STUDIES ON THE GENETIC ORIGIN OF MAMMALIAN MITOCHONDRIAL RNAs

by

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

August, 1978

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DOCTOR OF PHILOSOPHY (1978) McMASTER UNIVERSITY
(Biochemistry) Hamilton, Ontario

TITLE: Studies on the Genetic Origin of Mammalian Mitochondrial RNAs

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NUMBER OF PAGES: xvi, 247 Appendix pp 1-19

ABSTRACT

The nature and genetic origin of mammalian mitochondrial poly(A)-containing RNA and tRNAs was examined using isolated mitochondria to label these molecules.

The synthesis of poly(A)-containing RNA by isolated mitochondria from Ehrlich ascites cells and rat liver was studied. Isolated mitochondria incorporate [^{3}H]AMP or [^{3}H]UTP into an RNA species that adsorbed on oligo(dT)-cellulose columns or Millipore filters. Hydrolysis of the poly(A)-containing RNA with pancreatic and T_1 ribonucleases released a poly(A) sequence that had an electrophoretic mobility slightly faster than $4S_e$. In comparison, ascites-cell cytosolic poly(A)-containing RNA had a poly(A) tail that had an electrophoretic mobility of about $7S_e$. Sensitivity of the incorporation of [^{3}H]AMP into poly(A)-containing RNA to ethidium bromide and to atractyloside and lack of sensitivity to immobilized ribonuclease added to the mitochondria after incubation indicated that the site of incorporation was mitochondrial. The poly(A)-containing RNA from ascites cell mitochondria sedimented with a peak of about 18S, with much material of higher S value. After denaturation at 70°C for 5 min the poly(A)-containing RNA from both ascites cell and rat liver mitochondria separated into two components of 12S and 16S on a 5-20% (w/v) sucrose density gradient at 4°C, or at 4° and 25°C in the presence of formaldehyde. Poly(A)-containing RNA from ascites cell mitochondria synthesized in the presence of ethidium bromide sedimented at 5-10S in a 15-33% (w/v) sucrose density gradient at 24°C. The poly(A) tail of

this RNA was smaller than that synthesized in the absence of ethidium bromide. The size of the poly(A)-containing RNA (approx. 1300 nucleotides) is about the length necessary for that of mRNA species for the products of mitochondrial protein synthesis.

The sedimentation and electrophoretic properties of Syrian hamster cytosolic and mitochondrial methionyl- and leucyl-tRNAs were compared under denaturing conditions. Mitochondrial leucyl-tRNA could be separated into three species by chromatography on RPC-5. Their apparent molecule weights as determined by polyacrylamide slab gel electrophoresis were 23 000 for one species and 24 000 for the other two compared to the five cytosolic leucyl-tRNA species whose apparent molecular weights ranged from 26 000 to 28 000. Mitochondrial leucyl-tRNAs sedimented more slowly than their cytosolic counterparts, again indicating a lower molecular weight. The apparent molecular weights of the mitochondrial methionyl-tRNAs were identical or only slightly lower than their cytosolic counterparts as determined by polyacrylamide slab gel electrophoresis.

Individual tRNAs for arginine, asparagine, leucine, lysine, methionine, proline and valine were charged in isolated rat liver mitochondria and shown to be distinct for their cytosolic counterparts by chromatography on RPC-5. By electrophoresis on urea polyacrylamide slab gels it was found that all these mitochondrial aminoacyl-tRNAs were about 70-76 nucleotides long. The unique mitochondrial asparaginyl- and prolyl-tRNAs, not previously identified in mammalian cells, were shown to hybridize to mtDNA. Mitochondrial leucyl-tRNA was separated into 3 peaks on RPC-5 and the first species was shown to be different

than a combination of the other two by molecular size and partial RNase T_1 digestion patterns. Each was coded by a separate gene on mtDNA as shown by partial additivity of hybridization. Separate genes for mitochondrial $tRNA_m^{Met}$ and $tRNA_f^{Met}$, separated by RPC-5 chromatography, were also demonstrated. These results bring to 21 the number of individual tRNAs coded by mammalian mtDNA.

ACKNOWLEDGEMENTS

I am deeply indebted to the following people for their contribution to this investigation and to the preparation of this thesis:

Dr. K.B Freeman, for his patience and invaluable guidance and for his crititical evaluation throughout my research and in the preparation of this thesis.

Drs. B.M Ferrier, H.P. Ghosh and S. Mak for their valuable discussions and suggestions throughout this work.

Drs. P.M. Strasberg, K. A. Webster and R.B. Wallace and Mr. R.W. Yatscoff for their numerous advice and discussions.

Ms. Hansa Patel for her excellent technical assistance.

Mrs. B.Bell for drawing the figures.

In addition, I would like to thank Dr. L.J. King for giving me the much welcomed opportunity to indulge in extra-curricular activities which, in their own small way, will have, I hope, contributed to changing the political image of McMaster University.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BudR	5-bromo-2'-deoxyuridine
CHO	Chinese hamster ovary
Ci	curie
CPM (cpm)	counts.min^{-1}
DEAE	diethylaminoethyl
DNA	deoxyribonucleate
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetate
fMet	N-formylmethionine
fMetA	N-formylmethionyl-adenosine
g	relative centrifugal force (1g = 980 cm sec^{-2})
H strand	heavy strand
L strand	light strand
MAK	Methylated albumin kieselguhr
MetA	methione-adenosine
mRNA	messenger ribonucleate
mtDNA	mitochondrial deoxyribonucleate
µCi	micro curie
poly(A)	polymeric adenylate
poly(A)+RNA	polymeric adenylate containing ribonucleate

poly(A)-RNA	ribonucleate lacking polymeric adenylate
RNA	ribonucleate
RNase	ribonuclease
RPC	reverse phase chromatography
rRNA	ribosomal ribonucleate
S	sedimentation coefficient
S_E	electrophoretic mobility
SDS	sodium dodecyl sulfate
TK	thymidine kinase
Tris	tris (hydroxymethyl) amine methane
tRNA	transfer ribonucleate
$tRNA_m^{met}$	non-formylated methionyl transfer ribonucleate
$tRNA_f^{met}$	formylated methionyl transfer ribonucleate
$tRNA^{leu}$	leucyl transfer ribonucleate
$tRNA^{pro}$	prolyl transfer ribonucleate
$tRNA^{asp}$	asparaginyl transfer ribonucleate
UTP	uridine triphosphate

INTRODUCTION

The most important of the numerous characteristics which distinguish eukaryotic from prokaryotic cells is the presence of the internal organelles, mitochondria and chloroplasts. A number of theories of two basic types, "endosymbiotic" (Margolis, 1970) and "episomal" (Raff & Malher, 1972), have been suggested to account for their origin. Although the question is not resolved, the origin and development of eukaryotes must have depended in large measure on these organelles which are the major sites of cellular energy production.

Mitochondria and chloroplasts are characterized by their semi-autonomy fron the nuclear-cytoplasmic system, that is by the presence of a separate protein-synthesizing system dependent on both nuclear and organellar DNA. The central problem of their biogenesis is the origin of their protein components, the segregation of these from other proteins in the cell and of the regulation of the two protein-synthesizing systems so that a functional organelle results. The cellular origin of mitochondria and chloroplasts has intrigued biologists since their discovery (see Lehninger (1965) for a historical review of mitochondria), but it is only in recent years that progress has been made in understanding their biogenesis. This has been mainly at the level of elucidation of their genetic capacity.

This thesis is concerned with aspects of the genetic capacity of mammalian mitochondria. In this Introduction, the mitochondrial genome and its role are described and differences between various species

examined. It appears that mitochondrial autonomy is limited by the capacity of the mitochondrial genome in coding for proteins. This will be examined first, followed by a description of the RNA transcripts and of the genome itself.

I. Mitochondrial semi-autonomy: characterization and genetic origin of mitochondrial proteins

It is now well established that most mitochondrial proteins are coded by the nuclear genome, synthesized on cytosolic ribosomes and then subsequently transferred by unknown mechanisms to the mitochondrial matrix and the inner and outer mitochondrial membranes (see Schatz & Mason (1974) for review). The contribution of mitochondrial protein synthesis is relatively small, being less than 10% of the total mitochondrial protein, but these proteins play a key role in the functional ability of mitochondria (Schatz & Mason, 1974). Here, I will consider the characterization of and the genetic origin of these proteins.

A. Number and molecular weight range of mitochondrially-synthesized proteins

Mitochondrial protein synthesis can be studied in whole cells or in isolated mitochondria. Most studies have used the former approach which is based on the specificity of inhibitors of translation. The most common are cycloheximide, which inhibits cytosolic but not mitochondrial protein synthesis (Loeb & Hubby, 1968; Ashwell & Work, 1968; Beattie et al, 1967) or chloramphenicol, which inhibits mitochondrial but not cytosolic protein synthesis (Kalf, 1963; Kroon, 1963). Other inhibitors which have been employed include anisomycin (Lizardi & Luck, 1972),

emetine (Grollman,1966; Perlman & Penman, 1970) and pederine (Brega & Baglioni, 1971) as specific inhibitors of cytosolic protein synthesis, and erythromycin (Mason & Schatz, 1973) and Tevenel (Wallace et al, 1975b), the sulfamoyl analogue of chloramphenicol, as inhibitors of translation in mitochondria. Analysis of mitochondrial proteins synthesized in vivo involves inhibition of cytosolic protein synthesis, labelling and characterization of the labelled proteins in the mitochondrial fraction. A drawback of this approach is the secondary effects of inhibitors. For example, both chloramphenicol (Freeman & Haldar, 1967,1968; Haldar & Freeman, 1968; Freeman, 1970) and cycloheximide (Garber et al, 1973) inhibit respiration, while emetine at high concentrations blocks mitochondrial protein synthesis (Ibrahim et al, 1974). Further, inhibition of cytosolic protein synthesis leads to an apparent lack of mitochondrial protein in yeast and Neurospora crassa (Schatz & Mason, 1974; Poyton & Kavanagh, 1976) and mammalian cells (Costantino & Attardi, 1977; Yatscoff & Freeman, 1977).

In early work, mitochondrially-synthesized proteins were analysed by polyacrylamide disc gel electrophoresis. Despite the poor resolution and reproducibility, in part because of the difficulty in solubilizing the very hydrophobic mitochondrial proteins (Schatz & Mason, 1974), it was shown that the mitochondrial proteins had apparent molecular weights between 5,000 and 50,000 (Galper & Darnell, 1971; Coote & Work, 1971; Costantino & Attardi, 1973; Wallace et al, 1975). In most cases, enumeration of the mitochondrially-synthesized proteins was not possible by this method. This has been overcome by the recent introduction of polyacrylamide slab gel electrophoresis and autoradiographic techniques.

Jeffreys & Craig (1975, 1976ab) used such a system to determine that HeLa cell mitochondria synthesize between 8 and 11 proteins with molecular weights between 10,000 and 50,000. Yatscoff & Freeman (1977) were able to enumerate the components synthesized in CHO cell lines and later to detect interspecific variations (Yatscoff et al, 1978a), extending the results of Jeffreys & Craig (1976a). Intraspecific variations in human cell lines were also detected (Yatscoff et al,1978b). The number of bands observed varied between 10 and 13 and the molecular weight range was about the same as detected by previous workers. The number is probably minimal. Some bands might still represent the aggregation or polymerization of smaller products, as suggested by others (Tzagaloff & Akai, 1972; Michel & Neupert, 1973, 1974; Wheeldon et al, 1974; Kuntzel & Blossey, 1974). Better resolution will have to await improved techniques, such as two-dimensional gel electrophoresis. It is unlikely, however, that the range of some 8 to 15 proteins synthesized within mitochondria of mammalian cells will be substantially altered. The number in yeast might be up to 21 (Douglas & Butow, 1976).

B. Identification of mitochondrially-synthesized proteins

The basic protocol used to identify products of mitochondrial protein synthesis has been to 1) label whole cells with a radioactive amino acid in the presence of an inhibitor of cytosolic protein synthesis, 2) isolate mitochondrial enzymes either by standard purification procedure or by immunochemical precipitation with antiserum to the purified enzyme and 3) identify the labelled proteins by electrophoretic separation. This method has allowed the identification of a number of mitochondrial products. These are summarized in Table 1 for yeast and Neurospora.

TABLE 1

Proteins synthesized on mitochondrial ribosomes in yeast and Neurospora

Complex (subunits)	Mol. Wt. (approx.) ($x10^{-4}$)
Cytochrome oxidase (7)	4
	3
	2
ATPase (9)	3*
	2
	1
	0.8*
Cytochrome bc_1 (7)	3
Small ribosome (22)	3.5

* Not found in ATPase complex of Neurospora

Adapted from Borst (1977)

In yeast, Tzagaloff and his co-workers have shown that 4 of the 9 components of the mitochondrial ATPase complex were mitochondrially-synthesized (Tzagaloff & Meagher, 1972; Tzagaloff et al, 1974). Although the ATPase complex of Neurospora has a similar subunit composition (Jackl & Sebald, 1974), only two subunits are synthesized in mitochondria. The yeast ATPase contains a small subunit (subunit 9) which binds the inhibitor DCCD (dicyclohexylcarbodiimide) and which is mitochondrially-synthesized (Wachter et al, 1977). In contrast, in Neurospora, the similar DCCD-binding subunit of the ATPase complex is translated extramitochondrially (Sebald et al, 1977). Both of these proteins have now been sequenced and show considerable homology in spite of their different genetic origin (Wachter et al, 1977; Sebald et al, 1977). Another subunit synthesized in yeast mitochondria, a peptide of molecular weight of 29,000, is cytoplasmically translated in Neurospora (Sebald, 1977).

The site of synthesis of cytochrome b of the cytochrome bc_1 complex has also been shown to be mitochondrial. In Neurospora, it is dimeric in nature with apparently identical subunits of molecular weights of 32,000 (Weiss & Ziganke, 1974ab; Weiss, 1976; Weiss, 1976; Weiss & Ziganke, 1977). A mitochondrial tranlation site has also been established for it in yeast (Katan & Groot, 1975, 1976) and Locusta migratoria (Lorenz et al, 1974). In contrast, Marjanen & Ryrie (1976) have suggested that 3 components of the complex are mitochondrially-synthesized in yeast.

The cytochrome oxidase enzyme complex of yeast and Neurospora is the best characterized complex which has subunits of mitochondrial

origin. In these two organisms, the enzyme consists of 7 non-identical subunits, ranging in molecular weight from 4,600 to 40,000 (Sebald et al, 1973; Poyton & Schatz, 1975). The three largest subunits are mitochondrially-synthesized (Mason & Schatz, 1973; Rubin & Tzagaloff, 1973; Weiss et al, 1971, Sebald et al, 1972, 1973).

Groot (1974) identified a 35,000 molecular weight protein of the small ribosomal subunit of yeast mitochondria as being synthesized within the organelle. A similar protein has also been shown to be mitochondrially-synthesized in Neurospora (Lambowitz et al, 1976). This raises to 9 the number of mitochondrially-synthesized proteins now identified in Fungi.

In mammalian cells, the situation is slightly different. Reports on the number of mammalian cytochrome oxidase subunits have varied. Usually, 6 subunits have been observed, using different systems of analysis (Yamamoto & Orii, 1974; Eytan et al, 1975 ; Bucher & Penniall, 1975; Phan & Malher, 1976), but 7 (Yamamoto & Orii, 1974; Downer et al, 1976) and 8 subunits (Penniall et al, 1976) have been reported. Early work with mammalian cells suggested a mitochondrial origin of at least some of the subunits of the cytochrome oxidase complex because formation of the enzyme was inhibited by chloramphenicol and Tevenel (Schatz & Mason, 1974). Jeffreys & Craig (1977) indicated that one subunit of the complex, with a molecular weight of 20,000, was of mitochondrial origin, but the complex isolated consisted of only 4 subunits with molecular weights lower than 25,000. On the other hand, Yatscoff et al (1977) were able to show that the two largest subunits of the 6 components of bovine heart cytochrome oxidase were synthesized within mitochondria. These are the only mitochondrially-synthesized proteins identified so

far in mammalian cells. While the mitochondrial ATPase complex has been studied in both bovine heart (Knowles & Penefsky, 1972ab; Brooks & Senior, 1972; Capaldi, 1973; Serrano et al, 1976) and rat liver (Catteral et al, 1973), the site of translation of the 5 to 10 subunits has not been determined. It can be suggested that polypeptides corresponding to those synthesized in yeast and/or Neurospora would be expected to have the same translation site in mammalian cells.

C. Genetic origin of mitochondrially-synthesized proteins

The identification of mitochondrially-synthesized proteins does not resolve the question of their genetic origin. Observations that inhibitors of transcription such as ethidium bromide or rifamycin also inhibited mitochondrial protein synthesis (Schatz et al, 1972; Kroon & de Vries, 1971; Grant & Poulter, 1973; Stegeman & Hoober, 1974) gave rise to the notion that all mitochondrial messages for these proteins originated from transcription of the mitochondrial genome. However, this is far from proven (see Section IIC and D for further discussion), in spite of recent progress, especially with yeast.

In this organism, studies of mitochondrial genetics have been a major tool in approaching the problem of the genetic origin of mitochondrially-synthesized proteins. A number of cytoplasmic mutants have been isolated, most of which confer resitance to inhibitors of mitochondrial protein synthesis or to oligomycin. A survey of these mutants is beyond the scope of this Introduction but there are recent reviews (Borst et al, 1977; Linnane & Nagley, 1978). More relevant are results showing a direct relationship between these mutations and proteins identified as being synthesized within mitochondria. Fig. 1

shows the present functional map of the yeast mitochondrial genome indicating the functions controlled by the main genetic loci which have so far been physically mapped and the corresponding proteins.

There is now strong evidence that the OXI loci are the genes for subunits of cytochrome oxidase. Cabral et al (1978) showed that the OXI-1 locus codes for subunit II of cytochrome oxidase. In mutants mapping in this locus, subunit II was replaced by a shorter, mitochondrially-made polypeptide not detected in the wild-type. This polypeptide cross-reacted significantly with an anti-serum against subunit II. Eccleshall et al (1978) have used a temperature-sensitive mitochondrial mutant affecting the OXI-3 region to show that this locus codes for subunit I of cytochrome oxidase, thus confirming an earlier result (Slonimski & Tzagaloff, 1976). Preliminary data indicate that the OXI-2 locus codes for subunit III (Cabral et al, 1978). Other, non-genetic evidence that these loci code for these subunits is presented below.

There is evidence that other loci represent the genes for other mitochondrial proteins. Mutations in the OLI-1 region affect the aggregation behavior of subunit 9 of the ATPase complex (Groot Obbink et al, 1976; Tzagaloff et al, 1976). A mutation in this region also produced an amino acid exchange in this protein (Wachter et al, 1977) strongly indicating that the OLI-1 locus is in the structural gene for this subunit. Groot Obbink et al (1976) have also obtained evidence that the OLI-2 locus affects ATPase subunit 6. The other two ATPase subunits synthesized in mitochondria have not yet been linked to other loci such as PHO-1 or 2 or OLI-3 (see Fig. 1). The COB loci could be one or two genes for cytochrome b, if the two subunits identified in Neurospora are present in yeast and are coded by separate genes. In a

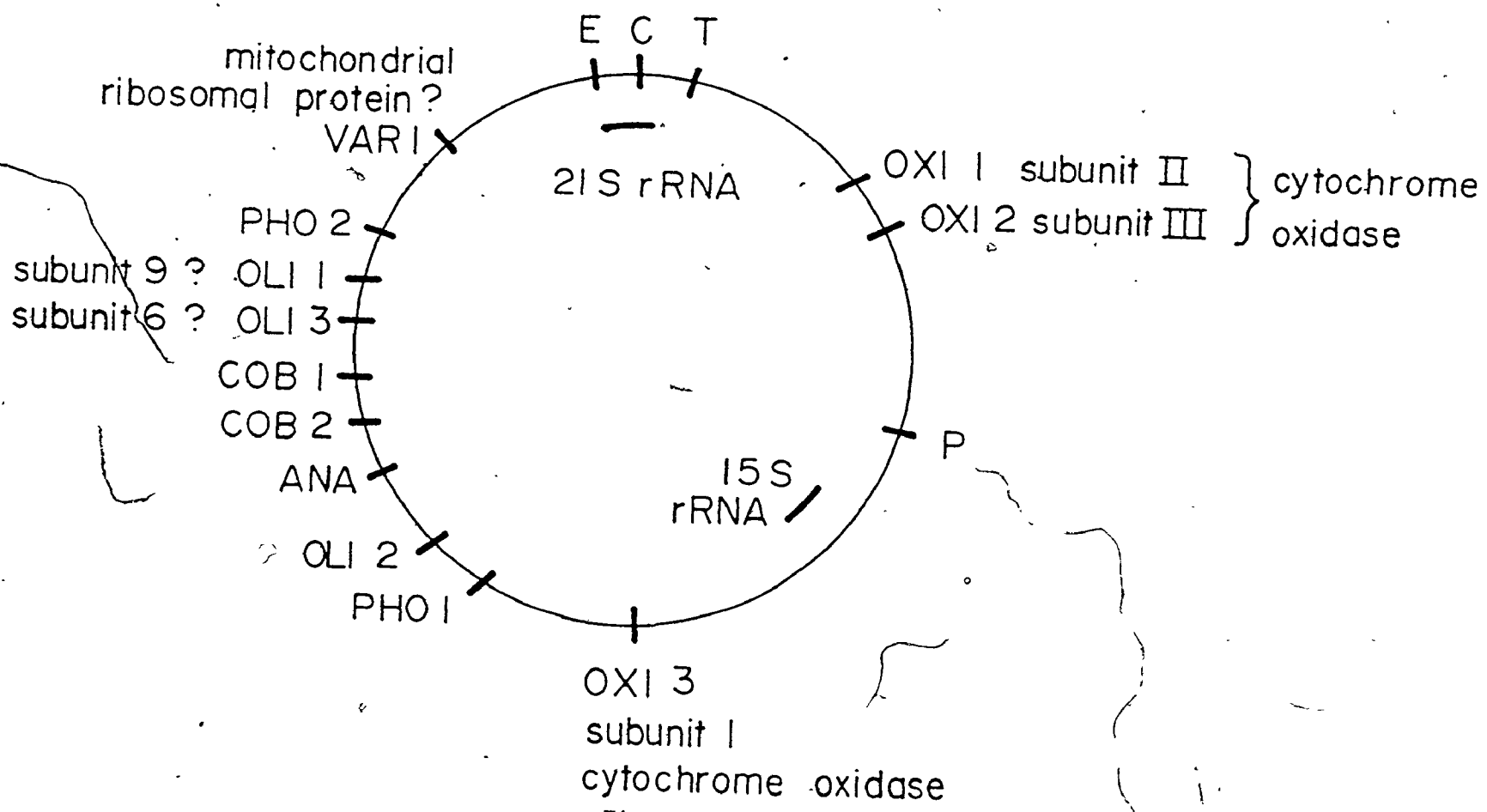


Figure 1

Location of genetic loci and corresponding mitochondrially-coded proteins on the genetic map of S. cerevisiae. E, C, P and OLI are loci for erythromycin, chloramphenicol, paromycin and oligomycin resistance respectively; T is a locus affecting the stability of rho$^+$ mtDNA; OXI-1 to 3 are three loci involved in the biosynthesis of cytochrome oxidase; PHO-1 and 2 are loci involved in the biosynthesis of the ATPase complex; COB-1 and 2 are loci involved in the biosynthesis of cytochrome b; ANA is an antimycin-resistance locus. The position of the rRNA genes is given for reference (adapted from Borst et al, 1977)

of these results will be presented in Section IIC.

Work with mammalian cells has not progressed as fast as in yeast, the main difficulty being the absence of mitochondrial mutants which could serve as genetic markers. Only recently have methods been developed to produce and isolate mammalian cytoplasmic mutants. These are all resistant to drugs. Only some studies will be considered here.

Spolsky & Eisenstadt (1972) selected a chloramphenicol-resistant strain from HeLa cells after treatment of cells with ethidium bromide. These cells were found to be resistant at the level of isolated mitochondrial protein synthesis. Cytoplasmic inheritance of chloramphenicol-resistance was then demonstrated by Bunn et al (1974) and Wallace et al (1975a) by fusion of enucleated chloramphenicol-resistant cells with sensitive strains. The maintainance of the resistance marker provided by the enucleated cells in the cytoplasmic hybrid ("cybrid") indicated cytoplasmic inheritance, possibly a mutation in mtDNA. Mitotic segregation of this marker in hybrid cells has also been observed further indicating cytoplasmic inheritance (Bunn et al, 1977; Wallace et al, 1977).

Wallace & Freeman (1975) have used another method for selecting Tevenel-resistant cells. They made use of LMTK$^-$ cells. This cell line lacks cytosolic thymidine kinase and therefore specifically incorporates the base analog bromodeoxyuridine (BrdU) into mtDNA. While BrdU-treatment did not seem to increase the yield of cells resistant to Tevenel, the cell line did give rise to a higher proportion of resistant cells than the corresponding wild-type L cell. It remains to be demonstrated that the resistance arose from a mutation in mtDNA. Similarly, Yatscoff & Freeman

(personal communication) have obtained CHO TK$^-$ cells resistant to levenel.

Recently, Eisenstadt's group have used mutagenesis with ethyl methane sulfonate followed by selection in 3 to 5 µg oligomycin/ml to obtain oligomycin-resistant strains of CHO and mouse (LMTK$^-$) culture lines (Kuhns et al, 1978). Most of the LMTK$^-$ resistant lines were obtained after enucleation, followed by mutagenesis. It is not clear whether these cells have a cytoplasmic mode of inheritance of oligomycin resistance, although the procedure of enucleation prior to mutagenesis would be expected to yield strictly mitochondrial mutants.

A possible mitochondrial marker has been detected by Yatscoff et al (1978b) who observed that HeLa cell mitochondria synthesize a unique protein not found in other human cell lines, except KB and HEP-2 which are probably HeLa contaminants. The reverse also holds and it should be possible, through enucleation and appropriate hybridization experiments, to determine the genetic origin of the proteins that differ.

It has been found that mammalian mtDNAs recombine (Coon et al, 1973; Dawid et al, 1974; Hurak et al, 1974). This means that when sufficient markers are available, mapping of mtDNA should be possible.

II. Transcriptional products of the mitochondrial genome

The mitochondrial origin of a number of mitochondrially-synthesized proteins implies by definition the transcription from mtDNA of corresponding mRNAs. The presence of a DNA-dependent RNA polymerase was indicated by early studies (Luck & Reich, 1964) and this enzyme has now been fairly well characterized in a number of systems (Kuntzel &

Schafer, 1971; Wu & Dawid, 1972; Dawid & Wu, 1974; Eccleshall & Riddle, 1974; Gallerani & Saccone, 1974; Scragg, 1976). The nature of the mitochondrial transcripts however remains incompletely specified.

While it is clear that mtDNA codes for a set of rRNAs, the number of tRNAs transcribed is unresolved. The lack of identification of most mitochondrially-synthesized proteins has only been one factor leading to a lag in the characterization of the mRNA species. The observations concerning the nature and origin of mitochondrial rRNA, tRNA and mRNA will now be reviewed.

A. Mitochondrial ribosomes and ribosomal RNA

The major criterion for defining ribosomal RNAs is that they are the major species associated with ribosomes and that there are two high molecular weight forms each derived from one ribosomal subunit. Mitochondrial ribosomes were first isolated independently by Rabinowitz et al (1966) and O'Brien & Kalf (1967) from rat liver. They have now been characterized in a whole series of organisms and have been the subject of a recent and extensive review by O'Brien (1976). The main features of mitochondrial ribosomes are summarized in Table 2.

Substantial differences are observed between mitochondrial ribosomes of lower eukaryotes and plants and animals, and from cytosolic ribosomes. Their major distinguishing features are the following: 1) they sediment at the same rate as their cytosolic counterparts, except in mammalian cells where they sediment more slowly; 2) they require a higher Mg^{++} concentration for stability than their cytosolic counterparts; 3) they are sensitive to inhibitors of bacterial but not cytosolic protein synthesis (Avadhani et al, 1975) and 4) the rRNAs from the faster

TABLE 2

Summary of the properties of cytosolic and mitochondrial ribosomes and rRNAs

Organism	Ribosome		rRNAs		G+C	
	Cyto	Mito	Cyto	Mito	Cyto	Mito
	(S value)		(S value)		%	
Animals	80	60-55	28,18	21-15,17-12	65	45-38
Fungi	80-77	80-72	25,17	24-21,19-15	50	38-23
Protists	80	80	28,18	21,14	46	29
Higher Plants	80	80-70	26,18	26,18	55	55

cyto: cytosolic; mito: mitochondrial

sedimenting ribosomes are larger (21-24S and 14-16S) than those from slower sedimenting ribosomes (16-17S and 12-13S).

Mitochondrial ribosomal proteins have been found to be distinct from the proteins of cytosolic ribosomes. This was first shown by disc gel electrophoresis (Chi & Suyama, 1970; Vasconcelos & Bogorad, 1971) and immunological precipitation (Hallermeyer & Neupert, 1974), then by two-dimensional gel electrophoresis (O'Brien et al, 1974; Leister & Dawid, 1974; de Vries & van den Bogert, 1976). All mitochondrial ribosomal proteins, except one protein of the small subunit, are synthesized in the cytosol (Schatz & Mason, 1974). Mitochondrial ribosomal proteins seemed to have evolved much faster than their cytosolic counterparts (Leister & Dawid, 1975; Matthews et al, 1978). This suggests that different genetic constraints, for example the origin of their rRNAs, are imposed on these proteins.

It is now well established that the mitochondrial rRNAs are coded by the mitochondrial genome. Saturation hybridization data indicated that the genome has one cistron each for the large and small rRNAs (Aloni & Attardi, 1971c; Dawid, 1972; Reijnders et al, 1973; Borst & Grivell, 1973; Reboul & Vignais, 1974). This was confirmed by the determination of their map position in a number of organisms, as described in Section III of this Introduction.

It should come as no surprise that variations in physico-chemical properties among mitochondrial ribosomes of the major eukaryotic grpups are also found in the rRNAs (Table 2). Sedimentation analysis and electron microscopy indicate that the rRNAs of lower eukaryotes and plants are considerably larger than those of animal sources. There appears to be a correlation between the smaller size and higher G+C

content of the rRNAs. The small size of animal mitochondrial rRNAs is particularly apparent. Except for plants, mitochondrial rRNAs have a lower G+C content than their cytosolic counterparts (Freeman et al, 1973; Cunningham & Gray, 1977). Fungal mitochondrial rRNAs are about the same size as cytosolic rRNAs, while plant rRNAs seem to have a higher molecular weight than cytosolic rRNAs of the same species (Leaver & Harmey, 1973; Quetier & Vedel, 1974; Pring & Thornbury, 1975; Cunningham & Gray, 1977). A recent comparison of the sequences of oligonucleotides produced by RNase T_1 digestion of wheat embryo 18S mitochondrial rRNA with that of other small rRNAs of prokaryotes and chloroplasts indicates that this rRNA may be "prokaryotic" in nature (Bonen et al, 1977). Except for one report (Attardi & Attardi, 1971), mitochondrial rRNAs are less methylated than cytosolic rRNAs (Vesco & Penman, 1969; Dubin, 1974; Klootwijk et al, 1975; Lambowitz & Luck, 1976; Cunningham & Gray, 1977). A low degree of methylation would be in agreement with the low degree of methylation and modification of mitochondrial tRNAs (see next Section).

In contrast to bacterial and cytosolic ribosomes, and possibly those of plant mitochondria (Leaver, 1975), mitochondrial ribosomes from a number of lower eukaryotes and animal sources seem to be devoid of 5S RNA. Lizardi & Luck (1972) first suggested that Neurospora ribosomes lacked such a component, but Kroon et al (1976) found that a 5S RNA associated with 80S ribosomes hybridized to mtDNA. This observation has been challenged by Michel et al (1976, 1978) who concluded that 73S particles were the only functional mitochondrial ribosomes in Neurospora and that the 80S ribosomes and 5S RNA were cytosolic contaminants. A "5S RNA" might have gone undetected, if, like the rRNAs,

mitochondrial "5S RNA" of animal cells was actually smaller than the cytosolic or prokaryotic RNA. Dubin et al (1974) reported the isolation of a "3S RNA" of low G+C content and low degree of methylation in hamster BHK cells which could represent a "5S RNA" equivalent. Its association with mitochondrial ribosomes has not yet been reported.

B. Mitochondrial tRNAs

Unique mitochondrial tRNAs different from cytosolic tRNAs have been demonstrated in several ways: 1) differential charging of mitochondrial tRNAs by aminoacyl-tRNA synthetases from mitochondria and cytosol; 2) unique chromatographic behavior; and 3) hybridization to mtDNA.

Aminoacyl-tRNA synthetases: While there were a number of early studies of mitochondrial aminoacyl-tRNA synthetase activity (Reis et al, 1959; Roodyn et al, 1961; Craddock & Simpson, 1961; Davis & Novelli, 1958), the first convincing demonstration of their existence came from Wintersberger (1965). He showed, using [^{14}C] leucine and phenylalanine, that a synthetase preparation from yeast mitochondria could charge homologous RNAs more efficiently than heterologous, that is, cytosolic RNAs. The reverse, charging of cytosolic RNA by the cytosolic enzymes, was also more efficient. The same conclusion was reached with Tetrahymena pyriformis (Suyama & Eyer, 1969), Neurospora (Barnett et al, 1967; Barnett & Brown, 1967; Epler & Barnett, 1967), again in yeast (Schneller et al, 1976a), rat liver (Buck & Nass, 1968, 1969) and calf brain (Charezimski & Borkowski, 1973). Only in HeLa cells was charging of some mitochondrial tRNAs by the two synthetase preparations about the same (Lynch & Attardi, 1976). Other similar reports in Phaseolus vulgaris (Guillemaut et al,

1975) and Tetrahymena (Suyama & Hanada, 1976) can be explained by cytosolic contamination of the mitochondrial fraction. Generally, however, the differences found are strong evidence for the uniqueness of mitochondrial tRNAs and synthetases.

Another approach was used by Gross and co-workers. They found that in a temperature-sensitive mutant of Neurospora both mitochondrial and cytosolic leucyl-tRNA synthetases were affected (Printz & Gross, 1967; Gross et al, 1968). They suggested that one nuclear gene might code for at least one subunit of both enzymes (Weeks & Gross, 1971). In a more recent paper, Beauchamp et al (1977) presented immunological evidence that the two enzymes have little, if any, structural homology but that they are probably coded by adjacent genes. Lack of structural homology of the mitochondrial with the cytosolic enzyme has also been demonstrated in yeast (Schneller et al, 1976b) and in Tetrahymena (Chiu & Suyama, 1973, 1975). The distinctiveness of mitochondrial aminoacyl-tRNA synthetases was also demonstrated by Wallace et al (1975b) using the CHO tsH1 mutant (see Section IA) with a temperature-sensitive cytosolic leucyl-tRNA synthetase. At the non-permissive temperature, cytosolic activity was reduced but mitochondrial synthetase activity could be measured by following the incorporation of labelled leucine into mitochondrial proteins. A similar approach was used by Aujame et al (1978) (see Appendix) to demonstrate mitochondrial asparaginyl-tRNA synthetase activity in another CHO mutant cell line and a unique mitochondrial asparaginyl-tRNA.

Chromatographic properties of mitochondrial tRNAs: The demonstration of specific mitochondrial synthetases has allowed the isolation and characterization of specific mitochondrial tRNAs. Table 3 summarizes

TABLE 3

tRNA species found in various mitochondrial systems

Organism	tRNA species	Analytical technique
Neurospora	Ala, Arg, Ash, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr, Val	Counter-current RPC DEAE·Sephadex Hydroxylapatite
Saccharomyces	Ala, Arg, Asp, Gln, Glu, Gly, Cys, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr, Ser, Val	RPC-5
Tetrahymena	Arg, Leu, Lys, Phe, Val	RPC-5
Rat liver	Asp, Leu, Ser, Trp	MAK
HeLa	Ala, Arg, Asp, Cys, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Met, Trp, Tyr, Val	Acceptor activity

Adapted from Avadhani et al (1975)

results in different organisms on the charging of tRNA by the corresponding amino acids. Results in this thesis with hamster and rat liver and in CHO cells are not included.

Studies in Neurospora and especially in yeast have again been the most extensive. Amino acid acceptor activities for 18 amino acids were found associated with Neurospora mitochondria (Barnett & Brown, 1967). The uniqueness of mitochondrial aminoacyl-tRNAs was initially shown by chromatographic separation of the tRNAs on either DEAE-cellulose or hydroxyapatite or by counter-current distribution (Barnett et al, 1967; Epler & Barnett, 1967). Brown & Novelli (1968) and Epler (1969), utilizing reverse-phase chromatography (RPC) showed the separation of 15 Neurospora mitochondrial aminoacyl-tRNAs from their corresponding cytosolic tRNAs. Further, one of the two methionyl-tRNAs (Brown & Novelli, 1968) could be formylated (Epler et al, 1970). The initiator tRNA ($tRNA_f^{met}$) has now been sequenced (Heckman et al, 1978). It bears little resemblance other sequenced initiator tRNAs found in either the cytosol, prokaryotes or chloroplasts.

In yeast, charging by 19 amino acids has now been demonstrated by Rabinowitz's group (Martin & Rabinowitz, 1978). Charging with asparagine was not observed. These mitochondrial tRNAs were found to be chromatographically distinct from the cytosolic tRNAs on RPC-5 both by Rabinowitz's group (Martin & Rabinowitz, 1978) and Stahl's group (Accoceberry & Stahl, 1971; Accoceberry et al, 1973; Schneller et al, 1973; Schneller et al, 1975a). They were able to identify isoaccepting species sometimes coded by different genes (see below). These groups differ in the number of peaks of mitochondrial tyrosyl- and leucyl-tRNA

(Schneller et al, 1975a; Martin & Rabinowitz, 1978) found but this could depend on different growth conditions used in these studies (Baldacci et al, 1976, 1977).

In a series of experiments with rat liver mitochondria, Buck & Nass (1968, 1969) demonstrated unique mitochondrial aminoacyl-tRNAs and presumably synthetases for aspartate, leucine, tyrosine, serine and valine. The aminoacyl-tRNAs could be separated from their cytosolic counterparts by MAK chromatography. Although multiple mitochondrial peaks were seen, including the case of leucyl-tRNA, it was unclear whether some represented cytosolic contamination. Separation of mitochondrial and cytosolic phenylalanyl-tRNA was not observed (Buck & Nass, 1969). but the two were resolved by Lietman (1968). Similar studies with Tetrahymena mitochondria showed that the mitochondrial leucyl-tRNA was distinct from cytosolic leucyl-tRNA and could be resolved into three peaks by RPC-5 chromatography (Chiu et al, 1974).

While all these studies used isolated mitochondrial synthetases to charge tRNAs, Wallace & Freeman (1974) used another approach. They incubated mouse liver mitochondria under conditions optimum for mitochondrial protein synthesis, and therefore probably for synthetase activity, and were able to isolate methionyl-tRNA. Further, the methionyl-tRNA could be separated on RPC-5 into 4 peaks, 2 of which were formylated.

Origin of mitochondrial tRNAs: The isolation of mitochondrial tRNAs chromatographically distinct from their cytosolic counterparts does not necessarily imply that these are coded by the mitochondrial genome. In fact, an import of tRNAs in conjunction with nuclear-coded synthetases was suggested in early studies (Suyama, 1967). To resolve this question, either charged tRNAs or 4S RNA from mitochondria have been hybridized to

mtDNA.

In Neurospora, the hybridization of mitochondrial leucyl-tRNAs to mtDNA has been reported (Terpstra et al, 1977b). Other tRNAs have not been examined, but in yeast, all the charged species were found to hybridize to mtDNA (Martin & Rabinowitz, 1978). In some cases, some were found to be coded by more than one gene: this is the case for methionyl-tRNA ($tRNA_m^{met}$ and $tRNA_f^{met}$) (Martin et al, 1976b; Martin & Rabinowitz, 1978), cysteinyl- and threonyl-tRNA (2 each) (Martin & Rabinowitz, 1978), and possibly also leucyl-tRNA (2) (Martin et al, 1977b). A few other aminoacyl-tRNAs, separating in multiple peaks, may yet be shown to be coded by more than one gene (Martin & Rabinowitz, 1978).The synthetase preparation was found to charge both $tRNA^{glu}$ and $tRNA^{gln}$ with glutamate. The latter is presumably then amidated to form glutaminyl-$tRNA^{gln}$. Each of these tRNAs are coded by distinct genes (Martin et al, 1977a). The total number of identified mitochondrial tRNA cistrons in yeast is now 22. Only a gene for $tRNA^{asn}$ has not been identified.

In HeLa cells, mitochondrial tRNAs could be charged with 16 amino acids and sites for 17 tRNAs on mtDNA were shown by hybridization (Lynch & Attardi, 1976). tRNAs for asparagine, glutamine, histidine and proline were not isolated. None of the 16 aminoacyl-tRNAs were characterized by chromatography. However, methionyl-tRNA could be formylated indicating the possible existence of two species and seryl-tRNA hybridized to both strands of mtDNA. Of the 17 tRNAs, 12 hybridized to the heavy (H) strand and 5 to the light (L) strand. In earlier studies, Nass & Buck (1970) had shown that rat liver mitochondrial leucyl-tRNA and phenylalanyl-tRNA hybridized to the H strand, while tyrosyl- and seryl-tRNAs

hybridized to the L strand of mtDNA.

In Tetrahymena, Chiu et al (1974) found that the three peaks of leucyl-tRNA hybridized to mtDNA. While there was no additivity of hybridization, the melting temperature of the tRNA-DNA hybrids differed for at least two species. In addition, one species recognized CUG only, another CU(U,C,G) and the third CUC. From these results, they concluded that there were two $tRNA^{leu}$ genes on the mitochondrial genome. In contrast, while distinct mitochondrial valyl-, lysyl- and arginyl-tRNAs could be charged by the homologous synthetases, these tRNAs did not hybridize to mtDNA (Chiu et al, 1975). They suggested that some tRNAs might be imported from the cytoplasm into mitochondria.

The question of import of tRNAs raised by this study also arose from studies on the 4S RNA fraction. This mitochondrial component was recognized early in the study of mitochondrially-associated RNA (Attardi & Attardi, 1967; Dubin & Brown, 1967; Rifkin et al, 1967; Suyama, 1967; Knight & Sugiyama, 1969; Zylber & Penman, 1969). More recent studies indicate that this fraction has unique characteristics compared to cytosolic 4S RNA, in particular a low degree of methylation and base modification (Chia et al, 1976; Davenport et al, 1975; Schneller et al, 1975b). It consists mostly if not exclusively of mitochondrial tRNAs (Feldman & Kleinow, 1976ab; Lynch & Attardi, 1976; Martin et al, 1977b). Inhibition of mitochondrial 4S RNA synthesis by ethidium bromide (Zylber & Penman, 1969) as well as early hybridization experiments (Suyama, 1967; Attardi & Attardi, 1967) suggested that at least some of the 4S RNA components were transcribed from mtDNA.

Saturation hybridization experiments with mitochondrial 4S RNA

of known specific radioactivity initially demonstrated 20 genes on yeast mtDNA (Reijnders & Borst, 1972; Schneller et al, 1975c). More recently, yeast 4S RNA has been hybridized to restriction fragments and the number of genes were calculated to be 26 (Van Ommen et al, 1977). In Neurospora, the estimate from similar experiments was 25 (Terpstra et al, 1977b). Only 11 genes on HeLa cell mtDNA (Aloni & Attardi, 1971c) and 15 on Xenopus mtDNA (Dawid, 1972) were initially found. In an attempt to visualize the position of the 4S RNA genes on the mitochondrial genome of HeLa cells, Wu et al (1972) and later Angerer et al (1976) prepared 4S RNA covalently coupled to ferritin. This was hybridized to both the H and L strands of mtDNA and examined by electron microscopy. Nineteen regions of hybridization, 12 on the H strand and 7 on the L strand were observed implying 19 genes. These numbers are remarkably close to those obtained by hybridization of individual aminoacyl-tRNAs discussed earlier (Lynch & Attardi, 1976). Using the same procedure, Dawid et al (1976) were able to locate 15 tRNA genes on the H strand of Xenopus mtDNA and 5-6 on the L strand, raising the number of 4S RNA genes on this genome from 15 to 22. Hybridization of the 4S RNA fraction has also been carried out on rat liver mtDNA restriction fragments and between 16 and 23 genes were suggested (Saccone et al, 1977).

The number of genes calculated- or the number of tRNA genes identified- is not sufficient to code for all the tRNAs necessary to recognize all codons in the genetic code, if they are all used in mitochondrial protein synthesis. According to the wobble hypothesis (Crick, 1966), 32 tRNAs would be necessary.

The initial saturation values (Aloni & Attardi, 1971c; Dawid,

1972; Reijnder & Borst, 1972; Schneller et al, 1975c) could have been underestimated for a number of reasons. One possible explanation is an overestimation of the molecular weight of the 4S RNA fraction assumed for calculations of gene numbers. For exemple, Dawid (1972) took a molecular weight of 28,000. Dubin & Friend (1972) suggested, on the basis of sucrose gradient and gel electrophoretic analyses under denaturing and non-denaturing conditions, that hamster BHK-21 mitochondrial 4S RNA was smaller than cytosolic 4S RNA, with a molecular weight calculated at 19,000. In the case of Xenopus, if this value were taken, the number of 4S RNA genes would be raised to 22 instead of 15.

Another explanation is the possible overlap of genes. This could also explain the non-additivity of hybridization of Tetrahymena leucyl-tRNAs (Chiu et al, 1974)and of some yeast isoaccepting species (Martin & Rabinowitz, 1978). It would also lead to an underestimation of the number of genes seen under the electron microscope (Wu et al, 1972; Angerer et al, 1976; Dawid et al, 1976).

Recently, the mitochondrial 4S RNA fraction has been analysed by two-dimensional gel electrophoresis in yeast (Martin et al, 1977b), Locusta migratoria (Feldman & Kleinow, 1976ab) and Neurospora (de Vries et al, 1978). In yeast this method revealed 26 spots which all hybridized to mtDNA. Some of them have been identified by charging of individual spots. Since some contained more than one species, it is possible that the actual number of tRNA genes in yeast mtDNA might be more than 26, but still short of 32. In Locusta, there were 27 spots (Feldman & Kleinow, 1976ab), and in Neurospora, 25 with 17 minor spots which could represent cytosolic contamination (Terpstra et al, 1977b).

The general picture which emerges from these various studies is an apparent deficit in the number of mitochondrially-coded tRNAs possibly necessary to read all codons. This result could reflect the following:

1) Absence of charging of some tRNAs because of inappropriate charging conditions.

2) Mitochondrial tRNAs are smaller than cytosolic tRNAs. Consequently, some of the saturation hybridization experiments significantly underestimated the actual number of genes present, as discussed above.

3) Not all amino acids are incorporated into mitochondrially-synthesized proteins. Some tRNAs might not be necessary for mitochondrial protein synthesis (Costantino & Attardi, 1973).

4) If all amino acids are used, and assuming that all codons are read, mitochondrial tRNAs could read more codons than expected from the wobble hypothesis, either through unusual base-pairing of the type suggested by Topal & Fresco (1976a b) or by utilizing a "two-out-of-three" reading scheme (Mitra et al, 1977; Lagerkvist, 1978). The consequence of this proposition would be the absence or reduced number of isoaccepting species and corresponding genes for a number of tRNAs.

5) Genes could overlap.

6) Finally, mitochondrial are not fully autonomous with respect to tRNAs and some tRNAs are imported from the cytosol as suggested by Suyama (Suyama, 1967; Chiu et al, 1975; Suyama & Hanada, 1976).

All of these alternatives, based on present results, are still possible. One of the objectives of this thesis was to determine whether some of these are valid.

C. Mitochondrial messenger RNAs

Early suggestions that the mitochondrial genome coded for mRNA came from inhibitor studies. Ethidium bromide was shown to inhibit mitochondrial translation in isolated mitochondria (Kroon & de Vries, 1970; Schatz et al, 1972) and a number of inhibitors of prokaryotic transcription were also found to affect transcription of the mitochondrial genome (Stegeman & Hoober, 1974; Dube et al, 1973). Using a temperature-sensitive yeast mutant for nuclear mRNA, Malher & Dawidowicz (1973) showed that mitochondrial protein synthesis continued normally at the non-permissive temperature but was inhibited by ethidium bromide, suggesting that the mitochondrial translation apparatus used only endogenous messages coded by the mitochondrial genome.

A number of approaches are now being utilized to characterize the origin, function and nature of mitochondrial mRNA. For an RNA molecule to be considered a mitochondrial mRNA, the following criteria must be satisfied: 1) association with mitochondrial polysomal structures; 2) size sufficient to code for mitochondrially-synthesized proteins; 3) translation of the RNA should yield mitochondrially-synthesized proteins; and 4) hybridization to mtDNA.

The last two criteria taken together provide unambiguous proof of the transcriptional origin of mRNA on mtDNA and of its role, while the first two are mainly indicative. However, because of the small amounts of presumptive mitochondrial mRNAs, attempts at translation in vitro have been limited.

Transcription of mtDNA: Direct transcription of isolated mtDNA either by mitochondrial RNA polymerase or by heterologous enzymes has been reported.

RNA transcripts, when translated in an E. coli cell-free system yielded products similar by gel electrophoresis and immunoprecipitation to membrane proteins labelled in vivo (Scragg, 1974). Others have transcribed mtDNA from Artemia salina (Schmitt et al, 1974) or rat liver (Gallerani & Saccone, 1974) but did not demonstrate that mRNAs were produced.

Coupled-transcription-translation: Another approach to demonstrate mitochondrial messages has been to transcribe mtDNA in an E. coli coupled transcription-translation system. Translational activity was observed but the SDS gel electrophoretic patterns of labelled polypeptides were totally different from those of polypeptides obtained in vivo (Blossey & Küntzel, 1972; Chang & Weissbach, 1973; Scragg & Thomas, 1975). This has been attributed to the inability of the RNA polymerase from E. coli to recognize the correct initiation sequences on mtDNA (Blossey & Küntzel, 1972) or of the E. coli ribosomal system to translate mitochondrial mRNA correctly (Moorman et al, 1978). Using yeast mtDNA, Moorman et al (1978) showed with indirect immunoprecipitation that antigenic determinants of cytochrome oxidase were among the products synthesized. There was no immunoprecipitation when a mtDNA fragment with the gene for 21S rRNA and some flanking sequences only (see Fig. 1) was used. On the other hand, restriction fragments from mtDNA corresponding to the OXI-1 and OXI-2 regions were shown to be transcribed into RNA coding for cytochrome oxidase subunits II and III. The RNA synthesized in the coupled system was short, sedimenting at 6-9S in formaldehyde-containing sucrose gradients. The size of the transcripts probably explains the small size of the polypeptides precipitated by anti-cytochrome c oxidase antisera.

As an alternative to the cell-free system, plasmids containing either Eco RI restriction fragments of mouse mtDNA (Chang et al, 1975) or of hamster mtDNA (Miller et al, 1977) were also apparently incorrectly translated, if at all, in E. coli cells. The transcripts were shown to be mainly from the L strand of mouse mtDNA plasmids (Chang et al, 1975), whereas most stable products of mammalian cell mtDNA seem to be transcribed from the H strand (see next Section).

Translation in vitro: Mitochondrial RNA from yeast has been extracted and translated in vitro . Early results (Eggitt, 1976) were inconclusive but two or possibly three messages have now been identified (Moorman et al, 1976, 1977; Borst et al, 1977). Yeast total RNA was fractionated into discrete size classes by sucrose gradient centrifugation and their ability to stimulate the synthesis of specific proteins analysed by immunoprecipitation. RNA species in the 11-12S region were resolved and were shown to hybridize to mtDNA (see Section III, Fig. 2). A 12S RNA could code for an ATPase subunit and 11S RNA, which hybridized to both the OXI-1 and OXI-3 regions, could code for two of the subunits of cytochrome oxidase. As yeast mitochondrial RNA hybridizes to many restriction fragments of mtDNA (Van Ommen & Groot, 1977), it should be possible to eventually show the translational role of other mRNAs.

Attempts at setting up a completely homologous yeast system for translating mitochondrial RNA have been unsuccessful. Eukaryotic cell-free systems (wheat germ, reticulocyte and Xenopus oocytes) were also incapable of translating yeast mitochondrial RNA (Moorman et al, 1977). In view of the difficulties encountered in heterologous systems, the reconstruction of a completely homologous system should be of great

importance in the identification of mitochondrial mRNAs

Attempts at translating mitochondrial poly(A)-containing RNA (see below) have been made in yeast (Padmanaban et al, 1976; Hendler et al, 1976) and Neurospora (Kroon et al, 1976) where it appears to code for subunits of cytochrome oxidase, and in rat liver (Kisselev et al, 1977). These results will be considered further in the Discussion (Part I).

Poly(A)+RNA: The presence of a poly(A) sequence in mitochondrial RNA was first described by Perlman et al (1973) in HeLa cells and by Avadhani et al (1973) in Ehrlich ascites cells. In HeLa cells, this poly(A) consisted of a 3'-end tail of about 60 nucleotides as compared to the 150-250 nucleotides long cytosolic poly(A) tail (Hirsch & Penman, 1973). A similar situation has been found in other animal cell lines.

By comparison with cytosolic mRNAs, such RNA molecules would be expected to serve as mitochondrial messages and be found in mitochondrial polysomes which had been identified previously.

Intramitochondrial polysomal structures sedimentating between 73S and 260S were first isolated by Küntzel & Noll (1967) in Neurospora and by Avadhani et al (1971) in Euglena. High molecular weight RNA-containing particles were isolated from mitochondria of HeLa cells (Ojala & Attardi, 1972), yeast (Malher & Dawidowicz, 1973; Cooper & Avers, 1974) and Ehrlich ascites cells (Avadhani et al, 1973; Lewis et al, 1976a). In a number of cases, these particles were resistant to RNase treatment and therefore not necessarily polysomes (Ojala & Attardi, 1972; Michel & Neupert, 1973, 1974). It was suggested that RNase resistance resulted from the hydrophobic properties of the protein products and their tendancy to associate with each other and with membranes (Avadhani et al, 1975). The

involvement of these particles in protein synthesis (Avadhani & Buetow, 1972; Allen & Suyama, 1972) and electron microscopic studies showing up to heptamers (Vignais et al, 1972; Charret & Charlier, 1973; Kuriyama & Luck, 1973; Cooper & Avers, 1974) are convincing evidence that they represent mitochondrial polysomes. The isolation of poly(A)+RNA from these polysomal structures would obviously make them good candidates for mRNAs. In fact, poly(A)+RNA has been isolated from mitochondrial polysomal structures in HeLa cells (Hirsch & Penman, 1974a; Ojala & Attardi, 1974) and Ehrlich ascites cells (Avadhani et al, 1973).

Further evidence that poly(A)+RNA serves as mRNA comes from its characterization. In HeLa cells, poly(A)+RNA could be resolved into 8 species ranging from 24S to 16S (Hirsch & Penman, 1973). A similar result was obtained in hamster and insect mitochondria (Hirsch et al, 1974). Attardi and his co-workers were able to dissociate poly(A)+RNA from HeLa cell mitochondria into 12 species ranging from 12S to 7S (Ojala & Attardi, 1974c). All of these species hybridized to mtDNA, and in HeLa cells, mostly to the H strand (Ojala & Attardi, 1974c). The size and number of poly(A)+RNAs would be sufficient to code for mitochondrially-synthesized proteins.

In contrast to HeLa cells (Perlman et al, 1973; Hirsch & Penman, 1973, 1974ab; Ojala & Attardi, 1974abc) where ethidium bromide completely inhibited the synthesis of mitochondrial poly(A)+RNA, in Ehrlich ascites cells (Avadhani et al, 1973, 1974) and in rat liver (Gaitskhoki et al, 1973) partial resistance of synthesis to this inhibitor was observed. It was concluded that some of the intramitochondrial poly(A)+RNA had an extramitochondrial origin. Kinetic experiments showing a lag of 15-20

min before the appearance of labelled poly(A)+RNA in Ehrlich ascites mitochondria (Avadhani et al, 1973) were also interpreted as evidence for transport of nuclear RNA into mitochondria.

