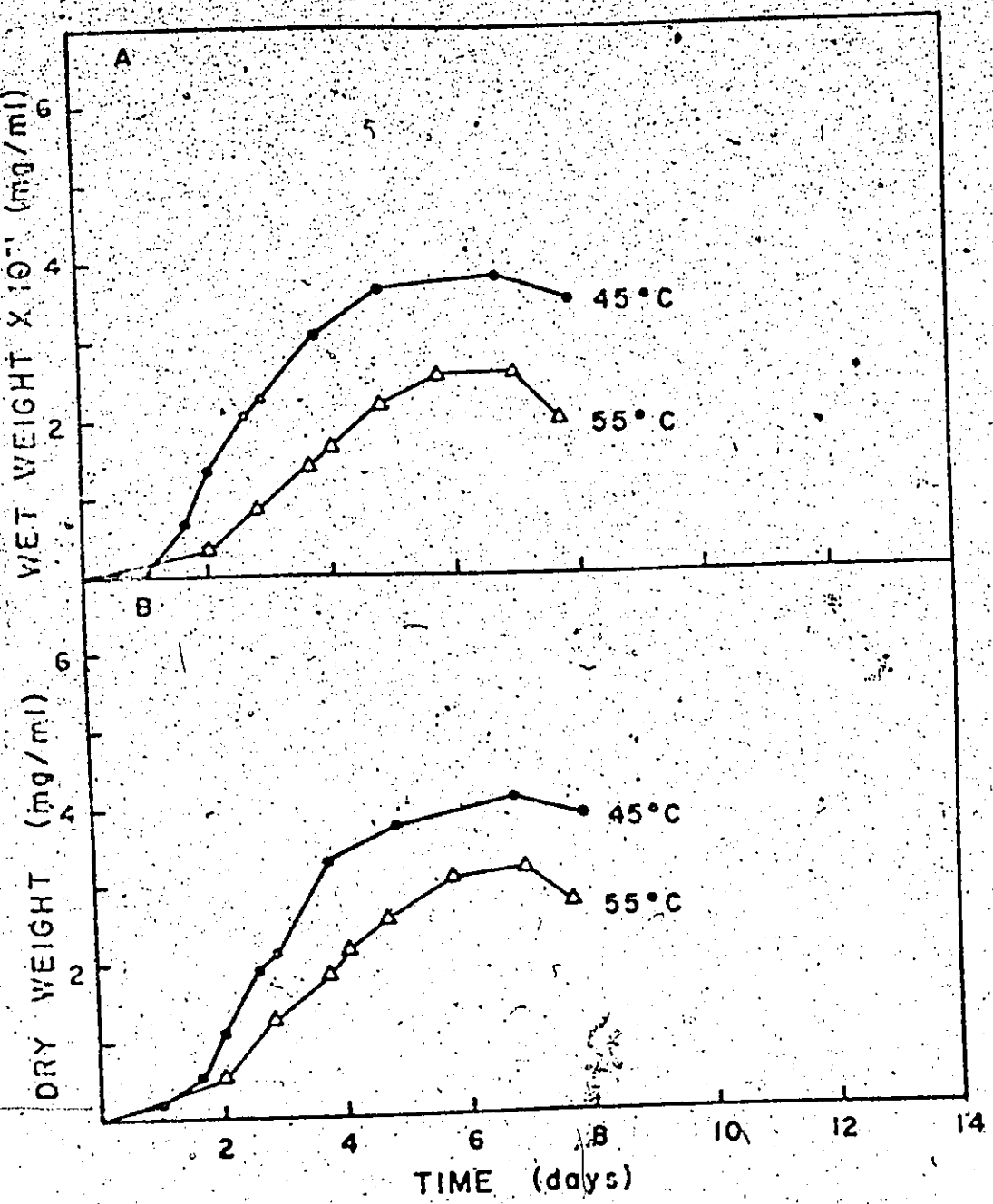

Figure 3. The effect of temperature on the growth of Humicola lanuginosa. (A) Wet weight of cell mass. (B) Dry weight of cell mass. Standard deviations for each point are similar to those shown in Figure 4.

Chaetomium, 45°C and 55°C only were examined. From both Figure 3 and Table IV, Humicola appears much less suited for this study. Both the growth rates and RNA contents were lower than those of Chaetomium, and the maximum standing crop was also relatively small. By these criteria, further growth experiments for Humicola were not undertaken and Chaetomium was selected for the next step in the investigation.

As discussed above, the RNA content data of Table IV does not give an adequate picture of the real situation. In order to investigate the relationship between RNA content and incubation time, the following experiment was carried out. The growth curve for Chaetomium at 45°C was repeated, using dry weight as the measure of cell mass and the RNA content was determined. It can be seen from Figure 4 that the RNA content peaks between 1 and 2 days, or in very early log phase. From these results it was decided that ribosomes and rRNA should be isolated approximately 36 to 40 hr after inoculation of the growth medium with spores. At this point, the mycelia contain not only the highest percentage of RNA but, perhaps, also the least amount of degradation products, as the RNA content has not yet begun to decline.

2. Electron Microscopy

At the outset of this investigation, no published data was found confirming the existence of mitochondria in thermophilic fungi. Since that time, the only information to appear was a citation of a personal communication from S.F. Conti & W. Samsonoff, by Tansey & Brock (1972) that thermophilic fungi contain membrane-bound intracellular organelles such as nuclei and mitochondria. Therefore, Chaetomium was examined by

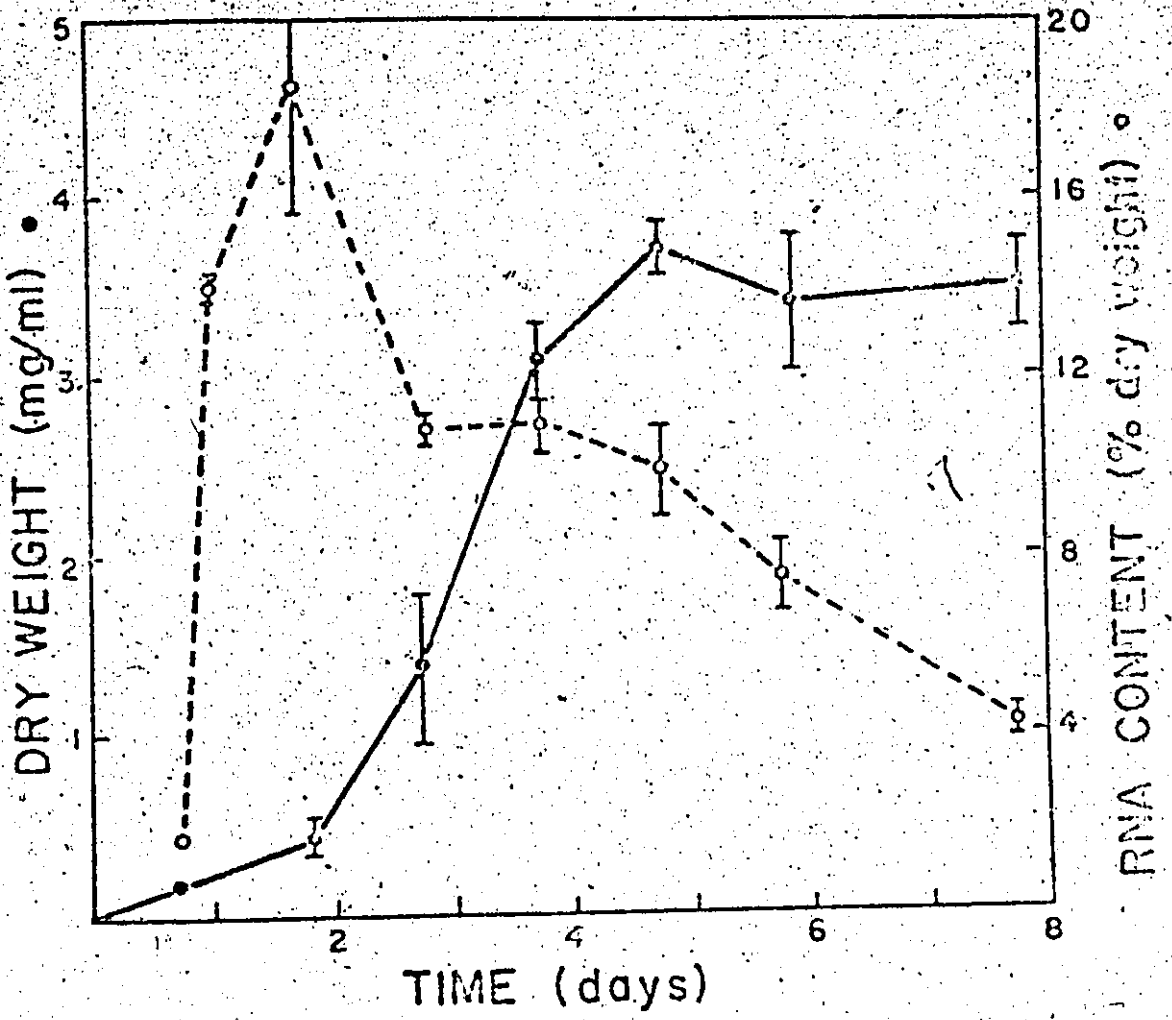

Figure 4. The changes in RNA content during growth of Chaetomium thermophile var. coprophile at 45°C. •——•, Growth curve measured by dry weight of cell mass; o----o, RNA content expressed as a percentage of the dry weight. Error bars indicate standard deviations for each point.

Figure 5. Longitudinal and cross sectional view of conidia-bearing fixed hyphae of Chaetomium. (M) Mitochondria, (N) Nucleus, (V) Vacuole, (ER) Endoplasmic reticulum, (cW) Cell wall. Magnification: 37,500 x.


CW

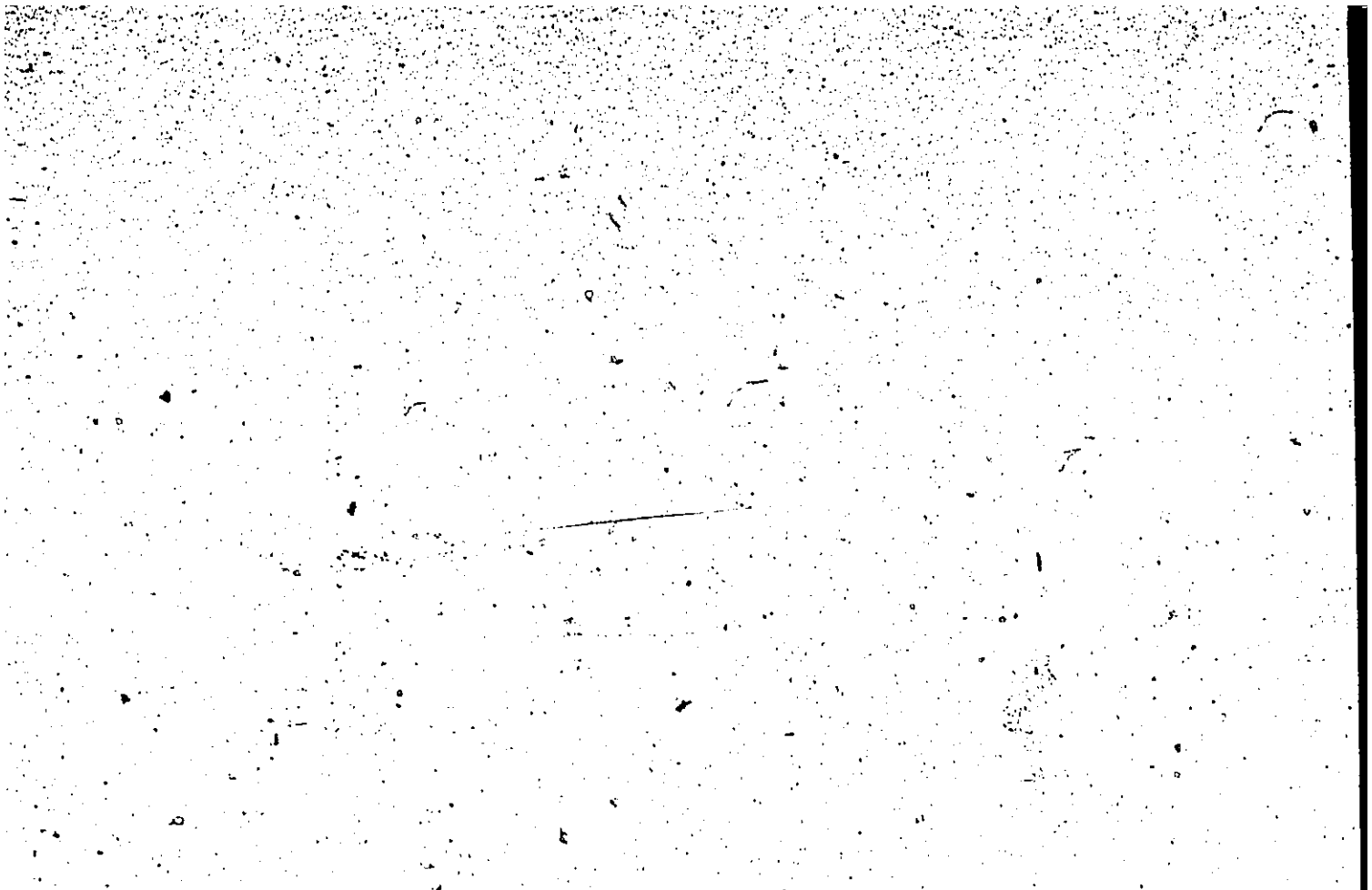

Figure 6. Cross sectional view of glutaraldehyde-and [illegible] hypha of Chaetomium. Typical as in Figs. 5, [illegible] 61,500 x.


