PART I

CHARACTERIZATION OF THE RNA FROM THE MITOCHONDRIAL FRACTION OF HELA CELLS

PART II

PROPERTIES OF MEMBRANE-BOUND RIBOSOMES IN HELA CELLS

Thesis by

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ABSTRACT

PART I of this thesis is concerned with the characterization of RNA components from the mitochondrial fraction of HeLa cells. Mitochondria prepared by differential centrifugation followed by buoyant density fractionation in sucrose gradient are contaminated by elements of the rough endoplasmic reticulum. In order to identify RNA components of mitochondrial origin in this fraction the following criteria were used: association of newly synthesized RNA with mitochondria (recognized by cytochrome oxidase assay), insensitivity in situ to ribonuclease digestion, linear kinetics of labeling after [^{3}H]-uridine pulses, sensitivity of their synthesis to ethidium bromide, and, especially, capacity to hybridize with purified closed-circular mitochondrial DNA.

It has been found that the RNA synthesized on a mit-DNA template consists of both rapidly labeled heterogeneous RNA components, varying in sedimentation constant from 4 to 50 S or more, and discrete RNA species, migrating at 16 S, 12 S, and 4 S in sucrose gradient. Analysis of the sedimentation behavior of the 16 S and 12 S species under denaturing conditions has indicated that they are represented by continuous polynucleotide chains. From their migration in polyacrylamide gels relative to ribosomal RNA markers, the respective molecular weights of 16 S and 12 S have been estimated to be 0.7×10^6 and 0.4×10^6 daltons. The 16 S, 12 S, and 4 S RNA's appear to be methylated. Both the discrete and heterogeneous mit-DNA coded RNA components have

a base composition clearly different from that of ribosomal RNA and cytoplasmic messenger RNA and complementary, as concerns the A and U content, to that of the heavy strand of mit-DNA.

The results of an investigation concerning the properties of the ribosomes associated with the endoplasmic reticulum in HeLa cells are reported in PART II. From the distribution of ribosomal RNA among the subcellular fractions, it has been estimated that 10-15% of the total ribosomes in HeLa cells are membrane-bound; 65-70% of these can be recovered in the form of polysomes after membrane lysis with sodium deoxycholate. The 18 S and 28 S RNA components of the membrane-bound ribosomes have similar kinetics of labeling and identical sedimentation properties and nucleotide composition to the homologous components of free ribosomes, these results pointing to a common nuclear origin. The ribosomal RNA of membrane-bound ribosomes is made acid-soluble to about the same extent as the ribosomal RNA of free polysomes under the conditions of ribonuclease digestion in situ employed here. Treatment with EDTA releases about 85-90% of the small ribosomal subunits and 70% of the large ribosomal subunits, a situation which is similar to that which has been observed for rat liver microsomes. These results suggest that the great majority of the ribosomes in the mitochondrial fraction of HeLa cells are extramitochondrial, i. e. bound to the endoplasmic reticulum, but the existence of a small number of intramitochondrial ribosomes is not excluded.

TABLE OF CONTENTS

GENERAL INTRODUCTION

The discovery of DNA in cytoplasmic organelles (mitochondria, chloroplasts, and centrioles (see for review 1, 2)) of eukaryotic cells has provided the physical evidence for the existence of cytoplasmic genetic systems already recognized circumstantially through examples of cytoplasmic inheritance. It is now well-established that mitochondria from all organsims contain DNA. Mitochondrial DNA from plants and lower eukaryotic organisms has been isolated in general in the form of linear molecules of various sizes. For example, the mitochondrial DNA from Tetrahymena appears to consist of a population of linear molecules of about 17μ (3), while heterogeneous molecules of linear DNA up to 25-26μ have been obtained from Neurospora (4) and up to 60μ, from the red bean, Phaseolus vulgaris (5). The size and structure of yeast DNA have been open to question due to conflicting reports from various laboratories (6, 7, 8, 9, 10, 11) although the most recent report suggests that mitochondrial DNA of yeast consists of 25μ circles which are easily degraded during isolation (12). In contrast to the situation obtaining for the mitochondrial DNA from lower eukaryotic and plant cells, a striking observation has been that the DNA from animal cells over a broad evolutionary range, from sea urchin (13) to man (14), consists of closed circular duplex molecules with a contour length of about 5μ and a molecular weight of about 10^7 daltons (see for review 2); in addition, two complex forms of mitochondrial DNA, catenated oligomers and circular oligomers (which appear to be multimers of the basic 5μ unit (15)), have been demonstrated (16, 17, 18, 19). From experiments measuring the renaturation kinetics of mitochondrial DNA from chick liver (20), it appears that the sequence length (that is, that amount

of mitochondrial DNA in which every sequence is represented once) of mitochondrial DNA is very close to the physical length. Furthermore, the complete reannealing of a mixture of denatured mitochondrial DNA monomers and dimers from human leukemic leukocytes and the absence of heterologous regions in the renatured monomers or dimers provide additional evidence that the mitochondrial DNA population is essentially homogeneous (15). The homogeneity in size and base sequence of mitochondrial DNA from animal cells suggests that the informational content of this DNA has reached an evolutionary minimum.

As concerns the informational role of mitochondrial DNA, the idea was suggested at the end of the last century that mitochondria are capable of semi-autonomous growth and division (21, 22). This idea has received impetus from the demonstrations of the existence in mitochondria of DNA and active enzyme systems for DNA replication (23, 24, 25, 26, 27, 28, 29) and transcription (30, 31, 32, 33, 34) and of an intrinsic protein synthesizing system (35, 36, 37, 38, 39, 40). The mitochondrial protein synthesizing system in yeast exhibits both in vivo and in vitro a pattern of sensitivity towards various inhibitors which is different from that of the cytoplasmic system and which resembles rather that of bacterial systems (40, 41, 42, 43). This pattern of sensitivity towards inhibitors of bacterial protein synthesis is suggestive of the presence of bacterial-type ribosomes in mitochondria. Indeed ribosomes with distinctive ribosomal RNA components having sedimentation coefficients similar to bacterial ribosomal RNA species have been shown to occur in mitochondria from Neurospora and yeast (44, 45, 46, 47, 48,

49, 50); the spectrum of the proteins from mitochondrial ribosomes of Neurospora crassa, fractionated on carboxymethyl cellulose columns, appears to be quite different from that of the cytoplasmic ribosomes of this organism (51). Mitochondria from various organisms have specific transfer RNA species (52, 53, 54) including N-formyl-methionyl tRNA (55, 56), the initiator of protein synthesis in bacteria, and the existence of a full complement of tRNA's as well as tRNA synthetases in the mitochondria of Neurospora crassa has been reported (52, 57). As concerns the origin of the information for the synthesis of the components of the mitochondrial protein synthesizing system and the constituents of the mitochondrial membranes, several types of observations have pointed to a dual control, nuclear and mitochondrial. This evidence is summarized below:

(a) Mitochondrial - In yeast and Neurospora there is evidence from RNA-DNA hybridization experiments that the specific mitochondrial ribosomal RNA species are coded by mitochondrial DNA (4, 58). Preliminary evidence for a cytoplasmic genetic involvement in the determination of resistance to the antibiotics erythromycin and lincomycin (40, 59) , mutations which presumably reflect changes in ribosomal proteins as in E. coli (60), is suggestive of at least a partial control by mitochondrial DNA of the synthesis of mitochondrial ribosomal proteins; however, evidence has also been presented that most, if not all, of the ribosomal proteins from the mitochondrial ribosomes of Neurospora crassa and yeast are synthesized by cytoplasmic ribosomes (51, 61). Mitochondrial leucyl-tRNA of rat liver has been shown to anneal with mitochondrial DNA implying that this DNA may be the

template from which mitochondrial tRNA is transcribed (62). The classic non-chromosomal respiratory deficient mutants of Neurospora ("poky") and yeast (cytoplasmic "petites") have provided various types of evidence for the role of mitochondrial DNA in controlling mitochondrial structure and function. It has been shown that cytoplasmic petites (rho^- cells) contain mitochondrial DNA with a greatly altered base composition resulting in changes in buoyant density (63). Cytochromes aa_3, b, and c_1 are absent from rho^- cells (64), and their synthesis in wild-type yeast is inhibited by various antibiotics which affect specifically mitochondrial protein synthesizing systems (40); however, it is possible that these results reflect the control, by a protein coded by mitochondrial DNA, of the synthesis of these cytochromes or on their incorporation into the mitochondrial membranes. As a matter of fact, one of the several components of wild-type structural protein in yeast mitochondria has been reported to be missing in preparations from a cytoplasmic petite mutant (65, 66, 67). Further support for the hypothesis of the involvement of mitochondrial DNA in mitochondrial assembly comes from the observation of a preferential transcription of mitochondrial DNA during adaptation to oxygen in yeast, in concomitance with mitochondrial development (68). In Neurospora two presumably mitochondrial DNA mutations resulting in the "poky" phenotype have been reported to lead to alterations in the primary structure of the mitochondrial structural protein (69); however, this observation needs to be confirmed.

(b) Nuclear - That the nucleus must provide the major part of the information required for the synthesis of mitochondrial constituents

can be concluded from the small size of the mitochondrial DNA genome. This is particularly true in the case of animal cell mitochondrial DNA whose 15,000-16,000 base pairs are grossly inadequate to code for all the components of a protein synthesizing system (including 3 species of ribosomal RNA, 50-60 species of ribosomal proteins as in bacteria (70), at least 20 tRNA species and amino acyl tRNA synthetases, and several protein factors) and/or for all or most of the components of the mitochondrial membranes. Furthermore, definitive evidence has been presented for the nuclear control of the structural gene for cytochrome C in yeast (71), and there is genetic evidence for the nuclear control of the structural genes for several other mitochondrial enzymes in yeast (see 2), for mitochondrial malic dehydrogenase in Neurospora (72), maize (73), and man (74), for β-hydroxybutyric dehydrogenase in Paramecium (75), and for one mitochondrial specific tRNA synthetase in Neurospora (76). Biochemical evidence has indicated that all or the major part of the mitochondrial soluble enzymes and possibly also of the structurally-bound enzymes and of the structural protein are synthesized by the cytoplasmic protein synthesizing system and then transported to the mitochondria (77, 78, 79, 80, 81, 82). In in vitro protein synthesizing systems involving mitochondria, after pulse labeling with radioactive amino acids, radioactivity is found to be associated only with less soluble protein (39, 83, 84, 85, 86), in particular, with the structural protein of the inner mitochondrial membrane (85, 86). As concerns mitochondrial phospholipids, there is evidence that fundamental steps in their biosynthesis occur in the endoplasmic reticulum and that they then

must be transported to the mitochondria (87).

The mitochondrial system has the potential to serve as a relatively simple model for the study of replication, transcription, and translation processes occurring in eukaryotic cells and for regulation of these phenomena. One obvious way of approaching the problem of the informational role of mitochondrial DNA is to analyze the primary gene products of mitochondrial DNA. Accordingly, my advisor, Dr. Giuseppe Attardi, suggested to me in 1966 that I begin a study of cytoplasmic DNA-directed RNA synthesis in HeLa cells. The lack of available methods for purification of mitochondria from elements of rough endoplasmic reticulum has necessitated various approaches to determine the characteristics and origin of the RNA from the mitochondrial fraction of HeLa cells, as will be discussed in Part I of this thesis. The properties of the ribosomes contained in the mitochondrial fraction will be described in Part II.

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PART I

CHARACTERIZATION OF THE RNA FROM THE MITOCHONDRIAL FRACTION OF HELA CELLS

CHAPTER 1

A Membrane-Associated RNA of Cytoplasmic Origin in HeLa Cells

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INTRODUCTION

The investigations described in Chapter 1 represent the early experiments that were designed to answer the question of whether there exists a fraction of RNA in HeLa cells which is of cytoplasmic origin. In these investigations advantage was taken of the fact that the RNA species synthesized in the HeLa cell nucleus arrive in the cytoplasm with a delay of 20 to 30 minutes (1), so that labeled RNA appearing in the cytoplasm at earlier times should represent species synthesized in situ. For this reason most of the analysis was carried out on cells exposed to short pulses with a radioactive RNA precursor. Furthermore, since a minor contamination of the cytoplasm by labeled nuclear material would have easily mimicked an in situ synthesis, it was considered necessary, first of all, to modify the cell fractionation procedure so as to reduce to the minimum the chance of contamination of the cytoplasmic fraction by nuclear fragments or material leaked from the nucleus and, second, to separate the membranous structures of the cytoplasm from the bulk of cytoplasmic RNA of nuclear origin associated with free monomers and polysomes. To avoid rupture of nuclei and subsequent release of nuclear RNA components, conditions of cell breakage, involving swelling of the cells for a short time in hypotonic medium followed by mild homogenization, were adopted. As concerns fractionation of the cytoplasmic structures themselves, since the great majority (85-90%) of the ribosomes and polysomes in HeLa cells are non-membrane-bound (2), by differential centrifugation, the membrane fraction (which contains, in addi-

tion to mitochondria, elements of rough and smooth endoplasmic reticulum) can be separated from the bulk of the free polysomes and monomers containing RNA components of nuclear origin. The main result of this section is that there is indeed an RNA fraction associated with membranous structures which, for its sedimentation properties, nucleotide composition, and metabolic behavior, is clearly distinct from the messenger RNA of free polysomes. On the basis of its linear kinetics of appearance in the cytoplasm and its homology to cytoplasmic DNA, this RNA appeared to be of cytoplasmic origin.

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A MEMBRANE-ASSOCIATED RNA OF CYTOPLASMIC ORIGIN IN HELA CELLS

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The genetic evidence of the existence in yeast[1] and molds[2] of cytoplasmic determinants controlling the structure and function of mitochondria and the demonstration that mitochondria from all organisms contain DNA[3–7] have indicated that transcription and translation processes dependent on cytoplasmic genes occur in eukaryotic cells. More recent observations in *Neurospora* have suggested that a cytoplasmic, presumably mitochondrial, genetic determinant controls the structural proteins of different membrane systems (mitochondria, microsomes, nuclear membrane)[8, 9]; these structural proteins, from their amino acid composition, fingerprinting pattern, and immunological behavior, appear to be identical or to have a common component.[8, 9] These findings suggest that cytoplasmic genes may have a wider role in the cell than hitherto suspected and may direct a considerable fraction of mRNA and protein synthesis.

These considerations prompted an investigation of cytoplasmic DNA-directed RNA synthesis in HeLa cells. The evidence obtained indicates that, in these cells, polysomes associated with cytoplasmic membranes contain an mRNA fraction which is distinct from mRNA of free polysomes, and appears to be of cytoplasmic origin.

Materials and Methods.—(a) *Cells:* Conditions of growth of HeLa cells in suspension have been described previously.[10] These cultures were free of any detectable PPLO contamination.

(b) *Buffers:* The buffers used are: (1) T: 0.01 *M* tris buffer (pH 7.1); (2) TM: 0.01 *M* tris buffer (pH 7.1), 0.00015 *M* $MgCl_2$; (3) TKM: 0.01 *M* tris buffer (pH 7.1), 0.01 *M* KCl, 0.00015 *M* $MgCl_2$; (4) SMET:[11] 0.07 *M* sucrose, 0.21 *M* d-mannitol, 0.0001 *M* EDTA, 0.001 *M* tris buffer (pH 7.2).

(c) *Labeling conditions:* Exponentially growing HeLa cells (1–3 × 10^5 cells/ml) were exposed for various times to H^3-5-uridine (17.3–30.0 c/mM, 1.25–6.25 μc/ml). Long-term labeling of phosphatidyl choline was carried out by growing cells for 48 hr in the presence of C^{14}-choline chloride (25 μc/ml). For incorporation of P^{32}-orthophosphate, the cells were washed twice and resuspended in phosphate-free Eagle's medium (with dialyzed serum); carrier-free-orthophosphate was utilized at 50 μc/ml.

(d) *Preparation of subcellular fractions:* In order to minimize the possibility of aggregation of free polysomes with membranes, the Mg^{++} concentration in the homogenization medium was reduced to the minimum compatible with stability of polysomes, 1.5 × $10^{-4}M$.[12] The labeled cells were washed three times with NKM (0.13 *M* NaCl, 0.005 *M* KCl, 0.001 *M* $MgCl_2$) and then resuspended in 6 vol TKM. After 3 min the suspension was homogenized with an A. H. Thomas homogenizer (10–15 strokes), sucrose was added to 0.25 *M*, and the homogenate centrifuged at 1160 × *g* for 3 min to sediment nuclei, unbroken cells, and big cytoplasmic debris. The supernatant (*total cytoplasmic fraction*) was spun at 8100 × *g* for 10 min; the pellet was resuspended in 0.25 *M* sucrose in T buffer and, after a spin at 1000 × *g* for 2 min to sediment any possible residual nuclei and a small amount of aggregated debris, was recentrifuged at 8100 × *g* for 10 min to yield the *first membrane fraction*. The first 8100 × *g* supernatant was centrifuged at 15,800 × *g* for 20 min to separate the *second membrane fraction* (pellet) from the *free polysome fraction*. Any deviations from this procedure are specified in the legends of the figures.

For sedimentation analysis, subcellular fractions were centrifuged for 70–80 min at 24 krpm at 3°C in the SW 25 rotor of the Spinco L ultracentrifuge through a 15–30% (w/w) sucrose gradient in TKM prepared above 3 ml of 64% (w/w) sucrose.

Separation of cytoplasmic fractions on the basis of density was carried out by centrifuging them for 16 hr at 24 krpm at 3°C in the SW 25 rotor through a 1.0 to 2.0 *M* sucrose gradient in T buffer.

(e) *Extraction and analysis of RNA:* Conditions for RNA extraction and sedimentation analysis, isotope determinations, and base composition analysis have been described in detail elsewhere.[13]

(f) *Determination of the labeling of the pools of uridine nucleotides:* A cell suspension was exposed to H^3-5-uridine (25.4 c/mM, 3.3 μc/ml). At various intervals, aliquots were removed and quickly cooled; the cells were washed three times with NKM, then precipitated with 0.5 N $HClO_4$. The $O.D._{260}$ and the H^3 cpm of the acid-soluble fraction were measured. The samples were then neutralized with KOH, the insoluble $KClO_4$ was separated by centrifugation, and the labeled components of the acid-soluble pool fractionated by Dowex 1-X8 (formate form) chromatography.[14] For all times of labeling analyzed (4, 7, 10, 17 min), more than 90% of the cpm were found to be associated with UMP, UDP and UDP derivatives, and UTP.

(g) *Extraction of total and cytoplasmic DNA:* Total HeLa and *E. coli* DNA were extracted by the Marmur procedure.[15] For the isolation of cytoplasmic DNA, 20 ml packed HeLa cells were gently homogenized in SMET buffer; the nuclei were separated, and the total cytoplasmic fraction was treated according to the Marmur procedure, followed by CsCl density gradient centrifugation. About 1% of total cell DNA was recovered. As this figure is higher than that reported for the proportion of mitochondrial DNA in cultured mammalian cells (0.2%),[5] it is likely that this "cytoplasmic" DNA preparation is contaminated by nuclear DNA.

(h) *RNA-DNA hybridization* experiments were performed as described previously.[16]

Results.—(a) *Distribution of rapidly labeled RNA among cytoplasmic fractions:* Figure 1*A* shows the sedimentation pattern of the total cytoplasmic fraction from HeLa cells. A considerable amount of fast-sedimenting material has been prevented from pelleting by the dense sucrose layer at the bottom of the tube. These fast-sedimenting components contain the bulk of cytoplasmic phospholipids (about 85% of phosphatidyl choice) (Fig. 2), and 10–15% of total cell rRNA; they are known to include mitochondria, vesicles and tubules of the smooth and rough E.R., and other membranaceous structures that electron-microscopic examination has revealed in HeLa cells.[17, 18] One recognizes in the middle portion of the gradient, in Figures 1*A* and 2, a band of polysomes, which presumably correspond to the free

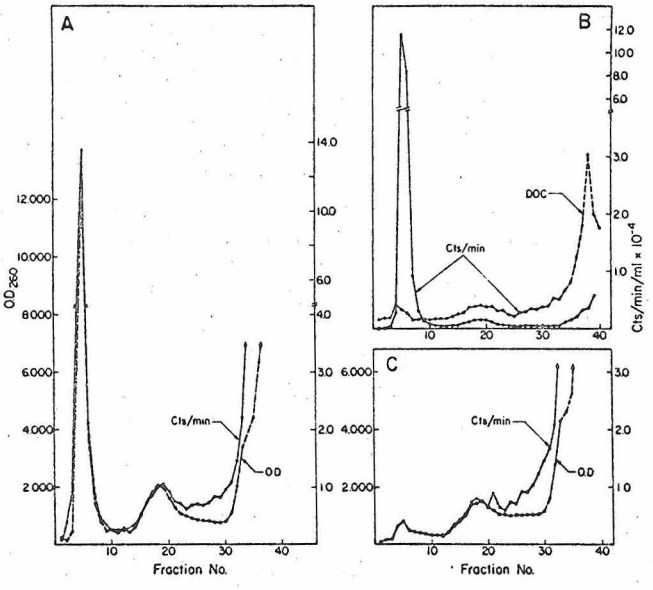


FIG. 1.—Sedimentation pattern of cytoplasmic fractions from HeLa cells labeled for 30 min with H^3-5-uridine. (*A*) Total cytoplasmic fraction. (*B*) Membrane fraction separated by centrifugation at 15,800 × *g* for 20 min and analyzed as such (●) or after treatment with 1% NaDOC (▲). (*C*) 15,800 × *g* supernatant. 15–30% sucrose in TKM (over 3 ml 64% sucrose), 80 min, 24 krpm.

polysomes which are seen in the cytoplasmic matrix in electron-microscopic pictures. Centrifugation for 5 hr in a 0.75 *M*–2.0 *M* sucrose gradient of this polysome fraction prepared (as described in *Materials and Methods*) from C^{14}-choline labeled cells reveals essentially no lipoprotein material in correspondence with the polysome peak.

After a 30-min pulse with H^3-uridine, about twice as much of the newly synthesized RNA appears in the region of the fast-sedimenting material as is associated with free polysomes (Fig. 1*A*).

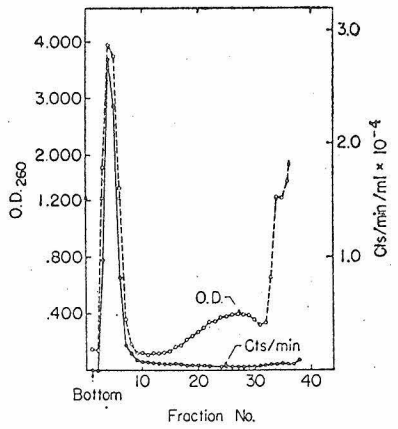


FIG. 2.—Sedimentation pattern of total cytoplasmic fraction from HeLa cells uniformly labeled with C^{14}-choline. Same gradient as in Fig. 1, 70 min, 24 krpm.

Differential centrifugation of the total cytoplasmic extract separates the membrane fraction, contaminated by a small amount of free polysomes (Fig. 1*B*), from the bulk of these (Fig. 1*C*). Treatment of the membrane fraction with 1% NaDOC releases almost all H^3, which now sediments in the upper two thirds of the gradient in the region corresponding to polysomes, monomers, and lighter components (Fig. 1*B*). The H^3 sedimenting in the polysome region shows the sensitivity to RNase characteristic for free polysomes,[19] suggesting that it is associated with polysomal structures.

In the experiments described below, the cytoplasmic membranaceous material was separated into two subfractions, as explained in *Materials and Methods*. The first membrane fraction contains the bulk of mitochondria,[11] contaminated by other membranaceous structures, some of which are rich in RNA. The second membrane fraction consists of slower-sedimenting mitochondria and other membrane elements, and a relatively small amount of free polysomes.

As the bulk of membrane-associated RNA was found to be in the first membrane fraction, all the investigations described below were carried out with this fraction.

(b) *Buoyant density distribution in sucrose gradient of the membrane components:* As shown in Figure 3, these components band in a region of the sucrose gradient corresponding to $\rho = 1.17$–1.195. Mitochondria, as revealed by the OD_{415} due to cytochrome *c*, band at $\rho \sim 1.19$, in agreement with the known density of these structures in sucrose gradient.[11] The buoyant density of the structures carrying the pulse-labeled RNA depends on the medium utilized for homogenization and sucrose gradient, in particular, on the Mg^{++} concentration. After complete removal of Mg^{++} by EDTA in the homogenization medium, the structures containing newly synthesized RNA band at $\rho \sim 1.18$ and are fairly well separated from the mitochondrial band (Fig. 3*A*). If the homogenization medium and sucrose gradient contain neither EDTA nor Mg^{++}, the material carrying labeled RNA is displaced towards higher densities (Fig. 3*B*); if Mg^{++} (1.5×10^{-4} *M*) is added to the homogenization medium, this material almost completely overlaps the mitochondrial band (Fig. 3*C*). It is known that divalent cations cause shrinkage (with resulting increased density) and aggregation of vesicles deriving from E. R., presumably as a result of the reduction of the surface potential of these structures.[20] From their

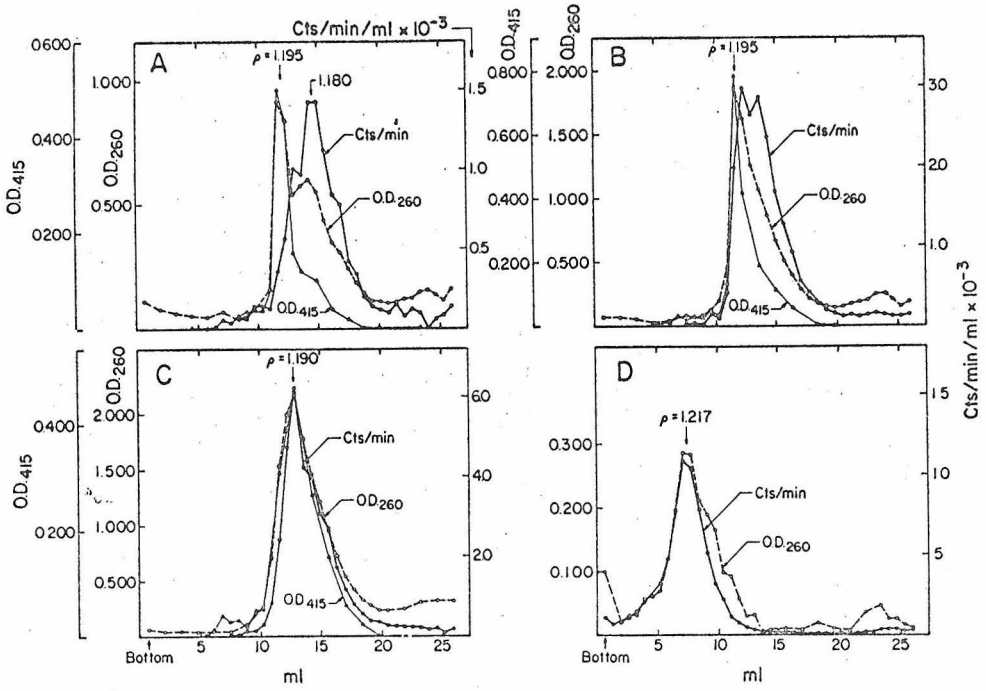
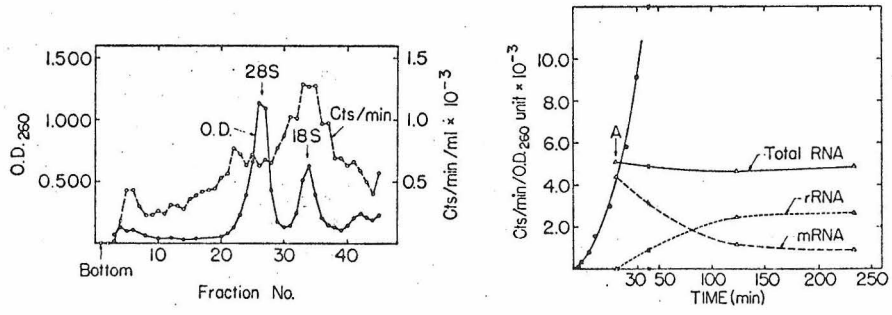


FIG. 3.—Equilibrium centrifugation in sucrose density gradients of membrane fractions from HeLa cells labeled for 15 min with H^3-5-uridine. The first membrane fraction was isolated from 3 batches of cells, homogenized, respectively, in SMET (*A*) or in T (*B*) or in TM buffer (*C*). (*D*) Nuclei obtained from the same cell suspension were extensively broken by homogenization, centrifuged at 1160 × *g* for 3 min, and the supernatant analyzed. 1.0–2.0 *M* sucrose in T buffer, 16 hr, 24 krpm.



(*Left*) FIG. 4.—Sedimentation pattern of membrane-associated RNA extracted from HeLa cells exposed for 7 min to H^3-5-uridine. 5–20% sucrose in acetate-NaCl buffer[13] (over 3 ml 64% sucrose), 8 hr, 25 krpm.

(*Right*) FIG. 5.—Effect of actinomycin D on the labeling of membrane-associated RNA. HeLa cells were exposed to H^3-5-uridine, and samples removed at different times; to one aliquot of the suspension actinomycin D (7.5 μg/ml) was added at 20 min, and samples removed from it at 104 and 230 min. From each sample the first membrane fraction was isolated as in Fig. 3*C*, and the H^3 associated with it measured. From the membrane fractions isolated at 20, 104, and 230 min, the RNA was extracted, and the proportion of mRNA (>6*S*) and rRNA estimated as described previously.[13]

density in sucrose gradients and their sensitivity to divalent cations, it seems likely that the structures containing newly synthesized RNA are vesicles of the rough E. R. As shown in Figure 3*D*, the material released from HeLa cell nuclei by extensive breakage bands at a higher density than the membrane components derived from the total cytoplasmic fraction; this observation confirms the cytoplasmic derivation of these components.

(c) *Sedimentation pattern of membrane-associated RNA:* Figure 4 shows the sedimentation profile of membrane-associated RNA extracted from cells exposed to H^3-uridine for 7 min. The OD_{260} reveals the two rRNA components in the ratio expected for ribosomes (~2.5).[21] Appropriate experiments have failed to show any difference in sedimentation properties between rRNA species from the membrane fraction and from free polysomes. For their sedimentation behavior the labeled RNA components are presumably of the messenger type; they are distributed over the whole gradient from the 6*S* to the 50*S* region, with a broad peak centered around 18*S*. The proportion of components sedimenting faster than 28*S* appears to be appreciably greater than in the mRNA extracted from free polysomes. After about 25 min labeling, radioactivity starts appearing in the 18*S* rRNA, and approximately 30 min later in the 28*S* rRNA; at the same time labeled rRNA components appear also in the free polysomes.

(d) *Base composition of cytoplasmic mRNA fractions:* Table 1 shows the

TABLE 1

NUCLEOTIDE COMPOSITION OF FRACTIONS OF HELA CYTOPLASMIC MESSENGER RNA*

Fraction	Moles (%) A	C	U(T)	G	GC%	A/U	A/G
Membrane-associated mRNA:†							
9–25*S*	33.9	24.5	22.6	18.9	43.4	1.50	1.79
26–48*S*	31.4	23.9	25.3	19.4	43.3	1.24	1.62
Free polysome mRNA:							
10–38*S*	24.8	21.4	27.9	25.8	47.2	0.89	0.96
"Toal" cytoplasmic mRNA:[10]							
9–40*S*	25.7	25.4	27.6	21.1	46.5	0.93	1.22
Total HeLa DNA[10]	29.8	20.0	30.1	20.1	40.1	0.99	1.48
Human mitochondrial DNA‡					45		

* Isolated from 30 min P^{32} pulse-labeled cells.
† The data represent averages of two analyses.
‡ From leucocytes of leukemic donor; GC content estimated from buoyant density in CsCl (J. Vinograd, personal communication).

"apparent" base composition of the mRNA extracted from the membrane fraction and from free polysomes after a 30-min P^{32} pulse. One can see that both the heavier (>25*S*) and lighter (9*S*–25*S*) components of the membrane-associated mRNA have a base composition strikingly different (especially for the high A content) from that of free polysomal mRNA. The base composition previously found[10] for "total" cytoplasmic mRNA (that is, the RNA extracted, after a 30-min pulse, from free polysomes and that portion of the membrane-associated polysomes which is released by 0.5% NaDOC) is also shown: the pattern of base ratios appears to be roughly that of a mixture of the two classes of mRNA.

(e) *Metabolic behavior of cytoplasmic mRNA fractions:* As shown in Figure 5, addition of actinomycin D (7.5 μg/ml) immediately stops the labeling of membrane-associated RNA; in contrast, the flow of radioactivity into free polysomal RNA (in particular, into rRNA) continues for several hours, although at a decreasing

rate, up to a final fourfold increase (not shown in figure). Sedimentation analysis of the RNA extracted from the membrane fraction after different times of exposure to the drug reveals a fairly rapid decline (estimated half-life less than 1 hr) of the mRNA fraction, which is compensated for by the appearance of labeled rRNA; this becomes the predominant labeled component (more than 65%) after about 1-$^1/_2$ hr actinomycin action. In contrast, the free polysome-associated mRNA is relatively stable: in fact, only about 20% of free polysomes break down during the 4-hr exposure to actinomycin D, and it is uncertain whether this is due to dissociation of polysomes or destruction of mRNA[22, 23]; furthermore, the absolute amount of labeled mRNA in the intact polysomes does not decrease appreciably during this time. These results suggest that membrane-associated mRNA has a considerably faster turnover than the free polysome-associated mRNA.

(f) *Kinetics of labeling of cytoplasmic mRNA fractions:* The observed differences in base composition, sedimentation properties, and metabolic behavior indicated that the membrane-associated mRNA and the free polysome mRNA represent two distinct mRNA populations. In order to obtain some information concerning the site of synthesis of these two mRNA classes, an analysis of the kinetics of appearance of label in the two fractions was carried out. Figure 6 shows that the membrane-

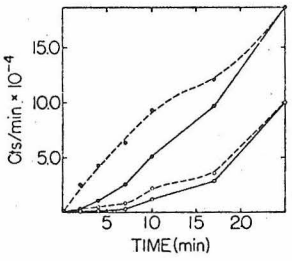


FIG. 6.—Flow of radioactivity into the membrane-associated RNA and free polysome RNA. HeLa cells were exposed to H^3-5-uridine, and samples removed at different times; from each sample the first membrane fraction and the free polysomes were separated as in Figs. 1 and 3, and the total H^3 associated with them measured and corrected for small variations in the yield of total OD_{260}. Correction for differences in UTP pool equilibration with external H^3-uridine was made by assuming that the amount of label in the RNA synthesized after a given incubation time is proportional to the integral of the curve of the UTP pool labeling. ◍, Membrane-associated RNA. ○, Free polysome-associated RNA. Dashed lines are used for the corrected kinetics curves.

associated mRNA becomes labeled much faster than the free polysome-associated mRNA. In order to correct these kinetics for incomplete equilibration of the UTP pool with H^3-uridine, the labeling of this pool after different times of exposure of the cells to the H^3 precursor was analyzed. After correction for differences in pool equilibration, it appears that the increase of label in the membrane-associated messenger is linear from zero time, whereas the appearance of label in the free polysome mRNA still shows an appreciable delay (Fig. 6). A reasonable interpretation of these results is that the membrane-associated mRNA is cytoplasmic in origin (in particular, synthesized in the membranaceous structures themselves); on the other hand, the slower appearance of labeled mRNA in free polysomes could reflect the time required for the equilibration of the pool of precursor mRNA molecules and/or for the processing of these mRNA molecules in the nucleus and their transport into the cytoplasm.

(g) *RNA-DNA hybridization experiments:* As a more direct approach to the problem of the site of synthesis of the membrane-associated mRNA, hybridization experiments were carried out between this mRNA and the free polysome mRNA, on the one hand, and total HeLa DNA and "cytoplasmic" DNA, on the other. It appears in Table 2 that the membrane-associated mRNA has a markedly greater sequence homology with "cytoplasmic" DNA than with total DNA. The amount

TABLE 2

COMPARATIVE HOMOLOGY OF MESSENGER RNA FRACTIONS WITH "CYTOPLASMIC" AND TOTAL HELA DNA

	Membrane-Associated mRNA		Free Polysome mRNA	
	Cpm in hybrid per μg DNA*	Input cpm in hybrid (%)	Cpm in hybrid per μg DNA*	Input cpm in hybrid (%)
Experiment 1:				
"Cytoplasmic" DNA	206	32.4	0.7	0.8
Total DNA	21	5.7	0.4	0.8
Experiment 2:				
"Cytoplasmic" DNA	97	26.0	0.8	1.3
Total DNA	15	5.2	0.4	0.8

Each annealing mixture contained 10 μg/ml DNA and 10 μg/ml total RNA, except in expt. 2 where 20 μg/ml DNA and 20 μg/ml free polysome RNA were used. Incubation at 70°C for 4 hr; RNase digestion with 10 μg/ml, 60 min at 22°C; washing of hybrid on S&S membranes at 55°C.[16] Expts. 1 and 2 utilized two different samples of membrane-associated mRNA (30 min and 20 min H^3 pulse, respectively).

* Recovered after Sephadex chromatography; the data are corrected for nonspecific background estimated with *E. coli* DNA (amounting to 10–15% of the highest hybrid value in each experiment).

of specific mRNA present in the hybridization mixture (estimated to be 2–5% of total RNA[24]) is presumably nonsaturating for the quantity of "cytoplasmic" DNA utilized, but closer to saturating levels for the relatively small fraction of cytoplasmic DNA present in the total DNA preparation. Therefore, the genuine cytoplasmic DNA present in total DNA may give per μg DNA a higher level of hybrid than that contained in the "cytoplasmic" DNA preparation. The observed difference in degree of homology of membrane-associated mRNA with "cytoplasmic" and total DNA is thus a minimum value. The free polysome-associated mRNA appears to hybridize with the two DNA preparations to a much closer extent than the membrane-associated mRNA. This hybridization with "cytoplasmic" DNA presumably occurs with contaminating nuclear DNA. The difference in hybridization of free polysome mRNA with "cytoplasmic" and total DNA is probably insignificant: it may derive from a small contamination of free polysomes by membrane-associated polysomes which would have a large effect on the hybridizing capacity of free polysomal mRNA, since the membrane-associated mRNA has tenfold specific activity and much greater hybridization efficiency. The high proportion of membrane-associated mRNA that hybridizes with "cytoplasmic" DNA (25–30% under the conditions utilized here), as compared to that observed for free polysome mRNA, shows that the cytoplasmic DNA contains a high concentration of genes homologous to the membrane-associated mRNA. This result strongly suggests that the membrane-associated mRNA is, in part at least, the product of cytoplasmic (presumably mitochondrial) genes.