Further, Avadhani et al (1973,1974) showed significant hybridization to nuclear DNA of poly(A)+RNA labelled in vivo and isolated from mitochondrial polysomes. RNA labelled in isolated mitochondria and isolated from polyribosomes was not polyadenylated and hybridized only to mtDNA. The latter RNA separated into 3 peaks with mobilities between 12 and 7S (Avadhani et al, 1974). The inability of isolated mitochondria to polyadenylate mitochondrial RNA is surprising in view of the presence of a poly(A) polymerase in rat liver mitochondria (Jacob & Schnindler, 1972; Jacob et al, 1972, 1974; Rose et al, 1975). If the results of Avadhani et al and of Gaitskhoki et al are correct, they suggest that animal mitochondria contain two types of mRNA, one which is polyadenylated and has a nuclear origin and one which is not polyadenylated and is transcribed from the mitochondrial genome. This conclusion depends on 1) the correct interpretation of the kinetic data; 2) demonstration of the nature of the RNA hybridizing to nuclear DNA; and 2) the inability of isolated mitochondria to synthesize poly(A)+RNA. The latter in particular could be artifactual and such a demonstration would weaken the idea of import of mRNA into mitochondria. This problem will be examined in this thesis.

III. The mitochondrial genome

In the last 14 years since mtDNA was first isolated (Luck & Reich, 1964, Kalf, 1964; Schatz et al, 1964; Nass et al, 1965), a wealth of information has been accumulated on the size, structure, composition and replication of mtDNA (see Borst (1972) for a review). Genetic methods and the recent introduction of restriction endonuclease analysis now allow mapping of the mitochondrial genome for its transcriptional products.

A. General characteristics of mtDNA

Some of the properties of mtDNA are summarized in Table 4. Mitochondrial DNAs fall essentially into 3 categories: in vertebrates and other animals (metazoans), it is a closed circular structure with a contour length of approximately 5 μm, corresponding to a molecular weight of 10^7; in lower organisms such as Fungi and protists, it is not necessarily circular and the molecular weight ranges from 2 to 5×10^7; and in higher plants, it is circular and still larger, having a molecular weight of up to 7×10^7.

The G+C content of mtDNA from one organism to another varies considerably and there is no clear relationship between G+C content and phylogenic position of an organism. This is particularly evident in Fungi. Neurospora mtDNA has a 42% G+C content (Terpstra et al, 1977a), but in yeast it is only 18% (Bernardi et al, 1972) and in Allomyces macrogynus, a phycomycete generally considered to be more primitive than the ascomycetes, it is 42% (Dizikes & Burke, 1978). There is more consistency among mammals where it is 45 $\pm$ 5% (Vanyshin & Kirnos, 1977).

TABLE 4

Mitochondrial DNAs

Species	Structure	G+C %	Mol. Wt. ($x10^{-6}$)
Animals (from flatworm to man)	Circular	45 (± 5)*	9-12
Higher Plants	Circular		70
Fungi			
Ascomycetes			
Yeast (*Saccharomyces*)	Circular	18	49
Neurospora	Circular	40	38
Phycomyces			
A. macrogynus	Circular		52
Protozoa			
Acanthamoeba	Circular		27
Malarial parasite	Circular		18
Paramecium	Linear		27
Tetrahymena	Linear		30-36

* except *Drosophila*
(adapted from Borst (1977)

In contrast, in Drosophila melanogaster, it is only 22% (Goldring & Peacock, 1977) and that of Euglena gracilis is of the same order (Fonty et al, 1972).

As the sizes of mtDNAs are well characterized, it is possible to determine whether the mtDNAs could code for all the RNA molecules identified as transcripts. For this it is assumed that the number of proteins presumably coded by the mitochondrial genome in different organisms is essentially the same and calculations are based on the nine proteins synthesized in mitochondria of yeast (Table 1). This is probably an underestimate. Table 5 shows the percentage of mtDNA coding for rRNA, tRNA and proteins (mRNA) in yeast and human. About 20% of the genome is accounted for in yeast and 80% in human. It would thus appear that the genome of animal cells cannot code for many more than some 15 proteins. The striking difference between yeast and animal cells is reflected in the differences in genome organization in these organisms.

B. Genome organization

Restriction maps of mtDNAs have now been obtained for a number or organisms. These maps have been used to determine the position of genes by hybridization by the method of Southern (1975), electron microscopy and genetic methods. Maps are shown in Fig. 2 for yeast and Neurospora and Fig. 3 for Drosophila, Xenopus, rat and human. Some major characteristics of the mitochondrial genome are apparent from a comparison of these maps.

1) There is no evidence of duplication of rRNA genes as found in nuclear DNA (Amalric & Attardi, 1972) or chloroplast DNA (Herrmann et al, 1976).

TABLE 5

Genes on mtDNA for rRNA, 4S RNA and proteins

Organism	DNA mol. wt ($\times 10^{-6}$)	Number of copies		% DNA* coding for			
		rRNA	tRNA	rRNA	tRNA	proteins	Total
Human	9.6	1	< 26	18.5	13	46.5**	78
Yeast	49	1	≤ 26	8.1	2.5	9.1**	19.7

* calculated as duplex DNA
** calculated from values in Table 1
(adapted from Borst (1977)

Figure 2

A. Transcription map of yeast mtDNA.

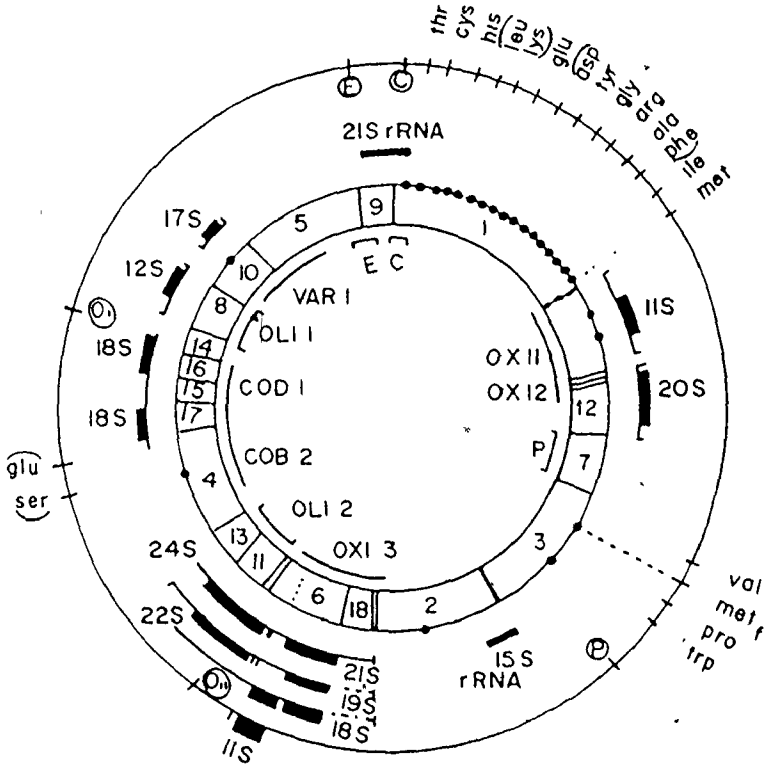
Outer circle: Position of the tRNA genes as determined by Martin et al (1977a) and Martin & Rabinowitz (1978). O_1 and O_{11} correspond to OLI-1 and OLI-2 respectively.

Inner circles: The circular map gives the fragments obtained by digesting S. carlbergensis mtDNA with a mixture of endonucleases Hind II+III and Eco RI. The dotted lines indicate extra Eco RI or Hind Hind III recognition sites present in the mtDNA of the strain KL14-4A, which have been used to get more precise map positions for some RNA species. The black dots represent 4S RNAs, two of which (shown by the dotted line with the outer circle, represent the two methionyl-tRNA species. The outer circle gives the position of larger mitochondrial RNAs, including 21S and 15S rRNAs. The inner circle gives the position of the genetic loci, taken from Fig. 1. (from Borst et al, 1977).

B. Transcription map of Neurospora crassa mtDNA

Outer circle band: Eco RI cleavage sites are drawn with solid lines within the circle. Hind III cleavage sites are indicated by arrows outside the circle, while Bam Hl sites are indicated by arrows inside the circle. The hatched regions within the circle indicate the position of the rRNA genes. Inner circle: position of the tRNA genes. One dot is tentatively equated with one tRNA gene. (from Terpstra et al, 1977a).

A. YEAST



B. NEUROSPORA CRASSA

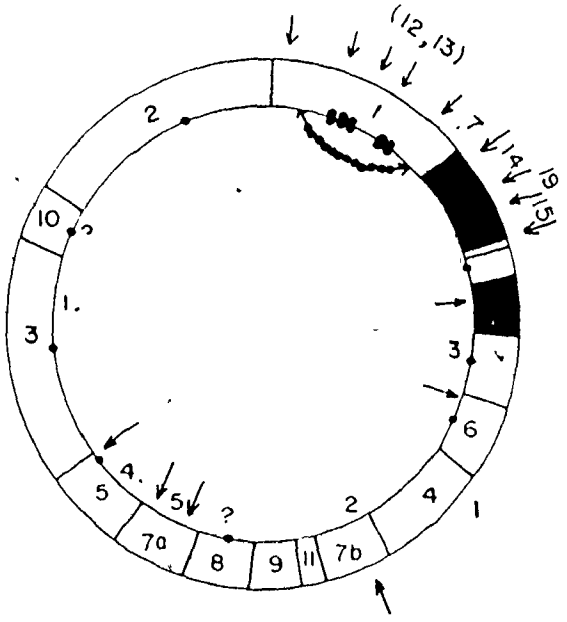


Figure 2

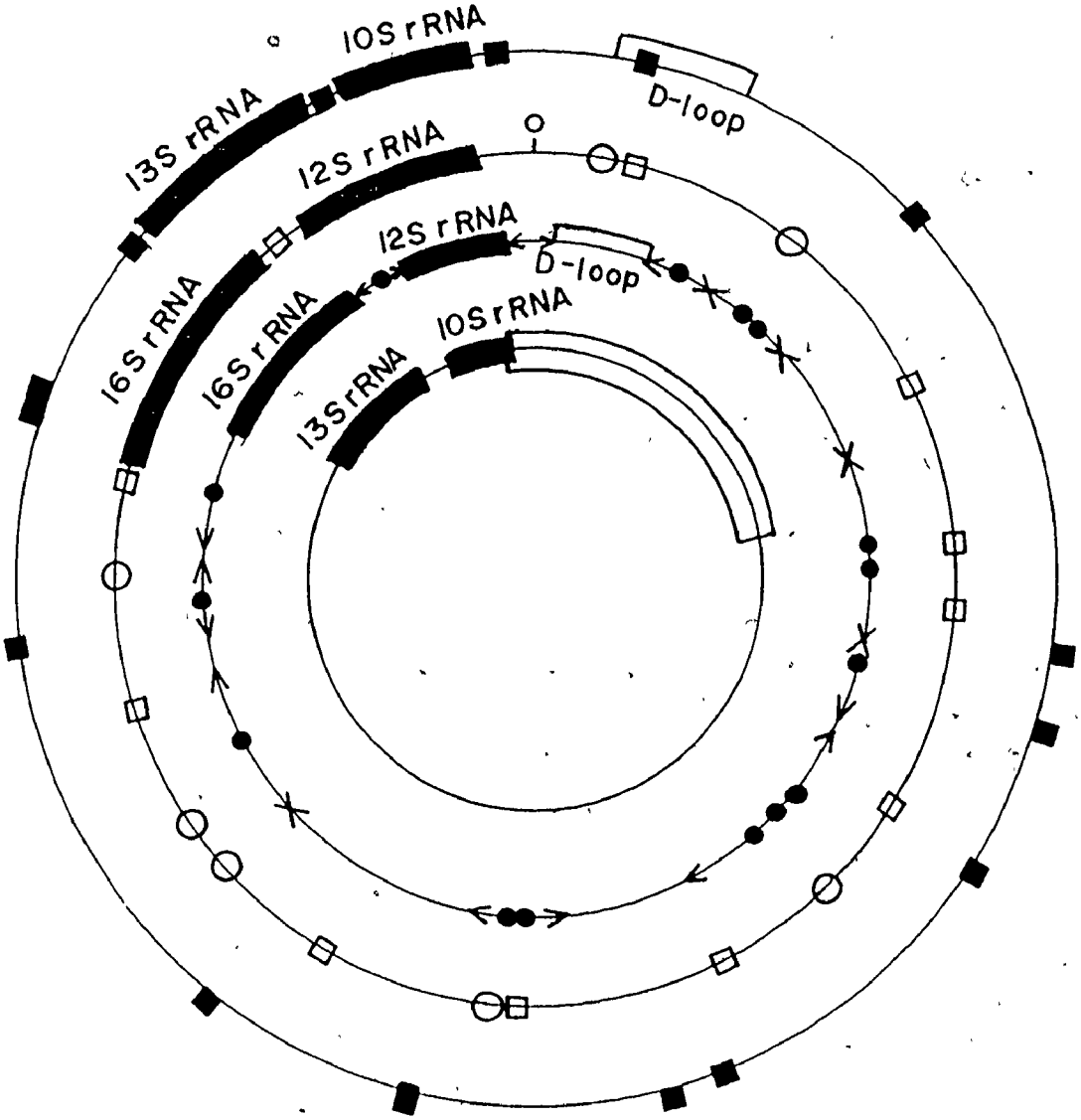


Figure 3

Localization of genes and other markers on the mitochondrial genome of different animals.

Inner circle: Drosophila melanogaster. The position of the rRNA genes of the A+T-rich region are indicated.

Second circle: rat liver. The position of the rRNA genes, of 4S RNA genes and of the D-loop are indicated.

Third circle: HeLa cell. In addition to the position of the rRNA genes and of the origin of replication (o), tRNA genes on the H strand (squares) and the L strand (circles) are shown.

Outer circle: Xenopus laevis : The rRNA genes, D-loop and tRNA genes found on the H strand are indicated.
(data adapted from Kroon & Saccone, 1976 and more recent references mentioned in the text)

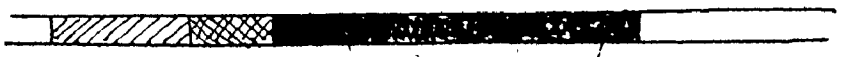
2) In both yeast and Neurospora, tRNA genes are clustered in one or more regions, but in animal cells, tRNA genes are distributed over the entire genome in what appears to be a similar arrangement (Dawid et al, 1976). As mentioned earlier, the tRNA genes are scattered on both strands of the genome in HeLa cells (Wu et al, 1972; Angerer et al, 1976; Lynch & Attardi, 1976), rat liver (Nass & Buck, 1970) and Xenopus (Dawid et al, 1976). This has not yet been examined in yeast and Neurospora.

3) The position of the rRNA genes in yeast is unusual in that the genes for the small and large rRNAs are separated by almost half the length of the genome. In contrast, they are adjacent to each other in Neurospora (Terpstra et al, 1977b), as in Drosophila (Klukas & Dawid, 1976), Xenopus (Ramirez & Dawid, 1978), rat liver (Kroon et al, 1977), mouse L cells (Battey & Clayton, 1978) and HeLa cells (Wu et al, 1972; Angerer et al, 1976). In some of these cells, the mitochondrial rRNAs map within several hundred bases of the origin of DNA replication with the smaller rRNA nearest the origin.

The 5'→3' direction of transcription of the H strand is known to be from small to large rRNA in Drosophila (Klukas & Dawid, 1976), Xenopus (Ramirez & Dawid, 1978), mouse L cell (Battey & Clayton, 1978) and HeLa cell (Attardi et al, 1976) which is opposite to the start of replication. The two rRNA genes are separated by a few hundred bases which codes for an RNA which might be a tRNA in Neurospora (Terpstra et al , 1977b), Xenopus (Dawid et al, 1976), rat liver (Saccone et al, 1977) and HeLa cell (Wu et al, 1972). Including the tentative position assigned for 5S RNA in Neurospora (Kroon et al, 1976), adjacent to the large rRNA gene, and with the exception of yeast mtDNA, the order of these genes is highly conserved and similar to that observed in prokaryotes (Lund

et al, 1976), eukaryotes (Wellauer & Dawid, 1977) and chloroplasts (Herrmann et al, 1976; Bedrook et al, 1977; Whitfield et al, 1978). The arrangement of the rRNA genes in mtDNA of plants is not yet known.

The difference in genome organisation between yeast and Neurospora is surprising in view of their possible close phylogenetic relationship, but an explanation can be provided by a consideration of the significance of intramolecular heterogeneity of G+C content found in mtDNAs. Yeast mtDNA, and possibly that of Euglena (Fonty et al, 1972) which has not been mapped, are unique in having extensive heterogeneity. In yeast, at least half of the genome has a G+C content lower than 5%, being present in approximately 70 interspersed segments (Prunell & Bernardi, 1974, 1977; Prunell et al, 1977). The larger A+T clusters are located within a certain region of the map, with smaller ones spread over the rest of the genome (Sanders & Borst, 1977). Prunell & Bernardi (1974) have presented a model of the yeast genome to account for this heterogeneity: A+T-rich sequences would constitute untranscribed "spacers" between "gene" sequences and G+C-rich smaller regions in the following fashion



	G+C-rich cluster	site cluster	gene	spacer
MW	3×10^4	2×10^4	$\approx3.5\times10^5$ (av.)	$\approx4\times10^5$ (av.)
G+C	60%	45-62%	26%	< 5%

With the possible exception of plant mtDNA (Quetier & Vedel, 1977), most other mtDNAs seem to have limited heterogeneity, possessing only one A+T-rich region constituting up to about 30% of the genome. In comparison with yeast, this region would not code for structural genes. An

A+T-rich region has been observed in Neurospora (Terpstra et al, 1977a), Drosophila (Klukas & Dawid, 1976; Goldring & Peacock, 1977) and rat liver (Shugalh et al, 1977).

In yeast, differences in mtDNA of different strains can be accounted for by major insertions and deletions in the A+T-rich sections (Sanders & Borst, 1977). This is in line with the suggestion (Bernardi et al, 1976) that the "spacers" are preferentially involved in the rearrangement of genes. Fonty et al (1978) recently presented results providing the first evidence for physical recombination of mtDNA in crosses of wild-type yeast cells. Analysis of restriction fragments indicated that recombination was very frequent in crosses and suggested that unequal crossing-over events occured in the "spacers" of "allelic" parental genetic units.

Thus the presence in yeast of A+T rich "spacer" sequences could be connected to the separation of the rRNA genes. In contrast, in organisms as distantly related as Drosophila, Xenopus and Neurospora, where there is only one A+T-rich sequence, the gene arrangement of the rRNAs has been conserved. The arrangement in yeast must represent a later independent evolution through gene recombination.

4) The mapping of mRNAs corresponding to specific mitochondrially-synthesized proteins has only started.

The yeast transcriptional map of Van Ommen & Groot (1977) is reproduced in Fig. 2. These workers fractionated 13 discrete components whose coding regions correspond closely to the positions of known genetic markers. Two of these have been shown to code for specific proteins (see Section IIC).

Battey & Clayton (1978) have mapped 9 transcripts on mouse L cell mtDNA. From their proximity to the origin of replication and their size, two of these correspond to the rRNAs. The 7 other transcripts, all coded by the H strand, cover approximately 44% of the genome. Preliminary results have been presented for HeLa cell mtDNA (Ojala et al, 1977).

The results in yeast are important in a number of ways. First, a number of RNAs of different sizes appear to be coded by the same region, particularly in the OXI-1 locus. A logical conclusion is that processing of RNA occurs, assuming the absence of aggregation or degradation of RNA during isolation and fractionation. Secondly, the sum of the calculated molecular weights of the non-overlapping RNAs, plus the rRNAs and 4S RNAs (tRNAs) account for at least 47% of the genome. This is in good agreement with the results of Hendler et al (1976) obtained by DNA-RNA hybridization, but greater than the 20% of the genome (Table 5) or 30% if some 20 proteins are mitochondrially-synthesized in yeast (Douglas &Butow, 1976) are considered, which is actually required to code for all the structural genes of known mitochondrial products. The difference could reflect either that the transcripts are actually polycistronic mRNAs for proteins not yet identified and/or that transcripts go through extensive processing.

These possibilities are important in view of Bernardi's model which implies:

1) monocistronic messages, with untranscribed "spacers".

2) coordinate expression of genes apart from each other through the interaction of regulatory proteins with operator sites having identical nucleotide sequences.

3) that transcription in yeast and animal cells occurs differently: in

animal cells, both strands are fully transcribed into RNAs which are then processed.

4) the yeast mitochondrial genome has a "eukaryotic" type of organisation in being an interspersed system of repetitive sequences.

5) recombination occurs at the level of the "spacer" sequences.

These predictions have been discussed at length by Borst et al (1977) and their conclusions can be summarized as follows:

1) The first prediction does not fit the data of Van Ommen & Groot (1977) in that spacers are transcribed. Further, Van Ommen et al (1977) have shown that not all tRNA genes are preceded by a "site cluster".

2) Experiments in Borst's group do not support the evidence of an interspersed system of repetitive sequences and of genes which are transcribed monocistronically. There is no evidence for reiterated sequences, at least as determined by DNA-DNA renaturation experiments.

3) The alternative to untranscribed A+T-rich regions is control of gene expression at the level of RNA processing and involving the A+U-rich sites in the RNA. Such a model would also account for insertions and deletions in these regions of the DNA (Fonty et al, 1978). If regulation of gene expression did take place at the level of RNA processing, the yeast mitochondrial genetic system could still be considered a "mini-replica" of the nuclear system.

In higher eukaryotes, complete symmetrical transcription of mtDNA occurs (Aloni & Attardi, 1971ab; Murphy et al, 1975) and the differential control of steady state concentrations of individual RNAs must occur exclusively at the level of processing. There is now increasing evidence that such processing occurs. In hamster mitochondria, Cleaves et al (1976)

reported the isolation of a 20S RNA precursor to the 17S rRNA. In HeLa cells, the total size of presumptive mitochondrial mRNAs is 1.5 times the size of the genome strand (H strand) which codes for them (Ojala et al, 1977; Amalric et al, 1978). Battey & Clayton (1978) observed a number of low yield transcripts of high molecular weight in mouse L cell. These hybridized to restriction fragments whose coding capacity was insufficient for their total size, suggesting that they may act as precursors. In addition, in Neurospora mitochondria, rRNAs are first transcribed as a 32S precursor which is then cleaved to give both large and small rRNAs (Kuriyama & Luck, 1973, 1974).

RNA processing signifies the presence of non-coding regions. The possibility that such sequences are present on the mitochondrial genome has been raised by other analyses of mtDNA.

In goat and sheep, Upholt & Dawid (1976, 1977) demonstrated the rapid evolution of the D-loop region (initial duplication loop of mtDNA) (see Fig. 3). It seems to consist of an evolutionary conserved area flanked by two regions of high evolutionary instability. This suggested that the D-loop contained conserved sequences important for specific functions, while other regions of this loop evolve rapidly. The presence of such regions in this and other parts of the genome could explain the heterogeneity of mtDNAs found within species and between related species. Heterogeneity could be in the form of additions, deletions and/or sequence divergence giving rise to differences in size within a population of mtDNA or in varying restriction patterns. In Drosophila melanogaster, size variations can be exclusively accounted by differences in the size of the A+T-rich region (Fauron & Wolstenholme, 1976).

Extensive restriction pattern differences have been found within a given population in yeast (Sanders et al, 1976; Sanders & Borst, 1977) and plants (Quetier & Vedel, 1977) and to a lesser extent in mouse L cells (Robberson et al, 1977) and other mammalian cells, including human cell lines (Potter et al, 1975; Parker & Watson, 1977; Robberson et al, 1977) and rat (Franscisco & Simpson, 1977; Buzzo et al, 1978; Hayashi et al, 1978), as well as between related species such as goat and sheep (Upholt & Dawid, 1977). In mammals, there is maternal inheritance of mtDNA (Hutchinson et al, 1974; Buzzo et al, 1978), so that variations in the mitochondrial genome within an animal population are conserved through natural "cloning". Thus, in rats, only 2 populations of mtDNA have been found without, as yet, any evidence that the transcriptional or translational products differ in the two populations. These observations argue for a limited number of "non-functional" regions in animal mtDNA at least.

The notion of processing and the presence of "non-functional" sequences introduces further limits on the possible coding capacity of mtDNA. Overlapping of genes and/or symmetrical transcription could increase the expected capacity. Both strands of mtDNA have genes in rat liver (Nass & Buck, 1970; Aaij et al, 1970), Xenopus (Dawid et al, 1976) and yeast (Rabinowitz et al, 1976; Hendler et al, 1976) and complete symmetrical transcription has been suggested in human cells (Aloni & Attardi, 1971ab, Young & Attardi, 1974; Murphy et al, 1975).

It has also been suggested that some mitochondrial RNAs may be of nuclear origin and imported (Suyama, 1967; Dawid, 1970, 1972). Isolated rat liver mitochondria take up synthetic polynucleotides (Swanson, 1971) and nuclear HnRNA (Gaitskhoki et al, 1973b; Kisselev et al, 1975b) which serve as templates for protein synthesis. Various groups have confirmed

these observations in other systems (Kyriakidis & Georgatsos, 1973; Grivell & Metz, 1973; Borst & Grivell, 1973). Dimitriadis & Georgatsos (1974) incubated purified globin mRNA with isolated mitochondria from Tetrahymena and showed that polypeptides with the properties of globin were apparently synthesized within mitochondria. Borst & Grivell (1973) observed transport of synthetic polynucleotides with Xenopus mitochondria only, but not those of rat, yeast or Tetrahymena . The presence of mitochondrial RNA complementary to nuclear DNA has been suggested in yeast (Humm & Humm, 1966; Wintersberger & Vielhauser, 1968; Fukuhara, 1970; De Kloet et al, 1971), Ehrlich ascites cell (Avadhani et al, 1973, 1974), rat liver (Gaitskhoki & Kisselev, 1974; Gaitskhoki et al, 1977) and Tetrahymena (Chiu et al, 1975). Some of these results can be explained by cytosolic contamination or non-specific hybridization. While import has been shown to be possible in vitro, the question is whether it takes place in vivo. The bulk of the evidence indicates that mitochondrial transcripts code for the mitochondrially-synthesized proteins. If import does occur, it should be clear from this discussion that it does not constitute a major process.

IV. Approaches to the study of mitochondrial transcriptional products

There clearly remains major unresolved problems on mitochondrial biogenesis, not the least of which is to establish the exact genetic content of mtDNA, particularly in mammalian cells. This is primarily a question of identifying the primary gene products, which, to a large extent, depends on the appropriate choice of approach. In the study of mitochondrial transcription, two approaches have been used: studies

in vivo and *in vitro*.

Studies in vivo. These depend on the identification of RNA species homologous to mtDNA. Most of the results described previously have been achieved by this method. Mitochondrial RNA is labelled in whole cells, usually in the presence of inhibitors of nuclear RNA synthesis such as camptothecin or actinomycin (Perlman *et al*, 1973; Ojala & Attardi, 1974abc) or under conditions where only the synthesis of nuclear RNA is affected (Malher & Dawidowicz, 1973; Spradling *et al*, 1977). Alternatively, RNA is extracted and labelled *in vitro*, such as with ^{125}I (Avadhani *et al*, 1975; Van Ommen *et al*, 1977) or, in the case of individual tRNAs with their respective radioactive amino acid using aminoacyl-tRNA synthetase preparations (Martin & Rabinowitz, 1978). All these methods suffer the possibility of cytosolic contamination which can be a major drawback in the identification of mitochondrial products. This and other problems have already been mentioned in previous Sections and need not be repeated here. Battey & Clayton (1978) have used a novel approach of labelling mtDNA and hybridizing it to unlabelled mitochondrial RNA. Here the difficulty is in identifying the hybridized RNA.

Studies in vitro. Transcription of the mitochondrial genome *in vitro* can be followed in one of 3 ways: 1) with isolated mtDNA translated with homologous or heterologous DNA-dpendent RNA polymerases; 2) with a coupled transcription-translation system and isolated mtDNA; and 3) with isolated mitochondria.

Some of the results obtained and problems associated with the first two approaches have already been considered in previous Sections. A recent review also deals with them at length (Saccone & Quagliariello,

1975). The third approach of using isolated mitochondria has not been widely used in the study of mitochondrial RNA although it was one of the first approaches used to study mitochondrial protein synthesis (Craddock & Simpson, 1961). A major objection to the use of isolated mitochondria might be that activities within mitochondria depend on coordinated activities of the cell's two genetic systems. Nevertheless, the approach is direct: the system constitutes a natural homologous transcription-translation system, which may or may not be coupled, under the appropriate conditions of incubation; if the mitochondrial fraction synthesizes RNA and the activity is due to mitochondria and is DNA-dependent, such experiments demonstrate RNA synthesis and transcription from mtDNA. There is much evidence that isolated mitochondria constitute an appropriate system to study mitochondrial transcription.

Since 1964, a number of laboratories have shown that isolated mitochondria, mostly in yeast, Neurospora and rat liver, incubated in appropriate media with a labelled precursor, are capable of synthesizing radioactive RNA by a mechanism having all the characteristics of transcription (Wintersberger, 1964; Luck & Reich, 1964; Neubert & Helge, 1965; Neubert et al, 1965, Saccone et al, 1967, 1968, 1969; Fukamachi et al, 1970, 1972). Synthesis was not dependent upon added DNA or RNA and was usually completely insensitive to DNase and RNase. Incorporation of labelled nucleotide precursors into RNA was sensitive to atractyloside, an inhibitor of the carrier in the inner mitochondrial membrane for ADP and ATP (Saccone et al, 1967; Fukamachi et al, 1972) as well as to ethidium bromide, actinomycin D and acriflavin (Neubert et al, 1965; Saccone et al, 1969; Fukamachi et al, 1970, 1972), indicating a DNA-dependent process. These characteristics among others differentiate

mitochondrial RNA synthesis from that of the nuclear fraction or from possible bacterial contamination. A number of properties of isolated mitochondria depend on the state of the mitochondrial fraction. The permeability of the mitochondrial membrane can be altered by using phosphate-swollen mitochondria. Under such conditions, RNA synthesis becomes partly sensitive to DNase and RNase (Neubert & Helge, 1965). Mitochondrial preparations also incorporate all four nucleotides equally, in contrast to mitochondria incubated in iso-osmotic medium where [^{3}H] ATP incorporation is much more significant than that of the other nucleotides (Saccone et al, 1968, 1969). Sensitivity to actinomycin also depends on the preparation of the mitochondrial fraction and the osmolarity of the incubation medium (Neubert & Helge, 1965; Fukamachi et al, 1972). These results point to the danger of artifacts if the proper incubations or preparations of mitochondria are not used.

The nature of the RNA synthesized has only been examined in a few cases. In general, the RNA appeared heterodisperse. In yeast, labelled RNA sedimented on sucrose gradients as rRNA-like RNA (23S and 16S) and tRNA (4S) (Wintersberger, 1966). It was shown by Groot & Van Harton Loesbrock (1976) that the RNA synthesized was not exclusively rRNA. Some RNA hybridized to restriction fragments of yeast mtDNA which do not code for rRNA. Suyama & Eyer (1968), with Tetrahymena mitochondria, observed RNA around 18 to 14S, but no tRNA. With rat liver mitochondria, Saccone et al (1969) showed radioactivity in the 14 to 8S region, while Fukamachi et al (1972) found polydisperse material with peaks in the 16S region. This RNA was shown to contain rRNA with electrophoretic mobilities of 21S and 13S on agarose-acrylamide gels (Fukamachi et al, 1970). Other

evidence that rRNA was being synthesized has come from competition experiments with cold mitochondrial rRNA (Saccone, 1973). Eighty-five percent of the RNA synthesized by isolated rat liver mitochondria was found to hybridize to mtDNA, preferentially to the H strand, although it also hybridized to a significant extent to the L strand (Aaij et al, 1970). The polydispersity of the rat liver profiles (Fukamachi et al, 1972) indicates that the RNA is not solely rRNA. In Ehrlich ascites cells, Avadhani et al (1973) isolated RNA from mitochondrial polysomes which hybridized to mtDNA. While they concluded that this RNA was mitochondrially-transcribed mRNA lacking a poly(A) tail, RNA labelled in vivo had a different size and transcriptional origin and had a poly(A) tail (see Section IIC). Other indications that RNA of a messenger nature might be synthesized by isolated mitochondria has come from kinetic studies showing short half-lives of the synthesized RNA (Lederman & Attardi, 1970; Gadaleta & Saccone, 1974). In terms of tRNA, only Wallace & Freeman (1974) have used isolated mitochondria to label specific tRNAs (see Section IIB).

Thus, while isolated mitochondria appear to synthesize RNA identical to those synthesized in vivo, a better characterization of these products remains to be done.

Objectives and Rationale

The general aim of this thesis is to further investigate whether, for RNAs, mammalian mitochondria are completely autonomous or import nuclear-coded species. While the origin of mitochondrial rRNA (with the exception of "5S RNA", Section IIA) is essentially resolved, the nature of the mRNAs is still unclear and the number and genetic origin of

mitochondrial tRNAs is not yet known.

These problems will be examined using isolated mitochondria to label the RNAs. The specific questions which I wish to answer are the following:

1) Mitochondrial mRNAs

The results of Avadhani et al (1973, 1974) raise important questions: Is there any mRNA imported from the cytosol? Does mitochondrial mRNA possess a poly(A) tail? As discussed above, Avadhani et al (1973, 1974) suggested that poly(A)+RNAs are imported into mitochondria. One of their major arguments for this conclusion was their inability to detect poly(A)+ RNA synthesis by isolated Ehrlich ascites cell mitochondria. This result contradicts results in whole cells by other groups. To resolve this, the possible synthesis of poly(A)+RNA by isolated mitochondria was re-examined. These studies are presented in Part I.

2) Mitochondrial tRNAs

The number and genetic origin of mitochondrial tRNAs has not yet been resolved. As discussed in Section IIB, a number of alternatives are possible to explain present results. Resolution of these questions depends on the ability to isolate, characterize and determine unambiguously the genetic origin of all mitochondrial tRNAs. tRNAS for a number of amino acids were not observed when mitochondrial tRNAs were charged by isolated aminoacyl-tRNA synthetases. This might reflect a limitation of the technique. Isolated mitochondria might serve as an appropriate system to charge individual tRNAs. Under conditions of protein synthesis, it would be expected that tRNAs for all amino acids would be charged. The more specific questions which will be considered are:

a) What are the sizes of mitochondrial tRNAs and how do they compare with those of their cytosolic counterparts? This question is raised in part by the results of Dubin & Friend (1972). Further calculation of the number of tRNA genes by saturation hybridization requires a knowledge of the size of mitochondrial tRNAs.

b) Do isolated mitochondria incorporate all amino acids into proteins? This is equivalent to asking whether mitochondria have tRNAs corresponding to each amino acid.

c) Do mitochondria contain distinctive tRNAs for all amino acids and are the tRNAs coded by the mitochondrial genome?

d) As isoaccepting species might be necessary to read all codons, are there such species for some amino acids and are they coded by separate genes?

PART I

THE SYNTHESIS OF POLY(A)+RNA BY ISOLATED MITOCHONDRIA

METHODS

Growth and maintainance of cells

Ehrlich ascites cells were maintained in 8-10 week old Swiss-Webster mice as described by Haldar & Freeman (1968) and were collected from 7 to 10 days after peritoneal injection. The yield was approximately $5x10^8$ cells per mouse.

Isolation of mitochondria

Ehrlich ascites cells from up to 12 mice were washed twice with 200 ml of ice-cold 0.137 M NaCl/ 0.027 M KCl/ 0.015 M KH_2PO_4/ 0.081 M Na_2HPO_4, pH 7.4 (PBS), twice with ice cold 0.3 M sucrose/ 2 mM EDTA/ 2 mM Tris-HCl, pH 7.4 (Medium B) and then suspended in 10 ml of Medium B before disruption with an Ultra-turrax as described by Freeman (1965). After disruption, nuclei and cell debris were removed at 2,000g for 4 min in a Sorval RC2-B refrigerated centrifuge and the pellet homogenized briefly in 10 ml of Medium B and recentrifuged. The combined supernatants were centrifuged first at 2,000g for 4 min to remove further nuclei or nuclear fragments, then at 5,000g for 10 min. The mitochondrial pellet was washed once in 0.25 M sucrose/ 2 mM EDTA, pH 7.4 (SE) before incubation.

Mitochondria were isolated from livers of 150 g Sprague-

Dawley rats essentially as described by Fukamachi et al (1972). The livers were washed in SE, minced and homogenized with a Thomas teflon homogenizer. Nuclei, unbroken cells and red blood cells were removed by 2 centrifugations at 1000g for 10 min in a Sorval R2-B refrigerated centrifuge. The supernatant was then centrifuged at 6,500g for 10 min. The pellet was resuspended, centrifuged again at 1,000g, then at 6,500g twice for 10 min. The mitochondrial fraction was resuspended in a small volume of SE prior to incubation. This procedure yielded 5 to 10 mg of mitochondrial protein per g of liver. This is 25 to 50% of that reported by others (Borst et al, 1967; Chia et al, 1976) and is the result of the third 1,000g centrifugation.

Incubation of isolated mitochondria

Mitochondrial fractions were incubated in 6 ml of the hypo-osmotic medium described by Fukamachi et al (1972), unless otherwise specified. The final incubation medium contained 3 mg protein/ml and 4 mM KCL/ 7 mM $MgCl_2$/ 5 mM KH_2PO_4/ 7.5 mM succinate/ 0.45 mM malate/ 7.5 mM pyruvate/ 0.1 mM of each of the unlabelled nucleotides/ 33.4 mM sucrose/ 0.27 mM EDTA, pH 7.4. Either [^{3}H] UTP (50 µCi/ml) or [^{3}H] AMP (25 µCi/ml) were used to label the RNA. Incubations were in acid-washed 125 ml Erlenmeyer flasks at 30°C usually for 30 min, using a shaking water-bath set at 80-100 oscillations/min. Following incubation, mitochondria were recovered by centrifugation, washed once with SE and the RNA extracted.

Isolation of RNA

RNA was isolated by the cold-phenol method used by Fukamachi et al (1972) to obtain total RNA from rat liver mitochondria. This is a modification of a method of Kirby (1965). About 1 mg rat liver rRNA isolated by the method of Kirby (1965) was used in each extraction as carrier. RNA was recovered by precipitation with 2.5 volumes of 95% (v/v) ethanol at -20°C overnight.

Isolation of poly(A)+RNA

Poly(A)+RNA was recovered by adsorption on and elution from oligo(dT)-cellulose as described by Hirsch & Penman (1973). RNA was dissolved in a small volume of binding buffer (0.4 M NaCl/ 1 mM EDTA/ 10% glyceral/ 0.1% SDS/ 10 mM Tris-HCl, pH 7.4) and applied to a column of 100 mg oligo(dT)-cellulose previously equilibrated with the same buffer. After washing with 10 ml of buffer, the poly(A)+RNA was eluted with low salt elution buffer (0.1% SDS/ 1 mM EDTA/ 10 mM Tris-HCl, pH 7.4). The eluate was made up to 0.1 M NaCl and the RNA precipitated with 95% (v/v) ethanol or used directly.

Alternatively, poly(A)+RNA was adsorbed on Millipore filters according to method B of Lee et al (1971). In this case, the RNA was suspended in 10 volumes of ice-cold buffer (0.5 M KCl/ 1 mM $MgCl_2$/ 10 mM Tris-HCl, pH 7.4). After 10 min at 0°C, the solution was filtered through a Millipore filter (0.45 µm) previously soaked with and washed twice with 10 ml of the same ice-cold buffer. The filters were then dried and counted for radioactivity.

Isolation of poly(A)

Poly(A)+RNA was dissolved in sterile water and buffer and then enzyme added to give a final solution of 20 µg pancreatic RNase A and 10 units RNase T_1 in 100 µl of 0.45 M KCl/ 15 mM Tris-HCl, pH 7.4 in a 1 ml Eppendorf microtube. After digestion for 30 min at 37°C, 1 ml of 0.1 M NaCl/ 2% SDS/ 25 mM sodium acetate, pH 8.5 was added and RNA extracted with an equal volume of water saturated phenol at room temperature. The RNA was precipitated from the aqueous phase with 2.5 volumes of 95% (v/v) ethanol at -20°C overnight.

Denaturation of poly(A)+RNA

Total RNA was dissolved in 0.1% SDS/ 1 mM EDTA/ 10 mM Tris-HCl, pH 7.4, heated at 70°C for 5 min (Ojala & Attardi, 1974b) and placed at 0°C for rapid cooling. The solution was then adjusted to 0.4 M NaCl and the poly(A)+RNA adsorbed on an oligo(dT)-cellulose. After elution from the column as described above, the poly(A)+RNA was either analysed directly or denatured again depending on the experiment.

SDS-polyacrylamide gel electrophoresis

Poly(A) and poly(A)+RNA were analysed on 6x60 mm gels containing 14% acrylamide/ 0.25% bis-acrylamide prepared and run in Buffer E (1 mM EDTA/ 0.2% SDS/ 36 mM NaH_2PO_4/ 30 mM Tris-HCl, pH 7.4) according to Loening (1969). Electrophoresis was usually for 3 h at 8 V/ cm. Alternatively, poly(A)+RNA was run on 10% polyacrylamide gels containing 5% formaldehyde. Details of the gel composition are given in Methods to Part II.

Sucrose density-gradient centrifugation

A number of different conditions were used to analyse poly(A)+RNA. (1) An isokinetic, 15 to 33% (w/v) sucrose density-gradient in 0.1 M NaCl/ 1 mM EDTA/ 0.2% SDS/ 10 mM Tris-HCl, pH 7.0 at 24°C for 16 h at 69,000g (Bartoov et al, 1970). In this case, poly(A)+RNA eluted from oligo(dT)-cellulose column was used directly for centrifugation.

(2) A linear 5 to 20% (w/v) sucrose density gradient in 0.25 mM EDTA/ 0.2% SDS/ 1 mM Tris-HCl, pH 7.4 with or without 1% formaldehyde at 24°C and 108,000g for 18 h.

(3) A linear 5 to 20% (w/v) sucrose density-gradient in 0.25 mM EDTA/ 0.1 M LiCl/ 0.2% SDS/ 1 mM Tris-HCl, pH 7.4 with or without formaldehyde at 4°C and 108,000g for 18 h.

Poly(A)+RNA was either layered directly over the gradient following elution from the oligo(dT)-cellulose column or denatured for 5 min at 70°C in either one of the 5% sucrose buffer solutions prior to centrifugation. In some cases, the poly(A)+RNA was first precipitated with 95% (v/v) ehtanol at -20°C, dissolved in 0.25 mM EDTA/ 0.2% SDS/ 1 mM Tris-HCl, pH 7.4 with or without 1% formaldehyde and denatured before centrifugation.

Centifugation was in the SW 41 rotor of the Beckman L2-65B centrifuge.

Gradients were dripped from the bottom and fractions of approximately 0.4 ml were collected and counted for radioactivity.

Alkaline hydrolysis

The procedure followed was that of Fukamachi et al (1972). RNA

was precipitated with 5% trichloroacetic acid, washed twice with 5 ml of 2% potassium acetate in 95% (v/v) ethanol, followed by ethanol-ether (3:1, v/v) and ether. Hydrolysis was performed in 2 ml of 0.3 N KOH for 18 h at 37°C. The reaction was stoppoed by acidifying to pH 6.0 with 6 N $HClO_4$ at 0°C. Precipitated material was removed by centrifugation and the supernatant neutralized with KOH and left for 2 to 3 h at 0°C. After removing further precipitate by centrifugation, the solution was lyophilized. The resulting nucleotides were resuspended in 0.2 ml of 0.5 N NH_4OH and separated by descending chromatogrphy on Whatman No. 1 paper with isobutyric acid-ammonia-water (66:1:33, v/v/v) as solvent for 18 h. The paper was dried, cut and counted for radioactivity.

Preparation of marker rRNA

Cytosolic rRNA was obtained from Chinese hamster ovary cells. Cells were grown in 200 ml of suspension culture as described by Wallace et al (1975). rRNA was extracted after overnight labelling of $5x10^5$ cells/ml with 1 µCi [^{14}C] uridine/ml by method 1 of Kirby (1965) in the presence of 1 mg carrier yeast rRNA.

Preparation of poly(A) from [^{3}H] adenosine-labelled cytosolic poly(A)+RNA

A Swiss-Webster mouse, containing 7-day Ehrlich ascites cells was injected with 150 µCi [^{3}H] adenosine. After 24 h, the cells were recovered, disrupted and the RNA extracted from the 10,000g supernatant by method 1 of Kirby (1965). Poly(A)+RNA was recovered on an oligo(dT)-cellulose column and poly(A) obtained as described for mitochondrial RNA.

Measurement of radioactivity

Samples of RNA, poly(A)+RNA or poly(A) were dried on Whatman 3MM filter disks, washed with two changes of ice-cold 5% trichloroacetic acid, followed by ethanol-ether (1:1, v/v) and ether. Radioactivity was measured in a toluene-based scientillation solution (Haldar & Freeman, 1968). Millipore filters and paper chromatograms were also counted in this solvent. Fractions from sucrose density gradients were counted with 0.3 ml H_2O and 3 ml PCS. Gels were cut in 1 mm slices using a Gilson aliquogel fractionator, digested overnight in 0.15 ml NCS-toluene (2:3, v/v) and counted with 3 ml of naphtalene-dioxane scintillation solvent (Haldar & Freeman, 1968). A Nuclear Chicago Mark I liquid scintillation spectrometer was used for counting.

Protein determination

Protein was routinely measured by optical density, assuming that a solution of 1 mg protein/ml in 1 N NaOH has an absorption of 1 at 280 nm. Identical results were obtained by the method of Lowry et al (1951) with bovine serum albumin as the standard.

Buffers

Medium B: 0.3 M sucrose
2 mM EDTA
2 mM Tris-HCl, pH 7.4

SE: 0.25 M sucrose
2 mM EDTA pH 7.4

PBS: 0.137 M NaCl
27 mM KCl
15 mM
81 mM Na_2HPO_4, pH 7.4

Buffer E: 36 mM NaH_2PO_4
1 mM EDTA
0.2% SDS
30 mM Tris-HCl, pH 7.4

MATERIALS

The source of most materials is tabulated below.

Material	Source
Acrylamide	Eastman Organic Chemicals Co.
[^{3}H] AMP (22 Ci/mmol)	Amersham/Searle
[^{3}H] adenosine (26 Ci/mmol)	" "
ATP	Boehringer Mannheim Co.
Atractyloside	Calbiochem
Bis-acrylamide	Eastman Organic Chemicals Co.
CTP	Boehringer Mannheim Co.
Deoxyribonuclease I	Worthington Biochemical Corp.
Ethidium bromide	Calbiochem
GTP	Boehringer Mannheim Co.
Oligo(dT)-cellulose	Collaborative Research
[^{3}H] poly(A) (18.7 Ci/mmol)	Schwartz-Mann
PCS scintillation fluid	Amersham/ Searle
Ribonuclease A	Worthington Biochemical Corp.
Immobilized RNase A (500 units/mg)	Worthington Biochemical Corp.
T_1 Ribonuclease (500,000 units/ml)	Sigma
[^{14}C]uridine (50mCi/mmol)	Amersham/Searle
UTP	Boehringer Mannheim Co.
[^{3}H] UTP (17 Ci/mmol)	Amersham/Searle

RESULTS

RNA synthesis by isolated mitochondria

The incorporation of label into poly(A)+RNA was routinely measured by following the incorporation of radioactivity into RNA that bound to oligo(dT)-cellulose, which is known to bind poly(A)-containing RNA selectively (Edmonds & Caramela, 1969; Aviv & Leder, 1972). Fig. 4A and B show typical results for both [^{3}H]UTP and [^{3}H]AMP incorporation into poly(A)+RNA by Ehrlich ascites cell mitochondria over a one hour time course. The incorporation into total acid-insoluble material was essentially linear over a one hour period as observed previously by Fukamachi et al (1972) for rat liver mitochondria. On the other hand, the incorporation of [^{3}H]AMP into material binding to oligo(dT)-cellulose (Fig. 4A) was rapid during the first 10 min and little further incorporation was observed during the next 50 min. In contrast, the incorporation of [^{3}H] UTP showed a lag, then was linear until 30 min before levelling off. The difference in the first 10 min between the incorporation of the two precursors into presumed poly(A)+RNA might be a reflection of the post-transcriptional synthesis of the poly(A) tail. The levelling off of [^{3}H]UTP incorporation and the plateau observed for [^{3}H]AMP incorporation could be due to the rapid turnover of either poly(A) or poly(A)+RNA or both, but not to a decline in RNA synthesis since total RNA synthesis continued.

Fukamachi et al (1972) observed that [^{3}H]ATP incorporation into

Figure 4

Incorporation of [^{3}H] AMP (A) and [^{3}H] UTP (B) into total and poly(A)+RNA by isolated ascites cell mitochondria. Mitochondria were isolated and incubated with 25 μCi of [^{3}H] AMP/ml or 50 μCi [^{3}H] UTP/ml in a total volume of 6 ml at 30°C. At each time interval a 0.5 ml sample was taken total RNA and poly(A)+RNA were isolated and the incorporation into each as trichloroacetic acid-precipitable counts was determined. ●, Incorporation into total acid-insoluble material; o, into poly(A)+RNA; □, into poly(A)+RNA in a separate experiment.

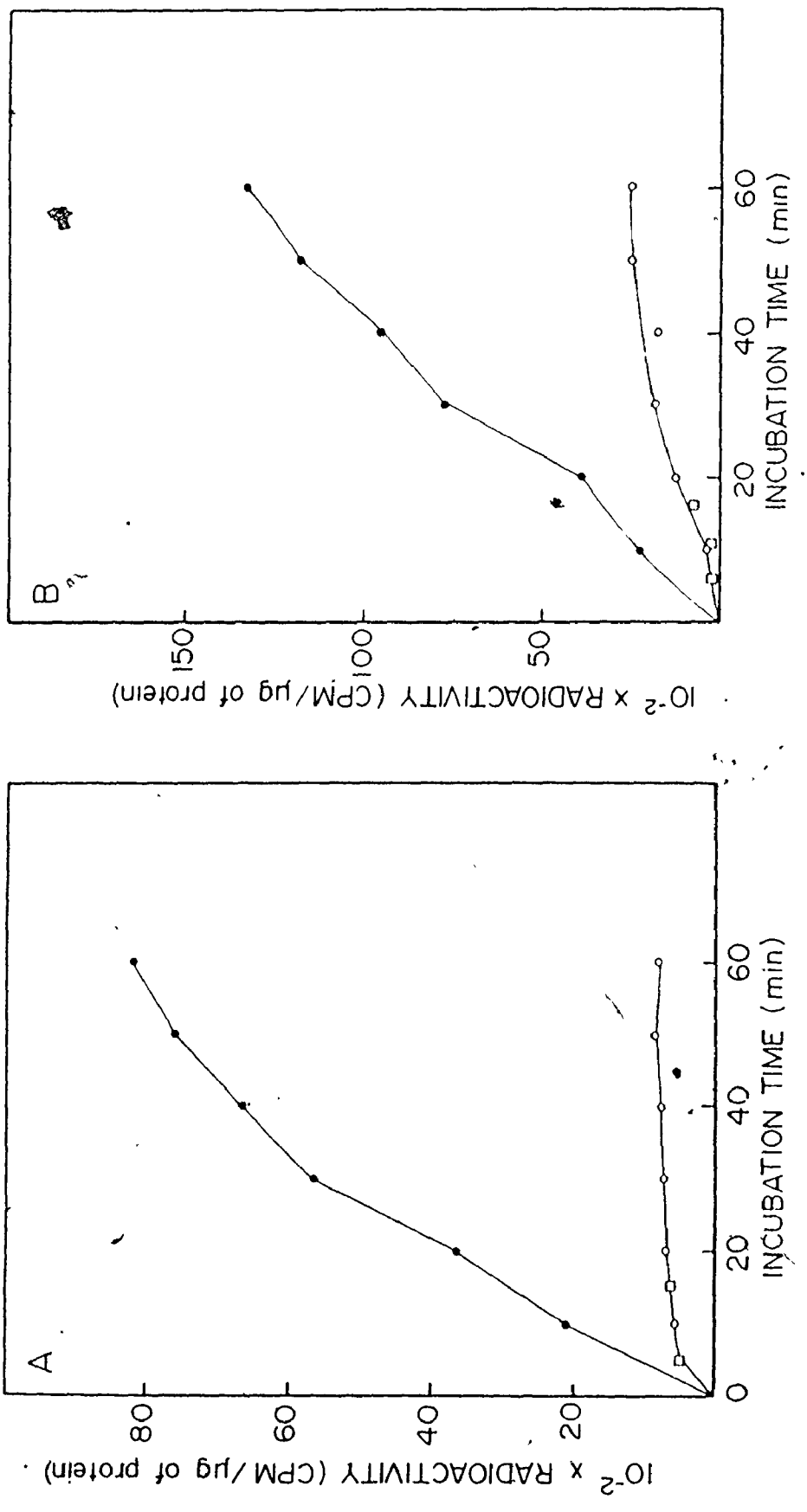

A
B
10^{-2} x RADIOACTIVITY (CPM/µg of protein)
INCUBATION TIME (min)
0
20
40
60
80
100
150

Figure 4

RNA represented only 15% of the total insoluble incorporation by isolated rat liver mitochondria. Thus, the difference in incorporation between total [^{3}H]AMP-labelled acid-insoluble material and that binding to oligo(dT)-cellulose could reflect the incorporation of [^{3}H] AMP into non-RNA products. For this reason, it was important to determine whether [^{3}H] AMP-labelled material, binding to oligo(dT)-cellulose was indeed RNA. Acid-precipitable material from the total RNA extracted from isolated Ehrlich ascites cell mitochondria also yielded an unidentified compound running between ADP and ATP as observed by Fukamachi et al (1972), but, as shown in Fig. 5, [^{3}H] 2'(3') AMP was the major component detected in the hydrolysis of presumed poly(A)+RNA, indicating that it was RNA.

While binding to oligo(dT)-cellulose is a characteristic of poly(A) and poly(A)+RNA, conclusive evidence that the RNA bound to oligo(dT)-cellulose was poly(A)+RNA and not RNA non-specifically bound, was necessary.

In addition to oligo(dT)-cellulose binding, poly(A) is usually measured by its ability to bind to Millipore filters or to poly(U)-Sepharose (Molloy et al, 1974). As shown in Table 6, the first two methods gave essentially identical results. Approximately 25% of the [^{3}H] UTP-labelled RNA from Ehrlich ascites cell mitochondria was reproducibly retained by oligo(dT)-cellulose or on Millipore filters; with [^{3}H] AMP as the precursor, about 30% was retained after a 30 min incubation. However, over a series of experiments, this value ranged between 10% (see Fig. 4A) to 35%. It is possible that such variations depended on the amount of [^{3}H] AMP-labelled material which was not RNA and was extracted with the RNA.

Figure 5

Paper chromatographic profile of a poly(A)+RNA hydrolysate. Ehrlich ascites cell mitochondria were isolated and incubated with 25 μCi [^{3}H] AMP/ml. Poly(A)+RNA was isolated and hydrolysed as described in the Methods. Paper chromatography was for 18 h on Whatman No. 1 paper with isobutyric acid-ammonia-water (66:1:33, v/v/v) as the solvent. Markers were run on the same paper and their positions determined with ultraviolet light. Background counts were not subtracted.

