MAMMALIAN MITOCHONDRIAL RIBONUCLEATES

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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

March, 1971.

DOCTOR OF PHILOSOPHY (1971) McMASTER UNIVERSITY
(Molecular Biology) Hamilton, Ontario.

TITLE: Mammalian Mitochondrial Ribonucleates

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NUMBER OF PAGES: x, 180.

SCOPE AND CONTENTS: Mitochondria from mammalian cells synthesize protein. To characterize this system, high molecular weight RNA and ribosomal components from mitochondria of rat, mouse and human sources were investigated. Novel ribosomal RNA and ribosomal components were found in these organisms which are unique compared with their counterparts in the cytoplasm or bacteria in respect to their physical properties, chemical composition and conformation. The RNA has sedimentation coefficients of 15 and 13 S, electrophoretic mobilities of about 21 and 12.5 S_E and 20.5 and 11.0 S_E for rodent and human cells respectively, and a high A+U content. The mitochondrial ribosomal RNA was transcribed from the mitochondrial DNA since its synthesis was inhibited by ethidium bromide. These findings support the hypothesis that mitochondria originated from an autonomous prokaryotic ancestor which established a symbiotic relationship in primitive eukaryotic cells resulting in the contemporary eukaryotic cell in which the original prokaryotic symbiont lost part of its autonomy.

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LIST OF ABBREVIATIONS

A	=	adenylate
C	=	cytidylate
cpm	=	counts per minute
cyto.	=	cytoplasm
cyt-RNA	=	cytoplasmic ribonucleate
cyt-rRNA	=	cytoplasmic ribosomal ribonucleate
DNA	=	deoxyribonucleate
DNase	=	deoxyribonuclease
DOC	=	deoxycholate
EDTA	=	ethylene diamine tetra acetate
G	=	guanylate
g	=	gram
mRNA	=	messenger ribonucleate
Mito.	=	mitochondria
M-DNA	=	mitochondrial deoxyribonucleate
mit-RNA	=	mitochondrial ribonucleate
mit-rRNA	=	mitochondrial ribosomal ribonucleate
μ	=	micron = $1x10^{-6}$ meter
nm	=	nano meter = $1x10^{-9}$ meter
RNA	=	ribonucleate
RNase	=	ribonuclease
rRNA	=	ribosomal ribonucleate

SDS	=	sodium dodecyl sulphate
STINS	=	sodium tri-isopropyl naphthalene sulphonate
Tris	=	tris (hydroxymethyl) amino methane
tRNA	=	transfer ribonucleate
U	=	uridylate
UTP	=	uridine triphosphate
U.V.	=	ultra violet
TD RNA	=	temperature dependent RNA

ACKNOWLEDGEMENTS

I sincerely thank the following people for contributions to this thesis:

Dr. K.B. Freeman for his support, guidance and critical insight during this investigation and in preparation of this manuscript,

Dr. R.S. Mitra for skilled help in some of the experiments and for many stimulating discussions,

Dr. R.H. Hall, Dr. A.D. Dingle, Dr. L. Prevec and Dr. I.D. Spenser for useful suggestions throughout the research programme,

Mr. S. Fukamachi and Mr. J. Monahan for helpful discussions,

Mrs. Y. Bartoov for drawing the figures,

Miss. B. McFarlane for typing the manuscript.

I am deeply indebted to the Research Unit in Biochemistry, Biophysics and Molecular Biology of McMaster University and the Ontario Government for the award of fellowships covering the period of the research herein.

I INTRODUCTION

The biogenesis of mitochondria and chloroplasts has intrigued biologists since their discovery. In particular the question of the possible autonomy of these organelles in eukaryotic cells was raised almost a century ago. Altman (1890) suggested on morphological grounds that mitochondria represented modified bacteria existing as symbionts within the cell. This idea was not considered seriously until the last decade when many separate lines of investigation indicated that mitochondria are semi-autonomous organelles.

Experiments of Luck (1963, 1964, 1965)

For many years, conflicting evidence has led to suggestions that the membranous structure of mitochondria arises either *de novo*, from other membranous structures or organelles or from pre-existing mitochondria. Utilizing a choline-requiring auxotroph of *Neurospora crassa*, Luck (1963, 1964) labelled the membranes of mitochondria with [^{3}H]choline and then followed the fate of the label in mitochondria after the *Neurospora* were transferred to a culture medium containing non-radioactive choline where they grew for several generations. Electron-microscopic radioautography of isolated mitochondria showed that the distribution of radioactivity in mitochondria followed a Poisson

distribution over many generations, suggesting that newly formed mitochondria were produced by the division of pre-existing mitochondria. Similar results were obtained when this mutant was grown on a medium relatively deficient in choline (Luck 1964). Mitochondria from these cells contained less lipid than normal and therefore had a higher density. Heavy and light mitochondria were separable by isopycnic sucrose density gradient centrifugation. After transfer of the cells from a choline-poor to a choline-rich medium, the lipid content of the mitochondria increased substantially. Analysis of the mitochondrial populations before, during, and after they had reacquired their normal phospholipid content revealed the presence of only one mitochondrial population. If new mitochondria arose de novo or from other membranous structures, one would expect a population of heavy lipid-poor mitochondria and also a population of light lipid-replete mitochondria after transfer to a high-choline medium.

These experiments, however, do not indicate anything about the processes involved in the biogenesis of mitochondria, in particular they provide neither the information on where in the cell the genes for mitochondrial proteins are located nor where these proteins are synthesized. The experiments are nevertheless suggestive that mitochondria could be autonomous because their increase depends on pre-existing mitochondria. What then is the evidence for autonomy?

Cytoplasmic Inheritance

The phenomenon of cytoplasmic inheritance, which has been established for decades [reviewed by Wilkie (1964)], suggested that cytoplasmic organelles might possess a genetic system independent of the nuclear one.

The "petite" mutant in yeast and the poky mutant of Neurospora both lack respiratory ability which is expressed phenotypically in degenerate mitochondrial profiles and in the loss of cytochrome a a_3, b and c_1 (Mitchell & Mitchell, 1952; Sherman & Slonimski, 1964). The pattern of cytoplasmic inheritance of respiratory-deficient mutants in Neurospora is reflected by the phenomenon of maternal inheritance. "Male"and"female"haploid cell (conidia and protoperithecia) unite to form diploid zygotes. An important characteristic of this mating is that the cytoplasm of the male cells appears to be either physically or physiologically excluded from the zygote. The"female"ganete contributes both nucleus and cytoplasm to the zygote whereas the male contributes only the nucleus. After sporulation haploid cells are obtained. Chromosomal mutations show a typical 1:1 segregation in the haploid spores. The mitochondrial defective cells however, show maternal inheritance. When a female with a cytoplasmic mutation is crossed with a normal male, all the spores are mutants. On the other hand, a cross between a mutant male and wild-type female results in normal haploid spores, since the abnormal cytoplasm has been excluded from the zygote (Mitchell & Mitchell, 1952).

The life cycle of yeast is such that it can exist and

multiply in stable form in either the haploid or diploid state. There are two yeast mating types, α and a. Crosses between α and a haploid cells result in a diploid zygote, which may multiply as a diploid. Under certain cultural conditions diploid cells sporulate. During this process the two meiotic divisions result in the formation of four haploid spores, the tetrad. The four cells of a tetrad may be separated by microdissection and cultured individually. Thus the pattern of genetic propagation may be directly determined by analysis of the four spores. When a chromosomal mutant is crossed with a wild-type cell of opposite mating type, the diploid zygote would be normal if the mutation was recessive or would have the defect if the mutation was dominant. After sporulation, two of the cells of the tetrad would be the wild-type and two would be mutants.

When yeast cells are exposed to acriflavine, respiratory-deficient mutants (petite) can be induced. The petites are of two general types, suppressive and neutral. If a neutral petite is crossed with a wild-type strain, the diploid zygote and offspring are all normal. After sporulation all the haploid cells are also normal. Conversely, a highly suppressive petite crossed with a normal strain produces diploid cells that are almost all petites, and all cells of the tetrad are also petites (Ephrussi, 1953). Cytoplasmic petites therefore do not follow Mendelian segregation but rather depend on transfer of a cytoplasmic hereditary factor that is now thought to be mitochondrial DNA.

A formal indication that the suppressive petite character

is indeed non-nuclear was obtained by Wright & Lederberg (1957) on a strain of yeast (Var. **ellipsoideus**) where haploid cell fusion occurs without nuclear fusion. This produces heterokaryons in which nuclei of each parental strain exist together in a common cytoplasm. During subsequent vegetative growth homokaryon cells may again be produced, bearing the haploid nuclei of the original parental strains. In crosses between strains marked with wild-type and mutant nuclei, the suppressive petite character was successfully transferred from association with one genetically marked nucleus to another, an indication that the suppressive character was transferrable even where transmission of nuclear genes did not take place.

Cytoplasmic inheritance although closely linked to abnormalities in mitochondria does not directly demonstrate that it is the mitochondria that possess the cytoplasmic genetic information. The relation between cytoplasmic inheritance and the autonomous nature of mitochondria has been most conclusively established by the demonstration that mitochondria contain a unique type of DNA.

Mitochondrial DNA (M-DNA)

The presence of DNA in mitochondria of a wide variety of invertebrate and vertebrate tissue was demonstrated by electron microscopy (Nass, Nass & Afzelius, 1965), by autoradiography of cell labelled with tritiated thymidine (Guttes & Guttes, 1964; Negata, et al., 1967; Scherbaum, 1960), and perhaps most conclusively

by isolation from mitochondria of unique DNA molecules differing in buoyant density from nuclear DNA of the same species (Table 1). The difference in buoyant density implies a difference in base ratios which has been confirmed by direct analysis. Estimates of the molecular size of mitochondrial DNA depended on two techniques, measurement of the contour length of circular DNA molecules seen in the electron microscopy, and measurement of the rate of renaturation of DNA which has been denatured by exposure to high pH. As shown in Table 1 the molecular weights of mitochondrial DNA from all vertebrates so far examined group around $1x10^7$ daltons. They are circular and have a contour length of about 5 μ. On the other hand, fungi such as yeast, Neurospora, unicellular eukaryotes such as Tetrahymena, and various plants all seem to have substantially larger mitochondrial DNA molecules which are non-circular when isolated. It seems quite likely that all mitochondrial DNAs are circular but that the larger the molecule the more difficult it is to find conditions which will allow isolation without damage. It must be realized, therefore, that the molecular weight values for the larger types of mitochondrial DNA are decidedly tentative. However these data suggested that the genetic information contained by mitochondrial DNA of plants, fungi and lower eukaryotes are considerably greater than that of vertebrates and echinoderms. A large porportion of the mitochondrial genome appears to have been lost by the vertebrate line during evolutionary development. It would be of interest to know the size of mitochondrial DNA of invertebrates to see if it is the same as vertebrates.

Table 1

Physical and Chemical Properties of Mitochondrial and Nuclear DNAs from Various Organisms

Organism and Reference	Density in CsCl[1] (g/cm^3) Mito. (M)	Nuclear (N)	Tm in SSC[2] (°C) M	N	Method	G+C[3] (moles %) M	N	Shape of M-DNA[4] ( ) Avg. contour length (μ)	Estimate M.W.x10^6 (daltons)
PROTISTA									
Physarum polycephalum									
Sonensheim & Holt (1968)	1.686	1.700			A	26	41	L	37
Evans (1966)	1.686	1.700			A	26	41	OC	26
Euglena gracilis									
Edelman *et al.* (1966)	1.691	1.707			A	32	48		
Ray & Hanawalt (1965)	1.690				A	31			3
Paramecium aurelia									
Suyama & Preer (1965)	1.706	1.693			A	47	34		
Tetrahymena pyriformis									
strain GL									
Suyama (1966)	1.684	1.688			A	24	30		40
strain HSM & 611									
Parsons & Dickson (1965)	1.671	1.685			A	12	25		

Suyama (1966)	1.683	1.683			A	23	23		
strain ST									
Suyama (1966)	1.686	1.692	79.5		A	26	33		
FUNGI					B	25			
Saccharomyces cerevisiae									
Guérineau *et al.* (1968)	1.683	1.698			A	23	39	30%C	
								70%L	> 40
Avers *et al.* (1968)	1.684	1.700			A	24	41	C(5)&L	
Tewari *et al.* (1966)	1.679	1.693	75	84	A	21	34		
					B	14	36		20
					C	24	39		
Corneo *et al.* (1966)	1.685	1.700			A	25	41		
Sinclair *et al.* (1967)	1.682	1.697			A	23	38	L	10
Moustacchi *et al.* (1966)	1.683	1.699			A	23	40		
Saccharomyces carlsbergensis									
Hollenberg *et al.* (1969)	1.683	1.699	73.5		A	23	40		
					B	10		C(27)&L	
Neurospora crassa									
Luck & Reich (1964)	1.701	1.712			A	42	53	L	66

ECHINODERMATA									
Sea urchin (L. pictus)									
Piko et al. (1967;1968)	1.704	1.694	86.8	84.0	A	44	34	CC(4.45)	
					B	43	36		
OSTEICHTHYES									
Carp									
Van Bruggen et al. (1968)	1.703	1.697			A	44	38		
					B	43	36		
AMPHIBIA									
Frog (Rana pipiens)									
David (1965; 1966) and									
Wolstenholme & David									
(1967)	1.702	1.702	88	88.5	A	43	43	TC&OC(5.6)	10
					B	46	47		
Toad (Xenopus laevis)									
as for frog	1.704	1.702	87.3	88	A	45	43		
BIRDS					B	44	46	TC&OC(5.4)	10
chick									
Rabinowitz et al. (1965)	1.707	1.698			A	48	39		

Borst et al. (1967 b&c)	1.706	1.701	90.0	87.5	A	49	42		
					B	51	44	C(5.35)	11
Pigeon									
Borst et al. (1967a)	1.707	1.700			A	48	41		
Duck									
Borst et al. (1967a) and Kroon et al. (1966)	1.711	1.700			A	52	41	C(5.1)	
MAMMALS									
Rabbit									
Borst et al. (1967a)	1.703	1.701			A	44	42		
Guinea pig (liver)									
Corneo et al. (1966)	1.700	1.700			A	41	41		
Borst & Ruttenberg (1966)	1.702	1.700			A	43	41	C(5.6)	
Mouse (liver)									
Kroon et al. (1966)	1.701	1.701			A	42	42	C(5.1)	
Sinclair & Stevens (1966)	1.699	1.699			A	40	40	C(4.96)	9.5
Mouse (brain)									
Du Buy et al. (1966)	1.701	1.702			A	42	43		

Mouse (L cells)									
Nass (1968; 1969b)	1.698	1.703	84.7	87	A	39	44	C(4.7)&CD	9.1
					B	38	43		
					C	41	44		
Rat (liver)									
Schneider & Kuff (1965)	1.701	1.703	85.6	87	A	42	44		
					C	40	42		
Sinclair et al. (1967)								C(5.1)	10
Sheep (heart)									
Kroon et al. (1966)	1.703	1.704	87	87	A	44	44	C(5.4)	
					B	43	43		
Ox									
Kroon (1966)	1.702	1.704			A	43	45	C(5.3)	
Human									
Corneo et al. (1967)		1.700			A		41		
Chang liver cells									
Hudson & Vinograd (1967)	1.706	1.700			A	47	41	C	
Koch & Stokstad (1967)	1.688	1.699			A	29	40		

Leukemic leukocytes							
Clayton & Vinograd (1967; 1969)	1.700	1.689		A	41	31	C&CD
HeLa cells							
Corneo et al. (1968) and							
Radloft et al. (1967) and							
Vesco & Penman (1969)	1.707	1.700		A	48	41	C(4.81)&CD
HIGHER PLANTS [5]							
Red bean							
Wolstenholme et al. (1968)	1.707	1.693					L 119 max.
Spinach							
Wells & Birnstiel (1967)	1.695	1.692					
Sweet pea							
Wells & Birnstiel (1967)	1.695	1.692					
Sweet potato							
Suyama & Bonner (1966)	1.706	1.692					
Peanut							
Breidenbach et al. (1967)	1.716	1.705					

[1] These densities were usually obtained by using E. coli DNA (1.710 g/cm^2) as reference

[2] Tm = Midpoint of melting curve (see Marmur & Doty, 1962). SSC = 0.15 M NaCl and 0.015 M sodium citrate pH 7.0.

[3] The guanine + cytosine moles % values were calculated: (A) from the density values using the formula of Schildkraut et al. (1962), (B) from the Tm values using the formula of Marmur & Doty (1962) and (C) from direct analysis.

[4] These symbols were used for the shape of M-DNA. L = linear. C = circular, CD = circular dimer, TC = twisted circle, OC = open circle and CC = close circle

[5] The nuclear DNA of higher plants may contain up to 6% methyl-C. The replacement of C by methyl-C lowers the density of the DNA (Kirk, 1967).

The number of DNA molecules per mitochondrion is somewhat indefinite. In vertebrate liver there are on average 4 or 5 molecules per mitochondrion(Borst et al., 1967b) but in some strains of yeast the value may be considerably greater (Fukuhara, 1967). It is generally assumed that all the DNA molecules in one mitochondrion are identical. This assumption is based partly on the narrow distribution of the buoyant density of any one type of mitochondrial DNA and partly upon renaturation characteristics of alkali denatured mitochondrial DNA. Mitochondrial DNA is a double-stranded molecule with the usual Watson-Crick base pairing; the two strands are readily dissociated in alkali and the rate of completeness of renaturation can be used as a measure of the molecular complexity of the mixture. With mitochondrial DNA from Xenopus laevis there was no detectable molecular heterogeneity (David & Wolstenholme, 1968) and comparison of the kinetics of renaturation of chick liver mitochondrial DNA with those of other DNA's of various size led Borst et al. (1967b) to conclude that only one predominant species of mitochondrial DNA exists.

If this assumption is correct and therefore in vertebrates all 5 micron mitochondrial DNA molecules are identical, then the amount of genetic information contained in mitochondrial DNA must be greatly limited. Five microns corresponds to 15,000 base pairs, or a molecular weight of about 10^7 daltons. This amount of DNA could not possibly provide enough information for the formation of all mitochondrial components. In comparison, the genome of Escherichia coli contains 200 times and rat or human haploid genomes

$2x10^5$ as many base pairs. Even if all the information stored in the mitochondrial DNA codes for proteins it can code only for 5000 amino acids, or for about 30 proteins of molecular weight of 20,000 each, which is about 10% of the known mitochondrial proteins. Insufficient genetic information for complete mitochondrial autonomy exist even in organism such as yeast or Neurospora with five times more information than in vertebrates. On the basis of the limited size of mitochondrial DNA alone, we can conclude that mitochondria cannot be totally independent organelles.

At this point the question of the origin of the mitochondrial DNA arises. Is the unique type of DNA found in mitochondria replicated there, enabling the mitochondria to maintain their genetic continuity, or is this DNA a part of the nuclear genome where it is replicated and then transferred to the mitochondrial compartment for some functional purpose? Do mitochondria possess a DNA replicative apparatus? If they do, what is the nature of this replication?

Replication of Mitochondrial DNA

Many studies have provided evidence that mitochondria are able to replicate their own DNA. Electron microscopic-radioautography demonstrates that cells of protista and animals may incorporate [^{3}H]thymidine into mitochondrial DNA independent of incorporation into nuclear DNA. (Guttes & Guttes, 1964; Nagata, Shibata & Nawa, 1967; Meyer & Bis, 1966; Parsons, 1965; Stone & Miller, 1965). Preferential labelling of mitochondrial DNA compared to nuclear DNA has been demonstrated in anaerobically-grown yeast in which

mitochondria are developing in response to exposure to oxygen (Moumolou, Perrodin & Slonimski, 1968: Rabinowitz, Getz & Swift, 1968; Rabinowitz et al., 1969). Smith et al. (1968) have studied the temporal separation of mitochondrial and nuclear DNA synthesis in synchronized cultures of yeast. Mitochondrial DNA synthesis was stepwise, as was nuclear DNA synthesis, but it occurred earlier than nuclear DNA synthesis.

The demonstration that isolated mitochondria from vertebrates and invertebrates can incorporate deoxyribonucleotides into DNA (Brewer, de Vries & Rusch, 1967: Neubert, Oberdisse & Bass, 1968; Parsons & Simpson, 1967, 1968; Wintersberger, 1966, 1968) leaves little doubt that replication of mitochondrial DNA occurs within the mitochondria. This is further shown by the electron microscopic demonstration of the presence of replicating forked molecules of circular mitochondrial DNA, isolated from rat liver mitochondria (Kirschner, Wolstenholme & Gross, 1969), like the ones which had been observed by Cairns (1963) in the E. coli chromosome. It was postulated by Cairns that local unraveling of the double-stranded helix enables each strand to act as a template for DNA synthesis. A molecule in the process of replicating would therefore consist of forked double circle, the circumference of each circle have an identical length. This finding also suggests that the nature of the mitochondrial DNA replication is semiconservative but more conclusive evidence has been obtained by injecting [^{3}H]bromodeoxy-uridine into rats. Mitochondrial DNA was isolated from rat liver and shown by isopycnic sedimentation in alkaline CsCl to consist of

a light and heavy strand, the heavy strand being radioactive (Gross & Rabinowitz, 1969). Studies with [^{3}H]bromouridine have also shown that DNA synthesis in isolated mitochondria is by a semiconservative mechanism (Karol & Simpson, 1968).

DNA Polymerase

Replication of mitochondrial DNA independent of replication of nuclear DNA requires that mitochondria contain their own DNA polymerase. DNA polymerase activity has been found in purified isolated rat liver mitochondria (Parsons & Simpson, 1967) and in yeast mitochondria (Wintersberger, 1966). Solubilized and partially purified mitochondrial DNA polymerase from rat liver was shown to be different in its enzymatic properties, and behavior on DEAE-cellulose chromatography than the respective DNA polymerase from the nucleus (Kalf & Ch'ih, 1968; Meyer & Simpson, 1968). The mitochondrial and nuclear enzymes of yeast have different sedimentation properties (Iwashima & Rabinovitz, 1969). It is not known yet whether the enzyme isolated from the mitochondria is involved in the repair or the replicative synthesis of mitochondrial DNA. It is, however, of interest that rat liver mitochondrial DNA polymerase is more sensitive to the inhibiting effect of ethidium bromide than is the nuclear enzyme (Meyer & Simpson, 1969), especially in view of the fact that ethidium bromide is a very potent agent producing cytoplasmic petite mutants in yeast (Slonimski, Perrodin & Croft, 1968).

The data mentioned above indicate that mitochondria possess

the mechanism for DNA replication and, indeed, that mitochondrial DNA is replicated in the mitochondria in a semiconservative way. Is mitochondrial DNA transcribed and the RNA translated in the mitochondria?

Transcription of Mitochondrial DNA

Luck and Reich (1964) demonstrated that isolated mitochondria from Neurospora contained a DNA-dependent RNA polymerase. This has also been shown for mitochondria from a variety of tissues, for rats, pigeons and a number of transplantable tumors (Kalf, 1964; Kroon et al., 1967 and South & Mahler, 1968; Neubert, Helge & Merker, 1968). RNA polymerase activity in isolated mitochondria is characterized by its independence on the presence of four nucleoside triphosphates and its resistance to Actinomycin D unless mitochondria had been swollen (Neubert & Helge, 1965).

Mitochondrial Protein Synthesis

McLean, Cohn, Brandt & Simpson (1958) were the first to show that mitochondria of rat skeletal muscle are capable of protein synthesis independent of cytoplasmic ribosomes. They found an equivalent rate of incorporation of radioactive leucine into mitochondrial and microsomal protein, in vivo. The independence of the mitochondrial protein synthesizing system was further proved by demonstrating that isolated mitochondria from unicellular eukaryotic cells to animals and plants are capable of protein synthesis [reviewed by Roodyn & Wilkie (1968)]. Although there was

considerable debate as to whether the observed synthesis was due to mitochondria or contaminating bacteria or endoplasmic reticulum, it is now clear that mitochondria do synthesize protein (Halder & Freeman, 1969). In order to determine whether the protein synthesizing system of mitochondria is basically different from the cytoplasmic one or whether it represents a different site of protein synthesis only, an attempt was made to characterize the two systems using different inhibitors of protein synthesis. It was found that, like in bacteria, chloramphenicol inhibits protein synthesis by isolated mitochondria of rat liver (Kroon, 1963, 1965: Wheeldon & Lehninger, 1966; Ashwell & Work, 1968) of Tetrahymena pyriformis (Mager, 1960) and of yeast (Wintersberger, 1965), but does not inhibit protein synthesis by cytoplasmic ribosomes of eukaryotic cell (Bretthauser et al., 1963; Von Ehrenstin & Lipmann, 1961). In contrast, cycloheximide irhibits protein synthesis in the cytoplasmic ribosome cell sap system but has no effect on protein synthesis by isolated mitochondria (Beattie et al., 1967; Borst et al., 1967a; Loeb & Hubby, 1968), even at high concentrations (Ashwell & Work, 1968). The selective inhibition of these two drugs is not limited to studies in vitro only.

Clark-Walker & Linnane (1966) have shown that chloramphenicol inhibits the growth of yeast when cultured on nonfermentable substrates such as lactate or ethanol. When grown on glucose, however, chloramphenicol does not inhibit growth but does prevent the development of mitochondria. These yeast have the phenotype of respiratory-deficient mutants in that they have lost their

capacity for oxidative metabolism and lack cytochromes aa_3, b, and c_1. They suggested that the selective effect of chloramphenicol was on mitochondrial protein synthesis. This assumption is consistent with the observation that acriflavine which induces cytoplasmic mutation in yeast also prevents the formation of cytochrome aa_3 and b (Nagao & Sugimara, 1965). In vivo, cycloheximide has a very pronounced inhibitory effect on the incorporation of radioactive amino acids in all the cell fractions of rat liver (Beattie, 1968; Schiefer, 1969), locust flight muscle (Sebald et al., 1969), Krebs ascites tumour cells (Ashwell & Work, 1968), and Neurospora crassa (Sebald, Schwab & Bucher, 1969). However, each of the above workers showed that cycloheximide has the smallest effect on the synthesis of the least-soluble mitochondrial protein.

The selective inhibition, in vivo and in vitro, of the mitochondrial protein synthesizing system and of the cytoplasmic protein synthesizing system by chloramphenicol and cycloheximide respectively indicates that the two systems are basically different. Before considering the origin of this difference it is worth noting that the phenomenon of selective inhibition of the two protein synthesizing systems within cells by chloramphenicol and cycloheximide has been used to investigate which of the mitochondrial proteins are synthesized by the mitochondrial system and which by the cytoplasmic one. The details of this investigation are beyond the scope of this thesis and for reviews one can read "The Biogenesis of Mitochondria" by Ashwell & Work (1970). The interesting data which came from this investigation were that only 10% or less of the total mitochondrial

proteins are synthesized in the mitochondria and that they are insoluble and probably located in the cristal membranes. So far none of them has been identified with known mitochondrial proteins. These results are in agreement with the limited information in mitochondrial DNA, and again reflect the fact that mitochondria cannot be organelles with an absolute autonomy.

Nature of the Mitochondrial Protein-synthesizing System

The results from the above lines of investigation indicate that mitochondria possess DNA which they replicate and transfer to new mitochondria thus preserving their genetic continuity. The information in the DNA is not enought to give mitochondria a complete autonomy but probably is sufficient to maintain their separate genetic identity within the eukaryotic cell. In order to understand the function of mitochondrial DNA it is necessary to determine those components of mitochondria whose synthesis is directly dependent on this unique DNA. With this view in mind one should consider the fact that the mitochondrial protein synthesizing system is different from the cytoplasmic one and resembles the bacterial system as far as the inhibition of these systems by chloramphenicol but not by cycloheximide. It is known that the two well defined protein synthesizing systems, in the eukaryotic cytoplasm and in prokaryotes, operate basically on the same principle, and that both systems consist of the same type of the following major components: ribosomes containing 40% protein and 60% RNA, messenger RNA, aminoacyl transfer RNA and initiation,

propagation and termination factors. There are small differences in operation between the two systems which probably derives from the differences in specific components. The questions that now arise in respect to the mitochondrial system are: first, whether this system is also ribosomal differing in only specific details or whether it operates on a complete new principle? Second, if the first possibility is correct then does the mitochondrial system resemble the bacterial one, as indicated by the chloramphenicol inhibition, or is it unique? Third, do mitochondria synthesize the components of the protein synthesizing system or are they imported from the cytoplasm? These questions were the basis of the investigation reported here. Since there are many aspects of the protein synthesizing system that could be studied it was necessary to focus on the major components of this system: the ribosomes and ribosomal RNA (rRNA) to answer the above questions. The aim of this investigation, therefore was: 1. to find whether mitochondria contain ribosomes and rRNA. 2. If they do contain ribosomes and rRNA are these different from the cytoplasmic ones, similar to the bacterial ones or completely unique. 3. If they contain unique rRNA is this rRNA transcribed from mitochondrial or nuclear DNA.

Mitochondrial Ribosomes and rRNA

Before surveying what was known about mitochondrial ribosomes and RNA at the start of this investigation a short summary on the nature of ribosomes and their RNA from eukaryotic and prokaryotic cells, is necessary (for review: Darnell, 1968;

Click & Tint, 1967; Leaver & Key, 1970). Eukaryotic cells contain ribosomes which in monomeric form have a sedimentation constant of about 80 S. The monomeric ribosome can be separated into two subunits with sedimentation constants of 60 and 40 S. The 60 S ribosomal subunit when extracted from animal cells with cold phenol yields 28 and 5 S RNA. The 28 S RNA when heated separates into two components sedimenting at 28 and 7 S (Pene, Knight & Darnell, 1968). RNA extracted from 60 S ribosomal subunits of plant cells with cold phenol sediments at 25 S. The 40 S subunits of both plants and animals contained 18 S RNA. Prokaryotic cells contain a 70 S ribosomal monomer which can be separated into 50 and 30 S subunits. After phenol extraction the 50 S subunits yield 23 and 5 S RNAs. The 30 S subunits yield 16 S RNA. Loening (1968) showed that in animals, while the smaller rRNA component (18 S) is common to all of them and has a molecular weight of 0.7×10^6 daltons, the higher rRNA component (28 S) has evolved with each major step of chordate evolution from about 1.4×10^6 daltons in sea urchins to 1.75×10^6 in humans.

In order to investigate whether mitochondria contain ribosomes and rRNA it is necessary to know whether mitochondria contain RNA or not. During the last few years, a large number of reports have appeared on mitochondrial RNA. In the process of cell fractionation by differential centrifugation, mitochondria are isolated after nuclei but before the microsomal fraction which contains cytoplasmic ribosomes, and as such any mitochondrial RNA had previously been suspected of being due to microsomal

contamination (Novikoff, 1957). But repeated washing of the mitochondrial pellet and its treatment with ribonuclease to free it from microsomal RNA contamination have been performed by several workers to get true mitochondrial RNA value. Table 2 gives the values of RNA contents of mitochondria from various sources obtained by different authors.

The RNA content of rat liver mitochondria is less than that found for yeast, plant or tumour cell mitochondria. The minimum value of rat liver mitochondrial RNA obtained was 6.6 µg/mg mitochondrial protein by O'Brien & Kalf (1967a), representing only 1.6% of the total liver RNA based on the data that 1 g of fresh rat liver contains 11×10^{10} mitochondria, 5×10^{9} mitochondria contain 1 mg mitochondrial protein (Nass, Nass & Hennix, 1965) and on the RNA content of the albino rat liver (Davidson, 1960). If mitochondria contain ribosomes then it is possible to calculate from these data the maximum amount of ribosomes that one can expect per mitochondrion. Assuming that all the 6.6 µg of RNA found in 5×10^{9} mitochondria from rat liver is rRNA then a mitochondrion contains 132×10^{-17} g of rRNA. If the minimum molecular weight of the postulated mitochondrial rRNA is estimated to be around 10^{6} daltons then the number of ribosomes/mitochondrion = Avogadro's number $\times \frac{\text{amount of rRNA/mitochondrion}}{\text{molecular weight}} = 6 \times 10^{23} \times \frac{132 \times 10^{-17}}{10^{6}}$ = 792. This amount of ribosomes per mitochondrion is small with comparison to 10,000 ribosomes per E. coli cell with an average size of rat liver mitochondria (1.0 to 3.0 µ) calculated by Carpenter (1967) however it seems to be sufficient for detection.

Table 2

RNA Content of Mitochondria From Different Tissues

Reference	Tissue	Amount (µg/ml mitochondrial protein)
Roodyn, Reis & Work (1961)	Rat liver	14.0
Truman & Korner (1962)	"	13.8
Muntwyler, Seifter & Harkness (1950)	"	13.4
Kroon (1965)	"	13.0
Rendi (1959)	"	12.0
McLean, Cohn, Brandt & Simpson (1958)	"	10.0
Laird, Nygaard, Ris & Barton (1953)	"	9.6
Nass, Nass & Hennix (1965)	"	9.0
Siekevitz & Watson (1956)	"	7.0
O'Brien & Kalf (1967a)	"	6.6
Das & Mukherjee (1964)	Seedlings of *Vigna Sinensis*	47.0
Pollard, Stemler & Blaydes (1966)	Cauliflower	21.0
Wintersberger & Tuppy (1965)	Yeast	48.0
Freeman (1965)	Ascites cells	54.0
Nass (1969a)	L cells	38.0

Early attempts to isolate mitochondrial ribosomes from rat liver were unconvincing (Rendi, 1959). The mitochondrial ribonucleoprotein particles showed a significantly higher RNA content and rate of protein synthesis compared to those of intact mitochondria. The demonstration that chloroplasts contained ribosomes by Lyttleton (1962) encouraged further investigation. Ribosome-like particles were successfully demonstrated in electron micrographs of mitochondria for vertebrate and invertebrate organisms (André & Marinozzi, 1965; Bernhard, 1969; Luck, 1964; Swift, 1965; Swift & Adams, 1966; Swift et al., 1964; Swift, Rabinowitz & Getz, 1968; Watson & Aldridge, 1964). After suitable fixation, particles which bind uranyl ions, as do cytoplasmic ribosomes, can be seen within the mitochondrial matrix area. The staining can be abolished by prior treatment with pancreatic ribonuclease (RNase). The ribosome-like particles seen in mitochondria by electron microscopy appear to be smaller in most organisms than the 80 S ribosomes in the cytoplasm. Their size suggests that they may be more closely related to the 70 S bacterial ribosomes. The paucity of these ribosome-like particles has, however, made their isolation difficult, and the results obtained are in controversy.

Neubert (1966) failed to isolate mitochondrial rRNA from rat liver which led him to favour the idea that structures other than classical ribosomes accounted for mitochondrial protein synthesis. On the other hand Truman (1963) and Klaév (1964; 1966) isolated ribosomal particles from rat liver and muscle mitochondria and found that their sedimentation constant in sucrose density

gradients and therefore their size was not distinguishable from the cytoplasmic ribosomal monomers and subunits. Rabinowitz et al. (1966) also reported 80 S mitochondrial ribosomes and 28 and 18 S rRNA from chick embryo heart and liver and concluded that mitochondrial ribosomes are identical with the cytoplasmic ones. It should be mentioned, however, that the results obtained by Rabinowitz, Truman and Klaév could be explained on the basis of cytoplasmic ribosomal contamination as will be discussed below.

Since the presence of mitochondrial ribosomes and rRNA are uncertain, an attempt was made in this investigation to isolate and characterize the rRNA first and only then to look for the more complex ribosomes.

Isolation of Mitochondrial Ribosomes and rRNA - Problems and Possible Solutions

1. Cytoplasmic Contamination

The technique of obtaining the mitochondrial fraction is based on a method in which certain centrifugal forces are applied to a cell homogenate so that particles with the size of mitochondria are separated from other organelles. Cytoplasmic ribosomes which are attached to the endoplasmic reticular membrane will co-sediment with the mitochondrial fraction if in the process of the cell homogenization the membrane is sheared to a size similar to that of mitochondria. Also, from the fact that the mitochondrial outer membrane has most of the enzyme activities characteristic of the endoplasmic reticulum (Harris et al. 1960), the possibility arises

that in some cases the two are attached and could sediment together. These possibilities and the fact that small amounts of ribosome-like particles were seen in electron micrographs of mitochondria indicate the real danger of contamination of the mitochondrial fraction with cytoplasmic ribosomes which might mask the identification of the mitochondrial ribosomes and rRNA.

There are three methods that one can use to avoid possibility of cytoplasmic contamination: a) To develop a technique for the isolation of pure mitochondria free of cytoplasmic ribosomes and smooth and rough endoplasmic reticulum. b) To hydrolyze the cytoplasmic ribosomes with pancreatic RNase under conditions in which the enzyme will not penetrate the mitochondria and then to inactivate the enzyme before isolation of mitochondrial ribosomes or rRNA. c) To label specifically the presumed mitochondrial rRNA with a radioactive precursor. In this case the labelling of other species of RNA in the cell which might be extracted with the mitochondrial rRNA must be inhibited with a drug. Though the first method requires a long period of investigation it seems to be the only way to obtain a clear cut result, because only in this way can the nature of the mitochondrial ribosomes and rRNA be determined directly, that is from a preparation of pure mitochondria. On the other hand in the second and the third approaches the nature of mitochondrial ribosomes and rRNA is determined indirectly and there are two hazards. First, the RNase or the drug might modify the native mitochondrial ribosomes or rRNA. Second, some of the cytoplasmic contamination might not be eliminated by the drug or

the RNase treatments. Because of these open questions the results obtained from the indirect methods should be accepted only when they are in agreement with the direct one.

Rat liver was choosen as the source of mitochondria to purify extensively. Rat liver contains a large number of mitochondria and it is therefore possible to sacrifice substantial amounts of them for the sake of purifying the rest. Of the two indirect methods the second one was chosen.. There is no certain way to remove or inactivate RNase and any result could be from hydrolysis of mitochondrial or cytoplasmic RNA. On the other hand, Perry (1963) showed that low levels of Actinomycin D selectively suppressed the synthesis of nuclear rRNA. Nuclear rRNA is a precursor of cytoplasmic rRNA and the only one which would be extracted with mitochondrial rRNA in methods which extract rRNA and not mRNA. Mitochondrial RNA might be synthesized under these conditions because mitochondria are reported to be impermeable to the antibiotic (Neubert & Helge, 1965). Alternatively it could be that the amount of DNA coding for the RNA is smaller than that for cytoplasmic rRNA (Perry & Kelley, 1969), thus permitting the synthesis of mitochondrial RNA. Tissue culture cells (usual malignant in nature) were choosen for the indirect method because of their fast growth compared with the normal rat liver tissue and also because the cells would get a homogeneous exposure to the antibiotic.

2. Paucity

As discussed above, electron micrographs of mitochondria

show a small amount of ribosome-like particles and there is a small amount of RNA in the mitochondrial fraction. In order to overcome this problem mitochondrial rRNA has to be labelled with a radioactive precursor, and isolated in the presence of cold cytoplasmic rRNA carrier to give sufficient material to work with.

3. Degradation of RNA

Rahman (1966) showed that the mitochondrial fraction contains three ribonucleases. One of them is acid ribonuclease and has pH optimum at around 5.0 and the other two are alkaline ribonucleases with pH optimum at 8.0 and 9.0 respectively. De Duve et al., (1955) reported that acid ribonucleases along with a group of acid hydrolases are associated not with mitochondria but with the lysosomes which usually contaminate the mitochondrial pellet.

Since the aim of this investigation is to isolate mitochondrial rRNA it is important to remove and/or inactivate these ribonucleases in the process of isolation of the RNA. There are a few approaches that one can use to minimize the RNase activity:

a) Since for the direct study of mitochondrial rRNA the mitochondrial fraction has to be pure, as discussed above, the acid RNase which is located in the lysosomes will be removed and therefore the mitochondrial alkaline RNase could be inactive if acidic pH is used during the isolation of RNA.

b) In the process of the extraction of RNA, RNases are partially inhibited by the phenol which denatures and extracts proteins but since phenol does not completely inactivate nucleases

(Littauer & Sela, 1962; Kidson, Kirby & Ralph, 1963) it will be necessary to further denature the RNases with the detergent sodium dodecyl sulphate (SDS) or to absorb the RNases, which are basic proteins with sodium magnesium lithoflurosilicate (Macaloid) or with aluminum silicate (Bentonite) which are negatively charged (Stanley & Bock, 1965; for review Barlow & Mathias, 1966). SDS is also necessary to disrupt the mitochondrial membrane and release RNA.

c) Addition of non-labelled carrier RNA will minimize the chance of the mitochondrial RNA degradation by these RNases.

To minimize mechanical shearing, the mitochondrial RNA has to be extracted under mild conditions such as a long phenol extraction at a low temperature.

4. Isolation Methods

The most suitable method for isolation of the possible mitochondrial rRNA (mit-rRNA) seems to be method 1 of Kirby (1965), in which cytoplasmic-rRNA (cyt-rRNA) is isolated specifically without contamination of messenger RNA (mRNA) or of transfer RNA (tRNA). This method would have to be modified to be performed at 4°C rather than room temperature and to use further RNase inhibitors as discussed above. For a quick extraction method the hot phenol method of Penman (1966) can be used. In this method the possibility of contamination with DNA and mRNA that might be present in a crude mitochondrial fraction is higher because all nucleates can be extracted by it, therefore, results obtained by the hot phenol extraction should be accepted only when they are in agreement with

the cold phenol extraction method. For both methods SDS is included to disrupt mitochondrial membranes and to inhibit RNases.

Characterization of Mit-rRNA

To prove that mit-rRNA are unique species, different from cyt-rRNA, the physical and chemical properties of the RNAs must be examined. The physical properties of the presumed mit-rRNA would, in addition to the comparison with the properties of cyt-rRNA, establish whether the RNA extracted from the mitochondria is ribosomal-type RNA, that is, whether it is indeed high molecular weight RNA consisting of two species or not. In addition nucleotide or base composition is the most direct method of chemical characterization and might further distinguish the RNAs.