GW

electron microscopy to establish the presence and quantity of mitochondria in thermophilic fungi.

Figure 5 shows both a longitudinal and cross-sectional view of osmium-fixed hyphae. Chaetomium shows the regular features found in many fungi. Of special interest are the dense particles scattered throughout the cytoplasm, which appear to be ribosomes. In some areas they seem to be clustered about structures resembling endoplasmic reticulum. However, the most clearly defined organelles evident in this preparation are mitochondria, each with typical cristae and matrix.

Figure 6 shows a longitudinal section of the same culture, fixed in glutaraldehyde-osmium tetroxide. Although swollen, the mitochondria appear intact, and seem to be present in much larger numbers than in the previous figure. In addition, very long segments of endoplasmic reticulum are visible, some of which apparently surround the mitochondria.

These results indicate that thermophilic fungi have mitochondria, which occur with a frequency similar to that seen for mesophilic fungi (Grove & Bracker, 1970).

3. Fungal Cytosol Ribosomes

a. Sedimentation analysis

When prepared in TKM medium, and run on sucrose density gradients containing TKM, Chaetomium cytosol ribosomes show only one peak, which coincidents with the 80 S peak of wheat germ ribosomes. (These were provided by Dr. H.P. Ghosh, of this department.) This same, single peak was found to run ahead of the 70 S peak of E. coli ribosomes, again in a position approximately equal to 80 S (Figure 7). This data was not

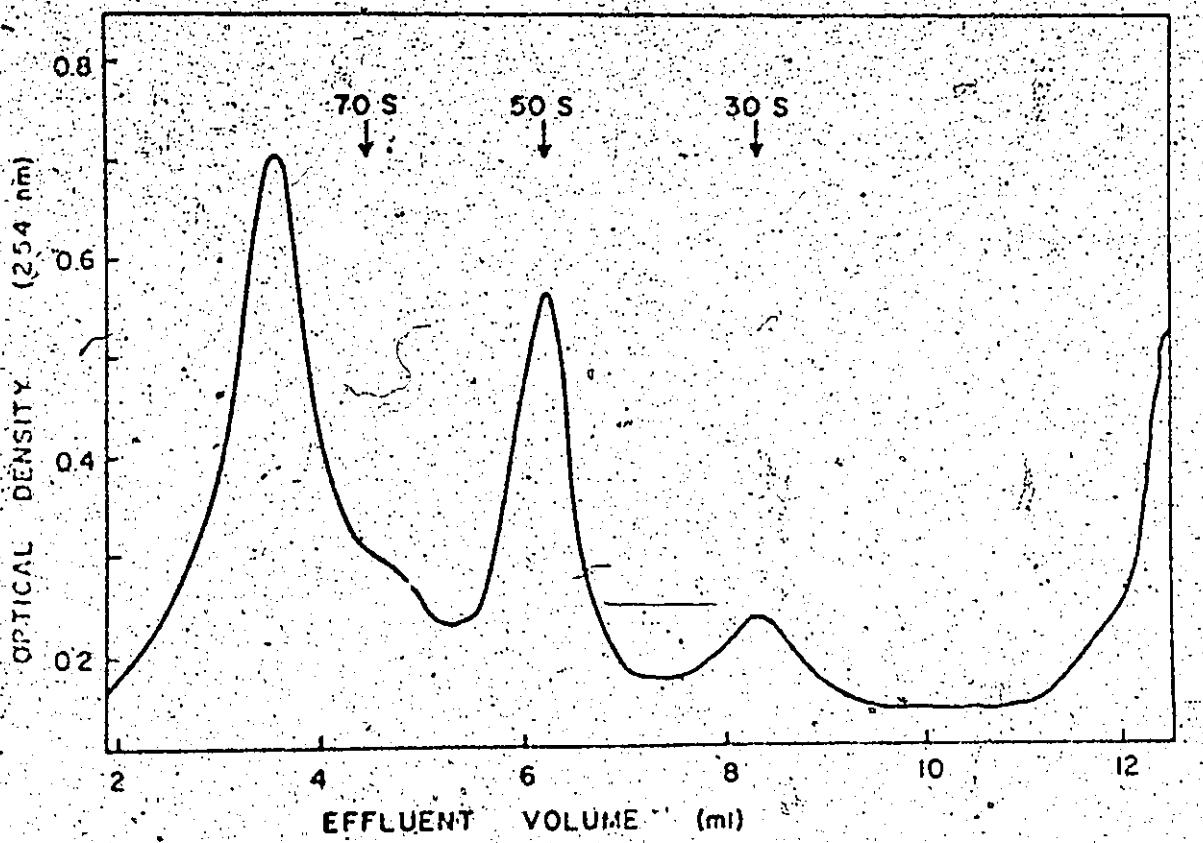

Figure 7. Sedimentation pattern of cytoplasmic ribosomes from Chaetomium, mixed with E. coli ribosomes. The absorbance profile was monitored with a chromatronix photometer as described in the methods.

sufficient to establish the exact S-value of the cytosol ribosome, but it is a good indication that it is about 80 S and is close to the reported values of 77 – 80 S for ribosomes of yeast and fungi (Borst & Grivell, 1971), rather than the 70 S ribosomes of prokaryotes.

b. Thermal denaturation studies

One aim of this study was to compare the thermal stability of Chaetomium cytosol and mitochondrial ribosomes with respect to the stability of their component rRNAs. Rat liver rRNA was selected as the control and internal standard for the RNA work because its physico-chemical properties are well characterized. With this in mind, rat liver ribosomes were also selected as the control for the characterization of Chaetomium ribosomes.

Figure 8 shows typical melting profiles obtained with 80 S ribosomes from the cytosol of rat liver and Chaetomium. In this case, 80 S peak regions were collected from sucrose density gradients and diluted by direct addition of T_m buffer. In other experiments, 80 S ribosomes were dialysed overnight against T_n buffer, or crude ribosomes were suspended directly in T_m buffer without prior centrifugation on sucrose gradients. To facilitate comparison, in any one experiment, both rat liver and Chaetomium ribosomes were prepared by identical methods, and denaturation was carried out in the same buffer. The absolute value of the observed T_ms varied from 1-5°C from experiment to experiment, but the T_ms for both ribosomes always shifted in the same direction, so that the relative difference between the two was conserved with the fungal rRNA T_m always being the highest.

The second interesting feature of the melting profiles is that

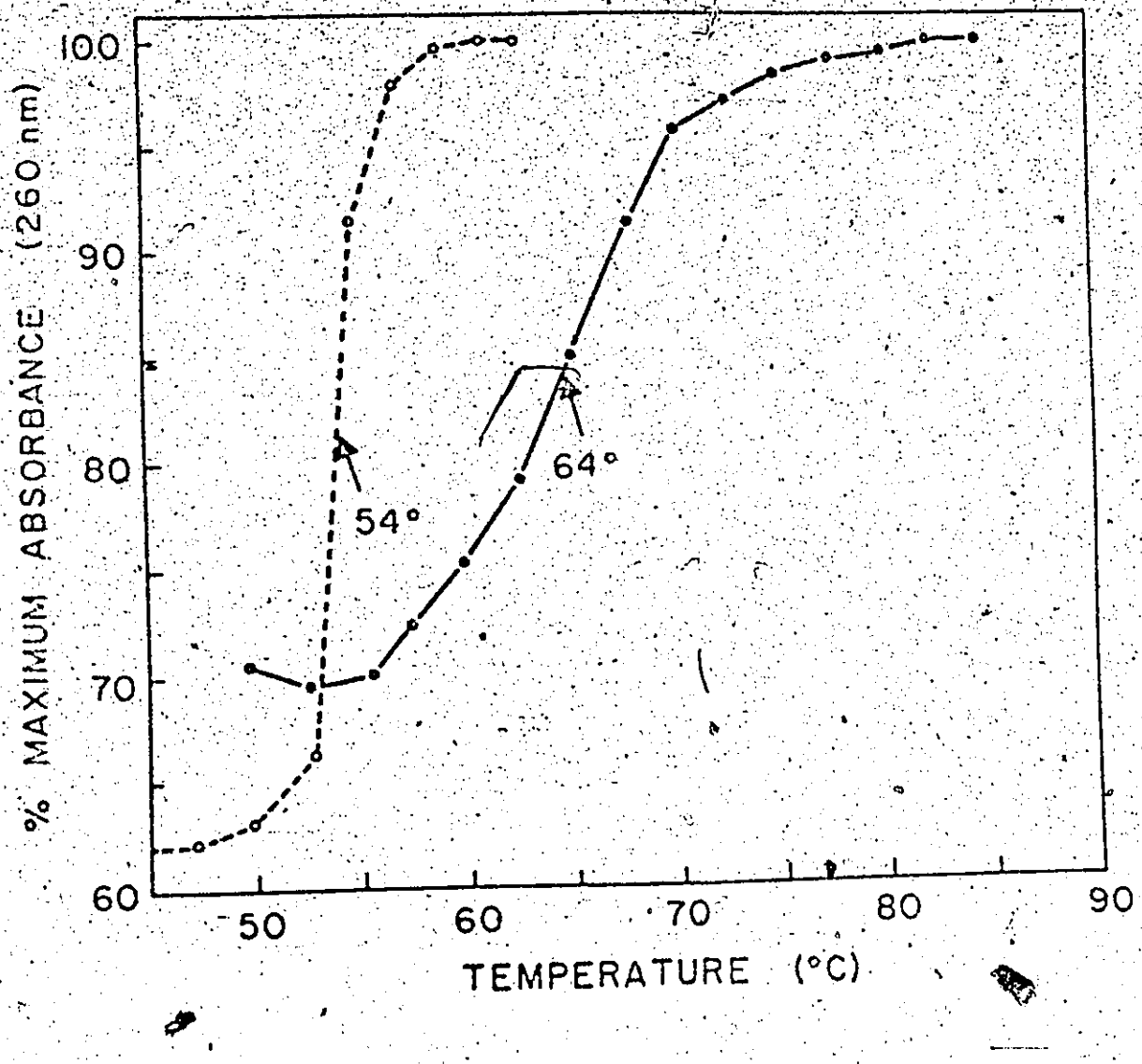

Figure 8. Thermal denaturation profiles of cytoplasmic ribosomes from rat liver (o----o), and Chaetomium (o——o). Peak fractions from the 80 S regions of sucrose density gradients were diluted with T_m buffer to give initial absorbancies of 0.250 - 0.350. To facilitate comparison, maximum absorbancies were set equal to 100%. Arrows indicate calculated T_ms.

not only are the thermophile ribosomes more thermostable as judged by [illegible] alone, but the fungal ribosomes seem to be able to maintain at least partial structure over a broader temperature range than the rat liver particles.

4. Studies on Chaetomium Microsomal Ribonuclease Activity

Initial attempts to isolate cytosol rRNA from Chaetomium were based on the method of Edelman, Verma & Littauer (1970) in which ribosomes, prepared from a Triton X-100 lysed microsomal fraction were resuspended in TKM and extracted with TKM-saturated phenol-chloroform. SDS was present as an RNase inhibitor. In four separate preparations using this method, the product showed RNA profiles approximating that seen in Figure 9. As only two peaks were expected, there must have been considerable degradation. It was also found that storage of the RNA under ethanol for 4 to 8 weeks did not present further degradation. The simplest explanation of this observation is that a ribonuclease (RNase) contaminates the RNA preparation.

The persistent occurrence of this RNase activity was more than an inconvenience. Although the major peaks seen on gels and sucrose gradients always showed the same molecular weights and sedimentation coefficients, it was impossible to decide if these values were indicative of native rRNA, or whether they had been altered by degradation. It was also impossible to firmly establish that Chaetomium ribosomes contain only two high molecular weight rRNAs. Therefore, an investigation of the properties of this ribonuclease was necessary in order to devise a new method for the isolation of undegraded rRNA.