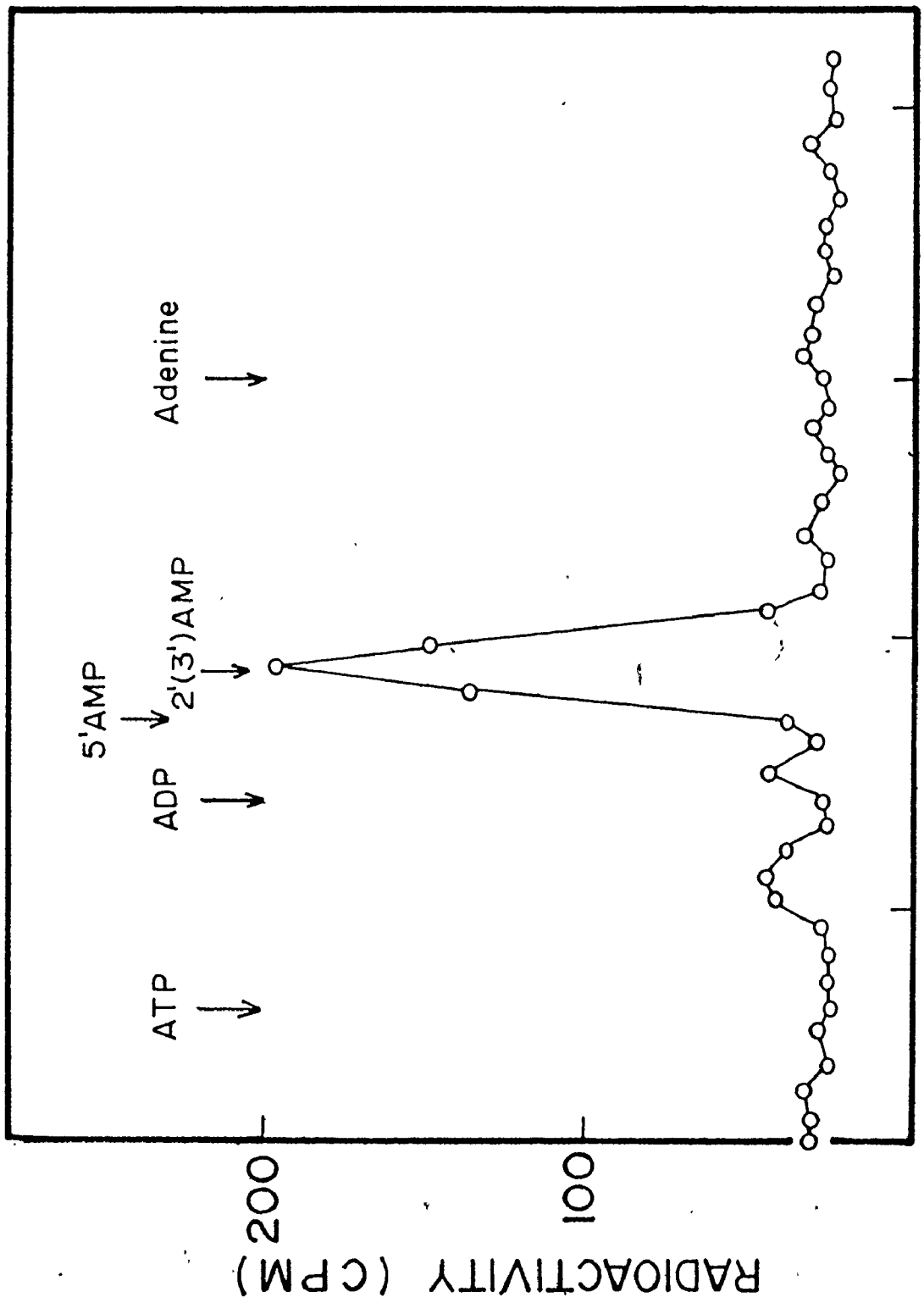

RADIOACTIVITY (CPM)
200
100
0
10
20
30
40
DISTANCE (cm)
ATP
ADP
5'AMP
2'(3')AMP
Adenine

Figure 5

TABLE 6

Incorporation of [^{3}H]UTP and [^{3}H]AMP into ascites cell and rat liver mitochondrial total RNA, poly(A)+RNA and poly(A)

Mitochondria were incubated and RNA and poly(A)+RNA isolated from 6 ml incubations (30°C, 30 min) as described in the Methods. The control incorporation was about 70,000 cpm for each of [^{3}H]UTP (incubations with 50 µCi/ml) and [^{3}H]AMP (incubations with 25 µCi/ml) and 14,000cpm for the incubation with ethidium bromide in the case of Ehrlich ascites cells and 8,000 cpm in the case of rat liver.

Type of RNA	Method of Fractionation	Label [^{3}H]UTP % control	Label [^{3}H]AMP % control
Ascites cell			
control			
Total		100	100
poly(A)+RNA	oligo(dT)-cellulose	27	28
	Millipore filter	24	31
poly(A)	oligo(dT)-cellulose	-	3
	Millipore filter	-	negligible
+ ethidium bromide (3 µg/ml)			
Total			100
poly(A)+RNA	oligo(dT)-cellulose		22
	Millipore filter		-
poly(A)	oligo(dT)cellulose		8.4
	Millipore filter		negligible
Rat liver			
control		-	100
poly(A)+RNA	oligo(dT)-cellulose	-	5
poly(A)	oligo(dT)cellulose	-	0.51

-, No determination was made.

With rat liver mitochondria, incorporation into [^{3}H] AMP-labelled poly(A)+RNA by isolated mitochondria was usually of the order of 5%. As well, total incorporation into acid insoluble material was up to ten fold lower than for Ehrlich ascites cell mitochondria. For this reason, most experiments were performed using Ehrlich ascites cells and not rat liver.

Characterization of the poly(A) tail of poly(A)+RNA

The presence of poly(A) in the material binding to and eluting from oligo(dT)-cellulose was established by hydrolysis with pancreatic RNase and T_1 RNase in 0.45M KCl. The [^{3}H] AMP-labelled RNase-resistant material was extracted with phenol at alkaline pH, bound to and eluted from oligo(dT)-cellulose and characterized by polyacrylamide gel electrophoresis. As shown in Fig. 6, the RNase-resistant material of the RNA extracted from Ehrlich ascites cell mitochondria migrated with a peak of slightly faster mobility than $4S_E$ with some more rapidly running material. In comparison, Ehrlich ascites cell cytosolic poly(A) obtained from cytosolic, oligo(dT)-cellulose-binding RNA and isolated by the same procedure described for mitochondrial poly(A), had a peak of $7S_E$ (Fig. 6). Under the same conditions of hydrolysis, the profile of synthetic [^{3}H] poly(A) (Schwartz-Mann), as determined by gel electrophoresis, was not altered. The fact that a cytosolic $7S_E$ peak was found, as observed by other workers (Edmonds et al, 1971; Adesnik et al, 1972; Greenberg & Perry, 1972), indicates that the $4S_E$ poly(A) peak was not the product of partial digestion of contaminating cytosolic poly(A). Further proof that the observed peak was indeed poly(A) was given by the absence of a peak [^{3}H] UTP-labelled RNA was treated in the same manner as shown in

Figure 6

Polyacrylamide gel electrophoresis of the poly(A) tail of Ehrlich ascites cell mitochondrial and cytosolic poly(A)+RNA. Mitochondria were isolated and incubated with 25 μCi of [^{3}H] AMP/ml or 50 μCi of [^{3}H] UTP/ml in a total volume of 6 ml at 30°C for 30 min. The RNA and poly(A)+RNA were isolated and the poly(A)+RNA digested with T_1 and pancreatic RNases, as described in the Methods. The poly(A) was recovered, re-adsorbed and eluted from oligo(dT)-cellulose and characterized on 14% polyacrylamide gels. The cytosolic poly(A) was obtained as described in the Methods. Electrophoresis was for 3.5 h at 8 V/cm. The gels were sliced and counted for radioactivity as described in the Methods. The marker was E. coli tRNA run on a separate gel and localized by scanning the gel at 260 nm on a Gilford spectrophotometer. •, Cytosolic poly(A); o, mitochondrial poly(A) labelled with [^{3}H] AMP; Δ, mitochondrial poly(A) labelled with [^{3}H] UTP.

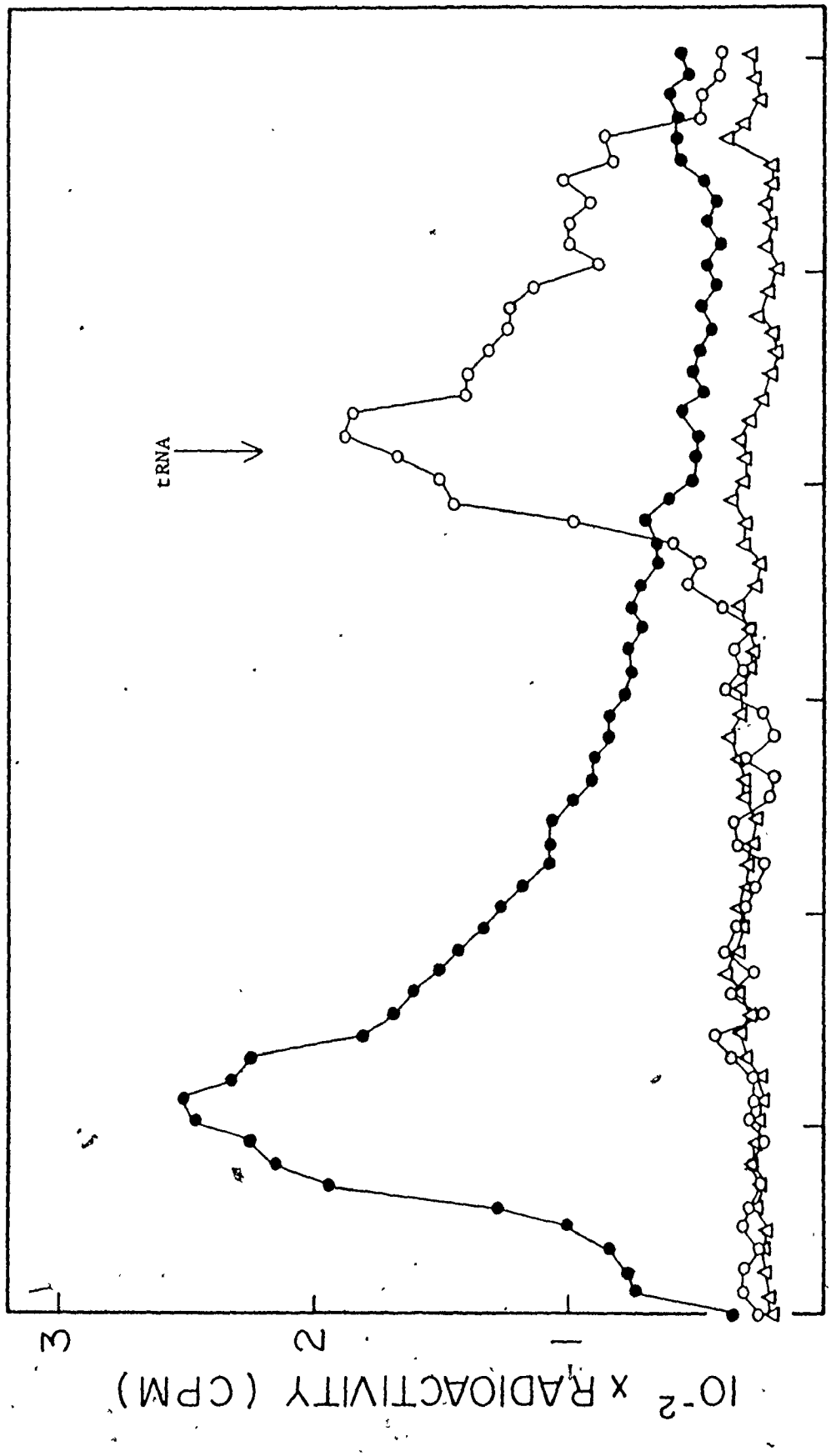

tRNA
DISTANCE FROM ORIGIN (mm)
0
10
20
30
40
50
60
1
2
3
10^{-2} x RADIOACTIVITY (CPM)

Figure 6

Fig. 6.

From the above, it can be concluded that the $4S_E$ peak observed from Ehrlich ascites cell mitochondrial poly(A)+RNA is poly(A).

Fig. 7 shows that a similar $4S_E$ peak was isolated in a similar experiment with rat liver mitochondria. The heterogeneity of the peak was probably the result of contamination by oligonucleotides as the RNase-resistant material was not, in this case, bound to oligo(dT)-cellulose following RNase digestion. A similar phenomenon was found for Ehrlich ascites cell mitochondrial poly(A). The material running faster than the $4S_E$ peak in Fig. 6 was most likely poly(A) and not undigested oligonucleotides as it was not labelled with [^{3}H] UTP and was retained by oligo(dT)-cellulose. This point was not pursued further in the case of rat liver mitochondria because of the low incorporation in this system.

While the poly(A)+RNA from Ehrlich ascites cell mitochondria could be bound equally by oligo(dT)-cellulose and Millipore filters, the corresponding poly(A) segment was found to bind only to the former (Table 6). Diez & Brawerman (1974) had previously indicated that Millipore filters did not retain short poly(A) stretches. The present results suggest that the length of the poly(A) stretch is not the only reason for retention of RNA or lack thereof on Millipore filters and that some other factor, such as interaction of poly(A) and at least part of the rest of the RNA, may be involved in binding.

The presence of free poly(A) was tested for by running undigested [^{3}H] AMP-labelled poly(A)+RNA on polyacrylamide gels under denaturing conditions. This was necessary because the poly(A) may be involved in the formation of aggregates due to the lack of secondary structure at

Figure 7

Polyacrylamide gel electrophoresis of the poly(A) tail of rat liver mitochondrial poly(A)+RNA. Rat liver mitochondria were isolated as described in the Methods. Incubation of mitochondria, isolation of poly(A)+RNA and poly(A), gel electrophoresis and counting were as in Fig. 6, except that poly(A) was not fractionated a second time on oligo(dT)-cellulose following RNase hydrolysis of poly(A)+RNA.

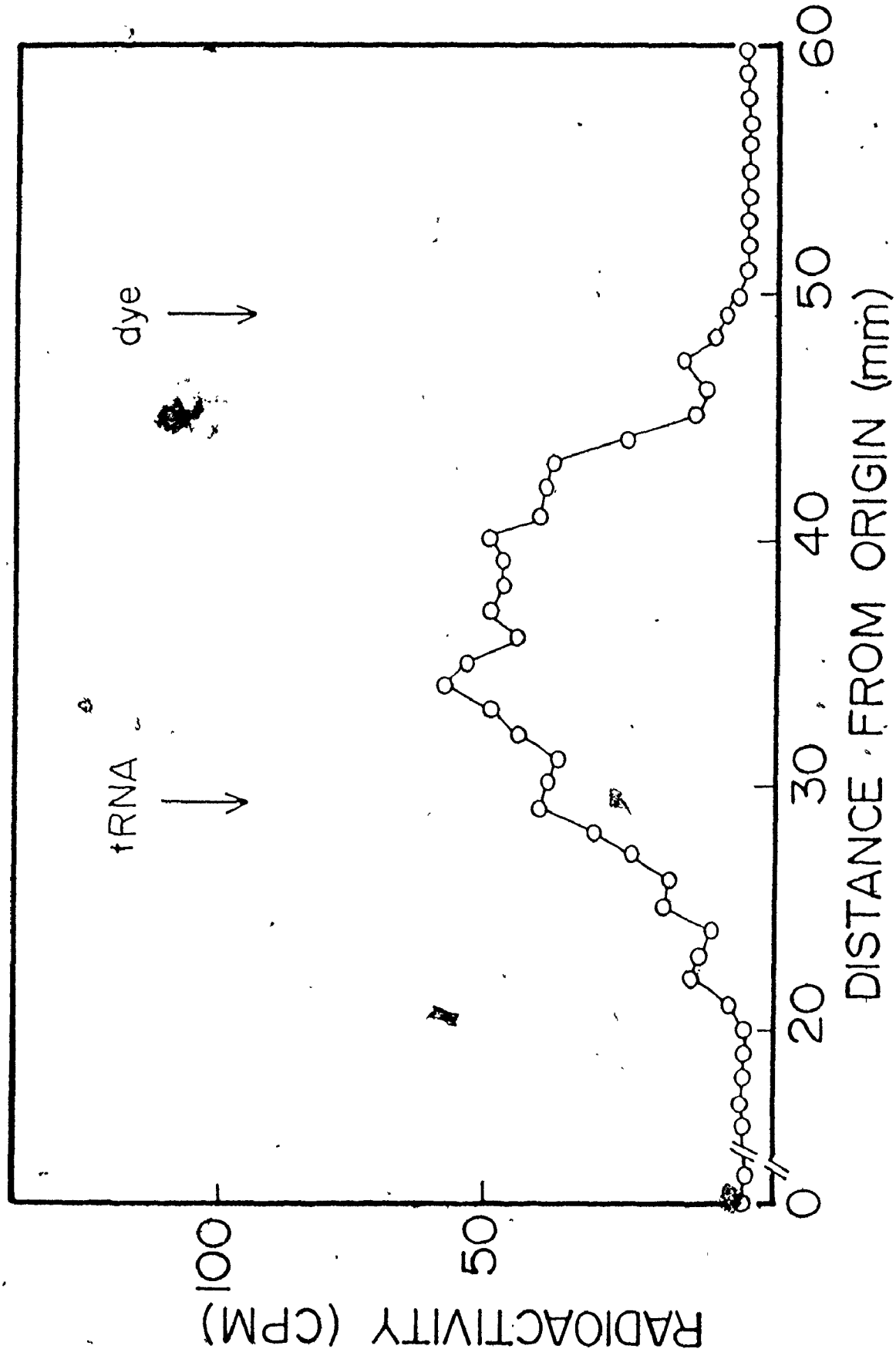

RADIOACTIVITY (CPM)
100
50
0
20
30
40
50
60
DISTANCE FROM ORIGIN (mm)
tRNA
dye

Figure 7

neutral pH (Rich et al, 1961), making it available for base pairing with complementary sequences (Ojala & Attardi, 1974b). On a 10% acrylamide gel containing 5% formaldehyde, all the material was retained at the top of the gel with no peak corresponding to the $4S_E$ poly(A) (results not shown). This result is similar to that of Hirsch & Penman (1974a) for HeLa cell mitochondrial poly(A)+RNA.

Mitochondrial origin of poly(A)+RNA

The difference in size between the mitochondrial and cytosolic poly(A) tail suggested that the poly(A)+RNA was not synthesized at the same site as the cytosolic poly(A)+RNA, but it was still possible that the poly(A) and poly(A)+RNA were synthesized in nuclei or in some other organelle contaminating the mitochondrial fraction. To prevent possible synthesis by nuclei, [^{3}H] AMP was used instead of [^{3}H] ATP, as isolated nuclei incorporate nucleotides from nucleoside triphosphates whereas the nucleoside monophosphate would be phosphorylated in mitochondria.

The site of synthesis was examined with selective inhibitors. As shown in Table 7, atractyloside, an inhibitor of adenine nucleotide translocation into mitochondria (Winkler & Lehninger, 1968; Vignais & Vignais,1970) and ethidium bromide, an inhibitor of labelling of RNA in mitochondria (Zylber et al, 1969; Fukamachi et al, 1972; Hirsch & Penman, 1974ab), both inhibited [^{3}H] AMP incorporation into poly(A)+RNA by Ehrlich ascites cell mitochondria up to 80%. The characterization of the RNA whose synthesis was not inhibited by ethidium bromide is given below. The atractyloside and ethidium bromide concentrations (216 μM and 3 μg/ml of incubation medium respectively) were chosen to give the

TABLE 7

Effect of Inhibitors on [^{3}H]AMP Incorporation

The experiment was performed as for Table 6. In one experiment, the mitochondria were washed after the incubation with Medium B and then incubated in Medium B for 15 min at 30°C in the presence of 1 unit of immobilized RNase/ml.

Inhibitor	Fractions	
	Total	poly(A)+RNA
	% control	
Control	100	100*
+ 210 µM atractyloside		
ascites cell mitochondria	22	20
rat liver mitochondria		
hypo-osmotic	30	28
iso-osmotic	32	17
+ 3 µg ethidium bromide/ml (ascites)	20	20
+ immobilized RNase (ascites)	81	90

* poly(A)+RNA was 10% of total incorporation in the case of Ehrlich ascites cell mitochondria and 5% in the case of rat liver mitochondria.

highest inhibition of total incorporation as previously determined by Fukamachi et al (1972) for isolated rat liver mitochondria. As shown in Table 7 also, atractyloside inhibited the incorporation of [^{3}H] AMP into poly(A)+RNA by isolated rat liver mitochondria. The origin of the difference in inhibition between iso-osmotic and hypo-osmotic media (Table 7) is not known, but could reflect leakage of mitochondria incubated in hypo-osmotic medium, which would decrease the ability of atractyloside to inhibit entry of adenine nucleotides. The inhibition of incorporation into total acid-insoluble material shown in Table 7 are similar to those of Fukamachi et al (1972) for rat liver mitochondria isolated in sucrose-EDTA. It should be noted that under the conditions of incubation, rat liver nuclei are completely insensitive to both atractyloside and ethidium bromide (Fukamachi et al. 1972), further indicating that the observed synthesis of poly(A)+RNA could not be caused by contaminating nuclei. The results therefore are consistent with a mitochondrial site of synthesis.

A further proof of the mitochondrial origin of poly(A)+RNA was obtained by the following experiment: following labelling of RNA by isolated Ehrlich ascites cell mitochondria, they were suspended in Medium B, divided in half and incubated with and without 1 unit of immobilized pancreatic RNase. Under the conditions of digestion in low salt (0.3M sucrose/ 2 mM EDTA/ 2mM Tris-HCl, pH 7.4), poly(A) is known to be hydrolysed (Beers, 1960). At various times aliquots were taken and the trichloroacetic acid-precipitable counts determined and as shown in Fig. 8, some 20% of the radioactivity was lost. Further, as summarized in Table 7, the radioactivity in the total acid-insoluble material after 15 min decreased by approximately 19% while only 10% of the [^{3}H] AMP-

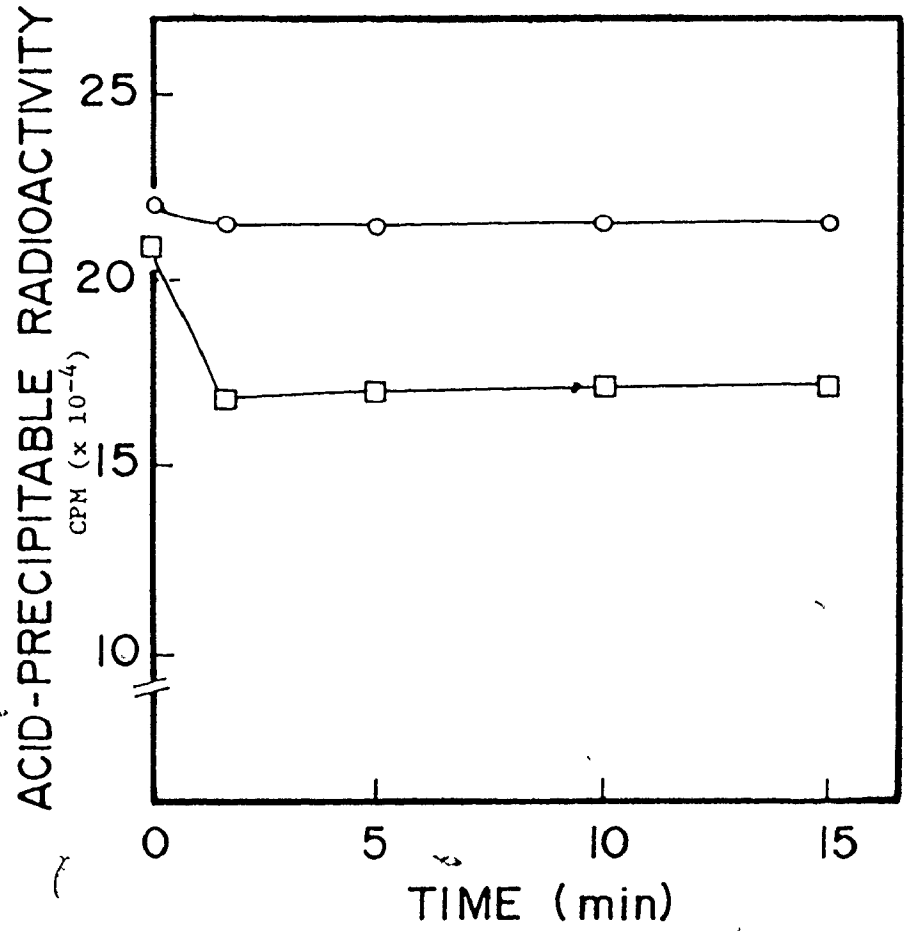


Figure 8

Effect of immobilized RNase on the incorporation of [^{3}H] AMP by isolated Ehrlich ascites cell mitochondria. Mitochondria were isolated and incubated as described in the Methods. Following incubation, they were recovered by centrifugation, resuspended in 12 ml of Medium B, divided in half and incubated with and without 1 unit of immobilized pancreatic RNase. Aliquots of 100 µl were taken at each time point, spotted on Whatmann 3MM filters and acid-precipitated as described in the Methods. o, -immobilized RNase; □, + immobilized RNase.

labelled poly(A)+RNA was lost. This indicates that the poly(A)+RNA was protected within a membrane. The 10% loss could be the result of RNA turnover, contamination with cytosolic poly(A)+RNA or digestion of mitochondrial poly(A)+RNA by the RNase because of partial disruption of mitochondria during the experiment. The results further substantiate that the synthesized poly(A)+RNA is not cytosolic in origin.

Characterization of poly(A)+RNA

Having demonstrated the mitochondrial origin of the isolated poly(A)+RNA, the RNA was then characterized by sucrose density gradient centrifugation. As shown in Fig. 9, both [^{3}H] AMP and [^{3}H] UTP-labelled poly(A)+RNA from Ehrlich ascites cell mitochondria gave similar patterns on a 15 to 33% sucrose density gradient in 0.1M NaCl under non-denaturing conditions. There was a broad peak at about 18S and much RNA of higher S values. In contrast, the material which was not retained by oligo(dT)-cellulose consisted of either free nucleotides, oligonucleotides, 4S RNA or some other acid-insoluble material. As shown in Fig. 10, there was no RNA corresponding to either mitochondrial rRNA or possible poly(A)-RNA of a size similar to poly(A)+RNA, as was found by Avadhani et al (1973, 1974).

The high S values of the poly(A)+RNA (Fig. 9) could be a reflection of the true size of the RNA, but because poly(A)+RNA tends to form aggregates (Ojala & Attardi, 1974b), the size was futher examined by characterizing denatured poly(A)+RNA.

When the [^{3}H] AMP-labelled poly(A)+RNA was denatured by heating at 70^{o}C for 5 min, as described in the methods, cooled rapidly and

Figure 9

Characterization on sucrose-density gradients of ascites cell mitochondrial poly(A)+RNA synthesized in the presence or absence of ethidium bromide. Mitochondrial poly(A)+RNA, obtained as described in Table 6, was centrifuged for 16 h at 69,000g at 24°C in a 15-33% (w/v) sucrose-density gradient in 0.1 N NaCl/0.2% SDS/ 1 mM EDTA/ 10 mM Tris-HCl, pH 7.0. The markers were [^{14}C]uridine-labelled cytosolic rRNA from CHO cells run in a parallel tube. The fraction size was 0.22 ml. ●, [^{3}H] AMP-labelled poly(A)+RNA; ▵, [^{3}H] UTP-labelled poly(A)+RNA; o, [^{3}H] AMP-labelled poly(A)+RNA synthesized in the presence of 3 µg of ethidium bromide.

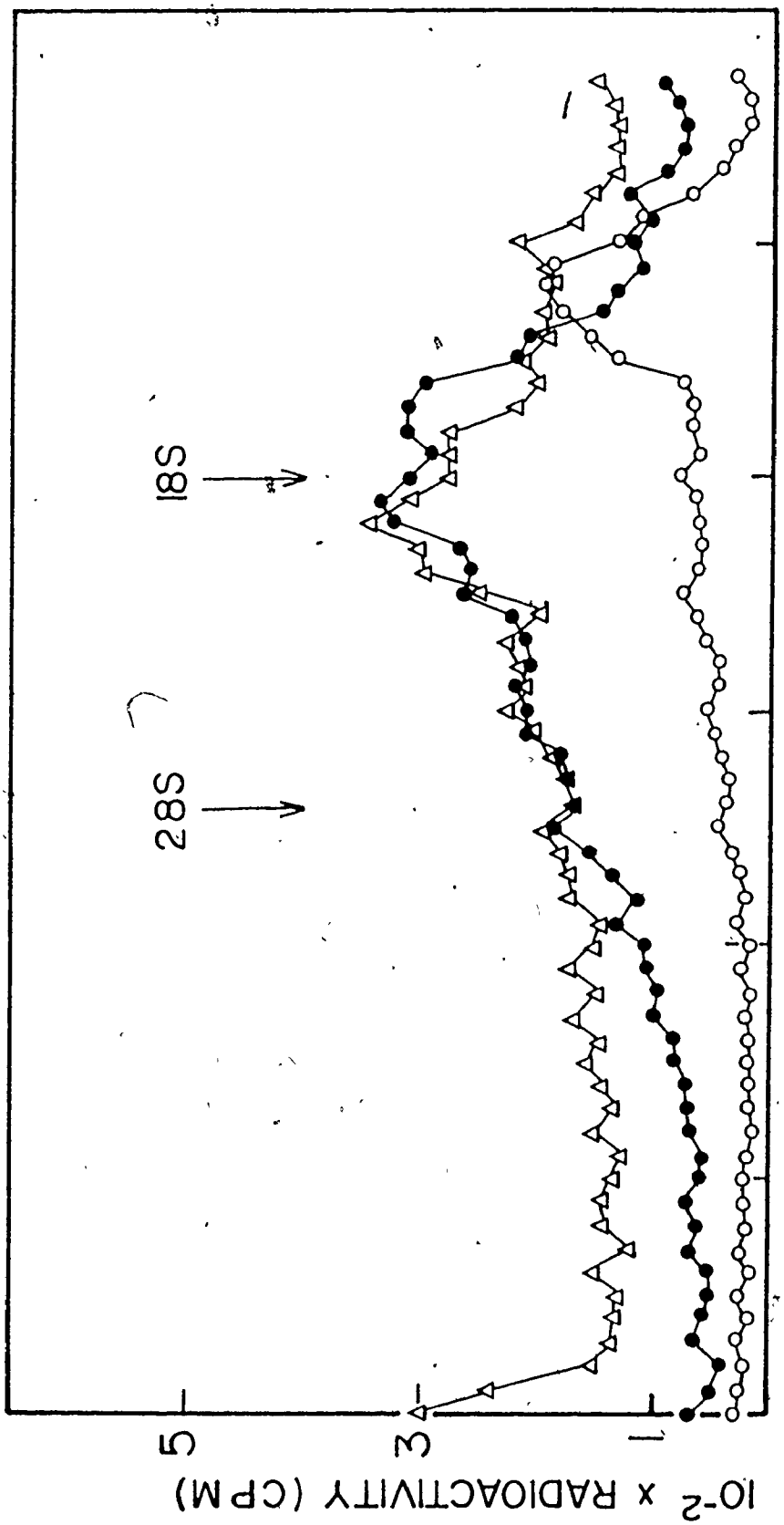


Figure 9

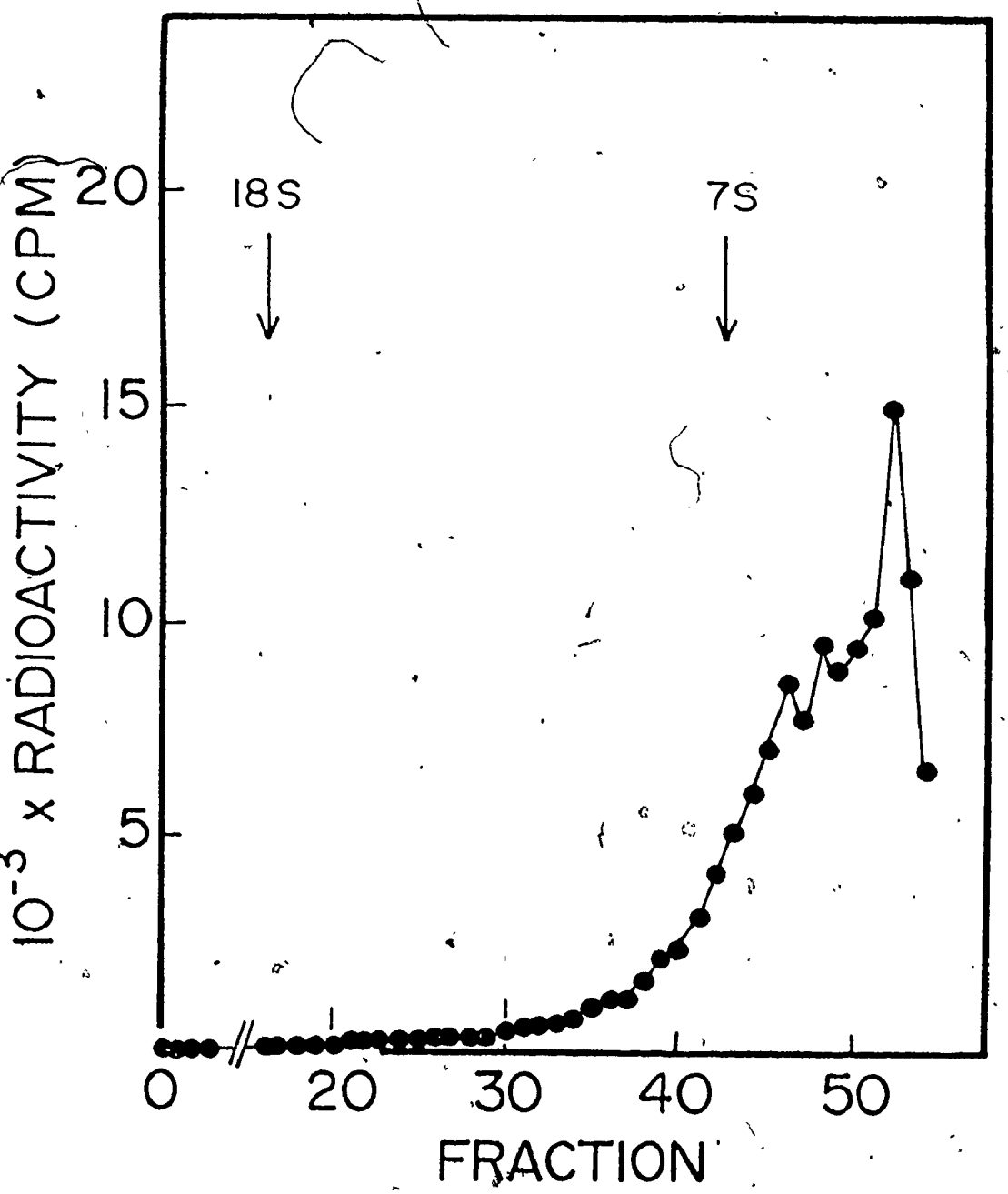


Figure 10

Characterization on sucrose-density gradients of ascites cell mitochondrial [^{3}H] AMP-labelled material not binding to oligo-(dT)-cellulose. Mitochondrial RNA was labelled with [^{3}H] AMP as described in the Methods. It was denatured and passed through oligo(dT)-cellulose. The material eluting at high salt was denatured again and ran on a 5-20% sucrose density gradient in 0.25 mM EDTA/ 0.2% SDS/ 1 mM Tris-HCl, pH 7.4 at 24°C and 108,000g for 18 h.

centrifuged on a 5 to 20% sucrose gradient in 0.25mM EDTA/ 1mM Tris-HCl, pH 7.4, at 24°C, a broad peak at 12S was obtained as shown in Fig. 11. As the low S value could reflect degradation at 24°C, the same poly(A)+RNA was centrifuged on a 5 to 20% sucrose density gradient in 0.1M LiCl, at 4°C. Two major peaks at 16S and 12S were observed (Fig.12). The profile obtained with [^{3}H] UTP-labelled poly(A)+RNA was very similar to that of the [^{3}H] AMP-labelled RNA (Fig. 12). As a further precaution against aggregation, [^{3}H] AMP-labelled poly(A)+RNA was denatured in the presence of 1% formaldehyde and centrifuged at 24°C and 4°C in the presence of formaldehyde. As shown in Fig. 13A and B, the two major peaks at 16S and 12S were observed as well as some heterogenous RNA with a lower sedimentation rate.

Rat liver mitochondrial poly(A)+RNA labelled with [^{3}H] AMP also showed broad peaks between 12S and 16S with some other possible components when centrifuged under denaturing conditions (Fig. 14).

Characterization of poly(A)+RNA synthesized in the presence of ethidium bromide

In the presence of ethidium bromide, up to 20% as much poly(A)+RNA as controls was synthesized (Table 6 and 7). This could represent cytosolic contamination or residual or partial synthesis of mitochondrial RNA. The observed labelling reflects the synthesis of both the poly(A) tail and the rest of the poly(A)+RNA (Table 6), but the poly(A) tail was a higher proportion (35%) of the radioactivity of poly(A)+RNA than in the absence of ethidium bromide. This would be expected if ethidium bromide inhibited mitochondrial transcription but not the synthesis of poly(A).

Figure 11

Characterization of denatured [^{3}H] AMP-labelled poly(A)+RNA on a sucrose-density gradient. Total [^{3}H] AMP-labelled ascites cell mitochondrial RNA was obtained as described in Table 6. It was denatured at 70°C for 5 min and the poly(A)+RNA adsorbed and eluted from oligo(dT)-cellulose and denatured again, as described in the Methods. Centrifugation was on a 5-20% (w/v) sucrose-density gradient in 0.25 mM EDTA/ 0.2% SDS/ 1 mM Tris-HCl, pH 7.4 at 24°C and 108,000g for 18 h. The markers are from denatured [^{14}C] uridine-labelled CHO cell cytosolic rRNA.

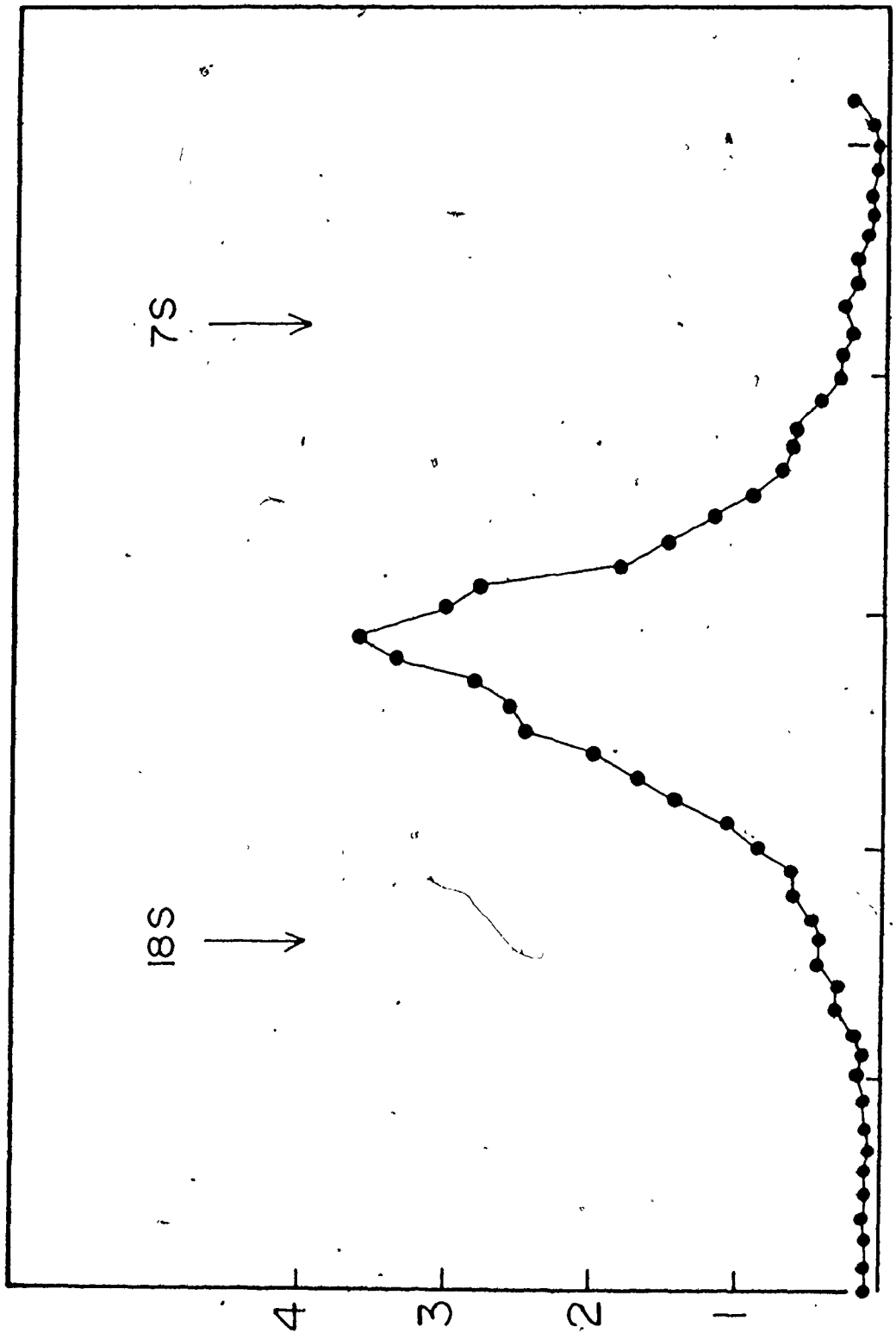


Figure 11

Figure 12

Characterization of denatured ascites cell mitochondrial poly(A)+RNA on a sucrose-density gradient. Denatured poly(A)+RNA, labelled with either [^{3}H] AMP or [^{3}H] UTP, was obtained as described in Table 6. Centrifugation was on a 5-20% (w/v) sucrose-density gradient in 0.1 M LiCl/ 0.25 mM EDTA/ 0.2% SDS/ 1 mM Tris-HCl, pH 7.4, at 4^{o}C and 108,000g for 18 h. •, [^{3}H] AMP-labelled poly(A)+RNA; o, [^{3}H] UTP-labelled poly(A)+RNA

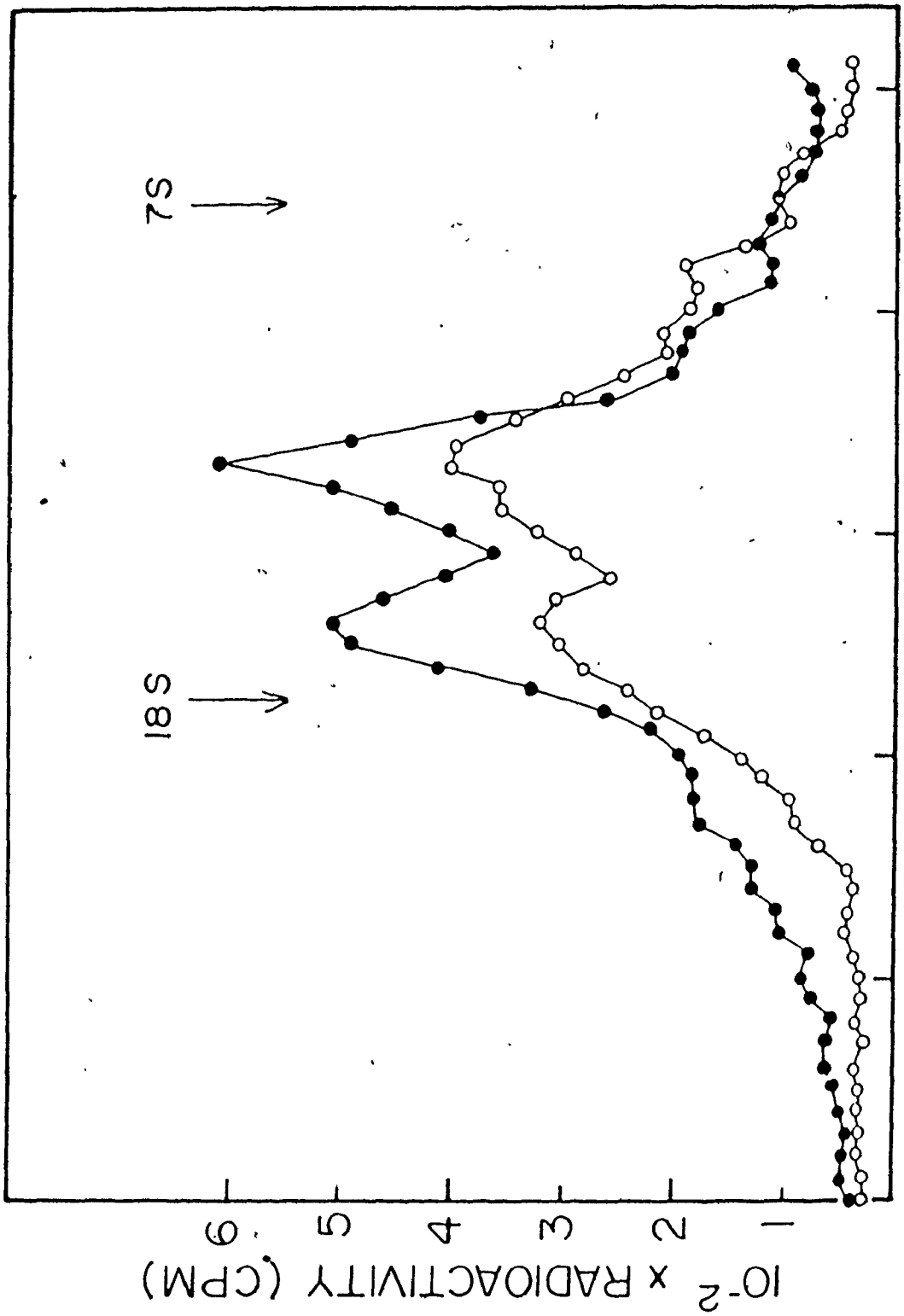

7S
18S
0
10
20
30
40
50
FRACTION
1
2
3
4
5
6
10^{-2} x RADIOACTIVITY (CPM)

Figure 12

Figure 13

Characterization of denatured [^{3}H] AMP-labelled ascites cell mitochondrial poly(A)+RNA on sucrose-density gradients in the presence of formaldehyde. [^{3}H] AMP-labelled poly(A)+ RNA was obtained as described in Table 6. Prior to centrifugation, it was denatured for 5 min at 70°C in the presence of 1% formaldehyde as described in the Methods. Centrifugation was in (A) 0.25 mM EDTA/ 0.2% SDS/ 1 mM Tris-HCl, pH 7.4, at 24°C and 108,000g for 18 h, and (B) 0.1 M LiCl/ 0.25 mM EDTA/ 0.2% SDS/ 1 mM Tris-HCl, pH 7.4 at 4°C and 108,000g for 18 h.

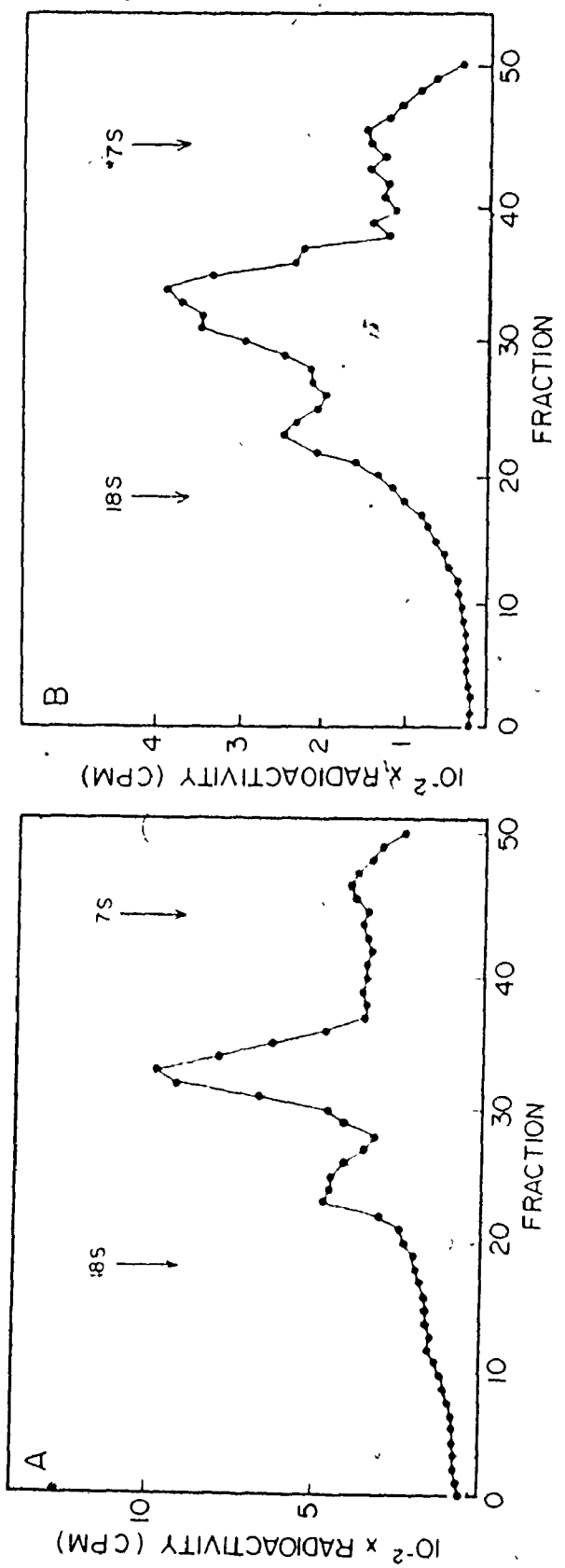


Figure 13

Figure 14

Characterization of denatured [^{3}H] AMP-labelled rat liver mitochondrial poly(A)+RNA on sucrose-density gradients in the presence of formaldehyde. [^{3}H] AMP-labelled poly(A)+RNA was obtained as described in Table 6, denatured and centrifuged as in Fig. 13A.

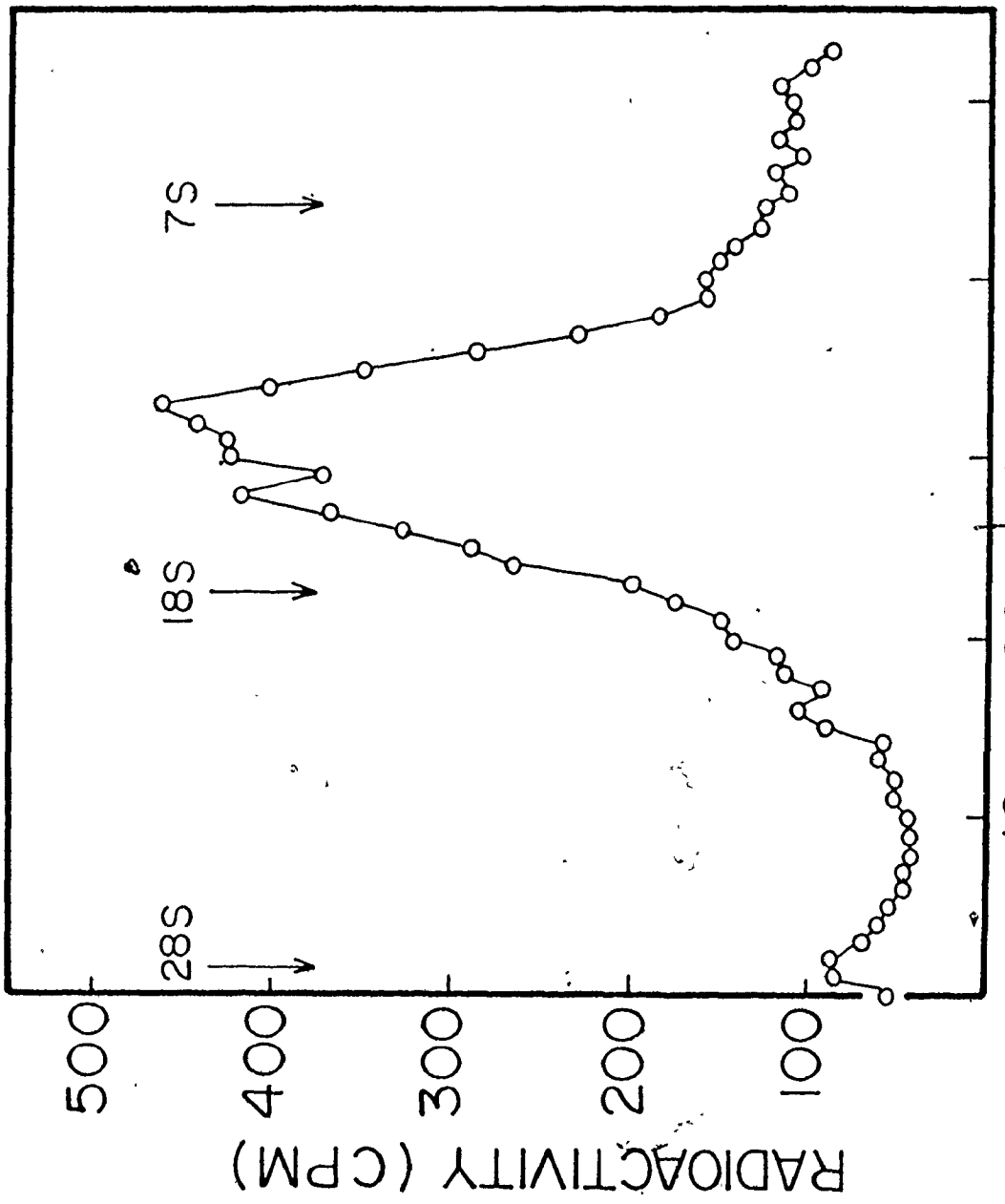

RADIOACTIVITY (CPM)
500
400
300
200
100
28S
18S
7S
10
20
30
40
50
FRACTION

Figure 14

This was examined further by characterizing the poly(A)+RNA and its poly(A) tail. Fig. 9 shows that the poly(A)+RNA now sedimented at about 5 to 10S on a 15 to 33% sucrose density gradient. On a 5 to 20% sucrose density gradient which gives greater resolution, peaks at 6S, 12S and a shoulder above 12S were observed as shown in Fig. 15. On a 14% acrylamide gel, some of the ethidium bromide-resistant poly(A)+RNA ran slower than $4S_E$ (Fig.16) but some did not enter the gels. This is in contrast to the poly(A)+RNA synthesized in the absence of ethidium bromide which does not enter the gels. Similar results were observed by Hirsch & Penman (1974a) with HeLa cell mitochondrial RNA. This result would be expected from the profile of the 5 to 20% sucrose density gradient which indicates that much of the RNA synthesized in the presence of ethidium bromide is shorter than that synthesized in its absence. However, the presence of a 12S peak would imply that inhibition of mitochondrial transcription is not complete. As shown in Fig. 16, the poly(A) tail was also shorter than the major $4S_E$ peak of poly(A) synthesized in the absence of the inhibitor. The smaller size of poly(A)+RNA, combined with a slightly shorter poly(A) tail would account for the greater proportion of radioactivity found in poly(A) extracted after incubations in the presence of the drug (35%) than in its absence (10%).

Figure 15

Characterization on sucrose-density gradients of ascites cell mitochondrial poly(A)+RNA synthesized in the presence of ethidium bromide. [^{3}H] AMP-labelled poly(A)+RNA synthesized in the presence of 3 µg of ethidium bromide/ ml was obtained as described in the Methods. Centrifugation conditions were as in Fig. 11. Neither the poly(A)+RNA nor the marker CHO cell cytosolic rRNA were denatured prior to centrifugation.

