THE SYNTHESIS OF RNA BY ISOLATED RAT LIVER MITOCHONDRIA

THE SYNTHESIS OF RNA
BY ISOLATED RAT LIVER MITOCHONDRIA

By

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SCOPE AND CONTENTS:

Mitochondria contain DNA which is distinct from nuclear DNA. The capacity of isolated rat liver mitochondria to synthesize RNA and the types of RNA synthesized were examined in order to determine the genetic function of mitochondrial DNA.

It was demonstrated that isolated rat liver mitochondria synthesize RNA, incorporating [^{3}H]UTP, [^{3}H]ATP, [^{3}H]CTP and [^{3}H]GTP in a DNA-dependent reaction. In addition, the DNA-independent incorporation of [^{3}H]CTP and [^{3}H]ATP suggested metabolic turnover of the CCA end of mitochondrial tRNA.

Analysis of the newly-synthesized RNA by sucrose density gradient centrifugation and agarose-polyacrylamide gel electrophoresis demonstrated that mitochondrial ribosomal RNA was synthesized in a DNA-dependent process. It is concluded that one of the genetic functions of mitochondrial DNA is to code for mitochondrial ribosomal RNA.

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LIST OF ABBREVIATIONS

AMP = adenosine monophosphate

ADP = adenosine diphosphate

ATP = adenosine triphosphate

cpm = counts per minute

CMP = cytidine monophosphate

CTP = cytidine triphosphate

dATP = deoxyadenosine triphosphate

dCTP = deoxycytidine triphosphate

dGTP = deoxyguanosine triphosphate

dUTP = deoxyuridine triphosphate

DNA = deoxyribonucleic acid

DNase = deoxyribonuclease

EDTA = ethylenediaminotetra-acetic acid

EGTA = ethylene glycol bis (2-aminoethylether)N,N'-tetra-acetic acid

GMP = guanosine monophosphate

GTP = guanosine triphosphate

hr = hour

min = minute

mRNA = messenger RNA

nm = nano meter ($1x10^{-9}$ meter)

NMP = nucleotide monophosphate

NTP = nucleotide triphosphate

RNA = ribonucleic acid

RNase = ribonuclease

SDS = sodium dodecyl sulphate

TES = N-tris (hydroxymethyl) methyl-2-amino ethane-sulphonic acid

tris = tris (hydroxymethyl) amino-methane

tRNA = transfer RNA

UMP = uridine monophosphate

UDP = uridine diphosphate

UTP = uridine triphosphate

° = degrees centrigrade

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I INTRODUCTION

While the nucleus of plant and animal cells has long been considered the exclusive site for DNA containing the complete genetic blueprint (Allfrey, 1959), there have been many reports that cytoplasmic organelles contain genetic systems distinct from the nuclear one (Gibor and Izawa, 1963; Pitelka, 1961; Ris, 1961). The mitochondrion is one such organelle. Mitochondria are present in all plant and animal cells so far examined. Mitochondria contain unique species of DNA, ribosomal, transfer and possibly messenger RNA, DNA and RNA polymerases and ribosomes and are capable of synthesizing DNA, RNA and protein (Roodyn and Wilkie, 1968; Ashwell and Work, 1970a; Rabinowitz and Swift, 1970).

Mitochondrial DNA

The presence of DNA in mitochondria has been demonstrated by electron microscopy (Nass and Nass, 1963a) and by autoradiography (Guttes and Guttes, 1964; Nagata, Shibata and Nawa, 1967). Mitochondrial DNA has been isolated and characterized by many workers. It differs in a number of features from nuclear DNA of the same species. The molecular weight of mammalian mitochondrial DNA is around 10^7 daltons. It is about 5 μ long, has a closed circular form (Sinclair, Stevens, Gross and Rabinowitz, 1967) and differs from nuclear

DNA in buoyant density (Schneider and Kuff, 1965). A characteristic property of mitochondrial DNA is its rapid rate of renaturation (Borst, Kroon and Ruttenberg, 1966). The number of DNA molecules per mitochondrion is unknown. In vertebrate liver there are on average four or five molecules per mitochondrion (Borst, Ruttenberg and Kroon, 1967). It is generally assumed that all the DNA molecules in one mitochondrion are identical.

Unicellular eukaryotic species such as yeast (Hollenberg, Borst, Thurning and Van Bruggen, 1969; Sinclair, Stevens, Sanhavi and Rabinowitz, 1967; Van Bruggen, Runner, Borst, Ruttenberg, Kroon and Schuurmansstekhoven, 1968), Neurospora crassa (Wood and Luck, 1969) and Tetrahymena pyriformis (Suyama, 1966) and plants such as red bean Phaseolus vulgaris (Wolstenholme and Gross, 1968) seem to have substantially larger DNA molecules in their mitochondria. Hollenberg et al. (1969) recently found circular DNA molecules that were over 26 μ long in yeast. Wood and Luck (1969) isolated 25 μ long linear molecules from Neurospora mitochondria. Wolstenholme and Gross (1968) found 20 μ long linear DNA molecules in red bean mitochondria.

In addition to the simple circular form, animal mitochondrial DNA has been shown to exist in two complex forms; a circular dimer of double circumference and a catenate form where single-length molecules are connected to each other as links in a chain (Hudson and Vinograd,

1967; Clayton and Vinograd, 1969; Nass, 1969). Their significance is not known although the circular dimer is found in higher concentration in certain types of leukemia (Clayton and Vinograd, 1969).

The size of DNA isolated from mitochondria is very small compared to both bacterial and eukaryotic nuclear DNA, being 200 times smaller than Escherichia coli DNA and 200,000 times less than the rat or human haploid genomes. One 5 μ mitochondrial DNA molecule contains 15,000 base pairs and can code for only 5,000 amino acids or 30 polypeptides with molecular weight of 20,000. Since there are some 300 known mitochondrial proteins, this amount of DNA could not possibly provide enough information for the formation of all mitochondrial components (Roodyn and Wilkie, 1968).

Synthesis of Mitochondrial DNA

Characterization of mitochondrial DNA in comparison to the nuclear DNA of the same species has shown the presence of a unique DNA in mitochondria, directly demonstrating that a cytoplasmic genetic system is present in mitochondria. However, the above studies do not show how and where mitochondrial DNA is synthesized.

Gross and Rabinowitz (1969) studied the synthesis of mitochondrial DNA of rat liver in vivo, using [^{3}H]-5-bromo-2'-deoxyuridine as a precursor. Radioactively-labelled mitochondrial DNA had a higher buoyant density

by 0.010 g/ml in cesium chloride centrifugation than previously existing unlabelled mitochondrial DNA, indicating that approximately 20 % of the thymine residues were substituted with [^{3}H]bromouracil in one DNA strand (Kohnlein, Thorsett and Hutchinson, 1966). If replication of DNA is semiconservative, one would expect the buoyant density difference between two strands to increase by a factor of 2 on denaturation. The buoyant density difference between radioactivity and absorbance at 260 nm doubled (0.020 g/ml) when the radioactively-labelled DNA was denatured and the strands were separated, demonstrating that radioactivity was present in only one strand. From this experiment, it was concluded that the synthesis of mitochondrial DNA was by a semiconservative mechanism.

Is mitochondrial DNA replicated in mitochondria, enabling the mitochondria to maintain their genetic continuity, or are there nuclear copies of mitochondrial DNA which are replicated in nuclei and then transferred to mitochondria? Do mitochondria possess a DNA-synthesizing apparatus? A direct approach to answer these questions would be to see whether isolated mitochondria can synthesize DNA. DNA polymerase activity has been found in isolated mitochondria from rat liver (Helge and Neubert, 1965; Parsons and Simpson, 1967) and yeast (Wintersberger, 1966a and b). Isolated mitochondria incorporated thymidine triphosphate into DNA in a reaction requiring the presence of the four deoxynucleoside triphosphates (Wintersberger,

1966a; Parsons and Simpson, 1967). Parsons and Simpson (1967) used a hypo-osmotic incubation medium containing Mg^{2+} and phosphate, necessary for oxidative phosphorylation in mitochondria, and succinate, pyruvate and malate as energy sources. Incorporation of [^{3}H]deoxyribonucleotides was resistant to DNase in the reaction medium (Wintersberger, 1966a; Parsons and Simpson, 1967), presumably because the mitochondrial membrane is impermeable to DNase. The product has been identified as mitochondrial DNA by its characteristic buoyant density (Iwashima and Rabinowitz, 1969; Wintersberger, 1966b; Mitra and Bernstein, 1970), and by its ability to renature rapidly (Parsons and Simpson, 1967). Density labelling experiments in cesium chloride using 5-bromodeoxyuridine triphosphate instead of thymidine triphosphate as precursor demonstrated that the observed synthesis of DNA *in vitro* was due to replication (Karol and Simpson, 1968).

A DNA polymerase has been solubilized and partially purified from mitochondria. DNA polymerase purified from yeast mitochondria required all four deoxyribonucleoside triphosphates, Mg^{2+} and DNA for activity. Yeast mitochondrial DNA was a better template for the mitochondrial DNA polymerase than for the nuclear enzyme (Wintersberger and Wintersberger, 1970b). The enzyme from rat liver mitochondria could be differentiated from the soluble nuclear DNA polymerase by DEAE-cellulose chromatography (Kalf and Ch'ih, 1968; Meyer and Simpson,

1968) while the mitochondrial and nuclear enzymes of yeast had different sedimentation properties (Iwashima and Rabinowitz, 1969). The rat liver mitochondrial DNA polymerase-template complex was more sensitive to ethidium bromide than was the nuclear enzyme complex (Meyer and Simpson, 1969). All these studies suggested that DNA polymerase from mitochondria is different from the nuclear counterpart. While it is not known whether the isolated DNA polymerase is the enzyme responsible for replication of mitochondrial DNA, studies on DNA synthesis by isolated mitochondria establish that mitochondrial DNA maintains a genetic continuity, replicating in mitochondria and not arising from the nucleus.

Site of Genes for Mitochondrial Proteins

Since the amount of genetic information in mitochondrial DNA is insufficient to code for all mitochondrial proteins, most of them must be coded for by nuclear genes. This has been shown for cytochrome c. Chromosomal mutants of yeast have been isolated in which the primary structure of cytochrome c is altered by a single amino acid change (Sherman, Stewart, Margoliash, Parker and Campbell, 1966). Studies of the kinetics of labelling cytochrome c with amino acids in mammalian cells are consistent with microsomal synthesis (Freeman, Haldar and Work, 1967; Kandenbach, 1969; Gonzalex-Cadavid and Campbell, 1967). A typical precursor-product relationship

is present with microsomal cytochrome c being labelled first and mitochondrial cytochrome c later. These studies suggest that those mitochondrial proteins which are coded for by nuclear genes are synthesized in the cytoplasm.

In order to determine where mitochondrial proteins are synthesized, inhibitors which act specifically on protein synthesis in mitochondria or on that in the cytoplasm have been used. It was found that chloramphenicol inhibits protein synthesis by isolated mitochondria of rat liver (Kroon, 1963; Ashwell and Work, 1968; Freeman, 1970), and of yeast (Wintersberger, 1965), but does not inhibit protein synthesis in the cytoplasm of eukaryotic cells (Bretthauser, Marcus, Chalaupka, Halvorson and Bock, 1963). In contrast, cychloheximide inhibits protein synthesis in the cytoplasm (Sisler and Siegel, 1967) but has no effect on protein synthesis by isolated mitochondria (Ashwell and Work, 1968; Loeb and Hubby, 1968). Using chloramphenicol and cycloheximide, the site of synthesis of mitochondrial proteins has been examined. For example, Küntzel (1969) demonstrated that cycloheximide inhibited the incorporation of labelled amino acid into mitochondrial ribosomal proteins, whereas chloramphenicol has no significant effect showing that mitochondrial ribosomal proteins of Neurospora were synthesized in the cytoplasm.

In whole cells, it has been found that cycloheximide had a very pronounced inhibitory effect on the incorporation of radioactive amino acids into all cell fractions.

However, cycloheximide inhibited least the synthesis of the insoluble mitochondrial proteins (Beattie, 1968; Sebald, Hofstotter, Racker and Bucher, 1969a and 1969b; Ashwell and Work, 1968; Neupert and Ludwig, 1971). This suggested that while there are some protein(s) synthesized in mitochondria these represent only a small portion of the total mitochondrial proteins, reflecting the limited amount of mitochondrial genetic information.

Protein Synthesis by Isolated Mitochondria

Studies of protein synthesis *in vivo* suggested that mitochondria synthesized a small portion of mitochondrial protein but did not demonstrate whether the proteins are coded for by mitochondrial DNA. Are the proteins translated from mitochondrial RNA coded for by mitochondrial DNA or translated from messenger RNA transported from the nucleus to mitochondria? Demonstration of the synthesis of a protein by isolated mitochondria would establish its site of synthesis. Protein synthesis in isolated mitochondria has been extensively studied by following the incorporation of labelled amino acids into an acid-insoluble form (Roodyn, Reis and Work, 1961; Roodyn, 1962; Roodyn, Suttie and Work, 1962; Kroon, 1964; Kroon, 1965; Roodyn, 1965). Roodyn *et al*. (1961) used an iso-osmotic incubation medium containing Mg^{2+} and phosphate, necessary for oxidative phosphorylation, and succinate as an energy source. Incorporation of labelled

amino acid was inhibited by incubating under anaerobic conditions, in the presence of 2,4-dinitrophenol, potassium cyanide or in the absence of inorganic phosphate or an oxidizable substrate (succinate or α-oxoglutarate), indicating that oxidative phosphorylation by mitochondria provides energy for the incorporation process. Two unique features of the synthesis of protein by isolated mitochondria are the insensitivity to RNase (McLean, Cohn, Brandt and Simpson, 1958; Roodyn et al., 1961) and the sensitivity to chloramphenicol (Kroon, 1963; Wintersberger, 1965; Ashwell and Work, 1968; Freeman, 1970). At one time there was much doubt as to whether the synthesis of protein by isolated mitochondria was a true mitochondrial activity or was due to contaminating bacteria. Von der Decken, Low and Sandell (1966) suggested that isolated rat liver mitochondria are not able to synthesize protein and that all the activity measured was attributable to micro-organisms in the mitochondrial preparations. The conclusion was based mainly on their experiments with sterile mitochondrial preparations, which did not incorporate any amino acids. However, Kroon, Saccone and Botman (1967) clearly demonstrated that [^{14}C]leucine was incorporated into protein by isolated rat liver mitochondria under sterile condition. It was then shown that the lack of incorporation by mitochondria observed by Von der Decken et al. (1966) was due to the high osmolarity of their incubation medium

and that mitochondria incubated under iso-osmotic conditions could incorporate amino acids (Haldar and Freeman, 1969). Another possible source of the incorporation is microsomal contamination. The insensitivity of the amino acid incorporation by isolated mitochondria to RNase and its sensitivity to chloramphenicol are considered sufficient for rejecting microsomal contamination (McLean et al., 1958; Roodyn et al., 1961). Therefore it is concluded that isolated mitochondria synthesize protein.

That the mitochondrial membrane was the major location of proteins synthesized in mitochondria was suggested by Roodyn (1962) and Wheeldon and Lehninger (1966), who found radioactivity in insoluble proteins of membrane fractions after incubation of isolated mitochondria. In order to define the site more precisely, Neupert, Bridiczka and Bücher (1967) labelled isolated mitochondria of rat liver with radioactive amino acid, separated the inner and outer membranes, and calculated that the outer membrane had a specific radioactivity less than 5 % of that of the inner membrane. It was concluded that amino acids are incorporated into proteins of the inner membrane.

Mitochondrial Ribosomes, Ribosomal RNA and tRNA

The experiments in vivo described above suggested that some protein(s) are synthesized in mitochondria. The demonstration that isolated mitochondria synthesize protein established the capability of mitochondria

to synthesize protein, supporting the work of experiments *in vivo*. If mitochondria synthesize protein, the question of the nature of its protein-synthesizing machinery arises. Is the protein-synthesizing machinery in mitochondria similar to or different from the cytoplasmic counterpart?

During the last few years, a large number of reports have appeared on the nature of the mitochondrial protein-synthesizing machinery. As with prokaryotes, chloraplasts and the cytoplasm of eukaryotes, mitochondria contain ribosomes, ribosomal RNA and tRNA. Since, in the process of cell fractionation by differential centrifugation, mitochondria are isolated after nuclei and before the microsomal fraction which contains cytoplasmic ribosomes, ribosomal RNA and tRNA, the origin of RNA associated with mitochondrial RNA was previously suspected of being due to cytoplasmic contamination. The nature of the mitochondrial ribosomes, ribosomal RNA and tRNA was masked by their cytoplasmic counterparts. But repeated washing of the mitochondrial pellet and development of techniques such as sucrose density gradient centrifugation has allowed the isolation of mitochondria free from cytoplasmic contamination and the characterization of the mitochondrial protein-synthesizing machinery from many organisms. Mitochondrial ribosomes and ribosomal RNA are compared with those from the corresponding cytoplasm and *E. coli* in Table 1 and 2, respectively.

Table 1

S Values of Mitochondrial Ribosomes Isolated from Different Species

Organism and Reference	S Values		
	Mitochondria	Cytoplasm	E. coli
Neurospora crassa			
Kuntzel and Noll (1967)	73	77	70
Rifkin, Wood and Luck (1967)	81	81	
Yeast			
Schmitt (1969)	80	80	70
Stegeman *et al.* (1970)	75	80	
Vignais, Huet and Andre (1969)	80	80	
Morimoto and Halvorson (1971)	80	80	
Rat Liver			
O'Brien and Kalf (1967)	55	78	
Ashwell and Work (1970b)	50-55	80	70
Bartoov (1971)	55		
Mouse Liver			
Georgatsos *et al.* (1968)	78	78	
Beef, Pig and Rabbit Liver			
O'Brien (1969)	70		
Mouse L Cells			
Bartoov (1971)	55	76	

Table 2

S and "S" Values and G+C Content of Mitochondrial RNA Isolated from Different Species

Organism and Reference	S Values			"S" Values			G+C % Content	
	Mit.	Cyt.	E. coli	Mit.	Cyt.	E. coli	Mit.	Cyt.
Neurospora crassa								
Kuntzel and Noll (1967)	21 16	25 16	- 16				37	49
Dure et al. (1967)	23 16	25.6 16	23					
Wood and Luck (1969)	25 19	28 18					35	50
Rifkin et al. (1967)	25 19	28 18						
Yeast								
Wintersberger (1967)	23 16		23 16					
Rogers et al. (1967)	22.4 17.8	24.6 16.2	22.6 18.9					
Leon and Mahler (1968)	23 16	25 17						
Steinschmeider (1969)	25 16	28 19						

(continued)

Fauman et al. (1969)	22	26	23				26	47
	15	17	26					
Forrester et al. (1970)	21	25	23				26	
	15	17.5	16					
Stegeman et al. (1970)				25	26	23		
				17	19	16		
Morimoto and Halvarson (1971)							30.2	52.6
Mammals								
Rat Liver								
Bartoov et al. (1970)	15	28		21.0	28		46.5	63.7
and Bartoov (1971)	13	18		12.5	18			
Mouse L cells								
Bartoov et al. (1970)	15	28		21.0	28		39.6	61.6
and Bartoov (1971)	12.5	18		12.5	18			
Human HeLa cells								
Knight (1969)	27	28						
	18	18						
Vesco and Penman (1969)				21	28		41.7	
and Zylber et al. (1969)				12	18			
Attardi and Attardi (1971)	16	28		21	28			
	12	18		12	18			

(continued)

Human KB cells						
Bartoov (1971)			20.5 11.0	28 18		
Human BHK-21 cells						
Dubin and Brown (1967) and Dubin (1969) and Dubin and Montenecourt (1970)	(27) 17	28 18			38	46.9
Montenecourt et al. (1970)	16.8 13.4	28 18				

Mit. = mitochondria

Cyt. = cytoplasm

"S" = electrophoretic mobility taking the S values of cytoplasmic ribosomal RNA as references of "28 S" and "18 S".

Neurospora

The mitochondrial ribosomes of Neurospora are not clearly differentiated in size from the cytoplasmic ones but mitochondrial ribosomal RNA is different from the cytoplasmic ribosomal RNA. Mitochondrial ribosomal proteins can be distinguished from those of the cytoplasm and E. coli, since the elution patterns from carboxymethyl cellulose of the 30-40 different ribosomal proteins were different (Küntzel, 1969).

Mitochondria in Neurospora have been shown to contain tRNA's that differ from the cytoplasmic tRNA's (Barnett and Brown, 1967; Barnett, Brown and Epler, 1967; Brown and Novelli, 1968; Epler, 1969; Epler and Barnett, 1967). Epler (1967) showed distinct and separable species of mitochondrial and cytoplasmic tRNA for 15 amino acids. Neurospora mitochondria also contained aminoacyl-tRNA synthetases, which had different chromatographic behavior from their cytoplasmic counterparts (Barnett et al., 1969; Brown and Novelli, 1968).

Yeast

Mitochondrial ribosomes were reported to be similar to cytoplasmic ribosomes in sedimentation properties but mitochondrial ribosomal RNA was different from the cytoplasmic ribosomal RNA in sedimentation rate and base composition but similar to E. coli ribosomal RNA in sedimentation rate. Aminoacyl-tRNA's that differ

from their cytoplasmic counterparts have been reported in yeast mitochondria (Wintersberger, 1965).

Mammals

The agreement of the work of O'Brien and Kalf (1967), Ashwell and Work (1970) and Bartoov (1971) suggested that mammalian mitochondrial ribosomes have a smaller sedimentation rate (55 S) than that of ribosomes in the cytoplasm and E. coli. The agreement of the work of Bartoov et al. (1970), Bartoov (1971), Vesco and Penman (1969), Zylber et al. (1969), and Attardi and Attardi (1971) demonstrated that mammalian ribosomal RNA has sedimentation rates of 15-16 S and 12-13 S and electrophoretic mobilities of "21 S" and "12 S" taking the S values of cytoplasmic ribosomal RNA as references of "28 S" and "18 S". Mitochondrial ribosomal RNA has a lower G + C content than the cytoplasmic ribosomal RNA. All these studies suggested that mammalian mitochondrial ribosomes and ribosomal RNA are distinct from the cytoplasmic counterparts.

Aminoacyl-tRNA's different from their cytoplasmic counterparts have been reported in rat liver. Buck and Nass (1969) demonstrated that six species of tRNA (leucyl-, tyrosyl-, aspartyl-, valyl-, and seryl-tRNA) different from cytoplasmic aminoacyl-tRNA's of rat liver in chromatographic behavior on methylated albumin-coated kieselguhr were found exclusively in mitochondria. It

was also found that cytoplasmic tRNA synthetases were unable to acylate species of mitochondrial tRNA. Leitman (1968) reported a phenylalanyl-tRNA in mitochondria of rat liver which was chromatographically separable from the phenylalanyl-tRNA of the cytoplasm.

Mitochondrial Messenger RNA

While mitochondria from all species so far examined have been shown to contain unique ribosomes, ribosomal RNA, tRNA's and aminoacyl-tRNA synthetases which are different from their cytoplasmic counterparts, there are a few reports of messenger-like RNA in mitochondria. The presence of a rapidly-labelled extramitochondrial RNA in HeLa cells, which hybridizes with mitochondrial DNA, has been reported by Attardi and Attardi (1967, 1968). The suggestion was made that the RNA might leave the mitochondria and become associated with polysomes bound to the endoplasmic reticulum. Attardi and Attardi (1969) reported that most of the RNA homologous to mitochondrial DNA in HeLa cells is represented by 4 S RNA and by species with sedimentation rates between 9 S and 15 S with a prominent 12 S component. However, it is not known whether the 9 S to 15 S components are utilized within the mitochondrion, or whether, on the contrary, they include a fraction of mitochondrial RNA exported to the rough endoplasmic reticulum. Because of the later work of Attardi (Attardi and Attardi, 1971) in which the 12 S

component was shown to be a mitochondrial ribosomal RNA species the interpretation of a messenger RNA that leaves the mitochondrion must be considered unproven.

Similarity Between Mitochondrial and Bacterial Protein Synthesis

There are several similarities in the protein-synthesizing systems of mitochondria and bacteria. Chloramphenicol inhibits protein synthesis by isolated rat liver mitochondria (Kroon, 1963; Ashwell and Work, 1968; Freeman, 1970), yeast mitochondria (Wintersberger, 1965) and bacteria (Roodyn and Wilkie, 1968) but does not inhibit protein synthesis in the cytoplasm of eukaryotic cells. In contrast, cycloheximide has no effect on protein synthesis by isolated mitochondria (Ashwell and Work, 1968; Loeb and Hubby, 1968) and bacterial protein-synthesizing systems (Roodyn and Wilkie, 1968) but inhibits protein synthesis in cytoplasm (Sisler and Siegel, 1967). N-Formylmethionyl-tRNA is present in mitochondria (Smith and Marcker, 1968; Galper and Darnell, 1969; Halbreich and Rabinowitz, 1971) but not in the cytoplasm. N-Formylmethionyl-tRNA is involved in the initiation of protein synthesis in bacteria (Clark and Marcker, 1966; Lengyel and Soll, 1969). All these observations suggest that there is some evolutionary relationship between mitochondria and bacteria. One suggestion is that mitochondria are derived from symbiotic bacteria. It

would be of interest if there are similarities between mitochondria and bacteria in the synthesis of DNA and RNA.

The Site of Genes for Mitochondrial RNA

Studies on the physical and chemical properties of mitochondrial RNA using sucrose density gradient centrifugation, polyacrylamide gel electrophoresis, column chromatography and the nucleotide composition establish that mitochondria contain unique RNA different from cytoplasmic RNA, but these studies do not show the origin of mitochondrial RNA. Is mitochondrial RNA transcribed from mitochondrial DNA or is it transcribed from nuclear DNA and transported into mitochondria or do both processes occur? In order to determine the origin of mitochondrial RNA, there are four possible approaches (a) hybridization between mitochondrial RNA and mitochondrial DNA and nuclear DNA. (b) selective inhibition of the synthesis of RNA from nuclear DNA templates. (c) selective inhibition of synthesis of RNA from mitochondrial DNA templates. (d) studies of RNA synthesis by isolated mitochondria.

In order to determine whether mitochondrial RNA is transcribed from mitochondrial DNA or from the nuclear DNA, hybridization studies could be made between mitochondrial RNA and the two DNAs. If mitochondrial RNA specifically hybridizes with mitochondrial DNA, then mitochondrial RNA must be coded for by mitochondrial

DNA. Wood and Luck (1969) showed that mitochondrial ribosomal RNA from Neurospora hybridized with mitochondrial but not nuclear DNA, and that cytoplasmic ribosomal RNA did not hybridize with mitochondrial DNA. Saturation levels of up to 10 % were obtained, indicating at least 3 genes for mitochondrial ribosomal RNA. Casey, Fukuhara, Getz and Rabinowitz (1969) observed hybridization of mitochondrial leucyl-tRNA of yeast with mitochondrial DNA. Mitochondrial fMet - tRNA of yeast hybridized with yeast mitochondrial DNA (Halbreich and Rabinowitz, 1971).

However, Wintersberger and Viehhauser (1968) found that mitochondrial ribosomal RNA of yeast hybridized not only with mitochondrial DNA (9 %) but also with yeast nuclear DNA (2.5 %) and suggested that the yeast nuclear DNA contains nucleotide sequences complementary to yeast mitochondrial RNA. This result indicates the presence of at least 2 genes and 160 genes for mitochondrial ribosomal RNA in mitochondrial DNA and in nuclear DNA respectively, since the molecular weight of yeast mitochondrial ribosomal RNA, yeast mitochondrial DNA and yeast nuclear DNA is respectively 2.02×10^6 daltons (Loening, 1968), 5×10^7 daltons (Hollenberg, Borst and Van Bruggen, 1970) and 1.3×10^{10} daltons (Ogur, Minckler, Lindegren and Lindegren, 1952). It is an open question whether the mitochondrial ribosomal RNA

used by Wintersberger and Viehhauser was free from cytoplasmic RNA. If mitochondrial RNA hybridizes with both mitochondrial DNA and nuclear DNA, the site of synthesis cannot be unambiguously determined by hybridization studies.

Cohen, Hollenberg and Borst (1970) interpreted the above result as meaning that the yeast mitochondrial ribosomal RNA contained two types of RNA, one type hybridizing only with mitochondrial DNA, the other hybridizing only with nuclear DNA. To check this possibility, these workers prehybridized RNA from yeast mitochondrial preparations with yeast mitochondrial DNA, then recovered the RNA from the hybrid and determined whether this RNA would also hybridize with yeast nuclear DNA. They found that about 7 % of the total mitochondrial DNA hybridized with mitochondrial ribosomal RNA so that the total molecular weight of the mitochondrial RNA hybridized with one copy of mitochondrial DNA corresponded to 3.5×10^6 daltons ($5 \times 10^7 \times 0.07 = 3.5 \times 10^6$). Since 0.01 % of yeast nuclear DNA hybridized with this mitochondrial RNA, the total molecular weight of the mitochondrial RNA hybridized with one copy of the nuclear DNA corresponds to 1.3×10^6 daltons ($1.3 \times 10^{10} \times 0.0001 = 1.3 \times 10^6$), which is equivalent to 37 % of the amount hybridized to mitochondrial DNA ($\frac{1.3 \times 10^6}{3.5 \times 10^6} \times 100 = 37$ %). This experiment suggests that there is less than one gene for yeast mitochondrial

ribosomal RNA in each yeast nuclear DNA genome. It was not conclusively shown whether hybridization between mitochondrial RNA and nuclear DNA was due to contamination of nuclear DNA with mitochondrial DNA or homology of nucleotide sequences between the two. This experiment, however, does not rule out the possibility that there are species of mitochondrial RNA that are coded for in the nucleus. It shows that DNA-RNA hybridization results such as those of Wintersberger and Viehhauser (1968) must be treated with caution.

In mammals, Attardi and Attardi (1969) found that the main species of RNA (12 S) in the mitochondrial fraction of HeLa cells hybridized with mitochondrial DNA. As an approach to determining the site of synthesis of the mRNA associated with the endoplasmic reticulum, Attardi and Attardi (1967, 1968) carried out hybridization experiments between this mRNA and the free polysome mRNA, on the one hand, and total DNA and mitochondrial DNA on the other. They suggested that the endoplasmic reticulum-associated mRNA is synthesized in mitochondria and exported to the endoplasmic reticulum, observing that the mRNA hybridized more efficiently with mitochondrial DNA (20 %) than with total cellular DNA (5 %). They could not, however, show specific hybridization between the endoplasmic reticulum-associated mRNA and mitochondrial DNA, due to possible contamination of mitochondrial DNA with the nuclear DNA and contamination of free polysomes

with endoplasmic reticulum-associated polysomes. Clearly RNA and DNA from highly purified subcellular fractions are required to obtain meaningful results. Experiments have not yet been carried out with mammals as have been done with yeast to see whether there is any homology in nucleotide sequence between mitochondrial RNA and nuclear DNA.

Another approach to finding the site of synthesis of mitochondrial RNA would be to use an inhibitor which would preferentially react with the nuclear DNA, inhibiting the synthesis of RNA from nuclear but not mitochondrial DNA templates. A low level of actinomycin D (0.1 µg/ml) was used to preferentially suppress cytoplasmic ribosomal RNA synthesis (Dubin and Montenecourt, 1970). The synthesis of mitochondrial ribosomal RNA was resistant to actinomycin D, suggesting that it was synthesized from mitochondrial DNA templates. This approach has some disadvantages. For example, actinomycin D at this level does not affect the synthesis of mRNA in the nucleus (Penman, Vesco and Penman, 1968).

A simpler approach would be to use an inhibitor which would act specifically on the synthesis of RNA in mitochondria. Ethidium bromide at concentrations lower than 5.4 µg/ml has a greater affinity for circular mitochondrial DNA than for nuclear DNA (Bauer and Vinograd, 1968). The tertiary structure of circular DNA is extensively altered when ethidium bromide is intercalated.

Selective inhibition of the synthesis of mitochondrial RNA by ethidium bromide was used as a criterion for concluding that mitochondrial RNA was transcribed from mitochondrial DNA (Zylber, Vesco and Penman, 1969).

RNA Synthesis by Isolated Mitochondria

The direct approach would be to study RNA synthesis by isolated mitochondria. If the mitochondrial fraction synthesizes RNA and the activity is due to mitochondria and is DNA-dependent, such experiments would directly demonstrate that RNA synthesized by isolated mitochondria is transcribed from mitochondrial DNA. DNA-dependent RNA synthesis would be shown by inhibition by actinomycin D, acriflavin or ethidium bromide.

At the start of this study little was known about mitochondrial RNA or its site of synthesis. Since isolated mitochondria had been found to synthesize protein and DNA, use of this approach would be a direct way to demonstrate which RNA species are coded by and transcribed from mitochondrial DNA. Several studies, described below, using this approach have been reported. Many of these were published while this work was in progress. However, in none of the work by others has there been a definitive demonstration of the synthesis of known species of mitochondrial RNA.

RNA synthesis in isolated mitochondria was first investigated by Wintersberger (1964), who observed

linear incorporation for 60 min of [^{3}H]uridine into acid-insoluble material by rat liver mitochondria isolated in 0.25 M sucrose-1 mM EDTA-20 mM tris-HCl, pH 7.4 and purified on a urographin gradient. The acid-insoluble product was solubilized by treatment with alkali or hot acid, indicating that it was RNA. Incorporation was inhibited by actinomycin, indicating that the RNA synthesis was DNA-dependent. However, uridine is poorly incorporated into RNA by rats (Hammarsten, Reichard and Saluste, 1950) raising the possibility that the incorporation observed by Wintersberger was not mitochondrial.

