A COMPARATIVE STUDY OF WHEAT MITOCHONDRIAL AND CYTOSOL RIBOSOMAL RNA

by

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ABSTRACT

Mitochondrial ribosomal RNAs have been isolated from viable wheat embryos germinated for 24 hr in the presence of large amount of [^{32}P]orthophosphate. The two high-molecular-weight mitochondrial [^{32}P]rRNAs ("26S" and "18S") were separated by sedimentation in sucrose density gradients, characterized in terms of size and nucleotide composition, and shown to be distinct in these respects from their cytosol homologues [1,2]. T_1 ribonuclease "fingerprints" of the individual, purified mitochondrial and cytosol 26 S and 18 S [^{32}P]rRNA species have confirmed these results [3]. T_1 ribonuclease oligonucleotide "catalogues" of the cytosol and mitochondrial 18 S rRNA species have been compiled and shown to be distinct from one another. Comparison of these two "catalogues" with those existing for several bacterial and blue-green algal 16 S rRNAs has demonstrated the prokaryotic nature of the wheat mitochondrial 18 S RNA [4,5], the first time that such information has been obtained. Oligonucleotide "catalogues" (both T_1 and pancreatic) have also been obtained for wheat embryo mitochondrial and cytosol 5 S RNAs, and have demonstrated that these species are distinct [3], the first time that a distinct 5 S RNA species has been localized in the mitochondrion.

[1] Cunningham, R.S. & Gray, M.W. (1977) Biochim. Biophys. Acta, in press
"Isolation and Characterization of ^{32}P-Labeled Mitochondrial and Cytosol Ribosomal RNA from Germinating Wheat Embryos"

[2] Gray, M.W. & Cunningham, R.S. (1977) Can. J. Biochem., in press
"Hypermodified Alkali-Stable Dinucleotide Sequences in Each of the High-Molecular-Weight (26S and 18S) Ribosomal RNA Species of Wheat"

[3] Cunningham, R.S., Bonen, L., Doolittle, W.F. and Gray, M.W. (1976) FEBS Lett. 69, 116-122
"Unique Species of 5 S, 18 S, and 26 S Ribosomal RNA in Wheat Mitochondria"

[4] Cunningham, R.S., Gray, M.W., Doolittle, W.F. and Bonen, L. (1976) in Proceedings of the CNRS Colloquium "Nucleic Acids and Protein Synthesis in Plants" (Weil, J.H. and Bogorad, L. eds.), Strasbourg, in press

[5] Bonen, L., Cunningham, R.S., Gray, M.W. and Doolittle, W.F. (1977) Nucleic Acids Res., in press
"Wheat Embryo Mitochondrial 18 S Ribosomal RNA: Evidence for its Prokaryotic Nature"

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LIST OF ABBREVIATIONS

A_{260} unit	an amount of material having an absorbance of 1.0 at 260 nm when dissolved in 1.0 ml and measured in a 1 cm light path (1 A_{260} = 40μg)
CTA-Br	hexadecyltrimethylammonium bromide (also cetyltrimethylammonium bromide)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DEAE-cellulose	O - (diethylaminoethyl) cellulose
EDTA	ethylenediaminetetraacetic acid
RNA	ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer RNA
mRNA	messenger RNA
iRNA	NaCl-insoluble RNA [operationally defined as that portion of the total cellular RNA which is insoluble in 1 - 3 M NaCl at 0 - 5° (consists primarily of high-molecular-weight (16 - 18 S and 23 - 28 S) rRNA)]
sRNA	NaCl-soluble RNA [operationally defined as that portion of the total cellular RNA which is soluble in 1 - 3 M NaCl at 0 - 5°

	(consists primarily of 5 S rRNA and tRNA)]
RNase	ribonuclease
S	Svedberg unit (1 S = 1 X 10^{-13} sec)
SLS	sodium lauryl (dodecyl) sulphate
TEMED	N, N, N', N' - tetramethylethylenediamine
Tris	tris (hydroxy methyl) aminomethane
UV	ultraviolet
mt	prefix for "mitochondrial" (e.g., mtDNA = mitochondrial DNA; mt-RNA = mitochondrial RNA)
n	prefix for "nuclear" (e.g., nDNA = nuclear DNA)

Abbreviations for nucleotide derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (1970), Biochemistry 9, 4022-4027. N, general abbreviation for ribonucleoside; A, C, G, U, Ψ, adenosine, cytidine, guanosine, uridine; pseudouridine; pN and pNm, nucleoside 5'-monophosphate and $O^{2'}$-methylnucleoside 5'-monophosphate, respectively (e.g., pA and pAm, adenosine 5'-monophosphate and $O^{2'}$-methyladenosine 5'-monophosphate, respectively). In the case of base-modified nucleosides, a superscript indicates the position of the substituent on the heterocycle, whereas a subscript indicates the level of

substitution. Thus, m_2^6A = $\underline{N}^6$, $\underline{N}^6$-dimethyladenosine. In referring to oligonucleotides derived from T_1 or pancreatic RNase hydrolysates, the internucleotide phosphate group is omitted (e.g., CCCG is properly CpCpCpGp or C-C-C-Gp).

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SECTION I: GENERAL INTRODUCTION

A. INTRODUCTORY PARAGRAPHS

The mitochondrion has been recognized as an obligate constituent of all eukaryotic cells (those cells containing a membrane-bound nucleus) for nearly a century (Lehninger, 1965) and its role in cellular energy metabolism was established by Lehninger and co-workers three decades ago (Kennedy and Lehninger, 1948). However, a new surge of interest in these organelles followed the discovery by Nass in 1962 that mitochondria contain DNA. Subsequent investigations established that the mitochondrion contains all the components of a functional protein-synthesizing system, including ribosomes, transfer RNA, presumptive messenger RNA, aminoacyl-tRNA synthetases, initiation, elongation and termination factors, as well as distinct DNA and RNA polymerases. These observations led to the concept of the mitochondrion as a semi-automonous organelle within the eukaryotic cell, and this in turn raised novel questions concerning the degree of independence and types of interactions of this organelle with the nucleus (and with other intracellular organelles, such

as the chloroplasts of higher plants). In addition, the new evidence of the partial genetic autonomy of the mitochondrion reawakened the relatively dormant controversy over its evolutionary origin. This latter topic will be discussed in depth in the introduction to SECTION III.

Numerous excellent reviews on the mitochondrial nucleic acids, mitochondrial protein synthesis, and mitochondrial biogenesis have been published in recent years (Borst, 1972; Schatz and Mason, 1974; Mahler, 1973; Paoleti and Riou, 1973; Avadhani, Lewis, and Rutman, 1976; Saccone and Kroon, 1976) and it is not the purpose of this General Introduction to attempt to cover all the data that has been analyzed in these articles. I will, however, survey what is known of the mitochondrial nucleic acids, emphasizing the latest developments. One section of this introduction will be devoted to protein biosynthesis in the mitochondrion and another to mitochondrial biogenesis. These chapters will of necessity be brief, since these topics are not the main concern of this thesis. In addition, a discussion of several relevant topics such as plant mitochondrial rRNAs, 5 S rRNAs, and the prokaryotic features of the mitochondrion will be reserved

for later sections of this thesis where they relate directly to the experimental work.

B. MITOCHONDRIAL NUCLEIC ACIDS

(1) Mitochondrial DNA

As early as 1924 the kinetoplasts of the parasitic flagellate Trypanosoma and related genera were recognized as Feulgen-positive, DNA-containing cytoplasmic organelles (Bresslau and Scremin, 1924). However, although we now recognize that the kinetoplast is a specialized mitochondrion it was then considered a distinct organelle found only in the trypanosomids and bodonids (Pitelka, 1963). Chèvrement and his co-workers were the first to present evidence that "true" mitochondria contained DNA (Chèvrement et al., 1959; Chèvrement, 1963). They demonstrated that chick embryo fibroblasts exposed to certain conditions which inhibit mitosis (e.g., treatment with DNase II or cold) produced modified spherical mitochondria which were Feulgen-positive and incorporated tritiated thymidine. However, the unorthodox procedures used to obtain transformed mitochondria left many investigators unconvinced and it was not until the exacting analytical-morphological studies of Nass and Nass (Nass, 1962; Nass and Nass, 1963 a,b), also with chick

embryo tissues, that the idea of a mitochondrial-specific DNA gained general acceptance. Since these early studies, mtDNA has been found in all eukaryotic organisms examined and is considered an indispensable component of this organelle.

Mitochondrial DNA exists in the form of a closed circular duplex, the contour length of which varies from 5 μ (in animals) to 30 μ (in plants) (TABLE I). In some organisms intact circular mtDNA has not yet been isolated but these results are considered artifactual due to mechanical damage or enzyme degradation during the isolation procedure. For example, in Tetrahymena no circular mtDNA species has been detected, but circularity in vivo can be inferred from the fact that the nucleotide sequences of linear molecules (contour length 15 μ) are permuted (Borst and Grivell, 1973). The molecular weights of mtDNAs (TABLE I), determined both by direct observation (electron microscopy) and by studies of renaturation kinetics, range from 70 million daltons in pea down to 10 million daltons in animal mitochondria. The close agreement of values calculated by these two methods suggests that there is no sequence heterogeneity in the mtDNA of a given organism (Talen et al., 1975).

References (TABLE I)

(1) Borst (1972)

(2) Borst (1977)

(3) Goddard and Cummings (1975)

(4) Bohnert and Hermann (1974)

(5) Hollenberg *et al.* (1973)

(6) Bernard *et al.* (1975)

(7) Kolodner and Tewari (1972)

(8) Vedel and Quetier (1974)

TABLE I

PHYSICAL PROPERTIES OF MITOCHONDRIAL DNA

Organism	Length (μm)	Conformation	Mol. Wt. (x 10^{-6})	Buoyant Density (g/cc)	G + C (mol %)	Ref.
Animals (flatworm - man)	5-6	circular	9-12	1.686-1.711	40-43	1,2
Protozoa						
Tetrahymena	15	linear	30-36	1.684		2,9
Paramecium	14	linear	30-35	1.699		3
Acanthamoeba	12.8	circular	26	1.690	31	4
Plasmodium	9	circular	18			2
Fungi						
Saccharomyces	25	circular	50	1.679	18	5
Neurospora	20	circular	41	1.701	40	6
Kluyveromyces	10	circular	22			2
Plants						
Pea	30	circular	70 (74)*	1.706		7
Potato	28	linear	60 (100)*	1.706	47	8

* The bracketed values were obtained through renaturation kinetic experiments

The G + C content (and hence the buoyant density) of mtDNA varies considerably from organism to organism (TABLE I), from a low of 18% in yeast (Bernardi et al., 1974) to a high of 47% in both potato and HeLa cells (Vedel and Quetier, 1974; Clayton and Vinograd, 1967). An increase in G + C content and a decrease in size of mtDNA appears to be a general phenomenon as one ascends the evolutionary ladder. The notable exception is higher plant mtDNA, which has both the highest G + C content (47%) and the largest size (30 μ). However, since higher plants are neither the most highly-evolved nor the most primitive organisms (as is quite evident from classical taxonomic studies), considerable caution must be exercised when interpreting the significance of such data (G + C content and size) in evolutionary terms.

Borst (1970) has suggested that the information content of mtDNA is equivalent to its genome size and renaturation studies support this view. If this is in fact the case, then the potential information content of mtDNA varies considerably from the 5 μ molecules in animals to the 30 μ molecules in plants. The smallest mtDNAs are of sufficient size to code for the large

and small mitochondrial rRNAs, at least two dozen tRNAs, and a limited number of mitochondrial proteins. By simple extrapolation, the mtDNAs of lower fungi and higher plants could encode 6 - 7 times this amount of genetic information. However, it is believed that much of the mtDNA of these latter organisms consists of spacer and regulatory regions and does not contain structural genes. The amount of AT-rich regions found in yeast (> 50%; Prunell, 1974) and Neurospora (Bernard et al., 1975) mtDNA demonstrates the possibility of a large excess of spacer regions in these molecules. Since there is no evidence of such regions in higher plant mtDNA, Leaver and Pope (1976) suggest that these latter molecules may code for additional plant mitochondrial-specific translation products. While the upper limit of genetic information encoded in mtDNA is unknown, it is believed (Borst, 1972) that the minimum amount needed for a functional mitochondrial genetic system is 10 million daltons.

RNA/DNA Hybridization studies have demonstrated the presence of rRNA and tRNA genes on all mtDNAs examined. In addition, genetic analyses have indicated that hydrophobic subunits of certain inner mitochondrial membrane

enzyme complexes (cytochrome c oxidase, cytochrome bc_1, and oligomycin-sensitive ATPase) are also coded for by mtDNA. Recently, poly(A)-containing mtRNAs which are likely to be mitochondrial mRNAs were shown to hybridize specifically to mtDNA of HeLa cells (Attardi et al., 1976), rat liver (Cantatore et al., 1976) and yeast (Hendler, et al., 1975). However, it has only been recently that the possibility of constructing a "map" of mtDNA has become feasible. Mitochondrial genetics has offered a powerful experimental approach in the past but is limited to those organisms amenable to genetic manipulation, and the map produced from such techniques does not give the actual physical distances of the various markers from one another. The refinement of electron microscopic techniques has allowed visualization of hybrids between labelled RNAs and single-stranded mtDNA and subsequent mapping of the rRNAs and tRNAs of HeLa cells (Wu et al., 1972; Attardi et al., 1976). "Denaturation mapping" gives information on the localization of the AT-rich regions in the mtDNA (Bernard et al., 1975). However, the most powerful technique developed to date of mapping mtDNA is that using restriction endonucleases. By cleaving the mtDNA molecule with two or

more of these site-specific endonucleases, a number of fragments can be produced which can be orientated with respect to one another to produce a physical map. The location of the various transcription products (e.g., rRNA, tRNA, mRNA), can be ascertained by direct hybridization with individual fragments. The use of this technique has allowed the positioning of rRNA and tRNA genes on a number of different mitochondrial DNAs and will eventually lead to a map locating all of the structural and regulatory genes, as well as the spacer regions. One bit of information that has already been obtained is that the gene arrangement of rRNA and tRNA cistrons on mtDNA is not a constant feature. For example, in yeast (Sanders et al., 1975) and rat liver (Saccone et al., 1976) the two rRNA genes lie almost diametrically opposed on the circular mtDNA while in HeLa cells (Attardi et al., 1976) and Neurospora (Bernard et al., 1976) they are adjacent, separated by only small "spacer" regions. The location of the tRNA genes also varies depending upon the organism examined. In yeast, they are grouped together and are probably transcribed in a single precursor, as is the case in bacteria. In HeLa cells, however, they are distributed throughout the entire mitochondrial genome. It thus

appears that the initial belief in the constancy of the arrangement and polarity of mtRNA genes (Dawid, 1976) is unfounded and that in the future we will find additional variations in structural organization and possibly also in mtDNA function. Additional studies are also required to clarify the role of the extra sequences in plant and fungal mtDNAs.

(ii) Mitochondrial Ribosomes and Ribosomal RNA

a. Physical Properties and Composition of Mitoribosomes

Mitochondrial ribosomes (mitoribosomes) were first discovered in rat liver by Rabinowitz *et al.* (1966) and O'Brien and Kalf (1967) and subsequently identified in many other organisms. However, it was not until 1970 that Swanson and Dawid demonstrated that these ribosome-like particles actually catalyzed poly U-directed polyphenylalanine synthesis *in vitro*. Since these initial studies, mitoribosomes have been isolated from all organisms examined, ranging from lower fungi to higher plants and mammals (TABLE II), and it has been demonstrated unequivocally that they participate *in vivo* in mitochondrial protein synthesis.

References (TABLE II)

(1) Attardi and Ojala (1971)

(2) Attardi and Attardi (1971)

(3) Borst and Grivell (1971)

(4) Greco *et al.* (1974)

(5) Sacchi *et al.* (1973)

(6) Avadhani and Buetow (1972)

(7) Chi and Suyama (1970)

(8) Reijnders *et al.* (1972)

(9) Reijnders *et al.* (1973)

(10) Kuriyama and Luck (1974)

(11) Datema *et al.* (1974)

(12) Agsteribbe *et al.* (1974)

(13) Pring (1974)

(14) Leaver and Harmey (1973)

(15) Gray (unpublished data)

TABLE II

PHYSICAL PROPERTIES OF MITOCHONDRIAL AND CYTOSOL RIBOSOMES AND RIBOSOMAL RNA

Organism	Ribosomes (S value)		Ribosomal RNAs						Ref.
			S value		G + C (mol %)		Mol. Wt. (x 10^{-6})		
	cyto	mito	cyto	mito	cyto	mito	cyto	mito	
Animals									
Human (HeLa)	80	60	28, 18	16, 12	65	45		0.56, 0.36	1, 2
Rat Liver	80	55	28, 18	16, 13	64	47		0.50, 0.30	3, 4, 5
Protozoa									
Euglena	87	71	24, 20	21, 16	51	31			6
Tetrahymena	80	80	26, 17	21, 14	46	29	1.38, 0.64	0.93, 0.43	7
Fungi									
Saccharomyces	80	74		23, 16	48	23		1.30, 0.70	8, 9
Neurospora	77 77	73 80	28, 18 25, 18	25, 19 24, 17	48	35		1.28, 0.72	10 11, 12
Plants									
Maize	80	78					1.26, 0.70	1.26, 0.75	13
Turnip	80	78			52	44	1.30, 0.70	1.15, 0.70	14
Wheat					55.8	54.8	1.3 , 0.70	1.3 , 0.79	15

Mitoribosomes have been characterized according to several parameters, including size and sedimentation coefficient, protein and RNA composition, and susceptibility to changes in cation concentration and to inhibitors of protein synthesis. They differ in all these respects from their cytosol homologues. The sedimentation coefficients vary among mitoribosomes, but in general they are lower than those of the homologous cytoribosomes (TABLE II). The mitoribosomes have been classified into two groups based on size differences (Dawid, 1972). In the first group are the animal mitoribosomes which sediment between 55 S and 60 S, while in the second group are the 72 - 75 S mitoribosomes of several fungi. However, as with most arbitrary classifications based upon limited information, further examination of additional organisms has demonstrated some anomalies and even long-accepted sizes have recently been disputed. The protozoan Tetrahymena pyriformis contains mitoribosomes with a sedimentation coefficient of 80 S, identical to that of the cytoribosomes (Curgy et al., 1974), and plant mitoribosomes sediment only marginally slower than their cytosol counterparts

(Leaver and Harmey, 1973; Pring, 1974). Also, recent studies in Neurospora by Kroon and co-workers (Datema et al., 1974; Agsterribbe et al., 1974) suggest that the mitoribosomes of this organism are even larger than the corresponding cytoribosomes (80 S compared to 77 S) and that the reported value of 73 S (Kuriyama and Luck, 1974) is an artifact of the preparation procedure. The dispute over this issue remains to be resolved.

Direct measurement of ribosome size has been made using electron microscopy. Kleinow et al. (1974) have demonstrated that in the locust thoracic muscle the mitoribosomes are smaller (270Å X 210Å X 215Å) than the cytoribosomes (295Å X 210Å X 255Å). This is consistent with the sedimentation data. On the other hand, while the dimensions of Tetrahymena mito- and cytoribosomes suggest that the mitoribosomes are considerably larger (275Å X 230Å, cytoribosomes; 370Å X 240Å, mitoribosomes, Curgy et al., 1974), sedimentation data indicate identical sizes. In electron micrographs the 55 S mitoribosomes of rat liver appear smaller (169Å X 199Å) and less elongated than 80 S cytosol ribosomes (194Å X 247Å) (Aaij et al., 1972). However, hydrodynamic studies (DeVries and Van der Koogh-Schurring, 1973) have suggested that the

physicochemical properties of the rat liver mitoribosome are determined by a very low charge/mass ratio and high protein content, and that the volume of the 55 S particle is actually larger than, and its molecular weight equal to, that of the E. coli 70 S ribosome. Obviously, artifacts in sedimentation velocities and in electron micrograph preparation make a determination of actual and relative ribosome sizes difficult.

Mitoribosomes consist of two subunits, as do their cytosol counterparts, but the former ribosomes vary considerably more in size than do the latter. This reflects the variation in the subunit sizes. Animal mitoribosomes have subunit sedimentation coefficients of 30 S and 40 S (Borst, 1972), fungi 30 - 40 S and 50 S (Avadhani et al., 1976), and higher plants 40 - 44 S and 60 S (Leaver and Harmey, 1973; Pring, 1974). Tetrahymena mitoribosomes are unique in having identically-sized subunits of 55 S. Cytoribosome subunit sizes vary to a lesser degree, from 52 - 66 S and 37 - 46 S for the large and small subunits, respectively. Mitoribosomes require a higher Mg^{2+} concentration in order to prevent dissociation into subunits and in this respect, and in their sensitivity to

various antibiotics, they resemble bacterial ribosomes more closely than they do their cytosol homologues (cf. SECTION III for further discussion).

Mitoribosome composition both in terms of protein and RNA is distinct and differs from that of cytoribosomes. Van den Bogert and de Vries (1976) have resolved mitochondrial and cytosol ribosomal proteins from Neurospora using two-dimensional gel electrophoresis techniques, and have demonstrated a unique set of proteins associated with each type of ribosome. The number and mobility of these ribosomal proteins on gels vary greatly. The Neurospora mitoribosome contains 39 and 30 proteins (from the large and small subunits, respectively) while the cytoribosomes have 31 and 21 protein components in the corresponding subunits. Leister and Dawid (1974) have resolved 84 proteins from Xenopus mitoribosomes and 71 components from the cytosol counterpart, most migrating distinctively on two-dimensional gels. Additional strong evidence of the uniqueness of mitoribosomal proteins has been presented by Hallermayer and Neupert (1974), who demonstrated that antibodies prepared against Neurospora mitoribosomal proteins would precipitate only Neurospora

mitoribosomes and not the cytosol counterparts, whereas antibodies to Neurospora cytoribosomal proteins would precipitate Neurospora cytoribosomes but not mitoribosomes. Some dispute still exists as to what constitutes a ribosomal protein but it appears that although most, if not all, mitoribosomal proteins are coded for by nuclear DNA and synthesized in the cytoplasm, these proteins are distinct from their cytosol counterparts.

b. Physical Properties and Composition of Mitochondrial Ribosomal RNA

Mitoribosomes contain large and small subunit RNAs, both of which also differ from their cytosol homologues. As with the mitoribosomes themselves, sizes and base compositions of their constituent RNAs differ considerably among eukaryotes (TABLE II). Sedimentation velocities of animal mitochondrial rRNAs range from 12 - 13 S (small subunit rRNA) and 16 - 17 S (large subunit rRNA), those of fungal rRNAs range from 14 - 16 S and 21 - 24 S, and those of plant rRNAs from 18 - 18.5 S and 24 - 26 S. The cytosol rRNA components, on the other hand, are more conservative in size and vary only between 25 S and 28 S

for the large species and 17 S and 18 S for the small species. It is interesting to note that the variation in size of mitochondrial rRNA corresponds to the variation in size of the respective mtDNAs: that is, an increase or decrease in size of the mtDNA is coupled to a similar change in the size of the rRNAs for which it codes.

The G + C content of all mitochondrial rRNAs examined so far (excepting possibly those of higher plants) is lower than that of the cytosol counterparts (TABLE II). For example, the G + C content of yeast mitochondrial rRNA is 23%, the lowest value known for a ribosomal RNA, while that of yeast cytosol rRNA is 48%. A similar difference exists in the mammalian system, where we find G + C contents of 45% and 65% for the mitochondrial and cytosol rRNAs, respectively, of HeLa cells. This low G + C content of many mitochondrial rRNAs may be the cause of the extreme sensitivity of the conformation of these rRNAs to changes in temperature and ionic conditions (Grivell et al., 1971; Dawid and Chase, 1972). This results in an anomalous behavior of these molecules during sedimentation and polyacrylamide gel electrophoresis and stresses caution in accepting the assessed molecular weights determined by these methods. The thermal instability of mt-RNA raises the question of how mitoribosomes can function at

temperatures optimum for the function of their cytosol homologues. Freeman et al. (1973) suggest that mitochondrial ribosomal proteins and membranes as well as a possibly higher cation concentration might stabilize the rRNA and ribosomes. However, this does not explain why there should be a lower G + C content in the mitochondrial rRNA.

Mitochondrial ribosomes do not contain a 5.8 S "satellite" rRNA which is non-covalently bound to the large subunit rRNA of eukaryotic ribosomes (Pene et al., 1968; Payne and Dyer, 1972). Most species thus far examined also do not contain any mitochondrial 5 S rRNA molecule and this will be discussed in detail in SECTION IV.

As has been indicated in the previous chapter, mt-RNA is coded for on mtDNA and there is only one copy of each rRNA species per genome. The physical location of the two rRNA genes relative to each other on the DNA molecule depends upon the organism. For example, on yeast mtDNA the two rRNA genes are well separated from one another while in Neurospora they are adjacent. This difference could explain why a precursor molecule containing both rRNAs

has not yet been detected in yeast but has been found in Neurospora. In the latter study Kuriyama and Luck (1973) demonstrated that an initial transcript of 32 S (2.4 million daltons) is cleaved into pieces 1.6 and 0.9 million daltons in size, which are further processed into the mature 25 S and 19 S species (1.28 and 0.72 million daltons, respectively). As in the case of the mature mitochondrial ribosomal RNAs, the mitochondrial precursor rRNA has a low G + C content.

The mitochondrial ribosomes are the products of two distinct and separate genetic systems, as are several of the oligomeric enzyme complexes found in the inner mitochondrial membrane (these will be discussed briefly in the chapter on mitochondrial biogenesis). The ribosomal proteins are coded for by nuclear DNA, synthesized on cytoribosomes, and transported into the mitochondrion. The mt-rRNA, on the other hand, is transcribed from mtDNA. The two components combine by a poorly-understood process within the mitochondrion to form the functional mito-ribosome.

c. Modification of Mitochondrial Ribosomal RNA

Prokaryotic and eukaryotic cytosol ribosomal RNAs have been examined extensively for the presence of minor constituents. It has been demonstrated that these RNA molecules contain a small number of modified nucleosides (base-methylated, sugar-methylated, and pseudouridine) and that the modifications are added post-transcriptionally and occur at specific sites within the molecule (i.e., modification is non-random) (Maden, 1971). In addition, the type and extent of the modification appears to reflect the evolutionary position of the organism. Eukaryotic cytosol rRNA is more highly-methylated (1.2 to 1.7 methyl groups per 100 nucleotides; Klootwijk and Planta, 1973a; Maden and Salim, 1974) than bacterial rRNA (0.7 methyl groups per 100 nucleotides; Dubin and Gunalp, 1967; Fellner, 1969). Also, the content of pseudouridine is higher in cytosol rRNA, ranging from 0.25 residues per 100 nucleotides in bacterial rRNA (Dubin and Gunalp, 1967) to 0.8 in yeast (Klootwijk and

Planta, 1973b) and 1.2 in HeLa cell rRNA (Jeanteur et al., 1968). Eukaryotic cytosol rRNA contains a preponderance of the sugar methylations (those where a methyl group replaces a hydrogen at the O-2' position on the ribose moieties) whereas bacterial rRNA preferentially contains base methylations (those where a methyl group substitutes a hydrogen on the purine or pyrimidine heterocyclic rings).

Much less is known about the type and extent of methylation in the mitochondrial rRNAs. This has not been due to lack of interest but because of the technical difficulties of (1) isolating mitochondrial RNA free from any contaminating, more highly-modified cytosol RNAs and (2) quantitating the extremely low levels of modified nucleosides in mt-RNA with existing detection techniques (either ultraviolet absorption or radioisotope incorporation). As a result, considerable controversy still exists. For example, Vesco and Penman (1969) reported that human mitochondrial rRNA was unmethylated, but the analyses of Attardi and Attardi (1971) suggest that it contains one methyl group per 100 nucleotides.

However, several recent reports suggest that mitochondrial rRNA is indeed methylated, albeit to a very low degree. Lambowitz and Luck (1975) demonstrated that when Neurospora RNA is labelled with [methyl-^{3}H]methionine in the presence of sodium formate (to prevent randomization of the methyl label throughout the purine skeletons), the mitochondrial rRNA contains 0.05 - 0.16 methyl groups per 100 nucleotides, compared to a value of 1.5 for the cytosol rRNA. Dubin (1974) analyzed hamster cell mitochondrial rRNA and found low levels of methylation in the 17 S (0.13 methyl groups per 100 nucleotides) and in the 13 S (0.37 methyl groups per 100 nucleotides) rRNA species. This is equivalent to two residues per 17 S molecule (one ribose-methylated residue, $O^{2'}$-methyluridine (Um), in the sequence Um-Up; one unidentified residue) and four residues per 13 S molecule (one N^6, N^6-dimethyladenosine (m^6_2A) residue; one 5-methyluridine (m^5U) residue; one base-methylated cytidine; a fourth residue unidentified). Klootwijk et al. (1975) also demonstrated low levels of methylation in yeast mitochondrial rRNA. They could detect no methylation in the small subunit rRNA (15 S) and only two methylations in the large subunit RNA (21 S), which they suggested are on the ribose moiety. (An identical conclusion has been reached by Lambowitz and Luck (1976) in

a more recent study of Neurospora mt-rRNA). In addition, Klootwijk et al. (1975) found no more than one pseudouridine per molecule of 15 S or 21 S mt-rRNA. It is interesting to note that although the methylation of mitochondrial rRNA is lower than that of eukaryotic cytosol rRNA, the predominant type of methylation is the same in both cases (i.e., sugar methylation). This is in contrast to the situation with bacterial rRNAs, where base methylation predominates, even though the overall degree of methylation is relatively low. Dubin (1974) cites this in support of his belief that, at best, the idea that mitochondria evolved from bacterial endosymbionts is a gross oversimplification. Mention should be made of a recent report suggesting a two- to three-fold higher level of methylation in mouse liver mitochondrial rRNA than in the corresponding cytosol rRNA species (Dierich et al., 1975). However, in contrast to most other work with mammalian mt-RNAs (including mouse liver), this study indicated that the mitochondrial rRNA being analyzed had sedimentation properties and base ratios very similar to those of the cytosol homologues. Therefore, these results need confirmation.

The biological significance of modified nucleosides in ribosomal RNA is still obscure. Ribose methylation is an

early event in eukaryotic ribosome biosynthesis (Salim and Maden, 1973) and may be necessary for the proper processing of the 45 S rRNA precursor into the mature 28 S and 18 S species (Vaughan et al., 1967). Some base methylation appears to occur later, during the maturation of the small subunit rRNA (Klootwijk et al., 1972; Salim and Maden, 1973) in both bacterial and eukaryotic rRNAs, and may play an important role in ribosome assembly or ribosome function (Dubin, 1974). The dinucleotide sequence m_2^6A-m_2^6Ap is particularly interesting since it is present in the smaller rRNA (16 S or 18 S) of yeast and several bacterial and mammalian species (Klagsbrun, 1973), and possibly plays a role in ribosome function (Helser et al., 1972) and rRNA maturation (Hayes et al., 1971; Klootwijk et al., 1972). It has been suggested by Steitz and Jakes (1975) that the sequence G-m_2^6A-m_2^6A-Cp, which is positioned approximately 25 nucleotides from the 3'-terminus of E. coli 16 S RNA (Ehresmann et al., 1971), is involved in mRNA recognition. Chao and Woese (unpublished data, cited by Zablen and Woese, 1975) have found that the sensitivity of bacterial species to the antibiotic kasugamycin is dependent on the presence of this methylated dinucleotide sequence.

The role of methylation in the functioning of mitochondrial rRNA is even less clear. Indeed, in at least two cases (yeast and Neurospora) the small subunit mt-rRNA appears to be completely devoid of methyl groups. This suggests that methylation is not a universal requirement for the proper assembly and functioning of a mitoribosome. Additional analyses will help to answer a number of questions. Firstly, the degree, type, position, and time of introduction into the molecule of mitochondrial RNA modifications may tell us something of their function within the ribosome both during and after its formation. Since modified sequences are strongly conserved among the rRNAs of the different prokaryotes (Sogin et al., 1972), suggesting they are of functional importance, identification of modified sequences within mt-rRNA should indicate centres of special structural and/or functional significance. For example, the sites of antibiotic sensitivity (chloramphenicol, erythromycin) may possibly be in those areas containing methyl groups. This has been shown to be the case in bacterial mutants in which resistance to certain antibiotics results from changes in rRNA methylation (Lai and Weisblum, 1971; Helser et al., 1971).

Secondly, a study of mitochondrial rRNA modification might help to establish the degree of similarity among bacterial, mitochondrial, and eukaryotic cytosol protein-synthesizing systems. This idea will be pursued further in the introduction to SECTION IV, but one example here will suffice to illustrate the point. As mentioned above, the dinucleotide sequence m_2^6A-m_2^6Ap is present in several prokaryotic and eukaryotic small subunit rRNAs and denotes a region of conservation in these molecules. The modified nucleoside m_2^6A is also present in hamster cell 13 S rRNA (Dubin, 1974) and although it apparently occurs in only one copy per molecule, it possibly designates an homologous conserved region. Further analyses could help to ascertain the degree of evolutionary divergence in this region of prokaryotic, mitochondrial, and eukaryotic cytosol rRNAs.

Thirdly, as suggested by Klootwijk et al. (1975), an examination of modification in mitochondrial rRNA should shed light on the amount of nuclear genetic input required to process a mitochondrial rRNA whose primary sequence is coded for on mitochondrial DNA.

(iii) Mitochondrial tRNA

There is now ample evidence that mitochondria contain specific transfer RNAs which are coded for by the mtDNA and which participate in mitochondrial protein synthesis (Borst, 1972; Rabinowitz and Swift, 1970). RNA/DNA Hybridization experiments suggest that the number of those tRNAs which are coded for by the mitochondrial genome varies with the organism. For example, in Tetrahymena mitochondria, Suyama and Hamada (1976) found only seven "native" tRNAs but in yeast the figure may be as high as 25 (Reijnders and Borst, 1972). However, the exact number of tRNA genes on any mtDNA is not known with certainty and any current estimations are probably low. In 1972, Dawid estimated the number of tRNA genes on Xenopus mtDNA to be 15, but in a more recent study (Dawid et al., 1976) employing improved detection techniques, he suggests a value of 22. Similarly, Wu et al. (1972) initially reported 12 mtDNA-specific tRNAs in HeLa cells but have since revised that figure upwards to 19 (Attardi et al., 1976). Therefore, it is conceivable that at least some mtDNAs will eventually be shown to code for a full complement of tRNAs (33 in the case that all 20 amino acids are involved in

mitochondrial protein synthesis (Crick, 1966) and less if some amino acids are not utilized in the synthesis of intramitochondrial proteins). Attardi and co-workers (1976) have also demonstrated that HeLa cell mt-tRNAs are coded for by both ("heavy" and "light" strands) of mtDNA.

Some conflict exists as to whether or not all mitochondrial tRNAs are of organelle origin. Lynch and Attardi (1976) have shown that all of the HeLa cell mt-tRNA hybridizes to mtDNA but Suyama and Hamada (1976) have demonstrated the presence in Tetrahymena of "imported", or nuclear-coded, tRNAs, which are distinct from their cytosol counterparts. They suggested a mechanism whereby the tRNA synthetase may be involved in the transport of its specific tRNA into the mitochondria. Clarification of this question awaits further study.

Mitochondrial tRNAs differ from their cytosol counterparts in a number of respects. Although Dawid and Chase (1972) concluded from polyacrylamide gel electrophoresis studies that Xenopus mt-tRNA has sizes comparable to the cytosol tRNA, Dubin and Friend (1972) reported that hamster cell mt-tRNA migrates more slowly than cyt-tRNA during gel electrophoresis at high temperatures, suggesting a greater tendency for the former tRNA to

unfold under such conditions. Base composition analyses have demonstrated a lower G + C content for mt-tRNA when compared to the cytosol tRNA of the same organism (Chia et al., 1976; Martin et al., 1976). The G + C content of locust mt-tRNA has recently been reported to be 30.7% (vs. 55.5% for locust cytosol tRNA), the lowest G + C content found yet for any tRNA [Feldmann and Kleinow (1976)]. In addition, the kind and degree of modification differ between the tRNA of the two subcellular compartments. The mt-tRNAs are less methylated with, for example the content of methyl groups in HeLa cell mt-tRNAs being only 2.8% compared to a value of 8.7% for the cytosol species (Davenport et al., 1976). Randerath et al.(1974) suggested a slightly higher ratio of methylation (mt-tRNA/cyt-tRNA) but this could have been due to contaminating cyt-tRNA present in their mt-tRNA preparation. Both the eukaryotic-specific, fluorescent nucleoside wyosine (W) (or a derivative) and the prokaryotic-specific nucleoside 4-thiouridine (s^4U) are absent from yeast mt-tRNA (Schneller et al., 1975). Klagsbrun (1973) reported the presence of a 2-methyladenine-tRNA methylase activity in HeLa cell mitochondria but none in the cytosol. 2-Methyladenine has not been detected in any cytosol tRNA but is

present in the tRNA of prokaryotic organisms (Hall, 1971). However, Davenport et al. (1976) did not find any 2-methyladenine in HeLa cell mt-tRNA and attribute Klagsbrun's results to an artifact in the assay technique.

The significance of the low G + C content and the methylation pattern of mt-tRNA is not yet understood.

(iv) Mitochondrial Messenger RNA

Mitochondrial messenger-like RNAs from several mammalian, insect and fungal systems have recently been isolated and characterized. Penman and co-workers (Hirsch and Penman, 1973, 1974) and Attardi and co-workers (Ojala and Attardi, 1974 a, b; Attardi et al., 1976) have shown that synthesis of poly(A)-containing [poly(A)+] RNA from total mtRNA or from mitochondrial polysomes is sensitive to ethidium bromide and that this poly(A)$^+$ RNA hybridizes specifically to mtDNA. The presumptive mRNA contains polyadenylate stretches of 50 - 70 nucleotides at the 3'-terminus, smaller than the 100 - 150 poly(A) stretch in eukaryotic cytosol mRNA (Brawerman, 1974; however, cf. the values of 50 - 60 nucleotides for yeast cytosol mRNA (McLaughlin et al., 1973) and 19 - 34 for silk moth

cytosol mRNA (Vournakis et al., 1974). Devlin (1976) reported the presence of eight distinct poly(A)$^+$ species ranging in size from 9 S to 22 S in RNA isolated from sea urchin mitochondrial polysomes, and showed that these discrete components were transcribed from m[illegible] Poly(A)$^+$ RNAs have also been isolated from yeast (Hendler et al., 1975) and Trichoderma (Rosen and Edelman, 1976) mitochondria. In these two organisms, the poly(A) segment was only 20 - 30 nucleotides long, which may explain earlier work reporting the absence of poly(A)$^+$ RNA from yeast mitochondria (Groot et al., 1974). (RNA containing such short poly(A) stretches is not efficiently retained on an oligo(dT) affinity column, such as that employed by the latter investigators). As with mammalian poly(A)$^+$ RNA, the synthesis of the yeast poly(A)$^+$ RNA was also sensitive to ethidium bromide and it hybridized preferentially to yeast mtDNA (Hendler et al., 1975). However, the conflict over the sensitivity of mt-poly(A)$^+$ RNA synthesis to ethidium bromide and the hybridization studies with mtDNA and nDNA (for discussion see Avadhani et al., 1976) have raised the question of whether or not intramitochondrial processes are the sole source of mt-poly(A)$^+$ RNA, or whether some nuclear-coded mRNA is

transported into the mitochondria for translation on mitoribosomes. Chemical analyses of hamster cell mt-poly(A)$^+$ RNA (Taylor and Dubin, 1975) have shown that it is less methylated than the cytosol counterpart (if at all), and that it lacks the "blocked" 5'-methylated terminus characteristic of eukaryotic cytosol mRNA (Adams and Cory, 1975). In this latter respect, the presumptive mt-mRNA resembles bacterial mRNAs, although all three types of mRNA (prokaryotic, mitochondrial, and eukaryotic cytosol) contain poly(A) (Nakazoto et al., 1975; Ohta et al., 1975).

Although it is evident that mtDNA codes for several mitochondrial-specific proteins [cf. sections on mtDNA (I.B.(i)) and mitochondrial protein biosynthesis (I.D.)] and that the mitochondria contain some poly(A)$^+$ RNA coded for by the mtDNA, this in itself does not prove that the latter is mt-mRNA. It is necessary to demonstrate unequivocally that this RNA not only hybridizes specifically to mtDNA but also that it codes for definable mitochondrial proteins. In preliminary experiments, Padnanabam et al. (1975) have shown that this is the case with poly(A)$^+$ RNA isolated from yeast mitochondria. This RNA, in an E. coli cell-free system, directs the synthesis of polypeptides

which can be specifically precipitated with antibody to cytochrome c oxidase. Thus, all the evidence to date indicates that mt-poly(A)$^+$ RNA is indeed messenger RNA.

C. MITOCHONDRIAL PROTEIN BIOSYNTHESIS

As early as 1958 amino acid incorporation into mitochondria was detected (McLean et al., 1958) and these observations were subsequently confirmed by other workers. However, it was only through the use of specific inhibitors of protein synthesis that the notion of a distinct protein synthesis system within the mitochondrion gained general acceptance. Several groups (Kroon, 1963; Roodyn, 1965; Wheeldon and Lehninger, 1966; Beattie et al., 1971) demonstrated that amino acid incorporation into mitochondria was not an artifact due to cytoribosome contamination, since it was sensitive to low concentrations of chloramphenicol and insensitive to cycloheximide (a specific inhibitor of cytosol protein synthesis). Since those initial studies it has been shown that mitochondria contain all the components of a functional protein-synthesizing system, components [including mRNAs, tRNAs and ribosomes; (cf. section I.B.), as well as initiation, elongation and termination factors (Avadhani et al., 1976)] which are distinct from their cytosol counterparts. In many respects, including antibiotic sensitivity and the utilization of formylmethioninyl-tRNA as initiator, mitochondrial protein

synthesis is remarkably similar to the same process in prokaryotes. However, recent analyses of mt-rRNAs indicate that this functional similarity may not be reflected in as great a degree of structural similarity as previously assumed (refer to SECTION IV).

Attempts to identify and characterize the products of mitochondrial protein biosynthesis have involved numerous approaches. In addition to inhibitor studies *in vivo*, these include labelling *in vitro* of isolated mitochondria (Poyton and Groot, 1975), genetic analyses of cytoplasmic mutants (Griffith, 1975), coupled transcription - translation systems (Scragg and Thomas, 1977), incorporation of mtDNA into bacterial plasmids (Clayton, 1976), and the translation *in vitro* of poly(A)$^+$ mt-RNA (Padmanaban *et al.*, 1975). All these approaches suggest that from 5 - 15 polypeptides are synthesized in mitochondria (Michel and Neupert, 1973; Lederman and Attardi, 1973), and that in all cases these are hydrophobic proteins which are components of certain inner mitochondrial membrane complexes (e.g., cytochrome c oxidase, cytochrome bc_1, and oligomycin-sensitive ATPase). These mitochondrially-synthesized proteins represent only a small portion of the total

mitochondrial protein complement (< 10%, Schatz and Mason, 1974), most of which is coded for on nuclear DNA, synthesized on cytoribosomes, and transported into the mitochondrion. There is little evidence to date that the larger mtDNAs (e.g., those of lower fungi and higher plants) code for additional translation products. However, 68% of the potential coding capacity of plant mtDNA has no known function and it is interesting to speculate that these mtDNAs contain cistrons for as-yet-unidentified translation products (Leaver and Pope, 1976).

D. MITOCHONDRIAL BIOGENESIS

Mitochondria, it appears, are not synthesized de novo but originate from pre-existing organelles in a manner reminiscent of the growth and division of bacterial cells (Attardi et al., 1975; Luck, 1965). This mitochondrial biogenesis, resulting in the formation of a functional respiratory chain and protein-synthesizing apparatus, involves the co-ordination of two distinct genetic systems, one cytoplasmic and the other mitochondrial (Schatz and Mason, 1975; Tzagoloff et al., 1973). The interplay between these two systems is not well understood but some information has been obtained through study of the inner mitochondrial membrane complexes, specifically cytochrome c oxidase. These are hybrids of cytosol- and mitochondrially-synthesized components.

It appears that mitochondrial translation products are not needed for the synthesis of mitochondrial proteins of cytoplasmic origin (Ebner et al., 1973; Poyton and Schatz, 1975). In contrast, protein synthesis on mitochondrial ribosomes, measured both in vivo and in vitro, may be controlled by proteins synthesized in the cytoplasm (Schatz and Mason, 1974). Poyton and Kavanagh (1976) have recently

suggested that the cytoplasmic "stimulatory proteins" in yeast act specifically to stimulate the synthesis of certain distinct mitochondrial translation products. They presented evidence which indicates that it is the cytoplasmically-synthesized subunits of cytochrome c oxidase which exert a positive effect on the synthesis of the mitochondrial-specific subunits. Although these results are preliminary, they offer an experimental approach to the study of mitochondrial - nuclear interactions, which is rapidly becoming the most interesting area in mitochondrial research.

SECTION II: WHEAT EMBRYO MITOCHONDRIAL RNA: ISOLATION, CHARACTERIZATION, AND ISOTOPIC LABELLING

1. INTRODUCTION

A. MITOCHONDRIAL RIBOSOMES AND RIBOSOMAL RNA IN HIGHER PLANTS

As indicated in the preceding General Introduction, considerable information has been accumulated on the size and composition of the mitochondrial ribosomes and rRNA in animals and lower fungi. However, to date, few studies have been devoted specifically to an analysis of the higher-plant mitochondrial protein-synthesizing system and its components, and the information obtained has been much less detailed. In part this is due to the fact that green plants possess yet another semi-autonomous subcellular organelle, the chloroplast, which contains its own distinctive protein-synthesizing system and which grossly contaminates mitochondrial preparations during subcellular fractionation. Since chloroplast ribosomes may constitute as much as 20 - 30% of the total ribosome population in a higher plant cell, compared to less than 1% in the case

of mitochondrial ribosomes, the isolation and definitive characterization of mitoribosomes from green plants has not been an easy task. In addition, however, lack of interest in plant mitochondrial nucleic acids may be attributable to a general negative bias which many investigators direct toward the plant kingdom. Such an attitude is not only unjustified but is unfortunate, since the higher plant offers an opportunity to study a number of unique problems associated with embryonic development and intracellular interactions. Thus, embryogenesis in higher plants passes from a period of rapid cell growth and division (in the developing seed) into a condition of dormancy (mature seed) which is subsequently followed by a resumption of cell growth and differentiation (during germination). Well-documented functional and structural changes in the mitochondrial population occur during germination in higher plants (Solomos et al., 1972; Malhotra and Spencer, 1973) but the control mechanisms underlying these changes, as well as their biochemical basis, remain poorly understood. In such a context, the germinating plant seed becomes a model system in which to examine some unique aspects of mitochondrial biogenesis.