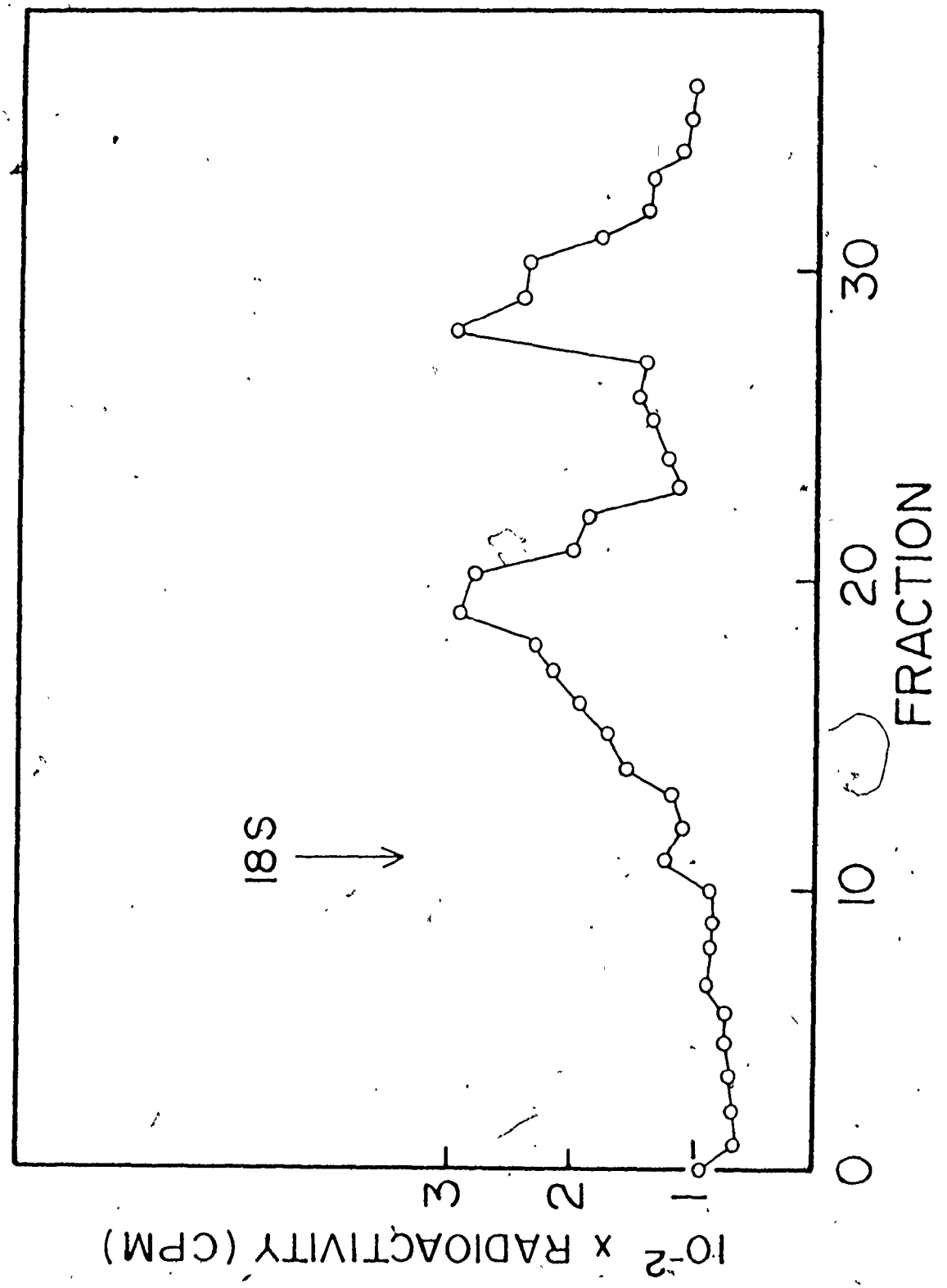


Figure 15

Figure 16

Polyacrylamide gel electrophoresis of ethidium bromide-resistant poly(A)+RNA and poly(A) from ascites cell mitochondria. [^{3}H]AMP-labelled poly(A)+RNA synthesized in the presence of 3 μg of ethidium bromide/ml was obtained as described in the Methods. Poly(A) was isolated as described in Fig. 6. Electrophoretic conditions were as in Fig. 6. ●-●, ethidium bromide-resistant poly(A)+RNA; o-o, poly(A) from ethidium bromide-resistant poly(A)+RNA; ▲ , poly(A)+RNA synthesized in the absence of ethidium bromide.

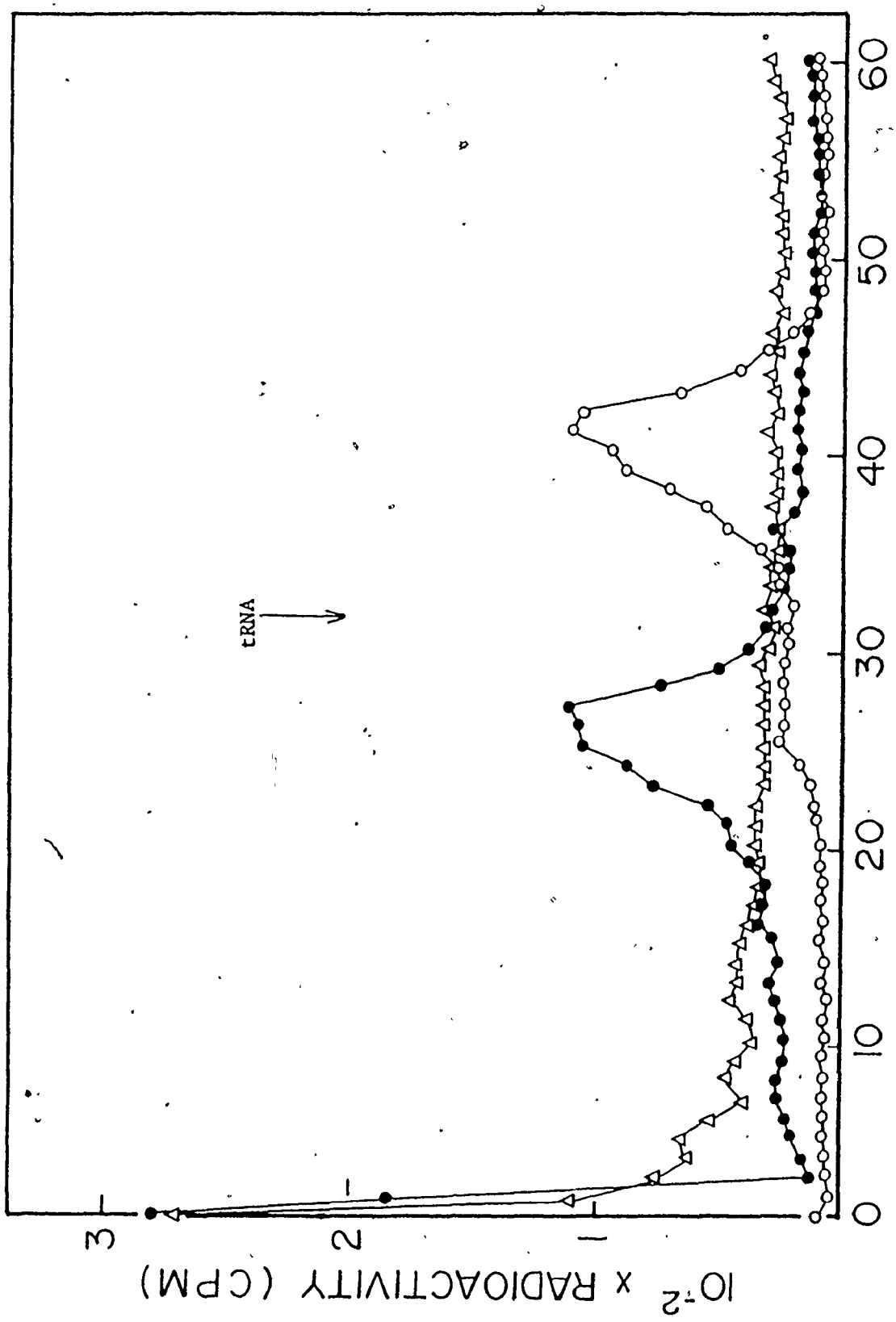

10^{-2} x RADIOACTIVITY (CPM)
3
2
1
0
tRNA
10
20
30
40
50
60
DISTANCE FROM ORIGIN (mm)

Figure 16

DISCUSSION

As outlined in the Introduction, the nature and metabolism of mitochondrial mRNA was approached through the isolation and characterization of mitochondrial poly(A)+RNA synthesized by isolated mitochondria. The results will be considered in relation to those of other workers *in vivo* and *in vitro* in order to assess the use and limitations of isolated mitochondria in such studies.

The evidence that the mitochondrial fraction synthesized poly(A)+RNA was that 1) a fraction of the labelled material bound to both oligo-(dT)-cellulose and Millipore filters indicating a poly(A) segment; 2) hydrolysis of this fraction gave rise to labelled 2'(3')-nucleotides showing that it was RNA and 3) RNase T_1 and RNase A digestion in the presence of high salt resulted in a fragment of fairly uniform size of about $4S_E$ that was labelled with [^{3}H] AMP but not [^{3}H] UTP and hence was the presumptive poly(A) tail. In comparison with the results of Hirsch & Penman (1973), a poly(A) tail with an electrophoretic mobility of $4S_E$ would correspond to a length of 57 nucleotides, but the average length of poly(A) found here, including the smaller fragments, would more likely be around 50 nucleotides. In contrast, the cytosolic $7S_E$ poly(A) tail represents between 145 and 210 nucleotides (Edmonds & Caramela, 1969; Sullivan & Roberts, 1973). The size of mitochondrial poly(A) found is similar to that reported by other workers in HeLa (Hirsch & Penman, 1973; Ojala & Attardi, 1974a), *Drosophila* (Hirsch *et al*, 1974), Ehrlich ascites

(Avadhani et al, 1973ab) and Krebs ascites cells (Gaitskhoki et al,1973a) and mouse and rabbit liver (Avadhani et al, 1975), as well as rat liver (Gaitskhoki et al, 1973a; Rose & Jacobs, 1976; Saccone et al, 1976; Cantatore et al, 1976).

The difference in size between cytosolic and mitochondrial poly(A) is an indication of a possible mitochondrial origin of poly(A)+ RNA. The inhibition of poly(A)+RNA synthesis by ethidium bromide (Fukamachi et al, 1970) and atractyloside (Saccone et al, 1967) and lack of significant hydrolysis by immobilized RNase supports the conclusion that the synthesis was in mitochondria. Synthesis of poly(A)+RNA by the mitochondrial fraction could arise from mitochondria, from the presence of microsomes (Rose & Jacobs, 1975) or nuclei, or from cytosolic elongation of pre-existing poly(A)+RNA (Rose et al, 1976: Brawerman & Diez, 1975). As pointed out previously, Fukamachi et al (1972) showed that under the incubation conditions used, neither nuclear nor microsomal RNA synthesis was significant. Further, at low ATP concentrations, isolated nuclei do not synthesize poly(A) (Jelinek, 1974). With [^{3}H]-AMP as the precursor, and in the absence of added ATP, it would be expected that neither nuclei nor nuclear fragments account for the synthesis of poly(A) by the mitochondrial fraction. Lack of digestion by immobilized RNase exludes cytosolic elongation. These criteria are sufficient to conclude that the poly(A)+RNA was synthesized in isolated mitochondria. Hybridization studies, which would have provided a definitive proof, were, however, not attempted.

Since this work was concluded, reports have been published substantiating this conclusion. In particular, two groups have reported the synthesis of poly(A) and poly(A)+RNA by isolated rat liver mitochon-

dria. Saccone et al (1976) obtained poly(A)+RNA which hybridized to various restriction fragments of mtDNA. Rose & Jacobs (1976) studied poly(A) polymerase activity in isolated mitochondria and found that the synthesis of poly(A), partially inhibited by atractyloside, appeared to be dependent on intramitochondrial pre-existing RNA to which it was covalently attached. In addition, the present results are in general agreement with those in vivo of other workers. The present results provide information on the question of post-transcriptional addition of poly(A) and of the nature of the poly(A)+RNA synthesized, and these will be considered now.

Post-transcriptional addition of poly(A)

The more rapid appearance of [^{3}H] AMP than [^{3}H] UTP in poly(A)+ RNA is consistent with a post-transcriptional addition of poly(A), as occurs in the nucleus. A dependence on pre-existing RNA for poly(A) synthesis was observed by Rose & Jacobs (1976). The time lag in appearance of poly(A)+RNA labelled with [^{3}H] UTP, which should reflect the synthesis of the whole molecule more than with [^{3}H] AMP, could result from processing from precursor to the final poly(A)+RNA products.

The effects of ethidium bromide inhibition are also consistent with a post-transcriptional addition of poly(A). In the presence of this drug which selectively inhibits transcription in mitochondria (Zylber et al, 1969), poly(A) synthesis should be either reduced or the poly(A) added to small fragments of RNA. With isolated Ehrlich ascites cell mitochondria, addition of ethidium bromide resulted in 80% inhibition of poly(A)+ RNA synthesis (Tables 6 and 7). The remaining poly(A)+RNA was mostly smaller than in the absence of the drug but a significant fraction had a

similar size (Fig. 15). This seems to be caused by incomplete inhibition of transcription rather than cytosolic contamination or import of RNA since the poly(A) tail was found to be shorter (Fig. 16) than in the absence of the drug. No free poly(A) was observed. Hirsch & Penman (1974a) also did not isolate any free poly(A) from HeLa cell mitochondria after ethidium bromide inhibition in whole cells, but the poly(A) formed was not reduced in length. Hirsch & Penman (1974a) also observed a transient decrease in the rate of poly(A) addition followed by a rise which was interpreted as resulting from an increase in abberent small RNA substrates similar to those seen here (Figs. 15 and 16). The absence of free poly(A) indicates that mitochondrial poly(A) polymerase needs a primer for polyadenylation as shown by Rose & Jacobs (1976). Recognition elements must reside at the 3' end of the RNAs. It is not clear, on the other hand, why Ojala & Attardi (1974a) found "free poly(A)" in HeLa cell mitochondria, from cells incubated in the presence of ethidium bromide. The presence of free poly(A) would be incompatible with present models of polyadenylation (Darnell et al, 1973; Brawerman, 1974).

Nature of poly(A)+RNA

In the cytosol, poly(A) is associated with the majority of mRNAs. The presence of a poly(A) tail on mitochondrial RNA makes it a good candidate for mitochondrial mRNA. For RNA to be of a messenger nature, it must be shown to be capable of coding for proteins. Studies of translation of mammalian mitochondrial poly(A)+RNA in vitro are difficult because of low availability of mRNA. However, it is possible to determine whether the size of poly(A)+RNA would be sufficient to code for the molecular weights of mitochondrially-synthesized proteins (see Introduction,

section IIC). This will be considered after a discussion of the size of the poly(A)+RNA.

The poly(A)+RNA synthesized by isolated mitochondria of both rat liver and Ehrlich ascites cells appeared as discrete peaks at around 16 and 12S when centrifuged under denaturing conditions in formaldehyde. HeLa cell mitochondrial poly(A)+RNA also had peaks in the same region with an additional 7S component (Ojala & Attardi, 1974b). A 7.5S component has also been observed in rat liver (Gaitskhoki et al, 1977; Kisselev et al, 1977).

The sedimentation profile of poly(A)+RNA (Fig. 14) was very similar to that obtained by Fukamachi et al (1972) for total rat liver mitochondrial RNA. These workers showed that a fraction of total RNA consisted of mitochondrial rRNA which was identified by gel electrophoresis (Fukamachi et al, 1970). Conceivably, the poly(A)+RNA fraction could be contaminated with mitochondrial rRNAs and Amalric et al (1978) and Spradling et al (1977) have observed a small amount of binding of 12S rRNA from HeLa cells and 16S rRNA from Drosophila cells to oligo(dT)-cellulose. However, non-specific binding of either rat liver or Ehrlich ascites cell mitochondrial rRNA to oligo(dT)-cellulose seems an unlikely explanation of the present results for the following reasons: 1) the amount which would be expected to bind would not be more than 5 to 10% (Spradling et al, 1977) especially when the RNA was denatured before fractionation. Even if a more significant fraction did bind to oligo-(dT)-cellulose, the remainder should be found in the eluting fraction. As shown in Fig. 10, this is not the case as only lower molecular weight RNAs, non-RNA molecules or nucleotides were present; 2) in both rat liver and Ehrlich ascites cells, the amount of

rRNA labelled by isolated mitochondria is low, even after a 1 h labelling (Fukamachi et al, 1972; Avadhani et al, 1973); 3) total cytosolic CHO rRNA and BHK mitochondrial rRNA both failed to bind to oligo(dT)-cellulose; 4) the molecular weight calculated for poly(A)+RNA from the size of the poly(A) and the percentage radioactivity associated with it corresponds to that determined from sedimentation analysis (see below). It is concluded that the fraction binding to oligo(dT)-cellulose consists mostly of poly(A)+RNA.

Only two peaks were observed on centrifuging the poly(A)+RNA under denaturing conditions. The broadness of the peaks suggests that they represent multiple RNA species. Attempts at further resolution on polyacrylamide disc gels were not succesful as large, non-reproducible aggregates were frequently obtained, whether or not formaldehyde or formamide was present to denature the RNA. Aggregation of poly(A)+RNA was also observed by Ojala & Attardi (1974b). It was suggested that such aggregation resulted either from the symmetrical transcription of the RNA and/or from the presence of the poly(A) tail and its interaction with the rest of the RNA. In HeLa cells, most of the mitochondrial poly(A)+RNA is transcribed from the H strand, with a 7S component transcribed from the L strand. Using CH_3HgOH/agarose slab gels, Amalric et al (1978) resolved HeLa cell mitochondrial poly(A)+RNA into 18 distinct bands. The tendancy of mitochondrial poly(A)+RNA to aggregate raises the possibility that some of the higher molecular weight peaks observed by Hirsch et al (1974) were artefacts. In fact, in a more recent paper, Spradling et al (1977) separated Drosophila cell mitochondrial poly(A)+RNA into 11 bands between 19S and 12S on urea slab gels.

Battey & Clayton (1978), in a unique approach, hybridized unlabelled poly(A)+RNA fractionated by poly(U)-Sepharose to labelled mouse L cell mtDNA and analysed the hybridized mtDNA after digestion of unhybridized DNA with S_1 nuclease. They showed that 7 mtDNA fragments coded for poly(A)+ RNA. The number and sizes of these transcripts were consistent with that of mitochondrially-synthesized proteins detected in that cell line (Lansman & Clayton, 1975).

Assuming that poly(A)+RNA from Ehrlich ascites cell and rat liver mitochondria when denatured was not partly hydrolysed, then its molecular weight would average 400,000. A similar size can be estimated from the size of the poly(A) tail. The poly(A) had about 10% of the radioactivity of the poly(A)+RNA (Table 6). Assuming that all the poly(A) was recovered after RNase digestion, and taking an average length of poly(A) of 50 nucleotides and that the content of A residues in mitochondrial RNA is 35% (Freeman et al, 1973), the average length of the poly(A)+RNA is about 1300 nucleotides for a molecular weight of about 400,000. In rat liver (see Results, Part III), the largest protein component synthesized in mitochondria has a molecular weight between 40,000 and 50,000 and the smallest is about 4,000, putting the minimal sizes of mRNAs necessary at 1200 and 100 nucleotides (not including the poly(A) tail, leader sequence and other non-translated segments) respectively. Thus, if the poly(A)+RNA is mRNA, this length is sufficient to code for proteins of the molecular weight synthesized in mammalian mitochondria (Yatscoff & Freeman, 1977; Yatscoff et al, 1978a). In mammalian cells, only Kisselev et al (1977) have shown that a mitochondrial 7.5S poly(A)+RNA isolated from rat liver mitochondrial polysomes can be translated. However, the hydrophobic polypeptide synthesized has not been identified as corresponding to any

mitochondrially-synthesized protein.

The synthesis by isolated mitochondria of poly(A)+RNA of a size sufficient to code for mitochondrially-synthesized proteins contrasts with the results of Avadhani et al (1973,1974). They did not detect synthesis of poly(A)+RNA by isolated mitochondria. Using their incubation conditions, it was found that total RNA synthesis was much lower than under the conditions used in this study but that there still was a significant fraction of labelled RNA that bound to oligo(dT)-cellulose. The absence of polyadenylation by isolated mitochondria observed by Avadhani et al (1973,1974) probably reflects the conditions of incubation or of the method of isolating the mitochondrial fraction. If this is the case, the present results contradict the interpretation that Avadhani and co-workers drew from their work that poly(A)+RNA was imported into mitochondria (see Introduction, section IIC). Further, the hybridization levels of mitochondrial poly(A)+RNA to nuclear DNA found by Avadhani et al (1973) were sufficiently low to be questionable. Gaitskhoki et al (1974, 1977) labelled rat liver mitochondrial poly(A)+RNA with ^{125}I in vitro and showed that it hybridized to nuclear DNA. In both cases, the possibility of cytosolic contamination cannot be entirely excluded, especially when labelling RNA in vivo or by iodination in vitro. Amalric et al (1978) have shown that unless extremely stringent conditions are used, such as RNase digestion of the mitochondrial fraction prior to RNA extraction, contaminating cytosolic RNAs, including poly(A)+RNA, are also extracted.

Contamination by cytosolic RNA however does not explain lack of hybridization to mtDNA as found by Avadhani and co-workers. Neither Penman's nor Attardi's group have given any evidence of poly(A)+RNA

import into HeLa cell mitochondria. The present results, while limited, do not support a need for RNA import either.

The present results do not give any information as to the presence of mRNAs lacking poly(A) (poly(A)-RNA), while those of other workers are contradictory. Amalric et al (1978) isolated an RNA fraction lacking poly(A) and hybridizing mostly to the H strand from a Triton X-100 extract of HeLa cell mitochondria, but this RNA was lacking from "polysomal structures". No poly(A)-RNA has been described by Penman's group (Perlman et al, 1973; Hirsch & Penman, 1974ab; Hirsch et al, 1974). On the other hand, Avadhani et al (1973, 1974) and Lewis et al (1976b) did isolate polysome-associated poly(A)-RNA hybridizing to mtDNA and Kisselev et al (1975) presented evidence of poly(A)-RNA associated with rat liver mitochondrial polysomes.

The existence of poly(A)-RNA in mitochondria would not be surprising. A poly(A) tail is now thought not to be necessary for translation per se (Revel & Groner, 1978) and mRNAs without poly(A) have been isolated from the cytosol of a number of eukaryotes. Furthermore, in yeast, mitochondrial mRNA might lack a poly(A) tail (Groot et al, 1974; Eggit & Scragg, 1976; Moorman et al, 1978), although this question is still considered controversial (Borst et al, 1977). A number of groups have suggested that fungal mitochondrial mRNAs possess a poly(A) tail of 30 nucleotides or less (Hendler et al, 1975; Kroon et al, 1976; Rosen & Edelman, 1976).

To summarize, it can be concluded that isolated mitochondria are an appropriate system for the study of mitochondrial RNA metabolism and of mitochondrial poly(A)+RNA specifically. A major problem is the choice of incubation medium, as shown by a comparison between the

present results and those of Avadhani's group. The slight heterogeneity of the poly(A) tail, the lack of a 7.5S poly(A)+RNA (Kisselev et al, 1977) and the possibility of aberrant effects of ethidium bromide on poly(A) synthesis point to other limitations of the approach. Nevertheless, the present results are generally consistent with in vivo results and further document the synthesis in mitochondria of poly(A)+RNA of a size sufficient to code for mitochondrially-synthesized proteins and thus be a mRNA. Import of nuclear-coded mRNA would not be needed.

PART II

THE SIZE OF MITOCHONDRIAL tRNAs

METHODS

Labelling and isolation of mitochondrial aminoacyl-tRNAs

Mitochondria from Syrian hamster or rat liver were isolated as described in Part I of Methods, except that the extra nuclear spin was omitted. tRNA was labelled by incubating mitochondria as described by Wallace & Freeman (1974). The incubation medium (pH 7.2) contained 0.05M KCl/ 0.05M $MgCl_2$/ 0.02M KH_2PO_4/ 0.1M sucrose/ 0.01M sodium succinate/ 2mM ADP/ 0.1mM of each of the 19 unlabelled amino acids/ 100 µg Tevenel/ml and 15 mg protein/ml. Mitochondria were preincubated for 4 min at 30°C before addition of either [^{35}S] methionine or [^{3}H] leucine to 10 µCi/ml to a final volume of 4.5 ml. After 20 min at 30°C at 80-100 oscillations/ min, mitochondria were centrifuged at 10,000g for 5 min at 4°C. The labelled aminoacyl-tRNA was isolated by resuspending mitochondria in 5nM magnesium acetate/ 1% SDS/ 0.1M sodium acetate, pH 5.0, followed by extraction twice with buffer-saturated phenol at 4°C. Total nucleic acid was then precipitated with 2 volumes of 95% (v/v) ethanol at -20°C overnight. Carrier tRNA (stripped *E. coli* or rabbit liver) was usually added to 100 µg except in preparations to be used for slab gel electrophoresis. This procedure yielded between 20,000 and 40,000 cpm of [^{3}H] leucyl-tRNA and up to 150,000 cpm of [^{35}S] methionyl-tRNA.

Labelling and isolation of cytosolic aminoacyl-tRNA

Livers from Syrian hamster or rat liver were homogenized in 3 volumes of 10mM KCl/ 1mM magnesium acetate/ 6mM mercaptoetnanol/ 10mM Tris-HCl, pH 7.5 as described by Wallace & Freeman (1974). After centrifugation at 30,000g for 10 min, the supernatant was passed through a Sephadex G-25 (fine) column equilibrated with the extraction buffer. The material eluting in the void volume, containing both tRNA and aminoacyl-tRNA synthetase, was used for acylation of cytosolic tRNA at 37°C for 10 min according to Wallace & Freeman (1974). The final incubation of 2 ml contained 1 ml of cytosolic extract and was 10mM KCl/ 1mM magnesium acetate/ 6mM mercaptoethanol/ 8mM ATP/ 4mM CTP/ 10mM Tris-HCl, pH 7.5, and either 50 µCi [^{3}H] methionine or 1 µCi [^{14}C] leucine/ml. The reaction was stopped with 9 volumes of 5mM magnesium acetate/ 1% SDS/ 0.1 M sodium acetate, pH 5.0, and aminoacyl-tRNA extracted as described for mitochondrial tRNA. Usually, about 20,000 cpm of [^{14}C] leucyl-tRNA and 100,000cpm of [^{3}H] methionyl-tRNA could be recovered.

Preparation of yeast penylalanyl-tRNA and leucyl-tRNA

Yeast aminoacyl-tRNA synthetase extract was prepared as described by Wimmer et al (1968) from baker's yeast obtained from a local bakery. Frozen yeast cake (25 g) was crushed with 30 ml of Superbrite (110 µm) glass beads in 15 ml of medium (10mM $MgCl_2$/ 30mM ammonium chloride/ 5mM mercaptoethanol/ 10mM Tris-HCl, pH 7.5) for 25-30 min at 4°C, using a mortar and pestle. The mixture was centrifuged at 2,000g at 4°C and the supernatant passed through a Sephadex G-25 (coarse) column equilibrated with 1mM mercaptoethanol/ 0.1mM EDTA/ 40% glycerol/ 10mM potassium phosphate, pH 7.5. The fraction eluting in the void volume was collected

and kept frozen at -20°C.

Acylation of commercial baker's yeast tRNA was also done essentially as described by Wimmer et al (1968). Mixtures contained the following components in a total volume of 1 ml: 15mM $MgCl_2$/ 30mM KCl/ 4 mM EDTA/ 3mM ATP/ 13mM mercaptoethanol/ 8 µM each of 19 unlabelled amino acids/ 120mM Tris-HCl, pH 7.5/ 1 µCi of either [^{3}H] phenylalanine or [^{3}H] leucine/ 0.8 mg protein of aminoacyl-tRNA synthetase mixture/ 5 A_{260} units of yeast tRNA. Incubations were for 15-20 min at room temperature (22°C), following which the aminoacyl-tRNAs were extracted as described for Syrian hamster and rat liver cytosolic tRNA.

RPC-5 chromatography of the extracted species indicated that leucyl-tRNA was made up of three species (Fig. 17A, the last two in order of elution being the major ones and in nearly equal amounts. Phenylalanyl-tRNA eluted as a single peak (Fig, 17B)

Purification of aminoacyl-tRNA

Labelled tRNA was dissolved in 0.3M NaCl/10mM $MgCl_2$/ 10mM sodium acetate, pH 4.5 and adsorbed onto a 4 cm column of DEAE-cellulose equilibrated with the same buffer in a Pasteur pipette. After washing with 5 ml of buffer, the tRNA was eluted from the column with 1M NaCl/ 10mM $MgCl_2$/ 10mM sodium acetate, pH 4.5 and precipitated with 2.5 volumes of 95% (v/v) ethanol. This procedure removed free amino acids as well as rRNA.

Nitrous acid modification of aminoacyl-tRNA

In order to study aminoacyl-tRNA by gel electrophoresis or by density gradient centrifugation at neutral pH (see Results), it

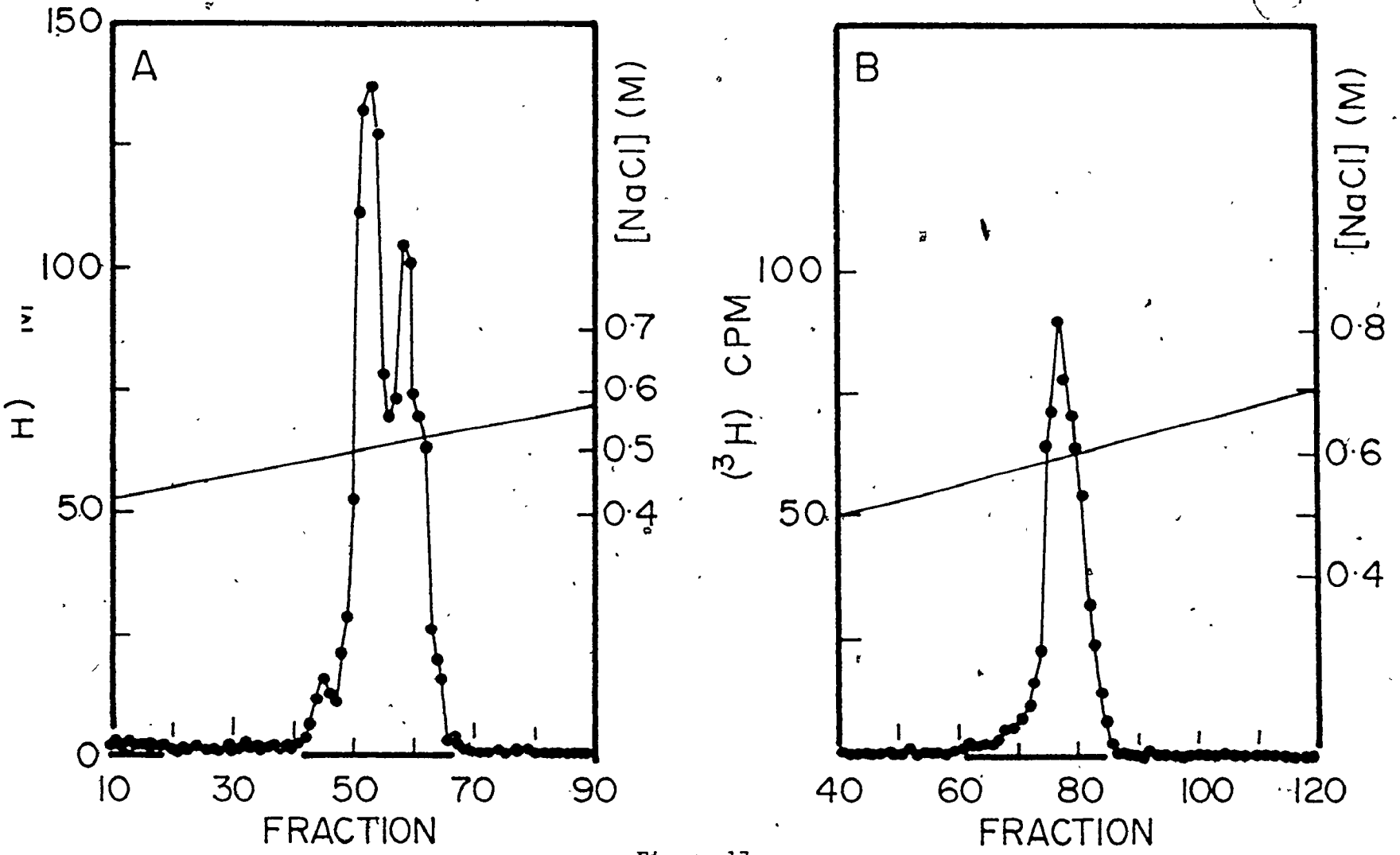


Figure 17

RPC-5 chromatographic profiles of yeast cytosolic [^{3}H] leucyl-tRNA (A) and [^{3}H] phenylalanyl-tRNA (B). Yeast aminoacyl-tRNAs were extracted and chromatographed as described in the Methods.

was necessary to stabilize the aminoacyl-tRNA bond to prevent deacylation. This was accomplished by the nitrous acid deamination of aminoacyl-tRNA as described by Clarkson et al (1973). Briefly, labelled aminoacyl-tRNA was suspended in 1ml of 10mM sodium acetate, pH 4.5 and 0.5 ml of saturated sodium nitrite and 5 µl glacial acetic acid were added. After incubation at 22°C for 15 min, 0.2 ml of 4M NaCl, pH 5.0 was added and labelled aminoacyl-tRNA was recovered by precipitation with 95% (v/y) ethanol.

Analysis of aminoacyl-tRNA

a. Stability: The stability of nitrous acid-treated and non-treated cytosolic and mitochondrial methionyl- and leucyl-tRNA was determined as follows. A sample of labelled aminoacyl-tRNA, either treated or non-treated, was dissolved in 100 µl of 10mM sodium acetate, pH 4.5, and incubated at 37°C. Aliquots of 10 µl were taken at various times and placed on Whatman 3 MM filters. Trichloroacetic acid-precipitable counts were determined after washing the filters in ice-cold 5% trichloroacetic acid, followed by ethanol/ether (1:1, v/v) and ether. The filters were then dried and counted for radioactivity. For analysis at neutral pH, the pH of the solution was adjusted to pH 7.0-7.5 by the addition of 0.1N NaOH and aliquots taken as above.

b. Formylated species: Methionyl-tRNA was analysed for formylated species essentially as described by Wallace & Freeman (1974). Samples were resuspended in a small volume of 10mM sodium acetate, pH 4.5 and RNase A added to 20 µg/ml. Digestion was for 30 min at 37°C. Aliquots were then spotted on Whatman No. 1 paper and subjected to electrophoresis at 60V/cm

dried and counted for radioactivity. Radioactive methionine was used as a marker. The position of methionyl-adenosine and formylmethionyl-adenosine were deduced from the results of Smith & Marcker (1968) for rat liver mitochondria.

c. RPC-5 chromatography: RPC-5 chromatography was performed as described by Pearson et al (1971). Acylated tRNA species were eluted at room temperature with a 200 ml linear gradient from 0.45 M NaCl to 0.7 M NaCl in 10mM $MgCl_2$/ 2mM mercaptoethanol/ 10mM sodium acetate, pH 4.5 for leucyl-tRNA and 0.4 to 0.7 M NaCl in the same buffer for methionyl-tRNA. Approximately 1 ml fractions were collected directly into scintillation vials using a Gilson microfractionator and counted for radioactivity by adding 3.5 ml PCS scintillation fluid.

Sucrose-density gradient centrifugation

Aminoacyl-tRNAs and HNO_2-aminoacyl-tRNAs were analysed by sucrose gradient centrifugation using three different conditions.

i) low pH, high salt gradients. Aminacyl-tRNAs were dissolved in 100 mM LiCl/ 10mM magnesium acetate/ 0.1% SDS/ 5% sucrose/ 2% formaldehyde/ 10 mM sodium acetate, pH 4.5. The solution was heated to 70°C for 5 min, cooled rapidly and layered over a 5 to 20% sucrose-density gradient in the same buffer.

ii) neutral pH, low salt gradients. HNO_2-aminoacyl-tRNAs were dissolved in 0.1% SDS/ 2% formaldehyde/ 5% sucrose/ 20mM sodium phosphate, pH 7.4. After heating at 70°C for 5 min and rapid cooling, samples were layered over a 5 to 20% sucrose-density gradient in the same buffer.

For both (i) and (ii), centrifugation was at 108,000g for 24 h at

24°C in a Beckman SW 41 rotor.

iii) low pH, low salt gradients at low temperature. Aminoacyl-tRNAs were dissolved in 0.1% SDS/ 2% formaldehyde/ 35mM sodium acetate, pH 4.5, the solution heated to 70°C for 5 min, cooled rapidly and layered over a 5 to 20% sucrose-density gradient in the same buffer. Centrifugation was as described for (i) and (ii), except that the temperature was kept at 4°C.

All gradients were dripped from the bottom of the tube and approximately 0.2-0.3 ml fractions collected and counted for radioactivity by addition of 0.3 ml H_2O and 3 ml PCS solubilizer.

Marker 7S RNA

Marker 7S (5.8S) RNA was released from [^{14}C] uridine-labelled CHO cell rRNA by denaturing as described for aminoacyl-tRNAs. The rRNA was extracted as described in Methods of Part I.

Polyacrylamide gel electrophoresis

i) Non-denaturing gels were run according to Loening (1969) as described in Methods of Part I.

ii) Electrophoresis of nitrous acid-treated aminoacyl-tRNA (HNO_2-aminoacyl-tRNA) was performed under denaturing conditions essentially as described by Dubin & Friend (1972). The acrylamide concentration was 10 or 15% with a ratio of acrylamide to bis-acrylamide of 40:1. The gels contained 10% glycerol and 5% formaldehyde. The gel buffer was 20mM sodium phosphate, pH 7.0 and the reservoir buffer 1mM EDTA/ 20mM sodium phosphate, pH 7.0. Samples were dissolved in 18% formaldehyde/ 0.1% SDS/ 20mM sodium phosphate, pH 7.0, heated for 5 min at 70°C

and cooled rapidly prior to applying to the gel. Marker 5S RNA when used was run on the same gel. Electrophoresis was at 10 mA/gel for 3 h.

Gels were fractionated into 1 mm slices on a Gilson Aliquogel fractionator, gel fractions treated with 0.4 ml 20% NH_4OH and counted for radioactivity as described in Methods of Part I.

iii) Electrophoresis of HNO_2-aminoacyl-tRNA was also performed on slab gels under denaturing conditions as described by Maniatis et al (1975). The final acrylamide concentration was 12% with an acrylamide to bis-acrylamide ratio of 29:1, in 4mM EDTA/ 7M urea/ 90mM Tris-borate, pH 8.0. tRNA samples were dissolved in 25 to 30 µl 98% formamide, heated to boiling for 2 min and cooled rapidly on ice. Electrophoresis was at 10 V/cm for 5 to 5.5 h. Dye markers were run with each sample. According to Maniatis et al (1975), Xylene cyanol FF and Bromophenol Blue migrate to positions corresponding to 58 and 13 nucleotide-long chain lengths respectively. Gels were prepared for fluorography as described by Bonner & Laskey (1974) and exposed to Royal-B X-Omat films at -70°C.

MATERIALS

L-(4,5-^{3}H) Leucine (38-57 Ci/mmol)	New England Nuclear
L-(^{14}C(U)) Leucine (270 mCi/mmol)	" "
L-(CH_3-^{3}H) Methionine (5 Ci/mmol)	" "
L-(^{35}S) Methionine (300 Ci/mmol)	"
L-(^{3}H) Phenylalanine (60 Ci/mmol)	" "
Pancreatic RNase A	Worthington Biochemical
Royal-B X-Omat film	Eastman Kodak Co.
RPC-5	Astro Enterprises
Rabbit liver stripped tRNA	Sigma
E. coli stripped tRNA	Sigma
Baker's yeast tRNA	Sigma
Urea (Ultra pure)	Schwartz-Mann
Xylene cyanol FF	Eastman Kodak Co.

Wheat embryo 5S RNA ([^{3}H] and [^{14}C] -labelled) was a generous gift of Dr. B.G. Lane, Dept. of Biochemistry, University of Toronto, Ontario

RESULTS

In order to determine the molecular weight of a specific mitochondrial aminoacyl-tRNA in comparison with its cytosolic counterpart it is necessary to use conditions in which each would have the same conformation. This can be achieved in dual-labelling experiments by separating the fully denatured aminoacyl-tRNAs by centrifugation or electrophoresis. Leucyl- and methionyl-tRNAs were chosen for three reasons: 1) labelled aminoacids can be obtained at very high specific activity; 2) the extent of incorporation of these amino acids into protein by isolated mitochondria is higher than any other amino acid, and 3) the cytosolic leucyl- and methionyl-tRNAs would be expected to have different molecular weights. Assuming full denaturation of the tRNAs under the electrophoretic and centrifugal conditions used, the validity of such studies depends on the purity and the stability of the aminoacyl-tRNAs.

Characterization of the aminoacyl-tRNAs

The purity of the mitochondrial and cytosolic aminoacyl-tRNA preparations was determined by chromatography on RPC-5 according to Pearson et al (1971). As shown in Fig. 18, mitochondrial [^{35}S] methionyl-tRNA eluted in three distinctive peaks at salt concentrations lower than those for the three cytosolic species labelled with [^{3}H] methionine. These results are similar to those with mouse liver mitochondrial methionyl-tRNAs (Wallace & Freeman, 1974) but differ in that the first peak

Figure 18

RPC-5 chromatographic profile of Syrian hamster liver [^{35}S] methionine-labelled mitochondrial methionyl-tRNA (o-o) co-chromatographed with [^{3}H] methionine-labelled cytosolic methionyl-tRNA (●–●). Labelled mitochondrial and cytosolic methionyl-tRNAs were prepared as described in the Methods. After elution from DEAE-cellulose and precipitation, samples were dissolved in a small volume of running buffer at the initial salt concentration, made up to approximately 0.5 M sucrose and applied to a packed column of RPC-5 previously equilibrated with buffer at the initial salt concentration. Fractions were collected and counted as described in the Methods

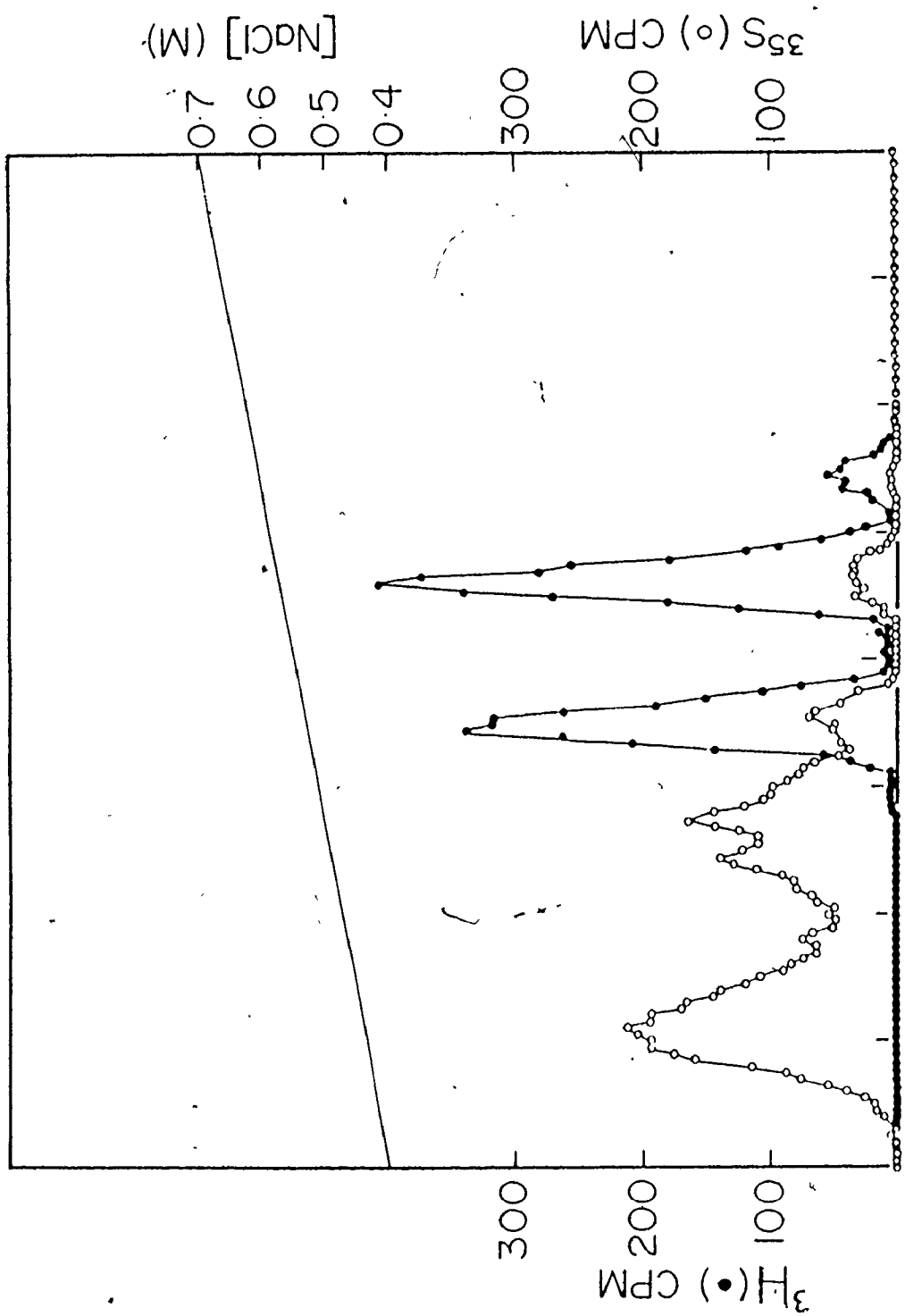

[NaCl] (M)
0·7
0·6
0·5
0·4
^{35}S (○) CPM
300
200
100
^{3}H (●) CPM
300
200
100
20
40
60
80
100
120
140
FRACTION

Figure 18

could not be separated into two species. This difference could reflect the absence of two separate species in hamster liver mitochondria or lack of separation by the chromatographic system. The result of a similar experiment with leucyl-tRNAs are shown in Fig. 19. Three major peaks of mitochondrial [^{3}H]leucyl-tRNA were found to elute at salt concentrations higher than the bulk of the cytosolic [^{14}C] leucyl-tRNA which appeared as at least five distinctive peaks. This confirms the results of Wallace (1975) who compared Syrian hamster mitochondrial leucyl-tRNA to CHO cell tsH1 cytosolic leucyl-tRNA.

The preparations of both leucyl- and methionyl-tRNAs from hamster mitochondria contained some species eluting with the cytosolic tRNAs. However, the distinctive patterns of mitochondrial leucyl- and methionyl-tRNAs obtained indicate that less than 10% of the label in these preparations was in cytosolic aminoacyl-tRNAs.

Having demonstrated the specificity of the labelling of mitochondrial aminoacyl-tRNAs by isolated mitochondria, the stability of the aminoacyl-tRNA bond, labile under most conditions of analyses, was studied. While the stability of this bond is greatest at low pH, as shown by comparison of the rate of deacylation at pH 4.5 and 7.5 (Figs. 20 & 21), deacylation has been reported to occur at pH 5.0 during aminoacyl-tRNA/DNA hybridization (Clarkson et al, 1973) as might be expected from the half-times of hydrolysis of aminoacyl-tRNAs at this pH (Hentzen et al, 1972). In order to study labelled aminoacyl-tRNA by gel electrophoresis and sucrose-density gradient sedimentation at neutral pH, the labelled preparations of aminoacyl-tRNAs were stabilized by treatment with nitrous acid to deaminate the aminoacyl moiety (Hervé & Chapeville, 1965) under conditions in which

Figure 19

RPC-5 chromatographic profile of Syrian hamster liver [^{3}H] leucine-labelled mitochondrial leucyl-tRNA (o-o) co-chromatographed with[^{14}C] leucine-labelled cytosolic leucyl-tRNA (●-●) Conditions were the same as in Fig. 18.

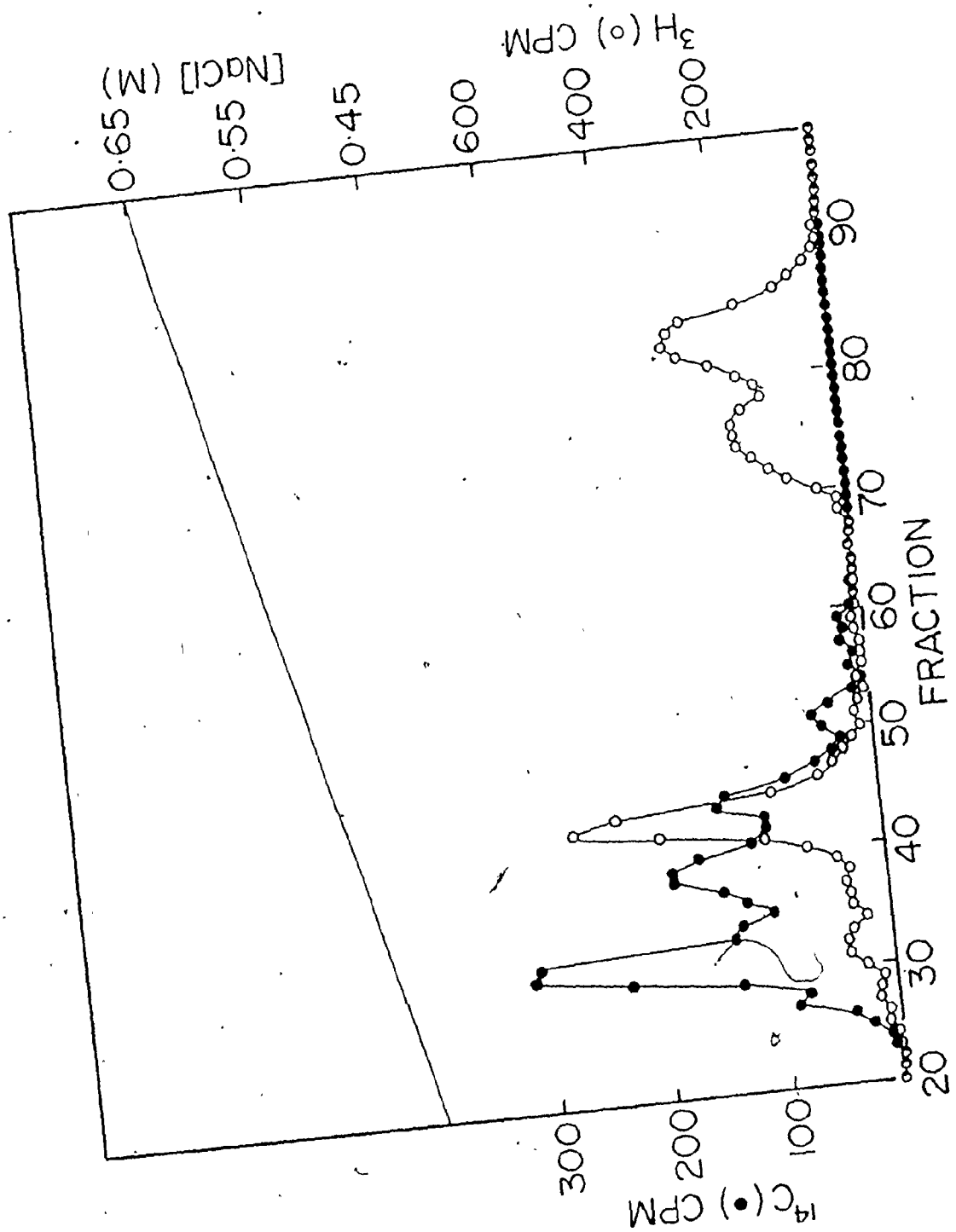

[NaCl] (M)
0·65
0·55
0·45
^{3}H (○) CPM
600
400
200
^{14}C (●) CPM
300
200
100
FRACTION
20
30
40
50
60
70
80
90

Figure 19

Figure 20

Stability of nitrous acid-treated and untreated Syrian hamster liver cytosolic (A) and mitochondrial (B) tRNAs.A sample of [^{3}H] methionine-labelled cytosolic methionyl-tRNA or [^{35}S]. methionine-labelled mitochondrial methionyl-tRNA, either treated with nitrous acid as described in the Methods or non-treated, was dissolved in 100 µl of 10 mM sodium acetate, pH 4.5, incubated at 37°C and 10 µl aliquots taken at various times and placed on Whatman 3 MM filters. Trichloroacetic acid-precipitable counts were determined as described in the Methods. For the assay at neutral pH, the pH was adjusted to 7.0-7.5 by the addition of NaOH. o-o, Methionyl-tRNA, pH 4.5; •-•, methionyl-tRNA, pH 7.5, ▲-▲, HNO_2-methionyl-tRNA, pH 4.5; ▲-▲, HNO_2-methionyl-tRNA, pH 7.5.

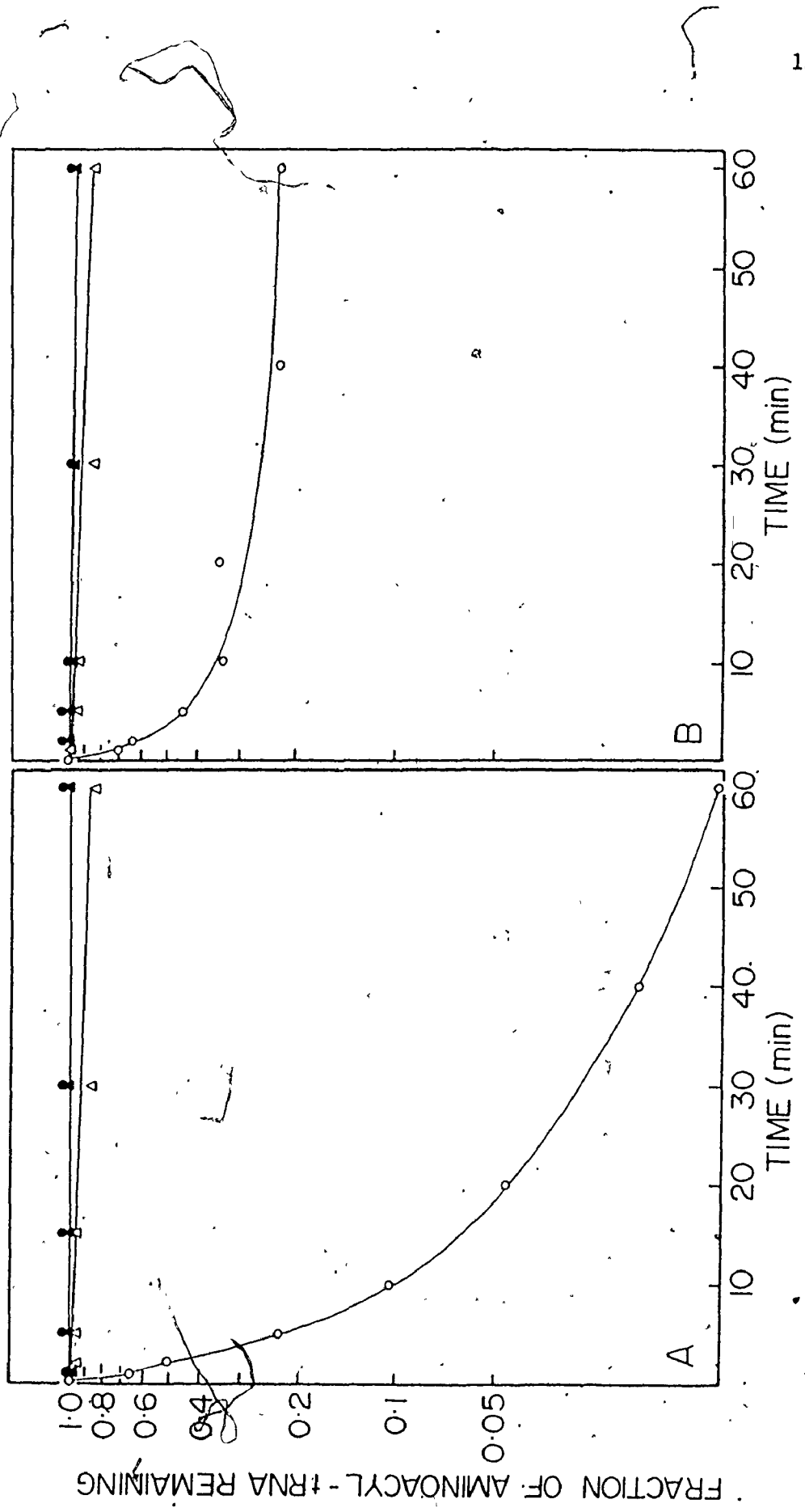

FRACTION OF AMINOACYL - tRNA REMAINING
1·0
0·8
0·6
0·4
0·2
0·1
0·05
A
B
10
20
30
40
50
60
TIME (min)

Figure 20

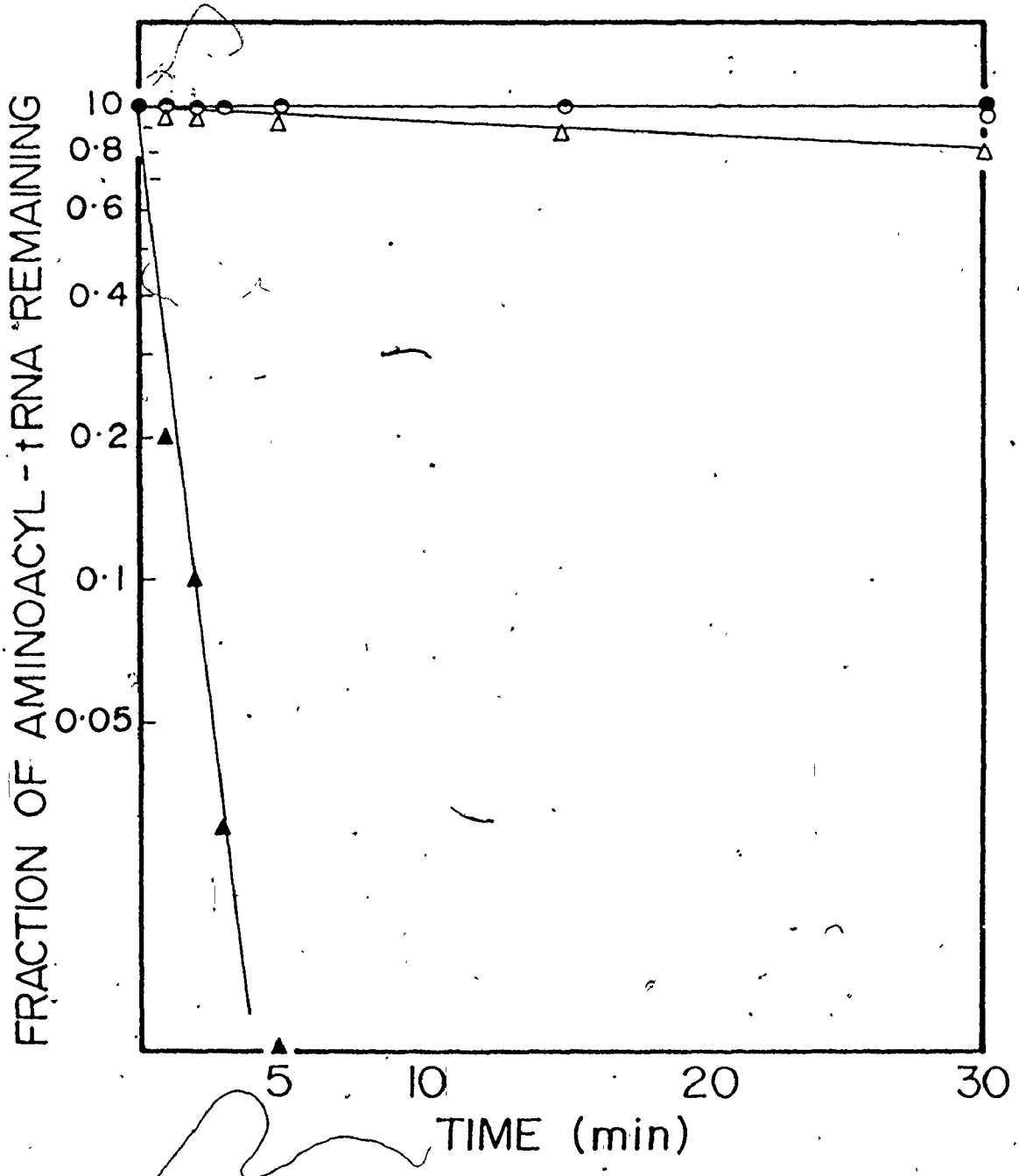


Figure 21

Stability of nitrous acid-treated and untreated Syrian hamster ^{3}H leucine-labelled cytosolic leucyl-tRNA. The experimental conditions were the same as in Fig. 20. o-o, Leucyl-tRNA, pH 4.5; ▲-▲, leucyl-tRNA, pH 7.5; ●-◆, HNO_2-leucyl-tRNA, pH 4.5; Δ-Δ, HNO_2-leucyl-tRNA, pH 7.5.

the ribonucleotides are not affected (Carbon, 1965) . The stability of cytosolic nitrous acid treated methionyl-tRNA (HNO_2-methionyl-tRNA) to deacylation at pH 7.5 as compared to that of untreated methionyl-tRNA is shown in Fig. 20. The cytosolic HNO_2-methionyl-tRNA was stable at this pH while the untreated methionyl-tRNA deacylated rapidly.