Luck and Reich (1964) demonstrated that isolated Neurospora mitochondria were capable of incorporating [^{3}H]GTP into an acid-insoluble form. The incorporation of [^{3}H]GTP reached maximal levels within 5 min, after which there was a progressive decrease in acid-insoluble radioactivity. The incorporation of [^{3}H]GTP was dependent on the presence of the other three ribonucleoside triphosphates and was inhibited by actinomycin D but not by DNase or RNase. The acid precipitate was fully solubilized by alkali yielding only radioactive GMP on electrophoresis. Incubation of the alkaline hydrolyzate with 5'-nucleotidase did not alter the level of the radioactivity of the GMP, which was therefore, identified as 2'(3')GMP. Thus [^{3}H]GTP was incorporated into internucleotide linkages. It was concluded that mitochondria of Neurospora posses an RNA-synthesizing

system which is DNA-dependent. However, the incorporation of [^{3}H]GTP continued for only 5 min and required the presence of the added ribonucleoside triphosphates, indicating that their mitochondrial preparations were probably damaged or swollen. Either the preparation by isopycnic centrifugation in sucrose gradients (0.97 to 1.9 M sucrose without buffer) or suspension in 0.02 M tris-HCl, pH 7.9 for incorporation studies could have caused this. The activity was considered to be mitochondrial and not due to nuclei because of insensitivity to DNase and RNase. Weiss (1960) had previously demonstrated that these enzymes decrease RNA synthesis by isolated rat liver nuclei.

The above finding suggests that a DNA-dependent RNA synthesis occurs in mitochondria of Neurospora, but is this general? Since nuclei are the main site of RNA synthesis in cells, synthesis of RNA by mitochondria must be clearly differentiated from it. Further as with protein synthesis by isolated mitochondria, bacterial and microsomal contamination must be excluded. As indicated by the work of Luck and Reich (1964), the method of isolating mitochondria, of their incubation and the characterization of the product as RNA are important considerations in these studies.

Two main groups, those of Neubert and of Saccone have studied the synthesis of RNA by isolated rat liver mitochondria in some detail.

In the experiments of Neubert and Helge (1965) and Neubert, Helge and Merker (1965) mitochondria were isolated in 0.25 M sucrose and incubated in an iso-osmotic medium containing nucleoside triphosphates and phosphoenol pyruvate and pyruvate kinase as an energy source. Incubations were for 5 min and RNA was recovered by acid precipitation. Neubert and Helge (1965) found that the incorporation of [^{14}C]UTP into RNA was not inhibited by RNase and actinomycin C_1, and was independent of the presence of other nucleoside triphosphates. In contrast, [^{14}C]UTP incorporation into RNA in isolated nuclei was inhibited by RNase and actinomycin C_1 and required the addition of the other three nucleoside triphosphates. These observations distinguished RNA synthesis by isolated mitochondria from that by nuclei. The insensitivity of the mitochondrial system to RNase, DNase and actinomycin was presumed to be due to the impermeability of the mitochondrial membrane to these compounds. The lack of a requirement for nucleoside triphosphates showed that mitochondria contain sufficient nucleotides, whose presence had been shown by Heldt and Klingenberg (1965), to maintain RNA synthesis.

The effect of mitochondrial permeability was tested by increasing permeability either with repeating washings in sucrose without EDTA, preincubating the mitochondrial preparations with inorganic phosphate in the absence of respiratory substrates, or by a brief

treatment with hypo-osmotic solutions. Mitochondria treated in any one of these ways retained their capacity to incorporate labelled UTP into RNA, but the incorporation was now inhibited 90 % by actinomycin C_1 and 30 % by RNase and DNase. The presence of the other three nucleoside triphosphates was necessary for RNA synthesis in these altered mitochondrial preparations. These results indicated the importance of integrity of mitochondrial preparations for studies on the characteristic properties of nucleotide incorporation by isolated mitochondria.

However, in the studies of Neubert there was a very low rate of [^{14}C]UTP incorporation with incubations of 5 min. The acid-insoluble radioactive counts after correction for background were around 20 cpm for control experiments with a background of 42 ± 3 cpm. Moreover in later work Neubert, Helge and Taske (1966) found linear incorporation for only 5 to 8 min with incorporation ceasing after 20 min. In addition they found a K_M of 27 µM and 60 µM for [^{3}H]UTP for isolated mitochondria and nuclei, respectively. Neubert, Helge and Merker (1965) further found an equal incorporation of UTP and GTP by mitochondria but a 3-fold higher incorporation of CTP. Zero-time controls with ATP were too high to allow its use as a precursor (Neubert, Helge and Merker, 1965). They also showed that the synthesis was sensitive to acriflavin. From the work of Neubert it can be concluded that isolated rat liver mitochondria synthesize

RNA in a DNA-dependent reaction. In addition Neubert, Helge and Merker (1965) have shown this to be the case for mitochondria from rat kidney, heart and brain and pigeon heart and breast muscle.

Saccone, Gadaleta and Quagliariello (1967) differentiated RNA synthesis by isolated mitochondria from that by nuclei, using atractyloside as an inhibitor of RNA synthesis. Atractyloside inhibits the carrier system in the inner mitochondrial membrane for ADP and ATP. This system enables the passage of ADP and ATP across the membrane into an internal nucleotide pool in the matrix, or vice-versa (Bruni, Contessa and Scalhella, 1965; Winkler, Bygrave and Lehninger, 1968; Vignais and Vignais, 1970). Atractyloside inhibited incorporation of [^{14}C]ATP into an acid-insoluble form by intact mitochondria isolated from rat liver, but had no effect on the incorporation by isolated nuclei of rat liver. If mitochondria were sonicated or swollen, there was no atractyloside-sensitive incorporation of [^{14}C]ATP. The inner membrane of mitochondria was probably damaged by these pretreatments. The selective inhibition by atractyloside of [^{14}C]ATP incorporation into RNA indicates that the incorporation in isolated mitochondria is not due to nuclei contaminating the mitochondrial preparation.

Although this experiment seems straight forward there are several difficulties involved. Saccone *et al.*

(1967) isolated the mitochondria in 0.25 M sucrose and incubated in an iso-osmotic medium containing ribonucleoside triphosphates and phosphoenol pyruvate and pyruvate kinase as the energy source. They followed RNA synthesis by measuring acid-insoluble radioactivity and attempted to demonstrate that the [^{14}C]ATP-labelled product was really RNA. It was completely solubilized by hot acid or by alkali, 70 % by RNase and snake venom phosphodiesterase at 37° for 2 hr and about 33 % was recovered by extraction with NaCl and alcohol precipitation. From these results it was concluded that [^{14}C]ATP was incorporated into RNA. However, it was found that [^{14}C]ATP incorporation was 10 times higher than [^{14}C]UTP or [^{14}C]GTP (Saccone, Gadaleta and Gimigliano, 1968) and that the incorporation of ATP was linear for 40 min, while that of UTP and GTP declined rapidly after 10-20 min (Saccone et al., 1968). They suggested that ATP was taken up across mitochondrial membranes by the atractyloside-sensitive, adenine nucleotide-specific transport mechanism, and that there was a permeability barrier for UTP and GTP, which caused the low incorporation. However, mitochondria sonicated to reduce the permeability barriers for these nucleotides still incorporated UTP and GTP poorly compared with ATP. In view of these difficulties it is an open question whether the ATP incorporated into an acid-insoluble form really represents RNA synthesis. This

possibility is strengthened by the latest studies of Saccone, Gadaleta and Gallerani (1969) in which they, in contrast to their earlier work, observed an equal incorporation of [^{3}H]ATP, [^{3}H]UTP, [^{3}H]CTP and [^{3}H]GTP into RNA by isolated rat liver mitochondria pretreated with phosphate. In these experiments RNA was extracted with phenol, purified by passage through Sephadex G-25 and precipitated with alcohol.

As with amino acid incorporation, the question of bacterial contamination is important in examining nucleotide incorporation by isolated mitochondria, since contaminating bacteria in isolated mitochondria might synthesize RNA. Kroon, Saccone and Botman (1967) showed that [^{14}C]ATP was incorporated into RNA by mitochondria isolated under sterile conditions. The presence of bacteria (10^3 - 10^5/mg of mitochondrial protein) did not affect the level of [^{14}C]ATP incorporation. They concluded that RNA synthesis by isolated mitochondria was not due to contaminating bacteria, but as indicated above because ATP was used as the precursor the results are not conclusive. Kroon *et al.* (1967) also found that when no precautions were taken to avoid gross bacterial contamination of mitochondria or reagents, or when the incorporation measured was low, bacterial synthesis could easily become significant.

Both Neubert and Saccone have attempted to demonstrate the type of RNA synthesized by isolated mitochondria. The RNA was heterodisperse sedimenting from 4 - 10 S (Neubert, Helge and Merker, 1968). Saccone, Gadaleta and Gallerani (1969) made a more detailed analysis. RNA synthesized with [^{3}H]UTP or [^{3}H]ATP as precursor for 10 min in intact or swollen mitochondria showed radioactivity in the 8-14 S region of a sucrose density gradient in a heterodisperse pattern. On incubation of mitochondria with [^{3}H]ATP for 30 min, more radioactivity appeared around the 4 S region. When RNA was hydrolyzed with alkali about 6 % of the [^{3}H]UTP, [^{3}H]CTP and [^{3}H]GTP incorporated was recovered as the nucleoside and the rest as the nucleotide. With [^{3}H]ATP 16 % was recovered as the nucleoside, indicating terminal addition of adenylate to tRNA. However, the RNA synthesized might be broken down to 4 S, because the larger RNA observed in 10 min incubations disappeared at 30 min. Thus, no definitive demonstration of the synthesis of RNA species characteristic of rat liver mitochondria has been observed.

Attempts have also been made to show the types of RNA synthesized in isolated mitochondria of yeast and Tetrahymena pyriformis. Wintersberger and Tuppy (1965) showed that [^{3}H]uridine and [^{3}H]ATP were incorporated by isolated yeast mitochondria into RNA that, on sucrose density gradients, have a pattern with radioactivity at

4 S and greater. No distinct species were definitively demonstrated. Suyama and Eyer (1968) found high molecular weight RNA around 14 to 18 S for RNA synthesized with [^{3}H]UTP in incubations of 5 to 20 min for mitochondria from T. pyriformis. After 20 min the RNA was smaller in size. It was not known whether the newly-synthesized RNA was messenger or ribosomal RNA.

The difficulty in characterization of the RNA synthesized by isolated rat liver mitochondria could arise for many reasons. The RNA could be degraded during isolation or, more important in this context, during the incubation. Do the mitochondria as isolated and incubated really carry out RNA synthesis as it occurs in vivo? In the studies to date RNA synthesis continued for only 20 min. Mitochondria were usually isolated in 0.25 M sucrose and incubated in iso-osmotic media using phosphoenol pyruvate and pyruvate kinase as an energy source. In contrast, protein synthesis continues for at least 1 hr (Roodyn et al., 1961) with mitochondria isolated in 0.3 M sucrose-2 mM EDTA, pH 7.2 and incubated with succinate as an energy source. DNA synthesis continues for 2 hr. (Parsons and Simpson, 1967) with mitochondria isolated in 0.25 M sucrose-1 mM EDTA, pH 7.0 and incubated with succinate, pyruvate and malate as the energy source. It is possible, therefore, that the rapid decline in RNA synthesis could be because mitochondria were damaged during isolation in unbuffered

sucrose and because of an insufficient energy source. This in turn might prevent the complete synthesis of characteristic species of mitochondrial RNA.

Objectives and Rationale

While mitochondria have been shown to contain unique DNA, RNA and a protein-synthesizing system, the site of synthesis of many mitochondrial components is not known. Is mitochondrial RNA transcribed from mitochondrial DNA or transcribed from the nuclear DNA and transported into mitochondria? A direct approach to investigate the site of genes for mitochondrial RNA is to demonstrate whether isolated mitochondria synthesize RNA. Further, if isolated mitochondria synthesize distinct species of RNA in a DNA-dependent process it would directly establish the site of their genes.

In the earlier studies, RNA synthesis by isolated mitochondria was followed by incorporation of labelled nucleotides into an acid-insoluble form and it was not clearly demonstrated that the labelled nucleotides were incorporated into RNA. Synthesis continued for a short period of time only and the synthesis of unique species of mitochondrial RNA was not demonstrated. The aims of the present work were as follows:

(i) To investigate a method for measuring RNA synthesis.

The most reliable method would be to quantitatively extract pure RNA from mitochondria after incubation with

labelled nucleotides and measure its radioactivity. This is technically difficult because of losses of RNA and the presence of possible contaminants. A simpler method is to obtain an acid-insoluble precipitate and demonstrate that labelled nucleotides are really incorporated into RNA by determining the recovery of 2'(3') nucleotides and nucleosides by paper chromatography after alkaline hydrolysis.

(ii) To establish a system in which isolated mitochondria synthesize RNA for a longer period of time.

The groups of Saccone and of Neubert, using iso-osmotic sucrose containing no buffering agent for isolating mitochondria, observed that RNA synthesis levelled off within 10-20 min. However, Roodyn et al. (1961) and Parsons and Simpson (1969) using iso-osmotic sucrose containing EDTA for isolating mitochondria observed that synthesis of protein and DNA by isolated mitochondria lasted for at least 1 hr and 2 hr, respectively. In the present work, isolation media containing a buffering agent such as EDTA or TES were examined. The incubation medium used by Roodyn et al. (1961) and Parsons and Simpson (1969) was chosen to study RNA synthesis.

(iii) To demonstrate the capacity of mitochondria to synthesize RNA.

Since nuclei, bacteria and the microsomal fraction (Wilkie and Smellie, 1967) synthesize RNA,

these contaminants must be eliminated as the site of RNA synthesis. The direct approach of isolating mitochondria completely free from nuclei, bacteria and the microsomal fraction is technically difficult. A simpler approach is to differentiate the characteristics of RNA synthesis by the isolated mitochondrial fraction from that by nuclei, bacteria and the microsomal fraction. Differential sensitivity to DNase, RNase, ethidium bromide and changes in the incubation medium were used to distinguish RNA synthesis by isolated mitochondria from that by isolated nuclei. Furthermore, since atractyloside inhibits transport of ATP across mitochondrial membranes only it should provide a useful test to differentiate between synthesis by mitochondria and that by nuclei. To eliminate RNA synthesis by bacteria, mitochondria were prepared with sterilized sucrose media and incubated in a sterilized medium. Bacterial contamination was checked before and after incubation. Differential sensitivity to actinomycin D, the time course of nucleotide incorporation and changes in the incubation medium were used to differentiate RNA synthesis by isolated mitochondria from that by the microsomal fraction.

(iv) To characterize the system to demonstrate the site of RNA synthesis.

The first approach is to sub-fractionate mitochondria and to determine the nucleotide incorporation in each fraction. The second approach is to incubate

isolated mitochondria with labelled nucleotides, sub-fractionate the mitochondria and count the radioactivity of RNA in each fraction. The third approach is to examine the characteristics of RNA synthesis by isolated mitochondria. The first and second approaches suffer from the difficulty of isolating pure sub-fractions. The third approach is indirect but simpler. In the present work, the third approach was taken, observing the effect of osmolarity, nucleotides, RNase, DNase and atractyloside on incorporation.

(v) To examine the DNA-dependence of RNA synthesis.

This was studied by examining the effect of actinomycin D, acriflavin and ethidium bromide on nucleotide incorporation.

(vi) To characterize the RNA synthesized and to demonstrate the site of genes for mitochondrial RNA.

The RNA synthesized was characterized by sucrose density gradient centrifugation and by polyacrylamide gel electrophoresis. Dependence of the synthesis of specific mitochondrial RNA species on mitochondrial DNA was examined by the sensitivity to actinomycin D and ethidium bromide.

II METHODS AND MATERIALS

I. Preparation of Rat Liver Mitochondria

Mitochondria were prepared from rat liver by a modification of the method of Schneider and Hogeboom (1950). All possible precautions were taken to avoid nuclear and bacterial contamination in the mitochondrial fraction. Mitochondria were isolated from livers of 125-150 g hooded rats of either sex fed *ad libitum*. The isolation medium was either 0.30 M sucrose-2 mM EDTA, pH 7.2, 0.25 M sucrose-2 mM EDTA, pH 7.4 (sucrose-EDTA) or 0.25 M sucrose-0.5 mM TES, pH 7.4 (sucrose-TES). For the experiments in section II of the results, 0.30 M sucrose-2 mM EDTA, pH 7.2 was used. For the experiments in section I, III, IV, and V of the results, sucrose-EDTA or sucrose-TES were used. All equipment and the isolation media were sterilized at 120° under steam at 16 lb/sq in. for 20 min to help eliminate bacteria as a source of [^{3}H]nucleotide incorporation. The pH of the medium was readjusted to 7.2 or 7.4 with KOH if necessary just prior to use. All procedures were performed at 0-4°. The rats, usually four, were killed by a blow to the head and exsanguinated. The livers were quickly removed with scissors and forceps and placed in ice-cold medium. After the volumes of the livers were measured, they were washed twice with isolation medium, minced as small as possible (less than 10 mm^3), washed twice and suspended in 3 volumes of isolation medium. The mince was homogenized with

four slow passes of a motor-driven Potter Teflon tissue grinder and diluted with an equal amount of isolation medium to give about a 12 % homogenate. After homogenization, nuclei, unbroken cells and red blood cells were removed by two centrifugations at 1,000 x g for 10 min. The supernatant was centrifuged at 6,500 x g for 8 min. The lipid layer was gently wiped out with tissue paper. The fluffy layer was carefully discarded. The mitochondrial pellet was washed twice by resuspension first in one-half and second in one-quarter of the original volume followed each time by centrifugation at 6,500 x g for 8 min. The final mitochondrial pellet was suspended in a small volume of the isolation medium. From 4 to 7 mg of mitochondrial protein was obtained per g of liver. Further washing of the mitochondrial fraction did not remove any more visible material and did not affect the characteristics of the incorporation.

2. Preparation of Rat Liver Nuclei

Rat liver nuclei were isolated by the method of Widnell and Tata (1964). All procedures were performed at 0-4°. Livers from three or four 150 g hooded rats, fed *ad libitum*, were pooled and washed twice in 0.32 M sucrose-3 mM $MgCl_2$, pH 7.4 (homogenizing medium). The tissue was finely minced with scissors, rinsed twice and homogenized in 3 volumes of medium by 25 slow up and down movements with a Potter Teflon tissue grinder at a pestle speed of 1,000 revolutions/min.

Clumps of connective tissue and attached unbroken cells were removed by filtration through two layers and then four layers of fine mesh cheesecloth. The homogenate was diluted from 12.5 ml to 20 ml with homogenizing medium, and then to a final sucrose concentration of 0.25 M with double-distilled water. Homogenizing medium (20 ml/tube) was layered underneath 22 ml of the homogenate, and a crude nuclear pellet was obtained by centrifugation at 700 x g for 10 min. The pellets from the first low speed centrifugation were pooled and resuspended in 52 ml of 2.4 M sucrose-1 mM $MgCl_2$, pH 7.4. For the final nuclear centrifugation, 13 ml of the suspension was layered over 22 ml of 2.4 M sucrose-1 mM $MgCl_2$ and centrifuged for 1 hr. at 38,000 x g in the SW27 swinging bucket rotor of the Beckman L2-65 ultracentrifuge. An almost colorless, gelatinous nuclear sediment was obtained and erythrocytes, whole cells and mitochondria formed a plug at the top of the tube. The latter was removed with a spatula, and the tubes were stood upside down for 10 to 15 min. and the inside walls wiped with tissue paper. The nuclear pellet was resuspended in a small volume of 0.25 M sucrose-1 mM $MgCl_2$, pH 7.4.

3. Incubation of Isolated Rat Liver Mitochondria

Mitochondria were incubated either in medium I, a medium modified from Roodyn, Reis and Work (1961), or in medium II, a medium modified from Parsons and Simpson (1967). Medium I contained three unlabelled nucleotides of AMP, CMP, UMP and GMP instead of AMP. The [^{3}H]nucleoside triphosphate was

used to follow incorporation of a particular nucleotide into RNA. Medium II contained four ribonucleotides of ATP, CTP, GTP and UTP instead of four deoxyribonucleotides of dATP, dCTP, dGTP and dUTP. RNA synthesis was measured by incorporation of the [^{3}H]nucleoside triphosphate in presence of the other three unlabelled nucleoside triphosphates. The final incubation medium after addition of the mitochondrial suspension is shown in Table 3. Medium I was used for the experiments in section II of the results with mitochondria isolated in 0.30 M sucrose-2 mM EDTA, pH 7.2 and medium II was used for the experiments in section I, III, IV and V with mitochondria isolated in either sucrose-EDTA or sucrose-TES. All components of the medium were sterilized except for the ribonucleoside phosphates. The components of the medium were mixed before use and the pH adjusted with KOH. An aliquot of mitochondrial suspension was added to start the reaction. The final concentration of mitochondrial protein was about 3 mg/ml. The [^{3}H]nucleoside triphosphate was added to 3.3 µCi/ml, the total volume of which was 0.6 ml or 3 ml and to 8.3 µCi/ml for alkaline hydrolysis experiments which contained 6 ml of medium. Incubations were performed in round-bottomed centrifuge tubes or in 50 ml Erlenmeyer flasks in a shaking water bath at 80-90 osillations/min.

4. Incubation of Isolated Rat Liver Nuclei

Nuclei were incubated as described by Weiss (1960). The medium contained in 0.6 ml: 100 mM tris-HCl, pH 8.1,

Table 3

Components of Incubation Media

Component	Medium I	Medium II	
		hypo-osmotic medium	iso-osmotic medium
KCl	40 mM	4 mM	4 mM
$MgSO_4$	8 mM	-	-
$MgCl_2$	-	7 mM	7 mM
KH_2PO_4	16 mM	5 mM	5 mM
Succinate	10 mM	7.5 mM	7.5 mM
Malate	-	0.45 mM	0.45 mM
Pyruvate	-	7.5 mM	7.5 mM
Three unlabelled NMP	1 mM each	-	-
Three unlabelled NTP	-	0.1 mM each	0.1 mM each
Sucrose	100 mM	33.4 mM	183 mM
EDTA (or TES)	0.67 mM	0.27 mM (0.07 mM)	0.27 mM (0.07 mM)
pH	7.2	7.4	7.4
Osmolarity	247 milliosmoles/l	128 milliosmoles/l	290 milliosmoles/l
Incubation Temperature	37°	30°	30°

5 mM $MgCl_2$, 10 mM cysteine, [^{3}H]UTP (1 μCi) plus 1 mM each of unlabelled ATP, CTP and GTP, or [^{3}H]ATP (0.25 μCi) plus 1 mM each of unlabelled CTP, GTP and UTP and 0.12 ml of the nuclear suspension. The incubations were carried out at 37° for 20 min. RNA synthesis was measured by the incorporation of [^{3}H]nucleotides into 5 % trichloroacetic acid-insoluble material as described below.

5. Measurement of [^{3}H]Nucleoside Triphosphate Incorporation

(a) Acid precipitation

After incubation of mitochondria in medium I, 0.6 ml samples were pipetted into 12 ml conical centrifuge tubes containing 0.2 ml of ice-cold saturated tetrasodium pyrophosphate and ice-cold trichloroacetic acid was added to a final concentration of 5 %. The precipitates were centrifuged at 2,000 x g for 5 min and washed twice with 5 ml of 5 % trichloroacetic acid at 0°. The acid-insoluble material was dissolved in 0.5 ml of a strong base (NCS solubilizer) for counting.

After the incubation of nuclei, the reaction was stopped with 0.2 ml of ice-cold saturated tetrasodium pyrophosphate. About 2 mg of crude yeast RNA was added as carrier and a precipitate was obtained by adding ice-cold trichloroacetic acid to 5 %. The precipitate was recovered by centrifugation, washed 2 or 3 times with 5 ml of 5 % trichloroacetic acid at 0° and dissolved as above for counting.

(b) Hot phenol-SDS extraction followed by acid precipitation

After incubation of mitochondria in medium II, 0.6 ml samples were pipetted into 12 ml thick-walled pyrex tubes containing 0.2 ml of ice-cold saturated tetrasodium pyrophosphate and centrifuged at 6,000 x g for 10 min. The supernatant was decanted and the pellet rinsed with 5 ml of isolation medium (sucrose-EDTA or sucrose-TES). RNA was isolated by a modification of the method of Penman (1966). The pellet was suspended in 2 ml of 0.1 N NaCl-0.025 M sodium acetate, pH 5.0 containing 2 % SDS and 2 mg of crude yeast RNA. The sample was vigorously shaken with a vortex mixer at 10° until mitochondria were lysed and a clear solution obtained. The solution was mixed with 2 ml of water-saturated phenol and heated at 60° for 2 min. After mixing, 2 ml of chloroform containing 1 % isoamyl alcohol was added and shaken. The emulsion was heated to 60° for 2 min and mixed well. After centrifugation at 1,000 x g for 4 min at 20°, the bottom phase was removed with a Pasteur pipette. The upper phase was re-extracted with 4 ml of the chloroform-isoamyl alcohol solution at 60° for 2 min. Trichloroacetic acid was added to the aqueous phase to 5 % at 0°. RNA was pelleted by centrifugation at 15,000 x g for 10 min, rinsed with 5 ml of 5 % trichloroacetic acid and dissolved in 0.5 ml of a strong base (NCS solubilizer) for counting. In preliminary experiments ethanol instead of trichloroacetic acid was used to precipitate the RNA. It was found that this also precipitated unincorporated

radioactive nucleotides which masked the incorporated radioactivity. For this reason acid precipitation was used.

6. Alkaline Hydrolysis

Acid-insoluble material was hydrolyzed with alkali in order to demonstrate that the [^{3}H]nucleotide was incorporated into RNA. If base- or ribose-labelled nucleotides are used as precursors, [^{3}H]nucleotide incorporated into inter-nucleotide linkages of RNA is recovered as the mixed [^{3}H] 2'(3') nucleoside monophosphate after alkaline hydrolysis and [^{3}H]nucleotide added to the terminal ends of pre-existing nucleotide chains is recovered as [^{3}H]nucleoside after alkaline hydrolysis. Alkaline hydrolysis was carried out by a modification of the method of Gebicki and Freed (1966). The acid-insoluble fraction recovered after incubation in medium I was treated successively twice with 5 ml of 2 % potassium acetate in 95 % ethanol, ethanol:ether 3:1 (v/v) and ether to extract lipids. The ether-dried material was dissolved in 2 ml of 0.3 N LiOH or 0.3 N KOH and hydrolyzed for 18 hr at 37°. Hydrolysis was terminated at 0° by acidifying to pH 1.0 with 6 N $HClO_4$. The precipitate was removed by centrifugation. The supernatant was neutralized to pH 7.0 with LiOH or KOH and left for 2 to 3 hr at 0°. The precipitate was removed by centrifugation. The solution was evaporated under vacuum at 35°. The $LiClO_4$ was removed by washing 3 times with ether-isopropanol (2:1). The remainder was dried overnight in a desicator and dissolved

in 0.2 ml of 0.5 n NH_4OH for paper chromatography.

7. Paper Chromatography

Descending paper chromatography was carried out using Whatman 3 MM paper (28 cm wide and 75 cm long). The solvent was 1 M ammonium acetate saturated with boric acid (pH 7.5) - 95 % ethanol (2:5, v/v). The system separates 5' nucleotides, 2'(3') nucleoside monophosphates, nucleosides and bases for any particular base type. The mobilities relative to uracil were as follows:

Nucleotide	Base	Nucleoside	2' and 3' Nucleotide	5' Nucleotide
uridylate	1.00	0.58	0.36	0.07
cytidylate and adenylate	0.92	0.50	0.26	0.06
guanylate	0.69	0.34	0.22	0.05

After running for 35 hr at room temperature, the paper was dried in air, and reference compounds were located under an ultraviolet lamp. The paper was cut into pieces 1 cm x 2 cm (1 cm in the running direction), placed in vials and counted with 2 ml of toluene scintillation liquid.

The alkaline hydrolyzate of the [^{3}H]ATP-labelled acid-insoluble products was further characterized by descending paper chromatography on Whatman No. 1 paper using isobutyric acid-ammonia-water (66:1:33, v/v/v), and 0.1 M

sodium phosphate (pH 6.8)-ammonium sulphate-n-propanol (100 ml: 6 g: 2 ml). The former was run for 20 hr and the latter for 16 hr at room temperature.

8. Isolation of RNA

RNA was extracted by three different methods to permit characterization of RNA.

(a) Hot phenol-SDS method

In this method total RNA is recovered. Mitochondria from a 6 ml incubation were centrifuged at 6,500 x g for 10 min and washed with 5 ml of 0.25 M sucrose-1 mM EDTA-10 mM tris-HCl, pH 7.4. The pellet was suspended in 1.1 ml of 0.5 M NaCl-50 mM $MgCl_2$-10 mM tris-HCl, pH 7.4, and 0.4 ml of 25 % SDS and 0.5 ml of 0.4 M EDTA, pH 7.4 were added to the suspension to completely solubilize the mitochondrial pellet. RNA was extracted with hot phenol-SDS as described earlier in the presence of 200 µg of rat liver cytoplasmic RNA as carrier and precipitated with 2 volumes of 95 % ethanol. The RNA precipitate was kept at -20° for at least 3 hr, recovered by centrifugation at 15,000 x g for 10 min, dissolved in 0.5 ml of 0.1 N sodium acetate, pH 6.0, and applied to a linear sucrose density gradient.

(b) Cold phenol-SDS method I

A method of Kirby (1965) which was modified by Bartoov et al., (1970) was used. This method was designed to isolate cytoplasmic ribosomal RNA without contamination of messenger RNA and tRNA (Kirby, 1965). The mitochondrial

fraction obtained by centrifuging the incubate at 6,500 x g for 10 min was suspended in 2 ml of isolation medium (sucrose-EDTA) at 0° and solubilized at 25° by the addition of 5 ml of a solution containing 2 % SDS, 0.1 % sodium magnesium lithoflurosilicate (Macaloid), 25 mM sodium acetate, and 1 % NaCl, pH 4.8. L-cell cytoplasmic ribosomal RNA (1 mg) labelled with [^{14}C]uridine was added as carrier and marker. One volume of phenol-cresol mixture (300 g of fresh distilled phenol, 42 ml of fresh distilled m-cresol, 33 ml of water and 0.3 g of 8-hydroxyquinoline) was added. Sodium triisopropylnaphthalene sulphonate was also added to 2.5 %. The mixture was stirred at 4° for 1 hr. The aqueous layer was extracted at 4° once with 1 volume and once with 0.5 volume of phenol-cresol mixture. The aqueous layer was made 3 % with NaCl and 20 % with sodium benzoate and m-cresol was added until turbidity occurred at about 30 %. After 1 hr at 0°, the washing solution (3 g of NaCl, 20 g of sodium benzoate, 10 ml of m-cresol and 100 ml of water) was added dropwise until the turbidity just disappeared. RNA was recovered by centrifugation at 10,000 x g for 10 min, washed twice with the washing solution, once with 1 % NaCl in 75 % ethanol, once with 75 % ethanol, and twice with absolute ethanol, and dried under vacuum overnight. For agarose-polyacrylamide gel electrophoresis, RNA was dissolved in 50 mM NaCl-1.5 mM $MgCl_2$-10 mM tris-HCl, pH 7.4.

(c) Cold phenol-SDS method II

After incubation, mitochondria were centrifuged at 6,500 x g for 10 min and washed twice with 5 ml of isolation medium (sucrose-EDTA).

The pellet was suspended in 2 ml of the isolation medium, and 1 mg of L-cell ribosomal RNA and 0.5 mg of E. coli tRNA were added as marker and carrier RNA. Mitochondria were disrupted by adding 4 ml of 25 mM sodium acetate-0.17 M NaCl-10 mM $MgCl_2$-2 % SDS-0.1 % Macaloid, pH 4.8. RNA was extracted at 4° with 7 ml of 4 % amino-salicylic acid-2% SDS-0.1 % Macaloid-25 mM sodium acetate, pH 4.8 and 14 ml of phenol-cresol mixture. Sodium tri-isopropylnaphthalene sulphonate was added to 2.5 %. The mixture was stirred at 4° for 1 hr. After recovery of the aqueous phase RNA was re-extracted at 4° with 1 volume of phenol-cresol mixture, precipitated in 2.5 volumes of 95 % ethanol and left overnight at -20°. RNA was washed twice with 1 % NaCl in 75 % ethanol, once with 75 % ethanol, twice with absolute ethanol, and dried under vacuum overnight. RNA was dissolved in 0.1 N sodium acetate, pH 6.0 and applied to a convex sucrose density gradient.

9. Centrifugation on Sucrose Density Gradients

(a) Linear sucrose density gradient

A linear gradient from 5 to 20 % sucrose in 0.1 N sodium acetate, pH 6.0 was prepared in a volume of 12 ml and 0.2 ml of RNA dissolved in 0.1 N sodium acetate, pH 6.0

was layered on top. Centrifugation was at 105,500 x g for 12 hr at 4° using the Beckman preparative ultracentrifuge model L2-65 with a SW41 swinging-bucket rotor.

After centrifugation the bottom of the tube was punctured and fractionated manually. Samples of 20 drops were collected and 0.3 ml of water added. The optical density of each fraction was read at 260 nm with 0.5 cm path length cuvettes and the solution transferred to a counting vial. To solubilize the RNA, 0.5 ml of water and 0.5 ml of a strong base (NCS solubilizer) were added. After mixing, samples were left overnight. Ten ml of dioxane scintillation liquid was added for counting.

