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PHYSICO-CHEMICAL PROPERTIES OF BISOSOMAL RNA
FROM THERMO菲ILIC FUNGI

by

ERIC R. HALL, B.Sc.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
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Master of Science

Master University
September, 1974

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TITLE: Physico-chemical Properties of Ribosomal RNA from Thermophilic Fungi

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SCOPE AND CONTENTS: Thermophilic fungi have the highest known growth temperature (60°C) of any eukaryotic organism. 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 Chondromyces thermophilus var. cuprophilus were compared to those of rat liver. Chondromyces 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 Chondromyces ribosome results from unique RNA-protein interactions rather than from novel characteristics of its ribosomal RNAs.

An examination of Chondromyces 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
Atherosclerotic plaques. These observations represent the first demonstration of mitochondrion ribosomal RNA with properties similar to those of its homologous extracellular fragments.
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LIST OF ABBREVIATIONS

EDTA = ethylene diaminetetraacetate
LCS = liquid chromatography system
MDH = malate dehydrogenase
rRNA = ribosomal ribonucleic acid
SDS = sodium dodecyl sulphate
STINS = sodium tri-isopropylolphthalene sulphonate
Tm = thermal transition midpoint
Tris = tris (hydroxymethyl) aminomethane
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I. INTRODUCTION

Mitochondria of eukaryotic organisms are known to contain a protein synthesizing system that is distinct from the corresponding cytoplasmic 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 cytoplasm. Mitochondria are also known to contain unique species of messenger RNA, transfer RNA, ribosomes, and ribonucleases. Reviews by Ashwell and Work (1970), Kuchel (1971), Boner (1972) and Eichler (1973) cover these areas and others in adequate detail. It is the unusual properties of mitochondrial ribosomes and their messenger RNAs which led to the investigations reported in this paper.

Mitochondrial Ribosomes

Early reports on the existence of a protein synthesizing apparatus in mitochondria met with a great deal of resistance. Many attributed the observations to contamination of the mitochondria with bacteria or with cytoplasmic ribosomes. But improvements in techniques 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. These are classified on the basis of physico-chemical properties, mitochondrial ribosomes.

* This term is used to indicate extra-organellar cytoplasm.
Mitochondria-free nuclear ribosomes are an essential component in the synthesis of mRNA and protein synthesis. The mitochondria-free nuclear ribosomes are known to be responsible for the production of mRNA and protein synthesis. In the presence of synthetic RNA, the mitochondria-free nuclear ribosomes are able to synthesize protein. The mitochondria-free nuclear ribosomes are also able to synthesize protein in the absence of synthetic RNA. The mitochondria-free nuclear ribosomes are essential for the synthesis of protein.

The mitochondria-free nuclear ribosomes have a specific inhibitor of protein synthesis. The mitochondria-free nuclear ribosomes are able to synthesize protein in the presence of this inhibitor.

However, the mitochondria-free nuclear ribosomes are not completely inhibited by this inhibitor. The mitochondria-free nuclear ribosomes are able to synthesize protein in the presence of this inhibitor. The mitochondria-free nuclear ribosomes are able to synthesize protein in the absence of this inhibitor.

The mitochondria-free nuclear ribosomes are essential for the synthesis of protein. The mitochondria-free nuclear ribosomes are able to synthesize protein in the presence of this inhibitor. The mitochondria-free nuclear ribosomes are able to synthesize protein in the absence of this inhibitor.
of such unusual composition can maintain functional similarities to other ribosomes in Intrazonal and, to date, ribosomes from animal mitochondria remain the least studied and the least understood in the field of mitochondrial biology.

At present, the second category of mitochondrial ribosomes is limited to those found in the protozoan Paramecium bursaria. Suyama (1970) originally characterized these ribosomes, finding their sedimentation coefficients (s20, w) to be identical to that of the external ribosomes. The two types could be distinguished, however, by their differing buoyant densities in CsCl and by the fact that in E. coli, the 80 S mitochondrial ribosome is transformed into a 55 S, doublet by peak at 55 S. They also found that there are cytosol ribosomes containing 28 S and 17 S ribosomal RNA, mitochondrial ribosomes and their polyribosomal subunits yielded RNA sedimenting at 21 S and 14 S. Recently, Scarlath et al. (1974) further authenticated the 80 S mitochondrial particle by demonstrating that its unique properties are shown by polyribosome gel electrophoresis and electron microscopy.

The final category encompasses those ribosomes isolated from the mitochondria of the aspergillus. Most authors have assumed that the mitochondrial ribosomes of yeast and fungi have sedimentation coefficients intermediate between those of the cytoplasm and those of E. coli. Morimoto & Halvorson (1971) were unable to accept they maintained that for yeast, 80 S was the correct sedimentation coefficient. In addition, Hara 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 (Lambert et al., 1974), a fact which may force revision of the previously accepted value of 71 s.

Although controversy may surround the s-value of mitochondrial ribosomes of amphibians, there is agreement on the s-value of the mitochondrial particles. Unlike their eukaryotic counterparts, amphibian mitochondrial ribosomes readily dissociate into subunits (at S and 3) when the magnesium concentration is less than 100 mEq/l.

The amphibian category is also unique in that mitochondrial ribosomal RNA (rRNA) bands have the largest sedimentation coefficients of any mitochondrial RNA's (23 S and 18 S), differing but little from their corresponding cytosol species (23 S and 17 S).