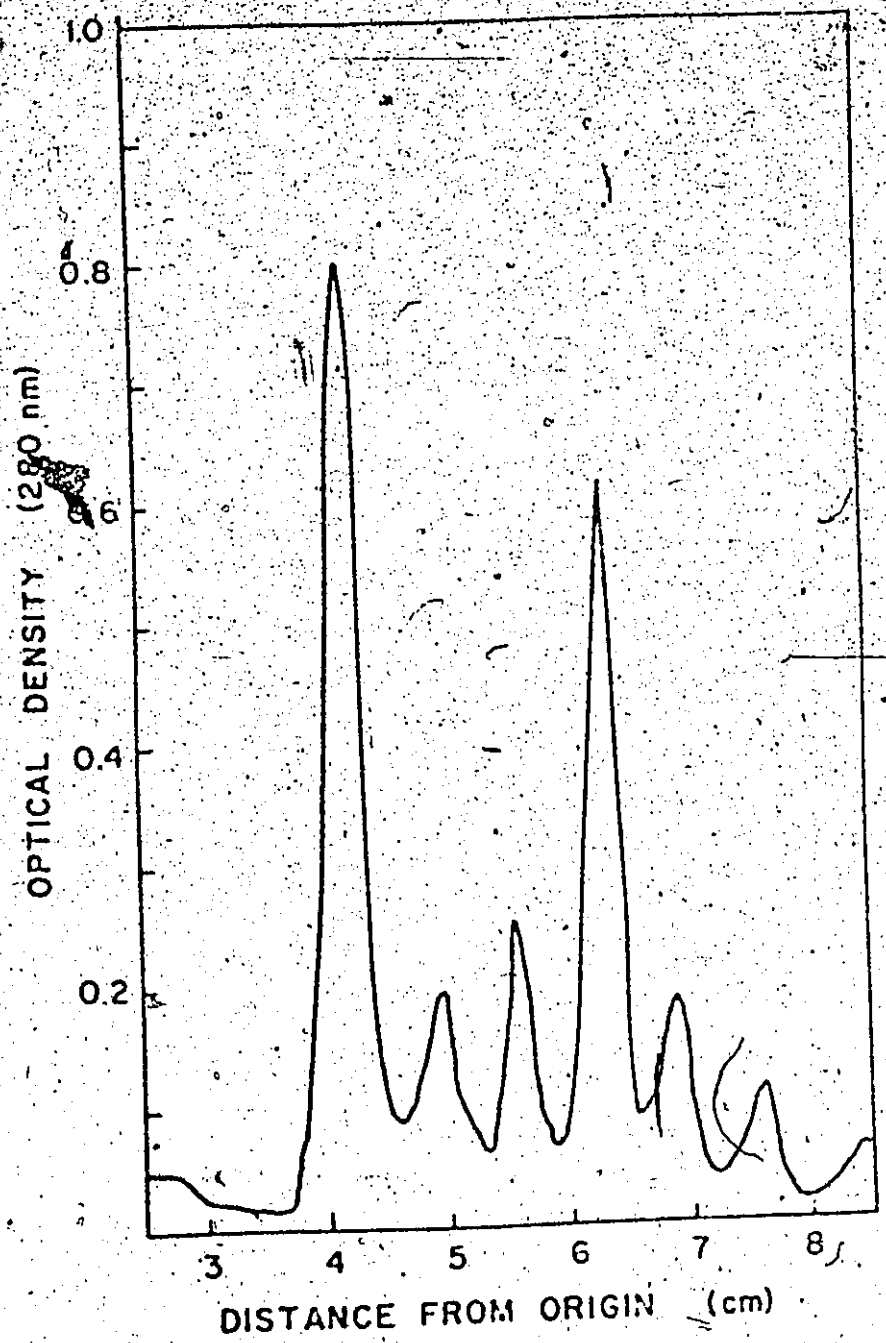

Figure 9. Gel electrophoresis of RNA prepared from cytoplasmic ribosomes of Chaetomium by the SDS-phenol-chloroform method of Edelman et al. (1970). Gels were 9.5 cm long and electrophoresis was in E-buffer, at 25°C, for 4 hr.

Figure 10 shows the preliminary time course studies for various concentrations of substrate and enzyme. By changing both variables independently, it was possible to alter the initial velocity of the reaction, but not the duration of the linear portion of the curves. However, all four curves are linear for at least 10 minutes, and therefore a ten minute incubation was chosen to be the single time point for the inhibition studies.

From Figure 11, it can be seen that of the four variables examined, only mercaptoethanol had no effect on the activity of the RNase. At Mg^{2+} concentrations above 8 mM and at Na^{+} concentrations above 250 mM, the enzyme activity is reduced to almost zero. The pH curve shows a definite optimum at pH 7 and a shoulder at pH 6.0. Below pH 6.0 and above pH 8.0 the RNase activity is inhibited significantly.

On the basis of these results, it was decided that the phenol-chloroform method was quite unsuitable, as the extraction was done in 2 mM Mg^{2+} at pH 7.5, close to optimal conditions for RNase activity. Therefore, the extraction medium was changed to medium D (Bartoov, 1971) which contains 10 mM Mg^{2+} and has a pH of 4.8. Medium D also contains other RNase inhibitors (SDS, Na-EDTA) whose effect was not examined. Although high pH and high Na^{+} concentrations were also found to effectively reduce RNase activity, they were excluded from the final extraction medium to prevent alkali hydrolysis and high salt precipitation respectively, during the extraction procedure. It was also noted that when RNA was extracted from cultures at later times in the growth phase, more degradation products were observed.

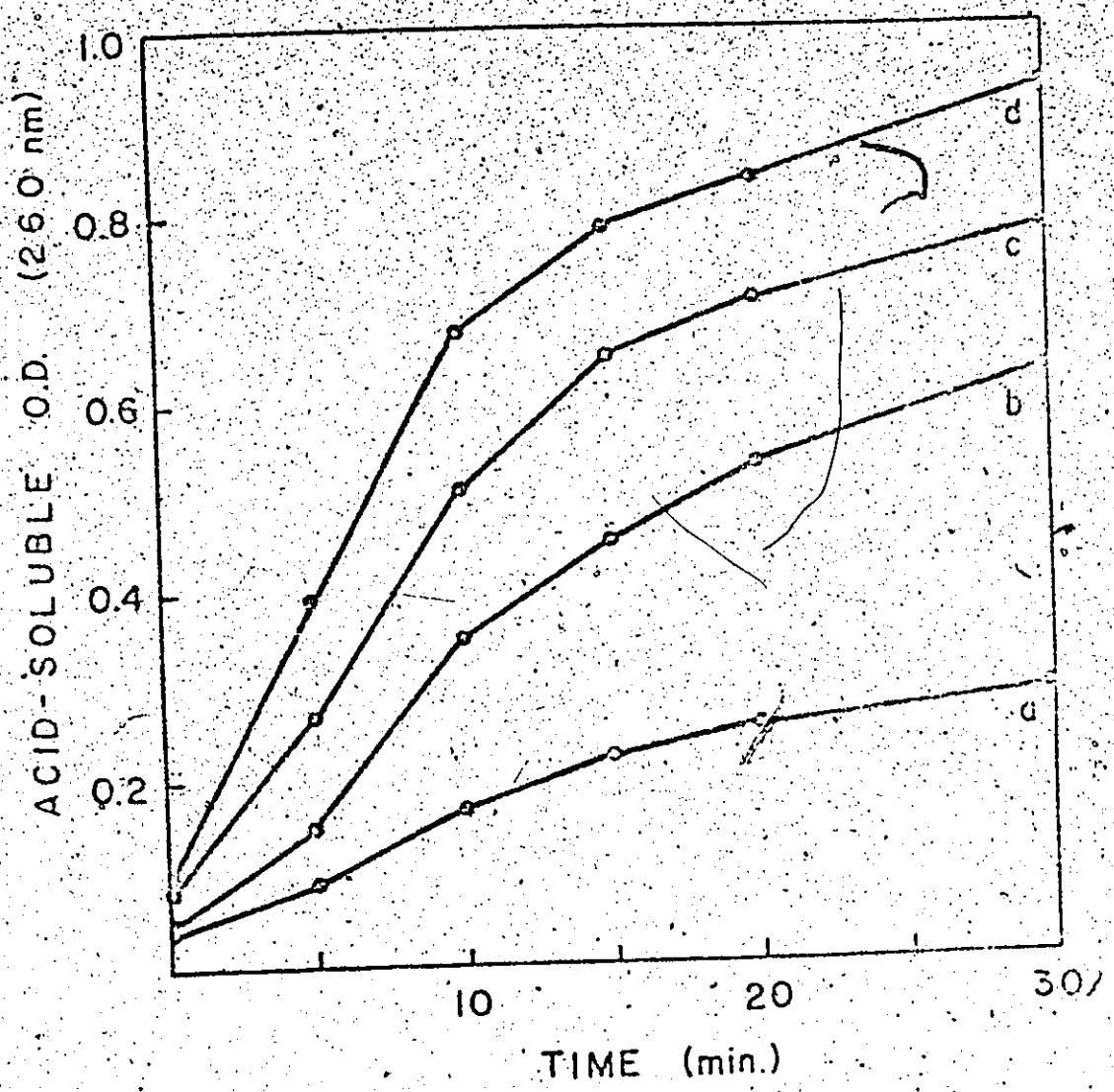

Figure 19. The effect of varying enzyme and substrate concentrations on the RNase activity of the microsomal fraction of Chaetomium. Incubation mixtures contained 0.1 M Tris-HCl, pH 7.0 and (a) 0.5 mg RNA and 0.05 mg microsomal protein, (b) 1.0 mg RNA and 0.05 mg microsomal protein, (c) 1.0 mg RNA and 1.0 mg microsomal protein, (d) 1.0 mg RNA and 1.5 mg microsomal protein.

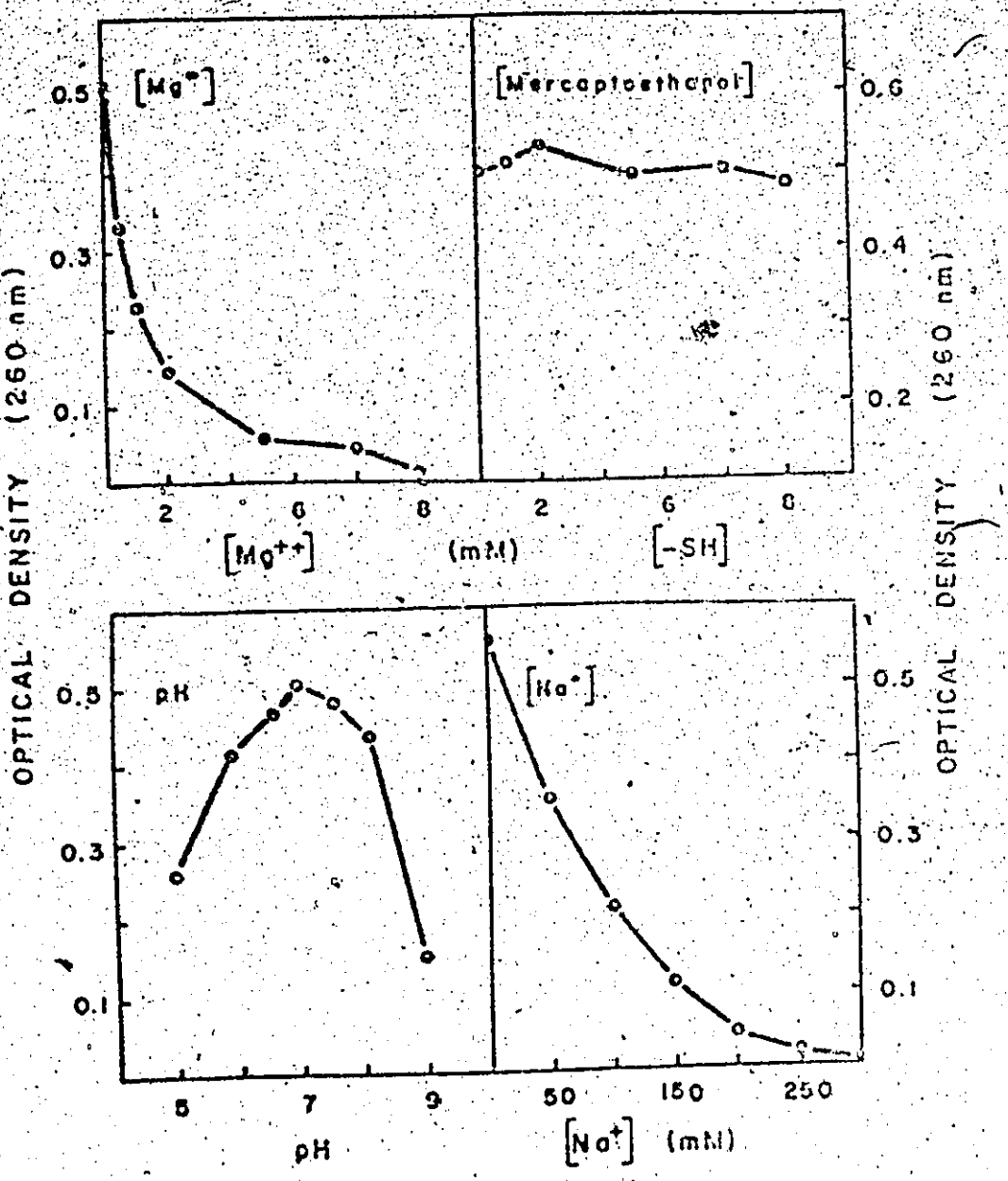

Figure 11. The effect of various conditions on the activity of micrococcal ribonuclease from Chaetomium. Each point represents the acid-soluble optical density after incubation at 37°C for 10 minutes.