In addition, the presence of a third genetic system (in the chloroplast) in higher plants allows a study of the interaction of three distinct genomes within the same cell. In particular, the opportunity to examine the regulation of function of two complementary, semi-autonomous, energy-transducing organelles is not provided by any other eukaryotic system.

Initially, mitochondria were isolated from plant material for studies of their energy-generating capacities (Stafford, 1951; Johnston et al., 1957; Honda et al., 1966; Bonner, 1967). The isolation methods involved the differential centrifugation of cellular homogenates, a procedure developed by Palade and coworkers (Hogeboom et al., 1948) which allowed the preparation of structurally-intact mitochondria. In these preparations, the monitoring of parameters such as P:O and respiratory control ratios (indicators of the degree of coupling of oxidative phosphorylation) was sufficient to ensure the biochemical integrity of the mitochondria. Contamination of these crude mitochondrial preparations by other cell components (e.g., nuclear fragments, plastids, microsomes) was high but could be tolerated as it did not affect the results of the analyses. In addition, plant tissues

containing a low content of these organelles could be used as a starting material and low yields could be accepted because relatively few mitochondria were needed for such studies.

However, analyses of mitochondrial nucleic acids and protein biosynthesis require considerably purer mitochondrial preparations since the crude mitochondrial fractions contain numerous nucleic acid contaminants. The presence of minor amounts of such material (nuclear fragments, endoplasmic reticulum, plastids and microorganisms) could significantly affect the results of such analyses, due to the inherently-low content of mitochondrial nucleic acids present in all organisms examined to date (less than 1 μg DNA per mg mitochondrial protein; Suyama and Bonner, 1966; Leffler et al., 1970; 3 - 20 μg RNA per mg mitochondrial protein; Pring, 1974). The RNA content of microsomes, on the other hand, is much higher and in the order of 200 μg/mg (O'Brien and Kalf, 1967). The traditional methods of assessing the purity of mitochondrial preparations used for nucleic acid studies are insufficient and may lead to erroneous conclusions. Electron microscopy of purified mitochondria may aid in

determining the physical integrity of the organelles but is incapable of quantitating any extramitochondrial contamination that may exist (De Duve, 1967). Assay of marker enzymes such as glucose 6-phosphatase (whose activity in mitochondrial fractions has been taken as an indication of contaminating fragments of endoplasmic reticulum; De Duve et al., 1955) does not always offer sufficient evidence for the purity of a mitochondrial preparation (Pollard et al., 1966). Therefore an analysis of the mitochondrial nucleic acids, demonstrating that they are distinct and different from any possible contaminants, is necessary.

A number of methods have been developed in an attempt to remove contaminating nucleic acids from crude mitochondrial fractions. These include: (1) buoyant density centrifugation (Guderian et al., 1972), which will separate nuclei and nuclear fragments, mitochondria, intact chloroplasts and microsomes on the basis of their differential densities; (2) deoxyribonuclease treatment (Suyama and Bonner, 1966; Tewari, 1971; Kolodner and Tewari, 1972), which selectively removes extra-mitochondrial (nuclear) DNA; (3) EDTA treatment (Sabatini et al.,

1966; Attardi et al., 1969), which dissociates contaminating cytosol ribosomes in microsomes or bound to the outer mitochondrial membrane; (4) ribonuclease treatment (Leaver and Pope, 1976), which degrades contaminating cytosol RNA; (5) digitonin treatment (Malkin, 1971), which selectively solubilizes microsomes and the outer mitochondrial membrane, thus eliminating contamination by extra-mitochondrial ribosomes; and (6) preferential suppression of cytosol RNA synthesis with actinomycin D, an inhibitor of nuclear DNA transcription (Vesco and Penman, 1969; Dubin and Montenecourt, 1970). In the present study a modification of the medium employed by Guderian et al. (1972) for the preparation of tobacco leaf mitochondria was used for the isolation (by differential centrifugation) of a crude fraction of wheat embryo mitochondria. Inclusion of relatively high concentrations of EDTA during subsequent buoyant density centrifugation facilitated removal of contaminating nucleic acids.

Early analyses of higher plant mitochondrial ribosomes date from 1965 when Kislev and coworkers observed ribosome-like particles in swiss chard (Beta vulgaris var. cicla) mitochondria by electron microscopy. Wilson et al. (1968)

also observed such particles in intact maize mitochondria and in addition found particles in mitochondrial lysates with a sedimentation coefficient of 66 S. However, these structures were not characterized and shown to be ribosomes. It is possible that the particles were 60 S ribosomal subunits, since maize mitoribosomes have since been shown to be sensitive to the K^+/Mg^{2+} ratio in the isolation medium and the 60 S subunit often represents the major species in maize mitochondrial ribosome preparations (Pring, 1974).

The first characterization of plant mt-RNAs was that reported by Pollard et al. in 1966. They isolated high-molecular-weight RNA from mitochondrial fractions of Brassica oleracea (cauliflower), analyzed it by sucrose density gradient centrifugation, and examined its nucleotide composition. The RNA sedimented at rates characteristic of ribosomal RNA, although precise sedimentation coefficients and molecular weights were not determined. The individual ("18 S" and "28 S") mt-RNA species had a relatively high G + C content and their overall nucleotide compositions were similar, although not identical, to those determined for the corresponding cytosol rRNA components. Since these mitochondria were isolated in the

presence of a high concentration of Mg^{2+} (10 mM), it is probable that the mt-RNA species analyzed were, in fact, cytosol contaminants. Gray (unpublished data) has shown that when mitochondria are prepared from commercial wheat germ using the procedure of Pollard et al. (1966), the RNA subsequently extracted consists largely of contaminating cytosol RNA, even when the crude mitochondrial fraction is further purified by buoyant density centrifugation. In 1968 Baxter and Bishop examined soybean mitochondrial RNA and observed two species with electrophoretic mobilities intermediate between those of the two cytosol rRNAs. They also reported that the large mtRNA species had a relatively low G + C content. However, the G + C content of this species is actually 52.4% (Baxter, personal communication), which is close to the G + C contents of the two cytosol rRNAs (57.4%, 25 S; 53.4%, 18 S) and high for mitochondrial rRNAs (22% to 47%; TABLE II). The RNA preparation analysed by these authors was degraded and the possibility of plastid contamination was not excluded. Thus, not much confidence can be placed on the reported sizes and nucleotide compositions of their mtRNA species. Vasconcelos and Bogorad (1971) reported the isolation of 70 S ribosomes from mung bean mitochondria, whereas 80 S ribosomes were obtained

from the cytosol. The molecular weights of the presumed mitochondrial rRNAs were stated to be 1.1 and 0.58 million daltons. However, no supporting data on the characterization were given and subsequent work by Leaver and Harmey (1973) has demonstrated these results to be incorrect. Vasconcelos and Bogorad possibly were dealing with plastid contaminants, since mung bean chloroplasts contain 70 S ribosomes and their rRNA components have molecular weights of 1.1 and 0.58 million daltons.

The first systematic study of plant mitoribosomes and mt-RNA was undertaken by Leaver and Harmey in 1973. These investigators analyzed five dicotyledonous plant species (turnip, mung bean, potato, cauliflower, and pea), and went to great lengths to ensure that their mitochondria were pure and intact, monitoring their preparations with the aid of electron microscopy, oxygen-electrode polarography and spectrophotometry. They also demonstrated (by buoyant density centrifugation) that contaminating nuclear DNA represented less than 5% of the DNA isolated from the mitochondrial fractions. The yield of mitochondrial protein was 35 to 45 μg/g mung bean hypocotyl or turnip root, and the yield of mitochondrial RNA was 10 -

20 μg/mg mitochondrial protein. The use of non-green plant materials (etiolated seedlings, storage tissues) for isolation of mitochondrial fractions minimized any problem with plastid contamination.

Characterization of the total plant mt-RNA by polyacrylamide gel electrophoresis in an EDTA-containing buffer (Leaver and Harmey, 1973) revealed two major components, the larger having a molecular weight of 1.12 - 1.18 million daltons and the smaller with a molecular weight of 0.69 - 0.78 million daltons, depending on the plant species. The cytosol homologues had molecular weights of 1.30 - 1.36 and 0.65 - 0.70 million daltons. Leaver and Harmey found that degradation of the 1.15 million dalton mt-RNA component occurred during electrophoresis in EDTA medium but that this breakdown could be prevented if the RNA was prepared in the presence of Mg^{2+} and fractionated in a Mg^{2+}-containing buffer. They also characterized the mt-RNA by sucrose density gradient centrifugation using E. coli rRNA markers. The two mitochondrial rRNAs sedimented faster than their bacterial homologues but between the two cytosol species. The sedimentation coefficients of the mt-RNA components were calculated to be 24 S and 18.5 S

(compared with 25 S and 18 S for their cytosol counterparts). Preliminary analysis of turnip rRNA nucleotide composition revealed G + C contents of 44% and 52% for the mitochondrial and cytosol species, respectively.

No 5.8 S "satellite" rRNA, a specific marker of eukaryotic cytosol ribosomes (Payne and Dyer, 1972), was present in total mt-RNA preparations. This provided additional evidence of the purity of the mitochondrial fractions. However, plant mitochondria did contain 5 S rRNA (in contrast to other systems; cf. SECTION IV) and presumptive transfer RNA (4 S).

Plant mitoribosomes were prepared by lysing the mitochondrial fraction with Triton X-100 and purifying the ribosomes by sedimentation in a linear sucrose density gradient. The mitoribosomes sedimented faster than E. coli ribosomes (70 S) but more slowly than the plant cytosol ribosomes (80 S) and were tentatively assigned a sedimentation coefficient of 77 - 78 S. An analysis of the rRNA obtained directly from the mitoribosomes confirmed the results found with the total mt-RNA. In the case of turnip mitoribosomes, the 1.15 million dalton rRNA component was shown to be restricted to the large, or 60 S, subunit, while the 0.70 million dalton component was

confined to the small, or 40 S, subunit.

The conclusions of Leaver and Harmey regarding the properties of higher plant mitoribosomes and mt-RNA were confirmed in a subsequent study carried out by Pring (1974) in maize. The mitoribosomes of this higher plant (a monocotyledon) also had a sedimentation coefficient of 78 S and dissociated into 60 S and 44 S subunits. The two major mt-RNA species had molecular weights (as determined by polyacrylamide gel electrophoresis, using E. coli rRNA as a standard) of 0.74 - 0.75 and 1.26 million daltons both before and after denaturation in formaldehyde. The cytosol rRNAs had apparent molecular weights of 0.70 and 1.26 million daltons, as determined by electrophoresis in non-denaturing gels, but 0.68 and 1.15 million daltons in formaldehyde gels, suggesting a preferential reaction of the larger cytosol rRNA component with formaldehyde. The UV absorbance ratio of heavy to light mt-rRNA was 1.5 although a value of 1.68 (based on molecular weight assignments of 0.75 and 1.26 million daltons) was expected. However, some preferential degradation of the large mt-rRNA species could have resulted from use of an EDTA-containing buffer during polyacrylamide gel electrophoresis. Pring extended his

analyses of maize mt-rRNAs by examining their mobilities under several denaturing and non-denaturing conditions of gel electrophoresis (Pring and Thonbury, 1975). He confirmed his previous results by demonstrating a dependence of electrophoretic mobility and hence apparent molecular weight of plant RNAs upon the electrophoresis conditions employed. The difference in sizes between the maize mt-rRNAs and those of the five dicotyledonous species studied by Leaver and Harmey was attributed to either differences in the conditions of non-denaturing gel electrophoresis employed in the two studies or to a genuine difference between the two groups (mono- and dicotyledons) of plants (Pring and Thonbury, 1975). Such diversity is found among the fungal and animal mt-rRNAs and might also be expected among the higher plants.

Concurrent with the above studies of Leaver and Pring, an independent investigation of the mitochondrial RNA species of wheat was undertaken by Gray (1974a). Initial studies were conducted using commercial wheat germ, which, as a naturally-dessicated tissue, seemed a particularly suitable source material for the amounts of mitochondria required for identification and characterization of the RNA species which might be present in these organelles. Freshly-milled

wheat germ was available locally at low cost and in unlimited quantity, and could easily be stored for extended periods without obvious deterioration. Selection of the wheat embryo system for comparative studies of higher plant cytosol and mitochondrial RNA was dictated in no small measure by the extensive information already available on the physical and chemical properties of wheat cytosol ribosomal and transfer RNA, generated largely through the efforts of B. G. Lane and his colleagues (Lane and Allen, 1961; Singh and Lane, 1964; Lane, 1965; Hudson et al., 1965; Kay and Oikawa, 1966; Wolfe and Kay, 1967; Wolfe et al., 1968; Wolfe and Kay, 1969; Dudock et al., 1969). Moreover, since viable embryos can be isolated in quantity in the laboratory and will germinate and develop normally for a considerable period of time, this system seemed well-suited for studies of the role of mitochondrial transcription and translation in the activation of pre-existing mitochondria and in the formation of new mitochondria during seed germination.

Although it is a metabolically-dormant tissue, wheat embryo was known to have a high content of mitochondria, displaying at least some aspects of functional competence

(e.g., the presence of active cytochrome oxidase; Johnston et al., 1957; Setterfield et al., 1959). However, initial attempts to isolate mitochondrial fractions from commercial wheat germ met with failure, even though the methods employed had been used successfully with numerous other plant tissues (e.g., Bonner, 1967). These negative results have since been attributed to the unique physiological state of the tissue. The structurally-immature membranes of nuclei and mitochondria in dormant embryos appear to be exquisitely sensitive to preparative damage, so that these organelles are largely broken when the tissue is disrupted in EDTA-containing medium. Thus, the crude "mitochondrial" fraction isolated by differential centrifugation of such homogenates consisted largely of nuclear fragments. Attempts to counter this effect by replacing EDTA with divalent cations (Mg^{2+}, Ca^{2+}), with the aim of stabilizing membranes during isolation (Johnston et al., 1957), led to massive contamination of the mitochondrial fraction with membrane-bound cytosol ribosomes. In the end, an homogenizing medium containing both EDTA and Mg^{2+} (Guderian et al., 1972) allowed the isolation of a crude mitochondrial fraction which could

then largely be freed of cytoribosome contamination by buoyant density centrifugation in EDTA-containing sucrose gradients. By this method, yields of 600 μg mitochondrial protein/g of embryos and about 20 μg RNA/mg mitochondrial protein were obtained. The specific activity of cytochrome oxidase in the gradient-purified mitochondrial preparations compared favorably with values reported for highly-purified mitochondria from other plant tissues (Stafford, 1951; Smillie, 1956; Solomos et al., 1972).

Resolution of wheat total mt-RNA by polyacrylamide gel electrophoresis under non-denaturing conditions revealed a pattern similar to that found in maize (another monocotyledon) (Pring, 1974). The largest mt-RNA species (which was also present in greatest amount) co-migrated with the cytosol large subunit rRNA (26 S, 1.3 million daltons), while the second largest (and second most prominent) mt-RNA species migrated slightly but distinctly more slowly than the cytosol small subunit rRNA (18 S, 0.7 million daltons). These two mt-RNA species (1.3 and 0.79 million daltons, relative to the cytosol rRNA species) had the size and solubility characteristics (insoluble in 1 M NaCl at 0°) of ribosomal RNA, and Gray (1974a) suggested that they represented

the constituent high-molecular-weight RNAs of wheat embryo mitoribosomes. The total mt-RNA also contained 5 S and 4 S RNAs, but unlike the situation with the cytosol total RNA, little 5.8 S "satellite" RNA was produced upon heat denaturation of mitochondrial total RNA. This indicated the absence of major contamination of mitochondrial rRNA with cytosol rRNA.

Two other, minor RNA species (0.70 and 0.44 million daltons) were reproducibly detected in wheat mitochondrial total and NaCl-insoluble RNA. The origin of these two components has not been resolved; however, since the larger species co-migrated with the cytosol 18 S rRNA, it is tempting to suggest that it was derived from contaminating cytosol ribosomes (the 0.44 million dalton component had no cytosol counterpart). Gray discounts this possibility on the basis that extensive treatment of the mitochondrial fraction with EDTA during its preparation would be expected to largely remove any contaminating cytoribosomes. The conspicuous absence in wheat mt-RNA of a 5.8 S RNA component, which is a specific marker of the large subunit of wheat cytoribosomes (Azad and Lane, 1973), lends support to this argument. The fact that small sub-

units are more readily removed by EDTA from membrane-bound ribosomes than are large subunits (Sabatini *et al.*, 1966; Attardi *et al.*, 1969) makes it even more unlikely that mt-RNA depleted of 5.8 S (and hence of 26 S) cytosol rRNA could contain any significant quantity of cytosol 18 S rRNA. Since the molar ratio of the two largest (1.3 : 0.79) wheat mt-RNAs was always observed to be less than the theoretical value for equal numbers of the two species, it is possible that the 0.7 and 0.44 million dalton RNA species represented discrete breakdown products of the 1.3 million dalton mt-RNA component (*cf.* Leaver and Harmey, 1973).

The ability to isolate relatively large quantities of mt-RNA from commercial wheat germ allowed Gray to carry out nucleoside composition analysis of the tRNA fraction, the first such analyses conducted in a higher plant system. Wheat mt-tRNA was found to have a high G + C content (54.8%), similar to the G + C content of wheat cytosol tRNA (55.8%). On the other hand, a preliminary search for pseudouridine and $O^{2'}$-methylnucleosides in the mt-rRNA indicated that levels of both were markedly lower than in the cytosol rRNA (Gray, unpublished). This latter observation is in agreement with the results obtained

in fungal and mammalian mitochondrial systems (Section I.B.(ii).c), whereas the similarity in nucleoside composition between wheat mitochondrial and cytosol rRNA stands in marked contrast to results obtained in other eukaryotic systems (TABLE II).

It was against this background of knowledge about the properties of the mitochondrial RNA of higher plants in general, and of wheat embryo in particular, that the present investigation was begun.

B. SCOPE AND SPECIFIC AIMS OF THE PRESENT INVESTIGATION

Although commercial wheat germ has proven to be an ideal source material for the isolation of relatively large quantities of plant mitochondria and mitochondrial nucleic acids, its non-viability limits the types of analyses which can be undertaken. For example, studies of mt-RNA metabolism [biosynthesis (including post-transcriptional modification and processing), degradation, and the regulation of these two processes] and the involvement of mitochondrial transcription and translation in plant development (e.g., the onset, regulation and role of mitochondrial protein synthesis in the activation of mitochondrial functions during germination and the part such activation plays in the initial development of the plant) are obviously impossible to do in such a system. Thus, it was necessary to establish the germinating wheat embryo as a model system in which to be able to study the above phenomena. In the context of the present investigation, it was of particular interest to determine whether this system could provide the isotopically-labeled RNAs required for more exacting characterization studies than can be carried out with unlabeled RNAs.

The specific aims of this investigation were:

(1) to isolate viable, dormant wheat embryos and, using the procedures developed for commercial embryos, confirm the previous conclusions about the properties of wheat mt-RNA. The methodology for mass isolation of viable wheat embryos existed (Johnston and Stern, 1958) and had been used successfully by other workers (e.g., Lau, 1973).

(2) to establish conditions for isotopic labeling of mt-RNAs in germinating wheat embryos, using the labeling protocols previously developed by Lane and co-workers (Lau _et al._, 1974) for bulk cellular RNA.

(3) to isolate [^{32}P]-labeled mitochondrial and cytosol rRNAs, demonstrate that the individual RNA species could be purified free of appreciable cross-contamination, and prove that such labeled RNAs indeed originated in the embryos and were not contributed by contaminating organisms.

(4) to characterize the [^{32}P]-labeled rRNAs and show that they had the same physical properties as the corresponding species from dormant embryos, and to carry out detailed analyses of major and modified nucleotide composition.

(5) to "fingerprint" the 26 S, 18 S, and 5 S cytosol and mitochondrial rRNA species (with the aim of obtaining

evidence as to the uniqueness of each of these molecules).
(6) to prepare [^{32}P]-labeled mitochondrial and cytosol rRNA of sufficiently-high specific activity to be able to carry out detailed sequence analyses ("T_1 oligonucleotide cataloguing") of the 18 S species of the two subcellular compartments, with the aim of gaining insight into their evolutionary relatedness to each other and to prokaryotic (bacterial, blue-green algal) and chloroplast 16 S rRNAs. This study was made possible through a close collaboration with other members of the Department of Biochemistry (Dr. W. F. Doolittle, L. Bonen) who possessed the necessary expertise in the area of T_1 oligonucleotide cataloguing.

2. MITOCHONDRIAL AND CYTOSOL RNA FROM DORMANT, VIABLE WHEAT EMBRYOS

A. MATERIALS

Pedigree wheat seed (*Triticum vulgare* var. Thatcher) was obtained from Early Seed and Feed Ltd., Saskatoon. Acrylamide and bisacrylamide were purchased from Eastern Kodak Co. and Canalco respectively. The former was recrystallized from acetone and the latter recrystallized from 95% ethanol. All chemicals and solvents were reagent grade.

B. METHODS

(i) Preparation of Viable Wheat Embryos

A modification of the method of Johnston and Stern (1957) was used to prepare viable embyos from pedigree wheat seed. Portions of seed (250 g) were ground in an aluminum Waring blender (commercial) for 12 seconds at low speed with a Powerstat (Type 116B, Superior Electric Co., Bristol, Conn., U.S.A.) setting of 85. No dry ice was used. The mixture was then transferred to the uppermost of a set of Endecott test sieves arranged from bottom to top in ascending order of pore size (10-, 14-, and 28-mesh, Canadian Standard Sieve, W.S. Tyler Co.) and shaken by hand

for approximately 30 seconds. The freed embryos passed through the 10- and 14-mesh sieves and were deposited on the 28-mesh sieve along with small fragments of endosperm and bran. This portion was retained for further processing. The material stopped by the 14-mesh sieve, mostly fragmented endosperm, was discarded. The intact wheat seed and large endosperm fragments remaining on the 10-mesh sieve were reground for a further 10 seconds as described above. The crude embryos were saved and the portion remaining on the 10-mesh sieve was reprocessed a third time (8 seconds).

The yield from 5 kg of wheat seed was approximately 150 g of crude embyos. To remove the bran, a hair dryer was fixed 3 feet above the table and the bran was blown from the mixture as it was shaken by hand in the 28-mesh sieve. The embryos were then separated from the fragmented endosperm by suspending the material in a mixture of cyclohexane/carbon tetrachloride (10/25, v/v), allowing the endosperm fragments to settle, and removing the embryos from the surface by vacuum suction. This procedure was repeated 4 - 6 times to remove all contaminating endosperm fragments. The purified embryos were air dried and stored at 0 - 4° in the presence of anhydrous $CaCl_2$. A final

yield of approximately 25 g of largely intact and viable embryos was obtained from 5 kg wheat seed.

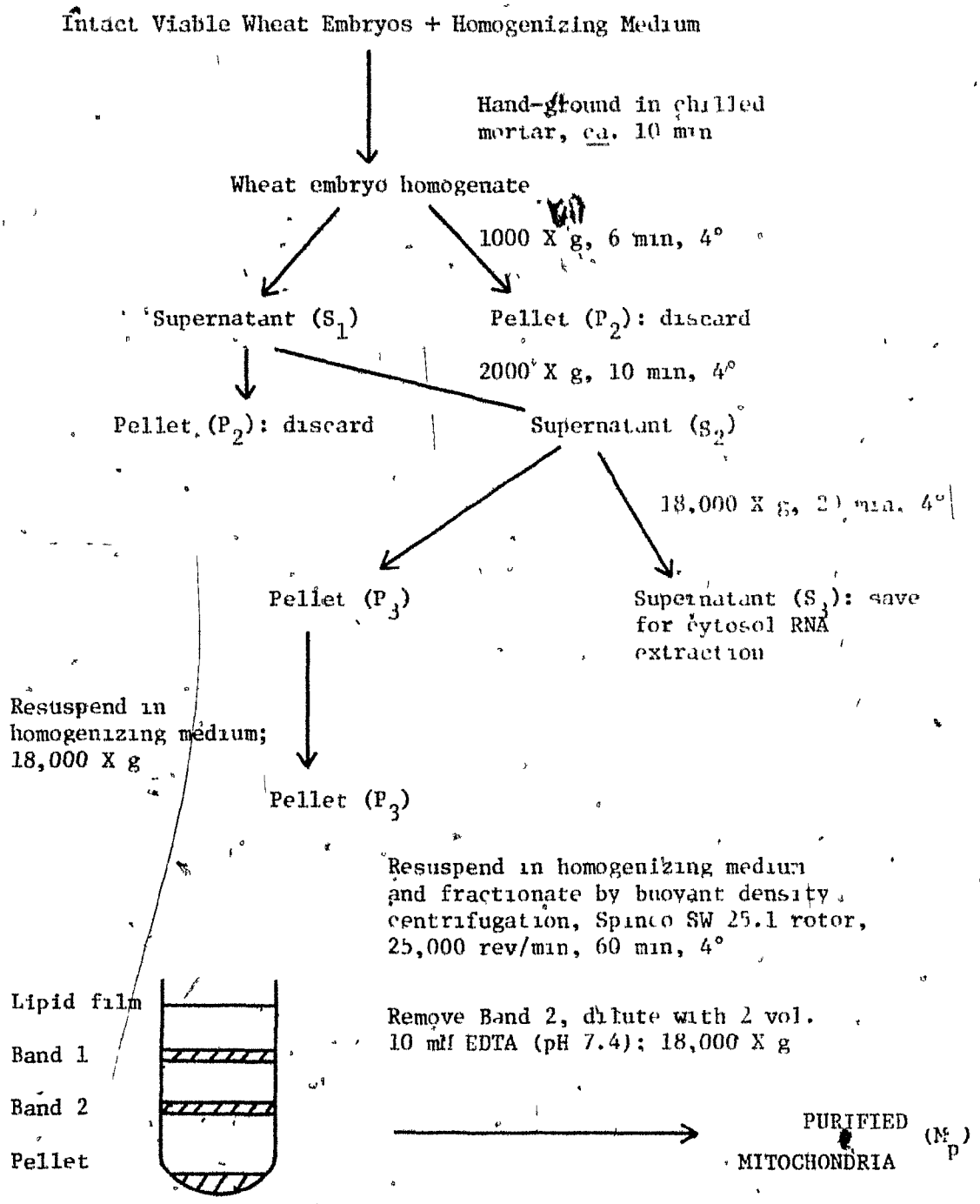
(ii) Isolation of Mitochondria

Mitochondria were prepared from dormant viable wheat embryos using a modification of a procedure developed by M.W. Gray (manuscript in preparation) for the large-scale preparation of a mitochondrial fraction from commercial wheat germ. Viable wheat embryos were suspended in homogenizing medium (0.25 M sucrose - 10 mM $MgCl_2$, 10 mM EDTA, 10 mM NaCl, 4 mM β-mercaptoethanol, 0.05 M Tris·HCl, pH 7.8 at 4°; 10 ml/g of embryos) and ground by hand for 10 min in a chilled mortar. The homogenate was centrifuged at 1000 X g for 6 min in an IEC International Refrigerated Centrifuge and the pellets (P_1), containing whole embryos and intact cells, were discarded. Supernatants (S_1) were filtered through cheesecloth and centrifuged at 2000 X g for 10 min. Pellets (P_2) were resuspended in fresh homogenizing medium for cytochrome c oxidase assays, while supernatants (S_2) were centrifuged at 18,000 X g in a Sorvall RC2-B centrifuge for 20 min. Supernatants (S_3) were saved for cytochrome c oxidase assays and cytosol RNA

extraction, while pellets were resuspended in fresh homogenizing medium and the mitochondria again sedimented at 18,000 X g for 20 min. The crude washed mitochondrial fraction (P_3) was resuspended in 24 ml homogenizing medium and further purified by isopycnic centrifugation in three discontinuous gradients, each consisting of 8 ml 1.2 M and 1.5 M sucrose in 10 mM EDTA (pH 7.4). The crude mitochondrial fraction was layered on the gradients, which were then centrifuged in a Beckman Model L ultracentrifuge in a Spinco SW 25.1 rotor for 60 min at 25,000 rpm. The bulk of the material above the 1.2 M - 1.5 M interface was removed by aspiration and the purified mitochondria (M_p) were carefully removed using a syringe fitted with a needle containing a right-angle bend (to avoid turbulence). The three pooled mitochondrial bands were slowly diluted (over a 20 min period) with 2 vol. 10 mM EDTA (pH 7.5) and centrifuged at 18,000 X g for 20 min. A schematic representation of the mitochondrial isolation prodedure is shown in Figure 1.

FIGURE 1

PROTOCOL FOR THE ISOLATION OF A PURIFIED MITOCHONDRIAL FRACTION FROM A WHEAT EMBRYO HOMOGENATE



(iii) Cytochrome c Oxidase Assays

The purification of the mitochondrial fraction was monitored by determining the specific activity of cytochrome c oxidase (cytochrome a a_3, EC 1.9.3.1), the terminal carrier in the electron transport chain and a marker enzyme of the inner mitochondrial membrane. Cytochrome c oxidase activity was measured by the method of Cooperstein and Lazarow (1951), in which the rate of oxidation of ferrocytochrome c is monitored by following the decrease in absorbance at 550 nm. Protein was assayed by the method of Lowry et al. (1951). First-order velocity constants and specific activities were determined as described by Wharton and Tzagoloff (1967). Specific activity was expressed as nmoles ferrocytochrome c oxidized per minute per mg protein.

(iv) Isolation of Mitochondrial and Cytosol RNA

a. Isolation of Total Cytosol RNA

Fifteen ml cytosol supernatant (S_3) were transferred to a glass centrifuge tube containing 100 mg NaCl, 1.5 ml bentonite (6.5 mg/ml) and 1.7 ml 20% SLS. Ten ml 90% phenol (aqueous) were added and the mixture was shaken for

10 min at room temperature on a horizontal shaker. The organic and aqueous phases were then separated by centrifugation at 1000 X g (2000 X rpm, IEC International Refrigerated Centrifuge, Model PR-6, rotor 269) for 10 min at 5°. The upper aqueous phase was removed with a Pasteur pipette and re-extracted for 10 min with an additional 8 ml 90% phenol. The phases were again separated as described above and the aqueous phase removed, combined with 2 vol. 95% ethanol, and stored at -20°.

b. Isolation of Total Mitochondrial RNA

The purified mitochondrial pellet (Mp) was suspended in 15 ml extraction buffer (0.05 M Tris. HCl (pH 7.5), 10 mM $MgCl_2$). Sodium chloride (100 mg), bentonite (1.5 ml of a 6.5 mg/ml suspension) and 20% SLS (1.7 ml) were added to the suspension and the RNA was extracted with phenol as described above for the cytosol RNA. The ethanol-precipitated mitochondrial RNA was stored at -20°.

c. Isolation of Mitochondrial and Cytosol NaCl-soluble and NaCl-insoluble RNA

In order to selectively precipitate ribosomal-type RNA, total nucleic acids (cytosol or mitochondrial) were dissolved in water (at a concentration of 1 - 4 mg/ml) and made .3 M with respect to NaCl by addition of solid salt. After 8 hr at 4°, the NaCl-insoluble RNA (iRNA) was recovered by centrifugation at 13,000 X g (20 min, 0°). The NaCl-soluble RNA (sRNA) was precipitated from the supernatant by addition of 2 vol. of 95% ethanol. The iRNA was subjected to two additional precipitations from 3 M NaCl. The bulk of the residual NaCl was removed by dissolving the final pellet in 0.1 M NaCl and reprecipitating the RNA with ethanol. All RNA was stored at -20° as an ethanol precipitate.

(v) Polyacrylamide Gel Electrophoresis

Electrophoresis of RNA samples was carried out in 2.4%, 2.8%, or 5% polyacrylamide gels by a modification of the method described by Loening (1967). The 2.4% and 2.8% gels were prepared from an aqueous stock solution containing 15%

acrylamide, recrystallized from acetone, and 0.75% bis-acrylamide, recrystallized from 95% ethanol (Acrylamide I). For 2.4% gels, Acrylamide I (3.6 ml) was added to a side-arm Erlenmeyer flask (125 ml) along with 11.2 ml distilled water and 7.5 ml of the 3E buffer [0.12 M Tris - 0.06 M sodium acetate - 0.003 M EDTA (pH 7.2)] of Bishop et al. (1967). The mixture was degassed under vacuum for approximately 30 seconds. With constant agitation, 50 µl TEMED (N,N,N',N'-tetramethylethylenediamine) were added, followed by 0.375 ml 10% ammonium persulphate. The mixture was then pipetted into 6 Plexiglass tubes (0.7 cm diameter, 7 cm length), each having dialysis tubing wrapped around the bottom to retain the gels during polymerization. Absorbent tissue was used to remove any solution clinging to the sides above the gel meniscus and the gels were left to polymerize for 30 min. If the gels were not used immediately, distilled water was layered on top and the tube covered with Parafilm. In this manner the gels could be stored for several days without altering their resolution properties. The 2.8% gels were prepared in the sequence described above but from a mixture containing different proportions of ingredients (4.2 ml Acrylamide I, 7.5 ml 3E buffer, 10.4

ml water, 50 µl TEMED, and 0.375 ml 10% ammonium persulphate). The mixture used to prepare 5% gels contained 7.5 ml Acrylamide II solution [15% recrystallized acrylamide, 0.375% recrystallized bisacrylamide], 7.5 ml 3E buffer, 7.35 ml distilled water, 50 µl TEMED, and 100 µl 10% ammonium persulphate.

Prior to sample application, gels were run in a Canalco electrophoresis apparatus (Canalco Industries Corp., Rockville, Maryland) for 1 hr at 5 mA/gel. The electrophoresis buffer was E buffer containing 0.2% SLS. Samples of RNA (10-20 µg) in < 50 µl E buffer containing 10-20% sucrose were layered on the gels with a micropipette and electrophoresis was carried out for either 2.5 - 3 hr (2.8% and 5% gels) or 3.5 - 4 hr (2.4% gels). The gels were then removed from the Plexiglass tubing and scanned in a Joyce Loebl UV scanner attached to a Sargent Model SRLG recorder.

The relative proportions of the individual DNA and RNA species resolved on polyacrylamide gels were determined by cutting out and weighing the areas under the peaks on the scanning paper.

C. RESULTS AND DISCUSSION

(i) Cytochrome C Oxidase Activities of Isolated Mitochondria

Cytochrome c oxidase activities in the various subcellular fractions produced during the isolation of mitochondria from dormant viable wheat embryos are given in TABLE III. These results are comparable to those obtained with non-viable, commercial wheat germ (M.W. Gray, in preparation). An analysis of this data in relation to that obtained with 24 hr-germinated wheat embryos is given in SECTION II. 2.C.(ii).B.

(ii) Characterization of Mitochondrial and Cytosol RNAs by Polyacrylamide Gel Electrophoresis

When cytosol total, NaCl-insoluble, and NaCl-soluble RNA fractions from dormant (unimbibed), viable wheat embryos were resolved by polyacrylamide gel electrophoresis, the distribution of UV-absorbing material (cf. FIGURE 2A, 3A, 20*) was qualitatively similar to that of the corresponding

*Since the polyacrylamide gel electrophoresis profiles of cytosol and mitochondrial total, NaCl-insoluble, and NaCl-soluble RNAs were qualitatively identical whether isolated from unimbibed (viable) or imbibed wheat embryos, only the profiles for the latter system are shown (FIG. 2, 3, 20).

LEGEND OF FIGURE 2

Polyacrylamide gel (2.8 %) electrophoresis of total nucleic acid fractions isolated from the cytosol [(A), ca. 0.3 A_{260} units] and mitochondria [(B), ca. 0.5 A_{260} units] of 24 hr - germinated wheat embryos. Profiles were obtained by scanning the gels with ultraviolet light as described in SECTION II.2.B.(v). Arrows in (B) indicate minor UV-absorbing components which were reproducibly observed in the gel electrophoretic profiles of wheat mitochondrial total nucleic acids. Panel (C) shows the profile obtained after mixing cytosol (ca. 0.2 A_{260} units) and mitochondrial (ca. 0.4 A_{260} units) nucleic acids.

FIGURE 2

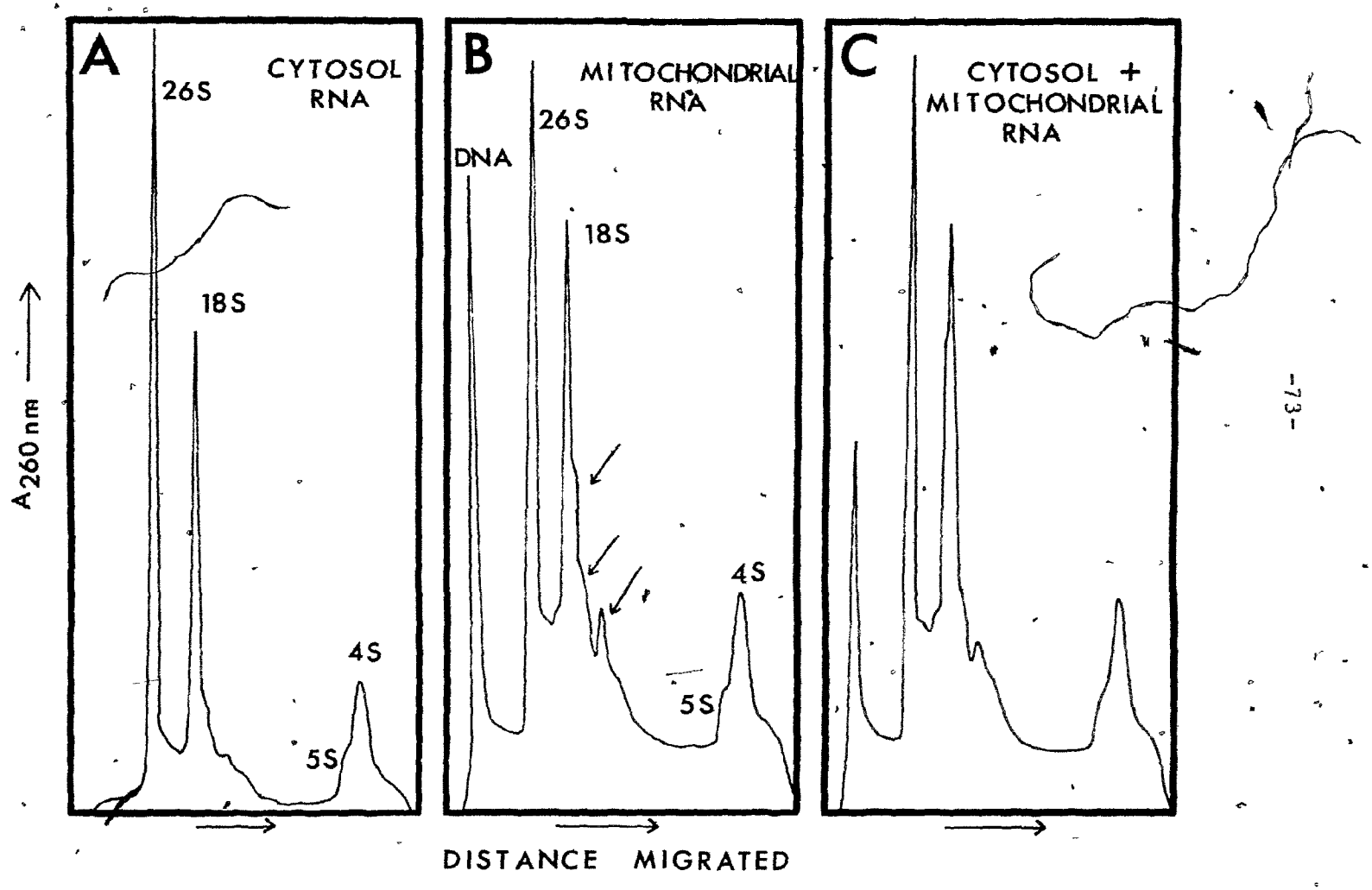
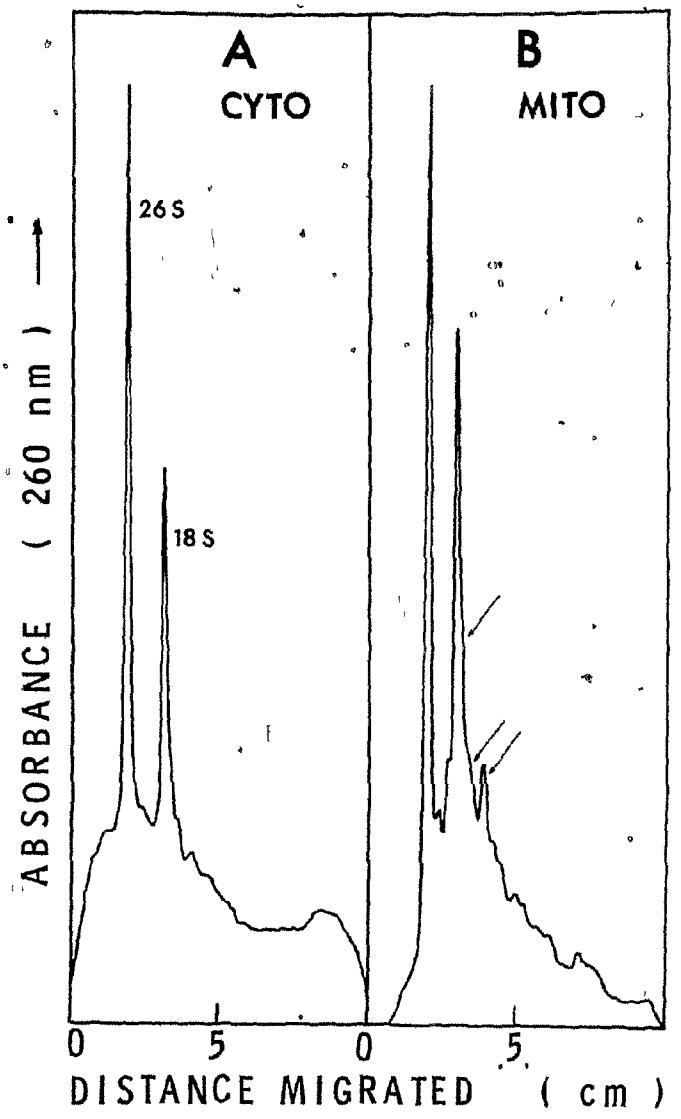

A
CYTOSOL RNA
26S
18S
4S
5S
B
MITOCHONDRIAL RNA
DNA
26S
18S
4S
5S
C
CYTOSOL + MITOCHONDRIAL RNA
$A_{260\,nm}$
DISTANCE MIGRATED

FIGURE 3



Polyacrylamide gel (2.8%) electrophoresis of NaCl- insoluble RNA (iRNA) fractions from the cytosol [(A) ca. 0.2 A_{260} units] and mitochondria [(B) ca. 0.4 A_{260} units] of 24 hr - germinated wheat embryos. Profiles were obtained by scanning the gels with ultraviolet light as described in SECTION II.2.B.(v). Arrows in (B) indicate minor UV-absorbing components which were reproducibly observed in the gel profiles of wheat mitochondrial iRNA.

RNA fractions prepared from commercial wheat germ (Gray, manuscript in preparation), with the two major species in the rRNA fraction representing the 26 S and 18 S RNA components of cytoribosomes. The polyacrylamide gel electrophoretic profiles of mitochondrial total, NaCl-insoluble, and NaCl-soluble RNA from dormant viable embryos (cf. FIGURES 2B, 3B)* were also essentially identical to those observed for the same RNA fractions from commercial wheat germ (Gray, manuscript in preparation). In addition to the peaks migrating in the positions of the 4 S and 5 S RNAs, the mitochondrial total RNA gel profiles contained two major components, one of which co-migrated with the cytosol 26 S rRNA species and a second which migrated slightly but distinctly more slowly than the cytosol 18 S rRNA (cf. FIGURE 2C*). [Due to the similar electrophoretic mobilities on polyacrylamide gels of the respective large- and small-subunit RNAs of higher plant cyto- and mitoribosomes (Leaver and Harmey, 1973; Pring and Thornbury, 1975), the wheat mitochondrial large and small rRNA species are here designated 26 S and 18 S, respectively. It should be emphasized that these assignments are made for the sake of convenience and are not

meant to imply that actual measurements of sedimentation coefficients have been carried out].

In addition to the two major mt-RNA species, presumed to be the high-molecular-weight components of wheat mitoribosomes, the gel profiles reproducibly contained several minor components having distinctive electrophoretic mobilities (cf. FIGURES 2B and 3B, arrows*). The larger of these components migrated as a shoulder on the leading edge of the mitochondrial 18 S RNA, in a position identical to that occupied by the cytosol 18 S rRNA (as evidenced by the mixing experiment, FIGURE 2C*). The possibility that this component represents residual cytosol rRNA contamination will be discussed in the following chapter (SECTION II.3.C.(iv).b.). DNA was also present in the wheat mitochondrial total RNA fraction (cf. FIGURE 2B*) and was removed (as expected, into the soluble phase; cf. FIGURE 2D*) during precipitation of the iRNA with 3 M NaCl (cf. FIGURE 3B*). The relative proportion of DNA in the total mitochondrial nucleic acid fraction often varied considerably and much of it was undoubtedly of nuclear origin. The reason for this conclusion is that in other plant mitochondria examined, DNA represents less than 5% of the total mito-

chondrial nucleic acids (e.g., Leaver and Harmey, 1973), whereas in the wheat system, it can amount to as much as 35% (cf: TABLE VIII). As mentioned above, the mitochondrial RNA contained both 4 S and 5 S components, consistent with the results obtained with the commercial wheat germ.

The yields of mt-RNA and mitochondrial protein from dormant viable embryos as well as the RNA/protein ratios are given in TABLE IX and will be discussed in the following section along with the results obtained for the germinating wheat embryos. In addition, an analysis of the mass ratios of the cytosol and mitochondrial high-molecular-weight RNAs (TABLE VIII) and an assessment of the degree of contamination (in dormant tissue) of the mitochondrial RNA fraction with cytosol RNA (TABLE VII) is also reserved for the following section.

The results presented here demonstrate that the mitochondrial nucleic acids isolated from the dormant, viable wheat embryos are essentially identical to those obtained from the non-viable, commercial wheat germ. Therefore any damage that may possibly occur during the isolation and storage of the commercial germ does not seem to affect the

types and proportions of RNA components subsequently isolated from purified mitochondria. These results also suggest that at least some of the components of an organellar protein-synthesizing system are present in at least a certain proportion of the mitochondria of dormant wheat embryos.

3. ISOTOPIC LABELING OF MITOCHONDRIAL AND CYTOSOL RNA IN GERMINATING WHEAT EMBRYOS

A. MATERIALS

[^{32}P]orthophosphate (carrier-free), [methyl-^{3}H]methionine, [^{3}H]uridine and Aquasol were all obtained from New England Nuclear (Boston, Mass.). Actinomycin D and ethidium bromide were purchased from Calbiochem (Los Angeles, Calif.). All other chemicals and solvents were reagent grade.