(b) Convex sucrose density gradient

On a convex density gradient the sedimentation constant for an unknown RNA is readily determined since the sedimentation constants are linearly related to the distance from the meniscus (Bartoov, 1971). A convex sucrose density gradient of 12 ml from 15 to 33 % sucrose in 0.1 N sodium acetate, pH 6.0 was prepared as described by Bartoov (1971). The gradient was prepared with 10 ml of 15 % sucrose solution in the mixing chamber and 12 ml of 40 % sucrose solution added dropwise to the mixing chamber at a rate of 1 ml/min with mixing. Centrifugation was at 192,500 x g for 12 hr at 4°, using the Beckman preparative ultracentrifuge model L2-65 with a SW41 swinging-bucket rotor. Radioactivity and optical density were measured as described for linear sucrose density gradients.

10. Electrophoresis of RNA on an Agarose-polyacrylamide Gel

The electrophoresis was carried out by a modification of the method of Watanabe, Prevec and Graham (1967) and Peacock and Dingman (1968). Agarose-polyacrylamide gel was prepared just before use. A solution containing 4.18 % acrylamide, 0.22 % methylene bisacrylamide, 0.05 % N,N,N',N'-tetramethylene diamine and 1 % SDS in 2 times concentrated reservoir buffer was kept at 0° for 30 min. Ammonium persulphate was added to 0.1 % and the solution mixed with an equal volume of boiling 0.5 % agarose. The mixture was immediately poured into plugged electrophoresis tubes, 6 x 65 mm, allowed to solidify for 3 hr, and used immediately. The plug was removed and 50 µl of RNA (about 30 µg) in 50 mM NaCl-1.5 mM $MgCl_2$-10 mM tris HCl, pH 7.4 was applied to the previous bottom surface of the gel. Electrophoresis was for 45 min at 200 volts (30 volts/cm and 20 mA/tube) in a reservoir buffer which contains 108 g of tris, 9.3 g of EDTA and 55 g of boric acid in 10 l with a final pH of 8.3. SDS was added to the buffer to 0.25 %. The upper chamber was at 25° initially. Ice-cold water was circulated around the lower reservoir which was at 2° initially.

The gel was sliced in 1 mm length with a gel slicer designed in our laboratory by Bartoov (1971). The RNA was extracted from the gel with 0.5 ml of water and 0.5 ml of NCS solubilizer overnight. For counting, 10 ml of a dioxane scintillation liquid was added to each vial.

11. Measurement of Radioactivity

All acid-insoluble fractions were dissolved in NCS solubilizer and washed into vials with 10 ml of dioxane scintillation liquid (twice with 3 ml and once with 4 ml). A small amount of water (0.5 ml) was added to keep the samples and naphthalene in solution at 4°. The radioactivity was counted at 4° using a Nuclear Chicago Mark I liquid scintillation counter. The efficiency of counting was about 30-35 % for [^{3}H]-labelled samples as determined with a standard [^{3}H]H_2O.

After paper chromatography, pieces of paper (1 cm x 2 cm) were counted in 2 ml of toluene scintillation liquid at room temperature using a Nuclear Chicago Unilux liquid scintillation counter. The efficiency of counting was 5-7 % for [^{3}H]-labelled samples.

The dioxane scintillation liquid contained 12 g of 2,5-diphenyloxazole, 0.55 g of 1,4-bis-(4-methy-5-phenyloxazole-2-yl) benzene and 310 g of naphthalene in a total volume of 1.0 l of dioxane.

The toluene scintillation liquid contained 4 g of 2,5-diphenyloxazole and 0.32 g of 1,4 bis-(4-methyl-5-phenyloxazol-2-yl) benzene in a total volume of 1.0 l of toluene.

Dual-labelled samples (^{14}C and ^{3}H) were counted on two channels of the Nuclear Chicago Mark I counter. Corrections for [^{14}C] counts in the [^{3}H] channel was by addition of an internal standard ([^{14}C]methanol), recounting

and computer calculation.

12. Thin Sectioning

Mitochondria from rat liver were isolated either in sucrose-EDTA or in sucrose-TES as described earlier. Small mitochondrial pellets were collected in conical centrifuge tubes. The mitochondria were fixed by layering on the pellet a solution of 1.5 % gluteraldehyde in 0.1 M sodium cacodylate, pH 7.3, 270 milliosmoles/l, for 1 hr at 0°. After removing the gluteraldehyde solution, post-fixation was done with 2 % osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4 for 2 hr at 0°. After dehydration with ethanol and propylene oxide, the specimens were embedded in epon. Thin sectioning was carried out with a LKB microtome. The embedded tissue was recovered on copper mesh grids and stained with uranyl acetate and lead citrate. Microscopy was with a Phillips 300 electron microscope.

13. Respiratory Studies on Mitochondria

Mitochondria were isolated in sucrose-EDTA or sucrose-TES and suspended in the isolation medium. Oxygen consumption was measured polarographically at 23° in a volume of 3 ml with a Yellow Springs biological oxygen monitor, model 53, and a Clark fixed voltage polarographic probe. The solubility of oxygen at 23° was taken as 825 mμmoles/3 ml. The incubation medium contained 45 mM

mannitol, 15 mM sucrose, 0.2 mM EDTA, 40 mM KCl, 10 mM $MgCl_2$ and 20 mM phosphate, pH 7.4.

ADP:O ratios were determined with 10 mM succinate and 10 mM β-hydroxybutyrate as substrates. ADP (0.05 M) was added in limiting quantities by microliter-syringe. Substrates and ADP were adjusted to pH 7.2 prior to addition to the mitochondrial suspension.

14. Measurement of ATP and ADP

The ATP and ADP content of mitochondria was measured by a modification of the fire-fly tail assay of Strehler and Totter (1954). Fire-fly lantern extract (50 mg) was diluted to 5 ml in water. Particulate matter was removed by centrifugation at 3,000 x g for 10 min at 0° and the extract stored at 0°. Standard ATP was prepared from 10^{-7} to 3 x 10^{-6} M ADP using 20 µl of conversion mixture to 1 ml of standard and incubated for 15 min at room temperature. The conversion mixture contained 0.1 M $MgSO_4$, 0.86 M KCl, 1.2 mM EDTA, 4.6 mg phosphoenol pyruvate/ml and 1.2 mg pyruvate kinase/ml. For the assay, 0.1 ml of the enzyme was added to 2 ml of water in a liquid scintillation vial, and then 0.1 ml of the sample was added and mixed. The vial was placed in a scintillation counter and counted for 0.1 min, 25 seconds after sample addition. The ATP content of the unknown was determined in the same way. The ADP content was determined from the difference between the ATP content and the total ADP plus

ATP content after conversion of the ADP to ATP as described above.

For measurement of ATP and ADP, mitochondria were isolated in sucrose-EDTA or sucrose-TES, and incubated at 30° for 5 min in 2 ml of a solution which contained 4 mM KCl, 7 mM $MgCl_2$, 5 mM KH_2PO_4, 7.5 mM succinate, 0.45 mM malate, 33 mM sucrose and 0.27 mM EDTA (or 0.07 mM TES), pH 7.4. The incubation was stopped by adding 0.14 ml of 6 N HClO to the medium. The pH of supernatant was neutralized with 3 N KOH after centrifugation. The volume of the supernatant was measured and a 20 times diluted supernatant was used for aliquots of sample.

15. Colorimetric Determination of Protein, RNA and DNA

Protein was measured by the method of Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin as the standard.

RNA was measured by the orcinol method of Mejbaum (1939) with a heating time of 45 min. Adenosine was used as the standard.

DNA was estimated by the method of Burton (1956) using a preparation of salmon sperm DNA containing 7 % phosphate as a standard.

16. Bacterial Counts

Mitochondria were isolated and incubated as described earlier. At the end of 1 hr incubation, 0.1 ml of mitochondrial incubate was spread on Tryptose Blood Agar Base plate. The number of bacterial colonies was counted after incubation at 37° for 24 to 48 hr. Bacterial contamination in the mitochondrial stock suspension was similarly checked.

17. Materials

All chemicals were reagent grade or the purest available. Nucleotides were obtained from either P-L Biochemicals, Milwaukee, Wis. or Boehringer, Mannheim Corp., N.Y., U.S.A. Pyruvate kinase and phosphoenolpyruvate were obtained from Boehringer, Mannheim Corp., N.Y., U.S.A. Firefly latern extract was obtained from the Sigma Chemical Corp., St. Louis, Mo., U.S.A. Actinomycin D was a gift of Merk, Sharp and Dohme Ltd. Rifampicin and rifamycin SV were gifts of Dr. P. Sensi of Lepetit Sp. A. Milan, Italy. Streptovaricin was a gift from Dr. S. Mizuno, National Institute of Health, Tokyo, Japan and Toyojozo Co., Tokyo, Japan. Atractyloside was a gift of Dr. A.L. Lehninger. DNase and RNase A type II were products of the Sigma Chemical Corp., St. Louis, Mo., U.S.A. NCS solubilizer, [5-^{3}H]UTP (2-4 Ci/mmole), [2-^{3}H]ATP (0.45 Ci/mmole), [8-^{3}H]GTP (11.8 Ci/mmole) and [5-^{3}H]CTP (4.39 Ci/mmole and [^{14}C]methanol (50 μCi/mmole) were obtained from Amersham/Searle Corp., Toronto, Ont., Canada. Agarose was obtained from Bausch and Lomb Co. Ltd., Toronto, Ont.

Acrylamide of the highest purity grade was obtained from Distillation Products, Rochester, N.Y. Sucrose for the gradient centrifugation was density gradient grade (RNase-free) and obtained from Mann Research Lab., New York, N.Y. Yeast RNA was obtained from the British Drug Houses Ltd., Poole, England. 1,4 Bis-(4-methyl-5-phenyloxazole-2-yl) benzene and 2,5-diphenyloxazole were products of Amersham/Searle Corp., Toronto, Ont., Canada.

III RESULTS

Section I. Characterization of the Mitochondrial Fraction

A number of features of the mitochondrial fraction prepared as described in the methods were examined. These included visual checks of structural features, the capacity for oxidative phosphorylation and the content of ATP, ADP, RNA and DNA. For these studies mitochondria were isolated in sucrose-EDTA or sucrose-TES, since most of the subsequent investigation was done with mitochondria isolated in these media.

1. Electron Microscopic Studies

Mitochondria (M) were clearly the main component of the preparation isolated in sucrose-EDTA (Fig. 1). Mitochondria were intact and in the condensed form (Hackenbrock, 1966) possibly with some contamination with endoplasmic reticulum (ER). Sucrose-TES isolation also yielded a preparation consisting largely of intact mitochondria but contaminated possibly with rough endoplasmic reticulum (RER) and peroxisomes (P) (Fig. 2). Both mitochondrial preparations appeared free from contamination with nuclei.

Higher magnifications are presented in Fig. 3 and Fig. 4. With mitochondria isolated in sucrose-EDTA and sucrose-TES, outer membrane (O), inner membrane (I), cristae (C) and matrix (Mx) are clearly seen.

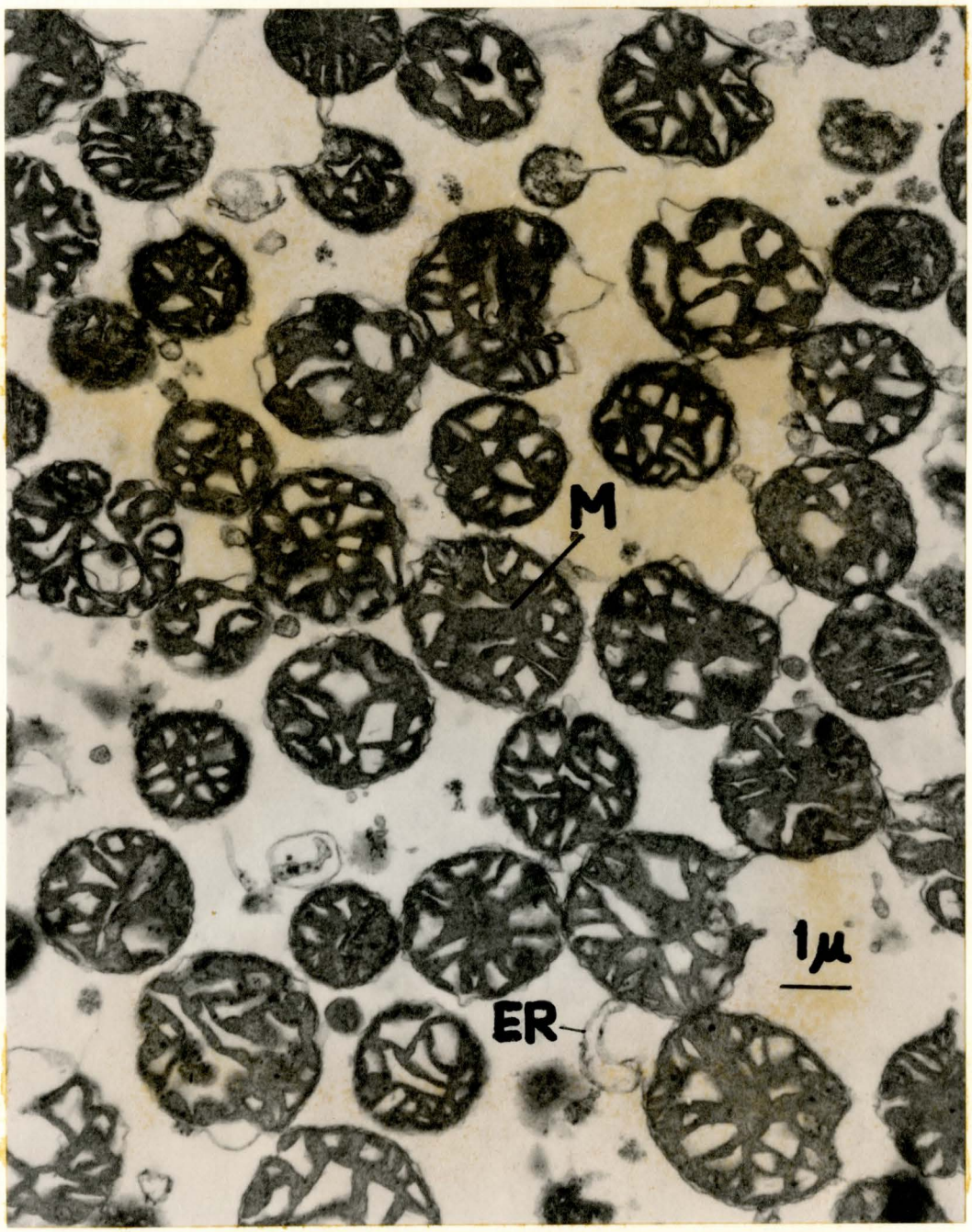

Figure 1. Low-power view of the mitochondrial fraction isolated in sucrose-EDTA. Mitochondria were isolated and the thin sectioning was performed as described in the Methods section. M, mitochondria; ER, endoplasmic reticulum. Magnification: 10,200 X.

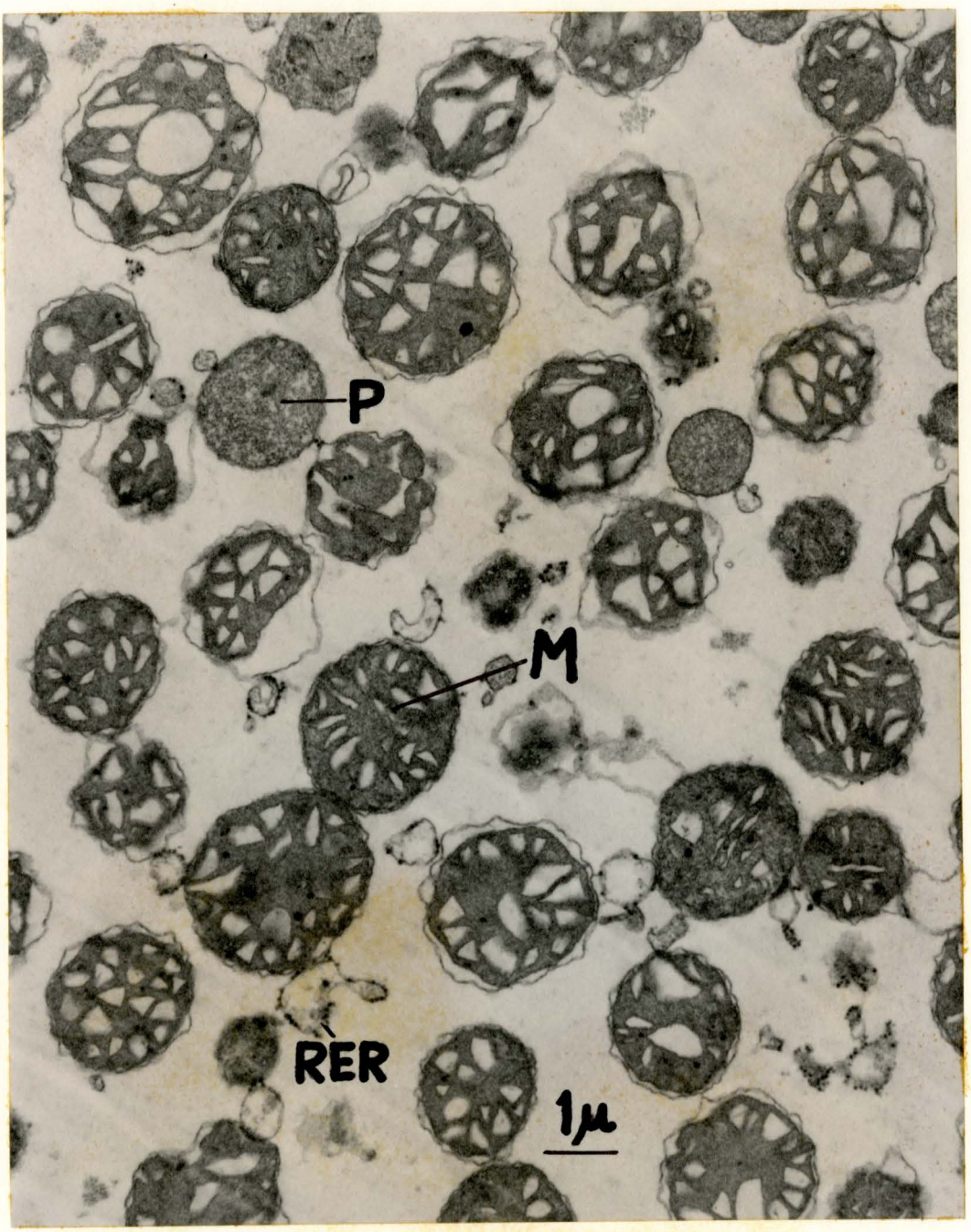

Figure 2. Low-power view of the mitochondrial fraction isolated in sucrose-TES. Mitochondria were isolated and the thin sectioning was performed as described in the Methods section. M, mitochondria; RER, rough endoplasmic reticulum; P. peroxisome. Magnification: 10,200 X.

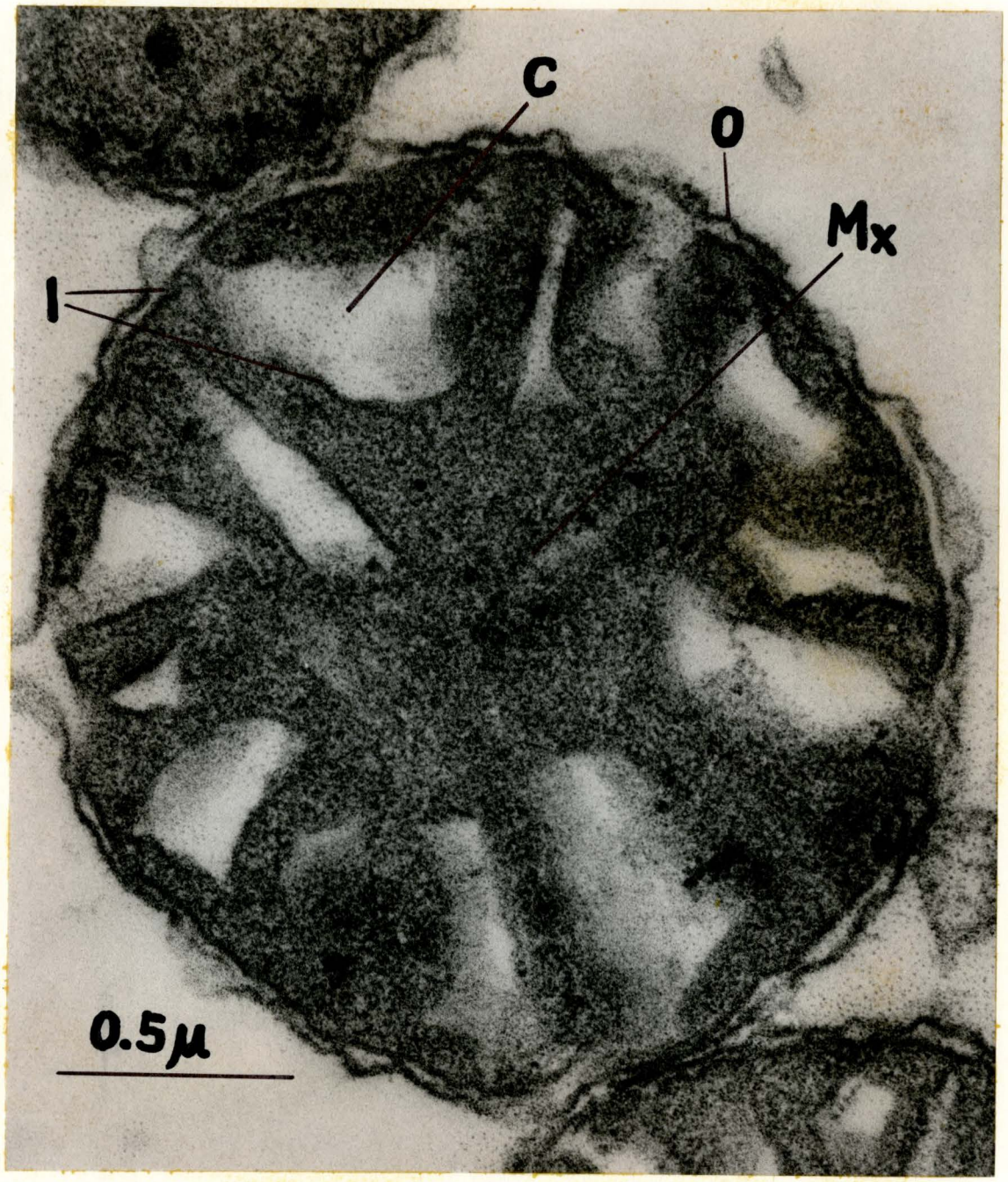

Figure 3. High-power view of mitochondria isolated in sucrose-EDTA. Mitochondria were isolated and the thin sectioning was carried out as described in the Methods section. O, outer membrane; I, inner membrane; C, cristae; Mx, matrix, Magnification; 72,000 X.

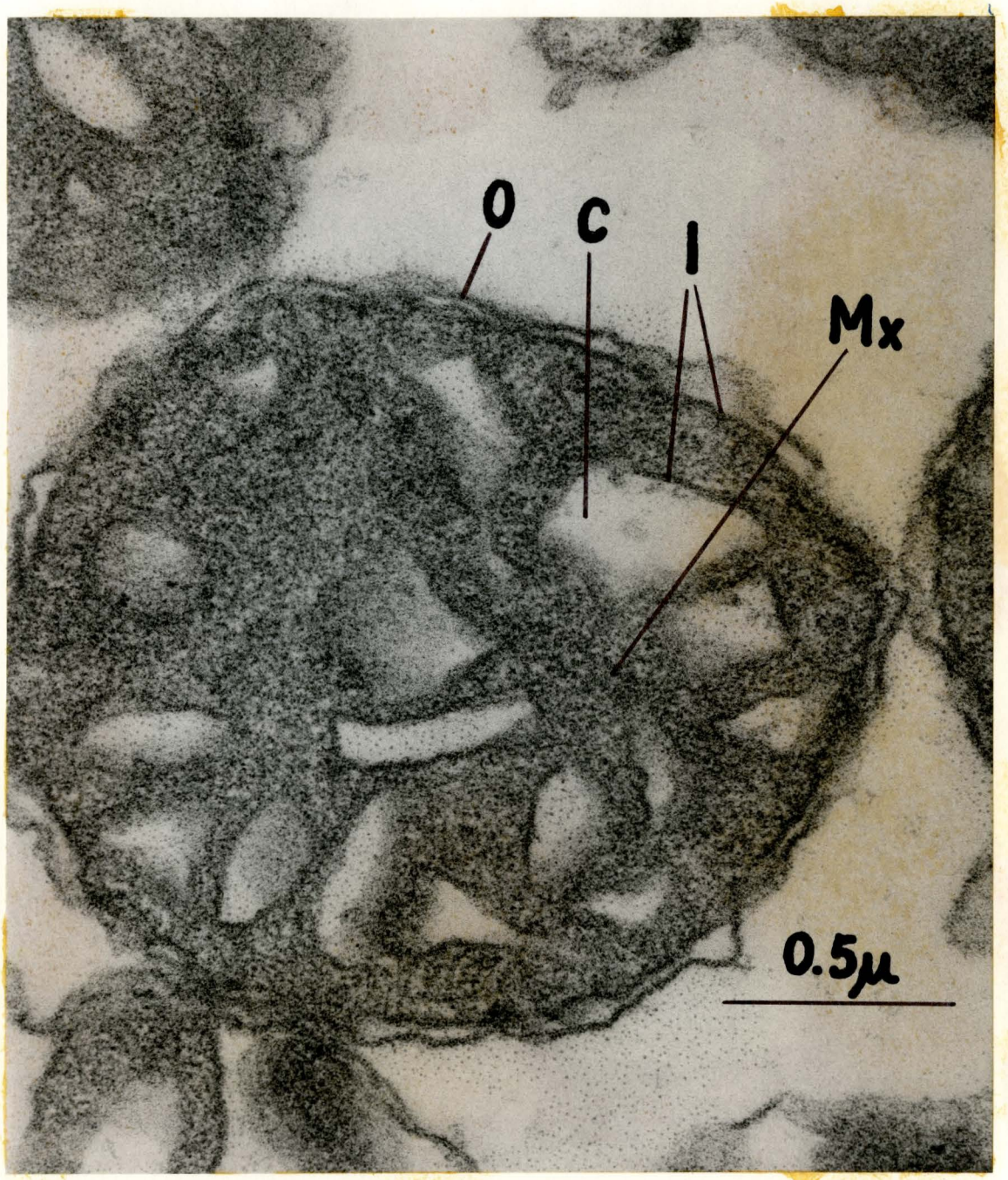

Figure 4. High-power view of mitochondria isolated in sucrose-TES. Mitochondria were isolated and the thin sectioning was carried out as described in the Methods section. O, outer membrane; I, inner membrane; C, cristae; Mx, matrix. Magnification: 72,000 X.

2. Respiratory Studies

Using succinate or β-hydroxybutyrate as substrate, the ADP:O ratios obtained, as shown in Table 4, were close to the theoretical values of 2 and 3, respectively.

The respiratory control ratios were above 3.0 in all cases.

3. Concentration of ATP and ADP

The ATP and ADP content of mitochondria determined as described in the methods are given in Table 5.

4. Content of RNA and DNA

The content of RNA and DNA in mitochondria are given in Table 6.

Section II. Incorporation of [^{3}H]Nucleotides by Mitochondria Isolated in 0.30 M Sucrose-2 mM EDTA, pH 7.2 and Incubated in Medium I

1. Incorporation of [^{3}H]Nucleotides into Acid-insoluble Material

The level of incorporation of the four [^{3}H] ribonucleoside triphosphates as well as [^{3}H]adenosine monophosphate into acid-insoluble material by isolated rat liver mitochondria is shown in Table 7. While all [^{3}H]nucleotides were incorporated, the [^{3}H]adenine

Table 4

ADP:O Ratios and Respiratory Control Ratios

Mitochondria were isolated in sucrose-EDTA or sucrose-TES. The ADP:O ratios and respiratory control ratios were determined as described in the Methods. Mean values are listed with standard deviations (N=4).

Mitochondria	Substrate	ADP:O	Respiratory Control Ratio
sucrose-EDTA	succinate	1.62±0.07	3.64±0.50
	β-hydroxybutyrate	2.26±0.12	3.12±0.14
sucrose-TES	succinate	1.61±0.03	3.75±0.45
	β-hydroxybutyrate	2.22±0.15	4.79±0.83

Table 5

ATP and ADP Content of Mitochondria

Mitochondria were isolated in sucrose-EDTA or in sucrose-TES. The content of ATP and ADP was determined as described in the Methods. Mean values are average of two assays.

Mitochondria	ATP	ADP
	10^{-9} moles/mg of protein	
sucrose-EDTA	12.3±0.2	4.0±0.5
sucrose-TES	11.4±0.5	4.2±0.7

Table 6

RNA and DNA Content of Mitochondria

Mitochondria were isolated in sucrose-EDTA or sucrose-TES. The content of RNA and DNA in isolated mitochondria was determined colorimetrically as described in the Methods. The mean value for sucrose-TES mitochondria is listed with the standard deviation (N=3).

Mitochondria	RNA	DNA
	µg/mg of protein	
sucrose-EDTA	11.0	0.47
sucrose-TES	8.9±0.9	0.43±0.2
0.30 M sucrose-2 mM EDTA, pH 7.2	12.7	0.36

Table 7

Incorporation of [^{3}H]Nucleotides by Isolated Rat Liver Mitochondria

Mitochondria isolated in 0.30 M sucrose-2 mM EDTA, pH 7.2 were incubated for 40 min in medium I and the acid-insoluble radioactivity measured.

[^{3}H]Nucleotide	Incorporation 10^{-15} moles/40 min/mg of protein
[^{3}H]ATP	1,200
[^{3}H]AMP	1,030
[^{3}H]UTP	123
[^{3}H]CTP	1,110
[^{3}H]GTP	27

nucleotides and [^{3}H]CTP were incorporated to the greatest extent followed by [^{3}H]UTP, with [^{3}H]GTP being incorporated least.

2. Lipid Extraction and Alkaline Hydrolysis of Acid-insoluble Material

As a first step in determining the nature of labelled acid-insoluble products, they were extracted with lipid solvents. From the results in Table 8, it can be seen that at least 59 %, 77 % and 65 % of the [^{3}H]ATP, [^{3}H]CTP and [^{3}H]GTP, respectively, were incorporated into acid-insoluble, lipid-extractable compounds, that is, not into RNA. Only 22 % of the incorporated [^{3}H]UTP could be extracted under these conditions.

In order to conclusively demonstrate that [^{3}H]nucleotides were incorporated into RNA, the acid-insoluble material was hydrolyzed with alkali after lipid extraction and the hydrolysis products examined by paper chromatography.

As shown in Table 9, all five [^{3}H]nucleotides were incorporated into RNA. Seventy-five percent of the incorporated [^{3}H]UTP was recovered as the mixed [^{3}H]2'(3')-UMP. In the case of [^{3}H]GTP, 42 % of the precursor was recovered as the mixed [^{3}H]2'(3')-GMP. However, on a percentage basis, there was a poor recovery of mixed [^{3}H]2'(3')-AMP and [^{3}H]2'(3')-CMP from the

Table 8

Effect of Lipid Extraction on the Level of [^{3}H]Nucleotide Incorporation

Mitochondria isolated in 0.30 M sucrose-2 mM EDTA, pH 7.2 were incubated for 40 min in medium I. The acid precipitate after washing at 0° 3 times with 5 % trichloroacetic acid was extracted successively 2 times with 2 % postassium acetate in ethanol, ethanol:ether 3:1 (v/v) and ether. Separate samples of the precipitate were counted at the end of each step. The acid-insoluble radioactivity was given a value of 100 % to compare with the activity remaining after lipid extraction.

[^{3}H]Nucleotide precursor	Precipitable radioactivity (percent) [^{3}H]ATP	[^{3}H]UTP	[^{3}H]CTP	[^{3}H]GTP
Precipitate				
Trichloroacetic acid	100	100	100	100
Potassium acetate in ethanol	47	87	14	48
Ethanol:ether, 3:1 (v/v)	49	82	23	39
Ether	41	78	23	35

Table 9

Recovery of 2'(3') Nucleotides After Alkaline Hydrolysis

Mitochondria isolated in 0.30 M sucrose-2 mM EDTA, pH 7.2 were incubated for 40 min in medium I with [^{3}H]nucleotide and extracted with 5 % trichloroacetic acid and lipid solvents. After hydrolysis in 0.3 N LiOH for 18 hr at 37° the products were chromatographed with 1 M ammonium acetate-saturated boric acid, pH 7.5: 95 % ethanol (2:5, v/v) and the radioactivity measured.

[^{3}H]Nucleotide	Radioactivity recovered as 2'(3') nucleotides (percent)
[^{3}H]ATP	15
[^{3}H]AMP	20
[^{3}H]UTP	75
[^{3}H]CTP	20
[^{3}H]GTP	42

[^{3}H]ATP-, [^{3}H]AMP- and [^{3}H]CTP-labelled products. In each case, the rest of the radioactivity ran as a spot in the region which cochromatographed with the corresponding nucleoside diphosphate.