Discussion.—The results presented in this paper have indicated that in HeLa cells a fraction of polysomes (amounting to about 10–15% of the total) are associated with membranes and contain a messenger fraction which, for sedimentation properties, base composition, and metabolic behavior, is distinct from mRNA of free polysomes; on the basis of the kinetics of its appearance in the cytoplasm, and especially of its sequence homology with cytoplasmic DNA, this mRNA appears to be of cytoplasmic origin, presumably synthesized on a mitochondrial DNA template. As to the membrane structures with which this RNA is associated, several lines of evidence suggest that they are distinct from mitochondria. In the absence of divalent cations, these structures band in sucrose density gradients at a lower density than the bulk of mitochondria; furthermore, they show the sensitivity to divalent cations described for elements of E. R.[20] Their relatively high content in

RNA (present in structures with the properties of typical polysomes) also speaks against their mitochondrial nature, in view of the low RNA content reported for mammalian mitochondria.[25, 26] The probable nuclear origin of the ribosomes serving the membrane-bound mRNA, which is suggested by the similar kinetics of appearance of labeled rRNA in bound and free polysomes, further supports the idea that the membrane-bound polysomes are extramitochondrial. On the basis of the present evidence it is thus tentatively concluded that the polysome-carrying membrane structures observed here correspond to the tubules and vesicles of the rough E. R. that electron microscopy has revealed in HeLa cells.[17, 18] The membrane-associated mRNA studied here would thus represent mitochondrial mRNA which is exported to the rough E. R. A fraction of this RNA, however, presumably remains inside the mitochondria since these organelles are able to support protein synthesis.[27, 28] Work is in progress to establish conclusively the nature of the cytoplasmic DNA template of the membrane-bound mRNA and to test the hypothesis of the possible involvement of this RNA in directing the synthesis of structural components of mitochondria and other cytoplasmic membranes.

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Abbreviations used: mRNA, messenger RNA; rRNA, ribosomal RNA; E. R., endoplasmic reticulum; NaDOC, sodium deoxycholate.

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CHAPTER 2

Mitochondrial Origin of Membrane-Associated Heterogeneous RNA in HeLa Cells

INTRODUCTION

The previous chapter described the existence of a membrane-associated RNA in HeLa cells having properties clearly distinct from those of the messenger RNA of free polysomes. The linear kinetics of labeling of this RNA and its sequence homology to cytoplasmic (presumably mitochondrial) DNA pointed to a cytoplasmic site of synthesis. In order to obtain direct evidence as to the template involved, it was necessary to perform hybridization experiments with this RNA and purified mitochondrial DNA. About this time Radloff, Bauer, and Vinograd (1) described the occurrence in the mitochondria of HeLa cells of closed circular DNA molecules which could be well-separated from linear or open circular DNA in cesium chloride-ethidium bromide density gradients. This procedure was utilized for the isolation of mit-DNA for hybridization experiments, and it was found that the RNA from the mitochondrial fraction synthesized during a short pulse with a radioactive RNA precursor has a high efficiency of hybridization with purified mit-DNA.

Further experiments also described in this chapter were aimed at determining the nature of the membranous structures with which this RNA is associated. This problem was complicated by the fact that the conventional preparation of mitochondria by differential centrifugation results in a mitochondrial pellet contaminated by other cytoplasmic organelles. In particular, although mitochondria could be separated from smooth membrane structures by the additional step of buoyant density centrifugation in sucrose gradient of

the crude mitochondrial pellet, it was found that at best a partial separation of mitochondria and elements of rough endoplasmic reticulum could be achieved due to their extensive overlapping in sedimentation properties or buoyant density in sucrose gradients. It was therefore necessary to find other means for distinguishing intramitochondrial from extramitochondrial RNA, and this chapter describes the use of ribonuclease digestion in situ for this purpose. In fact, intramitochondrial RNA should be protected from the action of nuclease if the mitochondrial membranes remain intact (2, 3). The results of this type of analysis have indicated that with increasing pulse length an increasing proportion of labeled heterogeneous RNA is associated with extramitochondrial structures. Several types of evidence suggest that a portion of the RNA synthesized on a mitochondrial DNA template may be exported to the rough endoplasmic reticulum where it becomes associated with ribosomes.

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*MITOCHONDRIAL ORIGIN OF MEMBRANE-ASSOCIATED HETEROGENEOUS RNA IN HELA CELLS**

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The occurrence in the cytoplasm of HeLa cells of a membrane-associated heterogeneous RNA distinct in sedimentation properties, base composition, and metabolic behavior from mRNA of free polysomes has been previously reported.[1] The kinetics of appearance of this RNA fraction and its sequence homology with cytoplasmic DNA clearly pointed to a cytoplasmic site of synthesis.

This paper reports the results of experiments aimed at identifying the DNA template involved and the nature of the membrane structures with which this RNA is associated. It was found that the membrane-associated heterogeneous RNA is synthesized at a high rate on mit-DNA; furthermore, the evidence obtained strongly suggests that this RNA is exported in part to the rough ER.

Materials and Methods.—(a) *Cells:* HeLa cells growing in suspension[2] were used.

(b) *Buffers:* The buffer designations are: T: 0.01 *M* tris buffer (pH 7.1); TKM: 0.01 *M* tris buffer (pH 7.1), 0.01 *M* KCl, 0.00015 *M* $MgCl_2$.

(c) *Labeling conditions:* Pulse labeling of RNA was carried out, as detailed below, with H^3-5-uridine (17.3–28.8 mc/μM) or C^{14}-2-uridine (30–50 μc/μM) in modified Eagle's medium (with 5% dialyzed calf serum) or with carrier-free P^{32}-orthophosphate (37 μc/ml) in medium containing 2×10^{-7} *M* phosphate. DNA was labeled by growing cells for 48 hr in the presence of H^3-thymidine (1.25 μc/ml; 17.7 mc/μM).

(d) *Preparation of subcellular fractions:* The outline of the procedure utilized has been described previously;[1] TKM was the homogenization medium. Buoyant density fractionation of the 8100 $\times$ *g* membrane components in 1.0–1.7 *M* sucrose gradient in T buffer (Spinco SW 25.1 rotor, 25 krpm, 18–20 hr) separated a main band containing mitochondria and rough ER from a lighter band of smooth membrane structures.[3]

(e) *Extraction and analysis of RNA* were carried out as described elsewhere.[4]

(f) *Isolation of closed circular mit-DNA* was carried out by centrifuging the SDS lysate of the 8100 $\times$ *g* membrane fraction in a CsCl-ethidium bromide density gradient.[5] In some experiments, the band of closed circular mit-DNA was rebanded.

(g) *RNA-DNA hybridization* experiments were performed as described previously.[6]

(h) *Cytochrome oxidase assay* was carried out by a modification of the procedure of Smith:[7] 0.1-ml aliquots of the sucrose gradient fractions were mixed in a cuvette with 2.9 ml 18 μM solution of reduced cytochrome C in 0.04 *M* PO_4 buffer, pH 6.2. The decrease of OD at 550 mμ at 25°C was measured at 10-sec intervals for 4–6 min.

Results.—(a) *Base sequence homology of membrane-associated RNA with mit-DNA:* Figure 1 shows the buoyant density profile in a CsCl-ethidium bromide

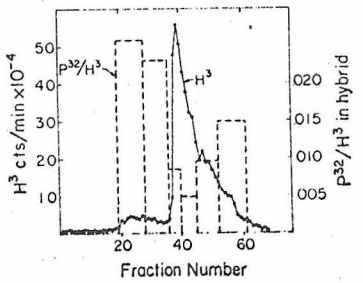

FIG. 1.—Comparative homology with membrane-associated heterogeneous RNA of different HeLa DNA components. The DNA from the 8100 $\times$ *g* membrane fraction of cells labeled with H^3-thymidine was analyzed in a CsCl-ethidium bromide density gradient.[5] Aliquots of pooled fractions were tested for hybridization capacity with membrane-associated RNA from 90-min P^{32}-labeled cells (1 μg DNA, 5 μg RNA). The results are expressed as P^{32}/H^3 in the hybrids. The data are corrected for nonspecific background estimated with *E. coli* DNA.

gradient of DNA from the 8100 × *g* membrane fraction from HeLa cells labeled with H^3-thymidine. The heavy band (fractions 19–28) contains closed circular mit-DNA.[5, 8, 9] The larger light band (fractions 36–60) consists of linear DNA molecules from nuclear contamination, but it would also contain any nicked or linear mit-DNA. The region between the light and dense bands corresponds to the position of the recently described middle band[8, 9] (not resolved here): this contains catenated dimers and higher oligomers of mit-DNA. The capacity of fractions from different regions of the gradient to hybridize with P^{32}-labeled membrane-associated RNA is also shown in Figure 1. It is apparent that the DNA from both the heavy band and the intermediate region has a three to four times higher specific hybridizability with this RNA than the DNA from the light band. This result indicates that mit-DNA is involved in hybridization with this RNA fraction. The fractions on the light side of the main band have a somewhat higher hybridization capacity than the fractions of the center of the band, presumably due to the trapping of mit-DNA which occurs when the gradient is heavily loaded with DNA (here 60 μg).

(b) *Rate of synthesis of mit-RNA:* Figure 2 shows the kinetics of labeling of the membrane-associated heterogeneous RNA and of the free polysome mRNA during prolonged exposure of HeLa cells to H^3-uridine; correction was made for incomplete equilibration of the whole cell UTP pool with the exogenous H^3-uridine at early times (up to 40 min).[1, 10] The labeling of the RNA of the membrane fraction proceeds linearly from zero time, in agreement with an *in situ* synthesis of this RNA. The slight increase in slope at about 30 minutes, if significant, may indicate the arrival at the membrane fraction of an mRNA component of nuclear origin starting at that time. The decrease in the rate of labeling

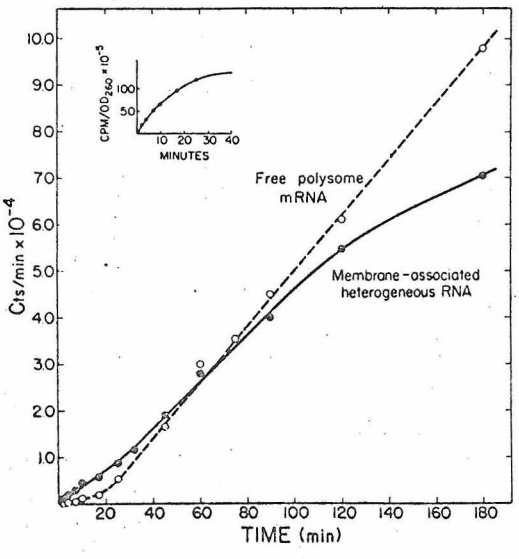

FIG. 2.—Flow of radioactivity into membrane - associated heterogeneous RNA and free polysome mRNA. HeLa cells (3 × 10^5/ml) were exposed to H^3-5-uridine (3.3 μc/ml) and 300-ml samples removed at different times; from each sample the 8100 × *g* membrane fraction was isolated and banded in sucrose gradient; free polysomes were isolated by centrifugation of the 15,800 × *g* supernatant of the cytoplasmic fraction in a 0.75 *M*–2.0 *M* sucrose gradient in TKM for 5 hr;[1, 3] the total H^3 cpm associated with these fractions was determined and corrected for small variations in the yield of total OD_{260}. RNA was released by SDS and analyzed in sucrose gradient in SDS buffer.[15] The proportion of ribosomal and nonribosomal components was estimated as in ref. 15. Determination of the labeling of the total cell uridine nucleotide pools (*insert*) and correction of the RNA labeling data for incomplete UTP pool equilibration with external H^3-uridine were performed as described previously.[1]

after about 100 minutes (see also Fig. 6) suggests a saturation of radioactivity of some components of this RNA fraction. The labeling of the free polysome mRNA, after a 20–30-minute acceleration period, increases at a constant rate, which presumably represents the rate of its arrival from the nucleus. It appears from Figure 2 that the initial rate of labeling of the membrane-associated RNA is equivalent to about two thirds of the rate of arrival at the cytoplasm of free polysome mRNA (see *Discussion*).

(c) *Export of mit-RNA:* (I) *Kinetics of appearance of labeled RNA in mitochondria and extramitochondrial membrane structures:* An appreciable fraction (10–15%) of polysomes in HeLa cells are associated with tubules and vesicles of rough ER:[3, 11, 12] the major part of these polysomes is recovered in the 8100 × *g* membrane fraction and can be released from the membrane components by NaDOC treatment.[1, 3] In both buoyant density fractionation and sedimentation velocity runs (Fig. 3), the distribution of membrane structures containing

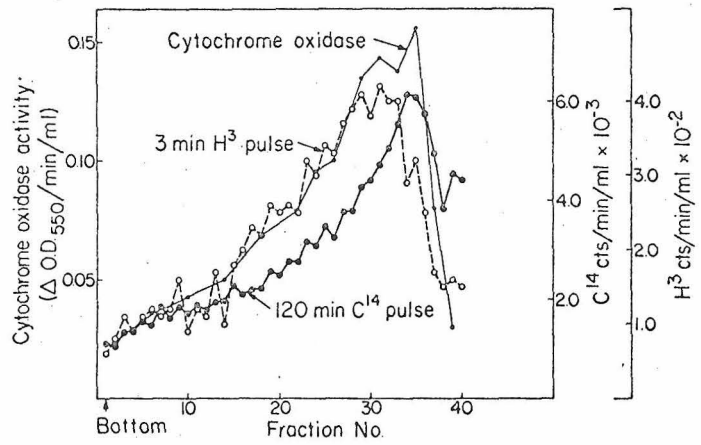

FIG. 3.—Sedimentation pattern of components of the 8100 × *g* membrane fraction isolated from a mixture of HeLa cells labeled for 3 min with H^3-uridine and for 120 min with C^{14}-uridine. 15–30% sucrose gradient in T buffer, SW 25.3 rotor, 7 krpm, 35 min.

RNA labeled during one hour or longer exposure to C^{14}-uridine was found to be significantly different from that of the cytochrome oxidase activity; on the contrary, the latter distribution coincided reasonably well with that of the structures containing RNA labeled for 3 minutes with H^3-uridine. These results suggested that with increasing pulse length labeled RNA accumulates in extramitochondrial structures, presumably elements of rough ER. In order to obtain further evidence on this point, the sensitivity to pancreatic RNase of the RNA in the isolated membrane components was investigated: intramitochondrial RNA *in situ* is protected from the action of nucleases.[13, 14] As an internal standard to monitor the extent of any possible mitochondrial damage, cells labeled for 3 minutes with H^3-uridine (and mixed after harvesting with cells labeled for various times with C^{14}-uridine) were used: in the membrane fraction from 3-minute labeled cells, the majority of the RNA synthesized on mit-DNA, if not

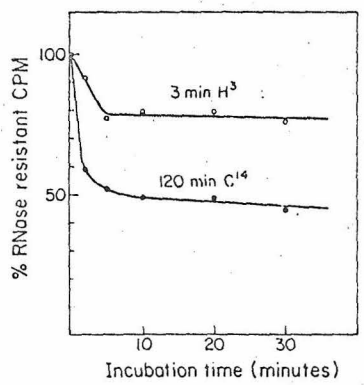

FIG. 4.—Kinetics of RNase digestion *in situ* of membrane-associated heterogeneous RNA. A portion of the 8100 × *g* membrane fraction utilized in the experiment of Fig. 3 was banded in a sucrose gradient: aliquots from the peak fraction of the main band were treated with 50 μg/ml pancreatic RNase at 2°C in 0.1 *M* NaCl, 0.01 *M* Na citrate.

all, would presumably be intramitochondrial. Conditions of RNase digestion were found which clearly distinguished in the membrane-associated RNA a fraction which was quickly digested, from the remainder which was inaccessible to the enzyme or only slowly attacked by it (Fig. 4).

Figure 5 shows the buoyant density distribution in sucrose gradient of the structures containing RNase-sensitive or RNase-resistant 3-minute H^3-labeled RNA and 30- or 120-minute C^{14}-labeled RNA. While the proportion of H^3 label sensitive to RNase is very similar in the two experiments, the proportion of C^{14} sensitive to RNase increases with pulse length. The significance of the 3-minute H^3-RNA fraction sensitive to RNase is not clear: it may result from the damage of a portion of the mitochondria, or it may reflect the rapidity with which one fraction of mit-RNA is exported (see below), or both. The distribution of RNase-sensitive C^{14}-containing structures is significantly displaced toward higher densities with respect to that of the RNase-resistant C^{14}-labeled structures (Fig. 5). An increase in RNase sensitivity of the C^{14}-labeled membrane-associated RNA was also observed after a cold chase or actinomycin treatment. It should be pointed out that for C^{14}-uridine pulses longer than 30 minutes, an increasing fraction of label is in rRNA. As described elsewhere,[3] the great majority (>97%) of the ribosomes of the 8100 × *g* membrane fraction are associated with the rough ER and ~60 per cent of the rRNA they contain is made acid-soluble under the conditions of RNase treatment used here. In the present experiments, the radioactivity associated with RNase-sensitive or RNase-resistant rRNA (estimated by assuming 60% sensitivity of the fraction of label in rRNA[15]) was subtracted from the total RNase-sensitive or RNase-resistant radioactivity: the difference would represent mainly label in heterogeneous RNA. The data thus obtained in different experiments (Fig. 6) indicate that with increasing pulse length or after a pulse chase an increasing proportion of labeled heterogeneous RNA is associated with extramitochondrial structures. The interpretation that the RNase-resistant fraction of the membrane-associated heterogeneous RNA corresponds to intramitochondrial RNA, and the RNase-sensitive fraction to mRNA of polysomes of the rough ER, is supported by a different type of observation. After labeling times longer than half an hour, a considerably greater proportion of label is found in heterogeneous RNA components in the membrane fraction than in free polysomes (Table 1): this "excess," estimated under the assumption that the polysomes of the rough ER receive newly synthesized rRNA and mRNA in the same relative proportion as free polysomes, is very close to the RNase-resistant fraction found in the experiments

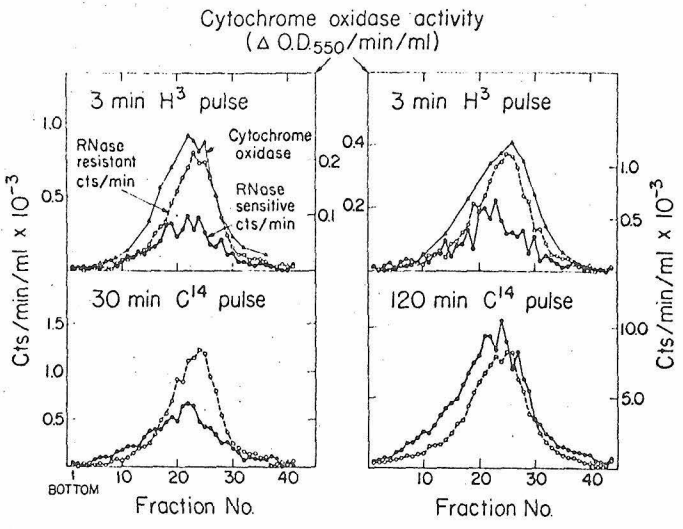

FIG. 5.—Buoyant density distribution in sucrose gradient of membrane structures containing RNase-sensitive or RNase-resistant RNA from a mixture of cells labeled for 3 min with H^3-uridine and for 30 or 120 min with C^{14}-uridine. The 8100 × *g* membrane fraction was banded in sucrose gradient: an aliquot of each gradient fraction was treated with 50 μg/ml pancreatic RNase for 10 min at 2°C as in Fig. 4. Correction for a low level of DNA labeling by C^{14}-uridine was made by subtracting the TCA-precipitable radioactivity resistant to hydrolysis by 0.5 *M* NaOH for 20 hr at 30°C.

of Figure 6, and very likely corresponds to the intramitochondrial RNA (Table 1). It appears from Figure 6 that the radioactivity of the intramitochondrial RNA becomes saturated after about 75 minutes: if this saturation reflects the turnover of a labile fraction (see *Discussion*), it may account for the decrease in the over-all rate of labeling of the membrane-associated heterogeneous RNA (Fig. 2).

(II) *Comparative homology with mit-DNA of membrane-associated RNA labeled during short and long pulses:* In order to test whether the accumulation of newly synthesized RNA in extramitochondrial structures results from export of mit-RNA or from arrival of RNA of nuclear origin, the capacity of membrane-associated RNA labeled during a very short H^3-uridine pulse to hybridize with mit-DNA was compared with that of membrane-associated RNA labeled during a two-hour C^{14}-uridine pulse (when a considerable fraction of it is extramitochondrial (Fig. 6)). In these experiments, because of the nuclear origin of the rRNA in the membrane-bound ribosomes,[1, 3, 16] labeled rRNA would not contribute to hybrid formation. If all the extramitochondrial nonribosomal RNA labeled in a long pulse were of nuclear origin, on the basis of the data of Figure 6 and Table 1 one would expect the ratio of H^3 to C^{14} in the hybrid to be about twice that in the input heterogeneous RNA. On the contrary, as is shown in Table 2, the heterogeneous membrane-associated RNA labeled in a long pulse has a degree of homology with mit-DNA which is similar to that of the RNA labeled in a short pulse. The slightly higher (from 0 to 25% in different experiments) hybridiza-

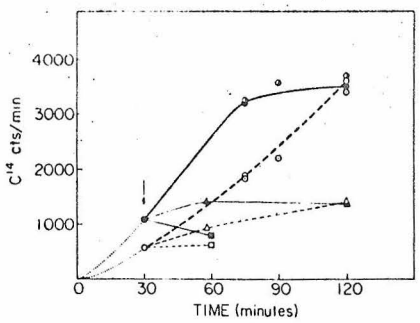

FIG. 6.—Distribution of membrane-associated heterogeneous RNA between RNase-sensitive and -resistant structures in continuous incorporation and pulse-chase experiments. The 8100 × *g* membrane fraction was isolated from mixtures of 1.2×10^8 cells labeled for 3 min with H^3-uridine (10 μc/ml) and 1.2×10^8 cells labeled for different times with C^{14}-uridine (0.06 μc/ml); the total RNase-sensitive and RNase-resistant fractions of membrane-associated RNA were determined as in Fig. 5, and corrected for the contribution of rRNA as indicated in the text. The C^{14} data are normalized for small variations in the yield of membrane components on the basis of total H^3-uridine incorporation (= C^{14} cpm per 1000 H^3 cpm), and for possible contribution to RNase sensitivity by mitochondrial damage on the basis of a constant 25% RNase sensitivity of H^3-RNA (see text).

◉—◉ RNase-resistant, ○—○ RNase-sensitive fraction, after labeling with C^{14}-uridine for different times; ▲—▲ RNase-resistant, △—△ RNase-sensitive fraction, after 30 min C^{14}-uridine pulse, then 0.01 *M* cold uridine chase; ▣—▣ RNase-resistant, □—□ RNase-sensitive fraction, after 30 min C^{14}-uridine pulse, then actinomycin (10 μg/ml) treatment; ⊙ polysome associated, and ◑ nonpolysome-associated heterogeneous RNA of the 8100 × *g* membrane fraction, after different exposure times to C^{14}-uridine, was estimated as explained in the legend of Table 1.

tion capacity of the seven-minute H^3-RNA compared to the two-hour C^{14}-RNA may be due to the fact that the sequences of mit-RNA are not represented in the same proportion in preparations labeled during a short and a long pulse, or to the late arrival at the membrane fraction of an mRNA component of nuclear origin: the latter possibility is suggested by the hybridization results obtained with total HeLa cell DNA (Table 2), and possibly by the change in slope at about 30 minutes in the labeling of the membrane-associated RNA (Fig. 2).

TABLE 1. *Polysome-associated and nonpolysome-associated heterogeneous RNA of the membrane fraction.*

Incubation time (min)	Fraction	Ribosomal RNA (% of total)	Heterogeneous RNA* (% of total)	Polysome-associated fraction of heterogeneous RNA† (%)	Non-polysome-associated fraction of heterogeneous RNA (%)	RNase-resistant fraction of heterogeneous RNA‡ (%)
75	Free polysome RNA	42	58			
	Membrane-associated RNA	21	79	36	64	63
90	Free polysome RNA	51	49			
	Membrane-associated RNA	28	72	38	62	58
120	Free polysome RNA	62	38			
	Membrane-associated RNA	44	56	48	52	49

* Includes small amount of tRNA and 5*S* RNA (5–10% of heterogeneous RNA).

† Calculated according to the formula $[(hRNA_F/rRNA_F) \times rRNA_M]/hRNA_M$, where hRNA and rRNA indicate heterogeneous and ribosomal RNA, respectively, and subscripts F and M refer to free polysomes and membrane fraction, respectively.

‡ Data derived from Fig. 6.

TABLE 2. *Comparative homology with mit-DNA of membrane-associated heterogeneous RNA labeled during short and long pulses.*

Membrane-associated RNA fraction	DNA	Cpm in Hybrid per μg DNA H^3	Cpm in Hybrid per μg DNA C^{14}	Per Cent Input Cpm in Hybrid H^3	Per Cent Input Cpm in Hybrid C^{14}	H^3/C^{14} in RNA Input	H^3/C^{14} in RNA Hybrid
Expt. 1: (7 min H^3-RNA + 120 min C^{14}-RNA)							
8–35*S*	Mitochondrial	8.3	9.5	11.4	9.1	0.72	0.88
35–50*S*	Mitochondrial	14.2	6.7	14.1	13.5	2.0	2.12
Expt. 2: (15 min H^3-RNA + 120 min C^{14}-RNA)							
8–50*S*	Total	7.5	5.2	8.6	9.6	1.6	1.43 (1.57*)
	"cytoplasmic"						
	Total cell	0.6	0.8	1.2	2.8	1.6	0.73
Expt. 3: (7 min H^3-RNA + 120 min C^{14}-RNA)							
10–26*S*	Mitochondrial 1	77	55	10.4	8.1	1.1	1.39
	Mitochondrial 2	97	69	13.3	10.3	1.1	1.40
	Total cell	2.5	4	0.3	0.6	1.1	0.62

Expt. 1: 3.6 μg HeLa mit-DNA and 10 μg RNA (8–35*S*) or 15 μg RNA (35–50*S*); *expt. 2:* 22 μg "cytoplasmic DNA"[1] or total HeLa DNA and 20 μg RNA; *expt. 3:* 2 μg mit-DNA (two preparations) or total HeLa DNA and 22 μg RNA (all in 2 ml). The data are corrected for nonspecific background estimated with *E. coli* DNA. The total cpm and the H^3/C^{14} in input RNA refer to heterogeneous RNA only, estimated as in ref. 15.

* Corrected for contribution of hybridization with contaminating nuclear DNA,[1] as estimated from the data obtained with total cell DNA.

Discussion.—The main conclusion of this paper is that mit-DNA is the template of the membrane-associated heterogeneous RNA of cytoplasmic origin previously described in HeLa cells.[1] Base sequence homology of this RNA with mit-DNA and association of the newly synthesized RNA with mitochondria (recognized by buoyant density properties and cytochrome oxidase activity) have led to this conclusion. Mit-RNA is synthesized at a high rate: if the intramitochondrial UTP pool equilibrates with exogenous H^3-uridine in parallel with the total cell UTP pool, this rate can be estimated to correspond to two thirds of the rate of entry into the cytoplasm of the free polysome mRNA. In *Neurospora* the pool of precursors for mit-DNA synthesis has been shown to turn over more slowly than the pool utilized for nuclear DNA synthesis, possibly because it is fed by an effectively large ribonucleotide pool deriving from turnover of RNA.[17] A similar situation in HeLa cells might lead to an underestimate of the relative rate of mit-RNA synthesis. Assuming mit-DNA to be of the order of 0.1 per cent of total HeLa cell DNA,[8] the rate of mit-RNA synthesis per unit DNA mass appears to be very high indeed. This RNA, however, does not accumulate in the cytoplasm in proportion to its synthesis, due to its metabolic instability.[1] It is possible that the saturation of radioactivity of intramitochondrial RNA after about 75 minutes of labeling reflects the turnover kinetics of a labile fraction. To what extent the high rate of transcription of mit-DNA is related to its informational role for protein synthesis is not known. Biochem-

ical and genetic evidence in *Neurospora*[17, 18] and yeast[16, 19] supports the idea that mit-DNA is genetically active. Several types of observations strongly suggest that mit-RNA does not remain confined inside mitochondria, but is transported in part to extramitochondrial structures. Biochemical and EM evidence has indicated that the great majority (>97%) of the ribosomes of the 8100 × *g* membrane fraction are associated with ER elements:[3] this would put an upper limit of 0.3–0.4 per cent of the total cell ribosomal content for the amount of intramitochondrial ribosomes. If a substantial part of mit-RNA functions as messenger for protein synthesis, as is suggested by its association with polysomal structures,[1, 3] it seems unlikely that it could be served by very few intramitochondrial ribosomes. Furthermore, RNA-DNA hybridization experiments have shown that the labeled heterogeneous RNA which accumulates in extramitochondrial membrane structures in a two-hour pulse has a degree of homology with purified mit-DNA which is similar to that of the intramitochondrial RNA. On the basis of the available evidence, it seems thus justifiable to conclude that mit-RNA in HeLa cells is exported in part for its utilization to elements of the rough ER, where it becomes associated with ribosomes of nuclear origin.[1, 3] The possibility that a fraction of this RNA functions inside the mitochondria is suggested by the published reports concerning mitochondrial protein synthesis.[20]

Abbreviations used: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; mit-DNA, mitochondrial DNA; mit-RNA, mitochondrial RNA; ER, endoplasmic reticulum; EM, electron-microscopic; NaDOC, sodium deoxycholate; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane; UTP, uridine triphosphate.

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ADDITIONAL RESULTS

Export of mitochondrial RNA - About 10-15% of the ribosomes and polysomes of HeLa cells are bound to endoplasmic reticulum and appear in the mitochondrial fraction. In order to obtain further information concerning the possibility that a portion of the RNA synthesized on a mit-DNA template is exported to the rough E.R. where it becomes associated with these ribosomes to form polysomes, two types of experiments were done:

(a) Hybridization of messenger RNA from membrane-bound polysomes with mit-DNA - Membrane-associated polysomes from cells labeled for 30 min with [^{3}H]-uridine were released by NaDOC from the mitochondrial fraction and purified by two cycles of sucrose gradient centrifugation (Figure 1). The broad O. D.$_{260}$ band which was observed in the region of the gradient corresponding to polysomes (120 to 350 S) after the first run (Figure 1a) has been shown to have the sensitivity to ribonuclease and EDTA expected for polysomal structures (4). The fractions corresponding to the polysomal band were pooled and rerun through a 23 to 55% sucrose gradient (Figure 1b), yielding a well-defined peak of polysomes separated from a small amount of faster and slower sedimenting material. RNA was extracted from the polysome peak with SDS and run through a sucrose gradient. The labeled messenger RNA components sedimenting between 5 and 33 S were pooled and tested for their capacity to hybridize with increasing amounts of mitochondrial and nuclear DNA. Figure 2 shows that the mRNA of bound polysomes hybridizes with

high efficiency with mit- DNA and, on the contrary, only to a very small extent with nuclear DNA.

(b) Effect of ethidium bromide on the labeling of the RNA of the membrane-associated polysomes - Membrane-associated polysomes were prepared from HeLa cells labeled for 60 min with [^{3}H]-uridine, in the presence or absence of 1 μg/ml ethidium bromide, by lysis of the mitochondrial fraction with NaDOC and centrifugation on a 15 to 30% sucrose gradient. As shown in Figure 3, ethidium bromide treatment results in a significant decrease in the labeling of the RNA of the membrane-bound polysomes (about 43% decrease in the polysomal region of the gradient from fractions 8-36). (In another experiment done under the same conditions a similar effect (28% decrease) was observed). The labeled material near the top of the gradient which shows a striking sensitivity to ethidium bromide presumably represents RNA components derived from lysis of mitochondria.

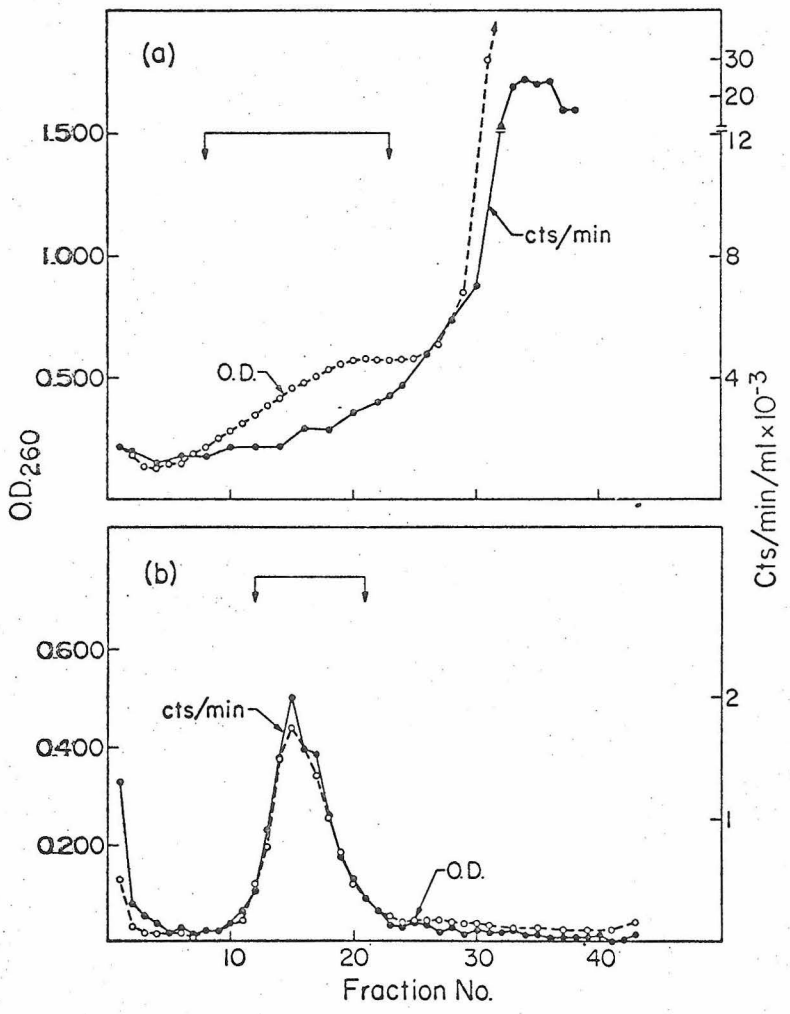

(a)
1.500
1.000
0.500
O.D.
cts/min
30
20
12
8
4
O.D.260
(b)
0.600
0.400
0.200
cts/min
O.D.
2
1
10
20
30
40
Fraction No.
Cts/min/ml x 10-3

Figure 1

FIGURE 1. Isolation of polysomes from the rough E. R. of HeLa cells. The mitochondrial fraction (containing the bulk of the elements of rough E. R.) was isolated by differential centrifugation from HeLa cells labeled for 30 min with [5-^{3}H]-uridine, treated with 1% Na DOC, and run on a 15 to 30% sucrose gradient in TKM (0. 01 tris buffer, pH 7.1, 0.01 M KCl, 0.00015 M $MgCl_2$) for 120 min at 24,000 rev. /min. in the Spinco SW 25.1 rotor (Figure 1a). The fractions corresponding to the portion of the gradient indicated by arrows were pooled, dialyzed for 30 min. against 200 volumes of TKM, then centrifuged through a sucrose gradient consisting of 5 ml. 23 % (w/w) sucrose, 24 ml. 23 to 55% sucrose gradient, and 10 ml. 55% sucrose, all in TKM, in the Spinco SW 25.2 rotor at 20,000 rev. /min. for 11.5 hr. (Figure 1b).

0------0 O. D. $_{260}$

●——● ^{3}H-cts. /min.

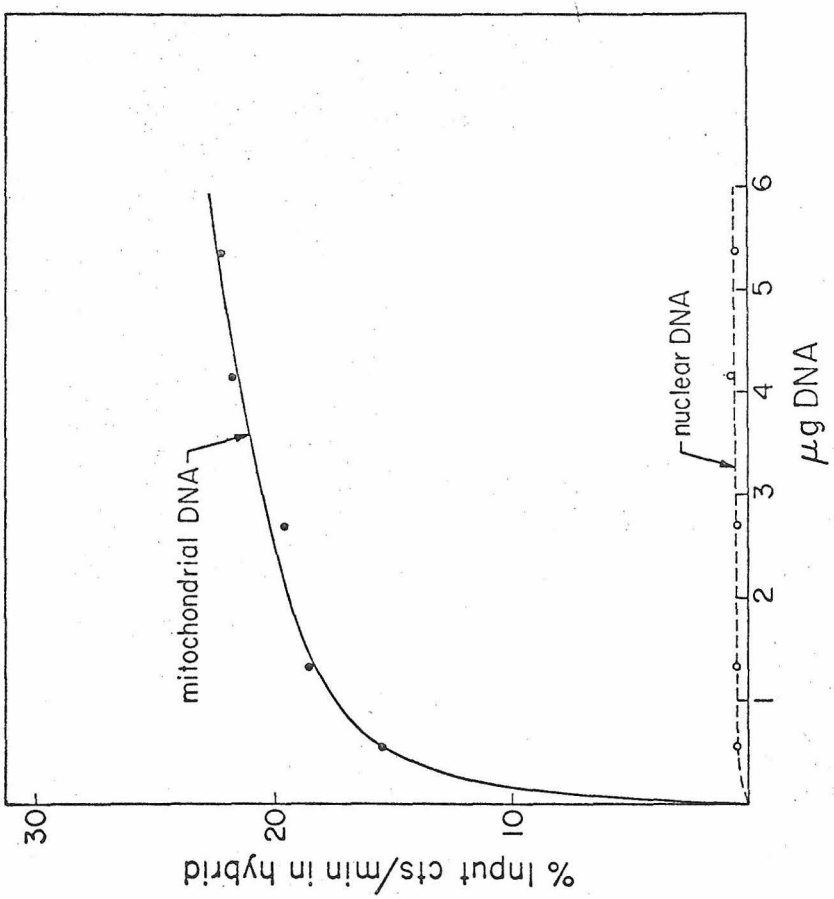

% Input cts/min in hybrid
30
20
10
mitochondrial DNA
nuclear DNA
1
2
3
4
5
6
μg DNA

Figure 2

FIGURE 2. Hybridization of mRNA from E. R. -bound polysomes with increasing amounts of mitochondrial or nuclear DNA. RNA was extracted by SDS from the polysomal band shown in Figure 1b and run in sucrose gradient in SDS buffer (0.01 M tris buffer, pH 7.0, 0.1 M NaCl, 0.001 M EDTA, 0.5% SDS). The RNA components sedimenting between 5 and 33 S were pooled, precipitated with ethanol, and dissolved in SSC/10 (SSC = 0.15 M NaCl, 0.015 M Na citrate). For hybridization experiments, nitrocellulose membrane filters charged with increasing amounts of heat denatured ^{14}C-labeled mit-DNA or nuclear DNA, were incubated in scintillation vials with 3 μg RNA in 2 ml. of 2 x SSC for 22 hr. at 66°C. After incubation, the membranes were extensively washed on both sides with 2 x SSC at room temperature, treated with pancreatic RNase (10 μg/ml., 60 min.), and again washed on both sides with 2 x SSC.