B. METHODS

(i) Germination and Labeling

Viable wheat embryos were prepared as described in SECTION II.2.B.(i). The labeling protocol was adapted from that of Lau *et al*. (1974). Embryos (2 - 8 g) were uniformly distributed among sterile Petri dishes (8.5 cm diameter; 0.5 g embryos/dish) on a layer of Whatman No. 1 filter paper. A sterile solution of 1% glucose (4 ml) containing 0.1 - 1.6 mCi/ml [^{32}P]orthophosphate was added to each dish, after which the embryos were placed in the dark at room temperature for 24 hr.

Three additional experiments were performed using different germination and labeling protocols. In the first experiment, wheat embryos were germinated in the presence of both [^{32}P]orthophosphate and [methyl-^{3}H]methionine in order to label methyl groups in the RNA chains as well as the phosphate backbone. The second experiment examined the effect of antibiotics on both mitochondrial and cytosol RNA transcription, and involved germinating the wheat embryos in the presence of ethidium bromide or Actinomycin D. The third experiment was an attempt to ascertain the presence or absence of a mitochondrial ribosomal RNA precursor by pulse-labeling germinating embryos in a medium containing [^{3}H]uridine. All incubations were done at room temperature in the dark.

In the double-labeling experiment, 2 g viable wheat embryos were distributed among 8 Petri dishes on a layer of Whatman No. 1 filter paper. Five ml 1% glucose solution containing 0.3 mCi [^{32}P]orthophosphate, 0.2 mCi [methyl-^{3}H]-methionine (5 Ci/mmole), 1 μmole adenosine and 1 μmole guanosine were added to each dish and the embryos were incubated for 24 hr. In Part A of the second experiment, 1 g of viable wheat embryos was added to each of 6 Petri

dishes (on filter paper) followed by 2.5 ml of a 1% glucose solution containing in two cases 65 µg Actinomycin D, in two cases 125 µg Actinomycin D, and in the final two cases no antibiotic (control). The embryos were incubated for 4 hr followed by the addition of the same solutions given above but this time also containing 0.8 mCi [^{32}P]orthophosphate in each case. Incubation was continued for a further 13 hr. In Part B of the second experiment, the effect of ethidium bromide on RNA transcription was determined. Three g viable wheat embryos were distributed among 12 Petri dishes and 3 ml of a 1% glucose solution were added to each dish. After preliminary incubation for 8 hr, ethidium bromide (15 µg in 3 ml 1% glucose) was added to each of 6 dishes and 1% glucose (3 ml) to the remaining 6 dishes. Following a further 2 hr incubation, 2 ml of a 1% glucose solution containing both ethidium bromide (10 µg) and [^{32}P]orthophosphate (0.10 mCi) were added to those samples already containing ethidium bromide. The 6 control dishes received 2 ml of 1% glucose containing only the [^{32}P]orthophosphate. The embryos were incubated for a final 6 hr. In the third experiment total cellular, cytosol, and mitochondrial RNAs were pulse-labeled in the

presence of [^{3}H]uridine. Viable wheat embryos (1 g) were distributed on filter paper in one Petri dish along with 4 ml of a 1% glucose solution. In a second dish 50 mg (ca. 100) wheat embryos were added followed by 2.5 ml glucose solution. Both samples were incubated for 6 hr. After incubation the 1 g embryo sample was transferred to a 15 ml test tube and 1.5 ml 1% glucose containing 1.5 mCi [^{3}H]uridine (40 Ci/mmole) was added. The embryos were distributed uniformly along the length of the tube such that they were all partially immersed in the solution. The embryos were then incubated for 60 min with the tube held in a horizontal position. The 50 mg sample was transferred to a 5 ml test tube. A 1% glucose solution (0.075 ml) was added along with 80 µCi [^{3}H]uridine (80 µl) and the embryos were distributed evenly on the tube bottom. The embryos were incubated a further 60 min.

(ii) Purity of the Mitochondria

a. Electron Microscopy

The final purified mitochondrial pellet [Mp] was fixed in 2% paraformaldehyde - 2.5% glutaraldehyde - 2% sucrose -

0.2 M sodium cacodylate (pH 7.4) for 1 hr at 4°. The pellet was then washed, post-osmicated for 1 hr at 4° (1% osmium tetroxide - 2% sucrose - 0.2 M sodium cacodylate), washed and stained with uranyl acetate (aqueous) for 8 hr at 4°. The material was then dehydrated through a graded series of alcohols, solvent-exchanged in propylene oxide, and embedded in Durcupran resin. The fixed, stained mitochondria were sectioned (600 Å in thickness) and scanned at a magnification of 22,000 X in a Zeiss EM 10 electron microscope.

b. Cytochrome c Oxidase Assays

The cytochrome c oxidase assays of the subcellular fractions from imbibed viable wheat embryos were determined as described in SECTION II.2.B.(iii).

(iii) Isolation of [^{32}P]-Labeled Mitochondrial and Cytosol iRNA

Mitochondria were purified as described in SECTION II. 2.B.(ii) except that the first centrifugation (1000 X g, 6 min) was omitted and the second centrifugation (2000 X g) was increased to 12 min. The isolation of labeled mitochon-

drial and cytosol RNA (total, NaCl-insoluble, and NaCl-soluble) was carried out as described previously for unlabeled RNA. Ultraviolet absorbance was measured in a Zeiss PM QII spectrophotometer and the radioactivity was determined by mixing RNA samples (< 200 µl) with 3 ml Aquasol and counting in a Nuclear-Chicago "Unilux" liquid scintillation spectrometer.

Total cellular pulse-labeled RNA was isolated directly from germinated wheat embryos. The 50 mg wheat embryo sample was ground in a small mortar with approximately 10 ml homogenizing medium (50 mM NaCl - 0.5% naphthalene 1,5-disulphonate - 10 mM Tris·HCl, pH 7.6). The mixture was then transferred to a centrifuge tube and diluted to 20 ml with homogenizing medium. Sodium tri-isopropylnaphthalene sulphonate (to 1%) and sodium 4-aminosalicylate (to 6%) were added and the mixture was shaken for 5 min at room temperature. An equal volume (20 ml) of phenol mixture (90 ml water-saturated phenol - 10 ml m-cresol - 0.1 g 8-hydroxyquinoline) was added and the suspension was shaken a further 15 min. After centrifugation of the mixture at 2000 X g (10 min), the lower phenol phase was removed with a Pasteur pipette and discarded. The aqueous phase

and interphase were made 0.5 M in NaCl and the mixture re-extracted for 15 min with an equal volume of the phenol mixture. The aqueous phase was removed (the phenol phase and interphase were discarded) and re-extracted 2 X with an equal volume of the phenol mixture. The total nucleic acids were then precipitated from the final aqueous phase by adding 2 volumes 95% ethanol. The precipitated RNA was stored at -20°.

(iv) Fractionation and Characterization of the Cytosol and Mitochondrial RNA

a. Separation of the Individual 26 S and 18 S rRNA Species by Sucrose Density Gradient Centrifugation

Due to technical difficulties, the large and small ribosomal RNA species could not be isolated in sufficient yield from polyacrylamide gels as had been done previously when preparing bacterial, blue green algal, and chloroplast 16 S ribosomal RNA for nucleotide sequence analyses (Doolittle and Pace, 1971). In addition, the RNA isolated from polyacrylamide gels was contaminated with material which caused streaking during subsequent electrophoresis

of T_1 RNase digests on DEAE-cellulose, and this material (acrylamide byproducts) could not be removed. In order to overcome this problem, cytosol and mitochondrial NaCl-insoluble RNAs were fractionated by sedimentation in sucrose density gradients (5 - 25%). Samples of RNA (200 - 250 µg) in 60 µl E buffer (electrophoresis buffer) containing 0.5% SLS were layered onto 12 ml gradients, which were then centrifuged for 18 hr at 24,000 rpm and 5° in the Spinco SW41 rotor. Alternatively, 0.5 - 1.5 mg samples of RNA in 0.5 ml E buffer were applied to 31 ml gradients for centrifugation in the Spinco SW 25.1 rotor (24,000 rpm, 18 hr, 5°). When the 26 S rRNA species was to be used for nucleotide composition analyses, cytosol iRNA was heated at 60° for 5 min prior to centrifugation. This procedure liberates the 5.8 S rRNA from its noncovalent association with the 26 S rRNA (Azad and Lane, 1973).

Fractions were collected by puncturing the bottoms of the gradient tubes with a 20 gauge hypodermic needle and collecting the drops (approx. 250 µl/fraction, 12 ml gradients; approx. 450 µl/fraction, 31 ml gradients). All operations were done at 4°. An aliquot (200 µl) of each fraction was diluted to 1 ml with water and the UV absorbance measured. Radioactivity was determined as

described in SECTION II.3.B.(iii). The fractions containing the separated species of 26 S and 18 S rRNA were pooled as indicated in FIGURES 5A and 5B, made 0.2 M in NaCl, and combined with 2 vol. 95% ethanol. The RNA precipitate was stored at -20°.

b. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of RNA samples and subsequent scanning at 260 nm was carried out as described in SECTION II.2.B.(iv). In order to determine the distribution of radioactivity in the gels, they were first frozen on dry ice and sliced (0.8 - 1 mm, Mickle Gel Slicer, Gomshall, England). The gel slices were then hydrolyzed overnight in a capped scintillation vial at 90° with 0.3 ml 30% hydrogen peroxide. The clear solution in each vial was mixed with 3 ml Aquasol and the radioactivity measured in either a Nuclear-Chicago "Unilux" (single label experiments) or a Philips four channel (double label experiments) liquid scintillation counter.

c. Nucleotide Composition Analysis

The nucleotide composition was determined for both purified mitochondrial and cytosol 26 S and 18 S [^{32}P]-labeled RNA species. Hydrolysis of [^{32}P]RNA (2 - 10 A_{260} units, 100 - 500 μg; sp. act. 1 X 10^6 cpm/A_{260} unit, 2 X 10^4 cpm/μg) with purified *Vipera russelli* phosphodiesterase (Lane *et al.*, 1963) and fractionation of the resulting 5'-nucleotides by two-dimensional paper chromatography (Singh and Lane, 1964) were carried out using the conditions described by Hudson *et al.* (1965) for unlabeled RNA. Hydrolysates were supplemented with markers of N^6-methyladenosine 5'-phosphate (pm^6A) and $O^{2'}$-methylpseudouridine 5'-phosphate (pΨm) before being applied to paper chromatograms. After chromatography, the major 5'-nucleotides (pA, pC, pG, pU) were located under ultraviolet light and were excised, along with corresponding areas of a blank chromatogram. Specific activities of the individual 5'-nucleotides were determined as described in TABLE IX. For detection of modified nucleotides, the remainder of each chromatogram was submitted to autoradiography (Fuji medical X-ray film; approximately 1 week exposure). Minor

nucleotides were readily identified by their position on the autoradiograms. The mole % of the modified and the major 5'-nucleotides is given in TABLE X .

(v) Assessment of Bacterial and Fungal Contamination

Three approaches were used to assess the possibility of bacterial and/or fungal contamination of purified mitochondrial fractions obtained from 24 hr-germinated wheat embryos. In the first, [^{3}H]uridine-labeled *Anacystis nidulans* (a blue-green alga) and [^{32}P]orthophosphate-labeled *Bacillus subtilis* (a bacterium) were mixed with unlabeled, imbibed embryos. Purified mitochondria were prepared and the radioactivity in the various subcellular fractions was measured. In the second approach, the imbibed embryos were vortexed in saline and samples of the supernatant were examined by light microscopy. Separate samples were plated on blood agar (for detection of bacteria) and sabarose agar (for fungi). Plates were incubated for several days at room temperature in the dark. Purified bacterial isolates (3 species) were each cultured in 30 ml beef heart infusion broth containing 1 mCi [^{32}P]orthophosphate for 24 hr at 37°. Labeled bacteria (> 1 X 10^{8} cpm)

were then mixed with unlabeled, imbibed wheat embryos and the mitochondrial fraction was prepared in the usual manner. Mitochondrial RNA was isolated and resolved on polyacrylamide gels, and the UV absorbance and radioactivity profiles were recorded. In the third approach, the purified mitochondrial fraction was examined under the electron microscope (as described in SECTION II.3.B.(ii)) for the presence of contaminating organisms.

C. RESULTS

(1) Germination and Labeling

As previously demonstrated by Lau *et al.* (1974), highly-labeled RNA can be obtained from laboratory-prepared, viable wheat embryos imbibed for 24 hr in the presence of [^{32}P]orthophosphate. By subjecting homogenates of such labeled embryos to the fractionation procedure described earlier, it was possible in the present study to isolate [^{32}P]-labeled mitochondrial as well as cytosol RNA. Although a systematic study of the [^{32}P]-labeling of mitochondrial and cytosol RNA in the initial stages of germination was not carried out, it was noted that there was little labeling of these RNA fractions at 8 hr, in contrast to 24 hr. As will be demonstrated later, the RNA in both subcellular fractions had the same qualitative and quantitative distribution of radioactivity among the 5'-nucleotide constituents after 24 hr of labeling.

In preliminary experiments, it appeared that there was a proportional increase in the specific activity of wheat embryo RNA over a [^{32}P]orthophosphate/embryo ratio of 3 - 12.5 mCi/g (and this range would probably extend

above 12.5 mCi/g). However, for two reasons, increases in specific activity were not simply obtained by adding larger amounts of [^{32}P]orthophosphate to smaller amounts of embryos. In the first place, compared to bacterial systems, incorporation of label into the RNA of germinating wheat embryos is relatively inefficient, and there was a limit to the quantity of [^{32}P]orthophosphate which could be safely used in each experiment (much more than 100 mCi constituted a health hazard). In the second place, a minimum quantity of embryos was required for efficient recovery of RNA, since high percentage losses were incurred when processing small quantities (< 4 g) of embryos. Taking these factors into consideration, the largest-scale preparations employed 8 g of embryos imbibed with 100 - 120 mCi [^{32}P]orthophosphate.

Some increase (up to 50% at 48 hr) in the specific activity of the RNA could be obtained by extending the time of embryo imbibition beyond 24 hr. However, for unknown reasons, but possibly associated with cellular changes during development, the degree of cytosol contamination also increased with time, as did the risk of bacterial and fungal contamination. Thus, the shorter labeling time of 24 hr was preferred.

(ii) Purity of the Mitochondrial Preparations

a. Electron Microscopy

Electron micrographs of purified mitochondria from 24 hr-imbibed wheat embryos (FIGURE 4) demonstrated mostly intact organelles, although some ruptured mitochondria were also present. Little non-mitochondrial material was evident and there was no apparent contamination with cytosol ribosomes (either attached to microsomes or to the outer mitochondrial membrane). No contaminating bacteria or fungal spores or hyphae were observed.

The mitochondria were of two types, as previously observed by Pring (1974) for maize. One type ("condensed") was smaller with a strong contrast between the matrix and cristae, while the other type was swollen and more homogenous in appearance ("homogenous"). Pring observed that the "condensed" mitochondria were present in highly-vacuolated coleoptile cells, a tissue which surrounds the primary leaf and which is metabolically less active than the plumule cells. The latter contain a larger proportion of "homogenous" mitochondria. No attempt was made here to examine separately the mitochondria present in the

FIGURE 4

Electron micrograph of sucrose gradient-purified mitochondria (M_p, FIGURE 1) from viable wheat embryos imbibed for 24 hr (X 53,200). The bar represents 0.5 μ.

various structurally-distinct regions of the developing embryo.

b. Cytochrome C Oxidase Activities

The cytochrome c oxidase activities measured in the various subcellular fractions from both unimbibed and imbibed wheat embryos are given in TABLE III. The specific activity of the initial homogenate of the 24 hr-germinated embryos was approximately double that for the dormant embryos. The reason for this difference is not known but may possibly be due to the preferential liberation of mitochondria during homogenization of the imbibed embryos, the activation of the enzyme during early germination, or even its de novo synthesis. TABLE III also shows that more than 40% of the cytochrome c oxidase activity remained in the post-mitochondrial supernatant (S_3) after sedimentation of intact organelles when unimbibed viable wheat embryos were used for the preparation of a mitochondrial fraction. An even higher percentage of the enzyme remained in the S_3 fraction when non-viable, commercial wheat germ was used (M.W. Gray, in unpublished results). In contrast, less than 10% of the cytochrome c oxidase activity remained in

TABLE III

CYTOCHROME OXIDASE ACTIVITY IN VARIOUS SUBCELLULAR FRACTIONS DURING ISOLATION OF MITOCHONDRIA FROM UNIMBIBED AND IMBIBED WHEAT EMBRYOS

Fraction	Unimbibed		Imbibed	
	Specific Activity*	Ratio	Specific Activity*	Ratio
S_1	25.8	(1.00)	49.3	(1.00)
S_2	29.6	(1.15)	38.0	(0.77)
S_3	11.2	(0.43)	3.6	(0.07)
P_2	32.7	(1.27)	71.6	(1.45)
P_3	41.7	(1.62)	143	(2.90)
M_p	152	(5.89)	535	(10.9)

*nmoles ferrocytochrome c oxidized/min/mg protein

The isolation of the various fractions from imbibed and unimbibed wheat embryos is described in the text and in FIGURE 1 . The specific activities were determined as described in the methods section of SECTION II.2.

the post-mitochondrial supernatant during fractionation of homogenates of imbibed, viable embryos, and the degree of enzyme purification in the final mitochondrial preparation from imbibed wheat embryos was approximately twice that achieved using unimbibed tissue. These differences could possibly result from development of the structurally-immature mitochondria present in the dormant tissue during the initial 24 hr after imbibition. Solomos et al. (1972) have shown that membranes are scarce in mitochondria present in dormant pea cotyledons but that these membranes develop further following germination. In pea, this results in a stabilization of the mitochondrial membrane and its lower susceptability to preparative damage, as demonstrated by Sato and Asahi (1975).

(iii) Purification of the Cytosol and Mitochondrial RNA

The yields of mitochondrial RNA + DNA and mitochondrial protein obtained from both unimbibed and imbibed viable wheat embryos are given in TABLE IV. The variability in yield was often considerable and the lack of sufficient data for a valid statistical analysis made it difficult, if not impossible, to draw meaningful conclusions when

TABLE IV

YIELD OF MITOCHONDRIAL PROTEIN AND MITOCHONDRIAL NUCLEIC ACIDS FROM VIABLE (DORMANT AND GERMINATED) WHEAT EMBRYOS

	UNIMBIBED*	IMBIBED*
Mitochondrial RNA (+DNA)/Wheat Embryos (μg/g)	80 ± 48 (n=5; 35-150)	146 ± 35 (n=11; 100-200)
Mitochondrial Protein/Wheat Embryos (mg/g)	3.2 (2.3, 4.1)	2.1 (1.9, 2.3)
Mitochondrial RNA (+DNA)/Mitochondrial Protein	37	55

* Mean ± S.D. The values in parentheses represent the range for n determinations.

comparing preparations from unimbibed and imbibed embryos. However, it does appear that the yields of mitochondrial RNA + DNA and protein from viable embryos are substantially higher than in the case of commercial wheat germ (12 µg and 0.6 mg/g wheat germ, respectively; M.W. Gray, unpublished data). This may possibly reflect the presence in viable embryos of more intact organelles which are less subject to rupture and loss during the isolation procedure, or a more efficient liberation of mitochondria during homogenization of viable embryos. The results of Leaver and Harmey (1973) with mung bean hypocotyls and turnip root (0.5 - 0.7 µg mitochondrial RNA and 35 - 45 µg mitochondrial protein/g tissue) and Pring (1974) with maize mesocotyl and coleoptile tissues (0.2 µg mitochondrial RNA and 70 µg mitochondrial protein/g tissue) indicate an even lower yield of mitochondrial nucleic acids and protein from these plant sources. This is probably attributable to the high water content of the latter tissues, in contrast to the relatively dry wheat embryos. The ratio of mitochondrial (RNA + DNA)/mitochondrial protein is also lower in the commercial wheat germ (20 µg/mg, M.W. Gray, unpublished data) than in the viable embryos, which may be due to larger amounts of non-mitochondrial

protein co-purifying with the organelles in the case of commercial germ. As pointed out in TABLE IV, however, high mitochondrial (RNA + DNA)/protein ratios may simply indicate the presence of a relatively large amount of contaminating DNA. Even lower RNA/protein ratios have been measured for other plant mitochondria (e.g., 3 μg/mg, maize mitochondria (Pring, 1974); 10 - 20 μg/mg, turnip root mitochondria (Leaver and Harmey, 1973). In these cases the differences may reflect actual mitochondrial composition differences, although variability in the efficiency of extraction of the RNA cannot be ruled out.

The purification of the individual 18 S and 26 S cytosol and mitochondrial RNA species required for nucleotide composition and/or sequence analysis involved several steps, each of which resulted in the loss of some material. This was of special concern in the purification of the mitochondrial species, in view of the relatively low amount of starting material (total mt-RNA). TABLE V follows the RNA purification in an experiment where highly-labeled [^{32}P]RNA was isolated for nucleotide sequence analysis. It is apparent from the TABLE that, in spite of the very large quantity of [^{32}P]orthophosphate (120 mCi) used in

the initial labeling of the germinating embryos, the specific activity of the cytosol and mitochondrial RNA was relatively low (ca. 4 X 10^6 cpm/A_{260} unit). This was a constant problem in attempting to obtain sufficient radioactive RNA for detailed sequence analysis. Little difficulty was encountered in purifying enough of the individual cytosol RNA species because of the large amount of cytosol iRNA available (4 X 10^9 cpm). However, loss of mt-RNA through the purification (especially during the sucrose density gradient step and subsequent removal of salt) was considerable and exceeded 50%. The amount of mitochondrial 18 S RNA recovered after purification (ca. 7 X 10^6 cpm) was at the lower limit of the amount required for sequence analysis.

A large proportion of the radioactivity present in the cytosol total RNA fraction was non-RNA material which remained in the soluble RNA fraction after precipitation of the iRNA with 3 M NaCl. This non-RNA material was removed during further purification of the soluble RNA (TABLE V; see also SECTION IV).

The UV spectral properties of all fractions of both the mitochondrial and cytosol RNA were characteristic of

TABLE V

ISOLATION OF CYTOSOL AND MITOCHONDRIAL 18 S AND 26 S RNA FROM WHEAT EMBRYOS GERMINATED IN THE PRESENCE OF [^{32}P]ORTHOPHOSPHATE

RNA Fraction	CYTOSOL			MITOCHONDRIAL		
	A_{260} Units	Radioactivity (cpm x 10^{-6})	Specific Activity (cpm X $10^{-6}/A_{260}$ unit)	A_{260} Units	Radioactivity (cpm x 10^{-6})	Specific Activity (cpm X $10^{-6}/A_{260}$ unit)
Total*	1330	11 250	8.5	32	128	4.0
Purified iRNA	1270	5 605	4.0	21	88	4.2
Crude sRNA*	236	2 130	9.0	7	9.1	1.3
Purified sRNA	225	772	3.4	-	-	-
Purified 18S	7	28 (16)	4.0 (2.3)	3	12.3 (7.0)	4.1 (2.3)
Purified 26S	16	-	-	5	-	-

* Includes DNA in the case of the mitochondrial fraction

NOTE: The above data were obtained in an experiment in which 8 g of wheat embryos were germinated for 24 hr in the presence of 120 mCi [^{32}P]orthophosphate. Radioactivity measurements and specific activities (with the exception of those values in parentheses) have been adjusted to account for isotope decay in the course of the experiment. Purified iRNA was subjected to multiple precipitation from 3 M NaCl. Crude sRNA refers to the RNA remaining in the supernatant and recovered by ethanol precipitation after the first NaCl fractionation of total RNA. Treatment of crude sRNA with 2-methoxyethanol followed by cetyltrimethylammonium bromide (to remove polysaccharides and polyphosphates) gave purified sRNA. The purified 18 S and 26 S RNAs were prepared by sedimentation of purified iRNA on sucrose density gradients. In the case of the cytosol 18 S and 26 S RNAs, the amounts listed in the above table represent the yield from further fractionation of 0.7% of the total purified cytosol iRNA available.

purified nucleic acid.

(iv) Fractionation and Characterization of the Cytosol and Mitochondrial RNA

a. Sucrose Density Gradient Centrifugation

The individual 26 S and 18 S rRNA species of wheat mitochondrial and cytosol iRNA were satisfactorally separated by sedimentation in sucrose density gradients. The UV absorbance and radioactivity profiles of such gradients are shown in FIGURE 5. The ratios of the large to small rRNAs (determined both from the radioactivity and optical density profiles) as well as the specific activities of the various rRNA species are given in TABLE VI.

When undenatured cytosol iRNA was fractionated in this manner the specific activities of the 26 S and 18 S rRNA species were found to be very similar (TABLE VI). In addition, the 26 S/18 S UV absorbance ratio was similar to that found after gel electrophoresis of cytosol iRNA and close to that expected (ca. 1.8 - 1.9) for equimolar ratios of the two RNA species (assuming that wheat embryo

LEGEND OF FIGURE 5

Resolution of wheat cytosol (A) and mitochondrial (B) NaCl-insoluble [^{32}P]RNA by sedimentation in linear 5 - 25 % sucrose density gradients [(A) ca. 6.5 A_{260} units; 12 ml gradient; Spinco SW 41 rotor; (B) ca. 3.3 A_{260} units; 31 ml gradient; Spinco SW 25 1 rotor]. On completion of centrifugation, the bottom of each gradient tube was punctured and approximately 0.25 ml (A) or 0.50 ml (B) fractions were collected dropwise. For determination of ultraviolet absorbance (- - -o- - -o- - -), 0.2 ml of each fraction was mixed with 0.8 ml water. For determination of radioactivity (——●——●——), 20µl of each fraction was mixed with 3 ml Aquasol and counted in a Nuclear-Chicago "Unilux" liquid scintillation spectrometer. The bars indicate the fractions pooled for isolation of the individual 26 S and 18 S species.

FIGURE 5

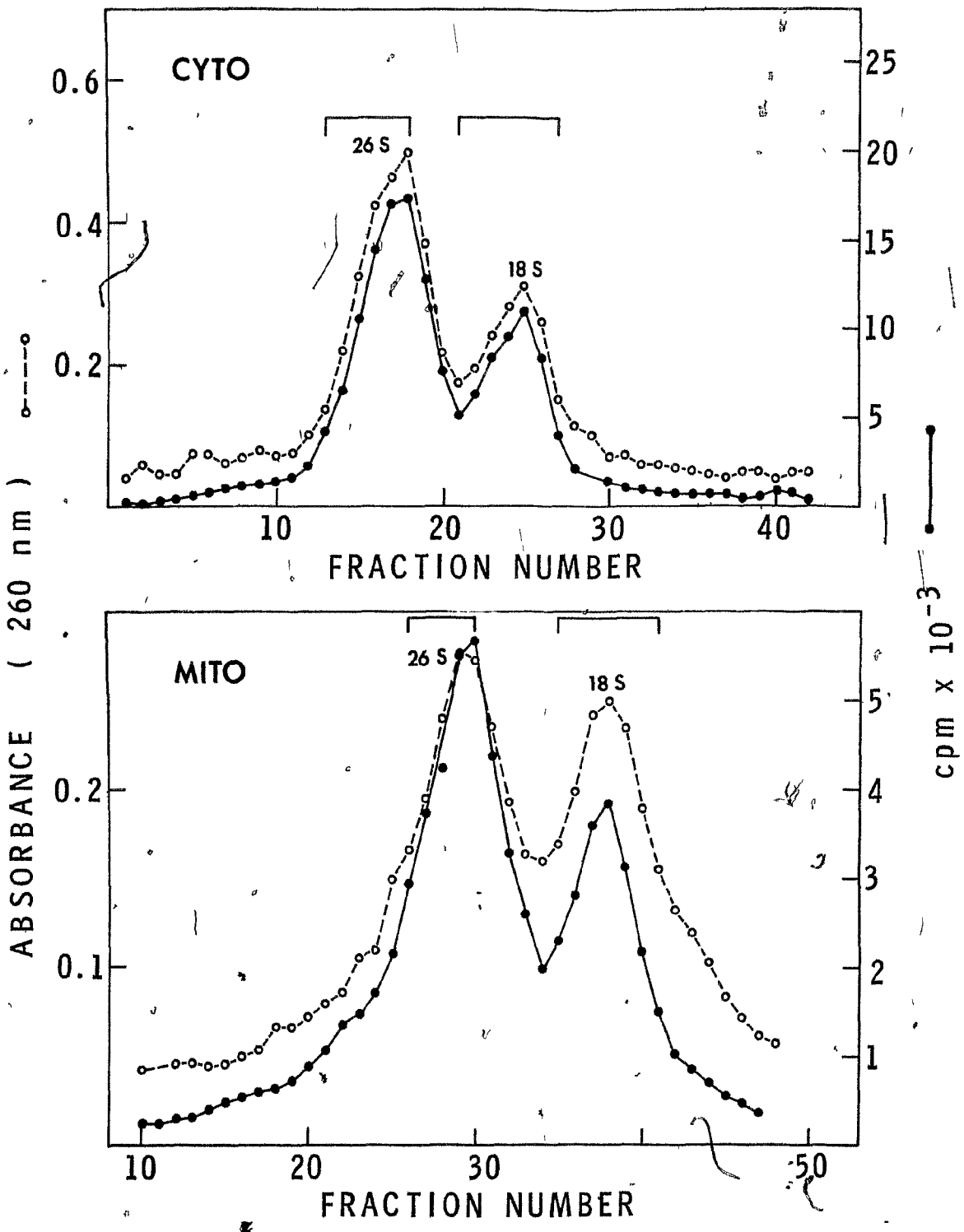

CYTO
26 S
18 S
0.6
0.4
0.2
25
20
15
10
5
10
20
30
40
FRACTION NUMBER
MITO
26 S
18 S
0.2
0.1
5
4
3
2
1
10
20
30
50
FRACTION NUMBER
ABSORBANCE (260 nm)
cpm x 10^-3

TABLE VI

OPTICAL DENSITY, RADIOACTIVITY AND SPECIFIC ACTIVITY RATIOS OF THE MITOCHONDRIAL AND CYTOSOL 26 S AND 18 S rRNA SPECIES SEPARATED BY SEDIMENTATION IN SUCROSE DENSITY GRADIENTS

	26 S/18 S (optical density)	26 S/18 S (counts/min)	26 S (specific activity) cpm X $10^{-6}/A_{260}$ unit	18 S (specific activity) cpm X $10^{-6}/A_{260}$ unit	26 S/18 S (specific activity)
CYTOSOL RNA*	1.85	1.90	3.42	3.32	1.03
MITOCHONDRIAL RNA*	1.15	1.85	1.82	1.09	1.68

*The mitochondrial and cytosol data were taken from separate experiments, each using different amounts of radioactive precursor. This is the reason for the lower specific activities in the case of the mitochondrial RNA, although it should be emphasized that when isolated simultaneously from the same batch of labeled embryos, the mitochondrial and cytosol RNA fractions have the same intrinsic specific activity.

cytosol 26 S and 18 S rRNAs have molecular weights of 1.3 and 0.7 million, respectively, corresponding to chain lengths of ca. 3600 and 2000 nucleotides; see Lau et al., 1974; Gray, 1974b). However, if the cytosol iRNA was heat-denatured prior to gradient fractionation, the 18 S rRNA component had a significantly lower specific activity than the 26 S rRNA component (data not shown). This result is not unexpected if the unheated RNA contained "hidden breaks" due to a selective degradation of preformed RNA, as has been suggested by Lau et al. (1974).

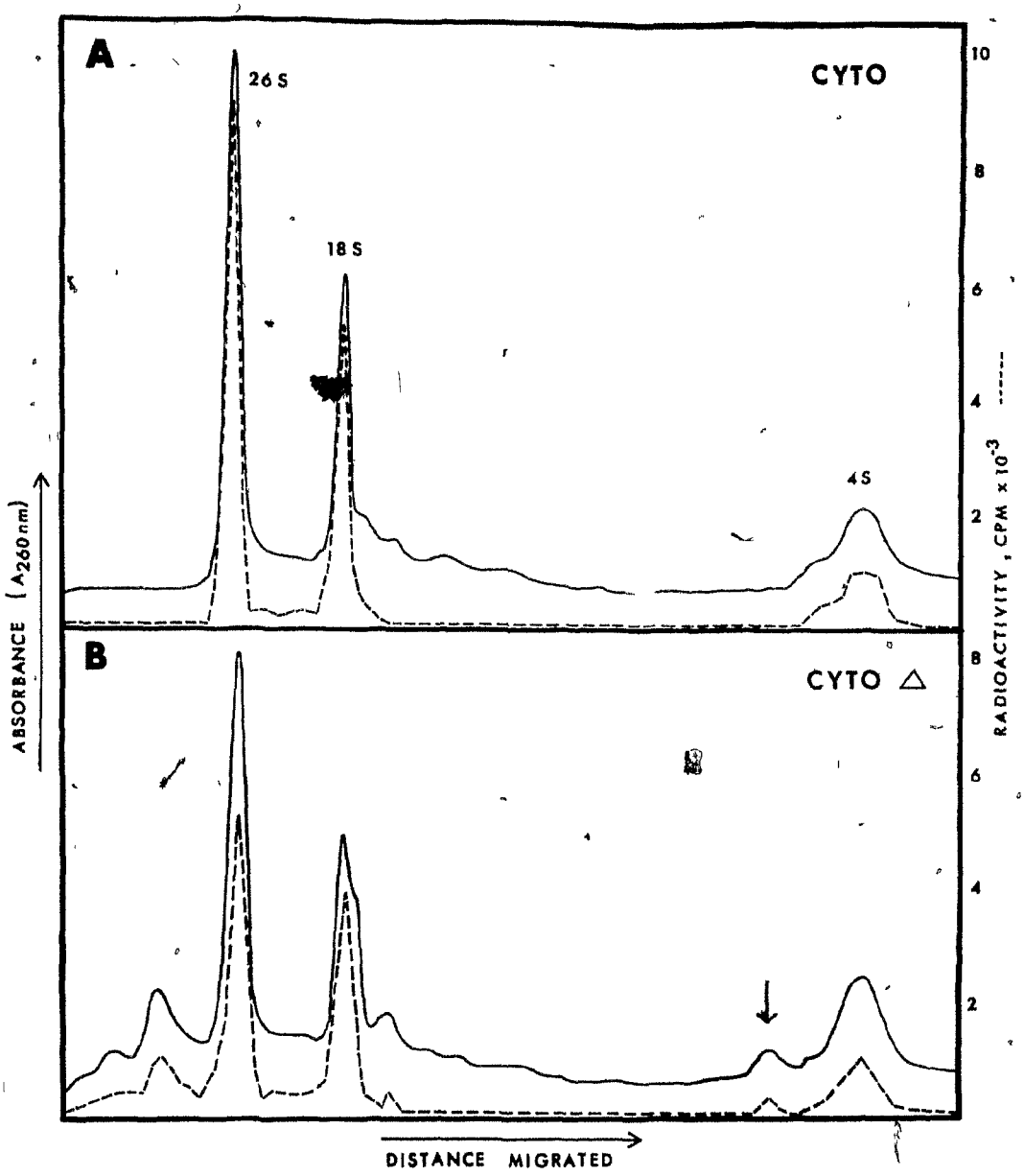
In contrast to the results with cytosol iRNA, there was a substantial difference in the specific activities of the two mitochondrial rRNA species isolated from sucrose gradients, with the specific activity of the 18 S component only about 60% that of the 26 S component. This difference reflects the fact that while the 26 S/18 S radioactivity ratio was comparable to that observed in the case of undenatured cytosol iRNA the mitochondrial 26 S/18 S absorbance ratio was only ca. 1:1. This suggests that "hidden breaks" present in isolated (unlabeled) mitochondrial 26 S rRNA are revealed even in the absence of heat denaturation.

These results indicate that the preformed RNA (both mitochondrial and cytosol) present in the dormant wheat embryo is either partially degraded before or during imbibition or, less likely, is subsequently subject to preferential breakdown during isolation. The newly-synthesized RNA is more stable. Similar results, indicating a preferential metabolic breakdown of "old" rRNA, have been obtained in bacterial systems (Doolittle, 1973). These observations were significant in the context of nucleotide composition and sequence analyses of the individual RNA species, since there was actually much less contamination of the (labeled) 18 S rRNA fractions with breakdown products of the (labeled) 26 S RNA species than would be indicated by the optical density profiles.

b. Polyacrylamide Gel Electrophoresis

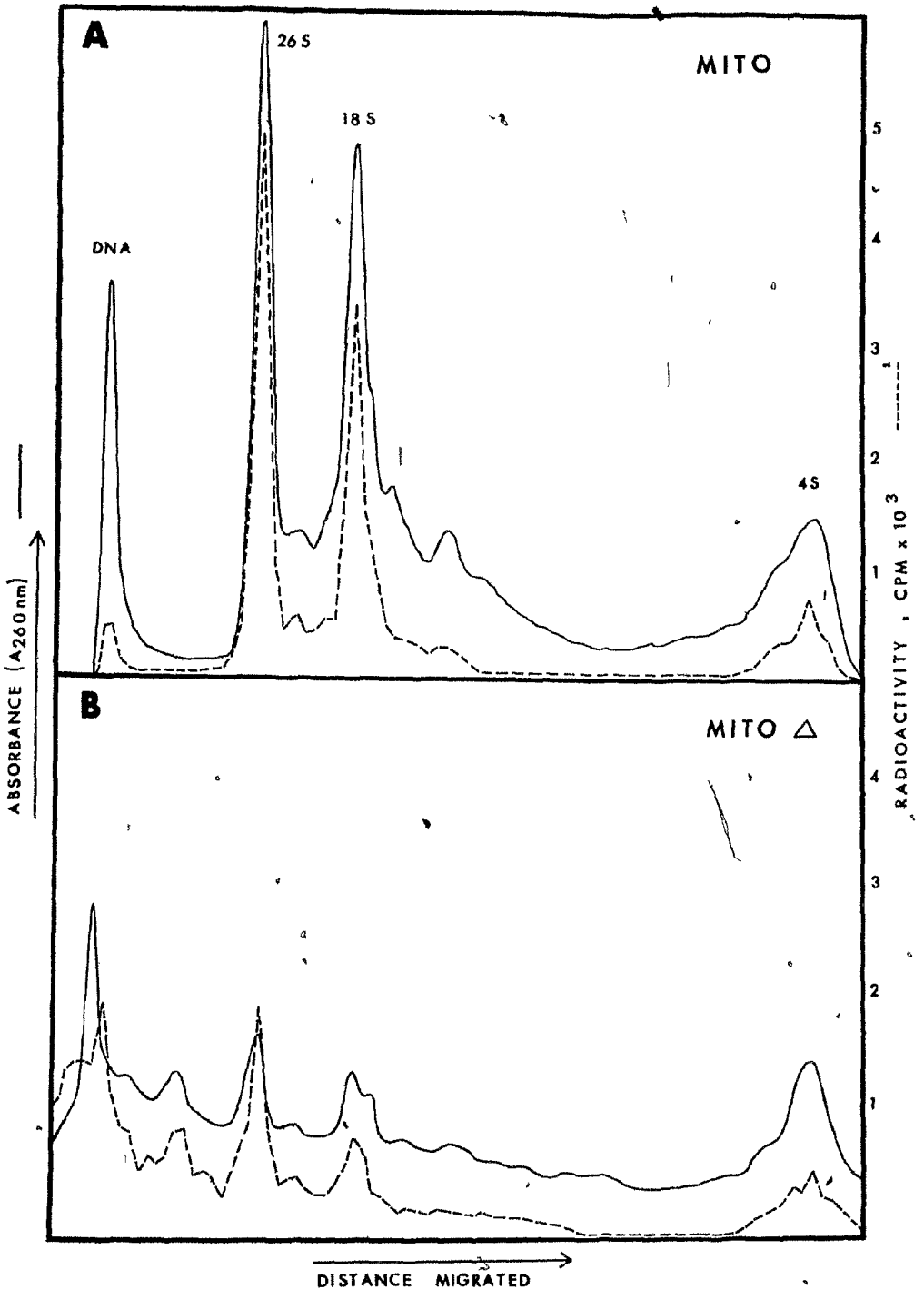
When cytosol and mitochondrial total nucleic acids from 24 hr-germinated wheat embryos were fractionated by polyacrylamide gel electrophoresis the distributions of UV-absorbing material were both qualitatively (FIGURES 6 A and 7 A) and quantitatively (TABLE VII) similar to those of the respective fractions obtained from dormant viable

FIGURE 6



Polyacrylamide gel (2.8 %) electrophoresis of unheated (A) and heated (60°, 5 min) (B) wheat embryo cytosol total [^{32}P]nucleic acids. Ultraviolet absorbance (solid line) profiles were determined as described in SECTION II.2.B.(v). Radioactivity (dashed line) profiles were determined by digesting gel slices (1 mm) overnight in 0.3 ml 30 % hydrogen peroxide at 90°, adding 3 ml Aquasol to the clear solution in each vial, and measuring the radioactivity in a Nuclear-Chicago "Unilux" liquid scintillation spectrometer.

FIGURE 7



Polyacrylamide gel (2.8%) electrophoresis of unheated (A) and heated (B) wheat embryo mitochondrial total [^{32}P] nucleic acids. Ultraviolet absorbance (solid line) profiles were determined as described in SECTION II.2.B.(v). Radioactivity (dashed line) profiles were determined as described in the legend of FIGURE 6.

TABLE VII

RELATIVE PROPORTIONS OF 26 S AND 18 S RNA AND DNA IN THE TOTAL NUCLEIC ACIDS ISOLATED FROM THE MITOCHONDRIA AND CYTOSOL OF UNIMBIBED AND IMBIBED WHEAT EMBRYOS

Mass Ratio	UNIMBIBED		IMBIBED	
	Cytosol	Mitochondrial	Cytosol	Mitochondrial
26 S/(18 S+18 S')	- -	1.1, 1.2	- -	1.07 ± 0.08 (n=13; 1.00-1.27)
26 S/18 S	1.8 ± 0.1 (n=3; 1.7-1.9)	1.58	1.9 ± 0.2 (n=5; 1.6-2.2)	1.3, 1.5
18 S/18 S'	- -	3.3	- -	4.2 ± 0.7 (n=5; 3.4-5.3)
(26 S+18 S+18 S')/DNA	- -	1.5	- -	9.1 ± 7.0 (n=13; 2.2-27.2)

In the above table, the designation " 18 S' " represents the prominent shoulder (migrating in the position of the cytosol 18 S RNA) on the leading edge of the mitochondrial 18 S RNA (cf. FIGURE 2). Total RNA from wheat embryo cytosol and mitochondrial fractions was resolved by electrophoresis in 2.4% polyacrylamide gels, and expanded UV absorbance profiles were obtained by scanning the gels in the Joyce Loebl UV scanner while setting the Sargent Model SRLG recorder at high speed (Gray, 1974b). The area under each peak in the tracing was determined by cutting out and weighing the peak; the values obtained were assumed to be proportional to the amount of each component in the RNA sample applied to each gel. In the case of the incompletely-resolved peaks (such as 18 S + 18 S'), an arbitary division was made by drawing a vertical line from the lowest point in the profile between the two peaks to the baseline. Where more than two determinations were made, the values listed above represent the mean; the values in parentheses indicate the number of determinations (n) and the range of values.

embryos. However, the 26 S/18 S mass ratio in the cytosol RNA (1.7 - 2.2) was appreciably higher than that previously determined for wheat germ bulk cellular NaCl-insoluble RNA (Gray, 1974b). That this higher ratio was not due to imbibition itself was confirmed by the results of an experiment in which parallel analyses of unimbibed and imbibed wheat embryos (from the same batch) gave identical 26 S/18 S ratios for the bulk cellular RNA. It remains to be determined whether the difference relates to subcellular fractionation (i.e., a qualitative distinction between cytosol and bulk cellular NaCl-insoluble RNA) or to differences in the preparation of the RNA (e.g., different ionic conditions at the phenol extraction stage). The 26 S/18 S mass ratio in the mitochondrial RNA hovered around 1.5 (TABLE VIII), which is close to that expected if the 26 S and 18 S peaks do indeed represent the two high-molecular-weight mitoribosomal RNA components, present in equimolar amounts. However, this leaves the minor components to be accounted for, and if the leading shoulder of the mitochondrial 18 S RNA (18 S') represents cytosol 18 S RNA contamination one would also expect the cytosol 26 S RNA component to be present. This would result in a 26 S/(18 S + 18 S') ratio of ca. 1.6,

TABLE VIII

DEGREE OF CYTOSOL RNA CONTAMINATION OF MITOCHONDRIAL RNA AS ESTIMATED BY THE RELATIVE PROPORTIONS OF 5.8 S RNA IN THE TOTAL CYTOSOL AND MITOCHONDRIAL RNA

5.8 S RNA/total iRNA	UNIMBIBED			IMBIBED		
	Cytosol	Mitochondrial	Mito/Cyto	Cytosol	Mitochondrial	Mito/Cyto
mg paper/A_{260} unit	18.3	4.5	0.25	19.2	4.9	0.26
cpm/A_{260} unit	--	--	--	540	126	0.23

Heat-denatured cytosol and mitochondrial iRNA (ca. 3 A_{260} units) was electrophoresed in 5.0% polyacrylamide gels (cf. FIGURE 8). Gels were scanned as described in TABLE VIII. The peak corresponding to 5.8 S RNA in the UV tracings was cut out, weighed, and related to the total amount of iRNA applied to each gel ("mg paper/A_{260} unit"). When [^{32}P]iRNA samples were fractionated, the gels were frozen after UV scanning and sliced for radioactivity measurements (cf. legend to FIGURE 6). The radioactivity in the peak corresponding to 5.8 S RNA could then be related to the total amount of iRNA fractionated ("cpm/A_{260} unit"). Assuming that any 5.8 S RNA detected in the mitochondrial iRNA was derived from contaminating cytosol iRNA, the proportion of the latter could be calculated by simple ratio ("Mito/Cyto").