Treatment with nitrous acid also stabilized mitochondrial methionyl-tRNA completely at pH 7.5 as shown in Fig.20B, as well as cytosolic leucyl-tRNA (Fig. 21) and mitochondrial leucyl-tRNA (results not shown).

In contrast to cytosolic methionyl-tRNA, untreated mitochondrial methionyl-tRNA deacylated very rapidly at pH 7.5 but plateaued at 30% (Fig. 20B). Paper electrophoresis of a pancreatic digest of this fraction of [^{35}S] methionyl-tRNA revealed formylmethionyladenosine arising from formylmethionyl-tRNA (Fig. 22A). The formyl group presumably stabilizes the aminoacyl bond of this tRNA. In contrast, total mitochondrial methionyl-tRNA yielded both formylmethionyl-adenosine and methionyl-adenosine (Fig. 22B). This differential stability was exploited in the analysis of mitochondrial formylmethionyl-tRNA. No attempt was made to determine which peak(s) separated on RPC-5 chromatography corresponded to formylmethionyl-tRNA, as was done by Wallace & Freeman (1974).

While the deamination treatment should not affect the ribonucleotides of the tRNAs, it was necessary to determine whether either the electrophoretic mobility or sedimentation rate of the tRNAs was affected. Electrophoresis under non-denaturing conditions of mitochondrial methionyl-tRNA, which, in relation to the above, corresponds to

Figure 22

Electrophoretic profiles of RNase digests of [^{35}S] methionine-labelled Syrian hamster liver mitochondrial methionyl-tRNAs. Mitochondrial methionyl-tRNAs were labelled and prepared as described in the Methods. The neutral pH-stable fraction of mitochondrial methionyl-tRNA was obtained after deacylation of methionyl-tRNA at pH 7.5 for 15 min at 37°C (Fig. 20B), precipitation and adsorption and elution from a DEAE-cellulose column. The aminoacyl-tRNAs were digested with RNase A (20 µg/ml) in 50 µl of 10 mM sodium acetate, pH 4.5 at 37°C for 30 min. A sample was subjected to paper electrophoresis, the paper dried and counted for radioactivity. Met, fMetA and MetA refer to methionine, formylmethionyl-adenosine and methionyl-adenosine respectively. A. Neutral pH-stable fraction of mitochondrial methionyl-tRNA. B. Total mitochondrial methionyl-tRNA.

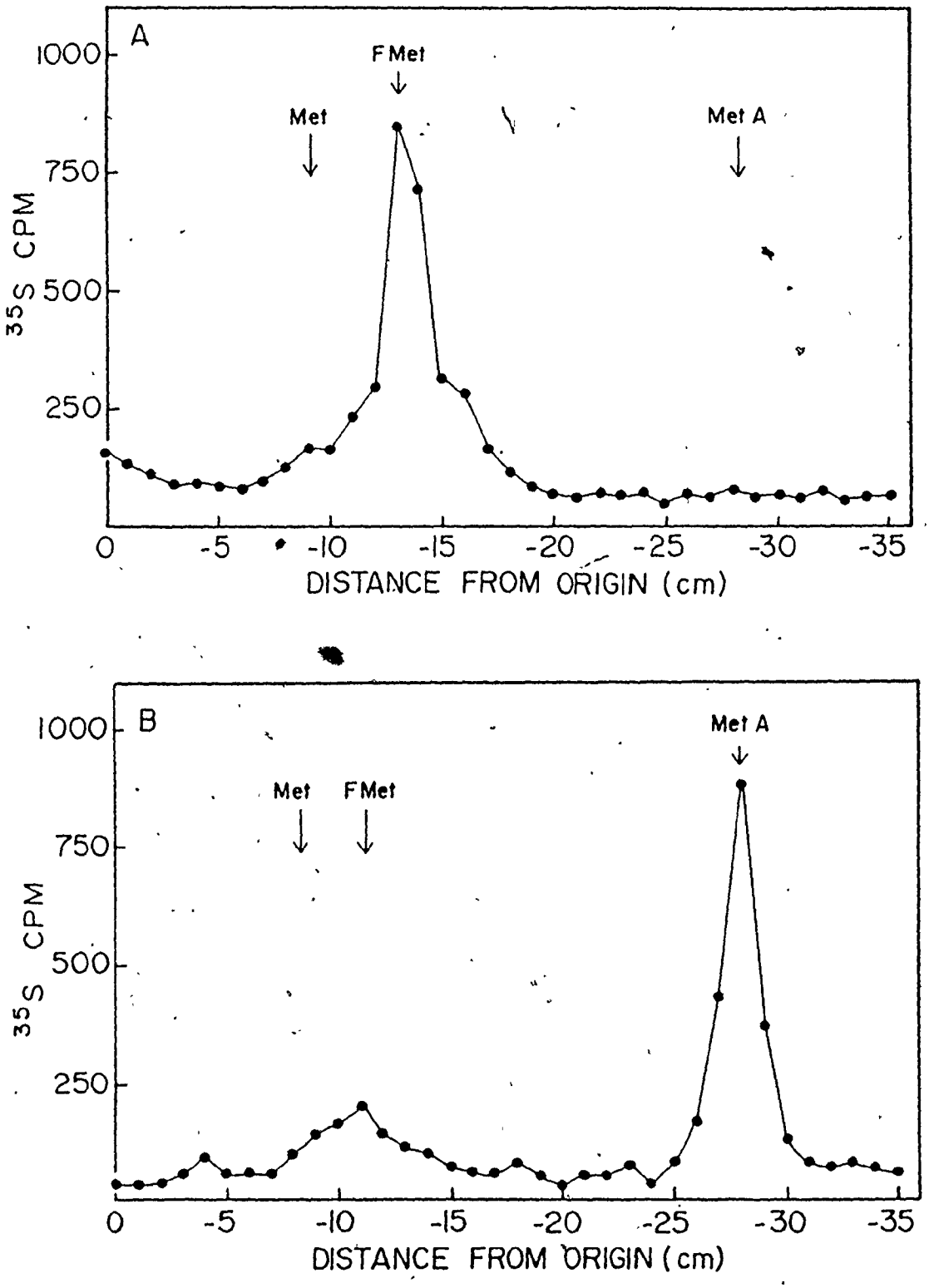

A
1000
750
500
250
35S CPM
Met
FMet
Met A
0
-5
-10
-15
-20
-25
-30
-35
DISTANCE FROM ORIGIN (cm)
B
1000
750
500
250
35S CPM
Met
FMet
Met A
0
-5
-10
-15
-20
-25
-30
-35
DISTANCE FROM ORIGIN (cm)

Figure 22

Figure 23

Polyacrylamide gel electrophoresis of mitochondrial (A) and cytosolic (B) methionyl-tRNAs of Syrian hamster liver treated and untreated with nitrous acid.
Mitochondrial and cytosolic methionyl-tRNAs were prepared as described in the Methods. Samples, either treated with nitrous acid as described in the Methods or untreated, were electrophoresed on 14% polyacrylamide gels for 3 h at 8 mA/ gel. Gels were then sliced and counted as described in the Methods. A. ●-●, Mitochondrial [^{35}S] methionyl-tRNA; o-o, mitochondrial HNO_2-[^{35}S] methionyl-tRNA. B. ●-●, cytosolic[^{3}H] methionyl-tRNA; o-o, cytosolic HNO_2-[^{3}H] methionyl-tRNA.

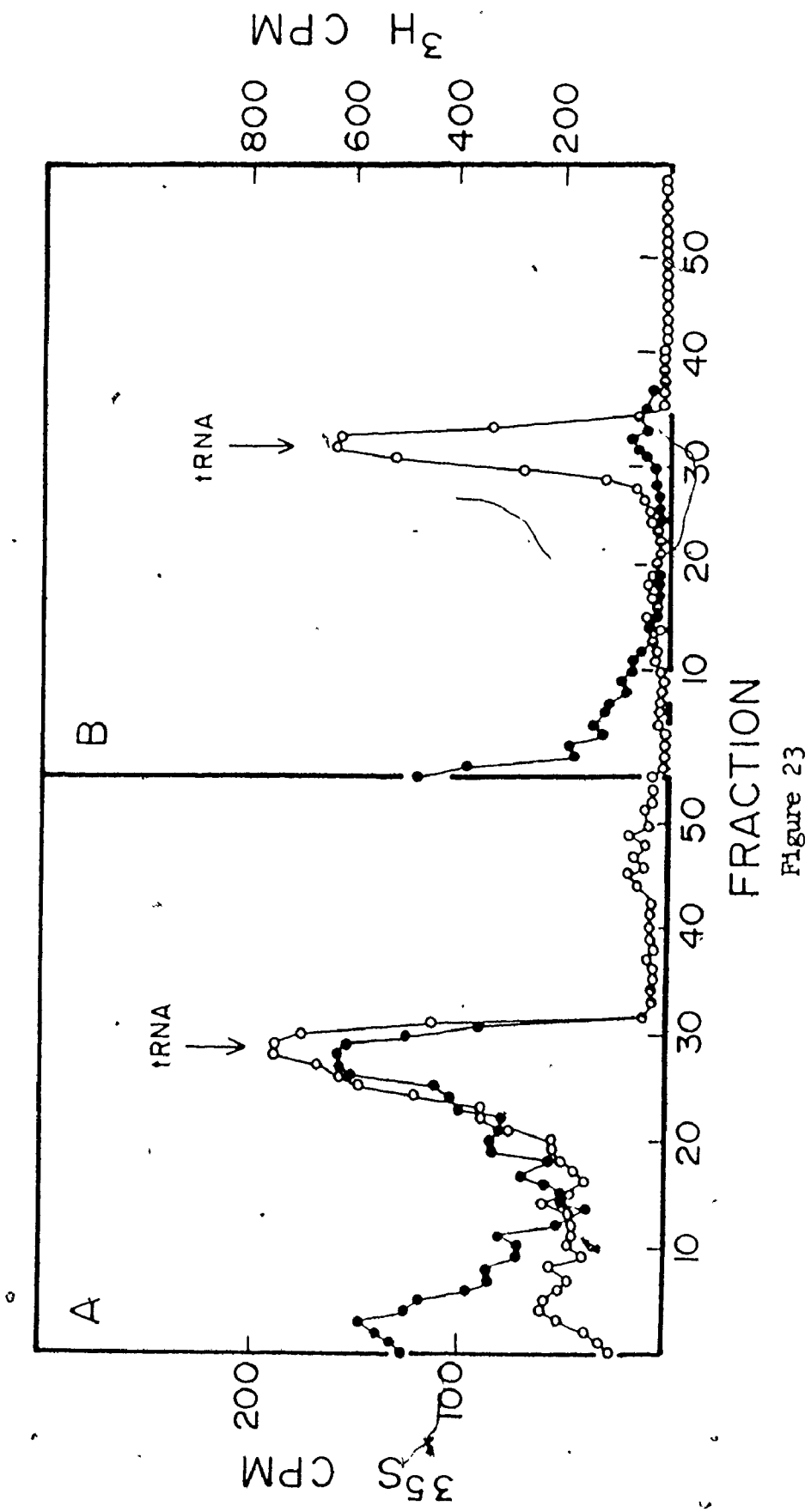

^{35}S CPM
200
100
A
tRNA
10
20
30
40
50
FRACTION
B
tRNA
10
20
30
40
50
^{3}H CPM
800
600
400
200

Figure 23

to rabbit liver tRNA (Fig. 23). The rest of the label appeared at the top of the gel. Electrophoresis of labelled leucyl-tRNA, whether cytosolic or mitochondrial did not reveal any band corresponding to tRNA (results not shown) as expected from the instability of the aminoacyl-bond at neutral pH (Fig. 21). On the other hand, cytosolic HNO_2-[^{35}S] methionyl-tRNA (Fig. 23) and HNO_2-[^{3}H] leucyl-tRNA (not shown) both have identical mobilities to rabbit liver tRNA. To determine whether the sedimentation rates were affected, [^{3}H] methionyl-tRNA and HNO_2-[^{35}S] methionyl-tRNA were run on low pH-high salt sucrose gradients. Both mitochondrial and cytosolic species were respectively found to co-sediment (results not shown). These results tend to indicate that the electrophoretic mobility, as observed by others with yeast aminoacyl-tRNA (Fradin et al, 1975) and the sedimentation rate of the aminoacyl-tRNAs were not affected by the nitrous acid treatment.

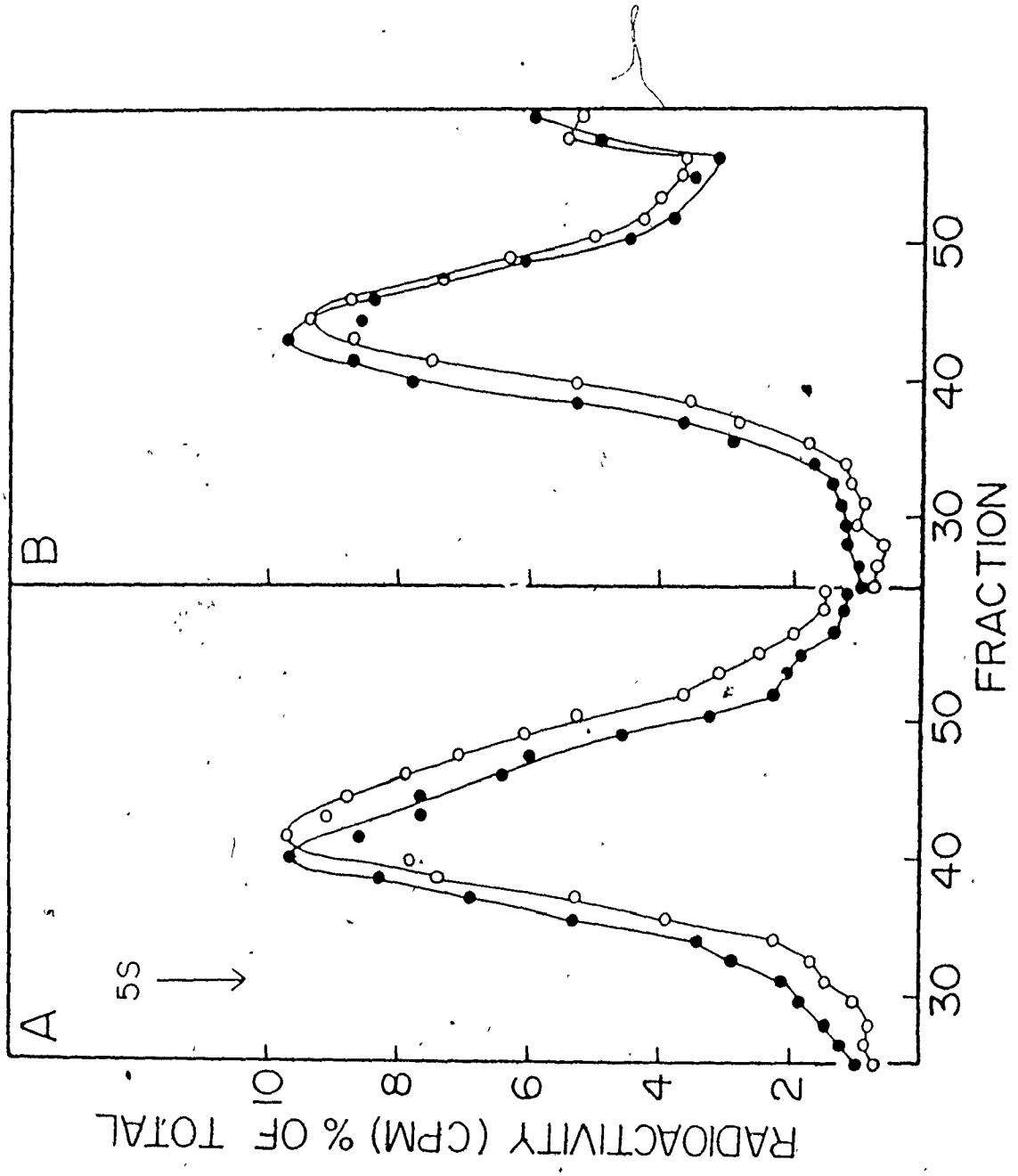
Sucrose gradient density centrifugation analysis

The size of mitochondrial and cytosolic tRNAs were compared under different conditions of sedimentation on sucrose density gradients containing formaldehyde. The aminoacyl-tRNAs were denatured by heating in formaldehyde at 70°C for 5 min, cooled rapidly and centrifuged on 5 to 20% sucrose density gradients in the presence of 2% formaldehyde.

Fig. 24A shows the profile of mitochondrial [^{35}S] methionyl-tRNA and cytosolic [^{3}H] methionyl-tRNA sedimented on a low pH-high salt (0.1M LiCl/ 10mM $MgCl_2$/ 10mM sodium acetate, pH 4.5) gradient. It is clear that the mitochondrial methionyl-tRNA sedimented more slowly than the cytosolic tRNA. A similar result was obtained if mitochondrial methionyl-tRNA was allowed to deacylate at pH 7.5 to yield formyl-

Figure 24

Sucrose density-gradient sedimentation profiles of Syrian hamster liver methionyl-tRNAs. The preparation and denaturation of the methionyl-tRNAs and HNO_2-methionyl-tRNAs were as described in the Methods. Sucrose density gradients (5-20%) in the presence of 2% formaldehyde were run either at pH 4.5 and high salt with methionyl-tRNAs (A) or at pH 7.4 and low salt with HNO_2-methionyl-tRNAs (B). Centrifugation was for 24 h at 108,000$\bar{g}$ at 24°C. Sedimetation is from right to left. •-•, Cytosolic [^{3}H] methionyl or HNO_2-[^{3}H] methionyl-tRNA; o-o, mitochondrial [^{35}S] methionyl or HNO_2-[^{35}S] methionyl-tRNA.


A
B
5S
RADIOACTIVITY (CPM) % OF TOTAL
0
2
4
6
8
10
30
40
50
30
40
50
FRACTION

methionyl-tRNA.

It was possible that in the presence of Mg^{++} ions, aminoacyl-tRNAs are not fully denatured on heating. Therefore, the experiments were repeated by heating and sedimentation in low salt buffers. HNO_2-methionyl-tRNAs were compared on sucrose density gradients at neutral pH and low salt (20mM phosphate, pH 7.4). Fig. 24B shows that the profile was similar to that obtained in the low pH-high salt buffer. The same result was also obtained if the tRNAs were denatured in 18% formaldehyde rather than 2% formaldehyde. Finally, methionyl-tRNAs were run at $4^{o}C$ in gradients having the same ionic strength as the neutral pH-low salt buffer but at low pH. Again as shown in Fig. 25B a similar difference in the rate of sedimentation of mitochondrial and cytosolic methionyl-tRNAs was observed.

Fig.26 shows the profiles of mitochondrial and cytosolic leucyl-tRNAs (A) and HNO_2-leucyl-tRNAs (B) sedimented at low pH-high salt and neutral pH-low salt respectively. Mitochondrial leucyl-tRNA sedimented more slowly than its cytosolic counterpart. At $4^{o}C$, the same difference was observed (Fig. 25A). In all cases, the difference between cytosolic and mitochondrial leucyl-tRNAs was larger (2 to 3 fractions in the low pH- high salt buffer) than that of the methionyl-tRNAs (1 fraction). The results suggest that the mitochondrial methionyl- and leucyl-tRNAs should co-sediment and this was found to be the case as shown in Fig. 27 .

In order to determine whether these results were specific for Syrian hamster tRNAs, comparisons were made with rat liver mitochondrial and cytosolic leucyl-tRNA and methionyl-tRNA. Fig. 28 A shows that the difference in sedimentation rate of rat liver mitochondrial and cyto-

Figure 25

Sucrose density gradient sedimentation profiles of Syrian hamster liver leucyl- (A) and methionyl-tRNAs (B) at low temperature. Aminoacyl-tRNAs were prepared and denatured as described in the Methods. Centrifugation was in 2% formaldehyde/ 0.1% SDS/ 35 mM sodium acetate, pH 4.5 at 4°C and 108,000g for 24 h. Sedimentation is from right to left. ●–●, Cytosolic tRNA; o–o, mitochondrial tRNA.

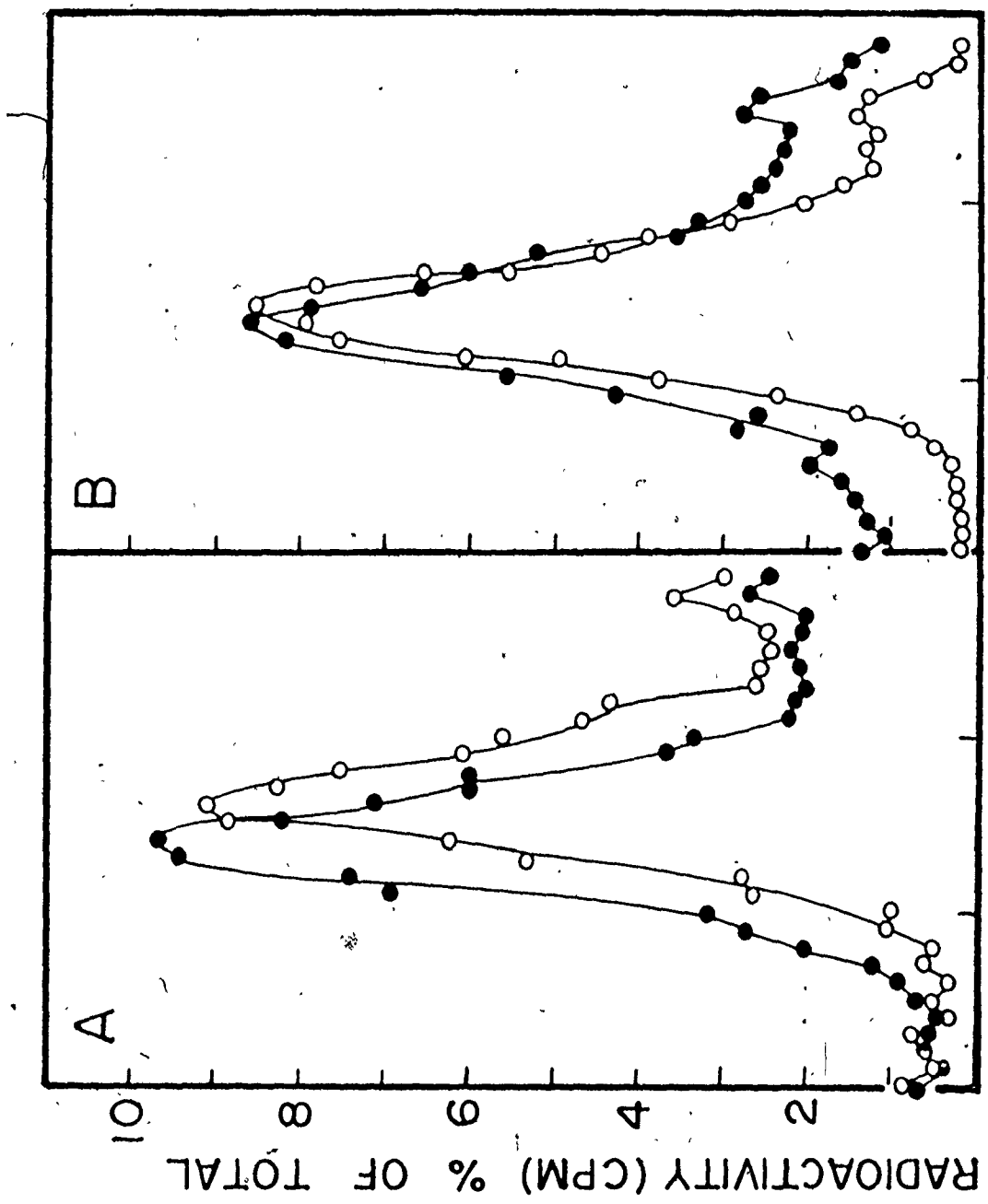


Figure 25

Figure 26

Sucrose density-gradient sedimentation profiles of Syrian hamster liver leucyl-tRNAs. The preparation and denaturation of leucyl-tRNA or HNO_2-leucyl-tRNA were as described in the Methods. Sucrose density gradients (5-20%) in the presence of 2% formaldehyde were run either at pH 4.5 and high salt with leucyl-tRNAs (A) or at pH 7.4 and low salt with HNO_2-leucyl-tRNAs (B). Centrifugation was for 24 h at 108,000 at 24°C. Sedimentation is from right to left. ●-●, Cytosolic [^{14}C] leucyl- or HNO_2-[^{14}C] leucyl-tRNA; o-o, mitochondrial [^{3}H] leucyl- or HNO_2-[^{3}H] leucyl-tRNA.

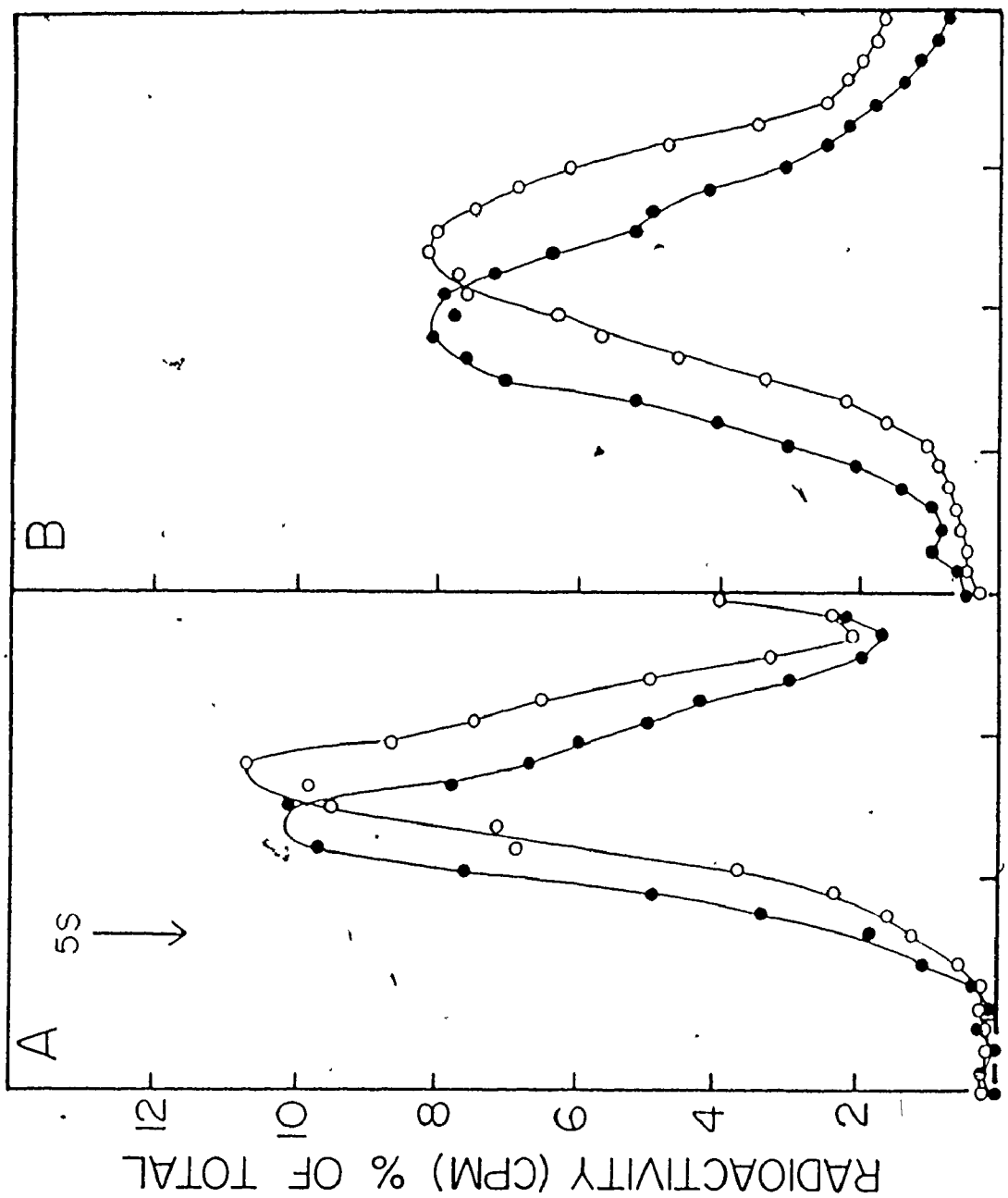

RADIOACTIVITY (CPM) % OF TOTAL
2
4
6
8
10
12
A
5S
B
30
40
50
30
40
50
FRACTION

Figure 26

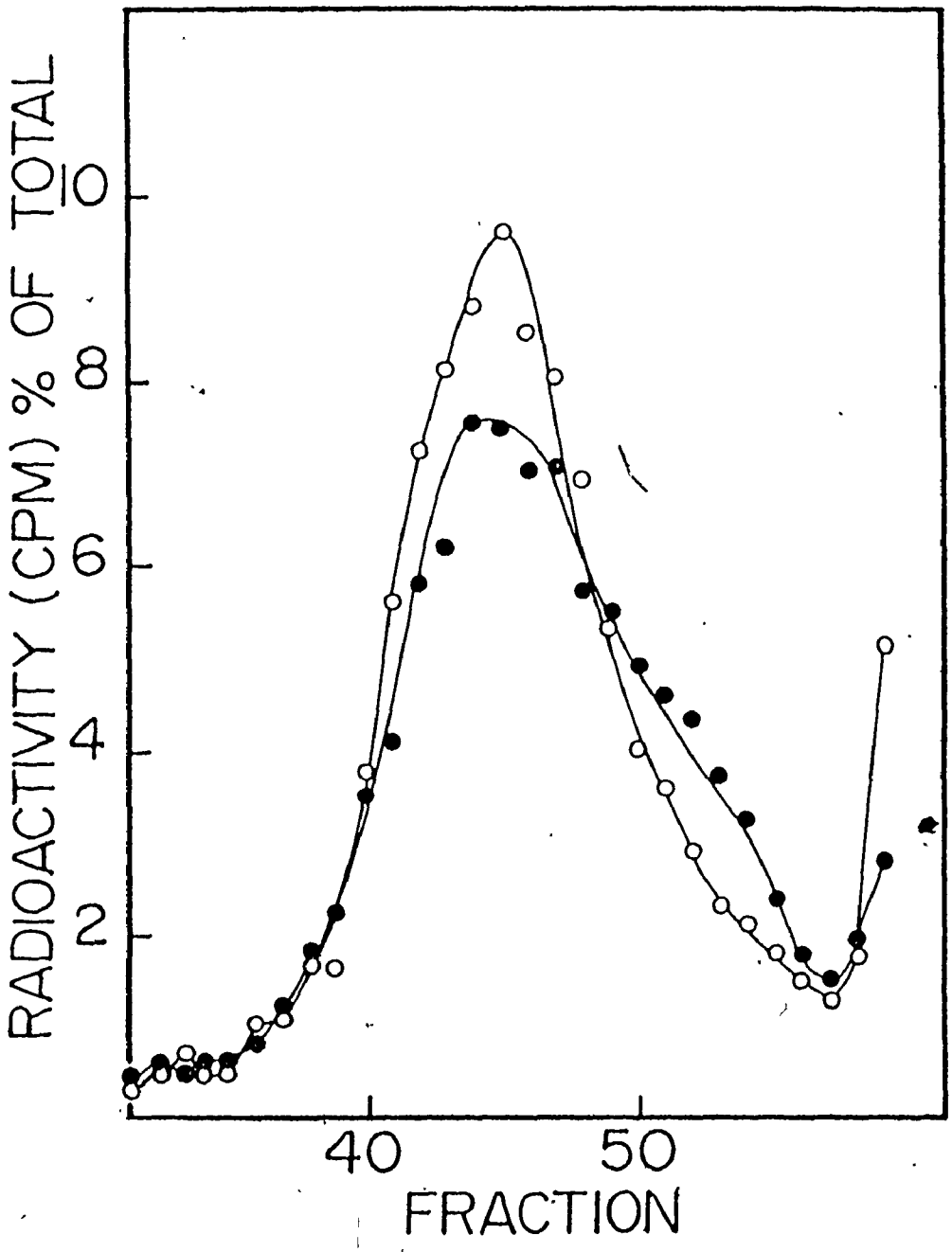


Figure 27

Sucrose density-gradient sedimentation profiles of Syrian hamster liver mitochondrial leucyl- and methionyl-tRNAs. Aminoacyl-tRNAs were prepared, denatured and centrifuged at pH 4.5 as described in Figs. 24A and 26A. •–•, Mitochondrial [^{35}S]methionyl-tRNA; o–o, [^{3}H] leu

Figure 28

Sucrose density gradient sedimentation profiles of rat liver leucyl-tRNAs (A) and of rat liver and Syrian hamster liver mitochondrial methionyl-tRNAs (B). Rat liver cytosolic and mitochondrial leucyl-tRNAs and rat liver and Syrian hamster liver mitochondrial methionyl-tRNAs were prepared and denatured as described in the Methods. Centrifugation was as in Fig. 27. A. o-o, Mitochondrial [^{3}H] leucyl-tRNA; •-•, cytosolic [^{14}C] leucyl-tRNA. B. •-•,Syrian hamster liver [^{35}S] methionyl-tRNA; o-o, rat liver [^{3}H] methionyl-tRNA.

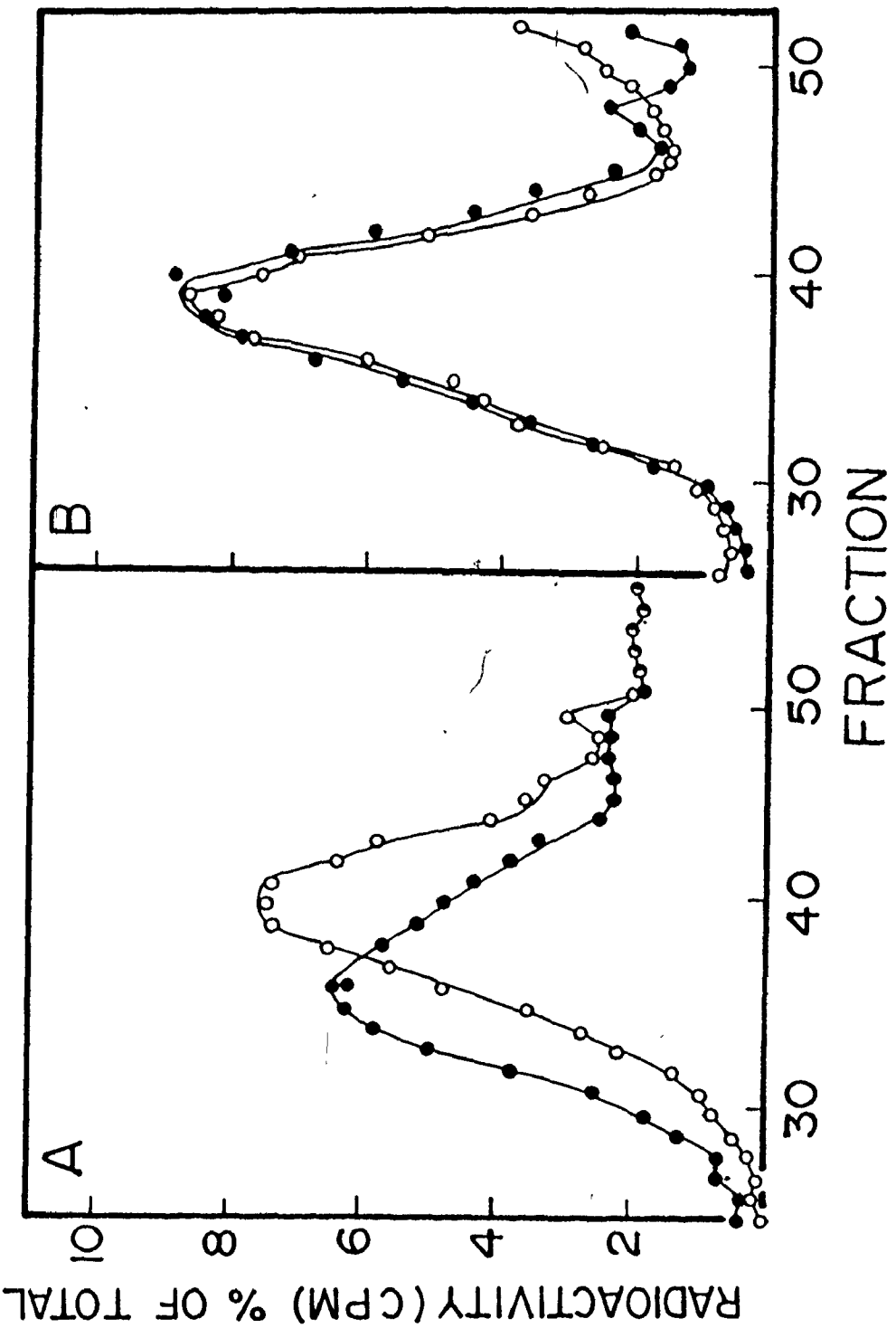

RADIOACTIVITY (CPM) % OF TOTAL
2
4
6
8
10
A
B
30
40
50
30
40
50
FRACTION

Figure 28

solic leucyl-tRNAs was similar to that of the Syrian hamster tRNAs (compare with Fig. 26 A). The same was true of the methionyl-tRNAs (results not shown). Further, Syrian hamster and rat liver mitochondrial methionyl-tRNAs co-sedimented (Fig. 28 B), Results with slab gel electrophoresis (vide infra) confirm these results.

Polyacrylamide gel electrophoresis of HNO_2-aminoacyl-tRNA

A true difference in size should be reflected in the mitochondrial tRNAs sedimenting slower on sucrose density gradients than their cytosolic counterparts and running faster on polyacrylamide gels.

Mitochondrial aminoacyl-tRNAs were first analysed under denaturing conditions in formaldehyde in order to compare with sucrose density gradient centrifugation analyses. Mitochondrial and cytosolic HNO_2-aminoacyl-tRNAs were denatured by heating in 18% formaldehyde at 70^0C for 5 min and compared by disc gel electrophoresis in gels containing 5% formaldehyde. Such conditions have been reported to give fully denatured molecules (Boedtker,,1968; Fenwick, 1968).The electrophoretic conditions used corresponded to the "high amperage, formaldehyde-treated" conditions used by Dubin & Friend (1972). Fig. 29 A shows the profile of mitochondrial HNO_2- [^{35}S] methionyl-tRNA and cytosolic HNO_2- [^{3}H] methionyl-tRNA run on the same 10% polyacrylamide gel. It can be seen that the mitochondrial peak corresponds exactly to that of the cytosolic tRNA. From the position of the 5S RNA marker (120 nucleotides long), assuming linearity of electrophoretic mobility and taking an average size of the cytosolic methionyl-tRNA of 75 nucleotides (Piper & Clark,1974; Simsek et al, 1974), each slice represents a difference in

Figure 29

Electrophoretic profiles of Syrian hamster liver aminoacyl-tRNAs in 10% polyacrylamide gels. Mitochondrial and cytosolic labelled aminoacyl-tRNAs were prepared as described in the Methods. All samples were treated with nitrous acid except for B where mitochondrial methionyl-tRNA was applied directly after extraction. Electrophoresis was for 3 h at 10 mA/gel. Gels were sliced and counted as described in the Methods. The 5S RNA marker, labelled with ^{14}C or ^{3}H, was run on the same gels as the samples. A. Methionyl-tRNAs. B. Cytosolic methionyl- and mitochondrial formylmethionyl-tRNA. C. Leucyl-tRNAs. ●-●, Cytosolic tRNA; o-o, mitochondrial tRNA.

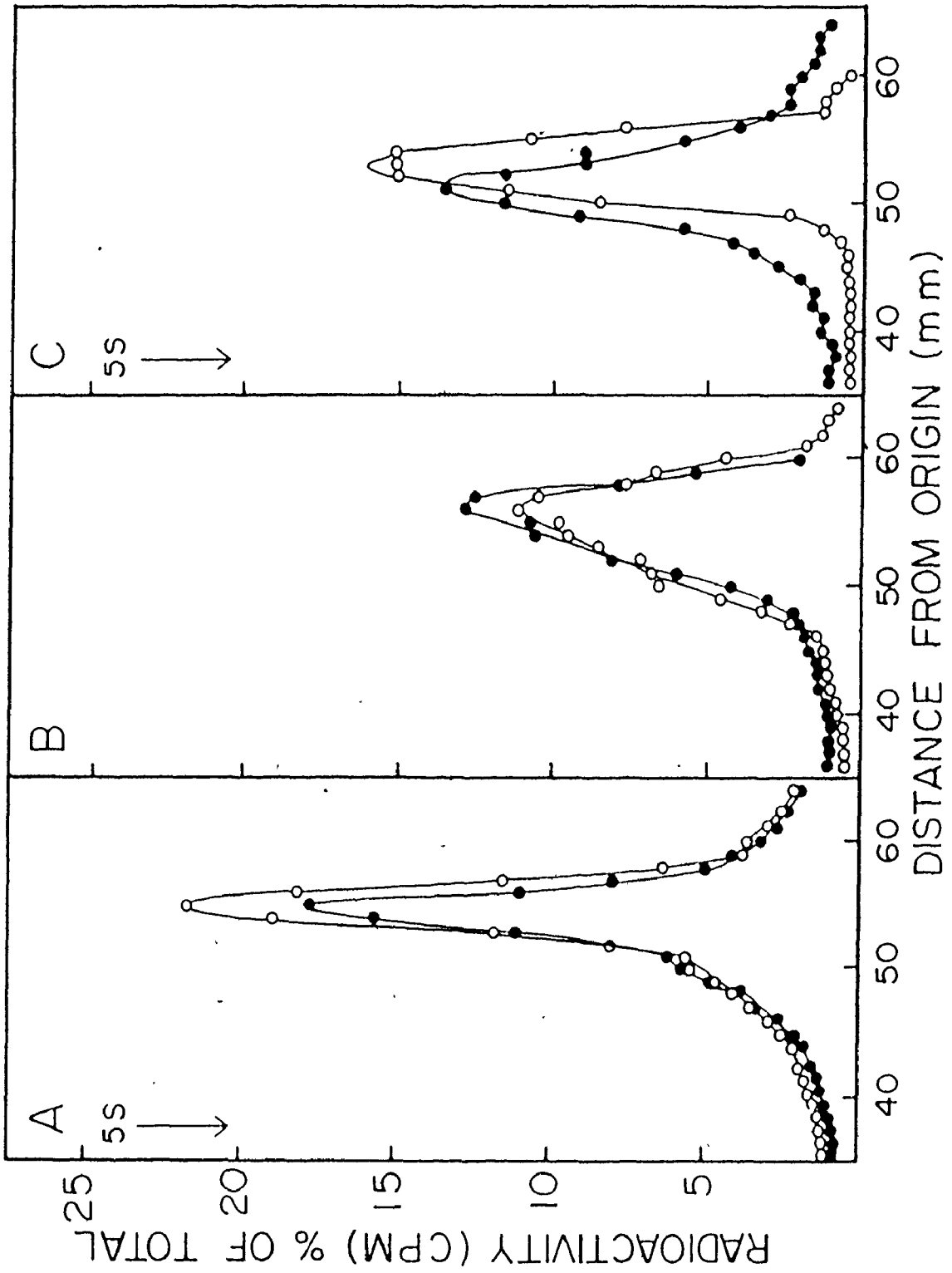

A
5S
B
C
5S
RADIOACTIVITY (CPM) % OF TOTAL
25
20
15
10
5
40
50
60
DISTANCE FROM ORIGIN (mm)

Figure 29

length of three nucleotides and smaller differences would not be detected. HNO_2-methionyl-tRNAs were therefore analysed under the same denaturing conditions but on more sensitive 15% polyacrylamide gels. Results are shown in Fig. 30 . On this gel (Fig. 30 A), mitochondrial HNO_2-[^{35}S] methionyl-tRNA migrated slightly ahead (1 slice) of the cytosolic methionyl-tRNA, a difference corresponding to no more than one or two nucleotides. In some experiments there was no separation. Fig. 29B 30B show that mitochondrial formylmethionyl-tRNA co-migrated with cytosolic HNO_2-[^{3}H] methionyl-tRNA on both 10% and 15% polyacrylamide gels. In contrast to the results with methionyl-tRNAs, Fig. 29C shows that mitochondrial HNO_2-[^{3}H] leucyl-tRNA migrated ahead of cytosolic HNO_2-[^{14}C] leucyl-tRNA on 10% polyacrylamide gels. Taking the position of the 5S RNA marker, the difference would represent a shorter length of the mitochondrial leucyl-tRNA of approximately 6 to 9 nucleotides compared to its cytosolic counterpart.

Because of the low resolution of this system in separating various tRNA species, the urea slab gel system (Maniatis et al, 1975) which has a greater resolving power was used. Mitochondrial and cytosolic HNO_2-aminoacyl-tRNAs were denatured by heating in 98% formamide and compared by gel electrophoresis on 12% polyacrylamide slab gels containing 7M urea. This system was shown to be linear for single and double stranded DNA chains as well as RNA molecules, including some tRNAs (Maniatis et al, 1975).

Fig. 31 shows the results obtained for both cytosolic and mitochondrial HNO_2-[^{35}S] methionyl-tRNA and HNO_2-[^{3}H] leucyl-tRNA. Yeast HNO_2-[^{3}H] phenylalnyl-tRNA (slot A) and yeast HNO_2-[^{3}H] leucyl-tRNA (slot B) were used as markers for 76 (RajBhandary et al, 1967) and 85

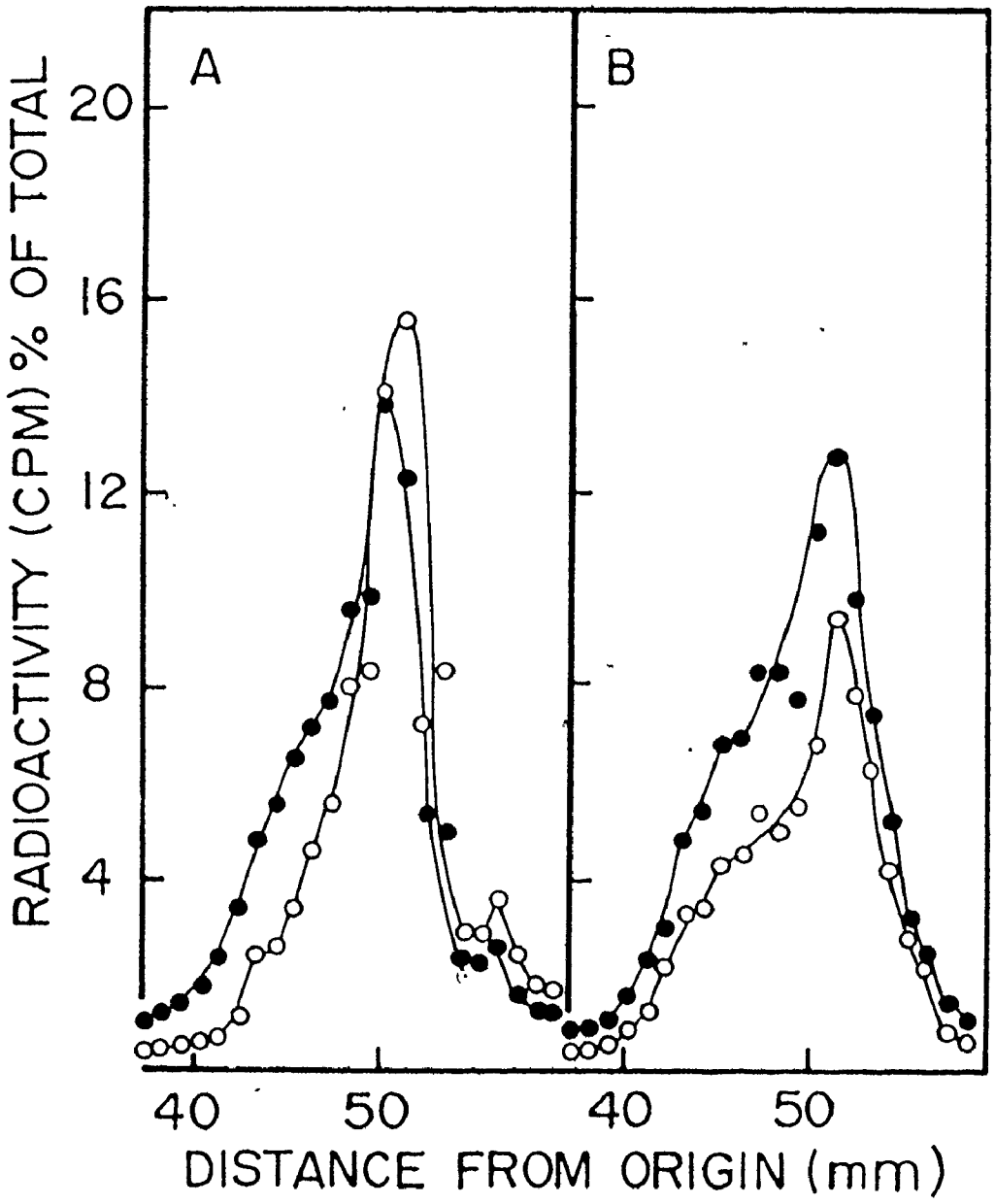


Figure 30

Electrophoretic profiles of Syrian hamster methionyl-tRNAs in 15% polyacrylamide gels. Conditions were as described in Fig. 29 except for the acrylamide concentration. A. Methionyl-tRNAs. B. Cytosolic methionyl- and mitochondrial formyl-methionyl-tRNAs. ●-●, Cytosolic tRNA; o-o, mitochondrial tRNA.

Figure 31

Slab gel electrophoresis of Syrian hamster liver mitochondrial and cytosolic aminoacyl-tRNAs.

Labelled mitochondrial and cytosolic aminoacyl-tRNAs were prepared as described in the Methods. All samples were treated with nitrous acid except for slot E where mitochondrial methionyl-tRNA was applied directly after extraction. Samples were resuspended in 98% formamide, brought to boiling for 2 min and cooled rapidly before applying to the gels. Electrophoresis was for 5.5 h at 10 V/cm. gels were then prepared for fluorography as described in the Methods. The marker dyes are X, Xylene cyanol FF and B, Bromophenol Blue. Nucleotide lengths were determined from Fig. 35. Slot A. yeast HNO_2-[^{3}H] phenylalanyl-tRNA; B, yeast HNO_2-[^{3}H] leucyl-tRNA; C, cytosolic HNO_2-[^{35}S] methionyl-tRNA; D, mitochondrial HNO_2-[^{35}S] methionyl-tRNA; E, mitochondrial [^{35}S] formylmethionyl-tRNA; F, cytosolic HNO_2-[^{3}H] leucyl-tRNA; G, mitochondrial HNO_2-[^{3}H] leucyl-tRNA; H, cytosolic HNO_2-[^{3}H] leucyl-tRNA and HNO_2-[^{35}S] methionyl-tRNA.