●———● ^{3}H-cts./min. hybridized with mit-DNA

0-------0 ^{3}H-cts./min. hybridized with nuclear DNA

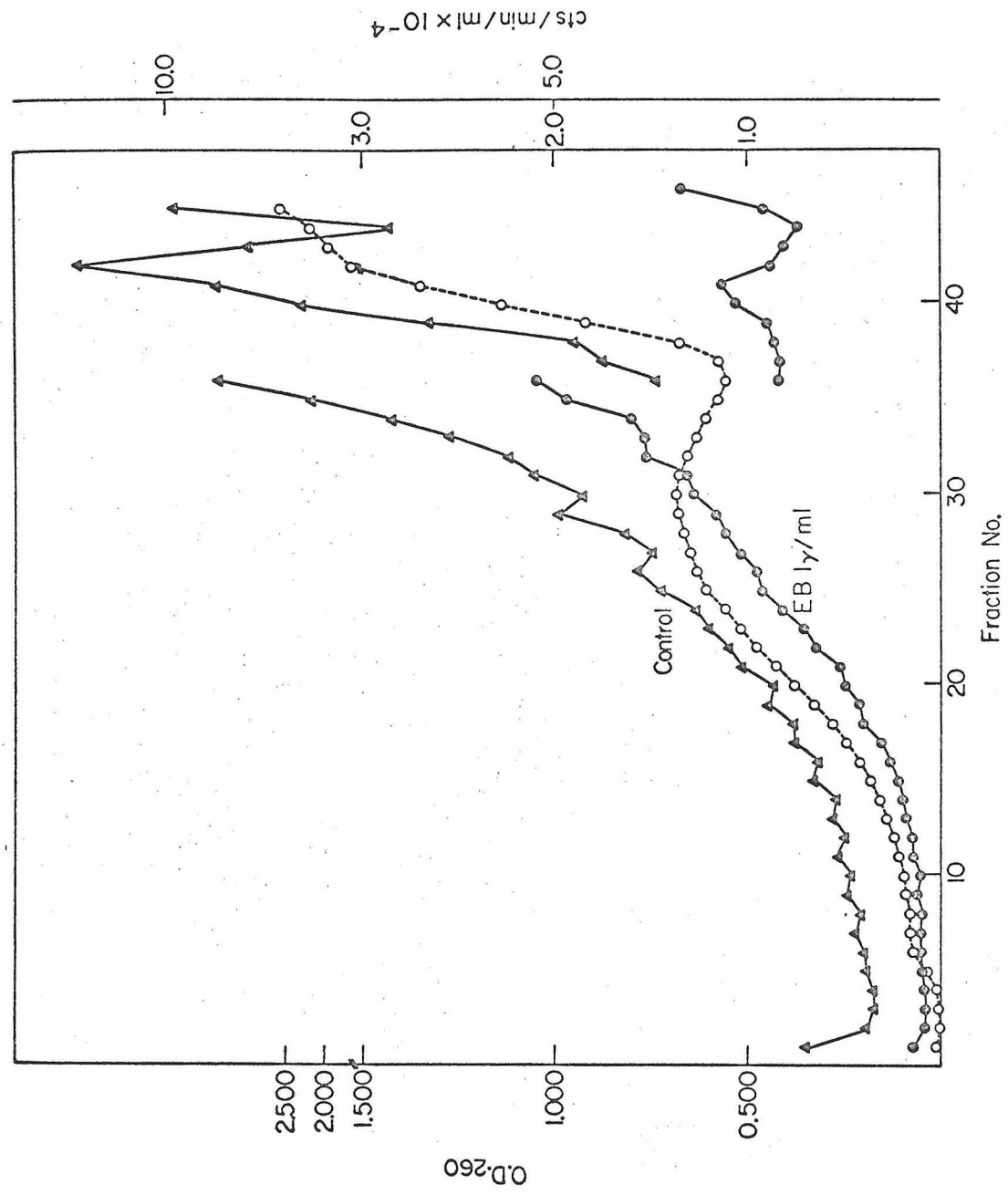
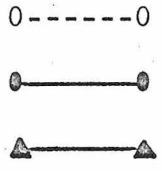

cts/min/ml x 10⁻⁴
10.0
5.0
3.0
2.0
1.0
O.D.260
2.500
2.000
1.500
1.000
0.500
10
20
30
40
Fraction No.
Control
EB 1γ/ml

Figure 3

FIGURE 3. Effect of ethidium bromide on the labeling of the RNA of the E. R. -bound polysomes. Mitochondrial fractions were prepared from HeLa cells labeled with [5-^{3}H]-uridine for 60 min. in the absence or presence of ethidium bromide (1 μg/ml. added 15 min. prior to the addition of the isotope) by differential centrifugation, lysed with 1% NaDOC, and run on 15 to 30 % sucrose gradients in TKM for 95 min. at 24, 000 rev. /min. in the Spinco SW 27 rotor. Only the O. D.$_{260}$ pertaining to the ethidium bromide sample is shown. The ^{3}H-cts. /min. of the control were plotted after normalization for the recovery of O. D.$_{260}$ in the polysome region.



ADDITIONAL DISCUSSION

Export of mitochondrial RNA - The results, presented in Chapter 2, showing the similarity in hybridization efficiency with mit-DNA of the RNA extracted from the mitochondrial fraction of cells labeled for 2 hr. with [^{14}C]-uridine (that is, at a time when about 50% of the heterogeneous RNA of the mitochondrial fraction is extramitochondrial, presumably associated with polysomes of the rough E. R.) as compared to the RNA labeled during very short pulses with [^{3}H]-uridine (when the great majority of the RNA of the mitochondrial fraction is intramitochondrial) suggested that mRNA of membrane-bound polysomes may have base sequence homology to mit- DNA. Further information concerning this hypothesis was obtained by carrying out hybridization experiments between mRNA isolated from membrane-bound polysomes and mit-DNA and by examining the sensitivity of the labeling of the RNA of the membrane-associated polysomes to ethidium bromide, an intercalating dye which extensively alters the tertiary structure of closed circular DNA and which has recently been shown in short term experiments to selectively inhibit the synthesis of both heterogeneous RNA components (see Chapter 4 of this thesis) and discrete RNA species (Chapter 4 of this thesis and (5)) synthesized on a mit-DNA template. Both types of experiments suggest that a portion of the RNA synthesized on mit-DNA is associated with polysomes, possibly those of the rough E. R.. It should be mentioned that Zylber, Vesco, and Penman have recently reported that ethidium bromide treatment has no effect on the labeling of RNA associated

with polysomes released by NaDOC from the crude mitochondrial pellet from HeLa cells (5). The discrepancy between the results of these authors and those described here (Additional results (b)) may be due to the use of a low dose of actinomycin D which selectively inhibits the synthesis of ribosomal RNA and may, therefore, interfere with polysome metabolism and also of a high concentration of cells by the cited authors.

There are two types of problems existing at present which complicate an analysis of the mRNA of E. R. -bound polysomes. First, although the great majority of the polysomes of the mitochondrial fraction are extramitochondrial (4), the existence of a small amount of intramitochondrial ribosomes capable of serving mitochondrial mRNA is suggested by the analogy with lower eukaryotic cells (6, 7, 8, 9, 10, 11, 12), by the protein synthetic capacity of animal cell mitochondria in vitro (13, 14, 15, 16), and by the reported presence in rat liver and HeLa cell mitochondria of specific tRNA species (17, 18, 19). In the present experiments any intramitochondrial polysomes would cosediment with the polysomes of the rough E. R. and could account for the hybridization capacity and sensitivity to ethidium bromide of the mRNA of the polysomes of the mitochondrial fraction. On the other hand, although the material labeled during a short pulse with an RNA precursor sedimenting in the polysomal region of a 15 to 30% sucrose gradient (see, for example, Figure 1a, Additional results) shows the complete sensitivity to low concentrations of ribonuclease expected for polysomal structures, it is not completely sensitive to EDTA treatment (4), which has been shown to distinguish polysomes

from cosedimenting non-polysomal structures (20). The possibility, therefore, exists that after NaDOC treatment, in addition to polysomes, other structures derived from membrane lysis sediment in the polysomal region of the gradient. Since these structures are labeled during a very short [^{3}H]-uridine pulse (4), it is likely that they derive from lysis of mitochondria. In view of these two problems, definitive evidence for the presence in the polysomes of the rough E. R. of mRNA of mitochondrial origin will have to wait for the development of a procedure to fractionate mitochondria and elements of the rough E. R.. Experiments of this type are in progress in Dr. Attardi's laboratory.

One might ask, in this connection, why the RNA synthesized on a mit-DNA template should be exported and translated by extramitochondrial protein synthesizing systems. Two main interpretations could be given for such an export. Thus, the exported RNA could represent mRNA for some mitochondrial protein which would have to be translated on cytoplasmic ribosomes either because of the incompetence of the intramitochondrial protein synthesizing machinery or because the newly synthesized protein has to perform some transport or regulatory role in connection with the synthesis on the cytoplasmic ribosomes of mitochondrial proteins specified by nuclear genes. Evidence has recently been presented for the synthesis of the mitochondrial ribosomal proteins by the cytoplasmic protein synthesizing machinery in Neurospora (21) and yeast (22); on the other hand, the existence of antibiotic resistance mutants manifesting cytoplasmic

inheritance in yeast has suggested that mit-DNA may contain the information for some ribosomal proteins (23). These observations, taken together, imply that some mRNA of mitochondrial origin for ribosomal proteins may be exported.

The second possibility is that the mit-RNA exported to the rough E. R. could function as a messenger for the synthesis of proteins destined for extramitochondrial structures such as membrane structural proteins. Support for this possibility comes from the work of Woodward and Munkres (24, 25) who prepared structural proteins with similar physical and immunological properties, amino acid composition, and peptide maps from the mitochondria, nuclei, E. R., and plasma membrane of Neurospora; furthermore, these authors isolated structural protein with an altered primary structure from all these sources in Neurospora strains carrying a mutation of a cytoplasmic, presumably mitochondrial, gene (25). Although evidence substantiating the similarity in physical properties, amino acid composition, and peptide maps among structural proteins of different cell membrane systems has been presented (26, 27), confirmation of the results of Woodward and Munkres concerning the possibility that a single cytoplasmic mutation leads to alterations in the primary structure of the structural proteins of these various membrane systems will be necessary before the idea that exported mit-RNA could serve as a messenger for all these structural proteins can be seriously considered.

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CHAPTER 3

Sedimentation Properties of the Majority RNA Species Homologous to Mitochondrial DNA in HeLa Cells

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INTRODUCTION

In Chapters 1 and 2 the properties of the newly synthesized mit-RNA were considered. In order to obtain some information concerning the characteristics of the bulk species of mit-RNA, HeLa cells were labeled for 48 hr. with [^{3}H]-uridine. After such a long labeling time with an RNA precursor, the rRNA of the contaminating rough E. R. contains the majority of the label in the mitochondrial fraction. Fortunately, as previously shown for rat liver microsomes (1), the bulk of the E. R. -bound ribosomes can be removed from this fraction by EDTA treatment.

In order to determine the distribution of the mit-RNA components in the sedimentation pattern of the long-term labeled RNA from the EDTA-treated mitochondrial fraction, hybridization with purified mit-DNA was used as an analytical tool. So that peaks of RNA hybridizable with mit-DNA in specific regions of the RNA sedimentation pattern could be distinguished over the background of hybridization of heterogeneous RNA components, it was necessary to scan the sedimentation pattern by annealing individual fractions with mit- DNA. Furthermore, in order to be able to measure the concentration of the various RNA species homologous to mit-DNA, a low RNA to DNA ratio (well below that required to reach DNA saturation with total mit-RNA) was used in the incubation mixtures. This analysis was made more meaningful by the high degree of resolution of RNA components obtainable by sucrose gradient centrifugation in the Spinco SW 25. 3 rotor.

As described in the accompanying publication, the sedimentation profile of the RNA homologous to mit-DNA shows a broad band between 9 and 15-16 S with a peak at 12 S, a prominent 4 S peak, and a small amount of faster sedimenting components hybridizable with mit-DNA in the 16 to 28 S region of the gradient.

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Sedimentation Properties of RNA Species homologous to Mitochondrial DNA in HeLa Cells

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Most of the RNA homologous to mitochondrial DNA in HeLa cells is represented by species with sedimentation coefficients between 9S and 15S—with a prominent 12S component—and by 4S RNA. No ribosomal RNA species hybridizable with mitochondrial DNA were detected: similarly, the 21S RNA present in crude mitochondrial preparations had no appreciable sequence homology to mitochondrial DNA.

WHEN exponentially growing HeLa cells are exposed to a labelled RNA precursor, radioactivity is very rapidly incorporated into RNA associated with a crude mitochondrial fraction[1–3]. After a short pulse of ^{3}H-uridine (up to 20–30 min), the labelled RNA is found in structures of the same size and buoyant density distribution as mitochondria (recognized from the cytochrome oxidase activity), and is inaccessible *in situ* to ribonuclease suggesting an intramitochondrial location; this RNA hybridizes with high efficiency with mitochondrial DNA (mit-DNA)[2,3], which is therefore presumably its template. After longer pulses, labelled RNA accumulates in increasing amounts in elements of the rough endoplasmic reticulum contaminating the mitochondria; RNA–DNA hybridization studies indicate that at least a part of the messenger RNA (*m*RNA) of the polysomes bound to the endoplasmic reticulum has sequence homology with mit-DNA and very likely has been exported to the rough endoplasmic reticulum from mitochondria[2,3]. The labelled RNA extracted from a crude mitochondrial fraction after a short ^{3}H-uridine pulse is represented by components sedimenting between 4*S* and about 50*S* (refs. 1 and 3). The kinetics of labelling of the various molecules suggests that this RNA is synthesized in the form of very large molecules and then converted to smaller ones, which accumulate particularly in the 30*S* to 33*S* region of the gradient, between 12*S* and 22*S*, and in the 4*S* region. No evidence was obtained for the synthesis in mitochondria of ribosomal RNA (*r*RNA) components[3]. In our study, the sedimentation properties of the majority species of mitochondrial RNA (mit-RNA) have been investigated after prolonged labelling with ^{3}H-uridine; hybridization with purified mit-DNA was used to determine the sedimentation profile of the RNA species homologous to mit-DNA.

RNA Species

After long exposure of HeLa cells to a radioactive RNA precursor, most of the label in the crude mitochondrial fraction is in the *r*RNA of the contaminating rough

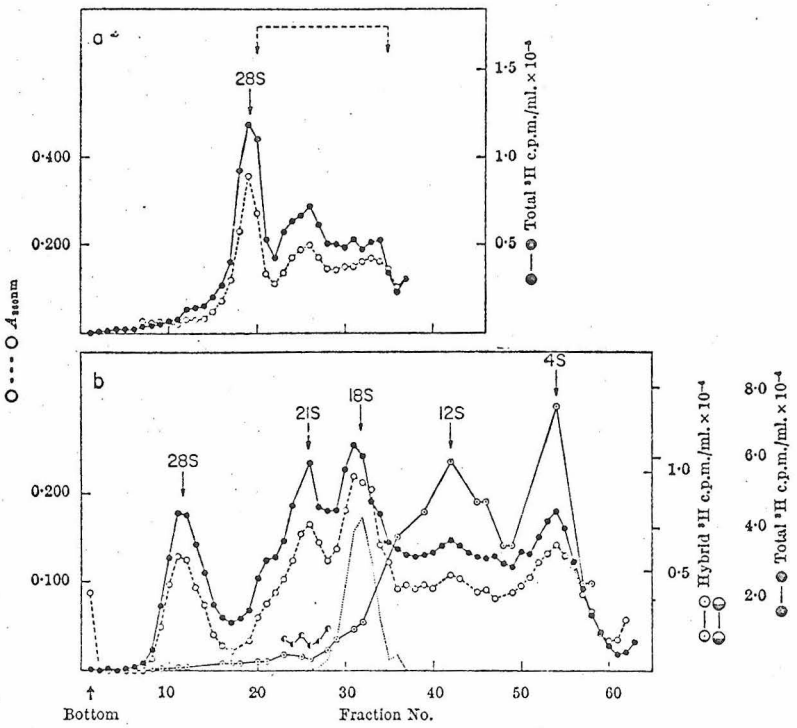

Fig. 1. Sedimentation profiles of total RNA, and RNA hybridizable with mit-DNA (at a low RNA to DNA input ratio), from the EDTA-treated crude mitochondrial fraction of HeLa cells. *a*, The crude mitochondrial fraction was isolated by differential centrifugation, as previously described[1-2], from a homogenate of HeLa cells labelled for 48 h with 5-³H-uridine. The final pellet, resuspended in 0·25 M sucrose in 0·01 M Tris buffer (*p*H 7·1), 0·00015 M $MgCl_2$, was treated with 25 μg/ml. ribonuclease-free pancreatic deoxyribonuclease (Worthington) for 30 min at 2° C. After addition of EDTA to 0·03 M, the suspension was centrifuged at 11,000*g* for 10 min, washed by centrifugation with 0·25 M sucrose in 0·01 M Tris (*p*H 7·1)—0·015 M EDTA, and then centrifuged to equilibrium in the Spinco *SW* 25·1 rotor at 25,000 r.p.m. for 20 h at 3° C in a 30 to 48 per cent sucrose gradient in 0·01 M Tris buffer (*p*H 7·1). RNA was extracted by sodium dodecyl sulphate (SDS)–phenol from the mitochondrial band (which was contaminated with rough endoplasmic reticulum elements) and then passed through a 5 to 20 per cent sucrose gradient in 0·01 M acetate buffer (*p*H 5·0)—0·1 M NaCl in the Spinco *SW* 25·1 rotor at 22,000 r.p.m. for 15·5 h at 3° C. *b*, The components corresponding to 4*S* to 28*S* in the pattern of *a* (indicated by arrows) were pooled, precipitated with ethanol, centrifuged, redissolved in SDS buffer: 0·01 M Tris (*p*H 7·0)—0·1 M NaCl—0·001 M EDTA—0·5 per cent SDS, and after addition of a small amount of ¹⁴C-labelled 18*S* RNA from free polysomes (. . .), passed through a 15 to 30 per cent sucrose gradient in SDS buffer in the Spinco *SW* 25·3 rotor at 25,000 r.p.m. for 25 h at 20° C. Components in individual or pooled fractions were precipitated with ethanol, centrifuged, dissolved in 0·05 M Tris buffer (*p*H 7·1)—0·025 M KCl— 0·0025 M $MgCl_2$ (TKM), treated with 25 μg/ml. ribonuclease-free pancreatic deoxyribonuclease for 60 min at room temperature, extracted with SDS-phenol, and passed through 'Sephadex *G*-100' columns equilibrated with 0·15 M NaCl—0·015 M sodium citrate (SSC). The fractions of the eluate containing the peak of radioactivity from each column were pooled and used for hybridization experiments. Nitrocellulose membrane filters charged with 3 μg ¹⁴C-thymidine labelled mit-DNA[7] (purified as described in Fig. 2 and denatured at 2 μg/ml. in 0·1 × SSC by heating at 100° C for 20 min) were incubated in scintillation vials with 0·5 μg RNA from each fraction (specific activity = 7,620 c.p.m./μg) in 2 ml. 2 × SSC at 67° C for 22 h, then washed with 2 × SSC at room temperature, treated with pancreatic ribonuclease (5 μg/ml., 60 min), and again washed with 2 × SSC. As a control for non-specific background, 0·5 μg RNA from each fraction was incubated with membrane filters loaded with 3 μg heat-denatured *E. coli* DNA, and the membranes were washed and treated with ribonuclease in the same conditions. The hybridization values obtained with mit-DNA were corrected for this non-specific background (amounting to 1 to 10 per cent depending on the portion of the gradient) and for recovery of DNA (about 90 per cent), and normalized for the differences in amount of RNA in various fractions of the gradient (○—○). One set of hybridization experiments (◐—◐) was performed with 0·25 μg RNA and 5 μg mitochondrial or *E. coli* DNA.

endoplasmic reticulum. As previously demonstrated for rat liver microsomes[1], however, one can remove from the rough endoplasmic reticulum elements almost all the small ribosomal subunits and about 70 per cent of the large subunits by EDTA treatment[5]. Fig. 1*a* shows the A_{260nm} and radioactivity sedimentation profiles of the RNA extracted from the EDTA-treated crude mitochondrial fraction of HeLa cells labelled for 48 h with ³H-uridine. One recognizes the peak of 28*S* RNA pertaining to the membrane-stuck 50*S* subunits of contamin ting endoplasmic reticulum bound ribosomes[5], a broad band in the region 14*S* to 24*S* and slower sedimenting components in the region 4*S* to 13*S*. After re-sedimenting the 4*S* to 28*S* components for longer times, a peak sedimenting at about 21*S* and another at about 18*S* appeared to be partially resolved over a background of heterogeneous RNA (Fig. 1*b*); furthermore, a shoulder at about 24*S*, a fairly pronounced peak at 12*S*, and a prominent peak at 4*S* could reproducibly be observed. The 18*S* peak presumably reflects the presence of a small amount of 30*S* subunits not removed by the EDTA, possibly pertaining to intramitochondrial ribosomes. The 21*S* and 12*S* components have been previously recognized in the A_{260nm} or long-term labelling profiles of RNA extracted from a crude mitochondrial fraction of HeLa cells[3,6].

Homology with Mitochondrial DNA

In order to determine the distribution of components homologous to mit-DNA, hybridization experiments were carried out between individual fractions of the RNA sedimentation pattern in Fig. 1*b* and purified closed

circular mit-DNA. The latter was isolated by two cycles of caesium chloride–ethidium bromide density gradient centrifugation[8] from the sodium dodecyl sulphate lysate of a crude mitochondrial fraction from cells labelled with ^{14}C-thymidine (Fig. 2). Fractions of the sucrose gradient pattern shown in Fig. 1*b*, after deoxyribonuclease digestion and 'Sephadex' chromatography, were annealed with mit-DNA, using an input ratio of RNA to DNA of 1 to 6. This ratio is much lower than that required to reach DNA saturation with the whole mit-RNA (see Fig. 4); therefore, the hybrid levels obtained, after normalization for the differences in amount of RNA in various fractions of the gradient, should reflect roughly the distribution of RNA homologous to mit-DNA. The sedimentation profile of the components homologous to mit-DNA has a broad band between 9*S* and 15*S*, with a peak at 12*S*, and a pronounced 4*S* peak (Fig. 1*b*); a small amount of faster sedimenting components hybridizable with mit-DNA can also be seen in the region 16*S* to 30*S*. No clear peaks of RNA hybridizable with mit-DNA are present in the regions where *r*RNA components are expected. Similarly, there is no peak of hybridizable RNA at 21*S* emerging over the low background of hybrids in the region 18*S* to 30*S*. The level of hybrid formed in the 21*S* region of the gradient corresponded to about 0·0035–0·0045 μg RNA bound per 3 μg mit-DNA. The amount of DNA pertaining to presumptive 21*S* sites in each hybridization assay, estimated under the assumption that each mit-DNA molecule (mol. wt. 10^7 daltons) contains one site for 21*S* RNA (mol. wt. approx. 9×10^5 daltons[10]), was equivalent to about 0·27 μg. Because at least 20 per cent of the mit-DNA molecules in these experiments were available for hybrid formation with RNA, one would have expected at least 0·05 μg RNA to be bound per 3 μg DNA at saturation. The absence of detectable hybridization of 21*S* RNA with mit-DNA was therefore not due to lack of available DNA sites. In fact, RNA–DNA hybridization experiments carried out at a still lower RNA to DNA ratio (1 to 20) gave similar results (Fig. 1*b*; see also insert in Fig. 3).

The possibility that the 21*S* RNA did not hybridize with mit-DNA because of a double stranded structure seemed also to be excluded. It was found that in mild conditions of ribonuclease digestion (0·15 M NaCl–0·015 sodium citrate, 0·3 μg/ml. RNA, 0·03 μg/ml. pancreatic ribonuclease, 30 min at 37° C)—which leave the double stranded reovirus RNA completely intact[11]—the RNA from the 21*S* region was made 48 per cent acid-soluble, that is, to the same extent as the RNA from the other portions of the gradient. Furthermore, heating at 100° C for 10 min and rapid cooling did not increase the ribonuclease sensitivity of the components sedimenting at 21*S*, nor did this treatment enhance their capacity to hybridize with mit-DNA.

At the low ratio of input RNA to DNA used in the hybridization experiments described so far, an RNA species present in relatively low concentration might have been difficult to detect. It was reasoned that working at RNA to DNA ratios at or near saturation of the DNA sites could represent a more favourable situation for the detection of RNA species, which, although present in relatively low amounts, were complementary to a fairly large portion of the mit-DNA—as conceivably could be the case for the large *r*RNA components. As shown in Fig. 3, however, even at a relatively high RNA to DNA input ratio (5 : 1) there is no evidence of peaks of RNA homologous to mit-DNA in the regions where *r*RNA components are expected; again in this experiment the 21*S* RNA does not have any appreciable sequence homology with mit-DNA. The distribution in the gradient of the RNA hybridized with mit-DNA at high RNA to DNA input ratio differs from the sedimentation profile of the RNA hybridizable at low RNA to DNA ratio (Fig. 1*b*) for the higher amounts of hybrids formed by components heavier than 15*S* and for the uniformly high

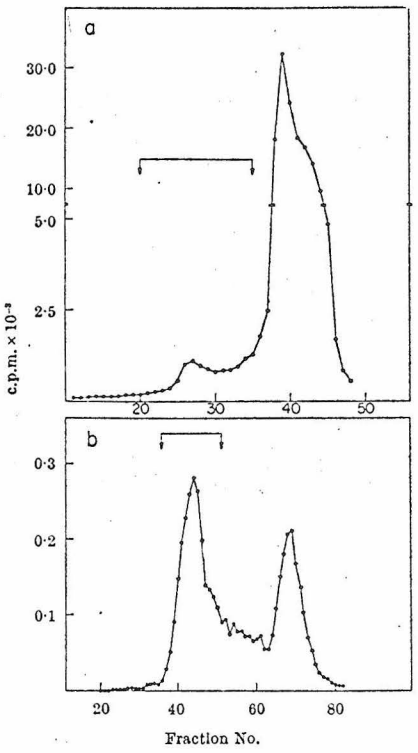

Fig. 2. Purification of HeLa closed circular mit-DNA by caesium chloride–ethidium bromide density gradient centrifugation. HeLa cells labelled for 28 h with ^{14}C-thymidine were homogenized in 0·01 M Tris buffer (*p*H 7·1)—0·01 M KCl—0·0001 M EDTA, and the crude mitochondrial fraction was separated by differential centrifugation, resuspended in 0·01 M Tris (*p*H 7·4)—0·01 M EDTA—1·2 per cent SDS, and left at room temperature for 45 min. Caesium chloride was added to give a final concentration of 1 M; the lysate was left in the cold for 30 min and then centrifuged at 15,800*g* for 15 min. Ethidium bromide was added to the supernatant to 200 μg/ml., and the solution was adjusted to 1·55 g/ml. with caesium chloride and centrifuged in the Spinco *SW* 41 rotor at 35,000 r.p.m. for 48 h at 20° C. Fractions were collected dropwise from the bottom of the tube. After assay of radioactivity, the fractions corresponding to the smaller heavy band (designated by arrows in *a*) were pooled and re-centrifuged in the same conditions in a caesium chloride–ethidium bromide density gradient. Two almost equal bands were obtained. The fractions containing the heavier band (designated by arrows in *b*), which consisted exclusively of closed circular mit-DNA[8,9], were pooled and, after passage through a Dowex 50*W*–*X*8 column to remove the dye[8], dialysed extensively against 0·1 × SSC.

level of hybridization between 4*S* and 15*S*. In these conditions, one would expect that the contribution of various RNA species to the hybrids would tend to reflect more the fraction of DNA complementary to each species than the concentration of the latter. The relatively high level of hybridization occurring with 16*S* to 28*S* components is therefore presumably to be attributed to the small amounts of heterogeneous RNA sedimenting in this region, detected also in the hybridization at a low RNA to DNA ratio (see Fig. 1*b* and insert in Fig. 3). Hybridization with heterogeneous components is probably also predominant in the region of 4*S* to 15*S* at high RNA to DNA ratio, and tends to cover here the contribution to the hybrids by 12*S* and 4*S* RNA.

Sites Available for Hybridization

In order to estimate the proportion of mit-DNA molecules available for hybridization in these experiments, mit-DNA was annealed with increasing amounts of RNA from the EDTA-treated crude mitochondrial fraction of cells labelled with ^{3}H-uridine: at the highest input ratio of RNA to DNA used (15 : 1), about 10 per cent of the DNA was hybridized, this being still below the saturation level (Fig. 4). Because mit-DNA is transcribed from the heavy strand[3,12], about 20 per cent of the *in vivo* transcrib-

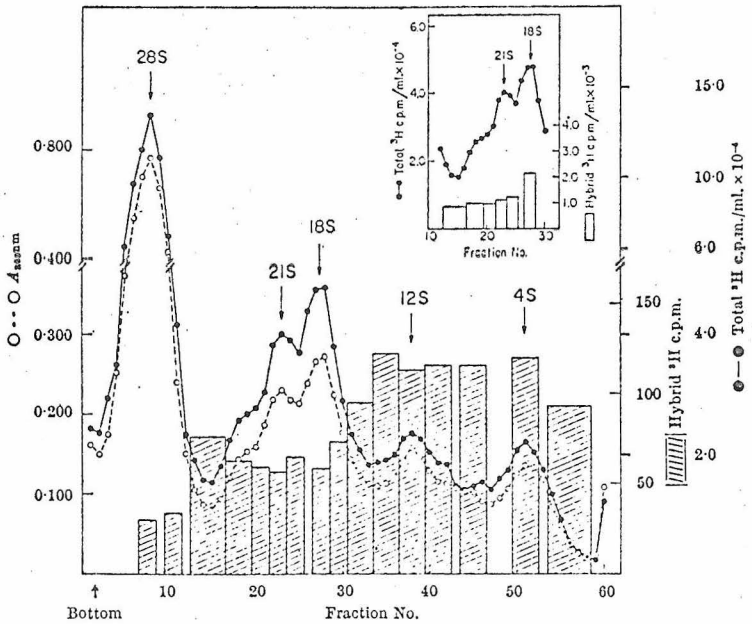

Fig. 3. Hybridization at a high RNA to DNA input ratio between mit-DNA and different components of the sedimentation pattern of RNA from the EDTA-treated crude mitochondrial fraction of HeLa cells. The RNA from the EDTA-treated mitochondrial fraction of HeLa cells labelled for 24 h with 5-³H-uridine was run on a 5 to 20 per cent sucrose gradient in acetate-NaCl buffer as in Fig. 1*a*: the 4*S* to 28*S* components collected by ethanol precipitation and centrifugation, were re-centrifuged through a 15 to 30 per cent sucrose gradient in acetate-NaCl buffer in the Spinco *SW* 25·3 rotor at 25,000 r.p.m. for 38 h at 3° C. Components in various fractions of the gradient (pooled in groups of 2 to 6, as shown) were subjected to deoxyribonuclease digestion in 0·05 M Tris (*p*H 7·1)—0·025 M KCl—0·0025 M $MgCl_2$ and 'Sephadex' chromatography as indicated in Fig. 1*b*. RNA from each sample (2·5 μg; specific activity = 4,800 c.p.m./μg) was incubated with 0·50 μg heat denatured ¹⁴C-thymidine labelled mit-DNA bound to a nitrocellulose membrane; conditions of incubation and treatment of the hybrids are described in Fig. 1*b* (ribonuclease digestion was at 10 μg/ml.). The hybridization values obtained with mit-DNA have been corrected for non-specific background (< 5 per cent) determined in separate experiments utilizing *E. coli* DNA, and for recovery of DNA. *Inset:* Results of hybridization assays carried out with the 18*S* to 26*S* RNA components at a low RNA to DNA input ratio (0·3 μg RNA, 5 μg mit-DNA per incubation mixture), corrected and normalized as in Fig. 1*b*, are plotted.

able DNA formed hybrids with RNA in this experiment. This is an underestimate of the proportion of mit-DNA molecules accessible for hybridization, for saturation of DNA was not reached. On the basis of a minimum proportion of 20 per cent of mit-DNA molecules being available for hybridization, and assuming one 21*S* site per mit-DNA molecule (from the sedimentation profile in Fig. 3, it appears that the 21*S* RNA was present in at least 50 times excess over the presumptive available 21*S* sites; this excess, by analogy with the *Escherichia coli* *r*RNA system, should be saturating[7]), saturation of 21*S* sites would involve binding of about 0·009 μg RNA per 0·5 μg DNA (about 45 c.p.m. in the experiment of Fig. 3). No evidence of such a level of hybridization in the form of a peak or shoulder can be seen in the 21*S* region. Because the available information indicates a considerable homogeneity of mit-DNA molecules in an animal cell population[13], these results are in agreement with those of the hybridization experiments carried out at low RNA to DNA ratio in suggesting that the 21*S* RNA is not synthesized on a mit-DNA template.

Non-mitochondrial Site for *r*RNA Synthesis

The results indicate that the bulk of the RNA homologous to mit-DNA in HeLa cells is represented by species sedimenting between 9*S* and 15*S* (with a prominent 12*S* component), and by 4*S* RNA. It is not known whether the 9*S* to 15*S* components are all intramitochondrial, or whether, on the contrary, they include a fraction of mit-RNA "exported" to the rough endoplasmic reticulum (it is likely, in fact, that the *m*RNA of polysomes bound to the endoplasmic reticulum would not be completely removed by the EDTA treatment[5]).

Our work suggests that the 12*S* component is homologous to mit-DNA and that it is therefore presumably synthesized on a mit-DNA template. In yeast mit-RNA the occurrence of a 12·7*S* component has been previously described[14], and maximum hybridization with mit-DNA has been found to occur with 12–13*S* components[15]. The apparent lack of homology between the 21*S* RNA and mit-DNA suggests a non-mitochondrial site of synthesis

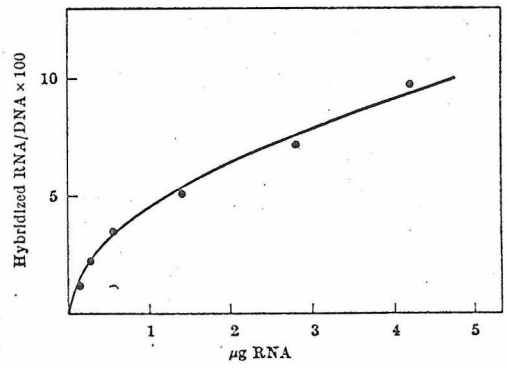

Fig. 4. Hybridization of HeLa mit-DNA with increasing amounts of RNA (4*S* to 30*S* components with the exception of those in the 28*S* region) from the EDTA-treated crude mitochondrial fraction of HeLa cells labelled for 48 h with 5-³H-uridine. Increasing amounts of RNA, isolated and subjected to deoxyribonuclease digestion and 'Sephadex' chromatography as described in Fig. 1*b* (specific activity = 6,840 c.p.m./μg), were incubated in scintillation vials with 0·28 μg ¹⁴C-labelled heat-denatured mit-DNA bound to nitrocellulose membranes. The hybridization values are corrected for non-specific background determined with *E. coli* DNA, as explained in Fig. 1*b*, and for recovery of DNA.

for this RNA species, but its origin and nature still have to be determined. The 4*S* RNA homologous to mit-DNA presumably contains at least some of the mitochondrial specific *t*RNA species which have been described in animal cells, including HeLa cells[16–19]. Sequence homology between mitochondrial leucyl-*t*RNA from rat liver and mit-DNA has been recently reported[20].

The lack, in this work, of any indication of *r*RNA components homologous to mit-DNA is in full agreement with previously reported observations concerning the absence in mit-RNA preparations (from cells pulse labelled with ^{3}H-uridine for 3 to 20 min) of homogeneous radioactive components identifiable as possible *r*RNA species or precursors[1,3]. The lack of mitochondrial-specific *r*RNA components coded by mit-DNA in HeLa cells, and presumably in other animal cells, which is strongly suggested by the experiments reported here, contrasts with the well documented occurrence in *Neurospora*[21–24] and yeast[14,25] of distinctive, bacterial-type *r*RNA species, which are capable of hybridizing with mit-DNA[24,25]. These observations suggest that in the evolution of eukaryotic cells there has been a reduction in the informational content of mit-DNA. In agreement with this idea are the numerous observations which indicate that, whereas mit-DNA from animal cells consists of basic units in the form of circular molecules about 5 μm in length and 10^7 daltons in molecular weight[13], mit-DNA from plant cells, protozoa, *Neurospora* and (in part) also yeast, is in the form of structures longer than 5 μm (refs. 24 and 26–29). The loss of information for the synthesis of *r*RNA in mit-DNA from animal cells would imply that in these cells ribosomes under nuclear control are utilized for the translation of mitochondrial messages. This could be accomplished by the import into the mitochondria of nuclear ribosomes, as suggested by the protein synthesizing capacity of isolated animal cell mitochondria[30], and/or by the export of mitochondrial *m*RNA to the cytoplasmic translating machinery, as implied by the observations made in HeLa cells[2,3].