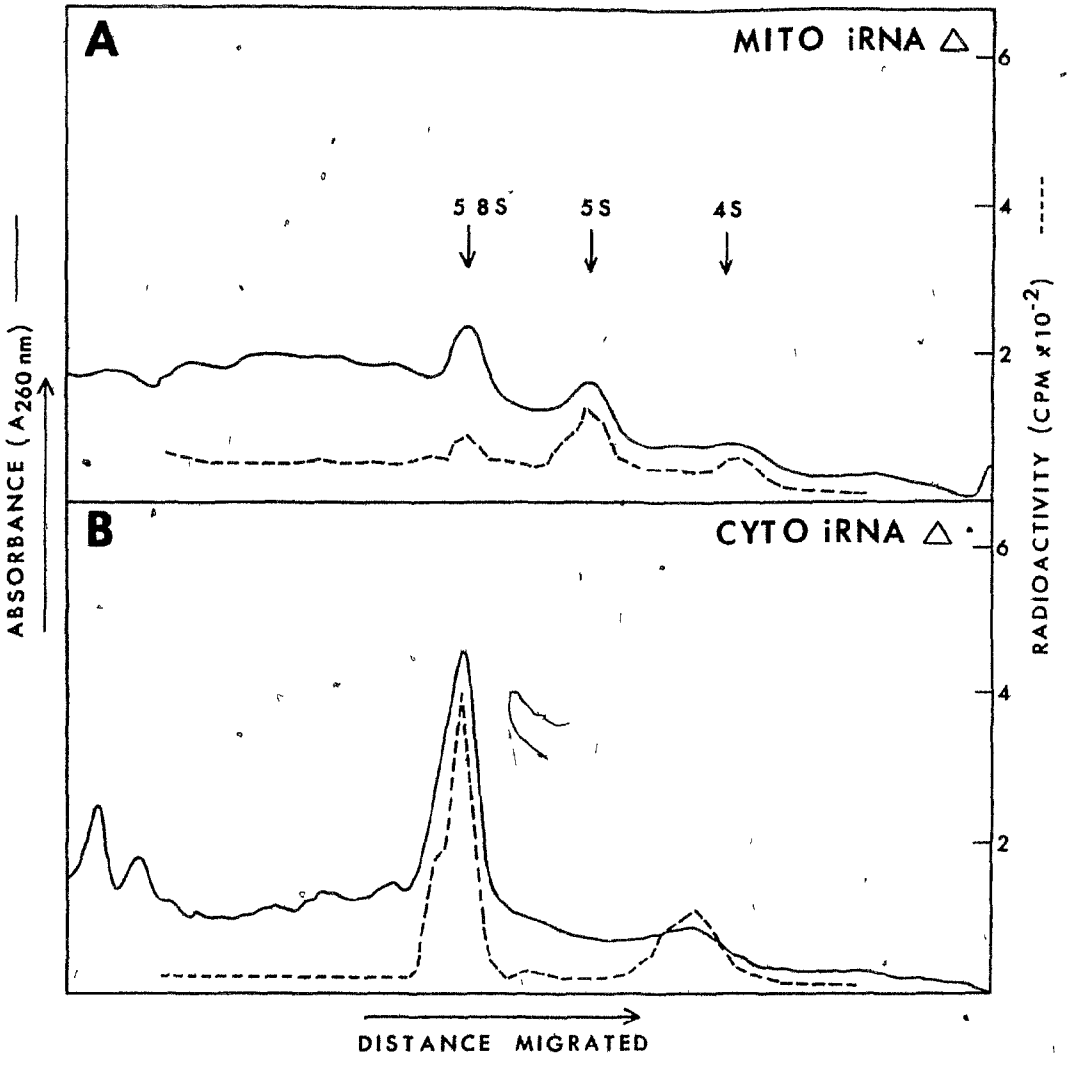
whereas a ratio closer to 1.0 was consistently obtained. Whether this was due to preferential extraction or degradation of particular RNA species remains to be resolved.

The radioactivity profiles paralleled those of UV absorbance in the case of cytosol and mitochondrial total (FIGURES 6 A and 7 A) and NaCl-insoluble RNA (not shown), indicating that after 24 hr incubation in the presence of [^{32}P]orthophosphate, the bulk of the radioactivity was present in the stable RNA species. It appears, however, that the RNA species migrating slightly faster than the cytosol and mitochondrial 18 S RNA components (which include the three minor peaks in the mitochondrial RNA fraction) had a lower specific activity than the two high-molecular-weight RNA species. Little or no non-RNA radioactive material was present in the gels and thus the large amount of such material present in total cytosol RNA (TABLE V) must have been of sufficiently-low molecular weight to cause it to run off the gel in the course of the electrophoresis. The DNA peak present in wheat total mitochondrial nucleic acids was labeled only slightly, in agreement with the late onset of cellular division following imbibition (Chen and Osborne, 1970). It should be noted that the

relative amount of DNA extracted varied considerably, with the (26 S + 18 S)/DNA mass ratio ranging from 2 - 27 ("imbibed embryos", TABLE VI).

Heat-denatured cytosol and mitochondrial total RNA isolated from imbibed embryos (FIGURES 6 B and 7 B) had UV absorbance profiles similar to those for the corresponding RNAs isolated from dormant viable tissue (not shown). Here, as in the case of the non-denatured nucleic acids, the radioactivity profiles paralleled the UV absorbance profiles. It is interesting to note that although the amounts of the 26 S and 18 S species decreased considerably upon heating, this was not due to large-scale degradation of the RNA but rather to aggregation. This was particularly evident in the case of the mitochondrial RNA (FIGURE 7 B). As expected, heat denaturation of cytosol RNA liberated the 26 S - associated (5.8 S) RNA (FIGURE 6 B, arrow), but no comparable species was detected in the heat-denatured mitochondrial RNA (FIGURE 7 B). However, when larger amounts (> 3 A_{260} units) of heated mitochondrial iRNA were electrophoresed on 5% polyacrylamide gels, a small radioactive peak appeared in the position of the cytosol 5.8 S RNA (cf. FIGURE 8 A and 8B), indicating

FIGURE 8



Polyacrylamide gel (5 %) electrophoresis of heated mitochondrial (A) and cytosol (B) iRNA (ca. 3 A_{260} units). Ultraviolet absorbance (solid line) profiles were determined as described in SECTION II.2.B.(v). Radioactivity (dashed line) profiles were determined as described in the legend of FIGURE 6.

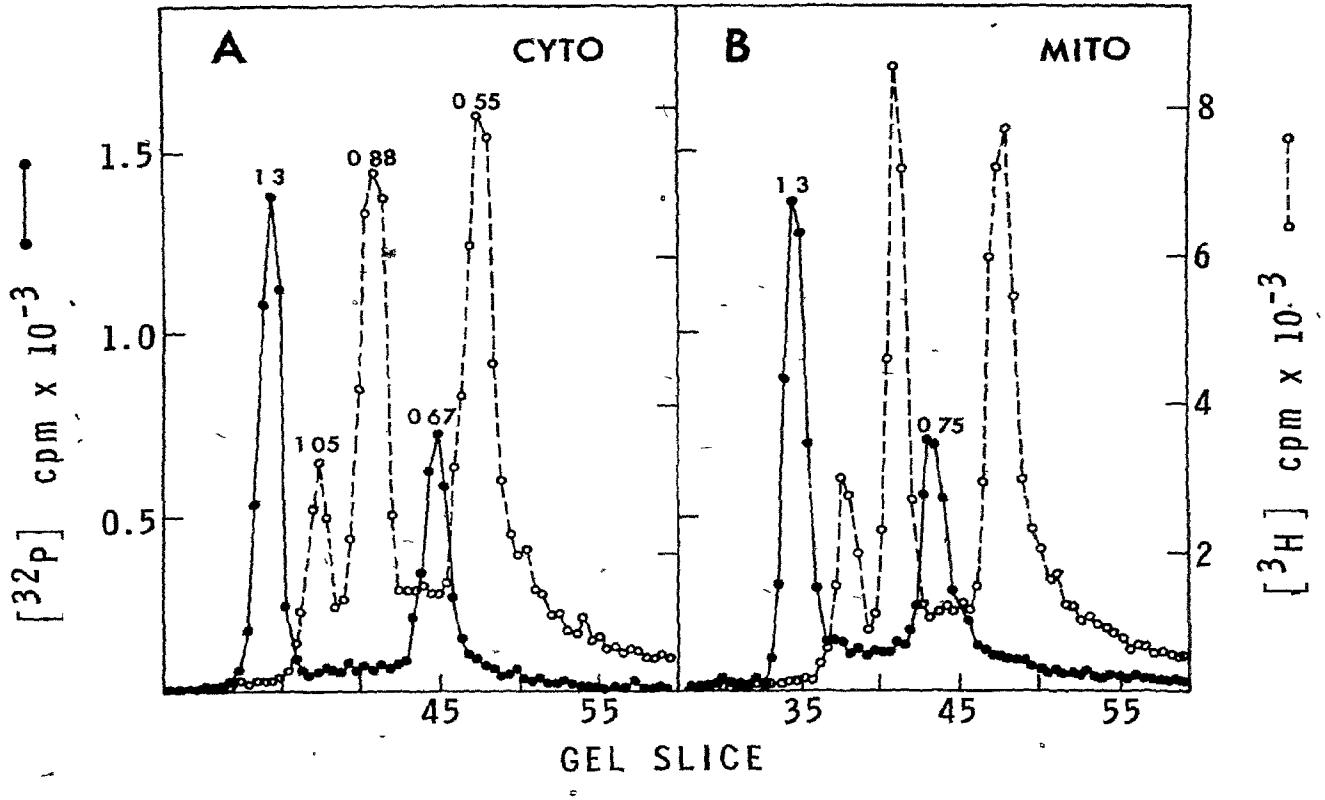
possible low level contamination of the mitochondrial iRNA with cytosol 26 S RNA.

In order to compare more exactly the sizes of the two major cytosol and mitochondrial RNA species, mitochondrial and cytosol NaCl-insoluble [^{32}P]RNA fractions were co-electrophoresed on non-denaturing polyacrylamide gels with Anacystis nidulans [^{3}H]rRNA (FIGURE 9). From the known molecular weights of the Anacystis rRNA species (1.05, 0.88, and 0.55 million daltons, the 0.88 million dalton component being a stable breakdown product of the 1.05 million dalton component; Doolittle, 1973), and assuming a linear relationship between electrophoretic mobility and log (molecular weight)$^{-1}$ (Bishop et al., 1967), the molecular weights were calculated to be 1.3 and 0.67 million daltons (cytosol) and 1.3 and 0.75 million daltons (mitochondrial) (average of two determinations; duplicate values were within 1% of the average). These results, which indicate a significant difference in apparent size between the mitochondrial and cytosol small rRNA species, are in agreement with the results of mixing experiments (FIGURE 2 C). In both cases a partial resolution between the two smaller rRNA species (but not between

LEGEND OF FIGURE 9

Co-electrophoresis of Anacystis nidulans [^{3}H]rRNA (---o---o-) and wheat embryo [^{32}P]rRNA (—●——●—) in 2.4 % polyacrylamide gels. (A), cytosol iRNA; (B) mitochondrial iRNA. Radioactivity profiles were determined as described in the legend of FIGURE 5 except that the radioactivity was measured in a Philips four-channel liquid scintillation spectrometer.

FIGURE 9


A
CYTO
B
MITO
1.3
1.05
0.88
0.67
0.55
1.3
0.75
1.5
1.0
0.5
8
6
4
2
45
55
35
45
55
GEL SLICE
[32P] cpm x 10-3
[3H] cpm x 10-3

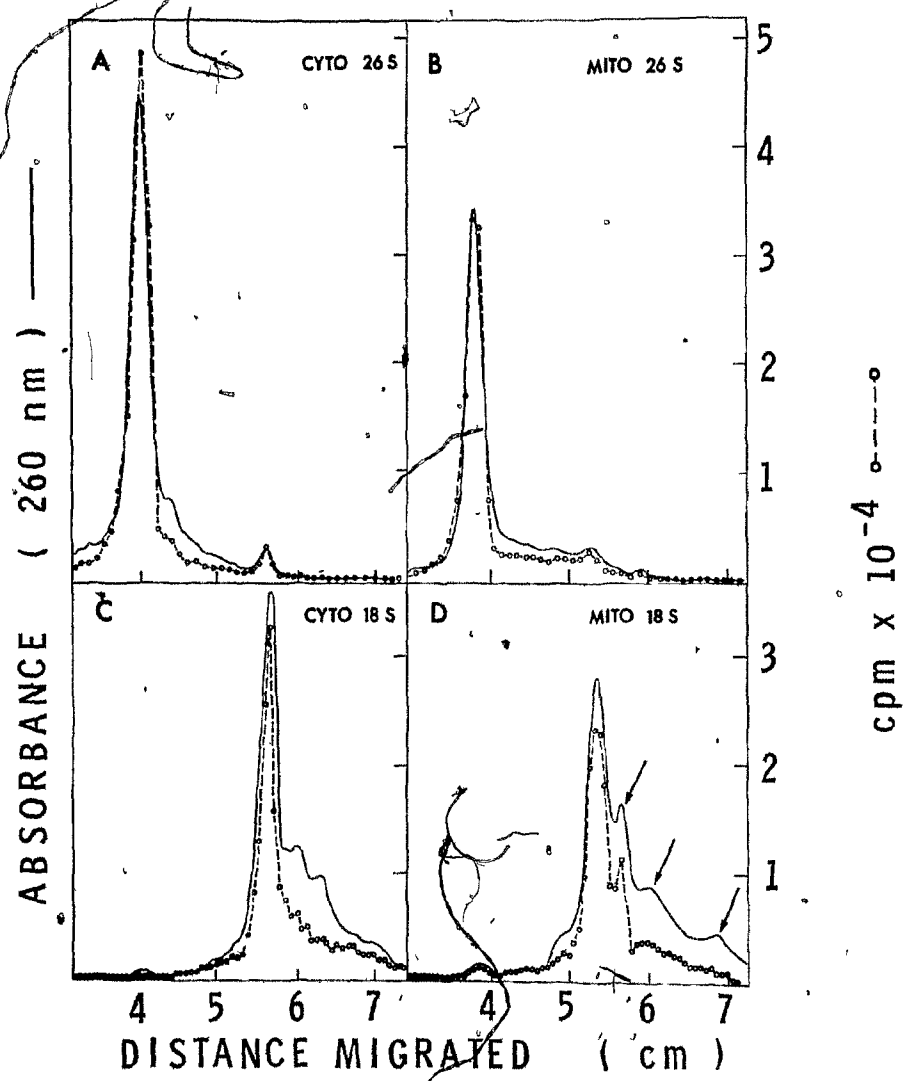
the two larger species) could be obtained. These molecular weights correspond closely to those obtained for the same (unlabeled) species isolated from mitochondrial and cytosol fractions of commercial wheat germ (Gray, 1974a) and indicate that the wheat mitochondrial RNA species are similar in size to the mitochondrial rRNAs of other higher plants (Leaver and Harmey, 1973; Pring, 1974).

When the individual 26 S and 18 S RNA species (separated on sucrose gradients) were electrophoresed on polyacrylamide gels, they were found not to be significantly cross-contaminated (FIGURE 10, A - D). The UV absorbance profiles of the two 26 S species (FIGURE 10, A and B) showed that each was essentially homogeneous. The UV absorbance profile of each of the two 18 S species (FIGURE 10, C and D) contained a number of prominent shoulders (which were less apparent in the radioactivity profiles) on the leading side of the main peak. In the case of the cytosol 18 S RNA (FIGURE 10 C), which in this particular instance had been isolated from heat-denatured iRNA, the shoulders undoubtedly reflect the selective breakdown during imbibition of pre-existing cytosol RNA (Lau *et al.*, 1974), as discussed in the preceeding chapter. They were largely absent when the 18 S RNA was isolated from unheated

LEGEND OF FIGURE 10

Polyacrylamide gel (2.4 %) electrophoresis of purified wheat embryo cytosol 26 S (A) and 18 S (C) rRNA and wheat embryo mitochondrial 26 S (B) and 18 S (D) rRNA. The RNA fractions were isolated from sucrose gradients, as indicated in FIGURE 5. Ultraviolet absorbance (solid line) and radioactivity (dashed line) profiles were determined as described in SECTION II.2.C.(v) and the legend of FIGURE 6. The arrows in the gel profile of mitochondrial 18 S RNA (D) indicate the minor RNA species similarly-designated in FIGURE 2 and 3.

FIGURE 10


A
CYTO 26 S
B
MITO 26 S
C
CYTO 18 S
D
MITO 18 S
ABSORBANCE (260 nm) ——
cpm x 10^-4 o---o
DISTANCE MIGRATED (cm)
4 5 6 7
1 2 3 4 5

iRNA. In the case of the mitochondrial 18 S RNA (FIGURE 10 D), the shoulders must also be due, at least in part, to degradation of pre-existing mitochondrial RNA. However, they also partly represent contaminating cytosol 18 S RNA.

It is apparent from the polyacrylamide gel electrophoresis profiles of mitochondrial total (FIGURE 7 A) and NaCl-insoluble (FIGURE 3 B) RNA that considerable UV-absorbing material migrated in regions other than those occupied by the two major high-molecular-weight RNA species (26 S and 18 S). This is in contrast to the situation observed with the cytosol RNA (FIGURES 2 A and 6 A) but similar to that found with mt-RNA isolated from both commercial wheat germ and dormant viable wheat embryos. The origin of this material is not known with certainty but it most probably represents a combination of contaminating cytosol RNA and unique mitochondrial RNA species (which might be additional mitoribosomal RNA components or, more likely, distinctive breakdown products of the larger mt-RNAs). Notably, a much smaller proportion of radioactivity than UV absorbance was found outside of the 26 S and 18 S peak regions in the gel electrophoresis profiles of mitochondrial RNA (FIGURE 7 A). The degree of cytosol

contamination of the mitochondrial RNA fractions is difficult to ascertain but if one assumes that the 18 S species (TABLE VII) is cytosol 18 S RNA, then a measure of the amount of contamination of the mitochondrial RNA can be calculated (ca. 20 - 25% for imbibed embryos, ca. 30% for unimbibed embryos; TABLE VII). Another method for evaluating contamination involves quantitating the amount of 5.8 S RNA present in the mitochondrial RNA fraction. The estimated contamination by this method is about 25%, based upon both UV absorbance and (in the case of germinating embryos) radioactivity measurements. The data on which such an analysis is based are presented in TABLE VIII. It should be emphasized that the results obtained by the above methods are not precise, due to the difficulty in resolving the two 18 S peaks in the former approach and in quantitating the small amounts of 5.8 S RNA in the latter approach. However, these values are in general agreement with those obtained by the analysis of modified nucleotides (cf. following chapter) and the qualitative examination of the primary T_1 ribonuclease fingerprints of these species (cf. SECTION III).

c. Nucleotide Composition Analysis

Under labeling conditions similar to those used in the present study, Lau et al. (1974) noted significant differences in the specific activities of the constituent 5'-nucleotides (pN) of wheat embryo [^{32}P]rRNA, implying unequal [^{32}P]-labeling of the separate precursor pools of ribonucleoside 5'-triphosphates in 24 hr-imbibed wheat embryos. Comparable results have been obtained in the present investigation for the [^{32}P]-labeled mitochondrial and cytosol 26 S and 18 S rRNA species isolated from 24 hr-imbibed wheat embryos (TABLE IX). The relative specific activities of the constituent 5'-nucleotides of all four isolated rRNA species were very similar, and in the order pA ≃ pU > pG > pC. The absolute specific activity (cpm/μmol) of each pN constituent was essentially the same for both mitochondrial and cytosol 26 S RNA, implying an equivalent extent of [^{32}P]-labeling of the four ribonucleoside triphosphates in the two (nuclear and mitochondrial) subcellular compartments. The specific activities of the pN constituents of cytosol and mitochondrial 18 S RNA were only 70 - 80% as great as the specific activities of the pN constituents of the corresponding 26 S RNAs,

TABLE IX

SPECIFIC ACTIVITIES OF THE CONSTITUENT 5'-NUCLEOTIDES OF WHEAT MITOCHONDRIAL AND CYTOSOL 26 S AND 18 S [^{32}P]rRNA

	Specific Activity (cpm/μmol pN X 10^{-4})*							
pN	Mito 26 S		Mito 18 S		Cyto 26 S		Cyto 18 S	
pA	5.91	(1.00)	4.28	(1.00)	5.99	(1.00)	4.85	(1.00)
pG	4.42	(0.75)	3.06	(0.71)	4.65	(0.78)	3.45	(0.71)
pC	3.97	(0.67)	2.78	(0.65)	4.15	(0.69)	3.13	(0.65)
pU	5.94	(1.01)	4.69	(1.10)	6.13	(1.02)	4.69	(0.97)

*Values in brackets are the relative specific activities of the pN constituents of the individual RNA species (the specific activity of pA was assigned a value of 1.00 in each case)

Phosphodiesterase hydrolysates of the individual [^{32}P]RNA species (isolated from embryos imbibed for 24 hr in the presence of [^{32}P]orthophosphate) were resolved by two-dimensional paper chromatography, as described in the text. The major 5'-nucleotides (pA, pG, pC, and pU) were eluted in 0.1 M HCl and their quantitative proportions were determined spectrophotometrically. For determination of radioactivity by liquid scintillation counting, aliquots (0.2 ml) of each eluate were mixed with 9 ml Aquasol (New England Nuclear) and 0.8 ml 0.1 M HCl (Lau et al., 1974). Specific activities were determined approximately three weeks after labeling of the RNA.

consistent with the postulate of a selective breakdown of preexisting (unlabeled) RNA during embryo imbibition. The pN constituents of mitochondrial 18 S RNA had slightly lower specific activities than the corresponding pN constituents of cytosol 18 S RNA, a difference which could be explained by a slightly greater extent of degradation of pre-existing mitochondrial RNA.

Because of these specific activity differences, suitable corrections had to be applied to the radioactivity measurements used to calculate the molar proportions of the nucleotide constituents of the various RNA species. When this was done, it can be seen (TABLE X) that there was little difference in the molar proportions of the major nucleotides or in the overall G + C content of the two 26 S species and the two 18 S species. However, a marked deficiency was apparent in the content of modified nucleoside constituents (pseudouridine and $\underline{O}^{2'}$-methylnucleosides) when the mitochondrial rRNA species were compared with their cytosol counterparts. The levels of modified nucleosides reported here for wheat mitochondrial 26 S and 18 S rRNA should be regarded as maximal levels, since some contamination with cytosol 26 S and 18 S rRNA cannot be excluded. While it is difficult at present to precisely quantitate such

TABLE X

NUCLEOTIDE COMPOSITION OF MITOCHONDRIAL AND CYTOSOL

26 S AND 18 S [^{32}P]rRNA

	mole %			
pN	Cyto 26 S	Mito 26 S	Cyto 18 S	Mito 18 S
pA	23.2	25.2	23.2	25.2
pG	31.4	30.6	29.9	31.6
pC	24.7	23.4	23.0	23.3
pU	17.0	19.3	20.0	18.9
pΨ	1.4	0.56	1.9	0.41
pAm*	0.72	0.25	0.68	0.19
pGm	0.52	0.22	0.39	0.12
pCm	0.52	0.26	0.28	0.13
pUm	0.49	0.17	0.57	0.14
pΨm	<0.01	<0.01	0.036	<0.01
pm^6_2A**	----	----	0.052	0.056
G + C***	55.2	53.6	54.5	57.1

*Includes any pm^6A which may be present

**Tentative identification based on chromatographic mobility

***Including modified 5'-nucleotides

TABLE X (continued)

Phosphodiesterase hydrolysates of the individual [^{32}P]RNA species were prepared and resolved as described in the text and in TABLE IX. Major and modified 5'-nucleotides were located on chromatograms as indicated in the text. In order to calculate molar proportions, radioactivity measurements (carried out as described in TABLE IX) were corrected for the differences in specific activity of the constituent 5'-nucleotides (TABLE IX). Modified 5'-nucleotides were assumed to have the same specific activity as their unmodified parents (e.g., pΨ, pΨm, and pUm the same as pU). Radioactivity recovered in the 5'-nucleotides listed in the above table accounted for at least 98.0% of the total radioactivity recovered from two-dimensional chromatograms.

It should be noted that in the two-dimensional paper chromatographic system used for the above analysis, pCm co-migrated with pΨ, while pAm largely overlapped pm^{6}A. In order to separate the individual components, the remaining portion of each pΨ + pCm and pm^{6}A + pAm spot was recovered by charcoal desalting (Gray and Lane, 1967). The mixture of pΨ + pCm was supplemented with a pN marker and electrophoresed on Whatman No. 1 paper in 0.025 M triethylammonium formate (pH 2.5). This resolved the marker 5'-nucleotides into three well-separated bands, containing, in order of increasing net negative charge, pA + pC (+ pCm), pG, and pU

TABLE X (continued)

(+ pΨ). Assuming equivalent recovery of pCm and pΨ at the charcoal desalting step, the relative proportion of counts in the pA + pC and pU bands was taken as a measure of the relative proportion of counts in pCm and pΨ in the unresolved mixture eluted from the two-dimensional chrmatograms. As expected, negligible radioactivity was associated with the pG band. The recovered pm^6A + pAm spot was supplemented with a pAm marker and the two compounds were resolved by paper chromatography in a borate-containing solvent (Plesner, 1955). The resulting chromatogram was submitted to autoradiography (3 weeks exposure) in order to assess qualitatively the relative distribution of ^{32}P between the two compounds. In each case, most of the radioactivity migrated as pAm.

contamination (see previous chapter), independent analyses of T_1 oligonucleotide sequences (SECTION III) and alkali-stable dinucleotide sequences [Gray, unpublished results] have suggested that contamination of mitochondrial 26 S [^{32}P]rRNA with its [^{32}P]-labeled cytosol counterpart can be as high as 15 - 20%. Although similar analyses of modified sequences in mitochondrial 18 S [^{32}P]rRNA have yet to be carried out, the gel electrophoresis profile of the particular sample of mitochondrial 18 S [^{32}P]rRNA analyzed here (FIGURE 10) suggests a comparable level of contamination of this RNA species with cytosol 18 S [^{32}P]rRNA. Thus, the actual levels of pseudouridine and $\underline{O}^{2'}$-methylnucleosides in wheat mitochondrial rRNA are likely to be even lower than those presented in TABLE X.

Although no systematic search for low levels of base-modified 5'-nucleotides other than pseudouridine 5'-phosphate was carried out, it is noteworthy that a compound having the chromatographic properties of $\underline{N}^6$, $\underline{N}^6$-dimethyl-adenosine 5'-phosphate (pm^6_2A) was detected in phosphodiesterase hydrolysates of both cytosol and mitochondrial 18 S rRNA, but not in hydrolysates of the two 26 S rRNA species. $\underline{O}^{2'}$-Methylpseudouridine (Ψm) was detected only in cytosol 18 S RNA. The presence of Ψm in cytosol 18 S

but not 26 S RNA implies that the dinucleotide sequence Ψm-Ap (Gray, 1974b) is confined to the 18 S species, a conclusion which has been confirmed by more extensive analyses to be reported elsewhere (Gray, and Cunningham, 1977).

(v) Assessment of Bacterial and Fungal Contamination

In the germinating wheat embryo system, incorporation of radioactive precursors into RNA and protein occurs relatively slowly, a reflection of the relatively slow rate of growth and division of the cells involved. This contrasts sharply with the rapid incorporation of these precursors into the RNA and protein of rapidly-dividing bacterial and fungal cells. For this reason, it was important to establish whether contaminating fungi or bacteria could have contributed significantly to the [^{32}P]RNA isolated from purified wheat embryo mitochondria, since it was not clear *a priori* to what extent such organisms would fractionate with mitochondria during differential and buoyant density centrifugation.

Since the wheat embryos themselves could not be rendered completely sterile prior to germination (although they were germinated under sterile conditions), the

possibility that bacterial and/or fungal contamination might affect the results of the RNA analyses had to be considered. This possibility was eliminated by a number of experiments and by a consideration of the properties of the mitochondrial RNA itself. In the first experiment, in which the blue-green alga A. nidulans (grown in the presence of [^{3}H]uridine) and the bacterium B. subtilis (grown in medium containing [^{32}P]orthophosphate) were mixed with 24 hr-imbibed embryos and a mitochondrial fraction prepared in the usual manner, it was found that very few of the microorganisms ($<$ 0.02%) co-purified with the mitochondria. Over 99% of these organisms sedimented during the initial low-speed centrifugation of the homogenate (and so were largely confined to P_1), while most of the remaining organisms contaminating the crude mitochondrial fraction (P_3) migrated to the pellet during the sucrose density gradient centrifugation step.

In the second experiment, contaminating organisms were actually isolated from imbibed embryos. Although microscopic examination and sample plating revealed no fungal contamination, three distinct bacterial species (one gram-positive, two gram-negative) were found. When

these bacteria were labeled with [^{32}P]orthophosphate, mixed with imbibed wheat embryos, and a mitochondrial fraction prepared, the results were similar to those for the experiment using A. nidulans and B. subtilis. Radioactive monitoring of the fractions demonstrated that most of the bacteria sedimented during the initial centrifugation and that only a small percentage co-purified with the mitochondria. The purified total mitochondrial RNA fraction contained less than 0.05% of the initial quantity of radioactivity added. Polyacrylamide gel electrophoresis of this RNA fraction gave the expected UV absorbance profile for mitochondrial RNA. However, no radioactivity was present in the gel. This indicates that even the minor contamination of the mitochondrial RNA fraction with radioactivity was not due to the presence of bacterial RNA. In any event, high-molecular-weight bacterial or fungal rRNA would not be expected to be isolated by the procedure used for the preparation of mitochondrial RNA, since breakage of the cell walls of these organisms is a pre-requisite for efficient extraction of rRNA. Finally, as discussed earlier, neither fungal nor bacterial contamination of purified mitochondria was detected by electron microscopy.

These experiments exclude the possibility of contaminating bacterial or fungal [^{32}P]RNA co-purifying with the mitochondrial RNA and influencing the subsequent analyses. In addition, the relatively large size of the wheat embryo mitochondrial rRNAs (26 S and 18 S) and the exact coincidence of the radioactivity and UV absorbance profiles (FIGURE 7) further discount the chance that labeled wheat mitochondrial RNA is in reality contaminating bacterial RNA. As shown in FIGURE 9, bacterial rRNAs are considerably smaller in size than the wheat embryo mitochondrial RNAs.

(vi) Simultaneous Labeling of Mitochondrial and Cytosol RNA with [^{32}P]Orthophosphate and [Methyl-^{3}H]Methionine

The use of [^{32}P]orthophosphate as a general label for RNA, and of [methyl-^{3}H]methionine as a specific label for methylated nucleoside constituents, has greatly facilitated the chemical characterization of the wheat cytosol rRNA components (Lau *et al.*, 1974). The availability of isotopically-labeled wheat mitochondrial RNA would similarly facilitate the chemical characterization of these distinctive RNA species. In the case of [^{32}P]-labeling, this has already been demonstrated in the chapter

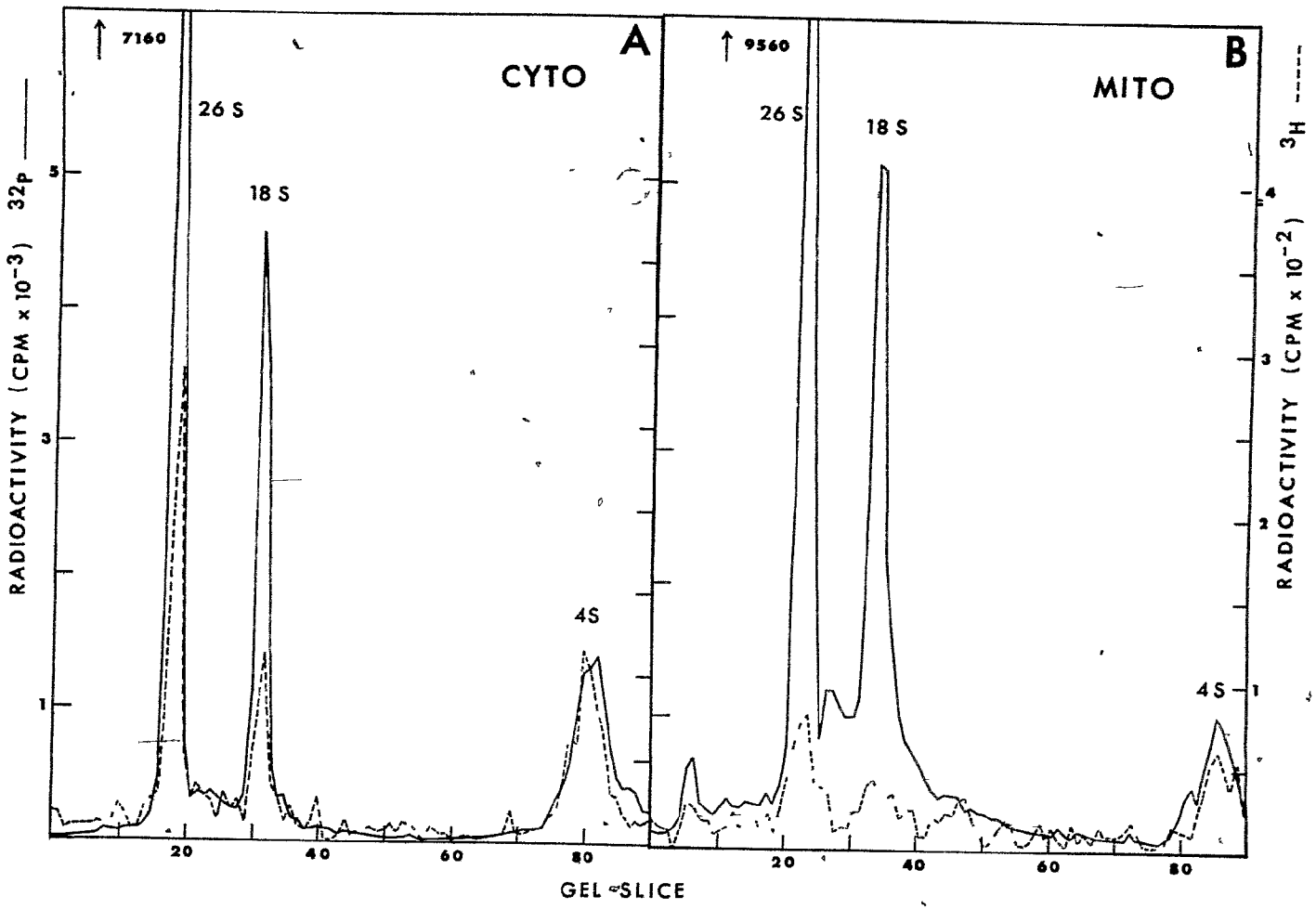
on the nucleotide composition analysis of mt-RNA (SECTION II.3.C.(iv).c.). The potential usefulness of methyl group labeling was examined in the following experiment, in which embryos were germinated in the presence of both [^{32}P]orthophosphate and [methyl-^{3}H]methionine.

FIGURE 11 shows the radioactivity profiles of the total cytosol RNA (A) and total mitochondrial nucleic acids (B) obtained from such an experiment, after polyacrylamide gel electrophoresis on 2.8% gels. As has been demonstrated previously (FIGURES 6, 7), [^{32}P]orthophosphate was efficiently incorporated into both the cytosol and mitochondrial RNA species but there was little labeling of the DNA present in the mitochondrial fraction. However, it is apparent from FIGURE 11 that there was differential [^{3}H]-labeling of the high-molecular-weight RNA species, with the mitochondrial species much less-heavily labeled (the low level of incorporation into the mt-RNA species made it impossible, however, to determine accurate ^{32}P/^{3}H ratios in this experiment). Since it has been shown in the case of the wheat cytosol rRNA species that 85% of the methyl label incorporated into the RNA is specifically in methyl groups under the labeling conditions used in this

LEGEND OF FIGURE 11

Polyacrylamide gel electrophoresis of the total cytosol (A) and mitochondrial (B) nucleic acids isolated from viable wheat embryos imbibed for 24 hr in a medium containing [^{32}P]orthophosphate and [methyl-^{3}H]methionine. Radioactivity (solid line, [^{32}P]; dashed line, [^{3}H]) profiles were determined as described in the legends of FIGURES 5 and 9.

FIGURE 11


7160
26 S
18 S
4S
CYTO
A
9560
26 S
18 S
4 S
MITO
B
RADIOACTIVITY (CPM x 10^-3) 32P
RADIOACTIVITY (CPM x 10^-2) 3H
GEL SLICE
20
40
60
80

experiment (Lau, 1973), this result would seem to imply a much lower degree of methylation of the mitochondrial 26 S and 18 S rRNA species, in agreement with results obtained by direct chemical analysis of $\underline{O}^{2'}$-methylated nucleoside components in the mt-rRNA (SECTION II.3.C.(iv).c.). While additonal experiments (including the establishment of conditions for more efficient methyl-labeling of the mitochondrial RNA species) will be necessary to eliminate artifacts (e.g., a markedly lower specific activity of the intramitochondrial pool of S-adenosylmethionine, compared to that of the cytosol pool), it should be pointed out that the relative degrees of [^{3}H]- and [^{32}P]-labeling appeared to be quite similar in the case of the low-molecular-weight RNA species (largely tRNA) of both the mitochondria and cytosol, in contrast to the situation with the high-molecular-weight RNA species. Admittedly, this argument is somewhat weakened by the low number of counts in the low-molecular-weight regions of the gels. In order to selectively label the methylated components of the wheat mt-rRNA species, it is evident that it will be necessary to use substantially higher levels of labeled precursor than those used by Lau *et al.* (1974) for selective methyl-labeling of wheat cytosol rRNA.

(vii) Effect of Actinomycin D and Ethidium Bromide on Transcription of Wheat Embryo Cytosol and Mitochondrial RNA

Actinomycin D is known to inhibit nuclear DNA-dependent RNA synthesis at concentrations which do not affect the synthesis of mitochondrial RNA. Accordingly, an attempt was made to explore the possibility of eliminating cytosol RNA contamination of the wheat embryo mt-RNA preprations by utilizing the differential effect of this drug. The results demonstrated a reduced specific activity of both the cytosol and mitochondrial RNA preprations when embryos were incubated in the presence of Actinomycin D. However, as expected, the specific activities of the cytosol RNAs decreased to a greater extent than did those of their mitochondrial counterprats. When using the higher inhibitor concentration (50 μg/ml), these decreases were ca. 55% and ca. 25%, respectively, of the control values. It has yet to be determined if the inhibition in the mitochondrial RNA fractions was due to reduced labeling of contaminating cytosol RNA (in which case the contamination would have represented ca. 45% of the total) or whether the drug also affects the transcription of wheat embryo mt-RNA. In order

to further assess the potential of this approach for obtaining cytosol RNA-free preparations of mitochondrial RNA, it will be necessary to determine if cytosol RNA synthesis can be inhibited to such an extent that contamination of the mitochondrial RNA fraction is reduced to a negligible level, without at the same time appreciably affecting the synthesis of mt-RNA.

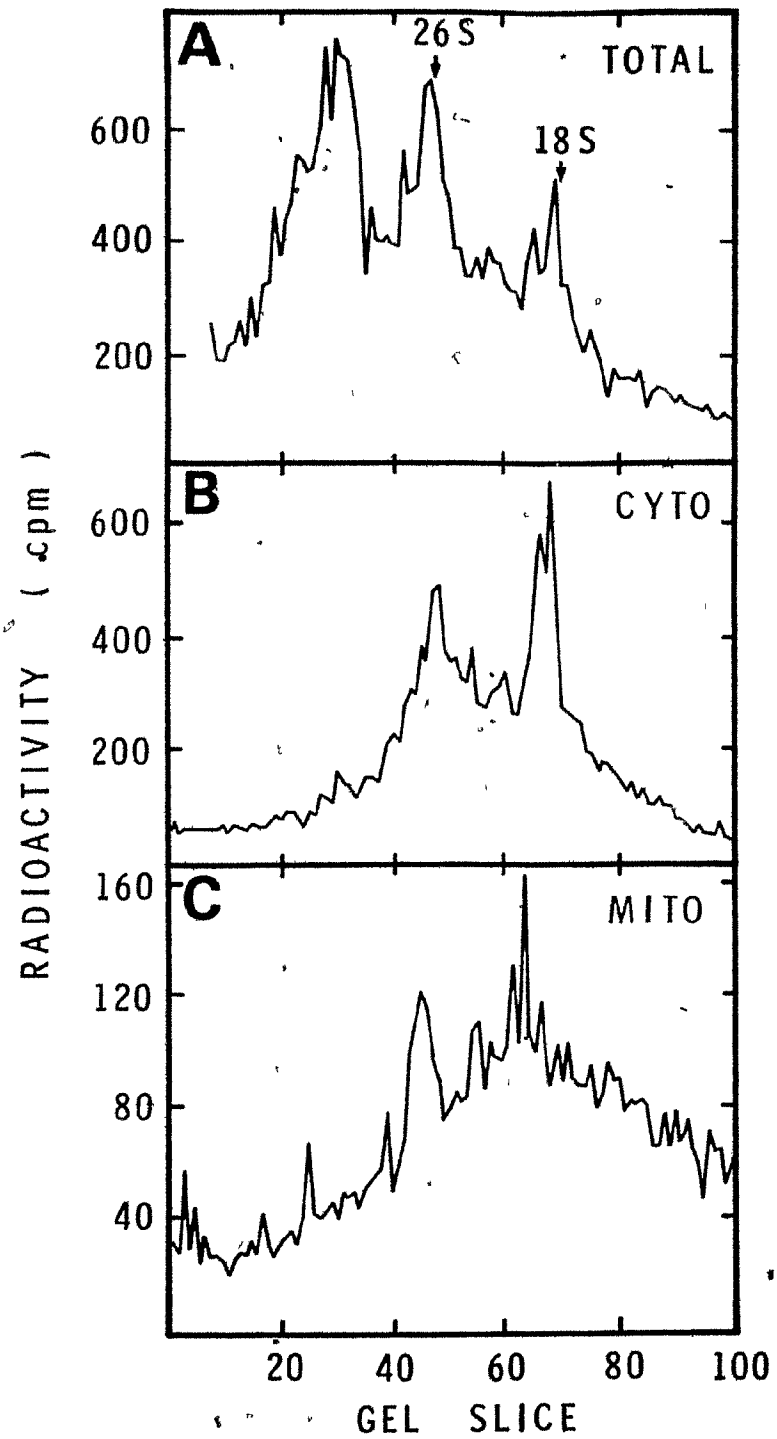
Ethidium bromide has been shown to completely inhibit the synthesis of HeLa cell mt-RNA at concentrations (0.2 μg/ml) which have little or no effect on the synthesis of all other species of cellular RNA or on DNA and protein synthesis (Zylber et al., 1969). On the other hand, in plants (radish root cells), nuclear as well as mitochondrial DNA synthesis has been shown to be inhibited at concentrations as low as 1 μg/ml (Lord, 1974). When wheat embryos were labeled in the presence of 5 μg/ml of ethidium bromide, no effect was observed on either cytosol or mitochondrial RNA synthesis. Although the insensitivity of nuclear RNA transcription was expected (on the basis of similar results in most other systems examined) the resistance of mitochondrial RNA transcription to this drug was not anticipated. This latter result could possibly have been due to

a failure of ethidium bromide (at the concentration employed) to enter the mitochondria of germinating wheat embryos or, less likely, to a unique resistance of wheat mt-RNA transcription to inhibition by this drug.

(viii) Isolation of Pulse-Labeled Total Cellular, Cytosol, and Mitochondrial RNA

The radioactivity profiles of total cellular, cytosol, and mitochondrial RNA (pulse-labeled for 1 hr in the presence of [^{3}H]uridine) following polyacrylamide gel electrophoresis in 2.4% gels are given in FIGURE 12 A, B, and C, respectively. The total cellular and cytosol RNA contained two radioactive peaks which migrated in the positions of the 18 S and 26 S rRNA species (the optical density profiles are not shown). In addition, a third component, which did not have any corresponding peak in the optical density profile and which migrated more slowly than the 26 S rRNA species, was present in the total cellular RNA. Although these results are preliminary and no chase experiments were performed with unlabeled uridine, it appears that this larger component may be the wheat cytosol rRNA precursor. Chen et al. (1971) have examined the process of cytosol

FIGURE 12



Polyacrylamide gel (2.4 %) electrophoresis of pulse-labeled ([^{3}H]-uridine) wheat embryo bulk cellular (A), cytosol (B), and mitochondrial (C) RNA. Radioactivity profiles were determined as described in the legend of FIGURE 6.

rRNA maturation (also in wheat) in some detail and have demonstrated the presence of a high-molecular-weight rRNA precursor in the total cellular RNA. They have suggested that the processing of the precursor molecule takes place in the nucleus (not in the cytosol) and that the smaller rRNA species is transported into the cytosol at a faster rate than the larger species. The results presented here are in agreement with these suggestions since there was no indication of an rRNA precursor component in the cytosol RNA (FIGURE 12 B), while the ratio of cpm 26 S/cpm 18 S was much lower than the expected 1.8.

The mitochondrial RNA radioactivity profile is more difficult to interpret. Under the conditions of RNA labeling and isolation employed here, there was no radioactive peak in the position expected of a large precursor molecule (FIGURE 12 C). This result could reflect a more rapid processing of a wheat mt-rRNA precursor or the absence of any high-molecular-weight precursor altogether. Kuriyama and Luck (1973) have demonstrated in Neurospora the presence of a short-lived 32 S mitochondrial RNA, possibly the precursor to the large and small mitoribosomal subunit RNAs. Support for this idea has come from mtDNA mapping experiments, which show that the Neurospora mt-rRNA

cistrons are adjacent to one another on the mitochondrial genome (Bernard and Kuntzel, 1976). However, no precursor has been found for the two yeast mt-RNAs, and it has recently been shown that the cistrons for these two molecules are well-separated on the yeast mitochondrial genome (Sanders et al., 1975). Thus, there is no reason a priori to expect either the presence or the absence of a wheat mt-rRNA precursor. Although a peak in the position of the 26 S rRNA species appeared in the mt-RNA radioactivity profile, the presence of an 18 S rRNA component could not be ascertained, due to the large amount of heterodisperse radioactive material migrating more slowly than the 26 S RNA. Bearing in mind that the [^{32}P]-labeled mt-RNA has been shown to be contaminated with the cytosol species (SECTION II.3.C.(iv).b.), it remains to be established that the 26 S and 18 S RNA components (if indeed present) labeled under such conditions are in fact of mitochondrial origin.

The heterodisperse material in the pulse-labeled mitochondrial RNA was largely absent from the total cellular and cytosol RNA preparations. It is interesting to speculate that this material might be mitochondrial mRNA. However,

additional pulse-chase experiments, using larger amounts of label, will be necessary in order to further explore this possibility.