5. Properties of Fungal Cytosol rRNA

a. Apparent molecular weight

With the introduction of medium D in the extraction procedure, it was possible to obtain intact rRNA from the fungus. Figure 12 shows the RNA profiles resulting from extraction methods I and II. When co-electrophoresed with rat liver cytosol or E. coli rRNA, whose molecular weights are assumed from Table II, both methods gave identical molecular weights calculated according to Loening (1967). The average and standard deviation of six determinations for the large component was 1.25 (±0.02) x 10^6. The value for the small component was calculated to be 0.70 (±0.02) x 10^6 on the basis of three determinations.

b. Sedimentation analysis

Sedimentation coefficients were determined by centrifugation with ^{14}C-labelled L-cell rRNA on convex sucrose density gradients. With gradients of this type, the S-values of the RNAs are linearly related to their distance from the meniscus at any time during centrifugation. A typical fractionation of Chaetomium cytosol rRNA by this method is shown in Figure 13. Assuming values of 28 S and 18 S for the two high molecular weight L-cell components, the corresponding values for the fungal cytosol rRNAs were calculated. Based on 4 determinations, the averages and standard deviations were calculated to be 23.8 ±0.3 and 17.4 ±0.2. Both buffer systems described in the methods were used, and the temperature differential between them (21°C) had no effect on the observed sedimentation coefficients.

c. Thermal denaturation analysis

Having previously established the extra thermostability of

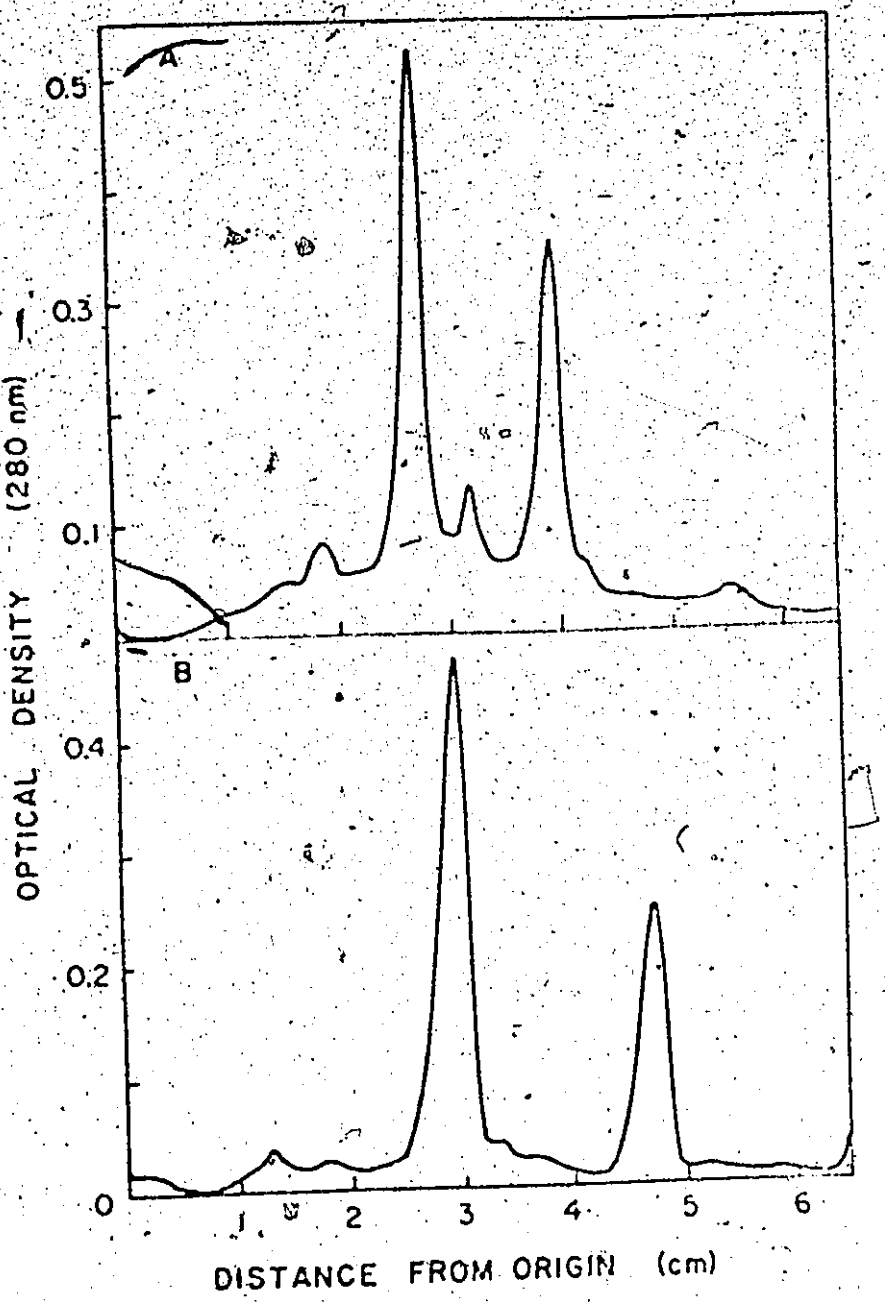

Figure 12. Gel electrophoresis of cytoplasmic rRNA from Chaetomium. Approximately 30 µg RNA were applied to 6.5 cm gels. Electrophoresis was at 25°C in E-buffer. (A) RNA isolated by method I from cytoplasmic ribosomes. The duration of electrophoresis was 3 hr. (B) RNA isolated by method II from hyphae ruptured in liquid nitrogen. The duration of electrophoresis was 3.6 hr.

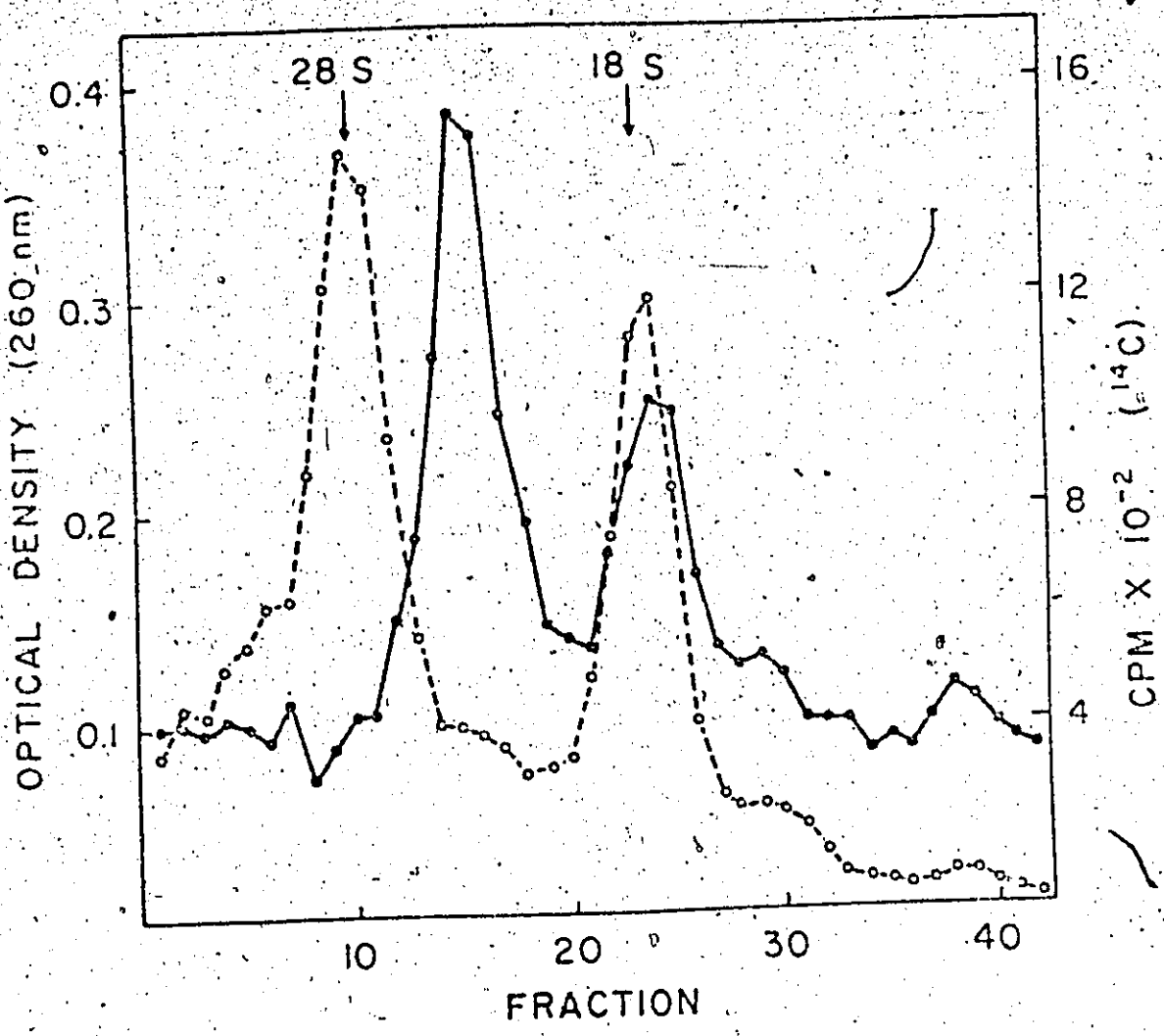

Figure 13. Fractionation of Chaetomium cytoplasmic rRNA on a convex sucrose density gradient in SDS. RNA was prepared from cytoplasmic ribosomes by method 1, and mixed with ^{14}C-labelled L-cell cytoplasmic rRNA for sedimentation analysis. ●——●, OD_{260}, Chaetomium cytoplasmic rRNA; o----o ^{14}C-L-cell rRNA.

Chaetomium cytosol ribosomes over those of rat liver, it was of interest to also examine the relative thermostability of their component rRNAs. The two high molecular weight rRNA components were isolated from rat liver and Chaetomium cytosol ribosomes, and their thermal denaturation profiles are shown in Figure 14. For each species of rRNA, melting curves were determined at both 260 and 280 nm. It has been shown that at 280 nm, the hyperchromic effect observed is due entirely to thermally induced transitions in GC base pairs of the ordered regions of the molecules (Klotz et al., 1963; Cox, 1966). However, if the hyperchromic effect is measured at 260 nm, transitions in both GC and AU pairs are detected. By monitoring at both wavelengths, the ratios of the amount of hyperchromicity measured at 280 nm, to that measured at 260 nm ($\%H_{280}/\%H_{260}$) gives an indication of the relative percentage of GC pairs in the ordered regions of two different RNA molecules. The term %H is defined as $(1-[OD^{T^\circ}/OD^{25^\circ}]) \times 100$.

From Figure 14 and the summary in Table V, it was seen that the fungal and rat liver rRNAs differed in two aspects. First of all, both rat rRNA species had higher T_ms than the corresponding fungal species, and this was true at both the wavelengths monitored. In addition, it was observed that for rat liver rRNA the %H was greater at 280 than at 260 nm. For Chaetomium rRNA, the %H was approximately equal at both wavelengths. This observation is reflected in the ratios of $\%H_{280}/\%H_{260}$ shown in Table V, where the rat liver values are significantly higher than those for Chaetomium. As explained above, the higher ratios for rat liver rRNA are indicative of a higher percentage of GC pairs in the ordered regions of these molecules, relative to the fungal cytosol rRNAs.

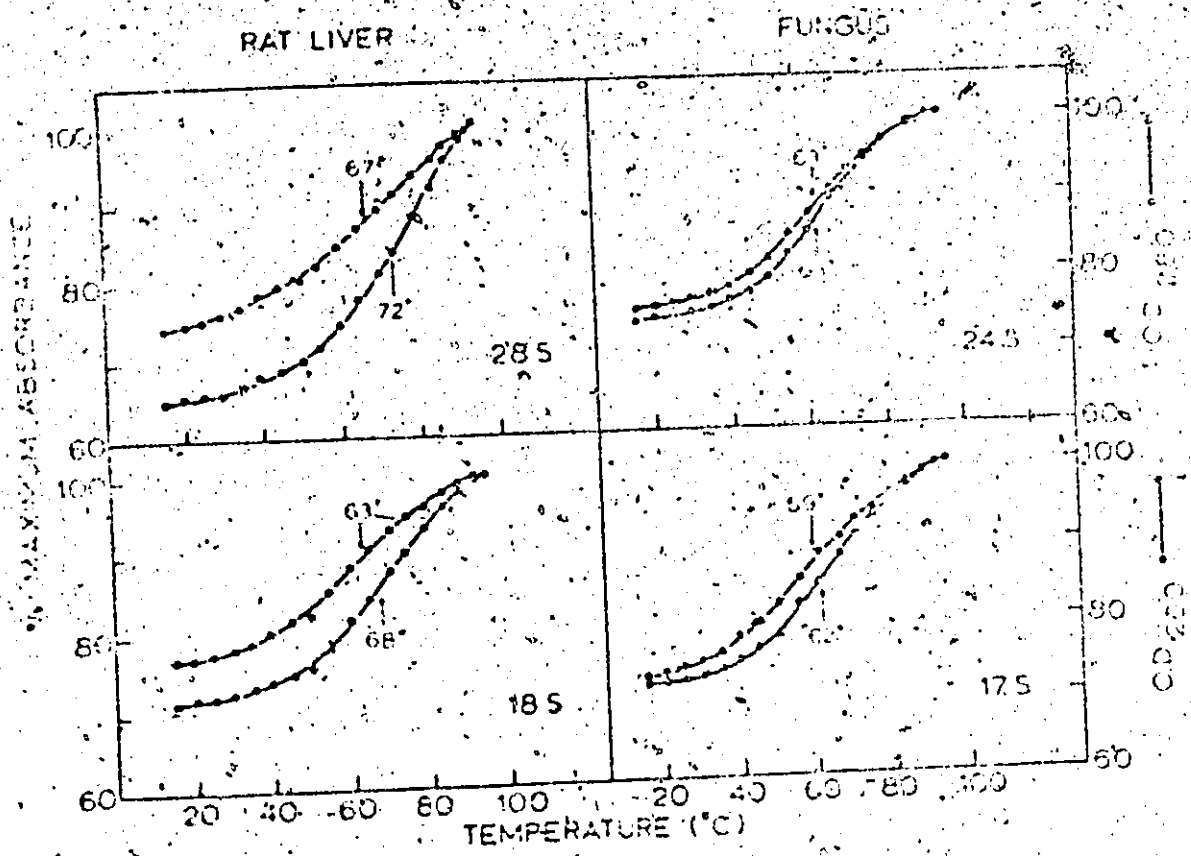

Figure 14. Thermal denaturation profiles of cytoplasmic rRNA from rat liver and Chaetomium. RNA was fractionated on sucrose density gradients, and precipitated with ethanol. After redissolving in RNA-T_m buffer, absorbancies were adjusted initially to 0.250 - 0.350. To facilitate comparison, readings at 95°C were set equal to 100% maximum absorbance. Arrows indicate the T_ms, averaged from 3 determinations.