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CHAPTER 4

Rapidly Labeled Heterogeneous RNA and Discrete RNA Components Identified by Sedimentation and Electrophoretic Analysis of RNA from the Mitochondrial Fraction of HeLa Cells

The first part of this chapter has been submitted for publication in the Journal of Molecular Biology. It is included here in the form in which it was submitted.

EXPRESSION OF THE MITOCHONDRIAL GENOME IN HeLa CELLS

I. Rapidly Labeled Heterogeneous RNA and Discrete RNA Components Identified by Sedimentation and Electrophoretic Analysis of RNA from the Mitochondrial Fraction

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Running title: RNA from the Mitochondrial Fraction

ABSTRACT

Both heterogeneous RNA components, varying in sedimentation constant from 4 S to more than 50 S, and discrete RNA components have been identified in the sedimentation pattern of RNA from the mitochondrial fraction of HeLa cells.

The labeling of heterogeneous RNA, in short-term experiments, has been shown to be almost completely sensitive to a concentration of ethidium bromide (1 µg/ml.) which does not appreciably affect nuclear RNA synthesis. This observation has provided additional support for the previously reached conclusion that mit-DNA is the template of this RNA fraction.

The discrete RNA components associated with the mitochondrial fraction have been shown to include: (a) 16 S, 12 S, and 4 S components, which for their kinetics of labeling, sensitivity of their synthesis to 1 µg/ml. ethidium bromide and lack of sensitivity to 0.04 µg/ml. actinomycin D, their nucleotide composition, and base sequence homology to mit-DNA appear to be synthesized on a mit-DNA template; (b) 28 S, 18 S, and 5 S components of nuclear origin, pertaining to ribosomes which are exclusively or in their great majority attached to contaminating rough endoplasmic reticulum; (c) 23 S and 21 S components, which appear to be of extramitochondrial origin. The base composition and methylation level of these components suggest that they may be degradation products of 28 S RNA.

Analysis of the sedimentation behavior of the 16 S and 12 S species under denaturing conditions has indicated that they are

represented by continuous polynucleotide chains. The 16 S and 12 S species move in polyacrylamide gel electrophoresis, relative to the 28 S and 18 S RNA markers, as expected, respectively, for a species slightly larger than 18 S RNA (i.e. with a molecular weight about 0.7×10^6) and for a species with a molecular weight of about 0.4×10^6. The 16 S and 12 S RNA appear to correspond to the "21 S" and "12 S" electrophoretic components previously described in HeLa cells. However, in contrast to what has been reported for the latter components, the 16 S and 12 S RNA have been found to be methylated; furthermore, these species appear to have a considerably longer half-life than previously surmised on the basis of their behavior in the presence of ethidium bromide.

The possible relationship between rapidly labeled heterogeneous RNA and mit-DNA coded discrete RNA species is discussed.

1. Introduction

Previous work from this laboratory has shown the occurrence in the mitochondrial fraction from HeLa cells of rapidly labeled heterogeneous RNA sedimenting between 4 S and more than 50 S. The linear kinetics of labeling of this RNA, its association with structures having the same sedimentation velocity and density distribution in sucrose gradients as mitochondria, its insensitivity *in situ* to RNase, and, most important, its sequence homology to closed circular mit-DNA† have clearly pointed to this DNA as its template (Attardi & Attardi, 1967, 1968, 1969a,b; Attardi, Aloni, Attardi, Lederman, Ojala, Pica-Mattoccia & Storrie, 1970).

Recently, several discrete RNA components have also been identified by polyacrylamide gel electrophoresis and sedimentation analysis of bulk RNA extracted from the mitochondrial fraction of human (HeLa), hamster and *Xenopus* cells (Dubin, 1967; Vesco & Penman, 1969a,b; Attardi & Attardi, 1969a,b; Dawid, 1969). In HeLa cells, in addition to 28 S and 18 S RNA, pertaining in their great majority if not exclusively to ribosomes of contaminating rough endoplasmic reticulum (Attardi, Cravioto & Attardi, 1969), and to 4 S RNA, two minor components with sedimentation coefficients of about 21 S and 12 S have been recognized by sedimentation analysis in sucrose gradient of the RNA from the mitochondrial fraction (Attardi & Attardi, 1969b). On the other hand, two main distinctive high molecular weight RNA components tentatively designated as "21 S" and "12 S" from their electrophoretic mobilities have been described in the electrophoretic pattern of RNA

from the mitochondrial fraction of HeLa cells (Vesco & Penman, 1969a). Previously, a "17 S" RNA had been found in the sedimentation pattern of RNA from the mitochondrial fraction of cultured hamster cells treated for a long time with actinomycin D (Dubin, 1967), and more recently this RNA has been shown to consist of two components (Dubin & Montenecourt, 1970).

The intercalating dye ethidium bromide has been recently reported to inhibit selectively the labeling, during a 2 hr exposure of the cells to [^{3}H]-uridine, of the "21 S" and "12 S" electrophoretic RNA species (Zylber, Vesco & Penman, 1969) and to a great extent also that of 4 S RNA from the mitochondrial fraction of HeLa cells. This effect has been interpreted to indicate an inhibition of the synthesis of these components on a closed circular mit-DNA template. On the other hand, a scanning of the sedimentation distribution of long-term labeled RNA from the mitochondrial fraction of HeLa cells for capacity to hybridize with purified closed circular mit-DNA at widely different RNA to DNA ratios has shown that, whereas the 4 S and 12 S components of the sedimentation analysis exhibit sequence homology to mit-DNA, the 21 S component does not hybridize with this DNA to any appreciable extent (Attardi & Attardi, 1969b). This result points to a non-mitochondrial site of synthesis for this RNA component.

In order to obtain further information concerning the origin of the heterogeneous and discrete RNA components present in the mitochondrial fraction from HeLa cells and to try to clarify the question of the relationship between the sedimentation and electrophoretic discrete

RNA species, we have investigated the kinetics of labeling of these components, their nucleotide composition and methylation level, the early effects of preferential inhibitors of mitochondrial RNA synthesis (ethidium bromide) and nuclear RNA synthesis (actinomycin D), and the electrophoretic behavior of individual components isolated by high resolution sucrose gradient centrifugation.

2. Materials and Methods

(a) Method of growth of cells and labeling conditions

The method of growth of HeLa cells in suspension has been previously described (Amaldi & Attardi, 1968). Labeling of RNA was carried out by exposing exponentially growing cells (2 to 3 x 10^5 cells/ml.) for various times to [5-^{3}H]-uridine (22.7 to 31.0 mC/μmole, 1.4 μC/ml.) in modified Eagle's medium with 5% dialyzed calf serum. To determine the degree of methylation of RNA, exponentially growing HeLa cells were collected by centrifugation, resuspended at a concentration of 2 x 10^5 cells/ml. in modified medium containing 10^{-3} M cold phosphate and 3 μg/ml. methionine, plus 5% dialyzed serum, and grown for 22 hr in the presence of ^{32}P-orthophosphate (1.75 μC/ml.) and L-[^{3}H-methyl] methionine (5.4 mC/μM, 2.5 μC/ml.). The concentration of methionine in the medium was sufficient to allow more than one generation of growth. To reduce to a minimum the labeling of the purine rings, 10^{-4} M adenosine and 10^{-4} M guanosine were added to the growth medium for 24 hr prior to the exposure of the cells to L-[^{3}H-methyl] methionine and during the 22 hr labeling period (Knight, 1969). In the experiments

in which the effects of ethidium bromide or actinomycin D were to be tested, the drug was added, at the concentration specified below, 15 min or 30 min, respectively, prior to addition of the radioactive RNA precursor.

(b) Subcellular fractionation

The cells, washed with 0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$, were homogenized in 6 vol. 0.01 M Tris buffer (pH 6.7 (25°C)), 0.001 M KCl, 0.00015 M $MgCL_2$ with a Potter-Elvehjem homogenizer. After addition of sucrose to 0.25 M and a low speed spin (1160 g_{av} for 3 min) to sediment nuclei and unbroken cells, the cytoplasmic extract was centrifuged at 8100 g_{av} for 10 min to pellet the crude mitochondrial fraction. The latter was resuspended in 0.25 M sucrose in 0.01 M Tris buffer (pH 6.7), 0.00015 M $MgCl_2$ (one-half of the vol. of the homogenate) and, after another low speed spin, sedimented at 8100 g_{av} for 10 min. The final pellet was resuspended in 0.25 M sucrose (2 to 4 ml. per ml. of packed cells), treated with 0.03 M EDTA for 10 min (to remove the bulk of ribosomes of contaminating rough E.R. (Attardi et al., 1969)), and centrifuged at 11,000 g_{av} for 10 min; the pellet was washed by centrifugation with 0.25 M sucrose in 0.01 M Tris buffer (pH 6.7), 0.015 M EDTA, and then fractionated on the basis of buoyant density by centrifugation through a 30 to 48% sucrose gradient in 0.01 M Tris buffer (pH 6.7) in the Spinco SW25.1 rotor at 25,000 rev./min for 17 to 19 hr at 3°C. The mitochondria, revealed by the profile of cytochrome oxidase activity, banded in a region of the gradient centered around $\rho \sim 1.17$ g/ml.; however, the mitochondrial band was contaminated with rough E.R. elements (Attardi et al.,

1969). The material of this band will be called mitochondrial fraction throughout this paper. Deviations from the procedure outlined above for the preparation and isopycnic separation of the mitochondrial fraction will be specified in the text and legends of figures.

The free monomer-polysome fraction was isolated by centrifuging the 15,800 g supernatant of the cytoplasmic extract at 105,000 g_{av} for 2 hr (Attardi et al., 1969).

(c) Extraction and analysis of RNA

The fractions corresponding to the mitochondrial band in the sucrose gradient were pooled, brought to 1% dodecyl SO_4, 0.1 M NaCl and mixed with 2 vol. ethanol. After standing at -20°C for 2 hr or longer, the mixture was centrifuged at 23,500 x g for 15 min; the resulting pellet was then dissolved in 5 ml. (per 1 to 2 gram cell equivalent of crude mitochondrial fraction) of dodecyl SO_4 buffer (0.5% dodecyl SO_4, 0.1 M NaCl, 0.01 M Tris buffer, pH 7.0, 0.001 M EDTA), mixed with an equal vol. of a mixture containing 2% dodecyl SO_4, 1% Na deoxycholate, 1% Na cholate, 2.6 mg/ml. bentonite, and extracted 3 times with water-saturated redistilled phenol containing 0.1% 8-hydroxy-quinoline, as previously described (Attardi, Parnas, Huang & Attardi, 1966). The RNA was precipitated 3 times with ethanol, finally dissolved in dodecyl SO_4 buffer, and run in a 15 to 30% (w/w) sucrose gradient in the same medium as specified below.

For RNA extraction from the free monomer-polysome fraction, the 105,000 g_{av} pellet was resuspended in 2 ml. 0.05 M Tris buffer, pH 6.7, 0.025 M KCl, 0.0025 M $MgCl_2$, with the aid of a glass homogenizer;

dodecyl SO_4 was then added to 1%, and the RNA precipitated with 2 vol. ethanol in the presence of 0.1 M NaCl. After centrifugation, the pellet was resuspended in dodecyl SO_4 buffer, and RNA was extracted and analyzed under the same conditions described above for RNA from the crude mitochondrial fraction.

For the analysis of the sedimentation properties of the 16 S and 12 S RNA under denaturing conditions, samples of these components, mixed with ^{14}C-labeled 18 S RNA marker (prepared from small subunits of free polysomes (Attardi et al., 1969)), were heated in 0.2 ml. of 18% neutralized formaldehyde, 0.001 M EDTA at 70°C for 5 min and quickly cooled to 0°C; for sedimentation analysis, the samples were diluted 2.5 times with 0.001 M EDTA and run through a 5 to 20% (w/w) sucrose gradient in 0.02 M phosphate buffer (pH 7.4), 0.1 M NaCl, 1% formaldehyde in the SW41 rotor at 41,000 rev./min for 22 hr at 4°C.

For electrophoretic analysis, polyacrylamide gels were polymerized (Loening, 1967) in plexiglas tubes of 0.6 cm internal diameter from a mixture of 2.7% (for analysis of high molecular weight RNA) or 10% (for low molecular weight RNA) (w/v) acrylamide (Eastman Chemical Co., recrystallized from chloroform) and 0.25% (v/v) ethylene diacrylate (K & K Laboratories) in polymerization buffer (0.04 M Tris, 0.02 M Na acetate, 0.002 M EDTA, adjusted to pH 7.4 with acetic acid). 8 cm long gels were prepared. RNA samples in 100 µl. 5% sucrose in electrophoresis buffer (i.e. polymerization buffer with 0.5% dodecyl SO_4), were layered onto the polyacrylamide gels. Electrophoresis was carried out at room temperature at 5 mA/gel for the time specified below. The gels

were fractionated with a gel smasher specially built according to the principles of the technique of Maizel (1966), by using 0.1% dodecyl SO_4 as eluant. The fractions, of about 1.8 ml., were shaken overnight at room temperature, then each fraction or a sample of it was mixed with 15 ml. Bray's (1960) scintillation fluid and counted in a Packard scintillation counter.

Analysis of nucleotide composition after alkaline hydrolysis of ^{32}P- or ^{3}H-labeled RNA components was carried out as previously described (Attardi et al., 1966).

3. Results

(a) Rapidly labeled mitochondria-associated‡ heterogeneous RNA

Figure 1 illustrates the effect of ethidium bromide on the labeling, during a 15 min [5-^{3}H]-uridine pulse, of the heterogeneous mitochondria-associated RNA. The $O.D._{260}$ profile shows a prominent 28 S RNA peak pertaining in its great majority, if not exclusively, to membrane-stuck 50 S subunits of contaminating rough E.R. elements (Attardi et al., 1969), a broad band centered around 18 S (consisting of components which are resolved in long sedimentation runs (see below)) and slower sedimenting material. The heterogeneous pulse-labeled RNA consists, as was mentioned earlier, of components sedimenting between 4 S and more than 50 S. After a 15 min pulse most of the labeled components sediment slower than 35 S. A broad peak at about 33 S is reproducibly recognizable after this labeling time (Attardi et al., 1970); furthermore, as will be seen below (Fig. 2),

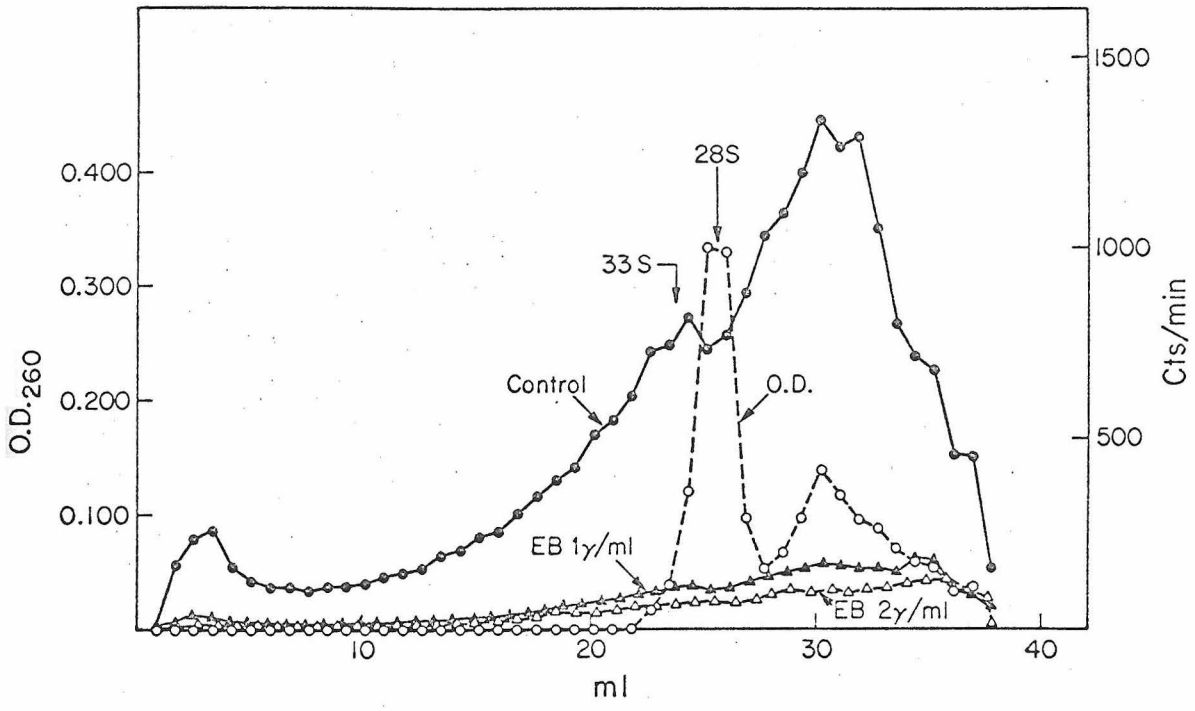
0.400
0.300
0.200
0.100
O.D.260
1500
1000
500
Cts/min
28S
33S
Control
O.D.
EB 1γ/ml
EB 2γ/ml
10
20
30
40
ml

Figure 1

Fig. 1. Effect of ethidium bromide on the labeling of heterogeneous mitochondria-associated RNA.

2×10^8 HeLa cells were exposed to [5-^{3}H]-uridine (22.7 C/mM, 1.4 μc/ml.) for 15 min in the absence (o——o) or in the presence of 1 μg/ml. (Δ——Δ), or 2 μg/ml. (Δ---Δ) ethidium bromide. The RNA was extracted from the mitochondrial fraction, which had been treated with EDTA and isopycnically separated in sucrose gradient (as detailed in Materials and Methods (b)), and run through a 5 to 20% (w/w) sucrose gradient in 0.01 M acetate buffer, pH 5.0, 0.1 M NaCl, above a 4 ml. cushion of 64% sucrose, in the SW27 Spinco rotor (2.54 x 8.83 cm buckets) at 20,000 rev./min for 10 hr at 3°C.

discrete components start emerging also in the region between 4 S and 25 S. A concentration of ethidium bromide of 1 µg/ml. inhibits uniformly by about 85% the labeling of different size components of the heterogeneous mitochondria-associated RNA; the inhibition is of about 90% at a concentration of the drug of 2 µg/ml. On the other hand, at 1 µg/ml. ethidium bromide has no appreciable effects for at least 4 hr on the labeling of total cell RNA (to which nuclear RNA synthesis contributes more than 95%), in agreement with data reported by others (Zylber et al., 1969) (Table 1). Other experiments utilizing actinomycin D have shown that concentrations of this drug that inhibit completely the synthesis of rRNA and to a great extent the arrival in the cytoplasm of free polysome mRNA do not affect appreciably the pulse labeling of the heterogeneous mitochondria-associated RNA (Attardi & Ojala, in preparation).

The nature of the rapidly labeled RNA components heavier than 28 S which are complementary to mit-DNA (Attardi & Attardi, 1968; Attardi et al., 1970) and ethidium-bromide sensitive (this work) is at present being investigated. The evidence obtained so far indicates that the sedimentation properties of these heavy components are not affected by recentrifugation in a sucrose gradient, by two additional extractions with hot phenol-dodecyl SO_4, or by DNase treatment.

(b) Discrete components recognized by sedimentation analysis of RNA from the mitochondrial fraction

Figures 2, 3, and 4 show the labeling of RNA components sedimenting slower than 28 S from the mitochondrial fraction of cells

Table 1

Effect of ethidium bromide on total [5-^{3}H]-uridine incorporation into HeLa cells

Labeling time (min)	^{3}H cts/min incorporated per 10^8 cells x 10^{-6}	
	Control	Ethidium Bromide*
30	7.15	8.29 (116)
90	24.1	22.4 (93)
240	31.9	37.4 (117)

HeLa cells, labeled at a concentration of 1.7 x 10^5 cells/ml. for different times with [5-^{3}H]-uridine (22.8 mC/μM; 1.4 μC/ml.) in the absence or in the presence of 1 μg/ml. ethidium bromide, were washed three times with 0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$, and resuspended in 20 ml. of the same salt solution. Duplicate 50 μl. samples of each cell suspension were precipitated with 5% trichloroacetic acid, and the insoluble residues collected by filtration through Millipore membranes, dried and counted in the scintillation counter.

*The figures in parentheses indicate the per cent with respect to the control values.

exposed to [5-^{3}H]-uridine pulses of various duration. The mitochondrial fraction had been treated with EDTA, as detailed in Materials and Methods (b), to remove the bulk of ribosomes of contaminating rough E.R. (Attardi et al., 1969). It is evident that sucrose gradient centrifugation in the Spinco SW25.3 or SW27 rotor with long buckets provides a good resolution of RNA components sedimenting slower than 28 S RNA. One can recognize in the O.D.$_{260}$ profile the prominent peak (partially pelleted) of 28 S RNA near the bottom of the tube; near the center of the gradient there is an 18 S peak (pertaining presumably to a small amount of 30 S subunits not removed by EDTA, estimated to represent between 10 and 15% of that originally present in the crude mitochondrial fraction), with a shoulder at about 16 S. On the two sides of the 18 S peak there are two somewhat smaller peaks at about 21 S and 12 S, and near the meniscus a pronounced peak at 4 S. A minor component not always well resolved and possibly present in variable amount can be seen at about 23 S, and it corresponds presumably to the shoulder previously observed on the heavy side of the 21 S component in the somewhat shorter sedimentation runs (Attardi & Attardi, 1969b).

After a 15 min [5-^{3}H]-uridine pulse (Fig. 2a), discrete peaks of radioactivity are already clearly recognizable over a high background of heterogeneous RNA. One can see a labeled peak at 17 S with a shoulder at about 16 S in correspondence with the shoulder in the O.D.$_{260}$ profile. The 12 S and 4 S components appear also to be labeled at this time; by contrast, no radioactivity appears to be associated with the 21 S component. After a 30 min pulse (Fig. 2b),

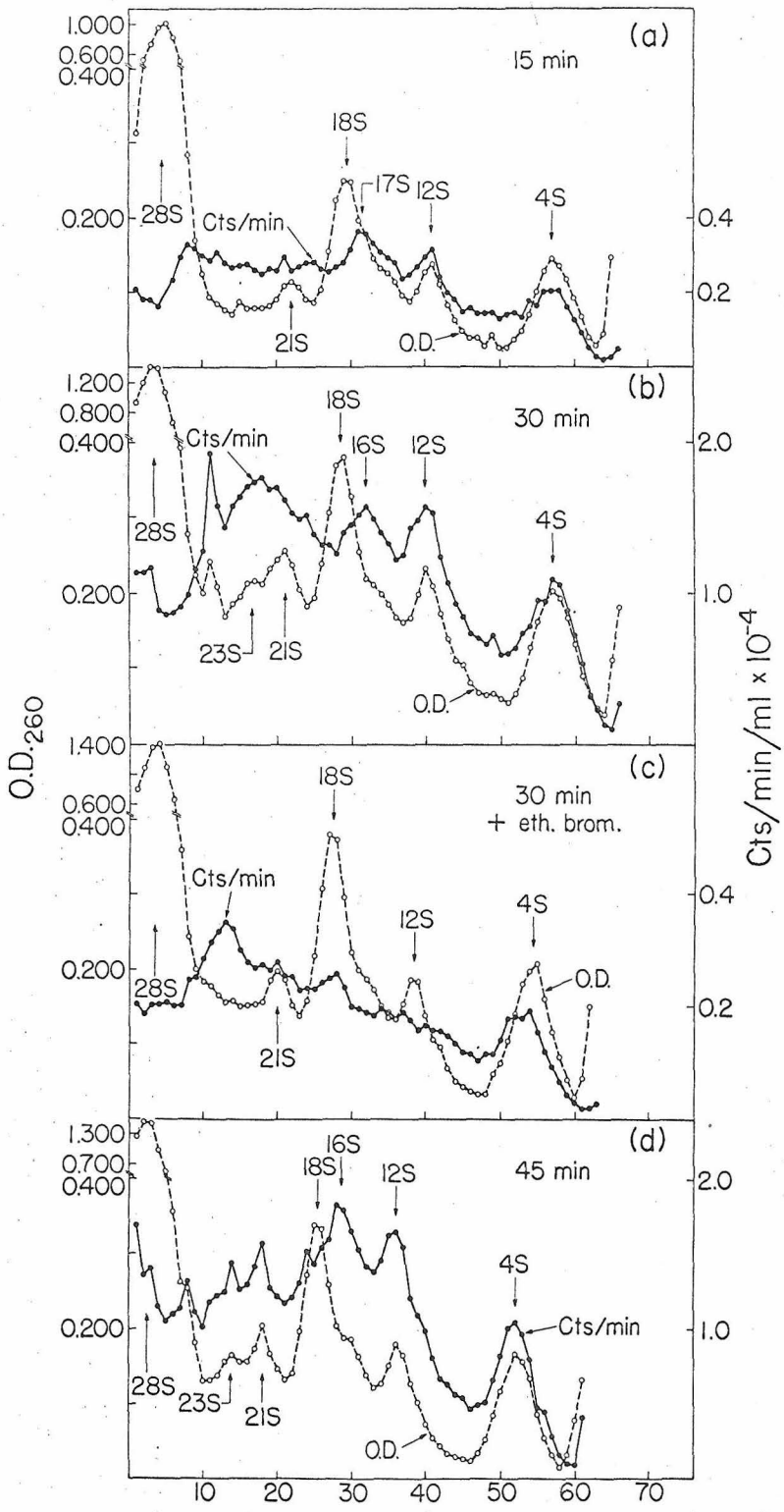
(a)
15 min
18S
17S
12S
4S
28S
Cts/min
21S
O.D.
(b)
30 min
18S
16S
12S
4S
28S
Cts/min
23S
21S
O.D.
(c)
30 min
+ eth. brom.
18S
12S
4S
28S
Cts/min
21S
O.D.
(d)
45 min
16S
18S
12S
4S
28S
23S
21S
Cts/min
O.D.
O.D.260
Cts/min/ml x 10-4
Fraction no.

Figure 2

Fig. 2. Sedimentation patterns of RNA components sedimenting slower than 28 S RNA from the mitochondrial fraction of HeLa cells exposed to [5-^{3}H]-uridine pulses of various duration in the absence or in the presence of ethidium bromide.

2 x 10^{8} HeLa cells were labeled for 15 min (a), 30 min (b), or 45 min (d) in the absence of ethidium bromide, or for 30 min in the presence of 1 µg/ml. ethidium bromide (c), with [5-^{3}H]-uridine (22.8 C/mM, 1.4 µc/ml.). The RNA was extracted from the EDTA-treated and isopycnically separated mitochondrial fraction, and run through a 15 to 30% (w/w) sucrose gradient in dodecyl SO_4 buffer in the SW25.3 Spinco rotor at 25,000 rev./min for 28 hr at 20°C.

O---O, O.D.$_{260}$; ●——●, cts/min

the three peaks of radioactivity observed earlier have become more pronounced. The labeled peak sedimenting behind 18 S RNA has its center now at about 16 S, suggesting a conversion of the early labeled 17 S RNA to somewhat slower sedimenting molecules; a shoulder at 18 S suggests the initial labeling of this component. If the 30 min [5-^{3}H]-uridine pulse is carried out in the presence of 1 µg/ml. ethidium bromide, the labeling of the 16 S component is completely inhibited, unmasking the initial labeling of 18 S RNA (Fig. 2c). (In a parallel analysis it was observed that the 18 S RNA of free ribosomes starts becoming labeled at about the same time.) Ethidium bromide also inhibits the labeling of the 12 S component and to a great extent (by about 81%) that of 4 S RNA; the labeling of the heterogeneous RNA is likewise strongly suppressed by the drug, as previously discussed. After a 45 min pulse (Fig. 2d), the 21 S and 23 S components appear to be labeled. After a 90 min pulse (Fig. 3a), the considerable labeling of the 18 S RNA species masks in part the 16 S component, which is recognizable now only as a shoulder. After this time of exposure of the cells to the radioactive precursor, all the labeled components in the gradient appear to be alkali-sensitive, thus excluding any contribution to the radioactive profile of the incorporation of label into DNA. If the 90 min pulse is carried out in the presence of 0.04 µg/ml. actinomycin D to block selectively rRNA synthesis (Penman, Vesco & Penman, 1968), the 28 S and 18 S RNA labeling is abolished, and the 16 S labeled peak stands out clearly (Fig. 3b). Under the same conditions, the labeling of the 12 S and 4 S peaks is likewise not

affected to any appreciable extent; by contrast, the labeling of the 21 S component appears to be greatly reduced. A different pattern is observed if the 90 min [5-^{3}H]-uridine pulse is carried out in the presence of 1 µg/ml. ethidium bromide. As shown in Figure 3c, this drug strongly inhibits the labeling of the heterogeneous RNA, and abolishes that of the 16 S, 12 S and of most of the 4 S (~76%) components. The label associated with the membrane-stuck 28 S RNA appears to be reduced by about 40% under the same conditions, without any appreciable decrease in the amount of this component, as judged by taking the O.D.$_{260}$ of 4 S RNA as a reference.

Effects of actinomycin D and ethidium bromide similar to those described above are observed after a 2 hr and 4 hr pulse (Fig. 4). It is apparent from Figure 4c that after 255 min treatment with ethidium bromide no 12 S O.D.$_{260}$ peak is present in the sedimentation profile of mitochondria-associated RNA. The lack of a shoulder or a trailing edge in the 18 S O.D.$_{260}$ peak suggests that the 16 S component has also to a great extent or completely disappeared. No obvious reduction in the O.D.$_{260}$ associated with the 16 S and 12 S RNA component was, on the contrary, observed after 105 min (Fig. 3) or 135 min treatment with ethidium bromide. After a 4 hr pulse the labeling of 28 S RNA was reduced by about 47%.

In an experiment in which the effect of ethidium bromide (1 µg/ml.) on the labeling of the RNA of the mitochondrial fraction during a 2 hr exposure to [5-^{3}H]-uridine in the presence of 0.04 µg/ml. actinomycin D was tested, almost 90% inhibition was observed.

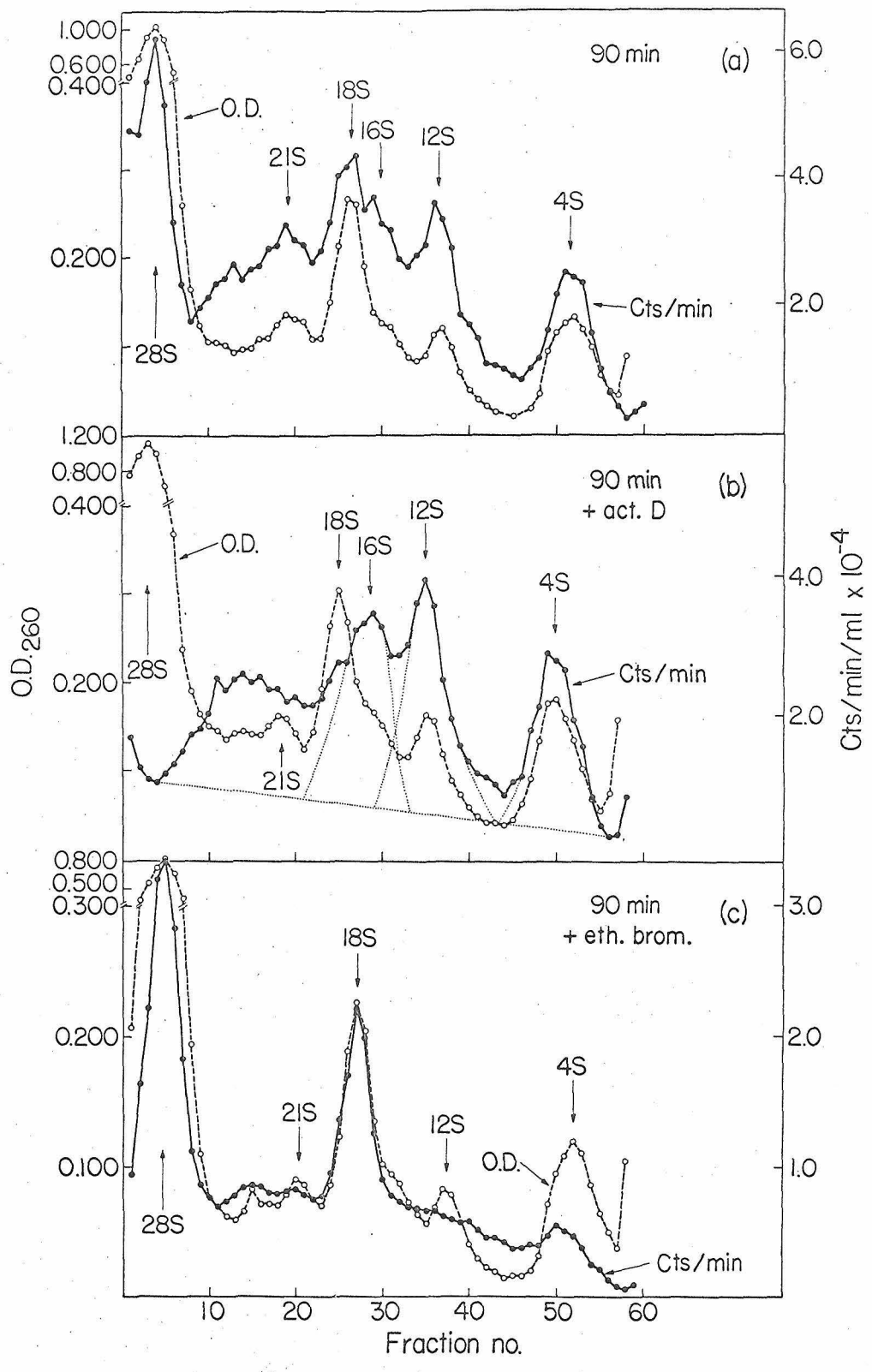
90 min
(a)
O.D.
18S
16S
12S
21S
4S
28S
Cts/min
90 min
+ act. D
(b)
O.D.
18S
16S
12S
4S
28S
21S
Cts/min
90 min
+ eth. brom.
(c)
18S
21S
12S
4S
O.D.
28S
Cts/min
O.D.260
Cts/min/ml x 10-4
Fraction no.

Figure 3

Fig. 3. Sedimentation pattern of RNA components sedimenting slower than 28 S RNA from the mitochondrial fraction of HeLa cells exposed to [5-^{3}H]-uridine for 90 min in the absence or in the presence of actinomycin D or ethidium bromide.

2.4 x 10^{8} HeLa cells were labeled for 90 min with [5-^{3}H]-uridine (22.8 C/mM, 1.4 µc/ml.) in the absence (a) or in the presence of 0.04 µg/ml. actinomycin D (b) or 1 µg/ml. ethidium bromide (c). RNA was extracted from the EDTA-treated and isopycnically separated mitochondrial fraction, and analyzed in sucrose gradient as in Figure 2.

O---O, O.D.$_{260}$; ●——●, cts/min;

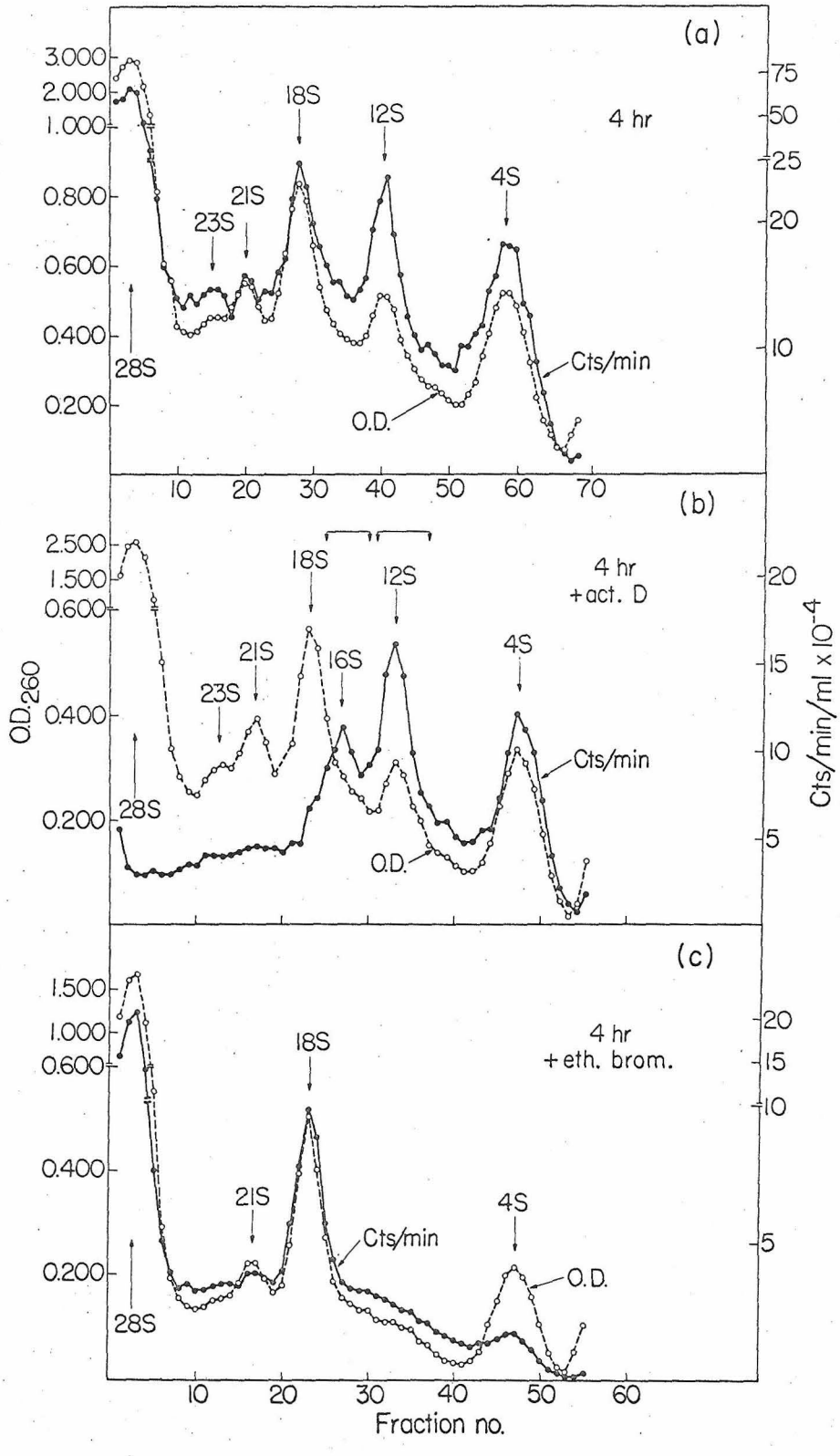
(a)
4 hr
18S
12S
4S
21S
23S
28S
Cts/min
O.D.
(b)
4 hr
+act. D
18S
12S
21S
16S
23S
4S
28S
Cts/min
O.D.
O.D.260
Cts/min/ml x 10⁻⁴
(c)
4 hr
+eth. brom.
18S
21S
4S
28S
Cts/min
O.D.
Fraction no.