The following four methods will be used to determine the physical and chemical properties of mitochondrial RNA:

1. Sucrose Density Gradient Centrifugation

In this technique the RNA molecules are sedimented in a tube by a centrifugal force through a sucrose density gradient. The sedimentation constant of a molecule will depend on its size (i.e. molecular weight) and shape (i.e. secondary and tertiary structure). The larger and more compact the molecules are the faster they will sediment to the bottom of the tube. If the molecular weight of a standard RNA molecule is known and its shape is assumed to be similar under identical conditions, to the tested one then the molecular weight of the latter can be determined.

Two different types of sucrose density gradients can be employed: a linear gradient like the one used by Gilbert (1963) and exponential gradient like the one used by Noll (1967). In the exponential gradient, but not in the linear one sedimentation is isokinetic along the tube and the sedimentation constant (S) of the molecules are linearly related to their sedimentation distance (X):

$$S = K_1X \qquad (1)$$

Gierer (1958) and Spirin (1961) have demonstrated that a logarithmic plot of the molecular weight (M) versus the sedimentation coefficient of the molecules resulted in an empirical linear relationship:

$$M = K_2S^{\alpha} \qquad (2)$$

Thus from (1) and (2) it is clear that the logarithmic plot of M versus X will result also in linear relationship:

$$M = K_3X^{\alpha} \qquad (3)$$

This phenomenon makes the exponential gradient a useful tool for determining the sedimentation constant and the molecular weight of an unknown RNA molecule compared with a standard one via interpolation, assuming that these RNAs have a similar conformation in solution.

2. Agarose-polyacrylamide Gel Electrophoresis

Loening (1967) showed that electrophoresis in a polyacrylamide gel of low concentration (2.0 to 2.5%) can be used to separate the two types of cyt-rRNA from different organism. In this technique the negatively charged RNA molecules are forced to migrate through the pores of the gel toward the anode in an electrical field. The

migration rate of the molecules in the gel as in the sucrose density gradient technique will depend on their size and shape but with an opposite effect. The smaller the molecule and the more compact it is the faster it will migrate through the gel pores. When compared under the same condition with standard RNA molecules whose molecular weight is known and assuming a similar shape the molecular weight can be determined. Because the amount of the labelled mit-rRNA will probably be very small its migration profile can not be detected by measuring its U.V. absorption at 260 nm with a densitometer. Instead the gel has to be sliced and the radioactivity of each slice determined. But, since the low concentration of the polyacrylamide gel employed by Loening (1967) is too soft for slicing, agarose has to be added to the gel to harden it. The addition of agarose does not effect the separation properties of the polyacrylamide gel (Peacock & Dingman, 1968). The sedimentation constants and the logarithm of the molecular weight of the RNA molecules is linearly related to the distance migrated (Loening & Ingle, 1967; Loening, 1968).

3. Chromatography on Methylated Albumin Kieselguhr (MAK) Column

RNA can be absorbed to a MAK column and then eluted with increasing concentration of NaCl solution. The chromatographic properties of the molecules on MAK column is dependent on the size, chemical composition (G+C content) and the shape of the molecules. The larger the molecules, the lower their G+C content is and the more unfolded they are, the stronger they will absorb to the column

requiring a higher concentration of NaCl for elution (Ellem, 1966; Hershey & Burgi, 1960; Mandell & Hershey, 1960; Sevoka & Cheng, 1962). Thus, tRNA elutes before rRNA (Osawa & Sibatani, 1967); the smaller rRNA component of both bacteria and mammals elutes slightly before, but in the latter case not separated from, the larger rRNA (Osawa & Sibatani, 1967; Ellem, 1966); bacterial rRNA elutes slightly before, but not separated from mammalian rRNA (Osawa & Sibatani, 1967). The effect of base composition and secondary structure have also been examined. Several classes of rapidly-labelled RNA from mammalian cells have been characterized according to their pattern of elution from MAK column. A possible rRNA precursor (Q_1) and DNA-like RNA precursor (Q_2) elute with NaCl solution after rRNA (Yoshikawa et al., 1964). These species are found only in the nucleus (Billing & Barbiroli, 1970) but in addition the nucleus and the cytoplasm contain DNA-like RNA (TD RNA) which can be eluted only at higher temperatures (Ellem & Sheridan, 1964) or with SDS (Ellem, 1966). This RNA has a G+C content of about 50% (i.e. DNA-like) compared to an average of 65% for rRNA (Ellem & Sheridan, 1964). The properties of the TD RNA resemble those of polyribosome-associated mRNA of mammalian cells (Penman, Vesco & Penman, 1968). It is rapidly labelled RNA, has a G+C content of about 50% and a sedimentation rate of 16-18 S and its synthesis is resistant to low concentration of Actinomycin D (Ellem, 1966). Since DNA-like RNA has both a lower G+C content and probably a more open structure than rRNA, it is not possible to distinguish the extent to which each of these characteristics accounts for its

tighter binding (Ellem & Rhode, 1969). These findings indicate that the basic binding to MAK columns is due to ionic forces, as the negatively charged phosphorous backbone of the RNA molecules bind to the positively charged methylated albumin molecules (Mandell & Hershey, 1960), but that hydrophobic interactions involving the bases are probably also important (Ellem & Rhode, 1969).

4. Nucleotide Composition

If the physical characteristic of the presumed mit-rRNA will be unique in comparison with the cytoplasmic one, there will be still the question whether the RNA extracted from mitochondria is a cytoplasmic RNA contaminant and its unique physical properties arise from physical changes, perhaps degradation or aggregation, that took place during extraction. In order to examine this possibility the nucleotide composition of the RNA should be determined. Nucleotide composition of RNA can be determined by the following methods: paper chromatography (Lane, 1963), paper electrophoresis (Davidson & Smellie, 1952) and by elution from Dowex 50-H^+ column (Katz & Comb, 1963).

Results obtained from all the different characterization methods will indicate whether mitochondria contain a unique rRNA or not, and will yield an estimation of its molecular weight and shape compared with the cyt-rRNA.

Characterization of Mitochondrial Ribosomes and Ribosomal Subunits

In order to find whether mitochondria possess a unique class of ribosomes and ribosomal subunits, the latter will be characterized by determining their sedimentation constants on isokinetic sucrose density gradient compared with the cytoplasmic ones.

The Origin of Mit-rRNA

In order to determine whether mit-rRNA is transcribed from mitochondrial DNA or from the nuclear DNA, hybridization studies can be made between mit-rRNA and the two DNAs. A simpler approach would be to use an inhibitor which would act specifically on mitochondrial DNA or on the mitochondrial RNA polymerase. Ethidium bromide at concentrations less than 5.4 µg/ml has a greater affinity to the mitochondrial circular DNA than to the nuclear DNA (Bauer & Vinograd, 1968). It also inhibits the rat liver mitochondrial DNA polymerase more than the nuclear DNA polymerase in vitro (Meyer & Simpson, 1969). A third possibility is to investigate whether isolated mitochondria are able to synthesize mit-rRNA and to see whether this synthesis is DNA-dependent and inhibited by ethidium bromide.

II METHOD AND MATERIALS

1. Growth of Tissue Culture Cells

a. Mouse L cells derived from Earle's original fibroblast line (Sanford, Earle & Likely, 1948), were grown in suspension in Joklik's modification of minimum essential medium (Eagle, 1959) with 5% (v/v) foetal calf serum and 1% (v/v) anti-PPLO (mycoplasma) agent.

b. Human KB cells were originally derived from an epithelial cheek carcinoma (Eagle, 1965). The established line was obtained by Dr. S. Mak from Dr. M. Green, St. Louis, Missouri. Cells were grown in suspension in Joklik's modification of minimum essential medium (Eagle, 1959) with 5% (v/v) horse serum.

c. Rat hepatoma cells, derived from a solid hepatoma (Thompson, Tomkins & Curran, 1966) were originally grown in monolayer culture in Swim's medium but were adapted to grow in suspension in Joklik's modification of minimum essential medium (Eagle, 1959) with 5% (v/v) foetal calf serum and 1% (v/v) anti-PPLO agent.

2. Isolation of Mitochondria

a. Isolation of rat liver mitochondria

The mitochondrial fraction was prepared from livers of hooded rats with a technique designed to eliminate as much of the endoplasmic reticulum and cytoplasmic ribosomes as possible. It is similar to the method of O'Brien & Kalf (1967a). Livers were

homogenized with a Potter teflon homogenizer in 8 volumes of ice cold 0.34 M sucrose, 2 mM tris-HCl, 2 mM EDTA, pH 7.4 (medium A). Nuclei and cell debris were centrifuged at 1,000 x g for 10 minutes at 2°C and washed with 4 volumes of medium A. The combined nuclear supernatant fractions were centrifuged at 1,500 x g for 10 minutes at 2°C. The nuclear supernatant fraction was layered over a 20 to 35% sucrose density gradient in 2 mM tris-HCl, 2 mM EDTA, pH 7.4 which filled 40% of the tube's volume for the SW 25.1, SW 25.2 or SW 27 rotors of the Beckman Model L2 ultracentrifuge. The gradient was formed over a 40% sucrose, 2 mM tris-HCl, 2 mM EDTA, pH 7.4, layer which occupied 15% of the tube's volume. The mitochondrial pellet was recovered by centrifugation for 2 hours at 25,000 x g_{av}. The pellet was washed 4 times with medium A by centrifugation at 6,500 x g for 10 minutes. The fluffy layer was decanted in all cases and the surface of the pellet washed twice by inversion before resuspension of the mitochondrial fraction.

b. Isolation of L cell mitochondria

Mitochondria were prepared from L cells disrupted with an Ultra-Turrax homogenizer essentially as described by Freeman (1965). About $1.0-2\times10^9$ cells were washed twice with 0.3 M sucrose, 2 mM EDTA, 2 mM tris-HCl, pH 7.4 (medium B) suspended in 10 ml of medium B and disrupted by homogenization for 90 seconds (30 seconds intervals) at 70 volts. KCl was added to a final concentration of 0.02 M. Nuclei and cell debris were removed by two centrifugations at 1,000 x g for 15 minutes at 2°C. The mitochondrial fraction was centrifuged at 6,500 x g for 15 minutes and washed 3 times with

medium B. The crude mitochondrial fraction was in some cases purified further by isopycnic gradient centrifugation (Freeman, 1965) for 3 hours at 165,000 x g_{av} on a linear 0.9-1.8 M sucrose density gradient in 2 mM tris-HCl, 2 mM EDTA, pH 7.4 using the SW 41 rotor of the Beckman Model L2 ultracentrifuge. The bulk of the fraction was recovered as a band at about 1.35 M sucrose and was designated as the mitochondrial fraction (Freeman, 1965). In addition nuclei and nuclear fragments sedimented to the bottom of the tube and an unidentified band was observed above the mitochondrial band.

c. Isolation of KB cell mitochondria

About 1.7×10^8 cells were washed twice with medium B, suspended in 10 ml of this medium and disrupted by homogenization with an Ultra-Turrax homogenizer for 55 seconds at 70 volts. Mitochondria were isolated as described for those of L cells.

d. Isolation of rat hepatoma mitochondria

About 1.6×10^8 cells were washed thrice with 0.25 M sucrose, 2 mM EDTA, pH 7.4 (medium C). Cells were kept in suspension for 10 minutes in between each centrifugation in order to enable the cells to swell. The cells were suspended in 5 ml of medium C and disrupted by homogenization with an Ultra-Turrax for 90 seconds (30 seconds intervals) at 75 volts. Mitochondria were isolated as described for those of L cells.

3. Electron Microscopy of the Isolated Rat Liver Mitochondria

The isolated mitochondria were negatively stained according to the method of Parsons (1967). The surface-spreading method was

not used. Instead formvar-coated grids were allowed to float on top of a drop of mitochondrial suspension in isolation medium (5 mg protein/ml) for 1 minute. The excess of the mitochondrial suspension was removed with a filter paper and the grid allowed to float on top of a 2% (w/v) potassium phosphotungstate solution, pH 6.2, for 2 minutes. The excess phosphotungstate solution was removed with a filter paper and the grid was allowed to dry. Specimens were examined in a Philips Electron Microscope Model 300.

4. Preparation of Mitochondrial-Ribosomal Fraction

a. Preparation of rat liver mitochondrial-ribosomal fraction

The mitochondrial fraction obtained from 15 g of rat liver containing about 100 mg protein was suspended in 6 ml of 10 mM tris-HCl, 50 mM KCl, 6 mM $MgCl_2$, pH 7.6 (TKM buffer) made 0.5% (w/v) with sodium deoxycholate (DOC) and 0.5% (v/v) with Triton X100. After 30 minutes at 0°C the DOC-Triton treated sample was centrifuged at 12,000 x g for 10 minutes. The supernatant containing the crude mitochondrial-ribosomal fraction was layered on 15-30% conver sucrose-TKM gradients and centrifuged at 165,000 x g_{av} in the SW 41 rotor of the Beckman Model L2 ultracentrifuge for 3 hours at 2°C. The gradients were fractionated and the ribosomal peak was collected and dialyzed for 4 hours against 50 ml of TKM buffer at 4°C. The dialyzed ribosomal fraction was layered on an identical sucrose density gradient for sedimentation analysis.

b. Preparation of L cell mitochondrial-ribosomal fraction

The mitochondrial fraction obtained from 1.6×10^8 cells

containing about 3 mg protein was washed twice with medium B and finally with 0.3 M sucrose in TEN buffer. The washed mitochondrial pellet was suspended in 1.0 ml of TEN buffer and was made 0.5% (w/v) with DOC and 0.5% (v/v) with Triton X100. After a 30 minute incubation at 0°C, the sample was centrifuged at 12,000 x g for 10 minutes. The supernatant containing the mitochondrial-ribosomal fraction was analysed by layering on a sucrose density gradient as described above except for the dialysis and subsequent centrifugation.

c. Preparation of L cell cytoplasmic-ribosomal fraction

An L cell cytoplasmic ribosomal fraction was prepared according to the method outlined by Perry and Kelley (1968). Cells growing exponentially were rapidly chilled by the addition of crushed, frozen saline solution consisting of 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na_2HPO_4, 0.02% (w/v) KH_2PO_4 pH 7.4 (PBS), centrifuged at 100 x g for 10 minutes followed by another two washings with PBS. To a pellet of 5×10^7 cells 1 ml of hypotonic medium containing 2 mM $MgCl_2$, 150 mM $(NH_4)Cl$, 0.05% (v/v) Triton X100, 10 mM tris-HCl pH 7.8 was added, incubated for 10 minutes and then homogenized with 10 strokes in a Dounce homogenizer. The homogenate was centrifuged at 27,000 x g for 10 minutes at 2°C. The sediment containing nuclei, mitochondria and cell debris was discarded and the supernatant fraction was analysed on a sucrose density gradient.

5. Labelling of RNA and Ribosomes

a. Mitochondrial RNA

Mitochondrial RNA of rat liver was labelled by the

intraperitoneal injection of four 100 g rats with total of 10 mCi of $^{32}P_i$ neutralized with tris or 5 mCi of [5-^{3}H]orotic acid 48 hours before the rats were killed. During that period the rats were starved. The mitochondrial RNA of the different tissue culture lines was labelled using a 10 times concentrated cell suspension (about 2-4x10^{6} cells/ml). About 40 ml of cells were labelled with 10 µCi [5-^{3}H]uridine, 1 µCi [2-^{14}C]uridine or 50 µCi of $^{32}P_i$/ml. In the last case 0.0001 M instead of the usual 0.01 M NaH_2PO_4 normally present in the Joklik's modified minimum essential medium (Eagle, 1959) was used. All labelling of the different tissue culture lines was done for 4 hours in the presence of 0.1 µg Actinomycin D/ml to prevent the synthesis of cytoplasmic ribosomal-RNA (Perry, 1963).

b. Cytoplasmic RNA

Rat liver cytoplasmic RNA was labelled by intraperitoneal injection of a 150 g rat with 2 µCi of [6-^{14}C]orotic acid for 20 hours before the rat was killed. Cytoplasmic RNA of L cells was labelled with 0.2 µCi/ml of [2-^{14}C]uridine at a concentration of 3.0x10^{5} cells/ml for 20 hours.

c. Labelling of ribosomes

Mitochondrial and cytoplasmic ribosomes were labelled as described for the labelling of their corresponding RNA.

6. Extraction of Ribonucleates

a. Extraction of mitochondrial ribosomal RNA (Mit-rRNA)

i. Rat liver mit-rRNA was extracted by Method 1 of Firby (1965) modified in three ways. In the first modification, 2 g rat

liver was extracted for 1 hour at 25°C with 20 ml of 0.5% (w/v) of disodium naphthalene-1,5-disulphonate and 20 ml of phenol-cresol mixture (300 g of fresh distilled phenol, 42 ml of fresh distilled m-cresol, 33 ml of double-distilled water and 0.3 g of 8-hydroxy quinoline). The aqueous layer was added to 1 volume of phenol-cresol mixture made 8% (w/v) with sodium tri-isopropyl naphthalene sulphonate (STINS), stirred and then the emulsion was added to the rat-liver mitochondria which had been suspended in 2 ml of medium A. Stirring was continued for 1 hour at 25°C. The aqueous phase was recovered and re-extracted with 0.5 volumes of phenol-cresol mixture for 30 minutes at 25°C. Cytoplasmic ribosomal RNA (cyt-rRNA) and mit-rRNA were sedimented by making the aqueous layer 3% (w/v) with NaCl, 10% (v/v) with m-cresol and 20% (w/v) with sodium benzoate with the increase in volume neglected. The solution was kept for 1 hour at 0°C and centrifuged at 10,000 x g for 10 minutes to recover the gelatinous pellet of RNA. The RNA was washed twice with a cold solution of 3% (w/v) NaCl, 10% (v/v) m-cresol and 20% (w/v) sodium benzoate, once with 1% (w/v) NaCl in 75% (v/v) ethanol, once with 75% (v/v) ethanol and twice with absolute ethanol, dried under vacuum overnight then stored at -20°C. The yield of RNA was 10 mg. In one experiment mitochondrial RNA was isolated by this method in the absence of carrier cytoplasmic RNA from mitochondria isolated from 100 rats.

In the second modification, the mitochondrial pellet was suspended in 2 ml of medium A and the mitochondria were disrupted by the addition of 5 ml of a solution at 25°C of 2% (w/v) SDS,

0.1% (v/v) Macaloid, 1% (v/v) NaCl, 10 mM $MgCl_2$ and 25 mM sodium acetate, pH 4.8 (medium D), which contained 1 mg rat liver cyt-rRNA/ml. After 1 minute, 1 volume of phenol-cresol mixture, made 5% (w/v) with STINS, was added and the mixture stirred for 1 hour at 25°C. The aqueous layer was re-extracted once with 0.5 volume of phenol-cresol mixture and the RNA was recovered as described above. These methods will be referred to as modification 1 and 2 of the Kirby method.

In the third modification the mitochondrial pellet was suspended in 10 ml of medium A at 0°C then disrupted with 10 ml of medium D at 25°C, containing 1 mg of L cell cyt-rRNA. After 1 minute, 1 volume of phenol-cresol mixture, made 8% (w/v) with STINS, was added and the mixture stirred for 1 hour at 4°C. The aqueous layer was re-extracted for 1 hour with 1 volume of phenol-cresol mixture at 4°C. The aqueous layer was made 3% (w/v) with NaCl, 20% (v/v) with sodium benzoate and m-cresol was added until turbidity occured [about 30% (v/v)]. After 1 hour at 0°C the first washing solution was added dropwise until the turbidity just disappeared. On centrifugation a gelatinous pellet of RNA was recovered. This was washed and dried as described above. This method will be referred to as the cold phenol-SDS technique.

To prepare Macaloid for medium D the following procedure was used. Twenty five g of Macaloid were suspended vigorously in 1500 ml of 0.01 M tris-Hcl, pH 7.0 (tris buffer) using a Waring blender. The suspension was centrifuged at 16,000 x g for 30 minutes. The pellet was resuspended in 1500 ml of tris buffer

followed by another centrifugation as above. The final pellet of Macaloid was suspended in tris buffer to a concentration of 0.9% (v/v) and was stored at 25°C.

ii. Tissue culture mitochondrial RNA was also extracted from about $2x10^8$ cells by the cold phenol-SDS technique. The mitochondrial pellet was suspended in 2 ml of the mitochondrial isolation medium (medium B or C) and 0.3 ml of freshly prepared post-mitochondrial supernatant from $1.1x10^7$ L cells was added. The mitochondria were then disrupted with 5 ml of medium D and immediately added to 12 ml phenol-cresol mixture made 8% (v/v) with STDS to which 5 ml of 0.5% (v/v) of disodium naphthalene-1,5-disulphonate was previously added. RNA (about 1 mg) was isolated as described above for the cold phenol-SDS technique.

RNA was also extracted from the mitochondrial fraction from $2x10^9$ tissue culture cells by the method of Penman (1966). The mitochondrial pellet was suspended in 1 ml of a high strength buffer containing 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M tris-HCl, pH 7.4. Post-mitochondrial supernatant from $2x10^7$ cells was added. Mitochondria were disrupted by the addition of SDS to a final concentration of 0.5% (v/v) and EDTA, pH 7.4, to 0.1 M in a total volume of 1.3 ml. One volume of water saturated phenol was added and heated to 60°C for 2 minutes. After cooling to room temperature an equal volume of chloroform containing 1% (v/v) isoamyl alcohol was added followed by heating again to 60°C for 2 minutes. After centrifugation at 1,000 x g for 5 minutes, the lower chloroform-phenol layer was removed. A total of three extractions with

chloroform-isoamyl alcohol at 60°C for 2 minutes were made. The aqueous layer was removed and the RNA (about 0.5 to 1.0 mg) was sedimented overnight by the addition of 2 volumes of 95% (v/v) ethanol at -20°C. This method involved a hot phenol-SDS extraction and will be so designated.

For use this RNA is centrifuged and dissolved in RSB buffer to a concentration of 1 mg/ml. The RNA could be reprecipitated with 2 volumes of ethanol and stored at -20°C. In some experiments the RNA was treated with RNase-free DNase (10 µg/ml).

iii. RNA was also extracted from the mitochondrial-ribosomal fraction of the sucrose gradient. Mitochondrial ribosomes were disrupted in the presence of L cell cyt-rRNA as carrier by the addition of 1 volume of medium D as described in the cold phenol-SDS technique. It was important to add to the phenol-cresol mixture a volume of 0.5% (w/v) disodium-naphthalene-1,5-disulphonate equal to the volume of the sucrose gradient fraction. This diluted the sucrose concentration in the aqueous layer and avoided a reversal of the phases. The mit-rRNA was isolated according to the cold phenol-SDS method.

b. Extraction of cytoplasmic ribosomal RNA (cyt-rRNA)

Rat liver cyt-rRNA was isolated by Method 1 of Kirby (1965), and stored as a dried powder at -20°C. Cyt-rRNA from L cells was isolated from a post-mitochondrial supernatant fraction. The RNA was extracted first with an equal volume of phenol-cresol mixture, re-extracted with 0.5 volume of phenol-cresol mixture made 5% (w/v) with SDS and the RNA precipitated, washed and

dried as described in the cold phenol-SDS method.

7. Analysis of RNA

a. Sucrose density gradient centrifugation

Sucrose density gradients from 15 to 40% sucrose in 0.1 M sodium acetate pH 6.0 were prepared as described by Noll (1967). This is a convex gradient which gives isokinetic sedimentation. As shown in Figures 1 and 2 the sedimentation constants of cyt-RNAs are linearly related to their distance moved from the meniscus and the logarithm of their molecular weight is linearly related to the logarithm of their distance moved from the meniscus respectively. This phenomenon simplifies the calculation of these characteristics for an unknown RNA. The gradient was prepared with 10 ml of 15% sucrose solution in the mixing chamber and 12 ml of 40% sucrose solution added dropwise at a rate of 1 ml/minute with mixing. From 100 to 400 µg of RNA dissolved in 0.1 M sodium acetate, pH 6.0 was layered on the gradient, centrifuged for 11 hours at 2°C at 180,000 x g_{av} in the SB 283 rotor of the International B-60 ultracentrifuge. Alternatively, linear sucrose density gradients from 15 to 30% sucrose in 50 mM NaCl, 1.5 mM $MgCl_2$, 10 mM tris-HCl, pH 7.4 (RSB buffer) made to 0.25% (w/v) with SDS, were used as described by Gilbert (1963). Centrifugation was for 16 hours at 20°C at 75,000 x g_{av}. The gradients were fractionated by puncturing the bottom of the tube and 0.2 ml fractions were collected dropwise. The optical density at 260 nm of each fraction was read in a 5 mm path length cuvet containing 0.3 ml and the radioactivity

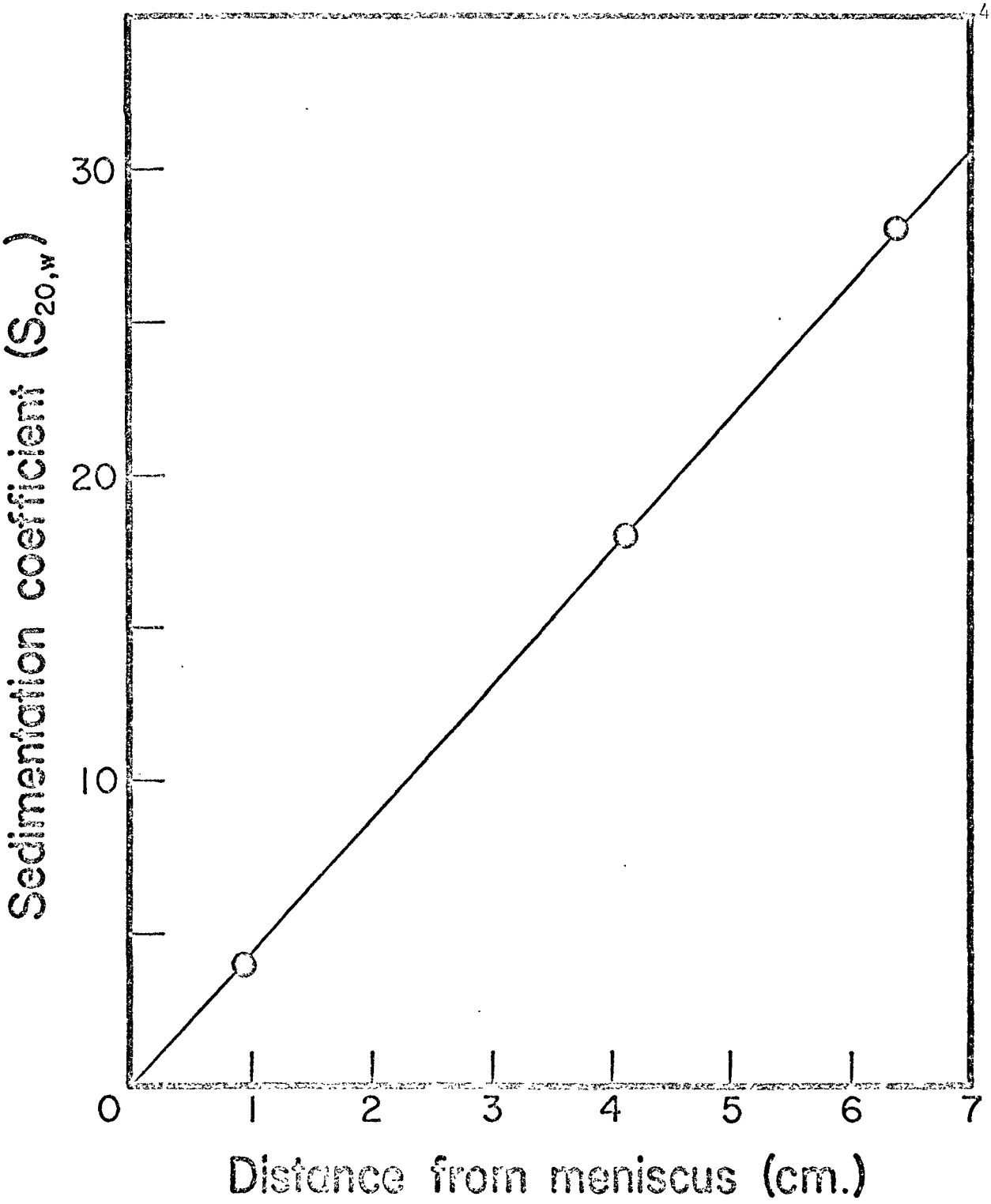

Figure 1. Relation between sedimentation constant and distance moved of rat liver cyt-RNAs in convex sucrose density gradient. A mixture of 200 µg of rat liver cyt-rRNA and tRNA were separated on a 15 to 40% convex sucrose density gradient by centrifugation for 11 hours at 2°C at 180,000 x g_{av} in the SB 283 rotor of the International B-60 ultracentrifuge. The values 28, 18 and 4 S were used for the sedimentation constant of the cyt-rRNA large and small components and tRNA respectively (Kirby, 1965).

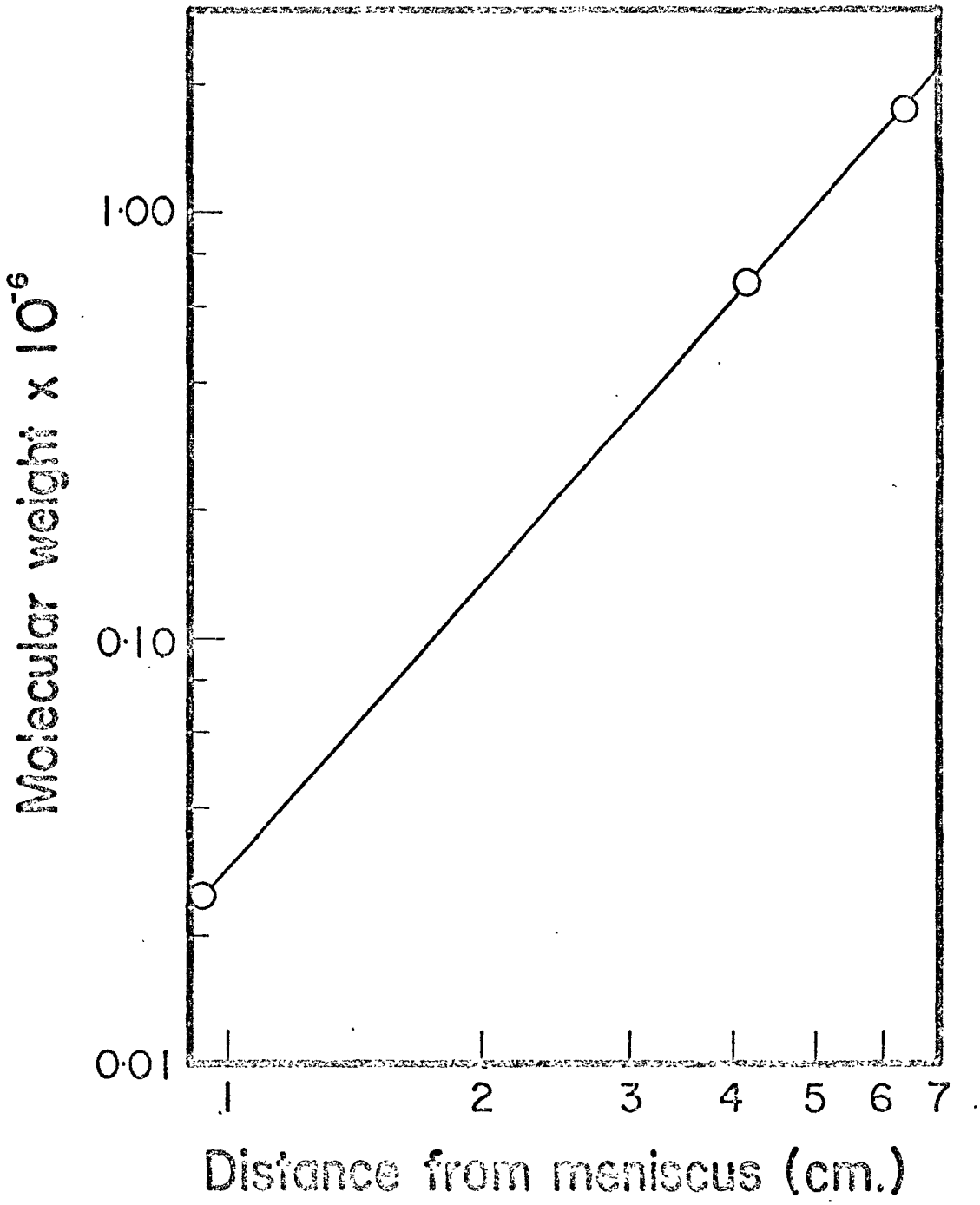

Figure 2. Relation between molecular weight and sedimentation distance of rat liver cyt-RNAs in convex sucrose gradient.
A mixture of 200 µg of rat liver cyt-rRNA and tRNA were separated on 15 to 40% convex sucrose gradient as described for Figure 1. The values 1.75×10^6, 0.7×10^6 and 2.5×10^4 daltons were used for the molecular weight of the cyt-rRNA large and small components and tRNA respectively (Loening, 1968).

was determined.

b. Chromatography on methylated serum albumin-coated kieselguhr (MAK) columns

Mitochondrial RNA, isolated from $8x10^8$ L cells by the cold phenol-SDS technique, was analyzed on a MAK column according to Osawa and Sibatani (1967). Hyflo supercel was washed with 0.1 N HCl, H_2O until neutral, 0.1 N NaOH, and again with H_2O until neutral and dried. Methylated albumin was prepared by dissolving 5 g of bovine serum albumin fraction V in 500 ml of absolute methanol to which 4.2 ml of 12 N HCl was added. The solution was kept in the dark for 3 days at 25°C and was shaken from time to time. The methylated albumin sediment was washed twice with absolute methanol and twice with peroxide-free ether (distilled over ferrosulphate), dried and stored at -20°C.

Thirty g of washed Hyflo supercel was suspended in 150 ml of 0.1 M buffered saline (0.1 M NaCl, 0.05 M sodium phosphate, pH 6.7) boiled for 1 minute then cooled to 35°C. Ten ml of 1% (w/v) methylated albumin was added, stirred and then poured quickly to make a 160 x 22 mm column which was jacketed and heated to 35°C. After the MAK settled down a layer of 0.5 cm of Hyflo supercel suspended in 0.1 M buffered saline was added, to serve as a mechanical barrier to the working portion of the column. The bed volume of the column was about 60 ml and the liquid displacement volume was about 54 ml. The column was washed with 100 ml of 0.1 M buffered saline then loaded with about 4 mg of RNA dissolved in 40 ml of 0.1 M buffered saline. Reservoirs of 400 ml of 1.2 M NaCl 0.05 M sodium phosphate, pH 6.7 and 800 ml of 0.1 M NaCl, 0.05 M sodium

phosphate, pH 6.7 were incubated at 35°C and connected to a Phoenix varipump Model 4000 which pumped a linear salt gradient through the column at a rate of 1 ml/minute. Three ml fractions were collected, the optical density at 254 mμ was recorded continuously by an ISCO UV analyzer Model 222 and an ISCO chart recorder Model 170. Fractions were checked for NaCl concentration with a conductivity bridge, Model 31 (Yellow Springs Instrument Co.) and counted for radioactivity.

c. Agarose-polyacrylamide gel electrophoresis

The gel electrophoresis method was a modification of the methods of Watanabe, Prevec & Graham (1967) and Peacock & Dingman (1968). To assure penetration of the cyt-rRNA into the polyacrylamide gel, the acrylamide and the methylenebisacrylamide have to be of the highest purity grade possible (Loering, 1967). A minimal amount of agarose was added to allow slicing at room temperature but not affect the separation properties of the polyacrylamide. For this the agarose must solidify first (Peacock & Dingman, 1968). Not every lot of agarose was suitable for this purpose since some lots contained impurities which bind the cyt-rRNA to the top of the gel. SDS was added to the ingredients of the gel and to the running buffer in order to inactivate possible RNase. A solution containing 4.18% (w/v) acrylamide, 0.22% (w/v) methylenebisacrylamide, 0.05% (v/v) N,N,N',N'-tetramethylene diamine and 1% (w/v) SDS in 2 times concentrated reservoir buffer was kept at 0°C for 30 minutes. Ammonium persulphate was added to 0.1% (w/v) and the solution mixed with an equal volume of a boiling solution of 0.5% (w/v)

agarose. The solution was poured into plugged electrophoresis tubes, 6 x 65 mm, allowed to solidify for 3 hours and used immediately. The plug was removed and about 30 µg of an RNA solution in 50 µl of RSB buffer or in 0.01 or 0.1 M sodium acetate, pH 6.0, was applied to the previous bottom surface of the gel. No difference in electrophoretic mobility was seen when the RNA was dissolved in any of these buffers. [^{14}C]cyt-rRNA was often run in the same tube as [^{3}H]mit-RNA to serve as a marker.

RNA samples eluted from the MAK column with a NaCl gradient were prepared for electrophoresis by adding 400 µg of Escherichia coli B tRNA dialysing against a 1000 fold volume of 0.05 M ammonium formate pH 6.7 in two steps of 4 hours each. The dialysed samples were lyophilyzed for 8 hours, dissolved in 1.5 mM $MgCl_2$, 10 mM tris-HCl, pH 7.4 and then applied to the gel.

Electrophoresis was for 45 minutes at 200 volts (30 volts/cm) at 20 mA/tube in the Disc electrophoresis apparatus of Buchler Instrument Co. Model 1004. Normally 8 tubes were run. Ice cold water was circulated around the lower reservoir which was at 0°C initially and the upper chamber was at 25°C initially. The reservoir buffer contained 108 g of tris, 9.3 g of EDTA and 55 g of boric acid in 10 l of water at a final pH of 8.3. SDS was added to the buffer to a concentration of 0.25% (w/v) before the electrophoresis.

Under these conditions the logarithm of the sedimentation coefficient of the 28, 18 and 4 S rat liver cyt-RNAs and their molecular weight are linearly related to their migration distance

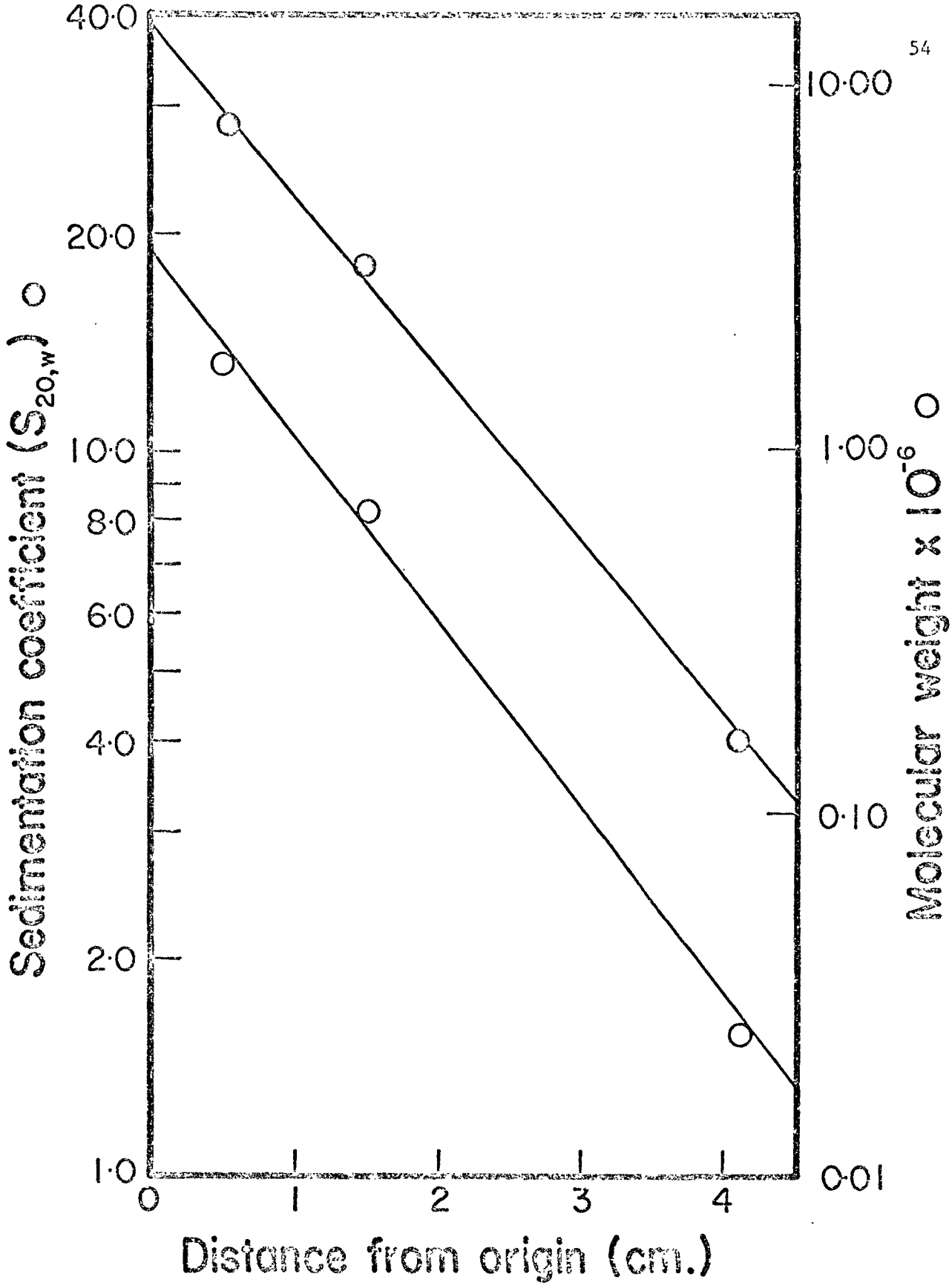

Figure 3. Relation between sedimentation constant, molecular weight and electrophoretic mobility of rat liver cyt-RNAs on agarose-polyacrylamide gel. A mixture of 30 µg of rat liver cyt-rRNA and tRNA were separated by electrophoresis in a 2.2% acrylamide and 0.25% agarose gel. Electrophoresis was for 2.5 hours at 40 Volts/cm, 5 mA/tube in 6.5 cm tubes.