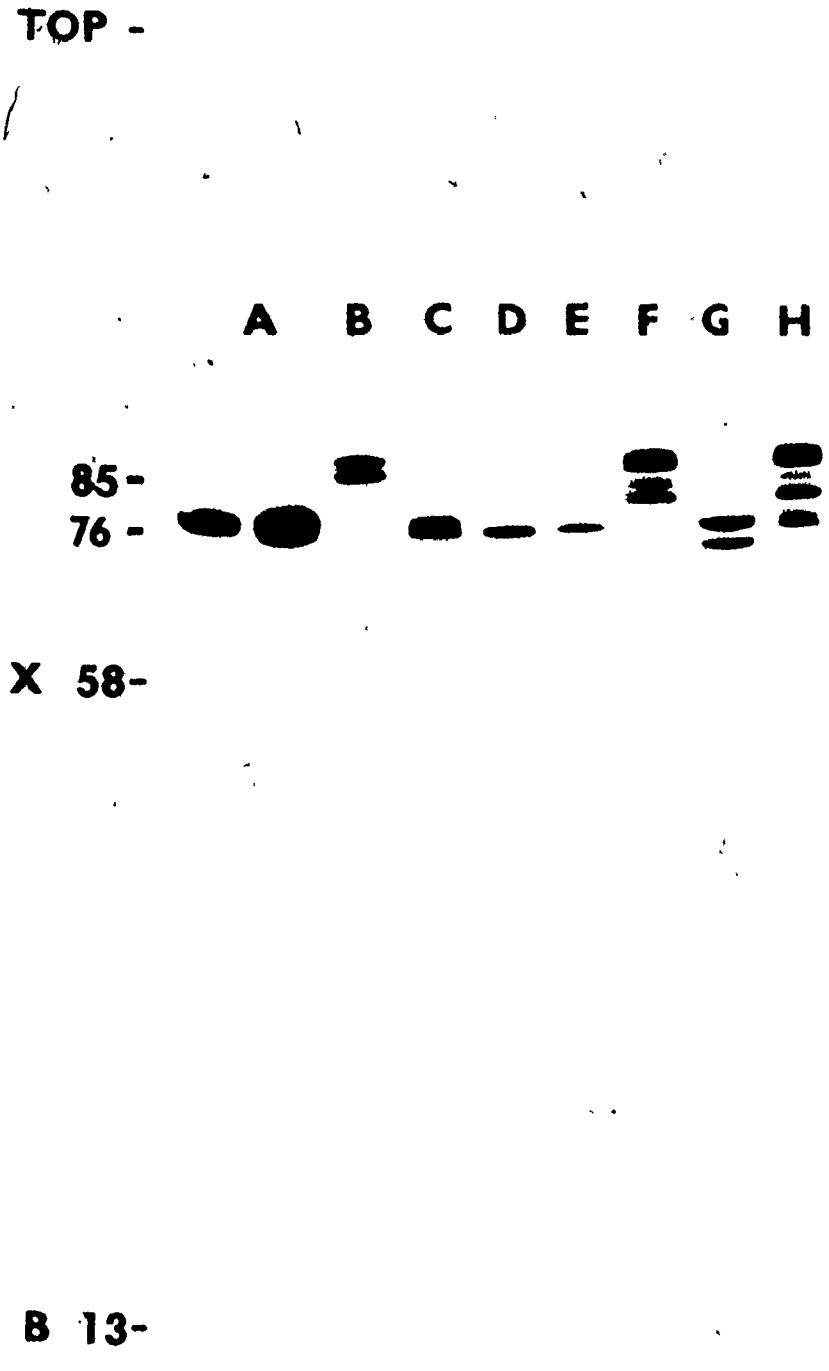


Figure 31

(Chang et al, 1973; Randerath et al,1975) nucleotides respectively. Yeast HNO_2-[^{3}H] leucyl-tRNA migrated as two bands, the two major peaks separated by RPC-5 chromatography (see Fig. 17A), the faster moving one corresponding to 85 nucleotides. The broadness of the yeast HNO_2-[^{3}H] phenylalanyl-tRNA band was due to the low specific activity of the preparation and did not reflect the presence of multiple species. (see Fig. 17B).

It can be seen that cytosolic HNO_2-[^{35}S] methionyl-tRNA (slot C) migrated as two bands, corresponding to the major peaks separated by RPC-5 chromatography (Fig. 18), while total mitochondrial HNO_2-[^{35}S] methionyl-tRNA (slot D) and [^{35}S] formylmethionyl-tRNA (slot E) ran as single bands. All had mobilities similar or identical to HNO_2-[^{3}H] phenylalnyl-tRNA. Mitochondrial HNO_2-[^{3}H] leucyl-tRNA split into two bands (slot E) which ran faster than any of the four bands (the separation of the last two is not clear on the print) observed for cytosolic HNO_2-[^{3}H] leucyl-tRNA (slot F). Precipitation of the three peak fractions of mitochondrial [^{3}H] leucyl-tRNA separated by RPC-5 chromatography (Fig. 19) ($tRNA_1^{leu}$, $tRNA_2^{leu}$, and $tRNA_3^{leu}$ in order of elution) and electrophoresis of each species shows that HNO_2-[^{3}H] leucyl-$tRNA_1^{leu}$ is the faster moving species (Fig. 32, slot C). A small amount of contaminating cytosolic HNO_2-[^{3}H] leucyl-tRNA is clearly visible in the mitochondrial preparation (Fig. 32, slot F).

Molecular weight determination of the mitochondrial tRNAs

An estimate of the molecular weights of the mitochondrial tRNAs can be made from 1) a double logarithmic plot of molecular weight against distance sedimented (Gierer, 1958; Noll,1967); 2) a plot of the logarithm

Figure 32

Slab gel electrophoresis of Syrian hamster liver mitochondrial leucyl-tRNA species.

Labelled mitochondrial and cytosolic aminoacyl-tRNAs were prepared as described in the Methods. Mitochondrial [^{3}H] leucyl-tRNA was chromatographed on RPC-5 and the peak fractions corresponding to each species pooled and the tRNA precipitated with 2.5 volumes of 95% (v/v) ehtanol at -20°C. All samples were treated with nitrous acid. Samples were resuspended in 98% formamide, brought to boiling for 2 min and cooled rapidly before applying to the gels. Electrophoresis was for 5.5 h at 10 V/cm. The gels were then prepared for fluorography as described in the Methods. The markers are the same as in Fig. 31. Slot A, yeast HNO_2-[^{3}H] leucyl-tRNA; B, cytosolic HNO_2-[^{3}H] leucyl-tRNA; C, mitochondrial HNO_2-[^{3}H] leucyl-$tRNA_1^{leu}$; D, mitochondrial HNO_2-[^{3}H] leucyl-$tRNA_2^{leu}$; E, mitochondrial HNO_2-[^{3}H]leucyl-$tRNA_3^{leu}$; F, mitochoddrial HNO_2-[^{3}H]leucyl-tRNA.

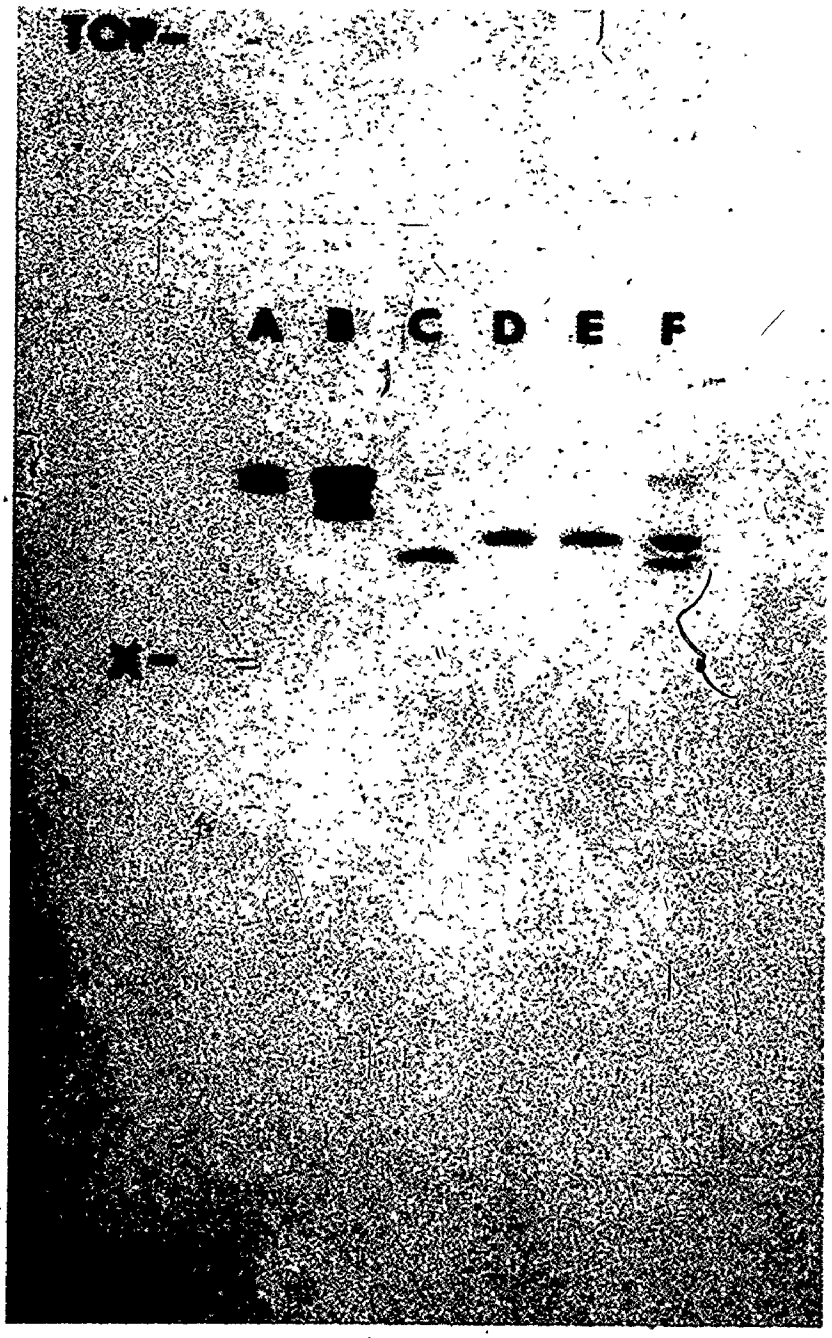


Figure 32

of molecular weight against distance migrated (Bishop et al, 1967; Loening, 1968, 1969) and 3) distance migrated expressed as the ratio of mobilities of each tRNA species to that of the marker Bromophenol Blue against the logarithm of chain length (or molecular weight, taking an average molecular weight of 320 per nucleotide) (Maniatis et al, 1975). All plots should be linear, indicating in the first case that sedimentation coefficients of denatured RNAs depend only on their molecular weight, and, in the other two cases, that electrophoretic mobilities are inversely proportional to the logarithm of the molecular weights of the RNA molecules analysed.

The electrophoretic molecular weight markers used were: wheat germ 5S RNA (40,000) (Hindley et al, 1972; Payne et al, 1973), yeast cytosolic phenylalanyl-tRNA (76 nucleotides, 24,000) (RajBhandary et al, 1967) and yeast cytosolic leucyl-tRNA (85 nucleotides, 27,000) (Chang et al,1973; Randerath et al, 1975). For sucrose gradient sedimentation analyses, markers were: wheat germ 5S RNA, Chinese hamster ovary 7S (5.8S) RNA (53,000) (King et al, 1970), cytosolic methionyl-tRNA (24,000-25,000) and cytosolic leucyl-tRNA (27,000-28,000), these last two being estimated from slab gel electrophoresis and by comparison with known sequences (Blank & Soll, 1971; Piper & Clark, 1974; Simsek et al, 1974).

Combined results of 3 to 5 experiments are given for sedimentation analyses and gel electrophoresis in formaldehyde. Peak fractions of radioactivity usually did not vary more than 2 fractions or slices per experiment. Plots for sedimentation and gel electrophoresis in formaldehyde analyses are shown in Fig. 33 and 34 respectively, while Fig. 35 shows the plot obtained from slab gel electrophoresis analyses. The results, expressed in terms of apparent molecular weights; are

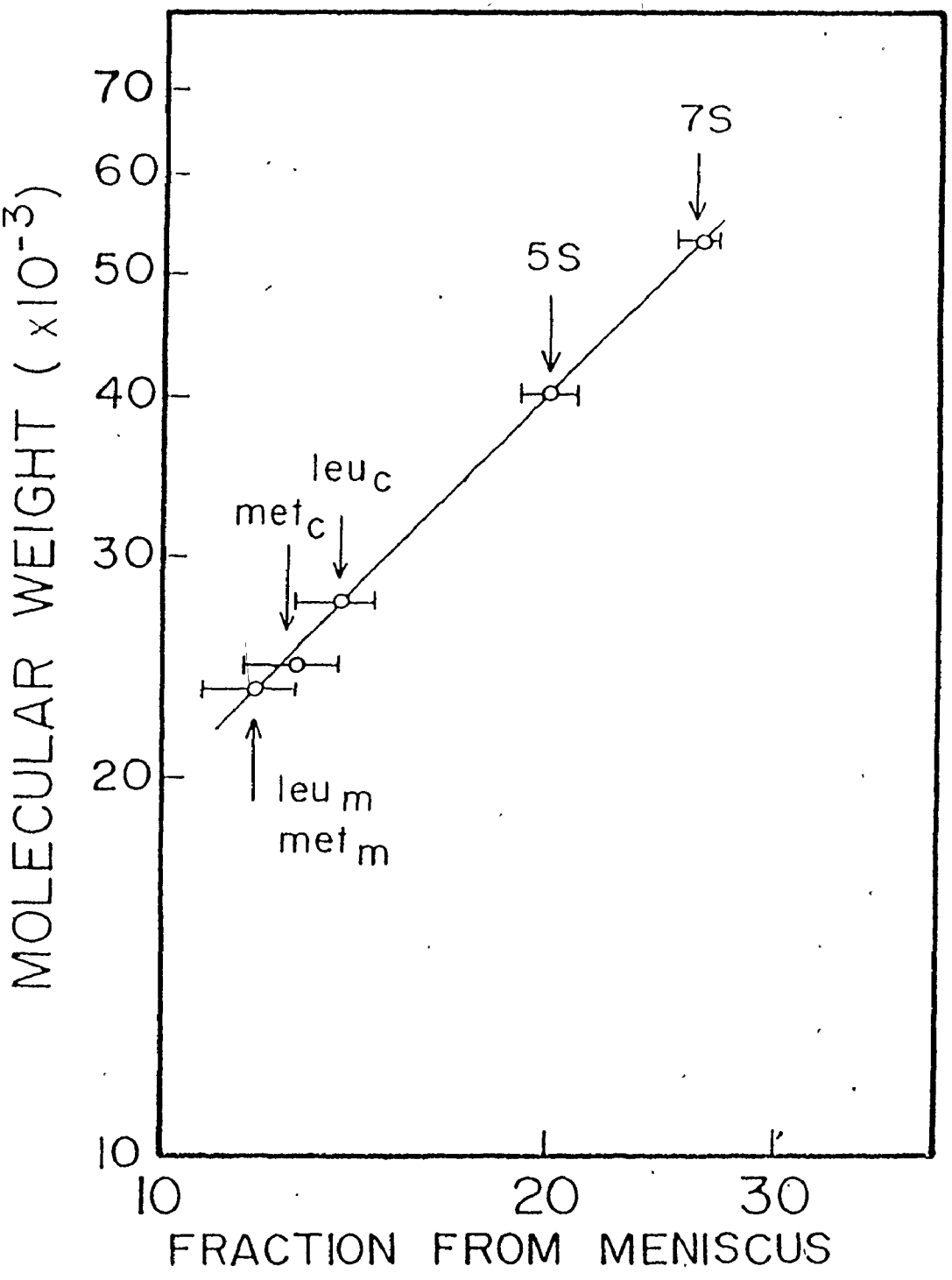


Figure 33

Double logarithmic plot of molecular weight vs. fraction for molecular weight determination of Syrian hamster liver aminoacyl-tRNAs from sucrose density gradient sedimentation analyses. Conditions of centrifugation were as in Figs. 24A and 26A. Points are the average of 3 to 5 experiments and bars indicate standard deviations. The molecular weights of 5S RNA and 7S RNA were taken as 4×10^4 and 5.3×10^4 res

Figure 34

Plot of log molecular weight vs. distance travelled for molecular weight determination of leucyl- and methionyl-tRNAs from polyacrylamide gel electrophoresis analyses. Conditions of electrophoresis were as in Fig. 29. Points represent the average of 2 or 3 experiments. Leu_c, cytosolic HNO_2-[^{14}C]leucyl-tRNA; Leu_m, mitochondrial HNO_2-[^{3}H] leucyl-tRNA; Met_c, cytosolic HNO_2-[^{3}H] methionyl-tRNA; met_m; mitochondrial HNO_2-[^{35}S] methionyl-tRNA.

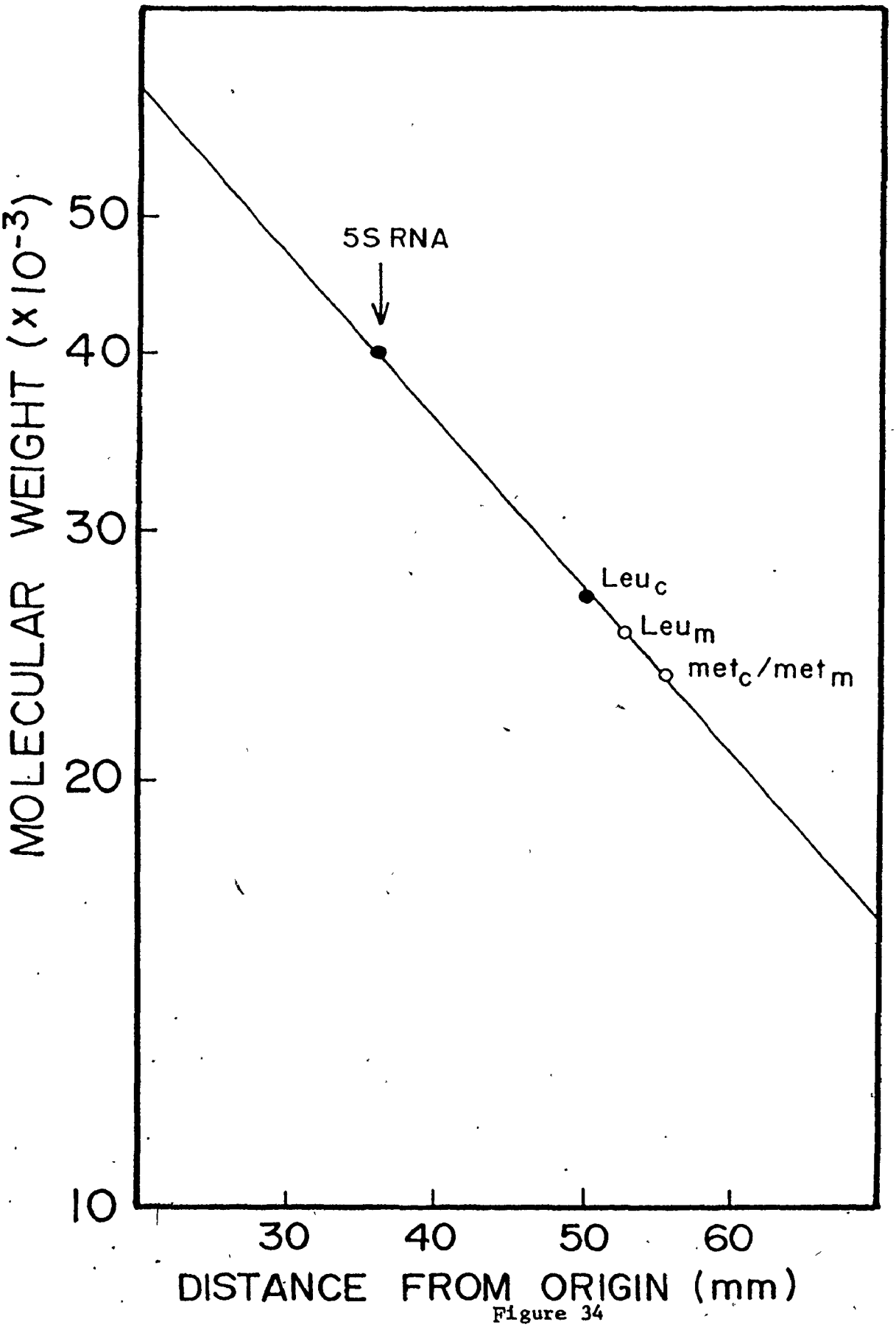

MOLECULAR WEIGHT (x 10-3)
50
40
30
20
10
5S RNA
Leuc
Leum
metc/metm
30
40
50
60
DISTANCE FROM ORIGIN (mm)

Figure 34

Figure 35

Semi-logarithmic plot of nucleotide length vs. mobility for molecular weight determination of leucyl- and methionyl-tRNAs from slab gel electrophoresis in 7 M urea. Conditions of electrophoresis were as in Fig. 31. Points are taken from two experiments. Leu_c, cytosolic HNO_2-[^{3}H] leucyl-tRNA; leu_y, yeast HNO_2-[^{3}H] leucyl-tRNA; phe_y, yeast HNO_2-[^{3}H] phenylalanyl-tRNA; met_c, cytosolic HNO_2-[^{35}S] methionyl-tRNA; met_m mitochondrial HNO_2-[^{35}S] methionyl-tRNA; leu_m, mitochondrial HNO_2-[^{3}H] leucyl-tRNA; $fmet_m$, mitochondrial [^{35}S] formyl-methionyl-tRNA.

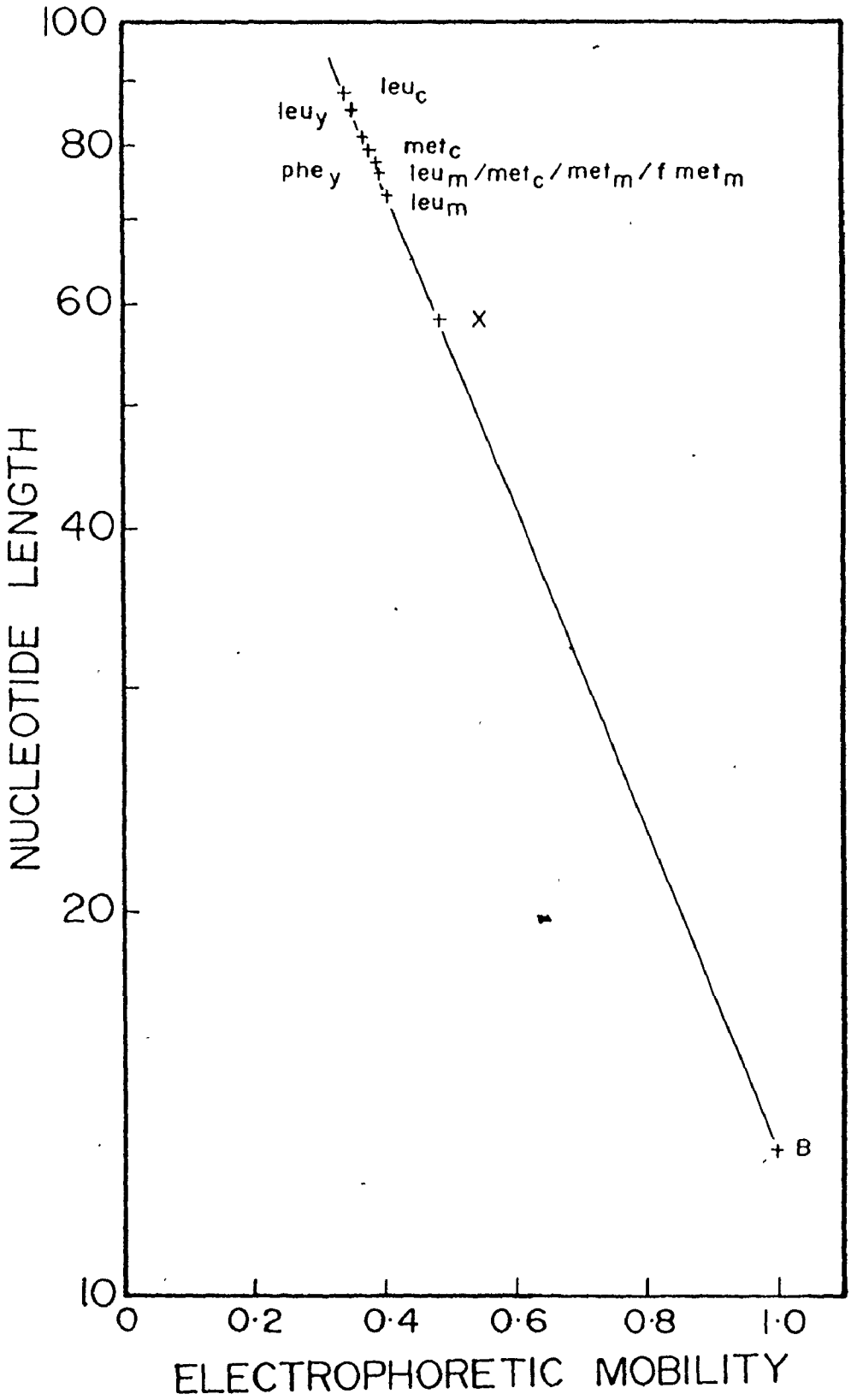

100
80
60
40
20
10
NUCLEOTIDE LENGTH
leu$_c$
leu$_y$
met$_c$
phe$_y$
leu$_m$/met$_c$/met$_m$/f met$_m$
leu$_m$
X
B
0
0·2
0·4
0·6
0·8
1·0
ELECTROPHORETIC MOBILITY

Figure 35

summarized in Table 8 .

TABLE 8

Apparent molecular weights of mitochondrial tRNAs

Species	Cytosolic	Mitochondrial	Procedure
Leucyl-tRNA	28,000 27,000 26,000 25,000	24,000 (2,3)* 23,000 (1)*	III
	27,000	25-26,000	II
	27-28,000	23-24,000	I
Methionyl-tRNA	24-25,000	24,000	III
	24,000	24,000	II
	24-25,000	23-24,000	I
Formylmethionyl-tRNA		24,000	II
		23-24,000	I

I: sedimentation in formaldehyde; II, electrophoresis in formaldehyde; III, slab gel electrophoresis in urea

* numbers indicate mitochondrial $tRNA^{leu}$ species, based on their order of elution from RPC-5.

DISCUSSION

As outlined in the Introduction, the object of this investigation was to determine whether there was any size differences between specific cytosolic and mitochondrial tRNAs and to determine the size of the latter tRNAs. Syrian hamster tRNAs were chosen for this study in order to compare results with those of Dubin & Friend (1972) on BHK-21 mitochondrial 4S RNA. These results have been extended to rat liver mitochondrial tRNAs.

Results on the three tRNA species examined, $tRNA_m^{met}$, $tRNA_f^{met}$ and $tRNA_s^{leu}$ have been summarized in Table 8. With the exception of the value obtained for mitochondrial leucyl-$tRNA_s^{leu}$ from electrophoresis in formaldehyde, which is discussed below, all the results are consistent with one another. Both mitochondrial methionyl- and formylmethionyl-$tRNA_s^{met}$ have apparent molecular weights of 24,000, equivalent to some 76 nucleotides long, while the mitochondrial leucyl-$tRNA_s^{leu}$ have, by the two other methods and more specifically from slab gel electrophoresis in 7M urea, apparent molecular weights of 23,000 and 24,000 for $tRNA_1^{leu}$ and $tRNA_2^{leu}$ and $tRNA_3^{leu}$ respectively. These last values compare with those of the cytosolic $tRNA_s^{leu}$ whose apparent molecular weights range from 25,000 to 28,000. The major cytosolic species, in the 28,000 molecular weight range, are some 85-87 nucleotides long, which is within the lengths of known $tRNA_s^{leu}$ such as in yeast (Chang *et al*, 1973; Randerath *et al*, 1975) and *E. coli* (Blank & Soll, 1971). From these results

it can be concluded that mitochondrial tRNAs are within the size range necessary for tRNAs to fit within the "clover leaf" model structure. The significance of these size characteristics of mitochondrial tRNAs will be considered later, in relation to the results obtained with other rat liver tRNAs, but particular aspects will be considered here.

The presence of formylmethionyl-tRNA

Analysis of mitochondrial formylmethionyl-$tRNA_f^{met}$ was facilitated by the stability of this species at high pH compared to that of methionyl-$tRNA_m^{met}$, which provided a means to selectively deacylate methionyl-$tRNA_m^{met}$. The size of the combined methionyl-tRNAs were indistinguishable from formylmethionyl-$tRNA_f^{met}$. Wallace & Freeman (1974) have shown that, in mouse liver, the mitochondrial methionyl-tRNA species eluting at the higher salt concentration were formylated. This is also the case in rat liver (see Part III). Although this has not been determined here, the analogy in the RPC-5 profiles of Syrian hamster, mouse and rat liver methionyl-tRNAs implies that the Syrian hamster species eluting at the higher salt concentrations are formylated.

The fact that formylated methionyl-tRNA can be obtained from isolated liver mitochondria incubated with labelled methionine confirms the existence of a transformylase in this organelle (Smith & Marcker, 1968). It is consistent with a role of formylmethionyl-$tRNA_f^{met}$ in the initiation of protein synthesis in mitochondria in contrast to the cytosol where the equivalent species is not formylated. This role was recently confirmed by partial sequence determination of a beef heart mitochondrially-synthesized subunit of cytochrome oxidase showing the presence of formylmethionine at the N-terminus (Buse & Steffens, 1976).

Comparison of the methods

The discrepancy between the results obtained by gel electrophoresis and sedimentation in formaldehyde was not expected. In the absence of conformational effects, polyacrylamide gels give a direct measure of molecular weights. In practice, conformational effects can be considerable and lead to quite inaccurate size determinations. Thus, Loening (1969) noted changes in relative mobilities of different ribosomal RNAs of varying G+C content with ionic strength. Borst et al (1970) found considerable temperature effects on the mobility of mitochondrial RNAs resulting in changes of up to 40% in their apparent molecular weights. Similar results have been observed on vertebrate and fungal mitochondrial rRNAs whose mobilities depend, under native conditions, on temperature and ionic conditions (Groot et al, 1970; Edelman et al, 1971; Forrester et al, 1971; Grivell et al, 1971; David & Chase, 1972; Mitra et al, 1972; Yu et al, 1972; Reijnders et al, 1973) and BHK 4S RNA (Dubin & Friend, 1972). These results were generally interpreted on the basis of the low G+C content of mitochondrial rRNAs (Freeman et al, 1973) leading to a larger unfolding of these molecules compared to those with a higher G+C content. However, a low G+C content does not entirely explain this behavior of mitochondrial RNAs. Dawid (1972) compared the thermal denaturation behavior of Xenopus mitochondrial rRNA and Drosophila cytosolic rRNA, which have equal G+C contents and found that Drosophila rRNAs melted at a higher temperature and over a narrower range than the mitochondrial rRNAs while their electrophoretic mobilities were not affected.

It was assumed that the secondary structure of all RNA molecules would be identical during electrophoresis in formaldehyde. This assumption

might not be valid in spite of the disruption of base pairing. Similar discrepancies as under non-denaturing conditions have been reported. Yeast (Grivell et al, 1971) and Xenopus (Dawid & Chase, 1972) mitochondrial rRNAS were shown to have lower mobilities than expected from sedimentation. This is exactly what is observed here with the mitochondrial leucyl-$tRNA_s^{leu}$. This contrasts with the observation on Xenopus 4S RNA of Dawid & Chase (1972) who could not detect any difference between cytosolic and mitochondrial 4S RNAs either by sedimentation or electrophoretic analysis in formaldehyde, or those of Dubin & Friend (1972) who calculated similar apparent molecular weights of 19,000 for BHK mitochondrial 4S RNA by both methods. This molecular weight would correspond to a length of 60 nucleotides, and such tRNAs could not possibly be accomodated within the generally accepted "clover-leaf" structure. However, it is not clear how this value was reached and the method employed might have lead to an underestimation of the size of mitochondrial 4S RNA.

Results using formaldehyde as a denaturing agent have to be considered with caution because of the possibility of intra-molecular cross-linking by formaldehyde through methylene bridges involving predominantly purine residues. (Staynov et al, 1972). In addition, single - stranded stacking is unimpaired after formaldehyde treatment. Depending on the base composition and sequence of the mitochondrial tRNAs, these effects could alter their conformation sufficiently to affect their mobility. Nevertheless, the results obtained here for mitochondrial methionyl-$tRNA_m^{met}$ and formylmethionyl-$tRNA_f^{met}$ indicate that such differences in base composition do not necessarily result in electrophoretic mobility discrepancies for all molecules. This observation might explain the results of Dawid & Chase (1972) if one considers that the

majority or the major species of mitochondrial tRNA found in 4S RNA are in fact (see Part III on rat liver tRNAs) essentially the same size as cytosolic species. This certainly seems to be the case for yeast mitochondrial tRNAs (Martin et al, 1977b)

It is clear in any case that electrophoresis in formaldehyde is not an appropriate method of analysis for precise molecular weight determinations of molecules whose G+C content is low, such as mitochondrial RNAs. A similar conclusion had previously been reached by Dawid & Chase (1972) for mitochondrial rRNA. The method seems applicable to other RNAs of higher G+C content (Boedtker, 1971).

While no unusual behavior could be detected for the tRNA species studied by sedimentation analysis in formaldehyde, the main disadvantage of this method is its lack of precision. Molecular weights have been estimated only within a 4-5% error (23,000 to 24,000 molecular weights for mitochondrial tRNAs). The error is compounded by the fact that the size of the cytosolic species used as markers is not known exactly and were determined on the basis of analysis by slab gel electrophoresis in urea. This last method, in which the tRNAs are first denatured in 98% formamide, gives the most reliable and accurate size determinations. Varriachio & Ernst (1975) have suggested that tRNAs of different base composition can have different electrophoretic mobilities in spite of identical sizes in such urea gel systems with acrylamide concentrations higher than 10%. For this reason, tRNA markers of known size and base composition were used. The fact that these molecules have mobilities corresponding to their sizes would justify the use of this system for the size determination of mitochondrial tRNAs. Small variations in mobility (in the pre gel of the ord of 1—

necessarily reflect real size differences. They could also reflect differences in base composition. This is the case for the two separable yeast cytosolic leucyl-tRNA species both of which are 85 nucleotides and differ in base composition by 21 residues (Chang et al, 1973; Randerath et al, 1975), and most likely also for Syrian hamster cytosolic methionyl-tRNA and some but not all of the Syrian hamster cytosolic leucyl-tRNAs. Larger differences in mobility, such as between mitochondrial and cytosolic leucyl-tRNAs must reflect a real lower molecular weight for the mitochondrial species.

Comparison with rat liver mitochondrial $tRNA^{leu}$

It is interesting to note that the size difference of mitochondrial and cytosolic $tRNAs^{leu}$ is not specific to Syrian hamster. Sedimentation results shown in this section (Fig. 28) indicate that it is also the case of rat liver mitochondrial $tRNAs^{leu}$. This is further confirmed by slab gel electrophoresis in urea (Part III). This observation would extend and be consistent with that of Jacovcic et al (1975) on the sequence homology of the mitochondrial $tRNA^{leu}$ cistron. These workers hybridized rat liver mitochondrial leucyl-tRNA to rat, mouse, guinea pig, monkey, chicken, and yeast mtDNAs and found that hybridization took place with rat, mouse and guinea pig mtDNAs only, implying a fair homology of the $tRNA^{leu}$ gene(s) among these species. Such an homology might also be found between rat and Syrian hamster, which, with mouse and guinea pig, are all members of the same Family.

PART III

CHARACTERIZATION AND GENETIC ORIGIN OF MITOCHONDRIAL tRNAs

METHODS

Protein synthesis by isolated mitochondria

Rat liver mitochondria were isolated and incubated as described for the isolation of mitochondrial aminoacyl-tRNAs from Syrian hamster (Part II), except the Tevenel was replaced by cycloheximide at a final concentration of 300 µg/ml. Incubations of 1 ml were in acid-washed tubes. Fifty µCi of labelled amino acids were added to each ml in all cases except where indicated. After 30 min at 30°C, mitochondria were centrifuged, resuspended in a small volume of Medium B and used directly.

For the time course of incorporation, all labelled amino acids were diluted with a 1 mM solution of cold amino acid to 10 Ci/mmol and incubated at 40 µCi/ml. Aliquots of 80 µl were removed at various times and spotted on Whatman 3MM filter disks which were boiled for 20 min in 5% trichloroacetic acid containing 1 mg cold amino acid/ml. After washing with ice-cold 5% trichloroacetic acid twice, followed by ethanol-ether (1:1. v/v) and ether, disks were counted as described previously (Methods, Part I).

Slab gel electrophoresis of mitochondrial proteins

Electrophoresis was performed in SDS-polyacrylamide slab gels

using the buffer system of Laemmli (1970). The amount of protein added to each gel was from 150 µg to 250 µg. The amount varied depending on the number of counts present in each fraction. All samples were diluted by a factor of 2 in buffer which after dilution contained 6% SDS/10% glycerol/0.5% 2-mercaptoethanol/0.01% Bromophenol Blue/0.1 M Tris-glycine-HCl, pH 6.8. Electrophoresis was on 25 cm 16 cm x 1.5 mm slab gels at 6 mA/cm^2, until the dye front migrated around 14.5 cm. The acrylamide concentration was 12.5%. Gels were prepared for fluorography as described by Bonner & Laskey (1974) and exposed to Royal-B X-Omat films at -70°C. Densitometric scanning of the developed fluorograms was performed with a Joyce Loebl scanning densitometer.

Proteinase digestion

In order to determine whether the label incorporated into mitochondrial proteins was the original labelled amino acid, the following procedure was used: Mitochondrial samples obtained as above were dialysed in EDTA-treated tubing against 1 l of 100 mM NaCl/10 mM Tris-HCl, pH 7.5, overnight at 4°C. Dialysed samples were then treated with proteinase K alone or with pronase B each at a final concentration of 50 µg/ml usually for 3 h at 30°C. Following digestion, aliquotes were spotted on Whatman No. 1 paper and analysed by descending chromatography with phenol-water (100:37, w/v) as the solvent. The paper was dried, cut into 1x2 cm strips and counted for radioactivity. Cold amino acid markers were run in parallel and their position determined by ninhydrin treatment.

Extraction of aminoacyl-tRNAs

A. Mitochondrial tRNAs

Labelled aminoacyl-tRNAs were extracted from rat liver mitochondria as described previously (Part II). Where indicated a modified procedure was used to obtain mitochondrial preparations free of contaminating labelled cytosolic aminoacyl-tRNAs. About 0.1 unit of immobilized RNase/ml was added at the beginning of the incubation. After 15 min, 100 mM EDTA, pH 7.5 was added to 10 mM and the incubation continued for another 5 min. Finally, 100 mM $MgCl_2$ was added to 10 mM prior to centrifuging of the mitochondria.

B Cytosolic tRNAs

Rat liver and yeast cytosolic aminoacyl-tRNAs were obtained as described in Methods of Part II.

Paper chromatography of aminoacyl-tRNA

Labelled aminoacyl-tRNA preparations were deacylated at pH 7.4 and aliquots spotted on Whatman No. 1 paper. Free amino acids were separated by descending paper chromatography and counted as described for the products of proteinase digestion.

RPC-5 chromatography

Labelled aminoacyl-tRNAs were purified on DEAE-cellolose as described for Syrian hamster liver tRNA. Chromatogrpphy on RPC-5 was performed as described by Pearson et al (1971). All species were eluted at room temperature except for asparaginyl-tRNAs which were eluted at 15-18°C because of their presumed instability (White et al, 1973).

Gel electrophoresis of aminoacyl-tRNAs

Labelled aminoacyl-tRNA preparations were deaminated and run on slab gels as described for Syrian hamster liver aminoacyl-tRNAs.

Fingerprint analysis of mitochondrial leucyl-tRNAs

Partial digestion with RNase T_1 of [^{3}H] leucyl-tRNAs recovered after chromatography on RPC-5 and deamination was performed according to the procedures of Domis-Keller et al (1977) and Simoncsits et al (1977).

In the first method, samples were dissloved in a small volume of water and 1-10 µl added to 20 µl of buffer containing 7M urea/ 1 mM EDTA/ 0.025% Xylene cyanol FF and Bromophenol Blue /20 mM sodium citrate, pH 5.0 and made up to 0.25 µg stripped E. coli tRNA/ml in 4 .1 ml siliconized platic Eppendorf tubes. These were placed in a water bath at 50°C for 5 min, then quickly chilled on ice. RNase T_1 (1 µl of 0.1 units/ml) was added to the second tube and two successive ten-fold serial dilutions made in the other two tubes using a fresh micropipette each time. Samples were incubated at 50°C for 15 min (or 20 min in later experiments). the reaction stopped on ice and the samples loaded on gels.

In the method of Simoncsits et al (1977), samples were suspended in 10 mM EDTA/ 0.1 M Tris-HCl, pH 7.5 with 0.2 units of RNase T_1 in 10 µl and incubated at 0°C for 30 min. Reactions were stopped by the addition of 15 µl formamide containing 0.025% Xylene cyanol FF and Bromophenol Blue. Samples were then heated to 100°C for 1 min, cooled rapidly on ice and loaded on gels.

Polyacrylamide slab gels contained 20% acrylamide/ 0.67% bis-acrylamide/ 7 M urea/ 1mM EDTA/ 0.07% ammonium persulfate/ 50 mM Tris-borate, pH 8.3 . Slab dimensions were 30 cm x 13.5 cm x 0.075 or 0.15 cm and the sample well dimemsions were 1 cm x 0.5 cm x 0.075 or 0.15 cm. The running buffer was 1 mM EDTA/ 50 mM Tris-borate, pH 8.3. The gels were pre-run at 800 V for 2 h prior to loading, and then at 800 V for 10 to 14 h, keeping the gels slightly warm during the run by cooling with ethylene glycol using a cooling unit.

After electrophoresis, gels were prepared for fluorography as described by Bonner & Laskey (1974), except for the 0.075 cm thick gels where the procedure was modified as follows: gels were immersed in a 7.5% acetic solution (acetic acid/methanol/ water, 1.5:1:17.5) (Weber & Osborne, 1969), followed by two immersions in 20 volumes of DMSO for 10 min each. The gels were then left in 4 volumes DMSO containing 20% PPO for 45 min and in water for 15 min before drying. Exposure was to Royal-B X-Omat films at -70°C for up to 45 days.

Extraction of mitochondrial DNA

Mitochondria were isolated from the livers of 150g Sprague-Dawley rats starved 24 h. After 4 washes in SE buffer, the mitochondria were resuspended in 10 ml lysis buffer (10 mM EDTA/ 100 mM NaCl/ 50 mM Tris-HCl, pH 7.5). Sarkosyl NL-30 was added to 2 %. Following lysis of the mitochondria at 0°C, 7 M CsCl in 0.1 mM EDTA/ 10 mM Tris-HCl, pH 8.0 (TE) was added to 1 M and the nucleic acids spun down at 38,000 rpm in a Beckman 65 rotor for 19 h at 20°C. The pellet was resuspended in approximately 1 ml of TE and layered over a CsCl-EtBr step gradient

as described by Storrie & Attardi (1972). Centrifugation was for 5 h at 20°C and 35,000 rpm in a Beckman SW 50.1 rotor. The lower band of the mitochondrial DNA was collected by suction into a plastic syringe after puncturing the centrifuge tube 1-2 mm below the band. These operations were performed under ultraviolet light to clearly visualise the DNA bands by their fluorescence emission.

Ethidium bromide was extracted from the DNA preparations by 6 sequential extractions with isoamyl alcohol. The EtBr-free DNA was spun down as before for total nucleic acids but in TE instead of lysis buffer. The pellet of mitochondrial DNA was resuspendend in 1/100 xSSC and kept frozen until use.

Cleavage of DNA with restriction endonuclease

The purity of mtDNA was checked from the pattern of restriction fragments obtained with Eco RI and Hind III endonucleases. Digestion buffers were 10 mM $MgCl_2$/ 150 mM NaCl/ 5 mM mercaptoethanol/ 10 mM Tris-HCl, pH 7.6 for Eco RI and 10 mM $MgCl_2$/ 60 mM NaCl/ 20 mM Tris-HCl, pH 7.4 for Hind III. DNA was incubated with sufficient endonuclease to ensure complete digestion within two hours (2 to 5 units of enzyme / µg of DNA). Digestions were carried out at 37°C. The reactions were stopped with 0.3 volumes of 70% sucrose/100 mM EDTA, pH 7.0. The resulting cleaved mtDNA was used directly for agarose gel electrophoresis.

Restriction fragments were separated by electrophoresis essentially as described by Kroon et al (1977). The buffer was 20mM sodium acetate/ 2 mM EDTA/ 40 mM Tris-acetate, pH 7.8. Samples containing up to 10 µg mtDNA were made 0.005% in Bromophenol Blue and loaded on the flat surface of the gel through the electrophoresis buffer.

Electrophoresis was at room temperature at 100 V. After electrophoresis the gels were stained with 0.5 µg ethidium bromide/ml in electrophoresis buffer and the DNA bands visualised under UV light.

Fig 36 shows typical patterns of mtDNA digestion with Eco RI and Hind III endonucleases. The patterns are similar to those obtained by others (Francisco & Simpson, 1977; Kroon et al, 1977; Buzzo et al, 1978; Hayashi et al, 1978). Two extra bands are faintly visible in the Eco RI digest indicating the presence of two types of DNA populations, as found by Hayashi et al (1978).

Extraction of nuclear DNA

Nuclear DNA was recovered from the nuclear pellet, obtained during isolation of the mitochondrial fraction, by centrifugation as for mtDNA. Fractions of the nuclear DNA pellet (up to 500 µg DNA) were resuspended by sonication (Cole-Parker ultrasonic cleaner, model 8845-2) in a small volume of 10 mM EDTA/ 10 mM Tris-HCl, pH 8.0 and applied to an EtBr-CsCl step gradient as described for mtDNA. The nuclear DNA was recovered from the top of the gradient and all further steps were as for mtDNA.

Hybridization

MtDNA was denatured in 1/100xSSC made up to 0.1 N NaOH for 20 min at 22°C. After rapid cooling, the solution was made up to 6XSSC, pH 7.0. Pre-soaked nitrocellulose filters (Schleicher & Schuell, 0.45 µm) were loaded under slow suction usually with 10 µg mtDNA and washed with 10 ml of 6xSSC, pH 7.0. Loaded filters were dried under vacuum at 80°C

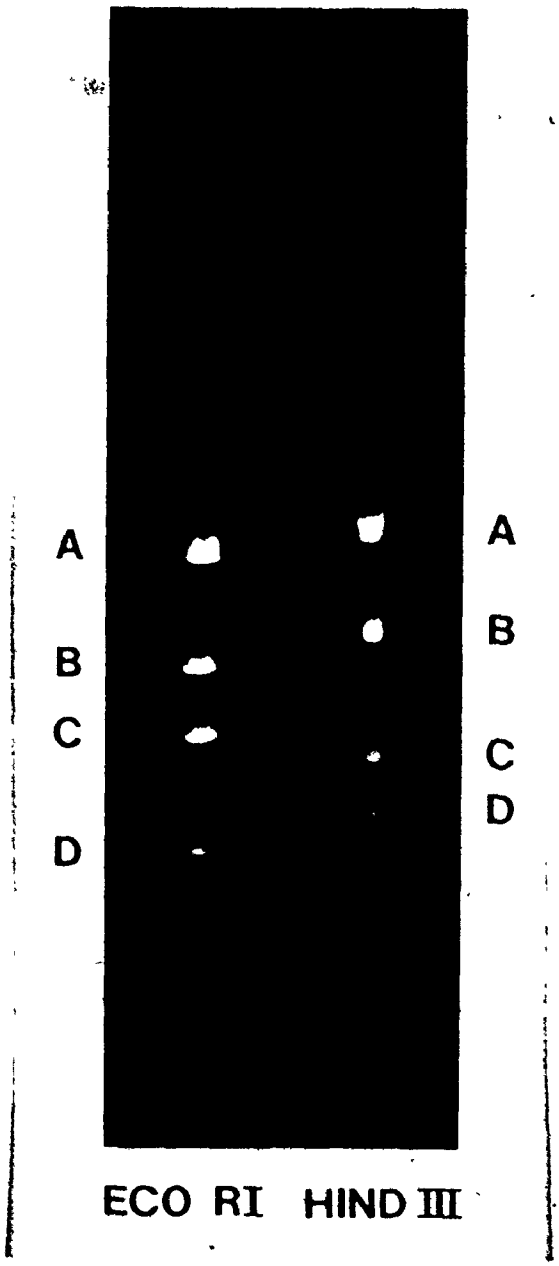


Figure 36

Agarose-gel electrophoretic analysis of the Eco RI and Hind II endonuclease restriction fragments of rat liver mtDNA.

for at least 4 h and kept in the dark in a dessicator until use. The same procedure was used for nuclear DNA except that it was further sonicated before loading on filters.

Hybridizations were in 2xSSC/0.1% SDS/ 10 mM sodium acetate, pH 5.0 with either 30 or 50% formamide for 18 h at 33°C in a final volume of 0.3 or 0.5 ml. Blank filters were added to all incubations. After hybridizations, RNA was recovered, filters were washed with incubation buffer and left to sit for 30 min at 33°C in the incubation buffer. The filters were then washed thoroughly with 2xSSS, pH 5.0 and incubated in 2xSSC, pH 5.0 with 25 units RNase T_1/ml for another 30 min at 33°C. In some cases, this step was omitted. The filters were washed again and left to sit in 2xSSC, pH 5.0 for up to 1 h at room temperature, before a final washing under slow suction with 10 ml of 2xSSC, pH 5.0 on both sides, dried and counted for radioactivity at 5% efficiency. This procedure gave background counts usually not exceeding 40 cpm.

Measurement of DNA

DNA was measured by scanning from 320 nm to 220 nm on a Beckman spectrophotometer, assuming that 40 µg DNA give a reading of 1 at 260 nm. The ratio of absorbance at 230:260:280 was usually close to 0.5:1:0.5 for both mtDNA and nuclear DNA.

Buffers

TE:	1 mM EDTA
	10 mM Tris-HCl, pH 8.0
SSC:	150 mM NaCl
	15 mM sodium citrate

MATERIALS

Cycloheximide		Sigma
Eco RI 3 x 10^4 units/μg protein		New England Biolabs
Hind III (105 units/μg protein)		" " "
Proteinase K (fungal) (20 mAnson units/μg)		EM Laboratories
Pronase B		
Sarkosyl NL-30		Geigy Co.,Toronto,Ontario
L-[^{14}C] Asparagine	200 mCi/mmol	New England Nuclear,Boston,MS.
[^{3}H] Asparagine	21.8 Ci/mmol	CEA, Saclay, France
L-[^{14}C( )] Arginine	292 mCi/mmol	New England Nuclear,Boston,MS.
L-[2,3-^{3}H] Arginine	18.1-21 Ci/mmol	" " " " "
L-[^{14}C(U)] Lysine	292 mCi/mmol	" " " " "
L-[4,5-^{3}H] Lysine	38-63 Ci/mmol	" " " " "
L-[^{14}C(U)] Valine	253 mCi/mmol	" " " " "
L-[2,3,4-^{3}H] Valine	22 Ci/mmol	" " " " "
[^{14}C] Proline	255 mCi/mmol	" " " " "
[^{3}H] Proline	111 Ci/mmol	" " " " "
[^{3}H] Histidine	10-25 Ci/mmol	" " " " "
L-[2,3-^{3}H(U)] Aspartate	15 Ci/mmol	" " " " "
L-[^{3}H(G)] serine	2.76 Ci/mmol	" " " " "
[^{3}H] Glutamine	21 Ci/mmol	Amersham/Searle
RNase T_1	(500,000 units/ml)	Worthington Biochemical Corp. Freehold, N.J.

PART III

RESULTS

Incorporation of labelled amino acids and charging of aminoacyl-tRNAs by isolated mitochondria

HeLa cell mitochondrial aminoacyl-tRNA synthetase preparations charge mitochondrial tRNA with all the amino acids except asparagine, proline, glutamine and histidine (Lynch & Attardi, 1976). The ability of isolated rat liver mitochondria to incorporate these amino acids into protein and into aminoacyl-tRNAs was examined. Incorporation of leucine, whose tRNA is known to be coded by the mitochondrial genome (Nass & Buck, 1970) and methionine, because of the possibility of isolating $tRNA_f^{met}$, was also examined, as well as the amino acids arginine, lysine and valine whose tRNAs were suggested to be imported into mitochondria of Tetrahymena (Chiu et al, 1975). Fig. 37 shows the incorporation curves of these a amino acids into hot trichloroacetic acid-precipitable material. Incubations were performed at a constant specific activity of 10 Ci/mmol of each of the labelled amino acids and at 40 μCi/ml of incubation. Fig. 37 shows that incorporation was linear for 30 min in all cases. Only glutamine was not incorporated, while the incorporation of histidine was very low. The level of incorporation of [^{3}H] aspartate is also shown. The lower incorporation of aspartate than that of asparagine indicates that the incorporation of the latter is not due to its deamidation to aspartate. The levels of incorporation are summarized in

Figure 37

Incorporation of [^{3}H]-labelled amino acids into protein by isolated rat liver mitochondria. Mitochondria were isolated and incubated with 40 µCi/ml of the different [^{3}H]-labelled amino acids at 10 Ci/mmol each in a total volume of 450 µl at 30°C. At each time interval, a 80 µl sample was taken, spotted on Whatman 3MM filter disks and the incorporation into hot-trichloroacetic acid -precipitable counts determined as described in the Methods. A and B represent the same experiment but plotted on a different scale. ●-●, Leucine; o-o, methionine; ●-.-●, proline; ▵-▵, valine; o-.-o, arginine; ●-.-●, lysine; ▲-▲, asparagine; □, asparate; ■, histidine; ▽, glutamine.

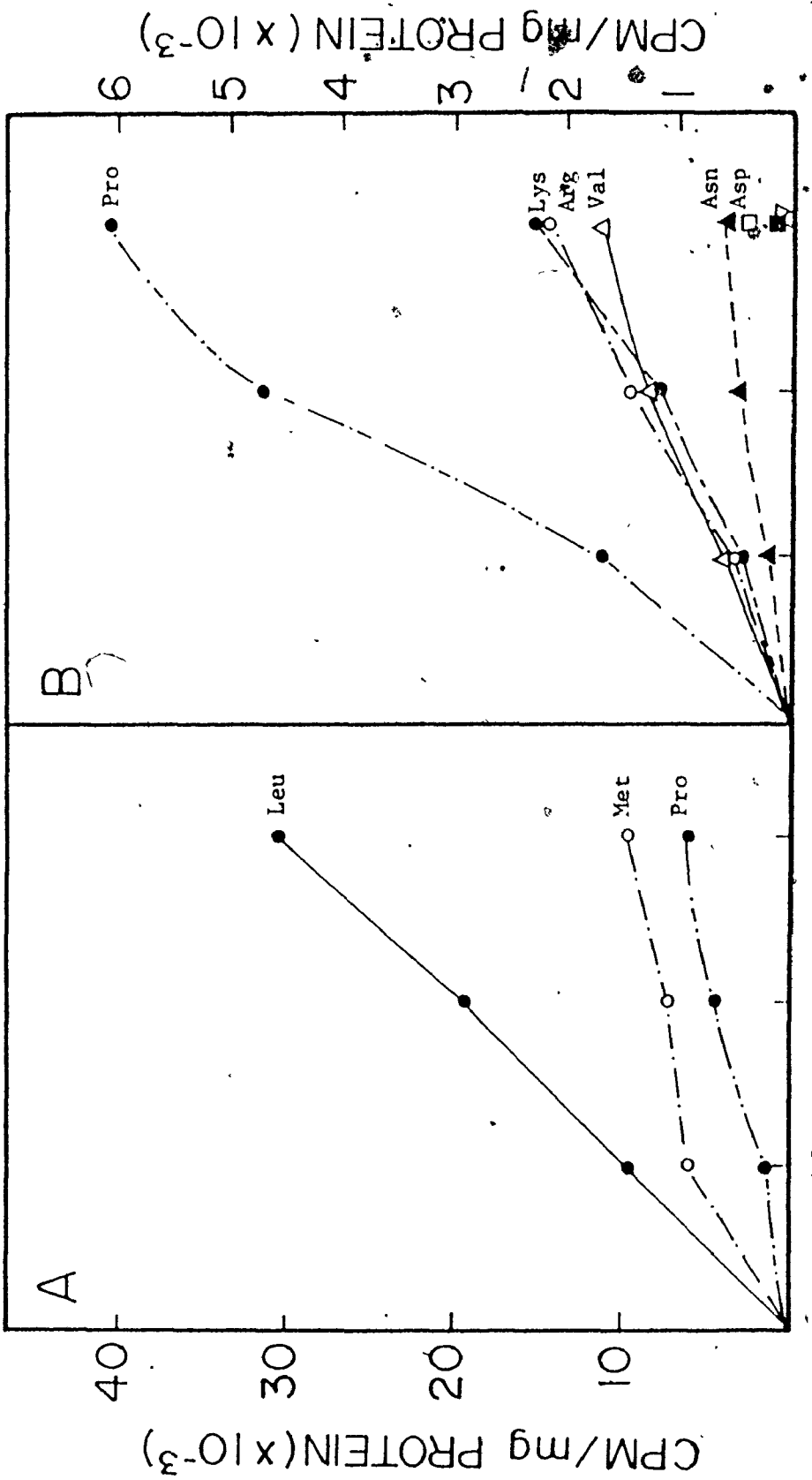

CPM/mg PROTEIN (x 10⁻³)
40
30
20
10
A
Leu
Met
Pro
10
20
30
TIME (min)
CPM/mg PROTEIN (x 10⁻³)
6
5
4
3
2
1
B
Pro
Lys
Arg
Val
Asn
Asp
10
20
30

Figure 37

Table 9, in relation to that of leucine which had the highest incorporation.