Figure 4

Fig. 4. Sedimentation pattern of RNA components sedimenting slower than 28 S RNA from the mitochondrial fraction of HeLa cells exposed to [5-^{3}H]-uridine for 4 hr in the absence or in the presence of actinomycin D or ethidium bromide.

5.9 x 10^8 (a) or 3.0 x 10^8 (b,c) HeLa cells were labeled for 4 hr with [5-^{3}H]-uridine (22.8 C/mM, 1.4 µc/ml.) in the absence (a) or in the presence of 0.04 µg/ml. actinomycin D (b) or 1 µg/ml. ethidium bromide (c). RNA was extracted from the EDTA-treated and isopycnically separated mitochondrial fraction and analyzed in 15 to 30% sucrose gradients in dodecyl SO_4 buffer (SW27 Spinco rotor with 1.59 x 10.16 cm buckets, 25,000 rev./min, 27 hr at 20°C). The components corresponding to the portions of the sucrose gradient pattern (b) indicated by arrows were used for reruns (Fig. 5).

0------0 O. D. $_{260}$

●———● ^{3}H cts. /min.

The material sedimenting in the 12 S and in the 16 S regions of the pattern of RNA from cells labeled for 4 hr in the presence of actinomycin D (indicated by arrows in Fig. 4b) was separately rerun in sucrose gradient under the same conditions. After the rerun, the 12 S RNA appears as a sharp peak in the expected position relative to the ^{14}C-labeled 18 S RNA marker (Fig. 5a). The 16 S component appears in the $O.D._{260}$ profile as a pronounced shoulder on the light side of a relatively small 18 S peak (Fig. 5b): the ^{3}H-radioactivity profile follows fairly closely the $O.D._{260}$ profile of the 16 S component as reconstructed by subtracting the contribution of the 18 S RNA, estimated from the ^{14}C-radioactivity pattern. The amount of $O.D._{260}$ associated with the 16 S component, as judged from the second sucrose gradient centrifugation pattern and taking into account the fraction of labeled 16 S utilized for rerun (about 75%), appears to be similar to that pertaining to the 12 S component. In various experiments, the amount of $O.D._{260}$ associated with the 4 S RNA peak was found to be on the average 10% higher than that associated with the 12 S component.

After denaturation by heat-formaldehyde treatment, the 12 S and 16 S RNA components sediment in sucrose gradient in the presence of formaldehyde as sharp peaks, with the 16 S moving about 25% faster than the 12 S (Fig. 6). By applying the formula relating molecular weight of unreacted RNA to sedimentation constant after reaction with formaldehyde (Boedtker, 1968), and neglecting the effects on the sedimentation rate of the radial increase in centrifugal force and in sucrose viscosity (which would in part compensate each other), it

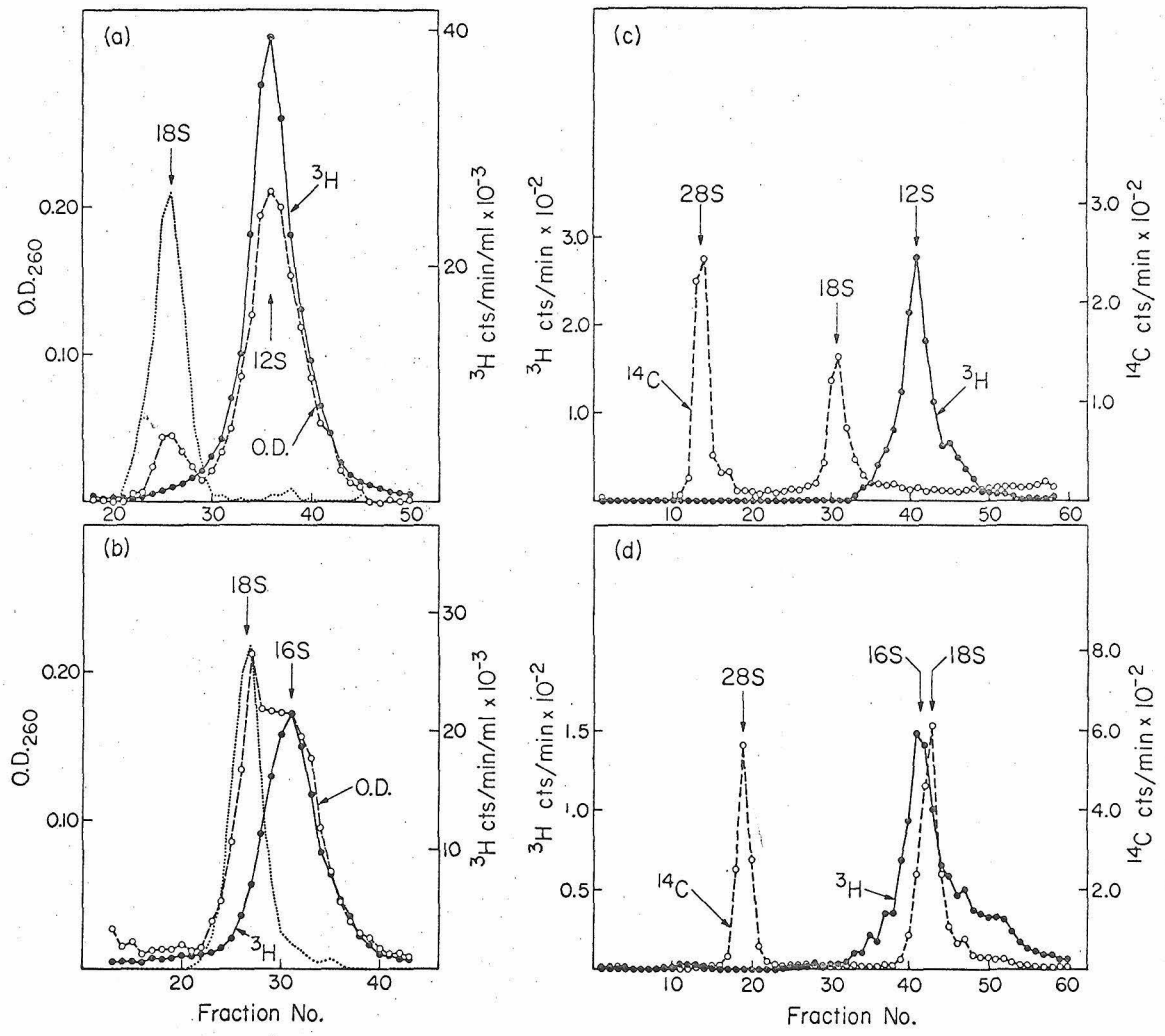
(a)
18S
^{3}H
12S
O.D.
O.D.$_{260}$
^{3}H cts/min/ml x 10^{-3}
(b)
18S
16S
O.D.
^{3}H
Fraction No.
(c)
28S
12S
18S
^{14}C
^{3}H
^{3}H cts/min x 10^{-2}
^{14}C cts/min x 10^{-2}
(d)
28S
16S
18S
^{14}C
^{3}H
Fraction No.

Figure 5

Fig. 5. Rerun in sucrose gradient and electrophoretic analysis of 16 S and 12 S RNA.

(a) and (b) The components corresponding to the portions of the pattern in Figure 4b indicated by arrows were collected by ethanol precipitation and centrifugation, and rerun, in the presence of ^{14}C-labeled 18 S RNA from isolated small subunits of free ribosomes (Attardi et al., 1969), through 15 to 30% sucrose gradients in dodecyl SO_4 buffer in the SW27 rotor (1.59 x 10.16 cm buckets) at 25,000 rev./min for 26 hr at 20°C. O---O, $O.D._{260}$; ⊙——⊙, ^{3}H cts/min.

(c) and (d) The fractions indicated by a double circle in (a) and (b) were analyzed by polyacrylamide gel electrophoresis (2.7% acrylamide gel) at 5 mA per 8 cm gel for 6 hr (16 S RNA) or for 4-1/2 hr (12 S RNA), in the presence of ^{14}C-labeled 28 S and 18 S RNA extracted from isolated subunits of free ribosomes. ⊙——⊙, ^{3}H cts/min; O---O, ^{14}C cts/min

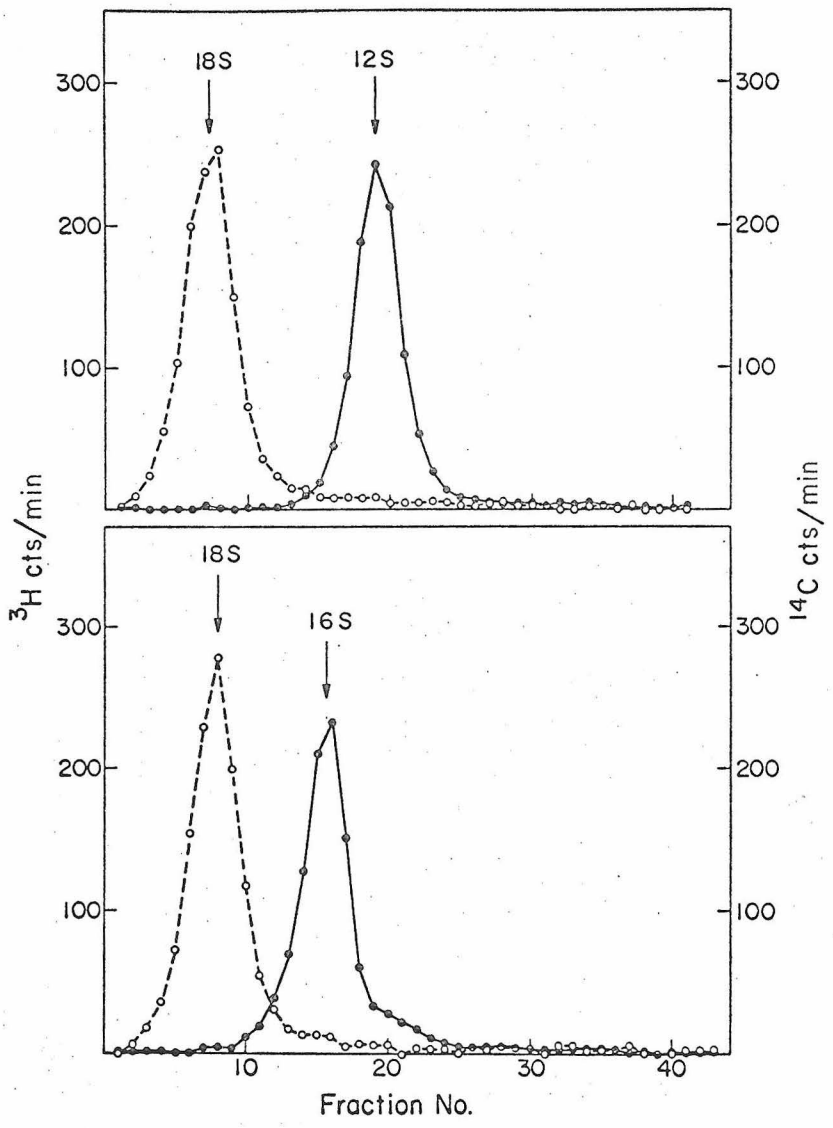
18S
12S
18S
16S
300
200
100
^{3}H cts/min
^{14}C cts/min
10
20
30
40
Fraction No.

Figure 6

Fig. 6. Sedimentation analysis of 16 S and 12 S RNA under denaturing conditions.

Purified RNA components were denatured with formaldehyde and run in a sucrose gradient in the presence of formaldehyde as specified in Materials and Methods (c). ^{14}C-labeled 18 S RNA from small subunits of free ribosomes was added as a marker.

0------0 ^{14}C cts. /min.

●——————● ^{3}H cts. /min.

can be estimated that the 16 S RNA would have a molecular weight about 1.7 times higher than that of the 12 S.

Rerun in separate sucrose gradients of the material sedimenting at 23 S and at 21 S in the pattern of 4 hr-labeled RNA showed, respectively, a clear 23 S $O.D._{260}$ peak with a small amount of non-resolved 21 S on the light side, and a fairly sharp 21 S $O.D._{260}$ peak with a shoulder on the light side corresponding to residual 18 S RNA (not shown). The sensitivity to drugs of the labeling of these components, in the experiment shown in Figure 4, could be better evaluated after rerunning them in sucrose gradient. Ethidium bromide reduced the labeling of the 23 S component by about 43% (as estimated in correspondence of the two peak fractions), whereas the labeling of the 21 S component appeared to be somewhat less affected (about 28%). The labeling of the 28 S RNA after a 4 hr pulse, as measured after a second sucrose gradient centrifugation, was inhibited by ethidium bromide by about 47%. In the presence of 0.04 µg/ml. actinomycin D, the labeling of the 23 S and 21 S RNA appeared to be incompletely suppressed (by about 70% in correspondence to the two peak fractions) as judged from the rerun patterns.

Table 2 shows the specific activities of various discrete RNA components from the EDTA-treated mitochondrial fraction of HeLa cells after different times of labeling with [5-^{3}H]-uridine or ^{32}P-orthophosphate. It appears that the 12 S and 4 S RNA become labeled much faster than the 28 S and 18 S RNA, with the specific activity of the 4 S RNA being significantly lower than that of the 12 S component. An analysis of the distribution

Table 2

Specific activities of discrete RNA components from the mitochondrial fraction and the free monomer-polysome fraction of HeLa cells after different labeling times

RNA component	Cts/min/O.D.$_{260}$ unit x 10^{-5}			
	45 min [5-^{3}H]-uridine	90 min [5-^{3}H]-uridine	240 min [5-^{3}H]-uridine	1320 min ^{32}P-ortho-phosphate
Mitochondrial Fraction				
12 S	0.73	2.52 1.99*	3.90 4.54*	2.36
4 S	0.51	1.68 1.35*	3.12 3.25*	2.14
18 S	0.12**	0.97**	1.95**	2.07
28 S	0.013**	0.63	1.64	2.09
Free monomer-polysome Fraction				
28 S				2.08

* culture treated with 0.04 µg/ml. actinomycin D

** culture treated with 1 µg/ml. ethidium bromide

Legend for Table 2

The data pertaining to discrete RNA components of the mitochondrial fraction were derived from the experiments illustrated in Figures 2, 3, 4 and 8 and from an experiment utilizing HeLa cells labeled for 45 min with [5-^{3}H]-uridine (under the same conditions described in Fig. 2b) in the presence of 1 µg/ml. ethidium bromide. The 28 S RNA from the free monomer-polysome fraction was isolated as described in Fig. 9b. In the 240 min and 1320 min labeling experiments, the specific activity was determined on the discrete components purified by a second cycle of sucrose gradient centrifugation.

of radioactivity among the alkaline digestion products of 4 S RNA labeled during 15 and 30 min [5-^{3}H]-uridine pulses showed that about 91% and, respectively, 85% of the label was associated with uridylic acid and therefore did not result from turnover of the -CCA end of tRNA components: the situation is thus analogous to that described for cytoplasmic 4 S RNA in HeLa cells and other cell types (Bernhardt & Darnell, 1969). After 22 hr exposure to ^{32}P-orthophosphate, in the presence of sufficient unlabeled phosphate to allow normal growth (Fig. 8), the specific activity of 4 S RNA has become substantially equal to that of the 18 and 28 S RNA of the mitochondrial fraction and of 28 S RNA of free polysomes, while the specific activity of 12 S RNA is still slightly higher (10 to 15%). The specific activity of the 16 S RNA component behaved similarly to that of 12 S RNA, although it was not analyzed in detail.

Figure 7 shows the kinetics of labeling of the 16 S, 12 S and ethidium bromide-sensitive 4 S components during 4 hr exposure of HeLa cells to [5-^{3}H]-uridine. The amount of radioactivity in the 16 S and 12 S RNA species was estimated from the control sedimentation patterns for labeling times up to 45 min and from the patterns pertaining to actinomycin D-treated cells for longer times. It appears that after a short initial lag the radioactivity incorporated into the three discrete components increases approximately linearly during the first 90 min, then at a slower rate up to 240 min (presumably due to exhaustion of the exogenous labeled precursor (see accompanying paper)). The labeling of the three discrete components proceeds in time in a

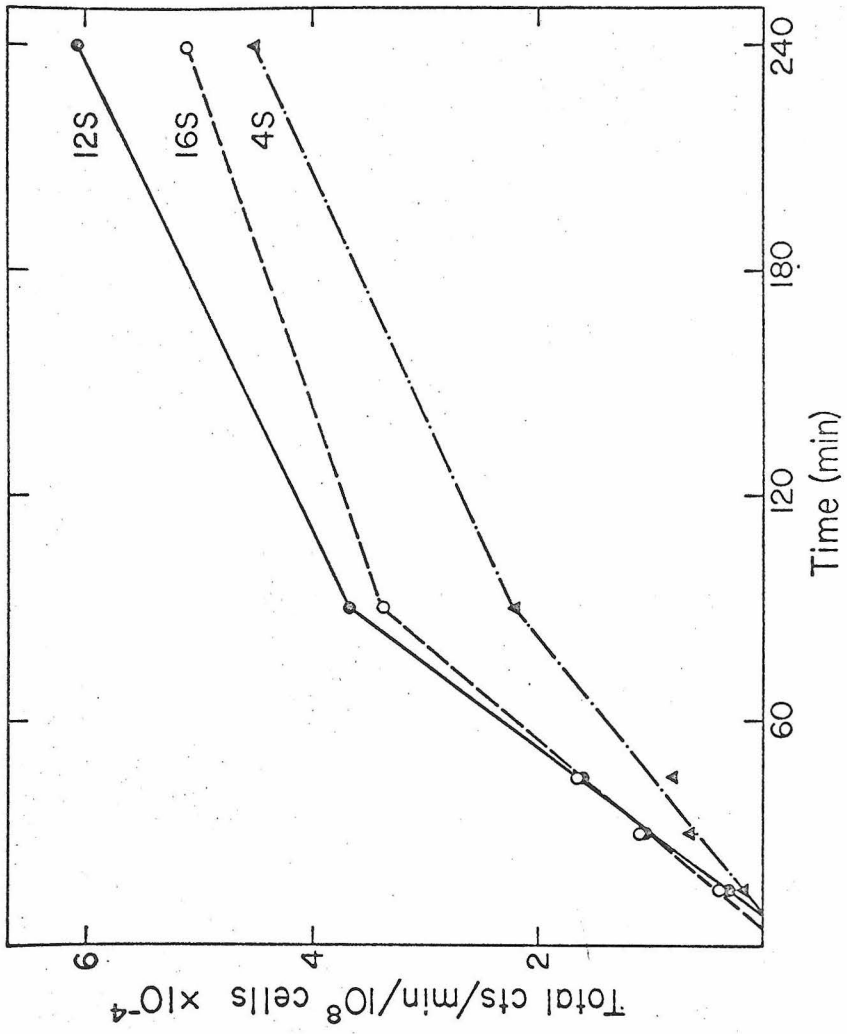
12S
16S
4S
Time (min)
60
120
180
240
2
4
6
Total cts/min/10^8 cells x10^-4

Figure 7

Fig. 7. Kinetics of labeling of 16 S, 12 S and ethidium bromide-sensitive 4 S RNA from the mitochondrial fraction of the HeLa cells exposed to [5-^{3}H]-uridine pulses of various duration.

The data are derived from the experiments shown in Figures 2 (a,b,d), 3b, 4b, and two other 4 hr pulse experiments. For the determination of the radioactivity associated with each discrete species a baseline was drawn from the 28 S region of the gradient to the furrow near the meniscus and the profiles of the 16 S and 12 S RNA reconstructed, as exemplified in Figure 3b; the validity of these determinations was verified in some experiments by rerun in sucrose gradient of the individual components. For 4 S RNA, only the fraction of radioactivity sensitive to ethidium bromide was plotted. The data are normalized for variation in the recovery of the mitochondrial fraction on the basis of the O.D.$_{260}$ associated with 4 S RNA.

●——●, 12 S RNA; O---O, 16 S RNA; ▲-·-▲, ethidium bromide sensitive 4 S RNA

proportional fashion (with the labeling of the 16 S RNA being almost equal to that of the 12 S RNA and the labeling of the ethidium bromide-sensitive 4 S component being 60 to 70% of the same) and extrapolates back to approximately the same initial time (5 to 8 min).

(c) Nucleotide composition of discrete RNA components from the mitochondrial fraction of HeLa cells

In order to determine the ^{32}P-nucleotide composition of the various discrete components detected in the sedimentation pattern of RNA from the mitochondrial fraction of HeLa cells, and also to establish whether and to what extent these components are methylated, exponentially growing cells were exposed for 22 hr to [^{3}H-methyl]-methionine and ^{32}P-orthophosphate in medium containing sufficient cold methionine and unlabeled phosphate to allow normal growth, in the absence (Fig. 8) or presence of 0.04 µg/ml. actinomycin D. To minimize the labeling of the purine rings arising from the contribution of the ^{3}H-methyl groups of methionine to the C1-carbon pool, adenosine and guanosine were added at a concentration of 10^{-4} M to the growth medium for 24 hr prior to exposure to the isotopes and during the labeling period. After 22 hr exposure of the cells to ^{32}P-orthophosphate, almost all the label in the sedimentation profile of RNA from the EDTA-treated mitochondrial fraction (Fig. 8) was found to be alkali-sensitive. The proportion of alkali-resistant ^{32}P (presumably in DNA) was about 3% in the 28 S region, 7% around 18 S, 6% in the 12 S region and less than 0.1% in the 4 S region. After 22 hr labeling with ^{32}P-orthophosphate in the presence of 0.04 µg/ml. actinomycin D, the 16 S, 12 S, and 4 S RNA were

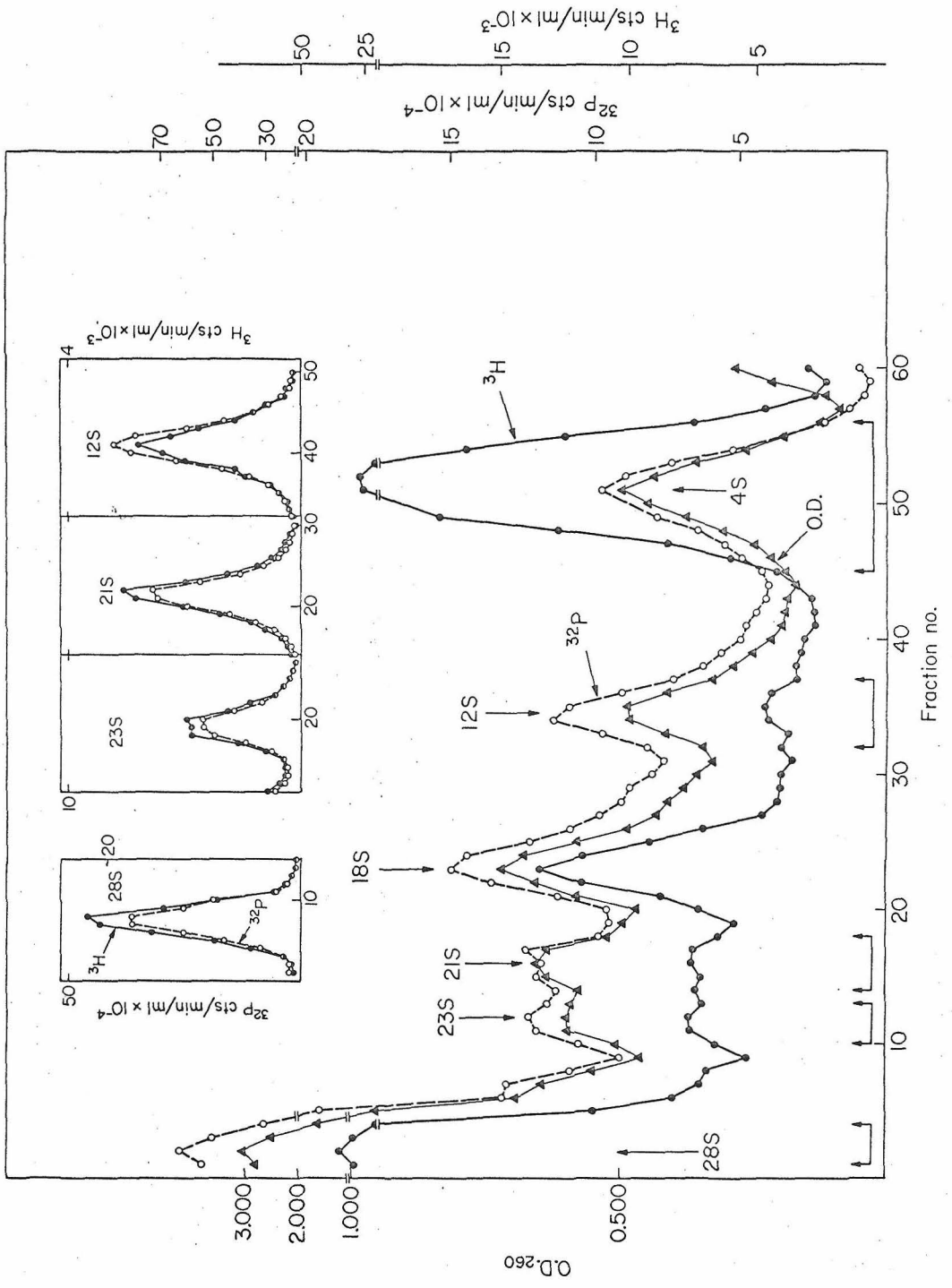
O.D.260
3.000
2.000
1.000
0.500
28S
18S
23S
21S
12S
4S
O.D.
^{32}P
^{3}H
Fraction no.
^{32}P cts/min/ml x 10^{-4}
^{3}H cts/min/ml x 10^{-3}
23S
21S
12S
28S
18S
^{32}P
^{3}H

Figure 8

Fig. 8. Sedimentation pattern of RNA components sedimenting slower than 28 S RNA from the mitochondrial fraction of HeLa cells labeled for 22 hr with [^{3}H-methyl]-methionine and ^{32}P-orthophosphate.

HeLa cells (which had been grown for 24 hr in the presence of 10^{-4} M adenosine and 10^{-4} M guanosine) were exposed for 22 hr, under the conditions specified in Materials and Methods (a), to [^{3}H-methyl]-methionine and ^{32}P-orthophosphate. The RNA was extracted from the EDTA-treated and isopycnically separated mitochondrial fraction, and run through a 15 to 30% (w/w) sucrose gradient in dodecyl SO_4 buffer in the SW27 Spinco rotor (1.59 x 10.16 cm buckets) at 25,000 rev./min for 25 hr at 20°C. The fractions corresponding to the 28 S, 23 S, 21 S, and 12 S components (indicated by arrows) were collected by ethanol precipitation and centrifugation and rerun in sucrose gradient under the same conditions specified above. (Centrifugation time was 23 hr.) (Inserts). All the ^{32}P data are corrected for decay to the same counting date. The spilling over of ^{32}P cts/min into the ^{3}H channel was 0.3-0.4%.

The components sedimenting in the 1 to 7 S region (indicated by arrows) were utilized for electrophoretic analysis (see Fig. 9a).

Δ——Δ, O.D.$_{260}$; O---O, ^{32}P cts/min; ●——●, ^{3}H cts/min

the main radioactive components in the sedimentation pattern of RNA from the crude mitochondrial fraction. The amount of label associated with 16 S and 12 S components, as determined after rerunning them in sucrose gradient, was approximately equivalent.

Table 3 shows the ^{32}P-nucleotide composition of the discrete RNA components purified by two consecutive cycles of sucrose gradient centrifugation. For comparison, the previously determined ^{32}P-nucleotide composition of pulse-labeled mitochondria-associated heterogeneous RNA is also given in Table 3. It appears that the 16 S and 12 S components have a base composition which is similar to that of the heterogeneous RNA. The 21 S and 23 S RNA, on the other hand, have a base composition of the high GC type, fairly similar to that of 28 S RNA present in the mitochondrial fraction. Notice that the major nucleotide composition of the latter RNA species is indistinguishable from that of the 28 S RNA from the free monomer-polysome fraction and that previously reported for total HeLa cell 28 S RNA (Amaldi & Attardi, 1969). Also shown in Table 3 is the base composition of the 4 S RNA purified by polyacrylamide gel electrophoresis of the low molecular weight components of RNA from the crude mitochondrial fraction of long-term ^{32}P-labeled cells and, for comparison, the base composition of the 4 S RNA purified from cytoplasmic ribosomes (Fig. 9). Mitochondria-associated 4 S RNA has a nucleotide composition characterized by relatively high A and U content, which resembles that of the 16 S, 12 S, and ^{32}P-pulse-labeled heterogeneous RNA (though differing from it significantly for the high U content). Clearly distinct from the base composition of

Table 3

Nucleotide composition of RNA components from the mitochondrial fraction and from the free monomer-polysome fraction of HeLa cells

RNA component	Moles per cent A	C	U+ψ	G	%GC	A/G	$^{3}H/^{32}P$*	Estimated number of methyl groups per 100 nucleotides
Mitochondrial Fraction								
30 min ^{32}P-pulse labeled heterogeneous RNA 9-25 S	33.9	24.5	22.6	18.9	43.4	1.79		
30 min ^{32}P-pulse labeled heterogeneous RNA 26-48 S	31.4	23.9	25.3	19.4	43.3	1.62		
16 S (act.)	32.0	25.7	23.4	18.9	44.6	1.69	0.053	0.9
12 S (act.)	32.7	26.3	22.6	18.3	44.6	1.79	0.060	1.0
12 S	30.5	26.6	22.1	20.8	47.4	1.47	0.047	0.8
5 S							n.d.	
4 S	27.5	22.2	28.9	21.4	43.6	1.29	0.276	4.6
28 S	16.5	31.5	16.3	35.7	67.2	0.46	0.082	1.4
23 S	18.9	31.1	18.1	31.9	63.0	0.59	0.066	1.1
21 S	18.5	30.6	17.9	32.9	63.5	0.56	0.069	1.1
Free monomer-polysome Fraction								
28 S	16.4	31.3	17.1	35.2	66.5	0.47	0.084	1.4**
5 S							n.d.	
4 S	17.6	26.9	21.6	33.9	60.8	0.52	0.607	10.1

Legend for Table 3

The fractions corresponding to the 28 S (1-4), 23 S (10-13), 21 S (14-18), and 12 S components (32-37) in the pattern of Figure 8, and the fractions corresponding to the 16 S and 12 S components in the sedimentation pattern of RNA from the mitochondrial fraction of HeLa cells labeled for 22 hr with [^{3}H-methyl]-methionine and ^{32}P-orthophosphate (see Materials and Methods (a)) in the presence of 0.04 µg/ml. actinomycin D were collected by ethanol precipitation and centrifugation, and rerun through 15 to 30% sucrose gradients in dodecyl SO_4 buffer in the SW27 rotor (1.59 x 10.16 cm buckets) at 25,000 rev./min for 23 hr at 20°C. After determination of the ^{3}H and ^{32}P acid insoluble radioactivity profiles (Fig. 8, inserts), portions of the peak tubes were used for alkali digestion and nucleotide composition analysis, as previously described (Attardi et al., 1966). The 4 S and 5 S from the mitochondrial fraction and from free ribosomes utilized for nucleotide composition analysis were isolated by polyacrylamide gel electrophoresis of the low molecular weight components (up to about 7 S) of the sedimentation patterns (Fig. 9a and 9b). The 28 S RNA from the free monomer-polysome fraction was isolated as described in Figure 9b.

The per cent of methylated nucleotides was calculated from the ^{3}H to ^{32}P ratios and from the known content of methyl groups of cytoplasmic 28 S RNA (Brown & Attardi, 1965) (see text). No correction was made for the small proportion of alkali-resistant ^{32}P cts/min.

Legend for Table 3 (cont.)

*Corrected for decay of ^{32}P and for differences in ^{3}H counting efficiency between samples acid-precipitated, collected on Millipore membranes, and counted in toluene-POP-POPOP and gel electrophoresis samples counted directly in Bray's solution.

**Brown & Attardi (1965).

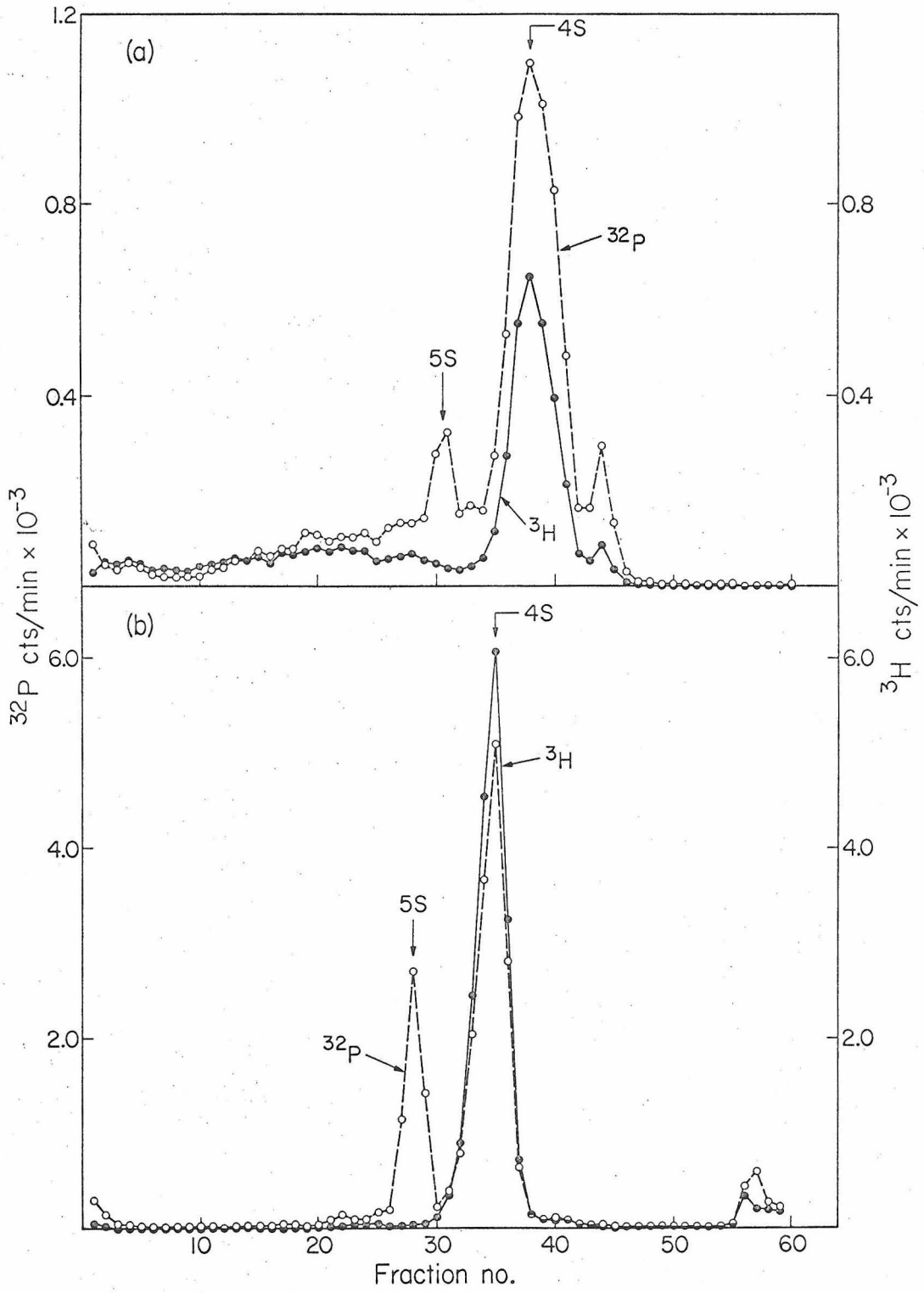
(a)
(b)
4S
5S
32P
3H
1.2
0.8
0.4
6.0
4.0
2.0
10
20
30
40
50
60
Fraction no.
32P cts/min × 10-3
3H cts/min × 10-3

Figure 9

Fig. 9. Electrophoretic analysis of low molecular weight RNA components from the mitochondrial fraction and from the free ribosome-polysome fraction of HeLa cells labeled for 22 hr with [^{3}H-methyl]-methionine and ^{32}P-orthophosphate.

(a) The components sedimenting in the 1 to 7 S region of the gradient in the pattern shown in Figure 8 were collected by ethanol precipitation and centrifugation and analyzed by polyacrylamide gel electrophoresis (10% acrylamide gel at 5 mA) for 3 hr.

(b) RNA was extracted from the free monomer-polysome fraction from the same experiment and analyzed in sucrose gradient as in Figure 8. The components sedimenting in the 1 to 7 S region of the gradient were collected by ethanol precipitation and centrigugation and analyzed by polyacrylamide gel electrophoresis as in (a).

●——●, ^{3}H cts. /min. O---O, ^{32}P cts. /min.

mitochondria-associated 4 S RNA is the base composition, of high GC type, of the 4 S RNA associated with cytoplasmic ribosomes, which is very close to that previously reported for 4 S RNA from the soluble fraction of HeLa cells (Hatlen, Amaldi & Attardi, 1969).