(Figure 3). These relationships make it possible to interpolate the sedimentation coefficient and the molecular weight of mitochondrial RNA with regard to those of the cytoplasmic RNA. The interpolated S values are designated S_E.

8. Analysis of Mitochondrial Ribosomes by Sucrose Density Gradient

From 0.4 to 0.8 ml of a DOC-Triton treated mitochondria from rat liver or L cells or from a cytoplasmic ribosomal fraction from L cells was loaded on an isokinetic convex gradient of 15-30% or 5-20% sucrose in TKM buffer. The gradient was made with a mixing chamber containing 9.1 ml of the light sucrose solution and with 12 ml of the heavy sucrose solution added dropwise. Centrifugation was at 165,000 x g_{av} for 3 hours at 2°C in the SW 41 rotor of the Beckman Model L2 ultracentrifuge. For determining the sedimentation rate of mitochondrial ribosomes, 0.1 ml of freshly prepared ^{14}C-labelled L cell cytoplasmic ribosomal fraction was added to the ^{3}H-labelled DOC-Triton treated mitochondria applied on the sucrose gradient. The gradients were fractionated and analyzed as with the RNA gradients described above.

9. Measurement of Radioactivity

a. From labelled RNA analyzed on a sucrose density gradient

^{32}P-Labelled RNA fractions were dried on Whatman 3MM paper and counted in toluene scintillation solution containing 0.5% (w/v) of 2,5 diphenyloxazole (PPO) and 0.05% (w/v) of 1,4-bis [2-(4-methyl-5-phenyloxazoly)] benzene (dimethyl-POPOP) (Calder

and Freeman, 1968). Fractions of ^{3}H- or ^{14}C- or dual-labelled RNA were digested overnight with 0.5 ml of water and 0.5 ml of NCS solubilizer and then counted with 10 ml naphthalene-dioxane scintillation solvent containing 15.5% (w/v) naphthalene recrystallized from alcohol, 0.6% (w/v) of 2,5 diphenyloxazole and 0.0275% (w/v) of 1,4-bis [2-(4-methyl-5-phenyloxazoly)] benzene in spectroquality p-dioxane (Bush & Hansen, 1965).

b. From labelled RNA in agarose-polyacrylamide gels

The gels were sliced in 1 mm widths with the device shown in Figure 4. Slices of ^{32}P-labelled RNA were dried at 100°C for 1 hour on Whatman 3 MM paper and counted in the toluene scintillation solution. Slices of ^{3}H- or ^{14}C- or dual-labelled RNA were digested overnight with 0.5 ml water and 0.5 ml of NCS solubilizer. Ten ml of naphthalene-dioxane scintillation fluid was added, the vials shaken for 1 hour and then counted. Over 98% of the counts were extracted from the slice as determined by removing the slice from the scintillation fluid and recounting.

c. From labelled RNA eluted from a MAK column

RNA from the fractions was counted by a modified technique of Trevavas (1967). To 2.5 ml of each fraction, 10 ml of a solution, containing 0.01% (w/v) yeast RNA in 0.05 M NaCl, 0.005 M Na_2HPO_4, pH 6.7 was added and shaken. Then 1 ml of 1% (w/v) of cetyltrimethyl (hexadecyl trimethyl) ammonium bromide was added, shaken again and incubated for 7 hours at 4°C. The insoluble cetyltrimethylammonium salt of the nucleate was collected on a Whatman 934 AH glass fiber filter and washed twice with 5 ml of distilled water at 25°C. The

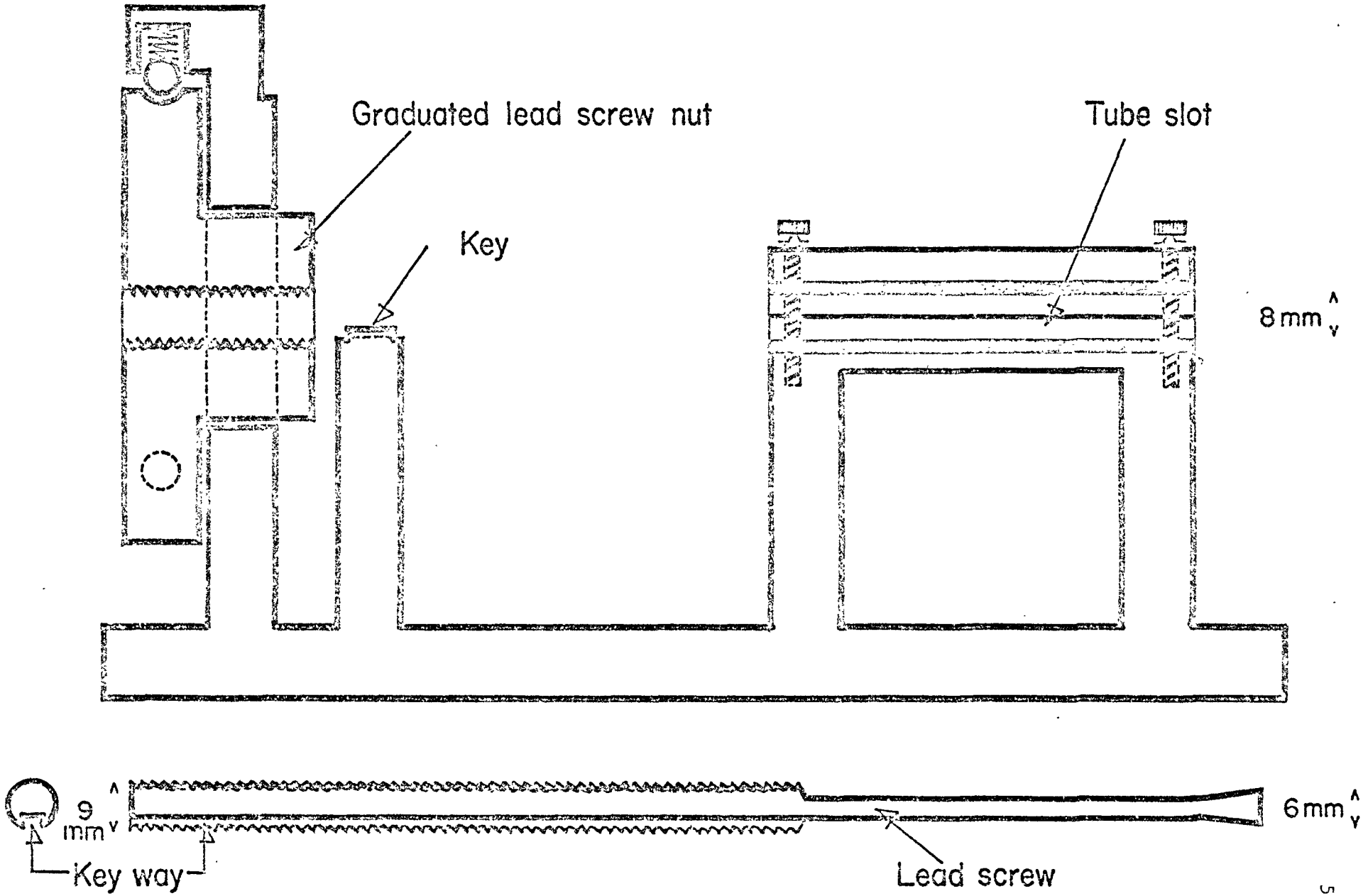

Figure 4. Gel removing apparatus in cross section. Scale 1:1.

filters were placed in counting vials and the RNA digested for 5 hours by adding 0.5 ml of NCS solubilizer and 0.5 ml toluene. Ten ml of toluene scintillation liquid was added and the vials counted.

d. Counting

Dual-(^{3}H and ^{14}C) or triple-(^{3}H, ^{14}C and ^{32}P) labelled samples were counted on two or three channels of the Nuclear Chicago Mark 1 counter respectively. Corrections for ^{14}C-counts in the ^{3}H-channel and for the ^{32}P-counts in the ^{14}C- and ^{3}H-channels were done by addition of an internal standard (^{14}C-methanol or $^{32}P_i$), recounting and calculation of the pure ^{3}H- and ^{14}C-counts using a computer.

10. Nucleotide Composition

About 0.5 mg of ^{32}P-labelled RNA was digested in 0.2 ml of 0.3N KOH for 18 hours at 37°C. Then 2N perchloric acid was added to pH 7.0. The $KClO_4$ precipitate was removed by centrifugation at 0°C. Nucleotides were separated by descending paper chromatography (Lane, 1963), using an ethanol-H_2O (5:2) solution for development, or by electrophoresis on Whatman 3 MM paper in 0.05 M ammonium formate buffer, pH 3.5, for 2 hours at 4,000 volts (Davidson & Smellie, 1952). The techniques gave identical results. The nucleotides were located by ultra-violet lamp, cut out and counted with the toluene-based scintillation solvent. No counts were found between the spots.

In one experiment unlabelled mitochondrial RNA, isolated in the absence of carrier cytoplasmic RNA, was analyzed for

nucleotide composition on a Dowex 50-H^+ column by the method of Katz and Comb (1963).

11. Chemical and Enzymatic Determinations

a. Analytical methods

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

RNA was measured by the orcinol method of Mejbaum (1939) with adenosine as the standard. The heating time was 45 minutes (Albaum and Umbreit, 1947).

DNA was estimated by the method of Burton (1956) with a preparation of salmon sperm DNA containing 7.0% phosphorous as a standard.

Inorganic phosphate was measured according to Chen et al. (1956).

b. Enzymatic activities

Malate dehydrogenase (EC 1.1.1. 37)

Total enzyme activity was measured by following the reduction of NAD^+ by malate at 340 nm according to the method of Poodyn et al. (1962). The enzyme fractions were solubilized in 0.2% (v/v) Triton X100 in 0.3 M sucrose. A unit of activity is defined as the amount of enzyme giving an increase in optical density at 340 nm of 1.0 per minute in a cuvet with a 1.0 cm light path containing 2.7 ml of solution.

Cytochrome oxidase (EC 1.9.3.1)

Cytochrome oxidase activity was measured by following the

oxidation of reduced cytochrome c at 550 nm according to Cooperstein and Lazarow (1951). A solution of 17 μM cytochrome c from horse heart in 0.03 M phosphate buffer pH 7.4 was reduced with a minimum of sodium dithionite crystals. The solution was shaken until a decrease in absorption at 550 nm was observed. The mitochondria were then added. A unit of activity is defined in the same way as a unit of activity of malate dehydrogenase.

Glucose-6-phosphatase (EC 3.1.3.9)

Glucose-6-phosphatase was determined by the release of inorganic phosphate as described by Roodyn, Reis and Work (1961). A unit of enzyme activity was defined as the release at 1 μg of orthophosphate/minute under the condition of assay.

Acid phosphatase (EC 3.1.3.2)

Acid phosphatase was estimated by the release of inorganic phosphate from β-glycerophosphate by the method of Gianetto and de Duve (1955). The enzyme was activated by freezing and thawing the fraction 10 times before the assay. A unit of enzyme activity was defined as for glucose-6-phosphatase.

12. List of Buffers and Media

Medium A: 0.34 M sucrose, 2mM tris-HCl, 2mM EDTA, pH 7.4.

Medium B: 0.30 M sucrose, 2mM tris-HCl, 2mM EDTA, pH 7.4.

Medium C: 0.25 M sucrose, 2mM EDTA, pH 7.4.

Medium D: 2% (w/v) SDS, 0.1% (w/v) Macaloid, 1% (w/v) NaCl, 10mM $MgCl_2$ and 25mM sodium acetate, pH 4.8.

Tris buffer: 0.01 M tris-HCl, pH 7.0.

TKM buffer: 10mM tris-HCl, 50mM KCl, 6mM $MgCl_2$, pH 7.6.

PBS: 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na_2HPO_4, 0.02% (w/v) KH_2PO_4, pH 7.4.

RSB buffer: 50mM NaCl, 1.5mM $MgCl_2$, 10mM tris-HCl, pH 7.4.

0.1 M Buffered Saline: 0.1 M NaCl, 0.05 M sodium phosphate, pH 6.7.

13. Materials

All chemicals where possible were reagent grade. Sucrose for the gradient centrifugation was ribonuclease-free. This and sodium deoxycholate were obtained from Mann Research Lab., New York, N.Y. Methylene-bisacrylamide was purified as described by Loening (1967). Agarose was obtained from Bausch and Lomb Co. Ltd., Toronto, Ont. Every batch was not suitable. Lot 962506 was used for all experiments reported here. Acrylamide of the highest purity grade and hexadecyltrimethylammonium bromide were obtained from Distillation Products, Rochester, N.Y. Joklik's modification of minimum essential medium, foetal calf serum and anti-PPLO agent were obtained from Grand Island Biological Co., Grand Island, N.Y. Macaloid was a gift of the Imperto Co., Las Vegas, Nev. Hyflo supercel was obtained from Fisher Scientific Co., Fair Lawn, N.J. Deoxyribonuclease, electrophoretically purified from ribonuclease was obtained from Worthington Biochemical Corp., Freehold, N.J. Ribonuclease A Type II A and bovine serum albumin Fraction V were obtained from Sigma Chemical Corp., St. Louis, Mo. t-RNA (stripped) from *E. coli* B was obtained from General Biochemical, Chagrin Falls, Ohio. Glass fiber filters 934AH, 2.4 cm were obtained from Reeve

Angle, Clifton, N.J. NCS solubilizer was obtained from Amershan/Searle Corp., Toronto, Ontario. Yeast ribonucleate was obtained from the British Drug Houses Ltd., Poole, England. Carrier free $^{32}P_i$ was obtained from Atomic Energy of Canada Ltd., Chalk River, Ont. and [^{14}C]methanol (50 mCi/mmole), [5-^{3}H]orotic acid (4 Ci/mmole), [5-^{3}H]uridine (2-5 Ci/mmole), [6-^{14}C]orotic acid (50 mCi/mmole) and [2-^{14}C]uridine (50 mCi/mmole) were obtained from Amersham/Searle Corp., Toronto, Ont.

III RESULTS

In order to understand how mitochondria maintain their genetic identity and to what degree they have autonomy within the eukaryotic cell, it is necessary to know the site of the genes and the site of synthesis of mitochondrial components. It is of particular interest to determine what mitochondrial DNA codes for, what proteins are made in mitochondria and the nature and origin of the protein syntheisizing system in mitochondria.

Mitochondria contain an independent protein synthesizing system which is different from the cytoplasmic one as far as its inhibition by chloramphenicol is concerned. Since the protein synthesizing systems of both the eukaryotic and the prokaryotic cell contain ribosomes and rRNA as essential components for their function, the aim of this investigation was to find answers to the following questions:

(a) Do mitochondria of mammalian cells contain rRNA?

(b) If they do contain rRNA is it similar to cytoplasmic rRNA, to bacterial rRNA or is it a unique class?

(c) If mitochondria from mammalian cells contain rRNA, do they also contain the more complex nucleoprotein particles, the ribosomes, consisting of rRNA and few dozen ribosomal proteins?

(d) If mitochondria from mammalian cells contain ribosomes are they similar to the cytoplasmic ones, to the bacterial ones or

are they unique?

(c) If mitochondria contain rRNA, is it coded for by mitochondrial or nuclear DNA?

For the initial studies it seemed that a logical approach would be to isolate and purify mitochondria from rat liver as described by Roodyn et al. (1961). Mitochondria were recovered from the post nuclear supernatant by five successive centrifugations at 8,000 x g for 10 minutes each, in 0.3 M sucrose, 2mM EDTA, pH 7.2. RNA was extracted by the modification 1 of Kirby's technique and then analyzed on a 15-30% convex sucrose density gradient. Two components were observed which sedimented like the carrier cyt-rRNA at 28 and 18 S. Also RNA extracted by the modification 1 of Kirby's technique from L cells mitochondrial fraction, purified by isopycnic gradient centrifugation, had the same sedimentation rate as the carrier cyt-rRNA except for a small amount of RNA sedimenting from 11 to 18 S and peak at about 21 S (Figure 5). These results were in agreement with those of Rabinowitz et al. (1966) for chick embryo and of Truman (1963) and Ilaév (1964; 1966) for rat liver and heart. They concluded that as far as the sedimentation constant of the mitochondrial ribosomes and rRNA was concerned they were undistinguishable from the cytoplasmic ones. At that point the following question arose. Was the observed rRNA in this and previous cases only from cyt-rRNA present as a contaminant in the mitochondrial fraction? If this was correct and the observed RNA was cyt-rRNA, then the mitochondrial RNA was either masked by the cyt-rRNA contaminant or simply does not exist as was concluded by Neubert (1966).

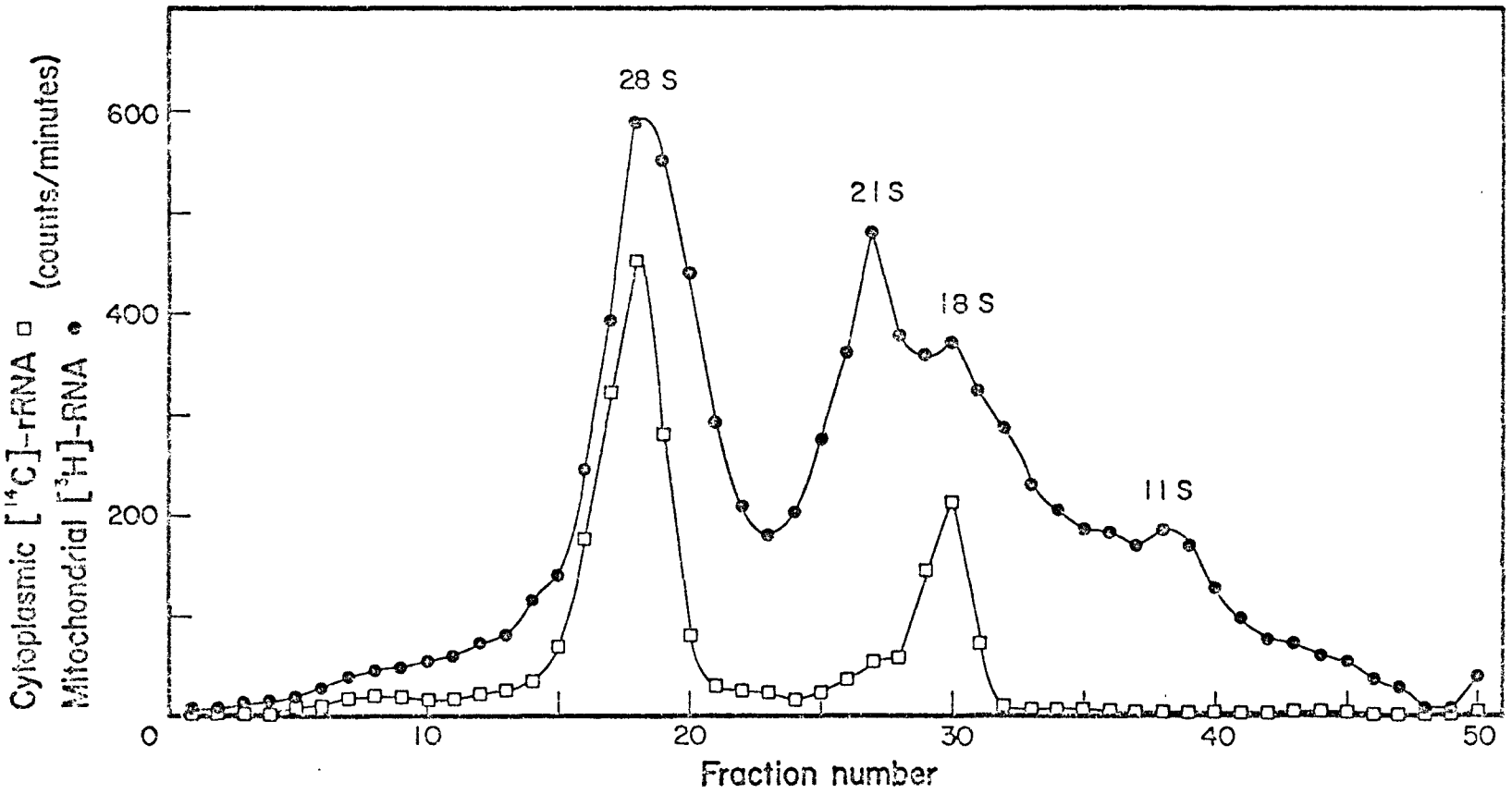

Figure 5. Fractionation of L cell mit-RNA on a convex sucrose density gradient. RNA of 2.7×10^8 cells was labelled with 1 µCi of [5-^{3}H]uridine/ml for 36 hours at a cell density of 2.5×10^5/ml. RNA was extracted from mitochondria which were purified by isopycnic gradient centrifugation by the modification 1 of Kirby's method in the presence of ^{14}C labelled rat liver cyt-rRNA as described in the method. RNA (200 µg) was centrifuged on 0.3-1.4 M isokinetic sucrose density gradient at 180,000 x g_{av} for 11 hours at 2°C in SB 283 rotor of the International B-60 ultracentrifuge and the radioactivity of each fraction was determined as described in the method.

To eliminate the possibility of cytoplasmic contamination, a better technique for isolation of mitochondria was developed as described in the Methods section. Two factors were considered important to obtain pure mitochondria. The first was the removal of as much as possible of the endoplasmic reticulum and ribosomes before the mitochondria were sedimented for the first time, so that they did not form aggregates with the mitochondria. This was achieved by layering the nuclear supernatant over a sucrose density gradient so that on centrifugation the mitochondria but not much of the endoplasmic reticulum and ribosomes sedimented. Secondly, successive washings with a sucrose-EDTA solution were used to remove the remaining contamination. EDTA complexes Mg^{2+} and hence dissociates ribosomes (Sabatani, Tashiro & Palade, 1966). Washings were very effective in lowering the RNA content whereas isopycnic gradient centrifugation was not. This was also observed by O'Brien & Kalf (1967a)

1. Isolation of Mitochondria

a. Purification of rat liver mitochondria

For devising the centrifugation purification method the purity of the mitochondria was followed by marker enzymes activities and a balance sheet drawn up. In addition, mitochondrial purity was determined by electron microscopy. The following enzymes which are known to be located in particular subcellular sites were used as a biochemical criterion for the purification of the mitochondria.

Malate dehydrogenase, located in the mitochondrial matrix

and the cytosome (Erdiczka et al., 1960; Marco et al., 1969: Witt et al., 1966), cytochrome oxidase, located in mitochondrial inner membrane (Schnaitman & Greenawalt, 1968), glucose-6-phosphatase, located in the endoplasmic reticulum (Parsons et al., 1967), acid phosphatase, located in lysosomes (Appelmans, Wattiaux & de Duve, 1955).

In Table 3 the specific activities of these enzymes as well as the percentage of activity during mitochondrial purification is presented. The results indicate that in the first nuclear spin about 50% of the mitochondria were trapped. This was avoided subsequently by rehomogenizing the nuclear pellet.

On the discontinuous sucrose density gradient step the bulk of the cytochrome oxidase activity spun down with the crude mitochondrial pellet. Most of the glucose-6-phosphatase activity was left in the supernatant solution but most of the acid phosphatase spun down with the mitochondria. The activities of the cytosome and the mitochondrial malate dehydrogenases separated between the mitochondrial pellet and the supernatant.

The crude mitochondrial pellet still contained lysosomes and some endoplasmic reticulum. These were removed by a series of washings with sucrose-EDTA. Between washes the tubes were inverted twice before decanting to dislodge the loosly packed layer of the more contaminated mitochondria and then thoroughly drained before resuspension of the pellet. The effect of these washings is reflected in the different enzyme activities as follows:

the mitochondrial fraction gradually lost less malate dehydrogenase and cytochrome oxidase activities until the last

Table 3

Recovery of Enzymatic Activities of Malate Dehydrogenase, Cytochrome oxidase, Glucose-6-Phosphatase and Acid Phosphatase During Purification of Rat Liver Mitochondria

Mitochondrial purification procedure	Enzymes							
	Malate dehydrogenase		Cytochrome oxidase		Glucose-6 phosphatase		Acid phosphatase	
	units/mg protein	%	units/mg protein	%	units/mg protein	%	units/mg protein	%
Homogenate	1.095	100.0	1.150	100.0	0.748	100.0	0.210	100.0
10 min × 1000 → Nuclei A	1.245	58.5	1.350	60.5	0.842	57.7	0.013	3.2
Supernatent A	1.908	50.8	1.842	48.2	1.415	56.8	0.211	30.0
gradient		100.0		100.0		100.0		100.0
35% 20%								
60 min × 25.000 → Supernatent B	0.634	48.1	0.149	11.0	0.720	71.4	0.062	41.0
Crude mitochondria	2.015	48.2	2.980	71.5	0.878	27.4	0.375	78.5
10 min × 1000 → Nuclei B	2.680	8.0	3.560	10.0	0.293	1.1	0.172	4.5

Crude Mitochondria								
→ Wash 1	0.548	5.5	1.517	15.4	1.555	20.5	0.559	49.5
→ Wash 2	1.940	7.1	3.050	11.1	0.915	4.3	0.318	10.2
→ Wash 3	1.405	1.4	0.800	0.8	2.640	3.4	0.557	4.9
6500 x g → Wash 4	0.667	0.7	0.667	0.7	2.525	3.5	0.183	1.7
→ Wash 5	2.180	1.4	--	0.0	3.480	3.1	0.488	2.9
→ Wash 6	5.520	8.0	--	0.0	1.648	3.1	0.202	2.6
Pure mitochondria	2.060	15.7	2.990	20.7	0.156	1.57	0.016	1.03
Total recovery		109.1		70.7		132.1		118.3

two washings, when more malate dehydrogenase activity was lost but none of the cytochrome oxidase activity. Probably after the fourth wash the mitochondria started to break up and the soluble mitochondrial malate dehydrogenase started to leak out from the mitochondrial matrix but no cytochrome oxidase activity was lost because it is bound to the mitochondrial inner membrane.

After the first two washings most of the acid phosphatase activity (lysosome) was removed and the rest was gradually removed through the subsequent washings. The loss of activity of glucose-6-phosphatase seemed to increase with washing. This might be due to the washing away of some of the mitochondrial outer membrane to which some endoplasmic reticulum could be aggregated (Parsons et al., 1967). This possibility was supported by the electron microscopy studies discussed below. A negative staining of the final mitochondrial fraction is shown in Figure 6. All the dark dense bodies are mitochondria which have lost their outer membrane during the sedimentation of the sucrose density gradient and the washings. This phenomenon was observed also by Kalf & Ch'ih (1966).

Electron microscopy was used to confirm the mitochondrial nature of the final pellet and to see whether this pellet was free of endoplasmic reticulum. The latter can be easily recognized by negative staining (Parsons, 1967). A general field view of five times washed mitochondria, stained in 2% phosphotungstate for 2 minutes is shown in Figure 6. The dark dense bodies are mitochondria in which the outer membranes were lost or damaged

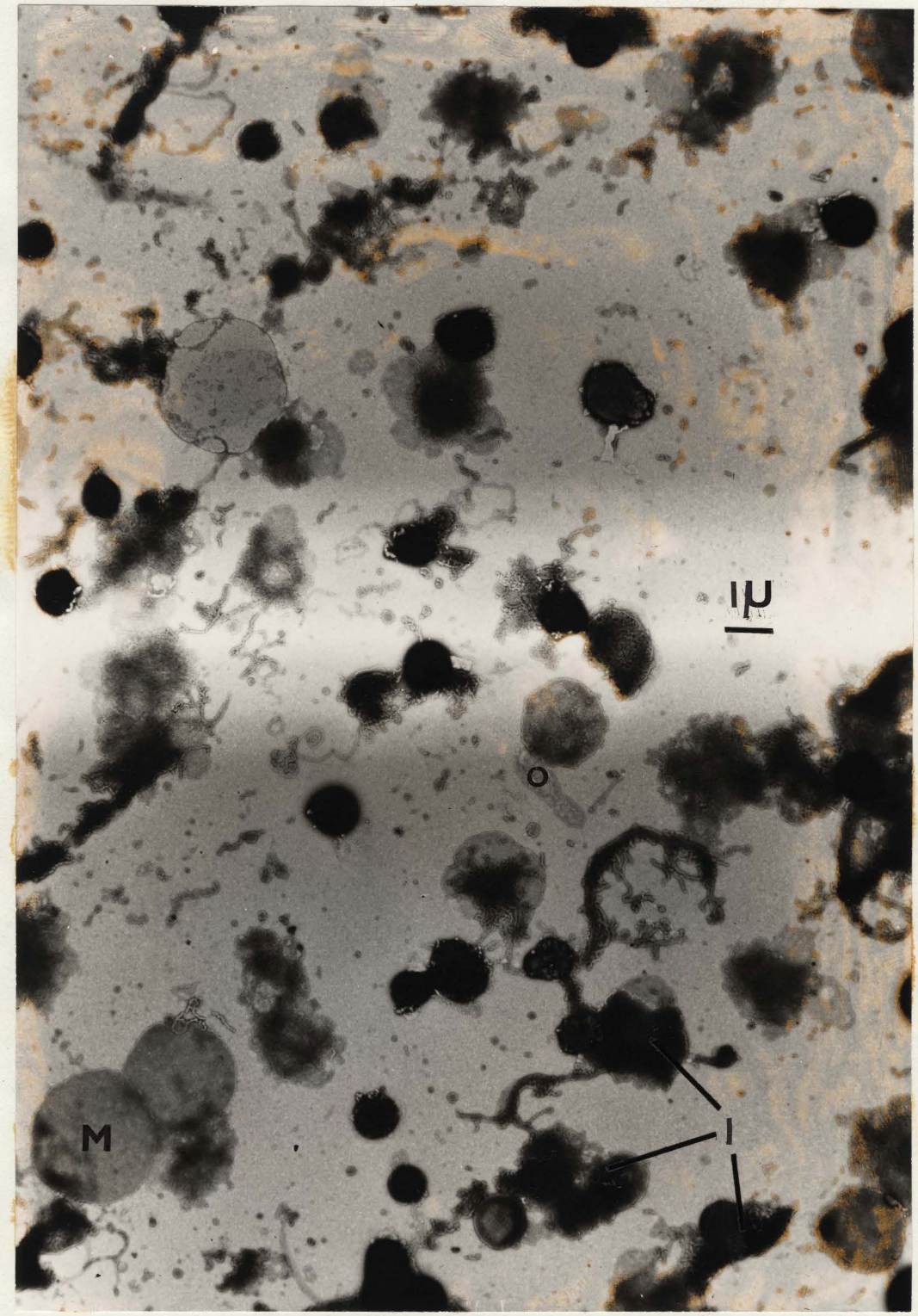

Figure 6. Low-power view of negatively stained, five times washed rat liver mitochondria. Although all the structure present are derived from mitochondria, only few intact mitochondria (M) can be seen. Mitochondria, usually, are lacking outer membranes (O) and their inner membranes (I) show a dense inner matrix. Magnification: 7,000 X.

allowing the phosphotungstate to penetrate and stain the mitochondrial matrix. There are some mitochondria which are intact and lightly stained, and whose background contains broken cristae. No smooth endoplasmic reticular membranes can be observed. For comparison, a general field of the crude mitochondrial pellet which had sedimented through the sucrose density gradient, from which the pure fraction was obtained is shown in Figure 7. In this picture though most of the small particles in the background are inner and outer mitochondrial fragments but there is still endoplasmic reticulum (E) and unidentified particles (X) present. Higher magnifications are presented in Figures 8 and 9 for crude mitochondria and in Figures 10 to 13 for pure mitochondria. The irregular pieces of membrane showing numerous projection of 90 Å knob-like subunits at the edges are inner membranes (I) or cristae. Round pieces of membrane showing no projecting subunits are outer membranes (O). The white pieces with round edges are contaminating endoplasmic reticulum. These figures clearly show that the final mitochondrial pellet was nearly completely free of endoplasmic reticulum while in the crude fraction contaminating membranes can be seen.

About 40 mg of purified mitochondrial pellet was obtained from 1 g of liver. The chemical analysis of the purified mitochondrial fraction showed that it contained 6.5 mg protein per g liver and 3.0 µg RNA and 0.24 µg DNA per mg of mitochondrial protein. These values were lower than any previously reported (Table 2). Rat liver mitochondria contain only 0.72% of the total liver RNA and 0.4% of the total liver DNA based on 1 g of rat liver

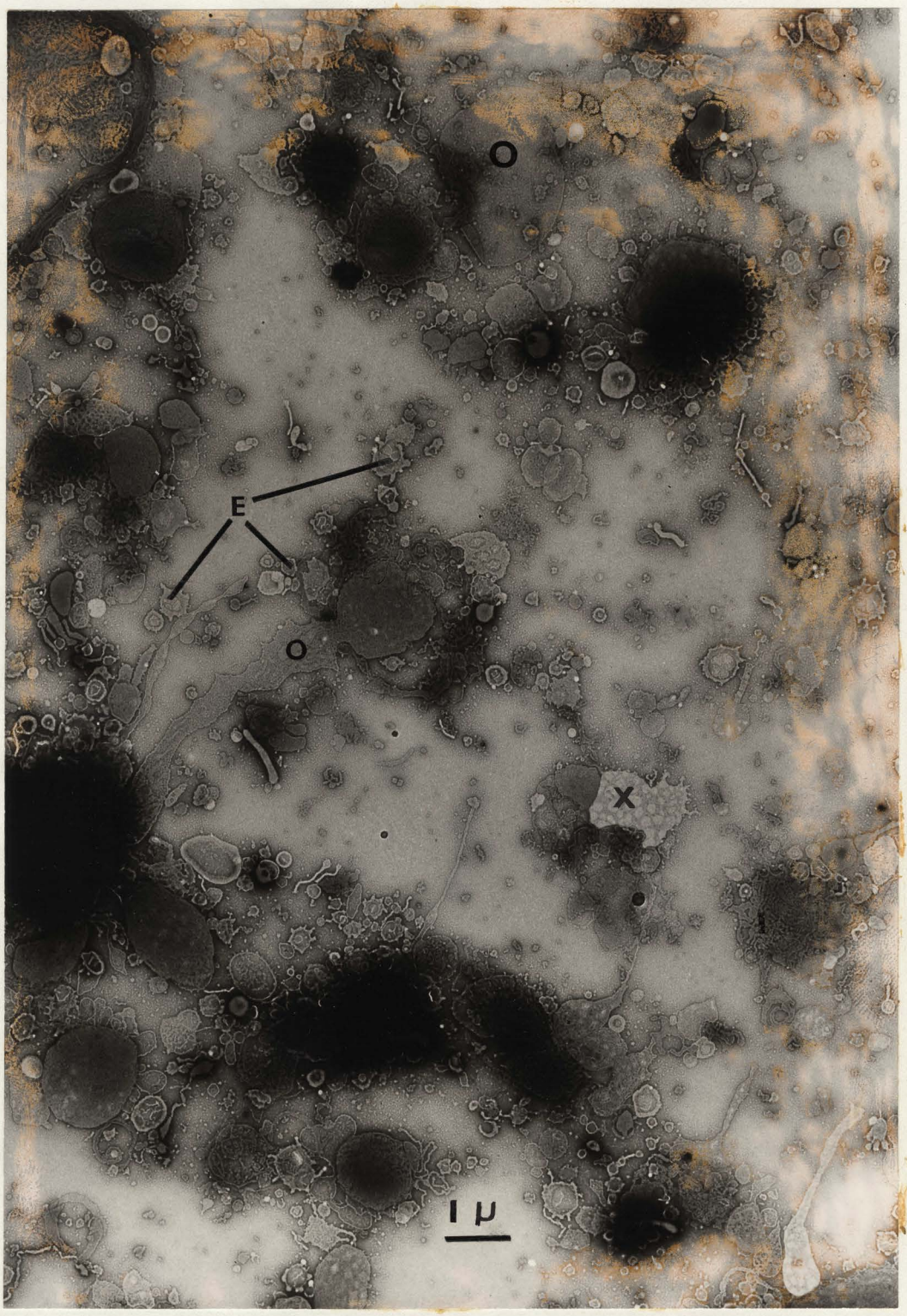

Figure 7. Low-power view of negatively stained crude rat liver mitochondria. Most of the small particles in the background are inner and outer mitochondrial fragments but endoplasmic reticulum (E) and unidentified particles (X) are present. Magnification: 10,000 X.

Figure 8. Low-power view of negatively stained crude rat liver mitochondria. Legend as in Figures 6 and 7. Magnification: 43,000 X.

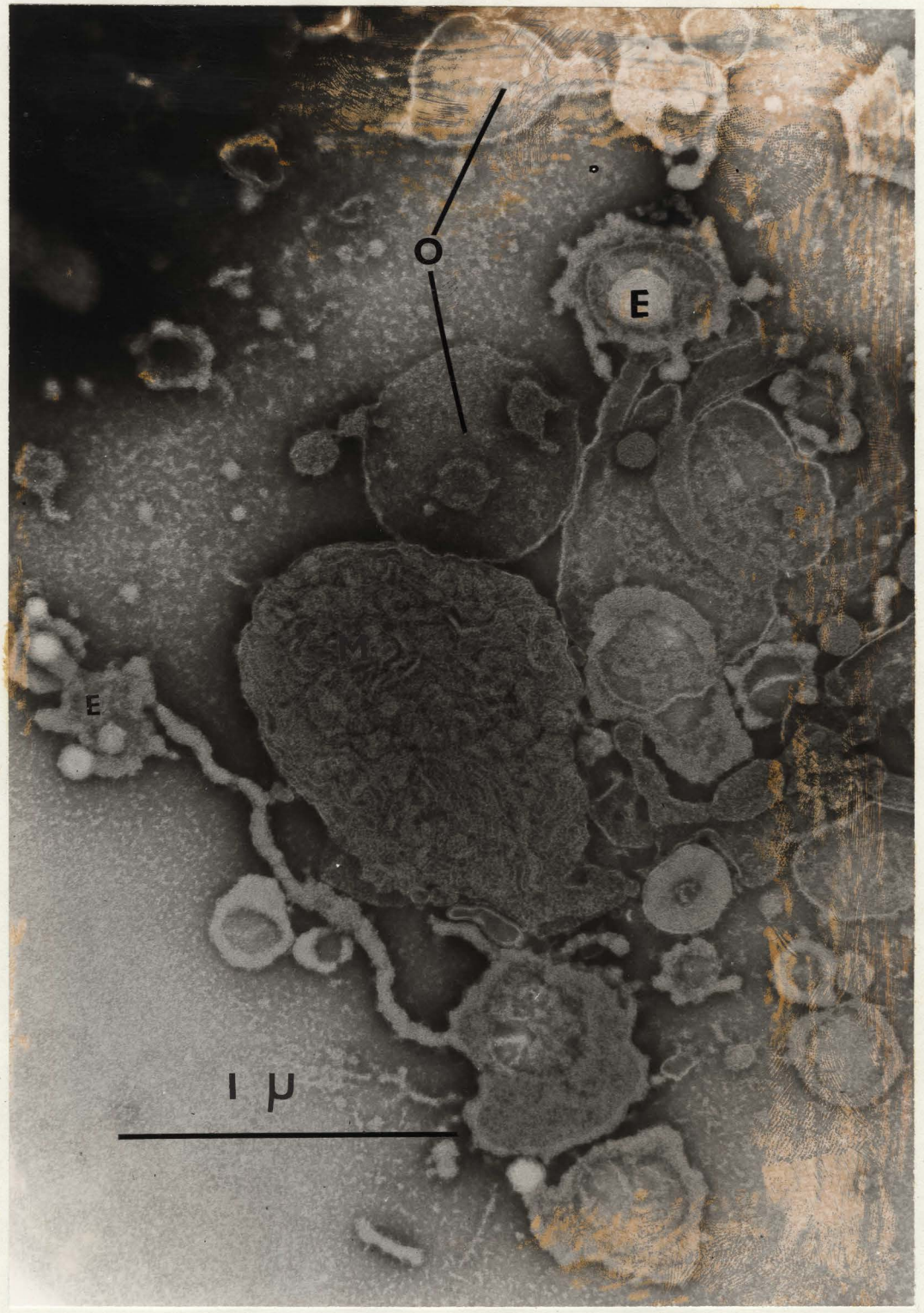

Figure 9. Low-power view of negatively stained crude rat liver mitochondria. Legand as in Figures 6 and 7. Magnification: 53,000 X.