Mitochondrial ribosomal RNA

In a reference to prokaryotic and eukaryotic ribosomes, Linde (1972) observed that the basic structure of 70S has been conserved through evolution; perhaps because a working alternative could not be found. In their studies, mitochondrial ribosomes must be studied. It may be unjustified to say that mitochondrial ribosomes are in fact the "working alternative" but certainly the unique physical and functional properties of mitochondrial RNA present quite interesting question 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. Robin Jones & Cleaves (1970) have found an unmethylated, ethanolic precipitable
sensitive species of RNA in hamster cell mitochondria, which sediments at 3 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 1 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 (Hedinger, 1967, 1969) that in neutral salt buffers, the rate of migration of rRNAs through polyacrylamide gels is linearly related to the logarithm of their molecular weights. But the appearance of the anomalies listed in Table 1 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 (Förrester et al., 1970; Grivell et al., 1971; Herzog et al., 1970; Edelman et al., ...)
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<tr>
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<td>coefficient (S)</td>
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<td>1.73 (1.25)</td>
<td>14 (17)</td>
<td>1.19 (0.82)</td>
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<td>15 (17)</td>
<td>0.72 (0.67)</td>
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<td>1.28 (1.28)</td>
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<td>Human (HeLa cells)</td>
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<td>12 (19)</td>
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<td>Attardi &amp; Attardi (1971)</td>
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* 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 rRNAs 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. Delcan 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 90°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 RNA by methods which are unaffected by the secondary structure of the RNA. The basis of their method is to examine the molecules by electrophoresis on polyacrylamide gels under highly denaturing conditions; either 6M urea at 0°C, or 9M formaldehyde at 30°C. The molecular weights determined by these methods were found to compare very well with values obtained from sedimentation equilibrium, ultracentrifugation studies, and from electron microscopy of the RNA crystallized in diethyl ether. The averages of the two methods (1.3 x 10^6 and 0.73 x 10^6) were very similar to the molecular weight reported for other mRNAs (Table I).

Although the unusually low G+C content of mitochondrial RNA may not entirely explain their behaviour on polyacrylamide gels, it seems to be the basis of their distinctive optical and emission properties.

Namba, Kay & Littauer (1961) have determined that like cytoplasmic RNA, mitochondrial RNA is stabilized by both hydrogen bonding and base stacking in helical segments of the molecules. The above changes induced when these molecules are heated are reflections of breakdown in this secondary structure. The thermal denaturation patterns of mitochondrial and cytosolic RNA have been studied by Edelman et al. (1971) and two observations are consistent. The first is that mitochondrial RNA have much lower thermal-denaturation midpoints (Tm) than their corresponding cytosolic-RNAs. The second is that judged from the ratios of the amount of hyperchrocity measured at 260 nm, to that measured at 260 nm (Fresco, Klotz & Richards, 1963; Col., 1966), mitochondrial RNAs have smaller percentages of GC pairs in the helical regions of their molecules than do the cytosolic 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 physical-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 & Bartoo, 1972). 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>
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* after Freeman, Htta and Barcoov (1971)
† when values are given for only one component, data is for total RNA
less compact structure than its cytosolic 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.

When mitochondrial rRNA from an organism living at high temperatures and 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 very high 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 thermostable form species, that there exists a lower limit to 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 physicochemical properties of mitochondrial and cytosolic rRNA from a thermostable fungus. Thermo-philic fungi will grow at temperatures up to 60°C, the highest for any eukaryotic organism. It might be expected that its ribosomal components would exhibit unusual properties, resulting in increased thermal stability.

Studies on Thermostable 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. According to Frieden (1960),
provides a concise summary of studies related to ribosomes and
ribosomal proteins from thermophiles. However, because of their
ability to thrive at more extreme temperatures, the life cycle of bacteria
have been more thoroughly investigated than other prokaryotes. As
mentioned previously, the upper temperature limit for growth of
coliform bacteria is near 60°C, whereas some thermophiles have
been found at temperatures over 80°C, such as in hot springs
(Brock, 1968).

A large number of reports have confirmed the potential
catalytic and synthetic activity of thermophiles beyond merely adapting to
resist thermal denaturation. Ribosomes isolated from thermophile
bacteria, Bacillus thermoproteolyticus (Marshall et al., 1964),
Thermus aquatics (Crick, Taylor & Brenner, 1970) and the species of
Clostridium (Irvine, Knight & Hinton, 1970), all showed significantly
higher thermal denaturation temperatures (Tm) than their counterparts
related aerobes, or those of E. coli. In particular, Price & Bassoli
(1967) found a positive correlation between the maximum growth tempera-
ture of 19 psychrophiles, crenophiles and thermophile species and
the Tm of their ribosomes. These physical studies, however, do not
necesarily relate to the biological function of the ribosomes. With
this in mind, Friedman, Axell & Kuhns (1971) and Hill et al. (1971)
preincubated ribosomes at high temperatures, removed aliquots at various
times, and assayed their ability to support poly U-directed phenyl-
alanine incorporation. In both cases, after preincubation for 30
minutes, thermophile ribosomes lost no more than 25% of their activity.
On the other hand, ribosomes from the thermophiles 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, RNA, and the effect of heat and polymers. Because the characterization of thermophilic RNA are of primary importance to this thesis, further discussion will be limited to properties of this component. Reports by Bottone et al. (1963) on _E. coli_ RNA and by Selke et al. (1970) on _T. maritima_ RNA are typical of the findings of other workers.

In both cases, RNA 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 RNA alone cannot account for this variation. Of interest also, was the nucleotide composition of the thermophilic RNA. The G+C content of _T. maritima_ pRNA was 61% higher than that of _E. coli_ RNA. The difference between the 20.8 at _T. maritima_ pRNA 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 thermostability.

Since the differences in the G+C content between the thermophiles and _E. coli_ are small and variable (77 & 15), 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, Stenberg & Holazo (1967) determined the base compositions of RNA isolated from mesophilic and thermophilic strains of the same genus.
The average GC content for the thermophilic was 60\%, a value statistically distinct from that of the mesophilic, 50\%. However, when the same study was done with other species of bacteria, no difference in GC content could be detected (Luria et al., 1957).

Although these studies did not integrate with the information on the origin and evolution of bacteria, they did reveal the importance of the GC content in the study of bacterial adaptation. The work of Cambell (1967) is particularly interesting. He found that the growth temperature of 10 different strains, differing in their GC content (12-52\%), was correlated with the GC content of the DNA. The GC content doubled as the growth temperature increased. In a linear relationship, the GC content increased by 2% for each 10\°C increase in growth temperature. By extrapolating the data, we can calculate that for a 20\°C increase, the GC content would double. The results can be expressed as a 10\°C increase in GC content. However, the difference in GC content between thermophilic and mesophilic strains could only be expected to increase in the expected manner. For thermophilic strains, the GC content is about 60\%, for mesophilic, 50\%.