As appears in Figure 8, the sedimentation profile of ^{3}H-labeled RNA shows, besides three major peaks pertaining to 28 S, 18 S and 4 S RNA, minor peaks in correspondence with the 23 S, 21 S, and 12 S RNA. In the RNA from actinomycin D-treated cells, the 16 S, 12 S, and 4 S species are clearly labeled with ^{3}H. Table 3 shows the ^{3}H to ^{32}P ratio found in various discrete RNA components, separated by sedimentation velocity in sucrose gradient (Fig. 8) or by polyacrylamide gel electrophoresis (4 S and 5 S, Fig. 9), from the mitochondrial fraction and from the free ribosome-polysome fraction. No detectable ^{3}H-radioactivity was found to be associated with 5 S RNA from either free ribosomes or the mitochondrial fraction (Fig. 9): this result is in agreement with the absence of methylation in this RNA species (Hatlen, Amaldi & Attardi, 1969), and, furthermore, indicates that the labeling of the purine rings has been completely prevented in the present experiments by the addition of adenosine and guanosine to the medium. From the reported methyl content of total 28 S RNA from HeLa cells, which is mainly derived from the free monomer-polysome fraction (1.4 per one hundred nucleotides (Brown & Attardi, 1965)), the methyl content of the different discrete components could be estimated. The 28 S RNA from the mitochondrial fraction is methylated to the same extent as the 28 S RNA from free ribosomes, while the 23 S and 21 S components

appear to be somewhat less methylated. The level of methylation of the 12 S RNA and 16 S RNA was found to be about two-thirds of that of 28 S RNA from the mitochondrial fraction; the level of methylation of the 12 S RNA from the actinomycin-treated cells appeared to be significantly higher than that from the control cells. The estimated average methyl group content of 4 S RNA from the free ribosome-polysome fraction is 10.1 per one hundred nucleotides, whereas the mitochondria-associated 4 S appears to have less than one-half as many methyl groups. The distribution of methyl groups among the four major nucleotides separated by Dowex 1-X8 chromatography was found to be substantially different in cytoplasmic and mitochondria-associated 4 S RNA.

(d) Electrophoretic mobilities of discrete RNA components from the mitochondrial fraction

In order to clarify the relationship between the discrete RNA species detected in the present work by sedimentation analysis and those previously described in the electrophoretic pattern of mitochondria-associated RNA from HeLa cells (Vesco & Penman, 1969a) and Xenopus cells (Dawid, 1969), individual components isolated by two cycles of sucrose gradient centrifugation were analyzed by polyacrylamide gel electrophoresis. As shown in Figure 5c, the RNA species sedimenting at 12 S moved through the gel at the same rate as the species which had been previously designated as "12 S" on the basis of its electrophoretic mobility relative to that of the 18 S and 28 S rRNA markers (Vesco & Penman, 1969a). On the contrary, the 16 S RNA component of the sedimentation pattern appeared to move through

the polyacrylamide gel slightly behind the 18 S RNA marker, that is, slower than expected from its sedimentation behavior, although not so slow as the "21 S" electrophoretic component previously described (Fig. 5d). An inverse relationship between logarithm of molecular weight and electrophoretic mobility has been shown to hold for various RNA species (Bishop, Claybrook & Spiegelman, 1967). Assuming molecular weights of 1.65 and 0.65 x 10^6, respectively, for the 28 S and 18 S RNA from HeLa cells (Petermann & Pavlovec, 1966), and excluding conformational influences, the molecular weights of the 16 S and 12 S species were estimated to be 0.7 x 10^6 and 0.4 x 10^6, respectively.

The 23 S RNA detected in the sedimentation pattern of RNA from the mitochondrial fraction (Fig. 4a) and purified by a second cycle of sucrose gradient centrifugation was found to move in gel electrophoresis like a fairly homogeneous component with a molecular weight of about 1.3 x 10^6. The 21 S RNA of the sedimentation analysis (Fig. 4a), equally rerun in sucrose gradient, moved in the gel as a broad band partially resolved into two components present in approximately equal amounts and corresponding to molecular weights of about 1.0 x 10^6 and 0.9 x 10^6. Analysis by gel electrophoresis of the 21 S RNA from cells labeled with ^{32}P-orthophosphate and [^{3}H-methyl]-methionine (Fig. 8) showed that these two components of different electrophoretic mobility have a different degree of methylation, with the faster moving component containing about twice as many methyl groups per unit length as the slower moving one.

Analysis by polyacrylamide gel electrophoresis of the low molecular weight RNA components from the mitochondrial fraction of cells labeled with ^{32}P-orthophosphate and [^{3}H-methyl]-methionine (Fig. 8) showed a prominent methylated peak and a small slower moving non-methylated component (Fig. 9a); some heterogeneous methylated components moving more slowly than the main peak and spread up to the origin could also be seen. The electrophoretic pattern obtained with low molecular weight components from free ribosomes (Fig. 9a) showed also a major methylated component corresponding to tRNA and a minor non-methylated component corresponding to 5 S RNA, in agreement with previously reported findings (Knight & Darnell, 1967; Weinberg & Penman, 1968; Hatlen et al., 1969). The absence in this pattern of the 28 S-associated small molecular weight RNA (Pêne, Knight & Darnell, 1968) is presumably due to the use in the present work of cold phenol extraction, i.e. conditions of extraction under which this RNA species, which is normally hydrogen bonded to 28 S RNA, remains associated with it. On the basis of their electrophoretic mobilities, as compared to those of the cytoplasmic components, the small non-methylated component in the mitochondrial fraction appears to be 5 S RNA, while the major methylated peak represents a class of molecules of average size corresponding to 4 S RNA. Notice the considerably lower degree of methylation of this RNA component as compared to cytoplasmic tRNA (see Table 3).

(e) Influence of the conditions of preparation of the mitochondrial fraction on the characteristics of the extracted RNA

The similarity in kinetics of labeling, response to drugs, and nucleotide composition between the 23 S and 21 S components and the 28 S RNA suggested the possibility that these minor components resulted from degradation of 28 S RNA occurring during the long manipulations required for the isolation of the mitochondrial fraction. Likewise, the apparent lack of equimolarity in amounts and rates of synthesis of 16 S and 12 S RNA could conceivably result from degradation processes. Also the discrepancy in electrophoretic mobility between the 16 S RNA described in the present work and the previously described "21 S" electrophoretic component could possibly be ascribed to the same cause. Therefore, the effect on the characteristics of the extracted RNA of changing the procedure of isolation and treatment of the mitochondrial fraction was investigated. Shortening drastically the isolation procedure by using a centrifugation of much shorter duration (90 min at 81,300 x g instead of 17 to 19 hr at 52,000 x g) did not change appreciably the types or the relative proportions of the discrete components revealed in the sedimentation analysis (Fig. 10a). Likewise, elimination of the EDTA treatment of the mitochondrial fraction had no appreciable effect on the sedimentation pattern of RNA, apart from the presence of larger amounts of 28 S, 18 S and 4 S RNA, the excess of 18 S RNA resulting in a partial masking of the 21 S component (Fig. 10b). Omission of the isopycnic centrifugation step (Fig. 10c) did, on the contrary, result in an apparent decrease in the 21 S component, which was also, in some

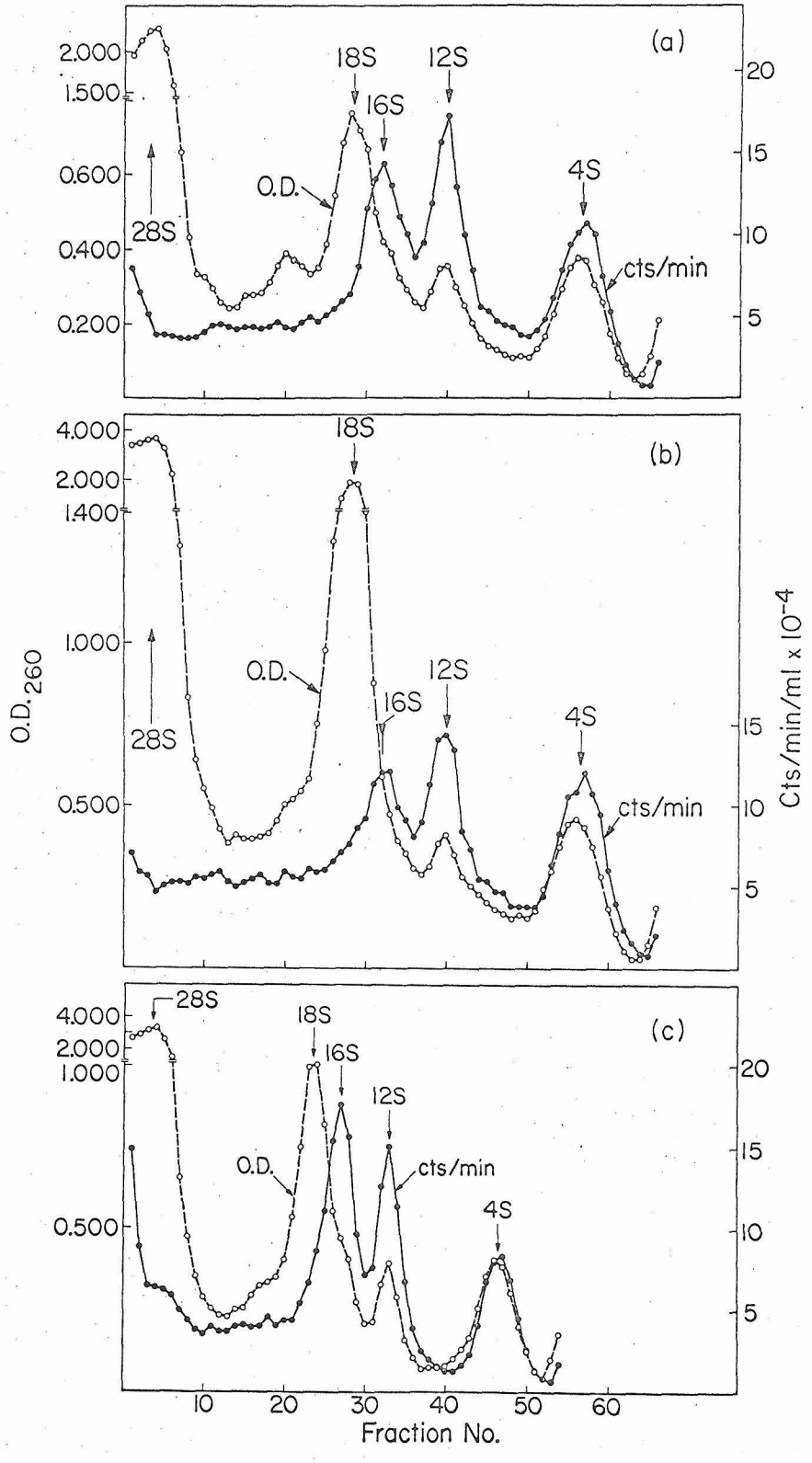
(a)
2.000
1.500
0.600
0.400
0.200
18S
16S
12S
4S
28S
O.D.
cts/min
20
15
10
5
(b)
4.000
2.000
1.400
1.000
0.500
18S
16S
12S
4S
28S
O.D.
cts/min
O.D.260
Cts/min/ml x 10-4
(c)
4.000
2.000
1.000
0.500
28S
18S
16S
12S
4S
O.D.
cts/min
10
20
30
40
50
60
Fraction No.

Figure 10

Fig. 10. Sedimentation patterns of RNA components sedimenting slower than 28 S RNA from the mitochondrial fraction isolated under different conditions from HeLa cells exposed to [5-^{3}H]-uridine for 4 hr in the presence of 0.04 µg/ml. actinomycin D.

In (a) and (b) the mitochondrial fraction was separated by differential centrifugation, and then subjected to a short (90 min) isopycnic centrifugation in sucrose gradient with (a) or without (b) pretreatment with EDTA; in (c) the mitochondrial fraction isolated by differential centrifugation was treated with 0.03 M EDTA and, after pelleting, utilized immediately for RNA extraction. RNA was extracted from the three fractions and analyzed in sucrose gradient as in Figure 8.

O---O, O.D.$_{260}$; ●——●, cts/min

experiments, less well resolved. Furthermore, the ratio of radioactivity in the 16 S to that in the 12 S component was higher under these conditions, varying between 1.2 and 1.4 in different experiments. However, the sedimentation properties of these components was not in the least affected. Likewise, the electrophoretic mobility of the major component (16 S) relative to the 18 S RNA marker remained absolutely unchanged.

4. Discussion

The results previously reported from this laboratory (Attardi & Attardi, 1967, 1968, 1969a,b; Attardi et al., 1970) have clearly indicated the occurrence in the sedimentation pattern of RNA from partially purified mitochondria of HeLa cells of rapidly labeled heterogeneous RNA with sedimentation constants up to 50 S or more, and of discrete RNA components. Evidence from RNA-DNA hybridization experiments has pointed to mit-DNA as the template of the rapidly labeled heterogeneous RNA and of some of the discrete components (Attardi & Attardi, 1969a,b; Attardi et al., 1970). The purpose of this work has been to obtain further evidence concerning the origin of the RNA components present in the mitochondrial fraction from HeLa cells, and to try to clarify the relationship between the discrete RNA components recognizable by sedimentation analysis and the electrophoretic RNA components described in other laboratories (Vesco & Penman, 1969a,b; Dawid, 1969).

(a) Origin of mitochondria-associated rapidly labeled heterogeneous RNA

The almost complete sensitivity to ethidium bromide, in short-term experiments, of the labeling of mitochondria-associated heterogeneous

RNA may reflect a direct effect of this drug on the transcription of a closed circular DNA template: in fact, this intercalating dye is known to distort the structure of supercoiled circular DNA (Radloff, Bauer & Vinograd, 1967; Crawford & Waring, 1967; Bauer & Vinograd, 1968). If this interpretation is correct, the selective inhibition by ethidium bromide of the labeling of the mitochondria-associated heterogeneous RNA provides additional support for the view that mit-DNA is the template involved: this view had been previously strongly suggested by the linear kinetics of labeling of this RNA fraction, its association with structures having the same sedimentation velocity and density distribution in sucrose gradients as mitochondria, its base composition complementarity to the transcriptionally active heavy mit-DNA strand, and its capacity to hybridize with mit-DNA (Attardi & Attardi, 1967, 1968, 1969a; Attardi et al., 1970; see also following paper). Also consistent with this conclusion is the lack of appreciable effects on the pulse labeling of this RNA of concentrations of actinomycin D which inhibit completely the synthesis of rRNA and to a great extent the arrival in the cytoplasm of mRNA of free polysomes (Attardi & Ojala, in preparation). The almost complete sensitivity to ethidium bromide of the pulse-labeled RNA from the mitochondrial fraction observed in the present work suggests that this fraction, as prepared here, is substantially free from contamination by RNA leaked from the nuclei or by nuclear fragments. Also after 2 hr exposure to [5-^{3}H]-uridine in the presence of 0.04 µg/ml. actinomycin D, the labeling of the RNA of the mitochondrial fraction was found to be inhibited by ethidium bromide to the extent of about 90%.

A considerably lower sensitivity to this drug had been previously reported for the labeling, during a 2 hr exposure to [^{3}H]-uridine, of heterogeneous RNA components present in the mitochondrial fraction from HeLa cells (Zylber et al., 1969): this is possibly due to the contamination of this fraction by nuclear material.

(b) Discrete RNA components present in the mitochondrial fraction

A considerable resolution of the discrete RNA species present in the RNA from the mitochondrial fraction of HeLa cells was achieved in the present work by sedimentation analysis in the Spinco SW25.3 or SW27 rotor with long buckets.

(i) 16 S, 12 S, and 4 S RNA

The 16 S and 12 S RNA detected in the sedimentation pattern of mitochondria-associated RNA from HeLa cells, on the basis of their size, nucleotide composition, kinetics of labeling and response of their synthesis to drugs, appear to be distinctive components of the mitochondrial fraction. Their major nucleotide composition is clearly different from that of rRNA and, on the contrary, closely related to that of the ^{32}P-pulse-labeled heterogeneous mitochondria-associated RNA: as is discussed in the accompanying paper, this base composition is complementary, as concerns the A and U content, to that of the heavy strand of mit-DNA.

The 12 S and 16 S RNA detected in the present work by sedimentation analysis appear to correspond to the "12 S" and "21 S" RNA previously described in the electrophoretic patterns of RNA from crude mitochondrial

fractions of HeLa cells (Vesco & Penman, 1969a) and Xenopus cells (Dawid, 1969). The kinetics of labeling of the 12 S and 16 S species, the actinomycin resistance and ethidium bromide sensitivity of their synthesis, and their base composition strongly support this identification. However, the large RNA species (16 S) has been shown in the present experiments to possess a somewhat greater electrophoretic mobility than that observed for the "21 S" RNA, moving just behind the 18 S RNA marker. It can be reasonably excluded that degradation phenomena during extraction are responsible for this difference, since this was observed even after immediate extraction of RNA from the mitochondrial fraction separated by differential centrifugation (Results, e). It should be mentioned that in cultured Golden and Chinese hamster cells the corresponding RNA component has been found to move in gel electrophoresis very close to the 18 S RNA marker (Vesco & Penman, 1969b; Dubin & Montenecourt, 1970). Since it seems unlikely that RNA species presumably homologous should have an equal size in HeLa cells and Xenopus cells, as judged from the previously reported electrophoretic mobilities (Vesco & Penman, 1969a; Dawid, 1969), and a smaller size in hamster cells, it is possible that the discrepancy is due to some unrecognized factors which influence the electrophoretic mobility of this RNA component in polyacrylamide gels. It should be mentioned that in a more recent work (Zylber et al., 1969), the "21 S" electrophoretic component of HeLa cells was found to move much closer to 18 S RNA than originally reported. The cited authors also found that the "21 S" component sediments in sucrose gradients slower than predicted by electrophoretic analysis (in the 18 S region).

A second discrepancy is that, in contrast to what had been reported for the "12 S" and "21 S" electrophoretic components, the 12 S and 16 S RNA were found in the present work to be methylated, though to a lesser extent than the 28 S RNA. The absence of 5 S RNA labeling by [^{3}H-methyl]-methionine indicates that no detectable labeling of the purine rings occurred under our experimental conditions. Furthermore, the observation that the 16 S and 12 S RNA, synthesized in the presence of a concentration of actinomycin D which blocks completely rRNA synthesis, are methylated excludes that the apparent methylation of these species results from breakdown products of 28 S or 18 S RNA cosedimenting with non-methylated components. Also in the non-drug-treated cells, the base composition and methylation level of 12 S RNA and the uniform ^{3}H to ^{32}P ratio over the whole 12 S peak rerun in sucrose gradient (Fig. 8) argue against the presence of a methylated contaminant of rRNA origin. The difference in the apparent methylation level of 12 S from actinomycin-treated cells as compared to the 12 S from control cells may be due to a different specific activity of the nucleoside triphosphate precursors under the non-growing conditions induced by the drug treatment. The reason for the discrepancy between our results and those of Vesco & Penman is not known, although the quite different conditions of labeling used by the cited authors (higher cell concentration and very high level of ^{32}P-orthophosphate in the medium, which would not be expected to allow normal growth) may be responsible for it. In the present experiments, the control cells exposed to ^{32}P-orthophosphate and [^{3}H-methyl]-methionine duplicated normally during the 22 hr labeling period.

The 12 S and 16 S species detected in the present work appear to correspond to the unresolved "17 S" RNA found by Dubin (1967) in the mitochondrial fraction from cultured hamster cells treated for a long time with a low dose (0.1 µg/ml.) of actinomycin D, and which has been recently shown to consist of two components having sedimentation and electrophoretic properties similar to those reported here (Dubin & Montenecourt, 1970). It should be mentioned that this "17 S" was found to be labeled with [^{14}C-methyl]-methionine (in the presence of 10^{-4} M adenosine and 10^{-4} M guanosine), although to a lesser degree than the 16 S and 12 S RNA were labeled with [^{3}H-methyl]-methionine in the present work, suggesting a low level of methylation.

A discrepancy between sedimentation and electrophoretic properties similar to that observed here for the 16 S RNA has also been reported by others for mitochondrial rRNA species of lower eukaryotic cells (Halvorson, Morimoto, Scragg & Nikhorocheff, 1970; Edelman, Verma & Littauer, 1970), and is presumably due to the less compact conformation of these species as compared to the rRNA markers. It is likely that the molecular weight determined on the basis of electrophoretic mobility reflects more closely the actual size of these RNA species than the sedimentation rate, although a slight tendency to overestimate the size by gel electrophoresis has been observed for RNA species with a low GC content (Loening, 1969). On the basis of their electrophoretic mobilities, the molecular weights of the 16 S and 12 S species were estimated to be 0.7×10^6 and 0.4×10^6, respectively. Analysis of the sedimentation behavior of these species under denaturing conditions

indicated that they are represented by continuous polynucleotide chains. From their relative sedimentation velocity after formaldehyde treatment a ratio of molecular weights of about 1.7 was calculated for the two species, in agreement with the ratio of sizes estimated on the basis of electrophoretic mobilities.

Under the ordinary conditions used in this work for the preparation of the mitochondrial fraction, involving isopycnic centrifugation in sucrose gradient, the 16 S and 12 S RNA were recovered in approximately equal amounts, as judged from the $O.D._{260}$ pertaining to the two components run twice in sucrose gradient, or from the radioactivity associated with them after long-term labeling in the presence of low doses of actinomycin D; also their rates of synthesis were apparently about equal, as estimated from their labeling during [5-^{3}H]-uridine pulses up to 4 hr. Similar results had been previously reported by Vesco & Penman (1969a) for the "21 S" and "12 S" electrophoretic components. That, however, these results may be due to a partial degradation or loss of the 16 S component is suggested by the observation that direct extraction of RNA from the crude mitochondrial fraction isolated by differential centrifugation consistently yielded 16 S RNA in appreciably higher yield relative to the 12 S RNA than observed under the ordinary preparative conditions. The ratio of label in the two species after a 4 hr pulse was, in different experiments, 1.2 to 1.4: on the basis of the molecular weight estimates derived from the electrophoretic mobilities of the two RNA components (0.7×10^6 and 0.4×10^6), this would correspond to a molar ratio of 0.70 to 0.80.

These observations suggest, therefore, that the two RNA species are synthesized in the cell in equimolar amounts.

We have confirmed the observation by Zylber et al. (1969) that the 12 S RNA and probably also the 16 S RNA have a fairly short half-life in the mitochondrial fraction in the presence of ethidium bromide. However, that the decay of these species under such conditions is, in part, a consequence of the abnormality induced by the drug treatment is suggested, in the first place, by the lack of any obvious decrease in the amount of 12 S and 16 S after 105 or 135 min treatment with ethidium bromide, as opposed to their complete, or almost so, disappearance after 255 min exposure to the drug; in the second place, by the observation that the specific activity of the 12 S RNA after 22 hr labeling with ^{32}P-orthophosphate was only slightly (10 to 15%) higher than that of 28 S RNA and 18 S RNA. Considering that a species with a physiological turnover as fast as that indicated by its complete, or nearly complete, disappearance after 4 hr ethidium bromide treatment would have after one cell duplication almost twice as high specific activity as the stable rRNA species, our results suggest that the 12 S RNA and, presumably, also the 16 S RNA have a fairly long lifetime in the mitochondrial fraction. The kinetics of labeling of the 16 S and 12 S RNA species observed in the present work and that of "21 S" and "12 S" electrophoretic components observed by Vesco and Penman (1969) also indicate a relative stability of these species. That, however, the 12 S and 16 S RNA species do have a certain turnover is suggested by the comparison of their labeling with that of the mitochondria-associated 4 S RNA. The latter has been previously reported to be

metabolically stable (Zylber & Penman, 1969; Knight, 1969); in agreement with this finding, the specific activity of this component after 22 hr labeling with ^{32}P-orthophosphate was found to be substantially equal to that of the stable rRNA species. In the present work, the specific activity of the mitochondria-associated 4 S RNA, both after relatively short pulses and after 22 hr labeling, was found to be significantly lower than that of the 12 S component.

The initial faster labeling of the 16 S, 12 S, and 4 S RNA as compared to the rRNA components of the crude mitochondrial fraction reflects their synthesis *in situ* as contrasted with the arrival from the nucleus of the 28 S and 18 S RNA (see below). The observation that the labeling of 16 S, 12 S, and the ethidium bromide-sensitive 4 S RNA (see below) proceeds in time in a proportional fashion and extrapolates back to the same initial time (5 to 8 min) is in keeping with the idea of a coordinated synthesis and accumulation of these components in the mitochondrial fraction and, furthermore, argues against any precursor to product relationship. The short initial lag is presumably due to the time required for intramitochondrial precursor pool equilibration with exogenous [5-^{3}H]-uridine (Attardi & Attardi, 1968) and for the possible processing of the discrete species from larger precursors. It is interesting to note that 7 min is the earliest time after exposure to [5-^{3}H]-uridine that the 16 S and 12 S components are clearly recognizable (Attardi *et al.*, 1970).

The kinetics of labeling, the sensitivity to ethidium bromide and the resistance to low doses of actinomycin D of the synthesis of the 16 S and 12 S components of the EDTA-treated crude mitochondrial

fraction strongly support the idea that these species are specified by mit-DNA. This conclusion is in agreement with the previously reported results of RNA-DNA hybridization experiments utilizing purified closed circular mit-DNA (Attardi & Attardi, 1969b). In those experiments, the sedimentation profile of RNA components homologous to mit-DNA had, in fact, revealed a broad band between 9 and 15 to 16 S with a peak at 12 S, and a prominent 4 S peak: the shoulder of RNA homologous to mit-DNA at 15 to 16 S presumably corresponds to the 16 S ethidium bromide-sensitive species which was not resolved in that analysis.

One can at present only speculate concerning the nature of the 16 S and 12 S components. The possibility that they represent mini-rRNA components, i.e. equivalent to the specific rRNA species present in the mitochondria of Neurospora and yeast (see for review Attardi & Attardi, 1969a), is being investigated. This possibility is made more plausible by the evidence presented here indicating that these species are methylated, that their half-life is considerably longer than previously surmised on the basis of their behavior in the presence of ethidium bromide, and finally that they are probably synthesized in equimolar amounts.

The 4 S RNA present in the EDTA-treated mitochondrial fraction has a base composition markedly distinct from that of cytoplasmic 4 S and resembling that of the 16 S and 12 S RNA and of the fast labeled heterogenous RNA. Its level of methylation is somewhat less than one-half of that of cytoplasmic 4 S RNA, and the pattern of methylated nucleotides in the two RNA populations is clearly different. In view of the evidence suggesting that about 20% of the mitochondria-associated

4 S RNA is cytoplasmic tRNA (see below), the actual methylation level of the mitochondria-specific 4 S RNA is probably lower than 4.6 nucleotides per 100 nucleotides. We have not confirmed the complex electrophoretic pattern with several distinct components described by Knight (1969) for the mitochondria-associated 4 S RNA in HeLa cells. Differences in base composition and methylation level between mitochondria-associated 4 S RNA and cytoplasmic tRNA similar to those observed in the present work have been reported recently in hamster cells (Dubin & Montenecourt, 1970). The 4 S RNA present in the mitochondrial fraction presumably contains at least some of the mitochondria-specific tRNA species which have been described in animal cells, including HeLa cells (Buck & Nass, 1968, 1969; Galper & Darnell, 1969; Smith & Marcker, 1969).

The labeling of mitochondria-associated 4 S RNA during [5-^{3}H]-uridine pulses up to 4 hr was found in the present work to be to a great extent (about 80%) sensitive to ethidium bromide. A sensitivity to this drug of the synthesis of HeLa mitochondria-associated 4 S RNA, as opposed to the resistance of the synthesis of cytoplasmic 4 S RNA, has been recently observed by others (Knight, 1969; Zylber & Penman, 1969). Furthermore, as mentioned above, mitochondria-associated 4 S RNA hybridizes with great efficiency with purified closed circular mit-DNA (Attardi & Attardi, 1969b). These observations strongly support the idea that the distinctive mitochondria-associated 4 S RNA is specified, as the 16 S and 12 S RNA components, by mit-DNA. Sequence homology of mitochondrial leucyl-tRNA from rat liver to

mit-DNA has been recently reported (Nass & Buck, 1969). The relatively small fraction of 4 S RNA labeled during [5-^{3}H]-uridine pulses up to 4 hr which is resistant to ethidium bromide (about 20%) presumably represents cytoplasmic tRNA (Zylber & Penman, 1969), possibly bound to EDTA-resistant membrane-stuck 50 S subunits.

(ii) 28 S, 18 S and 5 S RNA

Previous work from this laboratory (Attardi et al., 1969) has shown that a mitochondrial fraction from HeLa cells, partially purified by isopycnic centrifugation in sucrose gradient, is contaminated by elements of rough endoplasmic reticulum, which carry the great majority of the ribosomes present in this fraction. Treatment with a high concentration of EDTA removes about 70% of the large subunits of these ribosomes and 85 to 90% of the small subunits. The EDTA-resistant 28 S RNA pertains in its great majority, if not exclusively, to membrane-stuck 50 S subunits, which are presumably those which carry the more complete polypeptide chains (Attardi et al., 1969). Whether any of the EDTA-resistant 28 S and 18 S RNA pertains to intramitochondrial ribosomes is not known and is at present being investigated.

The EDTA-resistant 28 S and 18 S RNA present in the mitochondrial fraction and the 28 S and 18 S RNA of free ribosomes have been found to have identical sedimentation properties and a similar kinetics of labeling (this work and unpublished observations); furthermore, the 28 S RNA from the two cytoplasmic locations appears to have the same major nucleotide composition and level of methylation; finally, the 28 S and 18 S RNA from the mitochondrial fraction have been shown to

possess no base sequence homology to mit-DNA (Attardi & Attardi, 1969b). These observations point to a nuclear origin for these RNA species. Therefore, if any fraction of these EDTA-resistant rRNA species pertains to intramitochondrial ribosomes, these must be imported into the mitochondria. The significance of the partial inhibition by ethidium bromide of the labeling of membrane-associated 28 S RNA (about 40% after 90 min labeling, and about 47% after 4 hr labeling) is at present being investigated. A similar inhibition (about 20% for a concentration of the drug of 0.2 µg/ml.), in the absence of any effect on rRNA of free ribosomes, has been previously reported (Knight, 1969).

The small amount of 5 S RNA present in the EDTA-treated mitochondrial fraction presumably pertains in its great majority, if not exclusively, to residual membrane-stuck 50 S subunits of endoplasmic reticulum-bound ribosomes. That the 5 S RNA in the crude mitochondrial fraction is of nuclear origin is strongly suggested by the lack of effect of ethidium bromide (0.2 µg/ml.) on its labeling (Knight, 1969).

(iii) 21 S and 23 S RNA components

The 21 S and, less clearly, the 23 S RNA components have been reproducibly observed in the course of this work in the sedimentation pattern of RNA extracted from a partially purified mitochondrial fraction of HeLa cells. Their kinetics of labeling, their considerable sensitivity to actinomycin D and resistance to ethidium bromide and their lack of homology to mit-DNA (Attardi & Attardi, 1969b) have clearly indicated a non-mitochondrial site of synthesis for these components. Considerable attention was given to the possibility that

these components derive from degradation of 28 S RNA. RNA components with sedimentation constants of 24 S and 21 S have in fact been described as intermediary breakdown products of 28 S RNA from Ehrlich ascites tumor cells resulting from the activity of traces of RNase (Huppert & Pelmont, 1962). In the present work, the base composition of 21 S and 23 S components was found to be of the high GC type, fairly similar to that of 28 S RNA. Likewise, their degree of methylation was rather close to that of the major rRNA species. These observations would be in agreement with the idea of these components being degradation products of 28 S RNA. Also consistent with this possibility is the observation that the bulk of these components is recovered with contaminating elements of rough endoplasmic reticulum in experiments of subfractionation of the mitochondrial fraction (Storrie & Attardi, in preparation). The amount of the 21 S component did not increase in an obvious way relative to that of 28 S RNA with the interval of time passed between cell homogenization and RNA extraction (compare, for example, Figures 2 and 4 and 10a). This suggests that, if the 21 S component derives from degradation of endoplasmic reticulum-bound 28 S RNA, this degradation may occur very rapidly and concern only a small fraction of the 28 S, and that it may even occur <u>in vivo</u>. In disagreement with the idea of the 23 S and 21 S components being related to 28 S RNA is the observation that their labeling was somewhat less affected by ethidium bromide than the labeling of 28 S RNA and, in contrast to this, was not completely suppressed by actinomycin D: it is possible, however, that this behavior is due to overlapping of other RNA (heterogeneous) of similar

sedimentation rate. Electrophoretic analysis of the 21 S component has indeed provided evidence for its heterogeneity.

(c) Relationship between rapidly labeled heterogeneous RNA and mit-DNA coded discrete RNA species

Work carried out in this laboratory has shown that mitochondrial RNA is synthesized in HeLa cells, as in rat liver (Borst & Aaij, 1969), exclusively on the heavy strand of mit-DNA, and, moreover, that the whole or almost whole length of this strand (corresponding to about 5×10^6 daltons) is transcribed (see following paper). It is possible that this transcription takes place in the form of a continuous RNA chain: this would be in agreement with the occurrence, in the sedimentation profile of pulse-labeled mitochondrial RNA, of heavy molecules ethidium bromide-sensitive and homologous to mit-DNA. What proportion of the sequences transcribed from mit-DNA is represented in the discrete RNA species described above is not known. The sum of the molecular weights of 16 S and 12 S RNA (as estimated from their electrophoretic mobilities) is equivalent to a stretch of about 1.1×10^6 daltons. Furthermore, from the relative rates of labeling of the 12 S and ethidium bromide-sensitive 4 S species (under the reasonable assumptions that these rates reflect fairly closely the actual rates of synthesis of these components, and that all ethidium bromide-sensitive 4 S RNA is mit-DNA coded), one can estimate roughly that about 10 molecules of 4 S RNA (corresponding to a total molecular weight of about 0.25×10^6) are synthesized on a mit-DNA template per each 12 S molecule: if mit-DNA is transcribed as a continuous chain this would imply the existence of

about 10 cistrons for 4 S RNA per each 12 S cistron. Assuming 1 cistron for each 16 S and 12 S RNA and 10 cistrons for 4 S RNA, these discrete RNA species would account for about 25% of the total informational content of human mit-DNA.

One can ask what is the relationship between these discrete species and the mit-DNA coded heterogeneous RNA identified earlier in this laboratory. If the hypothesis of the continuous transcription of the mit-DNA heavy strand will prove to be correct, one would have to assume a precursor to product relationship between the rapidly labeled fast sedimenting mitochondrial RNA components and the discrete RNA species. The early kinetics of labeling of heterogeneous mitochondrial RNA of different sedimentation constant is indeed consistent with this precursor to product relationship (Attardi & Attardi, 1969a; Attardi et al., 1970). The heterogeneous components spread in the region 4 to 50 S could represent intermediates or waste products in the processing of the large precursor and/or incomplete nascent mitochondrial RNA chains. The heterogeneous RNA might also include mit-DNA coded mRNA species destined to be utilized inside the mitochondria or exported (Attardi et al., 1970). However, the understanding of the significance of the heterogeneous fast labeled RNA in relation to the discrete mit-DNA coded RNA components will have to wait for the elucidation of the mode of transcription of mit-DNA and of the processing of mitochondrial RNA.

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†Abbreviations used: mit-DNA, mitochondrial DNA; rRNA, ribosomal RNA; mRNA, messenger RNA; tRNA, transfer RNA; dodecyl SO_4, sodium dodecyl sulfate.

‡In the present work, the expression "mitochondria-associated" is used to indicate the presence of a certain component in the mitochondrial fraction isolated by differential centrifugation and buoyant density fractionation in sucrose gradient and containing the bulk of the cell mitochondria.

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ADDITIONAL RESULTS

Sedimentation analysis of [^{3}H]-thymidine labeled DNA from the mitochondrial fraction of HeLa cells - It was of interest to see the profile of DNA coextracted with RNA from the mitochondrial fraction prepared under the usual conditions of cell fractionation (see Materials and Methods, this chapter). For this purpose, the EDTA-treated mitochondrial pellet was isolated from HeLa cells labeled for 23 hr. with [^{3}H]-thymidine and subjected to buoyant density centrifugation in sucrose gradient. Nucleic acids were extracted with phenol-SDS from the mitochondrial band and centrifuged on a 15-30% sucrose gradient in SDS buffer (Figure 1). The O. D. $_{260}$ profile shows the bulk RNA components of the mitochondrial fraction: 28 S and 18 S ribosomal RNA, a small peak at 21 S with a shoulder at 23 S, and prominent peaks of RNA sedimenting at 12 S and 4 S. The ^{3}H-thymidine radioactivity shows a broad band of DNA centered around 19-20 S. This DNA presumably represents linear molecules, largely derived from nuclear contamination ; breakage of HeLa cells by homogenization in hypotonic buffer has been shown to result in contamination of the mitochondrial fraction by DNA of nuclear origin (1). The standard sedimentation coefficients (for NaDNA at infinite dilution) for the closed circular and open circular mit-DNA duplexes in HeLa cells are 37 S and 26 S, respectively (2). Under the ionic conditions used in the present sedimentation analysis, the closed circular mit-DNA has been reported to cosediment with 28 S RNA (1).