Table V

Thermal Denaturation Parameters of Chaetomium Cytosol rRNAs

RNA Source	Wavelength	T_m	ΔH	T_m	ΔH
Rat liver	260	66.6 (±0.8)	24.0 (±1.3)	62.8 (±2.0)	22.0 (±0.9)
	280	72.3 (±0.8)	33.4 (±1.8)	67.5 (±1.3)	27.1 (±2.9)
	280/260		1.39		1.22
Chaetomium	260	62.0 (±0.5)	25.5 (±0.7)	59.3 (±0.3)	26.8 (±1.3)
	280	63.2 (±1.0)	26.5 (±0.6)	61.7 (±1.5)	26.6 (±3.0)
	280/260		1.04		1.03

Table VI

Base Composition of Cytosol rRNA from Rat Liver and Chaetomium

Source	Component	Mole %				
		A	U	G	C	G+C
Rat Liver	Heavy	16.2	16.5	35.3	29.0	67.3
	Light	20.9	19.9	33.5	25.8	59.3
Chaetomium	Heavy	24.9	23.7	31.8	19.7	51.5
	Light	25.5	24.4	29.9	20.2	50.1

d. Base composition studies

The higher percentage of GC pairs in the ordered regions of rat liver rRNA, as inferred from the optical data, seems to be reflected in the overall base composition results shown in Table VI. Assuming a molar ratio of 2:1 for the heavy and light components respectively, the average G+C content for Chaetomium cytosol rRNA (51.0%), is very comparable to these values reported in Table II for mesophilic fungi (51%, 53.7, 49.7%).

e. Studies on the Method of Determining Base Composition of RNAs.

To standardize the liquid chromatography system (LCS) for the determination of base compositions, high molecular rRNA components were isolated from rat liver cytosol and from E. coli. In addition, a number of standard nucleoside mixtures, of two different base compositions, were prepared. The base compositions of these standard rRNAs and prepared mixtures, as determined by the LCS method, are shown in Table VII. Also shown are the base compositions determined by other methods, and the theoretical compositions of the prepared mixtures. In comparing the observed and predicted values, two discrepancies appeared to be consistent. Guanosine content was always higher than expected, and cytidine content was always lower. It was felt that this variation was not primarily due to an error in the determination of the extinction coefficients in the elution buffer, because the standard deviations for the observed guanosine and cytidine values were also much higher (5-11%) than those for adenosine and uridine (1-3%). However, to rule out this possibility, spectra of our standard nucleosides were determined and compared to published data (Pabst Laboratories Circular OR-10). All maxima and minima occurred at the proper wavelengths, and all reference ratios were identical to the

Table VII

Base Compositions of RNA from Different Sources

RNA Source	Heavy component U	C	A	C	Light component U	C	A	C
Rat liver cytosol rRNA:								
(1)	16.5	38.3	16.2	29.0	19.9	33.5	20.9	25.8
(2)	19.0	32.9	18.3	29.8	19.6	30.3	22.4	27.8
(3)	17.9	36.3	15.3	30.3	22.5	31.3	20.1	26.1
(4)	17.0	33.0	17.8	32.2	18.0	32.4	19.8	29.8
Escherichia coli rRNA:								
(1)	20.7	33.8	25.5	20.2	20.0	34.9	24.7	20.6
(5)	21.0	32.5	25.5	21.0	21.3	32.1	24.3	22.3
Prepared nucleotide mixtures:								
theoretical					25.0	25.0	25.0	25.0
observed					24.7	26.2	26.4	22.6
theoretical					29.0	12.5	20.8	37.0
observed					30.3	12.8	22.8	34.2

(1) from this study
(2) from Munro (1964)
(3) from Hirsch (1966)
(4) from Kirby (1965)
(5) from Edelman et al. (1970)

published values. When the extinction coefficients at 254 nm, in the elution buffer, were redetermined, no significant changes from the previous estimates could be found.

The second aspect considered was the possibility that the pH (4.5) of the elution buffer was fluctuating. Any change in buffer pH would be most drastically reflected in changes of the ε_{254} for cytidine, whose amino group pK is 4.5 (pKs of other nucleosides are out of this range). Because the LCS is run at 58°C, it was thought that temperature changes may affect the pH of the elution buffer. However, when the effect of temperature on the buffer pH was examined directly, over the range from 22 to 48°C, no trend in pH change was detected.

The final experiment, shown in Figure 15, was undertaken to determine directly the effect of temperature on the ε_{254} of cytidine in elution buffer. Over the temperature range from 25 to 57°C, it was found that the optical density and extinction coefficient at 254 nm, for cytidine, increased approximately 7%. Although the magnitude of the change was thought to be significant, its direction was opposite to that predicted by the base composition results.

7. The Isolation of Chaetomium Mitochondria

Preliminary attempts to isolate Chaetomium mitochondria, free from cytosol contamination, were made utilizing the method of Edelman et al. (1970). In this procedure, nuclei and cell debris are removed from the cell homogenate by centrifugation at 500 x g. A crude mitochondrial pellet is then obtained by centrifuging the post-nuclear supernatant at 26,000 x g. To further purify the mitochondria, the crude fraction is

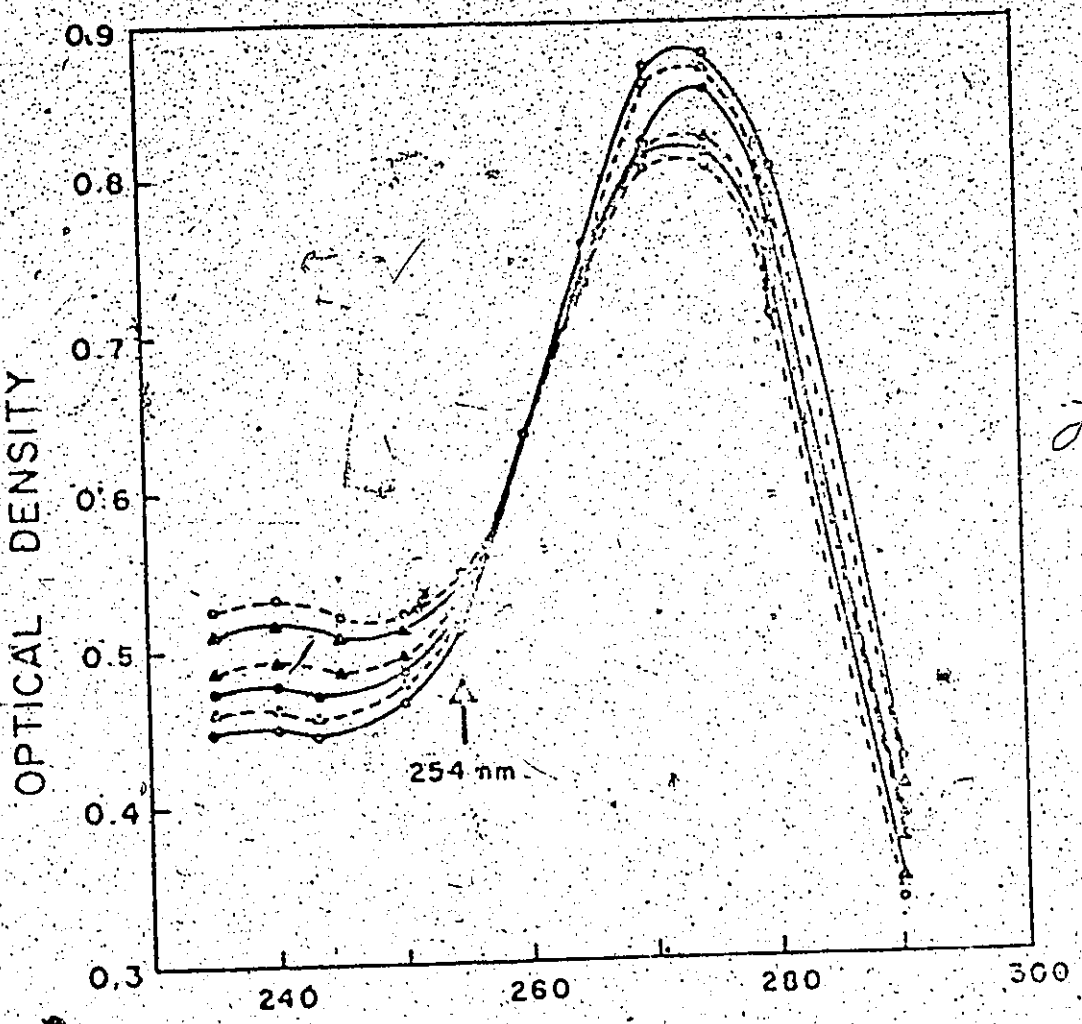

Figure 15. The effect of temperature on the ultraviolet absorbance spectrum of cytidine. A standard solution of cytidine in 0.2 M ammonium formate, pH 4.5 was examined in a Zeiss PMQ II spectrophotometer modified as described in the methods for thermal denaturation analyses. The ultraviolet spectra were determined at various temperatures following an equilibration time of 10 minutes. o——o, 25°C; ▲----▲, 30°C; o——o, 35°C; ▲----▲, 40°C; ▲——▲, 50°C; o----o, 57°C.

layered on top of an 8-step discontinuous sucrose gradient and, following centrifugation, the material at the 1.35-1.50 M sucrose interface was recovered and considered to be purified mitochondria.

The application of this method to Chaetonium gave less than satisfactory results. The initial preparation of the crude fraction at 26,000 x g resulted in a pellet containing unmanageably high amounts of non-mitochondrial material. The quantity of material in the pellet was too large for effective fractionation on the step gradient, and repeated attempts to do so never yielded any mitochondria at the 1.35-1.50 M interface, nor at the 1.50-1.65 M interface. All mitochondrial material appeared in the pellet. Further examination of the crude mitochondrial pellet (P-26) revealed that the bulk of the material was DNA, indicating that most of the contamination was of nuclear origin.

For developing a new isolation method suitable for Chaetonium, the location of the mitochondria at any step in the procedure was followed by assaying marker enzyme activities: cytochrome oxidase located in the inner mitochondrial membrane, and malate dehydrogenase located in the mitochondrial matrix.

Various changes in the differential centrifugation procedure were explored, but it was not possible to separate the mitochondria from the contamination. Treatment of the cell homogenate with deoxyribonuclease also had no positive effect. It was then decided that a slightly different approach would be taken. A crude mitochondrial fraction was obtained exactly as described by Edelman et al. (1970). The pellet was resuspended in isolation medium containing 10 mM EDTA. EDTA complexes with Mg^{2+}, thereby reducing contamination of the mitochondria with

cytosol ribosomes by causing them to dissociate. The homogenate was layered over a cushion of 2.0 M sucrose, and centrifuged at a relatively low (10,000 x g) centrifugal force. By this method, approximately 15-20% of the initial cytochrome oxidase activity was retained in the isolation medium layer, or at the interface. Most of the contaminating material passed through the 2.0 M sucrose layer. The upper layer was then removed and recentrifuged at 26,000 x g to pellet the partially purified mitochondria.