If the values for incorporation in Table 7 are corrected for the amount of label extracted by lipid solvents and for the amount recovered as [^{3}H]2'(3')-nucleotides, then the true level of incorporation into RNA is obtained. As is shown in Table 10, the level of incorporation of [^{3}H]ATP, [^{3}H]UTP and [^{3}H]CTP was nearly the same, and only the incorporation of [^{3}H]GTP was lower.

3. Chromatographic Analysis of Alkaline Hydrolyzate of the [^{3}H]ATP-labelled Product

As indicated above, [^{3}H]ATP-labelled acid-insoluble material gave rise to an unidentified compound on alkaline hydrolysis which ran close to ADP. The R_f values of this unidentified compound in three chromatographic solvents (Table 11) indicated that the unidentified compound was not AMP, ADP or ATP. Although the compound could not be identified, some properties of the total acid-insoluble material were further examined as described below.

Table 10

Calculated Level of [^{3}H]Nucleotide Incorporation

The value for incorporation into acid-insoluble material was corrected to the true level of incorporation into RNA by multiplying by the fraction removed by lipid solvents and by the fraction recovered as 2'(3') nucleotides.

Precursor	Acid-insoluble incorporation 10^{-15} moles/40 min/mg of protein from Table 7	Percentage remaining after extracted with lipid solvents from Table 8	Percentage recovered as 2'(3') nucleotides after lipid extraction from Table 9	Final level of incorporation into RNA 10^{-15} moles/40 min/mg of protein
[^{3}H]ATP	1,200	41	15	74
[^{3}H]UTP	123	78	75	72
[^{3}H]CTP	1,110	23	20	51
[^{3}H]GTP	27	35	42	4

Table 11

R_f Values of Unidentified [^{3}H]ATP-labelled Compound from Alkaline Hydrolysis

Alkaline hydrolyzates of the [^{3}H]ATP-labelled product were run in the following solvents: 1) 1 M ammonium acetate-saturated boric acid, pH 7.5: 95 % ethanol (2:5, v/v), 2) isobutyric acid: ammonia:water (66:1:33, v/v/v) and 3) 0.1 M sodium phosphate buffer, pH 6.8:ammonium sulphate:n-propanol (100 ml:6 g:2 ml).

Solvent	Unidentified Compound	ATP	ADP	5'AMP	2'(3') AMP	Adenosine
1	0.06	0.04	0.06	0.08	0.22	0.34
2	0.31	0.14	0.25	0.46	-	0.83
3	0.33	0.52	0.43	0.35	-	0.16

4. Acid- and Alkali-stabilities of the [^{3}H]UTP- and [^{3}H]ATP-labelled Acid-insoluble Products

The acid- and alkali-stabilities of the [^{3}H]UTP- and [^{3}H]ATP-labelled acid-insoluble products were examined by treating with 5 % trichloroacetic acid at 70° or with 0.5 N NaOH at 22°. In acid, there was a rapid hydrolysis of the [^{3}H]UTP-labelled acid-precipitable fraction, but the [^{3}H]ATP-labelled product was more stable (Fig. 5). Part of the [^{3}H]ATP-labelled product was hydrolyzed as rapidly as the [^{3}H]UTP-labelled product (first part of [^{3}H]ATP curve) and probably represents hydrolysis of RNA. Incubation in 0.5 N NaOH at 22° for 2 hr solubilized both products, the [^{3}H]UTP-labelled product being slightly more labile as shown in Fig. 6. Clearly the major [^{3}H]ATP-labelled product is more acid-stable and slightly more alkali-stable than RNA ([^{3}H]UTP-labelled acid-precipitable fraction).

From the recovery of 2'(3')nucleotides after alkaline hydrolysis and the results on the stabilities of the [^{3}H]ATP- and [^{3}H]UTP-labelled compounds in 5 % trichloroacetic acid at 70° and 0.5 N NaOH at 22°, it is concluded that while [^{3}H]UTP is incorporated principally into RNA, the synthesis of RNA as measured by the incorporation of [^{3}H]ATP is masked by the formation of another acid-insoluble compound.

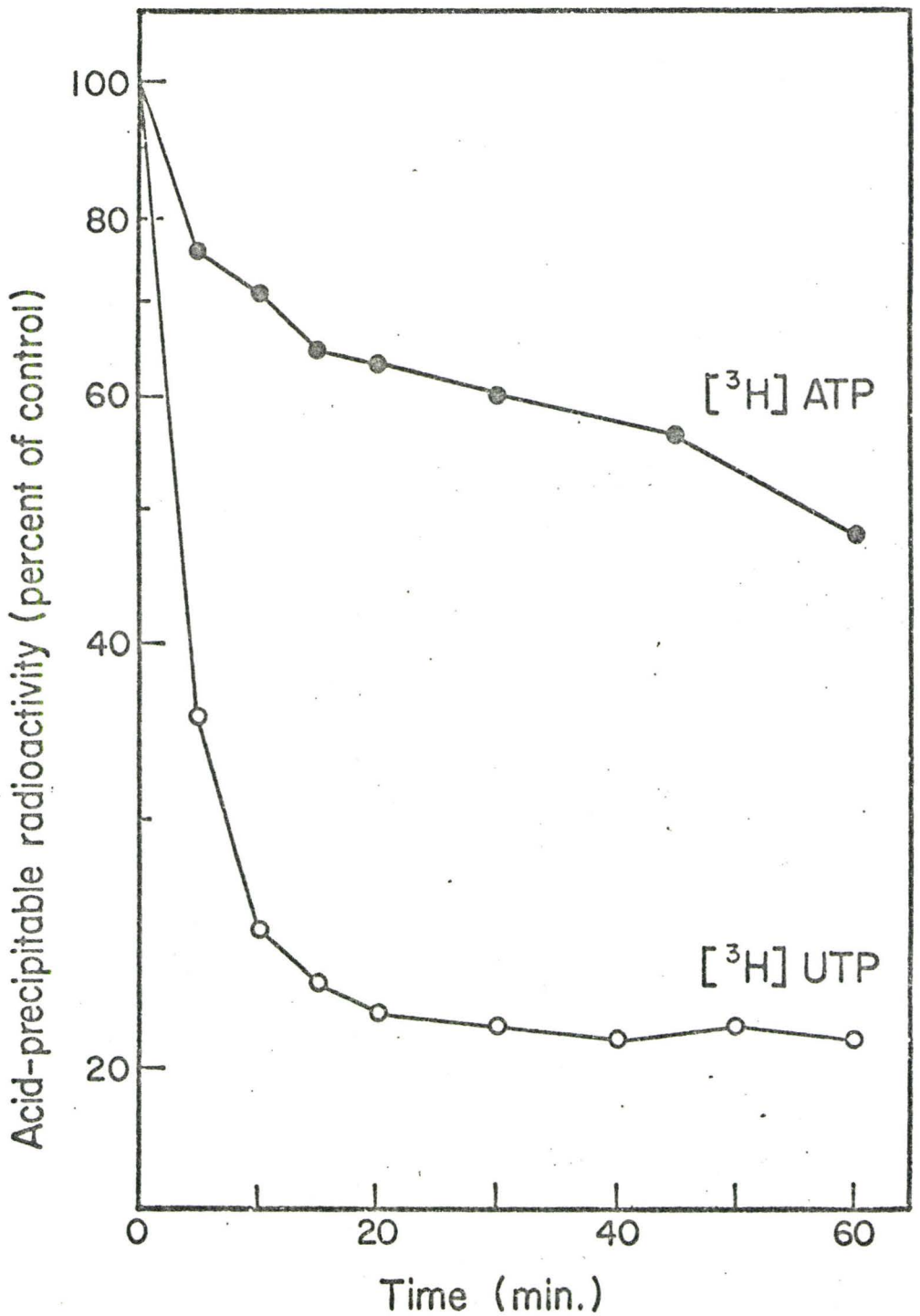

Figure 5. The effect of heating in acid on the acid precipitability of the [^{3}H]UTP- and the [^{3}H]ATP-labelled acid-insoluble products. Separate samples of a 20 min incubation for each point were heated for various lengths of time at 70° in 5 % trichloroacetic acid, cooled to 0°, washed 3 times with 5 % trichloroacetic acid and counted. o, [^{3}H]UTP; •, [^{3}H]ATP.

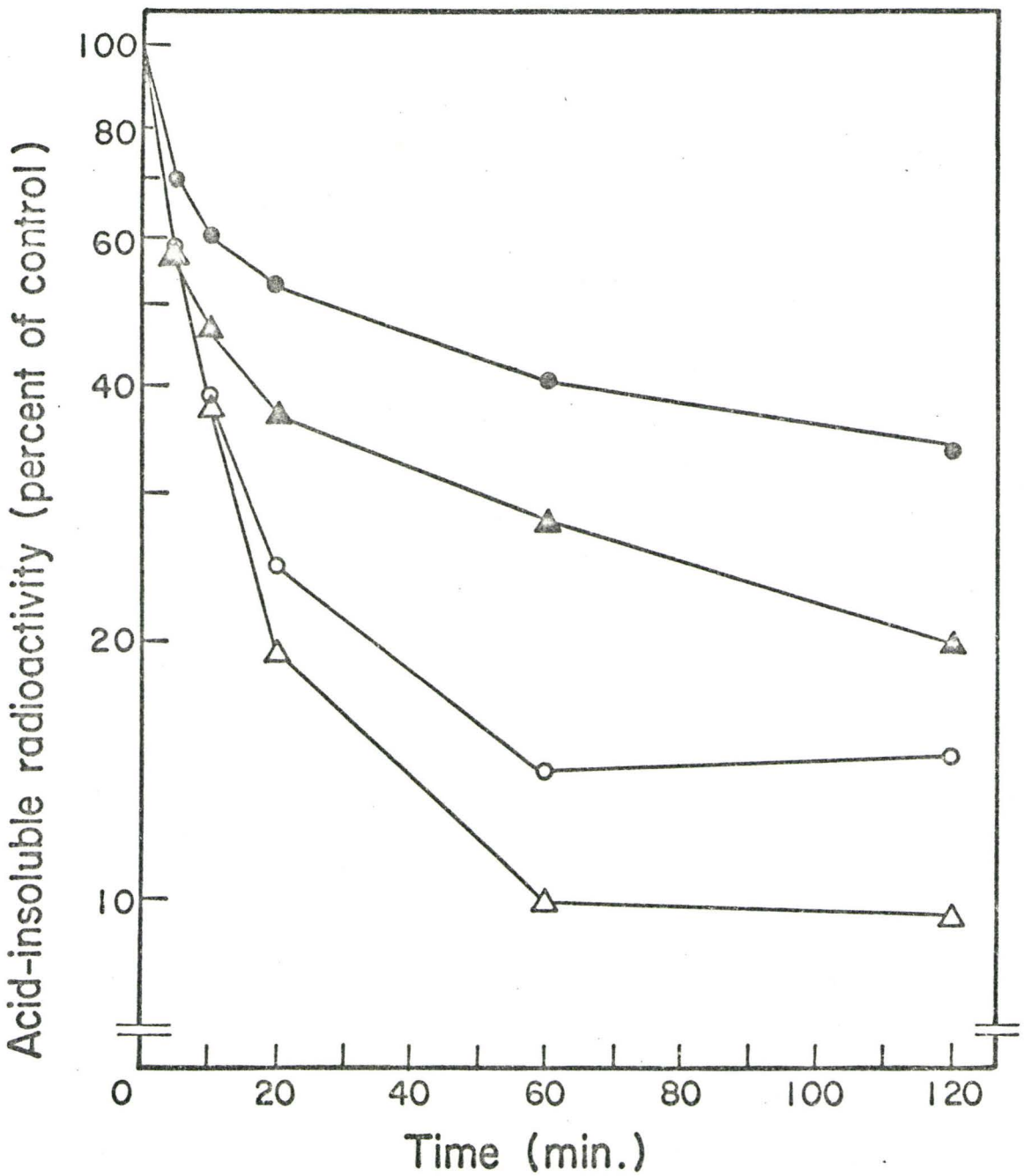

Figure 6. The effect of alkali treatment on the acid precipitability of the [^{3}H]UTP- and [^{3}H]ATP-labelled acid-insoluble products. Separate samples for each point were treated for various lengths of time with 0.5 N NaOH at 22°, washed 3 times with 5 % trichloroacetic acid and the precipitate counted. o, [^{3}H]UTP-labelled acid-insoluble product of a 20 min incubation; △, [^{3}H]UTP-labelled acid-insoluble product of a 60 min incubation; •, [^{3}H]ATP-labelled acid-insoluble product of a 20 min incubation; ▲, [^{3}H]ATP-labelled acid-insoluble product of a 60 min incubation.

5. Effect of the Medium on [^{3}H]UTP and [^{3}H]ATP Incorporation

The effect of the omission of various compounds from the incubation medium on the incorporation of [^{3}H]UTP into acid-insoluble material is shown in Fig. 7. The omission of phosphate, succinate and Mg^{2+} decreased incorporation. AMP, CMP and GMP were not absolutely required for incorporation. Actinomycin D had little effect on incorporation during the first 20 min of incubation, but subsequent incorporation was inhibited.

As shown in Fig. 8, the incorporation of [^{3}H]ATP into an acid-insoluble form required succinate and Mg^{2+} but not phosphate. CMP, GMP and UMP could be replaced by UMP alone, but omission of the ribonucleoside monophosphates caused a decrease in incorporation. Therefore, the requirements for the incorporation of [^{3}H]UTP and [^{3}H]ATP were different, supporting the above observation that [^{3}H]UTP and [^{3}H]ATP are incorporated into different acid-insoluble compounds.

6. Effect of DNase, RNase, Actinomycin D and Chloramphenicol on [^{3}H]UTP and [^{3}H]ATP Incorporation

There was a striking difference in the inhibition by pancreatic RNase of [^{3}H]UTP and [^{3}H]ATP incorporation. [^{3}H]UTP incorporation was inhibited by 59 %, while [^{3}H]ATP incorporation by only 11 % (Table 12). The sensitivity of [^{3}H]UTP incorporation to RNase lends weight to the supposition that [^{3}H]UTP is incorporated mainly

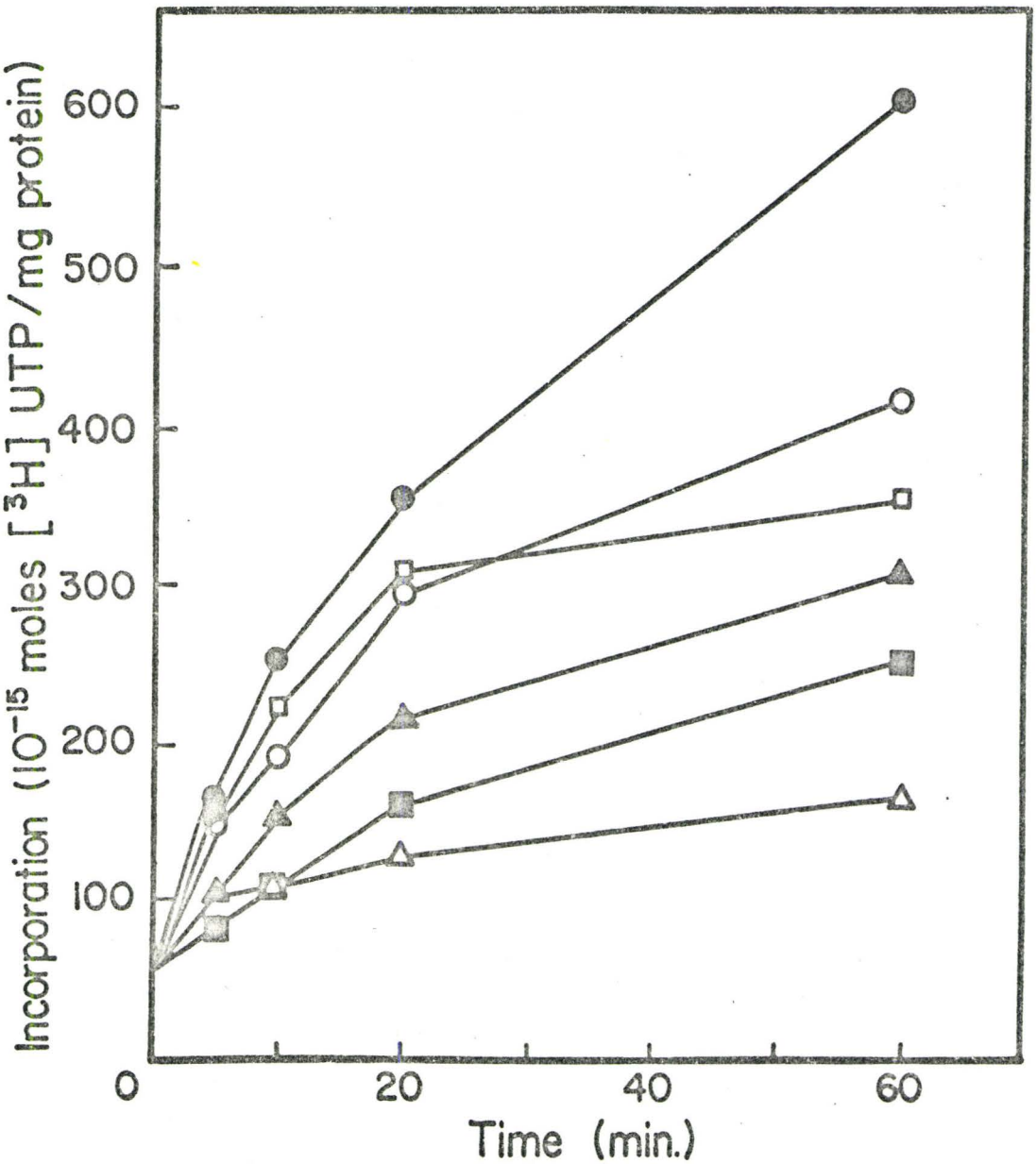

Figure 7. Time course of incorporation of [^{3}H]UTP. ●, Control; ○, ribonucleoside monophosphates omitted; ▲, Mg^{2+} omitted; ■, succinate omitted; △, phosphate omitted; □, actinomycin D (9.5 µg/ml).

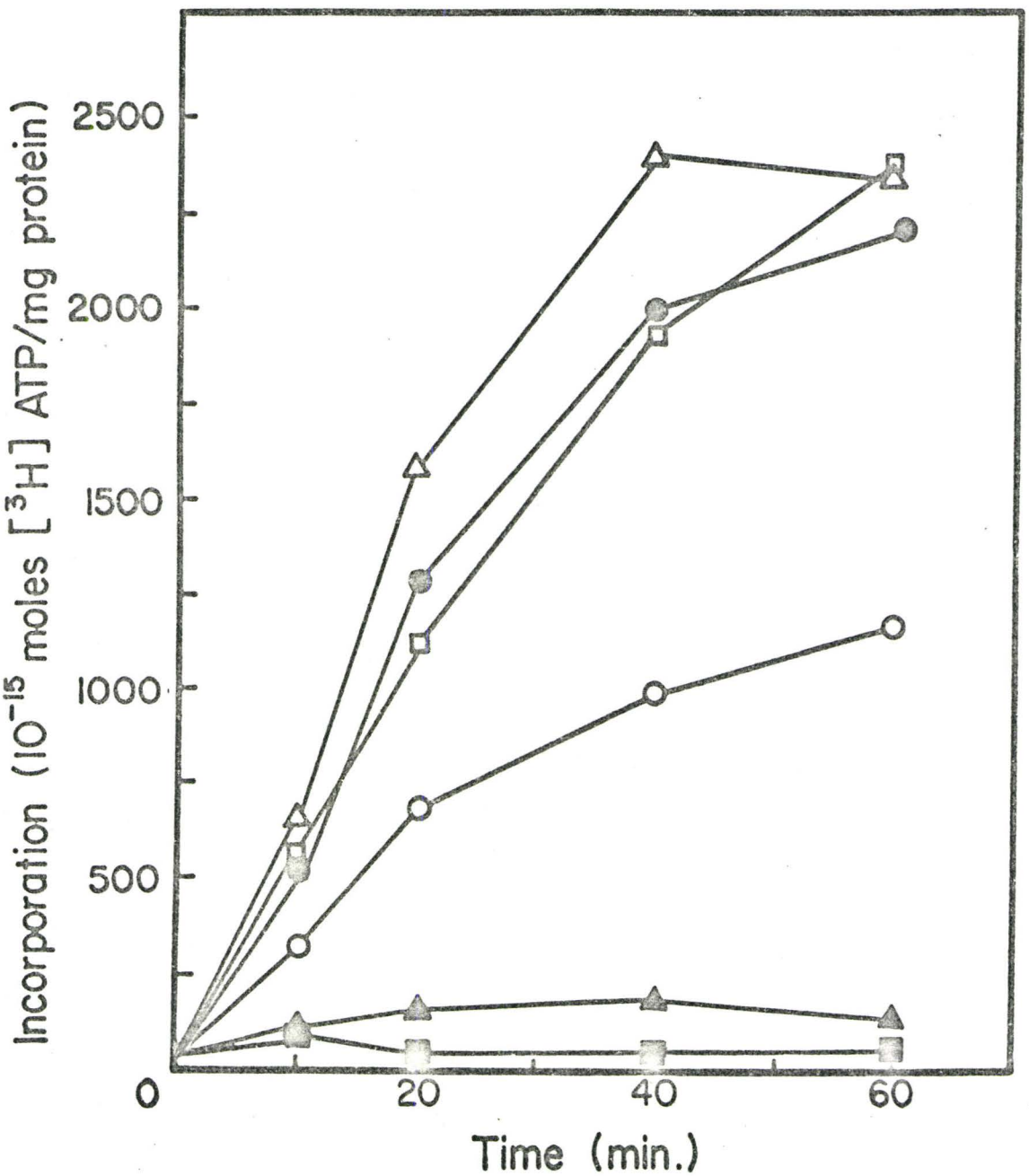

Figure 8. Time course of incorporation of [^{3}H]ATP. ●, Control; ○, ribonucleoside monophosphates omitted; ▲, Mg^{2+} omitted; ■, succinate omitted; △, phosphate omitted; □, UMP at same total concentration and instead of the mixture of UMP, CMP and GMP.

Table 12

Effect of DNase, RNase, Actinomycin D and Chloramphenicol on the Incorporation of [^{3}H]UTP and [^{3}H]ATP into Acid-insoluble Compounds by Isolated Rat Liver Mitochondria

Mitochondria isolated in 0.30 M sucrose-2 mM EDTA, pH 7.2 were incubated in medium I for 20 min in the presence or absence of inhibitors and the incorporation into acid-insoluble counts determined. The figure in brackets after the incorporation is the percentage of the control value.

		Incorporation	
		[^{3}H]UTP	[^{3}H]ATP
Incubation		10^{-15} moles/20 min/mg of protein	
Control		256	1,270
DNase	(83 µg/ml)	240 (94%)	1,260 (99%)
RNase	(36 µg/ml)	105 (41%)	1,130 (89%)
Actinomycin D	(9.5 µg/ml)	220 (85%)	1,230 (97%)
Chloramphenicol	(250 µg/ml)	256 (100%)	1,280 (101%)

into RNA.

DNase and chloramphenicol had relatively little effect on either [^{3}H]UTP or [^{3}H]ATP incorporation. As mentioned above (Fig. 7), actinomycin D had no effect on [^{3}H]UTP incorporation in 20 min and in addition had no effect on [^{3}H]ATP incorporation.

7. Effect of Atractyloside on Incorporation of [^{3}H]Adenine Nucleotides

The inhibitory action of atractyloside on [^{3}H]AMP and [^{3}H]ATP incorporation is shown in Table 13. Atractyloside at a concentration of 9.5 μM and 19.0 μM inhibited the incorporation into both RNA and the major acid-insoluble material as judged by the recovery of 2'(3')-AMP and the unidentified compound respectively.

8. RNA Synthesis by Isolated Rat Liver Nuclei

A possible source of RNA synthesis in isolated mitochondria is enzyme systems present in contaminating nuclei. In order to investigate this possibility, the characteristics of RNA synthesis by isolated nuclei were studied.

The effect of changes in the incubation medium, RNase, DNase, actinomycin D, ethidium bromide and atractyloside on the incorporation of [^{3}H]UTP or [^{3}H]ATP into RNA by isolated nuclei is shown in Table 14. The omission of ATP, CTP and GTP from the medium inhibited [^{3}H]UTP

Table 13

Effect of Atractyloside on the Incorporation of [^{3}H]ATP and [^{3}H]AMP into Acid-insoluble Products by Isolated Rat Liver Mitochondria

Mitochondria isolated in 0.30 M sucrose-2 mM EDTA, pH 7.2 were incubated in medium I with [^{3}H]ATP or [^{3}H]AMP in the presence or absence of actractyloside. After the incubation the acid- and lipid solvent-insoluble material was hydrolyzed in 0.3 N LiOH and the products chromatographed in 1M ammonium acetate-saturated boric acid, pH 7.5 - 95 % ethanol (2:5, v/v). Figures in brackets represent percent incorporation.

Experiment	Precursor	Time of Incubation (min)	Atractyloside (μM)	Unidentified compound	2'(3') AMP cpm	Total
1	[^{3}H]ATP	20	none	2705	313	3123
			9.5	612 (23)	206 (66)	891 (28)
2	[^{3}H]ATP	20	none	2672	328	3088
			9.5	307 (12)	131 (40)	591 (19)

(continued)

3	[^{3}H]ATP	60	none	1109	162	1335
			19.0	207 (18.6)	63 (38.9)	296 (22.2)
3	[^{3}H]AMP	60	none	931	308	1268
			19.0	189 (20.3)	94 (30.5)	283 (22.4)

Table 14

Effect of Inhibitors on the Synthesis of RNA by Rat Liver Nuclei

Nuclei were isolated, incubated with either [^{3}H]UTP or [^{3}H]ATP at 37° for 20 min and the acid precipitate washed and counted. Appropriate zero time values were also determined and subtracted from the value at 20 min. Each incubation has 0.34 mg protein/ml.

Incubation	Incorporation 10^{-12} moles/30 min/ mg of protein	percentage of control
Control with [^{3}H]UTP	12.2	100
- ATP, CTP, and GTP	1.11	9.1
+ DNase (83 μg/ml)	3.06	25.2
+ RNase (36 μg/ml)	1.43	11.7
+ actinomycin D (9.5 μg/ml)	3.34	27.4
+ ethidium bromide (0.5 μg/ml)	11.8	96.5
(1.0 μg/ml)	11.6	95.8
(3 μg/ml)	12.3	100.7
(9 μg/ml)	12.35	101.5
(45 μg/ml)	8.86	72.8
(225 μg/ml)	3.12	25.6
Control with [^{3}H]ATP	0.394	100
+ atractyloside (19.0 μM)	0.492	124

incorporation by 90 %. The addition of DNase, RNase and actinomycin D strongly decreased RNA synthesis. Ethidium bromide, however, at concentrations to 9 µg/ml had no effect on the reaction. Atractyloside also did not inhibit the incorporation of [^{3}H]ATP by nuclei.

The sensitivity of incorporation to DNase and the need for nucleoside triphosphates, clearly distinguished RNA synthesis by nuclei from that by mitochondria. The lack of inhibition by atractyloside in nuclei further differentiated RNA synthesis in nuclei from that in mitochondria.

9. Contamination with Bacteria

A second possible source of RNA synthesis in isolated mitochondria is contaminating bacteria. Aliquots (0.1 ml) taken from reaction mixtures after 1 hr of incubations were plated on blood agar medium. Bacterial counts ranged from 0 to 30 bacteria/0.1 ml. Mitochondrial stock suspensions usually contained no bacteria, but occasionally counts of 2 or 3 were obtained per 0.1 ml of the suspension.

10. Summary

The results show that all four [^{3}H]ribonucleoside triphosphates were precursors of RNA in isolated mitochondria. Acid precipitation alone of the incorporated radioactivity did not represent a true level of RNA synthesis for all

[^{3}H]nucleotides. A true level of RNA synthesis was, however, obtained by measuring the recovery of radioactive 2'(3') nucleotides after lipid extraction and alkaline hydrolysis.

The synthesis of RNA by isolated mitochondria was not due to the presence of contaminating nuclei or bacteria.

Section III Incorporation of [^{3}H]UTP by Mitochondria Isolated in Sucrose-TES or Sucrose-EDTA and Incubated in Medium II

In the studies to be described three changes in procedure were introduced.

(1) Mitochondria were isolated in 0.25 M sucrose-2 mM EDTA pH 7.4, (sucrose-EDTA) or 0.25 M sucrose-0.5 mM TES, pH 7.4 (sucrose-TES), to investigate the effect of the isolation medium on the nature of the incorporation.

(2) RNA was extracted by a hot phenol-SDS method before acid precipitation to study RNA synthesis.

(3) Incubations were carried out in hypo-osmotic and iso-osmotic media to study effects of osmotically-induced permeability changes on [^{3}H]nucleotide incorporation and inhibition of [^{3}H]nucleotide incorporation by enzymes and antibiotics.

Experiments in section II showed that [^{3}H]UTP was incorporated mainly into RNA. Therefore, [^{3}H]UTP was used as the precursor.

1. Effect of the Medium on [^{3}H]UTP Incorporation

The effect of deletions from the medium on incorporation is shown in Table 15. There was no absolute requirement for nucleoside triphosphates, Mg^{2+}, phosphate, or an energy source (malate, pyruvate and succinate). With mitochondria isolated in sucrose-TES, the incorporation was greater in the hypo-osmotic medium, but no difference was observed with mitochondria isolated in sucrose-EDTA. The incorporation was the same between pH 7.2 and 7.6 and was only 10 % of this level at pH 6.0.

The effect of excess amount of unlabelled UTP, UMP and uridine on [^{3}H]UTP incorporation was investigated. UTP and UMP reduced [^{3}H]UTP incorporation, but uridine had no effect (Table 16).

2. The Determination of K_M and V_{max} for [^{3}H]UTP Incorporation

The K_M and V_{max} for [^{3}H]UTP incorporation was determined using a constant amount of [^{3}H]UTP and an increasing amount of unlabelled UTP. Although this decreased the total counts measured, the amount of incorporation as measured in moles increased. The K_M for

Table 15

Effect of Changes in the Medium on Incorporation of [^{3}H]UTP into RNA

Mitochondria were isolated in sucrose-TES or sucrose-EDTA and incubated with [^{3}H]UTP for 20 min in the hypo-osmotic medium. RNA was isolated by the hot phenol-SDS method and precipitated with 5 % trichloroacetic acid and counted.

	Incorporation (cpm/mg of protein)	
Mitochondria	Sucrose-TES	Sucrose-EDTA
Complete	140	328
- Nucleotides	139	290
- Mg^{2+}	64.8	229
- Phosphate	40.8	260
- Malate, succinate and pyruvate	137	318

Table 16

Effect of UTP, UMP and Uridine
on the Incorporation of [^{3}H]UTP

Mitochondria were isolated in sucrose-TES and incubated with [^{3}H]UTP in the hypo-osmotic medium for 20 min or 60 min. An excess amount of unlabelled UTP (1,060 times), UMP (1,000 times) and uridine (1,000 times) was added before the incubation was started. RNA was extracted and counted as described in the methods.

	20 min	60 min
	cpm/mg of protein	
Control	246	1,030
+ UTP	22.2	-
+ UMP	52.8	157
+ Uridine	238	1,035

[^{3}H]UTP was 114 μM in the hypo-osmotic medium for mitochondria isolated in sucrose-TES and the V_{max} was 142 x 10^{-15}moles/mg of protein/min (Fig. 9).

3. Time Course of Incorporation of [^{3}H]UTP

The time course of incorporation of [^{3}H]UTP by mitochondria isolated in sucrose-TES and incubated in the hypo-osmotic medium and in the iso-osmotic medium is shown in Fig. 10a and 10b, respectively. There was an initial lag in incorporation, but after 10 min, the rate of incorporation increased and continued at a linear rate for the next 50 min. Both acriflavin and actinomycin D at 40 μg/ml strongly inhibited incorporation. Ethidium bromide (1 μg/ml) was slightly inhibitory during the first 20 min, but subsequent incorporation was inhibited completely. DNase had no effect but RNase inhibited in the first 20 min of the incubation after which the rate of incorporation approximated control values. In the iso-osmotic medium actinomycin D inhibited incorporation completely, and RNase had the same effect as in the hypo-osmotic media. After preincubation of the mitochondria for 10 min in 20 mM phosphate, pH 7.4 at 20°, a 79 % and 44 % increase in incorporation at 10 and 20 min, respectively, was observed, but thereafter incorporation levelled off.