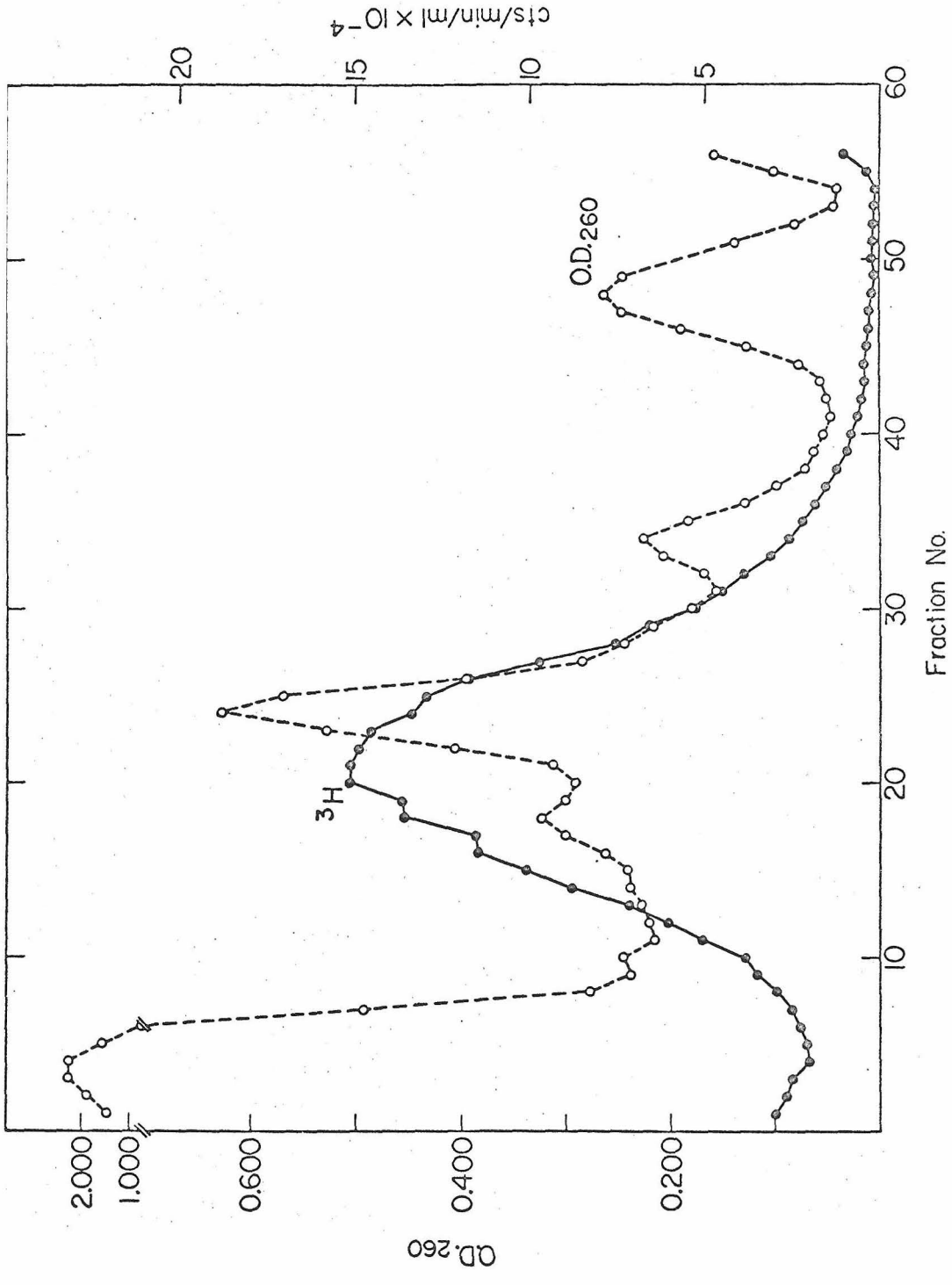
cts/min/ml x 10⁻⁴
20
15
10
5
O.D.260
3H
2.000
1.000
0.600
0.400
0.200
O.D.260
10
20
30
40
50
60
Fraction No.

Figure 1

FIGURE 1. Sedimentation profile of nucleic acids extracted from the mitochondrial fraction of HeLa cells labeled with[^{3}H]-thymidine. 1.5 x 10^8 HeLa cells were labeled for 23 hr. with[^{3}H-methyl]-thymidine (0.36 μC/ml.; 23 C/ mM). The mitochondrial fraction was prepared by differential centrifugation, treated with EDTA, and centrifuged on a 30 to 48 % sucrose gradient in 0.01 M tris buffer, pH 7.1, in the Spinco SW 25.1 rotor at 25,000 rev. / min. for 9.5 hr. at 3°C. Nucleic acids were extracted from the mitochondrial band by SDS-phenol and centrifuged on a 15 to 30% sucrose gradient in SDS buffer (0.01 M tris buffer, pH 7.0, 0.1 M NaCl, 0.001 M EDTA, 0.5% SDS) at 25,000 rev. /min. for 27 hr. at 20°C in the Spinco SW 27 rotor.

0------0 O. D. $_{260}$

●———● ^{3}H cts. /min.

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PART II

PROPERTIES OF MEMBRANE-BOUND RIBOSOMES IN HELA CELLS

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INTRODUCTION

The crude mitochondrial fraction from HeLa cells, conventionally prepared by differential centrifugation, is contaminated by a substantial amount of structures containing ribosomal RNA, presumably elements of the rough endoplasmic reticulum, which have been recognized by electron microscopy of animal cells growing in vitro (1, 2, 3). The latter can at best be partially separated from mitochondria by buoyant density centrifugation or sedimentation velocity in sucrose gradients due to the extensive overlapping in density and sedimentation properties of the two types of organelles. Partly because of the inescapable presence of these membrane-associated ribosomes in the mitochondrial fraction and partly because of the suggestion that a portion of the RNA of mitochondrial origin is contained in polysomal structures (see Part I, Chapter 2), an investigation was initiated on the properties of the membrane-associated ribosomes, in particular, with regard to their proportion to total cell ribosomes, their response to EDTA and ribonuclease treatment, and their association with mRNA. The results of these biochemical investigations, as well as of an electron microscopic study of the membrane-associated ribosomes in intact HeLa cells and in the mitochondrial fraction, performed by Barbara Cravioto, are discussed in the following publication.

It has been found that from 10 to 15% of the ribosomes in HeLa cells are bound to elements of the rough E. R.; the majority of these membrane-attached ribosomes can be recovered as polysomes (65 to 70%) after membrane lysis. Both electron microscopic and biochemi-

cal observations lead to the conclusion that few, if any, ribosomes are located in mitochondria in these cells.

In order to obtain some evidence as to the origin of the ribosomal RNA components of the membrane-associated ribosomes, preliminary experiments concerning the kinetics of labeling and the sedimentation properties of the rRNA from the membrane-associated ribosomes as compared to those of the rRNA of free polysomes were performed and are presented as additional results preceding a general discussion.

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Membrane-bound Ribosomes in HeLa Cells

I. Their Proportion to Total Cell Ribosomes and their Association with Messenger RNA

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I. Their Proportion to Total Cell Ribosomes and their Association with Messenger RNA

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The ribosomes associated with the endoplasmic reticulum in HeLa cells have been the object of an electron microscopic and biochemical investigation.

An appreciable amount of elements of rough endoplasmic reticulum, in the form of tubules, vesicles, and short cisternae scattered throughout the cytoplasm, has been observed by electron microscopic examination of thin sections of these cells. From the distribution of rRNA among the subcellular fractions, it has been estimated that from 10 to 15% (and possibly as many as 20%) of the total ribosomes in HeLa cells are associated with elements of rough endoplasmic reticulum. 65 to 70% of these ribosomes can be recovered as polysomes after sodium deoxycholate treatment and are presumably engaged in protein synthesis *in vivo*. Treatment with EDTA releases essentially all the small subunits and about 70% of the larger subunits of the endoplasmic reticulum-bound ribosomes: these respond, therefore, to EDTA treatment similarly to ribosomes of the rough endoplasmic reticulum from rat liver.

1. Introduction

In all animal cells ribosomes occur in two different topographical situations, namely, either attached to membranes of the endoplasmic reticulum or free in the cytoplasmic matrix. The proportion of bound and free ribosomes varies in different types of cells: in cells which are specialized for the synthesis of protein destined to be exported, like liver or pancreas, the major part of ribosomes are associated with the endoplasmic reticulum (Palade, 1956,1958); on the contrary, in rapidly multiplying cells, such as those in embryonic tissues or those growing *in vitro*, the great majority of ribosomes are free (see review by Porter, 1961). In addition to these two groups, the existence of a minor group of ribosomes in mitochondria has been postulated in animal, as in other eukaryotic cells, on the basis of direct electron microscopic and biochemical observations (Rendi & Warner, 1960; Truman, 1963; Watson & Aldridge, 1964; Swift, 1965; André & Marinozzi, 1965; Elaev, 1966; O'Brien & Kalf, 1967*a*,*b*; Dubin & Brown, 1967) and of indirect evidence bearing on the protein synthesizing capacity of these organelles (see, among others, Roodyn, Reis & Work, 1961; Roodyn, Suttie & Work, 1962; Truman & Korner, 1962; Kroon, 1963*a*,*b*,*c*; Wheeldon & Lehninger, 1966); the occurrence of intramitochondrial ribosomes with distinctive rRNA components has been reported in *Neurospora* (Küntzel & Noll, 1967; Rifkin, Wood &

Luck, 1967; Duro, Epler & Barnett, 1967) and yeast (Rogers, Preston, Titchener & Linnane, 1967; Wintersberger, 1967).

The ribosomes bound to endoplasmic reticulum in secretory cells have been the object of numerous investigations concerning their involvement in the synthesis and transport of protein (Siekevitz & Palade, 1960; Redman, Siekevitz & Palade, 1966; Henshaw, Bojarski & Hiatt, 1963; Howell, Loeb & Tomkins, 1964), their mode of attachment to the membranes (Sabatini, Tashiro & Palade, 1966; Blobel & Potter, 1967*b*), and their relationship with free ribosomes (Moulé, Rouiller & Chauveau, 1960; Webb, Blobel & Potter, 1964; Loeb, Howell & Tomkins, 1965,1967; Cammarano, Giudice & Lukes, 1965; Manganiello & Phillips, 1965). The membrane-bound ribosomes of animal cells growing *in vitro*, though recognized by electron microscopists (Epstein, 1961; Journey & Goldstein, 1961; Fuse, Price & Carpenter, 1963), have, on the contrary, been disregarded in most biochemical investigations. The occurrence of bound ribosomes in these cells, which lack, in general, an obvious secretory activity, suggests that the attachment of ribosomes to membranes is not exclusively related to the synthesis of proteins to be exported. The possibility that membrane-bound polysomes may be involved in the synthesis of membrane proteins is suggested by observations made in differentiating hepatocytes of newborn rats (Dallner, Siekevitz & Palade, 1966). Animal cells growing *in vitro* are favorable material for the study of the functional role not immediately related to secretion of the attachment of ribosomes to membranes. The association with membranes of an appreciable fraction of ribosomes and mRNA in these rapidly multiplying cells has also relevance for the study of the mRNA metabolism and of the dynamics of polysome assembly and function. Evidence suggesting that polysomes of the rough endoplasmic reticulum in HeLa cells contain mRNA of mitochondrial origin has been recently reported (Attardi & Attardi, 1968). As a preliminary to the study of the physiological significance of membrane-bound ribosomes in HeLa cells, in particular, of their possible involvement in membrane protein synthesis, an electron microscopic and biochemical investigation has been carried out on these ribosomes, with special regard to their proportion to total cell ribosomes, their attachment to the membranes and their association with mRNA. It has been found that, in these cells, from 10 to 15% (and possibly as many as 20%) of the ribosomes are attached to elements of the endoplasmic reticulum. The majority of these membrane-bound ribosomes (65 to 70%) are recovered as polysomes after sodium deoxycholate treatment.

2. Materials and Methods

(a) *Cells and method of growth*

The method of growth of HeLa cells has been previously described (Amaldi & Attardi, 1968). The cultures used here were free of any detectable contamination by pleuropneumonia-like organisms (Mycoplasma).

(b) *Buffers*

The buffer designations are: (1) T: 0·01 M-Tris buffer (pH 7·1); (2) TM: 0·01 M-Tris buffer (pH 7·1), 0·00015 M-$MgCl_2$; (3) TKM: 0·01 M-Tris buffer (pH 7·1), 0·01 M-KCl, 0·00015 M-$MgCl_2$; (4) SMET (Parsons, Williams & Chance, 1966): 0·07 M-sucrose, 0·21 M-D-mannitol, 0·001 M-Tris buffer (pH 7·1), 0·0001 M-EDTA; (5) TKV: 0·05 M-Tris buffer (pH 7·1), 0·025 M-KCl, 0·001 M-EDTA; (6) low ionic strength TKV: 0·01 M-Tris buffer (pH 7·1), 0·01 M-KCl, 0·001 M-EDTA; (7) acetate–NaCl buffer: 0·01 M-acetate buffer (pH 5·0), 0·1 M-NaCl; (8) sodium dodecyl sulfate buffer (Gilbert, 1963): 0·01 M-Tris buffer (pH 7·0), 0·1 M-NaCl, 0·001 M-EDTA, 0·5% sodium dodecyl sulfate.

(c) *Labeling conditions*

Pulse labeling of RNA was carried out by exposing exponentially growing HeLa cells (2 to 3 $\times$ 10^5 cells/ml.) for various times to [5-^{3}H]uridine (17·3 to 28·8 mc/μmole, 0·3 to 10·0 μc/ml.) or [2-^{14}C]uridine (30 to 52 μc/μmole, 0·025 to 0·07 μc/ml.) in modified Eagle's medium with 5% dialyzed calf serum. Long-term labeling of RNA was carried out by growing cells for 24 to 26 hr in the presence of [5-^{3}H]uridine (0·3 to 1·25 μc/ml.), unless otherwise specified. DNA was labeled by growing cells for 24 hr in the presence of [^{3}H-*methyl*]thymidine (22·6 mc/μmole, 0·25 μc/ml.).

(d) *Preparation and analysis of subcellular fractions*

All operations described below were carried out at 2 to 3°C. The labeled cells were washed three times with 0·13 M-NaCl, 0·005 M-KCl, 0·001 M-$MgCl_2$ and then resuspended in 6 vol. TKM. After 2 min, the suspension was homogenized with an A. H. Thomas homogenizer (motor-driven pestle, ~1600 rev./min, 8 to 10 strokes). Under these conditions of homogenization, at most 60 to 70% of the cells were broken: these relatively mild conditions of cell breakage were chosen so as to minimize the rupture of nuclei and the subsequent release of labeled nuclear RNA components. (Less than 1% of the total cell [^{3}H]thymidine-labeled DNA was found in the cytoplasmic fraction under these conditions.) After addition of sucrose to 0·25 M, the homogenate was centrifuged at 1160 g_{av} for 3 min to sediment nuclei, unbroken cells and large cytoplasmic debris. The supernatant (*total cytoplasmic fraction*) was spun at 8100 g_{av} for 10 min; the pellet thus obtained (including any loose fluffy layer) was resuspended in 0·25 M-sucrose in TM (one-half of the volume of the homogenate) and, after a spin at 1100 g_{av} for 2 min to sediment any residual nuclei, recentrifuged at 8100 g_{av} for 10 min. The pellet (and any fluffy layer) was resuspended in 0·25 M-sucrose in T buffer (1·0 or 2·0 ml., for material deriving from 1·0 to 2·5 $\times$ 10^8 cells) (Mg ions were omitted at this stage and in the following steps aimed at fractionation of the intact membrane components, in order to reduce the possibility of aggregation): this represented the 8100 *g membrane fraction*, which contained the bulk of mitochondria and of elements of rough endoplasmic reticulum, in addition to smooth membrane components. The first 8100 *g* supernatant was centrifuged at 15,800 g_{av} for 20 min to separate a small amount of slower sedimenting mitochondria and other membrane elements from the supernatant fraction containing the great majority of *free polysomes* and all free monomers and "native" ribosomal subunits.

Buoyant density fractionation of the 8100 *g* membrane components was carried out by centrifugation through a 30 to 48% (w/w) sucrose gradient in T buffer in the Spinco SW 25·1 rotor for 18 to 20 hr at 25,000 rev./min. For the analysis of the membrane-associated polysomes, the 8100 *g* membrane fraction was treated with 1% NaDOC† and centrifuged through a 15 to 30% (w/w) sucrose gradient in TKM (25 ml., prepared above 3 ml. of 64% (w/w) sucrose in TKM) in the SW25·1 rotor for 90 to 110 min at 24,000 rev./min; in some experiments the NaDOC-lysed membrane fraction was treated with EDTA (10^{-3} to 10^{-2} M), and centrifuged through a 15 to 30% sucrose gradient (over 3 ml. of 64% sucrose) in low ionic strength TKV at the speed and for the time indicated above. For the study of the effect of EDTA on the untreated 8100 *g* membrane fraction, a suspension of this in 0·25 M-sucrose in T buffer (1·0 to 2·5 ml. for material deriving from about 1·3 $\times$ 10^9 cells) was brought to 3×10^{-2} M-EDTA, kept in the cold for 10 min, and then centrifuged at 11,000 g_{av} for 10 min. The supernatant fraction was carefully sucked up; the pellet was rinsed with 0·5 ml. of 0·25 M-sucrose in T buffer containing $1{\cdot}5 \times 10^{-2}$ M-EDTA (which was pooled with the supernatant fraction) and resuspended in 4·0 ml. of the same medium: the suspension was immediately recentrifuged at 11,000 g_{av} for 10 min. The final pellet was resuspended in 1·0 or 2·0 ml. of 0·25 M-sucrose in T buffer, and either run on a 30 to 48% sucrose gradient in the same buffer (see above), or treated with 1% NaDOC and run on a 15 to 30% sucrose gradient (over 3 ml. of 64% sucrose) in TKV for 8 hr at 25,000 rev./min. Isolation of the ribosomal subunits released from the membrane fraction by EDTA treatment was carried out by centrifuging the EDTA supernatant fraction through a 15 to 30% sucrose gradient in TKV in the SW25·2 rotor for 17 hr at 25,000 rev./min.

† Abbreviations used: NaDOC, sodium deoxycholate; rRNA, ribosomal RNA; mRNA, messenger RNA; tRNA, transfer RNA.

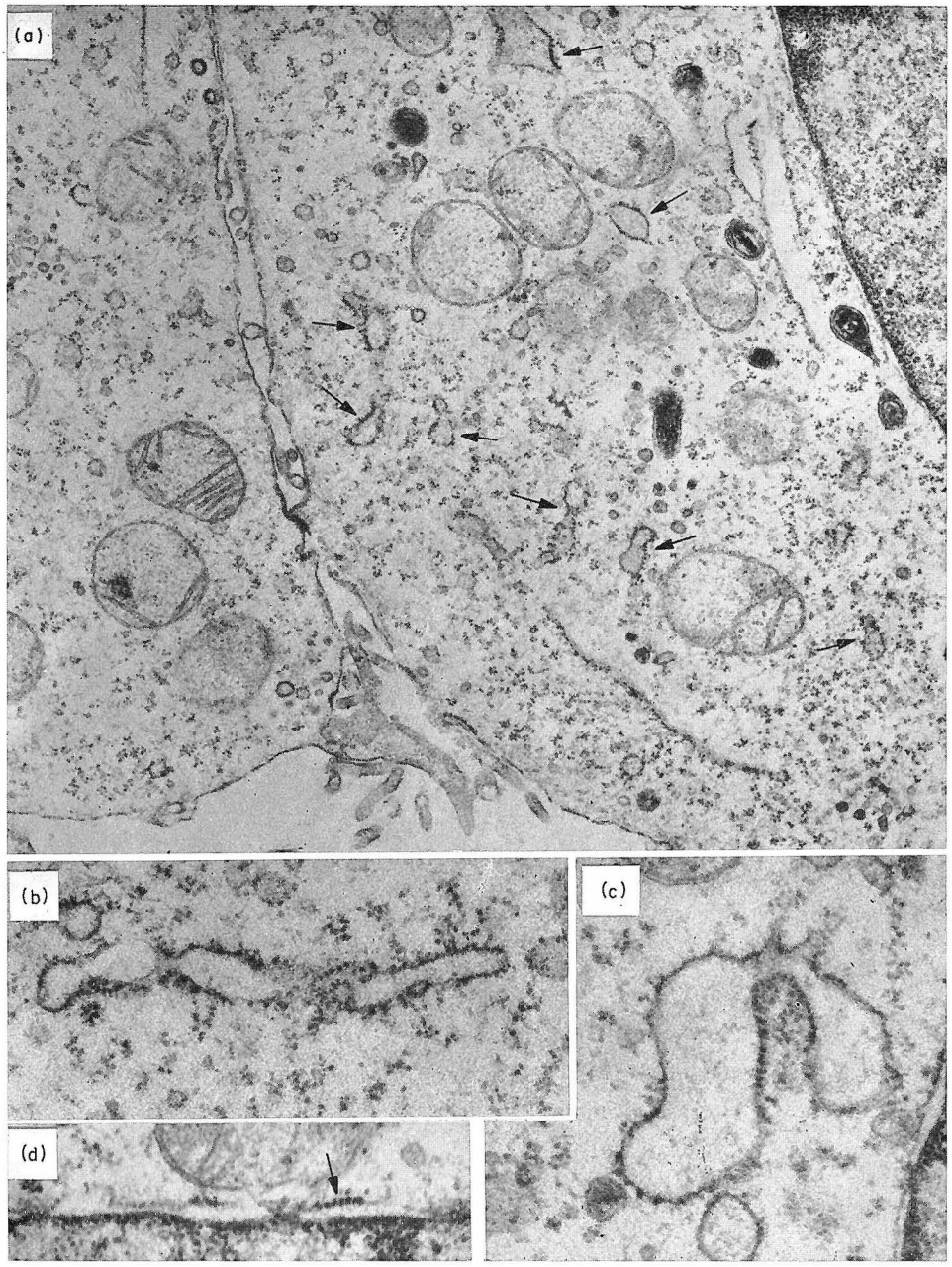

PLATE I. (a) Portion of the cytoplasm of two adjacent HeLa cells. Arrows point to elements of rough endoplasmic reticulum. Note the abundance of free polysomes in the cytoplasmic matrix. × 16,250.

(b) Cisterna of rough endoplasmic reticulum. Note rows of ribosomes along the edges and a rosette in the right half, at a point where the limiting membrane has been cut tangentially. × 35,000.

(c) Branched cisterna of rough endoplasmic reticulum. × 35,000.

(d) A row of ribosomes attached to the outer nuclear membrane. × 35,000.

[*facing p. 50*

Separation of free polysomes from monomers and native ribosomal subunits and from soluble components was carried out by centrifuging 3 to 5 ml. of the 15,800 *g* supernatant of the total cytoplasmic fraction through a sucrose gradient consisting, from the meniscus to the bottom, of 6 ml. 23% (w/w) sucrose, 12 ml. 23 to 55% sucrose gradient, and 6 ml. 55% sucrose, all in TKM (SW25·1 rotor, 25,000 rev./min, 5 hr). For better resolution of free monomers and native ribosomal subunits, the 15,800 *g* supernatant fraction was centrifuged through a 15 to 30% sucrose gradient in TKM for 8 hr at 25,000 rev./min. "Derived" ribosomal subunits were obtained from the free polysome–monomer fraction (pelleted by centrifuging the 15,800 *g* supernatant fraction at 105,000 g_{av} for 90 min) by treatment for 10 min with 10^{-2} M-EDTA, and separated on a 15 to 30% sucrose gradient in TKV as described above for the subunits from the 8100 *g* membrane fraction.

(e) *Extraction and analysis of RNA*

RNA was generally released from the membrane components and their NaDOC lysis products by treatment with 1% sodium dodecyl sulfate and from free polysomes with 0·5% sodium dodecyl sulfate, precipitated with 2 vol. of ethanol in the presence of 0·1 M-NaCl, dissolved in sodium dodecyl sulfate buffer, and run through a 15 to 30% (w/w) sucrose gradient (over 3 ml. of 64% sucrose) in sodium dodecyl sulfate buffer in the SW 25·1 rotor for 14 hr at 20,000 rev./min, 20°C.

For the analysis of radioactivity, a portion of each fraction was precipitated in the cold with 15% trichloroacetic acid by using 200 μg bovine serum albumin as a carrier, and the precipitate collected on a Millipore membrane. The isotope-counting procedures have been described elsewhere (Attardi, Parnas, Hwang & Attardi, 1966).

(f) *Cytochrome oxidase assay*

Cytochrome oxidase assay was carried out by a modification of the procedure of Smith (1954). 0·1-ml. portions of the sucrose gradient fractions were mixed in a cuvette with 2·9 ml. 18 μM-solution of reduced cytochrome *c* in 0·04 M-PO_4 buffer, pH 6·2. The decrease of O.D. at 550 mμ at 25°C was measured at 10-sec intervals for 4 to 6 min.

(g) *Electron microscopy*

HeLa cells were fixed, either in suspension or as a pellet, in sodium acetate–barbital buffer (pH 7·4–1% OsO_4 (Palade, 1952) for 1·5 hr at 2 to 3°C. For the preparation and fractionation of membrane components to be utilized for electron microscopy, the procedure described in Materials and Methods (d) was used, with the difference that the buoyant-density centrifugation was carried out in 30 to 48% sucrose gradient in 0·01 M-phosphate buffer, pH 7·0, to avoid interference in fixation by Tris buffer (Parsons *et al.*, 1966): the components corresponding to different portions of the buoyant-density pattern were diluted 4 times with 0·01 M-phosphate buffer, pH 7·0, and pelleted by centrifugation in the Spinco 40 rotor for 60 min at 20,000 rev./min; the pellets were then fixed with sodium acetate–barbital buffer–1% OsO_4 for 1 hr at 2 to 3°C. In all cases, after dehydration with a graded series of ethyl alcohols, the embedding was made in Araldite 502 (CIBA Company, Inc., Kimberton, Pa.). Sections 600 to 900 Å thick were cut with glass knives on an LKB ultrotome and stained with uranyl acetate (64% in methyl alcohol, 20 min) and lead citrate (0·4% adjusted to pH 12, 2 to 4 min) (modified from Reynolds, 1963). The specimens were examined in a Philips electron microscope.

3. Results

(a) *Electron microscopy of sections of HeLa cells*

An electron microscopic examination of thin sections of the HeLa cells used in the present study has shown the presence of numerous elements of both rough and smooth endoplasmic reticulum scattered throughout the cytoplasm (Plate I(a)). The rough elements appear as sections, at various angles, of tubules and vesicles of different size and shape and of short cisternae. Where the rough elements are cut tangentially,

one can see ribosomes arranged in rows, spirals or rosettes (Plate I(b)). The outer nuclear membrane sometimes shows attached ribosomes (Plate I(d)). Most of the rough elements appear to be isolated; sometimes, however, they are grouped and may also be in communication (Plate I(c)).

Most ribosomes in HeLa cells, as in other rapidly multiplying cells, are free in the cytoplasm, mainly in the form of aggregates (polysomes) of various size (Plate I(a)).

(b) *Fractionation of the 8100 g membrane components*

The 8100 *g* membrane fraction contains, as was mentioned earlier, the bulk of mitochondria and elements of rough endoplasmic reticulum, in addition to smooth membrane components. The latter could be separated from rough endoplasmic reticulum and mitochondria on the basis of buoyant-density centrifugation in sucrose gradient.

A large number of experiments were performed to try to obtain a satisfactory resolution of rough endoplasmic reticulum from mitochondria by differential centrifugation or by sedimentation velocity or buoyant-density fractionation in a sucrose gradient. For these experiments, a variety of media was utilized both for homogenization (TKM, low ionic strength TKV, SMET) and sucrose gradients (T buffer, with or without addition of EDTA or CsCl (according to an adaptation of the procedure of Dallner *et al.* (1966)). These experiments gave disappointing results. At best, a partial separation of the two types of organelles was obtained: this was due to the extensive overlapping in their sedimentation properties (Attardi & Attardi, 1968) and density (Fig. 1). Figure 1 shows the results of a typical buoyant-density centrifugation of the 8100 *g* membrane fraction in a 30 to 48% sucrose gradient in T buffer. The O.D.$_{260}$ analysis reveals a broad band occupying the region of the gradient corresponding to ρ values from 1·16 to 1·20 g/ml. and a smaller band centered around $\rho \sim 1{\cdot}14$ g/ml. (It should be pointed out that the O.D.$_{260}$ of membrane components only in part represents true absorption, in part being caused by light scattering.) The main band contains mainly mitochondria and elements of rough endoplasmic reticulum, as revealed by the cytochrome oxidase assay and by electron microscopic examination of sections of the pelleted components; in the light band, on the other hand, the electron microscopic analysis reveals smooth membrane structures. Both after short and long exposure of the cells to labeled RNA precursors, the structures containing the newly synthesized RNA are found in the region of the main O.D.$_{260}$ band; very little labeled RNA appears in correspondence with the band of smooth membrane components. It was previously shown (Attardi & Attardi, 1968) that after a very short pulse (3 min) with [^{3}H]uridine, the majority of the newly synthesized RNA in the membrane fraction, if not all, is intramitochondrial; with increasing pulse length or after a pulse–chase, an increasing proportion of labeled RNA is found to be associated with extramitochondrial structures, presumably elements of rough endoplasmic reticulum. In confirmation of the results of electron microscopic examination, the buoyant-density distribution in a sucrose gradient of the structures containing three-minute [^{3}H]uridine-labeled RNA overlaps extensively that of the structures containing RNA labeled during a two-hour pulse with [^{14}C]-uridine (Fig. 1), or during a 24- to 48-hour exposure to the precursor, which are in their great majority elements of rough endoplasmic reticulum (see below). Since the aim of the present work was to obtain the over-all picture of the properties of the endoplasmic reticulum-bound ribosomes in HeLa cells and to try to estimate their

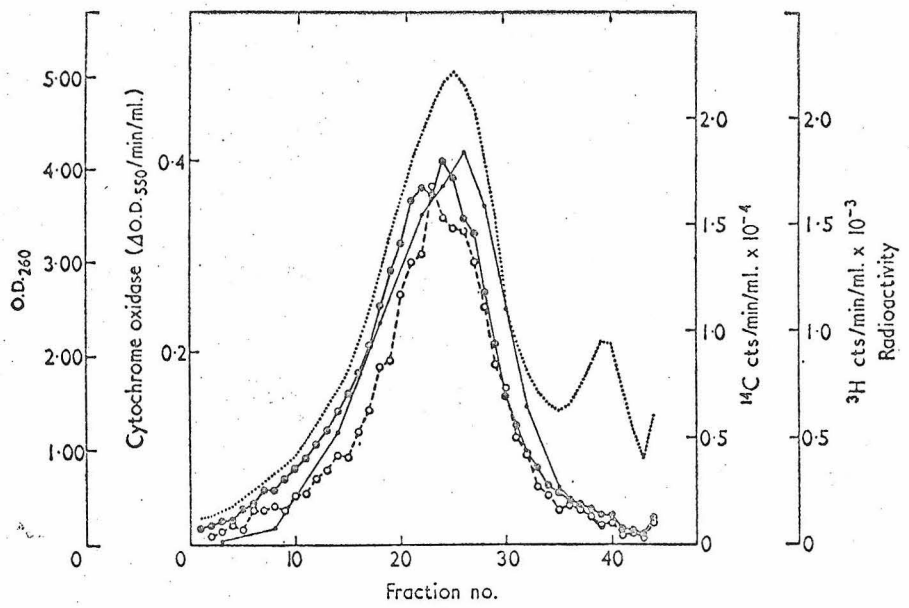

FIG. 1. Buoyant density distribution in sucrose gradient of components of the 8100 *g* membrane fraction.

The 8100 *g* membrane fraction was isolated from a mixture of $1{\cdot}2 \times 10^8$ cells labeled for 3 min with [^{3}H]uridine (28 mc/μmole, 10 μc/ml.) and $1{\cdot}2 \times 10^8$ cells labeled for 120 min with [^{14}C]-uridine (30 μc/μmole, 0·063 μc/ml.) and run to equilibrium on a 30 to 48% sucrose gradient in T buffer, as described in Materials and Methods (d). After O.D.$_{260}$ measurement, portions of each fraction were utilized for determination of radioactivity and for cytochrome oxidase assay (Materials and Methods (f)).

. . . . . , O.D.$_{260}$; --○--○--, 3 min [^{3}H]RNA cts/min; —◍——◍—, 120 min [^{14}C]RNA cts/min; —·——·—, cytochrome oxidase activity.

relative proportion to free ribosomes–polysomes, and since even a moderate enrichment of the membrane components in elements of rough endoplasmic reticulum would have been achieved only by introducing great losses of these structures and by selecting a particular fraction, it was considered necessary to use for this study the total components of the main band of the buoyant-density pattern in a sucrose gradient. This approach seemed to be justified since the other known potential source of ribosomes, mitochondria, was expected to contribute only a relatively small percentage of the total ribosomes of the membrane fraction, in view of the low amount of mitochondrial RNA (Truman & Korner, 1962; Elaev, 1966: Kroon, 1966; O'Brien & Kalf, 1967*a*,*b*). This expectation has been verified by appropriate controls (Results (e) and (f)).

(c) *Distribution of rRNA among different cytoplasmic fractions*

Table 1 shows the distribution of rRNA between the post-membranous (15,800 *g*) cytoplasmic supernatant (which contains free polysomes, monomers and "native" ribosomal subunits) and the 8100 *g* membrane fraction. (The 15,800 *g* pellet obtained from the 8100 *g* supernatant fraction contains about 10% of the free polysomes and 10 to 15% of the membrane-associated rRNA but was not systematically analyzed.) A considerable variability in the yield of rRNA associated with the two fractions and in their relative proportions was observed (Table 1): this was mainly due to

TABLE 1

Yield of ribosomal RNA and ribosomes from different cytoplasmic fractions of HeLa cells

Fraction	Ribosomal RNA	Subunits released by EDTA	Polysomes (120–350 s)
	(O.D.$_{260}$ units/10^8 cells)		
Postmembranous fraction	22·6[a] (17·3–39·0)	23·3	16[d]
8100 *g* membrane fraction	2·9[b] (1·4–4·0)	3·3[c] (2·9–3·7)	2·2[a] (1·6–2·7) [67%[e] (64–71%)]
	$\frac{\text{8100 } g \text{ membrane fraction rRNA}}{\text{total rRNA}^f} \times 100 = 11{\cdot}5\,(6{\cdot}3\text{–}18{\cdot}1)^a\%$		

RNA was extracted by sodium dodecyl sulfate from the 15,800 *g* supernatant fraction and from the mitochondria–endoplasmic reticulum components of the 8100 *g* membrane fraction (banded in a sucrose gradient) and analyzed in a sucrose gradient in sodium dodecyl sulfate buffer. Ribosomal subunits were released from the free polysome–monomer fraction and from the 8100 *g* membrane fraction by EDTA treatment and isolated in a sucrose gradient in TKV. Membrane-associated polysomes were released by treatment of the 8100 *g* membrane fraction with 1% NaDOC and separated by centrifugation in a sucrose gradient in TKM; RNA was extracted by sodium dodecyl sulfate from the pooled fractions of the polysome region (120 to 350 s) and of the lighter components (<120 s) and run on sucrose gradients in sodium dodecyl sulfate buffer for the determination (by O.D.$_{260}$) of the proportion of rRNA in the membrane fraction which pertains to polysomes. For experimental details, see Materials and Methods (d) and (e).

[a] Average and range of 5 experiments, [b] 8 experiments and [c] 2 experiments.

[d] This amount was calculated from the average rRNA yield in the postmembranous fraction, assuming that 70% of it is in polysomes (see Results (c)).

[e] Average and range of the proportion of rRNA in the membrane fraction which pertains to polysomes.

[f] This represents the sum of the rRNA present in the 8100 *g* membrane fraction and in the 15,800 *g* supernatant; the small amount of free polysomes and membrane components sedimenting at 15,800 *g* would not affect significantly the ratio considered here (see text).

differences in the effectiveness of homogenization, which resulted in a varying degree of fragmentation of the cytoplasm with ensuing variable losses in the low-speed centrifugations. These losses would tend to affect preferentially the membranous structures (Blobel & Potter, 1967*a*); thus, although in the present work the minimal centrifugal force and time of centrifugation required to sediment nuclei were used, it is likely that the relative amount of membrane-associated rRNA was somewhat under-estimated. It appears from Table 1 that from 10 to 15% (and possibly as much as 20%) of the cytoplasmic rRNA is associated with membrane components. As is also shown in Table 1 and as will be discussed further below, the great majority of rRNA of the 8100 *g* membrane fraction can be accounted for by the ribosomal particles isolated from this fraction. Therefore, the average proportion of the rRNA recovered from the 15,800 *g* supernatant fraction and from the 8100 *g* membrane fraction should reflect the approximate distribution of free and membrane-associated ribosomes, respectively, in the total cytoplasmic fraction and, keeping in mind the above-mentioned cautions concerning the recovery, also in the living cell. From 65 to 70% of the ribosomes of the 8100 *g* membrane fraction can be recovered as polysomes after NaDOC treatment (see Results (d)). Of the ribosomes present in the 15,800 *g* supernatant fraction, approximately 70% are in the form of polysomes,

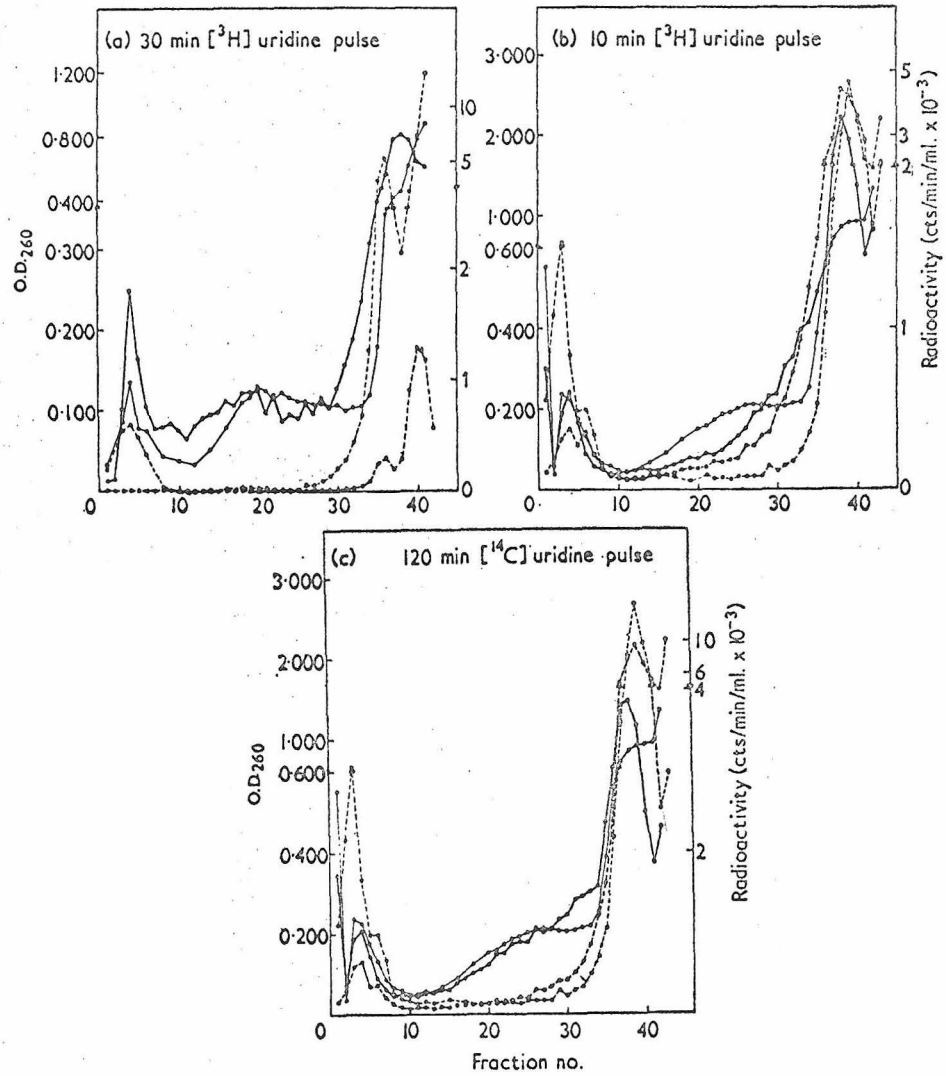

FIG. 2. RNase and EDTA sensitivity of polysomes released from the 8100 *g* membrane fraction by NaDOC.