The differences in incorporation reflect either transport of the amino acids or conditions internal to the mitochondria, since the specific activity was the same for all amino acids. In all cases, incorporation was completely inhibited by Tevenel, an inhibitor of mitochondrial protein synthesis (Wallace et al, 1975). This suggests that incorporation represented labelling of mitochondrially-synthesized proteins. This was examined by slab gel electrophoresis using the system of Laemmli (1970). Fig. 38 shows the pattern obtained for proline (lane B), lysine (lane C), valine (lane D), leucine (lane E), asparagine (lane F) and arginine (lane G) compared to CHO TK$^-$ mitochondrial [^{35}S] methionine-labelled proteins (lane A). The pattern reveals that all amino acids are incorporated into the same proteins, with the exception of lysine where a slower moving band was present. Some 13 to 15 major bands are present, ranging between molecular weights of 50,000 and 4,000. As, reported by Yatscoff et al (1978a), the bands are similar, although not quite identical, to those of rat hepatoma (HTC) mitochondria labelled in whole cells. This is shown in Fig. 39 where it can be seen that there is at least one extra band of low molecular weight present in the pattern obtained from rat liver mitochondria compared to that of HTC mitochondria. This would indicate that most bands observed represent mitochondrially-synthesized proteins. However, the [^{3}H] proline-labelled pattern revealed a multiplicity of minor bands and the same type of pattern was obtained when labelling with [^{35}S] methionine (result not shown). These heterogenous bands probably reflect partial protein synthesis of the major bands, and, in the case of

TABLE 9

Incorporation of [^{3}H] amino acids into protein by isolated rat liver mitochondria

Amino acid	Incorporation (% Leucine)
Leucine	100
Methionine	32
Proline	20
Lysine	7.3
Arginine	7.3
Valine	5.6
Asparagine	1.8
Histidine	0.5
Glutamine	0.09

Incorporation is expressed as a percentage of [^{3}H] leucine incorporation which was 31,000 cpm/mg protein after a 30 min incubation at 30°C in the presence of 40 μCi [^{3}H] leucine/ ml at 10 Ci/mmol as for the other amino acids as described in the Methods and the legend to Fig. 36. Zero time controls or incubations in the presence of Tevenel were about 300-400 cpm/ mg protein in most cases.

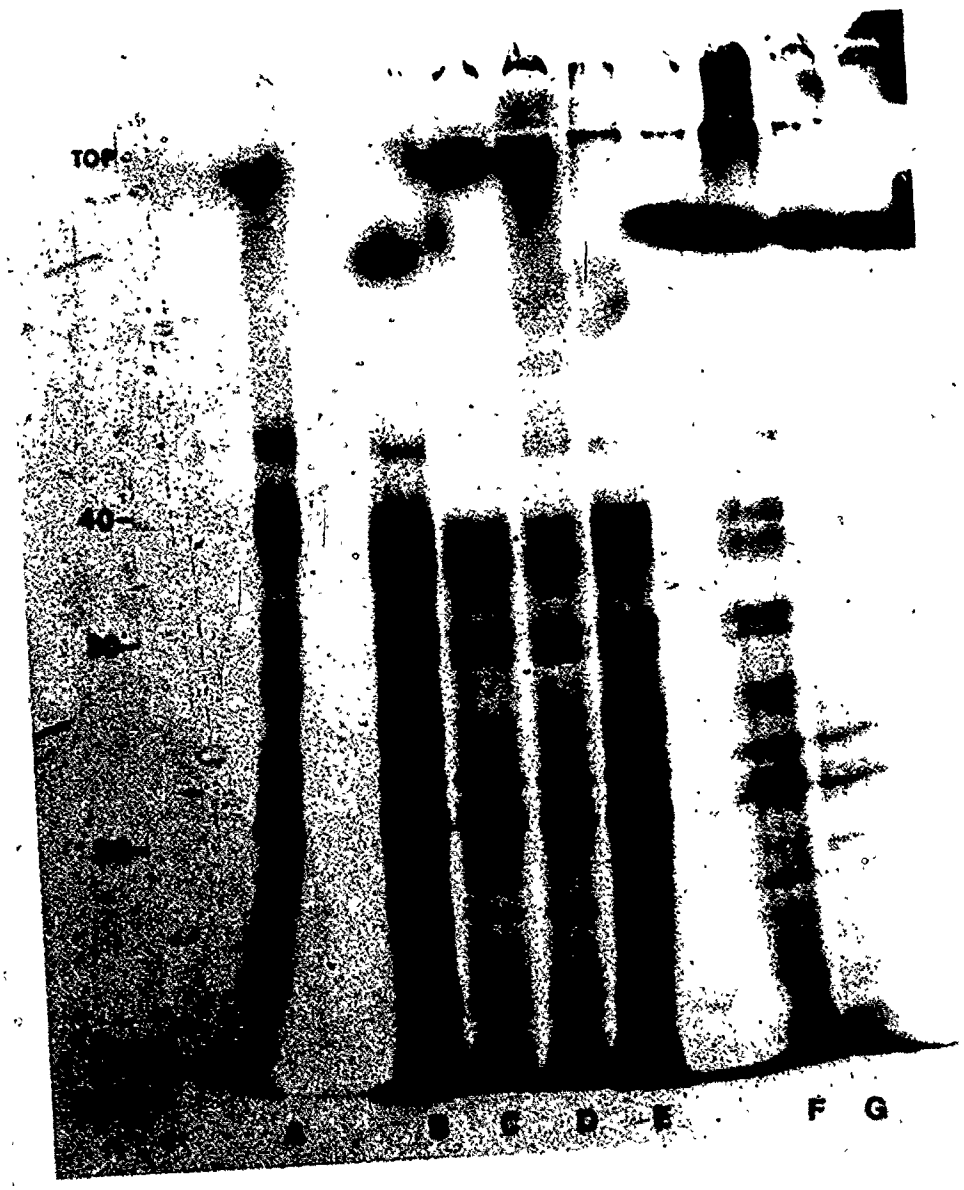


Figure 38

Fluorographic pattern of proteins synthesized by isolated rat liver mitochondria and separated by SDS-polyacrylamide slab gel electrophoresis. Mitochondria were incubated as described in the Methods with 50 µCi of [^{3}H]amino acid/ml, except in (B) and (F) where 100 µCi/ml were used. The sample of CHO TK^{-} cell mitochondrial proteins labelled with [^{35}S]methionine was a gift of R.W. Yatscoff, Dept. of Biochemistry, McMaster University. (A) CHO TK^{-} cell mitochondrial proteins; (B) proline; (C) lysine; (D), valine; (E), leucine; (F), asparagine; (G), arginine.

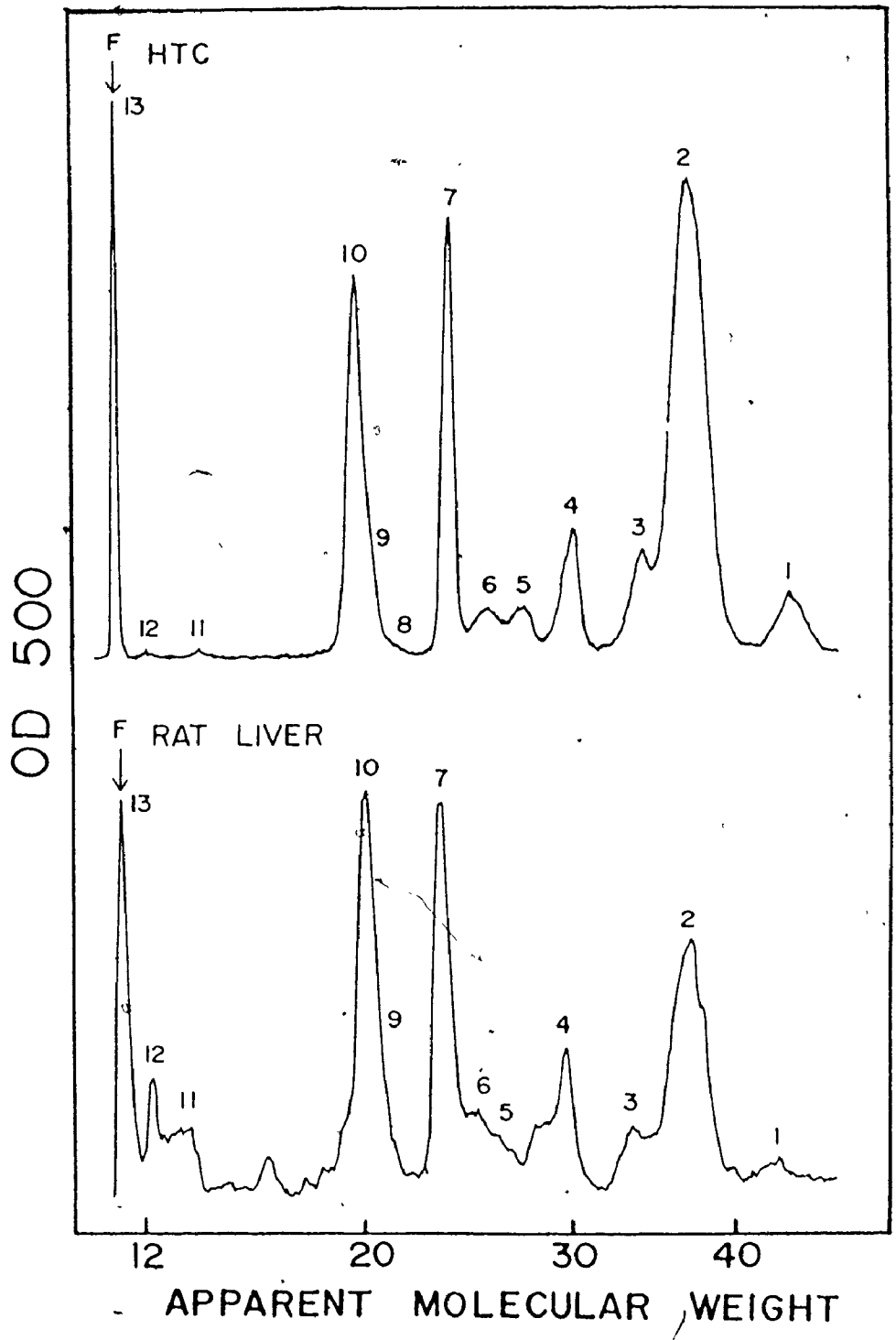


Figure 39

Densitometric traces of fluorograms of HTC mitochondrial proteins labelled in whole cells with [^{35}S] methionine and rat liver isolated mitochondria with [^{3}H] valine. The HTC mitochondrial protein sample was a gift of R.W. Yatscoff, Dpt. of Biochemistry, McMaster University, Ontario.

methionine, the incorporation of [^{35}S] formylmethionine into these proteins. These minor bands were not seen with the other amino acids because of the lower number of counts applied on the gels.

Ching et al (1977) showed that all amino acids except aspartate, cysteine, glutamate and proline were incorporated into whole cell products of mitochondrial protein synthesis in HeLa cells, but the nature of the incorporated amino acids was not determined. In the present case, the lower incorporation of [^{3}H] aspartate compared to [^{3}H] asparagine is an indication that the latter is indeed incorporated as asparagine. To determine the nature of the incorporated label, proteinase K and pronase B hydrolysis of proteins from mitochondria labelled with the radioactive amino acids was performed. The results for [^{3}H] leucine (A), [^{3}H] proline (B) and [^{3}H] asparagine (C) are shown in Fig. 40. While there was some material running close to the front, only the initial radioactive amino acids could be detected. The material running close to the front is most likely undigested di- or tripeptides because radioactivity in these peaks decreased when pronase B was added following digestion with proteinase K alone (Fig. 40) and with time of incubation (not shown). Similar results were obtained with the other amino acids (results not shown). These results suggest that charging of the corresponding tRNAs occurs under the conditions of incubations used. Indeed, mitochondrial tRNAs for all amino acids except histidine and glutamine could be recovered. Generally, charging paralleled the level of incorporation of each amino acid into protein, although this varied with the specific activity of the individual labelled amino acid. However, it was necessary to show that there were unique mitpchondrial aminoacyl-tRNAs.

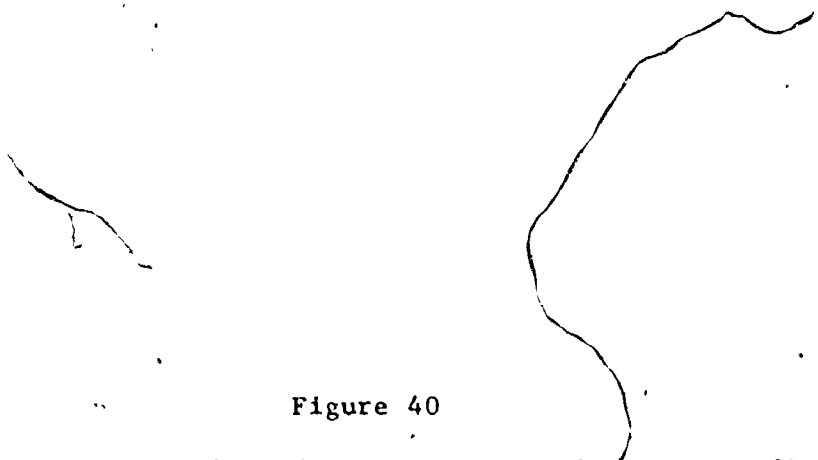


Figure 40

Paper electrophoretic profiles of proteinase K and pronase B digests of [^{3}H]-labelled rat liver mitochondrial proteins.

Mitochondria were isolated and incubated as in Fig. 37. A small amount of labelled mitochondrial protein was dialysed overnight against 0.1 M NaCl/ 10 mM Tris-HCl, pH 7.5 at 4°C and digested with proteinase K (50 µg/ml) or pronase B (50 µg/ml) for 3 h at 30°C. The solution was then spotted on Whatman No. 1 paper and chromatographed as described in the Methods. The paper was then dried, cut and counted for radioactivity. Unlabelled amino acids were run in parallel and spotted with ninhydrin. o-o, proteinase K digest; e-o, proteinase K + pronase B digest.
A. [^{3}H] leucine-labelled mitochondrial proteins; B, [^{3}H] proline-labelled mitochondrial proteins; C, [^{3}H] asparagine-labelled mitochondrial proteins.

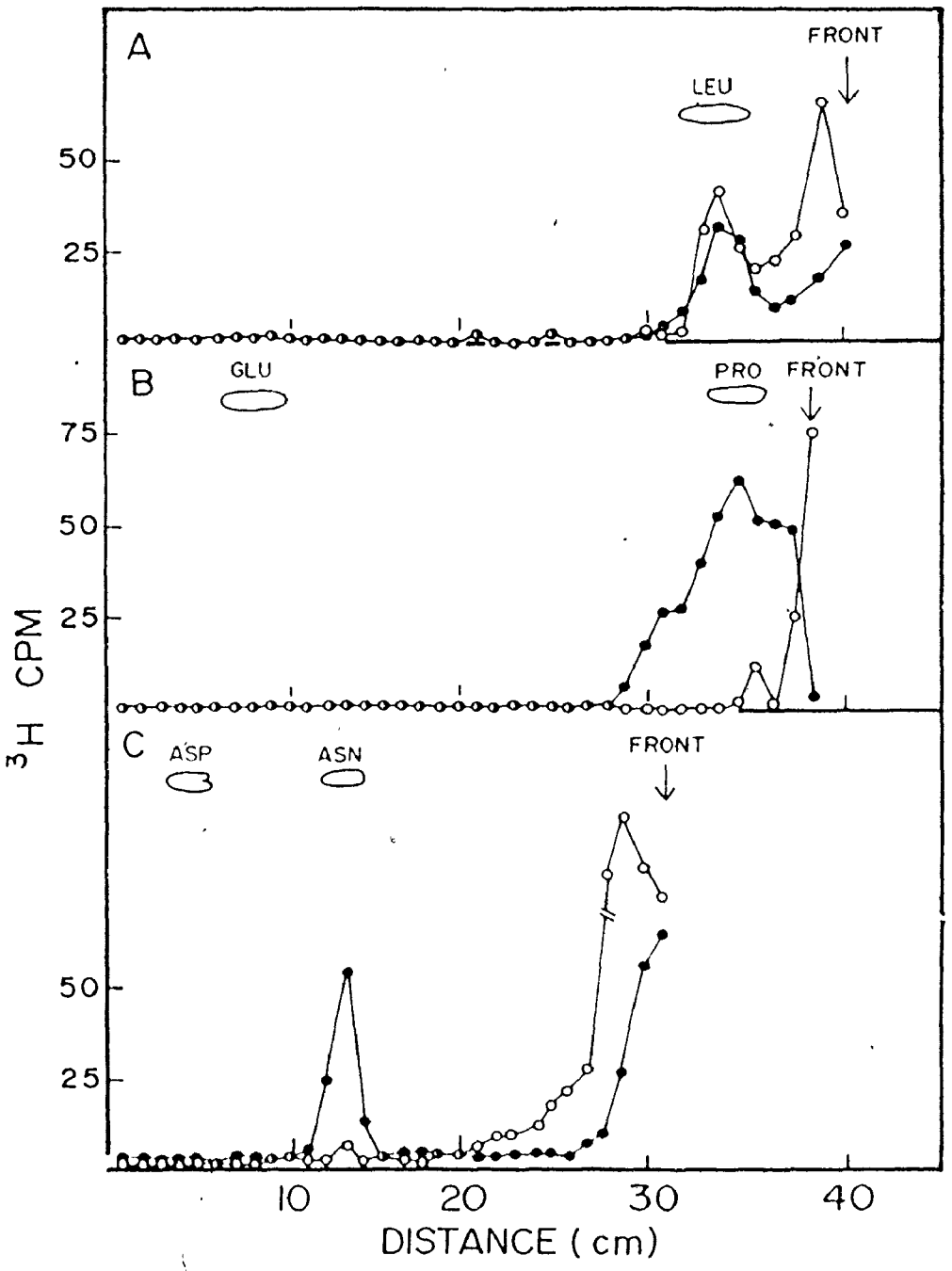

A
FRONT
LEU
50
25
B
GLU
PRO
FRONT
75
50
25
^{3}H CPM
C
ASP
ASN
FRONT
50
25
10
20
30
40
DISTANCE (cm)

Figure 40

Chromatographic analysis of mitochondrial aminoacyl-tRNAs

In order to distinguish the mitochondrial and cytosolic aminoacyl-tRNAs, they were co-chromatographed on RPC-5. The profiles for arginyl-, asparaginyl-, leucyl-, lysyl-, methionyl- and prolyl-tRNA are presented in Fig. 41. In all cases the mitochondrial aminoacyl-tRNAs differed from the corresponding cytosolic aminoacyl-tRNA.

Mitochondrial methionyl-tRNA separated into two broad peaks eluting at salt concentrations lower than the three cytosolic species. It is possible that the mitochondrial peaks contain more than one species which did not separate, since similar profiles of mitochondrial methionyl-tRNAs of both mouse (Wallace & Freeman, 1974) and Syrian hamster (Part II, Fig. 18) had more components. Analyses of the two mitochondrial peaks for terminal methionyl-adenosine and formylmethionyl-adenosine showed that the first peak consisted of methionyl-tRNA ($tRNA_m^{met}$) while the second peak was formylmethionyl-tRNA ($tRNA_f^{met}$), as shown in Fig. 42. The same order of elution was found for mouse liver mitochondrial methionyl-tRNA (Wallace & Freeman, 1974).

Mitochondrial leucyl-tRNA appeared as three peaks eluting after the bulk of the cytosolic species which separated into three major peaks with shoulders on either side of the first peak probably representing two other species. The mitochondrial elution profile is similar to that obtained with Syrian hamster liver mitochondrial leucyl-tRNA (Part II, Fig. 19).

Mitochondrial arginyl, asparaginyl-, lysyl- and prolyl-tRNAs all eluted before their cytosolic counterparts. These mitochondrial aminoacyl-tRNAs were extracted after incubation of isolated mitochondria

Figure 41

RPC-5 chromatographic profiles of rat liver mitochondrial aminoacyl-tRNAs co-chromatographed with cytosolic aminoacyl-tRNAs. [^{3}H] mitochondrial and [^{14}C] cytosolic rat liver aminoacyl- tRNAs were prepared as described in the Methods. Elution gradients were 0.4 to 0.7 M NaCl except for leucyl-tRNA where the gradient was 0.45 to 0.7. Except for mitochondrial methionyl- and leucyl-tRNAs, mitochondria were incubated in the presence of 0.1 units of immobilized RNase/ml. The running temperature was 22°C except for asparaginyl-tRNA where it was 18°C. (o-o), mitochondrial aminoacyl-tRNA; (•-•), cytosolic aminoacyl-tRNA.

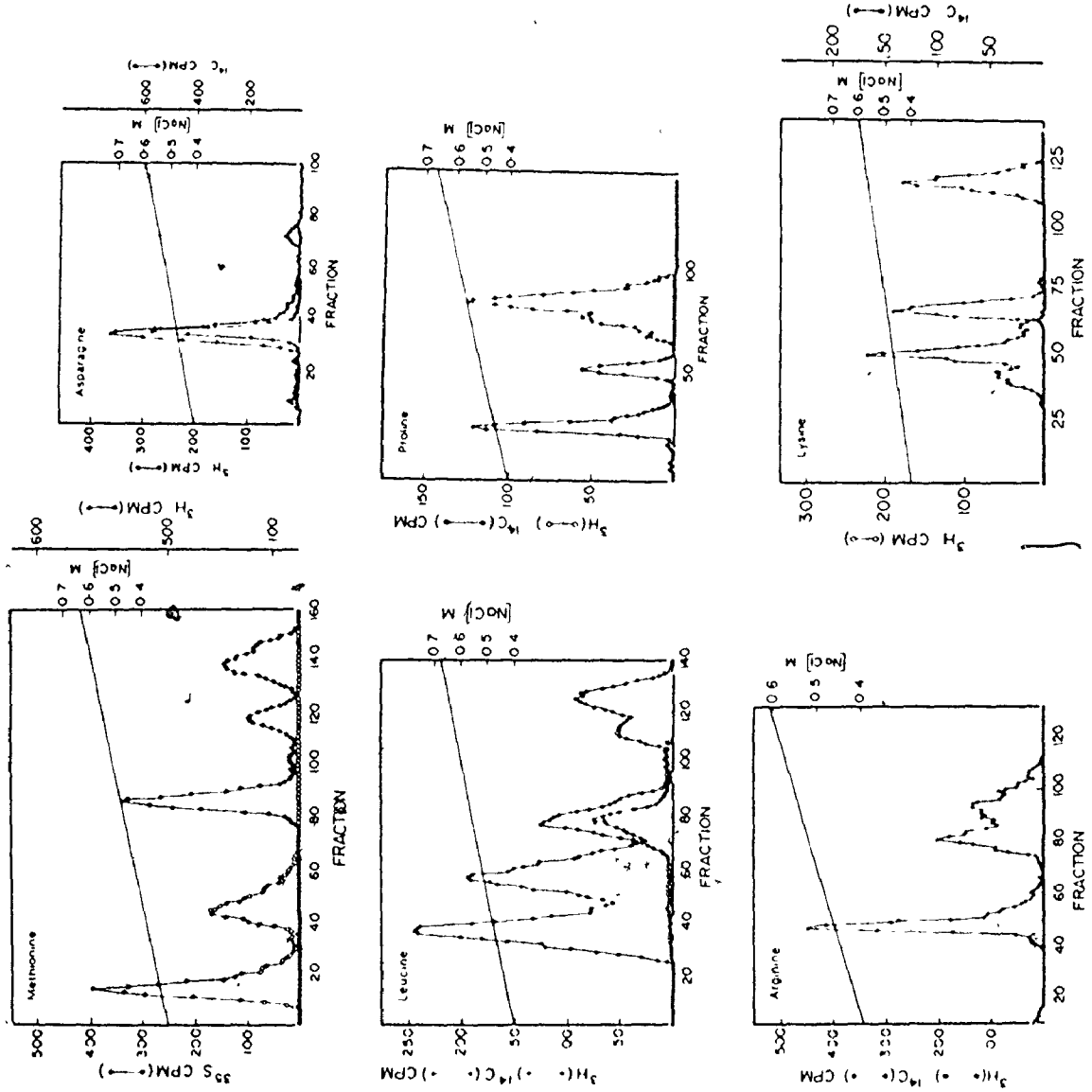

Asparagine
Proline
Lysine
Methionine
Leucine
Arginine
FRACTION
[NaCl] M
^{3}H CPM
^{14}C CPM
^{35}S CPM

Figure 41

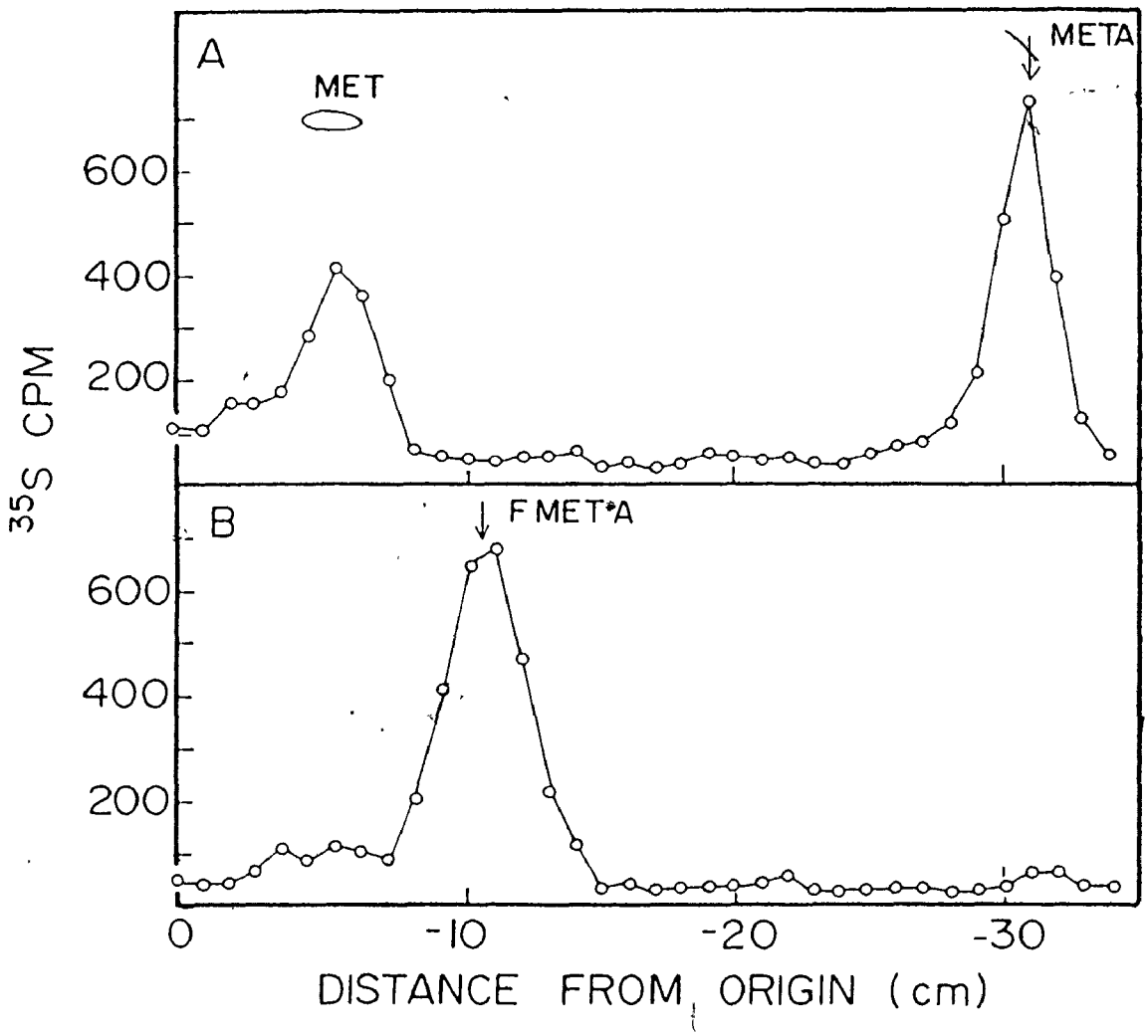


Figure 42

Electrophoretic profile of RNase digests of mitochondrial [^{35}S] methionyl-tRNAS separated by RPC-5 chromatography. Mitochondrial [^{35}S] methionyl-tRNA was prepared and separated on RPC-5 as in Figure 40. Peaks I and II in order of elution as in Figure 41 were recovered and digested with RNase A as described in the Methods. A sample was subjected to paper electrophoresis, the paper dried and counted for radioactivity. Met, fMetA and MetA refer to methionine, formylmethionyl-adenosine and methionyl-adenosine respectively. [^{35}S] methionine was run in parallel. The position of the other two markers was inferred from the results of Smith & Marcker (1968). A: Peak I; B: Peak II.

with immobilized RNase. If this digestion step was not included, the proportion of contaminating labelled cytosolic aminoacyl-tRNA was significant, as shown in Fig. 43 for valyl-tRNA. In this case, as for the four other aminoacyl-tRNAs, charging of the mitochondrial species was low compared to leucyl- and methionyl-tRNAs. Comparison of Fig. 43A and B indicates that RNase digestion removed all cytosolic contamination without affecting the single peak of mitochondrial valyl-tRNA. In the case of mitochondrial lysyl-tRNA, RNase digestion revealed a third minor peak (Fig. 41) previously hidden by contaminating labelled cytosolic lysyl-tRNA (result not shown). Mitochondrial prolyl-tRNA separated into a major and minor peak, while only one peak was observed for both arginyl- and asparaginyl-tRNAs. The separation of mitochondrial and cytosolic asparaginyl-tRNA was increased slightly if the temperature was increased to 20°C (result not shown). Running of this aminoacyl-tRNA at low temperature in order to prevent deacylation (White et al, 1973) does not seem warranted as significant deacylation did not occur at a higher temperature (33°C) under hybridization conditions (see below). This was not tested under chromatographic conditions.

Since neither prolyl- nor asparaginyl-tRNA from mammalian mitochondria had been reported, it was necessary to show that the tRNAs were indeed aminoacylated with proline and asparagine respectively. As shown in Fig. 44, only [^{3}H]asparagine (A) and [^{3}H] proline (B) were released on deacylation of the mitochondrial aminoacyl-tRNAs. This was also found for the cytosolic species. There is no evidence of any other labelled amino acid, such as aspartate or glutamate which could arise during metabolism. The two tRNAs are therefore $tRNA^{asn}$ and $tRNA^{pro}$.

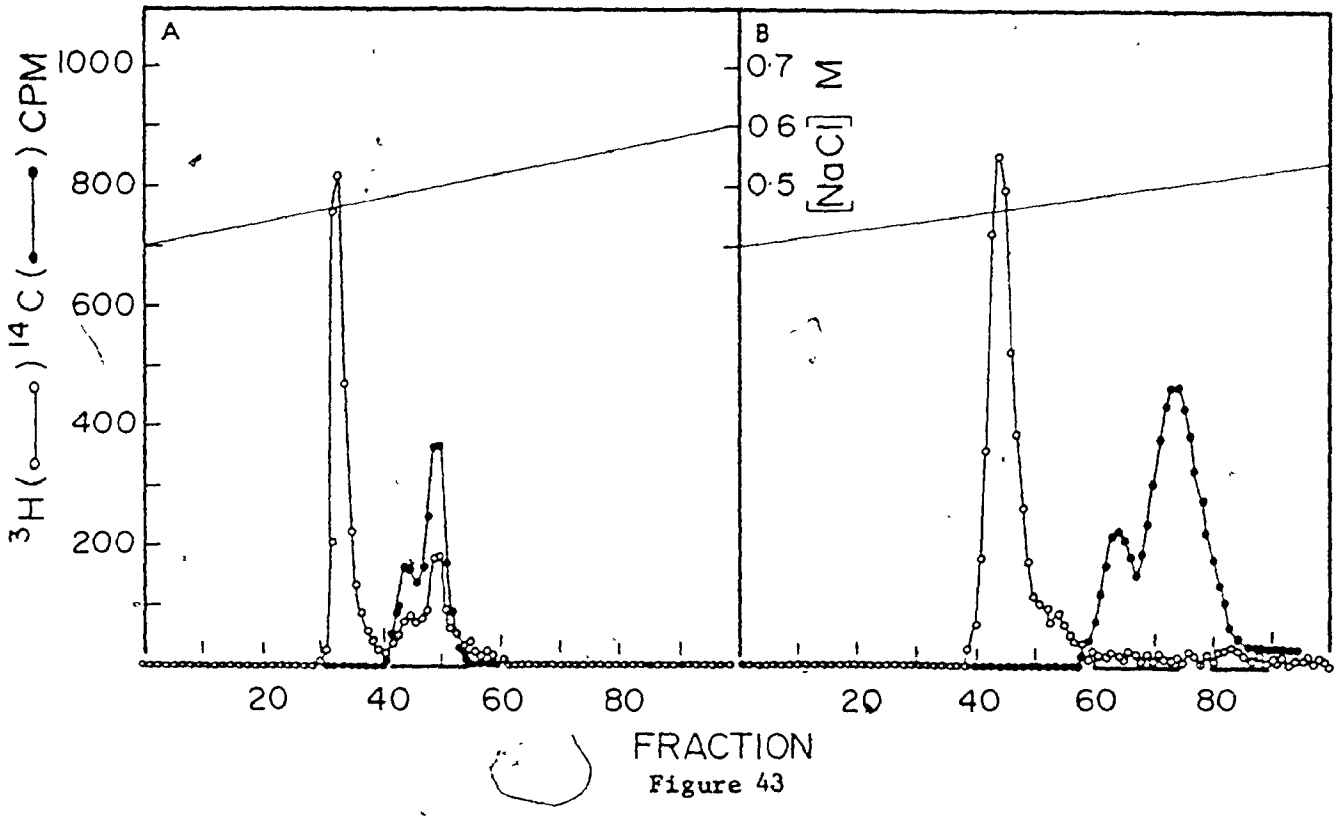


Figure 43

RPC-5 chromatography of mitochondrial (o-o) and cytosolic (•-•) valyl-tRNA. Mitochondria were incubated with [^{3}H] valine in the absence (A) or presence (B) of 0.1 units of immobilized RNase/ml and valyl-tRNA isolated and co-chromatographed with cytosolic [^{14}C] valyl-tRNA.

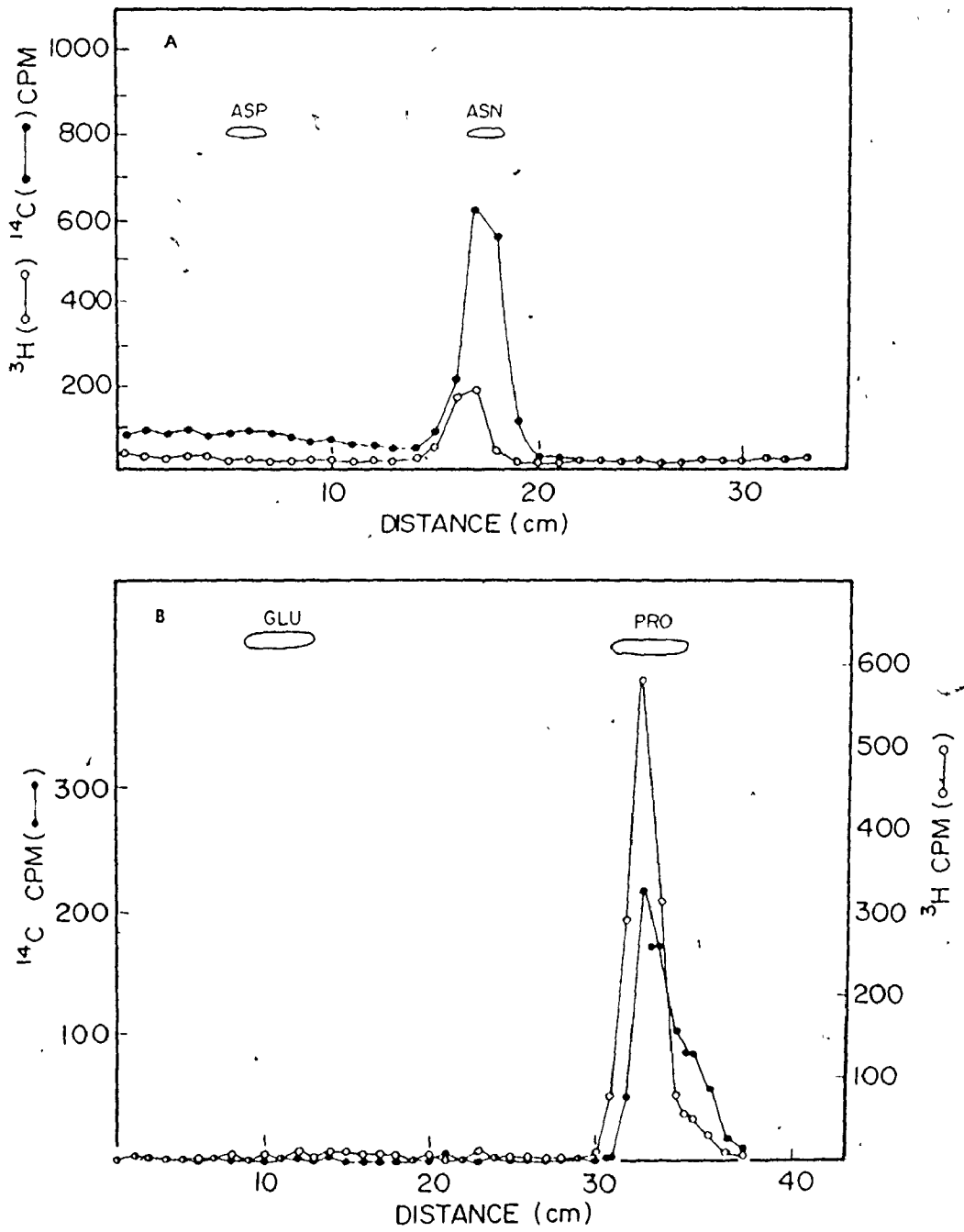


Figure 44

Identification of asparagine and proline in mitochondrial and cytosolic asparaginyl-tRNA (A) and prolyl-tRNA (B). Aminoacyl-tRNAs, either [^{3}H] mitochondrial or [^{14}C]-cytosolic, were hydrolyzed at pH 7.4 and submitted to paper chromatography as described in the Methods. Markers were run in parallel and their positions determined by ninhydrin treatment.

Polyacrylamide slab gel electrophoresis of aminoacyl-tRNAs

The urea polyacrylamide slab gel system of Maniatis et al (1975) used in Part II to determine the size of Syrian hamster liver mitochondrial HNO_2-aminoacyl-tRNAs was also utilized to analyse rat liver mitochondrial aminoacyl-tRNAs. Results are shown in Fig 45. All mitochondrial HNO_2-aminoacyl-tRNAs had higher mobilities than their cytosolic counterparts, the mobilities corresponding to lengths of 76 nucleotides or slightly shorter. The only exception was HNO_2-[^{3}H] lysyl-tRNA (A, slot i) whose apparent length was about 70 nucleotides. HNO_2-[^{3}H] leucyl-tRNA (A, slot c) was the only mitochondrial tRNA to separate into two bands, both of which ran faster than all but one of the 5 bands of cytosolic leucyl-tRNA. The separation is similar to that observed for Syrian hamster liver mitochondrial leucyl-tRNA but the mobilities of the rat liver species seem slightly lower than those of the Syrian hamster leucyl-tRNAs (Part II, Figs 31 and 32).

The smaller size of the Syrian hamster and rat liver mitochondrial leucyl-tRNAs compared to the major cytosolic species contrasts with yeast mitochondrial $tRNA^{leu}$ which is similar in size to its cytosolic counterpart, that is around 85 nucleotides long (Martin et al, 1977b). As the known sequences of isoaccepting species of rat liver cytosolic $tRNA^{ser}$ are all 85 nucleotides long (Ginsberg et al, 1971; Rogg et al, 1975), and as both the mitochondrial and cytosolic $tRNAs^{ser}$ of yeast appear to be about 85 nucleotides long (Martin et al, 1977b), the size of rat liver mitochondrial $tRNA^{ser}$ was determined. Mitochondrial HNO_2-[^{3}H] seryl-tRNA separated into two bands as shown in Fig. 46, slots b and c, with apparent lengths of 76 and lower than 70 nucleotides. The latter band had an unusually high mobility, but this was not caused by

Figure 45

Slab gel electrophoretic analysis of rat liver cytosolic and mitochondrial HNO_2-aminoacyl-tRNAs.

Cytosolic and mitochondrial HNO_2-aminoacyl-tRNAs were deaminated, resuspended in 98% formamide, brought to boiling for 2 min and cooled rapidly before applying to the gels. Electrophoresis was for 5.5 h at 10 V/cm as described in the Methods. The marker dyes were X, xylene cyanol FF and BB, Bromophenol Blue. The numbers refer to nucleotide length.

A, Slot a, yeast cytosolic HNO_2-[^{3}H] leucyl-tRNA; b, cytosolic HNO_2-[^{3}H] leucyl-tRNA; c, mitochondrial HNO_2- [^{3}H] leucyl-tRNA; d, cytosolic HNO_2-[^{35}S] methionyl-tRNA; e, mitochondrial HNO_2-[^{35}S] methionyl-tRNA; f, cytosolic HNO_2-[^{3}H] valyl-tRNA; g, mitochondrial HNO_2-[^{3}H] valyl-tRNA; h, cytosolic HNO_2-[^{3}H] lysyl-tRNA; i, mitochondrial HNO_2-[^{3}H] lysyl-tRNA.

B, Slot a, yeast cytosolic HNO_2-[^{3}H] leucyl-tRNA; b, cytosolic HNO_2-[^{3}H] asparaginyl-tRNA; c, mitochondrial HNO_2-[^{3}H] asparaginyl-tRNA; d, cytosolic HNO_2-[^{14}C] prolyl-tRNA; e, mitochondrial HNO_2-[^{3}H] prolyl-tRNA; f, cytosolic HNO_2-[^{3}H] arginyl-tRNA; g, mitochondrial HNO_2-[^{3}H] arginyl-tRNA.

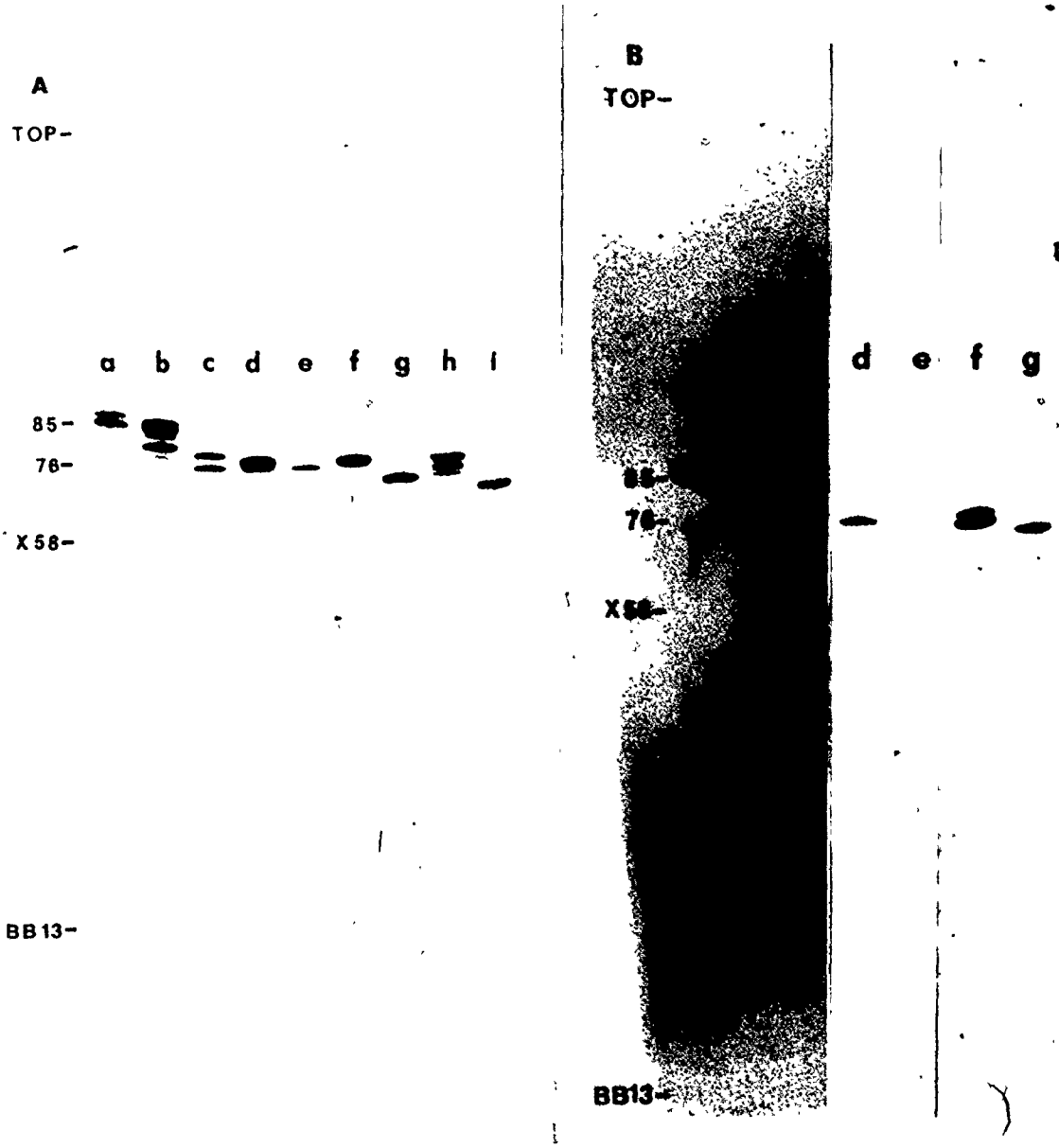


Figure 45

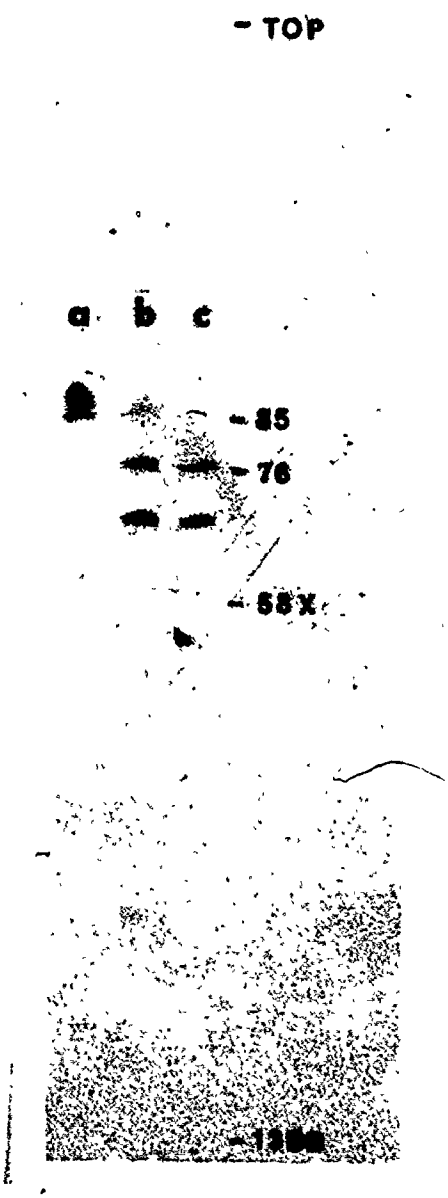


Figure 46

Slab gel electrophoretic analysis of rat liver cytosolic and mitochondrial seryl-tRNAs.

Cytosolic and mitochondrial HNO_2-[^{3}H] seryl-tRNAs were prepared and subjected to electrophoresis as described in Fig. 44. Slot a, cytosolic HNO_2-[^{3}H] seryl-tRNA; slob b, mitochondrial HNO_2-[^{3}H] seryl-tRNA isolated from a mitochondrial fraction incubated in the absence of immobilized RNase; sloc c, mitochondrial HNO_2-[^{3}H] seryl-tRNA isolated from a mitochondrial fraction incubated in the presence of immobilized RNase.

RNase digestion of the mitochondrial fraction prior to seryl-tRNA extraction as shown by a comparison of slots b(-RNase) and c(+RNase). This band also eluted at an unusually low salt concentration (lower than 0.3 N NaCl) on RPC-5 (result not shown). The mobilities of the mitochondrial tRNAs were clearly higher than that of the cytosolic species which appeared as a broad band (slot a) around 85 nucleotides. The breadth of the band was due to the low specific activity of the preparation and not to the separation of the species as can be seen from the mobility of the cytosolic contaminant in the mitochondrial preparation of slot b (-RNase).

The apparent lengths of the various cytosolic and mitochondrial species are summarized in Table 10. In the case of mitochondrial leucyl-tRNA, there is a discrepancy between the mobilities on 12% and 20% (see below) gels. The values in parenthesis are those estimated from a 20% gel (Fig. 47). These would be in accord with the size determined from the sucrose gradient centrifugation analyses (Part II, Fig. 28). However, Syrian hamster and rat liver leucyl-tRNAs were not run in parallel. The slower mobilities of the rat liver species on 12% polyacrylamide slab gels could be due to a slight difference in their base composition compared to Syrian hamster $tRNAs^{leu}$.

Fingerprint analysis of mitochondrial leucyl-tRNAs

The electrophoretic separation of the mitochondrial $tRNAs^{leu}$ and $tRNAs^{ser}$ suggests that in each case the isoaccepting species have distinct sequences. This was examined by obtaining sequence information on the $tRNAs^{leu}$ with the new RNA sequencing methodology (Donis-Keller et al, 1977;

TABLE 10

Apparent nucleotide lengths of cytosolic and mitochondrial aminoacyl-tRNAs

tRNA	Arginine	Asparagine	Leucine	Lysine	Methionine	Proline	Serine	Valine
Cytosolic	77 to 73	76	87 to 78	78 to 74	78 to 75	76	85	76
Mitochondrial	73	76-75	79(76)* 76(74)*	70	76-75	72	76 68-67	74

* values estimated from a 20% polyacrylamide slab gel (Fig. 46). All other values were calculated from analysis on 12% polyacrylamide slab gels (Figs. 44 and 45)

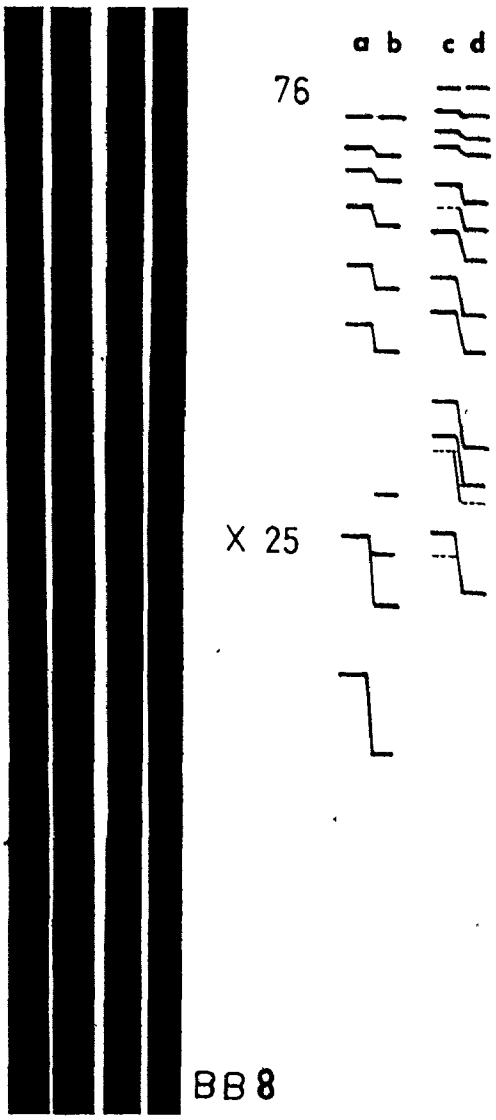


Figure 47

Fluorograms of partial RNase T_1 digests of mitochondrial HNO_2-[^{3}H] leucyl-tRNAs

Leucyl-$tRNA_1^{leu}$ (slots a and b) and leucyl-$tRNA_{2+3}^{leu}$ (slots c and d) were separated by chromatography on RPC-5, deaminated and partially digested with RNase T_1 as described in the Methods. Samples in slot a and c were run on the same 0.15 cm thick gel for 14 h. Samples in slot b and d were run on a 0.075 cm thick gel for 10 h. Partial digests were performed as described by Donis-Keller et al (1977) except for slot d where the method of Simonscits et al (1977) was used. Samples of approximately 8,000 cpm were used and exposure was upt to 45 days. A diagrammatic representation of the gel pattern is shown to the right as fainter bands were not reproduced clearly. Bands which are either not present in one of the gels or could represent artefacts are indicated by dashed lines.

Simoncits et al, 1977), utilizing the specific 3'-labelling as a marker of each fragment and the stability of the deaminated leucyl-tRNAs. As was the case with Syrian hamster mitochondrial leucyl-tRNAs, the first peak to elute on RPC-5 was also the leucyl-tRNA with the faster mobility ($tRNA_1^{leu}$), while the other two peaks ($tRNA_2^{leu}$ and $tRNA_3^{leu}$) both ran as the band of higher mobility, corresponding to 76 nucleotides on the gel shown in Fig. 46.

Partial RNase T_1 digests of HNO_2-[^{3}H] leucyl-$tRNA_1^{leu}$ (slots a and b) and HNO_2-[^{3}H] leucyl-$tRNA_{2+3}^{leu}$ (slots c and d) analysed on 20% urea polyacrylamide slab gels are shown in Fig. 47. The assumption was made that $tRNA_2^{leu}$ and $tRNA_3^{leu}$ differed only in their modified bases rather than in nucleotide sequence since they have the same electrophoretic mobility and elute close together on RPC-5. If this were the case, the number of bands obtained from an RNase T_1 digest should be equivalent to that expected for one species. About 15 G residues would be expected for a tRNA with a 43% G+C content such as in rat liver mitochondrial tRNAs (Chia et al, 1975). Since no bands are seen below a length of 20-25 nucleotides, the number of bands (9 for $tRNA_1^{leu}$ and up to 11 for $tRNA_{2+3}^{leu}$) indicates that the tRNAs have been cleaved at all G residues and that the equivalent of only one species is present in each case. It is not clear why fast migrating bands are not visible. These might be lost during the preparation of the gels for fluorography. Fixing with acetic acid or the use of thinner gels and a lower time of impregnation (slots b and d) did not lead to a recovery of the bands compared to the technique of Bonner & Laskey (1974).

The band patterns of $tRNA_1^{leu}$ and $tRNA_{2+3}^{leu}$ are nevertheless

distinct and the differences can not be explained by two tRNAs differing only in an added sequence. The tRNAs, consequently, differ in their sequences and must be coded by two different genes.

Hybridization of mitochondrial aminoacyl-tRNA to mtDNA

The hybridization curves for asparaginyl-, leucyl-, methionyl- and prolyl-tRNA are shown in Fig. 48. Assuming complete hybridization, the saturation levels indicate that most aminoacyl-tRNAs have a low specific radioactivity. In the case of leucyl-tRNA, up to 25 μg of mtDNA per filter had to be used to obtain levels of hybridized radioactivity significantly above background. Arginyl-, lysyl- and valyl-tRNA showed little hybridization probably because of very low specific radioactivities and further hybridizations were not attempted with these tRNAs. The mitochondrial origin of the first four tRNAs is further corroborated by the lack of hybridization to nuclear DNA and by the lack of hybridization of cytosolic aminoacyl-tRNAs to mtDNA, as summarized in Table 11.

Nass & Buck (1970) had already shown that rat liver mtDNA coded for leucyl-tRNA. To confirm the existence of at least two genes for mitochondrial leucyl-tRNA, suggested by the RNase T_1 partial digests, the individual leucyl-$tRNA_1^{leu}$ and $tRNA_{2+3}^{leu}$ were hybridized to mtDNA separately and together. As shown in Fig 47B, there was only partial additivity of hybridized radioactivity. In this particular experiment, RNase T_1 digestion to lower background levels was omitted. The low background obtained (Table 11) and the level of radioactivity hybridized, which is approximately 2.5 times that of filters loaded with 10 μg of mtDNA, suggest that the omission of this step does not affect the result.