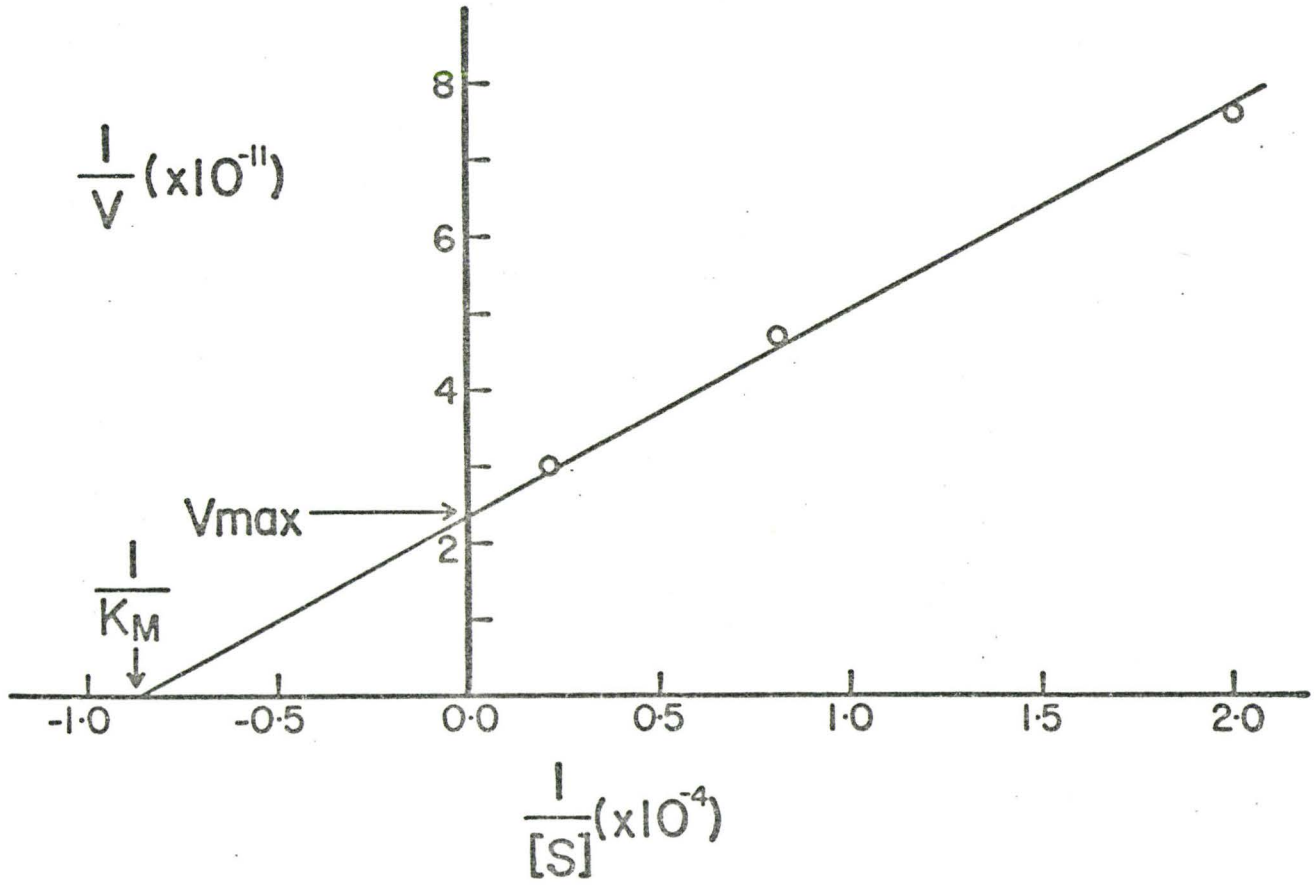

Figure 9. Reciprocal plot of the dependence of [^{3}H]UTP incorporation on substrate concentration. Mitochondria were isolated in sucrose-TES and incubated in the hypo-osmotic medium for 20 min. The same amount of [^{3}H]UTP and increasing concentrations of unlabelled UTP were added for each point.

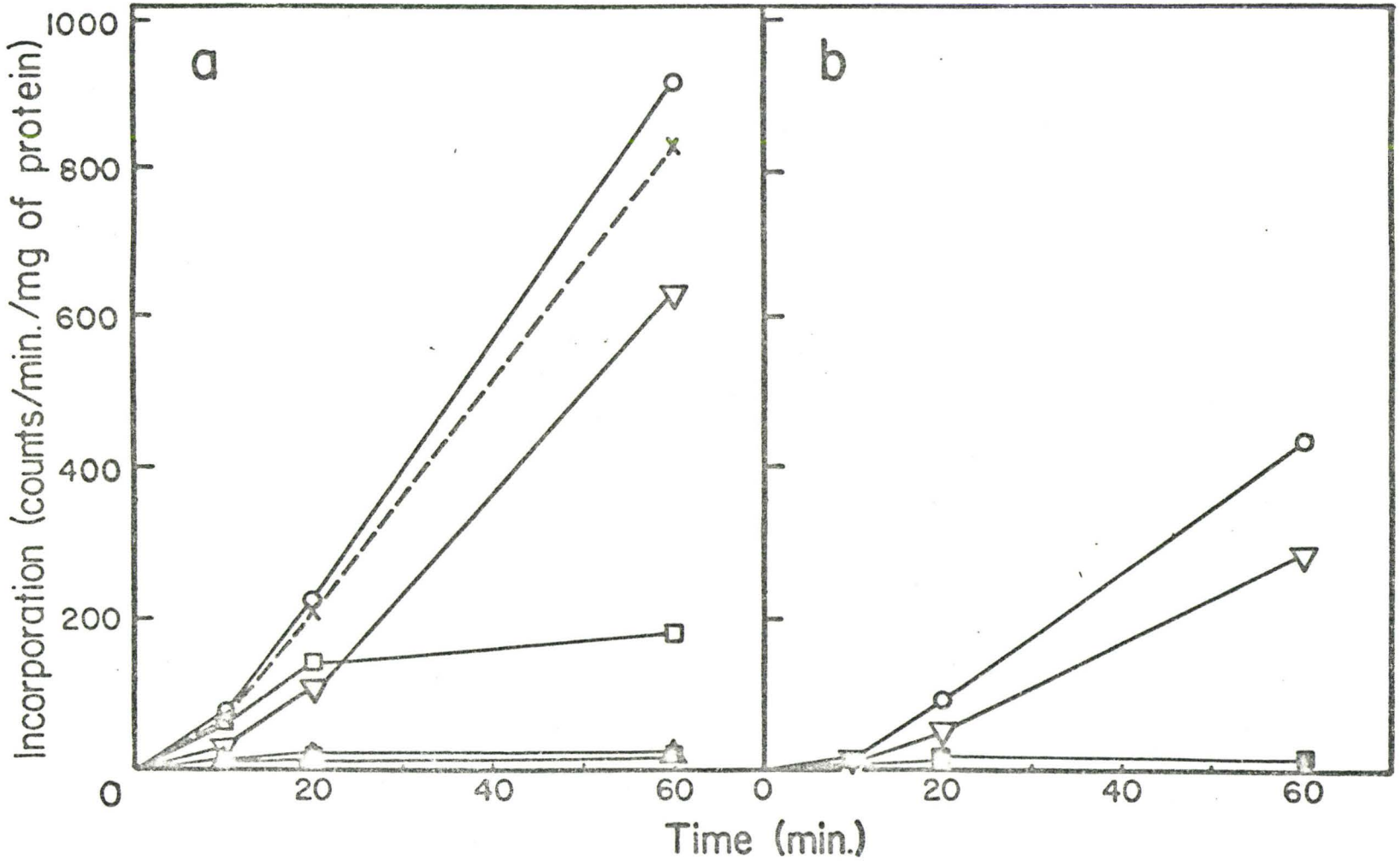

Figure 10. Time course of incorporation of [^{3}H]UTP into RNA by mitochondria isolated in sucrose-TES and incubated in a) the hypo-osmotic medium and in b) the iso-osmotic medium. o, Control; X, DNase (83 µg/ml); ▽, RNase (40 µg/ml); △, acriflavin (40 µg/ml); ■, actinomycin D (40 µg/ml); □, ethidium bromide (1 µg/ml).

The time course of incorporation with mitochondria isolated in sucrose-EDTA, and incubated in the hypo-osmotic medium and in the iso-osmotic medium is shown in Fig. 11a and 11b, respectively. In the hypo-osmotic medium, DNase had no effect, and acriflavin, actinomycin D and ethidium bromide inhibited strongly in the last 40 min of the incubation, but not in the first 20 min. In contrast, RNase inhibited in the first 20 min but not in the last 40 min. A similar time course of inhibition was observed in the iso-osmotic medium with actinomycin D and RNase.

With mitochondria isolated in sucrose-EDTA, the inhibitory effect of acriflavin and actinomycin D towards [^{3}H]UTP incorporation was more pronounced after the first 10 min of incubation. A possible explanation is that the mitochondria become more permeable to the antibiotics during the incubation. Therefore, mitochondria were preincubated in the hypo-osmotic medium at 30° for 10 or 20 min with the antibiotics before adding [^{3}H]UTP or without the antibiotics before adding [^{3}H]UTP and the antibiotics. These procedures led to a slightly increased inhibition by the antibiotics. However, the lag in inhibition in the first 10 min of incubation was not completely eliminated.

The characteristics of [^{3}H]UTP incorporation in mitochondria isolated in sucrose-EDTA and those in mitochondria isolated in sucrose-TES were different.

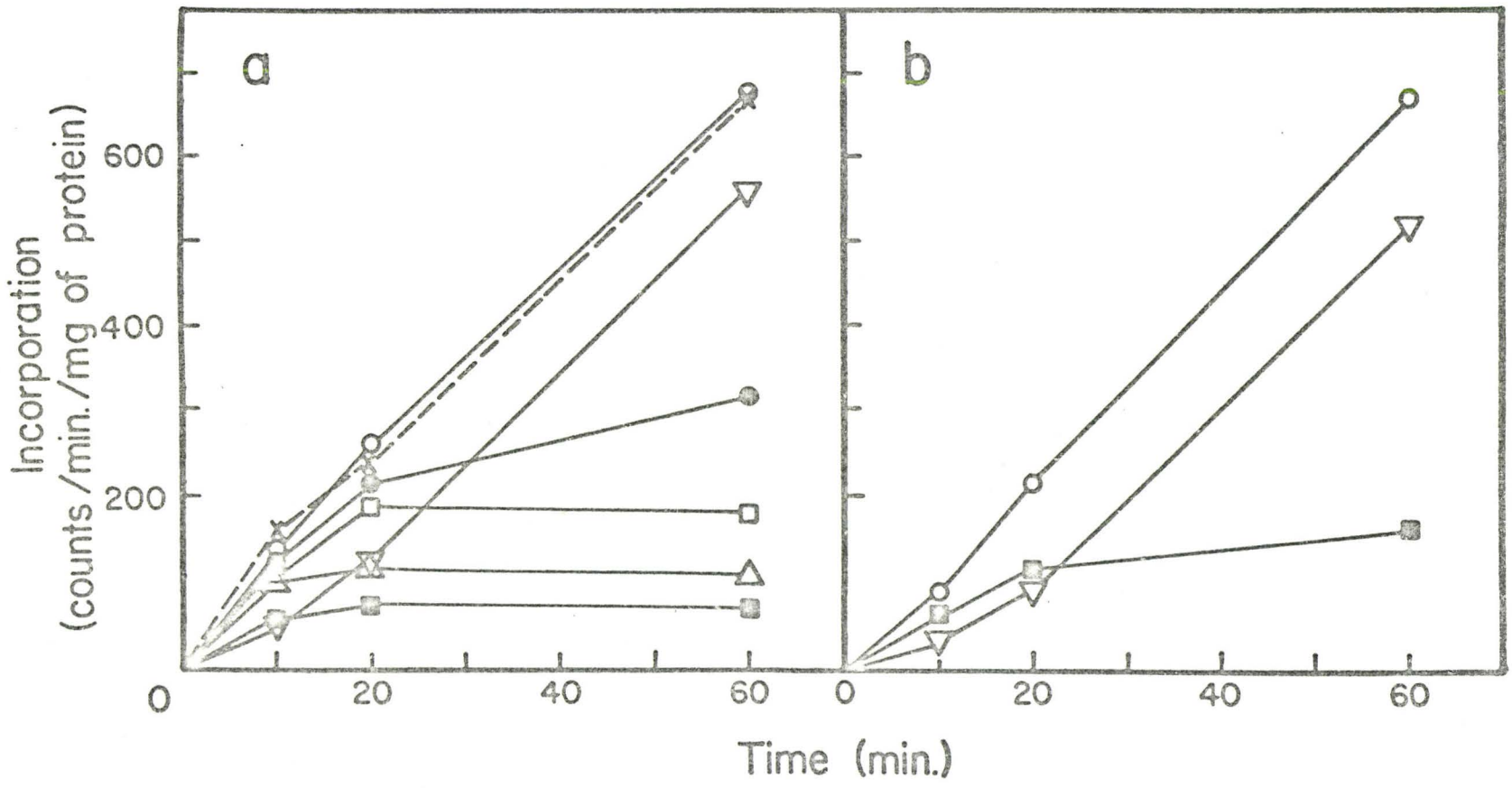

Figure 11. Time course of incorporation of [^{3}H]UTP into RNA by mitochondria isolated in sucrose-EDTA and incubated in a) the hypo-osmotic medium and in b) the iso-osmotic medium. o, Control; X, DNase (83 µg/ml); ▽, RNase (40 µg/ml); △, acriflavin (40 µg/ml); ■, actinomycin D (40 µg/ml); ●, ethidium bromide (0.33 µg/ml); □, ethidium bromide (1 µg/ml).

EDTA chelated Mg^{2+} and Ca^{2+}, but TES does not. In order to investigate whether chelation of Mg^{2+} by EDTA during the isolation of mitochondria could explain the difference, mitochondria were isolated in a 0.25 M sucrose- 2 mM EGTA, pH 7.4 (sucrose-EGTA). EGTA binds Ca^{2+} without removing bound Mg^{2+} (Settlemire, et al., 1968). The results are seen in Fig. 12, which can be compared to the results in Fig. 11a and 11b. The incorporation of [^{3}H]UTP by mitochondria isolated in sucrose-EGTA showed the same characteristics as that by mitochondria isolated in sucrose-EDTA in terms of sensitivity to acriflavin, actinomycin D and the change in the osmolarity of the medium.

4. Effect of Various Inhibitors at Different Concentrations

The effect of several antibiotics on RNA synthesis by mitochondria prepared in sucrose-TES and incubated in the hypo-osmotic medium is shown in Fig. 13. Both acriflavin and actinomycin D inhibited 50 % at about 1 µg/ml. About 0.5 µg of ethidium bromide/ml was necessary for 50 % inhibition. The antibiotics rifampicin rifamycin SV and streptovaricins did not inhibit the incorporation.

5. The Incorporation of [^{3}H]UTP into Internucleotide Linkages of RNA with Time and the Effect of RNase

The time course of RNA synthesis as judged

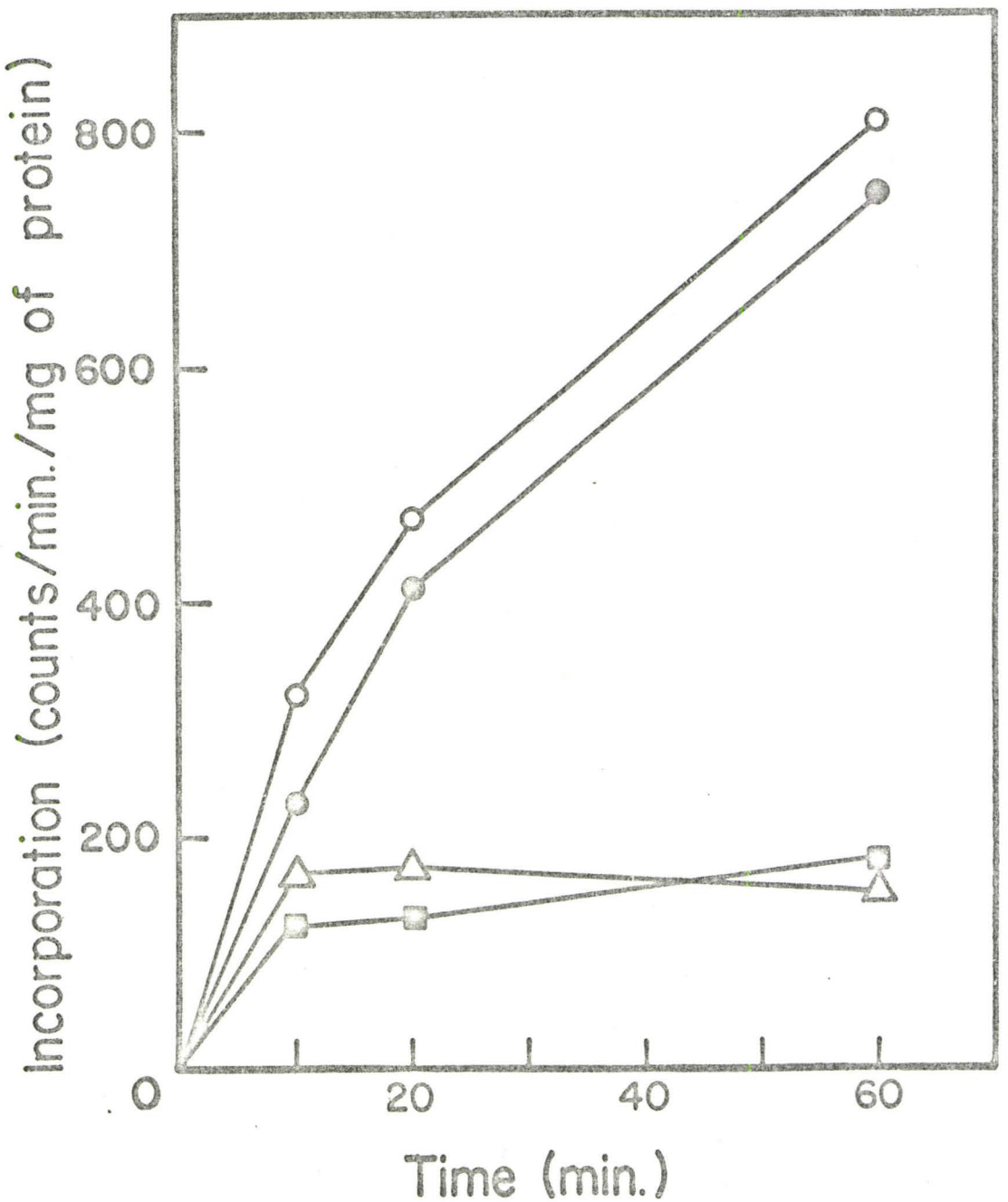

Figure 12. Time course of incorporation of [^{3}H]UTP into RNA by mitochondria isolated in sucrose-EGTA and incubated in the hypo-osmotic medium. In one experiment, mitochondria were incubated in the iso-osmotic medium (●). o, Control; △, acriflavin (40 µg/ml); ■, actinomycin D (40 µg/ml).

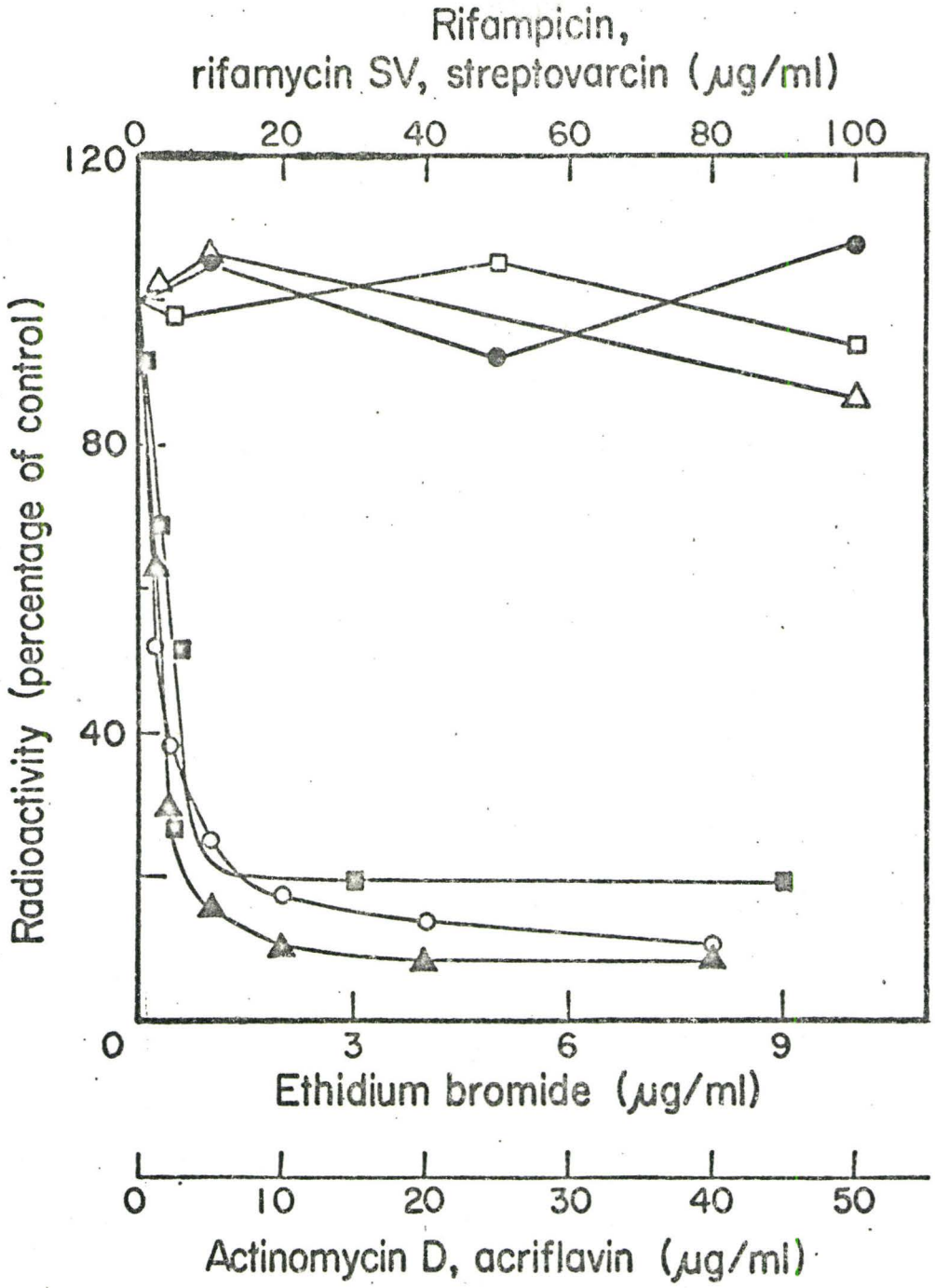

Figure 13. The effect of various inhibitors at different concentrations on incorporation of [^{3}H]UTP into RNA. Mitochondria were isolated in sucrose-TES and incubated in the hypo-osmotic medium for 20 min. △, Rifampicin; ●, rifamycin SV; □, streptovarcin; ▲, acriflavin; o, actinomycin D; ■, ethidium bromide.

by recovery of 2'(3')UMP and uridine after alkaline hydrolysis with mitochondria isolated in sucrose-TES and incubated in the hypo-osmotic medium and in the iso-osmotic medium are shown in Fig. 14a and 14b, respectively. Most of the radioactivity was recovered as 2'(3')UMP. The time course of RNA synthesis in both cases was similar to that shown in Fig. 10. The amount of 2'(3')UMP recovered increased with time, but there was not any striking increase of uridine recovered with time. The effect of RNase on recovery of 2'(3')UMP was also similar to that shown in Fig. 10. There was some inhibition at 20 min of incubation, but almost no effect in the next 40 min. With uridine, the amount recovered was too low to determine the effect of RNase.

Qualitatively, similar over-all observations were found for mitochondria isolated in sucrose-EDTA and incubated in the hypo-osmotic medium and in the iso-osmotic medium as shown in Fig. 15 a and 15b, respectively. RNase inhibited the recovery of 2'(3')UMP in the first 10 min, a result in agreement with that shown in Fig. 11. The amount of uridine recovered was reduced by RNase, particularly with the iso-osmotic medium where complete inhibition was observed.

6. Effect of RNase, Actinomycin D and Acriflavin

The effect of RNase, actinomycin D and acriflavin on the recovery of 2'(3')UMP and uridine in

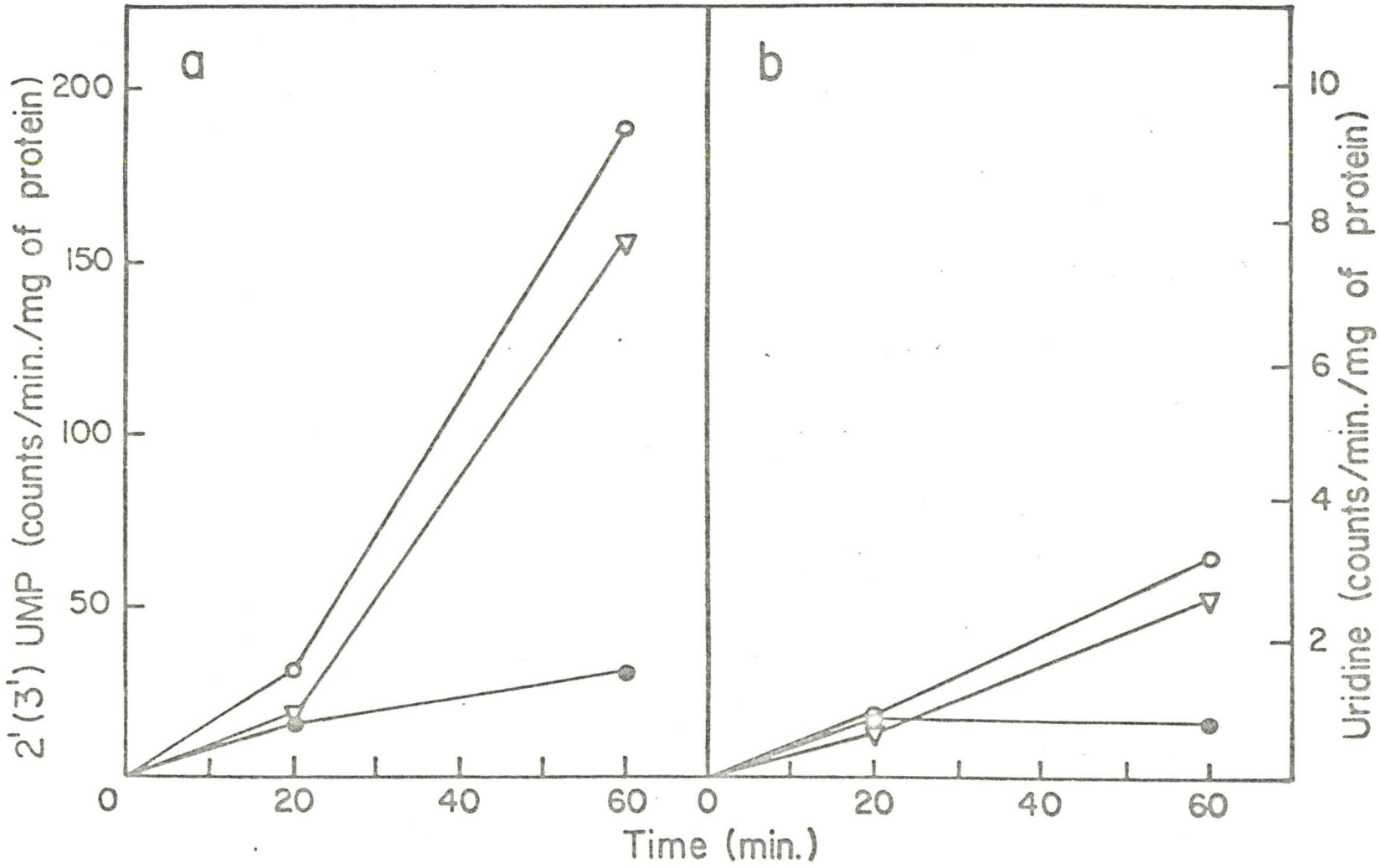

Figure 14. Time course of recovery of 2'(3')UMP and uridine after alkaline hydrolysis. Mitochondria were isolated in sucrose-TES and incubated in the presence or the absence of RNase (40 µg/ml) in a) the hypo-osmotic medium and in b) the iso-osmotic medium. o, 2'(3')UMP; ▽, 2'(3')UMP, RNase; ●, uridine.

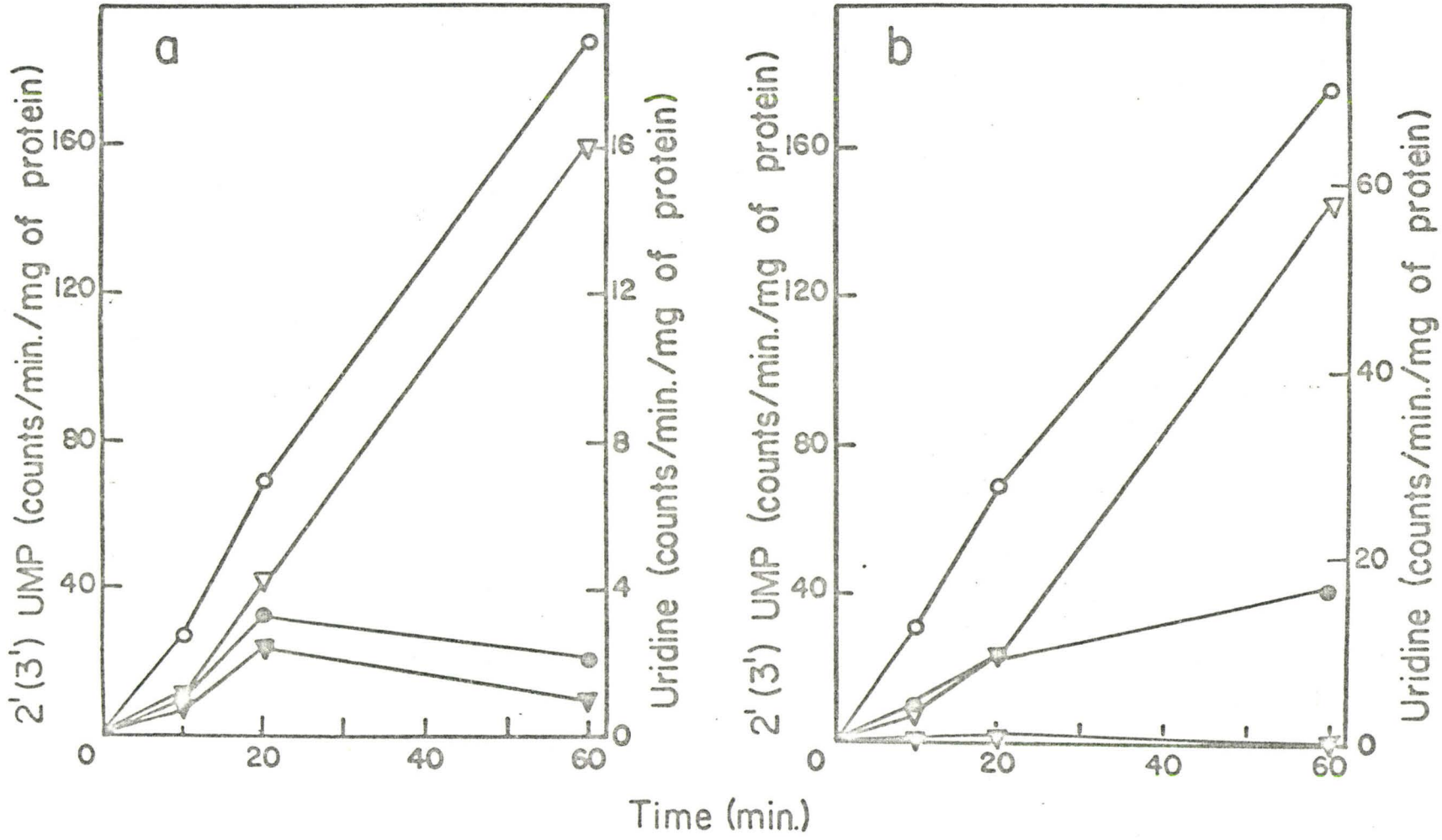

Figure 15. Time course of recovery of 2'(3')UMP and uridine after alkaline hydrolysis. Mitochondria were isolated in sucrose-EDTA and incubated in the presence or the absence of RNase (40 µg/ml) in a) the hypo-osmotic medium and in b) the iso-osmotic medium. o, 2'(3')UMP; ▽, 2'(3')UMP, RNase; •, uridine; ▼, uridine, RNase.

a 20 min incubation is presented in Table 17. With mitochondria isolated in sucrose-TES, RNase reduced the amount of 2'(3')UMP recovered in both incubation media. The recovery of uridine was too low to assess the effect of RNase. Actinomycin D reduced the amount of 2'(3')UMP in the hypo-osmotic medium, but to less extent in the iso-osmotic medium.

With mitochondria isolated in sucrose-EDTA, the amount of 2'(3')UMP recovered in both incubation media was reduced in the presence of RNase, actinomycin D and acriflavin. The recovery of uridine was decreased by RNase, particularly with the iso-osmotic medium. Actinomycin D did not decrease the amount of uridine recovered. Acriflavin increased the recovery of uridine.

The effect of adding RNase after stopping further [^{3}H]UTP incorporation with pyrophosphate at 0° was examined. RNase added in this way decreased the amount of observed [^{3}H]UTP counts incorporated to about the same extent as when it is present during the incubation (Table 18).

Table 17

Effect of RNase, Actinomycin D and Acriflavin

Mitochondria were isolated in sucrose-TES or sucrose-EDTA and incubated with [^{3}H]UTP for 20 min in the hypo-osmotic medium and iso-osmotic medium in the presence of RNase (40 µg/ml) and actinomycin D (40 µg/ml). RNA was hydrolyzed with alkali. The hydrolyzate was chromatographed on paper and counted.

Mitochondrial isolation medium	Incubation medium	Incorporation (cpm) 2'(3')UMP	uridine
Sucrose-TES	hypo-osmotic	318	8
	+ RNase	191	10
	+ actinomycin D	19	6
	iso-osmotic	191	9
	+ RNase	130	2
	+ actinomycin D	65	7
Sucrose-EDTA	hypo-osmotic	485	45
	+ RNase	283	30
	+ actinomycin D	122	43
	+ acriflavin	237	75
	iso-osmotic	481	54
	+ RNase	155	5
	+ actinomycin D	198	61
	+ acriflavin	268	97

Table 18

Effect of RNase Added after Incubation

Mitochondria were isolated in sucrose-TES and incubated with [^{3}H]UTP for 10 or 20 min in the hypo-osmotic medium. RNase was added to 40 µg/ml after [^{3}H]UTP incorporation was stopped with saturated tetrasodium pyrophosphate at 0°. RNA was extracted and counted as described in the Methods.