D. DISCUSSION

The utility of the wheat embryo system described in this section for the preparation of bulk cellular [^{32}P]rRNA suitable for nucleotide sequence analysis has previously been demonstrated by Lane and co-workers (Lau et al., 1974). However, the specific activity (0.3 - 0.5 X 10^6 cpm/A_{260} unit) of the [^{32}P]RNA generated under their labeling conditions was too low to permit a compilation of T_1 oligonucleotide catalogues (Pechman and Woese, 1972; Uchida et al., 1974; Woese et al., 1976). It should be emphasized again that in order to carry out nucleotide sequence analysis of [^{32}P]RNA by this method, it is essential that the labeled RNA have as high a specific activity as possible. The degree of resolution of oligonucleotides in the primary fingerprint is determined to a large extent by the amount of digested RNA which is electrophoresed on the cellulose acetate strip in the first dimension. However, each oligonucleotide must contain sufficient radioactivity to allow secondary and, if necessary, tertiary analyses to be carried out before isotopic decay. Thus, the complexity of the RNA digest is an important factor in defining a minimum specific activity value

for any [^{32}P]RNA species being analysed by this particular technique.* Accordingly, the incorporation studies of Lau *et al.* were extended and, by increasing the isotope/embryo ratio during imbibition, it was possible to obtain the individual, [^{32}P]-labeled 26 S and 18 S components of wheat cytosol and mitochondrial ribosomal RNA having specific activities of 2 - 3 X 10^6 cpm/A_{260} unit. Such RNA was found to be suitable for T_1 oligonucleotide cataloguing (*cf.* SECTION III), although the technical limitations alluded to in the present section have so far precluded the preparation of specimens of higher specific activity. Contamination of 26 S and 18 S rRNAs with each other, or of mitochondrial species with their cytosol counterparts, was below the level which would interfere with conventional fingerprinting techniques.

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*In theory, the required minimum specific activity value should decrease as the chain length of the RNA decreases, since correspondingly larger amounts of a less complex digest (such as that obtained from 5 S rRNA) should be able to be fingerprinted without loss of resolution and with provision of adequate label in each separated oligonucleotide. In practice, increasing levels of contaminating substances (isolated along with the RNA) tend to interfere with resolution of the fingerprint as increasing amounts of digest are applied. Therefore, the purity of the RNA specimen being fingerprinted imposes an additional constraint on the specific activity required.

The data presented in this section of the thesis suggest that there is limited degradation of preformed (unlabeled) rRNA in both the cytosol and mitochondria during the first 24 hr of imbibition of viable emvryos. In contrast, newly-synthesized ([^{32}P]-labeled) cytosol and mitochondrial rRNA can be isolated in a relatively-pure and undegraded form. Notably, the absence of [^{32}P]RNA components the size of bacterial 23 S and 16 S rRNA in either the cytosol or mitochondrial iRNA indicates that bacterial and/or proplastid contamination of either sub-cellular fraction is negligible (confirming independent experiments, in the case of the purified mitochondria).

The availability of wheat mitochondrial and cytosol [^{32}P]rRNA permitted an examination of the nucleotide composition of the separated 26 S and 18 S components. As reported previously by Lau et al. (1974), and confirmed here, there is unequal incorporation of [^{32}P]orthophosphate into the 5'-nucleotide (pN) constituents of wheat embryo RNA during the first 24 hr of germination, and this must be taken into consideration during determinations (based on radioactivity measurements) of nucleotide composition by hydrolytic methods (such as snake venom phosphodiesterase degradation) which do not result in randomization of the

[^{32}P]label among the nucleotide products. Under the conditions described in this thesis, there did not appear to be any significant quantitative or qualitative differences in the efficiency of [^{32}P]-labeling of the different ribonucleoside triphosphates in the nucleus and mitochondria. The relative specific activities of the pN constituents of wheat cytosol and mitochondrial 26 S and 18 S RNA were quite reproducible in different experiments, and similar values were also obtained for wheat cytosol tRNA isolated at the same time. It should be noted that the relative specific activities obtained here are somewhat different from those reported by Lau et al. (1974). As in their work, however, it was found that among the purine nucleotides, the specific activity of pA exceeded that of pG, while among the pyrimidine nucleotides, the specific activity of pU was greater than that of pC. In both investigations, pC had the lowest specific activity of the 5'-nucleotide constituents of the [^{32}P]RNA specimens analyzed.

When suitable corrections were made for the specific activity differences noted above, the major nucleotide compositions of the wheat mitochondrial 26 S and 18 S RNA components were found to be very similar to those of their cytosol counterparts. While it is not possible to tell at

this point whether the small differences which were observed are statistically significant, it is evident that in wheat, the mitochondrial rRNA does not have a substantially lower G + C content than the cytosol rRNA, in contrast to the situation in other mitochondrial systems examined to date (cf. GENERAL INTRODUCTION). Whether this conclusion holds for other higher plants remains to be determined. On the other hand, wheat mitochondrial rRNA is significantly less methylated and contains less pseudouridiene than wheat cytosol rRNA, a characteristic which it shares with protist (Klootwijk et al., 1975; Lambowitz and Luck, 1976) and animal (Dubin, 1974) mitochondrial rRNA. Because of the possibility of residual low-level contamination of the mitochondrial rRNA species with their cytosol counterparts, the actual levels of modified nucleosides in wheat mitochondrial rRNA are probably even substantially lower than the data presented in TABLE X suggest. By analyzing the mitochondrial rRNA species for alkali-stable oligonucleotide sequences that are diagnostic of either cytosol 18 S or 26 S rRNA (Lau et al., 1974; Gray, 1974b; Gray and Cunningham, 1977) it should be possible to quantitate such contamination, and at the same time to determine the precise

content and distribution of modified nucleosides in wheat mitochondrial rRNA.

Although it has been assumed that the wheat mitochondrial rRNA species are transcriptional products of wheat mitochondrial DNA (as is the case in every other mitochondrial system studied so far), it remains to be formally demonstrated that this is so. The availability of isotopically-labeled wheat mitochondrial 26 S and 18 S rRNA should permit a determination of the transcriptional origin of these species and their frequency of occurrence and distribution on the mitochondrial genome. Together with the results of nucleotide sequence analysis (to be described in the following section), the analyses reported in this section of the thesis illustrate the particular usefulness of the germinating wheat embryo for studies of the structure and function of higher-plant mitochondrial RNA.

SECTION III: NUCLEOTIDE SEQUENCE ANALYSIS OF MITOCHONDRIAL AND CYTOSOL 26 S AND 18 S RIBOSOMAL RNA

1. INTRODUCTION

Between 1850 and 1890 many cytologists observed granular inclusions in the cytosol of a wide range of tissues, and many of these inclusions were undoubtedly mitochondria. However, although the initial discoverer of the mitochondrion remains unknown, the German scientist, Kölliker, deserves special mention for his detailed studies of the orderly-arranged granules in the sarcoplasm. Over a period of many years during the latter half of the last century, Kölliker characterized these structures and in 1880 was the first to succeed in separating them from the rest of the cytoplasm (cf. Lehninger, 1965). Today we know these granules to be mitochondria and recognize that such structures (called organelles) are present in all cells which contain a nucleus (eukaryotic cells).

A great deal is now understood about the structure and function of mitochondria. They are small, specialized, membranous organelles which are the site of fatty acid and Krebs cycle oxidations and the accompanying oxidative

phosphorylation of ADP. Often the mitochondrion has been termed the "powerhouse" of the cell since it is here that the respiring eukaryotic cell converts potential chemical energy into a biochemically-useful form (ATP). However, mitochondria persist even in cells which lack the ability to carry on respiration-coupled, energy-yielding reactions (e.g., respiratory-deficient yeast cells) or which are grown for generations in an anaerobic environment. It thus appears that, with the possible exception of some trichomonads and Pyronympha (which seem to lose their mitochondria under certain growth conditions), these organelles are indispensable components of eukaryotic cells.

The debate on the origin of mitochondria has had a long history and at one time or another these organelles have been postulated to arise de novo from the plasma membrane, the nuclear membrane, the endoplasmic reticulum and the cytoplasmic matrix (Lehninger, 1965). It is now well-established, however, that mitochondria originate from pre-existing organelles (Luck, 1965) rather than de novo. The question of the ancestral origin of these organelles, on the other hand, remains very much in dispute. In the late nineteenth century, Altmann proposed that mitochondria had their origin in an endosymbiotic association of prim-

itive bacteria with the ancestral eukaryotic cell (proto-eukaryote). In his book "Elementarorganismen", published in 1890 (and cited by Lehninger, 1965), he describes these "bioplasts" as the elementary particles of cellular life, similar to bacteria, living in colonies in the cytoplasm. However, Altmann's thesis was neglected or ridiculed throughout most of this century (Pirie, 1973) and it was not until the early 1960's, with the discovery that mitochondria contain DNA and a functional protein-synthesizing system, that this idea began to gain in popularity. It has now become the dominant hypothesis concerning the evolutionary origin of mitochondria and in its most explicit form (Margulis, 1970; Stanier, 1974) this "endosymbiont" hypothesis proposes that the organelles of eukaryotes (chloroplasts as well as mitochondria) evolved from prokaryotes living as endosymbionts in the cytoplasm of protoeukaryotic cells to which they were not phylogenetically related. However, the endosymbiont theory has not gone unchallenged and a second, or "direct filiation", hypothesis (Cavalier-Smith, 1975; Uzzell and Spolsky, 1974; Raff and Mahler, 1975) proposes that a single protoeukaryotic genome gave rise to functionally-distinct DNAs which were compartmentalized within the nucleus and organelles.

Claims for the prokaryotic origin of chloroplasts have gained general acceptance as a result of qualitative analyses (showing, for instance, structural and functional similarities between chloroplasts and contemporary blue-green algae) and quantitative analyses (showing, for instance, extensive homology between chloroplast and prokaryotic ribosomal RNAs; Zablen et al., 1975; Bonen and Doolittle 1975, 1976). However, although mitochondria also display many characteristics which suggest they are more closely related to free-living prokaryotes than to the eukaryotic cell of which they form a part, the evidence is less compelling (refer to TABLE XI for the similarities and differences between mitochondria and prokaryotes) and Raff and Mahler (1975), among others, have argued against a prokaryotic origin for mitochondria. They maintain that all similarities between prokaryotes and mitochondria represent only retained primitive characteristics which are devoid of phylogenetic significance. Demonstrated homologies between bacterial and mitochondrial proteins are, they feel, merely evidence for the evolutionary conservatism of the latter, and arguments for the prokaryotic nature of mitochondrial ribosomes have, in particular, been overstated. Indeed, mitochondrial ribosomes do

TABLE XI A

Some Similarities Between Mitochondria and Contemporary Free-Living Prokaryotes

1. Like prokaryotic DNA, but unlike the DNA of the eukaryotic nucleus, mitochondrial DNA exists in the form of covalently-closed, circular duplexes devoid of attached basic proteins (histones).
2. Mitochondrial and prokaryotic protein syntheses are sensitive to many of the same inhibitors (e.g., chloramphenicol, erythromycin, lincomycin) but insensitive to inhibitors of cytoplasmic protein synthesis (e.g., cycloheximide, anisomycin).
3. Like the corresponding bacterial process but unlike cytoribosomal protein synthesis, mitochondrial protein synthesis uses N-formylmethionyl-tRNA in chain initiation.
4. Mitochondrial chain initiation and elongation factors are interchangeable with their bacterial but not with their cytosol counterparts.
5. Methylation of rRNA and tRNA is lower in both mitochondrial and prokaryotic species than in the corresponding eukaryotic cytosol RNAs. Neither bacterial nor mitochondrial $tRNA^{Phe}$ contains the nucleoside wyosine, a specific marker of eukaryotic cytosol $tRNA^{Phe}$ (however mitochondrial tRNAs may contain the prokaryotic tRNA marker, 2-methyladenine).
6. As with prokaryotic mRNA, mitochondrial mRNA does not appear to contain the "blocked" 5'-terminus found in eukaryotic mRNA.
7. The phospholipid, cardiolipin, is present in both bacterial and inner mitochondrial membranes. It is absent from other eukaryotic membranes.
8. Both bacterial and mitochondrial RNA polymerases, but not eukaryotic nuclear RNA polymerases, are inhibited by rifampicin.

TABLE XI B

Some Differences Between Mitochondria and Contemporary Free-Living Prokaryotes

1. Analogy of inhibition patterns between prokaryotic and mitochondrial translation systems is not perfect. For example, fusidic acid interacts with one of the elongation factors in both bacteria and in the cytoplasm of Neurospora but does not inhibit the mitochondrial factor in this organism.
2. While spinach chloroplast and E. coli ribosomal subunits form active hybrids, yeast mitochondrial and E. coli subunits do not.
3. Mitochondrial rRNAs differ considerably from their prokaryotic and cytosol homologues in size and base compositions.
4. Mitochondrial rRNA methylations are predominantly on the ribose moiety, a situation similar to that found in the cytosol rRNAs. In bacterial rRNAs base methylation is more common.
5. Although similar quantitatively, the methylation of mitochondrial tRNA is qualitatively distinct from that of prokaryotic tRNA. Mitochondrial tRNA does not contain 4-thiouridine (s^4U), a nucleoside present in bacterial but not eukaryotic cytosol tRNA.
6. Mitochondrial ribosomal proteins differ from their cytosol and prokaryotic homologues in mobility on polyacrylamide gels and in immunological properties.

differ substantially from their bacterial homologues (and among themselves) in size and protein content, and the rRNAs they contain show similarly "non-prokaryotic" (and variable) sizes, base compositions, and transcriptional organizations (Raff and Mahler, 1975; Sanders et al., 1975). In an alternative hypothesis, Raff and Mahler suggest that in the ancestral protoeukaryote, respiratory enzymes became sequestered in a membrane-enclosed structure (derived from the plasma membrane), which subsequently incorporated a stable plasmid containing the appropriate genes required for elaboration of a mitochondrial translation system.

In distinguishing autogenous from endosymbiotic origins, quantitative measurements of homology between mitochondrial, cytosol and prokaryotic DNA should prove especially useful. In particular, regions of these DNA molecules that are the least susceptible to evolutionary change would be ideal for comparative studies. It is in these regions that evolutionary divergence (since the separation of prokaryotes from eukaryotes) would have been minimal and would not have resulted in a complete "randomization" of the sequence. Obvious candidates for

such studies are the ribosomal RNA cistrons or their direct transcription products, the large and small rRNAs. These rRNAs are coded for by the separate genomes of prokaryotes (23 S and 16 S rRNAs), eukaryotic nuclei (cytosol 25 S - 28 S and 17 S - 18 S rRNAs), and eukaryotic organelles (chloroplast 23 S and 16 S and mitochondrial 16 S - 26 S and 13 S - 18 S rRNAs). The strong functional analogies and obvious indispensability of these molecules leave little doubt that they represent a family of evolutionary homologues suitable for molecular taxonomic and phylogenetic analyses. Indeed, several groups have demonstrated by RNA/DNA hybridization studies that rRNA cistrons on both mtDNA and nDNA have been much more conserved during evolution than the remaining regions of the molecule (Dawid, 1972; Groot et al., 1975). Gerbi (1976) has also demonstrated by hybridization competition studies that, at least in eukaryotes, this conservation is especially great in distinct areas of the rRNA molecules. Pigott and Carr (1971, 1972) have utilized RNA/DNA hybridization in an attempt to assess the relatedness of the chloroplast of Euglena gracilis to several existing prokaryotes. In their study, the rRNAs of the

blue-green algae showed the greatest degree of homology with the rRNA cistrons of E. gracilis chloroplast DNA while a much lower degree of homology was shown by other bacterial rRNAs. Homology between the E. gracilis cytosol and chloroplast rRNAs was barely detectable.

Groot et al. (1975) have examined the degree of sequence conservation among the mtDNAs from various organisms and also among the respective cytosol rRNAs. Their results indicate a greater rate of divergence in the mt-rRNA cistrons than in the homologous cistrons in the nuclear DNA. It was not, however, determined whether the mt-rRNAs were more related to cytosol or prokaryotic rRNA. This experiment remains to be done.

DNA/RNA Hybridization studies will give a qualitative assessment of homology between rRNA molecules from various organelles and organisms. However, there are several limitations which prevent an accurate assessment of the quantitative differences between such molecules by this technique. Firstly, the sensitivity of the technique is low and small differences in sequence would pass unnoticed. For example, below a certain number, nucleotide differences between two molecules being compared would not be detected

since the molecules would exhibit indistinguishable hybridization kinetics. On the other hand, above a certain number of nucleotide changes, no similarity would be detected by hybridization, even though such similarity existed. A second limitation of this technique is that in order to draw meaningful comparisons among a group of molecules, each molecule in the group must be compared with every other. The logistical problems which this could involve should be self-evident. In addition, since hybridization procedures vary among investigators, data reported from one laboratory would not necessarily be relevant and comparable to that obtained in another. A third limitation is that the position and sequence of conserved regions, which could be of important phylogenetic significance, would go unnoticed. Also, modifications which may reflect on the more conserved features would not be measured by this procedure.

The most exact method of ascertaining the degree of homology between two or more rRNAs is to compare the sequences of these molecules. In this case, not only qualitative, but also quantitative, comparisons can be made. Regions of exceptional conservation and areas where

the molecules have diverged significantly can be pinpointed, as well as the type and position of modifications. In addition, data obtained in one laboratory can readily be compared with that obtained in another. However, deducing the entire sequence of a ribosomal RNA molecule, even the smaller species, is a formidable task. For example, it has taken Fellner and his associates ten years to deduce the sequence of E. coli 16 S rRNA (and even that is not yet complete). Use of this technique for determining homology among several rRNAs would obviously be eminently impractical. Fortunately, it is not necessary to sequence entire molecules in order to obtain quantitative data on their degree of relatedness. The partial sequence technique developed by Sanger et al. (1965) has recently been refined by Woese and co-workers for use in the detection of sequence homology between ribosomal RNAs and has been useful in constructing bacterial phylogenies (Pechman and Woese, 1972; Woese et al., 1975; Woese et al., 1976). This method, called "oligonucleotide cataloguing", involves (1) complete T_1 ribonuclease digestion of purified [^{32}P]-labeled rRNA to produce over 500 Gp-terminated oligonucleotides ranging in length from 1 to about 15 residues, (2) separation of these

oligonucleotides by two-dimensional ionophoresis, (3) their sequencing with other, site-specific nucleases, and (4) comparison of the resulting oligonucleotide catalogues with similar catalogues obtained for other rRNAs.

Bonen and Doolittle (1975, 1976) and Zablen et al. (1975), utilizing this technique, have concluded that the chloroplast 16 S rRNAs of Porphyridium (a red alga) and Euglena are "prokaryotic" in nature, bearing strong (80 - 90%) homology to prokaryotic (and in the case of Porphyridium, specifically blue-green algal) rRNAs, while sharing with cytosol 18 S rRNAs less than the minimal homology detectable by these methods (60%). Such results can be most simply (although not exclusively; cf. Discussion) understood on the assumption that chloroplasts derive from photosynthetic prokaryotes incorporated as endosymbionts into the cytosol of "protoeukaryotic" cells.

The possibility of obtaining highly-labeled wheat embryo mitochondrial and cytosol rRNA has allowed construction of similar "T_1 oligonucleotide catalogues" for these molecules. Comparison of these catalogues with one another and with those already available for several bacterial, blue-green algal and chloroplast rRNAs has

allowed us to assess the degree of homology among them and to determine whether, in spite of its apparent non-prokaryotic size, the wheat embryo mitochondrial 18 S rRNA is indeed prokaryotic in nature.

It should be noted that, in addition to RNA sequencing, quantitative assessment of homology between prokaryotes and eukaryotes can also be obtained by comparing the sequences of homologous proteins. Fridovich (1974) has recently done this with the enzyme superoxide dismutase. However, an exact nucleotide sequence provides much finer detail than an amino acid sequence, which does not allow deduction of silent mutations and does not provide that additional information about the structure and function of an RNA which is given by a nucleotide sequence. In addition, the ribosomal RNAs are the only homologous gene products known to be coded for by nuclear, mitochondrial, chloroplast, and prokaryotic genomes. Thus, sequence analysis of rRNA offers decided advantages over protein sequencing for phylogenetic comparisons.

B. MATERIALS

The enzymes pancreatic RNase (RNase A) and T_1 and Ustilago RNases were purchased from Worthington Biochemical Corp. (Freehold, New Jersey) and Sankyo Co. Ltd. (Tokyo), respectively. DEAE-cellulose paper was obtained from Whatman (Clifton, New Jersey) and the cellulose acetate strips from Gelman (Ann Arbor, Michigan). The marker dyes Orange G and Acid Fuchsin were products of Fisher Scientific Co. (Fair Lawn, New Jersey) and Xylene Cyanole was supplied by Eastman Kodak Co. (Rochester, New York). The ^{14}C-labeled ink was obtained from Schwarz-Mann (Orangeburg, New York). Fuji Co. (Tokyo) supplied the X-ray film (Fuji Rx Medical) and yeast tRNA was the product of Pabst Laboratories Inc. (St. Louis, Missouri). Varsol was obtained from ESSO. Additional materials (e.g., Aquasol, [^{32}P]orthophosphate) were obtained as indicated in the previous sections. All chemicals and solvents were of analytical reagent grade and the origin of the equipment is indicated in the text.

C. METHODS

(i) Preparation of 26 S and 18 S rRNA for T_1 RNase Hydrolysis

Prior to enzyme hydrolysis, it was essential to remove all residual salt and sucrose from the RNA samples, since the presence of these substances can cause considerable streaking during two-dimensional ionophoresis and thus prevent adequate separation of the oligonucleotide spots. An initial attempt to remove salt using chromatography on CF-11 cellulose (Doolittle and Pace, 1971) was not successful. The RNA did not bind efficiently and that portion which did bind could only be partially eluted subsequently. Therefore, another approach was employed, in which the RNA pellets were washed repeatedly with small volumes of cold 70% isopropanol. This resulted in satisfactory removal of salt and sucrose, although at the expense of some RNA. The RNA pellets were vacuum-dried, dissolved in 0.5 ml water, lyophilized, and stored at -20°.

(ii) T_1 RNase Oligonucleotide Cataloguing

T_1 RNase oligonucleotide cataloguing is a technique pioneered by Sanger and co-workers (Sanger *et al.*, 1965)

for the partial nucleotide sequence analysis of ribosomal RNAs. It has since been refined by Woese and collaborators (Pechman and Woese, 1972; Woese et al., 1975; Woese et al., 1976) and used to detect primary sequence homology and to establish phylogenetic relatedness among various bacterial 16 S ribosomal RNAs. The technique utilizes two-dimensional "fingerprinting" methods to produce a number of Gp-terminating oligonucleotides. These oligonucleotides are then subjected to secondary enzymatic digestion and, when necessary, the resultant products undergo a third or "tertiary" hydrolysis. If required, additional tertiary analyses are performed, after which the sequence of the original T_1 oligonucleotides can usually be deduced. The data is tabulated and this "catalogue" is then compared with similar catalogues produced from the homologous RNAs of other organisms.

In this study, the two-dimensional fingerprints were prepared from the four large ribosomal RNA species (mitochondrial and cytosol 18 S and 26 S) and T_1 oligonucleotide catalogues were compiled for mitochondrial and cytosol 18 S rRNAs.

a. Primary Analyses

For T_1 RNase hydrolysis, a sample of each species of purified RNA (10 - 150 µg, lyophilized) was dissolved in 10 µl water and taken up into a drawn, 50 µl capillary (digestion) tube. Using Microcap micropipettes, carrier RNA (if necessary) and the required amount of T_1 RNase (RNA and enzyme concentrations are given in TABLE XII) were added directly into the tip of the digestion tube. The solution was then mixed well by pipetting onto a Petri dish, drawn back into the digestion tube, and the tube end sealed in a flame. The total digestion volume never exceeded 20 µl. The mixture was incubated in a 37° waterbath for 15 min following which the digest was ejected onto a Petri dish (cooled on dry ice). The drops were successively "stacked" in order to minimize the area of contact with the dish; this subsequently permitted a more complete transfer of the sample to the cellulose acetate strip. The frozen sample was finally lyophilized.

Fractionation in the first dimension was carried out on a cellulose acetate strip (1" X 24"). The strip was wetted by first passing one side of it over the surface of the urea-acetate electrophoresis buffer (7 M urea adjusted

TABLE XII

CONDITIONS OF ENZYMATIC DIGESTION OF MITOCHONDRIAL AND CYTOSOL RNAs AND THE RESULTANT OLIGONUCLEOTIDES

A. PRIMARY DIGESTIONS WITH RIBONUCLEASE T_1

Amount of RNA(μg)	Enzyme (1 mg/ml)[a]/RNA (w/w)	Carrier Yeast tRNA (5 mg/ml)	Incubation
10	1 / 7	ca. 2 μl (10 μg)	15 min 37°
30 - 150	1 / 15 - 20	-	15 min 37°

B. SECONDARY DIGESTIONS WITH RIBONUCLEASES U_2 AND A

	RNase A		RNase U_2	
Oligonucleotide Composition	G, UG isopliths	>UG isopliths	G isoplith	>UG isopliths
Enzyme Concentration	1 mg/ml[a]	5 mg/ml[a]	4 units/ml[b]	4 units/ml[b]
Incubation	2 hr 37°	2 hr 37°	1 hr 37°	1.5 - 2.5 hr 37°

C. SECONDARY AND TERTIARY DIGESTIONS WITH RIBONUCLEASES U_2 AND T_1

	RNase U_2 (overcutting)	RNase T_1 (overcutting)	
Oligonucleotide Composition	G, U_xG isopliths	G to U_3G isopliths	$\geq U_4G$ isoplith
Enzyme Concentration	8 units/ml[c]	100 μg/ml[d]	100 μg/ml[d]
Incubation	4 - 6 hr 37°	6 hr 37°	overnight, 37°

[a] in water

[b] in 0.1 M sodium acetate - 1.5 mM EDTA (pH 5.5)

[c] in 0.1 M sodium acetate - 1.5 mM EDTA (pH 4.5)

[d] in 2.5 mM Tris·HCl (pH 7.5)

to pH 3.5 with acetic acid - 0.003 M EDTA), contained in a small dish, and then immersing it completely in the buffer. Wetting was done in this manner in order to avoid the inclusion of air bubbles. The strip was then placed on a bed of tissuepaper and the origin (spotting area), supported about 10 cm from one end by two pipettes, was blotted dry. The strip was covered with tissuepaper (excluding the origin) and dampened with a small amount of buffer in order to prevent the cellulose acetate from drying (in which case it becomes very brittle). The lyophilized digest was dissolved in a small volume (preferably < 1 μl) of distilled water and, using a small drawn capillary tube, spotted on the origin. This was done by applying the sample in several small drops, allowing each one to dry before application of the following. Maintaining the sample digest in a small area on the origin was necessary to ensure adequate resolution of the oligonucleotides during the subsequent electrophoresis. Any residual hydrolysate still left on the Petri dish was rinsed with water and also spotted in the manner described above. A marker dye (Xylene Cyanole FF, Orange G, Acid Fuchsin) was applied to both edges of the cellulose acetate strip at the origin.

The tissue was then removed and the strip blotted on both sides (care being taken to avoid any buffer streaming through the applied spot). The cellulose acetate strip was passed through Varsol (to prevent evaporation of the buffer), with the excess allowed to drip off, and then placed on a clean Mylar sheet in the flat bed electrophoresis apparatus (Model FP-30A, Savant Instruments Inc., New York) with the origin at the cathode end. The ends of the strips were connected to the electrode buffer chambers with filter paper wicks (2 thicknesses Whatman #1 which had been soaked in buffer and blotted). To avoid evaporation and crystallization of the urea-acetate buffer, a small amount of Varsol was layered on top of it in each buffer chamber. A second Mylar sheet and a glass plate were then set on top of the cellulose acetate strip and care was taken to ensure that neither the strip nor the wicks were exposed to the air. Finally, the voltage was set at 5000 V (High Voltage Power Supply, Model HV - 10,000, Savant) with no detectable amperage (< 2 mA) and the run conducted at 50°F (with circulating ethylene glycol - water bath). The length of the run was approximately 2.5 hr, or until the fastest red dye had ran off onto the wick and the yellow dye had

almost reached the end (the majority of the oligonucleotides in the T_1 digests migrated between the yellow and blue dyes).

Fractionation in the second dimension was carried out on a sheet of DEAE-cellulose paper 36 inches in length and 18 inches in width. The origin was marked with a pencil 6.5 inches from the end and the paper then hung over the Plexiglass electrophoresis rack (for Electrophoresis Tank Model LT-48A, Savant) with the transfer area positioned over a Plexiglass plate and held secure by two Plexiglass rods. The paper was now ready for sample transfer. Upon completion of the first-dimensional run, the cellulose acetate strip was removed from the flat plate chamber and blotted. The positions of the oligonucleotides were determined with the aid of a Geiger counter (Model ASA-2, Wm. B. Johnson and Assoc. Inc., Montville, N.J.) and the strip was then placed on the DEAE-paper transfer line. The oligonucleotides were eluted from the cellulose acetate to the DEAE-paper by layering 4 strips of Whatman #1 filter paper (saturated with water) on top of the cellulose acetate and slowly applying pressure with a second Plexiglass plate. To ensure a more complete transfer, the top Plexiglass plate was removed, the filter paper re-wet with water from a

pipette, and pressure re-applied. The acidic oligonucleotides are held in place by the basic groups on the DEAE-paper at the same relative positions that they occupied on the cellulose acetate strip. The filter papers and cellulose acetate strips were then removed and the transfer of counts monitored with the Geiger counter (ca. 90% of the radioactivity should be transferred).

Urea was washed from the transfer area with ca. 200 ml 100% ethanol in order to ensure good resolution in the second dimension. The DEAE-paper was then air-dried and the entire sheet was wet with the second dimension buffer (0.1 M pyridine formate, pH 2.35) first by squirting buffer just above and below the transfer area and allowing the two fronts to meet and then evenly wetting the remainder of the paper. Care was taken at this stage since DEAE-paper is extremely fragile when wet and will tear readily. Marker dye was spotted on the sides of the paper at sample level and the rack and paper were then placed in the cooled electrophoresis tank (Model LT-48A, Savant), maintained at 30°F using Varsol as a coolant, with the transferred region several inches above the cathode buffer chamber. The run was conducted at approximately 2000 V (ca. 150 mA).

Ionophoresis was continued until the blue dye reached the third bar of the rack (ca. 16 - 18 hr). The rack was then carefully removed from the tank and the paper allowed to air-dry. The paper was trimmed to fit the size of the X-ray film (14" X 17"), the edges marked with radioactive [^{14}C] ink, and the oligonucleotide spots located by radiography (exposure time 2 - 4 hr for initial hydrolysate containing 10 X 10^6 cpm). After developing, the film was aligned with the paper using the [^{14}C]marker, the oligonucleotide spots cut out, and the radioactivity of each determined by scintillation spectrometry (BBOT - toluene scintillation cocktail; Sogin et al., 1971). The spots were then washed in three changes of toluene and air-dried in preparation for secondary and tertiary analyses.

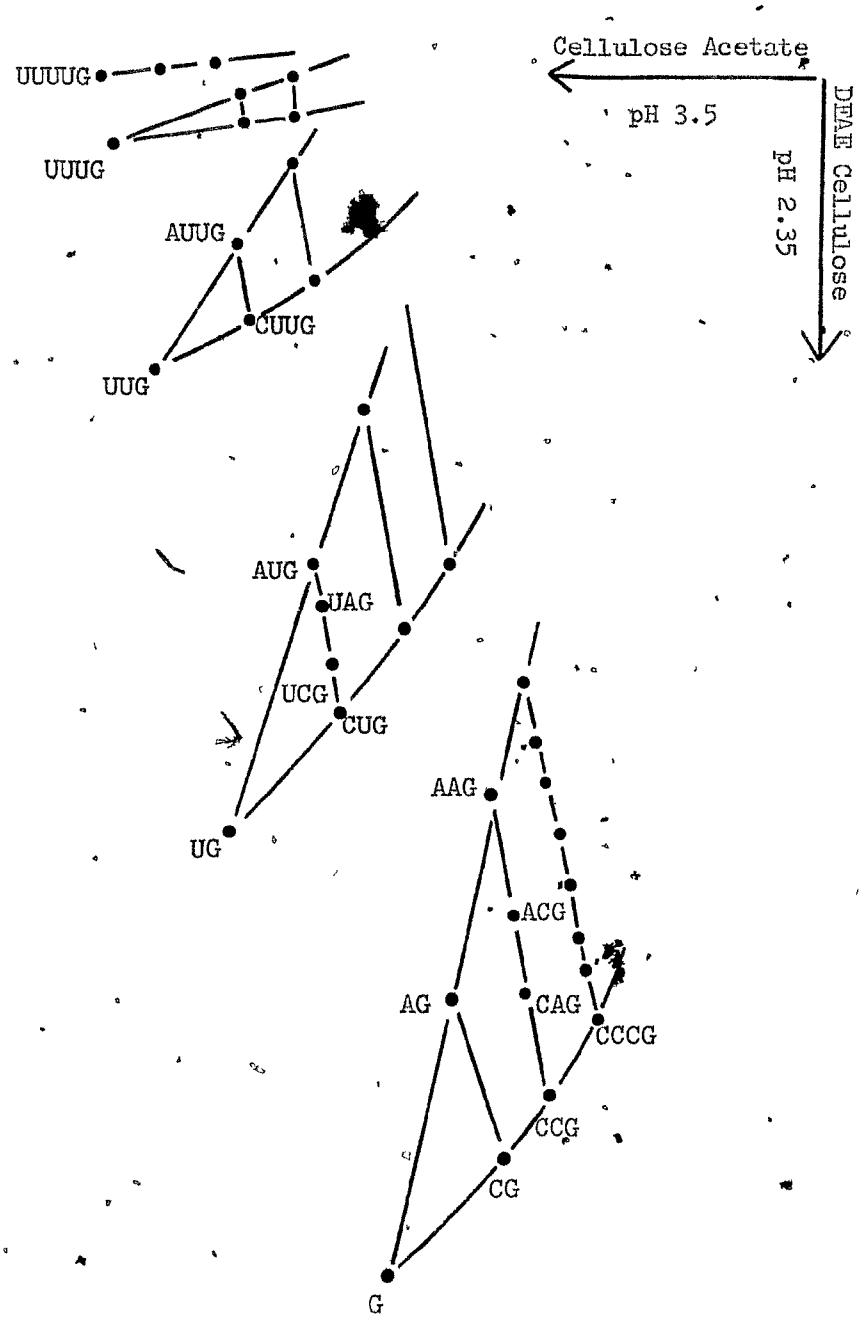
b. Secondary Analyses

Although the sequence of a number of small oligonucleotides can be deduced from their positions on the primary fingerprint, it is necessary to subject the vast majority of oligonucleotides to further enzymatic digestion in order to determine their sequence. The enzymes used in these analyses and the conditions of hydrolysis are depend-

ent upon the nucleotide composition and size of a particular oligonucleotide. An idea of these parameters can be obtained from the position that the oligonucleotides occupy on the primary fingerprint. FIGURE 13 is a schematic illustration of the relationship between the composition of an oligonucleotide and its position of the two-dimensional electrophoretogram as produced by the first- and second-dimensional buffer systems used in this study. The oligonucleotides are grouped into triangular-shaped regions called "isopliths" and the spots within a given isoplith contain the same number of uridylate (Up) residues. Those oligonucleotides in the lower-right (fastest-moving) isoplith contain no Up residues while those in the preceeding isoplith contain one and those in the isoplith above that contain two, and so on.

Within a given isoplith oligonucleotides containing the same number of residues are arranged in nearly vertical lines ("isomeric"). The composition of these oligonucleotides can be partially deduced by considering the respective mobilities of uridylate (Up), adenylate (Ap), and cytidylate (Cp) residues. In the first dimension the order of migration is Up > Ap > Cp while in the second dimension

FIGURE 13



Schematic illustration of the relationship between the composition of an oligonucleotide and its position on the primary fingerprint after two-dimensional ionophoresis of a T_1 ribonuclease hydrolysate of RNA.

this order reverses and we have Cp > Ap > Up. It is also often possible to deduce the sequence when two or more isomers exist. It was thus by analysis of the primary fingerprints that preliminary data on the size, composition, and sequence of the various oligonucleotides could be obtained and meaningful secondary digestions undertaken. This was especially important in the studies involving the mitochondrial 18 S RNA since the relatively low specific activity of this RNA prohibited much experimentation.

The two enzymes normally used in this study for the secondary analyses of T_1-generated oligonucleotides were pancreatic ribonuclease (RNase A) and Ustilago ribonuclease (U_2). These enzymes are quite specific in their actions, with the former enzyme cutting the polynucleotide chain to leave a 3'-phosphate attached to a pyrimidine residue, and the latter enzyme cutting at the 3'-end of purine residues (at pH 5.5). The concentration of the enzyme and the time of incubation depended upon the size and presumptive composition of the oligonucleotide and the usual conditions employed are given in TABLE XII. For non-A-containing oligonucleotides of the form C_xU_xG, secondary digestion using overcutting with U_2 and T_1 was employed.

During U_2 overcutting, the pH of incubation was changed from 5.5 to 4.5, a higher concentration of enzyme used and the incubation time lengthened. This alters the specificity of U_2 so that, in addition to cleaving after A and G residues, cleavage also occurs after pyrimidines leaving partial products. During T_1 overcutting (also called T_3 digestion), longer incubation times result in cleavage after A and U residues. Although an oversimplification, this tends to leave stretches beginning with a 5'-C residue. The specificity and mode of employ of U_2 and T_1 overcutting is described in detail by Uchida et al. (1974) and Woese et al. (1976), respectively.

The enzymatic digestions were carried out with the oligomers still on the DEAE-paper. Sufficient enzyme solution was applied to each spot so that the paper appeared thoroughly moistened (glistening) and the digestions were carried out in Petri dishes sealed with Parafilm. Following hydrolysis, the digests were stored in the freezer.

Separation of the secondary hydrolysis products was effected by ionophoresis of the digests on DEAE-cellulose paper in either 0.5% pyridine - 5% acetate acid, pH 3.5 (PA tank, Model LT-20A, Savant) or 6.5% formic acid

(Formate tank, Model LT-48A Savant) (refer to TABLE XIII for the usual approach). The enzyme digests were transferred to the DEAE-paper sheets (46" X 18" or 9" X 32.5") by folding them into small balls (several thickenings) and carefully pressing them into the paper. Moistening the balls with a small amount of sterile water and exerting pressure with the gloved thumb was usually sufficient to ensure attachment. Nucleotide and oligonucleotide markers obtained from the primary fingerprint were also inserted. The spots were allowed to dry and the sheet hung on the electrophoresis rack and soaked with buffer (care being taken not to loosen the inserted spots). Marker dye was applied and the rack and paper then lowered into the appropriate electrophoresis tank and ionophoresis carried out as described in TABLE XIII.

c. Tertiary Analyses

Often the analyses of secondary digestion products are not sufficient to determine unequivocally the sequence of the original T_1 oligonucleotide. In this case, one or more tertiary analyses must be undertaken. These include either subjecting a secondary digestion product to further

TABLE XIII

THE USUAL PROCEDURES EMPLOYED TO ANALYZE OLIGONUCLEOTIDES GENERATED WITH RIBONUCLEASE T_1

Oligonucleotide Composition	Ribonuclease	Buffer System
A_xC_xG (from G isoplith)	A, U_2 (regular)	0.5% pyridine, 5% acetic acid (pH 3.5)
$A_xC_xU_xG$ (from ≥UG isoplith)	A U_2 (regular)	0.5% pyridine, 5% acetic acid (pH 3.5) 6.5% formic acid
C_xU_xG (non-A-containing oligonucleotides)	U_2 (overcutting), T_1 (overcutting)	6.5% formic acid

ribonuclease digestion and/or re-running it in another solvent system. For example, tertiary T_1 and U_2 overcutting are often necessary, since secondary digestion with RNase A and U_2 (at pH 5.5) does not give the sequence of pyrimidine stretches. In addition, tertiary RNase A and U_2 regular digestion is sometimes needed in order to obtain the exact sequence of a secondary partial piece. Tertiary re-runs in another solvent system will often clarify the sequence of a product.

TABLE XII gives the conditions of the additional enzyme digestions which, as in the case of the secondaries, were also done right on the DEAE-paper and in sealed Petri dishes.

D. RESULTS

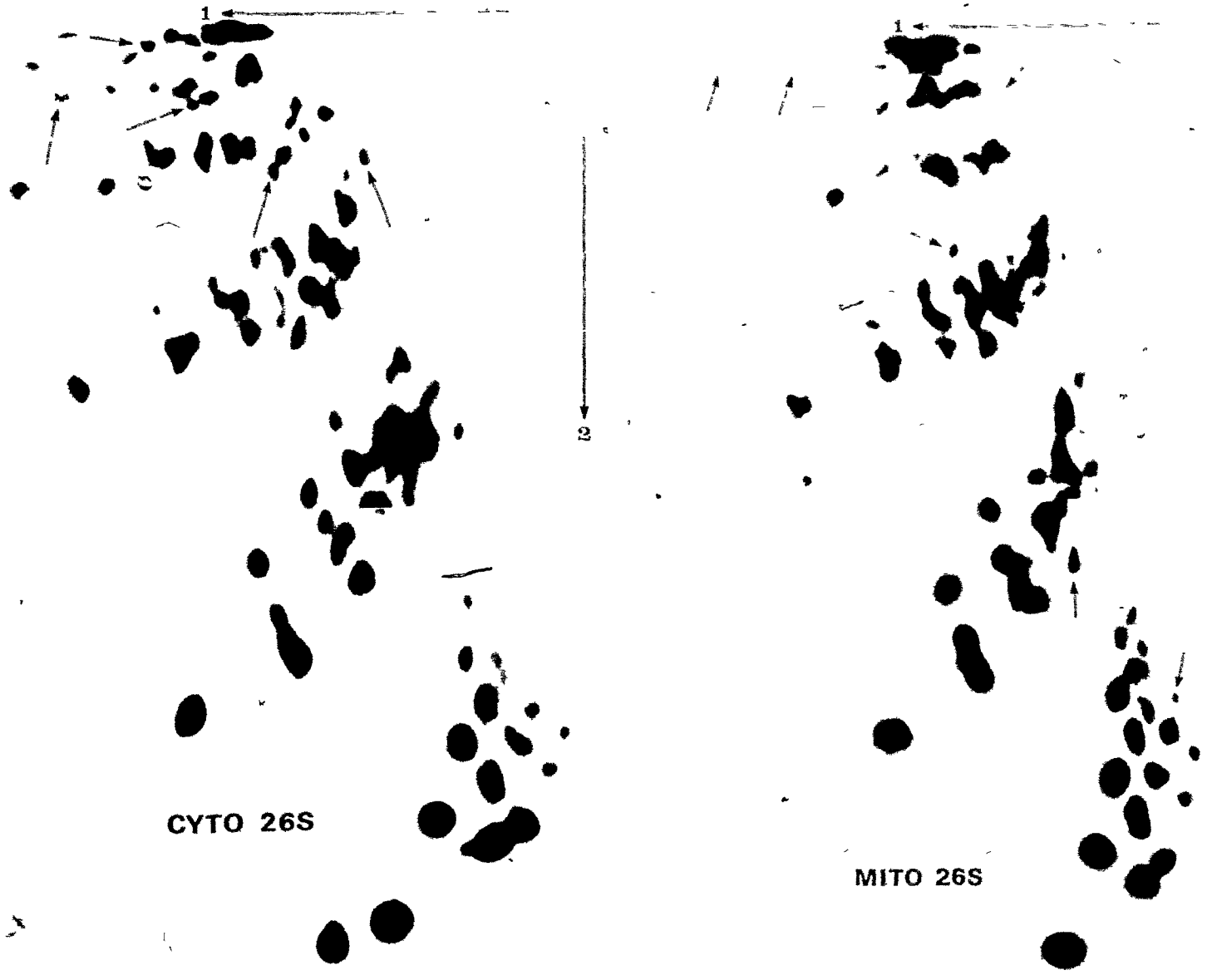
(1) Primary Fingerprints of Mitochondrial and Cytosol 26 S and 18 S RNA

The primary T_1 fingerprints of the wheat embryo cytosol and mitochondrial rRNA species are shown in FIGURES 14, A and B (26 S rRNAs) and 15, A and B (18 S rRNAs). The resolution of the individual oligonucleotides following two-dimensional paper ionophoresis varied from excellent, as in the case of the two 18 S rRNA species, to poor, as shown for the mitochondrial 26 S rRNA species. The success of the separation diminished with (1) the amount of RNA initially applied to the cellulose acetate strip and (2) the quantity of extraneous non-RNA material present in the sample (e.g., salt, sucrose, polyacrylamide). A comparison of FIGURES 14 A and 14 B demonstrates that cytosol and mitochondrial 26 S rRNAs are distinct molecular species and, although the T_1 fingerprints of these large RNAs are complex, each contains a number of unique oligonucleotides not present in the other (a few such oligonucleotides are indicated by arrows). Other (similarly-migrating) oligonucleotides showed significant quantitative differences in molar yield (as determined by radioactivity

LEGEND OF FIGURE 14

T_1 Ribonuclease fingerprints of the purified wheat embryo cytosol (left) and mitochondrial (right) 26 S rRNA species. Enzymatic hydrolysis of the two rRNA species and subsequent ionophoresis of the digest were conducted as described in SECTION III.C.(ii) a. Several unique oligonucleotides present in each 26 S rRNA but not in the other are indicated by arrows. Since the cytosol 26 S RNA was prepared from undenatured ribosomal RNA, its fingerprint includes oligonucleotides derived from the 5.8 S RNA species

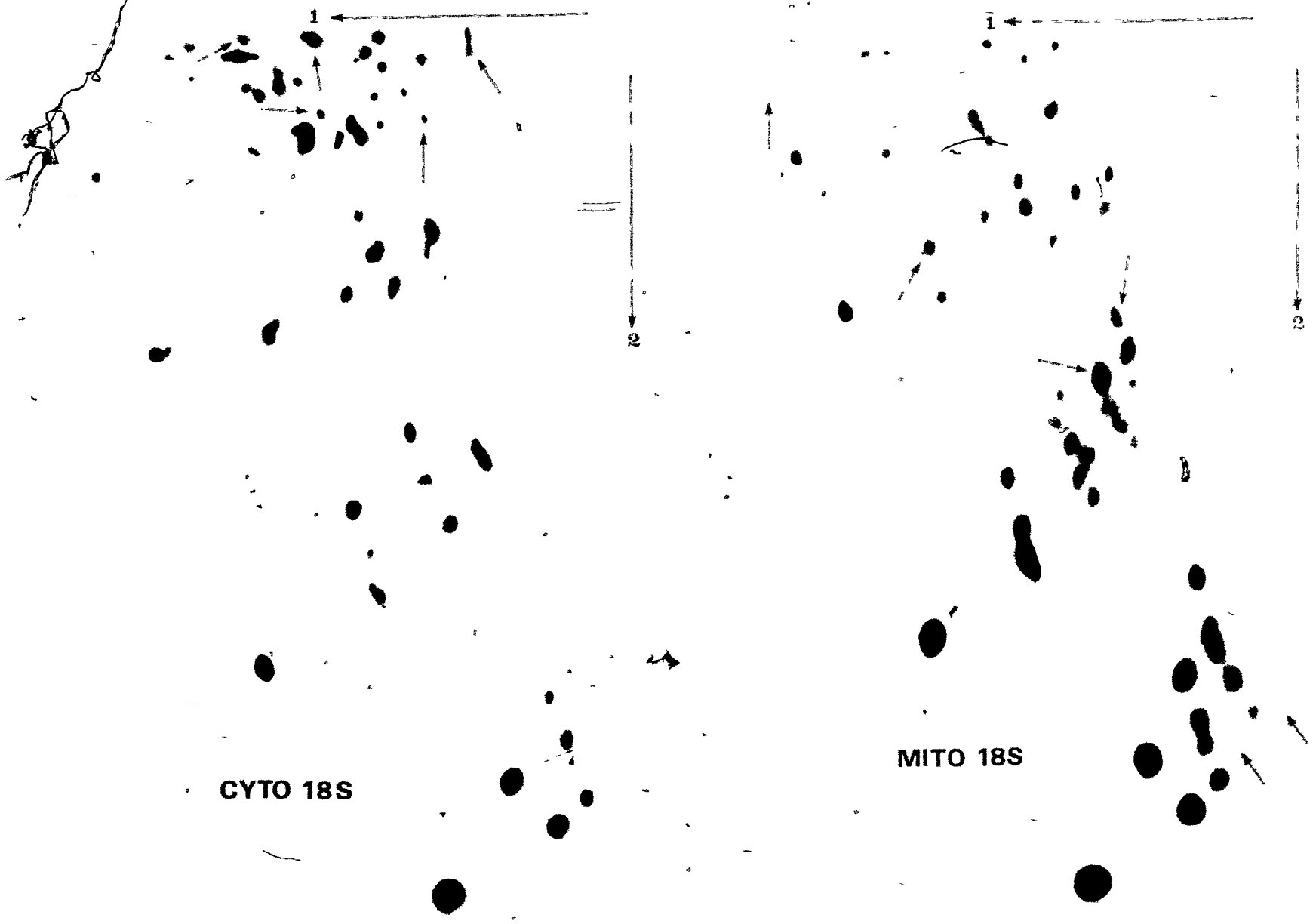
FIGURE 14



LEGEND OF FIGURE 15

T_1 Ribonuclease fingerprints of the purified wheat embryo cytosol (left) and mitochondrial (right) 18 S rRNA species. Enzymatic hydrolysis of the two rRNA species and subsequent ionophoresis of the digest were conducted as described in SECTION III.C (ii).a Several unique oligonucleotides present in each 18 S rRNA but not in the other are indicated by arrows.