In Table II, the maximum growth temperature is 90-100\°C. Since we are applying this temperature difference of 20-30\°C to the relationship of Face & Campbell, we might expect an increase in the GC content of 10\% from thermophilic strains of 1.6-4.3\%. Although the increase is small, it should still be detectable, and it would probably make the GC 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 autotrophs 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 fitness 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 cytoplasmic sources, Leontiev (1969) hypothesized a pattern of evolution based upon the molecular weights of rRNAs. Leontiev'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 $1.69 \times 10^6$ and $0.56 \times 10^6$. Even those organisms which are similar to eukaryotes show rRNA molecular weights comparable to those of bacteria. Actinomyces, which bud like yeast, and blue-green algae, which in the past were regarded as plants, had respective molecular weights $(1.11 \times 10^6 \& 0.56 \times 10^6; 1.07 \times 10^6 \& 0.56 \times 10^6)$ coinciding well with those of E. coli $(1.07 \times 10^6 \& 0.56 \times 10^6)$.

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

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were apparent. All the eukaryotic organisms from the lower plants to the mammals, seem to share the smaller rRNA component of molecular weight \(0.70 \times 10^6\). 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 \(1.40 \times 10^6\) in the sea urchin to \(1.75 \times 10^6\) in human cells. Among the plants and protozoa, the size of the large subunit remained constant at \(1.30 \times 10^6\). Lovett and Mock (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 cytoplasmic rRNAs from thermophilic fungi.
and to determine the evolutionary implications of the results according to Leenheer (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 rRNA.

Perhaps the rRNA of thermophilic fungi will be of the intermediate category between the prokaryotic and eukaryotic classes.
1. Growth of Fungi

*Humicola lanuginosa* (Griffin and Maulbanc) Bunce (1961) and
*Chaetomium thermophile var. coprophile* (Cooney and Emerson, 1964) were
obtained from Dr. H.R. Tansey, Dept. of Microbiology, Indiana University.
Mycelia were grown in liquid cultures of Emerson Ypsa medium (1.52
soluble starch, 0.4% Bacto-Yeast extract, 0.17 K₂HPO₄, 0.05% MgSO₄) 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. broth 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 μg fungal mycelia (wet
weight) per ml, and 0.5 ml aliquots (5 μg) 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
(Hunto 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 HClO₄ 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 HClO₄. RNA was hydrolyzed by incubation in 4 ml of 0.1 N KOH at 37°C for 2 hr. After cooling in ice, protein and RNA were precipitated by adding 2.5 ml of 1.2 N HClO₄. 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 HClO₄. The RNA content of the combined supernatant solutions was then measured using either ultraviolet absorption at 260 nm (εₓ = 7,600), or the orcinol method of Mojab (1939), with adenosine as the standard.

3. Electron Microscopy of Fungal Hyphae

Fungal 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, Verza, & Littauer (1969). The outer membrane was removed by a digitonin wash as described by De Vries & Van der Kooij-Schuring (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 bacteriostatic 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 (Visher, 200 mesh), and ground vigorously with slow addition of 2.5 volumes of SET medium (0.01 M sucrose, 1 mM EDTA, 10 mM Tris-Cl, pH 7.5). The homogenate was centrifuged at 300 x g for 15 minutes. The supernatant was saved, and the pellet was resuspended and recentrifuged as described above. The combined supernatant solutions were centrifuged at 20,000 x g for 20 minutes to obtain a crude mitochondrial pellet. The mitochondria were washed by resuspension in SET (10)T medium (SET medium, containing 10 mM EDTA) with the aid of a sonication homogenizer, and 20 ml portions were layered over a sucrose cushion of 5 ml of 7 M sucrose, 1 mM EDTA, 10 mM Tris-Cl, pH 7.5. The tubes were centrifuged for 15 minutes at 10,000 x g and 3°C in the HB-1 rotor of a Sorvall RC2-B centrifuge. The upper layer and the band at the interface were recovered, combined and centrifuged 20 minutes at 20,000 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-Cl, 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 SW55 rotor in a Spinco preparative ultracentrifuge, the upper layers of the gradient were aspirated, and the band at the 1.15-1.25 g/ml sucrose interface was recovered directly with a Pasteur pipette. Similar mitochondrial fractions were combined and diluted with 2.5 volumes of 0.3 M HEPES, 10 mM KCl, pH 7.5, and centrifuged at 10,000 g for 90 minutes. The purified mitochondria were then re-suspended, with homogenization, in 5 ml of ST medium (0.34 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 supernatant was diluted with 15 ml of ST medium, and mitochondria were pelleted at 10,000 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 Johnson-Fredd and Arndt and (1972). Livers were homogenized with a Potter-Elvejem homogenizer in 10 volumes of ice-cold STEM medium (0.25 M sucrose, 10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, pH 7.6). The homogenate was centrifuged at 2,000 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 STEM was added to the supernatant to a final concentration of 2%, 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 STEM buffer (STEM medium minus sucrose) and then suspended in STEM with the aid of a teflon homogenizer. Following a clarifying spin
(30 minutes, 26,000 x g), the ribosomal suspension was layered over 9 ml of 1.0 M sucrose in TES and centrifuged for 4 hr at 150,000 x g in the 60Ti rotor. The ribosomal pellets that obtained were either covered with STM buffer or suspended in TES at concentrations greater than 10 mg/ml. In both cases, ribosomes were stored at -70°C.

(b) Preparation of the mitochondrial-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 isolated mitochondrial-ribosomal fraction

Mitochondria, purified as described above, were suspended in 8 ml of ice-cold TES (10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 50 mg/ml dextran sulphate/ml, pH 7.6), and lysed by the addition of 2% Triton X-100 in TES to a final concentration of 2%. After 10 minutes at 0°C, the lysate was layered over 1 ml of 1.5 M sucrose in TES and spun for 4 hr at 150,000 x g in a type 65 rotor in a Beckman Model L3 ultracentrifuge, at 4°C. The pellets were rinsed, resuspended in TES 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 MgCl₂...
and 25 mM sodium acetate, pH 4.8, Fairley, 1971]. After 1 minute, 7.0 ml of phenol-cresol mixture (200 g distilled phenol, 42 g distilled cresol, 11 ml water and 0.3 g 8-hydroxyquinoline), made 5% in STINS (sodium tri-
propane sulfonate-citrate-sulfate), was added and the mixture stirred in 80 minutes at room temperature. The aqueous layer was re-extracted once with an equal volume of phenol-cresol, and the MMA was precipitated by the addition of 3% (w/v) NaCl, 60% (w/v) sodium benzoate and 10% (w/v) cresol. After 1 hr at 0°C, the MMA was precipitated by centrifugation at 10,000 g for 10 minutes. The MMA was washed twice with ice-cold 1M, 2M, 3M sodium benzoate, 10% methanol, once with 1.5 M NaCl in 1M ethanol, once with 75% ethanol, twice with absolute ethanol, dried overnight under vacuum, and stored desiccated, at -20°C.