	10 min	20 min
	cpm/mg of protein	
Control	171	362
RNase	66.6	226

Section IV. Comparison of Incorporation of the Four [^{3}H]Ribonucleoside Triphosphates by Mitochondria Isolated in Sucrose-TES or Sucrose-EDTA and Incubated in Medium II

In this section the incorporation of [^{3}H]UTP, [^{3}H]ATP, [^{3}H]CTP and [^{3}H]GTP into RNA is compared. Preparation and incubation of mitochondria, isolation of RNA, and alkaline hydrolysis of acid-insoluble products were the same as in Section III.

1. Incorporation of the four [^{3}H]Ribonucleoside Triphosphates

The incorporation of the four [^{3}H]ribonucleoside triphosphates into RNA is shown in Table 19. The relative extent of incorporation of the precursors based on the recovery of 2'(3') nucleotides was CTP > ATP = UTP >> GTP and based on the recovery of nucleosides was in the same order being CTP > ATP > UTP >> GTP. As with the alkaline hydrolysis in section II, an unidentified compound which ran close to the nucleoside diphosphate on paper chromatograms of the alkaline hydrolyzates was observed for each [^{3}H]ribonucleotide. In the case of ATP it represented a high proportion of the total acid-insoluble counts.

2. Turnover of Incorporated [^{3}H]CTP and [^{3}H]UTP

As shown in Table 20, following the addition of excess unlabelled CTP at 20 min, the incorporated [^{3}H]CTP decreased with the counts at 60 min being 42 %

Table 19

Incorporation of [^{3}H]Ribonucleoside Triphosphates into RNA

Mitochondria were isolated in sucrose-TES or sucrose-EDTA and incubated for 20 min in the hypo-osmotic medium. The concentration of the radioactive nucleoside triphosphate [5-^{3}H]UTP, [5-^{3}H]CTP, [2-^{3}H]ATP and [8-^{3}H]GTP was 8.3 μM and that of three unlabelled nucleoside triphosphates 100 μM. The RNA was hydrolyzed with alkali and subjected to paper chromatography.

	Incorporation 10^{-15} moles/mg of protein							
	Sucrose-TES mitochondria				Sucrose-EDTA mitochondria			
Precursor	[^{3}H]UTP	[^{3}H]ATP	[^{3}H]CTP	[^{3}H]GTP	[^{3}H]UTP	[^{3}H]ATP	[^{3}H]CTP	[^{3}H]GTP
Product								
2'(3')Nucleotide	123	144	238	31.2	110	141	178	27.5
Nucleoside	2.7	75	157	1.8	10.6	54.8	102	1.1
Unidentified	19.2	301	66	34.8	17.6	273	22	41.8

Table 20

Effect of CTP and UTP on the Incorporation of [^{3}H]CTP and [^{3}H]UTP

Mitochondria were isolated in sucrose-TES and incubated in the hypo-osmotic medium. An excess amount (1,000 times) of unlabelled CTP or UTP was added 20 min after the incubation started and the incubation continued for another 40 min. Radioactive counts in 5 % trichloroacetic acid precipitate after a hot phenol-SDS extraction were taken as a measure of the incorporation of [^{3}H]CTP and [^{3}H]UTP into RNA.

	Control at 20 min	Unlabelled CTP or UTP added at 20 min and incubation continued for 40 min	Control at 60 min
Precursor		cpm/mg of protein	
[^{3}H]CTP	424	178	957
[^{3}H]UTP	246	278	1,110

of the 20 min value. In contrast, excess [^{3}H]UTP added at 20 min did not change the level of the incorporated counts. Apparently, some product containing [^{3}H]CTP had a higher rate of turn-over while the product containing [^{3}H]UTP was stable.

3. Effect of the Osmolarity of the Medium and Actinomycin D on the Incorporation of [^{3}H]Ribonucleoside Triphosphates

The effect of the osmolarity of the medium and of actinomycin D at 40 µg/ml is shown in Table 21. The results with mitochondria isolated in sucrose-TES show that the incubation in the iso-osmotic medium greatly reduced the incorporation of [^{3}H]CTP and [^{3}H]GTP. The incorporation of [^{3}H]UTP was partially reduced in the iso-osmotic medium. The incorporation of [^{3}H]ATP was greater in the iso-osmotic medium. Less inhibition of [^{3}H]nucleotide incorporation in the iso-osmotic medium was observed with mitochondria isolated in sucrose-EDTA.

Actinomycin D inhibited incorporation of [^{3}H]UTP, [^{3}H]ATP, [^{3}H]CTP and [^{3}H]GTP by mitochondria isolated in sucrose-TES. Of the four nucleotides, the amount of 2'(3')CMP recovered was the least reduced by actinomycin D. The amount of nucleoside obtained with any of the precursors was not affected by actinomycin D.

The sensitivity to actinomycin D of incorporation of [^{3}H]nucleotides by mitochondria isolated in sucrose-EDTA was similar to that of mitochondria isolated in

Table 21

Effect of the Osmolarity of the Medium and Actinomycin D on the Incorporation of [^{3}H]Ribonucleoside Triphosphates into RNA

Mitochondria were incubated for 20 min in the hypo-osmotic medium and in the iso-osmotic medium in the presence and absence of actinomycin D (act. D) at 40 µg/ml using [^{3}H]UTP, [^{3}H]ATP, [^{3}H]CTP or [^{3}H]GTP as the precursor in each incubation.

	Incorporation (percentage of total counts in the hypo-osmotic medium)					
	Sucrose-TES mitochondria			Sucrose-EDTA mitochondria		
Precursor	Total	2'(3')nucleotide	nucleoside	Total	2'(3')nucleotide	nucleoside
[^{3}H]UTP - hypo-osmotic	100	98	2	100	91	9
" + act. D	8	6	2	33	24	9
iso-osmotic	61	59	2	105	95	10
" + act. D	22	20	2	50	39	11
[^{3}H]ATP - hypo-osmotic	100	69	31	100	73	27
" + act. D	54	22	32	49	25	24
iso-osmotic	145	110	35	175	137	38

(continued)

" + act. D	79	41	38	69	34	35
[^{3}H]CTP - hypo-osmotic	100	59	41	100	60	40
" + act. D	70	31	39	74	31	43
iso-osmotic	7	6	1	28	23	5
" + act. D	3	2	1	13	6	7
[^{3}H]GTP - hypo-osmotic	100	97	3	100	93	7
" + act. D	11	5	6	25	18	7
iso-osmotic	17	15	2	76	67	9
" + act. D	7	2	5	14	7	7

sucrose-TES.

4. Effect of Atractyloside on [^{3}H]ATP and [^{3}H]UTP Incorporation

The effect of atractyloside on [^{3}H]ATP incorporation is shown in Table 22. The incorporation into RNA recovered as 2'(3')-AMP after alkaline hydrolysis was inhibited 50 % at about 3 mμmoles/mg of protein and 2 mμmoles/mg of protein for mitochondria isolated in sucrose-TES and sucrose-EDTA, respectively, in the iso-osmotic medium. The incorporation into the unidentified compound and recovery of adenosine was inhibited to about the same extent.

The specificity of this inhibition was examined by testing the effect of atractyloside on [^{3}H]UTP incorporation. As seen in Fig. 16, at low concentrations of ATP, atractyloside inhibited [^{3}H]UTP incorporation, but as the concentration of ATP was raised, the inhibition was eliminated.

Section V. Types of RNA Synthesized by Mitochondria Isolated in Sucrose-EDTA and Incubated in Medium II

1. Incorporation of [^{3}H]UTP

(a) Incubation for 20 min

Mitochondria isolated in sucrose-EDTA were

Table 22

Effect of Atractyloside on [^{3}H]ATP Incorporation

Mitochondria were isolated in sucrose-TES or sucrose-EDTA and incubated for 20 min in the hypo-osmotic medium and iso-osmotic medium with [^{3}H]ATP as the precursor in the presence or absence of atractyloside. The RNA was extracted, hydrolyzed with alkali and chromatographed on paper as described in the Methods.

		Incorporation (percent of total control)			
Mitochondria	Medium	Total	Unidentified compound	2'(3')-AMP	Adenosine
Sucrose-TES	Iso-osmotic	100	51.0	40.9	8.1
	+ Atractyloside				
	(1.47 μM; 0.47 mμmoles/mg protein)	87.1	46.0	34.6	6.5
	(2.94 μM; 9.94 mμmoles/mg protein)	77.8	39.8	32.2	5.8
	(14.7 μM; 4.7 mμmoles/mg protein)	44.9	25.3	15.6	4.1
	(70.1 μM; 224 mμmoles/mg protein)	8.7	6.6	1.4	0.7

(continued)

	Hypo-osmotic	100	56.0	33.0	11.0
	+ Atractyloside				
	(14.7 μM; 4.7 mμmoles/mg protein)	49.6	27.8	17.4	4.4
Sucrose-EDTA	Iso-osmotic	100	58.0	34.4	7.5
	+ Atractyloside				
	(3.04 μM; 0.6 mμmoles/mg protein)	77.0	44.7	25.5	6.8
	(15.2 μM; 3 mμmoles/mg protein)	37.9	23.1	11.3	3.5
	(76 μM; 15 mμmoles/mg protein)	19.0	13.0	3.8	2.2
	(216 μM; 94.5 mμmoles/mg protein)	8.4	2.9	4.1	1.4
	Hypo-osmotic	100	55.2	27.4	17.3
	+ Atractyloside				
	(15.2 μM; 3 mμmoles/mg protein)	28.3	15.8	5.3	7.2

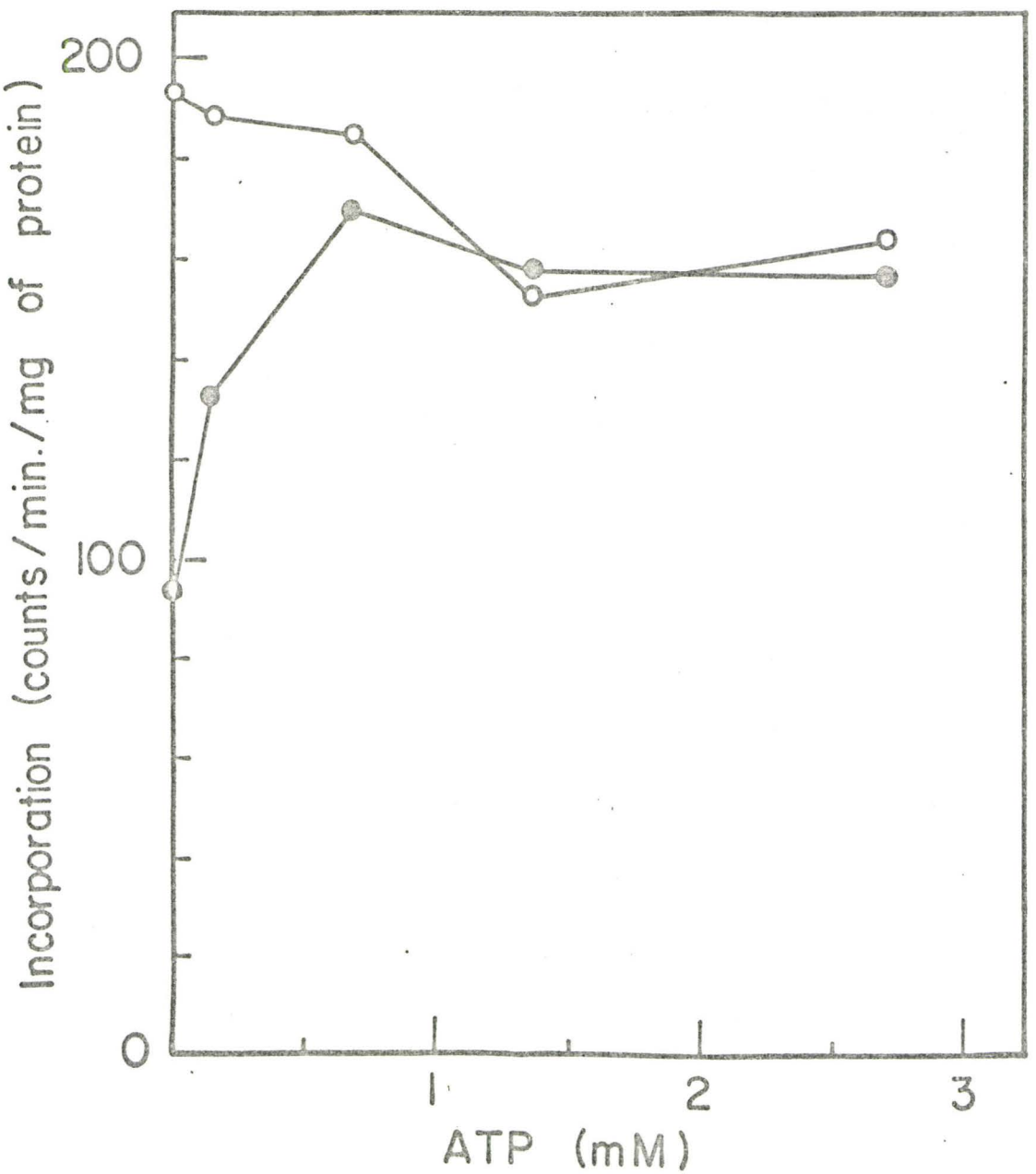

Figure 16. The effect of ATP and atractyloside on the incorporation of [^{3}H]UTP into RNA. Mitochondria were isolated in sucrose-EDTA and incubated in the hypo-osmotic medium for 20 min with different concentrations of ATP. o, Control; •, atractyloside, 28 μM (7.5 mμmoles/mg protein)

incubated with [^{3}H]UTP for 20 min in the hypo-osmotic medium. RNA was extracted by the cold phenol-SDS method II and the RNA was analyzed by convex sucrose density gradient centrifugation. Most of the radioactivity stayed on top of the gradient. On an expanded scale the synthesis of actinomycin D sensitive high molecular weight RNA can be seen (Fig. 17 and Fig. 18).

(b) Incubation for 1 hr

Mitochondria isolated in sucrose-EDTA were incubated with [^{3}H]UTP for 1 hr in the hypo-osmotic medium. RNA was extracted by the hot phenol-SDS method and analyzed by linear sucrose density gradient centrifugation (Fig. 19). There was a radioactive peak close to the meniscus and the remaining counts were observed as a heterodisperse pattern with radioactive RNA present up to 30 S. On an expanded scale, a peak at 16 S and a shoulder at 13 S were apparent. Possible peaks at 18 and 20 S were also seen.

In another experiment, mitochondria isolated in sucrose-EDTA were incubated with [^{3}H]UTP for 1 hr in the hypo-osmotic and the iso-osmotic medium in the presence and absence of actinomycin D. RNA was extracted by the cold phenol-SDS method II and analyzed by convex sucrose density gradient centrifugation. With the hypo-osmotic medium, most of the radioactivity stayed on top of the gradients. By plotting on an expanded scale, a heterodisperse pattern of high molecular weight RNA

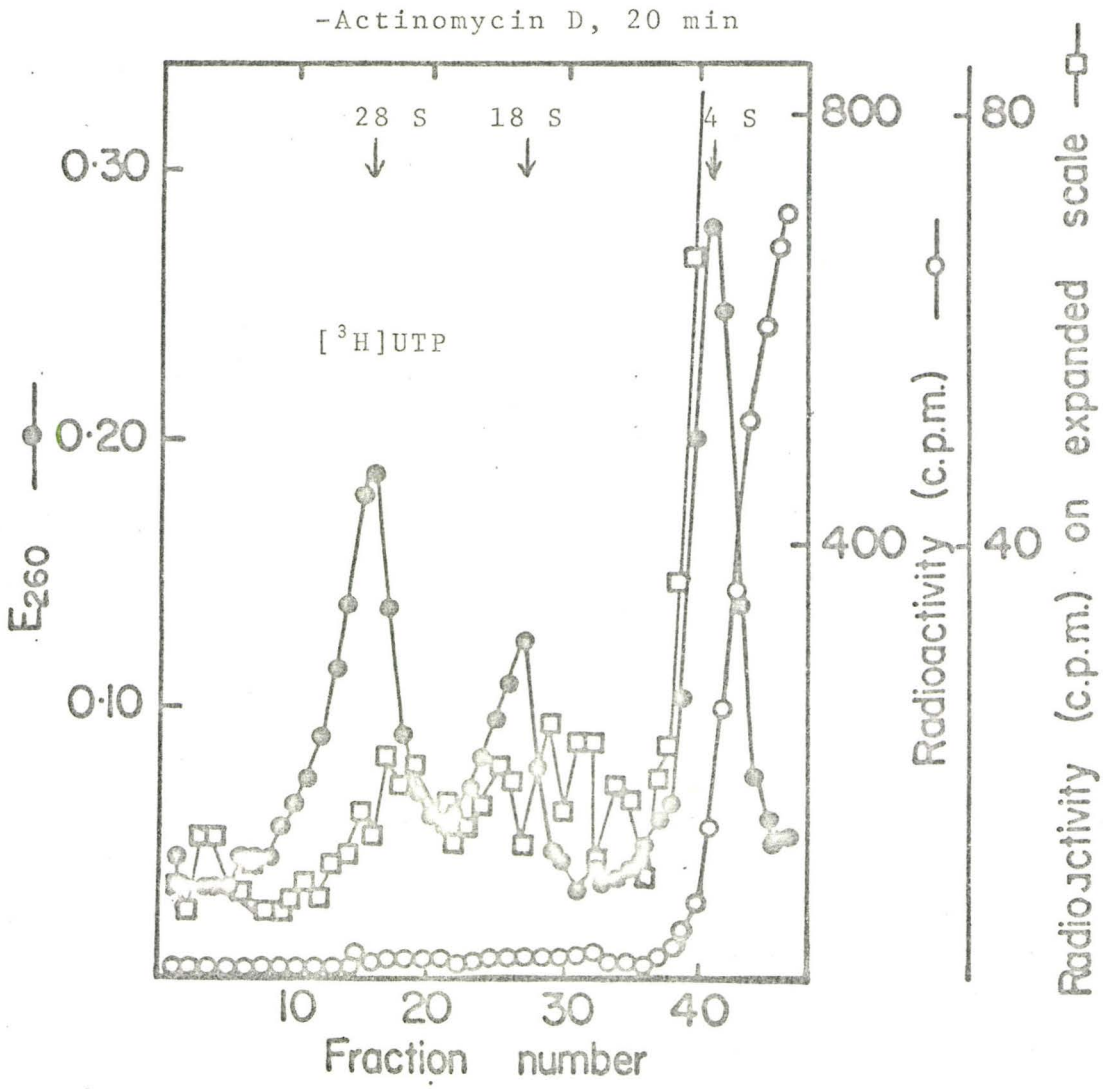

Figure 17. Sedimentation on a convex sucrose density gradient of RNA synthesized with [^{3}H]UTP for 20 min in the hypo-osmotic medium. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP for 20 min in the hypo-osmotic medium. RNA was extracted by the cold phenol-SDS method II. Optical density at 260 nm was read for the marker cyptoplasmic ribosomal RNA (28 and 18 S) and E. coli tRNA (4 S) and the radioactivity counted, as described in the Methods section. •, Cytoplasmic ribosomal RNA and E. coli tRNA; o, mitochondrial RNA; □ ,. mitochondrial RNA on an expanded scale.

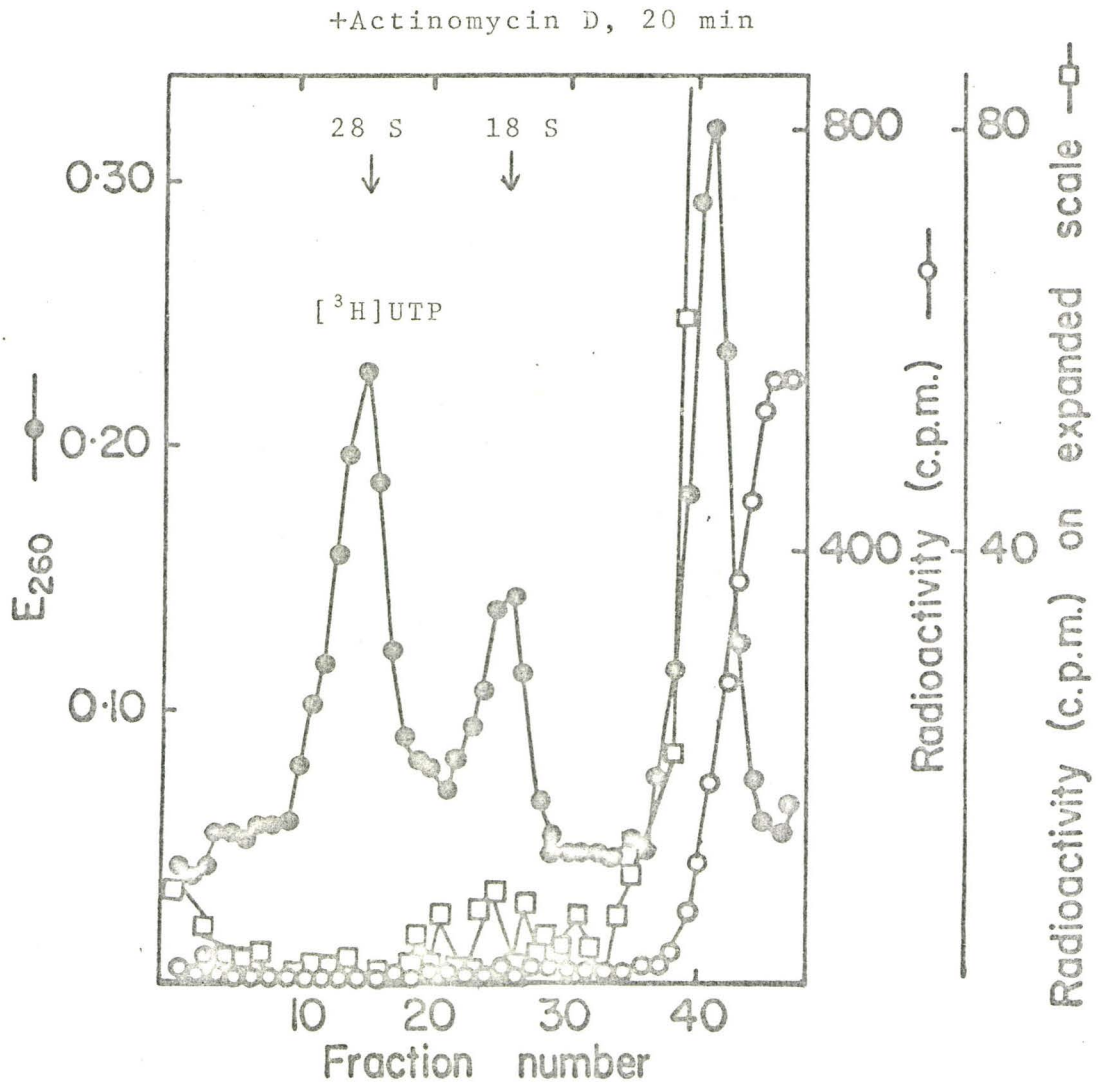

Figure 18. Sedimentation on a convex sucrose density gradient of RNA synthesized with [^{3}H]UTP for 20 min in the hypo-osmotic medium in the presence of actinomycin D. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP for 20 min in the hypo-osmotic medium in the presenceof actinomycin D (40 µg/ml). Legend is as in Figure 17. ●, Cytoplasmic ribosomal RNA and E. coli tRNA; O, mitochondrial RNA; □, mitochondrial RNA on an expanded scale.

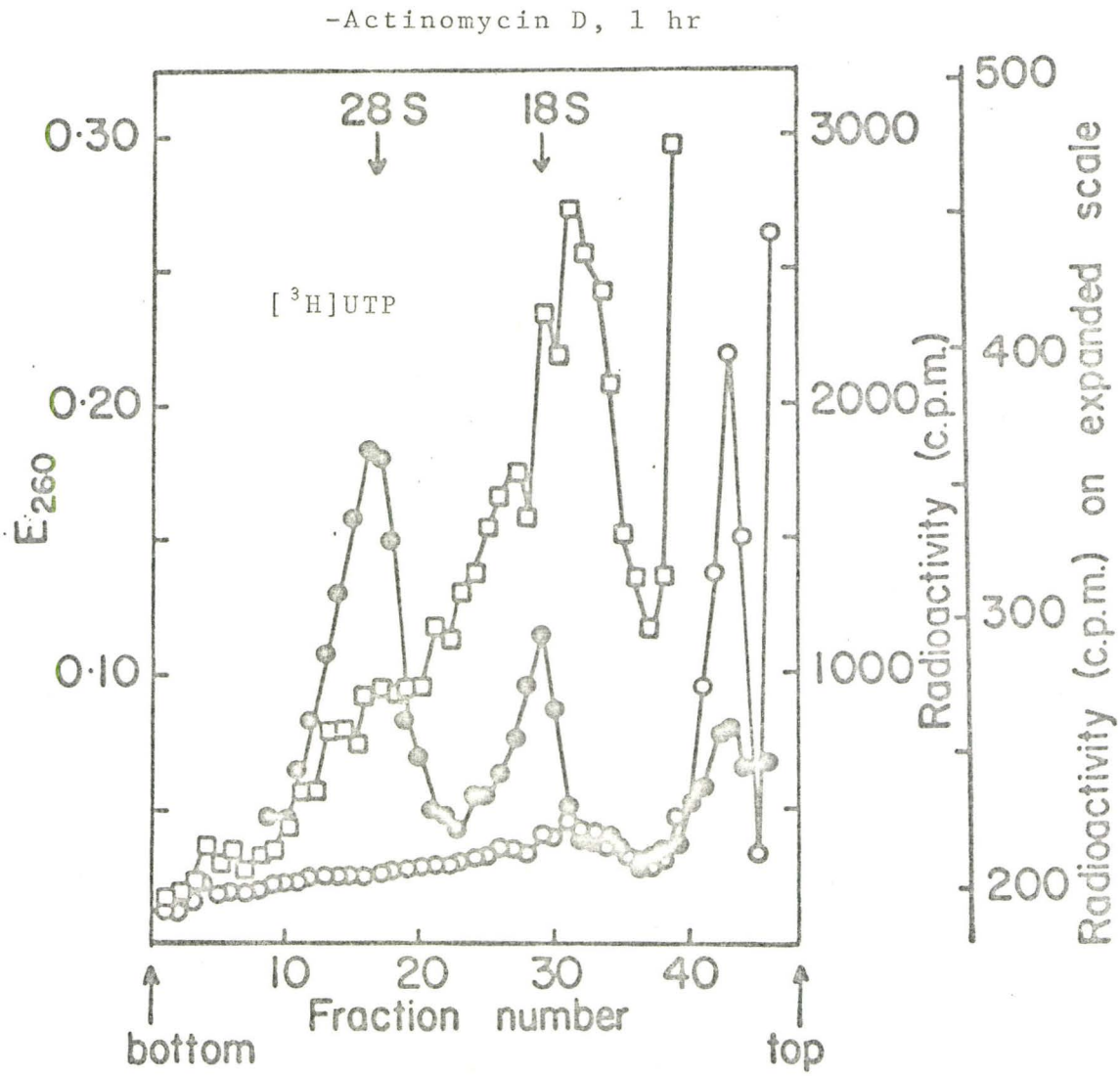

Figure 19. Sedimentation on a linear sucrose density gradient of RNA synthesized with [^{3}H]UTP for 1 hr in the hypo-osmotic medium and extracted by the hot phenol-SDS method. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP for 1 hr in the hypo-osmotic medium. RNA was extracted by the hot phenol-SDS method. Optical density at 260 nm was read for the marker cytoplasmic ribosomal RNA (28 and 18 S) and the radioactivity counted as described in the Methods section. •, Cytoplasmic ribosomal RNA; o, mitochondrial RNA; □, mitochondrial RNA on an expanded scale.

was apparent with a peak at 16 S (Fig. 20). Actinomycin D inhibited the synthesis of the high molecular weight RNA (Fig. 21). With the iso-osmotic medium, the synthesis of the heterodisperse RNA was also apparent with a possible peak at 16 S (Fig. 22). Its synthesis was sensitive to actinomycin D, but to a lesser extent than that in the hypo-osmotic medium (Fig. 23).

A portion of the sample used for sucrose gradient analysis was precipitated in ice-cold 5 % trichloroacetic acid and radioactive counts in the trichloroacetic acid precipitate were measured. Most of the radioactive counts became acid-soluble in 5 % trichloroacetic acid (Table 23). The acid-soluble counts were further examined by paper chromatography. This technique indicated that the acid-soluble counts represented UTP, UDP and UMP.

The possibility that [^{3}H]UTP was being incorporated into ribosomal RNA was studied. Mitochondria isolated in sucrose-EDTA were incubated with [^{3}H]UTP for 1 hr in the hypo-osmotic medium. RNA was extracted by the cold phenol-SDS method I and analyzed by linear sucrose density gradient centrifugation (Fig. 24). There were radioactive peaks at 16 and 13 S with a possible peak at 20 S.

The possibility that [^{3}H]UTP was being incorporated into ribosomal RNA was also examined by agarose-polyacrylamide gel electrophoresis. Mitochondria isolated in

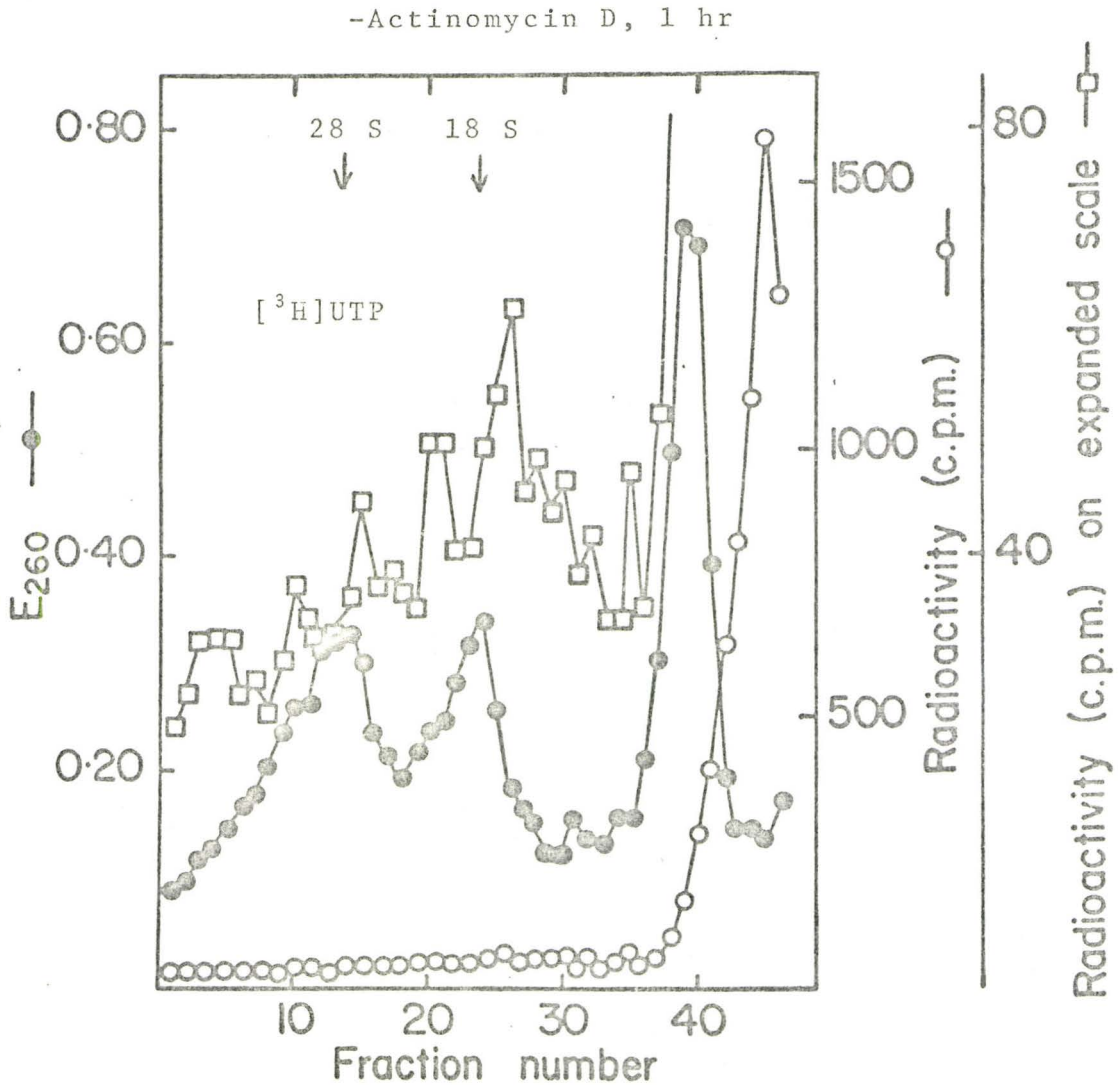

Figure 20. Sedimentation on a convex sucrose density gradient of RNA synthesized with [^{3}H]UTP for 1 hr in the hypo-osmotic medium. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP for 1 hr in the hypo-osmotic medium. Legend as in Figure 17. ●, Cytoplasmic ribosomal RNA and E. coli tRNA; o, mitochondrial RNA; □, mitochondrial RNA on an expanded scale.