FIGURE 15



measurements). In spite of these differences, low-level (15 - 20%) contamination of mitochondrial 26 S rRNA by the homologous cytosol species was apparent both from the fingerprints and from separate studies of alkali-stable dinucleotide sequences (Gray, unpublished data).

Wheat embryo cytosol and mitochondrial 18 S rRNAs are also distinct molecular species, showing readily-distinguishable fingerprints (FIGURE 15, A and B; again a few unique oligonucleotides are indicated by arrows). In this case there appeared to be no significant (<10%) contamination of either 18 S species by the other. However, the degree of contamination of the mitochondrial RNA did seem to vary from preparation to preparation (cf. SECTION II.3.C.(iv).b.). An especially interesting result from the T_1 fingerprint analysis of the mitochondrial 18 S rRNA was the detection in the G isoplith of two oligonucleotides which migrated in positions occupied by two modified "universal" oligonucleotides in prokaryotic 16 S rRNA fingerprints. Prokaryotic "universal" sequences" will be discussed further in the following chapter.

T_1 Ribonuclease fingerprints of the high-molecular-weight rRNA species from two other organisms have been

published. Verma et al. (1971) demonstrated that the fingerprint patterns of the homologous cytosol and mitochondrial species in Aspergillus nidulans differed from one another, although the resolution of the oligonucleotides in the slower-moving isoplrths was poor and therefore complicated the analysis. In a study of mouse liver rRNAs, Dierich et al. (1975) found that the fingerprint patterns of the homologous cytosol and mitochondrial rRNA were remarkably similar, in contrast to the results found for wheat embryo and Aspergillus. However, it remains to be firmly established that these authors were working with mitochondrial rRNA devoid of any significant cytosol rRNA contamination. The fingerprints presented in this thesis represent the first ones obtained for the mitochondrial rRNA species from a higher plant, and they demonstrate clearly the existence of different species of 26 S and 18 S RNA in the mitochondria and cytosol of wheat.

(ii) T_1 Oligonucleotide Catalogues of Mitochondrial and Cytosol 18 S rRNA

The T_1 oligonucleotide [chain length (N) $\geq$ 5] catalogues of the wheat embryo cytosol and mitochondrial 18 S rRNAs

are given in TABLES XIV and XV, respectively. The oligomer compositions were deduced from the secondary (2°) and tertiary (3°) analyses described in the chapter on methods and the molar quantitation was determined by radioactivity measurements, as described in TABLE XIV. The entire sequence of some oligonucleotides could be deduced after secondary analysis (e.g., RNase A hydrolysis of the oligonucleotide AAACAAAG gives the partial products AAAC and AAAG, which is sufficient to assign a definite sequence). In most cases, however, tertiary analysis was necessary. The following is one example of how this was done:

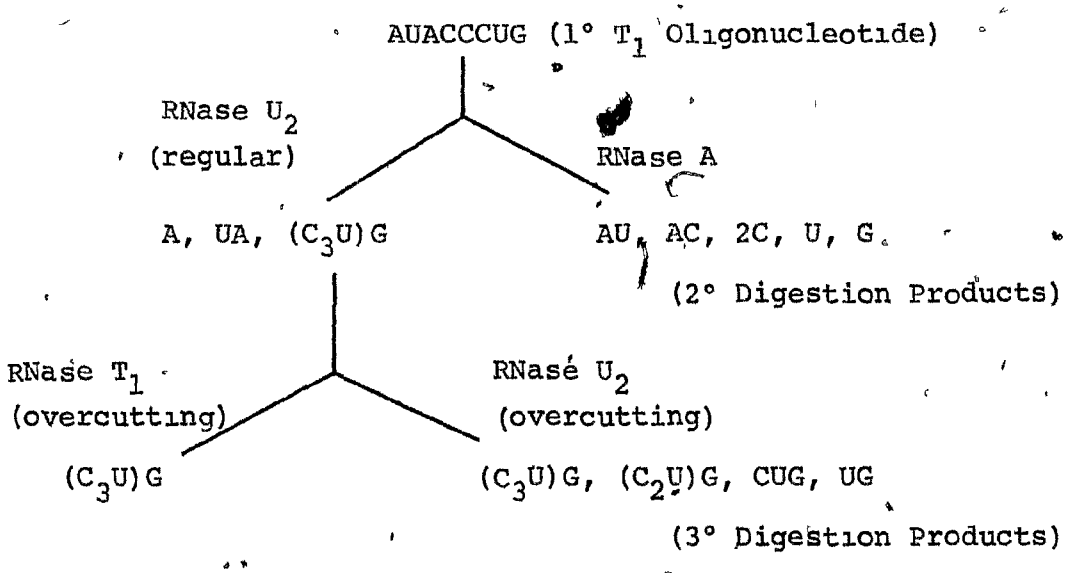


TABLE XIV

T_1 RIBONUCLEASE CATALOGUE OF WHEAT EMBRYO CYTOSOL 18 S rRNA

Pentamers

CCCCG (0-1)
CACCG
CCAAG
ACACG
(A,C)CAG (0-1)
AAACG
CCUAG
CAUCG (2)
CCAUG[4]
UAC*AG[4]
$C_{1-2}(A_{1-2}U^*)G$
CN*AUG[3]
AUCCG
AACUG
CUCUG (1-2)
CCUUG
UUCCG (0-1)
(CU)UCG (0-1)
CUAUG
UAAUG
AAUUG
UUCUG
UUUAG
A*UUUG

Hexamers

CCCCCG (0-1)
AUACC*G
CCAUCG
ACUCCG
AAACUG
ACAUAG
AAC*CUG
CCUUCG
UCCCUG[2]
$(C_3U_2)G$
(CU)CUA*G[2]
CAUUCG
UUAACG
AUUCCG

Hexamers (cont.)

AUCCUG
CAUUAG
CAAUUG
AAUUAG
CUU,U,CG[2] (0-1)
$(C_2U_2)U^*G$[2] (0-1)
UAAUUG
UA,UUA,G
CAUUUG
(CU)UUU*G
(UC)UUUG
ACUACG
unsequenced (0-1)
(modified)

Heptamers

UAAC*AAG
AUAACCG
AUAAAAG
CCUCACG[2] (1-2)
ACCUCAG
CUCAAAG
AAAUUAG
(UC)ACUCG
$N_{0-1}A$,(UC)A,U*G
(U_2C)AAAG
(UC)AUCACG[2]
$A(U_2C_2)$ACG[2]
ACUAUCG[2]
A(CU)UCA*G[4]
CCCUU*AG[2]
UCCAUUG
CUUAUAG
UAUAUAG
AUUCCUG[1]
UAU(AC*,U)G

Heptamers (cont.)

ACUUAUG (0-1)
AUAUAUG[2] (0-1)
(CU)A,UAUG (0-1)
UUCA,UA,G[4]
UUCUUAG[2]
AUUUAUG

Octamers

AAC,AC,NN*,G[3,4]
AACAACUG
AU,NNN*C_x,G
CUAACUAG
AUAA*CUCG
AU(UC)AAAG[4]
ACCUA,UA,G[4]

Nonamers

CCA,CCA,CA,G
UACACACCG[1]
CUCCUACCG
CCUACCA G
A_3N,C_{1-2}NN*G (1-2)
CAUAU(UC*)G
UAUUCAACG[2]
CCUUAAUUG
UUAAUUCCG[2]
UAAUUCUAG
(C_1U_4)AAAG[2]
AU(UC 2)AUUG[4]

Decamers

AAACUUAAAG
AACCAAUUUG
A,CA,CCUUA,UG[4]
$(C_{2-3}U_4)$CUUG
AAU(U_4,C_{1-2})G[2]
CUUUAUAUAG[4]
A(UUUCA,UUA)G[2]
UUUCAAA$_{0-1}$CA 1G
C 2U5AAG[4]

Undecamers

AA_{1-2}CAA 2CCCCG[2]
CAAUAACAUG*[4]
AAACUUACCAG[1]
A(CUA,CA)(UC_2)AAG[4]
A_{0-1}(UAACA3UCA)UAG[2]
CCUCAAUA(CU*)G
AAAC(UA*,UCUCAA),CG[4]
$(C_3U)AU(C_xU_2)G$[4]
...UCAAC(U3C)G[4]
CUCAUUAAAUCAG[1]
C_xA_3,C_xAAA,UUA,UCCUG[2]
AA,UAAA,UA,CAACCG[2]
CA(UUA,UUA,UA)G[4]
..UUA,UUAA,UCAA,G[2]
UUUA,CACCUUA,UCA,G[2]
AAAU,AU(U_xC_x)G
CAA,UA,UCUAA,CG
(C_xU)CUUA$_{1-2}$CG[4]

[1]Sequence very probably, but not certainly, as indicated.
[2]Sequence uncertain. Secondary and tertiary digestion products most consistent with the sequence as indicated.
[3]Secondary digestions produced unidentified modified dinucleotide.
[4]Sequence highly speculative.

TABLE XIV (cont'd)

This table lists all oligonucleotides larger than tetramers present in wheat cytosol 18 S rRNA. An asterisk indicates the presence of a modified nucleoside. An asterisk preceded by a bar over two or more nucleosides indicates a modified secondary product in which the exact position of the modification is unknown. N denotes an unknown modified nucleoside and the subscript x indicates that the number of copies of the preceding nucleoside is unknown. Molar yields were determined by measuring the radioactivity in several uniquely-migrating spots of known size, calculating the specific activity (cpm/nucleotide) and using this information to deduce the molar amounts of the remaining oligonucleotides. The molar yields were ca. unity except where indicated by the values in parentheses. An estimation of the size of the cytosol 18 S rRNA can be made by dividing the total cpm present in all the spots by the calculated specific activity. (The size of the wheat cytosol 18 S rRNA determined by this method is ca. 2000 nucleotides).

It should be noted that calculation of molar yields and specific activities of oligonucleotides might be expected to be complicated by the non-uniform labeling of the pN constituents of the RNA, as discussed in the previous section. Since the specific activity of pA residues was found to be essentially the same as that of pU residues, and since each T_1 oligonucleotide contains one pG residue, the non-uniform labeling of oligomers arises from variation in the number of pC residues, as well as from the nature of the adjacent pN constituent (whether pA, pC, pG or pU) from which the 3'-terminal phosphate group is derived. However, such non-uniform labeling did not appear to significantly affect the quantitation (at least of oligomers of chain length $N \geq 5$, most of which were present in single copies), since precise determination of molar yields was already subject to the limitations of the experimental technique (e.g., incomplete liberation of some oligonucleotides during the initial T_1 RNase hydrolysis, incomplete recovery of oligonucleotides due to streaking or overlapping on the fingerprint, etc.).

TABLE XV

T_1 RIBONUCLEASE CATALOGUE OF WHEAT EMBRYO MITOCHONDRIAL 18 S rRNA COMPARED TO THOSE OF THE HOMOLOGOUS PROKARYOTIC, CHLOROPLAST, AND WHEAT EMBRYO CYTOSOL rRNAs

Oligonucleotide sequence	*R. spheroides* 16S	*E. coli* 16S	*B. subtilis* 16S	*G. alpicola* 16S	*Porphyridium* chloroplast 16S	Wheat cytosol 18S
Pentamers						
CG*CCG	1	1	1	1	1	0
CCCCG	2	0	0	0	0	1
CCCAG	1	1	1	1	1	0
CCAAG (0-1)	0	0	0	0	0	1
ACACG	1	1-2	1	1	1	1
AACCG	0	1	0	0	0	0-1
AACAG	0	0	1	0	1	0
AAAAG	0	0	0	1	1	0
CUCCG	0	0	0	0	0	0
CCCUG	0	0	0	0	0	0
CUCAG	1	2	1	1	1	(0)
CUACG	0	0	0	0	0	0
CCAUG	1	1	0	0	0	(0)
CAUCG	1	0	0	1	1	1
ACUCG (1-2)	0	0	0	0	0	0
UCAAG (1-2)	1	1	0	1	(1)	0
UAACG	1-2	1	1	0	0	0
CAAUG	0	0	1	1	1	0
AUCAG	2	1	1	1	1	1
ACAUG	1	0	0	0	0	(0)
AAUCG	1	1	1	~2	1	0
AACUG	1	2-3	0	0	1	1
UAAAG (~2)	2	3-2	2	~2	2	0
AAUAG	1	0	1	0	1	0
CCUUG	1	1	1	0	1	1
CUCUG[1]	1	0	1	0	1	1
CUUAG	0	0	0	0	1	0
UUACG	0	0	0	0	0	0
UACUG	0	0	0	0	1	0
ACUUG[1]	0	1	0	0	0	0
UAUAG	0	0	0	2	0	0
AUUAG	1	2	1	2-3	2-3	0
AAUUG	2	1	1	1-2	1	1
CUUUG	0	1-2	0	(0)	0	0
UCUUG	0	2 1	1	2	0	0
UUCUG	0	0	0	0	1	1
UUUCG	0	0	0	0	0	0
UUUUG	0	0	0	0	0	0
unsequenced (0-1)	0	0	0	0	0	0
Hexamers						
CCACCG	0	0	0	0	0	0
CCCAAG	0	0	0	0	0	0
CACACG	1	1	1	1	1	0
ACCAAG	0	0	0	0	0	0
AAAAAG	0	0	0	0	0	0
CCUCCG	0	0	0	0	0	0
CCUCAG	0	0	0	0	(1)	0
UACCCG	0	0	0	0	0	0
CCUAAG	0	0	0	0	(1)	0
UACCAG	0	0	0	0	0	0
CUAACG	0	1	1	0	1	0
CCAAUG[1]	0	0	0	0	0	0
AUCCAG	0	0	0	0	0	0
AAUCCG	0	0	0	1	0	0
AACCUG (0-1)	0	1	0	0	0	0
CUAAAG	0	0	0	0	0	0
UAAACG (0-1)	1	1	1	1	1	0
AUCAAG	0	0	0	0	0	0
ACAAUG	1	0	0	0	0	0
AACAUG	0	0	0	0	0	0
AAAUCG	0	0	0	0	0	0
AAAAUG	0	0	0	0	0	0
CUCUCG	0	0	0	0	0	0
CCUACG	0	2	0	0	0	1
CCCUUG	0	1	0	0	0	0
UCUACG[2]	0	0	0	0	0	0
UCCAUG (?)	0	0	0	0	0	0
CCUAUG (?)	0	0	0	0	0	0
AUCCUG	1	0	1	1	1	(1)
UAAUCG	1	1	1	1	1	0
AUCAUG	0	1	0	0	0	0
AUACUG	1	1	0	0	0	0
AAUCUG	0	0	0	1	1	0
U,A,AUAG	0	0	0	0	0	0
AAUAUG	0	0	0	0	0	0
AAAUUG	0	0	0	0	0	0
UCUUCG	0	0	0	0	(1)	?
CCUUUG[1]	1	0	0	0	0	0
UUA,UA,G (~2)	0	0	0	0	0	(1)
UUUUC	0	0	1	0	0	0
CUUUUG[1]	0	0	0	0	0	?
Heptamers						
ACCACG	0	0	0	0	0	0
CCAAAAG[1]	0	0	0	0	0	0
AACAAAG	0	0	1	0	1	0
CAACACC	1	1	1	(0)	0	0
ACCAAUG	0	0	0	0	0	0
AAAUCCG	0	0	0	0	0	0
CUCCUAG	0	0	0	0	0	0
AUCUACG	0	0	0	0	0	0
A(UA,CA)U	0	0	0	0	0	0
CCUUUCG[2]	0	0	0	0	0	0
(A/C)UCUUG[2]	0	0	0	0	0	0
CUUUCAG	0	0	0	0	0	0
AUUCUUG[2]	0	0	0	0	0	0
ACUUAUG[2] (0-1)	0	0	0	0	0	?
ACUAUUG[2] (0-1)	0	0	0	0	0	0
AAUUUCG[2]	0	0	0	0	0	0
AAUUUUG[2]	0	0	0	0	0	0
AUUUUUG[1]	0	0	0	0	0	(0)
$(CU_3)UCG$	0	0	0	0	0	0
$(CU_2)UCG$	0	0	0	0	0	0

Light copy

TABLE XV (cont.)

Oligonucleotide sequence	R. spheroides 16S	E. coli 16S	B. subtilis 16S	G. alpicola 16S	Porphyridium chloroplast 16S	Wheat cytosol 18S
Octamers						
CCA,CA,AAC	0	0	0	0	0	0
AAACAAAG	0	0	0	0	0	0
A_{0-1}(CCA,CA)CUG	?	?	?	?	?	0
C_{0-1}(UU,AU)ACG[3]	0	0	0	0	0	0
C_{0-1},ACUCAA,CG[1]	0	0	0	0	0	0
AUACCCUG	1	1	1	0	0	0
A_{0-1}(CAA$_{1-0}$,C_{0-1},UA)UCG	0	0	0	0	0	0
$C_{0-1}A_{0-1}$,C_{0-1}UA,CAA$_{0-1}$,UG	?	?	?	0	?	0
CCCUUAUG	0	0	0	0	0	0
(C_2U)UUUCG	0	0	0	0	0	(0)
AAU,C_{1-2},U_2,G	0	0	0	0	0	(0)
UUCUA,UA,G	0	0	0	0	0	0
(C_2U_2)G[2]	0	0	0	0	0	0
(CU)UUAUU	0	0	0	0	0	0
Nonamers						
CCAAAA$_{1-3}$G	0	0	0	0	0	0
A(A/C)ACCUG	0	0	0	0	0	0
A [illegible]	0	0	0	0	0	0
A [illegible]	?	?	?	?	?	?
C [illegible] 2	0	0	0	0	0	0
[illegible]	0	0	0	0	0	(0)
CUAACUCCG[1]	1	1	0	1	1	0
UA(CUA,CCA)G[1]	0	0	0	0	0	0
UUUAAUUCC	1	1	1	1	0	0
unsequenced (1)	0	0	0	0	0	0

Oligonucleotide sequence	R. spheroides 16S	E. coli 16S	B. subtilis 16S	G. alpicola 16S	Porphyridium chloroplast 16S	Wheat cytosol 18S
Decamers						
A_{0-1}(C_{3-2}U)A AAG[2]	0	0	0	0	0	0
AAACUCAAAG	0	0	1	1	1	0
C_{0-2}A,C_{2-3}A,UCCUG	0	0	0	0	0	0
C_{0-2}A,C_{2-3}A,UCA,UG	?	0	0	0	0	0
CUUUCA,CA,CG[2]	0	0	0	0	0	0
CA,UCCUA,UA,G[1]	0	0	0	0	0	0
CA,(C_{0-1}A)$_{0-1}$((UC)C_{0-1}UUA$_{1-2}$)CG	0	0	0	0	0	0
CA,(C_{0-1}A)$_{0-1}$,CUUA$_{1-2}$,UG	0	0	0	?	?	0
UCAAAAUCUG	0	0	0	0	0	0
CUACUACUUG[1]	0	0	0	0	0	0
Undecamers and larger						
CCUA(CCUA,UA)G	0	0	0	0	0	0
CUUUCA,UA,CA,G	0	0	0	0	0	0
AACAACAACCUG[2]	0	0	0	0	0	0
(A_{0-2},UUUCA$_{2-1}$),CCA,UUG	0	0	0	0	0	0
CA,CUUCAAAA,CUA,G	0	0	0	0	0	0
unsequenced (2-3)	0	0	0	0	0	0

This table lists all oligonucleotides larger than tetramers present in wheat mitochondrial 18S rRNA and indicates which of these are also present in (1) R. spheroides 16 S (Zablen and Woese, 1975), (2) E. coli 16 S (Uchida et al., 1974), (3) B. subtilis 16 S (Woese et al., 1976), (4) G. alpicola (a unicellular blue-green alga) 16 S (Bonen and Doolittle, 1976), (5) Porphyridium (a eukaryotic red alga) chloroplast 16 S (Bonen and Doolittle, 1975), and (6) wheat cytosol 18 S rRNA. Numerals indicate number of copies of coincident sequences. (1) and (0) inidicate that coincidence is not certain but very probable, or very improbable, respectively. For those wheat mitochondrial 18 S oligonucleotides which (because of low specific activity) cannot now be completely sequenced, results of secondary and tertiary analyses are indicated. It should be realized that comparisons of secondary and tertiary digestion products to those expected for (known) prokaryotic and chloroplast 16 S rRNA-derived ologonucleotides of similar primary fingerprint position is usually sufficient to exclude sequence coincidence. Instances in which coincidence is possible are indicated by '?'. A/C denotes A or C. Asterisk indicates modified nucleoside. The 'unsequenced' pentamer may be modified. Refer to TABLE XIV for definition of superscripts 1, 2, and 3. Molar yields were determined as described in TABLE XIV. (The estimated size of wheat mitochondrial 18 S rRNA as determined by the method described in TABLE XIV is ca. 1700 nucleotides.)

In the above scheme, the secondary products indicated the nucleotide composition (A_2, C_4, U_2, G) and the partial sequence [AUAC(C_2U)G]. Tertiary digestion of the partial product (C_3U)G was necessary to position the U, thus giving the entire sequence.

As is apparent from an examination of both TABLES XIV and XV, several oligonucleotides (especially the larger ones) could not be sequenced in their entirety. This was mainly due to the coincident migration of oligomers on the primary fingerprint (i.e., two or more oligomers present in the same spot). It was not always easy to determine which secondary fragments were associated with which oligomer. This problem was complicated by the fact that in some fingerprints used for sequence analyses, the oligonucleotides were not as well separated as expected, due to streaking in the second dimension. Another problem was that the amount of radioactivity remaining by the time the tertiary cleavage products were obtained was insufficient to allow further analysis. It should be emphasized again that the specific activity of the 18 S rRNA species was relatively low in spite of the large quantity of [^{32}P]orthophosphate (> 100 mCi) used in the labeling experiment.

In order to obtain even the results presented here, two independent preparations of the 18 S rRNAs had to be analysed.

An additional difficulty arose in the analysis of the cytosol 18 S rRNA oligonucleotides. Here, the presence of a large number of modified nucleotides, for which appropriate markers were not available, precluded a determination of the composition as well as the sequence of many of the oligonucleotides containing modifications. This difficulty was not unexpected, since Lau et al. (1974) had previously shown that each molecule of cytosol 18 S rRNA (chain length ca. 2000 nucleotides) contains about 36 pseudouridylate and 30 $O^{2'}$-methylnucleoside residues. This high level of modification (3 - 4 modified nucleotides per 100) is similar to that found for other eukaryotic 18 S rRNAs (cf. SECTION I.B.(ii).c). A similar problem was not encountered in the analysis of the mitochondrial 18 S rRNA because of the low level of modification in this species. Only three modified oligonucleotides (G*CCG, CC*CCG, $C\sim_1$($\overline{NN}$*,AU)ACG, the asterisks denoting modification) were detected during sequence analysis of the latter RNA species. It should be noted that sequence analysis suggested a much lower level of modified nucleotides in

wheat mitochondrial 18 S rRNA than did nucleotide composition analysis (TABLE X, SECTION II.3.C.(iv).c) (~0.1-0.2 vs. ~1.0 modified nucleotides per 100). As discussed earlier, contaminating cytosol 18 S rRNA could have contributed in large measure to the pseudouridine and $O^{2'}$-methylnucleoside residues detected in wheat mitochondrial 18 S RNA.

It should also be noted here that the modified dimer m_2^6A-m_2^6A (found in the kasugamycin-sensitive sequence; Helser et al., 1972) is probably present in the cytosol 18 S rRNA, in the sequence m_2^6A-m_2^6ACCUG. The modified dimer is found in the 16 S rRNA of a large number of prokaryotes, including E. coli (Fellner, 1969), as well as in the 17 - 18 S rRNA of a number of eukaryotes, including yeast (Klootwijk and Planta, 1973) and HeLa cells (Maden and Salim, 1974). In the latter case, the dimer is present in the sequence m_2^6A-m_2^6ACUG, which is very close to the presumptive wheat cytosol homologue. Although a similar oligonucleotide was not detected in T_1 fingerprints of wheat mitochondrial 18 S RNA, it is noteworthy that nucleotide composition analysis (TABLE X, SECTION II.3.C.(iv).c) has provided presumptive evidence for the presence of m_2^6A (at comparable levels) in

both the cytosol and mitochondrial 18 S rRNAs of wheat. Dubin (1974) has reported the presence of one m_2^6A residue per molecule of hamster cell 13 S mt-rRNA (the small-subunit RNA of hamster mitoribosomes). The tentative identification of m_2^6A in wheat cytosol 18 S but not 26 S rRNA is in agreement with the results of previous analyses of these RNA species (Lau et al., 1974).

TABLE XV, in addition to presenting the catalogue of the wheat embryo mitochondrial 18 S rRNA, also indicates the presence or absence of identical oligonucleotide sequences in (1) three bacterial (Rhodopseudomonas spheroides, Escherichia coli, and Bacillus subtilis) 16 S rRNAs (Woese et al., 1976; Uchida et al., 1974; Zablen and Woese, 1975), (2) a blue-green algal (Gloeocapsa alpicola) 16 S rRNA (Bonen and Doolittle, 1976), (3) a red algal (Porphyridium) chloroplast 16 S rRNA (Bonen and Doolittle, 1975), and (4) wheat embryo cytosol 18 S rRNA. It should be emphasized that in the case of the latter comparison, the incompleteness of the cytosol 18 S rRNA catalogue was not a handicap, since in all cases where the oligonucleotide sequence was not known, the secondary digestion products were sufficient to rule on the question of coincidence.

TABLE XVI summarizes, for each oligonucleotide size class ($N \geq 5$) and for all size classes taken together, (1) the number of oligonucleotide sequence coincidences observed between wheat mitochondrial 18 S and each of the six RNAs to which it is compared, and (2) the (mean) number of sequence coincidences expected to arise by chance from any two RNAs of unrelated random sequence but of similar size. (Mean expectations for $N = 5$ and $N = 6$ were determined by a computerized Monte Carlo simulation [1000 trials], while those for $N > 7$ were approximated as described by Bonen and Doolittle, 1975).

Wheat embryo mitochondrial 18 S rRNA is clearly prokaryotic in nature, sharing with each of the (bacterial, blue-green algal, and chloroplast) 16 S rRNAs significantly more than the number of oligonucleotide sequences expected by chance. Even when scoring of coincidence is restricted to oligonucleotides with sequences indicated (in TABLE XV) as certain or very probable, the likelihood that the levels of coincidence observed arise at random (i.e., do not reflect homology) is less than 0.000001. On the other hand, the number of sequence coincidences observed between mitochondrial 18 S rRNA and cytosol 18 S rRNA is little

TABLE XVI

SUMMARY OF THE NUMBER OF SEQUENCE COINCIDENCES FOR OLIGONUCLEOTIDES OF SIZE N $\geq$ 5 BETWEEN WHEAT MITOCHONDRIAL 18 S AND THE HOMOLOGOUS PROKARYOTIC, CHLOROPLAST, AND WHEAT EMBRYO CYTOSOL rRNAs

Oligonucleotide Size Class	*R spheroides* 16S	*E coli* 16S	*B. subtilus* 16S	*C. alpacola* 16S	*Porphyridium* chloroplast 16S	Wheat cytosol 18S
Pentamers						
Observed	20	19	17	16	22	11
Expected	11	14	11	12	13	10
Hexamers						
Observed	7	9	6	6	9	3-5
Expected	4	3	3	3	3	4
Heptamers						
Observed	1	1	2	0	1	0-1
Expected	0 4	0.4	0.3	0 4	0 5	0 6
Octamers						
Observed	1-3	1-3	1-3	0-1	0-2	0
Expected	0 06	0.06	0.06	0 06	0.08	0 1
Nonamers						
Observed	2-3	2-3	1-2	2-3	1-2	0-1
Expected	0.02	0.02	0.02	0 02	0 02	0.02
Decamers						
Observed	0-1	0	1	1-2	1-2	0
Expected	0.003	0.002	0.003	0 005	0.004	0 006
All Size Classes						
Observed	31-35	32-35	28-31	25-28	34-38	14-18
Expected	15	17	13	15	16	15
Observed minus expected	16-20	15-18	15-18	10-13	18-22	(-1)-3

The data on which this table is based are presented in TABLE XV. Where ranges are indicated, lower values include only coincidences of sequences indicated (in Table XV) as certain or very probable; higher values include, in addition, those oligonucleotides for which sequence coincidence is considered possible (denoted '?' in TABLE XV).

if any greater than that expected by chance, and the base sequence homology between them is therefore at or below the limits detectable by these methods (60%) (Bonen and Doolittle, 1976).

There exist a number of oligonucleotides which occur in most of the prokaryotic 16 S rRNAs and thus appear to be remarkably well-conserved in these molecules. Woese et al. (1975), in comparing catalogues of 16 S rRNAs from 26 diverse bacterial species to that of E. coli 16 S rRNA, have defined 26 oligonucleotides ($N \geq 5$) to be "universal" among bacteria (present in E. coli and at least 24 other species). FIGURE 16 shows the presence or absence of these "universals" in both wheat embryo cytosol and mitochondrial 18 S rRNA catalogues. The large number of universals (17) found in the latter catalogue is another indication of the strong prokaryotic affinity of this molecule. The cytosol catalogue contains very few (4) of these universals. In addition to the universal sequences, Woese et al. (1975) also defined a further 27 sequences to be "conserved" (present in the 16 S rRNAs of E. coli and at least 14 other species) and showed that these sequences (plus the universals) occurred in nine relatively restricted regions within the known primary sequence of E. coli 16 S rRNA.

FIGURE 16

PRESENCE OR ABSENCE OF "UNIVERSAL" OLIGONUCLEOTIDES IN THE T_1 CATALOGUES OF WHEAT EMBRYO CYTOSOL AND MITOCHONDRIAL "18S" RIBOSOMAL RNA

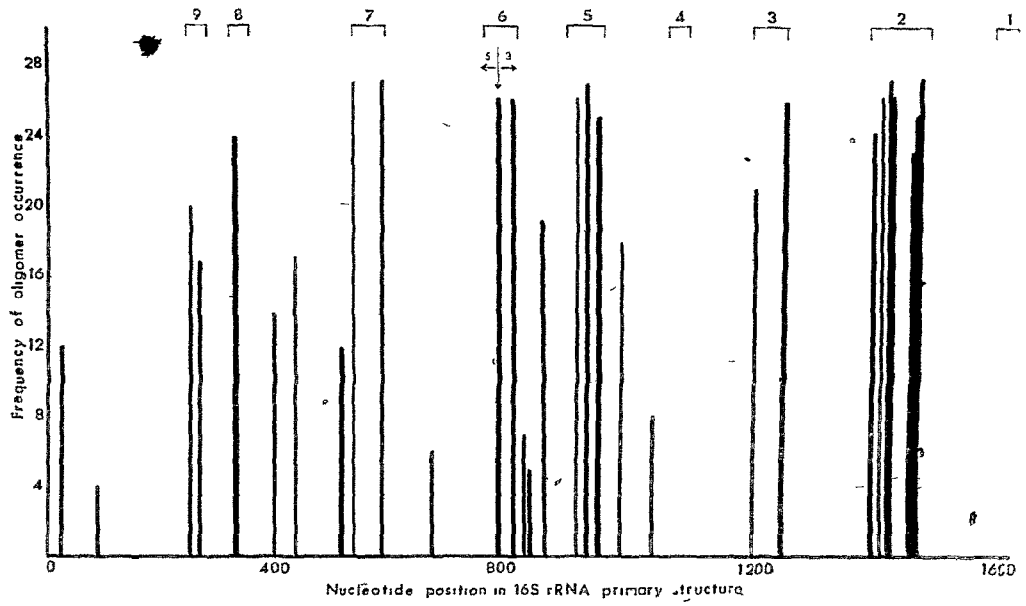
"UNIVERSAL" SEQUENCE	CYTOSOL "18S"	MITOCHONDRIAL "18S"
G*CCG		•
CC*CCG		•
CAACG		
C*AACG		
ACACG	•	•
CACAAG		•
UCCCG		
CUCAG		•
AUCAG	•	•
AAUCG		•
UAAAG		•
AAAUG		
UAAACG		•
AAUACG		
CAACUCG		•
U*AACAAG		
CCACACUG		(•)
UACACACCG	?	(•)
UUAAG		
AUUAG		•
AAUUG	•	•
UUCCCG		
UAAUCG		•
UAAUACG		
CUACAAUG		(•)
AUACCCUG		•
UUUAAUUCG		•
	3 4 / 27	15 18 / 27

? indicates possibly present
(.) indicates probably present

Wheat mitochondrial 18 S rRNA shares at most 35 oligonucleotide sequences ($N \geq 5$) with E. coli 16 S rRNA. However these comprise, in addition to 17 (64%) of the universal sequences, ten (36%) of the conserved sequences, but only eight (13%) of the approximately 60 E. coli oligonucleotides defined as neither universal nor conserved. (Wheat mitochondrial 18 S RNA also retains the modified tetramer G*CCG found in all prokaryotic rRNAs catalogued by Woese and co-workers (Woese et al., 1975)).

Of the 28 universal or conserved sequences common to wheat mitochondrial 18 S and E. coli 16 S rRNAs, 22 can be assigned unambiguous (unique) positions in the primary sequence of the latter (FIGURE 17). Of these 22, more than two-thirds (15) appear in the 3'-terminal one-half of the molecule, and nearly one-third (7) are clustered in the most highly-conserved region identified by Woese et al. (1975) (region 2, comprising only 7% of the total 16 S rRNA length). A similar clustering of conserved sequences (especially in region 2) has been reported for chloroplast rRNAs (Bonen and Doolittle, 1975; Zablen et al., 1975) and, together, these results suggest that at least some of the functional constraints on rRNA structure are similar in prokaryotes and organelles.

FIGURE 17



The position in the primary sequence of E. coli 16 S rRNA of T_1 oligonucleotides which are coincident between this RNA species and wheat mitochondrial 18 S rRNA. The width of the vertical bars is proportional to the lengths of the wheat mitochondrial oligonucleotides that they represent. The height of each bar indicates the number of other prokaryotic 16 S rRNAs in which that particular oligonucleotide is also found. The bracketed regions represent the prokaryotic 16 S rRNA conserved regions (Woese et al., 1975).

E. DISCUSSION

As has been noted in the introduction to this section, mitochondrial and prokaryotic ribosomes display a number of striking functional homologies. On the other hand, comparative studies among a wide range of eukaryotes have failed to reveal any obvious structural homologies between mitochondrial and prokaryotic ribosomal RNAs. The sizes of mt-rRNAs vary, from smaller than the homologous prokaryotic RNA species to possibly even larger than the cytosol homologues of the same organism. There is a remarkable variation in the G + C content of mitochondrial rRNA, ranging from a low of 23% in yeast (Reijnders, et al., 1972) to a high of 55% in wheat (Gray, 1974a). In contrast, size and G + C content are relatively constant among a wide range of prokaryotic rRNAs (Pace, 1973). In some instances, it might even be argued that a particular mt-RNA is more closely-related to its cytosol counterpart than to the prokaryotic homologue. For example, wheat mitochondrial rRNAs have base compositions and sizes (as determined by polyacrylamide gel electrophoresis under non-denaturing conditions) similar to the rRNAs found in the cytosol. Also, a comparative analysis of the finger-

prints of mammalian cytosol and mitochondrial 18 S and 26 S rRNA species (Dierich et al., 1975; cf. SECTION III. C.(i)) suggests very few sequence differences between these species. In addition, Dubin and Shine (1976) have recently analysed the 3'-terminal sequence of mitochondrial 13 S rRNA from hamster cells and have found it to be similar to the corresponding terminus of eukaryotic cytosol 18 S rRNA but different from that of its prokaryotic homologue. Therefore, the outcome of the study reported here was by no means predictable at the outset, and it was both surprising and pleasing to have obtained such definitive results.

The data which have been presented in the RESULTS section demonstrate unequivocally that wheat mitochondrial 18 S rRNA is indeed prokarytic in nature, sharing with bacterial, blue-green algal, and chloroplast 16 S rRNA more sequence homology than would be expected by chance alone, while having little or no homology (at the level of detection by these methods) with its wheat cytosol homologue. Although these results can be most easily interpreted in terms of the endosymbiotic hypothesis of the origin of mitochondria, they do not by themselves

prove it, and other interpretations are possible. For example, only if it could be demonstrated that the rate of nucleotide substitution has remained constant in nuclear, mitochondrial, and prokaryotic rRNA cistrons would the data presented here necessarily indicate the point in evolutionary history when these homologues diverged from one another. Otherwise, the sequence similarity between wheat mitochondrial 18 S rRNA and prokaryotic 16 S rRNAs might only represent the specific retention of primitive characteristics from a common ancestor, as has been suggested by Uzzell and Spolsky (1974). Indeed, nucleotide substitution has been shown not to be constant in different genomes. For example, nucleotide sequence analyses of the 5.8 S "satellite" rRNAs from yeast, humans, and plants (both dicotyledons and monocotyledons) demonstrate that two species of plants (even two monocotyledons) differ as much as do the yeast and human rRNA species (Philips and Carr, 1977). However, on the basis of overwhelming classical taxonomic data, it would be absurd to suggest that two species of monocotyledenous plants diverged from one another at the same time in evolution as did the yeast and human lines.

Therefore, when attempting to utilize the kinds of analyses presented here to ascertain evolutionary relatedness, we are faced with what at first glance may appear to be an insurmountable problem. However, by obtaining sequence information from other small-ribosomal-subunit RNAs, both cytosol and mitochondrial, this basic reservation about relating homology to evolutionary divergence might be overcome. For instance, if it can be demonstrated that the nuclear-coded rRNAs as a group are phylogenetically-related, as are the mitochondrial rRNAs, and that these two distinct groups of molecules have different roots among the prokaryotes, then the evidence supporting the endosymbiotic hypothesis would indeed be strong. In order to accommodate this data with the alternative ("direct filiation") hypothesis, one would necessarily have to argue for a type of convergent evolution between mitochondria and some species of bacteria, which appears highly unlikely.

In addition to establishing the prokaryotic nature of the wheat mitochondrial RNA molecule, the sequence analyses reported in this section demonstrate that regions of stringent conservation within the small-ribosomal-subunit RNA molecule (Woese et al., 1975) are not restricted to the

prokaryotes and chloroplasts but extend to the mitochondria as well. The fact that most of the homologous oligonucleotides found between the wheat mitochondrial 18 S rRNA and the E. coli 16 S rRNA are confined to the nine regions of conservation (as defined by Woese et al., 1975) lends additional support to the idea that these areas are of great functional significance. In light of the obvious overall functional equivalence of eukaryotic cytosol and prokaryotic ribosomes, it is interesting to find that the wheat cytosol 18 S rRNA (and also the cytosol homologues of Porphyridium and Chlamydomonas (Bonen and Doolittle, unpublished)) does not contain the same conserved sequences. Perhaps in prokaryotes, chloroplasts, and mitochondria these conserved regions serve in some ribosomal processes which do not occur in the eukaryotic cytosol. The cytosol small-ribosomal-subunit RNA may indeed contain regions of sequence conservation (Khan and Maden, 1976; Bonen and Doolittle, unpublished), but ones distinct from those found in the prokaryotic, chloroplast and mitochondrial homologues. Analysis of additional cytosol 18 S rRNAs from different organisms should help to answer this question.

The chain length of the wheat cytosol and mitochondrial 18 S rRNA species could not be determined with any degree of precision, due to the uncertainty in the sizes and in the number of copies of many oligomers. However, from semi-quantitative analysis of the relative proportions of the radioactivity in several uniquely-migrating oligomers, one has the definite impression that the mitochondrial 18 S rRNA has a lower chain length than the wheat cytosol 18 S rRNA. This is in marked contrast to the impression one gets from the behaviour of these two RNA species in non-denaturing polyacrylamide gels, where the mitochondrial 18 S RNA migrates distinctly more slowly (and has, therefore, an apparently-higher molecular weight) than its cytosol counterpart. The question of the relative sizes of the wheat mitochondrial and cytosol 18 S rRNAs therefore remains an unresolved question at the present time, but one well worth pursuing because of its obvious implications with regard to the structure, function, and evolutionary origin of these two RNAs. It should be noted

in this regard that the actual size of a molecule no less well-characterized than the E. coli 16 S rRNA is also still in doubt. Primary sequence analysis by Ehresmann et al. (1975) has suggested a length for this molecule of 1580 - 1600 nucleotides. However, sedimentation and diffusion data lead Hill et al. (1977) and others to a computed size of 1700-1750 nucleotides.

The results presented here should be extended to other cytosol and mitochondrial rRNAs both in order to establish phylogenetic trees and to further define regions of conservation which are not only common among the prokaryotes and the mitochondria but also possibly unique to mitochondrial rRNAs. Specifically, sequence analyses of small-ribosomal-subunit mt-RNAs of smaller size and lower G + C content would establish whether prokaryotic conserved areas are present in only the wheat mitochondrial 18 S rRNA or are a general feature of all mitochondrial rRNAs. Mammalian small-mitoribosomal-subunit RNA would be an obvious candidate, since it has a low sedimentation coefficient (12 S - 13 S), indicating a very small size, and many sequences present in the larger wheat molecule would be expected to be absent here. One may ask whether

these deleted sequences include conserved areas or only regions of apparently minor functional significance. The same question may be asked of protist (e.g., yeast, Neurospora) small-mitoribosomal-subunit RNA, which, although it is much closer in size to the wheat homologue, has a markedly lower G + C content.

The 3'- and 5'-terminal sequences of the rRNA molecules might be regions of major functional significance and it has indeed been shown that mRNA binds to the 3'-terminus of E. coli 16 S rRNA (Steitz and Jakes, 1975). This latter region of the molecule contains polypyrimidine sequences and appears to be a conserved region among bacteria. This particular sequence does not appear to be present in mitochondrial small-subunit RNA (at least in hamster 13 S rRNA, Dubin and Shine, 1976), the 3'-terminus of which more closely resembles the homologous region of eukaryotic cytosol 18 S rRNA. The determination of other 3'-terminal sequences isolated from mitochondrial small-subunit RNAs should shed more light on this matter.

SECTION IV: NUCLEOTIDE SEQUENCE ANALYSIS OF MITOCHONDRIAL AND CYTOSOL 5 S RIBOSOMAL RNA

A. INTRODUCTION

In 1963, Rosset and Monier identified 5 S ribosomal RNA as a constituent of the 50 S subunit of E. coli ribosomes. Since then, 5 S rRNA has been isolated from the cytoplasmic and chloroplast ribosomes of higher organisms (Monier, 1972). It is a remarkably highly-conserved molecule in size (varying in length only between 118 and 122 nucleotides) (Pace et al., 1973; Payne and Dyer, 1971), in base composition (it contains no modifications) (Monier, 1974), in primary sequence [mammalian sequences are identical (Walker et al., 1975); plant, human and yeast sequences are very similar (Payne et al., 1973)], and in secondary structure (Fox and Woese, 1975; Hori, 1976). It is indispensable to the proper assembly (Horne and Erdmann, 1972) and functioning (Erdmann et al., 1971) of the ribosomes of which it forms a part.

Although a 5 S RNA has been found to be a universal component of prokaryotic and eukaryotic cytoplasmic and chloroplast ribosomes, the situation in the case of

mitochondrial ribosomes is less clear. Purified ribosomes from fungal (Neurospora (Lizardi and Luck, 1971); yeast (Borst, 1972)), protist (Euglena (Avadhani and Buetow, 1974)), and animal (Zylber and Penman, 1969; Attardi et al., 1972) mitochondria lack a 5 S RNA component; isolation of 5 S RNA from the mitochondria of these organisms has been considered to be an indication of contamination by cytoplasmic ribosomes. Leaver and Harmey (1973, 1976), however, report the presence of a 5 S RNA species in the mitoribosomes of higher plants.

This failure to find a 5 S RNA in most mitoribosomes is puzzling. The presence of such a highly-conserved molecule in all other translation systems suggests that this RNA species plays a fundamental role, which is difficult to reconcile with its apparent absence from the mitoribosome. This state of affairs might be explained in one of the following ways:

(1) Firstly, the function involving the 5 S RNA may have been dispensed with in the mitochondrial translation apparatus. This appears unlikely since all the postulated reactions in which this molecule participates [for example, in the binding of tRNA to the ribosome (Forget and

Weissmann, 1967; Richter et al., 1973) and in the formation of the initiation complex (Avadhani and Buetow, 1973)] involve functions which are also prominent in the mitochondrial system.

(2) Secondly, the mitochondrial 5 S RNA may have evolved so that it is no longer recognizable as such, even though it still retains the 5 S RNA function. For example, the mitochondrial 5 S RNA homologue might be covalently continuous with the large ribosomal subunit RNA. In prokaryotes (Kossman et al., 1971) 5 S rRNA is transcribed as part of a large precursor containing both the large (23 - 25 S) and small (16 - 17 S) ribosomal RNAs. It is conceivable that, during evolution, the gene(s) for mitochondrial 5 S RNA has (have) become integrated into the cistrons coding for the large subunit RNA of mitoribosomes. No evidence, however, has been advanced to support this hypothesis (Avadhani et al., 1976).