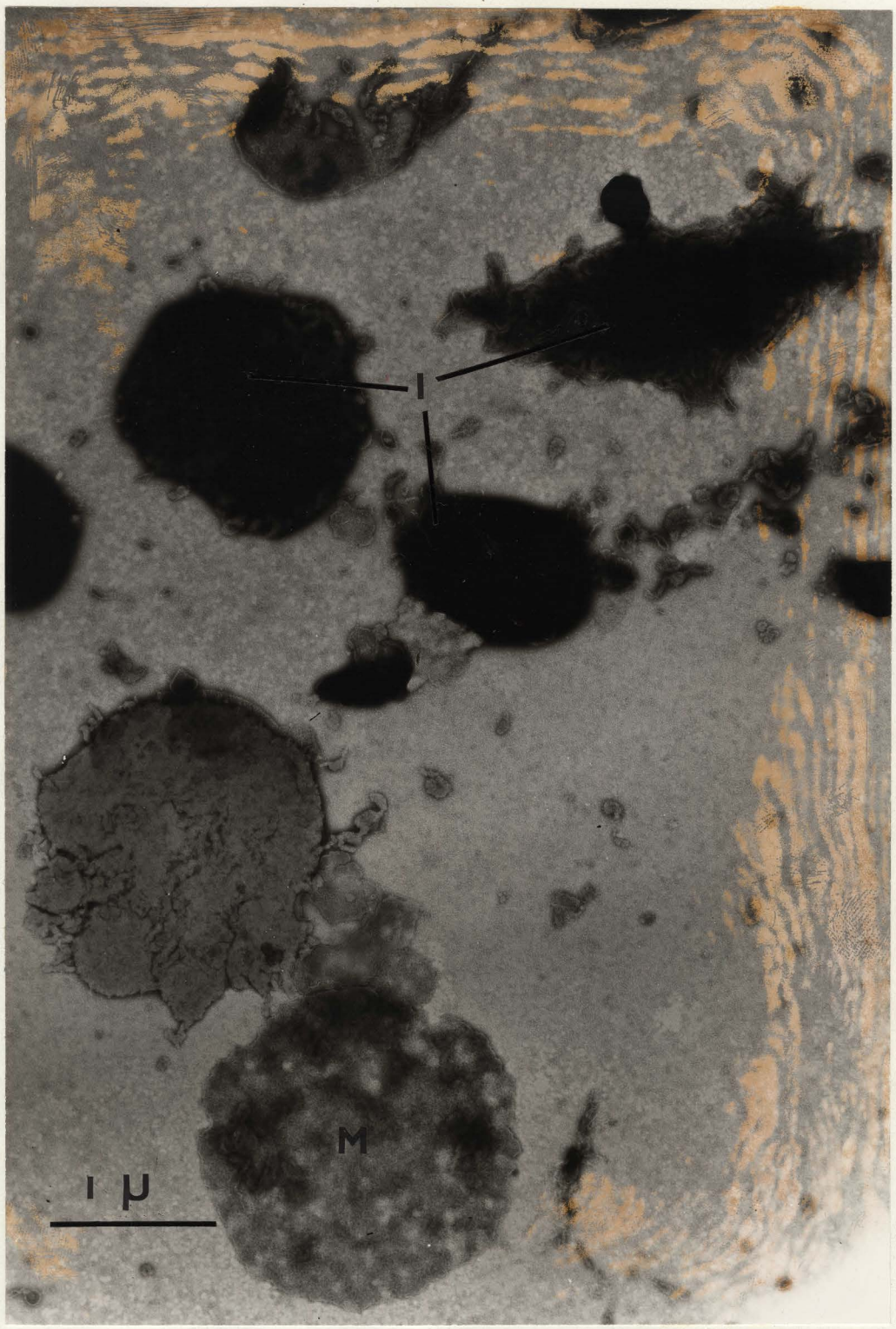

Figure 10. Low-power view of negatively stained five times washed rat liver mitochondria. Legend as in Figure 6. Magnification: 25,000 X.

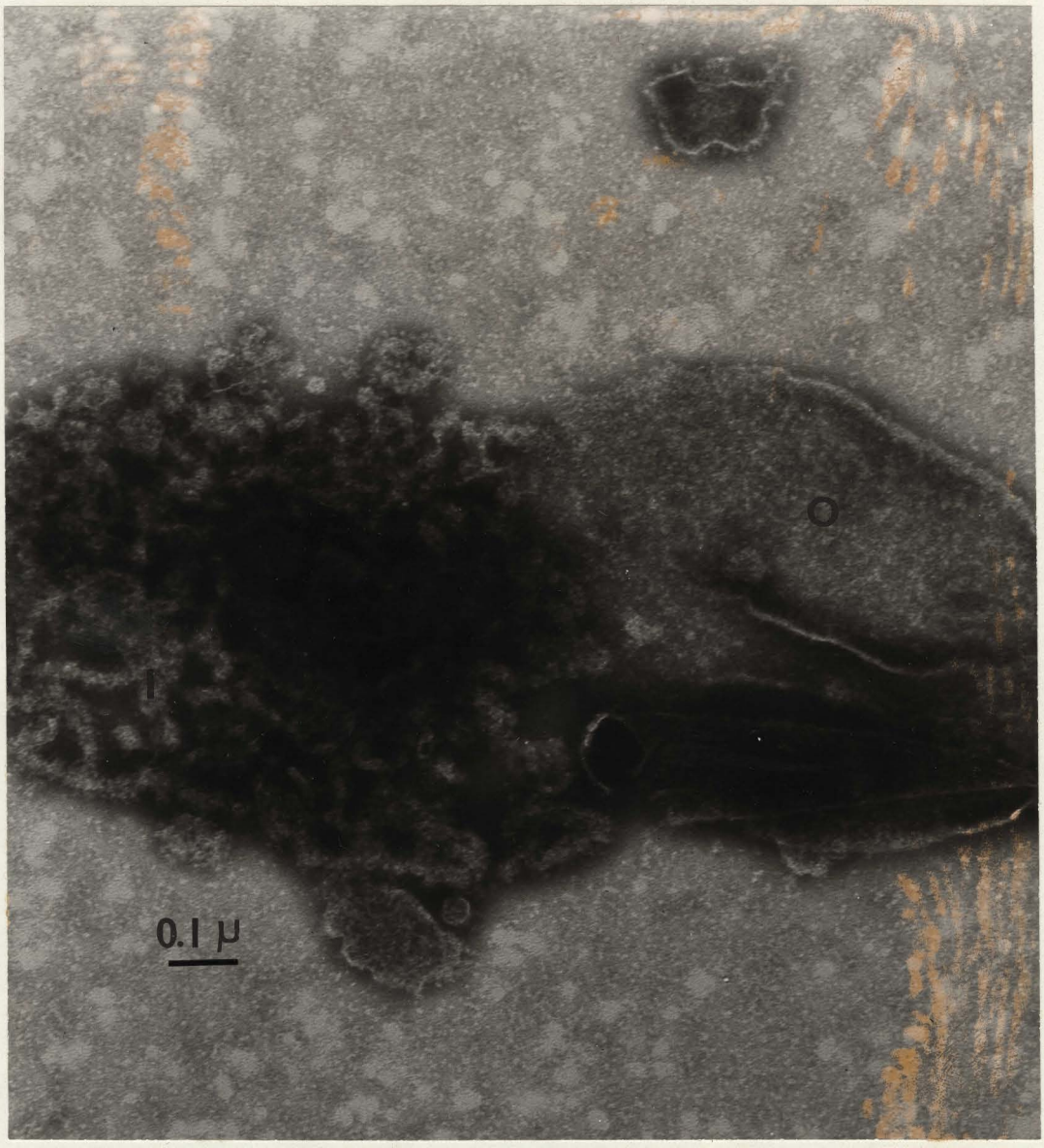

Figure 11. Higher-power view of negatively stained, a five times washed rat liver mitochondrion. Legend as in Figure 6. The outer membrane of the mitochondrion shown here is partly detached. Magnification: 90,000 X.

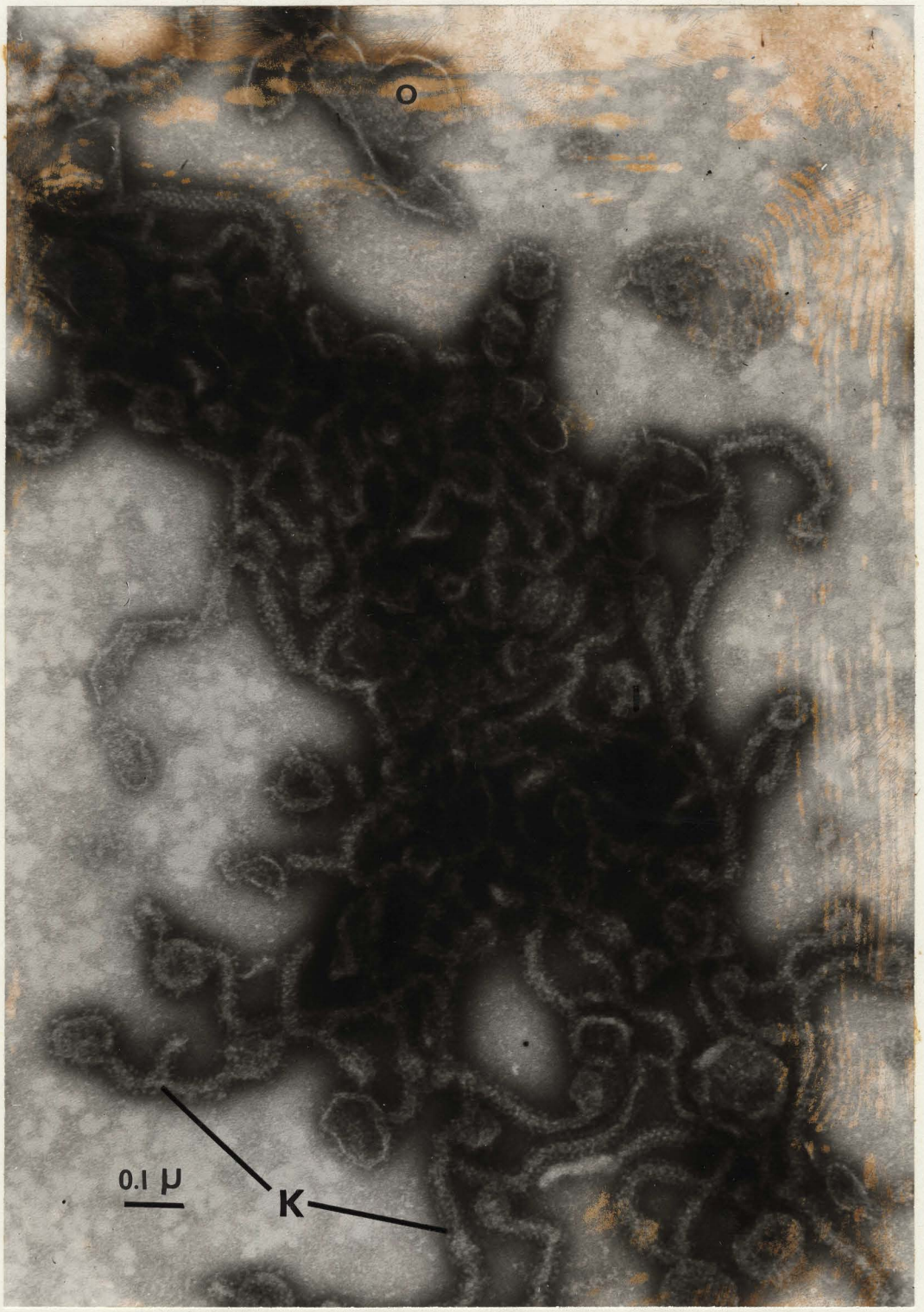

Figure 12. Higher-power view of negatively stained a five times washed rat liver mitochondrion. Legen as in Figure 6. Mitochondrial inner membranes are coated with projecting knob-like subunits (K) which are best seen lying in the plane of the object at the edge of the pieces of membrane. Magnification: 90,000 X.

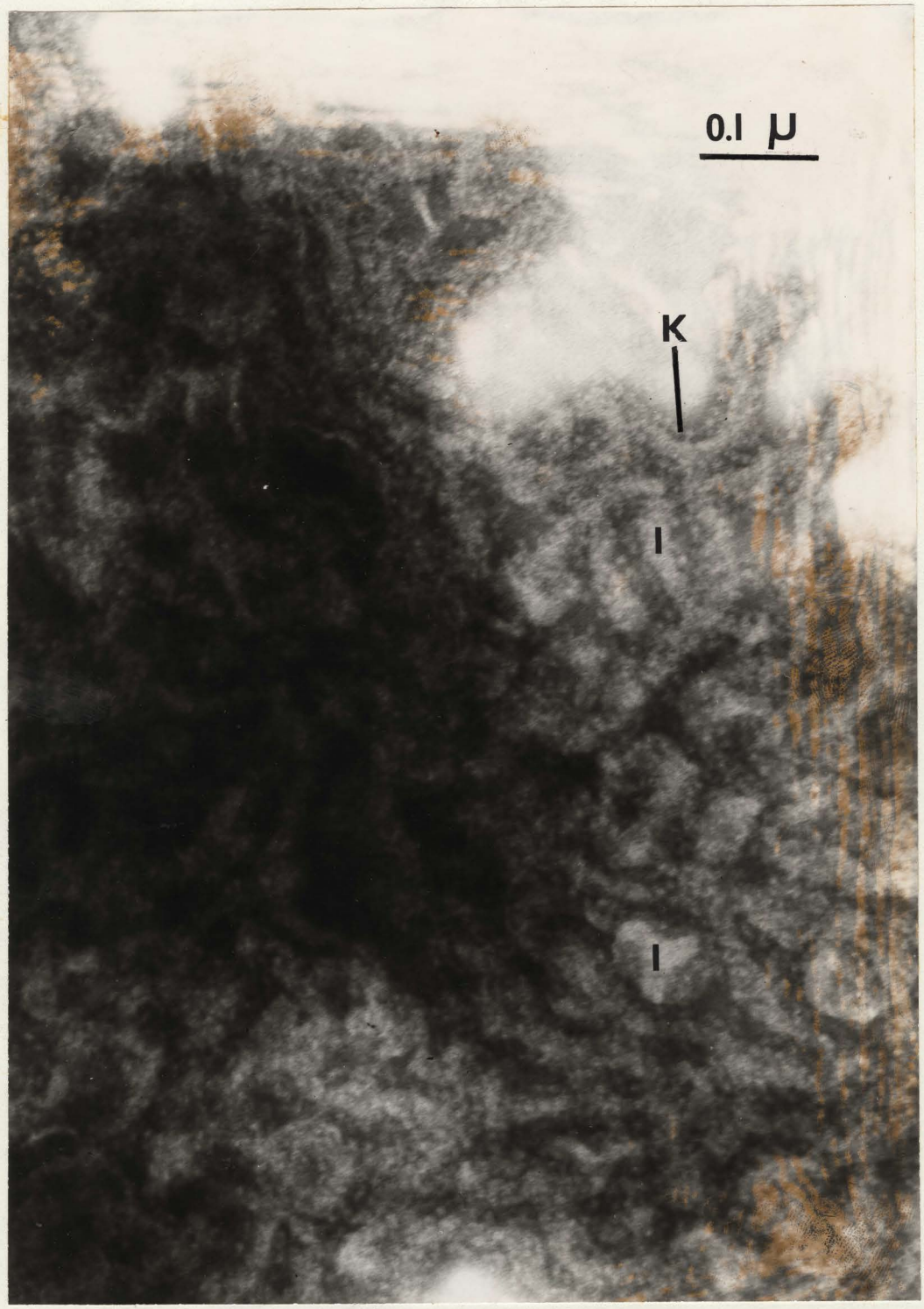

Figure 13. High-power view of negatively stained a five times washed rat liver mitochondrion. Legend as for Figure 12. The dimension of the head of the knob-like subunits is 90Å and the stem is approximately 35-40Å wide and 45Å long. Magnification: 175,000 X.

containing $11x10^{10}$ mitochondria, $5x10^{9}$ mitochondria containing 1 mg of protein (Nass, Nass & Hennix, 1965) and on the RNA and DNA content of albino rats which are 1% and 0.23% of the liver weight respectively (Davidson, 1960). Based on a 10% recovery of cytochrome oxidase activity, only 0.072% of the total liver RNA was present in the final pellet. In order to detect such a small amount of RNA it was necessary to label it with high specific activity radioactive precursors.

b. Mitochondria from tissue culture cells

The crude mitochondrial fraction isolated from $2x10^{8}$ cells of different cell lines contained only about 4 mg protein. This amount is too small to separate pure mitochondria from possible cytoplasmic contamination as described for rat liver mitochondria since 90% of the mitochondria are sacrificed for the sake of purity. One possible approach is to increase the number of cells but this is expensive and moreover it is inconvenient to disrupt large numbers of cells. Instead, cells were first incubated with a low concentration of Actinomycin D (0.1 µg/ml) to suppress the synthesis of cyt-rRNA but not that of the mit-RNA (see Introduction). This approach has some disadvantages. The Actinomycin D might affect the synthesis of the presumed mit-RNA and the mitochondrial fraction might contain other cellular components containing RNA whose synthesis is resistant to Actinomycin D. The tissue culture approach to the nature of mit-RNA is therefore indirect, compared with the direct approach with rat liver mitochondria where RNA is isolated from pure mitochondria. On the other hand the tissue

culture approach provided a much faster technique to isolate mitochondrial RNA and also more efficient labelling of it.

2. Isolation of RNA

a. Cytoplasmic ribosomal RNA (cyt-rRNA)

Cyt-rRNA was isolated from rat liver by Method 1 of Kirby (1965) with a yield of 5.5 mg cyt-rRNA per g rat liver. This was about 60% of the total liver RNA. The preparation contained 60% RNA and only 0.5% DNA and 0.23% protein. The rest was unidentified but could be detergents used for the isolation of the RNA or polysaccharides.

A modification of Kirby's method was required to isolate cyt-rRNA from tissue culture cells. It was necessary to add m-cresol to a final concentration of about 30% (v/v) in order to precipitate the cyt-rRNA, probably because its concentration was lower than that of rat liver cyt-rRNA. As will be seen in the figures to follow, the RNA isolated from both sources had typical 28 and 18 S cyt-rRNA. It was used as a carrier to isolated mit-RNA and hence as a marker for sedimentation, electrophoretic and chromatographic studies.

b. Mitochondrial-RNA

The minute amount of the labelled mit-RNA was always accompanied with cold cyt-rRNA in order to obtain a workable amount for extraction and handling, to minimize the possibility of RNase activity on the mit-RNA and also to serve as marker when mit-RNA was characterized.

Modification 1 and 2 of Kirby's method and the cold phenol-SDS method were employed when the extraction of mit-rRNA was specifically required, but when the total mitochondrial nucleate was needed the faster hot phenol-SDS method was used. Judgement of the success of these methods depended on the analysis of the properties of the mit-RNA.

3. Characterization of Mitochondrial RNA

a. Sucrose density gradient centrifugation of rat liver mit-rRNA

Sucrose density gradient centrifugation of RNA isolated from purified rat liver mitochondria with modification 1 of Kirby's method is shown in Figure 14 (5-[^{3}H]orotic acid-labelled RNA) and 15 ([^{32}P]labelled RNA). There were two major radioactive peaks which sediment at an average of 15.3 ± 0.17 and 12.8 ± 0.12 S, (28.0 and 18.0 S cyt-rRNA as the references standards) calculated from 6 determinations and from two different extractions of mit-RNA. There was always some hetrodisperse radioactive RNA with higher sedimentation values including peaks at 28 and 23 S. In all experiments, as shown in Figures 14 and 15, some mitochondrial 4 S but no cytoplasmic 4 S RNA was observed. No 4 S RNA would be expected in the method of Kirby (1965), in which ribosomal RNA is selectively recovered. If the 15.3 and 12.8 S are mit-rRNAs then their sedimentation constants are less than any rRNA from eukaryotic and prokaryotic organisms previously reported. It was therefore possible that they represent degradation products of higher molecular weight components. This possibility has not been

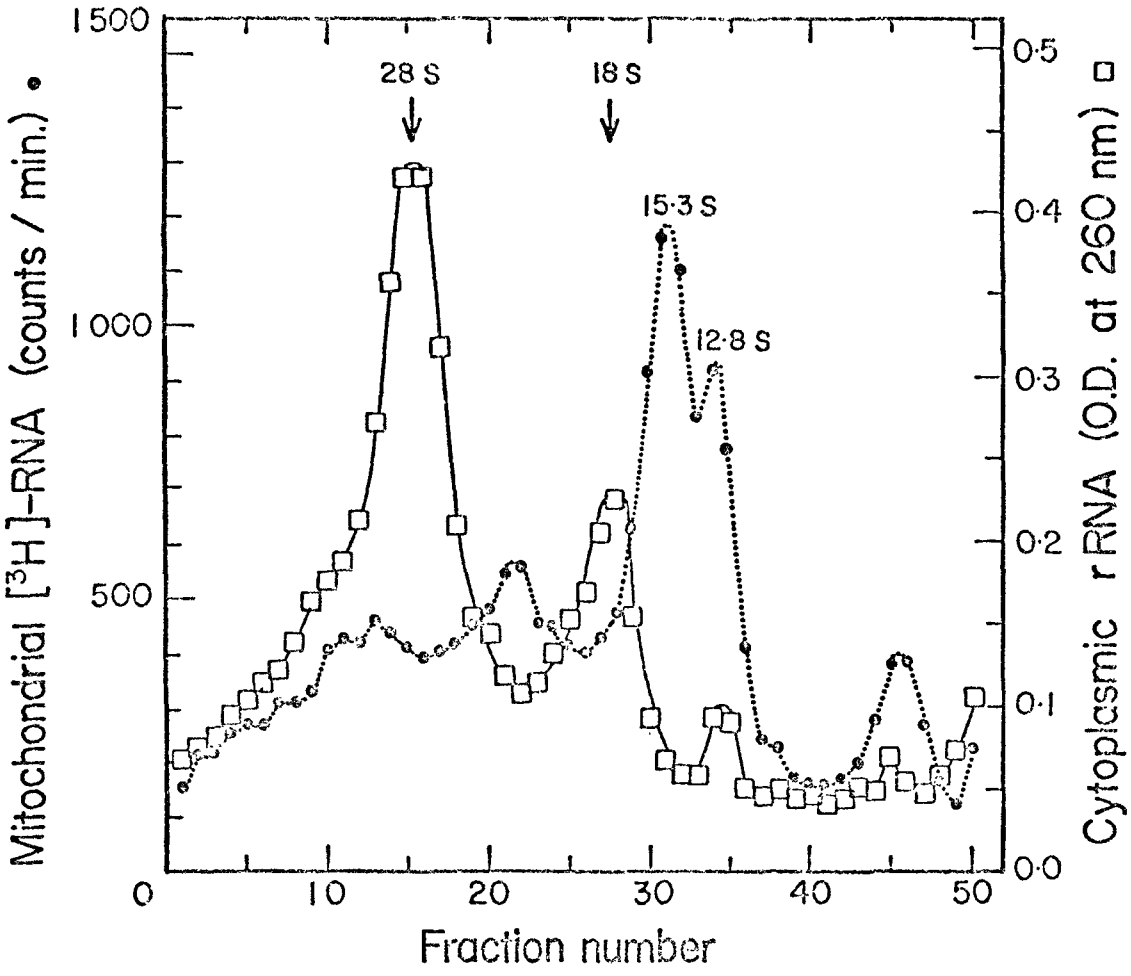

Figure 14. Separation of ^{3}H-labelled rat liver mit-RNA on a convex sucrose density gradient. RNA of four rats was labelled by intrperitoneal injection of a total of 5 mCi of [5-^{3}H]orotate 24 hours before the rats were killed. RNA was extracted from the purified mitochondria with 2 g of carrier rat liver by modification 1 of Kirby's method as described in the methods. RNA (400 µg) was centrifuged on an isokinetic sucrose density gradient and the E_{260} and the radioactivity of each fraction were determined.

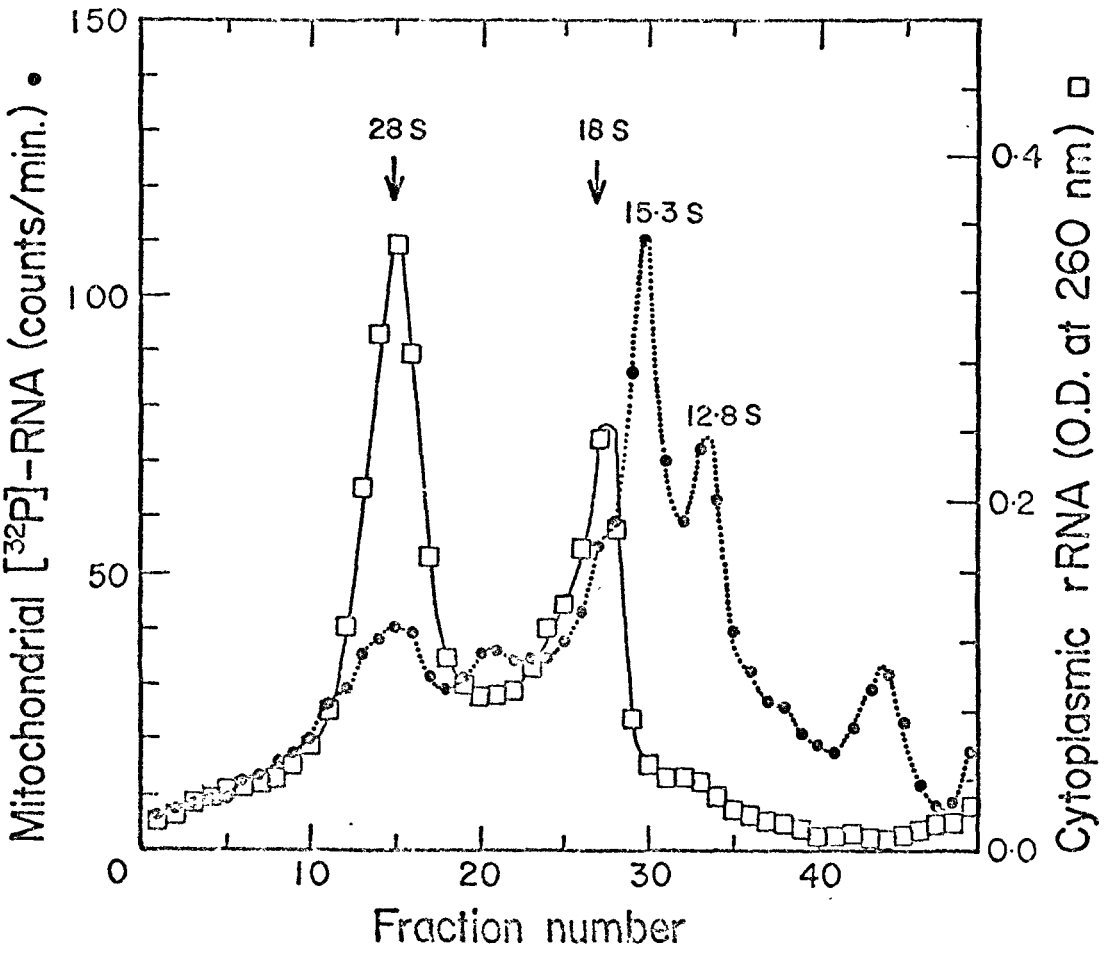

Figure 15. Separation of ^{32}P-labelled rat liver mit-RNA on a convex sucrose density gradient. RNA of four rats was labelled by intraperitoneal injection of a total of 10 mCi of $^{32}P_i$ 48 hours before the rats were killed. RNA was extracted from the purified mitochondria by modification 2 of Kirby's method as described in the methods. Sucrose density gradient centrifugation of 400 µg of RNA was as described in Figure 14.

completely excluded but was partially checked by recentrifugation of RNA fractions from the gradient to see if they, at least, were stable. In Figure 16 a typical analysis of mit-RNA on a sucrose density gradient is shown. RNAs from four fractions as indicated were precipitated with two volumes of ethanol after the addition of more carrier cyt-rRNA then recentrifuged on sucrose density gradients. The results are shown in Figure 17. Purified RNA components larger than 16 S were not degraded to 15.3 and 12.6 S RNA, nor were the latter degraded to smaller components, which indicated that once mit-RNA is isolated and separated by sucrose density gradient centrifugation, it is stable.

In one experiment an attempt was made to isolate unlabelled mit-RNA without carrier cyt-rRNA. Pure mitochondrial fraction was obtained from 106 rats containing 541 g liver and the mit-RNA was extracted by modification 1 of Kirby's method. No RNA precipitated when the aqueous layer was made 10% (v/v) with respect to m-cresol. In order to recover the RNA two volumes of cold ethanol were added. The precipitate obtained was mainly disodium naphthalene-1,5-dissulphonate. The precipitate was dissolved in 0.1 N sodium acetate pH 6.0 and the RNA was partially purified from the detergent by filtering the RNA-detergent solution through a Sephadex-G25 column. The RNA was precipitated from the first eluted fraction with two volumes of cold ethanol. When the RNA was analyzed on a sucrose density gradient no distinct species of RNA were obtained either because it was degraded or because the remainder of the detergent, which absorbs U.V. light, masked

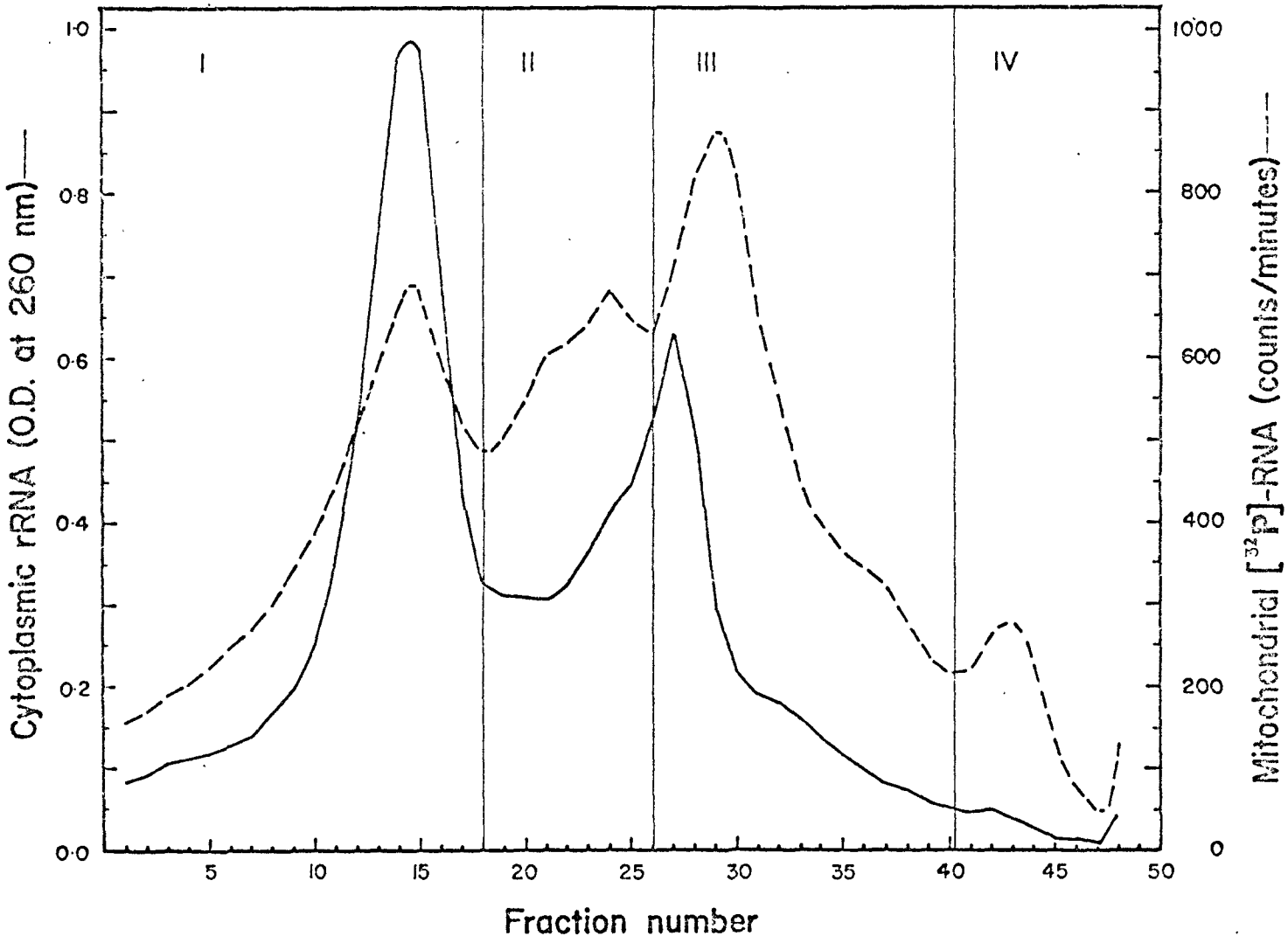

Figure 16. Fractionation of ^{3}H-labelled rat liver mit-RNA on a convex sucrose density gradient. Mit-RNA was extracted and analyzed on a sucrose density gradient as described in Figure 15. RNA from the four fractions as indicated of 3 parallel gradients was precipitated with two volumes of ethanol after addition of more carrier cyt-rRNA.

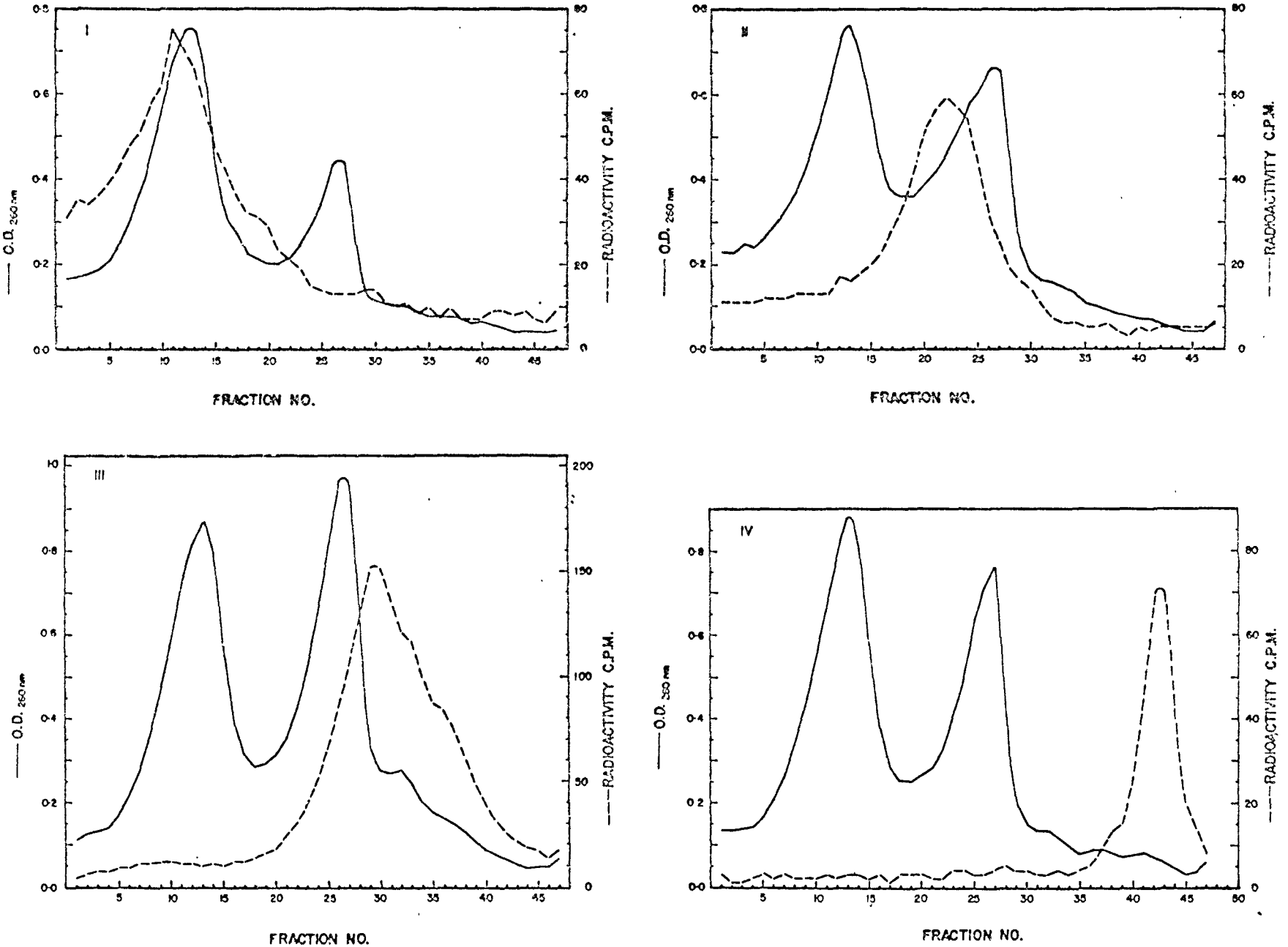

Figure 17. Re-centrifugation of Mit-RNA's fractions on convex sucrose density gradients. RNA obtained from fractions of sucrose density gradients (Figure 16) were re-centrifuged on similar gradients and analyzed for radioactivity and E_{260}.

its sedimentation profile. However, the nucleotide composition of the RNA was determined and the results are given below.

b. Sucrose density gradient centrifugation of L cell mit-RNA

Mit-RNA from L cells extracted with the cold phenol-SDS method from the crude mitochondrial fraction, of cells labelled with [^{3}H]uridine in the presence of 0.1 µg Actinomycin D/ml, sedimented on a linear sucrose density gradient at about 15.0 and 12.5 S as shown in Figure 18. No labelled 28 and 18 S cyt-rRNA was obtained which indicate that their synthesis was completely inhibited by the Actinomycin D. The synthesis of cytoplasmic rRNA is resistant to Actinomycin D (Perman, Vesco & Perman, 1968) but although the rRNA was probably present in the crude mitochondrial fraction, it was not extracted by the cold phenol-SDS method as would be expected from the selectivity of this method to the extraction of rRNA only. Thus, the results obtained from the indirect method are in good agreement with the results obtained directly from rat liver mit-RNA and therefore eliminate the possibility of effects of the antibiotic on the synthesis of mit-RNA or the possibility of observing other drug-resistant RNA species in addition to those of mitochondria.

The total nucleates extracted with the hot phenol-SDS method from the crude mitochondrial fraction of L cells, labelled in the presence of 0.1 µg Actinomycin D/ml, sedimented on a linear sucrose density gradient at about 15.0, 12.5 and 4 S as shown in Figure 19. It was more usual, however, to obtain a heterodisperse RNA throughout the sucrose density gradient and a component that

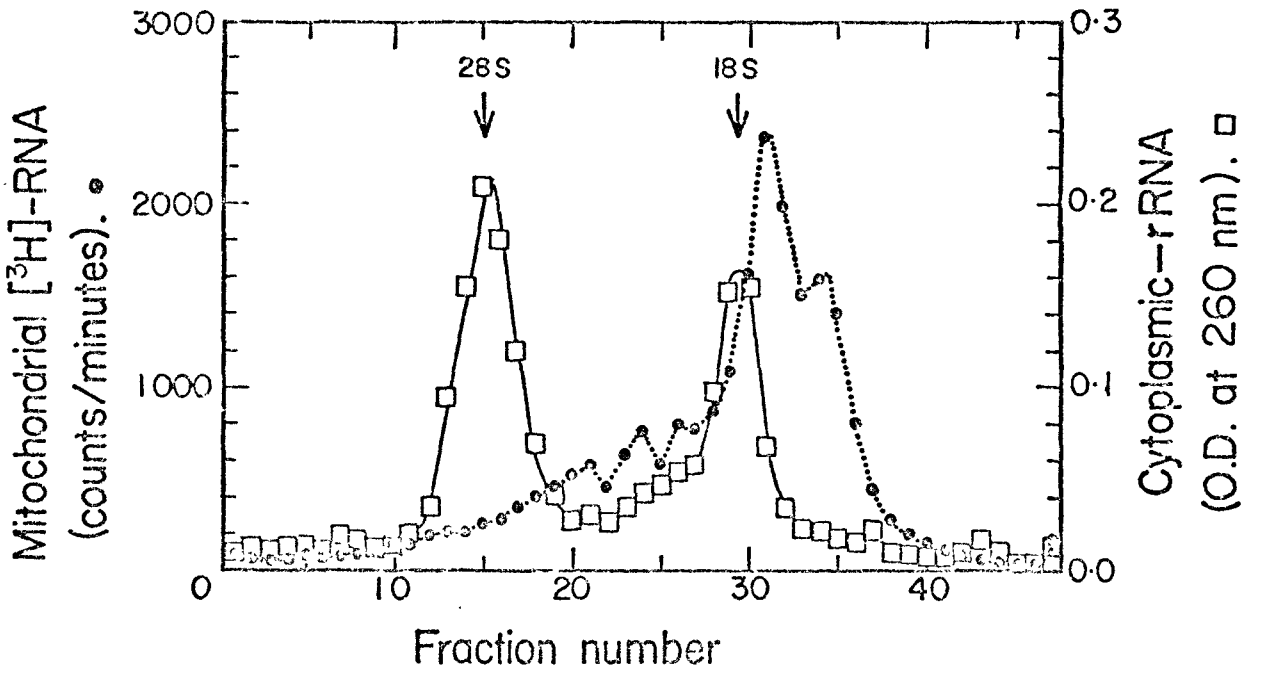

Figure 18. Separation of L cell mit-RNA, extracted by modification 2 of Kirby's method, on a linear sucrose density gradient. RNA of 2×10^8 cells was labelled with 10 µCi of [5-^{3}H]uridine/ml for 4 hours at a cell density of 5×10^6/ml in the presence of 0.1 µg Actinomycin D/ml. RNA was extracted by modification 2 of Kirby's method in the presence of L cell cyt-rRNA. RNA (200 µg) was centrifuged on a linear sucrose density gradient for 16 hours at 75,000 x g_{av} at 15°C.