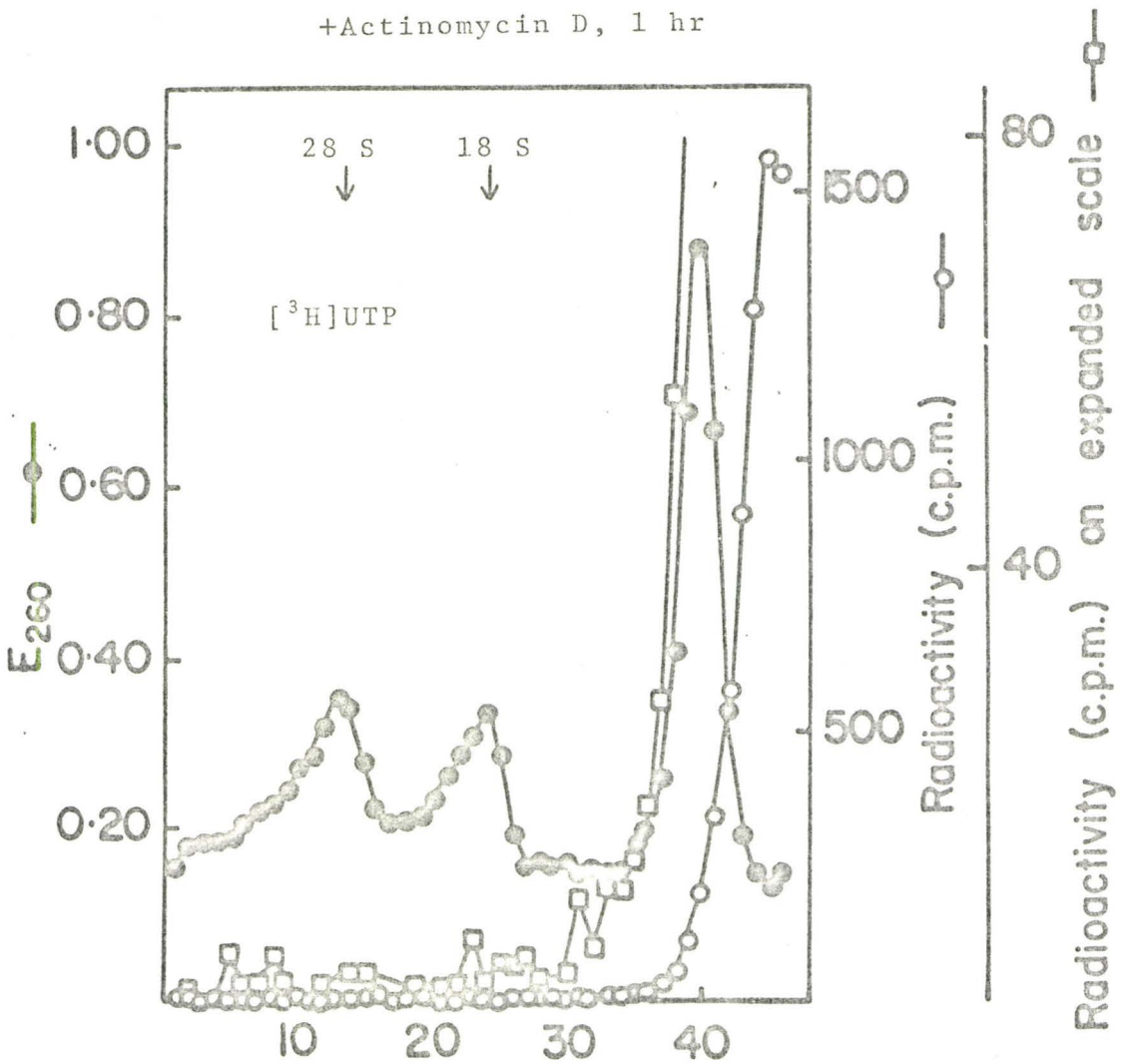

Figure 21. Sedimentation on a convex sucrose density gradient of RNA synthesized with [^{3}H]UTP for 1 hr in the hypo-osmotic medium in the presence of actinomycin D. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP for 1 hr in the hypo-osmotic medium in the presence of actinomycin D (40 µg/ml). Legend as in Figure 17. •, Cytoplasmic ribosomal RNA and E. coli tRNA; o, mitochondrial RNA; □, mitochondrial RNA on an expanded scale.

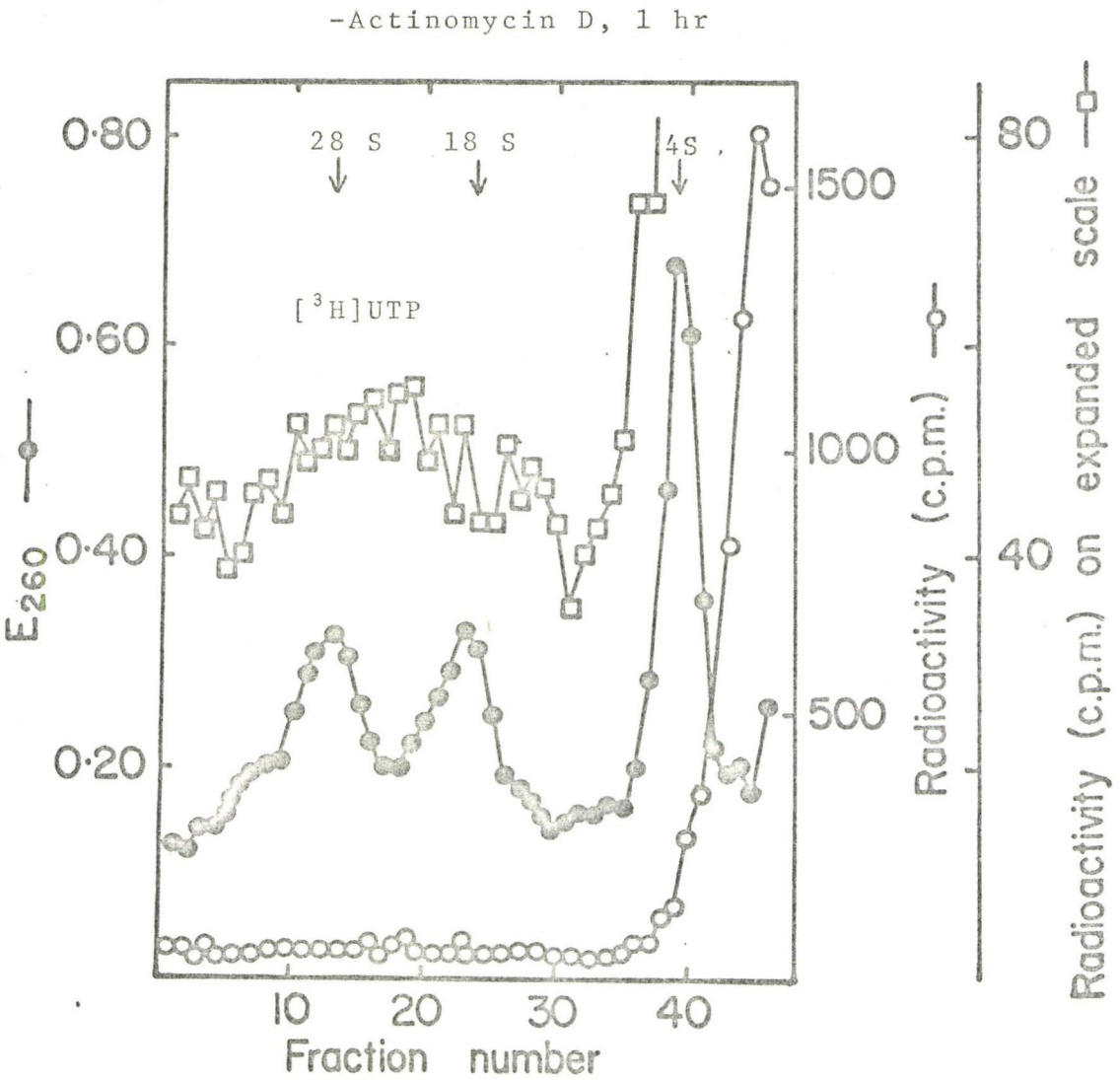

Figure 22. Sedimentation on a convex sucrose density gradient of RNA synthesized with [^{3}H]UTP for 1 hr in the iso-osmotic medium. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP for 1 hr in the iso-osmotic medium. Legend is as in Figure 17. •, Cytoplasmic ribosomal RNA and *E. coli* tRNA; o, mitochondrial RNA; □, mitochondrial RNA on an expanded scale.

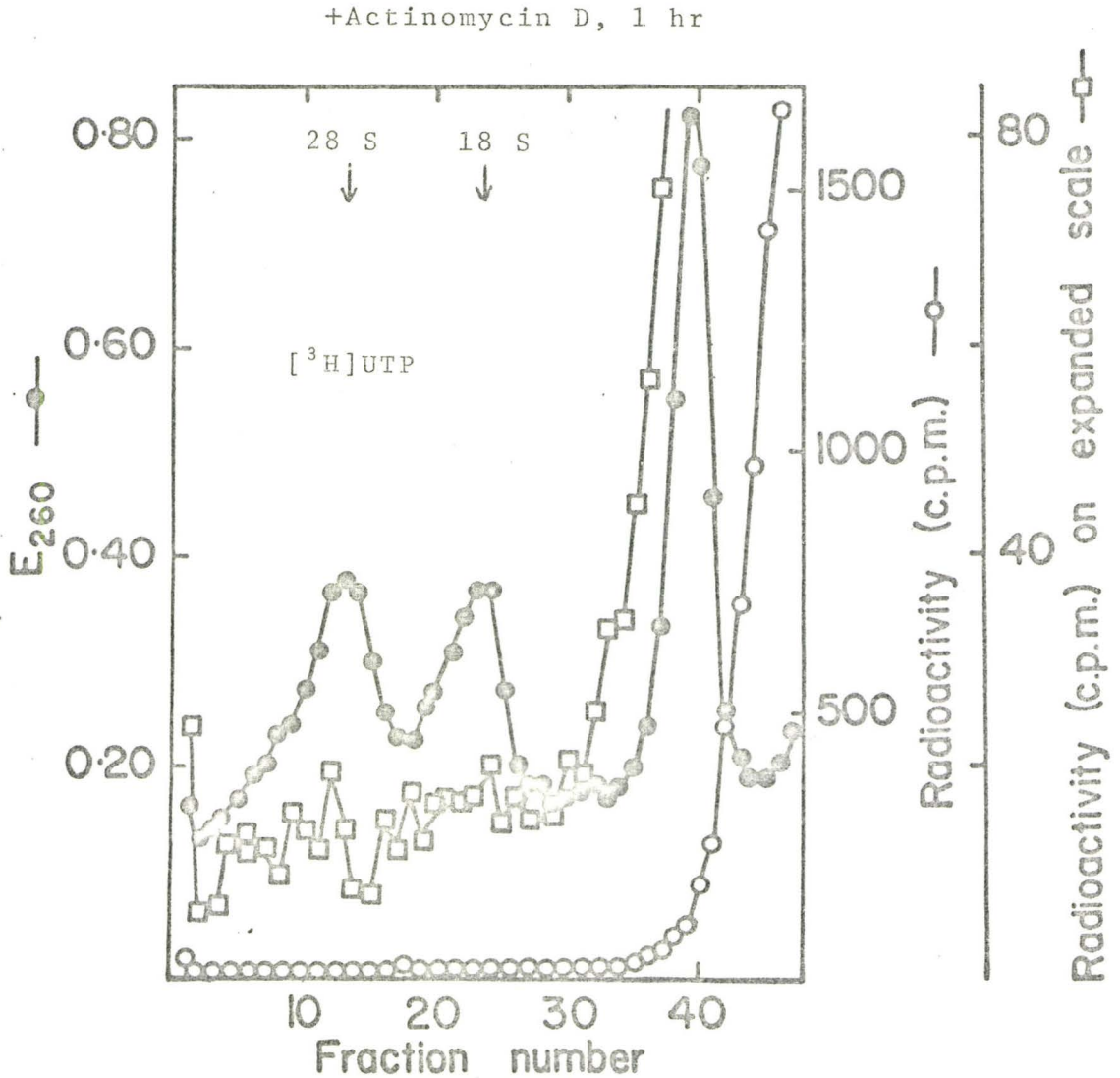

Figure 23. Sedimentation on a convex sucrose density gradient of RNA synthesized with [^{3}H]UTP for 1 hr in the iso-osmotic medium in the presence of actinomycin D. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP for 1 hr in the iso-osmotic medium in the presence of actinomycin D (40 µg/ml). Legend is as in Figure 17. •, Cytoplasmic ribosomal RNA and E. coli tRNA; o, mitochondrial RNA; □, mitochondrial RNA on an expanded scale.

Table 23

Solubility of RNA Sample in 5 %

Trichloroacetic Acid

A sample of RNA used for the sucrose density gradient analysis (Fig. 22 and 23) was precipitated in ice-cold 5 % trichloroacetic acid, washed two times with ice-cold 5 % trichloroacetic acid, and counted as described in Methods.

Actinomycin D	Original counts (cpm)	Trichloroacetic acid precipitable counts (cpm)	Trichloroacetic acid soluble* counts (cpm)
-	18480	1266	17114
+	12800	368	12432

* trichloroacetic acid soluble counts were obtained by substracting the trichloroacetic acid precipitable counts from original counts.

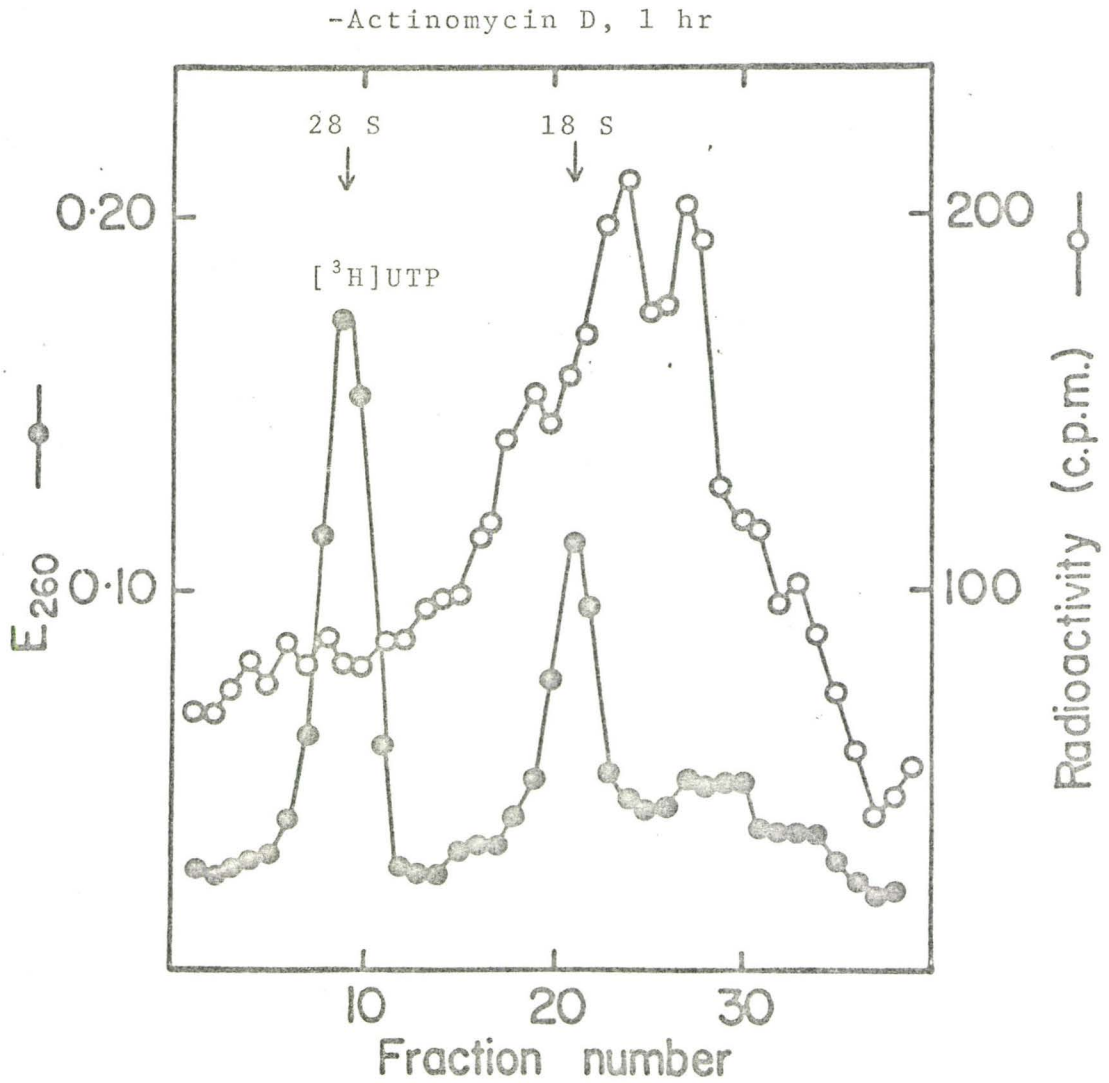

Figure 24. Sedimentation on a linear sucrose density gradient of RNA synthesized with [^{3}H]UTP for 1 hr in the hypo-osmotic medium and extracted by the cold phenol-SDS method I. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP for 1 hr in the hypo-osmotic medium. RNA was extracted by the cold phenol-SDS method I. Optical density at 260 nm was read for marker cytoplasmic ribosomal RNA (28 and 18 S) and the radioactivity counted as described in the Methods section. •, Cytoplasmic ribosomal RNA; o, mitochondrial RNA.

sucrose-EDTA were incubated with [^{3}H]UTP for 1 hr in the hypo-osmotic medium in the presence and absence of ethidium bromide. RNA was extracted by the cold phenol-SDS method I. Two major peaks with mobilities of "20.4 S" and "11.4 S" were observed (Fig. 25). In addition there was a small peak at "28 S" and a considerable amount of heterodisperse RNA. The synthesis of the "20.4 S" and "11.4 S" and heterodisperse RNA was inhibited by ethidium bromide (0.33 µg/ml) in the incubation medium. The synthesis of the "28 S" RNA was not inhibited by ethidium bromide.

2. Incorporation of [^{3}H]CTP

Mitochondria isolated in sucrose-EDTA were incubated with [^{3}H]CTP for 20 min in the hypo-osmotic medium in the presence and absence of actinomycin D. RNA was extracted by the cold phenol-SDS method II and analyzed by convex sucrose density gradient centrifugation.

The RNA sedimentation profile showed a peak sedimenting the same or slightly slower than tRNA from *E. coli* (Fig. 26). No significant synthesis of high molecular weight RNA was apparent. Actinomycin D did not change the pattern of the radioactive RNA (Fig. 27).

An aliquot of the sample used for sucrose gradient was subjected to alkaline hydrolysis and paper chromatography. The results of hydrolysis demonstrated that most of the radioactive counts represented RNA (Table 24).

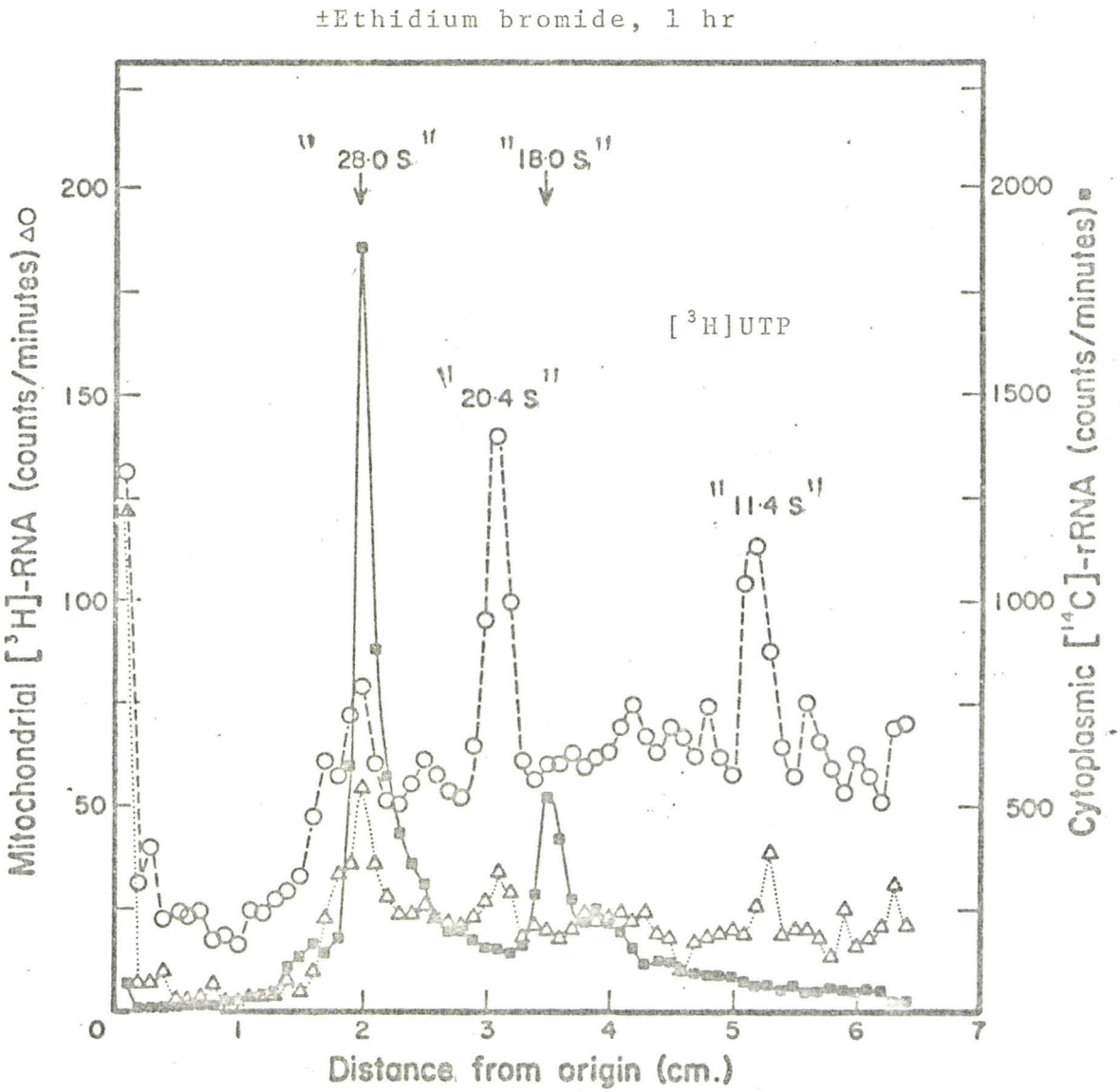

Figure 25. Electrophoretic pattern on an agarose-polyacrylamide gel of RNA synthesized with [^{3}H]UTP for 1 hr. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]UTP in the hypo-osmotic medium for 1 hr. ■ , Cytoplasmic ribosomal RNA; o, RNA synthesized by isolated mitochondria; Δ , RNA synthesized by isolated mitochondria in the presence of ethidium bromide (0.33 µg/ml).

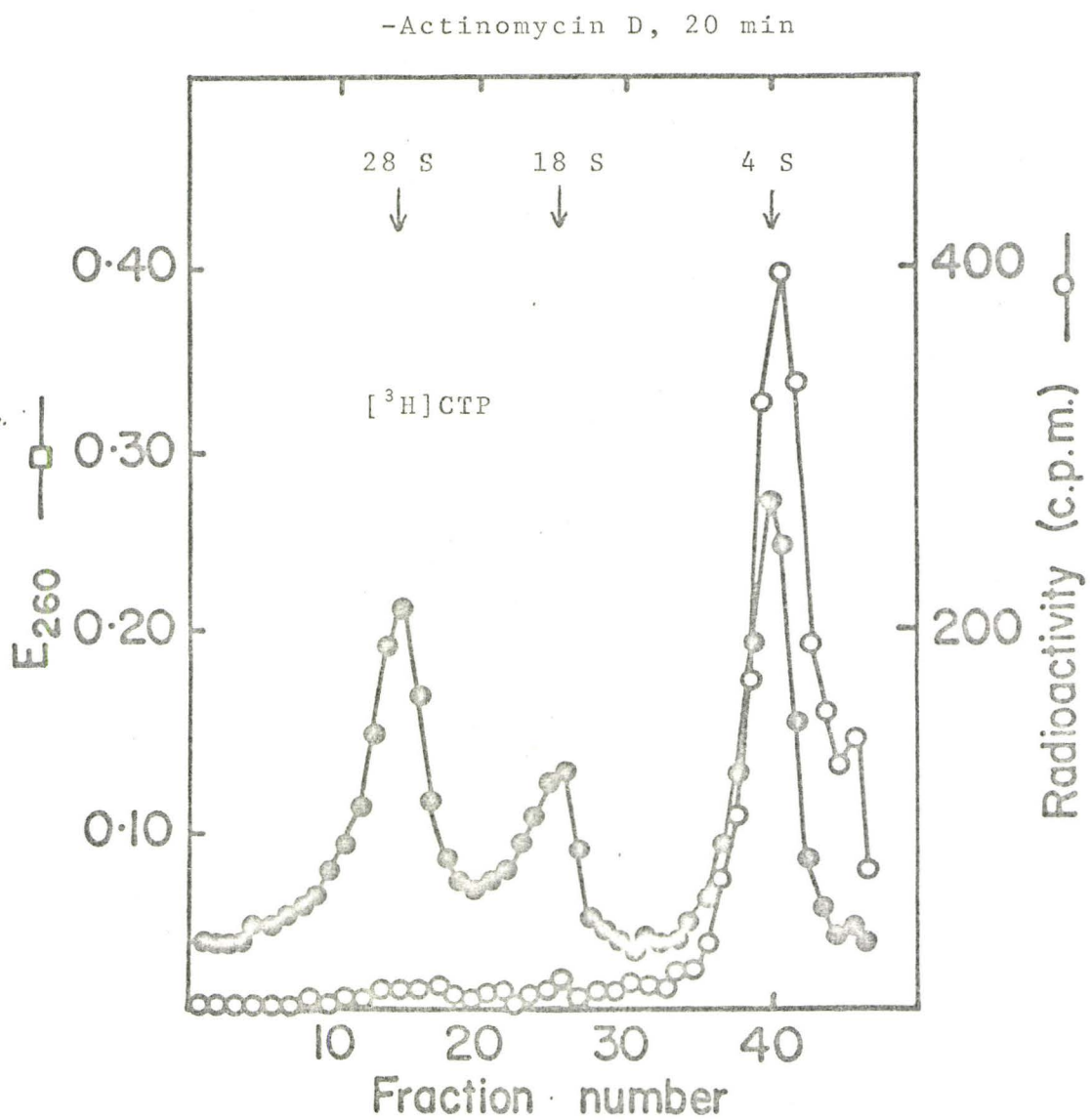

Figure 26. Sedimentation on a convex sucrose density gradient of RNA synthesized with [^{3}H]CTP for 20 min. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]CTP for 20 min in the hypo-osmotic medium. Legend is as in Figure 17. •, Cytoplasmic ribosomal RNA and E. coli tRNA; o, mitochondrial RNA.

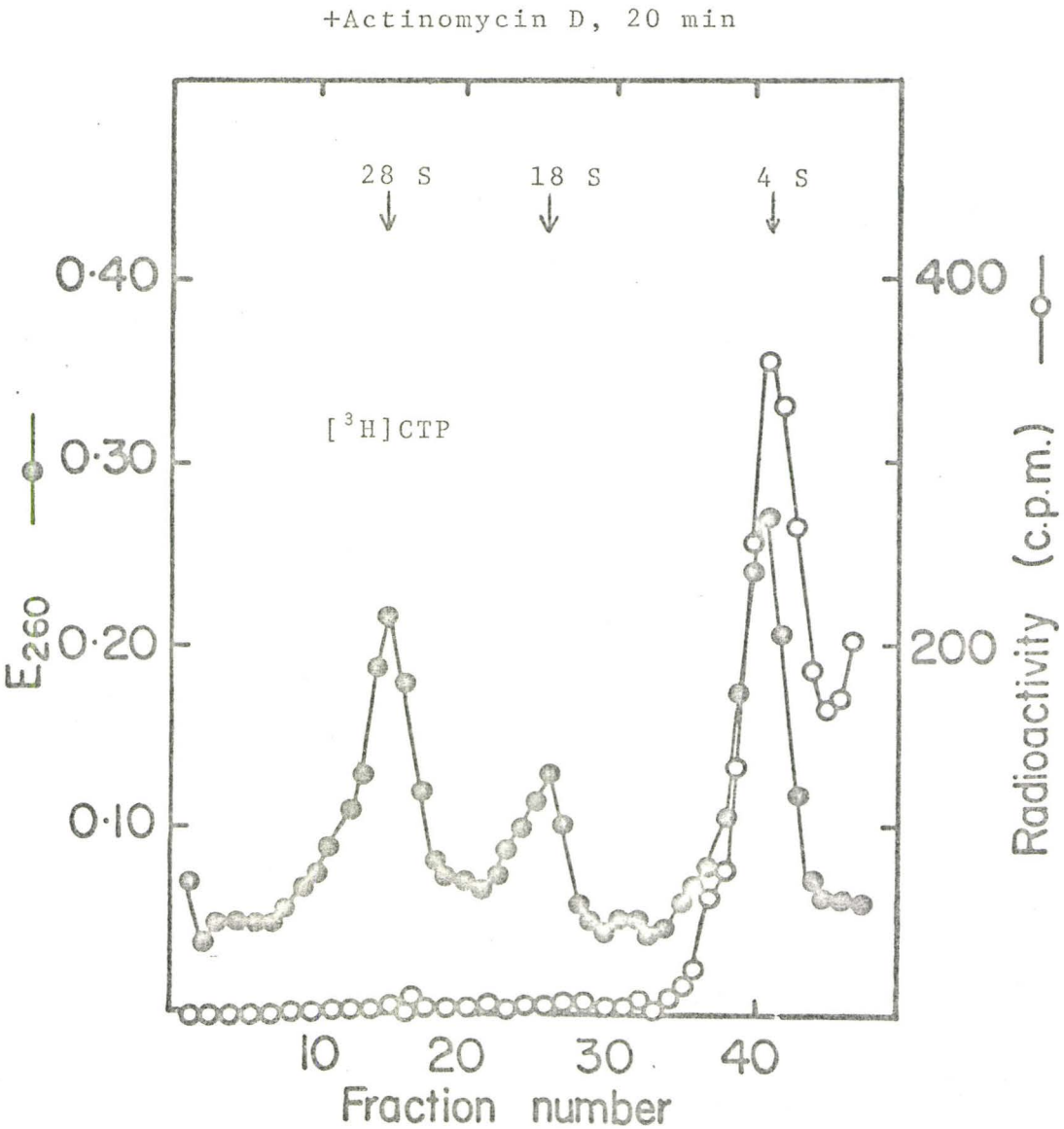

Figure 27. Sedimentation on a convex sucrose density gradient of RNA synthesized with [^{3}H]CTP for 20 min in the presence of actinomycin D. Mitochondria were isolated in sucrose-EDTA and incubated with [^{3}H]CTP for 20 min in the hypo-osmotic medium in the presence of actinomycin D (40 µg/ml). Legend is as in Figure 17. •, Cytoplasmic ribosomal RNA and E. coli tRNA; o, mitochondrial RNA.

Table 24

Alkali Hydrolysis of RNA Sample from [^{3}H]CTP Incorporation

A sample of RNA from the incubation with [^{3}H]CTP used for the sucrose density gradient analysis (Fig. 25 and 26) was hydrolyzed with alkali, chromatographed on paper and counted as described in the Methods.

Actinomycin D	Total	2'(3')CMP	Cytidine	Unidentified
		(percent of total control)		
-	100	46	33	21
+	79	28	29	22

IV DISCUSSION

The results in the preceding chapter described the synthesis of RNA by isolated mitochondria. The discussion will consider the following points

(i) Measurement of RNA synthesis: a reliable technique to demonstrate that [^{3}H]nucleotides are incorporated into RNA is developed.

(ii) RNA synthesis by isolated mitochondria: the capacity of mitochondria to synthesize RNA is differentiated from RNA synthesis by nuclei, bacteria and the microsomal fraction.

(iii) Site of RNA synthesis in mitochondria: consideration of the effect of osmolarity, nucleotides, RNase, DNase and atractyloside indicates that the site of RNA synthesis is inside the inner mitochondrial membrane.

(iv) DNA-dependent and DNA-independent RNA synthesis: the effects of actinomycin D, acriflavin and ethidium bromide on [^{3}H]nucleotide incorporation demonstrate both a DNA-dependent and DNA-independent synthesis of RNA.

(v) Nature of RNA synthesized and general discussion: the nature of RNA synthesized is discussed in relation to the mitochondrial DNA-coding function.