It is also possible that the mitochondrial 5 S RNA homologue has undergone a reduction in size, with all non-essential sequences being deleted. Such a "non-classic" 5 S RNA might easily be missed by the usual

techniques of detection (e.g., because it migrates with tRNA during polyacrylamide gel electrophoresis). There are some reports which, while not confirming this theory, are consistent with it. Chi and Suyama (1970), for example, have found small RNAs in the mitoribosomes of Tetrahymena, in addition to the 21 S and 14 S RNAs. Dubin et al. (Dubin and Friend, 1972; Dubin et al., 1974) have isolated a "3 S" RNA from hamster cell mitochondria and have suggested that it might be the mitochondrial equivalent of 5 S RNA. It is unmethylated and resembles the larger structural RNAs of mitoribosomes in base ratio and molar abundance. However, it has not been shown to be specifically associated with the large ribosomal subunit, nor has its presence been demonstrated to be essential for the proper functioning of the ribosome. Attardi's group (Gray and Attardi, 1973) have reported that HeLa cell mitochondria contain a similar RNA species, estimated to be 10 - 15 nucleotides smaller than the mitochondrial 4 S RNA and present in one copy per mitoribosome.

Recently, Attardi et al. (1976) have mapped a 4 S RNA in the spacer region between the 16 S and 12 S rRNA cistrons in HeLa cell mitochondrial DNA. Dawid et al. (1976) have observed the same situation in Xenopus laevis mito-

chondrial DNA. Although this molecule may be a mitochondrial homologue of 5 S rRNA, it is noteworthy that a tRNA gene, rather than a 5 S rRNA gene, is found in the analogous region of the E. coli chromosome (i.e., the region between the cistrons for the 23 S and 16 S rRNAs).

(3) Finally, the absence of a 5 S RNA from mitoribosomes could be simply an artifact of the isolation technique. Recent work from Kroon's laboratory (Agsteribbe et al., 1974; Datema et al., 1974) suggests that the native size of Neurospora mitochondrial ribosomes is 80 S and not 73 S, as has previously been reported. It is claimed that this difference is due to the loss of a 5 S RNA - protein complex from native 80 S ribosomes when EDTA-containing buffers are used in the isolation procedure. Treatment of mammalian large ribosomal subunits with EDTA does in fact result in the release of a 5 S rRNA - protein complex (Blobel, 1971; Horne and Erdmann, 1973; Dyer et al., 1976). This view is disputed by Neupert's group (Michel et al., 1976) which claims that Kroon et al. are observing contamination by cytoplasmic ribosomes, due to the use of a magnesium-containing buffer during mitoribosome isolation. While the presence of Mg^{2+} is indeed known to increase the

content of cytoribosomes in mitochondrial fractions (Kellems et al., 1975) a recent study (de Vries and Van den Bogert, 1976) of the ribosomal proteins of Neurospora "80 S" mitoribosomes (isolated by Kroon's procedure) has shown that there is little, if any, contamination by cytoplasmic 77 S ribosomes. Kroon et al. have yet to show that the 5 S rRNA they have isolated from Neurospora mitoribosomes is distinct from the cytosol 5 S rRNA. Until this is done, the controversy will remain unresolved.

It is interesting, in light of the failure to find a mitochondrial 5 S rRNA in all other systems (excluding the current dispute over the Neurospora 5 S rRNA), that Leaver and Harmey (1973, 1976) report the presence of a 5 S rRNA in higher-plant mitochondria. Plant mtDNA contains potentially more genetic information than the mtDNA from other sources (see GENERAL INTRODUCTION) and it is conceivable that in addition to retaining a number of messenger RNA genes that have been lost or transferred to the nucleus in other systems, plant mtDNA still codes for a conserved, full-size 5 S rRNA molecule. However, the putative plant mitochondrial 5 S rRNA has not been chemically characterized and shown to be structurally

distinct from its cytosol homologue. Until this is done, it is difficult to rule out entirely the possibility that one is dealing with an artifact.

Preliminary results with commercial wheat embryos (Gray, 1974a) indicated that a 5 S rRNA was also present in purified mitochondria of this plant and an analysis of the RNA from dormant and germinating viable embryos confirmed these observations. The production of highly-labeled wheat embryo mitochondrial and cytosol [^{32}P]RNA for the purpose of T_1 oligonucleotide cataloguing of the two 18 S rRNA species (SECTION II.3) permitted a more careful examination of this presumptive mitochondrial 5 S rRNA species than had been feasible with the unlabeled species. In this section, I describe the isolation, purification, and characterization of 5 S [^{32}P]RNA from purified mitochondria and from the cytosol of 24 hr-germinated wheat embryos. The results of oligonucleotide cataloguing studies demonstrate that wheat embryo mitochondria contain a 5 S RNA structurally distinct from the 5 S RNA present in the cytosol.

B. MATERIALS

Sephadex G-100 was supplied by Pharmacia Fine Chemicals (Uppsala, Sweden). All other enzymes, chemicals, solvents, and equipment were obtained as indicated in the previous sections of this thesis.

C. METHODS

(i) Isolation of [^{32}P]-Labeled Cytosol and Mitochondrial 5 S RNA

a. Purification of Cytosol and Mitochondrial sRNA

When cytosol [^{32}P]RNA was recovered from the aqueous phase of phenol extracts by ethanol precipitation, the RNA was found to be contaminated by a large amount of [^{32}P]-labeled, non-nucleic acid material. This material (probably inorganic polyphosphates) was recognized by its resolution from the RNA during polyacrylamide gel electrophoresis. The contaminating material was soluble in 3 M NaCl and therefore fractionated with the sRNA. To ensure that it would not subsequently interfere in nucleotide sequence analysis, the non-RNA [^{32}P]material was removed

by treating the crude sRNA fraction with 2-methoxyethanol (Kirby, 1956) and subsequently recovering the RNA by precipitation as the cetyltrimethylammonium (CTA) salt (Ralph and Bellamy, 1964).

Total cytosol sRNA (45 A_{260} units, prepared as described in SECTION II) was precipitated with ethanol, recovered by centrifugation, dried *in vacuo*, and dissolved in 2.5 ml water. Phosphate buffer (2.5 M K_2HPO_4/33% H_3PO_4, 25/1.25; 2.5 ml) and 2-methoxyethanol (2.5 ml) were added. The mixture was vortexed periodically for 5 min and then centrifuged at 2000 X g for 10 min at 5°. The upper organic phase was removed, combined with 0.5 vol. cold water and 0.5 vol. 1% cethyltrimethylammonium bromide (CTA-Br), and stored for 8 hr at 4°. The precipitate of CTA-RNA was then sedimented at 27,000 X g for 20 min and washed 3 X with water. The CTA-RNA was converted to the sodium salt by washing 3 X with 80% ethanol - 0.1% sodium acetate. The Na-RNA was dissolved in 2 ml water and the solution was made 0.1 M in sodium acetate, combined with 2 vol. 95% ethanol, and stored for 8 hr at -20°. This procedure was repeated once more and the RNA was stored as an ethanol precipitate. Recovery was *ca.* 85%.

The mitochondrial sRNA contained little, if any, of the non-RNA [^{32}P] contaminant. For this reason, and because of the small amount of mitochondrial sRNA available, it was not subjected to the above procedure.

b. Gel Filtration Chromatography on Sephadex G-100

The method of Azad and Lane (1973) was used to purify 5 S rRNA from the other components in the sRNA fractions. Approximately 40 A_{260} units of purified cytosol [^{32}P] sRNA was dissolved in 2 ml 0.05 M sodium acetate (pH 5.1) [buffer A] and heated for 5 min at 60°, to dissociate a small amount of aggregated RNA resulting from the 2-methoxyethanol treatment. The sample was layered on a Sephadex G-100 column (2.6 cm X 95 cm) pre-equilibrated with buffer A and eluted in the same buffer at a flow rate of 18 ml/hr. Fractions (4.5 ml) were collected (LKB Ultrorac Fraction Collector Type 7000, Stockholm) and the optical density and the radioactivity of each fraction were measured.

In order to monitor the purification of mitochondrial 5 S RNA, approximately 40 A_{260} units of unlabeled cytosol sRNA were mixed with 6.5 A_{260} units of mitochondrial [^{32}P]-

sRNA. Because of the possibility that heat treatment of the mitochondrial sRNA might cause aggregation (since heating total mitochondrial RNA in solution has been shown to result in substantial aggregation; SECTION II), the RNA sample was not heated, but was otherwise fractionated as described above. However, to ensure that no mitochondrial 5 S rRNA was in fact aggregated prior to application to the Sephadex column, the radioactive material eluting at the void volume of the column was recovered by ethanol precipitation, dissolved in buffer A, heated to 60° for 5 min, and chromatographed as described above.

The fractions were pooled as indicated in FIGURES 18 & 19. The volumes were reduced by flash evaporation in order to increase the RNA/solvent ratio and thus facilitate subsequent precipitation of the RNA. After addition of two vol. 95% ethanol, the solution was stored for 8 hr at -20°, after which the RNA was collected by centrifugation and washed 2 X with 70% isopropanol to remove the residual salt.

c. Polyacrylamide Gel Electrophoresis

The 2.8% polyacrylamide gels were prepared as described previously (SECTION II. 2.). The 10% gels were prepared as described previously for the 2.4% gels (SECTION II.2.) but using different amounts of ingredients [15 ml Acrylamide II, 7.5 ml 3E buffer, 25 µl TEMED, and 50 µl 10% ammonium persulphate] and the samples were run for about 3 hr at 5 mA/gel.

(ii) T_1 Oligonucleotide Cataloguing

T_1 RNase digestion of wheat embryo mitochondrial and cytosol 5 S RNA was carried out essentially as described in SECTION III using the RNA and enzyme concentrations given in TABLE XVII. The primary two-dimensional ionophoresis and subsequent secondary and tertiary analyses of the separated oligonucleotide spots were performed as described previously for the cytosol and mitochondrial 18 S rRNA sequence analyses.

TABLE XVII

CONDITIONS OF ENZYMATIC DIGESTION OF MITOCHONDRIAL AND CYTOSOL 5 S RNAs AND THE RESULTANT OLIGONUCLEOTIDES

A. PRIMARY DIGESTION WITH RIBONUCLEASE T_1

45 - 60 µg RNA; 1 µg enzyme (1 mg/ml water) / 15 - 20 µg RNA; 15 min , 37°

B. PRIMARY DIGESTION WITH RIBONUCLEASE A

60 - 80 µg RNA; 1 µg enzyme (5 mg/ml water) / 4 µg RNA; 30 min , 37°

C. SECONDARY AND TERTIARY DIGESTIONS WITH RIBONUCLEASES U_2, A, AND T_1

(i) RNAse U_2 -- Conditions as described in Table X

(ii) RNase A -- Conditions as described in Table X

(iii) RNase T_1 (with all RNase A-generated oligonucleotides): enzyme concentration 25 µg/ml; 2 hr , 37°

(iii) RNase A Oligonucleotide Cataloguing

The mitochondrial and cytosol 5 S RNA samples were digested with RNase A for 30 min at 37° using the RNA and enzyme concentrations given in TABLE XVII. The enzymic hydrolyses and the two-dimensional ionophoresis were performed as described for the T_1 "fingerprinting". Care was taken during transfer of the oligonucleotides from the cellulose acetate strip to the DEAE-cellulose sheet to ensure that the origin (which contained cytidylate) was also tranferred. The second-dimensional run was terminated when the blue dye reached the first bar of the rack (for ca. 10 hr at 1500V) to retain uridylate.

Since RNase A cuts at the 3'-end of both uridine and cytidine residues (pyrimidines) the size of the resultant oligonucleotides will, on the average, be less than in the case of T_1 RNase digestion where the enzymatic cleavage is more specific. This in conjunction with the fact that RNase A produces oligonucleotides containing fewer sequence possibilities (G and A stretches terminating with a pyrimidine compared to A, C, and U stretches terminating with G for T_1 oligonucleotides) normally simplifies the sequence determination. Two enzymes are used for secondary analyses,

T_1 and U_2 reg. RNases (concentrations given in TABLE XVII), and the rerunning of secondary digestion products in a second solvent system is sometimes necessary. The digestion on DEAE paper and subsequent transfer for ionophoresis were carried out as described for secondary analyses of T_1 oligonucleotides.

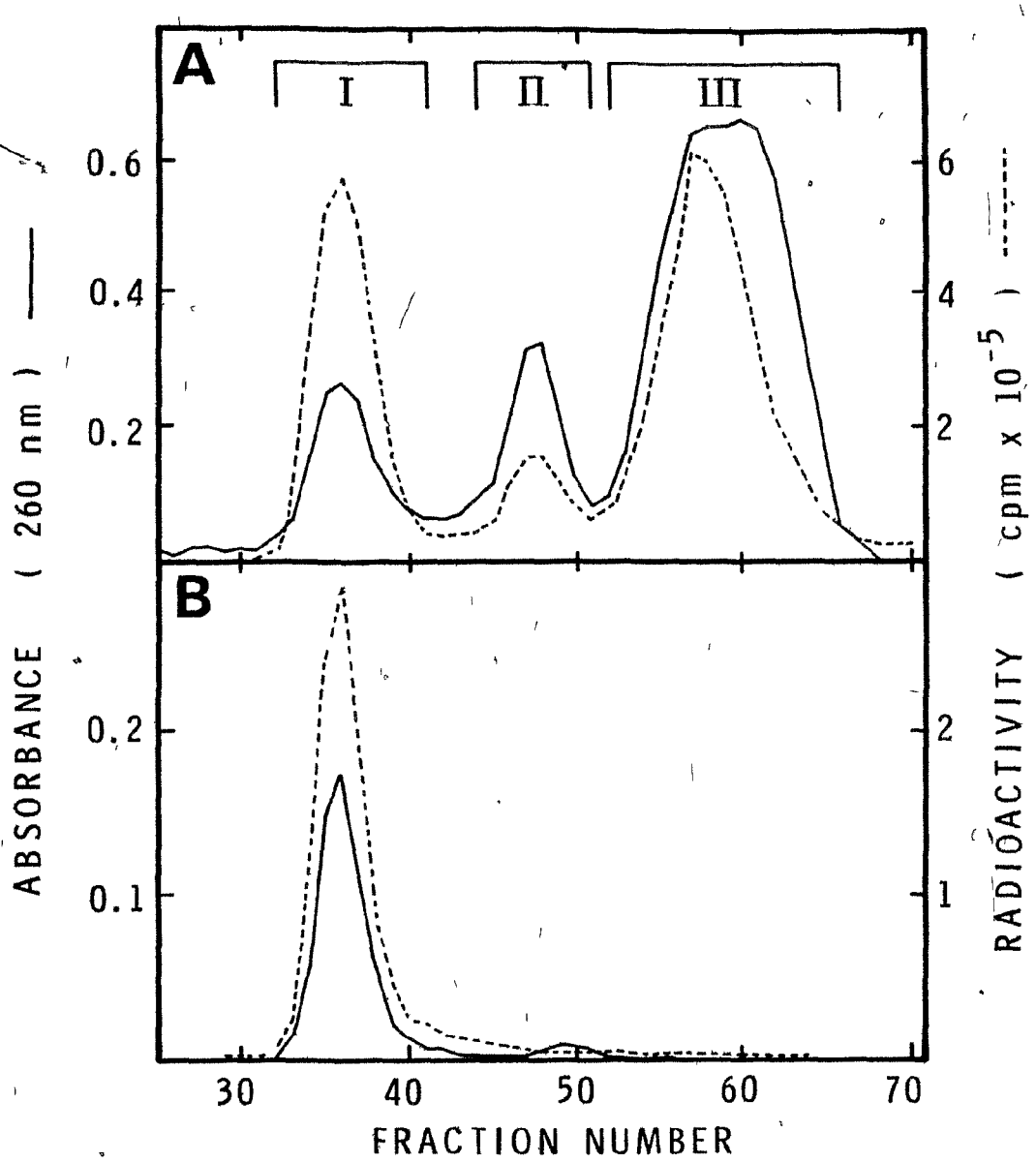
D. RESULTS

(i) Gel Filtration Chromatography

The fractionation of mitochondrial and cytosol [^{32}P]-sRNAs on Sephadex G-100 columns is shown in FIGURES 18 and 19, respectively. The elution profiles were very similar to that previously reported for bulk cellular [^{32}P] sRNA from germinating wheat embryos (Azad and Lane, 1975). In both cases the radioactivity profiles closely paralleled those of UV absorbance. However, the relative proportions of the radioactive peaks differed. Mitochondrial sRNA peak I (which eluted immediately after the void volume of the column) contained a substantially larger proportion of the total radioactivity than did the corresponding cytosol sRNA peak. When the mt-sRNA peak I material was heated and again chromatographed on the Sephadex column, it eluted in the same position (FIGURE 18B). This indicated that peak I was not an aggregate of smaller molecules (or, less likely, that if it was aggregated RNA, it was not dissociated upon heating).

In a further attempt to characterize the peak I material (obtained from both mitochondrial and cytosol sRNA),

FIGURE 18

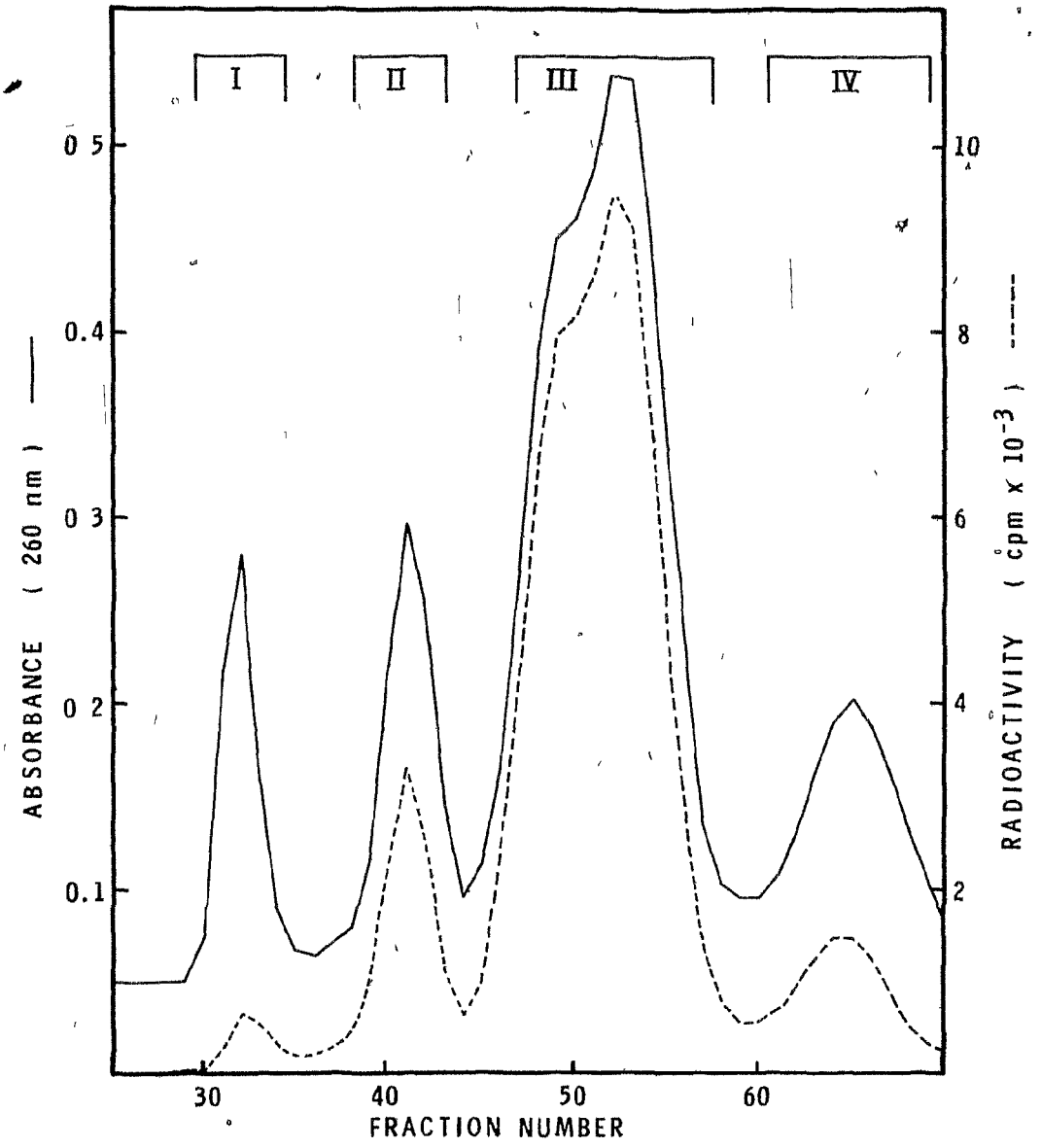


Distribution of ultraviolet absorbance (solid line) and radioactivity (dashed line) following Sephadex G-100 chromatography (A) of wheat embryo mitochondrial [^{32}P]sRNA (ca. 6.5 A_{260} units mt-sRNA + ca. 40 A_{260} units unlabeled bulk cellular sRNA). Panel (B) shows the profile obtained after Peak I from (A) was recovered, heat-denatured, and re-chromatographed. The conditions of fractionation are given in the text (SECTION IV.C.(i).b.). Ultraviolet absorbance was recorded directly from undiluted fractions and radioactivity was determined essentially as described in FIGURE 5. The heterodisperse RNA, 5 S RNA, and tRNA (eluting in Peaks I, II, and III, respectively) were pooled as indicated.

it was digested with T_1 RNase under conditions similar to those described previously (SECTION III) and then subjected to two-dimensional paper ionophoresis. Under these conditions, the radioactivity in mt-sRNA Peak I was largely unhydrolyzed and remained on the transfer line during ionophoresis in the second dimension. It fulfilled all the criteria of being DNA, since it was present in the void volume of the column (indicating large size), it was not hydrolyzed by T_1 RNase, and it was bound tightly to the DEAE-cellulose paper and did not migrate during ionophoresis (the second dimension). The presence of this large amount of labeled DNA would explain the higher proportion of radioactivity in Peak I of the mitochondrial sRNA when compared to that obtained from the cytosol sRNA. This latter material contained no DNA. (The presence of DNA in mitochondrial but not cytosol sRNA was confirmed by polyacrylamide gel electrophoresis; see SECTION IV.D.(ii), following).

In addition to DNA, the fingerprint of mt-sRNA Peak I material revealed a complex pattern of spots, and although the resolution was poor, it appeared that this represented contaminating fragments of high-molecular-weight mito-

FIGURE 19



Distribution of ultraviolet absorbance (solid line) and radioactivity (dashed line) following chromatography of wheat embryo cytosol [^{32}P]sRNA (ca. 40 A_{260} units) on Sephadex G-100. The conditions of fractionation are given in the text (SECTION IV.C.(1).b.). Ultraviolet absorbance was recorded directly from undiluted fractions and radioactivity was determined essentially as described in FIGURE 5. The heterodisperse RNA, 5 S RNA, and tRNA (eluting in Peaks I, II, and III, respectively) were pooled as indicated.

chondrial rRNAs. These fragments apparently purified with the mt-sRNA during salt fractionation but were large enough in size to be excluded from the Sephadex beads and thus eluted in the void volume of the column. Fingerprints of the Peak I material from cytosol sRNA also showed a complex pattern of oligonucleotides suggestive of contamination of this fraction with high-molecular-weight rRNA. Here, as expected, no DNA was present. It is interesting to note that heated bulk cellular sRNA from commercial wheat germ did not yield any Peak I material when chromatographed on Sephadex G-100 (not shown). The presence of Peak I in the cytosol sRNA from germinated embryos suggests that there is some breakdown of high-molecular-weight RNA during the germination process. The low specific activity of Peak I when compared to Peaks II (5 S rRNA) and III (tRNA) probably reflects the preferential degradation of unlabeled, high-molecular-weight cytosol rRNA during germination (Lau _et al._, 1974). Azad and Lane (1975) have also reported the presence of low-specific-activity, high-molecular-weight heterodisperse RNA in bulk cellular $[^{32}P]$sRNA from germinating wheat embryos.

The elution profiles of mitochondrial and cytosol sRNA each contained a symmetrical peak of radioactivity in the position of 5 S rRNA (Peak II). It is noteworthy that in both cases, there was an exact coincidence between the position of the radioactivity in Peak II and that of the unlabeled 5 S rRNA marker (contributed by added bulk cellular sRNA, in the case of the mt-sRNA fractionation). The separation of 5 S RNA from the following peak (tRNA) was very satisfactory and it was thus possible to isolate both mitochondrial and cytosol 5 S RNAs completely free from any tRNA contamination.

Peak III contained the tRNAs and in the case of the cytosol sRNA fractionation the radioactivity profile closely paralleled that of UV absorbance (FIGURE 18). The high left-hand shoulder was reproducible and an indication that tRNAs of different size classes were partially resolved on this column. Azad (1973) failed to show a similar shoulder when he chromatographed bulk cellular sRNA from germinating wheat embryos under similar conditions. However, Azad did not obtain the degree of resolution between the 5 S and tRNA fractions that was seen here and thus his failure to find a shoulder could simply reflect

this reduced resolution. The mitochondrial sRNA fractionation produced a tRNA peak with the UV absorbance profile representing a combination of a small amount (ca. 3 A_{260} units) of mt-tRNA and a larger amount (ca. 35 A_{260} units) of carrier bulk cellular tRNA. However, the radioactivity profile represented exclusively mitochondrial-specific tRNA (the term "mt-tRNA" being used in the sense that it was isolated from the purified mitochondrial fraction). It can be seen from FIGURES 18 and 19 that the size distribution of the mt-tRNAs was much narrower than in the case of the cytosol tRNA species, with the mitochondrial tRNAs on average appearing to be larger. This may reflect either a greater conformational instability of the mt-tRNAs under these conditions of fractionation (resulting in an unfolded structure with a higher apparent molecular weight) or real size differences between the two subcellular compartments of wheat tRNAs. It has been shown by other workers (Dubin and Friend, 1972) that animal mt-tRNAs, due to their relatively high A + U content, are more unstable to denaturing agents than are their cytosol counterparts, and that this results in an anomalously slow migration when they are electrophoresed in polyacrylamide gels. While these preliminary results

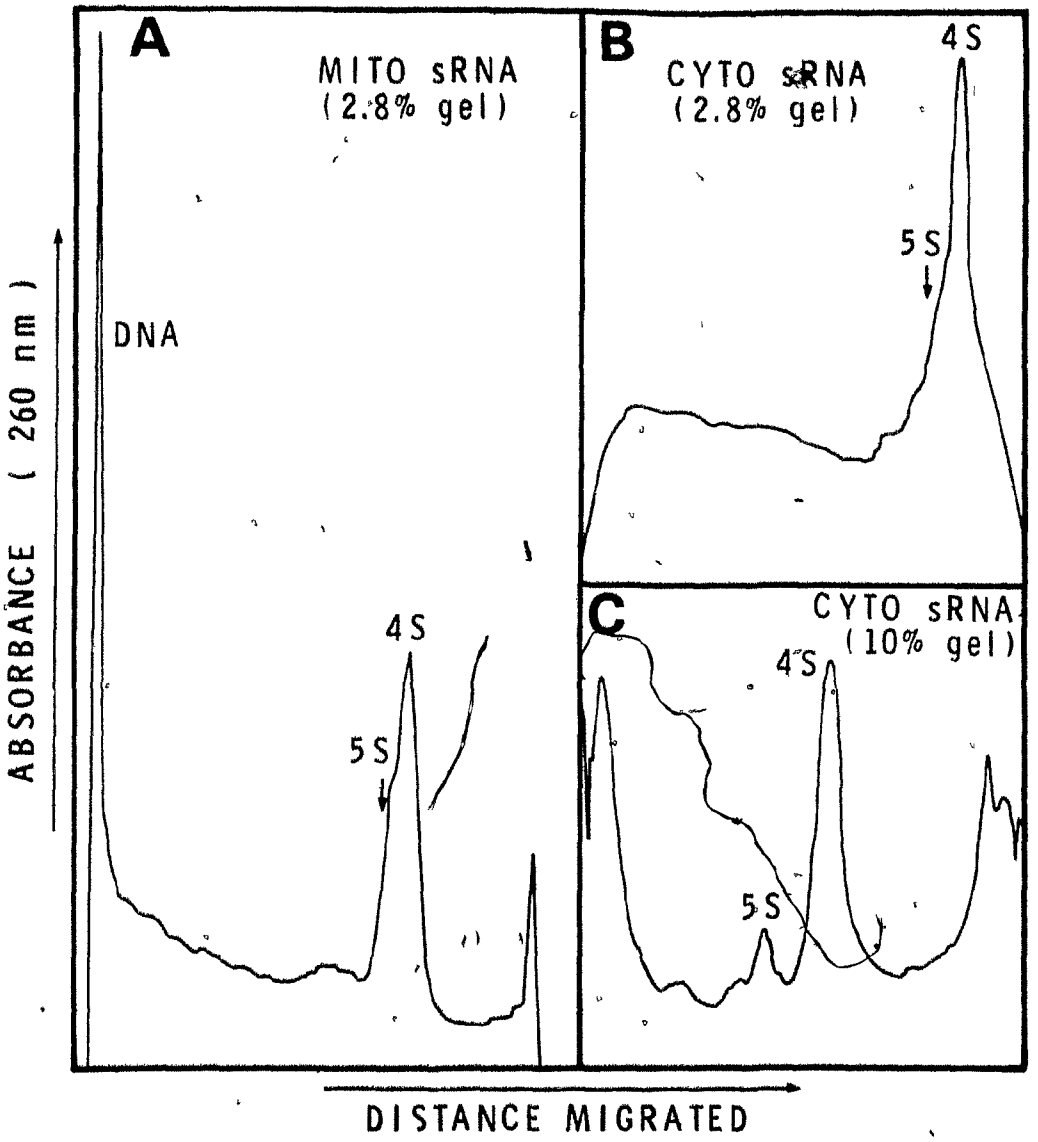
suggest that the tRNA extracted from purified wheat embryo mitochondrial fractions does not simply reflect cytosol contamination, additional experiments are required to confirm the observed chromatographic differences and to provide definitive evidence of structural differences between wheat mitochondrial and cytosol tRNA isoacceptors.

Peak IV of the cytosol sRNA fractionation profile possibly represents degraded RNA of very low molecular weight. The fractionation of mt-sRNA was terminated before the presence or absence of a similar peak could be ascertained.

(ii) Polyacrylamide Gel Electrophoresis

The UV absorption profiles of purified wheat embryo mitochondrial and cytosol sRNA after resolution on 2.8% polyacrylamide gels are shown in FIGURE 20, A and B, respectively. The cytosol sRNA appeared as a single, rapidly-migrating peak (tRNA) under these conditions, with a prominent shoulder (representing 5 S rRNA) on the heavier side. This is similar to results obtained with the comparable fractions isolated from commercial wheat germ and from dormant viable embryos. When electrophoresed on 10%

FIGURE 20



Polyacrylamide gel electrophoresis of purified wheat embryo cytosol [(B), 2.8% gel; (C), 10% gel] and mitochondrial [(A), 2.8% gel] sRNA fractions. Ultraviolet absorption profiles were determined as described in SECTION II.2.C.(v).

polyacrylamide gels (FIGURE 20, C), the cytosol sRNA fraction was completely resolved into a larger, faster-migrating peak (tRNA), and a smaller, slower-migrating peak (5 S rRNA) (present in a ratio of ca. 85:15). The mitochondrial sRNA fraction, in addition to the tRNA - 5 S rRNA peak, also contained DNA, which remained close to the origin during electrophoresis in 2.8% gels (FIGURE 20, A). The relative amount of DNA in this fraction can vary considerably, from less than 50% of the total sRNA up to and surpassing 80%. The bulk of this DNA is undoubtedly nuclear DNA which co-purifies with the mitochondrial fraction. As in the case of the cytosol sRNA, resolution of the mt-sRNA fraction on gels of smaller pore size revealed two well-separated components migrating with mobilities characteristic of tRNA and 5 S rRNA.

FIGURE 21 (A-C) shows the resolution on 10% polyacrylamide gels of the material in the three major peaks obtained by chromatography of cytosol sRNA on Sephadex G-100. Peaks II and III contained electrophoretically-pure 5 S rRNA and tRNA, respectively, while Peak I consisted exclusively of polydisperse, higher-molecular-weight material. These results are similar to those reported previously by

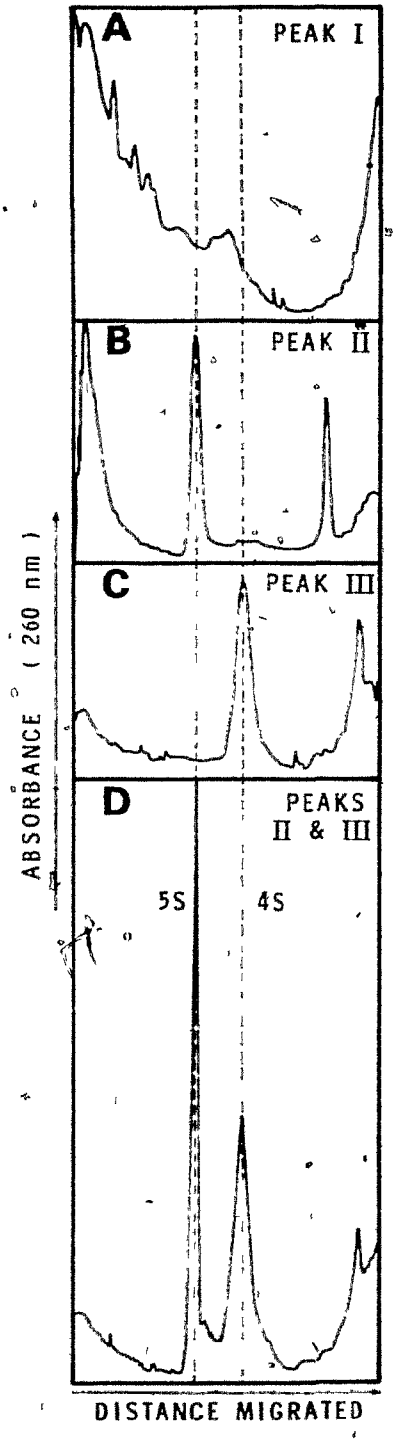


FIGURE 21

Polyacrylamide gel (10%) electrophoresis of purified wheat embryo cytosol heterodisperse (A), 5 S (B), 4 S (C), and 5 S + 4 S (D) RNAs. The RNA fractions were isolated from Sephadex G-100 columns as indicated in FIGURE 19 and ultraviolet absorbance profiles were determined as described in SECTION II.2.C.(v).

Azad (1973) for bulk cellular sRNA from germinating wheat embryos. Mixing experiments (Peak I and Peak II; FIGURE 21, D) confirmed the relative electrophoretic positions assumed by purified 5 S rRNA and tRNA when these were run on separate gels. Since all of the 5 S rRNA fraction from mt-sRNA was required for nucleotide sequence analysis, none of the Peak II material from the mt-sRNA fractionation was subsequently analyzed on polyacrylamide gels. However, the correspondence in the profiles of labeled mt-sRNA and unlabeled bulk cellular sRNA during Sephadex G-100 chromatography indicated that the apparent sizes of the mitochondrial and cytosol 5 S RNA components were very similar.

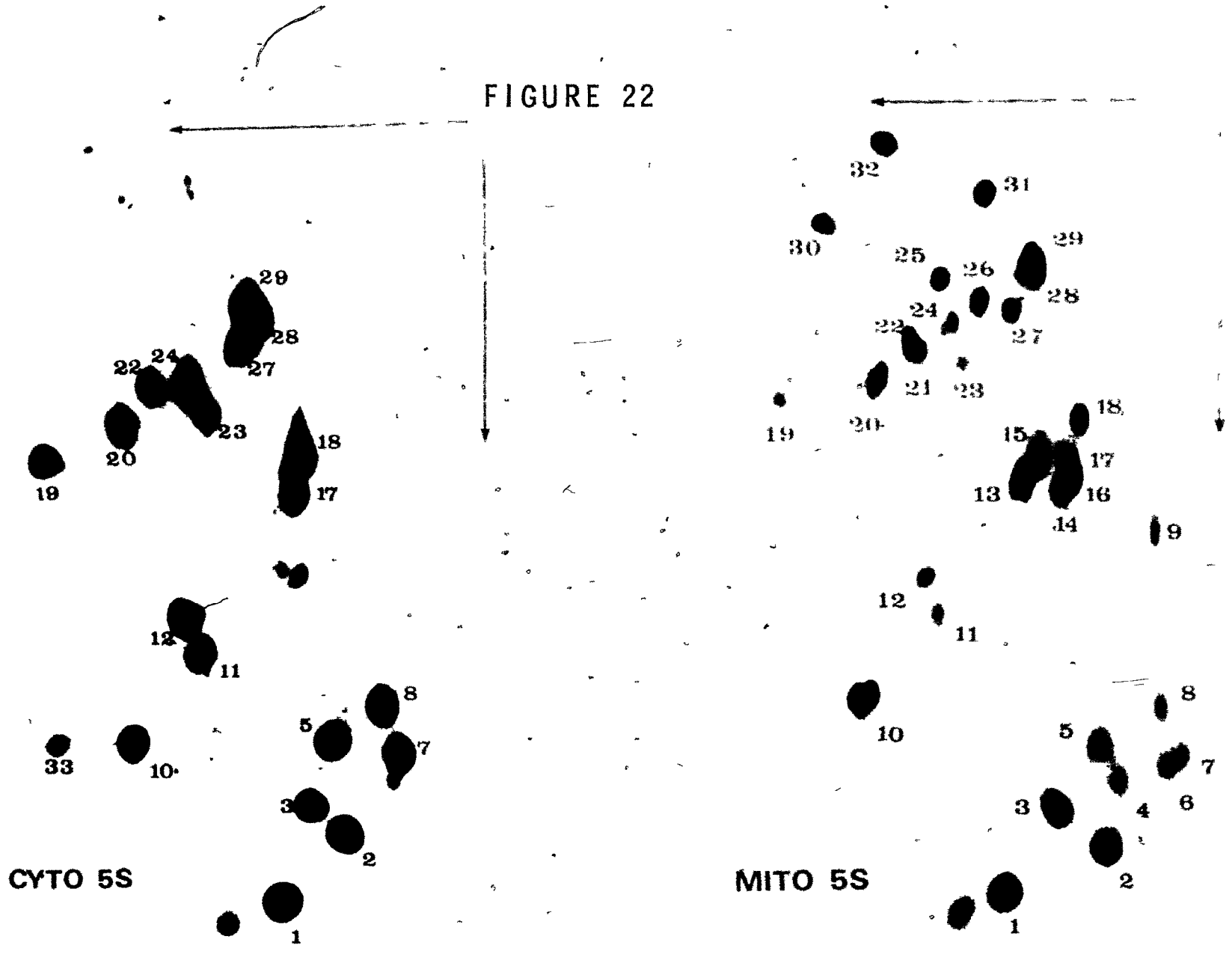
(iii) T_1 and RNase A Oligonucleotide Cataloguing

The primary fingerprints of wheat embryo cytosol and mitochondrial 5 S RNAs are presented in FIGURES 22 (T_1 fingerprints) and 23 (RNase fingerprints). The oligonucleotide spots were numbered beginning with the smallest in the fastest-moving "isoplith" of the mitochondrial species and progressing to the largest in the slowest-moving "isoplith". Similarly-migrating nucleotides in the finger-

LEGEND OF FIGURE 22

T_1 Ribonuclease fingerprints of wheat embryo cytosol (left) and mitochondrial (right) 5 S RNAs. Enzymatic hydrolysis of the two RNA species and subsequent two-dimensional ionophoresis of the digest were conducted as described in SECTION IV.C.(ii). The sequences of the oligonucleotides in the numbered spots on the fingerprints are listed in TABLE XVIII.

FIGURE 22


CYTO 5S
MITO 5S
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
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28
29
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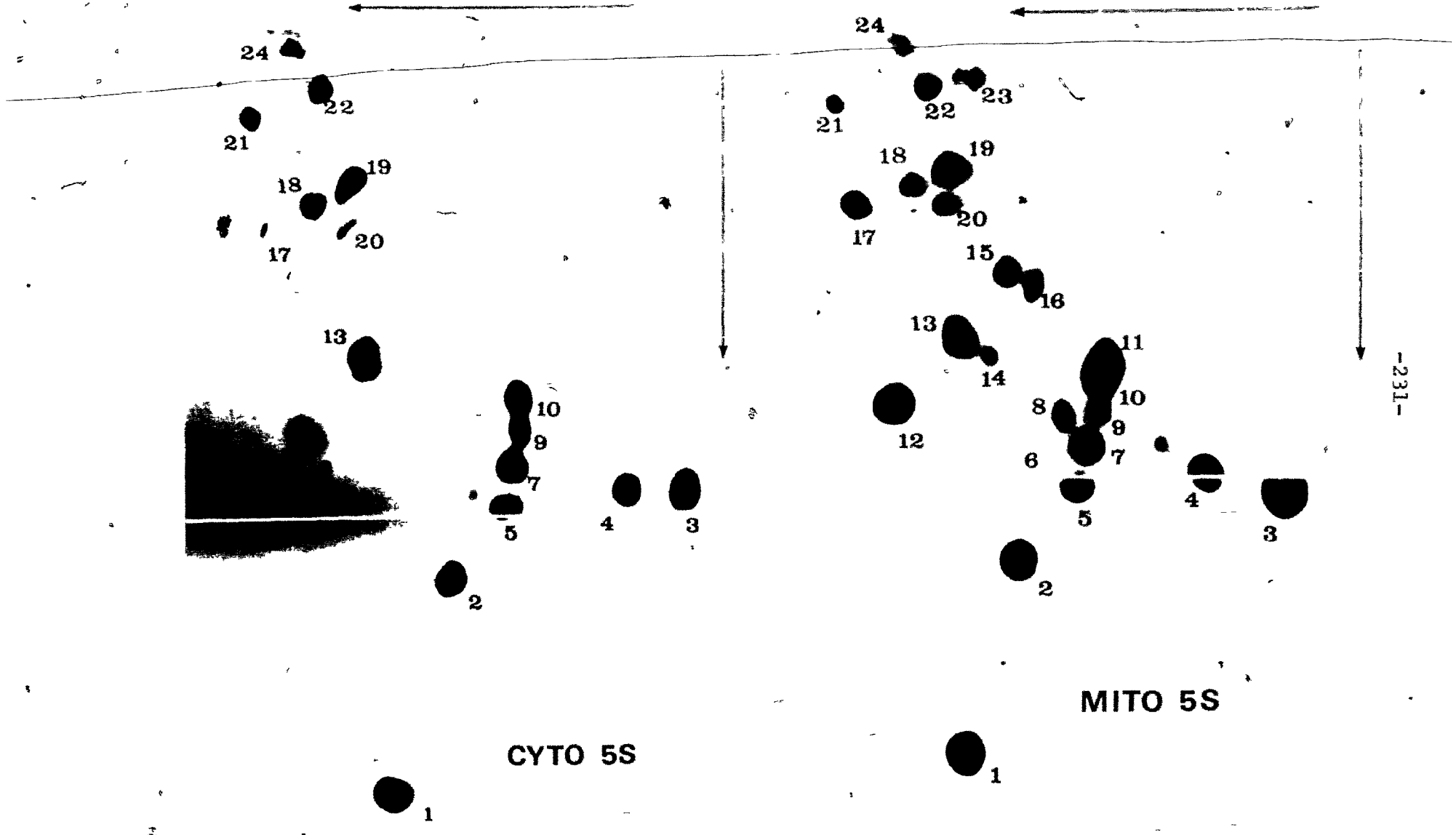
31
32
33

LEGEND OF FIGURE 23

Pancreatic ribonuclease fingerprints of wheat cytosol (left) and mitochondrial (right) 5 S RNAs. Enzymatic hydrolysis of the two RNA species and subsequent two-dimensional ionophoresis of the digest were conducted as described in SECTION IV.C.(ii). The sequences of the oligonucleotides in the numbered spots on the fingerprints are listed in TABLE XVIII.

FIGURE 23


24
22
21
19
18
20
17
13
10
9
7
5
4
3
2
1
CYTO 5S
24
22
23
21
19
18
20
17
15
16
13
14
11
10
8
9
12
6
7
5
4
3
2
1
MITO 5S

prints of cytosol 5 S RNA were given the same number as those in the corresponding fingerprints of mitochondrial 5 S RNA. It can be seen that the T_1 (FIGURE 22A) and RNase A (FIGURE 23A) fingerprints of the wheat mitochondrial 5 S RNA show, respectively, 7 and 13 oligonucleotide spots not present in the corresponding fingerprints of cytosol 5 S RNA (FIGURES 22B and 23B). In addition, the mitochondrial fingerprints contain all of the oligonucleotides found in the corresponding cytosol fingerprints. The wheat mitochondrial 5 S RNA preparation thus appears to contain a mixture (ca. 1:1) of a unique mitochondrial 5 S rRNA species and "contaminating" cytosol 5 S rRNA. This conclusion was confirmed by secondary and, in the case of cytosol 5 S RNA, tertiary nuclease digestion of all the oligonucleotides. The low number of counts precluded the possibility of doing tertiary analyses with the mitochondrial 5 S RNA preparation.