Method 2: In a modification of the method of Long and O’Shea (1971), 10-30 μg of filtered fungus was quickly frozen in a Teflon tube containing liquid nitrogen. The mycelia were then ground to a fine powder and then the nitrogen was completely evaporated. The mycelial cells were suspended in 10 ml of medium D. The suspension was centrifuged with phenol-cresol and the ribosomal RNA was precipitated as described in Method 1.

(b) 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) sucrose, 2% (w/v) SDS, 0.12% (w/v) Macaloid, 25 mM sodium acetate, pH 4.8 and two volumes of phenol-cresol made 5% in STINS. 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
3 M NaCl and 2.5 volumes of cold 95% ethanol and was kept overnight at
-20°C. The RNA was washed twice with 1 M NaCl in 70% ethanol, once with
70% 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 1 of Krik (1967),
dried under vacuum and stored desiccated at -20°C.

d) L. cell ribosomal RNA
Ribosomes, prepared by the method of Hirs et al. (1967),
were suspended in medium B, and their RNA was extracted as described
above for fungal cytoplasmic ribosomal RNA.

7. Radioactive Labelling of Ribosomal and RNA
   (a) E. coli

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

(b) L. cell cytoplasmic RNA
L cells were grown and their RNA extracted as described by Fante,
Hilton and Freierman (1970). Suspension cultures of 250-350 ml were labelled
for 48 hr with 10 uCi of 2^-C-galactose.

5. Sucrose Density Gradient Centrifugation
   (a) Analysis of ribosomes

Isokinetic sucrose gradients from 15-30% sucrose in TKI 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 chromosomes in 0.5 ml of 0.3M buffer were layered on top of the gradient and centrifuged for 3 hr at 3°C at 195,000 x g in the SS-34 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 microphototometer, held 0.1 ml and then the solution was transferred to test tubes 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 Schott Photometric Model 200 UV photometer. The gradients were forced through the photometer by pumping water onto the top of the gradient, at a rate of 0.7 ml/min, with an Isco Model 131 density gradient fractionator.

(b) Analysis

Isokinetic sucrose gradients from 12-37 sucrose were prepared according to Mell (1967), using either or two buffer systems. In the first system, the sucrose solutions were buffered with 0.1 M sodium acetate, pH 6.0. Approximately 200 ml of 50% sucrose solution was layered on top of the gradient and centrifuged for 16 hr. at 3°C and 142,000 x g in the SS-34 rotor.

In the second system, the sucrose solutions were in 0.1 M NaCl, 1 mM EDTA, 0.1 M (w/v) S phoenix (irissu-4Cl), pH 7.5 (Stromer and Attardi, 1971). Centrifugation was for 16 hr. at 25°C at 71,000 x g in a Spinco SW-27 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 nm, each fraction was counted for radioactivity.

**Agarose-Polyacrylamide Gel Electrophoresis**

Electrophoresis of RSA was carried out by the method of the
3 methods of the mode, molecular weight, and its gelation
behavior. A solution containing 0.1 M Tris (pH 7.6) and 0.1
M NaCl was used to dissolve the gelatin at 60°C for 30 minutes. An agarose gelatin solution was added to 0.17
M Tris (pH 7.6) at 0°C and the solution was mixed with an equal volume of a boiling
solution at 0.3% (w/v) agarose in distilled water. The mixture was
immediately poured into a plugged electrophoresis box at 60°C for 30
minutes, and allowed to solidify for 2 hr. We used a 2 x 20 cm gel. The
gel was removed to form a cell at the top of the tube and the RSA
(30 µg of RSA in 0.1 N sodium acetate, pH 7.0) was applied to the
surface of the gel.

Two buffer systems were used and both were at 0.1 M.

The first buffer was Schlesinger (1967) containing 10
mM Tris, 10 mM Mops, 0.1 mM EDTA, pH 7.4. In this system, electrophoresis was
carried out at 25°C and 7 volts/cm for 0.5 A/cm².

The second buffer was 10 mM Tris (pH 7.4), containing
10 mM Tris, 10 mM Mops, 0.1 mM EDTA, pH 7.4. Gel formation was allowed to
proceed for 2 hr, then the gel was cut into 2 x 20 cm strips.

In all cases, gels were pre-run for at least 5 min at 50 V.

following electrophoresis of the samples, the gels were stained at 25°C
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. Plan of Composite Analysis

DNA base composition was determined by the method of Noller (1973). Aqueous extracts were prepared by homogenizing the tissue in a medium containing 0.3 M mannitol, 0.1 M potassium acetate, 0.01 M Tris-Cl, pH 8.0, and 100 units of RNase-free RNase. The mixture was incubated at 37°C for 16 hr.

Chromatography analysis was performed on a Whatman 541 filter paper and on a Whatman 1 after high performance liquid chromatography. An aliquot of the mixture was applied to the column and chromatographed with a 4 ml mixture containing 0.5 M mannitol, 0.1 M potassium acetate, and 0.01 M Tris-Cl, pH 8.0.

11. Thermal Denaturation

(a) Ribonuclease

Ribonuclease purified on sucrose gradients was further dialyzed overnight against thermal denaturation buffer [10 mM Tris HCl, pH 7.5, 60 mM NaCl, 1 mM EDTA, pH 7.5, and 1% 1-mercaptoethanol] at 4°C. The ribonuclease was then treated with 200 units of the following reagents: 200 units of RNase A, 200 units of RNase T1, and 200 units of RNase T2. The ribonuclease was then analyzed for its thermal stability using a Beckman Densitometer 4000.
Direct addition of Tris buffer (10mM Tris-HCl, 95mL EC, 1mM MgCl₂, pH 7.6). Thermal denaturation profiles were then obtained with a Perkin Elmer 12 spectrophotometer equipped with a water jacketed cuvette holder, connected to a circulating water bath. The temperature was raised by heating the center of a Burrell, 22-K digital bath [19], in a water bath filled with paraffin oil. Solutions were degassed before use, either by gentle heating or by bubbling helium through them. Optical density measurements were made at 267 nm, at intervals of 0.1°. The temperature was raised at a rate of 0.15°C/min. Measurements were corrected for solvent expansion (Mandel and Yphantis, 1964).