Measurement of RNA Synthesis

The synthesis of RNA by isolated mitochondria can be studied by the incorporation of radioactively-labelled nucleotides into RNA. While the simplest method for measuring RNA synthesis has been to determine acid-precipitable radioactivity (Wintersberger, 1964; Luck and Reich, 1964; Neubert and Helge, 1965; Neubert, Helge and Merker, 1965; Saccone, Gadaleta and Quagliariello, 1967; Saccone, Gadaleta and Gimigliano, 1968; Kroon, Saccone and Botman, 1967), for this to be a valid index, it must be shown that the radioactive nucleotides are incorporated specifically into the RNA of the acid-insoluble material. The incorporation of [^{3}H]nucleotides into RNA was clearly demonstrated by recovery of the [^{3}H]2'(3') nucleotides and [^{3}H]nucleosides after alkaline hydrolysis and paper chromatography. In addition to [^{3}H]2'(3') nucleotides and [^{3}H]nucleosides, unidentified acid-precipitable material was recovered, indicating that [^{3}H]nucleotides were incorporated not only into RNA but also into other acid-insoluble compounds. The different stabilities of the [^{3}H]UTP-labelled and [^{3}H]ATP-labelled acid-insoluble material in hot acid or in alkali showed that a large portion of [^{3}H]ATP was incorporated into an acid-insoluble material that was not RNA. Treatment of the acid-insoluble material

with lipophilic solvents or hot phenol-SDS extraction before acid precipitation removed a large proportion of the non-RNA radioactivity. The recovery of 90 % of the remaining radioactivity as [^{3}H]2'(3') UMP and [^{3}H]uridine demonstrated that acid precipitation following hot phenol-SDS extraction was sufficiently reliable to permit a study of [^{3}H]UTP incorporation into RNA without the need for laborious alkaline hydrolysis and paper chromatography. The major product of [^{3}H]ATP incorporation was an acid-stable, alkali-labile compound which on alkaline hydrolysis gave rise to a product that was not ATP, ADP, 2'(3') AMP or 5' AMP. It was not further characterized

Saccone et al. (1967; 1968) studied RNA synthesis by the incorporation of radioactive nucleotides into an acid-insoluble form and found a much greater incorporation of [^{14}C]ATP than [^{14}C]UTP. In light of the above results it would be difficult to conclude that these results accurately reflect RNA synthesis.

Synthesis of RNA by Isolated Mitochondria

Synthesis of RNA by isolated mitochondria has been investigated by various workers and reported to level off within 10-20 min, but the reason for this was not studied (Neubert et al., 1966; Saccone et al., 1968 and 1969). This is an exceedingly short time when

compared to at least 2 hr for DNA synthesis (Parsons and Simpson, 1967) and 1 hr for protein synthesis (Roodyn et al., 1961) with mitochondria isolated in sucrose-EDTA. In the present work, RNA synthesis by mitochondria isolated in sucrose-EDTA or sucrose-TES continued for at least 1 hr using incubation media modified either from that of Roodyn et al. (1961) or from that of Parsons and Simpson (1967). These media differ in containing either nucleoside mono- or tri-phosphates. Since changes in the incubation media had small effects on incorporation, it is suggested that the better incorporation observed here resulted from preparing mitochondria in buffered iso-osmotic sucrose. Previous workers had used unbuffered, iso-osmotic isolation media. The improved incorporation more readily allowed characterization of the incorporation process and of the nature of the RNA product.

It was demonstrated by electron microscopy that the mitochondrial fraction which incorporated [^{3}H]nucleoside triphosphates into RNA consisted principally of mitochondria. Alone, this fact does not prove that mitochondria per se synthesized the RNA. Small amounts of nuclei, bacteria or the microsomal fraction, which are active in RNA synthesis, could contaminate the mitochondrial fraction and be the source of incorporation. In order to demonstrate that it was the mitochondria that synthesized RNA, these possibilities had to be

eliminated. Several lines of evidence demonstrate that nuclei were not responsible. Electron microscopy showed that mitochondria were the main component in the mitochondrial fraction and that no nuclei or nuclear fragments were apparent. The mitochondrial fraction isolated in sucrose-EDTA or sucrose-TES contained 0.47 and 0.43 µg DNA/mg of mitochondrial protein respectively, indicating little nuclear contamination, since rat liver mitochondria from which DNA free from nuclear DNA was obtained, contain 0.2 to 0.5 µg DNA/mg of protein (Leffler et al., 1970).

The characteristics of RNA synthesis by the mitochondrial fraction and nuclei were compared. RNA synthesis by the mitochondrial fraction was insensitive to DNase, sensitive to ethidium bromide and atractyloside, and was not inhibited by the absence of the other three ribonucleoside triphosphates. In contrast, RNA synthesis by isolated nuclei was sensitive to DNase, insensitive to ethidium bromide and atractyloside, and was inhibited by the absence of the other three ribonucleoside triphosphates. It is, therefore, concluded that the synthesis of RNA observed with the mitochondrial fraction was not due to contaminating nuclei.

Another possible source for RNA synthesis in the mitochondrial fraction is bacterial contamination.

To prevent bacterial contamination mitochondria were isolated with sterilized media and equipment. Bacterial counts were between 0 and 30/0.1 ml of the incubation mixture after a 1 hr incubation. An estimate of incorporation of [^{3}H]UTP by 30 bacteria/0.1 ml might be approximated as follows: E. coli contains 50 µg RNA/10^9 cells (1 mg wet weight). The UMP content of the RNA is 3.8×10^{-17} moles/cell, assuming that one quarter of the nucleotides are UMP. If [^{3}H]UTP with a specific activity of 2 Ci/mmole is used and if the uridylate is incorporated into bacterial RNA only, 0.05 cpm/cell would be obtained at a counting efficiency of 30 %. Thirty bacteria would give 1.5 cpm. Since usually more than 200 cpm/0.1 ml were obtained after a 1 hr incubation of mitochondria, bacterial contamination is in all probability not significant. This conclusion is supported by the inhibition of [^{3}H]UTP incorporation by actinomycin D whereas this antibiotic does not inhibit RNA synthesis by intact E. coli (Hurwitz, Furth, Malamy and Alexander, 1962). Rifampicin did not inhibit but does so in many bacteria (Calvori, Frontali, Leoni and Teece, 1965; Hartmann et al., 1967). Bacteria were also ruled out as a source of RNA synthesis for the following reason: bacteria can utilize external nucleosides but not external nucleoside triphosphates for RNA synthesis. Nucleotides would be utilized only after dephosphorylation (Price, Darmstadt, Hinds and Zamenhof, 1967). If the

incorporation were due to bacteria, excess unlabelled uridine should reduce [^{3}H]UTP incorporation. It did not, indicating that bacteria were not the source of RNA synthesis.

Since Wilkie and Smellie (1967) reported that the microsomal fraction incorporated ribonucleotides into a polynucleotide material, contamination with the microsomal fraction could be another source for RNA synthesis. Electron microscopic pictures showed some contamination of mitochondria with endoplasmic reticulum. The RNA content in the mitochondrial fractions isolated in sucrose-EDTA and sucrose-TES was 11 µg and 8.9 µg/mg of mitochondrial protein respectively, compared to 3.0-14.0 µg RNA/mg of protein obtained by other workers for rat liver mitochondria (Bartoov, 1971) and did not clearly indicate whether or not the isolated mitochondria were contaminated with some microsomal fraction which contains 200 µg RNA/mg of protein (Hess and Logg, 1963). Wilkie and Smellie (1967) reported that [^{3}H]UTP incorporation in the microsomal fraction was insensitive to actinomycin D and that the product degraded within 30 min during the incubation. In the present work, [^{3}H]UTP incorporation was sensitive to actinomycin D and continued for 1 hr. RNA synthesis by isolated mitochondria was not dependent on the presence of the other three nucleotides. In contrast [^{3}H]UTP incorporation in the microsomal fraction was increased by the addition of

the three other nucleotide triphosphates. For these reasons it seems unlikely that contaminating microsomal fraction was responsible for RNA synthesis in the mitochondrial fraction. By elimination, therefore, mitochondria are implicated as being responsible for the observed RNA synthesis and the site within mitochondria at which synthesis may occur will be now considered.

Site of RNA Synthesis in Mitochondria

Mitochondria may be structurally divided into outer membrane, inner membrane, the space between the two membranes and the space inside the inner membrane (matrix) (Stoner and Sirak, 1969). The site of RNA synthesis within mitochondria was investigated by examining the effect of osmolarity, nucleotides, RNase, DNase and atractyloside.

The osmolarity of the incubation medium affected the relative incorporation of the nucleotides as shown below (calculated from Table 21).

Mitochondria Precursor	Sucrose-TES	Sucrose-EDTA
	fraction of 2'(3') nucleotide recovered iso-osmotic medium/hypo-osmotic medium	
[^{3}H]UTP	0.62	1.06
[^{3}H]ATP	1.59	1.88
[^{3}H]CTP	0.12	0.38
[^{3}H]GTP	0.15	0.72

With mitochondria isolated in sucrose-TES, there was less incorporation of [^{3}H]UTP, [^{3}H]CTP and [^{3}H]GTP and an increased incorporation of [^{3}H]ATP in the iso-osmotic medium compared to the hypo-osmotic medium. Variation in incorporation with osmolarity probably reflects a control of nucleotide permeation by the inner mitochondrial membrane but not by the outer mitochondrial membrane since the outer membrane seems permeable to nucleotides (Winkler and Lehninger, 1968). Thus, the high incorporation of [^{3}H]ATP in iso-osmotic medium may be attributed to an adenine nucleotide-specific translocating enzyme located in the inner membrane (Winkler, Bygrave and Lehninger, 1968; Winkler and Lehninger, 1968; Vignais and Vignais, 1970). The lower incorporation of [^{3}H]UTP, [^{3}H]CTP and [^{3}H]GTP in the iso-osmotic medium probably reflects the lack of an active translocating enzyme system for these nucleotides across the inner mitochondrial membrane. In the hypo-osmotic medium the mitochondria swell (Stoner and Sirak, 1969) changing their permeability properties and thus possibly allowing more readily the diffusion of [^{3}H]UTP, [^{3}H]CTP and [^{3}H]GTP into mitochondria. As discussed below, mitochondria contain about 100 times more endogenous adenine nucleotides than those of the other three base types. [^{3}H]ATP probably does not diffuse into mitochondria but is taken up by the specific transport process. Duée and Vignais (1969b) found that

external radioactive adenine nucleotides enter into mitochondria by the specific exchange process and that, in addition, there is a slow rate of diffusion out of mitochondria of adenine nucleotides. The effect of osmolarity with mitochondria isolated in sucrose-EDTA was similar but less marked than with mitochondria isolated in sucrose-TES, perhaps indicating that the former mitochondria were more permeable to nucleotides. Indirectly, these results indicate that RNA synthesis occurs inside the inner membrane. Kalf and Faust (1969) demonstrated that an isolated inner membrane preparation synthesizes RNA.

In our system, RNA synthesis by isolated mitochondria did not require the simultaneous presence of four ribonucleotides although Neubert et al. (1965) showed such a requirement with swollen mitochondria. This may be explained by the fact that intact isolated mitochondria contain sufficient endogenous ribonucleotides; those isolated in sucrose-EDTA contained 12.3×10^{-9} moles ATP and 4.0×10^{-9} moles ADP/mg of mitochondrial protein and those isolated in sucrose-TES contained 11.4×10^{-9} moles ATP and 4.2×10^{-9} moles ADP/mg of mitochondrial protein. Heldt and Klingenberg (1965) found in isolated rat liver mitochondria 11.3×10^{-9} moles ATP, 3.13×10^{-9} moles ADP, 1.54×10^{-9} moles AMP, 0.23×10^{-9} moles GTP + GDP + GMP, 0.13×10^{-9} moles UTP + UDP + UMP and 0.09×10^{-9} moles CTP + CDP + CMP per

mg of mitochondrial protein.

Schnaitman, Erwin and Greenawalt (1967) reported that the site of the adenine nucleotide pool was inside the inner membrane of mitochondria. The lack of a requirement for the simultaneous presence of four ribonucleotides for RNA synthesis suggests that RNA synthesis occurs inside the inner membrane proximal to the site of the nucleotide pools.

The effect of RNase and DNase on incorporation lends further support to the hypothesis that the site of RNA synthesis is inside the inner membrane. It has been suggested that the mitochondrial membrane is impermeable to RNase, because protein synthesis by isolated mitochondria was not inhibited by RNase (Roodyn et al., 1961). In the present work, RNA synthesis in the last 40-50 min of the incubation was insensitive to RNase, demonstrating that synthesis takes place at a site inaccessible to the enzyme. RNase added after the incubation reduced the amount of the [^{3}H]UTP incorporated. The sensitivity to RNase in the first 10-20 min is possibly explained by the action of RNase after the incubation. Alternatively, the sensitivity to RNase could be due to the presence of a small amount of swollen mitochondria. Neubert et al. (1965) observed sensitivity to RNase with swollen mitochondria.

RNA synthesis by isolated mitochondria is DNA-dependent as discussed later but was not inhibited by DNase. The mitochondrial membrane is probably impermeable to DNase, preventing it from reaching the site of mitochondrial DNA which is inside the inner membrane (Nass and Nass, 1963; Nass, Nass and Afjelius, 1965). Insensitivity to DNase supports the contention that RNA synthesis takes place inside the inner membrane. DNase did not inhibit RNA synthesis (Neubert et al., 1965) and DNA synthesis (Parsons and Simpson, 1967) by isolated mitochondria.

Atractyloside specifically inhibits the translocation of ATP and ADP into and out of mitochondria across the inner membrane (Bruni et al., 1965; Winkler et al., 1968; Winkler and Lehninger, 1968; Vignais and Vignais, 1970). Inhibition of [^{3}H]ATP incorporation into RNA by atractyloside would demonstrate the site of the synthesis. The incorporation of [^{3}H]ATP into RNA was inhibited by atractyloside. RNA synthesis by isolated mitochondria, therefore, must occur inside the atractyloside barrier of mitochondria, that is, inside the inner membrane. Saccone et al. (1967) reached the same conclusion from data based on the incorporation of [^{14}C]ATP into acid-insoluble counts. However, since radioactive ATP incorporated into acid-insoluble material may not represent RNA synthesis, the result here was a more definite demonstration of the site of RNA

synthesis in mitochondria.

DNA-dependent and DNA-independent RNA Synthesis

Mitochondria contain DNA which is distinct from nuclear DNA (Introduction). The possibility that RNA synthesis by isolated mitochondria is dependent on mitochondrial DNA was explored by studying the effect of ethidium bromide, acriflavin and actinomycin D on [^{3}H]nucleotide incorporation into RNA. With mitochondria isolated in sucrose-TES 50 % inhibition of RNA synthesis was observed at 0.5 µg ethidium bromide/ml, 1 µg acriflavin/ml or 1 µg actinomycin D/ml. Ethidium bromide alters the tertiary structure of circular DNA (Bauer and Vinagrad, 1968) and thus preferentially inhibits synthesis of mitochondrial RNA (Zylber et al., 1969). Saccone et al. (1969) observed 50 % inhibition of RNA synthesis by 2 µg acriflavin/ml with swollen mitochondria. Neubert et al. (1965) found that RNA synthesis was inhibited 85 % by 12 µg actinomycin C/ml when mitochondria were swollen. Saccone et al. (1969) reported that a higher level (35 µg/ml) of actinomycin D was required to obtain 50 % inhibition with swollen mitochondria. Although differences may be a function of permeability, it is difficult to compare the reported sensitivity to actinomycin D, because of the different incubation times, isolation media and incubation media used in each study. Nonetheless, the results showed

that [^{3}H]UTP incorporation into RNA by isolated mitochondria was dependent on mitochondrial DNA.

DNA-dependent RNA synthesis was demonstrated for mitochondria isolated in sucrose-EDTA and in sucrose-TES. In the first 10 min of incubation, [^{3}H]UTP incorporation by mitochondria isolated in sucrose-EDTA was less sensitive to actinomycin D and acriflavin than was incorporation by mitochondria isolated in sucrose-TES. Although this could reflect a permeability barrier in the mitochondria isolated in sucrose-EDTA these mitochondria are more permeable to nucleotides as discussed earlier. The permeability properties of mitochondria are controlled in part by Mg^{2+} which prevents swelling of mitochondria (Vigers, 1970). Since EDTA binds Mg^{2+} and Ca^{2+} but TES does not, experiments were carried out with mitochondria isolated in sucrose-EGTA which chelates Ca^{2+} but not Mg^{2+} (Settlemir, et al., 1968). These experiments showed that the differences between mitochondria isolated in sucrose-EDTA and mitochondria isolated in sucrose-TES were not due to the chelation of Mg^{2+} by EDTA. The reason for the differences was not further examined.

All four ribonucleoside triphosphates, [^{3}H]UTP, [^{3}H]ATP, [^{3}H]CTP and [^{3}H]GTP were incorporated into RNA, but not all the nucleotide incorporation was DNA-dependent. Using the recovery of 2'(3') nucleotide as a criterion, the relative extent of incorporation was CTP > ATP = UTP >> GTP in the hypo-osmotic medium with

mitochondria isolated either in sucrose-EDTA or in sucrose-TES. Taking actinomycin D-sensitivity as measure of a DNA-dependent reaction, [^{3}H]nucleoside triphosphates incorporated in a DNA-dependent process into RNA can be calculated from Table 19 and 21.

Sucrose-TES Mitochondria	Incorporation sensitive to actinomycin D, 2'(3') nucleotide 10^{-15} moles/mg of protein	Incorporation insensitive to actinomycin D, 2'(3') nucleotide 10^{-15} moles/mg of protein
[^{3}H]UTP	115	8
[^{3}H]ATP	98	46
[^{3}H]CTP	112	126
[^{3}H]GTP	29	2.2
Sucrose-EDTA mitochondria		
[^{3}H]UTP	81	29
[^{3}H]ATP	93	48
[^{3}H]CTP	86	92
[^{3}H]GTP	22	5.5

Thus when expressed on the basis of actinomycin D-sensitivity an almost equivalent incorporation was observed for [^{3}H]UTP, [^{3}H]ATP, and [^{3}H]CTP, but [^{3}H]GTP incorporation was much less.

Other studies on the relative incorporation of nucleotides have not revealed these patterns because of the method of analysis of nucleotide incorporation. Thus Saccone et al. (1967, 1968) reported a high level of incorporation of ATP compared to UTP and GTP, following radioactivity in acid-insoluble materials. Later, Saccone et al. (1969) found an almost equivalent incorporation of all four [^{3}H]nucleoside triphosphates from experiments in which the RNA was purified.

There is some precedence and a possible explanation for the low level of [^{3}H]GTP incorporation. Neubert et al. (1965) also observed a low incorporation of [^{3}H]GTP. Parsons and Simpson (personal communciation) found that dGTP is incorporated to a much lower extent than the other deoxyribonucleoside triphosphates into DNA by isolated rat liver mitochondria and attributed this to a rapid degradation of dGTP to the nucleoside. Possibly [^{3}H]GTP is degraded by the same process.

In addition to the DNA-dependent synthesis of RNA, a DNA-independent incorporation of [^{3}H]CTP and [^{3}H]ATP into RNA was observed. A number of results indicate that the DNA-independent incorporation may be a reflection of the turnover of the CCA end of mitochondrial tRNA. While the recovery of radioactivity in [^{3}H]2'(3') nucleotide from the actinomycin D-insensitive product was low for precursors of [^{3}H]UTP, [^{3}H]ATP and [^{3}H]GTP, incorporation from [^{3}H]CTP yielded a high actinomycin

D-insensitive recovery of [^{3}H]2'(3') CMP. The recovery of [^{3}H]cytidine and [^{3}H]adenosine from [^{3}H]CTP and [^{3}H]ATP incorporation, respectively, was large compared to the recovery of [^{3}H]uridine after [^{3}H]UTP incorporation. The incorporated [^{3}H]CTP turned over, whereas the incorporated [^{3}H]UTP did not. RNA labelled with [^{3}H]CTP sedimented on a sucrose density gradient at a region comparable to tRNA from E. coli and actinomycin D did not change the profile of the labelled RNA. If one can assume that mitochondrial tRNA's have a CCA nucleotide end as do tRNA's of both prokaryotic and eukaryotic species (Madison, 1968), then these results, taken together, probably reflect the DNA-independent turnover of the CCA terminal end of mitochondrial tRNA.

There is little doubt that the DNA-independent incorporation of [^{3}H]CTP and [^{3}H]ATP takes place in mitochondria, because the osmolarity of the incubation medium affected the incorporation of [^{3}H]CTP, and the atractyloside inhibited the incorporation of [^{3}H]ATP and reduced the recovery of [^{3}H]adenosine. The enzymes responsible for this in mitochondria are not known. An enzyme has been observed in rat liver (Canellakis and Hebert, 1969; Daniel and Littauer, 1963 and 1965) and in E. coli (Preiss, Dieckmann and Berg, 1961; Furth, Hurwitz, Krug and Alexander, 1961) which catalyzes reversibly the incorporation of CMP and AMP from the CTP and ATP respectively into the terminal trinucleotide

sequence of the tRNA, yielding the tRNA---pCpCpA.

RNA synthesis by isolated mitochondria was studied by using antibiotics such as actinomycin D, acriflavin and ethidium bromide to determine the involvement of mitochondrial DNA in RNA synthesis. The effect of antibiotics such as rifampicin, rifamycin SV and streptovarcin was investigated to see whether mitochondria have properties common with bacteria in RNA synthesis. These antibiotics have been reported to inhibit bacterial RNA synthesis at the initiation step (Wehrli, Knusel, Schmid and Staehelin, 1968; Sippel and Hartmann, 1968; Mizuno et al., 1968a; DiMaruno et al., 1969), but RNA synthesis in mammalian nuclei is resistant (Hartmann et al. 1967; Wehrli, Nuesch, Knusel and Stachelin, 1968; Mizuno et al., 1968b). In this present study no sensitivity of mitochondrial RNA synthesis to any of these antibiotics was observed. The results of other workers are conflicting. Some have observed no inhibition with mitochondria from several species (Dubin and Montenecourt, 1970; Herzfeld, 1970; Wintersberger, 1970; Wintersberger and Wintersberger, 1970a; Yang and Criddle, 1970; Tsai, Michaelis and Criddle, 1971). Others have observed an inhibition (Shmerling, 1969; Gadaleta, Greco and Saccone, 1970; Gamble and McCluer, 1970; Küntzel and Schafer, 1971). It is quite possible that those preparations in which inhibition was demonstrated may have been contaminated with bacteria. Lack of inhibition of mitochondrial RNA synthesis by

any of these antibiotics may be due to impermeability of mitochondria to the antibiotics. This does not seem very likely here, since mitochondria were permeable to [^{3}H]UTP and actinomycin D under identical conditions, indicating that they would probably be permeable to all these antibiotics. Wintersberger (1970) and Tsai et al. (1971) found no inhibition with an RNA polymerase solubilized from yeast mitochondria. In contrast, Gadaleta et al. (1970) found inhibition with swollen rat liver mitochondria or with a solubilized preparation of RNA polymerase from rat liver mitochondria. Furthermore, studies in vivo on mitochondrial RNA synthesis in spinner cultures of hamster cells (Dubin and Montenecourt, 1970) did not reveal any inhibition by rifampicin. Clearly, further studies are needed to resolve these discrepancies Since rifampicin does not inhibit the elongation but specifically the initiation of RNA chains, its ineffectiveness in isolated mitochondria could be explained by the lack of initiation of new RNA chains. It is not known to what extent initiation takes place in isolated mitochondria. At present, from the results here, sensitivity to these antibiotics is not a feature common to bacteria and mitochondria.

Nature of RNA Synthesized and General Discussion

The logical question arising from the demonstration that isolated mitochondria synthesize RNA is what types

of RNA are produced. Characterization of the RNA after a 1 hr incubation by sucrose density gradient centrifugation revealed that high molecular weight RNA was synthesized. A definite peak at 16 S with a possible peak or a shoulder at 13 S was observed on sucrose gradients after hot phenol-SDS and cold phenol-SDS (method II) extraction. Definitive peaks at 16 and 13 S were clearly shown with cold phenol-SDS (method I). These S values coincided with those of ribosomal RNA in mitochondria of L cells (Bartoov et al., 1970) and of rat liver (Bartoov, 1971). The RNA profile suggested that isolated rat liver mitochondria synthesize mitochondrial ribosomal RNA. That ribosomal RNA was synthesized was confirmed by agarose-polyacrylamide gel electrophoresis. Two major peaks of mobility of "20.4 S" and "11.4 S" were observed. These values compare with the electrophoretic mobilities of ribosomal RNA reported for mitochondria from rat liver ("20.9 S" and "12.4 S", Bartoov, et al., 1970), L cells ("20.8 S" and "12.4 S", Bartoov et al., 1970). HeLa cells("21 S" and "12 S", Vesco and Penman, 1969 and Zylber et al., 1969), KB cells("20.4 S" and "11.2 S", Bartoov, 1971) and hepatoma cells("20.8 S" and "12.1 S", Bartoov, 1971).

In addition to the synthesis of mitochondrial ribosomal RNA, the synthesis of high molecular weight RNA with a heterodisperse pattern was observed on sucrose density gradients and on the polyacrylamide gel. The

synthesis of low molecular weight RNA was not demonstrated. The large amount of radioactivity on top of the sucrose gradients after cold phenol-SDS (method II) extraction was UTP, UDP and UMP but not RNA, as indicated by its acid solubility and paper chromatography. A similar conclusion was reached by Freeman (1971, personal communication) for the hot phenol-SDS extraction. The synthesis of a homologous species of mitochondrial RNA by isolated mitochondria has not been reported. The products of RNA synthesized by isolated mitochondria have been described as heterodisperse sedimenting from 4 to 10 S (Neubert et al., 1968) or from 8 to 14 S (Saccone et al, 1969).

Earlier in the discussion, it was concluded that two types of RNA synthesis occur, one DNA-dependent and another DNA-independent. Thus, ribosomal and heterodisperse RNA synthesis on mitochondrial DNA had to be demonstrated. This was accomplished by using actinomycin D or ethidium bromide which preferentially inhibits synthesis of mitochondrial RNA reacting with circular mitochondrial DNA (Zyler et al., 1969). The synthesis of "20.4 S" and "11.4 S" RNA was inhibited by ethidium bromide. It is, therefore, concluded that mitochondrial DNA codes for mitochondrial ribosomal RNA. The synthesis of heterodisperse RNA on sucrose gradient or on polyacrylamide gel was sensitive to actinomycin D or ethidium bromide, indicating that the

heterodisperse RNA was coded for by mitochondrial DNA.

The synthesis of high molecular weight RNA was observed during incubation. The recovery of 2'(3') UMP increased with time, but the amount of uridine recovered remained constant, suggesting that the RNA chains became longer as the time of incubation increased. The average chain length of RNA synthesized can be calculated from the ratio of nucleotide to nucleoside obtained from Figure 14 and 15.

Isolation Medium	sucrose-EDTA				sucrose-TES			
Incubation Medium	hypo-osmotic		iso-osmotic		hypo-osmotic		iso-osmotic	
Time (min)	20	60	20	60	20	60	20	60
Chain length	21	99	11	12	42	127	22	85

The maximum average chain length synthesized is much shorter than indicated by the sedimentation analysis. The latter method showed that most of the RNA sedimented faster than 10 S, that is, it had a chain length greater than 500 to 1,000 nucleotides. The two results are consistent if isolated mitochondria complete chains initiated before the incubation of the mitochondria. Alternatively, the average chain length could be heavily weighted by the synthesis of a large number of short chains or by terminal addition.

The amount of RNA synthesized for 1 hr can be calculated by using V_{max} = 142 x 10^{-15} moles/mg of protein/min from incorporation of [^{3}H]UTP and a value of 3.0 µg RNA/mg of protein. Assuming that each nucleoside triphosphate is incorporated into RNA in an amount equal to UTP and that the average molecular weight of nucleoside monophosphate is 330, 1.9 x 10^{-3} mg of nucleotides are incorporated per mg of RNA/hr. This means that the amount of RNA synthesized for 1 hr at the maximum rate corresponds to 0.37 % of total mitochondrial RNA.

The size of mitochondrial DNA coding for ribosomal RNA can be calculated. Assuming that only a single strand of mitochondrial DNA is transcribed (Borst and Aaij, 1969) and that the molecular weight of mitochondrial ribosomal RNA is 1.05 x 10^6 daltons (Bartoov, 1971), then 20 % of the mitochondrial DNA with a molecular weight of 10^7 daltons would code for ribosomal RNA. In agreement with this calculation, Aloni and Attardi (1971a) observed that mitochondrial DNA from HeLa cells hybridized 22 % with mitochondrial ribosomal RNA, indicating that there is one gene for each of the ribosomal RNAs. In the present studies, the synthesis of small molecular weight RNA such as tRNA coded for by mitochondrial DNA was not examined. Mitochondria contain tRNA's which are different from their cytoplasmic counterparts (Introduction). Each

species of tRNA contains about 75 base pairs (Rabinowitz and Swift, 1970). If one tRNA cistron for each amino acid were represented in mitochondrial DNA, 1,500 base pairs or 10 % of DNA with a molecular weight of 10^7 daltons would be used to specify tRNA's. As calculated before, 20 % of mitochondrial DNA codes for ribosomal RNA. What does the remaining 70 % of mitochondrial DNA code for? Although it is not known whether the heterodisperse RNA indicates the presence of mitochondrial messenger RNA, the remaining 70 % of mitochondrial DNA could possibly be used for transcription into mitochondrial messenger RNA with a total molecular weight of 3.5×10^6 daltons, corresponding to 3,500 amino acids or proteins with a total molecular weight of 4.0×10^5 daltons. It is theoretically possible that mitochondrial DNA codes for messenger RNA which may be translated into inner membrane protein, since mitochondria synthesize inner mitochondrial membrane protein (Neupert et al., 1967; Neupert and Ludwig, 1971; Roodyn, 1962).

Mitochondrial RNA was reported to be transcribed in vivo from the heavy strand of mitochondrial DNA (Borst and Aaij, 1969). Recently, however, Aaij, Saccone, Borst and Gadaleta (1970) reported that up to 12-15 % of RNA synthesized by isolated mitochondria was complementary to the light strand of mitochondrial DNA. Aloni and Attardi (1971b) demonstrated that both strands of mitochondrial DNA in vivo were transcribed but that

there was rapid degradation of RNA complementary to the light strand of mitochondrial DNA. Ribosomal RNA synthesized by isolated mitochondria is probably transcribed from the heavy strand of mitochondrial DNA, since Aloni and Attardi (1971a) reported that mitochondrial ribosomal RNA in HeLa cells hybridizes with the heavy strand of mitochondrial DNA.

Since mitochondrial DNA codes for ribosomal RNA and possibly for tRNA's and inner mitochondrial membrane proteins, the formation of complete mitochondria must rely on genetic information from the nucleus. For example, mitochondrial ribosomal proteins are synthesized in the cytoplasm and coded for by nuclear DNA (Küntzel, 1969). This suggests the dual control of nuclear and mitochondrial genes over the formation of the unique mitochondrial ribosomes. It is a challenging question how mitochondrial and nuclear genes are controlled and coordinated with one another for the synthesis of the mitochondrial components.

V SUMMARY

The synthesis of RNA by isolated rat liver mitochondria was studied. Mitochondria isolated in 0.30 M sucrose-2 mM EDTA, 0.25 M sucrose-2 mM EDTA, or 0.25 M sucrose-0.5 mM TES synthesized RNA, incorporating [^{3}H]UTP for at least 1 hr. [^{3}H]ATP, [^{3}H]CTP and [^{3}H]GTP were also incorporated into RNA.

RNA synthesis could not be followed by incorporation of labelled nucleotides into an acid-insoluble form without prior phenol-SDS extraction, because of the formation of other compounds besides RNA. A hot phenol-SDS method was used. Alkaline hydrolysis demonstrated that labelled nucleotides were incorporated into RNA in internal and terminal internucleotide linkages as judged by the recovery of 2'(3') nucleotide and nucleoside, respectively.

The possibility that the synthesis of RNA in the mitochondrial fraction was due to contaminating nuclei, bacteria or the microsomal fraction was examined. The effect of nucleoside triphosphates, DNase, ethidium bromide and atractyloside differentiated RNA synthesis by isolated mitochondria from that by isolated nuclei. Only an insignificant number of bacteria were present as determined by plate counts, and unlabelled uridine did not inhibit [^{3}H]UTP incorporation. The effect of actinomycin D and nucleoside triphosphates eliminated the microsomal fraction as contributing to the observed synthesis. Therefore,

RNA synthesis by isolated mitochondria was not due to contaminating nuclei, bacteria or the microsomal fraction.

The site of RNA synthesis in mitochondria was examined. The effect of osmolarity, nucleoside triphosphates, RNase and DNase in the incubation medium on RNA synthesis suggested that the site of synthesis was inside the inner mitochondrial membrane. This was clearly demonstrated by inhibition of [^{3}H]ATP incorporation into RNA by atractyloside.

Inhibition of [^{3}H]UTP, [^{3}H]ATP, [^{3}H]CTP and [^{3}H]GTP incorporation into RNA by actinomycin D, acriflavin and ethidium bromide demonstrated that the synthesis of RNA is dependent on mitochondrial DNA.

Besides the DNA-dependent RNA synthesis, a DNA-independent incorporation of [^{3}H]CTP and [^{3}H]ATP was observed. The DNA-independent process was probably the turnover of the CCA terminal nucleotides of tRNA in mitochondria.

RNA synthesized in a 1 hr incubation and analyzed by sucrose density gradient centrifugation showed the synthesis of a high molecular weight RNA in a heterodisperse pattern with peaks of 16 and 13 S, indicating the synthesis of mitochondrial ribosomal RNA. The actinomycin D-sensitivity showed that the synthesis was DNA-dependent.

The electrophoretic profile of RNA on an agarose-polyacrylamide gel demonstrated the synthesis of "20.4 S" and "11.4 S" RNA, that is mitochondrial ribosomal RNA. Ethidium bromide inhibited the synthesis of these species.

It was concluded that mitochondrial DNA codes for mitochondrial ribosomal RNA.

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