The T_1 and RNase oligonucleotide catalogues of wheat cytosol 5 S rRNA and the unique mitochondrial 5 S RNA species are shown in TABLE XVIII (the catalogues for the latter were determined by subtracting, from the catalogues of the mitochondrial 5 S RNA preparation, oligonucleotides contributed

TABLE XVIII

T_1 AND PANCREATIC RIBONUCLEASE CATALOGUES OF WHEAT EMBRYO CYTOSOL AND MITOCHONDRIAL 5 S RNA PREPARATIONS

T1 RNase products

Spot	Sequence	Molar yield Cytosol	Molar yield Mitochondrial
1	G	8-10	13-14
2	CC	3-4	~2
3	AG	2-3	1-3
4	ACG	0	1-2
5	AAG	2	0-1
6	CGCG	0	1
7	CAU(C_{1-2},U_{0-1})Y_{OH}	1	0
8	CACG	1	0
9	AAAACACCCG	0	1
10	UG	3-4	1-3
11	UAC	1-2	0
12	AUG	2	0
13	(C,A)AUC	0	1
	AAUCG	0	1
14	ACCUCG	0	1
15	CAAAUG	0	1-0 [1]
	(U,C)AAAC	0	1-2
16	CA,CUA,CG	0	1-0 [2]
17	AAC(C,U)CC	1	0-1
18	ACUAAAC	1	0
19	UUG	1	0
20	UUCG[3]	0	1
	CUCG	1	0
21	UACUG	0	1
22	UUAAG	1	0
23	UCCUCG	1	0
24	UACUAG	1	0
25	AAAUUG	0	1
26	CCAUAUG	0	1
27	ACCUCCUC[3]	1	0
28	A(UCCCA,UCA)G	1	0
29	A(CCA,UA,UCA)G	1	0
30	UCUUG	0	1
31	AUCCCAU(C,U)G	0	1
32	AUAUAUAUG	0	1
33	UG	1	0

Pancreatic RNase products

Spot	Sequence	Molar yield Cytosol	Molar yield Mitochondrial
1	U	10-11	12-11
2	AU	8	9
3	U	10	10
4	A	4	4
5	GC	3	3
6	AAU	0	0-1
7	GAC	1	2
	ACC	1	
8	AAAU[3]	0	1
9	AAGC	1	0
10	ACAAC	1	1-2
	AAAC	1	
11	CAAAAC	0	1
12	CU	4	7
13	CAU	2-3	2-2
	ACU		
14	GGC	0	0-1
15	GAAAU	0	1[4]
16	AC,C,AC	0	1
17	CGU	0-1	3-2
18	GGAU	1	0
19	AG,G,AU	1	0
	AAG,G,U	1	0
	CGAAU	0	1
20	GCGC	0-1	1
21	GGGU	1	0
22	AG,AG,G,U	1	0
23	AG,G_3,AC	0	1
24	AAG,G_3,U	1	0

This table lists sequences of oligonucleotides in the numbered spots on the fingerprints shown in Fig. 22 and 23. Cytosol molar yields were determined directly by measurement of radioactivity in spots. Mitochondrial molar yields were similarly determined after subtraction of the contribution of contaminating cytosol oligonucleotides. Sequences unique to the mitochondrial 5 S species are underlined.

[1] A total of two mitochondrial-specific oligonucleotides in this spot.
[2] A total of one mitochondrial-specific oligonucleotide in this spot.
[3] Sequence probable but not certain.
[4] Quantitation suggests more than one copy; 3'- or 5'-terminus may migrate in this position.

by "contaminating" cytosol 5 S rRNA). The computed length of the unique mitochondrial species is 125 - 145 nucleotides (exclusive of termini) and it contains no post-transcriptionally modified residues. The uncertainty in the length assessment of the molecule is due to uncertainty of the presence or absence (or the number) of several mitochondrial oligonucleotides. The mitochondrial 5 S RNA molecule contains the sequence GAUCCCAU(C,U)G, a closely-related variant of which occurs in the highly-conserved region (positions 28 - 59) of all 5 S rRNA molecules (Fox and Woese, 1975). This oligonucleotide differs by a single residue from the wheat cytosol 5 S sequence, GA(UCCCA,UCA)G. However, the sequence PyrGAAC, identified in every prokaryotic and chloroplast 5 S rRNA examined to date and believed to bind to the GTΨCPur sequence common to all prokaryotic tRNAs, is absent from the wheat mitochondrial 5 S RNA. The sequence AGAAC is possibly present but not the sequence PyrGAUC, both of which have been shown to substitute for PyrGAAC in cytosol 5 S rRNA (AGAAC in the 5 S RNA of plants, PyrGAUC in the 5 S RNA of vertebrates, insects and some yeasts). The homologous sequence in S. carlsbergensis, CGAUA, could conceivably be present in the wheat mitochondrial 5 S RNA

(the T_1 and RNase A cuts are consistent with this possibility). In addition, the mitochondrial 5 S oligonucleotide G(U,C)-AAAG is possibly a variant of the sequence CACUAAAG found in the cytosol 5 S RNA.

The computed length of the wheat cytosol 5 S rRNA is 117 - 126 nucleotides and, not surprisingly, the molecule shows very strong size and sequence homology with the 5 S rRNA of rye (Payne and Dyer, 1976). The T_1 catalogues of the two show only two differences [A(UCCCA,UCA)G in wheat, AUCCAUCAG in rye, and the anomalous (Payne et al., 1973) presence in rye 5 S rRNA of $(C_3U)G$], while the pancreatic catalogues show only one difference [CGGU in wheat, GGU in rye].

E. DISCUSSION

Leaver and Harmey (1973, 1976) have previously demonstrated the presence of a 5 S RNA component in the mitochondrial ribosomes of several higher plants (mung bean, artichoke and turnip), and this observation is of considerable interest in light of the failure to demonstrate a similar-sized molecule in other mitoribosomes (Borst, 1972; but cf. Agsteribbe et al., 1974). Until now, however, there has been no information to indicate whether higher-plant mitochondrial and cytosol 5 S rRNAs are distinct molecular entities; indeed, the two species cannot be distinguished by electrophoresis on 10% polyacrylamide gels (Leaver and Harmey, 1976). The most definitive means of showing that these molecules are unique is by direct chemical characterization and, preferably, primary sequence determination. In the case of the wheat mitochondrial and cytosol 5 S RNAs, extensive chemical characterization has been possible as a result of the ability to isotopically label these species in germinating embryos. [^{32}P]-Labeled 5 S RNAs of sufficiently-high specific activity could be isolated so that it was possible to assemble both T_1 and RNase A oligonucleotide catalogues. The results of these

analyses, presented in SECTION IV.D., confirm that the mitochondria of at least one higher plant contain a 5 S RNA species distinct from that found in the cytosol.

Although wheat cytosol 5 S RNA has been shown to reside in the large subunit of wheat cytoribosomes (Azad and Lane, 1973), it remains to be demonstrated that the unique mitochondrial 5 S RNA characterized here is localized in a wheat mitoribosome (and specifically, in the large subunit of such a particle). However, in all likelihood this is the case.

The sizes of the two wheat 5 S RNA species are likely quite similar in spite of the uncertainty in the length of the mitochondrial species (125 - 145 nucleotides) when it is computed from the sequence data. Both molecules migrate identically on polyacrylamide gels and elute in precisely the same position during chromatography on Sephadex G-100 columns. Additional reasons for concluding that the mitochondrial species is indeed the mitochondrial equivalent of cytosol 5 S rRNA include the fact that it contains no post-transcriptionally-modified residues (as would be present in any contaminating tRNA) but does possess the sequences GAUCCCAU(C,U)G, (a variant of which is found in the highly-conserved region of all 5 S rRNA molecules)

and (U,C)AAAG (which is similar to the sequence CACUAAAG found in the wheat cytosol 5 S rRNA). However, definitive information must await the isolation of purified wheat mitochondrial ribosomes and the demonstration that this 5 S RNA is an actual component of the large subunit.

It is interesting to note (especially in view of the prokaryotic nature of the wheat mitochondrial 18 S RNA) that the mitochondrial 5 S RNA does not contain the sequence PyrGAAC present in all prokaryotic and chloroplast 5 S rRNAs at positions 40 - 44 from the 5'-end of the molecule (some cytosol 5 S rRNAs do contain the sequence PyrGAAC, but not at positions 40 - 44). Nor does wheat mitochondrial 5 S RNA contain the sequence PyrGAUC present in the same position in most cytosol 5 S rRNAs. However, it does possibly contain AGAAC, which has been found in the same position in all higher plant cytosol 5 S rRNAs (including wheat) examined.

These results complicate speculation about the possible function of the mitochondrial 5 S RNA (within the organellar ribosome) as a potential binding site for organellar tRNA. Several authors (Dube, 1973; Erdmann et al., 1973; Richter et al., 1973) have convincingly demonstrated that the pro-

karyotic sequence PyrGAAC is essential in protein biosynthesis, being involved in antiparallel hydrogen-bonding with the sequence GTΨCPur ("common arm sequence"), which is common to all prokaryotic tRNAs. As noted above, this PyrGAAC sequence is absent in most eukaryotic cytosol 5 S rRNAs, being replaced by PyrGAUC. Only the initiator tRNAs of eukaryotes possess the complementary (antiparallel) sequence GAUCPur. Philips and Carr (1976) have suggested that the role of eukaryotic 5 S RNAs may be restricted to assisting in the binding of the initiation complex to 80 S ribosomes. The binding of "normal" transfer RNAs to 80 S ribosomes might be mediated by the 5.8 S rRNA, which contains the sequence PyrGAAC in all species examined. Higher plant cytosol 5 S rRNAs contain the sequence AGAAC in positions 40 - 44, so that all "normal" transfer RNAs (having the complementary sequence GTΨC) could possibly interact with this molecule, in contrast to the situation in other eukaryotes. On the other hand, it has been suggested that the common arm sequence in wheat cytosol initiator $tRNA^{Met}$ is GAU*CG (with U* a modified uridine; Ghosh et al., 1974), which would prohibit an antiparallel binding to AGAAC. Thus, in the cytosol of higher plants,

the situation seems to be the reverse of that found in most other eukaryotes.

If the wheat mitochondrial 5 S RNA does indeed contain AGAAC, this sequence may be the binding site for all wheat tRNAs involved in mitochondrial protein synthesis. Since the 5.8 S rRNA is not found in the mitoribosome, it obviously cannot function as a binding site. However, if the sequence AGAAC is not present in the mitochondrial 5 S RNA, we are left with only one other possible sequence alternative in this position which is also present in at least one other 5 S rRNA molecule. This is the sequence CGAUA, found in S. carlsbergensis cytosol 5 S rRNA (Hori, 1976). The RNase T_1 and A cutsdo not rule out the possibility of this sequence being present in wheat mitochondrial 5 S RNA. The role, if any, that such a sequence might play in tRNA binding is unknown.

The apparent 1:1 molar contamination of the mitochondrial 5 S RNA with the cytosol homologue is rather puzzling, since the estimated contamination of the mitochondrial RNA preparation with cytosol 18 S and 26 S rRNA species was only 20 - 30% (SECTION II). A number of possible explanations exist for the observation, none of which is entirely

satisfactory. First, it may be that contamination with cytosol 18 S and 26 S rRNA is in fact equivalent to that of the 5 S RNA species, but is not apparent due to degradation of the larger RNAs. It should be noted in this respect that the baseline in polyacrylamide gel radioactivity profiles of total mt-rRNA is rather high and may not be due only to mitochondrial RNA breakdown, but also to preferential degradation of higher-molecular-weight cytosol RNA. A second possibility is that the presence of relatively high concentrations of EDTA in the medium during purification of mitochondria not only removes the contaminating cytosol ribosomes attached to the outer mitochondrial membrane, but also releases a cytosol 5 S RNA - protein complex which may somehow remain associated with the mitochondria. It has been demonstrated in plants (Dyer et al., 1976) and animals (Blobel, 1971; Lebleu et al., 1971) that 5 S RNA - protein complexes can be dissociated from ribosomes by EDTA treatment. A third (also unlikely, although more interesting) possibility is that the presence of cytosol 5 S RNA in the mitochondrial preparation is not artifactual, and that wheat mitoribosomes consist of two populations, one containing a cytosol 5 S

rRNA and the other the unique mitochondrial species. In this case, one could postulate that the cytosol 5 S RNA is either imported from the cytosol or coded for on mt-DNA (notably, chloroplasts DNA codes for two 5 S rRNA molecules, Hermann et al., 1976). Finally, the possibility exists of selective loss of mitochondrial 5 S RNA during purification of mitochondria and/or isolation and purification of RNA. This, however, is unlikely.

Although it has been adequately demonstrated through the nucleotide sequence analyses reported here that wheat mitochondria contain a unique 5 S RNA species, these analyses are preliminary and should be expanded. However, in order to pursue such additional studies, it will be necessary to purify the mitochondria completely free from any contamination by cytosol RNA. This might be accomplished by treating the purified mitochondrial preparation with RNase (possibly using Enzite (Miles), a polysaccharide matrix to which Ribonuclease I is bound), which ideally should degrade only those RNA species external to the organelles, or by treating the mitochondria with digitonin, a steroid glycoside which has been shown in other systems

(Walkin, 1971) to selectively remove the outer mitochondrial membrane and any contaminating cytoribosomes. Also, it might be possible to selectively remove the cytosol 5 S rRNA as a high-molecular-weight complex with cytosol 18 S rRNA since it has been demonstrated by Azad and Lane (1975) that the wheat cytosol 5 S rRNA preferentially hybridizes to the cytosol 18 S rRNA under the same conditions in which the 5.8 S "satellite" RNA hybridizes to the cytosol 26 S rRNA.

Once wheat mitochondria are freed from any cytosol RNA, mitochondrial ribosomes could be purified from the organelle and the 5 S rRNA molecule shown to reside in the larger ribosomal subunit. This purified mitochondrial 5 S RNA could then be used to produce unambiguous T_1 and RNase A oligonucleotide catalogues and in addition, the complete sequence could be determined (using unlabeled 5 S RNA and post-labeling techniques, if necessary, due to the difficulty of incorporating sufficient [^{32}P]orthophosphate *in vivo*). A detailed comparison of the sequence of the mitochondrial 5 S RNA with those of prokaryotic and eukaryotic cytosol 5 S rRNA will further aid in identifying those regions of the molecules which are highly-conserved

and of probable functional importance (and also perhaps help resolve the confusion surrounding the tRNA binding sites). Since the mitochondrial 5 S RNA is a part of a distinctive protein-synthesizing system (mitochondrial) which differs from both the cytosol and prokaryotic counterparts, further study may provide additional clues about the process of protein biosynthesis in general.

Additional experiments might include hybridization analyses to determine the transcriptional origin of the mitochondrial 5 S RNA and its structural relation on the genome to the cistrons for the two higher-molecular-weight rRNAs. Also, the possibility that the mitochondrial 18 S RNA contains a specific binding site for the mitochondrial 5 S RNA (as has been shown for the wheat cytosol species; Azad and Lane, 1975; Oakden et al., 1977) could be explored.

SECTION V: GENERAL DISCUSSION: SIGNIFICANCE OF THE PRESENT INVESTIGATION AND POSSIBILITIES FOR FUTURE STUDIES

Although considerable attention has been directed towards the mitochondrion, in general, during the past decade, both in terms of its structure and its function, most of the specific research has been done with animal and lower fungal systems. In spite of some notable exceptions (e.g., Leaver and Harmey, 1973; Pring, 1974; Leaver and Pope, 1976), relatively few studies have been carried out using higher-plant mitochondria, and, as has been discussed in the introduction to SECTION II, this is unfortunate. Plant mitochondria reside in cells which are subject to unique developmental pressures (e.g., germination) and interaction with other cellular components not present in either animal or fungal tissues (e.g., chloroplasts). This offers an opportunity to study phenomena which may shed some light on the function of the mitochondria in general. Conforming with this idea, the studies reported in this thesis were an attempt to characterize some components of the mitochondrial protein-synthesizing system (specifically the ribosomal RNAs), with the goal of eventually being able

to answer some of the fundamental questions concerning this organelle.

The results of the comparative studies on wheat mitochondrial and cytosol rRNAs have been discussed in detail in the appropriate sections and I will only briefly summarize them here. The wheat mitochondrial high-molecular-weight RNAs (26 S and 18 S, presumptive components of the large and small mitoribosomal subunits, respectively) are similar in size to the corresponding cytosol species and to the homologous species found in other higher-plant mitochondria. On the other hand, they are larger than mitoribosomal RNAs isolated from most other eukaryotes (however, it remains to be established that the sizes of RNA species determined by polyacrylamide gel electrophoresis are indeed the actual sizes). The wheat rRNA species have an anomalously-high G + C content (compared to the value found for other mitochondrial rRNAs), similar to that found in the cytosol rRNA. However, in agreement with the results obtained from other systems, wheat mitochondrial RNAs contain a significantly smaller number of modified components than do the corresponding cytosol RNA species. The low degree of modification of mitochondrial RNA is undoubtedly of some

functional significance, although at present any suggestions are essentially speculative. Nucleotide sequence analysis of the cytosol and mitochondrial 18 S RNAs demonstrate for the first time the prokaryotic nature of a mitochondrial ribosomal RNA. Both the primary fingerprints of the 26 S RNAs and the sequence analyses of the 18 S species showed that, although apparently similar in size, the cytosol and mitochondrial homologues were distinctively different in sequence, with the mitochondrial 18 S RNA displaying remarkable sequence homology with the small-ribosomal-subunit RNA (16 S) of present-day free-living prokaryotes. These results have added significant additional supporting evidence to the theory that mitochondria did indeed evolve from endosymbionts of the ancestral protoeukaryote. Sequence analysis of the cytosol and mitochondrial 5 S RNA species showed unequivocally, for the first time, that plant mitochondria contain a 5 S RNA molecule distinct from that found in the cytosol. This is significant in view of the inability of other workers to find a similar molecule in the mitochondria of mammalian or fungal systems (or, in the latter cases, to demonstrate conclusively that it is not an artifact resulting from cytosol 5 S RNA contamination).

Even in the case of the 5 S rRNA reported to be present in other plant mitochondria, no chemical characterization was offered to demonstrate that this molecule was indeed distinct from that found in the cytosol.

The possibilities for further studies to extend the results presented in this thesis appear almost unlimited. Many, however, must first await a procedure for the isolation of mitochondrial rRNA species completely devoid of cytosol rRNA contamination. This might be accomplished by extracting the RNA from purified mitochondrial ribosomes, which would not only demonstrate the location of these RNA species (5 S, 18 S, and 26 S) but also provide material for definitive nucleotide composition analyses and, in the case of the 5 S RNA, open the way for determination of the entire sequence. In light of the demonstrated prokaryotic nature of the mitochondrial 18 S RNA, it will be very interesting to determine the degree of sequence homology of the wheat mitochondrial 5 S RNA with prokaryotic 5 S RNA in general, or even with the 5 S RNAs of specific groups of prokaryotes. It should also be possible to isolate wheat mitochondrial DNA and, with the use of restriction endonuclease and RNA/DNA hybridization experiments, to determine the number and

location of the cistrons coding for the mitochondrial ribosomal RNAs.

In addition to further studies involving the mitochondrial rRNAs, the *in vivo* labeling approach described in the thesis will allow analysis of both mitochondrial transfer RNA and messenger RNA. A search for wheat mitochondrial mRNA would be particularly interesting, since plant mtDNA is known to be an order of magnitude larger than the small mammalian mtDNAs and to be devoid of the large non-coding AT-rich regions present in yeast mtDNA. The possibility that plant mtDNA codes for additional products not found in the mitochondria from mammalian systems (and perhaps involved in unique plant cellular events such as germination or chloroplast-mitochondrial interactions) is an intriguing one and definitely warrants further study. For example, an analysis of the mitochondrial mRNA formed at different stages in embryogenesis and during germination may shed some light in this direction.

In conjunction with the nucleotide sequence analyses of the mitochondrial 18 S RNA it would be worthwhile to produce a similar set of data for the wheat chloroplast 16 S rRNA. A direct comparison of the degree of homology among

the three spatially-separated but functionally-equivalent small-ribosomal-subunit RNAs could then be made.

BIBLIOGRAPHY

Aalj, C., Nanninga, N., and Borst, P. (1972) Biochim. Biophys. Acta, 277, 140

Adams, J. M., and Cory, S. (1975) Nature 255, 28

Agsteribbe, E., Datema, R., and Kroon, A. (1974) In The Biogenesis of Mitochondria (Kroon, A. M., and Saccone, C., eds.), Academic Press, New York, 305

Attardi, G., Aloni, Y., Attardi, B., Ojala, D., Pica-Mattocia, L., Robberson, D. L., and Storrie, B. (1970) Cold Spring Harbour Symp. Quant. Biol. 35, 599

Attardi, G., Amalric, F., Ching, E., Costantino, P., Gelfand, R., and Lynch, D. (1976) In The Genetic Function of Mitochondrial DNA (Saccone, C., and Kroon, A. M., eds.), North-Holland, Amsterdam, 37

Attardi, B., and Attardi, G. (1971) J. Mol. Biol. 55, 231

Attardi, G., Costantino, P., England, J., Lynch, D., Murphy, W., Ojala, D., Posakony, J., and Storrie, B. (1975) In Genetics and Biogenesis of Mitochondria and Chloroplasts (Birky, C. W., Perlman, P. S., and Byers, T. J., eds.), Ohio State University Press, 3

Attardi, B., Cravioto, B., and Attardi, G. (1969) J. Mol. Biol. 44, 47

Attardi, G., and Ojala, D. (1971) Nature New Biol. 229, 133

Avadhani, N. G., and Buetow, D. E. (1972) Biochem. J. 128, 353

Avadhani, N. G., and Buetow, D. E. (1973) Biochem. Biophys. Res. Commun. 50, 443

Avadhani, N. G. and Buetow, D. E. (1974) Biochem. J. 140, 73

Avadhani, N. G., Lewis, F. S., and Rutman, R. J. (1976) Sub-Cell. Biochem. 4, 93

Azad, A. A. (1973) Ph.D. Dissertation, Department of Biochemistry, University of Toronto

Azad, A. A., and Lane, B. G. (1973a) Can. J. Biochem. 46, 1669

Azad, A. A., and Lane, B. G. (1973b) Can. J. Biochem. 51, 606

Azad, A. A., and Lane, B. G. (1975) Can. J. Biochem. 53, 320

Baxter, R., and Bishop, D. H. L. (1968) Biochem. J. 109, 13 p.

Beattie, D., Basford, R. E., and Koritz, S. B. (1967) Biochemistry 6, 3099

Bernard, U., Goldthwaite, C., and Küntzel, A. (1976) Nucleic Acids Res. 3, 3101

Bernard, U., Pühler, A., Mayer, F., and Küntzel, H. (1975) Biochim. Biophys. Acta 402, 270

Bernardi, G., Faures, M., Piperno, G., and Slonimski, P. P. (1970) J. Mol. Biol. 48, 23

Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1972) J. Mol. Biol. 26, 373

Blobel, G. (1971) Proc. Nat. Acad. Sci. USA 68, 1881

Bohnert, H. J., and Hermann, R. G. (1974) Eur. J. Biochem. 50, 83

Bonen, L., Cunningham, R. S., Gray, M. W., and Doolittle, W. F. (1977) Nucleic Acids Res., in press.

Bonen, L., and Doolittle, W. F. (1975) Proc. Natn. Acad. Sci. USA 72, 2310

Bonen, L., and Doolittle, W. F. (1976) Nature 261, 669

Bonner, W. D., Jr. (1967) Methods Enzymol. 10, 126

Borst, P. (1972) Ann. Rev. Biochem. 41, 333

Borst, P. (1977) Trends in Biochemical Sciences 2, 82

Borst, P., and Grivell, L. A. (1971) FEBS Lett. 13, 73

Borst, P., and Grivell, L. A. (1973) Biochimie 55, 801

Brawerman, G. (1974) Ann. Rev. Biochem. 43, 621

Bresslau, E. Z., and Scremin, L. (1924) Arch. Protistenk. 48, 509

Cantatore, P., De Giorgi, C., and Saccone, C. (1976) Biochem. Biophys. Res. Commun., in press

Cavalier-Smith, T. (1975) Nature 256, 463

Chen, D., Schultz, G., and Katchalski, E. (1971) Nature New Biol. 231, 69

Chèvrement, M. (1963) Symp. Intern. Soc. Cell Biol. 2, 323

Chèvrement, M., Chevrement-Comhaire, S., and Baekleland, E. (1959) Biochem. Pharmacol. 4, 67

Chi, J. C. H., and Suyama, Y. (1970) J. Mol. Biol. 53, 531

Chia, L. S. Y., Morris, H. P., Randerath, K., Randerath, E. (1976) Biochim. Biophys. Acta 425, 49

Clayton, D. A. (1976) In The Genetic Function of Mitochondrial DNA (Saccone, C., and Kroon, A. M., eds.), North Holland, Amsterdam, 47

Clayton, D. A., and Vinograd, J. (1967) Nature 216, 652

Cooperstein, S. J., and Lazarow, A. (1951) J. Biol. Chem. 189, 655

Crick, F. H. C. (1966) J. Mol. Biol. 19, 548

Cunningham, R. S., Bonen, L., Doolittle, W. F., and Gray, M. W. (1976) FEBS Lett. 69, 116

Cunningham, R. S., Bonen, L., Gray, M. W., and Doolittle, W. F. (1977) In Nucleic Acids and Protein Synthesis in Plants (Bogorad, L., and Weil, J. H., eds.), C.N.R.S., Strasbourg, in press

Cunningham, R. S., and Gray, M. W. (1977) Biochim. Biophys. Acta, in press

Curgy, J. J., Ledoigt, G., Stevens, B. J., and André, J. (1974) J. Cell Biol. 60, 628

Datema, R., Agsteribbe, E., and Kroon, A. M. (1974) Biochim. Biophys. Acta 335, 386

Davenport, L. W., Taylor, R. H., and Dubin, D. T. (1976) Biochim. Biophys. Acta 447, 285

Dawid, I. B. (1972a) Develop. Biol. 29, 139

Dawid, I. B. (1972b) J. Mol. Biol. 63, 201

Dawid, I. B. (1972c) In Mitochondria: Biogenesis and Bioenergetics; Biomembranes: Molecular Arrangements and Transport Mechanisms (Van Den Bergh, S. G., Borst, P., Van Deenan, L. L. L., Riemersma, J. C., Slater, E. C., and Tager, J. M., eds.), North-Holland, Amsterdam, 28: 35

Dawid, I. B., Klukas, C. K., Ohi, S., Ramirez, J. L., and Upholt, W. B. (1976) In The Genetic Function of Mitochondrial DNA (Saccone, C., and Kroon, A. M., eds.), North Holland, Amsterdam, 3

Dawid, I. B., and Chase, J. W. (1972) J. Mol. Biol. 63, 217

De Duve, C. (1967) Methods Enzymol. 10, 7

De Duve, C., Pressman, B. C., Crianetto, R., Wattiaux, R., and Appleman, F. (1955) Biochem. J. 60, 604

Devlin, R. (1976) Developmental Biology 50, 443

De Vries, H., and Van der Koogh-Schuuring, R. (1973) Biochem. Biophys. Res. Commun. 54, 308

Dierich, A., Wintzerith, M., and Mandel, P. (1975) Biochimie 57, 395

Doolittle, W. F. (1973) J. Bacteriol. 113, 1256

Doolittle, W. F., and Pace, N. R. (1971) Proc. Nat. Acad. Sci. USA 68, 1786

Dube, S. K. (1973) FEBS Lett. 36, 39

Dubin, D. T. (1974) J. Mol. Biol. 84, 257

Dubin, D. T., and Friend, D. A. (1972) J. Mol. Biol. 71, 163

Dubin, D. T., and Günalp, A. (1967) Biochim. Biophys. Acta, 134, 106

Dubin, D. T., Jones, T. H., and Cleaves, G. R. (1974) Biochem. Biophys. Res. Commun. 56, 401

Dubin, D. T., and Montenecourt, B. S. (1970) J. Mol. Biol. 48, 279

Dubin, D. T., and Shine, J. (1976) Nucleic Acids Res. 3, 1225

Dudock, B. S., Katz, G., Taylor, E. K., and Holley, R. W. (1969) Proc. Nat. Acad. Sci. USA 62, 941

Dyer, T. A., Bowman, C. M., and Payne, P. I. (1976) In Nucleic Acids and Protein Synthesis in Plants (Bogorad, L., and Weil, J. H., eds.), Plenum Press, New York, in press

Ebner, E., Mason, T. L., and Schatz, G. (1973) J. Biol. Chem. 248, 5369

Ehresmann, C., Fellner, P., and Ebel, J. P. (1971) FEBS Lett. 13, 325

Ehresmann, C., Stiegler, P., Makie, G. A., Zimmermann, R. A., Ebel, J. P., and Fellner, P. (1975) Nucleic Acids Res. 2, 265

Erdmann, V. A., Fahnestock, S., Higo, K., and Nomura, M. (1971) Proc. Nat. Acad. Sci. USA 68, 2932

Erdmann, V. A., Sprinzl, M., and Pongs, O. (1973) Biochem. Biophys. Res. Comm. 54, 942

Feldmann, H., and Kleinow, W. (1976) FEBS Lett. 69, 300

Fellner, P. (1969) Eur. J. Biochem. 11, 12

Forget, B. G., and Weissmann, S. M. (1967) Science 158, 1695

Fox, G. E., and Woese, C. R. (1975) J. Mol. Evol. 6, 61

Freeman, K. B., Mitra, R. S., and Bartoov, B. (1973) Subcell. Biochem. 2, 183

Fridovich, I. (1974) Life Sciences 14, 89

Gerbi, S. A. (1976) J. Mol. Biol. 106, 791

Ghosh, K., Ghosh, H. P., Simsek, M., and Rajbhandary, U. L. (1974) J. Biol. Chem. 249, 4720

Goddard, J. M., and Cummings, D. J. (1975) J. Mol. Biol. 97, 593

Gray, P. N., and Attardi, G. (1973) J. Cell Biol. 59, 120a

Gray, M. W. (1974a) Proc. Can. Fed. Biol. Soc. 17, 116

Gray, M. W. (1974b) Biochemistry 13, 5453

Gray, M. W., and Cunningham, R. S. (1977) Can. J. Biochem., in press

Gray, M. W., and Lane, B. G. (1967) Biochim. Biophys. Acta 134 243

Gseco, M., Cantatore, P., Pepe, G., and Saccone, C. (1973) Eur. J. Biochem. 37, 171

Griffith, D. E. (1975) In Genetics and Biogenesis of Mitochondria and Chloroplasts (Birky, C. W., Perlman, P. S., and Byers, T. J., eds.), Ohio State University Press

Grivell, L. A., Reijnders, L., and Borst, P. (1971) Eur. J. Biochem. 19, 64

Groot, G. S. P., Flavell, R. A., Van Ommen, G. J. B., and Grivell, L. A. (1974) Nature 252, 167

Groot, G. S. P., Flavell, R. A., and Sanders, J. P. M. (1975) Biochim. Biophys. Acta 378, 186

Guderian, R. H., Pulliam, R. L., and Gordon, M. P. (1972) Biochim. Biophys. Acta 262, 50

Hall, R. H. (1971) The Modified Nucleosides in Nucleic Acids, Columbia University Press, New York

Hallermayer, G., and Neupert, W. (1974) FEBS Lett. 41, 264

Hayes, F., Hayes, D., Fellner, P., and Ehresmann, C. (1971) Nature New Biol. 232, 54

Helser, T. L., Davies, J. E., and Dahlberg, J. E. (1971) Nature New Biol. 233, 12

Helser, T. L., Davies, J. E., and Dahlberg, J. E. (1972) Nature New Biol. 235, 6

Hendler, F. J., Padmanaban, G., Patzer, J., Ryan, R., Rabinowitz, M. (1975) Nature 258, 357

Hermann, R. G., Bohnert, H.-J., Driesel, A., and Hobom, G. (1976) In The Genetics and Biogenesis of Chloroplasts and Mitochondria (Bucher, Th., Neupert, W., Sebald, W. and Werner, S., eds.), North-Holland, Oxford, 351

Hill, W. E., Bakke, R., and Blair, D. P. (1977) Nucleic Acids Res. 4, 473

Hirsch, M., and Penman, S. (1973) J. Mol. Biol. 80, 379

Hirsch, M., and Penman, S. (1974) J. Mol. Biol. 83, 131

Hogeboom, G. H., Schneider, W. C., and Palade, G. C. (1948) J. Biol. Chem. 172, 619

Hollenberg, C. P., Borst, P., and Van Bruggen, E. F. J. (1970) Biochim. Biophys. Acta 209, 1

Honda, S. I., Hongladarom, T., and Laties, C. G. (1966) J. Exp. Botany 17, 460

Hori, D. (1976) Molec. Gen. Genet. 145, 119

Horne, J. R., and Erdmann, V. A. (1972) Molec. Gen. Genet. 119, 337

Horne, J. R., and Erdmann, V. A. (1973) Proc. Nat. Acad. Sci. USA 70, 2870

Hudson, L., Gray, M., and Lane, B. G. (1965) Biochemistry 4, 2009

Johnston, F. B., Nasatir, M., and Stern, H. (1957) Plant Physiol. 32, 124

Johnston, F. B., and Stern, H. (1957) Nature 179, 160

Jeanteur, Ph., Amaldi, F., and Attardi, G. (1968) J. Mol. Biol. 33, 757

Kay, C. M., and Oikawa, K. (1966) Biochemistry 5, 213

Kellems, R. E., Allison, V. F., and Butow, R. A. (1975) J. Cell Biol. 65, 1

Kennedy, E. P., and Lehninger, A. L. (1948) J. Biol. Chem. 172, 847

Khan, M. S. N., and Maden, B. E. H. (1976) J. Mol. Biol. 101, 235

Kirby, K. S. (1956) Biochem. J. 64, 405

Kislev, N., Swift, H., and Bogorad, L. (1965) J. Cell Biol. 25, 327

Klagsbrun, M. (1973) J. Biol. Chem. 248, 2612

Kleinow, W., Neupert, W., and Miller, F. (1974) J. Cell Biol. 62, 860-875

Klootwijk, J., and Planta, R. J. (1973a) Eur. J. Biochem. 39, 325

Klootwijk, J., and Planta, R. J. (1973b) Mol. Biol. Reports 1, 187-191

Klootwijk, J., Klein, I., and Grivell, L. A. (1975) J. Mol. Biol. 97, 337

Klootwijk, J., Van den Bos, R. C., and Planta, R. J. (1972) FEBS Lett. 27, 102

Kolodner, R., and Tewari, K. K. (1972) Proc. Nat. Acad. Sci. USA 69, 1830

Kossman, C. R., Stamato, J. D., and Pettijohn, D. E. (1971) Nature New Biol. 234, 102

Kroon, A. M. (1963) Biochim. Biophys. Acta 72, 391

Kuriyama, Y., and Luck, D. J. L. (1973) J. Mol. Biol. 73, 425

Kuriyama, Y., and Luck, D. J. L. (1974) J. Mol. Biol. 83, 253

Lai, C. J., and Weisblum, B. (1971) Proc. Nat. Acad. Sci. USA 68, 856

Lambowitz, A. M., and Luck, D. J. L. (1975) J. Mol. Biol. 96, 207

Lambowitz, A. M., and Luck, D. J. L. (1976) J. Biol. Chem. 251, 3081

Lane, B. G. (1965) Biochemistry 4, 212

Lane, B. G., and Allen, F. W. (1961) Biochim. Biophys. Acta 47, 36

Lane, B. G., Diemer, J., and Blashko, C. A. (1963) Can. J. Biochem. 41, 1927

Lau, R. Y. (1973) Ph.D. Dissertation, Department of Biochemistry, University of Toronto, Canada

Lau, R. Y., Kennedy, T. D., and Lane, B. G. (1974) Can. J. Biochem. 52, 1110

Leaver, C. J., and Harmey, M. A. (1973) Biochem. Soc. Symp. 38, 175

Leaver, C. J., and Harmey, M. A. (1976) Biochem. J. 157, 275

Leaver, C. J., and Pope, P. K. (1976) In Nucleic Acids and Protein Synthesis in Plants (Weil, J. H., and Bogorad, L., eds.), Plenum, London, in press

Lebleu, B., Marbaix, G., Huez, G., Temmerman, J., Burny, A., and Chantrenne, H. (1971) Eur. J. Biochem. 19, 264

Lederman, M., and Attardi, G. (1973) J. Mol. Biol. 78, 275

Leffler, H. T., II., Creskoff, E., Luborsky, S. W., McFarland, V., and Mora, P. T. (1970) J. Mol. Biol. 48, 455

Leister, D. E., and Dawid, I. B. (1973) Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 32, 647, abstract

Lehninger, A. L. (1965) The Mitochondrion, W. A. Benjamin, Inc., New York

Lizardi, P. M., and Luck, D. J. L. (1971) Nature New Biology 229, 140

Loening, U. E. (1967) Biochem. J. 102, 251

Lord, A. (1974) J. Ultrastruct. Res. 46, 117

Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265

Luck, D. J. L. (1965) J. Cell Biol. 24, 461

Lund, E., Dahlberg, J. E., Lindahl, L., Jaskunas, E. R., Dennis, P. P., and Nomura, M. (1976) Cell 7, 165

Lynch, D. C., and Attardi, G. (1976) J. Mol. Biol. 102, 125

Maden, B. E. H. (1971) Prog. Biophys. Mol. Biol. 22, 127

Maden, B. E. H., Vaughan, M. H., Warner, J. R., and Darnell, J. E. (1969) J. Mol. Biol. 45, 265

Maden, B. E. H., and Salim, M. (1974) J. Mol. Biol. 88, 133

Mahler, H. R. (1973) C. R. C. Critical Rev. in Biochem. 1, 381

Malhotra, S. S., and Spencer, M. (1973) Plant Physiol. 52, 575

Malkin, L. I. (1971) Biochemistry 10, 4752

Margulis, L. (1970) Origin of Eukaryotic Cells, Yale University Press, New Haven, Connecticut

Martin, R., Schneller, J. M., Stahl, A. J. C., and Dirheimer, G. (1976) Biochem. Biophys. Res. Commun. 70, 997

McLaughlin, C. S., Warner, J. R., Edmonds, M., Nakazato, H., and Vaughan, M. H. (1973) J. Biol. Chem. 248, 1466

McLean, J.-R., Cohn, G. L., Brandt, I. K., and Simpson, M. V. (1958) J. Biol. Chem. 233, 657

Michel, R., Hallermayer, G., Harmey, M., Miller, F., and Neupert, W. (1976) In The Genetics and Biogenesis of Chloroplasts and Mitochondria (Bucher, Th., Neupert, W., Sebald, W., and Werner, S., eds.), North-Holland, Oxford, 725

Michel, R., and Neupert, W. (1973) Eur. J. Biochem. 36, 53

Monier, R. (1972) In The Mechanism of Protein Synthesis and its Regulation (ed. L. Bosch), North-Holland, Amsterdam

Monier, R. (1974) In Ribosomes (Nomura, M., Tissieres, A., and Lengyel, P., eds.), Cold Spring Harbour Laboratory, 141

Nakazato, H., Venkatesan, S., and Edmonds, M. (1975) Nature 256, 144

Nass, M. M. K., and Nass, S. (1962) Exptl. Cell Res. 26, 424

Nass, S., and Nass, M. M. K. (1963a) J. Roy. Microscop. Soc. 81, 209

Nass, S., and Nass, M. M. K. (1963b) J. Cell Biol. 19, 613

Oakden, K. M., Azad, A. A., and Lane, B. G. (1977) Can. J. Biochem. 55, 99

O'Brien, T. W., and Kalf, G. F. (1967) J. Biol. Chem. 242, 2172

Ohta, N., Sanders, M., and Newton, A. (1975) Proc. Nat. Acad. Sci. USA 72, 2343

Ojala, D., and Attardi, G.(1974a) J. Mol. Biol. 82, 151

Ojala, D., and Attardi, G.(1974b) J. Mol. Biol. 88, 205

Pace, N. R. (1973) Bacteriological Rev. 37, 562

Pace, N. R., Pato, M. L., McKibbin, J., and Radcliffe, C. W. (1973) J. Mol. Biol. 75, 619

Padmanaban, G., Hendler, F., Patzer, J., Ryan, R., and Rabinowitz, M. (1975) Proc. Nat. Acad. Sci. USA 12, 4293

Paoletti, C. A., and Riou, G. (1973) Progress in Molecular and Subcellular Biology (Hahn, H., Pack, T., Springer, G. F., Szybalski, W., and Wallenfels, K., eds.), Springer-Verlag, New York: 202

Payne, P. I., and Dyer, T. A. (1971) Biochem. J. 124, 83

Payne, P. I., and Dyer, T. A. (1972) Nature New Biol. 235, 145

Payne, P. I., and Dyer, T. A. (1976) Eur. J. Biochem. 56, 251

Payne, P. I., Corry, M. J., and Dyer, T. A. (1973) Biochem. J. 135, 845

Pechman, K. J., and Woese, C. (1972) J. Mol. Evol. 1, 230

Pene, J. J., Knight, E., and Darnell, J. E. (1968) J. Mol. Biol. 33, 609

Phillips, D. O., and Carr, N. G. (1977) Taxon, in press

Pigott, G. H., and Carr, N. G. (1971) In Proc. II Inter. Cong. Photo., Stresa

Pigott, G. H., and Carr, N. G. (1972) Science 175, 1259

Pirie, N. W. (1973) Ann. Rev. Microbiol. 27, 119

Pitelka, D. R. (1963) Electron-microscopic Structure of Protozoa, Pergamon, Oxford

Plesner, P. (1955) Acta. Chem. Scand. 9, 197

Pollard, C. J., Stemler, A., and Blaydes, D. F. (1966) Plant Physiol. 41, 1323

Poyton, R. O., and Groot, G. S. P. (1975) Proc. Nat. Acad. Sci. USA 72, 172

Poyton, R. O., and Kavanagh, T. (1976) Proc. Nat. Acad. Sci. USA 73, 3947

Poyton, R. O., and Schatz, G. (1975) J. Biol. Chem. 250, 762

Pring, D. R. (1974) Plant Physiol. 53, 677

Pring, D. R., and Thornbury, D. W. (1975) Biochim. Biophys. Acta 383, 140

Prunell, A., and Bernardi, G. (1974) J. Mol. Biol. 86, 825

Rabinowitz, M., DeSalle, L., Sinclair, J., Stirewalt, R., and Swift, H. (1966) Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 25, 581, abstract

Rabinowitz, M., and Swift, H. (1970) Physiol. Rev. 3, 376

Raff, R. A., and Mahler, H. R. (1975) Symp. Soc. Gen. Microbiol. 25, 41-92

Ralph, R. K., and Bellamy, A. R. (1964) Biochim. Biophys. Acta 87, 9

Randerath, E., Chia, L. S. Y., Morris, H. P., and Randerath, K. (1974) Biochim. Biophys. Acta 336, 159

Reijnders, L., and Borst, P. (1972) Biochem. Biophys. Res. Commun. 47, 126

Reijnders, L., Kleisen, C. M., Grivell, L. A., and Borst, P. (1972) Biochim. Biophys. Acta 272, 396

Reijnders, L., Sloof, P., and Borst, P. (1973) Eur. J. Biochem. 35, 266

Richter, D., Erdmann, V. A., and Spintzel, M. (1973) Nature New Biol. 246, 132

Roodyn, D. B. (1962) Biochem. J. 85, 177

Rosen, D., and Edelman, M. (1976) Eur. J. Biochem. 63, 525

Rosset, R., and Monier, R. (1963) Biochim. Biophys. Acta 68, 653

Rubin, G. M., and Salston, J. E. (1973) J. Mol. Biol. 79, 521

Sabatini, D. D., Tashiro, Y., and Palade, G. E. (1966) J. Mol. Biol. 19, 503

Sacchi, A., Cerbone, F., Cammarano, P., and Ferrini, V. (1973) Biochim. Biophys. Acta 308, 390

Saccone, C., and Kroon, A. M. (1976) Concluding remarks. The Genetic Function of Mitochondrial DNA (Saccone, C., and Kroon, A. M., eds.), Academic Press, New York, 545

Saccone, C., Pepe, G., Cantatore, P., Terpstra, P., and Kroon, A. M. (1976) Ibid., 253

Salim, M., and Maden, B. E. H. (1973) Nature 244, 334

Sanders, J. P. M., Heyting, C., and Borst, P. (1975) Biochem. Biophys. Res. Commun. 65, 699

Sanger, F., Brownlee, G. G., and Barrell, B. G. (1965) J. Mol. Biol. 13, 373

Sato, S., and Asahi, T. (1975) Plant Physiol. 56, 816

Schatz, G., and Mason, T. L. (1974) Ann. Rev. Biochem. 43, 51

Schneller, J. M., Martin, R., Stahl, A. J. C., and Dirheimer, G. (1975) Biochem. Biophys. Res. Commun. 64, 1046

Scragg, A. H., and Thomas, D. Y. (1977) Molec. Gen. Genet. 150, 81

Setterfield, G., Stern, H., and Johnston, F. B. (1959) Can. J. Botany 37, 65

Singh, H., and Lane, B. G. (1964) Can. J. Biochem. 42, 1011

Smillie, R. M. (1956) Australian Jour. Biol. Sci. 9, 81

Sogin, M. L., Pechman, K. J., Lewis, L. B. J., and Woese, C. R. (1972) J. Bacteriol. 112, 13

Solomos, T., Malhotra, S. S., Prasad, S., Malhotra, S. K., and Spencer, M. (1972) Can. J. Biochem. 50, 725

Spiers, J., and Birnstiel, M. L. (1974) J. Mol. Biol. 87, 237

Stafford, H. A. (1951) Physiol. Plantarum 4, 696

Steitz, J. A., and Jakes, K. (1975) Proc. Nat. Acad. Sci. USA 72, 4734

Stanier, R. Y. (1974) Symp. Soc. Gen. Microbiol. 24, 219

Suyama, Y., and Bonner, W. D., Jr. (1966) Plant Physiol. 41, 383

Suyama, Y., and Hamada, J. (1976) In The Genetics and Biogenesis of Chloroplasts and Mitochondria (Bucher, Th., Neupert, W., Sebald, W., and Werner, S., eds.), North-Holland, Oxford, 763

Suyama, Y., and Miura, K. (1968) Proc. Nat. Acad. Sci. USA 60, 235

Swanson, R. F., and Dawid, I. B. (1970) Proc. Nat. Acad. Sci. USA 66, 117

Talen, J. L., Sanders, J. P. M., and Flavell, R. A. (1974) Biochim. Biophys. Acta 374, 129

Taylor, R. H., and Dubin, D. T. (1975) J. Cell Biol. 67, 428a

Tewari, K. K. (1971) Ann. Rev. Plant Physiol. 22, 141

Uchida, T., Bonen, L., Schaup, H. W., Lewis, B. J., Zablen, L., and Woese, C. (1974) J. Mol. Evol. 3, 63

Uzzell, T., and Spolsky, S. (1974) American Scientist 62, 334-343

Van den Bogert, C., and deVries, H. (1976) Biochim. Biophys. Acta 422, 227

Vasconcelos, A. C. L., and Bogorad, L. (1971) Biochim. Biophys. Acta 228, 492

Vedel, F., and Quetier, F. (1974) Biochim. Biophys. Acta 340, 374

Verma, I. M., Edelman, M., and Littauer, U. Z. (1971) Eur. J. Biochem. 19, 124

Vesco, C., and Penman, S. (1969) Proc. Nat. Acad. Sci. USA 62, 218

Vournakis, J. N., Gelinas, R. E., and Kafatos, F. C. (1974) Cell 3, 265

Walker, T. A., Betz, J. L., Olah, J., and Pace, N. R. (1975) FEBS Lett. 54, 241

Wharton, D. C., and Tzagoloff, A. (1967) Methods Enzymol. 10, 245

Wheeldon, L. W., and Lehninger, A. L. (1966) Biochemistry 5, 3533

Wilson, R. H., Hanson, J. B., and Mollenhauer, H. H. (1968) Plant Physiol. 43, 1874

Zylber, E., Vesco, C., and Penman, S. (1969) J. Mol. Biol. 46, 201.

Zylber, E., and Penman, S. (1969) J. Mol. Biol. 46, 201.