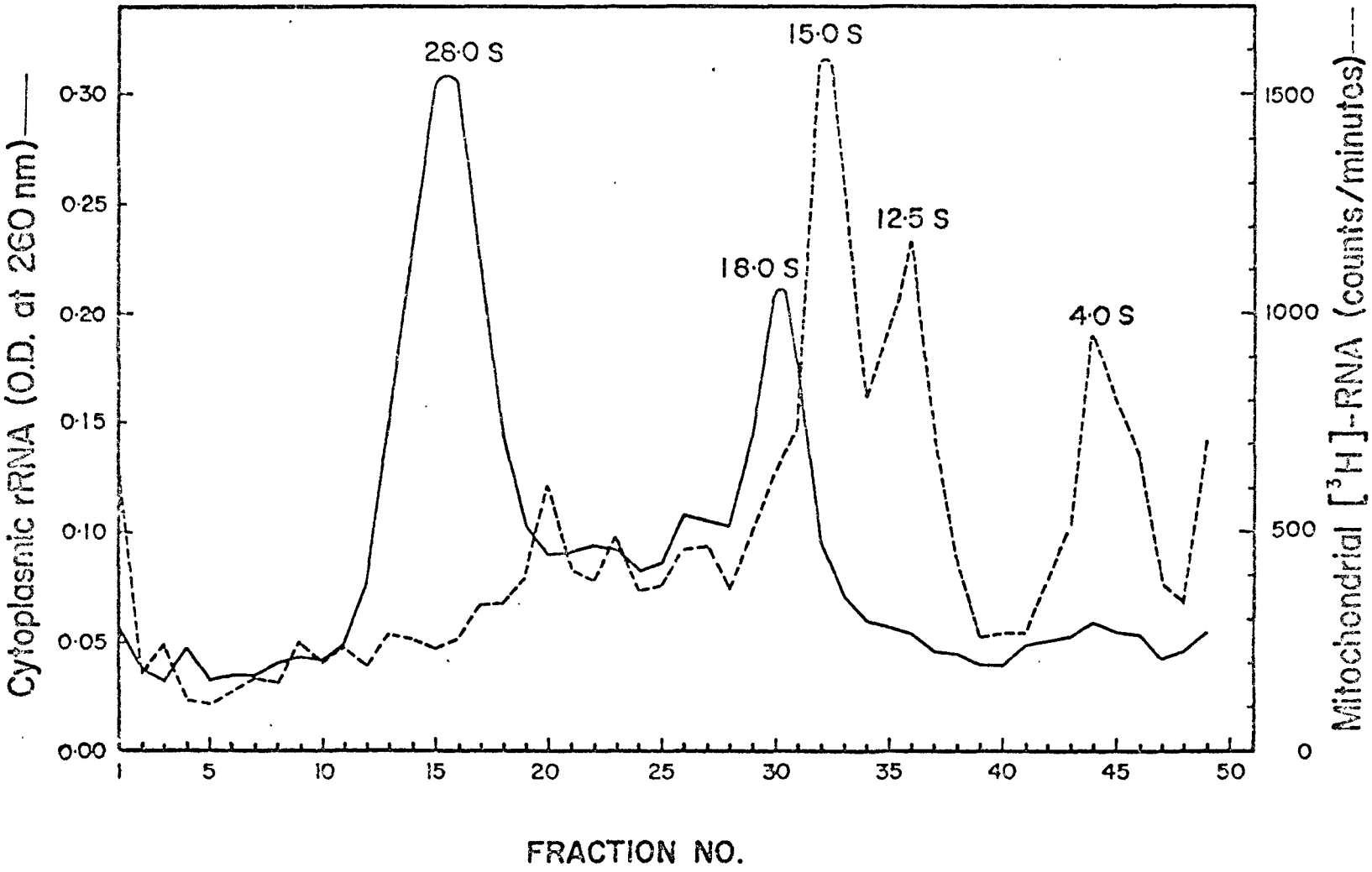

Figure 19. Separation of L cell mit-RNA, extracted by the hot phenol-SDS method, on linear sucrose density gradient. Methods were as for Figure 18 except that RNA of 5.4×10^8 cells were extracted by the hot phenol-SDS method.

sedimented at 21 S (Figures 20 and 21). The percentage of the 21 S component from the total labelled nucleic acid extracted by this method varied from one experiment to another (Figures 19-21). In this method of extraction (Figures 17, 20 and 21) the cytoplasmic carrier rRNA was usually more degraded when compared with the same RNA extracted with the modified Kirby method (Figures 14, 15 and 18). Figure 21 also shows that if cells were labelled in the presence of 1.0 µg of ethidium bromide, in addition to 0.1 µg of Actinomycin D/ml the synthesis of the 15.0 and 12.5 S RNA was inhibited completely while that of the heterodisperse and 4 S RNA was only partially inhibited.

The difference in the radioactive profile obtained from the hot phenol-SDS method and that of the cold phenol-SDS method suggests that the latter method extract mit-rRNA specifically. The fact that this RNA consists of two components of high molecular weight, found in any type of rRNA (Loening, 1968), strengthens this suggestion.

It should be noted that the S values calculated here for mit-rRNA are based on the 28.0 and 18.0 S values for the cyt-rRNA components as marker, these values were used by Kirby (1965). But these values vary from one investigator to another, for example, under essentially identical conditions of analysis, the sedimentation coefficients reported for the faster sedimenting rRNA component of both rabbit reticulocytes and rat liver vary from 24.5 to 30 S (Click & Tint, 1967).

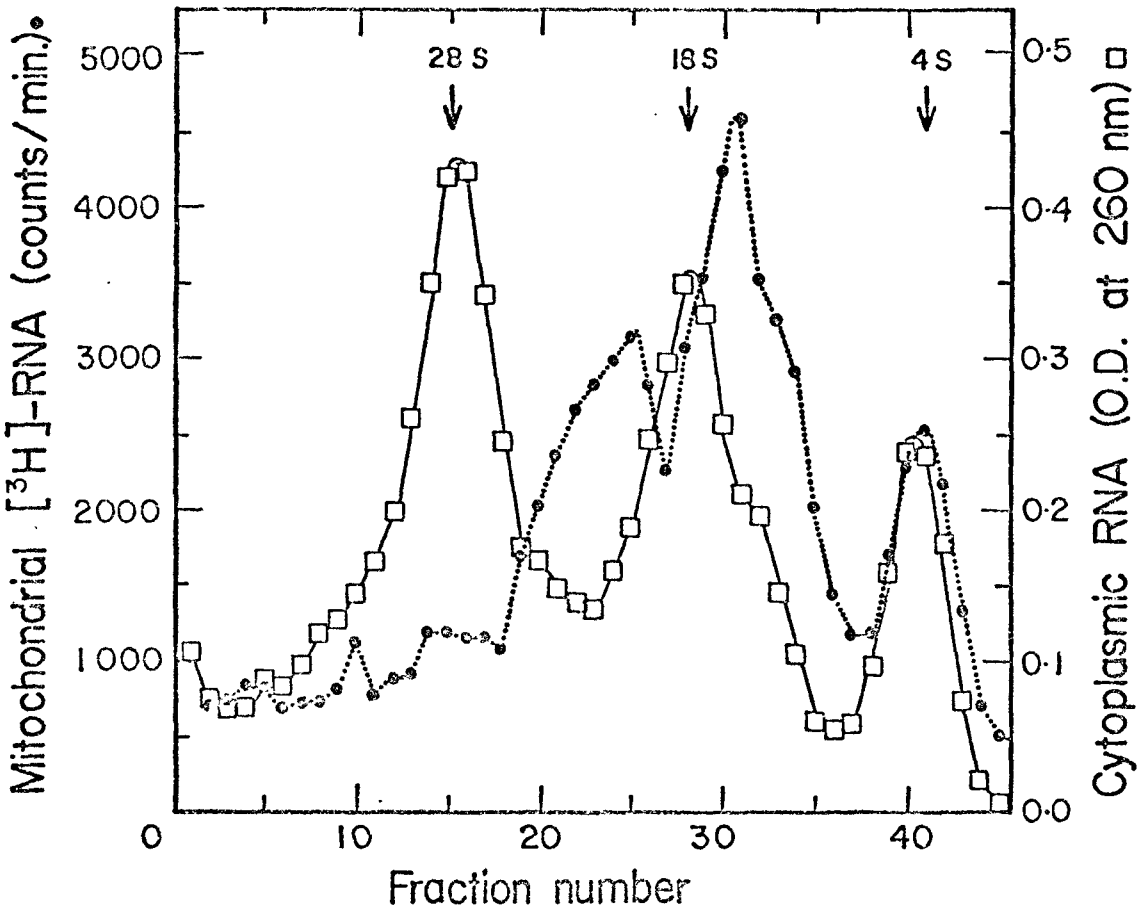

Figure 20. Sucrose density gradient centrifugation of L cell mit-RNA extracted by the hot phenol-SDS method. Legend as for Figure 19 except RNA was extracted from 1.8×10^8 cells in the presence of L cell cyt-RNA.

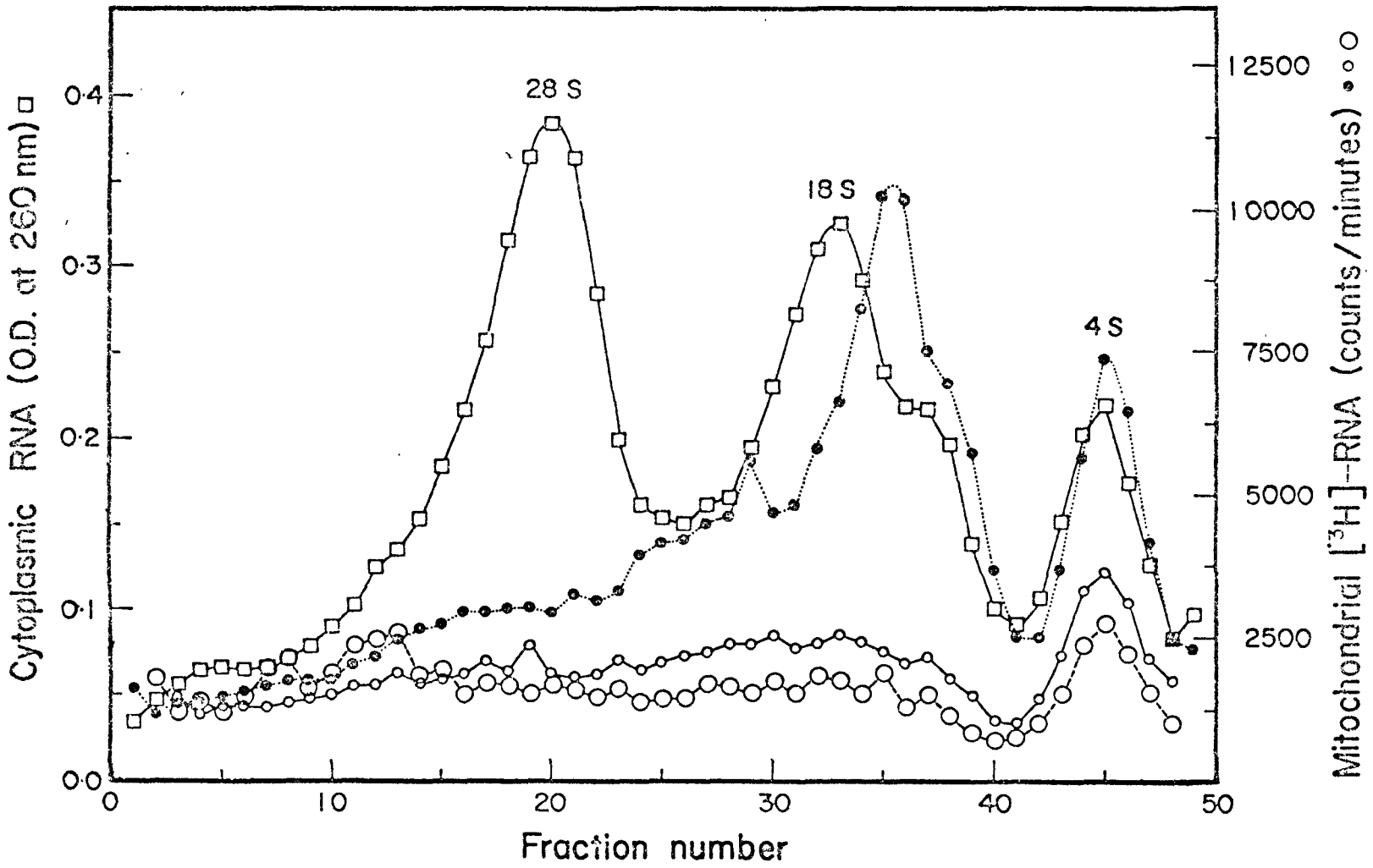

Figure 21. Sucrose density gradient centrifugation of RNA from L cell mitochondria extracted by the hot phenol-SDS method. Methods are as described for Figure 20. In one case cells were labelled in the presence of 1 µg and in another of 2 µg ethidium bromide/ml in addition to 0.1 µg of Actinomycin D/ml. □, L cells cyt-RNA; •, mit-RNA; o, mit-RNA from cells labelled in the presence of 1 µg ethidium bromide/ml; O, mit-RNA from cells labelled in the presence of 2 µg ethidium bromide/ml.

c. Agarose-polyacrylamide gel electrophoresis

Rat liver mit-RNA. Mit-RNA from rat liver extracted by the first or second modification of Kirby's method or the cold phenol-SDS method with rat liver cyt-rRNA as carrier, failed to enter the gel properly though the rat liver cyt-rRNA penetrated and separated as expected (Figure 22). Mit-RNA extracted with the cold phenol-SDS method in the presence of L cell cyt-rRNA as carrier, penetrated the gel and separated to two major components with average mobilities of 20.9 and 12.4 S_E compared with the 28.0 and 18.0 S_E cyt-rRNA, as shown in Figure 23. Because of the limit of accuracy values of 21 and 12.5 S_E will be used in subsequent discussion for rat liver mit-RNA. The ratio of the 21 S_E component to the 12.5 S_E component averaged 1.23 ± 0.03 as calculated from 4 electrophoretic runs and from 1 extraction of mit-RNA.

Mouse L cell mit-RNA. Mit-RNA from L cell extracted with the cold phenol-SDS method separated on the gel to two major components with average mobilities of 20.8 ± 0.2 and 12.4 ± 0.1 S_E as calculated from 6 electrophoretic runs and from 3 extractions (Figure 24). Again values of 21 and 12.5 S_E will be used for further discussion because of the limit of the accuracy. The ratio of the 21 S_E component to the 12.5 S_E component averaged to 2.08 ± 0.36 as calculated from 6 electrophoretic runs and from 3 extractions. As can be seen from Figure 24 the synthesis of mit-RNA were completely inhibited when cells were labelled in the presence of 1.0 µg of ethidium bromide in addition to the presence of 0.1 µg Actinomycin D/ml.

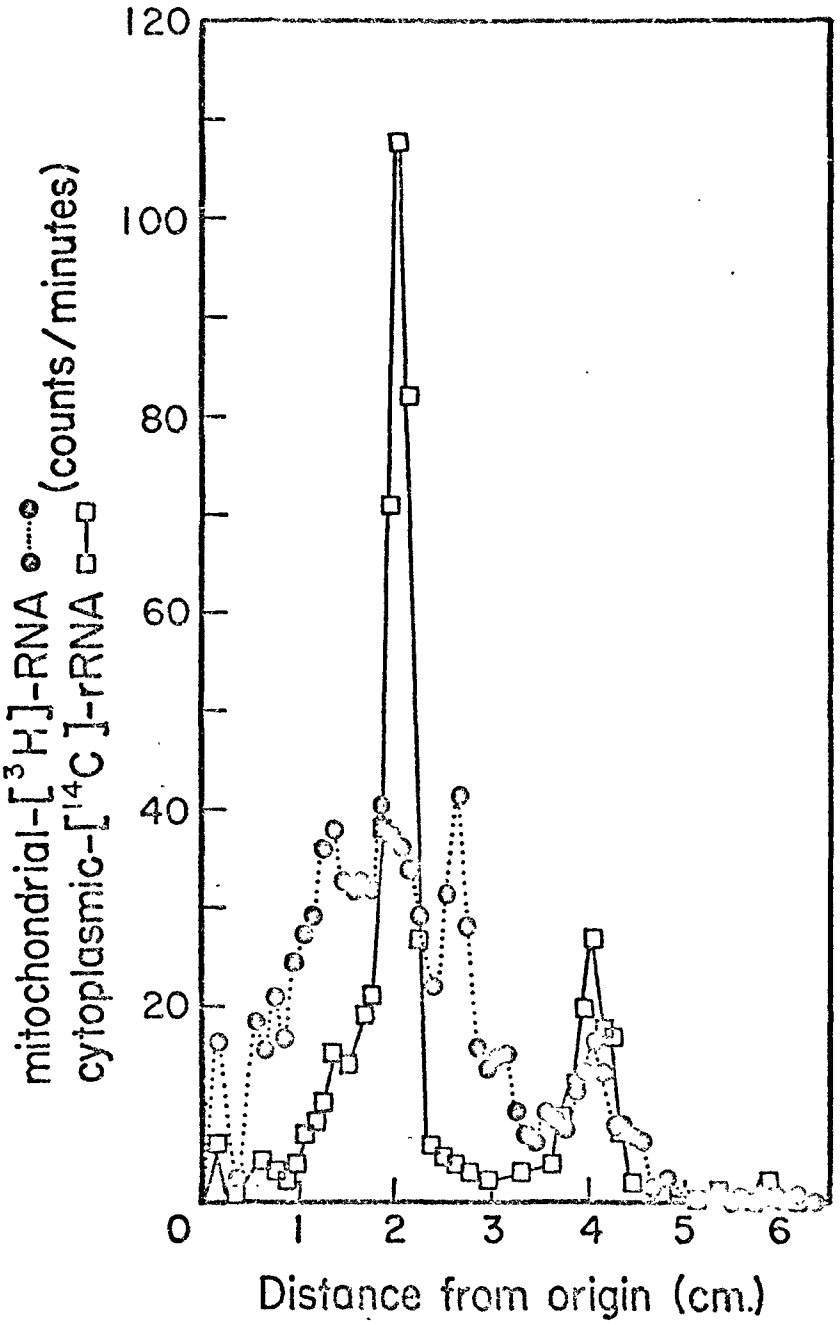

Figure 22. Gel electrophoresis of RNA from rat liver mitochondria extracted by the first modification of Kirby's method. RNA was labelled and extracted from mitochondria as described in Figure 5. RNA (30 µg) was electrophoresed as described in the methods.

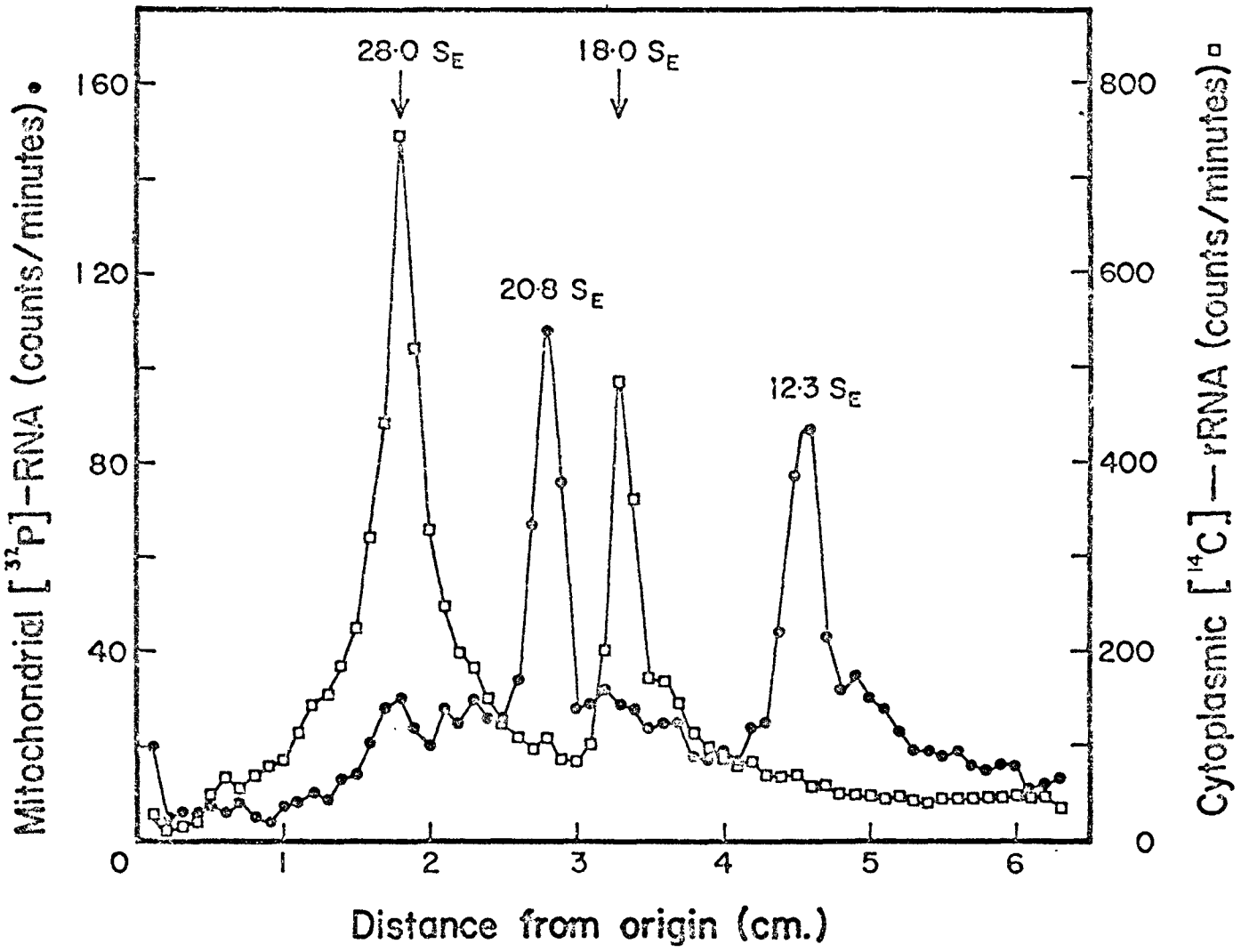

Figure 23. Separation of L cell cyt-rRNA and rat liver mit-RNA on agarose-polyacrylamide gel. RNA from rat liver mitochondria labelled with $^{32}P_i$ was extracted by the cold phenol-SDS method in the presence of L cell cyt-rRNA. [^{14}C] labelled L cell cyt-rRNA was added and subjected to simultaneously electrophoresis with the mit-RNA.

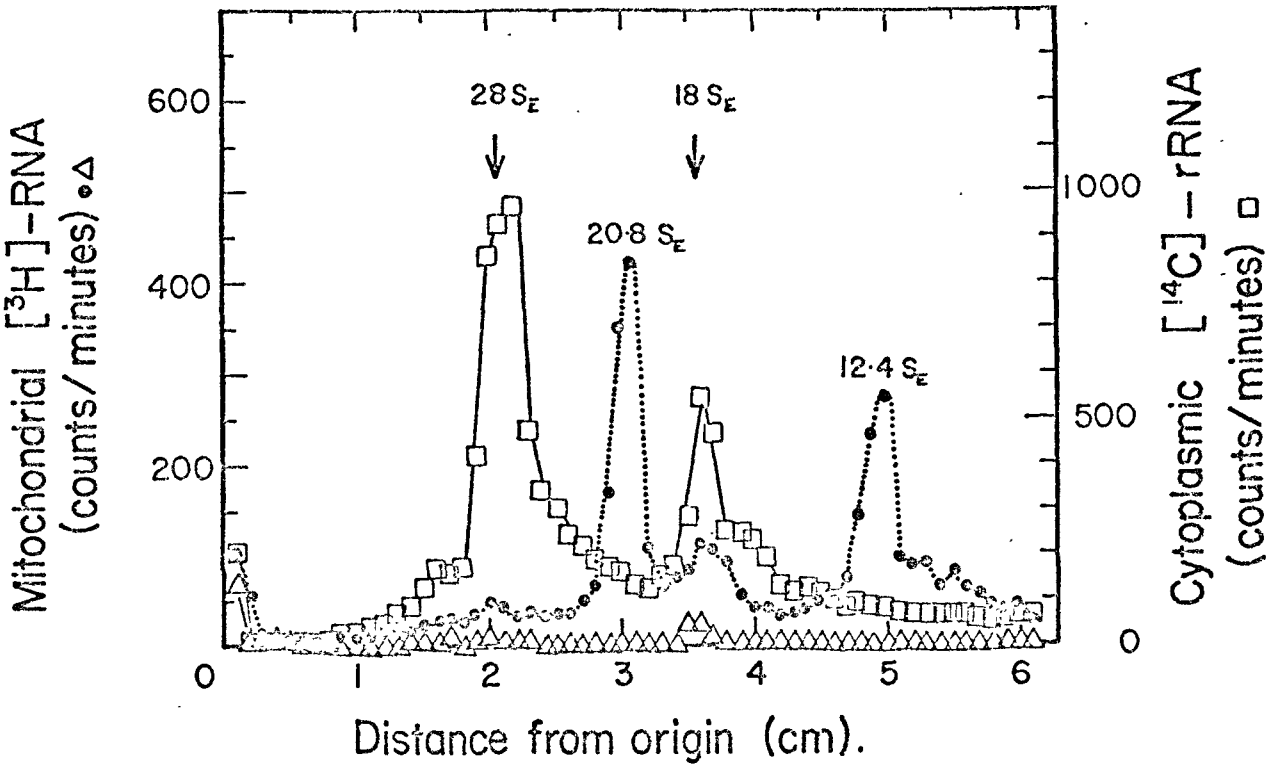

Figure 24. Gel electrophoresis of RNA from L cell mitochondria extracted by the cold phenol-SDS method. RNA was labelled and extracted from mitochondria of 1.8×10^8 cells in the presence of cyt-rRNA as described in the method. In one case mitochondrial RNA was labelled in the presence of 1 µg of ethidium bromide/ml in addition to 0.1 µg of Actinomycin D/ml. Cyt-rRNA labelled with [2-^{14}C]uridine was added as a marker to the sample of mit-RNA labelled in the absence of ethidium bromide. The mit-RNA labelled in the presence of ethidium bromide was subjected to electrophoresis separately. □, Cyt-rRNA; •, mit-RNA; △, mit-RNA from cells labelled in the presence of ethidium bromide.

Total nucleates isolated with the hot phenol-SDS method have the 21 and 12.5 S_E peaks but in addition a major peak at about 18-19 S_E appeared as shown in Figure 25. Further investigation showed that the 18-19 S_E component is DNA which originated from nuclear fragments found in the crude mitochondrial fraction. RNA extracted from mitochondria which had been purified by isopycnic sucrose density gradient step did not have this peak as shown in Figure 26. The 18-19 S_E component was present in the nuclear pellet obtained at the bottom of the tube on isopycnic centrifugation (Figure 26). The 18-19 S_E component was removed by incubating the mitochondrial fraction at 4°C for 4 hours or at 37°C for 10 minutes with 10 µg of RNase-free DNase/ml. This component was found to be resistant to pancreatic RNase and to 0.3 N KOH when compared with the 21 and 12.5 S_E components. The synthesis of the 18-19 S_E component was resistant to the incubation of the cells with ethidium bromide (1.0 µg/ml). The ratio of the 21 S_E component to the 12.5 S_E component when extracted with the hot phenol-SDS method was similar to the ratio obtained for these components when extracted by the cold phenol-SDS method and averaged 2.13 ± 0.38 calculated from 10 electrophoretic runs and from 4 extractions.

Because of the discrepancy between the sedimentation values of mit-RNA obtained from sucrose density gradient analysis and the mobility values obtained from the agarose-polyacrylamide gel electrophoresis analysis, an attempt was made to correlate the components. L cell mit-RNA components purified on sucrose density

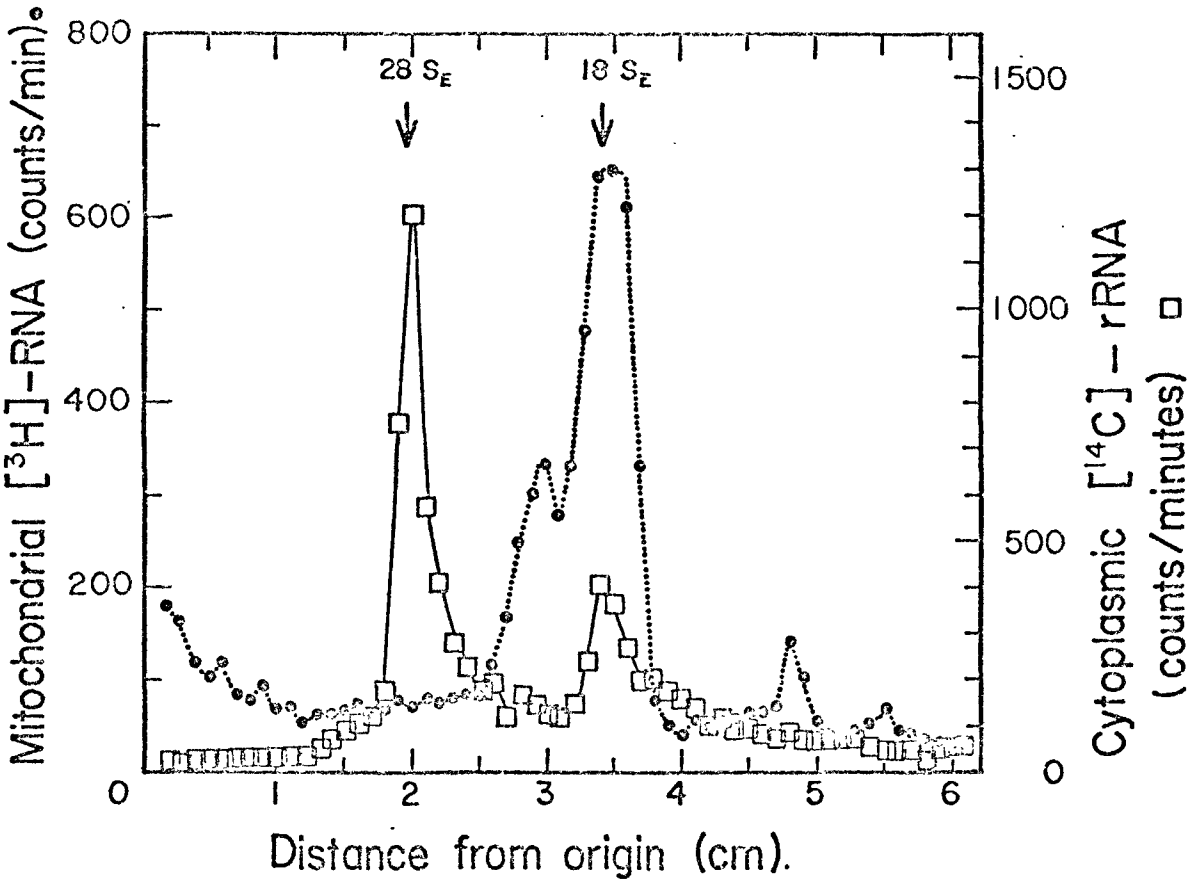

Figure 25. Gel electrophoresis of RNA from L cell mitochondria extracted by the hot phenol-SDS method. RNA was labelled and extracted from mitochondrial fraction as described in Figure 20 and subjected to electrophoresis as described in Figure 23. The cytoplasmic rRNA was subjected to electrophoresis in a parallel tube.

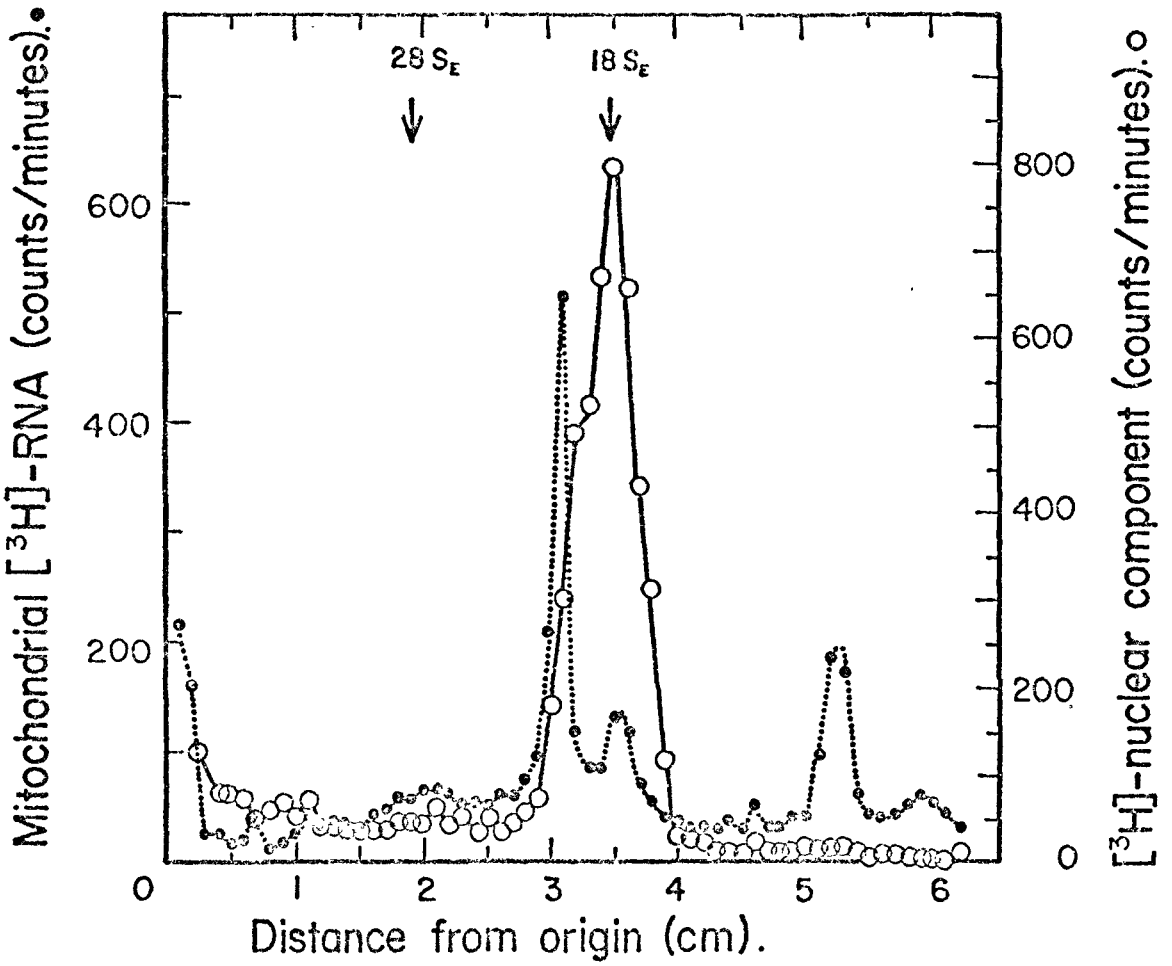

Figure 26. Gel electrophoresis of RNA isolated by the hot phenol-SDS method from the mitochondrial and nuclear fractions separated by isopycnic gradient centrifugation. The crude mitochondrial fraction from which the RNA was extracted and subjected to electrophoresis in Figure 25 was purified by isopycnic gradient centrifugation and RNA extracted by the hot phenol-SDS method from the resulting mitochondrial band and nuclear pellet. Electrophoresis was as described in Figure 23.

gradients were electrophoresed on agarose-polyacrylamide gels. The results indicated that the 12.5 S component ran at 12.5 S_E and the 15 S component at 21 S_E and the 21 S peak which appeared when total nucleate were extracted with the hot phenol-SDS method (Figure 20) ran at 18-19 S_E. Clearly the 21 S component was DNA, the possible nature of the retardation of the 15 S component will be considered in the Discussion.

These results, as with those obtained from the sucrose density gradient analysis, indicated that mit-RNA isolated indirectly from a crude mitochondrial fraction of L cells, labelled in the presence of 0.1 µg of Actinomycin D/ml, migrated on agarose-polyacrylamide gel electrophoresis at about the same S_E values as did mit-RNA isolated directly from the pure mitochondrial fraction of rat liver. This fact was further confirmed by running L cell mit-RNA labelled with [^{3}H]uridine, isolated with the hot phenol-SDS method and then treated with DNase simultaneously with rat liver [^{32}P]-labelled mit-RNA isolated with the cold phenol-SDS method. As shown in Figure 27, the 21 and the 12.5 S_E components of the two RNAs coincided. The only remarkable difference between the rat liver and L cell mit-RNA was the ratio of the 21 S_E to 12.5 S_E component as shown in Table 4.

Rat Hepatoma cell mit-RNA. [^{32}P]-labelled mit-RNA from hepatoma cells was isolated by the cold phenol-SDS method and analyzed on agarose-polyacrylamide gel electrophoresis. Two major components were obtained at 20.8 and 12.1 S_E as shown in Figure 28

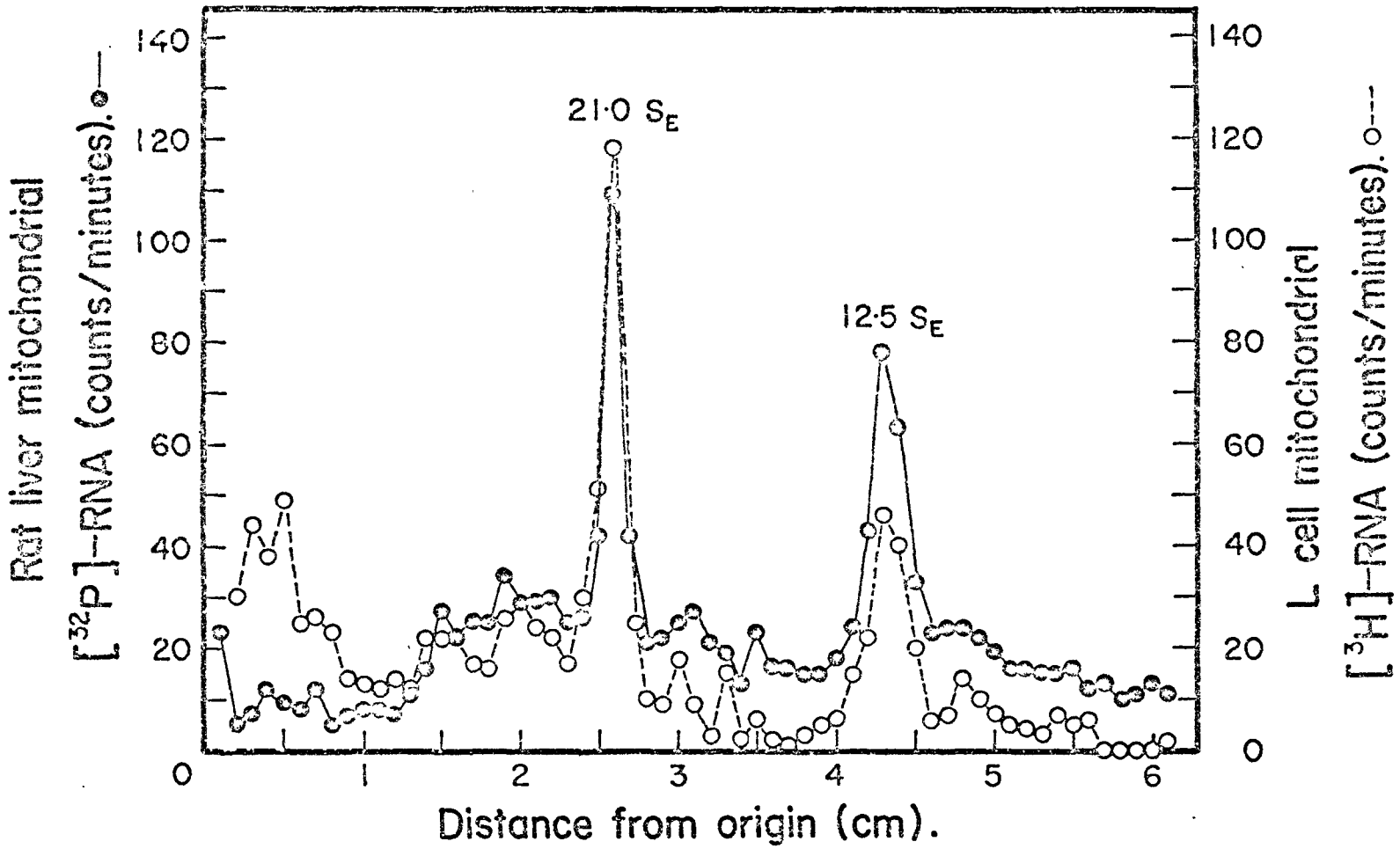

Figure 27. Gel electrophoresis of mit-RNAs from mouse L cell and rat liver. RNA from L cell mitochondria was labelled and extracted as described in Figure 20. Before electrophoresis the extract was incubated with 10 µg DNase/ml as described in the methods. Mit-RNA from rat liver was labelled and extracted as described in Figure 23. The two mit-RNAs were electrophoresed simultaneously.