(b) EDA

Thermal denaturation profiles of EDA were determined using essentially the same methods that described for Tris buffer. The Tris buffer was 10mM ethylendiaminetetraacetic acid, 50mM sodium acetate, pH 7.6. (Bedalov, Verma and Rittenberg, 1976), and absorption were monitored at both 280 and 250 nm.

12. Measurement of Radioactivity

Fractions from sucrose gradients containing [14C]-or [3H]-labeled samples were transferred to 10 mL glass shell vials and diluted with 0.5 mL of water. A 1 mL portion of PEG solubilizer (Beckman/Spinco) was added and the samples were counted at 15°C. A Nuclear Chicago N101 scintillation counter. Counting efficiencies for 7H were about 20% and for 14C, about 85-90%.
13. Chemical and Biochemical Reagents

(a) Chemical reagents

1) Protein concentrations were measured by the method of Lowry (1951). A solution of bovine serum albumin served as the standard.

II) DNA was measured by the method of Schlesinger (1969), with adenine as the standard.  The heating time was 5 minutes (Alwine et al., 1977).

II) RNA was estimated by the spectrophotometric method of Dacie (1954).

(b) Enzymatic methods

I) NADPH dehydrogenase and cycloheximide inhibition activities were measured as described by Fromm (1965). 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 ribosomal activity was assayed by a modification of the method of Argenziano and Rosenburg (1971). The reaction mixture contained: 8.8 ml of the appropriate buffer, 0.1 ml of a solution of 2 mg/ml cytoplasmic ribosomal RNA per ml of 0.1 M Tris-HCl, pH 7.0, and 0.4 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 5 \( \times \) (v/v) ethanol. The mixtures were kept at 0°C for 30 minutes and then filtered through glass filters. The acid-soluble optical density of the filtrate at 260 nm was read against a zero time blank, in a Zeiss PM II spectrophotometer.
Composition of Buffers and Solutions

SET medium: 0.42 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5.
SK10T medium: 0.44 M sucrose, 10 mM EDTA, 10 mM Tris-HCl, pH 7.5.
NT medium: 0.45 M sucrose, 10 mM Tris-HCl, pH 7.5.
TEM medium: 10 mM Tris-Cl, 10 mM HCl, 5 mM NaCl, pH 7.6.
Solution A: 25 mM Tris-Cl, 0.12 M NaCl, 1 % (w/v) NaN3, pH 7.5.
Solution B: 10 mM NaCl, 25 mM sodium acetate, pH 4.5.

Tris buffer:
1) Ribonuclease: 10 mM Tris-HCl, 95 mM HCl, 0.1% NaCl, pH 7.5.
2) KCl: 50 mM NaCl, 50 mM sodium acetate, pH 5.7.

Materials:
All chemicals and reagents used in this study were of the highest grade and the water used for all solutions was de-ionized and glass distilled. Dipicolinic (A grade) was obtained from Calbiochem, La Jolla, Calif.; Diethyl-1,1',3,3'-tetramethylthiourea, ethylène-bis-(o-amino-ethyl)-ether-ether-amide (purified as described by Lees, 1967) were obtained from the Eastman Kodak Co., Rochester, N.Y. Dayselylase (electrophoretically purified from ribonuclease), alkaline phosphatase (E. coli, chromatographically purified) and venom phosphodiesterase I were obtained from Worthington Biochemical Corp., Freehold, N.J.
RESULTS

1. Growth Experiments

Three species of therophilic fungi were obtained for this investigation: 

- *Heterobasidion annosum* (Ouflain 1959, Griffon & Roublanc) Raaij (1962),
- *Chaetomium thermophile* var. *yield* (Cooney & Emerson, 1964), and
- *Chaetomium thermophile* var. *peronophile* (Cooney & Emerson, 1964). These three are particularly suited for our study because, of the approximately twenty therophilic fungi known at that time, *Heterobasidion* and *Chaetomium* have the highest maximum growth temperature (18-20°C).

It was first necessary to determine the optimum time of growth and conditions of growth of the fungi for extraction of RNA. 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 starting crop. Presumably, a higher growth rate indicates a more active protein synthetic apparatus and perhaps a higher RNA content. The necessity of these preliminary experiments was due to the fact that as a colony of fungal growth 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 growth rates and crop yield, it was hoped that the problem of a paucity of ribosomes and RNA could be overcome.

The growth of *Chaetomium thermophile* var. *peronophile* was examined.
Figure 2. The effect of temperature on the growth of *Clostridium 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 (Conner & Emerson, 1966). At 30°C and 55°C, the fungus appears to be growing at or close to the temperature optimum 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 lag 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

<table>
<thead>
<tr>
<th>Temp. °C</th>
<th>Chaetomium thermophile var. coprophile:</th>
<th>Humicola lanuginosa:</th>
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<tbody>
<tr>
<td></td>
<td>Maximum Growth rate (g/day)</td>
<td>Maximum Standing crop (g/100 ml)</td>
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<tr>
<td></td>
<td>wet* weight</td>
<td>dry* weight</td>
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<tr>
<td>30°</td>
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<td>0.052</td>
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<td>40°</td>
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<td>50°</td>
<td>1.1</td>
<td>0.12</td>
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<tr>
<td>55°</td>
<td>0.56</td>
<td>0.07</td>
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</table>

*standard deviations were from ±15% of the indicated values.
Figure 3. The effect of temperature on the growth of *Nannocystis 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 48 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 thermostrophum var. coprophile at 45°C. ——, Growth curve measured by dry weight of cell mass; ——, 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 fungus-walled, fixed hypha of Claviceps. (1) Mitochondria; (2) Nucleus; (3) Vavilin; (4) Endoplasmic reticulum; (5) Cell wall. Magnification: 37,500 x.
Figure 6. Cross sectional view of pleurochloridecte and the hyphae of Chondromyces, stained as in Fig. 1. Magnification: 64,500 x.
electron microscopy to establish the presence and quantity of mitochondria in thermophilic fungi.