Figure 48

Hybridization curves of mitochondrial aminoacyl-tRNAs to mtDNA. Isolation of aminoacyl-tRNAs and hybridization conditions were as described in the Methods. A, [^{35}S] methionyl-tRNA; B, leucyl-tRNA; o-o, total unfractionated [^{3}H] leucyl-tRNA; ●-●, HNO_2-[^{3}H]leucyl-tRNA$_1^{leu}$; ▲-▲, HNO_2-[^{3}H] leucyl-tRNA$_{2+3}^{leu}$; Δ-Δ, HNO_2-[^{3}H] leucyl-tRNA$_1^{leu}$ + HNO_2[^{3}H] leucyl-tRNA$_{2+3}^{leu}$; C, [^{3}H] prolyl-tRNA; D. [^{3}H] asparaginyl-tRNA.

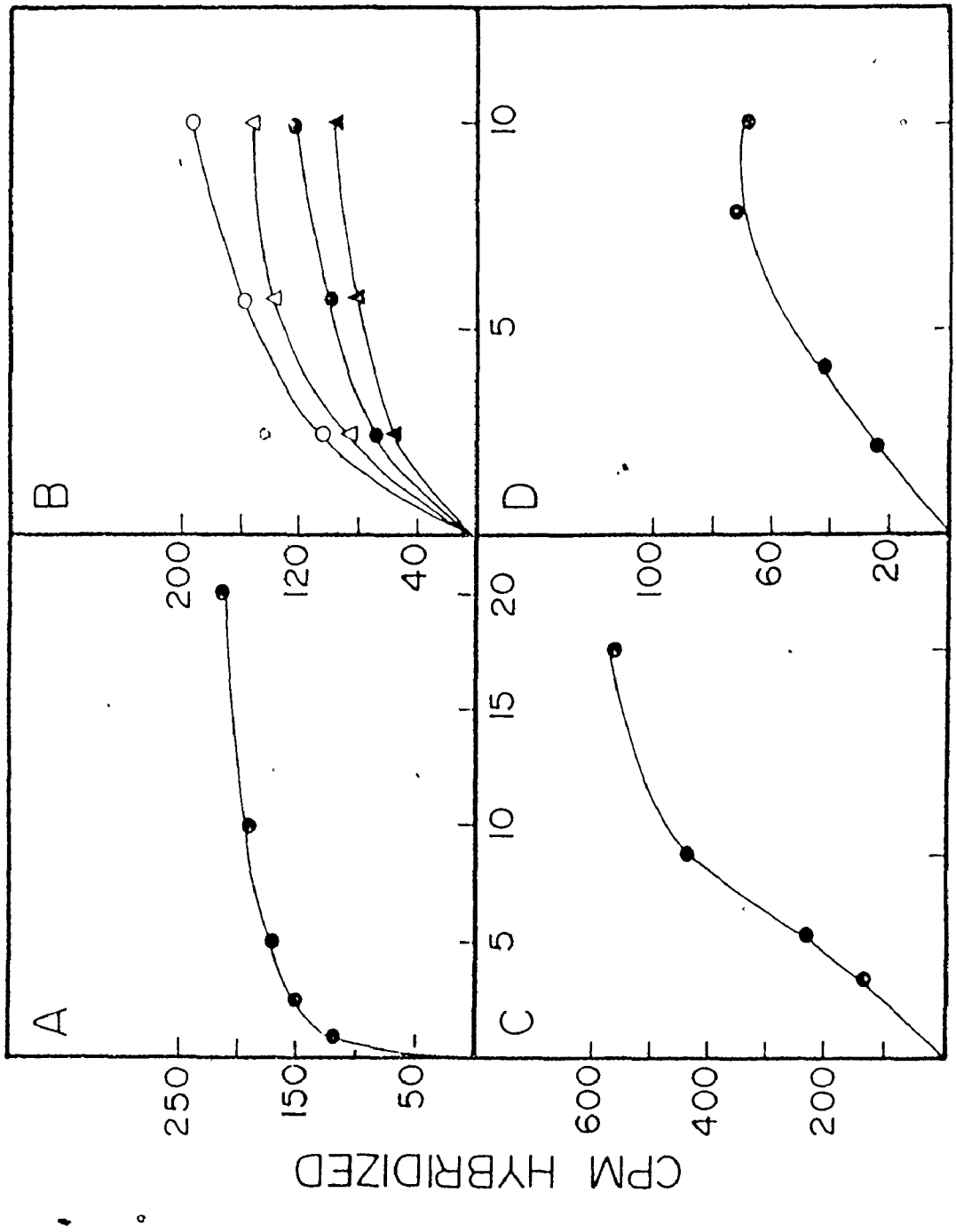


Figure 48

Table 11 Hybridization of Mitochondrial and Cytosolic Aminoacyl-tRNAs to mtDNA and Nuclear DNA

Source	tRNA	Input aminoacyl-tRNA (cpm)	mtDNA 10 µg (cpm)	mtDNA 25 µg (cpm)	Nuclear DNA 100 µg (cpm)	Blank Filter (cpm)
Mitochondria	Methionine					
	Total	10,000			-10	93
		10,000	198			45
	$tRNA_m^{Met}$	10,000[a]	120			
	$tRNA_f^{Met}$	10,000[a]	118			
	$tRNA_m^{Met} + tRNA_f^{Met}$	10,000[a]	222			
Mitochondria	Leucine					
	Total	5,000			6	26
		5,000	48	160		15
		10,000	75	194		29
	$tRNA_1^{Leu}$	5,000[abc]		104		19
		10,000[abc]		126		22
	$tRNA_{2+3}^{Leu}$	5,000[abc]		83		5
		10,000[abc]		101		14
	$tRNA_1^{Leu} + tRNA_{2+3}^{Leu}$	5,000[abc]		141		15
		10,000[abc]		151		25
Mitochondria	Asparagine	8,000	71			35
		6,600			8	40
Cytosolic	Asparagine	8,000	-4		28	29
Mitochondria	Proline	2,000	140			40
		8,000	431			65
Cytosolic	Proline	2,000			21	64
		1,000	2			25

Individual aminoacyl-tRNAs were either hybridized directly or separated by chromatography on RPC-5 ([a]) and in some cases the aminoacyl moiety deaminated with nitrous acid ([b]) before hybridization. Conditions of hybridization were as described in the experimental procedures. In some cases RNase T_1 digestion was omitted following hybridization ([c]). Levels of hybridization are expressed as cpm bound per filter with the cpm of the blank filters subtracted.

With methionyl-tRNA, a similar experiment summarized in Table 11 showed complete additivity, indicating that there is at least one gene for each of $tRNA_m^{met}$ and $tRNA_f^{met}$.

DISCUSSION

The major aim of this study was to determine whether mammalian mtDNA codes for tRNAs corresponding to each of the common amino acids and for tRNAs necessary to read all codons. The results obtained provide answers to some of the questions raised in the Introduction.

1) Incorporation of amino acids by isolated mitochondria

The results indicate that 7 of the 9 amino acids studied are utilized in mitochondrial protein synthesis. Further, the corresponding tRNAs were present as shown by the recovery of their aminoacyl-tRNAs from mitochondria. The mitochondrial nature of the labelled proteins observed was shown by the total inhibition of their synthesis by Tevenel and by the similarity of their electrophoretic pattern to that of rat hepatoma cells (Yatscoff et al, 1978a). There was at least one extra band in the pattern obtained with isolated mitochondria. This contrasts with results of other workers or with other cells (Lederman & Attardi, 1973; and Appendix) which do not reveal any such differences. The origin of the multiplicity of bands seen when labelling with [^{3}H] proline or [^{35}S] methionine is not known.

Neither histidinyl- or glutaminyl-tRNA could be recovered from isolated mitochondria incubated in the presence of the corresponding amino acids. Transport and/or internal pool effects could explain the levels of incorporation of these amino acids into proteins. Matzuzawa

(1974) found that the intra-mitochondrial pools of histidine and glutamine were among the largest of the amino acids. The present result on the lack of incorporation of glutamine contrasts with its very low but significant incorporation by HeLa cell mitochondria, either isolated (Costantino & Attardi, 1973) or in whole cells (Ching et al, 1977). In these studies, the nature of the label incorporated was not determined, as was done here, and it could possibly be another amino acid formed from the precursor administered. Regardless, it is possible that the lack of incorporation of glutamine reflects the existence in mammalian mitochondria of a process of aminoacylation of $tRNA^{gln}$ similar to that found in yeast mitochondria (Martin et al, 1977a) in which this tRNA is first aminoacylated with glutamate followed by amidation to form glutaminyl-$tRNA^{gln}$.

2) Hybridization of aminoacyl-tRNAs to mtDNA

The identification of mitochondrial $tRNA^{asn}$ and $tRNA^{pro}$ and the demonstration that they hybridize to mtDNA, together with the tRNAs for 16 amino acids previously shown to hybridize to mammalian mtDNA (Lynch & Attardi, 1976) brings to 18 the number of amino acids for which tRNAs have been identified in mammalian mitochondria. If the results with yeast (Martin & Rabinowitz, 1978) are included, it seems probable that the mitochondrial genome codes for tRNAs which recognize all amino acids in all organisms studied, with the possible exception of Tetrahymena (Chiu et al, 1975).

3) Isoaccepting species of mitochondrial tRNAs

If all codons are read, incorporation of all amino acids requires anticodons which recognise them, which in turn requires isoaccepting tRNA species for a number of amino acids. The observation that $tRNA_m^{met}$ and $tRNA_f^{met}$ are transcribed from distinct sequences in rat liver mtDNA extends the results in HeLa cells where hybridization of $tRNA_m^{met}$ was not shown (Lynch & Attardi, 1976) and those in yeast (Schneller et al, 1976 b). The results with leucyl-tRNA which indicate that there are two isoaccepting species coded by distinct genes are 1) leucyl-tRNA separated on urea polyacrylamide slab gels into two bands corresponding to $tRNA_1^{leu}$ and $tRNA_{2+3}^{leu}$ separated by chromatography on RPC-5; 2) the RNase T_1 digestion patterns of $tRNA_1^{leu}$ and $tRNA_{2+3}^{leu}$ were different, suggesting different sequences, and 3) hybridization of $tRNA_1^{leu}$ and $tRNA_{2+3}^{leu}$ was partially additive. This last observation suggests that the two genes might have common sequences or overlap in part. For this reason, lack of additivity is not a proof of the absence of distinct genes, as separable species may be sufficiently similar so that they cannot be completely differentiated by hybridization. The presence of two genes for $tRNA^{leu}$ seems to be a general feature of mtDNA. In Tetrahymena, Chiu et al (1974) concluded that there were two genes for $tRNA^{leu}$ although they did not observe additivity of hybridization of different species. Codon recognition experiments by Brown & Novelli (1968) initially indicated the presence of only one $tRNA^{leu}$ in Neurospora mitochondria, but, more recently, two isoaccepting species were separated by two-dimensional gel electrophoresis and shown to be coded by genes whose positions were determined on the restriction map of the mitochondrial genome (Terpstra et al, 1977b). The

The situation in yeast is ambiguous: Martin & Rabinowitz (1978) detected only one peak. Originally, Schneller et al (1975b) also identified one peak on RPC-5, but more recently Martin et al (1977b) concluded that there were two species possibly coded by two genes. The genetic map of yeast mtDNA also indicates the presence of two distinct genes (Dujon et al, 1977).

The separation of seryl-tRNA also into two bands on electrophoresis suggests that there are two isoaccepting species of $tRNA^{ser}$ coded by distinct genes. In HeLa cell mitochondria, the presence of two $tRNAs^{ser}$ was shown by the hybridization of seryl-tRNA to both strands of mtDNA (Lynch & Attardi, 1976). The other tRNAs characterized here probably consist of species coded by one gene only as in most cases only one peak on RPC-5 and one electrophoretic band were observed. These results add 4 tRNAs ($tRNA^{asn}$, $tRNA^{pro}$, $tRNA_m^{met}$ and one $tRNA^{leu}$) to the 17 found previously to hybridize to mtDNA (Lynch & Attardi, 1976). Angerer et al (1976) found 19 sites for tRNAs on HeLa cell mtDNA by electron microscopy of hybridized ferritin-labelled tRNAs, whereas Dawid et al (1976), with a similar technique, found 21-22 sites on Xenopus mtDNA. If tRNAs for glutamine and histidine are eventually found, it would bring the number of tRNAs coded by mammalian mtDNA to 23.

According to the Wobble hypothesis, 3 isoaccepting $tRNAs^{leu}$ and $tRNAs^{ser}$ are needed to read all leucine and serine codons. This is also the case for arginine, while only two would be needed to recognize all valine and proline codons. Possibly, post-transcriptional modifications of a single transcript, such as for $tRNA^{pro}$ and one of the $tRNAs^{leu}$ and $tRNAs^{ser}$ (seryl-tRNA separated into three peaks on RPC-5, not shown), or alternatively odd-base pairing, as suggested by Van Ommen et al (1977),

or a partial two-letter code (Lagerkvist, 1978), could lead to recognition of all codons. If further studies substantiate this idea, then fewer than 32 tRNAs would be necessary to read all possible codons in mitochondria. Sequence information not yet available and codon recognition experiments will be necessary to give a definitive answer. It is also possible, as suggested for methionyl-tRNA, that some isoaccepting species were not separated by RPC-5 chromatography under the present conditions of analysis.

Another approach to the determination of multiple isoaccepting species coded by distinct genes is to identify the location of these genes on the mitochondrial genome. In yeast, petite mutants have been used to obtain the map position of tRNAs (Martin et al, 1977a; Martin & Rabinowitz, 1978), but the method has not been refined sufficiently to localize isoaccepting species, with the exception of $tRNA_m^{met}$ and $tRNA_f^{met}$. Studies in mammalian mitochondrial genetics have not advanced to the stage to allow for such an approach. It might be possible to hybridize individual tRNAs to restriction fragments, a method which has only begun to be used with a few tRNAs (Borst et al, 1977; Terpstra et al, 1977b). At this time, the approach is dependent on preparations of aminoacyl-tRNAs of high specific activities. Experiments in this direction where attempted but where only partially successful, even though up to 30 µg of mtDNA was used in some experiments. It is possible that transfer, either by blotting (Southern, 1975) or electrophoretically (Arnheim & Southern, 1977), which were both tried, were not efficient with such high amounts of DNA and consequently did not compensate for the low specific activities of the aminoacyl-tRNAs. However, very tentatively,

methionyl-tRNA was assigned to band B of the Hind III restriction pattern (Fig. 35).

Sequence analysis of isoaccepting species would also provide proof of distinct genes if the sequences were found to be different. Here, the RNase T_1 partial digests of $tRNA_1^{leu}$ and $tRNA_{2+3}^{leu}$ were sufficient to conclude that these tRNAs have distinct sequences. Unfortunately, the method of partial digests by itself cannot be used to obtain the total sequence of aminoacyl-tRNAs since, in general, modified residues, although present in low amounts in mitochondrial tRNAs (Chia et al, 1976; Davenport et al, 1976), cannot be identified. Further, the disappearance of bands of high mobility remains to be overcome. However, in principle, the approach has the advantage of simplicity since unpurified tRNAs are used and it should be readily applicable for the determination of partial sequences of any tRNA.

4) The size of mitochondrial tRNAs

The size of rat liver mitochondrial tRNAs were about the same as those from Syrian hamster liver (Part II). Slab gel electrophoretic analyses confirm the centrifugation data on methionyl- and leucyl-tRNAs (Part II, Fig. 28). Most of the mitochondrial tRNAs were found to be about the same size or slightly smaller than their cytosolic counterparts. This excludes the possibility of an underestimation of the number of genes calculated from saturation hybridization of the mitochondrial 4S RNA fraction.

As pointed out earlier (Part II), the gel system does not entirely differentiate on the basis of size. Slight base composition differences

can account for variations in mobility, as is evident for yeast leucyl-tRNAs. Thus, the slightly higher mobility of many mitochondrial tRNAs compared to their cytosolic counterparts does not necessarily reflect a smaller size. This could also be true of mitochondrial $tRNA^{lys}$ whose apparent length (see Table 9) is about 70 nucleotides. On the other hand, both $tRNAs^{leu}$ and $tRNAs^{ser}$ were clearly smaller than the corresponding cytosolic species (or major species in the case of $tRNA^{leu}$), as observed also for the $tRNAs^{leu}$ of Syrian hamster livers. The mitochondrial $tRNA^{ser}$ band of highest mobility must correspond to a length of less than or close to 70 nucleotides. In addition, from the 20% polyacrylamide slab gels (Fig. 46), $tRNA_1^{leu}$ must be shorter than the other two $tRNAs^{leu}$. Such a difference could be associated with a reduction in size of the variable loop from 15 to 21 nucleotides to 4 or 5 nucleotides and/or a reduction in the size of the D loop. No other $tRNAs^{leu}$ and $tRNAs^{ser}$ of this size have been reported. In fact, in yeast and Neurospora (Martin et al, 1977b), both mitochondrial $tRNAs^{leu}$ and $tRNAs^{ser}$ are of the same size as their cytosolic counterparts (although one yeast mitochondrial $tRNA^{ser}$ might actually be shorter). Based on the results of Feldman & Kleinow (1976ab), it is possible that the $tRNAs^{leu}$ of Locusta migratoria may be smaller than the cytosolic species, contrary to the claim of Martin et al (1977b) that they are the same size as their cytosolic counterparts.

Rich & RajBhandary (1976) have classified tRNAs into three classes: class I would consist of tRNAs with four base pairs in the D stem and four or five in the variable loop (D_4V_{4-5}); class II of tRNAs with three base pairs in the D stem and four or five in the variable loop (D_3V_{4-5}), and class III of tRNAs with three base pairs in the D stem and a large variable loop (D_3V_N). The decrease in size of the mitochondrial $tRNAs^{leu}$

and $tRNAs^{ser}$ species raises the possibility that mammalian mitochondrial tRNAs are limited to class I and II tRNAs. Class III would be absent. Further experiments with $tRNA^{tyr}$, whose prokaryotic species, but not eukaryotic species, have long extra loops (Barrell & Clark, 1974; Sprinzl et al , 1978) would be necessary to confirm this.

Irrespective of these specific size differences, and in contrast to the observed smaller size of mammalian mitochondrial rRNAs compared to cytosolic rRNAs (see Introduction, Section IIA), mitochondrial tRNAs fall into the size range of other tRNAs. This might be because certain constraints are placed on the size of a tRNA molecule to accomodate the tRNA in the "clover-leaf" structure. No other tRNAs of known structure has been found to be as small as the mitochondrial 4S RNA of molecular weight 19,000 suggested by Dubin & Friend (1972). The size of the $tRNAs^{leu}$ and $tRNAs^{ser}$ still leaves these mitochondrial tRNAs longer or close to 70 nucleotides. The variable extra loop of about 3 to 5 nucleotides might be the minimum necessary to maitain the tertiary structure of a tRNA. Unless one assumes an unusual base composition, the $tRNA^{ser}$ of highest mobility would be at the extreme limit to be accomodated within the generally accepted "clover-leaf" structure.

CONCLUSION

The evidence presented leads to the conclusion that mammalian mitochondria do not import either mRNA or tRNA. In neither case have the results demonstrated absolutely mitochondrial autonomy, but the demonstration that isolated mitochondria synthesize poly(A)+RNA of a size sufficient to code for mitochondrially-synthesized proteins (Part I), and the isolation, characterization (Parts II and III) and determination of the genetic origin (Part III) of a number of mitochondrial tRNAs not previously identified in eukaryotes or in mammalian cells strongly suggest that this is indeed the case.

We are left with the question of why mitochondria would synthesize all the RNA species necessary for its protein-synthesizing apparatus, while, in contrast, most mitochondrial proteins are synthesized outside mitochondria. An answer may be provided by a consideration of the evolutionary origin of mitochondria. While solid experimental evidence to support the different theories of their origin is limited, the unexpected finding that mitochondrial $tRNA_S^{leu}$ and $tRNA_S^{ser}$ are significantly smaller than their cytosolic counterparts, in apparent contrast to the situation in Fungi, might introduce another aspect of the origin and evolution of mitochondria.

The size difference might be related to the low levels of methylation and other modifications of mitochondrial tRNAs. A number of bases found either in cytosolic tRNAs or typical of prokaryotes are

either absent or in lower amounts in mitochondrial tRNAs (Fairfield & Barnett, 1971; Chia et al, 1976; Davenport et al, 1976; Feldman & Kleinow, 1976a; Martin et al, 1977b). The base m^7G, which is present in all initiator $tRNA_f^{met}$ from E. coli, yeast and all mammalian sources tested, is not present in mitochondrial initiator tRNA of Neurospora (Heckman et al, 1978) and in the $tRNA^{leu}$ and $tRNA^{met}$ of Syrian hamster liver mitochondria (Wallace et al, 1978). Since the content of minor nucleosides in tRNA generally increases from lower to higher organisms, it could be taken as an evolutionary marker (Gross, 1973). Thus, the size difference might make sense from an evolutionary point of view if low levels of modification and a smaller size are both expressions of the "primitiveness" of mitochondrial tRNAs. Mitochondrial tRNAs would not only be more primitive than most present day eukaryotes, but also of chloroplasts, as shown by the comparison of the extent of modification of chloroplast $tRNA^{phe}$ with other $tRNAs^{phe}$ (Chang et al, 1976). Recently, Ehran et al (1977) suggested that all tRNAs evolved from the polymerization of an original oligonucleotide adaptor molecule, 6 to 20 nucleotide long, extending the hypothesis of Jukes (1966) of a single evolutionary origin of tRNAs. The possibility is then that the smaller size of mitochondrial $tRNAs^{leu}$ and $tRNAs^{ser}$ might represent an early stage in the evolution of tRNAs. A further evidence of the "primitive" character of mitochondrial tRNAs is the presence of formyl-methionyl-$tRNA_f^{met}$ and the amidation of glutamyl-$tRNA^{gln}$ to glutaminyl-$tRNA^{gln}$ in yeast mitochondria (Martin et al, 1977a). The same reaction is found in B. subtilis, but not in E. coli, the former being believed to be a more primitive bacterium than E. coli (Schwartz & Dayhoff, 1978).

Is the "primitiveness" of mitochondrial tRNAs related to the

evolution of mitochondria? From recent data (Bonen et al, 1977; Schwartz & Dayhoff, 1978), it seems likely that mitochondria must have arisen from symbiotic adaptations. Only this theory, as opposed to the "episomal" theory (Raff & Malher, 1972) or the "DNA-segregation" theory (Reijnders, 1975), explains satisfactorily the relative conservation of the main features of the inner mitochondrial membrane. The smaller size of mitochondrial $tRNAs^{leu}$ and $tRNAs^{ser}$ in mammalian cells compared to yeast (or Fungi) introduces the further possibility that mitochondria could have arisen from a multiplicity of original symbiotic events, as suggested by Woese (1977). Mitochondria of metazoans would either be the product of earlier events or result from the introduction of a symbiont having maintained some more "primitive" features than that leading to Fungi. The second suggestion is more likely. If the size of the poly(A) tail is taken as an evolutionary marker (Carlin, 1978), its absence or smaller size in mitochondrial mRNAs of yeast (and Fungi) compared to that of higher eukaryotes would imply a more recent origin of mitochondria of the latter group.

Since the conservation in genome organisation (at least for the rRNA genes) holds true also for the host nuclear DNA, as pointed out earlier (see Introduction, Section III), and implying a common origin of nuclear material in both the host and the symbiont, it could also signify that they originally coded for similar functions, some of which would have been lost from the mitochondrial genetic system in the course of adaptation of the symbiont. Most genes for proteins on the DNA of the mitochondrial symbiont would have been lost because specific proteins were able to pass across the mitochondrial membrane. With regards

to RNA, one must assume, to explain mitochondrial autonomy, that RNAs were not able to cross the mitochondrial membrane. Consequently, the smaller $tRNAs^{leu}$ and $tRNAs^{ser}$ were conserved as well.

The points raised here remain speculative. Sequencing of mitochondrial RNAs and proteins from different organisms will be necessary before any clearer picture can emerge.

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APPENDIX I

Biochemical and genetic approaches to the study of mammalian mitochondrial tRNAs[1]

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ABBREVIATIONS: CHO, Chinese hamster ovary; mtDNA, mitochondrial DNA; TK^-, thymidine kinase minus

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Aujame, L., Yatscoff, R.W. and Freeman, K.B. (1978) Biochemical and genetic approaches to the study of mammalian mitochondrial tRNAs Can. J. Biochem. 56

The possible existence of mammalian mitochondrial asparaginyl-tRNA has been examined using a variety of approaches. [^{3}H]Asparagine was incorporated into protein by mitochondria of the Chinese hamster ovary (CHO) cell line Asn-7, which has a temperature sensitive cytosolic asparaginyl-tRNA synthetase, either in the presence of cycloheximide or at a nonpermissive temperature. Isolated mitochondria of CHO TK^{-} cells also incorporated the amino acid into protein. In each case the number and electrophoretic mobility of the proteins was the same as mitochondrially-synthesized proteins of CHO TK^{-} cells labelled with [^{35}S]methionine. A tRNA^{Asn} could be charged in isolated CHO TK^{-} cell mitochondria and the asparaginyl-tRNA was found to elute before its cytosolic counterpart on an RPC-5 column and to have a higher mobility on polyacrylamide slab gels run under denaturing conditions. This is the first demonstration of a unique mitochondrial asparaginyl-tRNA.

Mitochondria from all sources examined have unique rRNAs, mRNAs and tRNAs but molecular hybridization studies have shown that vertebrate mtDNA appears to code for fewer than 20 tRNAs (1-6). In particular, only 17 mitochondrial tRNAs, charged by mitochondrial aminoacyl-tRNA synthetase preparations, hybridized to mtDNA of human HeLa cells (3). No conclusive evidence for charging of distinctive mitochondrial tRNAs for asparagine, glutamine, histidine and proline could be found. In contrast, only asparaginyl-tRNA has not been found in mitochondria of yeast (7). Charging of all mitochondrial tRNAs by mitochondrial synthetases of Neurospora crassa has been reported (8) but charging for tRNAs for asparagine, cysteine, glutamine, proline and tryptophan was too low to determine whether these tRNAs were distinctive mitochondrial components (9). These studies raised the possibliity that vertebrate mtDNA and perhaps other mtDNAs do not code for tRNAs for all amino acids or codons (10) resulting in either the importation of nuclear-coded tRNAs (11) or the incorporation of a limited number of amino acids into proteins. Isolated HeLa cell mitochondria incorporated only 12 amino acids into proteins to a significant extent (12). In contrast 16 amino acids, including the 4 whose tRNAs were not detected, were incorporated into whole cell products of mitochondrial protein synthesis in HeLa cells (13) but the nature of the incorporated amino acids was not determined. Aspartic acid, cysteine, glutamic acid and proline were not incorporated.

The results to date do not resolve the problem of the number or origin of mitochondrial tRNAs. This could reflect the limitations of the experimental techniques employed. Using a number of biochemical and genetic approaches we now show that CHO cell mitochondria incorporate

asparagine into protein and that these mitochondria possess a unique asparaginyl-tRNA not previously demonstrated in mitochondria from any source. The site of the gene for mitochondrial asparaginyl-tRNA will be described in a later paper.

Materials and Methods

Materials

The source of most chemicals, tissue culture media and RPC-5 have been described previously (14,15). L-[2,3-^{3}H]Asparagine (22Ci/mmol (1Ci=37GBq)) was purchased from the Commissariat a l'Energie Atomique, Gif-sur-Yvette, France, and L-[U-^{14}C]asparagine (>150μCi/mmol) was from New England Nuclear, Boston, MA.

Cells

The source and growth of the CHO TK^{-} and Asn-7 cell lines have been described previously (14).

Protein synthesis

The incorporation of [^{3}H]asparagine into protein by mitochondria of whole cells or isolated mitochondria from CHO TK^{-} cells follow methods described previously (14). Some details are given in the legends to Fig. 1 and 2. Proteins were separated by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis and visualized by fluorography (14).

The nature of the incorporated amino acid was identified as follows. Mitochondria were suspended in about 0.1 ml of 0.25 M sucrose, 2 mM EDTA, 2 mM Tris-HCl, pH 7.4 and dialyzed overnight at 4°C against 100 mM NaCl, 10 mM Tris-HCl, pH 7.5. The dialyzed sample (about 1 mg protein/ml) was digested with 50 μg proteinase K/ml for 3 h at 30°C and

chromatographed on Whatman no. 1 paper with phenol-water (100:37, w/v) for 18 h. After drying, the paper was cut into 1 cm x 2 cm pieces and counted in a toluene based scintillation solvent.

Asparaginyl-tRNA

tRNA for asparagine was charged in isolated CHO TK$^-$ cell mitochondria and mitochondrial [^{3}H]asparaginyl-tRNA extracted as described previously for methionyl-tRNA (15,16) except for the following changes. Incorporation mixtures for mitochondria contained 10 mg mitochondrial protein/ml, 45μCi [^{3}H]asparagine/ml and 10 units of immobilized ribonuclease in a total volume of 4.5 ml. The immobilized ribonuclease was added to remove possible contaminating cytosolic [^{3}H]asparaginyl-tRNA (17). After 15 min at 30°C, 100 mM EDTA, pH 7.5 was added to 10 mM and the incubation was continued for another 5 min. Then $MgCl_2$ was added to 10 mM, mitochondria recovered and [^{3}H]asparaginyl-tRNA extracted.

Cytosolic [^{14}C] asparaginyl-tRNA was isolated as follows. CHO TK$^-$ cells were resuspended in a small volume of asparagine-free Joklick minimal essential medium supplemented with 5% (v/v) dialysed fetal calf serum, essential amino acids and 60 mg aspartic acid/ml. After 30 min at 37°C, cycloheximide was added to 300 μg/ml and the cells incubated for 10 min. [^{14}C]Asparagine was then added to 2μCi/ml and incubation continued for 20 min. Cells were recovered and resuspended in 9 volumes of 10 mM $MgCl_2$, 10 mM sodium acetate, pH 5.0, washed twice with the same buffer, disrupted with an Ultra-Turrax (14) and centrifuged at 6,700 xg for 15 min. [^{14}C]Asparaginyl-tRNA was extracted from the supernatant as described previously (15,16).

Asparaginyl-tRNAs were chromatographed on RPC-5 and fractions

counted as described previously (15,16). The size of deaminated and denatured asparaginyl-tRNAs was determined as described elsewhere (15). [^{3}H]Asparagine was released from mitochondrial [^{3}H]asparaginyl-tRNA by incubation at 37°C for 15 min at pH 7.4 and identified by paper chromatography as described above.

Results

Incorporation of asparagine into mitochondrial protein

Proteins synthesized in mitochondria of all CHO cell lines with [^{35}S]methionine as label can be separated into 10 distinct components by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (14). This is shown in Fig. 1A for CHO TK$^-$ cells. The two fastest components ran at the front and band 9 appears split into 2 components. Although a splitting of this band was not seen in earlier work the possibility that it consisted of 2 components was suggested (14). [^{3}H]Asparagine was incorporated into the same components when cytosolic protein synthesis was inhibited in the CHO cell line Asn-7 both at a nonpermissive temperature (Fig. 1B) at which the temperature-sensitive cytosolic asparaginyl-tRNA synthetase is nearly inactive (14,18), and with cycloheximide (Fig. 1C). The former result eliminates possible secondary effects of cycloheximide (19). Incorporation was low partly due to the low specific activity of the [^{3}H]asparagine and the small difference in the mobility of component 6 in the C wells probably reflects differences in protein concentration in the C wells compared to A and B.

The incorporation of [^{3}H]asparagine in protein by mitochondria was further demonstrated using isolated mitochondria from CHO TK$^-$ cells.

The proteins synthesized by isolated mitochondria (Fig. 2B) correspond to those synthesized in whole cells with [^{35}S]methionine as label (Fig. 2A). In this gel, run at a higher concentration of acrylamide, band 9 appears to be split and additional bands were observed just ahead of components 4 and 7. These bands had not been detected previously (14) and their significance is not known. Their presence possibly reflects increased resolution obtained with the present lot of acrylamide.

It is possible that the [^{3}H]asparagine was incorporated as such or as aspartic acid or some other amino acid. It is unlikely to be aspartic acid because cells were labelled in the presence of an excess of this amino acid. Further, incorporation of [^{3}H]aspartic acid by mitochondria of whole cells could not be demonstrated as was also found for HeLa cells (13). The nature of the incorporated amino acid was demonstrated in a proteinase K hydrolysate of protein labelled with [^{3}H]asparagine at the nonpermissive temperature. [^{3}H]Asparagine but no [^{3}H]aspartate was obtained (Fig. 3A) but some labelled material ran close to the front. This is believed to be dipeptides not hydrolyzed by the enzyme.

Mitochondrial asparaginyl-tRNA

The incorporation of [^{3}H]asparagine into mitochondrially-synthesized protein suggested that mitochondria possess a distinct asparaginyl-tRNA synthetase and $tRNA^{Asn}$. The presence of a unique mitochondrial asparaginyl-tRNA was shown by charging the tRNA in isolated CHO TK^{-} cell mitochondria and demonstrating that the asparaginyl-tRNA was distinct from its cytosolic counterpart in its chromatographic and electrophoretic characteristics. As shown ~~in Fig~~. 4, mitochondrial [^{3}H]asparginyl-tRNA elutes before cytosolic [^{14}C]asparaginyl-tRNA on RPC-5. There was some cytosolic contamination in the mitochondrial sample. The

significance of the multiple peaks is not known. Hydrolysis of mitochondrial [^{3}H]asparaginyl-tRNA at pH 7.4 yielded mainly [^{3}H]asparagine (Fig.3B) indicating that the aminoacyl-tRNA was not aspartyl-tRNA or contained some other amino acid. Further aspartic acid is poorly incorporated into protein by isolated rat liver mitochondria (unpublished observations) and HeLa cell mitochondria (12).

Deaminated and denatured mitochondrial asparaginyl-tRNA had a slightly faster mobility than its cytosolic counterpart on polyacrylamide slab gels run under denaturing conditions (Fig. 5). Both asparaginyl-tRNAs were approximately 76 nucleotides long.

Discussion

Proteins of all sources examined have the standard 20 amino acids and it would be unusual if this were not the case for mitochondrially-synthesized proteins. Amino acid analyses of a variety of proteins synthesized in mitochondria of yeast (20,21) and of Neurospora (22-24) have shown the presence of at least 18 amino acids, it not being possible to identify asparagine and glutamine. Further, one subunit of bovine heart cytochrome c oxidase (EC 1.9.3.1) which is probably synthesized in mitochondria contains all the primary amino acids including asparagine and glutamine (25-27). Therefore it would be expected that asparagine would be incorporated into protein by mitochondria. The incorporation of [^{3}H]asparagine by HeLa cells had been reported previously (13,28) but it had not been demonstrated that the incorporated amino acid was actually asparagine. The present results show that asparagine is incorporated into distinctive mitochondrially-synthesized proteins in whole CHO cells and by isolated mitochondria and that in the former

case the incorporated amino acid was found to be asparagine. Of particular interest was the result with the Asn-7 cell line. The incorporation of asparagine into mitochondrial protein at the non-permissive temperature suggests that mitochondria contain a unique asparaginyl-tRNA synthetase which has not been affected by the temperature-sensitive mutation. until now, with the possible exception of Neurospora, the activity of this enzyme has not been detected in vitro (3,7-9). The importance of the genetic approach for the solution of this type of problem had been shown previously (19).

Although the results reported here and previously suggest that mitochondria should contain a $tRNA^{Asn}$, this tRNA has not been unambiguously detected using isolated mitochondrial tRNA and synthetases from HeLa cells (3), yeast (7) or Neurospora (8,9). With isolated mitochondria, asparaginyl-tRNA was formed and could be distinguished from its cytosolic counterpart by chromatography on RPC-5 and by electrophoresis. Its higher mobility on gels than the cytosolic asparaginyl-tRNA is consistent with earlier findings for mitochondrial leucyl-tRNAs and methionyl-tRNAs from Syrian hamster liver (15). It is not clear why asparaginyl-tRNA was not detected by others (3,7,9). This might have resulted from the lability of asparaginyl-tRNA synthetase. The aminoacylated tRNA apparently does not form via the amidation of aspartyl-$tRNA^{Asn}$ as may occur for mitochondrial glutamyl-$tRNA^{Gln}$ in yeast (7) since aspartate is poorly incorporated by mitochondria (unpublished observations, 12,13,28).

The utility of isolated mitochondria for charging mitochondrial tRNAs without the need for prior isolation of the tRNAs and aminoacyl-tRNA

synthetases was demonstrated by us previously (15,16). In the present case it has been the only successful approach used so far in demonstrating mitochondrial asparaginyl-tRNA. The genetic origin of this tRNA will be reported in a separate paper.

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List of Figures

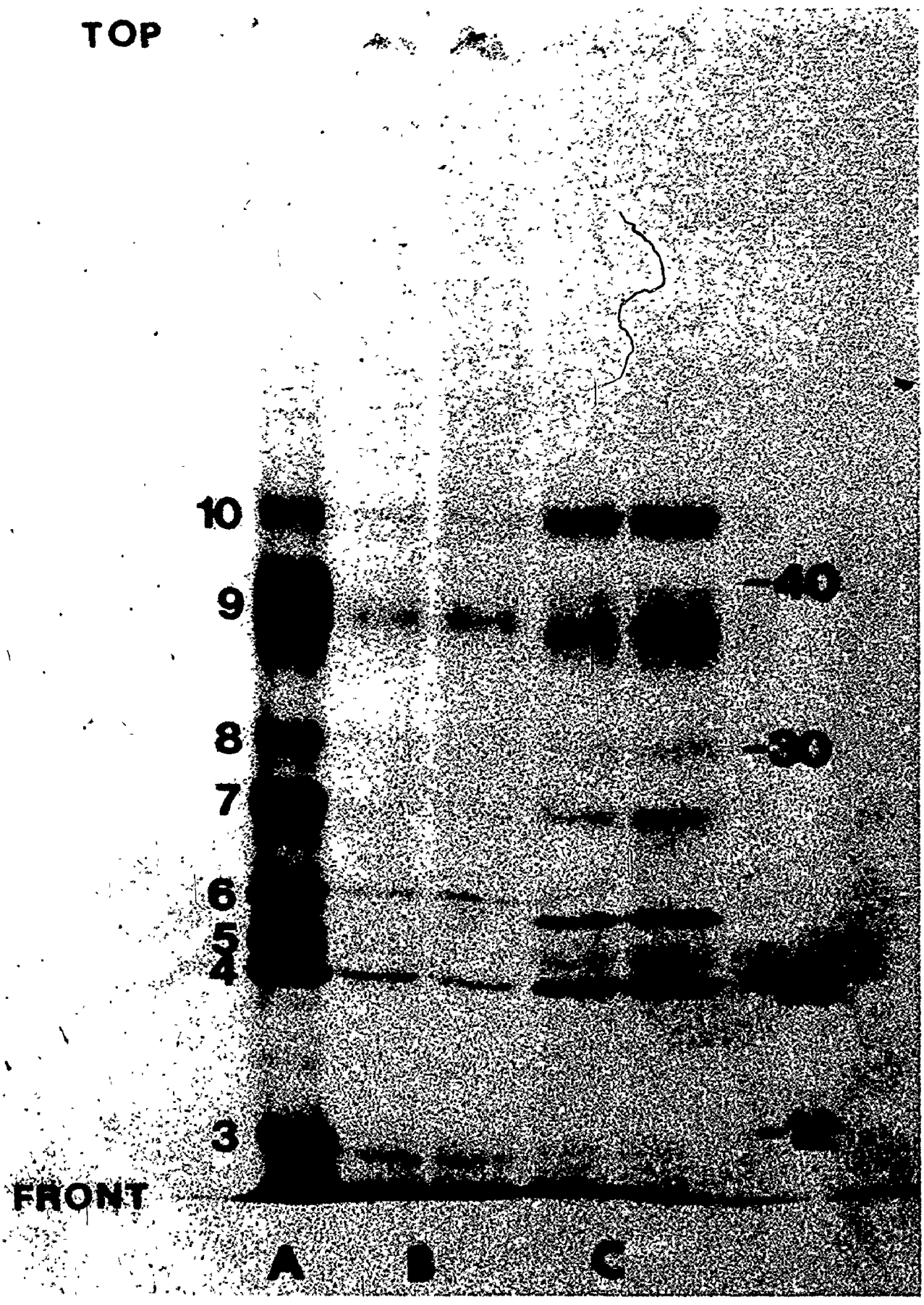
Fig. 1. Fluorographic patterns of proteins synthesized in mitochondria of CHO cell lines and separated by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis. A. Mitochondrial proteins of CHO TK$^-$ cells were labelled for 1 h at 37°C in the presence of 300µg cycloheximide/ml and 4µCi [^{35}S]methionine (400-600Ci/mmol)/ml as described previously. B. Mitochondrial proteins of Asn-7 cells were labelled at a nonpermissive temperature as follows. Approximately 2 x 10^9 cells were suspended in asparagine-free Joklik ~~mini~~mal essential medium, supplemented with 5% dialysed fetal calf serum, essential amino acids and 60 mg aspartic acid/ml and kept at 34° for 30 min. After 5 min at 42°C and 30 min at 39.5°C, [^{3}H]asparagine (22 Ci/mmol) was added to 40µCi/ml and cells incubated for 1 h. C. Mitochondrial proteins of Asn-7 cells were labelled in the presence of cycloheximide as follows. Approximately 2.5 x 10^8 cells were suspended in the same medium as in B. After 30 min at 33°C, cycloheximide was added to 300µg/ml and after a further 15 min [^{3}H]asparagine was added to 20µCi/ml. Cells were further incubated for 1 h. The isolation of mitochondria, electrophoresis in sodium dodecyl sulfate 12.5% polyacrylamide slab gels and fluorography have been described previously (14). Numbers to the left refer to individual bands of proteins synthesized in mitochondria and numbers on the right are the apparent molecular weights (14).

Fig. 2. Fluorographic patterns of proteins synthesized in mitochondria of CHO TK$^-$ cells or by isolated mitochondria and separated by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis. The concentration of acrylamide used in this gel was 13% compared to 12.5% in Fig. 1. A. Mitochondrial proteins of CHO TK$^-$ cells were labelled with [^{35}S]methionine as described in Fig. 1A. B. Isolated mitochondria of CHO TK$^-$ cells were labelled with [^{3}H]asparagine at a concentration of 45μCi/ml in the presence of 300μg cycloheximide/ml for 30 min at 30°C as described previously (14).

Fig. 3. Identification of [^{3}H]asparagine in mitochondrially-synthesized proteins of Asn-7 cells and mitochondrial [^{3}H]asparaginyl-tRNA of CHO TK$^-$ cell mitochondria. A. The same sample of mitochondrial protein of Asn-7 cells labelled with [^{3}H]asparagine at a nonpermissive temperature as described in Fig. 1B was hydrolyzed with proteinase K and chromatographed as described in the Materials and Methods. B. [^{3}H]-Labelled mitochondrial asparaginyl-tRNA of CHO TK$^-$ cells was isolated as described in the Materials and Methods, hydrolyzed at pH 7.4 and submitted to paper chromatography.

Fig. 4. RPC-5 chromatography of mitochondrial and cytosolic asparaginyl-tRNAs of CHO TK$^-$ cells. tRNAs were charged, isolated and chromatographed as described in the Materials and Methods. o—o, [^{3}H]Asparagine-labelled mitochondrial tRNA labelled in isolated mitochondria; •—•, [^{14}C]asparagine-labelled cytosolic tRNA labelled in whole cells.

Fig. 5. Slab gel electrophoretic analysis of mitochondrial and cytosolic asparaginyl-tRNAs. tRNAs were charged and isolated as described in the Materials and Methods and for yeast [^{3}H]leucyl-tRNA as described previously (15). The asparaginyl-tRNAs were then deaminated, resuspended in 98% formamide, brought to boiling for 2 min and cooled rapidly before applying to the gels. Electrophoresis was for 5.5h at 10 V/cm as described previously (15). The marker dyes were X, xylene cyanol FF and BB, Bromophenol Blue and the numbers refer to nucleotide length . A, Cytosolic deaminated [^{14}C]asparaginyl-tRNA: B, mitochondrial deaminated [^{3}H]asparaginyl-tRNA; C, yeast deaminated [^{3}H]leucyl-tRNA.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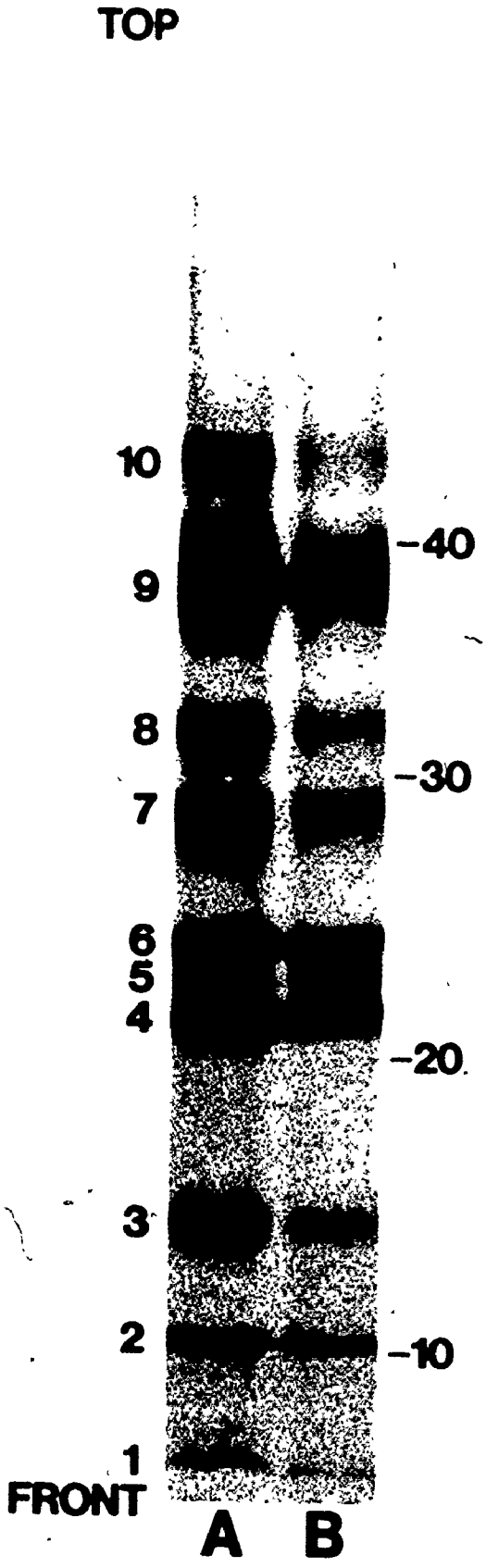
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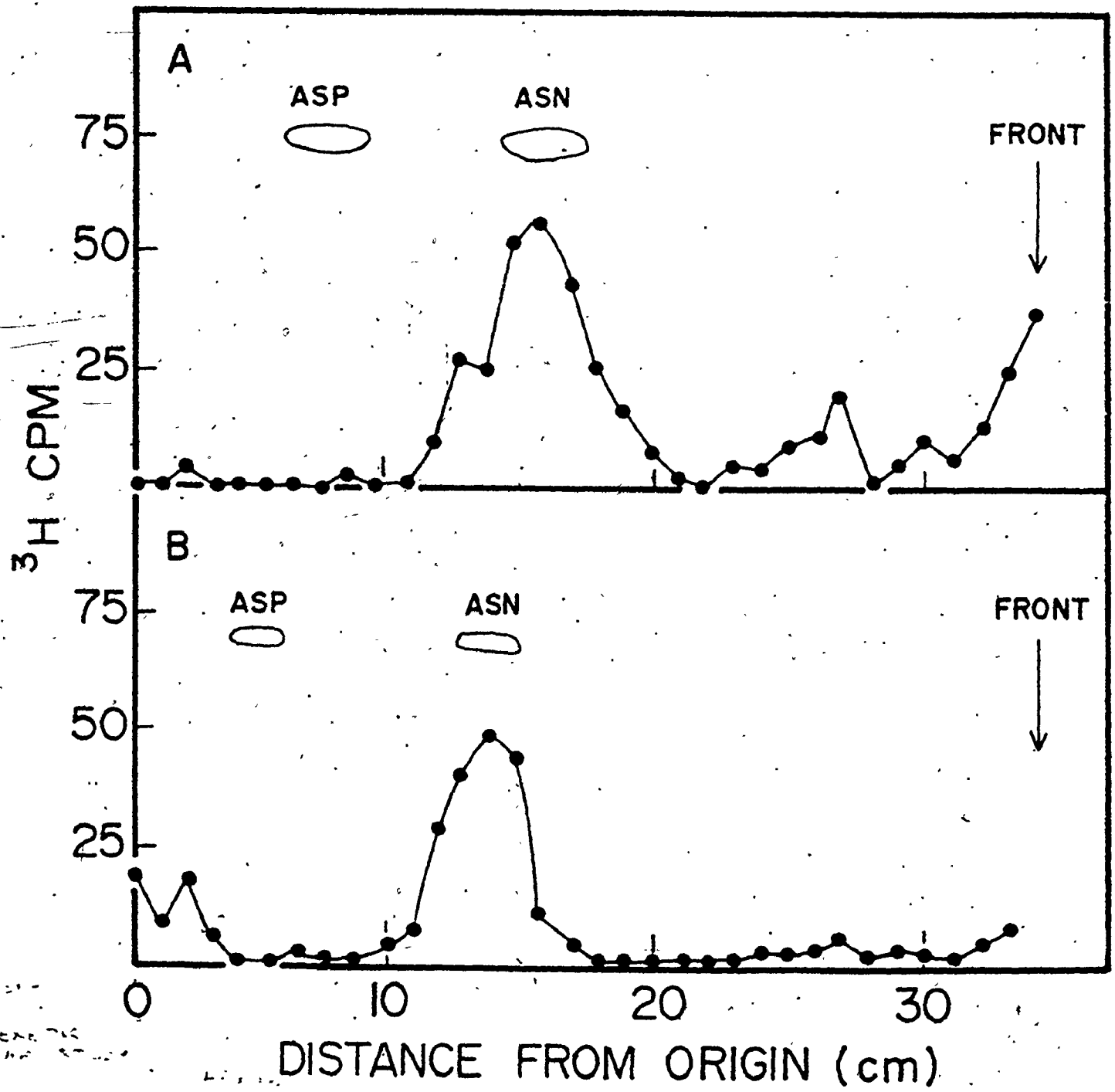
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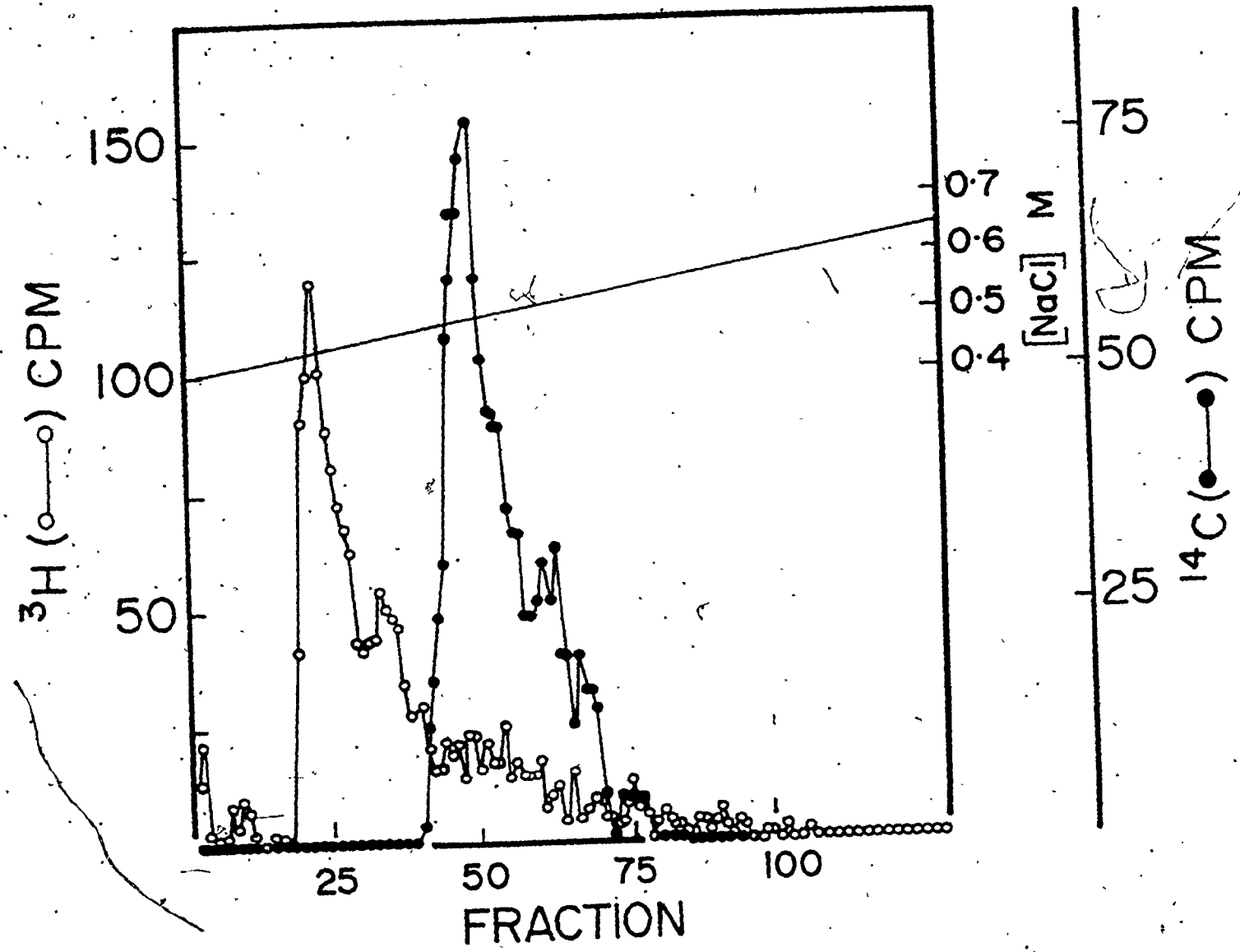

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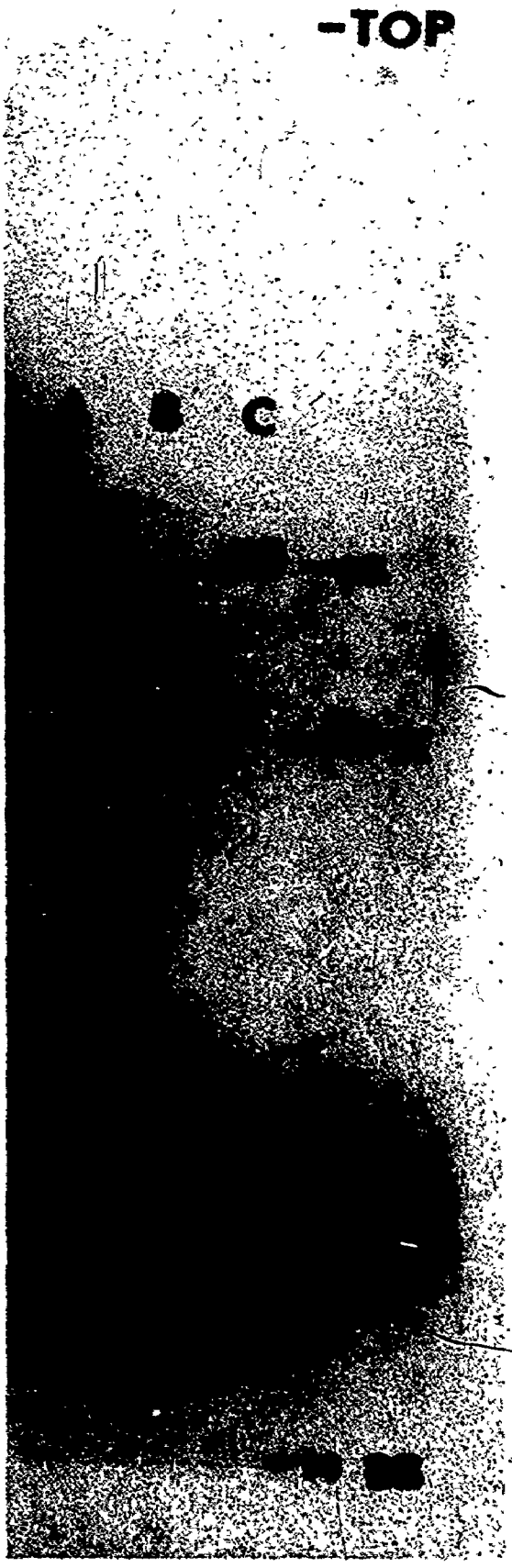
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