Table 4

Analysis of Mit-RNAs on Agarose-Polyacrylamide Electrophoresis

Source of Mit-RNA labelling isotope RNA extraction method	Mouse L cell ^{3}H cold phenol-SDS	Mouse L cell ^{3}H hot phenol-SDS	Rat liver ^{32}P cold phenol-SDS	Rat Hepatoma cell ^{32}P cold phenol-SDS	Human KB cell ^{14}C hot phenol-SDS
Number of electrophoretic runs (no. of experiments)	6 (3)	10 (4)	4 (1)	2 (1)	7 (1)
Means S_E of the larger mit-RNA component ± 1 S.D.	20.8 ± 0.2	21.0 ± 0.2	20.9	20.8	20.4 ± 0.3
Mean S_E of the smaller mit-RNA component ± 1 S.D.	12.4 ± 0.1	12.1 ± 0.4	12.4	12.1	11.2 ± 0.2
Mean of the relative amount of the incorporation of the radioactive isotope of the larger to the smaller mit-RNA component ± 1 S.D.	2.08 ± 0.36	2.13 ± 0.38	1.23 ± 0.03	1.34	1.8 ± 0.09

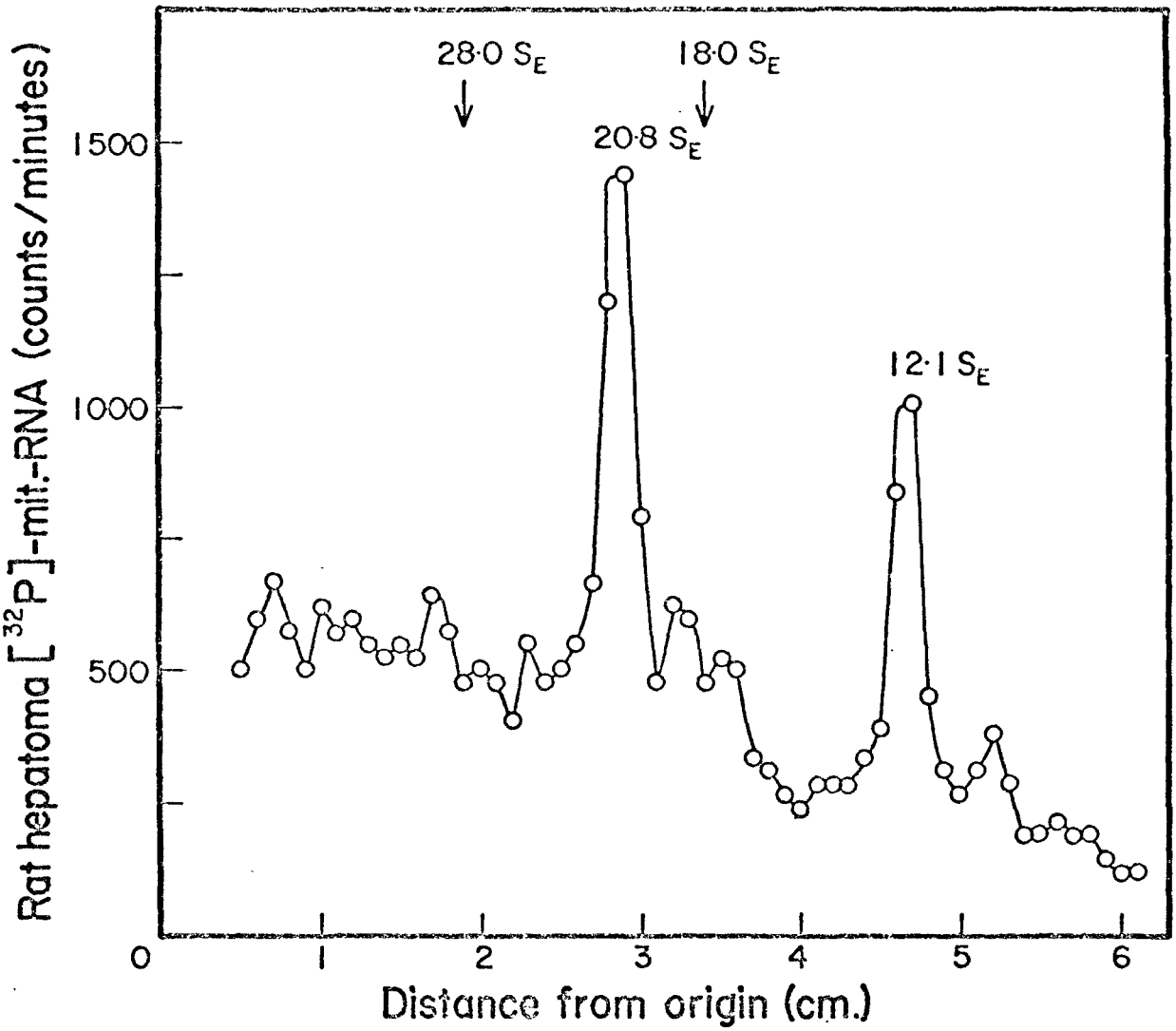

Figure 28. Gel electrophoresis of mit-RNA from rat hepatoma cells. RNA of 1.6×10^8 cells was labelled with 50 µCi of [^{32}P]P_i/ml for 4 hours at a cell density of 5×10^6/ml in suspension medium containing 0.1 mM NaH_2PO_4 in the presence of 0.1 µg of Actinomycin D/ml. RNA was extracted by the cold phenol-SDS method and analyzed (30 µg) on agarose-polyacrylamide gels.

that is close to if not the same as other rodent mit-RNA. The ratio of the 21 S_E component to the 12.1 S_E component was 1.34.

Human KB cell mit-RNA. Mit-RNA labelled with [^{14}C]uridine was isolated from human KB cells with the hot phenol-SDS method. Simultaneously electrophoresis with L cell mit-RNA showed components of 20.4 ± 0.3 and 11.2 ± 0.2 S_E as calculated from 1 extraction of the mit-RNA and from 7 electrophoretic runs (Figure 29). Because of the limit of accuracy values of 20.5 and 11.0 S_E respectively will be used. The ratio between the 20.5 S_E component and the 11.0 S_E component is 1.18 ± 0.09 calculated from 1 extraction of the mit-RNA and from 7 electrophoretic runs.

Comparison. An attempt at simultaneous electrophoresis of [^{3}H]-labelled mouse L cell mit-RNA, [^{32}P]-labelled rat liver mit-RNA and [^{14}C]-labelled human KB cell mit-RNA on one gel was made as shown in Figure 30. Though the gel was overloaded a clear separation was obtained between the rodent mit-RNA and the human KB mit-RNA.

The analytical results from agarose-polyacrylamide gel electrophoresis of mit-RNA, from the different tissues examined, are summarized in Table 4. Two points should be emphasized. First, all rodent mit-RNA whether extracted from the purified mitochondrial fraction, like in rat liver, or from a crude mitochondrial fraction, when cells were labelled in the presence of Actinomycin D, or whether extracted by the cold or the hot phenol-SDS method, the RNA contained two major components with mobilities of 21 and 12.5 S_E. On the other hand human KB cell

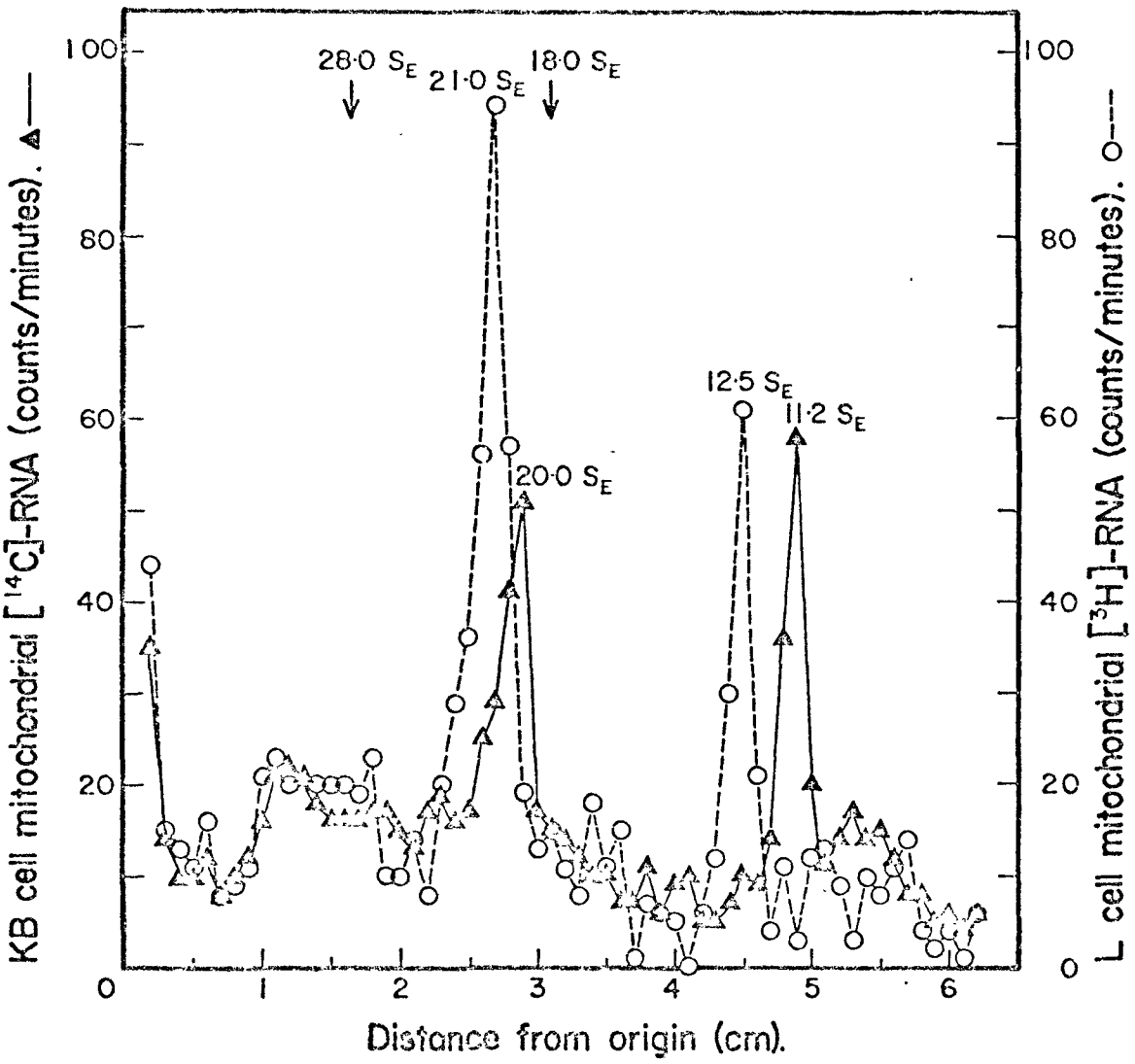

Figure 29. Separation of mit-RNAs from mouse L cell and from human KB cell on agarose-polyacrilamide gel. RNA from L cell mitochondria was labelled and extracted as described in Figure 27. RNA of 1.7×10^8 KB cells was labelled with 1 µCi of [2-^{14}C]uridine/ml for 4 hours at a cell concentration of 5×10^6/ml in the presence of 0.1 µg Actinomycin D/ml. RNA from KB mitochondria was extracted by the hot phenyl-SDS method then treated with 10 µg DNase/ml. The two mit-RNAs were electrophoresed simultaneously.

Figure 30. Gel electrophoresis of mit-RNAs from mouse L cell, rat liver and human KB cells on agarose-polyacrylamide gel. Mit-RNAs from L cell, KB cell and rat liver were labelled and extracted as described in Figures 27, 29 and 23 respectively. These RNAs were electrophoresed simultaneously and the radioactivity was determined. ▲, KB cell mit-RNA; ●, rat liver mit-RNA; ○, L cell mit-RNA.

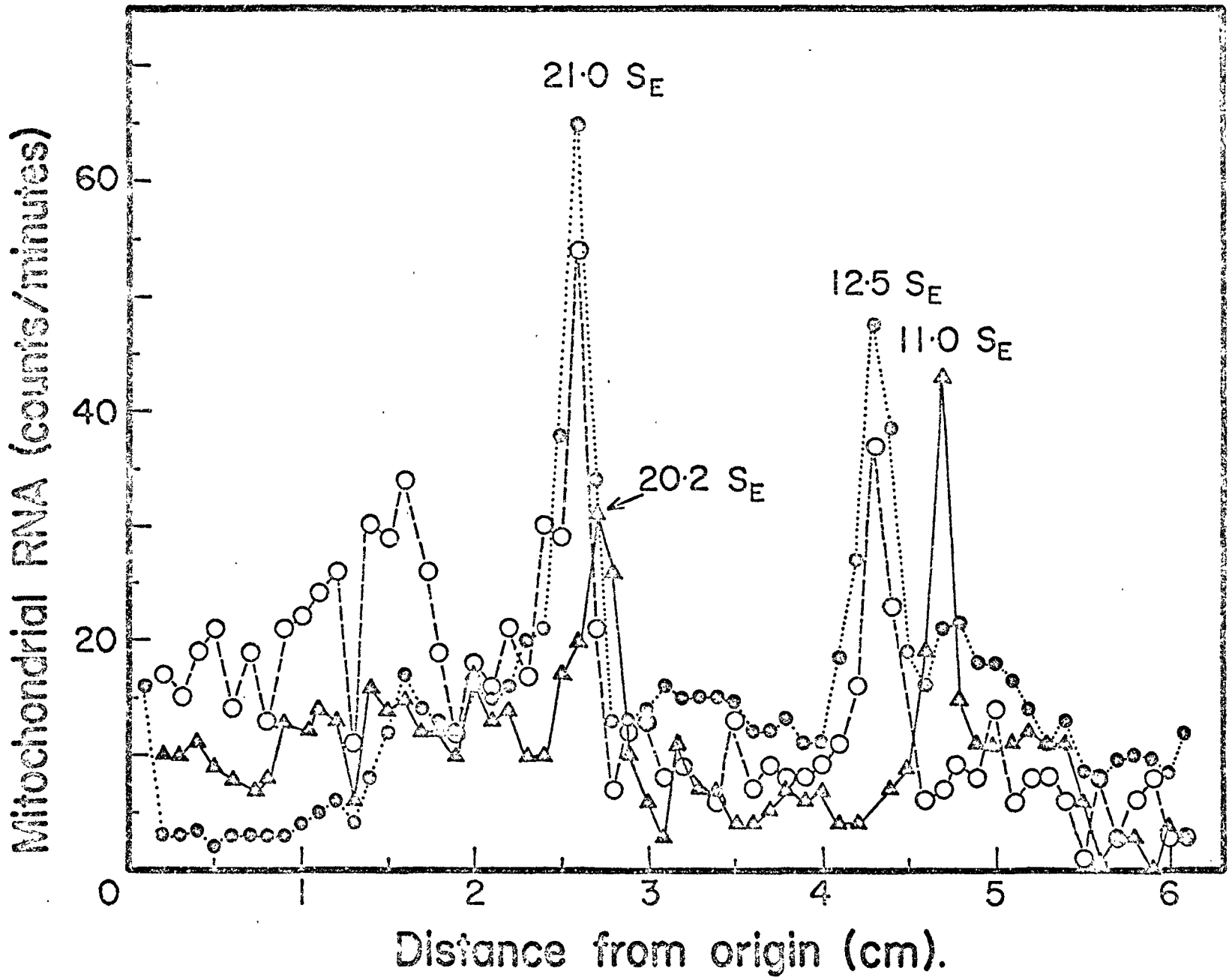

Figure 30. Gel electrophoresis of mit-RNAs from mouse L cell, rat liver and human KB cells on agarose-polyacrylamide gel.

mit-RNA migrated faster at 20.5 and 11.0 S_E. Secondly the ratio of the larger component to the smaller one in all tissue is just above 1 except for L cell where this ratio found to be over 2, whether the RNA was extracted by the cold or hot phenol-SDS method.

d. Chromatography on MAK columns

The elution profile of L cell mit-RNA, extracted with the cold phenol-SDS method, is shown in Figure 31. The bulk of the radioactivity was eluted with a peak at 0.95 M NaCl compared with cyt-rRNA which eluted at 0.89 M. No tRNA or DNA would be expected when RNA is extracted with the cold phenol-SDS method, but a small "DNA" peak was eluted at 0.66 M NaCl. A similar DNA peak has been observed by Billing & Barbiroli (1970), which they thought was a DNA-like RNA. Since 100% of the radioactivity was recovered from the column after elution of 150 fractions and since no messenger RNA (mRNA) is expected when rRNA is extracted with the cold phenol-SDS method, no attempt was made to elute mRNA which sticks to the column and can be eluted with SDS (Ellem, 1966) or with increasing amount of heat and therefore is called temperature dependent RNA (TD RNA) (Ellem & Sheridan, 1964). The recovery of RNA in various fractions as well as the result of MAK column chromatography of mit-RNA extracted with the hot phenol-SDS method is given in Table 5. Although the amount of mitochondrial high molecular weight RNA was extracted with both methods was about the same, 130,000 counts per minute (cpm) from the hot method and 116,000 cpm for the cold method, 58.5% of the RNA extracted with the hot method remained on the column. This corresponds to the

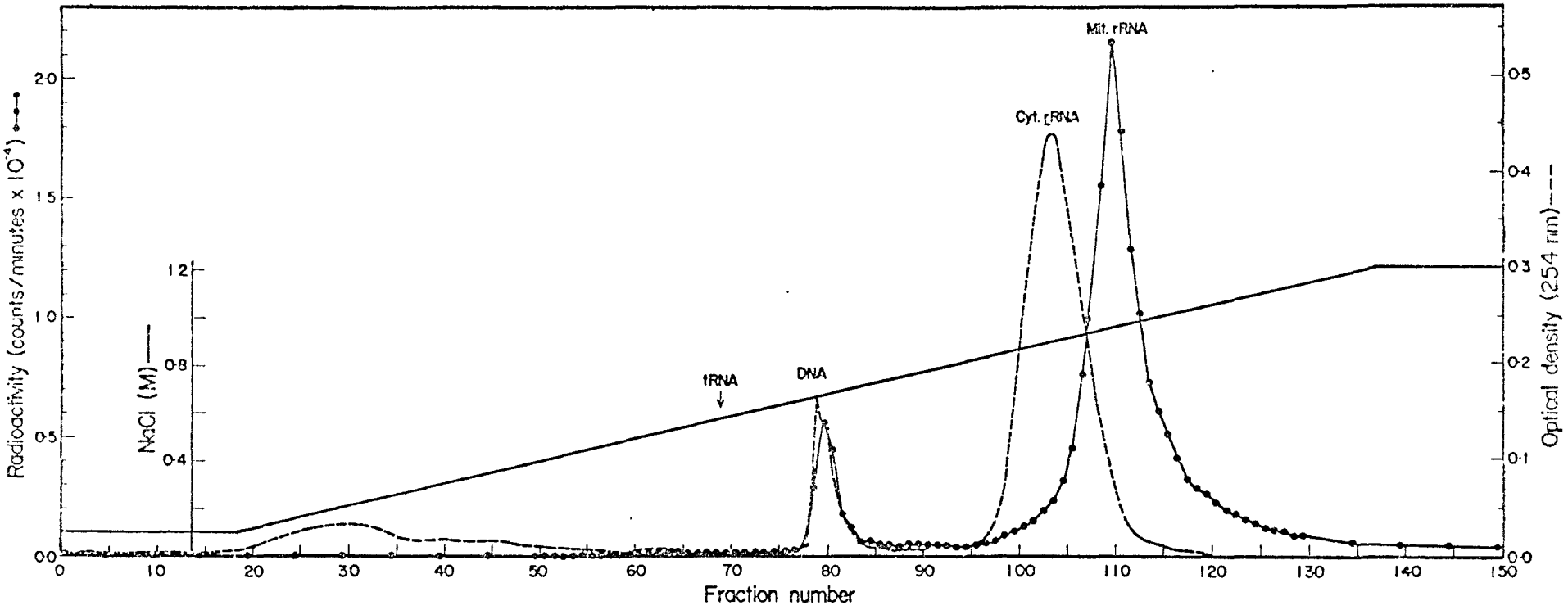

Figure 31. MAK column chromatography of mouse L cell mit-RNA. RNA of 8×10^8 cells was labelled as described in Figure 20. RNA was extracted from the crude mitochondrial fraction by the cold phenol-SDS method in the presence of cyt-rRNA. RNA (4 mg) was chromatographed and the E_{254} and the radioactivity of each fraction were determined as outlined in the methods.

Table 5

Elution of Radioactive Nucleates From MAK Columns

Extraction method	Recovery (per cent of total)							
	to tRNA	tRNA	to DNA	DNA	to Mit. RNA	Mit. RNA	after Mit. RNA	left on the column
Cold phenol-sodium dodecyl sulfate	--	--	2.6	9.0	6.4	70.8	11.1	0
Hot phenol-sodium dodecyl sulfate	2.9	2.9	1.3	5.8	6.1	20.8	1.5	58.5

Note: Each column fraction refers to the portion eluted by increasing concentrations of NaCl and is delineated by a particular nucleate. Thus the columns to tRNA, to DNA and to Mit-RNA refer to the percentage of counts eluting up to these compounds starting from the previous compound. A total of 194,000 cpm for the cold phenol-SDS extracted RNA and 554,000 cpm for the hot phenol-SDS extracted RNA were applied chromatographed.

heterodisperse TD RNA or mRNA that is synthesized by mammalian cells in the presence of low concentration of Actinomycin D (Penman, Vesco & Penman, 1968) and can be eluted only by drastic treatments as mentioned above.

In order to prove that the major peak obtained on the MAK column is mit-RNA, fractions from the gradient were collected, dialyzed, lyophilyzed then characterized by agarose-polyacrylamide gel electrophoresis as shown in Figure 32. The characteristic 21 and 12.5 S_E RNA species are seen in RNA obtained from fractions 105-115 and a 19 S_E peak was seen from nucleate obtained from fraction 78-83. The latter observation indicates that this radioactive peak could be DNA. A background radioactivity was observed on the gel (fraction 105-115). This could have arisen either from difficulty of the RNA penetrating the gel or to some degradation during the many steps in handling.

Since the analysis of mit-RNA by sucrose density gradient and by agarose-polyacrylamide gel electrophoresis indicated that the molecular size of this RNA is smaller than that of the cyt-rRNA, the result obtained by the MAK column where mit-RNA was eluted in a higher NaCl concentration than the cyt-rRNA, was unexpected. Characteristics other than the molecular size of the mit-RNA are probably responsible for its stronger adsorption to the column, and their nature will be considered in the Discussion. However, the above three analytical methods which characterized the physical properties of the mit-RNA indicate that the 15 and 13 S or the 21.0 and 12.5 S_E RNA components are probably the mit-rRNA

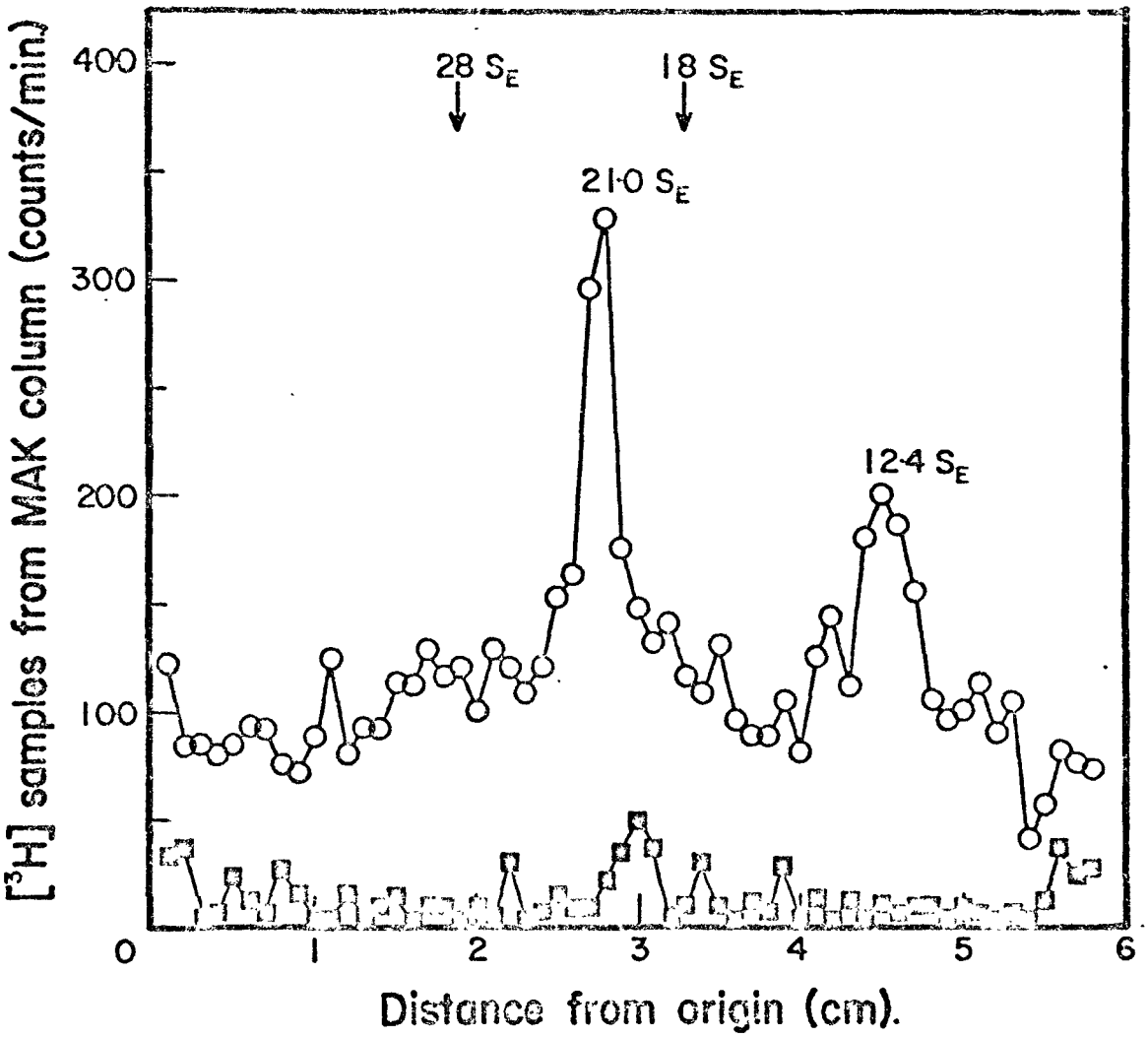

Figure 32. Gel electrophoresis of mouse L cell nucleates obtained from fractions eluted from MAK column. Mit-RNA from L cell was chromatographed on MAK column as described in Figure 31. Fractions from the NaCl gradient were collected, dialyzed, lyophilyzed and electrophoresed as described in the method. O. Profile of nucleate obtained from fractions 105-115; ■, profile of nucleate obtained from the fractions 78-83.

species for the following reasons:

1. They are extracted with the cold phenol-SDS method which is specific for ribosomal RNA even though mit-tRNA, DNA or hetero-disperse RNA, which were also extracted by the hot phenol-SDS method, were present in the crude mitochondrial fraction.
2. Ribosomal RNA isolated from any organism, so far reported, consists of 2 stable, well defined large and small components (Loening, 1968).
3. These components were eluted from a MAK column at a NaCl concentration at which rRNA is expected.

The only strong argument against the possibility that these RNA components are not mit-rRNA is that perhaps they actually are cyt-rRNA contaminants which in the process of isolation of mitochondrial fraction undergo physical changes like degradation which in turn yield the observed results. In order to eliminate this possibility the nucleotide compositions of these RNA components were examined. If these RNA components originated from cyt-rRNA, they have to contain the same nucleotide composition but if their nucleotide composition is different than that of the cyt-rRNA then mit-rRNA are unique species not derived from cyt-rRNA.

e. Nucleotide composition

The nucleotide composition of mit-RNA labelled with [^{32}P] and extracted with either modification 1 or 2 of Kirby's method or with the cold phenol-SDS method was determined by paper electrophoresis. The results from the analysis of rat liver

and mouse L cell mit-RNA compared with the nucleotide composition of the cyt-rRNA from these tissues obtained by Kirby (1965) and Lane & Tamaoki (1967) are given in Table 6. The determination of the nucleotide composition by [^{32}P]-labelled mit-rRNA was satisfactory because the result with direct nucleotide analysis of cold rat liver mit-rRNA obtained with a Dowex 50-H^+ column according to Katz & Comb (1963) was almost identical (Table 6). It is obvious from these results that the nucleotide composition of mit-RNA is fundamentally different from that of the cyt-rRNA and therefore mit-RNA cannot derive from it. Mit-rRNA from mouse L cells and rat liver and rat hepatoma contain more adenylate + uridylate and less guanylate + cytidylate than cyt-rRNA. On the other hand there are different nucleotide compositions for the 3 mit-rRNAs. The higher values of G and C and lower values of A and U in rat liver mit-rRNA could be due to cytoplasmic contamination. As shown in Table 7 the 19-8 S fraction (from Figure 16) did have values that approached closer to those of the tissue culture cells than that of the bulk mit-rRNA used in that experiment. However they were still not the same, leaving the possibility that mit-rRNA varies from species to species and from normal to malignant tissue. Differences in labelling techniques (48 hours for rat liver, 4 hours for tissue culture cells) or the contamination of tissue culture mit-rRNA with mRNA could also account for the results.

The main conclusion from Table 6 is that mit-rRNA is not derived from cyt-rRNA.

Table 6

The Nucleotide Composition of rRNA From Rat Liver, Rat Hepatoma Cell and Mouse L Cell Cytoplasm and Mitochondria

Source of rRNA ( ) No. of extractions	Nucleotide (moles %) Uridylate (U)	Adenylate (A)	Guanylate (G)	Cytidylate (C)	G+C
Rat liver cytoplasm					
- 28 S	17.0	17.8	33.0	32.2	65.2
- 18 S	18.0	19.8	32.4	29.8	62.2
Mouse L cell cytoplasm					
- 28 S	16.0	17.5	35.3	30.8	66.1
- 18 S	19.3	23.0	29.8	27.5	57.3
Rat liver mitochondria					
- ^{32}P (5)	23.2 ± 1.0	30.3 ± 1.2	21.1 ± 2.5	25.3 ± 1.5	46.4
- unlabelled	22.4	29.2	24.3	24.0	48.3
Rat hepatoma cell mitochondria					

- ^{32}P	27.5	31.5	16.4	24.5	40.9
Mouse L cell mitochondria					
- ^{32}P	26.3	34.1	17.4	22.2	39.6

Note: The values for the rat liver cyt-rRNA are from Kirby (1965) and those for L cell cyt-rRNA from Lane & Tamaoki (1967). Analysis of mitochondrial RNA by paper chromatography (Lane, 1963) gave identical results to those given above which were obtained by paper electrophoresis.

Table 7

Nucleotide Composition of Fractions of Rat-liver Mitochondrial RNA Separated By Sucrose Density Gradient Centrifugation

RNA labelled with $^{32}P_i$ isolated by the second modification of the method of Kirby, fractionated on a sucrose density gradient and the nucleotide composition determined. The nucleotide composition of original RNA and the calculated recovery using the percentage of RNA in each fraction are also given.

		Nucleotide (moles %)			
Fraction	RNA percent of total	uridylate	adenylate	guanylate	cytidylate
original RNA		23.5	29.6	20.1	26.8
39-25 S	28.5	22.4	27.9	23.0	26.7
25-19 S	25.5	21.3	27.6	23.5	27.6
19-8 S	39.9	22.4	30.9	19.9	26.7
8-1 S	6.2	24.3	32.6	19.0	24.0
Recovery		22.2	29.3	21.7	26.8

4. Molecular Weight

The molecular weight of the 21.0 and 12.5 S_E mit-rRNA components was calculated to be 0.96×10^6 and 0.33×10^6 daltons respectively, compared with 1.7×10^6 and 0.70×10^6 daltons of the 28.0 and 18.0 S_E cyt-rRNA markers respectively. This calculation was done according to the interpolation method of Loening (1968) and Peacock & Dingman (1968), based on the fact that the logarithm of the molecular weight of the RNA is linearly related to the distance of its mobility in the agarose-polyacrylamide gel electrophoresis (Figure 3). On the other hand the molecular weight of the 15.3 and 12.8 S mit-rRNA components was calculated from their sedimentation distance to be 0.49×10^6 and 0.33×10^6 daltons respectively. These data were obtained by interpolating the molecular weight of mit-rRNA compared with those of the cyt-rRNA based on the fact that the logarithm of the molecular weight of the RNA being linearly related to the logarithm of the distance of its sedimentation in the convex sucrose density gradient (Figure 2). The possible explanation for the discrepancy between the sedimentation and the mobility results, in the case of the larger mit-rRNA component, will be considered in the Discussion.

5. Mitochondrial Ribosomes

Having established the presence of a ribosomal-type of RNA in mitochondria, it was necessary to show the presence of mitochondrial ribosomes and that the mit-rRNA could be extracted from them. Mitochondrial ribosomes were isolated after lysis of

mitochondria with DOC and Triton-X100 and RNA was extracted from ribosomal peaks by the cold phenol-SDS method. The rational and and the problems were as follows:

Since mitochondrial ribosomes could be attached to the mitochondrial inner membrane or located in the mitochondrial matrix it was necessary to dissolve this membrane without affecting the complex structure of mitochondrial ribosomes. In addition in order to maintain the native structure of the ribosomal complex the ribosomes have to be isolated in a buffer with the right ionic strength and ratio of monovalent ions to divalent ones (for review, Peterman, 1968). An initial indication that mitochondria contain ribosome-like particles would be the presence in mitochondrial lysates of particles which sediment in sucrose density gradients in the range of known monomeric ribosomes. From a variety of isolation buffers which were tried for the isolation of mitochondrial ribosomes only the TKM buffer was successful. It was important that this buffer had a ratio of 10 between the monovalent to the divalent ions, a high concentration of Mg^{2+} (6mM). The presence of 6mM Mg^{2+} in the lysing medium caused a problem, since Mg^{2+} concentrations over 2mM form insoluble complexes with 0.5% (w/v)of sodium DOC which is usually used for isolation of cytoplasmic ribosomes. In order to overcome this problem it was found that if the mitochondrial fraction was first dissolved with 0.5% (w/v) Triton X100 and then made 0.5% (w/v) with sodium DOC then the insoluble magnesium DOC complex did not form.

As with mit-RNA, it was necessary to identify the mitochondrial ribosomes by labelling and excluding cytoplasmic contamination either by isolating pure mitochondria from rat liver or labelling L cells in the presence of 0.1 µg Actinomycin D/ml.

Ribosomes of rat liver were labelled by the intraperitoneal injection of four 100 g rats with a total of 5 mCi of [5-^{3}H]orotic acid 48 hours before the rats were killed. The ribosomes of about $2x10^{6}$ mouse L cells were labelled with 10 µCi of [5-^{3}H]uridine/ml for 4 hours at a cell density of $5x10^{6}$/ml.

In the process of isolating mitochondrial ribosomes, the RNase inhibitors Macaloid and SDS were not used because of their possible effect on ribosomal proteins. The SDS especially would disrupt ribosomes. Thus, although the characteristic sedimentation coefficient of mitochondrial ribosomes might not be affected by one or two nicks in the rRNA, it might not be possible to obtain native mit-rRNA from the ribosomes.

Rat liver mitochondrial ribosomes

In Figure 33 the sedimentation profile of a mitochondrial lysate on a convex 5-20% sucrose-TKM density gradient is shown. The sedimentation profile of the dialyzed [^{3}H]-labelled mitochondrial ribosomes on a convex 5-20% sucrose-TKM density gradient in the presence of [^{14}C]-L cell cytoplasmic ribosomes is shown in Figure 34. Two major components were observed, and their sedimentation coefficient were calculated by interpolation to be 55 and 35 S compared with the 76, 60 and 40 S L cell cytoplasmic

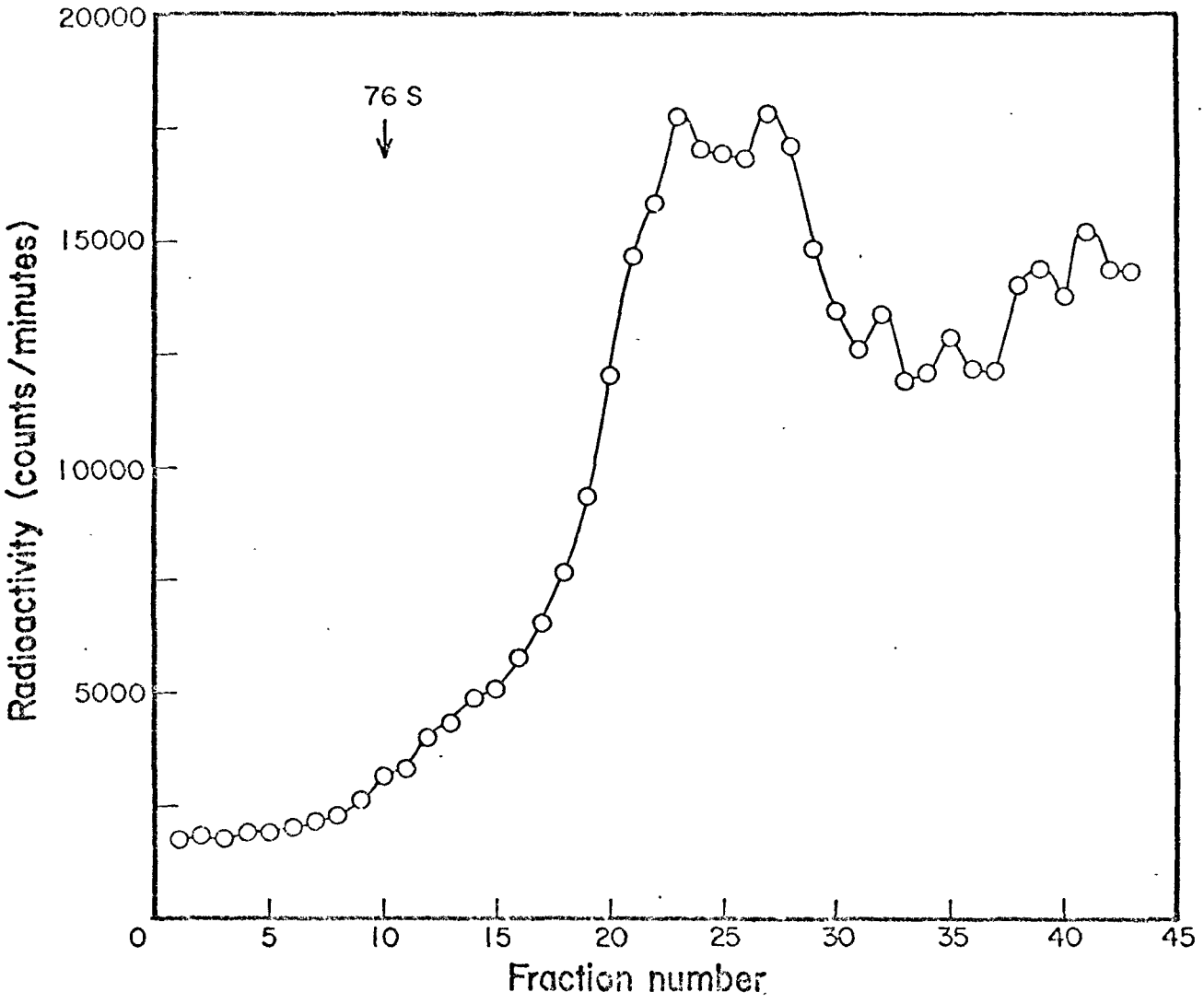

Figure 33. Sucrose density gradient centrifugation of mitochondrial lysate from rat liver. A pure mitochondrial fraction of rat liver labelled with [5-^{3}H]orotic acid was obtained as described in the methods. Mitochondria were lysed with 0.5% Triton X100 and 0.5% DOC in TKM buffer then centrifuged on a 5-20% convex sucrose-TKM density gradient. Fractions were collected and radioactivity determined.

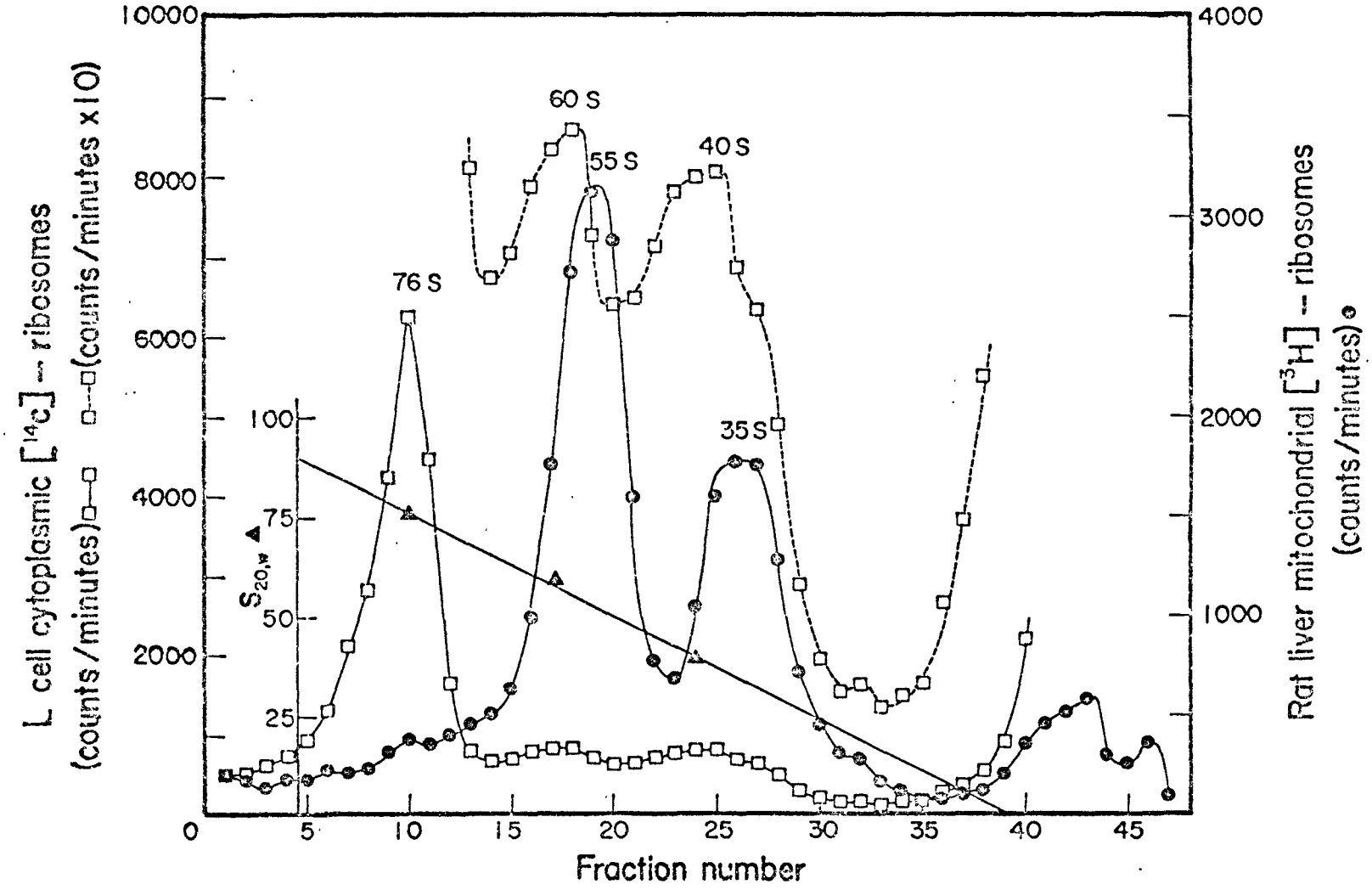

Figure 34. Separation of rat liver ribosomal fraction on sucrose density gradient. Rat liver ribosomal fraction was obtained from fractions 20-35 of the centrifugation of the mitochondrial lysate on the sucrose density gradient as described in Figure 33. The fractions were combined, dialyzed and recentrifuged on 5-20% sucrose density gradient in the presence of ^{14}C labelled L cell cytoplasmic ribosomes and subunits as described in the methods. The sedimentation coefficient of L cell cytoplasmic ribosomes and subunits were linearly related to their sedimentation distance (▲).

monomer and native subunits respectively according to Perry & Kelley (1968).