Before attempting further purification, it was necessary to re-examine the use of a discontinuous sucrose gradient for purifying Chaetomium mitochondria. In the experiment shown in Figure 16, an aliquot of partially purified mitochondria was layered on top of a continuous linear sucrose gradient and centrifuged for 1 hr at 50,000 x g. As judged by protein concentration, cytochrome oxidase activity and malate dehydrogenase activity, the isopycnic point for Chaetomium mitochondria is between 1.5 and 1.75 M sucrose, not between 1.35 and 1.50 M sucrose as was found for Aspergillus nidulans mitochondria by Edelman et al. As a result, the composition of the discontinuous sucrose gradient was changed to that shown in Figure 17. After fractionation of partially purified mitochondria on a gradient of this type, one main yellow band appeared at the 1.35-1.75 M sucrose interface, with less frequently-occurring minor bands at the 1.20-1.35 M and 0.90-1.20 M interfaces. Figure 17 also shows that the marker, cytochrome oxidase activity, peaks at the 1.35-1.75 M sucrose interface. Only mitochondria from this interface were used for the isolation of ribosomes and rRNA. Using this procedure, the yield was approximately 0.8 mg of mitochondrial

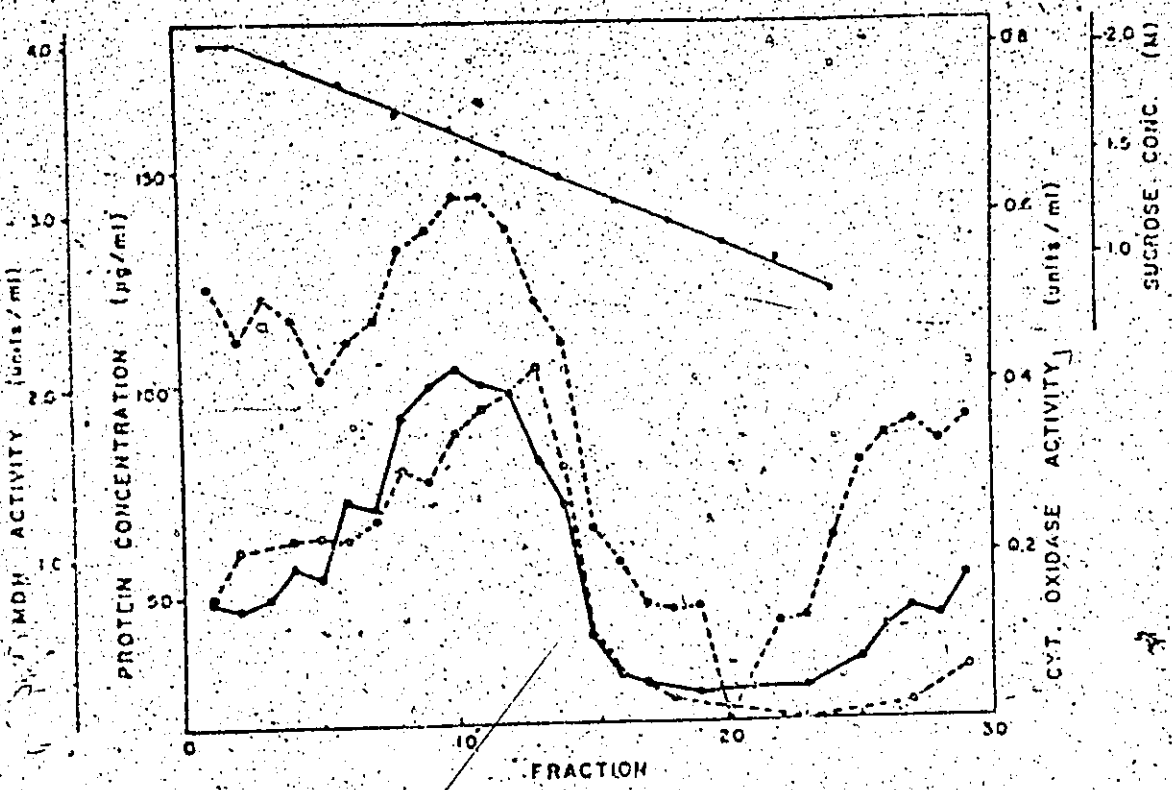

Figure 16. Isopycnic sucrose density gradient centrifugation of a crude mitochondrial fraction from Chaetomium. Approximately 30 mg of protein was layered onto a linear sucrose gradient in 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, and centrifuged at 50,000 x.g and 3°C in the SW25.1 rotor. o----o protein concentration, o——o malate dehydrogenase (MDH) activity, o----o cytochrome oxidase activity.

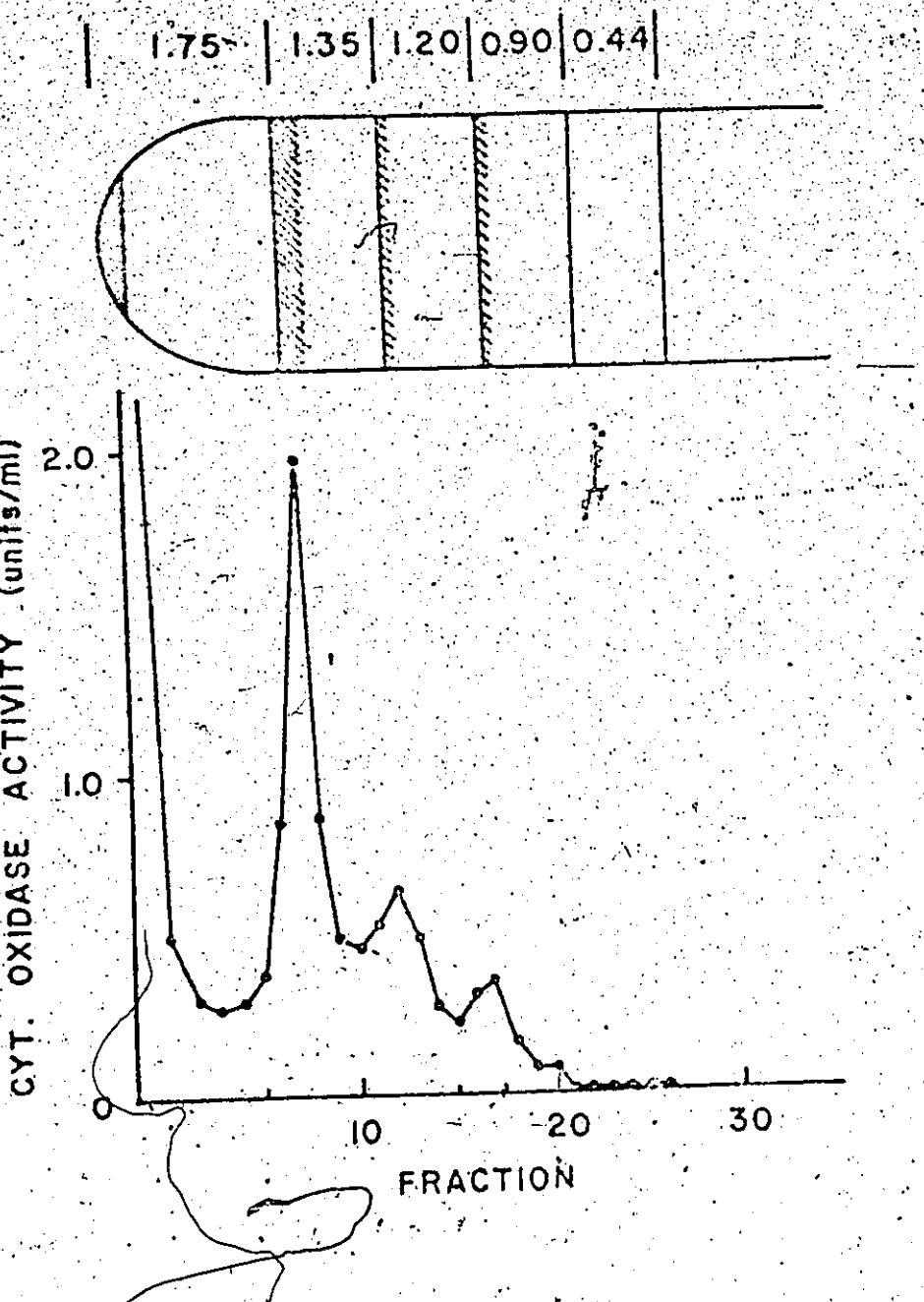

Figure 17. Purification of Chaetomium mitochondria on a discontinuous sucrose gradient. The gradient was prepared and run as described in the methods. The diagram at the top shows the appearance of the gradient after centrifugation, and indicates the molarity of sucrose in each step. The tube was punctured, the gradient was fractionated from the bottom and each fraction was assayed for cytochrome oxidase activity.

protein per gram, wet weight, of fungal mycelia.

Isolation and Characterization of Chaetomium mitochondrial rRNA

a. Sucrose gradient purified mitochondria

Three preparations of mitochondria, purified by centrifugation on sucrose density gradients, and which had been lysed and stored in sodium P as described in the methods, were pooled and their RNA was extracted. The yield was approximately 1 mg of RNA. When fractionated on an isokinetic sucrose density gradient at 25°C with ^{14}C-labelled L-cell rRNA, the fungal rRNA was resolved into two peaks whose sedimentation coefficients were calculated to be 22.8 S and 17.2 S (Figure 18).

The profile of this rRNA after polyacrylamide gel electrophoresis is shown in Figure 19. Two major peaks were seen. In two determinations, the apparent molecular weights of the larger component were calculated to be 1.28 and 1.29 $\times 10^6$. The small components gave identical molecular weights, 0.70×10^6, for both determinations. In addition, a shoulder was seen on the smaller component at slightly higher molecular weight. A peak at this position was not consistent with the pattern of degradation seen for fungal cytosol rRNA. The small, sharp peak near the top of the gel was DNA.

The base composition of the two components isolated on sucrose density gradients is given in Table VIII. The G+C content for both components was significantly lower than that seen for Chaetomium cytosol rRNA, but was very much higher than reported values (30-38%) for mesophilic fungi.

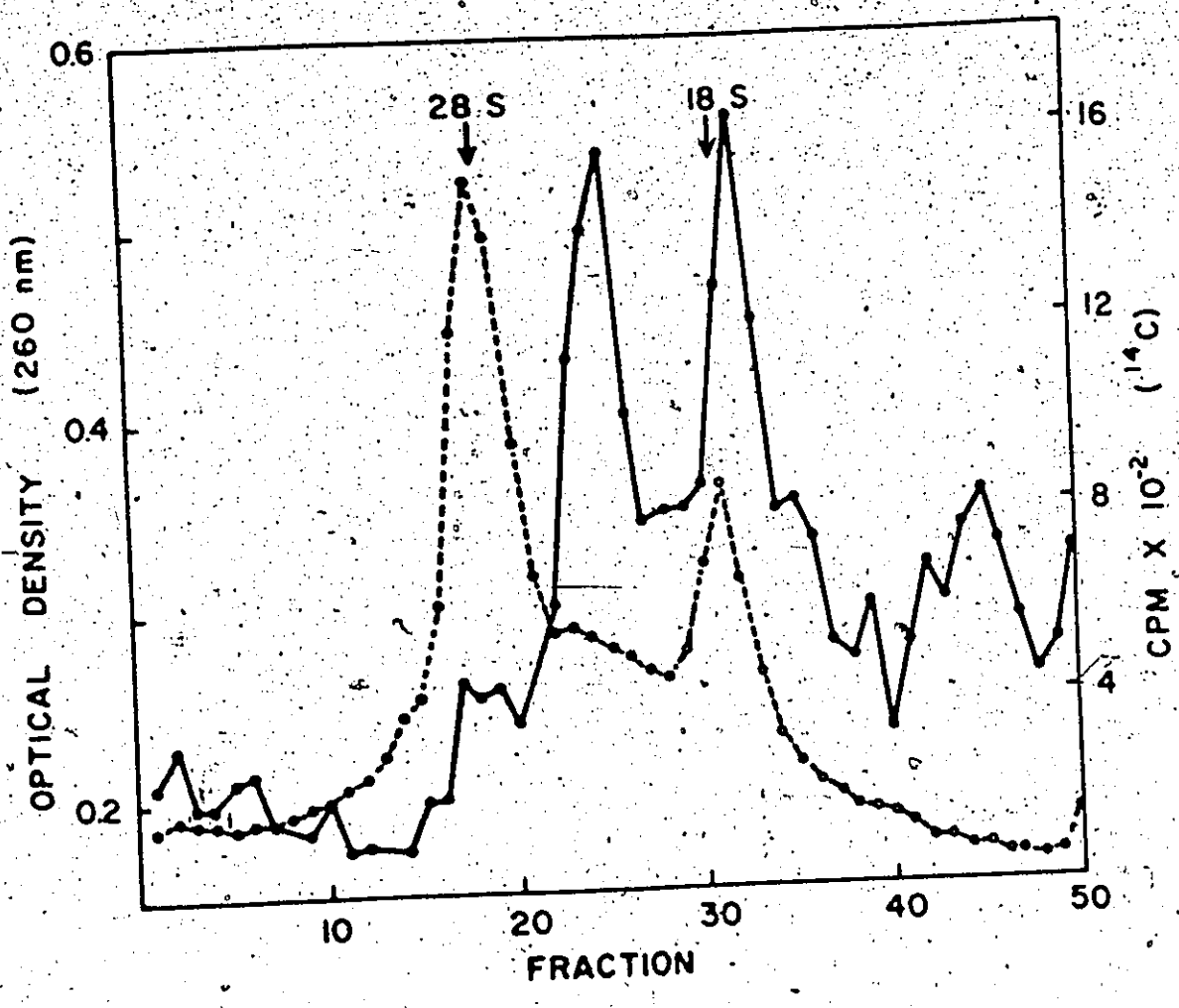

Figure 18. Sedimentation analysis of Chaetonium mitochondrial rRNA on a convex sucrose density gradient in buffer system two (p.28). RNA, isolated from density gradient-purified mitochondria, was mixed with ^{14}C-labelled L-cell cytoplasmic rRNA for sedimentation analysis. ●——●, OD_{260}, Chaetomium mitochondrial rRNA; o——o, ^{14}C L-cell rRNA.