Figure 5 shows both a longitudinal and cross-sectional view of acetone-fixed hyphae. Chaetomium shows the usual features found in any fungus. 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 TCM, medium, and run on sucrose density gradients containing TCM, Chaetomium cytosol ribosomes show only one peak, which co-sediments with the 80 S peak of yeast 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 Chlamydomonas, mixed with E. coli ribosomes. The absorbance profile was monitored with a chromatronic 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 & Griffiths, 1971), rather than the $70\ S$ ribosomes of prokaryotes.

b. Thermal denaturation studies

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

Figure 8 shows typical melting profiles obtained with $80S$ ribosomes from the cytosol of rat liver and Chaetocinum. In this case, $60S$ peak regions were collected from sucrose density gradients and diluted by direct addition of $T_m$ buffer. In other experiments, $60S$ ribosomes were dialysed overnight against $T_m$ 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 Chaetocinum ribosomes were prepared by identical methods, and denaturation was carried out in the same buffer. The absolute value of the observed $T_m$ varied from 1-5°C from experiment to experiment, but the $T_m$ for both ribosomes always shifted in the same direction, so that the relative difference between the two was conserved with the fungal tRNA $T_m$ 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 (○—○), and Chlamydomonas (□—□). Peak fractions from the 80 S regions of sucrose density gradients were diluted with Tm buffer to give initial absorbancies of 0.250 - 0.350. To facilitate comparison, maximum absorbancies were set equal to 100%. Arrows indicate calculated TmS.
not only are the thermophile ribosomes more thermostable as judged by
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.

Studies on Chaetomium Microsomal Ribonuclease Activity

Initial attempts to isolate cytoplasmic RNA from Chaetomium were
based on the method of Edelmann, Verma & Littauer (1970) in which ribo-

tomes, prepared from a Triton X-100 lysed microsomal fraction were
suspended in TME and extracted with TME-saturated phenol-chloroform.
SDS was present as an RNAase inhibitor. In four separate preparations
using this method, the product showed RNA profiles approximating those
seen in Figure 3. 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 prevent 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 sediementation
coefficients, it was impossible to decide if these values were indicative
of native RNA, or whether they had been altered by degradation. It
was also impossible to firmly establish that Chaetomium ribosomes contain
only two high molecular weight RNAs. Therefore, an investigation of
the properties of this ribonuclease was necessary in order to devise a
new method for the isolation of undegraded RNA.
Figure 9. Gel electrophoresis of RNA prepared from cytoplasmic ribosomes of Chlamydomonas 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 curve. 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 enzyme. At Mg$$^{2+}$$ concentrations above 8 mM and at Na$$^{+}$$ concentrations above 250 mM, the enzyme activity is reduced to almost zero. The curve shows a definite optimum at pH 7 and a shoulder at pH 8.0. Below pH 4.0 and above pH 8.0 the enzyme 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 E$$^{2+}$$-activity.

Therefore, the extraction medium was changed to medium B (Bartov, 1971) which contains 10 mM Mg$$^{2+}$$ and has a pH of 4.8. Medium B also contains other enzyme inhibitors (SDS, Macrogol) whose effect was not examined. Although high pH and high Na$$^{+}$$ concentrations were also found to effectively reduce E$$^{2+}$$ 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 E$$^{2+}$$ was extracted from cultures at later times in the growth phase, more degradation products were observed.
Figure 10. The effect of varying enzyme and substrate concentrations on the RNase activity of the microsomal fraction of Clapeyron. Incubation mixtures contained 0.1 M Tris-HCl, pH 7.0 and
(a) 0.3 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 microsomal ribonuclease from Chactomum. 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 12.25 (±0.02) x 10^4. The value for the small component was calculated to be 0.70 (±0.02) x 10^4 on the basis of three determinations.

   b. Sedimentation analysis

   Sedimentation coefficients were determined by centrifugation with 1^4C-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 Chlamydomonas. Approximately 30 μg RNA were applied to 0.5 cm gel. 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 11. Fractionation of Chlorella cytoplasmic RNA 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 RNA for sedimentation analysis.

- $\bullet$, $OD_{600}$, Chlorella cytoplasmic RNA; $\circ$, $^{14}$C-L-cell rRNA.
Chaperonin cytosolic ribosomes over those of rat liver, it was of interest to also examine the relative thermolability of their component rRNAs. The two high molecular weight rRNA components were isolated from rat liver and Chaperonin-cytosolic 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 (Mol et al., 1963; Cox, 1966). However, if the hyperchromic effect is measured at 260 nm, transitions in both GC and AT pairs are detected. By monitoring at both wavelengths, the ratio of the amount of hyperchromicity measured at 280 nm to that measured at 260 nm (R280/260) gives an indication of the relative percentage of GC pairs in the ordered regions of two different rRNA molecules. The term \( R \) is defined as 

\[
R = \frac{1}{1 - \frac{C_{280}}{C_{260}}} \times 100
\]

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 \( R \) 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 \( R \) was greater at 280 than at 260 nm. For Chaperonin rRNA, the \( R \) was approximately equal at both wavelengths. This observation is reflected in the ratios of \( R_{280}/R_{260} \) shown in Table V, were the rat liver values are significantly higher than those for Chaperonin. 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 cytosolic rRNAs.
Figure 14. Thermal denaturation profiles of cytoplasmic RNA from rat liver and Chloridium. RNA was fractionated on sucrose density gradients, and precipitated with ethanol. After redissolving in RNP buffer, absorbances were adjusted initially to 0.250 - 0.350. To facilitate comparison, readings at 95°C were set equal to 100% maximal absorbance. Arrows indicate the Tm's, averaged from 3 determinations.
<table>
<thead>
<tr>
<th>RNA Source</th>
<th>Wavelength</th>
<th>Tn</th>
<th>XII</th>
<th>Tb</th>
<th>XII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>260</td>
<td>66.6</td>
<td>24.0</td>
<td>68.8</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>(±0.8)</td>
<td>(±1.5)</td>
<td>(±2.0)</td>
<td>(±0.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>72.3</td>
<td>33.4</td>
<td>67.5</td>
<td>27.1</td>
</tr>
<tr>
<td></td>
<td>(±0.8)</td>
<td>(±1.8)</td>
<td>(±1.3)</td>
<td>(±2.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>260/240</td>
<td>1.39</td>
<td></td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>Chorionics</td>
<td>260</td>
<td>62.0</td>
<td>25.5</td>
<td>59.3</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>(±0.5)</td>
<td>(±0.7)</td>
<td>(±0.3)</td>
<td>(±1.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>63.2</td>
<td>26.5</td>
<td>61.7</td>
<td>26.6</td>
</tr>
<tr>
<td></td>
<td>(±1.0)</td>
<td>(±0.6)</td>
<td>(±1.5)</td>
<td>(±3.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>280/260</td>
<td>1.04</td>
<td></td>
<td>1.03</td>
<td></td>
</tr>
</tbody>
</table>
Table VI