RNA was extracted from the 55 and the 35 S components with the cold phenol-SDS method then analyzed on agarose-polyacrylamide gel electrophoresis. The results are shown in Figure 35. Five species of high molecular weight RNA were detected from the 55 S ribosomal component: 22.3, 16.7, 14.2, 12.4 and 10.7 S_E, only two of these species 14.2 and 10.7 S_E were found in the 35 S ribosomal component. The relationship between these RNA species and the mitochondrial ribosomal RNA isolated directly from the mitochondrial fraction is not yet understood but it is possible that these species represent a specific degradation pattern of mit-rRNA which took place in the process of analyzing the mitochondrial ribosomes.

L cell mitochondrial ribosomes

A mitochondrial lysate from 1.6×10^8 cells labelled in the presence of 0.1 µg Actinomycin D/ml was analyzed on 5-20% convex sucrose-TKM density gradient. The result is shown in Figure 36. The sedimentation constants of the three components obtained were calculated to be 55, 35 and 24 S by interpolation, using the linear relationship obtained between the sedimentation coefficient of L cell cytoplasmic ribosomes and ribosomal subunits and their sedimentation distance (Figure 34). There were not enough counts for extraction of RNA from these components. The finding that the amount of the smaller components of this profile is larger than the 55 S one could be due to the effect of Actinomycin

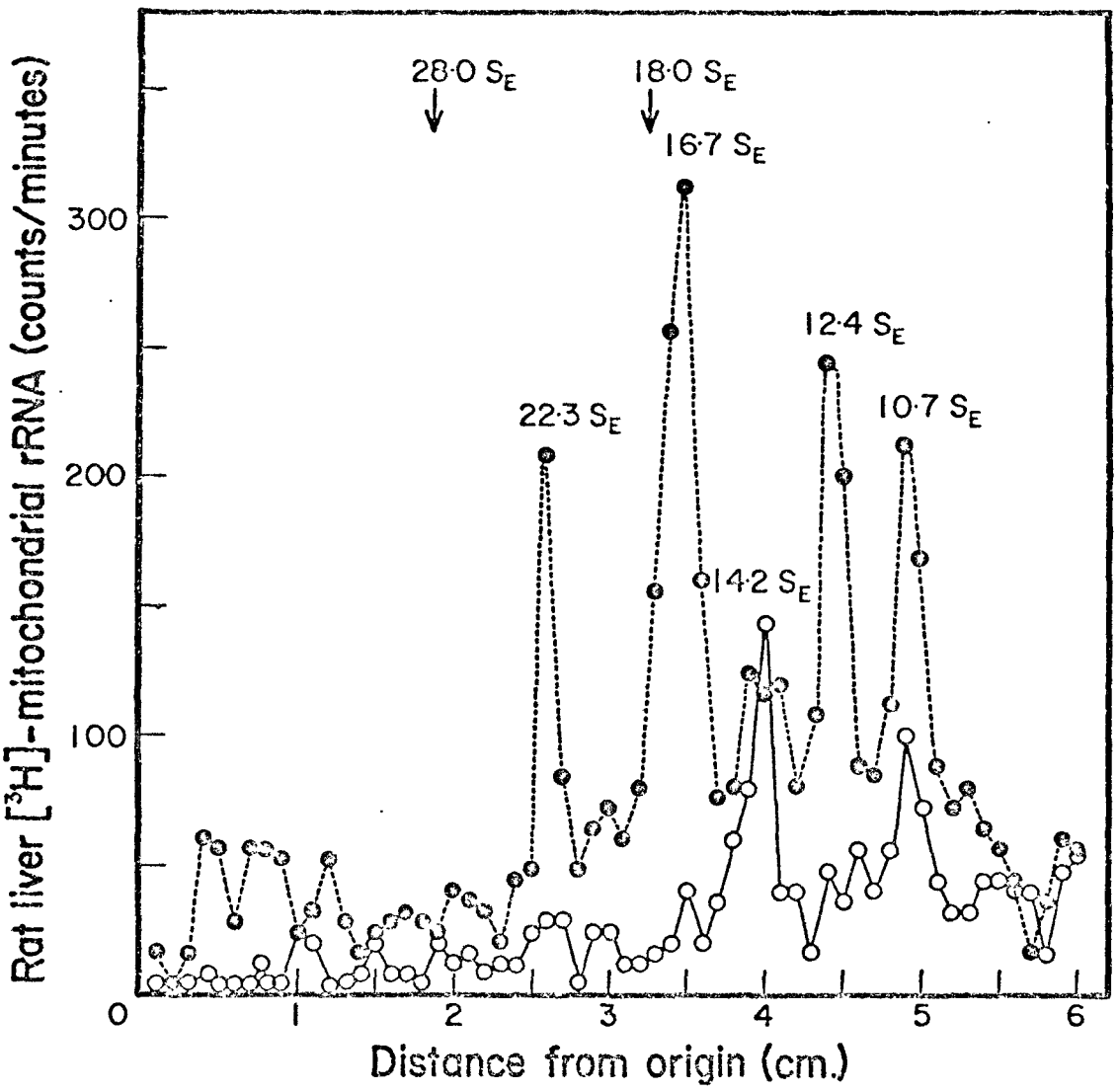

Figure 35. Gel electrophoresis of RNA extracted from rat liver mitochondrial ribosomal components. RNA was extracted from the 55 and the 35 S rat liver mitochondrial ribosomal components by the cold phenol-SDS method and electrophoresed as described in the method. ●, radioactivity of RNA extracted from the 55 S component; ○, radioactivity of RNA extracted from the 35 S component.

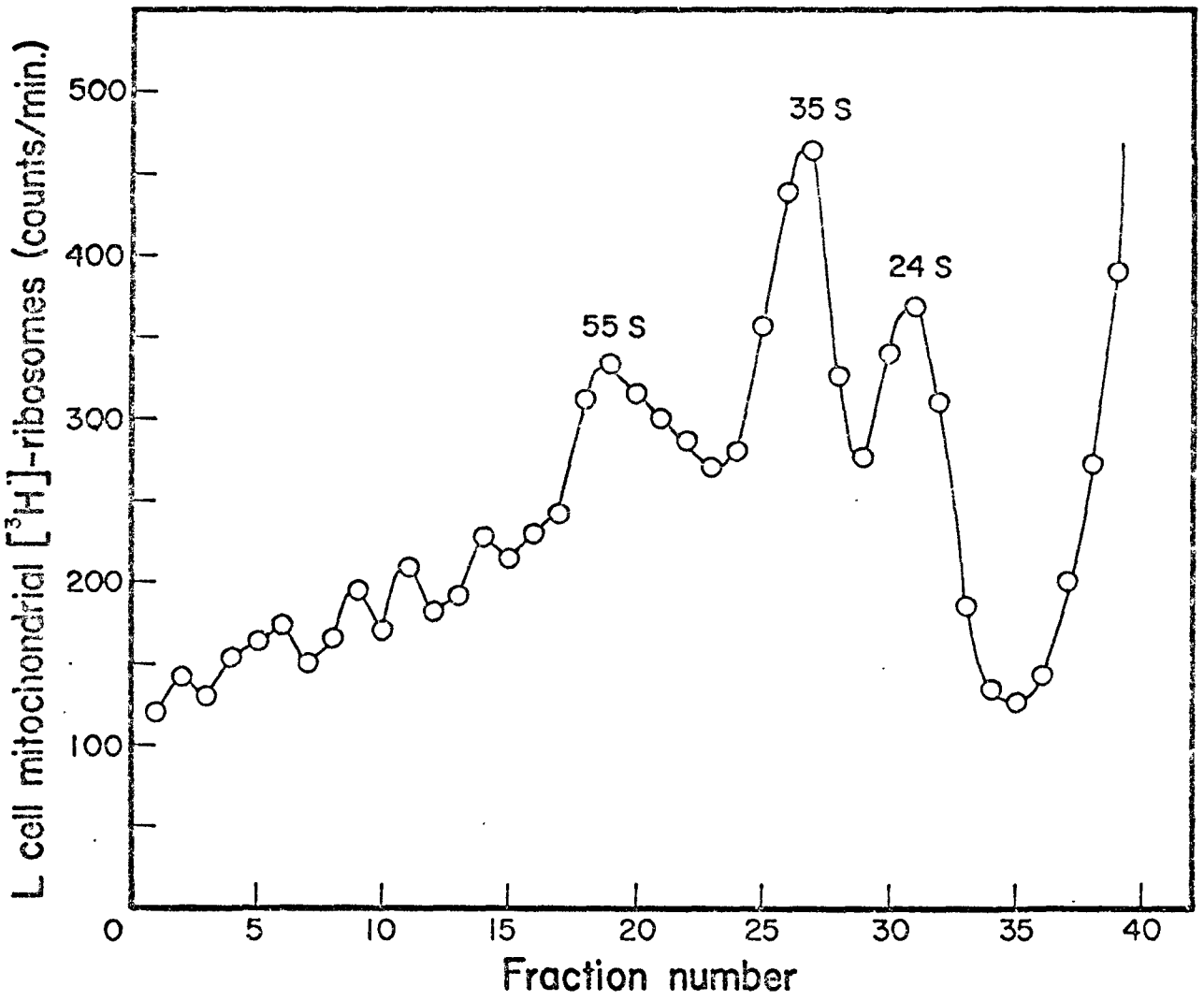

Figure 36. Sucrose density gradient centrifugation of mitochondrial Triton-DOC lysate of mouse L cell. Ribonucleoproteins of 1.6×10^3 cell were labelled in the presence of 0.1 µg of Actinomycin D/ml as described in the method. Crude mitochondrial fraction was isolated, lysed and centrifuged on 5-20% sucrose-TKM buffer density gradient as described in the method.

D since high molecular weight species from the polysome region which are normally present in a control cell seem to degrade to smaller components in its presence. Thus, it is possible that the 24 S component, which is missing from the ribosomal profile of rat liver mitochondria, is an artifact of the same effect.

These results indicate that in the range of known monomeric ribosomes, the 55 S component was the largest obtained either directly or indirectly from mitochondria of rat liver and mouse L cell respectively. Thus suggesting that the 55 S component might be a unique monomeric form of mitochondrial ribosomes and the 35 and 24 S components the ribosomal subunits.

IV DISCUSSION

The results show that mammalian mitochondria possess species of higher molecular weight RNA that are probably ribosomal. A conclusive demonstration of mitochondrial ribosomes was not possible but particles that could be ribosomes on the basis of their size were demonstrated. These results taken in conjunction with those of others for mit-rRNA from many species raise several questions of biological importance. The results presented here will be considered first and then the implication of this and other work on the autonomy, origin and evolution of mitochondria and mitochondrial DNA and rRNA in particular will be considered.

Mitochondrial Origin

The rRNA extracted from the mitochondrial fraction is clearly distinguished as a species distinct from cyt-rRNA by its sedimentation rate, electrophoretic mobility and nucleotide composition. These characteristics do not establish whether the RNA is normal constituent of mitochondria or a contaminant. The best evidence that these are constituents of mitochondria is their presence in highly-purified rat liver mitochondria. This finding precludes their origin from nuclei lysosomes, endoplasmic reticulum or cytoplasmic ribosomes as these cellular components were excluded by the enzymatic and electron microscopic criteria.

Although the RNA could be from an unidentified particle that co-sediments with mitochondria, this is highly unlikely in view of the demonstration of mitochondrial DNA and protein synthesis. Mitochondria, themselves, therefore contain these distinctive species of rRNA. This finding supports the demonstration of distinctive rRNA species in crude mitochondrial fractions of tissue culture cells utilizing Actinomycin D to suppress the synthesis of cytoplasmic rRNA. The RNA species obtained from the crude mitochondrial fraction of Actinomycin D-treated mouse L cells, human KB cells and rat hepatoma cells are similar to those obtained by others from Actinomycin D-treated hamster (BHK-21) cells (Dubin, 1967) and human (HeLa) cells (Vesco & Penman, 1969). In both of the latter cases only a broad peak of RNA sedimenting at about 17 S was observed on sucrose density gradient centrifugation but a separation into 21 and 12 S_E species was seen on electrophoresis of HeLa cell mit-RNA (Vesco & Penman, 1969) and that of hamster cells (Dubin & Montenecourt, 1970). These components could be an artifact of drug treatment because they are only clearly seen under these conditions, although Vesco & Penman (1969) and Knight (1969) did identify them in control cell cultures. A more serious problem is that the mitochondrial fractions obtained from cell cultures are not pure. Contamination from other cellular fractions, viruses or mycoplasma is possible. RNA species rich in A and U have been identified in tissue culture cells infected with mycoplasma and their sedimentation constant has been reported as 14-20 S (Levine et al., 1967; 1968) and 16 and 23 S (Markov et al., 1969). In the

experiments reported here this was probably not the case because there was no disturbance of RNA synthesis as reported by Levine et al. (1968) and because the cells were grown in the presence of an anti-mycoplasma drug. As stated above the finding of distinctive RNA species in highly purified rat liver mitochondria is the strongest evidence that the 15 and 13 S (21 and 12 S_E) rRNAs of tissue culture cells are truly mitochondrial. Their presence in tissue culture cells is therefore probably not due to mycoplasma, an artifact of drug treatment or contamination of the mitochondrial fraction.

The Unique Nature of Mammalian Mit-rRNA

The RNA isolated from mammalian mitochondrial fractions from different cells is considered to be ribosomal since it possesses properties which are common to all known rRNA. They all consist of two high molecular weight components, a larger and a smaller one, which are precipitated with molar concentration of salt and are eluted from a MAK column with a high concentration of NaCl. However, mammalian mit-rRNA has unique physical and chemical properties and shows certain inconsistencies between its physical properties and apparent molecular weight when compared to other rRNA.

a. Physical properties and nucleotide composition of mammalian mit-rRNA

The physical properties and the G+C content of the high molecular weight RNA isolated from different mammalian mitochondria are summarized in Table 8. All rodent mitochondria contained RNA

Table 8

Physical Properties and Chemical Composition of the Different Mammalian Mit-rRNA Examined

Source of mit-rRNA	rat liver	rat liver	L cell	hepatoma cell	KB cell
Labelling isotope	^{3}H or ^{32}P	cold	^{3}H	^{32}P	^{14}C
the nature of mit-rRNA determination	directly	directly	indirectly	indirectly	indirectly
extraction method of rRNA	modification 1 of Kirby's method	modification of Kirby's method	cold phenol-SDS	cold phenol-SDS	hot phenol-SDS
	modification 2 of Kirby's method		hot phenol-SDS		
	cold phenol-SDS				
S Values	15.3; 12.8	--	15.0; 12.5	--	--
S_E Values	21.0; 12.5	--	21.0; 12.5	21.0; 12.0	20.5; 11.0
Elution concentration with NaCl from MAK column	--	--	0.95 M	--	--
G+C (moles %)	46.8	48.3	39.6	40.9	--

sedimenting at 15 and 13 S on a sucrose density gradient and migrating at 21 and 12.5 S_E in agarose-polyacrylamide gels. These species were found regardless of the approach, direct or indirect, used for the isolation of the RNA, the method of extraction or the radioactive precursor. Mit-rRNA isolated from human KB cells migrates faster at 20.5 and 11.0 S_E and therefore is slightly smaller or more compact than its counterpart from rodent sources. Thus, the sedimentation coefficient and the S_E values of mammalian mit-rRNA are smaller than those of any other rRNA (Click & Tint, 1967; Loening, 1968).

The molecular weight of the smaller component was 0.33×10^6 daltons calculated both from sedimentation and electrophoretic studies. On the other hand the discrepancy between the S and S_E values of the larger mit-rRNA component is reflected in its molecular weight being 0.49×10^6 and 0.96×10^6 daltons from sedimentation and electrophoretic studies respectively. This discrepancy seems to be unique to mit-rRNA since animal cyt-rRNA and bacterial rRNA maintain the same relative size on sedimentation and electrophoresis (Loening & Ingle, 1967; Loening, 1969).

L cell mit-rRNA eluted from a MAK column at 0.95 M NaCl compared with rRNA from E. coli and the cytoplasm which elute at 0.75 and 0.85 M and 0.89 M NaCl respectively (Osawa & Sibatani, 1967). The strength of binding of rRNA from E. coli and the cytoplasm is directly related to their molecular weights but in contrast mit-rRNA binds more strongly than mammalian cyt-rRNA although its molecular weight is smaller than that of bacterial

rRNA. Thus, the inconsistency between the binding of the mit-rRNA to a MAK column and its molecular weight is another reflection of the unique nature of mit-rRNA.

The G+C content of rodent mit-rRNA is about 20% lower than that of its cytoplasmic counterpart (Table 6). It is A+U rich while rRNAs from most organism are G+C rich (Amaldi, 1969).

In order to explain the inconsistent behavior of mit-rRNA in physical analysis, a hypothesis is presented below which accounts for this behavior on the basis of a unique conformation of mammalian mit-rRNA compared with that of cyt-rRNA or bacterial rRNA.

b. Conformation of mammalian mit-rRNA

Information about the conformation of mit-rRNA was obtained indirectly from the hydrodynamic studies used to determine its molecular weight. The movement of molecules on sedimentation or electrophoresis depends on their molecular weight and on their conformation. In order to interpolate the molecular weight of unknown molecules from standard ones, as done in these studies, it is assumed that their conformations are identical. If these conformations differ, then striking discrepancies arise. For example, tobacco mosaic virus RNA and bacteriophage R17 RNA differ in molecular weight by a factor of two but have the same sedimentation coefficient when measured under identical conditions (Gesteland & Boedtker, 1964). Cyt-rRNA was chosen as standard here, since having a similar biological function as mit-rRNA it should have a similar conformation. However, from the

discrepancy of the molecular weight of the larger component of rodent mit-rRNA determined by sedimentation and electrophoresis, it seems that this assumption is wrong. The discrepancy can be explained if mit-rRNA has a more open conformation than that of cyt-rRNA or bacterial rRNA. An open conformation of mit-rRNA molecules would slow the movement of mit-rRNA both on sedimentation and electrophoresis compared with that of the standard cyt-rRNA. This would cause an over-estimation of the molecular weight of mit-rRNA calculated from gel mobility and an under-estimation when calculated from sedimentation, resulting in the observed discrepancy.

Further support for an open conformation of mit-rRNA is obtained from its nucleotide composition. In contrast to most rRNA which is G+C rich (Amaldi, 1969), rodent mit-rRNA is A+U rich (Table 6). Since the structure of the RNA molecule is largely dependent on its base pairing and since the melting temperature, T_m, of poly (A+U) is about 30°C lower than poly (G+C) (Cox, 1966), it is possible that under the conditions of analyzing mit-rRNA it has a lower proportion of its bases hydrogen bonded and thus possesses a more open conformation than the standard cyt-rRNA. Indeed Loening (1969) reported that the relative mobility and sedimentation of rRNA with a low content of G+C residues decreased in a low ionic strength buffer compared with that of rRNA rich in G+C, possibly because in the lower ionic strength buffer the A–U base pairs melt more readily.

The unique nucleotide composition of mit-rRNA and hence its suggested structure can explain its stronger binding to the MAK

column although its molecular size is smaller than that of cyt-rRNA. A similar phenomenon was observed in the chromatographic behavior of DNA-like RNA which has a lower content of G+C (50%) than cyt-rRNA (65%) (Ellem, 1966). DNA-like RNA although sedimenting around 16 to 18 S, binds strongly to a MAK column and can be eluted only at high temperatures (Ellem & Sheridan, 1964) or with SDS (Ellem, 1966). The stronger binding of mit-rRNA and DNA-like RNA than cyt-rRNA, although the latter is larger, indicates that the binding of RNA to MAK column is due not only to ionic forces (Mandell & Hershey, 1960) but that hydrophobic interaction involving the bases and the conformation of the RNA molecules are also important (Ellem, 1966; Ellem & Rhode, 1969). Thus on the basis of size, mit-rRNA should elute before cyt-rRNA but its base composition and suggested open structure make it adhere more strongly under the conditions of the chromatography (35°C and 0.1 to 1.2 M NaCl). The latter characteristics appeared to be more important because mit-rRNA eluted after the cyt-rRNA. However, although mit-rRNA has a lower G+C content than TD RNA it elutes more easily. This suggests that mit-rRNA must be relatively more folded than TD RNA and that its secondary structure is generally like other rRNA.

c. Molecular weight of rodent mit-rRNA

The possibility that mit-rRNA under the conditions of the physical analysis possesses a more open conformation than that of the standard cyt-rRNA, raises doubts about the accuracy of the calculated molecular weights of its components. From the strong binding of L cell mit-rRNA to the MAK column it seems that both

components of the mit-rRNA possess this unique conformation. However, there is a discrepancy in the molecular weight of the larger component only, when calculated from sedimentation rate and electrophoretic mobility. Therefore, either the smaller component possesses, under the conditions of these analytical methods, a similar conformation to that of the standard cyt-rRNA or for some unknown reason, its unique conformation does not effect its sedimentation rate or electrophoretic mobility. In any event it seems that 0.33×10^6 daltons is the molecular weight of the smaller component of rodent mit-rRNA.

If the relative amount of the two mit-rRNA components was known then the molecular weight of the larger component could be calculated from that of the smaller one assuming an equal molar proportion as occurs for all ribosomes (Click & Tint, 1967). However the ratio of radioactivity in the two components varied from 1.23 to 2.13 for rodent mit-rRNA (Table 4) indicating that factors in addition to the size of the RNA affected the ratio under the conditions of labelling. Since these factors are unmeasurable this approach can not be used. An exact measure of the molecular weight must use techniques not dependent on conformation. The best approach would be to completely denature the RNA with formaldehyde (Boedtker, 1968) or dimethylsulfoxide (Strauss et al., 1968) and analyze by sedimentation or electrophoresis.

The Origin of Mit-rRNA

In order to assess the degree of mitochondrial autonomy it

is important to investigate whether mit-rRNA is transcribed from mitochondrial DNA or from nuclear DNA and then transferred to mitochondria. Ethidium bromide at a concentration less than 5.4 µg/ml has a greater affinity for the circular DNA molecules of mammalian mitochondria than to the nuclear linear DNA (Bauer & Vinograd, 1968), and inhibits almost specifically rat liver mitochondrial DNA polymerase *in vitro* (Meyer & Simpson, 1969). If mit-rRNA is transcribed from the mitochondrial DNA its synthesis should be inhibited by low concentrations of ethidium bromide. Ethidium bromide at 1.0 µg/ml completely inhibited specifically the synthesis of mit-rRNA of L cells in suspension culture showing that it was transcribed from mitochondrial DNA. A selective inhibition of the synthesis of mit-rRNA of HeLa cells by ethidium bromide was also observed by Zylber *et al.* (1969).

In another experiment reported elsewhere (Fukamachi, Bartoov, Mitra & Freeman, 1970) isolated rat liver mitochondria (2 mg mitochondrial protein/ml) were incubated for 1 hour at 30°C with ribonucleoside triphosphates using [^{3}H]UTP (15 µCi/ml) as the radioactive precursor. One sample contained 0.33 µg/ml of ethidium bromide and another one was the control. The results, shown in Figure 37, clearly indicate that the 21 and 12 S_E mit-rRNA components isolated by the cold phenol-SDS method were transcribed from mitochondrial DNA since they were synthesized in mitochondria in a DNA-dependent process.

Other investigators have made use of DNA-RNA hybridization to show directly the location of the genes for mit-rRNA. Wood &

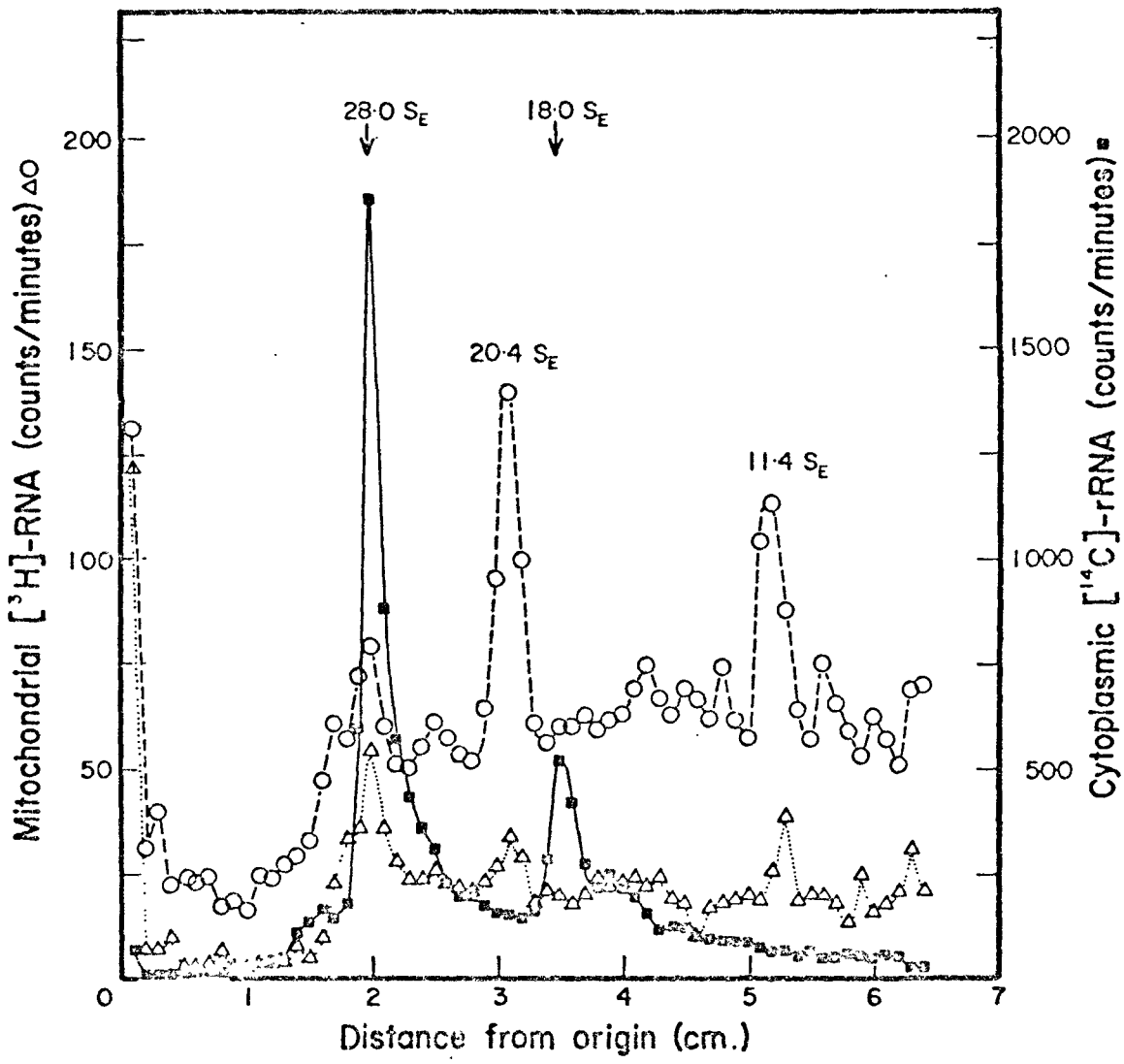

Figure 37. Electrophoresis profile of RNA synthesized by isolated rat liver mitochondria. Mitochondria were isolated from livers of hooded rats using 0.25 M sucrose, 2mM EDTA, pH 7.2 as the isolation medium. RNA was labelled with [^{3}H]UTP for 1 hour at 30°C, extracted with the cold phenol-SDS method. Electrophoresis on agarose-polyacrylamide gel was in the presence of L cell [^{14}C] cyt-rRNA as described in the methods. ■, L cell cyt-rRNA; O, mit-RNA; Δ, mit-RNA synthesized in the presence of ethidium bromide (0.33 μg/ml).

Luck (1969) have shown that mit-rRNA from Neurospora hybridizes specifically with mitochondrial DNA. The extent of hybridization at a saturation level of rRNA was 6.1% for the larger rRNA component and 2.8% for the smaller component. As the two types of RNA have molecular weight roughly in the ratio of 2 to 1 these values imply that there are an equal number of genes for each type of RNA on the mitochondrial DNA. From the molecular weight of mitochondrial DNA (6.6×10^7 daltons) they concluded that the genes for the 25 and 19 S rRNA are repeated at least four times in the mitochondrial DNA. In yeast, Wintersberger (1967) and Wintersberger & Viehhauser (1968) showed that mit-rRNA hybridizes specifically with mitochondrial DNA while the cyt-rRNA did not. Mit-rRNA from Tetrahymena pyriformis (Suyama, 1967) and from Xenopus laevis (Swanson & Dawid, 1970) were shown to hybridize with mitochondrial DNA. In these studies it has been firmly established that the cyt-rRNA does not compete with the mit-rRNA for sites on the mitochondrial DNA and there is therefore no sequence homology between the two types of rRNA. It is much less clear whether the mit-rRNA has any sequence homology with the nuclear DNA. It is in fact almost impossible to answer this question with certainty. Unless there are redundant sequences in the nuclear DNA which are complimentary to the mit-rRNA, it would be technically difficult to detect any homology. Although Wintersberger & Viehhauser (1968) have reported some degree of specific binding of mit-rRNA to yeast nuclear DNA, it must be remembered that the extreme complexity of nuclear DNA could result

in limited homology without any biological significance and that mit-rRNA is likely to be contaminated with cyt-rRNA. The reality of cyt-rRNA contamination is emphasized by the observation that yeast mit-rRNA which had been purified by hybridization with mitochondrial DNA hybridized very poorly with yeast nuclear DNA (Fukuhara et al., 1969).

No specific hybridization studies between mitochondrial DNA of mammals and their rRNA have been done, however, in HeLa cells Attardi & Attardi (1969) showed that mit-RNA species with sedimentation coefficient between 9-15 S hybridized with mitochondrial DNA while no cyt-rRNA species hybridized with it.

Borst & Aaij (1969) showed that mitochondrial DNA can be separated by alkaline CsCl equilibrium centrifugation into its complementary strands. This phenomenon indicates that the "heavy" strand of the mitochondrial DNA contains large amounts of thymidylate and guanylate which on centrifugation in alkaline CsCl gradients possess a high density (Sober, 1968). Mit-RNA from rat liver hybridizes with the "heavy" strand only. They concluded that in vivo only the "heavy" strand is the template for mit-RNA synthesis. Although this hybridization study was done on the overall mitochondrial RNA synthesized in a period of 6 hours in 1 month old rats, it seems from the results reported here with mit-rRNA from rat liver that it would contain high amounts of adenylate and cytidylate and thus be complimentary to the "heavy" mitochondrial DNA strand.

Mit-rRNAs Reported in the Literature

While this investigation was in progress, mit-rRNA was isolated from a variety of species from different families. A summary of these findings and those reported here (mostly published by Bartoov et al., 1970) is presented in Table 9. As can be seen from this table there is a lack of agreement on the exact sedimentation coefficient of mit-rRNA from the same organism. Generally, however, the sedimentation coefficient of mit-rRNA from fungi resemble that of bacterial rRNA being about 23 and 16 S. On the other hand mit-rRNA from protista and animalia possess smaller components than bacterial rRNA. It is possible that mit-rRNA of the higher organisms might be smaller, as is the DNA. There is a phylogenetic discontinuity in the size of mitochondrial DNA (Table 1). In Neurospora the DNA has a molecular weight of 66×10^6 daltons (Wood & Luck, 1969) but in Xenopus (Wolstenholme and David, 1968) and in L cells (Nass, 1969) it is 9×10^6 daltons. It is also possible that there is a phylogenic decrease in the size of mit-rRNA within the animal kingdom since mit-rRNA from Xenopus sediments on sucrose density gradients at 18 and 13 S (Swanson & David, 1970) and rat liver mit-rRNA sediment at 15 and 13 S. Human KB cell mit-rRNA might be smaller than the rodent mit-rRNA as judged by electrophoretic mobility. This contrasts with an increase in molecular weight of the larger rRNA of the animal cytoplasm which seem to evolve with each major step of animal evolution from about 1.4×10^6 daltons in sea urchins to 1.75×10^6 daltons in mammals (Loening, 1968).

Table 9

S and S_E Values of Mit-rRNA Isolated from Different Species

Organism & Reference	S Values			S_E Values			G+C% Content	
	Mito.	Cyto.	E. coli	Mito.	Cyto.	E. coli	Mito.	Cyto.
Protista								
Euglena gracilis Z								
Krawiec & Eisenstadt (1970)	14 (17.7)	25					27.4	54.4
	11 (14)	19						
Tetrahymena pyriformis (Strain ST)								
Suyama (1967) Chi et al. (1970)	21 (18)	26	23				27.9	43.2
	14	17	16				30.6	49.2
Neurospora crassa								
Kuntzel & Noll (1967)	21	25	--				37	49
	16	16	16					
Dure et al. (1967)	23	25.6	23					
	16	16	--					

Wood & Luck (1969) and	25	28					35	50
Rifkin et al. (1967)	19	18						
Saccharomyces cerevisiae								
Wintersberger (1967)	23		23					
	16		16					
Rogers et al. (1967)	22.4	24.6	22.6					
	17.8	16.2	16.9					
Leon & Mahler (1968)	23	25						
	16	17						
Steinschmeider (1969)	25	28						
	16	19						
Fauman et al. (1969)	22	26	23				26	47
	15	17	16					
Forrester et al. (1970)	21	25	23				26	
	15	17.5	16				26	
Stegeman et al. (1970)				25	26	23		
				17	19	16		

Aspergillus nidulans							
Edelman *et al.* (1970) and	23.5	26.5	24.0			32	51
Verma *et al* (1970	15.5	17.0	16.0				
Amphibia							
Xenopus loevis							
Dawid (1969) and	18-19	28		21	28	45	62
Swanson & Dawid (1970)	13	18		13	18		
Mammals							
Rat liver							
Rendi & Warner (1960)	23						
	16						
Kroon & Aaij (1968)	23	28					
	(16)	18					
Rakhimbekova *et al.* (1969)	24						
	15			18	28		
Bartoov *et al.* (1970) and	15	28		21.0	28	46.5	63.7
reported here	13	18		12.5	18		

Mouse L cells							
Bartoov *et al.* (1970) and	15	28		21.0	28	39.6	61.6
reported here	12.5	18		12.5	18		
Hamster BHK-21 cells							
Dubin & Brown (1967) and	(27)	28				38	46.9
Dubin (1969) and Dubin and Montenecourt (1970)	17	18					
Human HeLa cells							
Knight (1969)	27	28					
	18	18					
Vesco & Penman (1969) and				21	28	41.7	
Zylber *et al.* (1969)				12	18	43.3	
Human KB cells							
				20.5	28		
Reported here				11.0	18		
Higher Plants							
Soya bean							
Baxter & Bishop (1968)	2 species between 16-25	25	23				
		16	16			low	

Cauliflower				
Pollard et al. (1966)	28	28	57.7	56.2
	18	18	53.7	53.1

The results reported here are not in agreement with two other reports on the nature of mammalian mitochondrial RNA. Kroon and Aaij (1968) reported a 23 S species isolated from mitochondria of regenerating rat liver. Since there is a danger of bacterial contamination in regenerating liver it is possible that the 23 S RNA originated from bacterial rRNA. Attardi & Attardi (1969) found in HeLa cell mitochondria 21 S and 9-15 S components on sucrose density gradient centrifugation. The latter, which had a peak at 12 S, hybridized with mitochondrial DNA but the 21 S component did not. One interpretation of their result is that the 21 S peak is not mitochondrial and the 9-15 S RNA is the same RNA as seen here but is partly degraded. The 21 S peak could be a viral contaminant, degraded cyt-rRNA or may be the same as the 21 S component seen here. The latter appeared to be DNA in nuclei or nuclear fragments contaminating the crude mitochondria and could be removed by isopycnic gradient centrifugation or DNase treatment of the mitochondrial fraction. Attardi & Attardi (1969), however, had used both these treatments.

Table 9 also indicates that all mit-rRNAs are A+U rich whereas rRNA from most species is G+C rich (Amaldi, 1969).

Mammalian Mitochondrial Ribosomes

Paucity of Rat Liver Mitochondrial Ribosomes

The paucity of mitochondrial ribosomes which was observed from the electron microscopic studies was one of the problems that this investigation faced as was described in the Introduction.

From the amount of RNA in pure rat liver mitochondria and the approximate molecular weight of mit-rRNA it is possible to estimate the number of ribosomes present in a rat liver mitochondrion more accurately. Pure rat liver mitochondria contain 3 µg RNA/mg mitochondrial protein. If 90% of this amount is mit-rRNA, as was found in all organisms so far investigated (Darnell, 1968) and 1 mg mitochondrial protein contain 5×10^9 mitochondria (Nass, Nass & Hennix, 1965) then a mitochondrion contains 54×10^{-17} g of rRNA. The total molecular weight of the mit-rRNA averages 1.05×10^6 daltons. Then the number of rat liver mitochondrial ribosomes/mitochondrion = $\frac{NW}{M}$ = 310

where N - Avogadro's number

W - amount in g of rRNA/mitochondrion

M - Molecular weight of mit-rRNA.

For a comparison the number of ribosomes in one E. coli cell with an average size equal to a rat liver mitochondrion (1.0 to 3.0 µm) is 27,000. This is calculated from the average dry weight of E. coli being 33×10^{-14} g (Carpenter, 1967), 25% of the dry weight being RNA of which 90% is rRNA (Tissiéres & Watson, 1958), giving 7.4×10^{-14} g rRNA/cell. The molecular weight of E. coli rRNA is 1.66×10^6 daltons (Click & Tint, 1967) and therefore the number of bacterial ribosomes/cell is 27,000. Thus an autonomous organism the size of a mitochondrion contains 86 times more ribosomes in order to carry out its requirement for protein synthesis. This probably reflects the fact that mitochondria do not synthesize all of their proteins and also that the turnover

of mitochondria is slow compared to bacteria.

Since mitochondrial RNA in rat liver is only 0.72% of the total cell RNA and since 90% of this RNA is cyt-rRNA it can be roughly estimated that in liver there are about 100 to 200 times more cytoplasmic ribosomes than mitochondrial ones. In order to obtain a more accurate estimation as to the amount of mitochondrial ribosomes in 1 g of fresh liver in comparison to the cytoplasmic ones the following calculation can be made. One g of fresh liver contains 23.2 mg of microsomal RNA (Smuckler, 1968). If the molecular weight of rat liver cyt-rRNA is equal to 2.45×10^6 daltons (Loening, 1968) then the number of cytoplasmic ribosomes in 1 g of rat liver is 5.67×10^{15}. On the other hand 1 g of fresh rat liver contains 11×10^{10} mitochondria (Nass, Nass & Hennix, 1965). Since the number of ribosomes within rat liver mitochondria was calculated to be 310 then the number of mitochondrial ribosomes per 1 g of rat liver equal to 3.4×10^{13}. The amount of cytoplasmic ribosomes is then 165 times more than the mitochondrial ones in rat liver. This calculation emphasizes the paucity of mitochondrial ribosomes and rRNA and hence the problem of cytoplasmic contamination which can easily mask the mitochondrial ribosomes and rRNA.

The Unique Sedimentation Coefficient of Mammalian Mitochondrial Ribosomes

The presence of rRNA of unique size in mammalian mitochondria suggested that their ribosomes would also be unique. The preliminary

results indicated that the monomeric form of mammalian mitochondrial ribosomes sedimented at 55 S and the ribosomal subunits at 35 and 24 S. The fact that the 55 and 35 S components were found in both the direct and the indirect approaches and especially their presence in highly purified rat liver mitochondria is strong evidence that they are truly mitochondrial and not due to an artifact of drug treatment or contamination of the mitochondrial fraction. On the other hand the 24 S component found by the indirect approach in L cell mitochondria is completely missing from the sedimentation profile of rat liver mitochondrial ribosomes when centrifuged on a sucrose density gradient.

These results are not in agreement with those obtained early by Truman (1963), Elaév (1964, 1966) and Rabinowitz et al. (1966). These investigators isolated ribosomal particles from mitochondria of rat liver, rat muscle and chick embryo heart respectively which were identical in their sedimentation coefficient to those of the cytoplasm. It seems that the mitochondrial fractions in the above experiments were contaminated with cytoplasmic ribosomes which masked the identity of the mitochondrial ones.