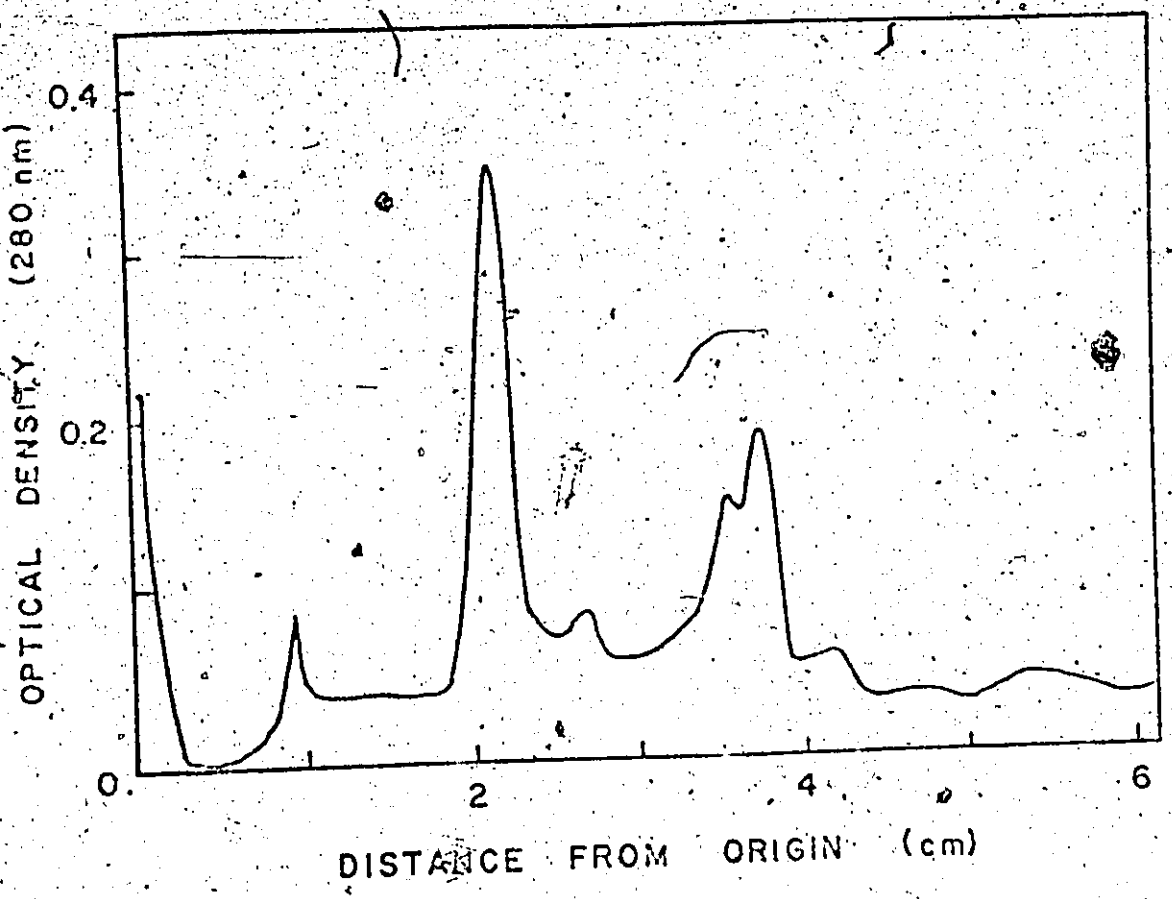

Figure 19. Gel electrophoresis of Chaetomium mitochondrial rRNA. RNA from density-gradient purified mitochondria was applied to a 6.5 cm gel and electrophoresis was in E-buffer for 3 hr at 25°C.

Table VIII

Base Composition of Chaetomium Mitochondrial rRNA

Component	Mole %				
	U	G	A	C	G+C
Heavy peak	25.1 (±0.5)	29.2 (±0.5)	27.5 (±0.3)	18.2 (±1.1)	47.4
Light peak	27.1 (±0.5)	26.9 (±0.3)	28.9 (±0.4)	17.2 (±0.4)	44.1

Table VI

Base Composition of Cytosol rRNA from Rat Liver and Chaetomium

Source	Component	Mole %				
		A	U	G	C	G+C
Rat liver	Heavy	16.2	16.5	38.3	29.0	67.3
	Light	20.9	19.9	33.5	25.8	59.3
Chaetomium	Heavy	24.9	23.7	31.8	19.7	51.5
	Light	25.5	24.4	29.9	20.2	50.1

b. Sucrose gradient-purified, digitonin-washed mitochondria.

It was possible that the results obtained for rRNA isolated from gradient-purified mitochondria could have arisen primarily from contamination of the mitochondria by cytosol ribosomes, perhaps attached to endoplasmic reticulum. Significant contamination by cytosol rRNA would definitely result in the unusually high G+C content seen for Chaetomium mitochondrial rRNA. To explore this possibility, a second preparation of mitochondrial rRNA was undertaken, incorporating a digitonin wash of the gradient-purified mitochondria, prior to extraction of the rRNA.

Low levels of digitonin have been shown to remove essentially all cytosol ribosome contamination of rat liver mitochondria (Malkin, 1971; De Vries & Van der Koogh-Schouring, 1973). At slightly higher levels, the entire outer mitochondrial membrane is removed (Schnaitman & Greenawalt, 1968), leaving the inner membrane and matrix intact.

For studies with Chaetomium, five preparations of mitochondria, washed in digitonin concentrations sufficient to remove outer membrane, were pooled and their rRNA was extracted. Following electrophoresis on E-buffered polyacrylamide gels, no peaks were seen in the ultraviolet profile. However, when centrifuged on a convex sucrose gradient, some high molecular weight material was observed (Figure 20). Although the rRNA appeared to be extensively degraded, the fractions approximating the location of rRNA (fractions 20 to 40) were pooled, and a base composition analysis was performed on the ultraviolet absorbing material (Table IX). The results indicate that the G+C content of rRNA from mitochondria prepared by both methods, is similar.

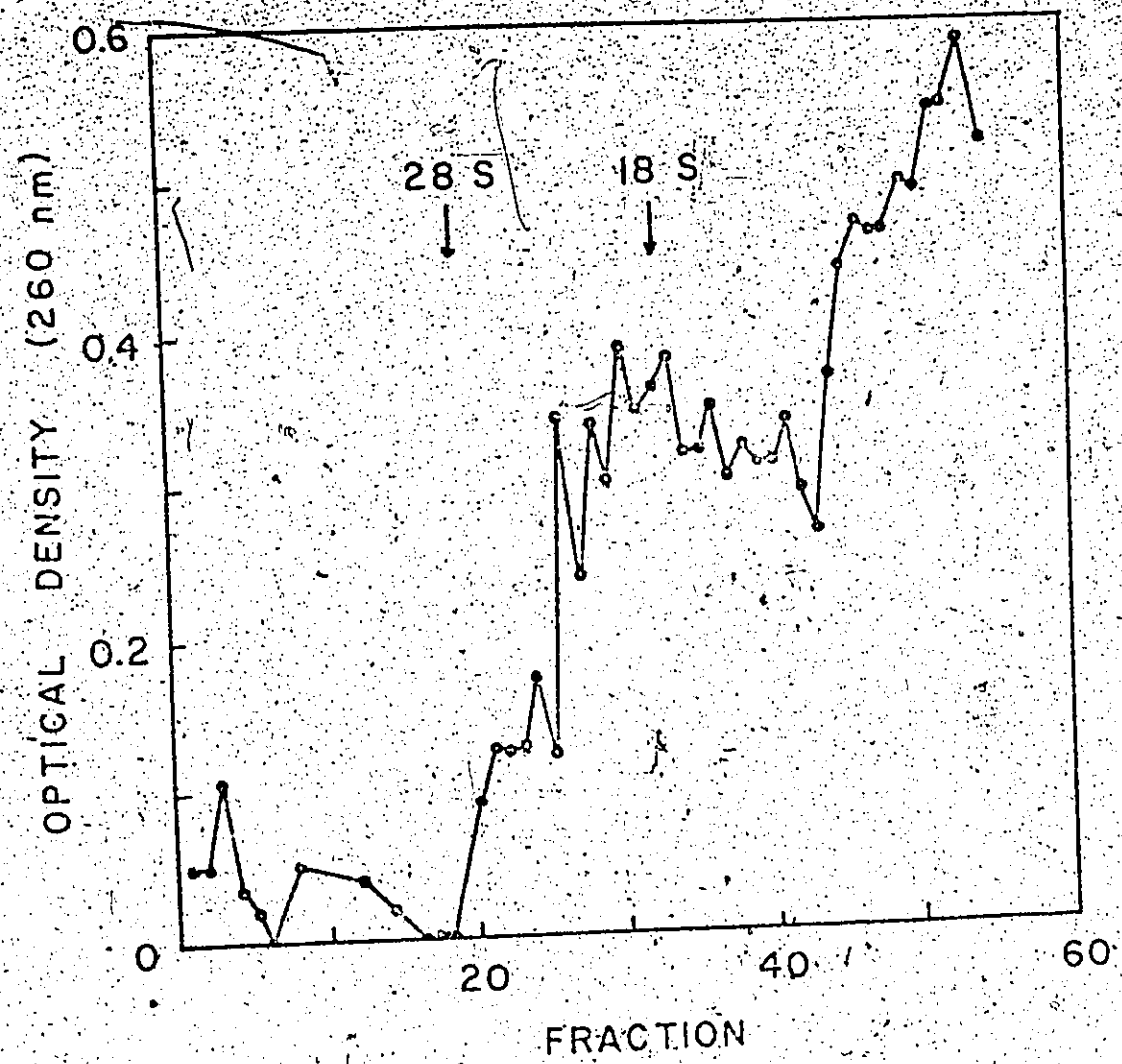

Figure 20. Sedimentation profile of rRNA from gradient-digitonin purified mitochondria from Chaetomium on a convex sucrose density gradient. Arrows indicate theoretical sedimentation coefficients at those positions.

Table IX

Base composition of tRNA from digitonin-washed Chaetomium mitochondria

Component	Moles %				
	U	G	A	C	G+C
Total	27.6	26.6	27.9	17.9	44.5

9. Molecular Weights of Humicola lanuginosa Cytosol rRNA

Cytosol rRNA of Humicola lanuginosa was isolated by method II. In two determinations, the apparent molecular weights were identical for the respective components, 1.31×10^6 and 0.69×10^6.

IV DISCUSSION

Thermostability of Chaetomium Cytosol Ribosomes

The results show that the cytosol ribosomes of Chaetomium thermophile var. coprophile exhibit a significantly greater thermostability when compared to rat liver cytosol ribosomes. However, this finding was not completely unexpected. As discussed above, the same observation has been made with the ribosomes of thermophilic bacteria. In addition, Miller & Shepherd (1973) have demonstrated this phenomenon in another thermophilic fungus, Penicillium duponti. Although the thermal denaturation data from all these studies may not be directly comparable, most of them show the same relationship between ribosome T_m and maximum growth temperature.

Pace & Campbell (1967) found a positive correlation between the maximum growth temperatures of a series of prokaryotic organisms and the T_ms of their ribosomes. In this study and in others, the maximum growth temperatures of the thermophilic organisms were closely approximated by the temperatures at which denaturation of their ribosomes is initiated. The work of Miller & Shepherd indicated that this observation applies also to thermophilic eukaryotic organisms. In addition, the studies reported here with Chaetomium have given the same results. It was observed that the temperature limit for the disruption of ribosomes was approximately [redacted]°C, only 2°C lower than the maximum growth temperature for this organism. As suggested by Mangiantini et al. (1965), the

possibility exists that ribosome stability may be the limiting factor for the growth of organisms at higher temperatures.

Attempts to demonstrate that the thermostability of Chaetomium cytosol ribosomes is accounted for by unusual properties of their component rRNAs, was unsuccessful. Although the T_m of the fungal ribosomes was approximately 10°C higher than that of rat liver cytosol ribosomes, almost the opposite relationship was found for their respective rRNAs. The average T_{m260} of rat liver rRNA was about 5°C higher than that of Chaetomium rRNA. When measured at 280 nm, the difference was 8°C. From the optical data and equation (5) of Cox (1966) it was possible to calculate the G+C content of the ordered regions of the four species of RNA. The values for rat liver were 62.8 (±0.4) and 54.9 (±3.2) for the large and small component respectively. For Chaetomium, the corresponding values were 45.3 (±2.0) and 47.8 (±0.7).

The base composition of the ordered regions of single-stranded polynucleotides is one factor which contributes to the observed T_ms of these molecules (Cox, 1966; Kallenbach, 1968). It is therefore reasonable to believe that the higher T_m for rat liver rRNA is due, at least partially, to a higher percentage of GC pairs per ordered region than for the fungal rRNA. This was reflected not only in G+C content per ordered region as calculated according to Cox, but also in the overall base compositions of the molecules, which closely approximate those of the ordered regions (Table VI).