Base Composition of Cytosol rRNA from Rat Liver and Chickens

<table>
<thead>
<tr>
<th>Source</th>
<th>Component</th>
<th>% A</th>
<th>% U</th>
<th>% G</th>
<th>% C</th>
<th>% T, G+U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Liver</td>
<td>Heavy</td>
<td>16.2</td>
<td>16.5</td>
<td>35.3</td>
<td>29.6</td>
<td>67.3</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>20.9</td>
<td>19.9</td>
<td>33.5</td>
<td>25.5</td>
<td>59.3</td>
</tr>
<tr>
<td>Chickens</td>
<td>Heavy</td>
<td>24.9</td>
<td>23.7</td>
<td>31.8</td>
<td>19.7</td>
<td>51.5</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>25.5</td>
<td>24.4</td>
<td>26.9</td>
<td>20.2</td>
<td>50.1</td>
</tr>
</tbody>
</table>
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 component, respectively, the average GC content for Charactomi nuclei, cytoplasmic rRNA (51.0%), is very comparable to these values reported in Table II for monophtilic fungi (51.0, 53.7; 49.7%).

6. Studies on the Method of Determining Base Composition of rRNA

To standardize the liquid chromatography system (LCS) for the determination of base compositions, high molecular rRNA components were isolated from rat liver cytoplasm and from E. coli. In addition, a number of standard nucleotide 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. Guanine 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 guanine and cytidine values were also much higher (5-115) than those for adenosine and uridine (1-32). 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

<table>
<thead>
<tr>
<th>RNA Source</th>
<th>Heavy component</th>
<th>Light component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U</td>
<td>C</td>
</tr>
<tr>
<td>Rat liver cytoplasmic RNA:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>16.5</td>
<td>38.3</td>
</tr>
<tr>
<td>(2)</td>
<td>19.0</td>
<td>32.9</td>
</tr>
<tr>
<td>(3)</td>
<td>17.9</td>
<td>36.3</td>
</tr>
<tr>
<td>(4)</td>
<td>17.0</td>
<td>33.0</td>
</tr>
<tr>
<td>Esthertibia cell RNA:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>20.7</td>
<td>31.8</td>
</tr>
<tr>
<td>(5)</td>
<td>21.0</td>
<td>32.5</td>
</tr>
<tr>
<td>Prepared nucleoside mixtures:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>theoretical</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>observed</td>
<td>24.7</td>
<td>26.2</td>
</tr>
<tr>
<td>theoretical</td>
<td>29.0</td>
<td>12.5</td>
</tr>
<tr>
<td>observed</td>
<td>30.3</td>
<td>12.8</td>
</tr>
</tbody>
</table>

(1) from this study
(2) from Munro (1964)
(3) from Hirsh (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 re-determined, no significant changes from the
previous estimates could be found.

The second aspect considered was the possibility that the pH
(7.3) of the elution buffer was fluctuating. Any change in buffer pH
would be most dramatically reflected in changes of the $\epsilon_{270}$ for cytidine.

Cytidine group pH is 4.5 (pHs 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 $\epsilon_{270}$ 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 Chlorella Mitochondria

Preliminary attempts to isolate Chlorella mitochondria, free
from cytoplasmic 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 Hitachi F2400 spectrophotometer modified as described in the methods for thermal denaturation analysis. The ultraviolet spectra were determined at various temperatures following an equilibration time of 10 minutes. ○—○, 25°C; ▲—▲, 30°C; ■—■, 35°C; ▲—▲, 40°C; ▲—▲, 50°C; ○—○, 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 Chlorella 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 Chlorella, the location of the mitochondria at any step in the procedure was followed by assaying darker 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 × 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 × 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 × 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 Chlorella. 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 Xg and 3°C in the SW25.1 rotor. ——— protein concentration, ——— malate dehydrogenase (MDH) activity, ——— cytochrome oxidase activity.
Figure 17. Purification of Chlamydomonas 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.
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 lyzed and stored in
medium B 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 14C-labelled i-cell rRNA, the fungal rRNA was resolved into two peaks whose sedimentation coefficients were calculated to be 22.6 S and 17.2 S (Figure 13).

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 x 10^6. The small component gave identical molecular weights, 0.70 x 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 rRNA.

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 (30-36%) for mesophilic fungi.
Figure 18. Sedimentation analysis of *Chaetomium* 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 Chlamydomonas mitochondrial RNA. RNA from density-gradient purified mitochondria was applied to a 6.5 cm gel and electrophoresis was in K-buffer for 3 hr at 25°C.
Table VIII
Base Composition of Chaetomium Mitochondrial rRNA

<table>
<thead>
<tr>
<th>Component</th>
<th>U</th>
<th>G</th>
<th>A</th>
<th>C</th>
<th>G+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy peak</td>
<td>25.1</td>
<td>29.2</td>
<td>27.5</td>
<td>18.2</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>(±0.5)</td>
<td>(±0.5)</td>
<td>(±0.3)</td>
<td>(±1.1)</td>
<td></td>
</tr>
<tr>
<td>Light peak</td>
<td>27.1</td>
<td>26.9</td>
<td>28.9</td>
<td>17.2</td>
<td>44.1</td>
</tr>
<tr>
<td></td>
<td>(±0.5)</td>
<td>(±0.3)</td>
<td>(±0.4)</td>
<td>(±0.4)</td>
<td></td>
</tr>
</tbody>
</table>

Table VI
Base Composition of Cytosol rRNA from Rat Liver and Chaetomium

<table>
<thead>
<tr>
<th>Source</th>
<th>Component</th>
<th>A</th>
<th>U</th>
<th>G</th>
<th>C</th>
<th>G+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>Heavy</td>
<td>16.2</td>
<td>16.5</td>
<td>38.3</td>
<td>29.0</td>
<td>67.3</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>20.9</td>
<td>19.9</td>
<td>33.5</td>
<td>25.8</td>
<td>59.3</td>
</tr>
<tr>
<td>Chaetomium</td>
<td>Heavy</td>
<td>24.9</td>
<td>23.7</td>
<td>31.8</td>
<td>19.7</td>
<td>51.5</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>25.5</td>
<td>24.4</td>
<td>29.9</td>
<td>20.2</td>
<td>50.1</td>
</tr>
</tbody>
</table>
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 C+C content seen for Charactbium 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 Hoogh-Schuurman, 1973). At slightly higher levels, the entire outer mitochondrial membrane is removed (Schnaitman & Greensvalt, 1968), leaving the inner membrane and matrix intact.