Recently 55 S mitochondrial ribosomes were demonstrated in rat liver by pulse labelling with [^{14}C]leucine (O'Brien & Kalf, 1967a & b; Ashwell & Work, 1970). Incubation in the presence of chloramphenicol inhibited the incorporation of [^{14}C]leucine into growing polypeptide chains associated with the 55 S peak and partially protected against the puromycin-induced release of the peptide chains from the ribosomes as it does in bacteria (Cannon,

1968; Weber & De Moss, 1969). Thus, the 55 S component seems to be the monomeric form of rat liver mitochondrial ribosomes.

A 60 S monomeric form of Xenopus laevis mitochondrial ribosomes and 43 and 32 S subunits were obtained by Swanson & Dawid (1970). They showed that the 60 S particle is active in polypeptide synthesis and accounted for the bulk of polypeptide synthesizing activity recovered from a fractionated mitochondrial extract. They isolated 21 and 13 S rRNA from the 43 and 32 S particles respectively. The attempt to isolate rRNA from the mitochondrial ribosomal particles obtained from rat liver in this investigation was not successful (Figure 35). The RNA isolated seemed to be degraded although the largest RNA species isolated from the 55 S particle was 22 S_E and the largest RNA species of the 35 S particles was 14 S_E. These S_E values are similar to those of mit-rRNA components isolated directly from the mitochondrial fraction, but the relationship between these species of RNA molecules and the ribosomal particles is not clear.

The S values obtained from mitochondrial ribosomes of different species by other investigators are presented in Table 10. Although there are conflicting results on the exact S values of mitochondrial ribosomes and subunits it seems that generally the overall results are in parallel to those obtained for the mit-rRNA. The mitochondrial ribosomes obtained from fungi are of the bacterial type while animal mitochondria which contain 5 μ circular DNA and small rRNA, also contain small 55-60 S ribosomes.

Table 10

S Values of Mitochondrial Ribosomes Isolated from Different Species

Organism & Reference	Isolation medium	Method of lysing mitochondria		S Values Mito.	Cyto.	E. coli
Tetrahymena pyriformis						
Chi & Suyama (1970)	10mM Tris-HCl	Triton X-100 (1.0%) or				
	0.1 M KCl (pH 7.4)	DOC (0.5%)				
	10^{-2} M $MgCl_2$		M	80	80	
			P	115		
	10^{-3} M $MgCl_2$		M	80	70	
	10^{-4} M $MgCl_2$		M	70	70	
	10^{-4} or 10^{-5} EDTA		S	55	60; 40	
Neurospora crassa						
Kuntzel & Noll (1967) and	10mM Tris-HCl	Triton X-100 (1%)	M	73	77	70
Kuntzel (1969a) and	0.1 M NH_4Cl		S	50; 37	60; 37	50; 30
Kuntzel (1969b)	10mM $MgCl_2$ (pH 7.5)		P	103; 134	108; 140	
				160; 186	169; 197; 215	

Rifkin et al. (1967)	10mM Tris-HCl	DOC (0.5%)	M	81	81	
	2mM $MgCl_2$		S	60; 45	61;47	
	50mM KCl (pH 7.6)					
Candida utilis						
Vignais et al. (1969)	10mM Tris-HCl	DOC (0.5%)	M	77-80	80	
	10mM $MgCl_2$ (pH 7.6)		S	52-54	60; 36	
				32-34		
			P	115	120; 154	
Candida krusei						
Kaempfer (1969)			M	76	80	70
			S	53; 35		
Saccharamyces cerevisiae						
Schmitt (1969)	20mM Tris-HCl	Initially DOC (1.25%)	M	80	80	70
	5mM $MgSO_4$	then Triton X-100 (0.5%)	S	60; 38	60; 38	
	50mM KCl (pH 7.4)					
Stegeman et al. (1970)	50mM Tris-HCl	Triton X-100				
	10mM $MgCl_2$	(2 %)	M	75	80	
	10mM KCl (pH 7.5)					

Xenopus laevis oocytes					
Swanson & Dawid (1970)	10mM Tris-HCl	Sonication	M	60	87
	40mM KCl			43; 32	60; 40
	10mM $MgCl_2$				
	6mM Mercaptoethanol				
Mouse liver					
Georgatsos et al. (1968)	50mM Tris-HCl	DOC (1 %)	M	78	78
	25mM KCl			55	
	5mM $MgCl_2$ (pH 7.6)				
Mouse L cells					
Reported here	10mM Tris-HCl	DOC (0.5%) Triton X-100	M	55	76
	50mM KCl	(0.5 %)	S	35;24	60; 40
	6mM $MgCl_2$ (pH 7.6)				
Rat liver					
Truman (1963)	30mM Tris-HCl	DOC (0.3%)	M	77	
	80mM KCl		S	44	
	5mM $MgCl_2$ (pH 7.6)		P	120	
Elaév (1964)	50mM Tris-HCl	DOC (1%)	M	83	83
	25mM KCl		S	54; 45	54; 45

	9mM MgCl					
	0.2M Sucrose (pH 7.6)					
Elaév (1966)	50mM Tris-HCl	DOC (1.25-1.5%)	M	83		
	25mM KCl		S	63; 46		
	10mM $MgCl_2$					
	0.2 M sucrose (pH 7.6)					
O'Brien & Kalf (1967)	1mM Tris-HCl	DOC (0.5%)	M	55	78	
	50mM KCl			40; 30		
	5mM $MgCl_2$ (pH 7.6)					
Ashwell & Work (1970)	10mM Tris-HCl	Any of the following	M	50-55	80	70
	10mM KCl	Brij-58 (0.2%)				50; 30
	10mM MgAc (pH 7.6)	DOC (0.1%) Triton X-100 (0.1%)				
		Nonident, NP 40 (0.5%)				
Results reported here	10mM Tris-HCl	DOC (0.5%) +				
	50mM KCl	Triton X-100 (0.5%)	M	55		
	6mM $MgCl_2$ (pH 7.6)		S	35		
Beef, Pig and Rabbit liver			M	52-55		
O'Brien (1969)	5mM $MgCl_2$ + ?			40		

Human HeLa cells

Perlman & Penman (1970)	10mM Tris-HCl	DOC-Brij-58	M	95	74
	0.1 M NaCl	(1 %)			
	10mM $MgCl_2$ (pH 7.4)				
	0.25M Sucrose RNase treated (1 ug/ml)		S	55	
	5mM EDTA treated		S	35	
	Cells incubated with 0.04 µg/ml of Actinomycin D		S	45	
	Actinomycin + 50mM EDTA		S	35	

M = Ribosomal monomer

S = Ribosomal subunits

P = Ribosomal polysomes

Mitochondrial Protein Synthesizing System

This investigation has been limited to the characterization of the two major components of mammalian mitochondrial protein synthesizing system, ribosomes and rRNA. However, while this work was in progress characterization of other components of this system like ribosomal proteins, tRNA and acylating enzymes was accomplished by others. As with mit-rRNA and ribosomes reported here these mitochondrial components are unique compared with their counterparts in the cytoplasm or from bacteria (for review, Rabinowitz & Swift, 1970). It was also shown that the unique mitochondrial ribosomal proteins are synthesized on the cytoplasmic ribosomes and that mitochondria of yeast, rat liver and human HeLa cells contain N-formylmethionyl-tRNA which is not present in the cytoplasm but is involved in the initiation of protein synthesis in bacteria (Smith & Marcker, 1968; Galper & Darnell, 1969). Thus, it seems that mammalian mitochondria possess a unique protein synthesizing system which is completely different from the cytoplasmic one and resembles that of the bacteria in only a few characteristics. Therefore, these findings raise the possibility of mitochondrial autonomy and control with respect to the proteins they synthesize.

Mitochondria, Organism or Functional Cell Organelle?

The observations that mitochondria contain DNA, a protein synthesizing system and that they increase in number by growth and division (see Introduction) indicate that mitochondria possess a certain degree of autonomy. From where did the mitochondrial

autonomy derive? Are mitochondria organelles that have evolved some degree of autonomy for their replication or role in the eukaryotic cell or did mitochondria originate from autonomous organisms (bacteria) which established a symbiotic relationship in the eukaryotic cell and subsequently lost some of their original autonomy? The presence of mitochondria gave the new type of eukaryotic cell an advantage in energy metabolism.

The results obtained in this investigation and by other investigators mentioned above strongly suggest that mitochondria originated from a prokaryotic ancestor, since the components of the mitochondrial protein synthesizing system like rRNA, ribosomes, tRNAs and acylating enzymes all are remarkably different than their counterparts in the cytoplasm. If mitochondria were just a functioning organelle in the cell it would be expected that the translation mechanism would have the same components as that of the cytoplasmic protein synthesizing system. Moreover the sensitivity to chloramphenicol and insensitivity to cycloheximide which is the reverse in the cytoplasm indicates that these two systems are different from each other.

The fact that the mitochondrial protein synthesizing system exhibit similarities to the bacterial system such as the sensitivity to chloramphenicol, the presence of N-formylmethionyl-tRNA, and the nature of mitochondrial ribosomes and rRNA of fungi (Table 9 and 10 respectively) indicates that mitochondria originated from a common ancestor to contemporary bacteria. The question now arises if mitochondria originated from a bacterial ancestor how did they

evolve to the present state where in mammalian cells they have lost over 99% of their original DNA?

Mitochondrial Evolution and Autonomy

The data gathered about the DNA, rRNA and ribosomes of mitochondria from different species of different families in Tables 1, 9 and 10 respectively, provide a basis for examining the evolution of mitochondria.

From Table 1, there are at least two different classes of mitochondrial DNA. Mitochondrial DNA in protista, fungi and perhaps higher plants is linear, being about $5x10^7$ daltons. It is linear either naturally or because of shearing of large circles during isolation. This DNA contains about 10 mole percent less G+C than the nuclear DNA, which contains between 35-45 mole percent in every eukaryotic cell. This may not apply to higher plants. Animal cells possess a different type of mitochondrial DNA which is smaller in size being about $1x10^7$ daltons and is usually circular in form of average circumference of 5 μm. This type of mitochondrial DNA does not differ significantly from the nuclear DNA in respect to its G+C content. Mit-rRNA and ribosomes (Table 9 and 10 respectively) also exist in two classes. Fungi possess mit-rRNA and ribosomes which are similar in size to their bacterial counterparts. Animals possess a unique smaller class of mit-rRNA and ribosomes. Within the animal kingdom the higher the animal in the evolutionary scale the smaller is the rRNA. All mit-rRNA have a 15 mole percent lower content of G+C than their

counterparts in the cytoplasm. Thus, mitochondria of animal cells possess smaller DNA, ribosomes and rRNA than those of fungi and possibly plants. The larger DNA codes not only for the larger rRNA and perhaps more copies of it but probably also for more mitochondrial proteins. It seems, therefore, that mitochondria of animal cells possess less autonomy than those of plants or fungi.

From the point of view of evolution two hypotheses could explain these two types of mitochondria. These are, first, a unique symbiotic relationship followed by branched evolution and second the establishment of two symbiotic relationships followed by evolution to maintain two types of mitochondria.

It follows from the first hypothesis that after the initial invasion, DNA and much autonomy was lost from the aerobic mitochondrial ancestors and a symbiotic form similar to that observed in fungi at present evolved. In this symbiotic form, mitochondria managed to maintain their original rRNA and ribosomes, which apparently are similar in some respects to those of the contemporary bacteria. At some point, still early in the evolutionary process, there was further loss of mitochondrial DNA; the decrease in DNA could have been either of redundant genes or the transfer of unique structural genes to the nucleus. This rearrangement of the old symbiotic form as seen in present day fungi resulted in more co-operation between the mitochondria and the cell and led to the present day animal cell and mitochondria. The newer symbiotic form might have been the cause or the result of the successful evolution of the animal cell from the primitive

protista cell. This trend of losing mitochondrial autonomy as a function of further evolutionary development is seen within the animal kingdom where ultimately human mit-rRNA is the smallest reported. This phenomenon of decreasing size of mit-rRNA is in contrast to the increase of the large component of the cyt-rRNA in higher animal cells (Loening, 1968). The unique character of mitochondria of protista, which possess DNA of the fungal type and rRNA and ribosomes of the animal type perhaps reflect its position in the evolutionary scale which is lower than the animal kingdom (Whittaker, 1969).

According to the second hypothesis two different invasions of mitochondrial ancestors into the eukaryotic cell occured, one leading to the form similar to that of fungi and another leading to the form similar to that of animals.

V SUMMARY

Highly purified mitochondria containing 3.0 µg of RNA/mg of mitochondrial protein were prepared from rat liver by differential centrifugation. These mitochondria were free of cytoplasmic contamination as judged by enzymatic and electron microscopic studies. RNA, labelled with $^{32}P_i$ or [^{3}H]orotate, was isolated from these mitochondria by a phenol-detergent extraction method. The RNA sedimented at 15 and 13 S on sucrose density gradient and migrated on agarose-polyacrylamide gel at 21 and 12.5 S_E. Identical results were also obtained from rodent tissue culture cells (rat hepatoma and L cells) which were labelled with $^{32}P_i$, [^{3}H] or [^{14}C]uridine in the presence of 0.1 µg Actinomycin D/ml to supress the synthesis of cytoplasmic ribosomal RNA. From the sedimentation rates, electrophoretic mobility and relative amounts of the two RNA components it was concluded that they are the mitochondrial ribosomal RNA species. The synthesis of mitochondrial RNA was found to be sensitive to ethidium bromide (1.0 µg/ml) indicating that the mitochondrial ribosomal RNA was synthesized on mitochondrial DNA. The high molecular weight RNA isolated from all the rodent mitochondria possesses a low content of G+C which is about 20 moles percent lower than the cytoplasmic ribosomal RNA. Human KB cell mitochondrial RNA migrated on gels faster than that of rodents having values of

20.5 and 11.0 S_E. L cell mitochondrial ribosomal RNA was found to bind to MAK column stronger than its cytoplasmic counterpart and was eluted with 0.95 and 0.89 M NaCl respectively.

Mitochondrial ribosomal components, labelled with [^{3}H]orotate, were detected in the highly purified rat liver mitochondria lysed with deoxycholate and Triton X100. They had sedimentation coefficient of 55 and 35 S. The L cell mitochondrial fraction labelled in the presence of 0.1 µg Actinomycin D/ml had in addition a component that sedimented at 24 S.

VI BIBLIOGRAPHY

Aaij, C. and Borst, P. (1970). Biochim. Biophys. Acta. 217, 560.

Albaum, H.G. and Umbreit, W.W. (1947). J. Biol. Chem. 167, 369.

Altmann, R. (1890). Die Elementaroganismen und ihre Beziehungen zu den Zellen. Leipizig.

Amaldi, F. (1969). Nature 222, 95.

André, J. and Marinozzi, V. (1965). J. Microscop. 4, 615.

Appelmans, F., Wattiaux, R. and De Duve, C. (1955). Biochem. J. 59, 438.

Ashwell, M.A. and Work, T.S. (1968). Biochem. Biophys. Res. Commun. 32, 1006.

Ashwell, M.A. and Work, T.S. (1970). Annual Review of Biochemistry 39, 251.

Attardi, B. and Attardi, G. (1969). Nature, 224, 1079.

Avers, C.J., Billheimer, F.E., Hoffmann, H.P. and Pauli, R.M. (1968). Proc. Natl. Acad. Sci. U.S. 61, 90.

Barlow, J. and Mathias, A.P. (1966). In "Procedures in Nucleic Acid Research" (G.L. Cantoni and D.R. Davies eds). P. 444, Harper and Row, Publishers.

Bartoov, B., Mitra, R.S. and Freeman, K.B. (1970). Biochem. J. 120, 455.

Bauer, W. and Vinograd, J. (1968). J. Mol. Biol. 33, 141.

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

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

Beattie, D.S. (1968). J. Biol. Chem. 243, 4027.

Bernhard, W.A. (1969). J. Ultrastruct. Res. 27, 250.

Billing, R.J. and Barbiroli, B. (1970). Biochim. Biophys. Acta, 217, 434.

Boedtker, H. (1968). J. Mol. Biol. 35, 61.

Borst, P. and Ruttenberg, G.U.C.M. (1966). Biochim. Biophys. Acta, 114, 645.

Borst, P., Kroon, A.M. and Ruttenberg, G.J.C.M. (1967a). In "Genetic Elements Properties and Function" (D. Shugar, ed.), p. 81, Academic Press and Polish Scientific Publishers, London and Warsaw.

Borst, P., Ruttenberg, G.J.C.M. and Kroon, A.M. (1967b). Biochim. Biophys. Acta, 149, 140.

Borst, P., Van Bruggen, E.F.J., Ruttenberg, G.J.C.M. and Kroon, A.M. (1967c). Biochim. Biophys. Acta, 149, 156.

Borst, P. and Aaij, C. (1969). Biochem. Biophys. Res. Commun. 34, 358.

Brdiczka, D., Pette, D., Brunner, G. and Miller, F. (1968). European J. Biochem. 5, 294.

Breidenbach, R.W., Castelfranco, P. and Criddle, R.S. (1967). Plant physiol. 42, 1035.

Bretthauser, R.K., Marcus, L., Chaloupka, J., Halvorson, H.O. and Bock, R.M. (1963). Biochemistry 2, 1079.

Brewer, E.N., De Vries, A. and Rusch, H.P. (1967). Biochim. Biophys. Acta, 145, 686.

Burton, K. (1956). Biochem. J. 62, 315.

Bush, E.T. and Hansen, D.L. (1965). In "Radioisotope Sample Measurement Techniques in Medicin and Biology", p. 395, International Atomic Energy Agency, Vienna.

Cairns, J. (1963). J. Mol. Biol. 6, 208.

Cannon, M. (1968). European J. Biochem. 7, 137.

Carpenter, P.L. (1967). In "Microbiology", p. 74, Saunders Co. London.

Chen, P.S., Taribara, T.Y. and Warner, H. (1956). Anal. Chem. 28, 1756.

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

Clark-Walker, G.D., and Linnane, A.W. (1966). Biochem. Biophys. Res. Commun. 25, 8.

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

Clayton, D.A., and Vinograd, J. (1969). Proc. Natl. Acad. Sci. 62, 1077.

Click, R.E. and Tint, B.L. (1967). J. Mol. Biol. 25, 111.

Cooperstein, S.J. and Lazarow, A. (1951). J. Biol. Chem. 189, 665.

Corneo, G., Moore, C., Sanadi, D.R., Grossman, L.J. and Marmur, J. (1966). Science, 151, 687.

Corneo, G., Zardi, L. and Polli, E. (1968). J. Mol. Biol. 36, 419.

Corneo, G., Ginelli, E. and Polli, E. (1967). J. Mol. Biol. 23, 619.

Cox, R.A. (1966). Biochem. J. 98, 841.

Darnell, J.E. Jr. (1968). Bacter. Rev. 32, 262.

Das, H.K. and Mukherjee, T. (1964). Biochim. Biophys. Acta, 93, 304.

Davidson, J.N. and Smellie, R.M.S. (1952). Biochem. J. 52, 599.

Davidson, J.N. (1961). In "The Biochemistry of the Nucleic Acids", p. 109, Nethuen, London.

Dawid, I.B. (1965). J. Mol. Biol. 12, 581.

Dawid, I.B. (1966), Proc. Natl. Acad. Sci. U.S. 56, 269.

Dawid, I.B. and Wolstenholme, D.R. (1968). Biophys. J. 8, 65.

Dawid, I.B. (1969). Fed. Proc. 28, 349.

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

De Camirande, G., Allard, C., Da Costa, H.C. and Cantero, A. (1954). Science, 119, 351.

Dubin, D.T. (1967). Biochem. Biophys. Res. Commun. 29, 655.

Dubin, D.T. and Brown, R.E. (1967). Biochim. Biophys. Acta, 145, 538.

Dubin, D.T. (1969). Fed. Proc. 28, 349.

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

Du Buy, H.G., Mattern, C.T.F. and Riley, F.L. (1966). Biochim. Biophys. Acta, 123, 298.

Dure, L.S., Epler, J.L. and Barnett, W.E. (1967). Proc. Natl. Acad. Sci. U.S. 58, 1883.

Eagle, H. (1965). Proc. Sci. Expt. Biol. ,ed. 89, 362.

Eagle, H. (1959). Science, 130, 432.

Edelman, M. Epstein, H.T. and Schiff, J.A. (1966). J. Mol. Biol. 17, 463.

Edelman, M., Verma, I.M. and Littauer, V.Z. (1970). J. Mol. Biol. 49, 67.

Elaćv, N.R. (1964). Biokimiya, 29, 413.

Elaćv, N.R. (1966). Biokhimija, 31, 234.

Ellem, K.A.O. and Sheridan, J.W. (1964). Biochem. Biophys. Res. Commun. 16, 505

Ellem, K.A.O. (1966). J. Mol. Biol. 20, 283.

Ellem, K.A.O. and Rhode, S.L. (1969). Biochim. Biophys. Acta, 174, 117.

Ephrussi, B. (1953). In "Nucleo-Cytoplasmic Relations in Micro-Organisms", New York, Oxford Univ. Press.

Evans, T.E. (1966). Biochem. Biophys. Res. Commun. 22, 678.

Fauman, M., Rabinowitz, M. and Getz, G.S. (1969). Biochim. Biophys. Acta, 182, 355.

Freeman, K.B. (1965). Biochem. J. 94, 494.

Forrester, I.T., Magley, P. and Linnane, A.W. (1970). FEBS letter, 11, 59.

Fukamachi, S., Bartoov, B., Mitra, R.S. and Freeman, K.B. (1970). Biochem. Biophys. Res. Commun. 40, 852.

Fukuhara, H. (1967). Proc. Natl. Acad. Sci. U.S. 58, 1065.

Fukuhara, H., Faures, M. and Genin, C. (1969). Mol. Gen. Genetics, 104, 264.

Galper, J.B. and Darnell, J.E. (1969). Biochem. Biophys. Res. Commun. 34, 205.

Georgatsos, J.G. and Papasarantopoulou, N. (1968). Arch. Biochem. Biophys. 126, 771.

Gesteland, R.F. and Boedtker, H. (1964). J. Mol. Biol. 8, 496.

Gianetto, R. and De Duve, C. (1955). Biochem. J. 59, 433.

Gierer, A. (1958). Z. Naturf. 13b, 358.

Gilbert, W. (1963). J. Mol. Biol. 6, 389.

Gross, N.J. and Rabinowitz, M. (1969). J. Biol. Chem. 244, 1563.

Guerineau, M., Grandchamp, C., Yotsuyanagi, Y. and Slonimski, P.P. (1968). Compt. Rend. 266, 1884.

Guttes, E. and Guttes, S. (1964). Science, 145, 1057.

Haldar, D. and Freeman, K.B. (1968). Can. J. Biochem. 46, 1099.

Haldar, D. and Freeman, K.B. (1969). Biochem. J. 111, 653.

Harris, R.A., Asbell, M.A., Asai, J., Jolly, W.W. and Green, D.E. (1969). Arch. Biochem. Biophys. 132, 545.

Hershey, A.D. and Burgi, E. (1960). J. Mol. Biol. 2, 143.

Hollenberg, C.P., Borst, P., Thuring, R.W.J. and Van Bruggen, E.F.J. (1969). Biochim. Biophys. Acta, 186, 417.

Hudson, B. and Vinograd, J. (1967). Nature, 216, 647.

Iwashima, A. and Rabinowitz, M. (1969). Biochim. Biophys. Acta, 178, 283.

Kaempfer, R. (1969). Nature, 222, 950.

Kalf, G.F. (1964). Biochemistry, 3, 1702.

Kalf, G.F. and Ch'ih, J.J. (1968). J. Biol. Chem. 243, 4904.

Karol, M.H. and Simpson, M.V. (1968). Science 162, 470.

Katz, S. and Comb, D.G. (1963). J. Biol. Chem. 238, 3065.

Kidson, C., Kirby, K.S. and Ralph, R.K. (1963). J. Mol. Biol. 7, 313.

Kirby, K.S. (1965). Biochem. J. 96, 266.

Kirk, J.T.O. (1967). J. Mol. Biol. 28, 171.

Kirschner, R.H., Wolstenholme, D.R. and Gross, N.J. (1968). Proc. Natl. Acad. Sci. U.S. 60, 1466.

Knight, E. (1969). Biochim. Biophys. Acta, 182, 562.

Koch, J. and Stakstad, E.L.R. (1967). European J. Biochem. 3, 1.

Krawiec, S. and Eisenstadt, J.M. (1970). Biochim. Biophys. Acta, 217, 120.

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

Kroon, A.M. (1965), Biochim. Biophys. Acta, 108, 275.

Kroon, A.M., Borst, P., Van Bruggen, E.F.J. and Ruttenberg, G.J.C.M. (1966). Proc. Natl. Acad. Sci. U.S. 56, 1836.

Kroon, A.M. (1966). In "Regulation of Metabolic Processes in Mitochondria" (J.M. Tagar, S. Papa, E. Quagliariello, and E.C. Slater, eds.), BBA Library, Vol. 7, p. 397, Elsevier, Amsterdam.

Kroon, A.M., Saccone, C. and Bottman, M.J. (1967). Biochim. Biophys. Acta, 142, 552.

Kroon, A.M. and Aaij, C. (1968). In "Biochemical Aspects of the Biogenesis of Mitochondria" p, 207 (Slater, E.C., Tager, J.M., Papa, S. and Quagliariello, E. eds.), Adriatica Editrice, Bari.

Kuntzel, H. and Noll, H. (1967). Nature, 215, 1340.

Kuntzel, H. (1969). Nature, 222, 142.

Kuntzel, H. (1969a). J. Mol. Biol. 40, 315.

Kuntzel, H. (1969b). FEBS letters, 4, 140.

Laird, A.K., Nygaard, A., Ris, H. and Barton, A.D. (1953). Expl. Cell Res. 5, 147.

Lane, B.G. and Tamaoki, T. (1967). J. Mol. Biol. 27, 335.

Lane, B.G. (1963). Biochim. Biophys. Acta, 72, 110.

Leaver, C.J. and Key, J.L. (1970). J. Mol. Biol. 49, 671.

Leon, S.A. and Mahler, H.R. (1968). Arch. Biochem. Biophys. 126, 305.

Levine, E.M., Burleight, I.G., Boone, C.W. and Eagle, H. (1967). Proc. Natl. Acad. Sci. U.S. 57, 431.

Levin, E.M., Thomas, L., McGregor, D., Hayflick, L. and Eagle, H. (1968). Proc. Natl. Acad. Sci. U.S. 60, 583.

Littauer, V.Z. and Sela, M. (1962). Biochim. Biophys. Acta, 61, 609.

Loeb, J.N. and Hubby, B.G. (1968). Biochim. Biophys. Acta, 166, 745.

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

Loening, V.E. and Ingle, J. (1967). Nature, 215, 363.

Loening, V.E. (1968). J. Mol. Biol. 38, 355.

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

Luck, D.J.L. (1963). J. Cell Biol. 16, 483.

Luck, D.J.L. (1964). J. Cell Biol. 24, 445.

Luck, D.J.L. and Reich, E. (1964). Proc. Natl. Acad. Sci. U.S. 52, 931.

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

Lyttleton, J.W. (1962). Exptl. Cell Res. 26, 312.

Mandell, J.D. and Hershey, A.D. (1960). Anal. Biochem. 1, 66.

Marco, R., Sebastian, J. and Sols, A. (1969). Biochem. Biophys. Res. Commun. 34, 725.

Markov, G.G., Bradvorava, I., Mintcheva, A., Petrov, P., Shisnokov, N. and Tsanev, R.G. (1969). Expl. Cell Res. 57, 374.

Marmur, J. and Doty, P. (1962). J. Mol. Biol. 5, 109.

Mager, J. (1960). Biochim. Biophys. Acta, 38, 150.

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

Mejbaum, W. (1939). Hoppe-Seyl. Z. 258, 117.

Meyer, R.R. and Ris, H. (1966). J. Cell Biol. 31, 76A.

Meyer, R.R. and Simpson, M.V. (1968). Proc. Natl. Acad. Sci. U.S. 61, 130.

Meyer, R.R. and Simpson, M.V. (1969). Biochem. Biophys. Res. Commun. 34, 238.

Mitchell, H.K. and Mitchell, M.B. (1952). Proc. Natl. Acad. Sci. U.S. 38, 442.

Mounolou, J.C., Perrodin, G. and Slonimski, P.P. (1968). In "Biochemical Aspects of the Biogenesis of Mitochondria", p. 133 (E.C. Slater, J.M. Tager, S. Papa and E. Quagliariello eds.), Adriatica Editrice, Bari, Italy.

Moustacchi, E. and Williamson, D.H. (1966), Biochem. Biophys. Res. Commun. 23, 56.

Muntwyler, E., Seifter, S. and Harkness, D.M. (1950). J. Biol. Chem. 183, 181.

Nagata, T., Shibata, O. and Nawa, T. (1967). Histochemistry 10, 305.

Nageo, M. and Sugimara, T. (1965). Biochim. Biophys. Acta, 103, 353.

Nass, S., Nass. M.M.K. and Hennix, V. (1965). Biochim. Biophys. Acta, 95, 426.

Nass, M.M.K., Nass, S. and Atzelius, B.A. (1965). Exptl. Cell Res. 37, 516.

Nass, M.M.K. (1968). In "Biochemical Aspects of the Biogenesis of Mitochondria", p. 27 (E.C. Slater, J.M. Tager, S. Papa, and E. Quagliariello, eds.), Adriatica Editrice, Bari, Italy.

Nass, M.M.K. (1969a). J. Mol. Biol. 42, 521.

Nass, M.M.K. (1969b). J. Mol. Biol. 42, 529.

Neubert, D. and Helge, H. (1965). Biochem. Biophys. Res. Commun. 18, 600.

Neubert, D. (1966). Arch Exptl. Pathal. Pharmakol. 253, 152.

Neubert, D., Oberdisse, E. and Bass, R. (1968). In "Biochemical Aspects of the Biogenesis of Mitochondria", p. 103, (E.C. Slater, J.M. Tager, S. Papa and E. Quagliariello, eds.), Adriatica Editrice, Bari, Italy.

Neubert, D., Helge, H. and Merker, H.J. (1968). In "Biochemical Aspects on the Biogenesis of Mitochondria:, p. 251, (E.C. Slater, J.M. Tager, S. Papa and E. Quagliariello, eds.), Adriatica Editrice, Bari, Italy.

Noll, H. (1967). Nature, 215, 360.

Novikoff, A.B. (1957). Symp. Soc. Exp. Biol. 10, 92.

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

O'Brien, T.W. and Kalf, G.F. (1967b). J. Biol. Chem. 242, 2180.

O'Brien, T.W. (1969). Fed. Proc. 28, 885.

Osawa, S. and Sibatani, A. (1967). In "Method in Enzymology", Vol. XII, p. 678, (L. Grossman and K. Moldave eds.), Academic Press, New York.

Parsons, J.A. (1965). J. Cell Biol. 25, 641.

Parsons, J.A. and Dickson, R.C. (1965). J. Cell Biol. 27, 77A

Parsons, D.F., Williams, G.R., Thompson, W., Wilson, D. and Chance, B. (1967). In "Mitochondrial Structure and Compartmentation", p. 29 (E. Quagliariello, S. Papa, E.C. Slater and J.M. Tager, eds.), Adriatica Editica, Bari, Italy.

Parsons, P. and Simpson, M.V. (1967). Science, 155, 91.

Parsons, D.F. (1967). In "Methods in Enzymology". Vol. X, p. 655, Academic Press, New York.

Parsons, P. and Simpson, M.V. (1968). In "Biochemical Aspect of the Biogenesis of Mitochondria" (E.C. Slater, J.M. Tager, S. Papa and E. Quagliariello, eds.), p. 171, Adriatica Editrice, Bari, Italy.

Peacock, A.C. and Dingman, C.W. (1968). Biochemistry, 7, 668.

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

Penman, S. (1966). J. Mol. Biol. 17, 117.

Penman, S., Vesco, C. and Penman, M. (1968). J. Mol. Biol. 34, 49.

Perlman, S. and Penman, S. (1970). Nature, 227, 133.

Perry, R. (1963). Exptl. Cell Res. 29, 400.

Perry, R.P. and Kelley, D.E. (1968). J. Mol. Biol. 35, 37.

Perry, R.P. and Kelley, D.E. (1969). J. Cell Biol. 43, 1039.

Peterman, M.L. (1964). In "The Physical and Chemical Properties of Ribosomes", p. 134, Elsevier Publishing Company, N.Y., N.Y.

Piko, L., Tyler, A. and Vinograd, J. (1967). Biol. Bull. 132, 68.

Piko, L., Blair, D.G., Tyler, A. and Vinograd, J. (1968). Proc. Natl. Acad. Sci. U.S. 59, 838.

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

Rabinowitz, M., Sinclair, J., De Salle, L., Haselkorn, R. and Swift, H.H. (1965). Proc. Natl. Acad. Sci. U.S. 53, 1126.

Rabinowitz, M., De Salle, L., Sinclair, J., Stirewalt, R. and Swift, H. (1966). Fed. Proc. 25, 581.

Rabinowitz, M., Getz, G.S. and Swift, H. (1968). In "Biochemical Aspect of the Biogenesis of Mitochondria", p. 155, (E.C. Slater, J.M. Tagar, S. Papa and E. Quagliariello, eds.), Bari, Italy.

Rabinowitz, M., Getz, G.S., Casey, J. and Swift, H. (1969). J. Mol. Biol. 41, 381.

Rabinowitz, M. and Swift, H. (1970). Physiol. Rev. 50, 376.

Radloff, R., Bauer, W. and Vinograd, J. (1967). Proc. Natl. Acad. Sci. U.S. 57, 1514.

Rahman, Y.E. (1966). Biochim. Biophys. Acta, 119, 470.

Rakhimbekova, L.S. and Gaitskoki, V.S. (1969). Molek. Biol. 3, 315.

Ray, D.S. and Hanawalt, P.C. (1965). J. Mol. Biol. 11, 760.

Rendi, R. (1959). Expl. Cell Res. 17, 585.

Rendi, R. and Warner, R.C. (1960). Ann. N.Y. Acad. Sci. 88, 741.

Rifkin, M.R., Wood, D.D. and Luck, D.J.L. (1967). Proc. Natl. Acad. Sci. U.S. 58, 1025.

Rogers, P.J., Preston, B.N., Titchener, E.B. and Linnane, A.W. (1967). Biochem. Biophys. Res. Commun. 27, 405.

Roodyn, D.B., Reis, P.J. and Work, T.S. (1961). Biochem. J. 80, 9.

Roodyn, D.B., Suttie, J.W. and Work, T.S. (1962). Biochem. J. 83, 29.

Roodyn, D.B. and Wilkie, D. (1968). In "The Biogenesis of Mitochondria", London; Methuen.

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

Sanford, K., Earle, W. and Likely, G. (1948). J. Natl. Cancer Ins. 9, 229.

Scherbaum, O. (1960). Ann. N.Y. Acad. Sci. 90, 565.

Schiefer, H.G. (1969). Z. Physiol. Chem. 350, 235.

Schildkraut, C.L., Marmur, J. and Doty, P. (1962). J. Mol. Biol. 4, 430.

Schmitt, H. (1969). FEBS letters, 4, 234.

Schnaitman, C. and Greenawalt, J.W. (1968). J. Cell Biol. 38, 158.

Schneider, W.C. and Kuff, E.L. (1965). Proc. Natl. Acad. Sci. U.S. 54, 1650.

Sebald, W., Hofstatter, T., Hacker, D. and Bücher, T. (1969). FEBS letters, 2, 177.

Sebald, W., Schwab, A.J. and Bücher, T. (1969). FEBS letters, 4, 243.

Sherman, F. and Slonimski, P.P. (1964). Biochim. Biophys. Acta, 90, 1.

Sinclair, J.H. and Stevens, B.J. (1966). Proc. Natl. Acad. Sci. U.S. 56, 508.

Sinclair, J.H., Stevens, B.J., Gross, N. and Rabinowitz, M. (1967). Biochim. Biophys. Acta, 145, 528.

Siekevitz, P. and Watson, M.L. (1956). J. Biophys. Biochem. Cytol. 7, 619.

Slonimski, P.P., Perrodin, G. and Croft, J.H. (1968). Biochem. Biophys. Res. Commun. 30, 232.

Smith, D., Tauro, P., Schweizer, E. and Halvorson, H.O. (1968). Proc. Natl. Acad. Sci. U.S. 60, 936.

Smith, A.E. and Marcker, K.A. (1968). J. Mol. Biol. 38, 241.

Smuckler, E.A. (1968). In "Structure and Function of Endoplasmic Reticulum in Animal Cells", p. 13 (Ed. by Gran, F.C.) FEBS Academic Press, London & New York.

Sober, H.A. (1968). In "Handbook of Biochemistry", p. H-14, Chemical Rubber Co., Cleveland.

Somenshein, G.E. and Holt, C.E. (1968). Biochem. Biophys. Res. Commun. 33, 361.

South, D.J. and Mahler, H.R. (1968). Nature, 218, 1226.

Spirin, A.S. (1961). Biochemistry, 26, 454 (USSR, English transl.).

Stanley, W.M. Jr. and Bock, R.M. (1965). Biochemistry, 4, 1302.

Stegeman, W.J., Cooper, C.S. and Avers, C.J. (1970). Biochem. Biophys. Res. Commun. 39, 69.

Steinschneider, A. (1969). Biochim. Biophys. Acta. 186, 405.

Stone, G.E. and Miller, O.L. (1965). J. Exptl. Zool. 159, 33.

Strauss, J.H., Kelly, R.B. and Sinsheimer, R.L. (1968). Biopolymers, 6, 793.

Sueoka, N. and Cheng, T.Y. (1962). J. Mol. Biol. 4, 161.

Suyama, Y. and Preer, J.R. Jr. (1965). Genetics, 52, 1051.

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

Suyama, Y. (1966). Biochemistry, 5, 2214.

Suyama, Y. (1967). Biochemistry, 6, 2829.

Swanson, F.R. and Dawid, I.B. (1970). Proc. Natl. Acad. Sci. U.S. 66, 117.

Swift, H., Adams, B.J. and Larsen, K. (1964). J. Roy. Microscop. Soc. 83, 161.

Swift, H. (1965). Am. Naturalist, 99, 201.

Swift, H. and Adams, B.J. (1966). J. Histochem. Cytochem. 14, 744.

Swift, H., Rabinowitz, M. and Getz, G.S. (1968). In "Biochemical Aspects of the Biogenesis of Mitochondria", p. 3 (E.C. Slater, J.M. Tager, S. Papa and E. Quagliariello, eds.), Adriatica Editrice, Bari, Italy.

Tewari, K.K., Vötsch, W., Mahler, H.R. and Mackler, B. (1966). J. Mol. Biol. 20, 453.

Thompson, E.B., Tomkins, G.M. and Curran, J.F. (1966). Biochemistry, 56, 296.

Tissiéres, A. and Watson, J.D. (1958). Nature, 182, 778.

Trewavas, A. (1967). Analit. Biochem. 21, 324.

Truman, D.E.S. and Korner, A. (1962). Biochem. J. 83, 588.

Truman, D.E.S. (1963). Exptl. Cell Res. 31, 313.

Van Bruggen, E.F.J., Runner, C.M., Borst, P., Ruttenberg, G.J.C.M., Kroon, A.M. and Schuurmans Stekhoven, F.M.A.H. (1968). Biochim. Biophys. Acta, 161, 402.

Verma, I.M., Edelman, M., Herzberg, M. and Littauer, U.Z. (1970). J. Mol. Biol. 59, 137.

Vesco, L. and Penman, S. (1969). Proc. Natl. Acad. Sci. U.S., 62, 218.

Vignais, P.V., Huet, J. and André, J. (1969). FEBS letters, 3, 177.

Von Ehrenstin, G. and Lipmann, F. (1961). Proc. Natl. Acad. Sci. U.S. 47, 941.

Watanabe, Y., Prevec, L. and Graham, A.F. (1967). Proc. Natl. Acad. Sci. U.S. 58, 1040.

Watson, M.L. and Aldridge, W.G. (1964). J. Histochem. Cytochem. 12, 96.

Weber, M.J. and De Moss, J.A. (1969). Bacteriol. 97, 1099.

Wells, R. and Birnstiel, M.L. (1967). Biochem. J. 105, 53P.

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

Whittaker, R.H. (1969). Science, 163, 150.

Wilkie, D. (1964). The Cytoplasm in Heredity, Methuen, London.

Wintersberger, E. (1965). Biochem. Z, 341, 409.

Wintersberger, E. and Tuppy, H. (1965). Biochem. Z. 341, 399.

Wintersberger, E. (1966). Biochem. Biophys. Res. Commun. 25, 1.

Wintersberger, E. (1967). Hoppe-Seyler's Z. physiol. Chem. 348, 1701.

Wintersberger, E. (1968). Nature, 220, 699.

Wintersberger, E. and Viehhauser, G. (1968). Nature, 220, 699.

Witt, J., Kronau, R. and Holzer, H. (1966). Biochem. Biophys. Acta, 128, 63.

Wolstenholme, D.R. and Dawid, I.B. (1967). Chromosoma, 20, 445.

Wolstenholme, D.R. and Gross, N.J. (1968). Proc. Natl. Acad. Sci. U.S. 61, 245.

Wolstenholme, D.R. and Dawid, I.B. (1968). J. Cell Biol. 39, 222.

Wood, D.D. and Luck, D.J.L. (1969). J. Mol. Biol. 41, 211.

Wright, R.E. and Lederberg, J. (1957). Proc. Natl. Acad. Sci. U.S. 43, 919.

Yoshikawa, M., Enkada, T. and Kawede, Y. (1964). Biochem. Biophys. Res. Commun. 15, 22.

Zylber, E., Vesco, C. and Penman, S. (1969). J. Mol. Biol. 44, 195.