Because the thermostability relationship seen for rat liver and fungal cytosol ribosomes was reversed for their component rRNAs, it is concluded that the extra thermostability of thermophile ribosomes is not

primarily due to any unusual properties of its rRNA. If the same assumption is made for ribosomal protein (Irwin et al. 1973) and assuming the absence of exogenous stabilizing factors, the thermostability is probably due to rRNA-ribosomal protein interactions (Pace & Campbell, 1967). It has been postulated by Spirin (1963) that protein can stabilize the RNA in ribosomes by shielding the negatively charged phosphate groups, or by filling in the grooves of helical regions to maintain the rigid structure of the helix and to preserve the spatial arrangement between adjacent helical regions.

It is noted that from the thermal denaturation studies, it appears that rat liver ribosomes are actually less thermostable than their component rRNAs. However, because solutions of different ionic composition are used for studies of intact ribosomes and of isolated rRNA, a direct comparison of ribosome T_ms and rRNA T_ms cannot be made.

Physico-chemical Properties of Chaetomium Cytosol rRNA

Characterization of Chaetomium cytosol rRNA has shown that its properties are very similar to those of cytosol rRNA from previously examined mesophilic fungi. The ribosomal nature of the RNA was established by its isolation from purified cytosol ribosomes, rather than from a cell homogenate or post-mitochondrial supernatant.

The largest discrepancy between Chaetomium rRNA and that of mesophilic fungi was observed during the comparison of sedimentation characteristics. Although the sedimentation coefficient of the light rRNA component (17.4 S) is identical to those of Neurospora and Aspergillus (17 - 18 S), the S-value of the heavy component (23.5 S) is significantly

smaller (25 - 28 S). In fact, the value for the Chaetomium heavy rRNA is identical to the value calculated by Edelman for the heavy mitochondrial component of Aspergillus nidulans. Even though the sedimentation coefficient is small, the observation was consistent, and changes in the ionic strength, or temperature of the sedimentation studies, had no effect on the observed S-values.

Examination of the apparent molecular weights of Chaetomium cytosol rRNA by polyacrylamide gel electrophoresis gave similar, but less definitive results. Again, the apparent molecular weight of the light component (0.70×10^6) compared well with the values for mesophiles ($0.67 - 0.72 \times 10^6$). On the other hand, the heavy component (1.25×10^6) was slightly smaller than the corresponding components of Aspergillus and Neurospora ($1.28 - 1.29 \times 10^6$). Although this difference is small and its statistical significance is unknown, it presents a situation analagous to that drawn from the sedimentation studies.

The thermal denaturation patterns of Chaetomium cytosol rRNA raise some interesting questions when compared to similar data available for the mesophiles Trichoderma, Neurospora and Aspergillus (Edelman et al., 1971). Adequate controls were not undertaken that would allow satisfactory comparison of the two sets of data, but because the same method was used in both cases, some analogies can be drawn.

As might be expected from the nature of the organism, the T_ms observed for Chaetomium were slightly higher (59.3 - 63.2°C) than those of the mesophiles (52 - 61°C). However, on the basis of the optical data, the G+C content of the ordered regions (45.3 - 47.8%) was calculated to be lower than the value of 54.5% in Neurospora (Edelman et al., 1970)

which has T_ms of 54.5°C. It is unlikely, therefore, that this slight difference in stability is caused by increased G+C content in the ordered regions of the thermophile rRNA.

Factors other than base composition, which affect the T_ms of single-stranded polynucleotides, include the length and base sequence of the ordered regions, base stacking and imperfect helix formation. Of these factors, only the length of the ordered region can be considered from the thermal denaturation patterns. When measured at 260 nm, the wavelength at which both AU- and GC transitions are detected, the amount of hyperchromicity seen for Chaetomium rRNA (25.5 - 26.8%) is marginally greater than that of the mesophiles (22 - 23%). This can be interpreted by assuming that the thermophile rRNA is more highly ordered than the mesophiles, containing either more short regions of helical structure, or an equivalent or smaller number of longer regions of helical structure. The latter situation, if correct, should impart a slight increase in thermostability to the rRNA of the thermophile.

One other difference noted in the characterization of Chaetomium rRNA, was the apparent discrepancy between the calculated base composition of the ordered regions and the overall base composition of the molecules. The anomaly was seen also for the rat liver rRNA control and was of approximately the same magnitude (3 - 4 mole % lower in the ordered regions). There is no *a priori* reason to assume that the base composition of the whole molecule would be the same as that of a particular region of the molecule. The study of Aspergillus rRNA by Edelman *et al.* (1970) revealed the same discrepancy of approximately the same size. It is possible that the method used for determining total base composition may

he suspect, especially in view of our inability to account for the variation of values for guanosine and cytidine by this method. However, in spite of changes in individual values for G and C, the total G+C content of the two reference rRNAs (rat liver cytosol and E. coli) was relatively constant and compared favourably to reported values (Table VII).

Molecular Weights of Thermophilic Fungal Cytosol rRNAs

The apparent molecular weights of cytosol rRNAs from Chaetomium thermophile var. coprophile (1.25×10^6 and 0.70×10^6) gave a first indication that the size of the heavy component of rRNA from thermophilic fungi might be less than those of other fungi. Of the range of molecular weights reported by Lovett and Haselby (1971), the smallest was 1.30 (± 0.07) $\times 10^6$; a value which was outside of the mean plus standard deviation for Chaetomium thermophile. However, the size of the corresponding heavy component from Humicola lanuginosa (1.31×10^6) was within the range reported by Lovett and Haselby.

On the basis of only two thermophiles, it is impossible to draw any meaningful conclusions about the size of rRNA from thermophilic fungi. The value determined for Chaetomium is definitely the smallest yet reported, but the larger Humicola species gives no further indication of a trend toward smaller molecular weights in the heavy component of rRNA from thermophilic fungi. A more likely conception is that the lower size limit (1.30×10^6) defined by Lovett & Haselby is incorrect, and that the value obtained for Chaetomium actually falls on a continuum of molecular weights which may end at some point less than 1.30×10^6.

Edelman et al. (1970) reported values of 1.29×10^6 and 0.72×10^6 for Aspergillus. In addition, Loening (1968) reports one other observation, from a species of Rhyzopus, of rRNA whose size was 1.28×10^6.

The Unique Nature of Chaetomium Mitochondrial rRNA

a. The evidence

Because of the similarity in properties between mitochondrial and cytosol rRNA, base composition data is usually used as a major criterion by which the two are distinguished. When the object of an investigation is to demonstrate possible similarities between the base compositions of mitochondrial and cytoplasmic rRNA, this distinction is obviously more difficult to make. The results obtained with Chaetomium have presented just such a difficulty.

By reason of the base composition data alone, it is possible to conclude that a unique species of rRNA has been isolated. The G+C content of rRNA from gradient-purified mitochondria was neither as high as that of the cytosol, nor as low as that seen for mitochondrial rRNA of other fungi. Because of this intermediate value (45%) for G+C content, it could be argued that the correct value is in fact much lower and that the observed estimate is simply a reflection of contamination of the preparation with cytosol rRNA. The likelihood of this occurrence was reduced by washing the mitochondria with digitonin to remove the outer mitochondrial membrane and most of the contaminating cytosol ribosomes. Even though no intact rRNA was isolated by this procedure, there was high molecular weight RNA present in the product; RNA whose base composition was identical (44.5% G+C) to the previously determined value.

Molecular weight determinations and sedimentation velocities give further indications that a unique species of RNA has been isolated from the mitochondrial fraction. In both cases, the values obtained for the light mitochondrial component are identical to those of the light cytosol component. For molecular weight studies, this was not unexpected, as Edelman et al. (1970) and Neupert et al. (1969) found that apparent molecular weights were the same for corresponding rRNA components from the mitochondria and cytosol of both Aspergillus and Neurospora (Table I). But for sedimentation studies, the light mitochondrial component has usually been found to have a smaller S-value than the cytosol component. Because the apparent molecular weight-sedimentation coefficient discrepancy is thought to be due to the low G+C content of mitochondrial rRNA, it is possible that this anomaly may not appear for Chaetomium because the G+C content of its mitochondrial rRNA is similar to that of the cytosol rRNA.

On the other hand, the heavier component from the mitochondrial fraction does appear to be unique in both molecular weight and S-value. On polyacrylamide gels, the heavy component has an apparent molecular weight of 1.29×10^6 compared to $1.25 (\pm 0.02) \times 10^6$ for the cytosol species. On sucrose density gradients, the mitochondrial component is observed to be slightly smaller than the cytosol component (22.8 S vs 23.8 S ± 0.3). The magnitude of this difference was not as large as that observed for other fungi, but, again, the higher G+C content of Chaetomium mitochondrial rRNA, if correct, may explain this.

Although the mitochondria used in this investigation were considered to be free of cytosol ribosomes, the properties of Chaetomium mitochondrial

rRNA will not be definitely established until the rRNA is isolated from purified mitochondrial ribosomes rather than from whole mitochondria. This would be particularly applicable for further study of the optical properties of mitochondrial rRNA, for which optical quantities of RNA are required. To simply verify the data obtained in the present study, labelling of the rRNA in vivo with ^{32}P-orthophosphate would be adequate. The mitochondria could be purified, the rRNA extracted, using previously prepared unlabelled cytosol rRNA as a carrier, and both hot and cold rRNAs could then be examined simultaneously on sucrose gradients or polyacrylamide gels. An experiment of this type would establish if the results obtained here were real, or whether they are simply the result of contamination of the mitochondria by cytosol ribosomes.

b. The implications

The results presented here for Chaetomium thermophile var. coprophile represent the first demonstration of mitochondrial rRNA from an animal or ascomycete source whose physico-chemical properties closely resemble those of its homologous cytosol rRNA. A recent report by Gray (1974) indicates that mitochondrial rRNA from wheat embryos shows sedimentation and electrophoretic properties identical to those of its cytosol counterparts. In addition, the G+C content (54.8%) was much higher than that found for any other organism, including Chaetomium.

If both studies are considered together, the results suggest that a low G+C content is not a universal characteristic of mitochondrial rRNA. The existence of at least two species of eukaryotes with relatively high G+C contents in their mitochondrial rRNA indicates that a low G+C content is not a prerequisite for the normal functioning of mitochondrial

ribosomes. However, the observations do not really elucidate the role of mitochondrial rRNA beyond what is already known.

The studies with Chaetomium cytosol ribosomes led to the speculation that ribosomal RNA does not play the major role in maintaining ribosome structure under conditions of thermal stress. But the discovery of mitochondrial rRNA from Chaetomium with a G+C content much higher than that seen for other fungi and animals, seemed to indicate that mitochondrial rRNA of low G+C content would not be sufficiently stable in a thermal environment to allow the ribosomes to function. This could be interpreted by assuming that although the rRNA does not alone define the stability of the ribosome, there is a lower limit of rRNA stability below which the ribosome cannot remain functional. Under normal temperature conditions, the low G+C content of other mitochondrial rRNAs does not approach this stability limit, but at higher growth temperatures the limit is reached, and the thermophilic organisms have evolved with more stable mitochondrial rRNA to remain viable.

The observations of Gray (1974) cast some doubt on the assumptions made above. The discovery of a mitochondrial rRNA in plants with a high G+C content is difficult to explain in terms of thermal stress. Wheat, although viable over a wide temperature range, cannot be considered to be subject to a thermal environment which is very different from that of the mesophilic fungi. Therefore, the high G+C content of its mitochondrial rRNA is puzzling. Perhaps the only way to understand the role of mitochondrial rRNA is to extend the survey of its properties even further. Studies of other thermophilic fungi and plants may yield mitochondrial rRNA with properties that do not fit the pattern discussed

here. It may also be found that other mesophilic fungal and animal sources do not conform to the low G+C content pattern. A thorough investigation of the varied eukaryotic organisms, and the resulting relationships seen between G+C content of the mitochondrial rRNA and the environment of the organism, will help to define the role of rRNA in the functioning of the mitochondrial ribosome.

V SUMMARY

Cytosol ribosomes were isolated from both rat liver and from the thermophilic fungus, Chaetomium thermophile var. coprophile. Both types sedimented at 80 S. Melting profiles were measured at 260 nm. Thermophile ribosomes exhibited a higher thermal denaturation midpoint (T_m=66°C) than the rat liver ribosomes (T_m=54°C). Fungal rRNA was extracted from the ribosomes and separated on sucrose gradients into two major species with sedimentation coefficients of 23.8 S and 17.4 S. Their corresponding molecular weights, determined by polyacrylamide gel electrophoresis, were 1.25×10^6 and 0.70×10^6. The melting temperatures (62.0°C and 59.5°C) were lower than those of rat liver rRNA (66.7°C and 62.8°C). This was interpreted on the basis of lower GC content in the fungal rRNAs (52%, 50%) than in the corresponding rat liver rRNA species (67%, 59%).

Mitochondrial rRNA was extracted from highly purified Chaetomium mitochondria. The two high molecular weight rRNAs had unique sedimentation coefficients (22.8 S, 17.4 S), molecular weights (1.29×10^6, 0.7×10^6) and GC contents (47%, 44%).

The molecular weights of the cytosol rRNAs of another thermophilic fungus, Humicola lanuginosa were found to be 1.31×10^6 and 0.69×10^6.

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