For studies with Charactbium, five preparations of mitochondria, washed in digitonin concentrations sufficient to remove outer membrane, were pooled and their rRNA was extracted. Following electrophoresis on 2-buffered polyacrylamide gels, no peaks were seen in the ultraviolet profile. However, when centrifuged on an invert 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 C+C content of rRNA from mitochondria prepared by both methods, is similar.
Figure 20. Sedimentation profile of rRNA from gradient-digironin purified mitochondria from Chondrichthyes on a convex sucrose density gradient. Arrows indicate theoretical sedimentation coefficients at these positions.
### Table IX

Base composition of tRNA from digitonin-washed *Chlamydomonas* mitochondria

<table>
<thead>
<tr>
<th>Component</th>
<th>U</th>
<th>G</th>
<th>A</th>
<th>C</th>
<th>G+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>27.6</td>
<td>26.6</td>
<td>27.9</td>
<td>17.9</td>
<td>74.5</td>
</tr>
</tbody>
</table>
9. Molecular Weights of *Humicola lanuginosa* Cytosol tRNA

Cytosol tRNA of *Humicola lanuginosa* was isolated by method II.

In two determinations, the apparent molecular weights were identical for the respective components, $1.31 \times 10^6$ and $0.69 \times 10^6$. 
IV. DISCUSSION

Thermostability of Chaetomium Cytoplasm Ribosomes.

The results show that the cytosol ribosomes of Chaetomium thermophile var. saprophyticus exhibit a significantly greater thermostability than 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 dupontii. Although the thermal denaturation data from all these studies may not be directly comparable, most of them show the same relationship between ribosome T° 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°s 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 52°C, only 2°C lower than the maximum growth temperature for this organism. As suggested by Mangiantini et al. (1967), 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 $T_{m28S}$ 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 (+1.2) for the large and small component respectively. For Chaetomium, the corresponding values were 49.3 (+2.0) and 47.6 (+0.7).

The base composition of the ordered regions of single-stranded polynucleotides is one factor which contributes to the observed $T_m$s of these molecules (Cox, 1966; Rallenchach, 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 Tm's and rRNA Tm's 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 rRNA 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 (23 – 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 x 10^6) compared well with the values for mesophiles (0.67 – 0.72 x 10^6). On the other hand, the heavy component (1.25 x 10^6) was slightly smaller than the corresponding components of Aspergillus and Neurospora (1.28 – 1.29 x 10^6). Although this difference is small and its statistical significance is unknown, it presents a situation analogous 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 Tm's 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 – 47.6%) was calculated to be lower than the value of 54.5% in Neurospora (Edelman et al., 1970)
which has T_m 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_m 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 wave length at which both AU- and GC-transitions are detected, the amount of hypochromicity 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 thermal stability 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 molecule. 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
be 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 C to G, the total G+C content of the two reference RNAs (rat liver cytosol and E. coli) was relatively constant and compared favourably to reported values (Table VII).

Molecular Weights of Thermophilic Fungal Cytosol RNAs

The apparent molecular weights of cytosol RNAs from Chaeotomium thermophilum, Cryptococcus (1.25 x 10^6 and 0.76 x 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 (1.02 x 10^6); a value which was outside of the mean plus standard deviation for Chaeotomium thermophilum. However, the size of the corresponding heavy component from Humicola lanuginosa (1.31 x 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 Chaeotomium 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 x 10^6) defined by Lovett & Haselby is incorrect, and that the value obtained for Chaeotomium actually falls on a continuum of molecular weights which may end at some point less than 1.30 x 10^6.
Leiman et al. (1970) reported values of $1.29 \times 10^6$ and $0.72 \times 10^6$ for Aspergillus. In addition, Loching (1968) reports one other observation, from a species of Rhynopus, of rRNA whose size was $1.28 \times 10^6$.

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 prevented 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 can be 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%) 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 Edelhoch et al. (1970) and Neupert et al. (1969) found that apparent molecular weights were the same for corresponding tRNA components from the mitochondria and cytosol of both Aspergillus and Neurospora (Table 1). 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 tRNA, it is possible that this anomaly may not appear for Chaetomium because the G+C content of its mitochondrial tRNA is similar to that of the cytosol tRNA.

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 $1.29 \times 10^6$ compared to $1.75 (0.32) \times 10^6$ 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 tRNA, 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 rRNA 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 thermophilum var. cedrophile represent the first demonstration of mitochondrial rRNA from an animal or a eukaryote 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 (54.82) 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 rRNA 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. Heat, 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 mesopholic fungal and animal
sources do not conform to the low C+C content pattern. A thorough
investigation of the varied eukaryotic organisms, and the resulting
relationships seen between C+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

Cytoplasmic ribosomes were isolated from both rat liver and from the thermophilic fungus, Chaetomium thermophile var. coprophilum. Both types sedimented at 80 S. Melting profiles were scanned at 260 nm. Thermophile ribosomes exhibited a higher thermal denaturation midpoint ($T_m = 64^\circ C$) than the rat liver ribosomes ($T_m = 54^\circ C$). 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 $1.25 \times 10^6$ and $0.70 \times 10^6$. The melting temperatures ($62.0^\circ C$ and $59.5^\circ C$) were lower than those of rat liver rRNA ($66.7^\circ C$ and $62.8^\circ C$). 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 ($1.29 \times 10^6$, $0.7 \times 10^6$) and GC contents (47%, 44%).

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


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