(a) The 8100 *g* membrane fraction was isolated from 6×10^7 cells labeled for 30 min with [^{3}H] uridine (25·4 mc/μmole, 1·25 μc/ml.), treated with 1% NaDOC, and divided into two equal parts: one-half was treated with 1 μg pancreatic RNase/ml. at 2 to 3°C for 15 min; the other half was used as a control. Each sample was run on a 15 to 30% sucrose gradient in TKM (over 3 ml. 64% sucrose) for 90 min at 24,000 rev./min. The superimposed O.D.$_{260}$ and radioactivity patterns of the two gradients are shown.

(b) and (c) The 8100 *g* membrane fraction was isolated from a mixture of 9×10^7 cells labeled for 10 min with [^{3}H]uridine (27·1 mc/μmole, 6·3 μc/ml.) and 9×10^7 cells labeled for 120 min with [^{14}C]uridine (51 μc/μmole, 0·025 μc/ml.), treated with 1% NaDOC and divided into two equal parts: one-half was treated with 10^{-3} M-EDTA; the other half was used as a control. Each sample was run on a 15 to 30% sucrose gradient in low ionic strength TKV (EDTA-treated sample) or in TKM (control) under the conditions indicated in (a). The superimposed O.D.$_{260}$ and ^{3}H-radioactivity patterns of the two gradients are shown in (b); the superimposed O.D.$_{260}$ and ^{14}C-radioactivity patterns, in (c). RNA was extracted by sodium dodecyl sulfate from the pooled fractions 11 to 33 and 34 to 43 of the control gradient and run on sucrose gradients in sodium dodecyl sulfate buffer for the determination (by O.D.$_{260}$) of the proportion of rRNA in the membrane fraction pertaining to polysomes (see Table 1).

(a) —○——○—, O.D.$_{260}$ and —◎——◎—, [^{3}H]RNA cts/min of control; --○--○--, O.D.$_{260}$ and --◎--◎--, [^{3}H]RNA cts/min after RNase treatment.

(b) and (c) —○——○—, O.D.$_{260}$ and —◎——◎—, [^{3}H]RNA cts/min (b), or [^{14}C]RNA cts/min (c) of control; --○--○--, O.D.$_{260}$ and --◎--◎--, [^{3}H]RNA cts/min (b) or [^{14}C]RNA cts/min (c) after EDTA treatment.

about 10% in the form of 74 s† monomers, and the rest in the form of 60 and 45 s ribosomal subunits in approximately equal number (the ratio of radioactivity in the two subunits after 24-hour labeling with [^{3}H]uridine was found to be 2).

(d) *Release of membrane-associated polysomes by treatment with sodium deoxycholate*

If the 8100 *g* membrane fraction is centrifuged through a 15 to 30% sucrose gradient in TKM, all material exhibiting O.D.$_{260}$ and radioactivity sediments rapidly to the bottom of the tube; however, it can be prevented from pelleting if a 64% sucrose cushion is used (Attardi & Attardi, 1967). Treatment of the membrane fraction with 1% NaDOC releases from these fast-sedimenting components almost all material contributing to the O.D.$_{260}$: a substantial amount of this now sediments in the form of a broad band in the region of the gradient corresponding to polysomes (120 to 350 s) and shows the characteristic RNase and EDTA sensitivity of these structures (Fig. 2); it is therefore reasonable to interpret this O.D.$_{260}$ band as consisting mainly of polysomes which have been liberated from their association with membrane components by the detergent. The small residue of fast-sedimenting material (blocked by the dense sucrose cushion) after NaDOC treatment is presumably represented mostly by structures resistant to the detergent, since the ratio of NaDOC to protein was more than adequate; a minor part of this material may be contributed by the advancing edge of the polysome band. NaDOC treatment also releases from the fast-sedimenting material most of the radioactivity incorporated into RNA: for increasing pulse length a steadily increasing proportion of the labeled membrane-associated RNA sediments after detergent action in the region of polysomes (from about 24% after a 5-min [^{3}H]uridine pulse to about 42% after a 2-hr [^{14}C]uridine pulse (Fig. 2(c)), with the radioactivity profile following progressivly more closely the O.D.$_{260}$ profile. The labeled components sedimenting in the polysome region show the sensitivity to RNase expected for polysomal structures (Fig. 2(a)); on the contrary, only a part of the label is associated with EDTA-sensitive structures (Fig. 2(b) and (c)). (Similar results were obtained by using EDTA concentrations from 10^{-3} to 10^{-2}.) The proportion of the label in the polysome region which is in structures sensitive to EDTA increases with pulse length: thus, it is about 32% after a 10-minute pulse with [^{3}H]uridine (Fig. 2(b)) and becomes more than 70% after a two-hour pulse with [^{14}C]uridine (Fig. 2(c)). The criterion of sensitivity to EDTA has been recently shown to distinguish polysomes from cosedimenting non-polysomal structures (Penman *et al.*, 1968). A reasonable interpretation of the above discussed results is that, after NaDOC treatment, in addition to polysomes, other structures derived from membrane lysis, which are resistant to EDTA and contain RNA in a form susceptible to RNase, sediment in the polysome region of the gradient. Since these EDTA-resistant structures become labeled after a very short [^{3}H]uridine pulse, when all or the great majority of newly synthesized RNA in the cytoplasm is in mitochondria (Attardi & Attardi, 1968), it is likely that they derive from lysis of these organelles. From the proportion of rRNA in the polysome band (120 to 350 s) and in the lighter components (<120 s) (Table 1), it can be estimated that from 65 to 70% of the ribosomes in the 8100 *g* membrane fraction are released in the form

† The value of 74 s used in this work for the sedimentation coefficient of HeLa ribosomes has been directly determined (Attardi & Smith, 1962); for the native ribosomal subunits the values of 60 and 45 s, and for the EDTA-derived particles the values of 50 and 30 s estimated by Girard, Latham, Penman & Darnell (1965) have been used.

of polysomes by NaDOC action. The labeled components sedimenting in the upper third of the gradient after this treatment presumably consist in part of free RNA or RNA-containing complexes released by the lysis of mitochondria, in part of single ribosomes possibly associated with mRNA.

(c) *RNase sensitivity of membrane-associated ribosomes*

In order to obtain information concerning the possible contribution of the presence of intramitochondrial ribosomes to the results described in the previous sections, the sensitivity of the membrane-associated ribosomes to pancreatic RNase was tested: in fact, intramitochondrial ribosomes *in situ* should be protected from the action of nucleases if the mitochondrial membranes are intact (Rifkin *et al.*, 1967). Conditions of RNase digestion were found under which more than 85% of the mRNA and more than 50% of the rRNA of free polysomes are quickly made acid-soluble, while the rest is only slowly degraded (Fig. 3 and Table 2). It appears from Table 2 that, under these conditions of RNase digestion, the rRNA of membrane-bound ribosomes is made acid-soluble to the same extent (55 to 60% after a 10-min treatment) as rRNA of free polysomes. Under the same conditions, the major part (about 75%) of the mitochondrial heterogeneous RNA labeled in a three-minute [^{3}H]-uridine pulse is not affected by the enzyme (Fig. 3 and Table 2). After solubilizing the mitochondrial membranes by NaDOC treatment, the intramitochondrial RNA becomes, on the contrary, accessible to RNase (more than 90% of the 3- to 30-min pulse-labeled RNA is quickly degraded to acid-soluble products even by low concentrations of the enzyme; see, for example, Fig. 2(a)). Since it can be reasonably assumed that the RNase-sensitive portion of the three-minute [^{3}H]RNA provides a

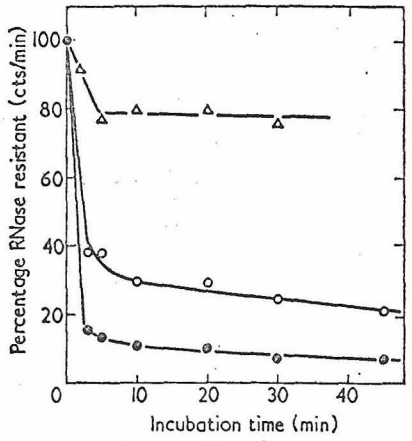

FIG. 3. Kinetics of RNase digestion *in situ* of RNA associated with mitochondria–endoplasmic reticulum components and of free polysome RNA from cells exposed for different times to [^{3}H]uridine.

Free polysomes were isolated by sucrose gradient centrifugation of the 15,800 *g* supernatant fraction from cells labeled with [^{3}H]uridine for 25 min (—◎——◎—) or 120 min (—○——○—); mitochondria–endoplasmic reticulum components were isolated by buoyant density centrifugation of the 8100 *g* membrane fraction from cells labeled with [^{3}H]uridine for 3 min (—△——△—). 50-μl. samples (each containing less than 4 μg RNA) from the peak fraction of the free polysome profile and of the cytochrome oxidase distribution, respectively, were diluted with 1·0 ml. 0·1 M-NaCl, 0·01 M-sodium citrate and treated for different times with 50 μg pancreatic RNase/ml. at 2°C. On the axis of ordinates the percentage of cts/min which remains acid-insoluble after this treatment is indicated. RNA was extracted by sodium dodecyl sulfate from the free polysomes and from the membrane components and analyzed for proportion of radioactivity in rRNA (Table 2).

TABLE 2

Ribonuclease sensitivity in situ *of RNA in different cytoplasmic fractions from HeLa cells*

Fraction	Labeling time (min)	% of total radioactivity in rRNA[a] (*A*)	% of total radioactivity made acid-soluble by RNase (*B*)	Estimated % of radioactivity in rRNA made acid-soluble by RNase[b] (*C*)
Free polysomes	25 (2[c])	n.d.	87·9	—
	120 (2)	62	69·4	58
	1440 (1)	≈ 90[d]	57·9	55
8100 *g* membrane fraction	3 (8)	n.d.	26·0 (28·7[f])	—
	1560 (2)	≈ 75[e]	57·2 (55·4[f])	59 (57[f]) [47–68[g]]

Free polysomes were isolated by centrifugation in sucrose gradient of the 15,800 *g* supernatant, and the mitochondria–endoplasmic reticulum components by buoyant density centrifugation of the 8100 *g* membrane fraction (see Materials and Methods (d)) from samples of 8×10^7 to $1{\cdot}9 \times 10^8$ HeLa cells labeled for different times with [^{3}H]uridine; in one experiment, a mixture of $1{\cdot}5 \times 10^8$ cells labeled for 3 min with [^{3}H]uridine (25·5 mc/μmole, 10 μc/ml.) and 5×10^7 cells labeled for 26 hr with [^{14}C]uridine (50 μc/μmole, 0·005 μc/ml.) was used. 50- to 100-μl. samples of fractions of the free polysome distribution and of the mitochondria–endoplasmic reticulum band (each containing <6 μg RNA) were diluted with 1·0 ml. 0·1 M-NaCl, 0·01 M-sodium citrate and treated for 10 min with 50 μg pancreatic RNase/ml. at 2°C. In the double labeling experiment, the RNase-resistant radioactive material was corrected for a low level of DNA labeling by [^{14}C]-uridine by subtracting the acid-insoluble radioactive material resistant to hydrolysis by 0·5 N-NaOH for 22 hr at 30°C. RNA was released from the free polysomes and from the membrane components by sodium dodecyl sulfate and analyzed in sucrose gradient in sodium dodecyl sulfate buffer. The proportion of radioactive material associated with the two major rRNA components was estimated as described by Girard *et al.* (1965).

n.d., Not detectable.

[a] This figure does not include 5 s RNA.

[b] This estimate was made by assuming that the non-ribosomal portion of RNA from free polysomes is digested by RNase to the same extent as the 25-min labeled RNA (~88%), i.e. $C = \dfrac{B - (100\text{-}A) \times 0{\cdot}88}{A} \times 100$, where the symbols are defined in the headings of the Table. For the non-ribosomal portion of RNA from the 8100 *g* membrane fraction, it was tentatively assumed that about 60% of it (as estimated from the fraction of heterogeneous RNA which was not released by EDTA, Fig. 4(b)) has the sensitivity to RNase of the 3 min-[^{3}H]uridine-labeled intramitochondrial RNA, and the rest is attacked by the enzyme to the extent of 88%, as is free polysome non-ribosomal RNA.

[c] The figures in parentheses indicate the number of experiments.

[d] This figure was estimated indirectly on the basis of the following available information: (1) almost 4% of free polysomal RNA is represented by tRNA (2 molecules per ribosome, Warner & Rich, 1964) and 5 s RNA (1 molecule per ribosome, Rosset, Monier & Julien, 1964); (2) 3 to 4% of free polysomal RNA is represented by mRNA (Soeiro, Vaughan, Warner & Darnell, 1968); and (3) the specific activity of mRNA after 24 hr growth in the presence of labeled RNA precursors is close to twice that of rRNA due to its turnover (Penman, Scherrer, Becker & Darnell, 1963; Attardi & Attardi, 1967). For the tRNA–5 s RNA components the above estimate was found to be very close to the amount directly determined from sucrose gradient analysis (~4·3%).

[e] This proportion was estimated directly from sucrose gradient analysis (see, for example, Fig. 4(b) and (c)).

[f] Data pertaining to the double labeling experiment.

[g] These figures represent the minimum and maximum proportion of radioactivity in rRNA solubilized by RNase as estimated under the two extreme assumptions that all the membrane-associated heterogeneous RNA has the RNase sensitivity of free polysome non-ribosomal RNA or intramitochondrial RNA, respectively.

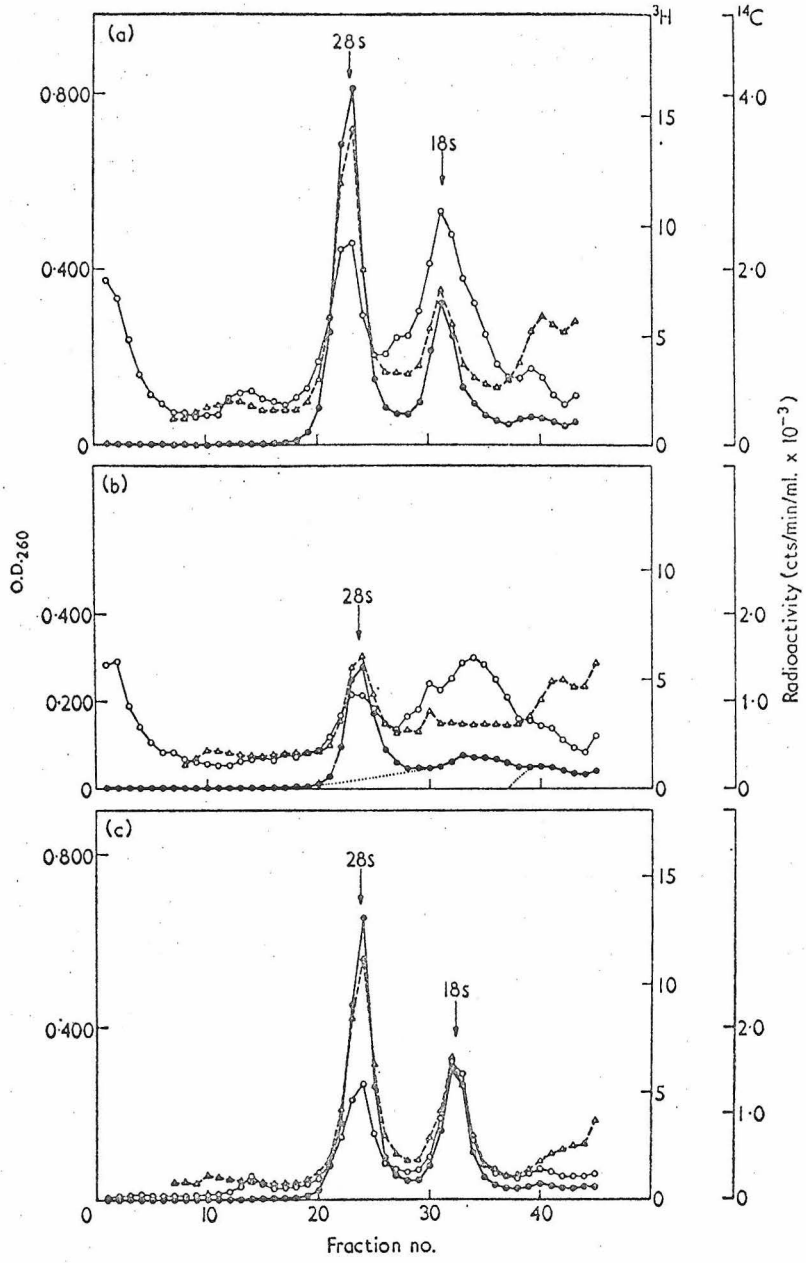

FIG. 4. Release of RNA from the 8100 *g* membrane fraction by EDTA treatment.

The 8100 *g* membrane fraction was isolated from a mixture of $1{\cdot}8 \times 10^8$ cells labeled for 75 min with [^{14}C]uridine (50 μc/μmole, 0·07 μc/ml.) and 7×10^7 cells labeled for 24 hr with [^{3}H]uridine (27·1 mc/μmole, 0·3 μc/ml.) and divided into two equal parts: one-half was used as a control; the other half was treated with 90 μmoles of EDTA, pelleted, and resuspended as described in Materials and Methods (d). Each sample was run on a 30 to 48% sucrose gradient. RNA was extracted by the sodium dodecyl sulfate method from the mitochondria–endoplasmic reticulum band of the control (a) and of the EDTA-treated sample (b) and from the material released into the EDTA supernatant fraction (c), and run on a 15 to 30% sucrose gradient in sodium dodecyl sulfate buffer, as detailed in Materials and Methods (e). In (b) the dotted line was used to estimate the amount of radioactivity associated with heterogeneous RNA.

--△--△--, O.D.$_{260}$; —○——○—, 75-min [^{14}C]RNA cts/min; —◉——◉—, 24-hr [^{3}H]RNA cts/min.

maximum estimate of the fraction of mitochondria which are accessible to the enzyme and that the ribosomes contained in these mitochondria would be as susceptible to RNase as free ribosomes, these results indicate that the great majority of the ribosomes of the membrane fraction are extramitochondrial, i.e. presumably bound to the endoplasmic reticulum. This conclusion was corroborated by the results of the analysis of the effects on these ribosomes of EDTA treatment, as described below.

(f) *Response of membrane-associated ribosomes to EDTA treatment*

Investigations carried out on rough microsomes from rat liver (Sabatini *et al.*, 1966) have shown that, if these are exposed to a concentration of EDTA sufficiently high (20 μmoles per 0·5 g tissue equivalent of microsomes), essentially all the small ribosomal subunits of the membrane-bound ribosomes are released; the detachment of the large subunits requires higher concentrations of EDTA and reaches a limiting value of 50 to 60% of the original 50 s content (as can be estimated from the reported proportion of the total membrane-bound rRNA released (65 to 70%), assuming 2·6 as the weight ratio of the two subunits (Amaldi & Attardi, 1968)) upon treatment with 100 μmoles or higher amounts of EDTA. A situation similar to that described for the ribosomes of rough endoplasmic reticulum from liver, as concerns response to EDTA treatment, appears to hold for the bulk of ribosomes of the membrane fraction from HeLa cells. In fact, as shown in Figure 4 and Table 3, the analysis of RNA extracted from the EDTA-treated membrane fraction reveals that about 30% of both the pre-existing and newly synthesized (i.e. labeled after a 75-min [^{14}C]-uridine pulse) 28 s RNA originally present in the membrane components remains associated with them after exposure to the chelating agent (from 35 to 75 μmoles per gram cell equivalent of membrane fraction). On the contrary, an examination of the O.D.$_{260}$ and of the 75-minute and 24-hour labeling profiles in the sedimentation pattern of RNA from the EDTA-treated membrane fraction fails to show a clear 18 s RNA component (Fig. 4(b)). The lower ratio of radioactivity in 28 s RNA to that in 18 s RNA after 75 minutes as compared to 24-hour labeling (Fig. 4(a)) is due to the asynchrony of arrival in the cytoplasm from the nucleus of the two newly synthesized rRNA components (Girard *et al.*, 1965). The broad band of heterogeneous

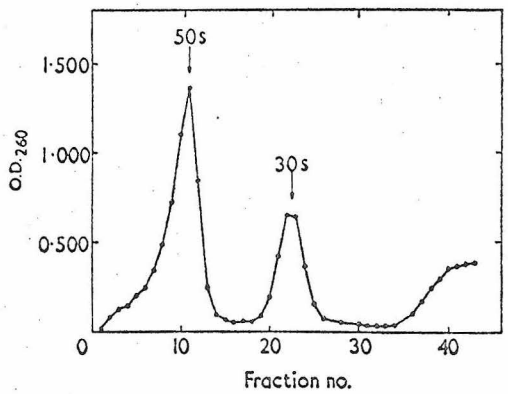

FIG. 5. Sedimentation pattern of ribosomal subunits released from the 8100 *g* membrane fraction by EDTA treatment. The 8100 *g* membrane fraction was isolated from $2{\cdot}6 \times 10^8$ cells; after treatment with 100 μmoles of EDTA, the membrane components were spun down and the supernatant fraction was run on a 15 to 30% sucrose gradient in TKV, as described in Materials and Methods (d).

TABLE 3

Release of RNA from the 8100 g membrane fraction by EDTA treatment

Experiment	Precursor	Labeling time (min)	Fraction (after EDTA treatment)	Ribosomal RNA (cts/min)			Heterogeneous RNA[a]
				Total	28 s	18 s	
1	[^{3}H]Uridine	3	pellet	n.d.	n.d.	n.d.	930 (89%)
			supernatant	n.d.	n.d.	n.d.	110 (11%)
	[^{14}C]Uridine	75	pellet	660	660 (31%	n.d.	8130 (80%)
			supernatant	3340	1440 (69%)	1900	2000 (20%)
2	[^{14}C]Uridine	75	pellet	630	630 (28%)	n.d.	11,000 (83%)
			supernatant	3400	1620 (72%)	1780	2280 (17%)
	[^{3}H]Uridine	1440	pellet	6550	6550 (31%)	n.d.	7000
			supernatant	21,950	14,250 (69%)	7700	≈1600

In experiment 1, the 8100 *g* membrane fraction was isolated from a mixture of 7×10^7 cells labeled for 3 min with [^{3}H]uridine (28 mc/μmole, 10 μc/ml.) and 7×10^7 cells labeled for 75 min with [^{14}C]uridine (30 μc/μmole, 0·063 μc/ml.). After treatment with 40 μmoles of EDTA, the membrane fraction was pelleted, resuspended, and run on a 30 to 48% sucrose gradient, as described in Materials and Methods (d). RNA was extracted by sodium dodecyl sulfate from the membrane components of the main band in the gradient and from the material released into the EDTA supernatant fraction, and run on 15 to 30% sucrose gradients in sodium dodecyl sulfate buffer, as detailed in Materials and Methods (e). The distribution of radioactivity between the two major rRNA components and heterogeneous RNA was estimated as described by Girard *et al.* (1965). The data of experiment 2, obtained in the same way, pertain to the RNA extracted from the EDTA-treated membrane fraction used in the experiment of Fig. 4.

n.d., Not detectable.

[a] Includes all non-ribosomal RNA from 7 s to more than 50 s.

TABLE 4

Ratio of 28 to 18 s RNA and of 50 to 30 s ribosomal subunits in different cytoplasmic fractions from HeLa cells

Fraction		Ratio of 28 to 18 s RNA[a]	Ratio of 50 to 30 s subunits[a]
	Total	2·65[c]	2·62[c]
8100 *g* Membrane fraction	Material released by EDTA	1·85	1·83
Free polysomes		2·47	2·75
Total ribosome–polysome fraction		2·43[d]	2·65[d]
Expected from molecular weight data[b]		2·5 to 2·6	

The RNA was extracted from the mitochondria–endoplasmic reticulum components of the EDTA-treated membrane fraction (banded in sucrose gradient), from the material released by EDTA and from free polysomes by sodium dodecyl sulfate, as detailed in Materials and Methods (e). The ribosomal subunits were isolated as described in Materials and Methods (d). The ratio of 28 to 18 s RNA in free polysomes and that of 50 to 30 s subunits in the EDTA supernatant fraction and in free polysomes were determined on the basis of optical density at 260 mμ after correction for the different base composition of the two rRNA components (Amaldi & Attardi, 1968). The ratio of 28 to 18 s RNA in the material released by EDTA was determined on the basis of distribution of radioactivity after 24-hr [^{3}H]uridine labeling (no correction for the different base composition of the two rRNA species was needed because the sum of U + C + ψ is almost identical in the two components, and C and U (and presumably ψ) have about the same specific activity after long exposure of the cells to labeled uridine (Salzman & Sebring, 1959). The slightly lower values obtained for the ratio of the two rRNA components, as compared to the ratio of the two ribosomal subunits, in free polysomes and in the total ribosome–polysome fraction may indicate a small conversion (less than 3%) of 28 s RNA to molecules sedimenting as 18 s RNA.

[a] Defined in the text.

[b] The figures given here represent the ratio of 28 to 18 s (of 50 to 30 s subunits) expected for equimolar amounts of the two rRNA species from their molecular weight ratio (Amaldi & Attardi, 1968).

[c] These figures represent the ratios determined on the material released by EDTA after correction for the amount of 28 s RNA (50 s subunits) remaining in the EDTA-treated membrane fraction (on the average, about 30% of total membrane-associated 28 s RNA (50 s subunits) (see Table 3)).

[d] Average values determined on the basis of O.D.$_{260}$ ratios and ^{32}P-labeling ratios (Amaldi & Attardi, 1968).

RNA sedimenting in the region 9 to 23 s and the heavier polydisperse components (up to more than 50 s) represent, in part at least, mitochondrial RNA (see Discussion).

Figure 5 shows the sedimentation pattern of the material released from the membrane fraction of HeLa cells by EDTA treatment: one recognizes the two ribosomal subunits and a small amount of slower sedimenting ultraviolet-absorbing material near the meniscus. The ratio of major to minor subunits (defined as the ratio of total number of nucleotides contained in the two classes) released by EDTA from the 8100 *g* membrane fraction is about 1·83 (Table 4). This ratio is considerably lower than that of 2·75 found for the subunits derived from free polysomes treated under the same conditions, and that of 2·65 previously reported for the subunits derived from the total ribosome–polysome fraction (Amaldi & Attardi, 1968): this difference can be accounted for completely by the amount of 28 s RNA remaining in the EDTA-treated membrane fraction, a finding which strongly suggests that this residual 28 s RNA is in the form of 50 s subunits. In the same way, the figure of 1·85

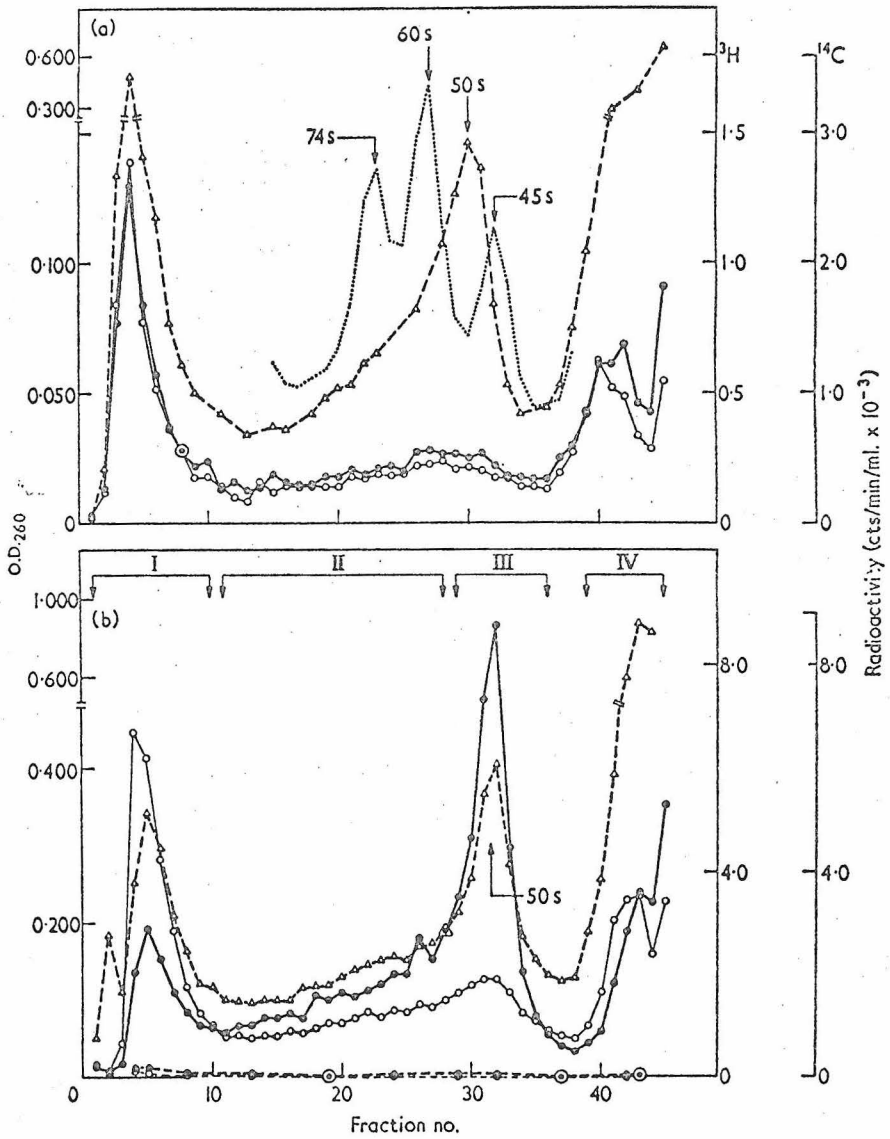

FIG. 6. Sedimentation pattern of the NaDOC lysate of EDTA-treated membrane fraction from cells exposed for different times to labeled uridine.

The 8100 *g* membrane fraction was isolated, in the experiment shown in (a), from a mixture of 3×10^7 cells labeled for 5 min with [^{3}H]uridine (27·1 mc/μmole, 6·3 μc/ml.) and 3×10^7 cells labeled for 30 min with [^{14}C]uridine (51 μc/μmole, 0·063 μc/ml.), and in the experiment shown in (b), from a mixture of $1{\cdot}0 \times 10^8$ cells labeled for 75 min with [^{14}C]uridine (50 μc/mole, 0·07 μc/ml.) and 4×10^7 cells labeled for 24 hr with [^{3}H]uridine (27·1 mc/μmole, 0·3 μc/ml.). After exposure to EDTA, the membrane fraction was pelleted, resuspended in 0·25 M-sucrose in T buffer (2·0 ml. in Expt. (a) and 1·0 ml. in Expt. (b)), treated with 1% NaDOC, and run on a 15 to 30% sucrose gradient (over 3 ml. 64% sucrose) in TKV, as detailed in Materials and Methods (d). In (a) a sample of the 15,800 *g* supernatant fraction was run at the same time on a 15 to 30% sucrose gradient in TKM to provide the O.D.$_{260}$ profile of free monomers and native ribosomal subunits.

For the determination of incorporation of labeled uridine into alkali-stable material, portions of

found for the ratio of 28 to 18 s RNA (defined as above) in the material released by EDTA, after correction for the residual membrane-associated 28 s RNA, becomes very close to the ratio of the two major rRNA species in free polysomes (Table 4). No obvious loss of intramitochondrial RNA components appears to occur as a result of EDTA treatment. Thus, under conditions which release from the membrane fraction almost all the 30 s ribosomal subunits, about 90% of the heterogeneous RNA labeled after a three-minute [^{3}H]uridine pulse (when 70% or more of the label is intramitochondrial (Attardi & Attardi, 1968)) and about 80% of the 75-minute ^{14}C-labeled heterogeneous RNA (of which at least 60% is intramitochondrial) remain in the EDTA-treated membrane fraction (Table 3). Although exposure of the membrane components to EDTA results in a slight decrease ($\sim$ 0·01 g/ml.) in density in sucrose gradient of the mitochondria–rough endoplasmic reticulum band (as revealed by the cytochrome oxidase, O.D.$_{260}$ and radioactivity profiles), no appreciable effect on the susceptibility *in situ* to RNase of the membrane-associated 75-minute [^{14}C]uridine pulse-labeled heterogeneous RNA was detected after this treatment: this finding speaks against the possibility of damage to mitochondrial membranes resulting in loss of intramitochondrial ribosomes.

The results analyzed above of the effects of EDTA treatment on membrane-associated ribosomes confirm the conclusion of the RNase-sensitivity experiments, indicating that the great majority of these ribosomes are bound to the endoplasmic reticulum. The amount of pre-existing heterogeneous RNA not removed by EDTA, estimated on the basis of the distribution of the 24-hour labeled material (Fig. 4(b) and (c)) and assuming that the specific activity of the heterogeneous RNA after this labeling time is about double that of rRNA (due to its turnover (Attardi & Attardi, 1967)), represents 9 to 10% of the total membrane-associated RNA. As mentioned above, a part of this RNA, and possibly the majority, is intramitochondrial (see Discussion).

The nature of the relatively minor portion of labeled heterogeneous RNA of the membrane fraction which is found in the supernatant fraction after EDTA treatment (about 20% after 75-min labeling), remains to be established; it seems likely that it contains mRNA of membrane-bound polysomes which has been released together with the ribosomal subunits.

(g) *Analysis of the material released by sodium deoxycholate from EDTA-treated membrane fraction*

In order to try to isolate the 50 s subunits which remain associated with membrane components after EDTA treatment (see preceding section), the 8100 *g* membrane

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individual fractions of (b) were treated with 0·5 N-NaOH for 22 hr at 30°C, neutralized with HCl, and then precipitated with 5% trichloroacetic acid.

In (b), the components corresponding to the portions of the sucrose gradient pattern indicated by arrows were utilized for RNA analysis (see Fig. 7).

(a) --△--△--, O.D.$_{260}$; —○——○—, 30-min [^{14}C]RNA cts/min; —◎——◎—, 5-min [^{3}H]RNA cts/min.

(b) --△--△--, O.D.$_{260}$; —○——○—, total 75-min [^{14}C]RNA cts/min; —◍——◍—, total 24-hr [^{3}H]RNA cts/min; --○--○--, alkali-resistant ^{14}C cts/min; --◍--◍-- alkali-resistant ^{3}H cts/min.

fraction from cells labeled for various times with [^{3}H]- or [^{14}C]uridine was treated with 3×10^{-2} M-EDTA, washed with $1{\cdot}5\times10^{-2}$ M-EDTA, lysed with 1% NaDOC, and run on a sucrose gradient. The O.D.$_{260}$ profile thus obtained (Fig. 6) showed a well-defined peak with the approximate sedimentation coefficient of 50 S (estimated by using the native ribosomal subunits 60 and 45 S present in the 15,800 *g* supernatant fraction as reference markers) and faster sedimenting components extending down to the region of 130 S and more (the heaviest material being prevented from pelleting by the dense sucrose cushion at the bottom of the tube). The radioactivity incorporated into RNA in a short pulse (which is mostly intramitochondrial) appeared to be distributed rather uniformly in the region of the gradient from 30 to about 130 S (Fig. 6(a)); there was a somewhat greater accumulation of label in the components sedimenting slower than 30 S and, furthermore, a considerable proportion of radioactive material sedimented with the material heavier than 130 S (presumably these fast sedimenting structures correspond to the rapidly labeled material released by NaDOC lysis of mitochondria which still sediments in the polysome region after EDTA treatment (Fig. 2(b) and (c)). No labeling of the 50 S peak was observed after exposing the cells to [^{14}C]uridine for up to 30 minutes; after a 75-minute pulse, some radioactivity appeared to be associated with the 50 S component (Fig. 6(b)). The labeling of the latter became progressively more pronounced with increasing time of exposure of the cells to the radioactive precursor (Fig. 6(b)). All the labeled components in the gradient appeared to be alkali-sensitive. It seemed likely that the 50 S peak consisted of large ribosomal subunits from membrane-bound ribosomes, which had not been detached by EDTA treatment. Consistent with this possibility was the delay in appearance of label in this peak, which agreed with the time of arrival of newly synthesized 28 S RNA to the endoplasmic reticulum (Attardi & Attardi, 1967). An analysis of the sedimentation pattern (O.D.$_{260}$ and 24-hr labeling profiles) of the RNA extracted from the 50 S peak region of the gradient showed indeed a well-defined 28 S RNA component and, in addition, heterogeneous material sedimenting in the region 7 to 28 S (Fig. 7(c)). A surprising finding, however, was that a very similar pattern, with a 28 S RNA peak and slower sedimenting heterogeneous material (Fig. 7(b)), was exhibited by the RNA extracted from the structures sedimenting between 65 and 130 S in the sucrose-gradient centrifugation analysis shown in Figure 6(b), and also by the RNA derived from the heaviest components (>130 S) (in the latter case some heterogeneous RNA with sedimentation coefficients from 30 to more than 50 S was also present) (Fig. 7(a)). The RNA extracted from the NaDOC-released components with S <20 S showed, on the contrary, a high peak sedimenting at about 6 S, with small amounts of heavier heterogeneous material and of 28 S RNA (Fig. 7(d)).

An analysis of the distribution of 28 S RNA among the various components liberated by NaDOC from the EDTA-treated membrane fraction (Fig. 7) revealed that only about 37% of the large rRNA species remaining in this fraction after exposure to EDTA could be recovered from the 50 S peak region (Fig. 6(b)), the remainder being mostly in structures sedimenting faster than 50 S (up to more than 130 S). Increasing the relative amount of NaDOC from 10 mg (Fig. 6(b)) to 45 mg (Fig. 6(a)) per gram cell equivalent of membrane fraction did not promote the release of free 50 S particles as judged from the relative amount of O.D.$_{260}$ in the 50 S peak. As in the case of the RNA extracted from the total membrane fraction after exposure to EDTA (Fig. 4(b)), an analysis of the O.D.$_{260}$ and of the 24-hour labeling profiles

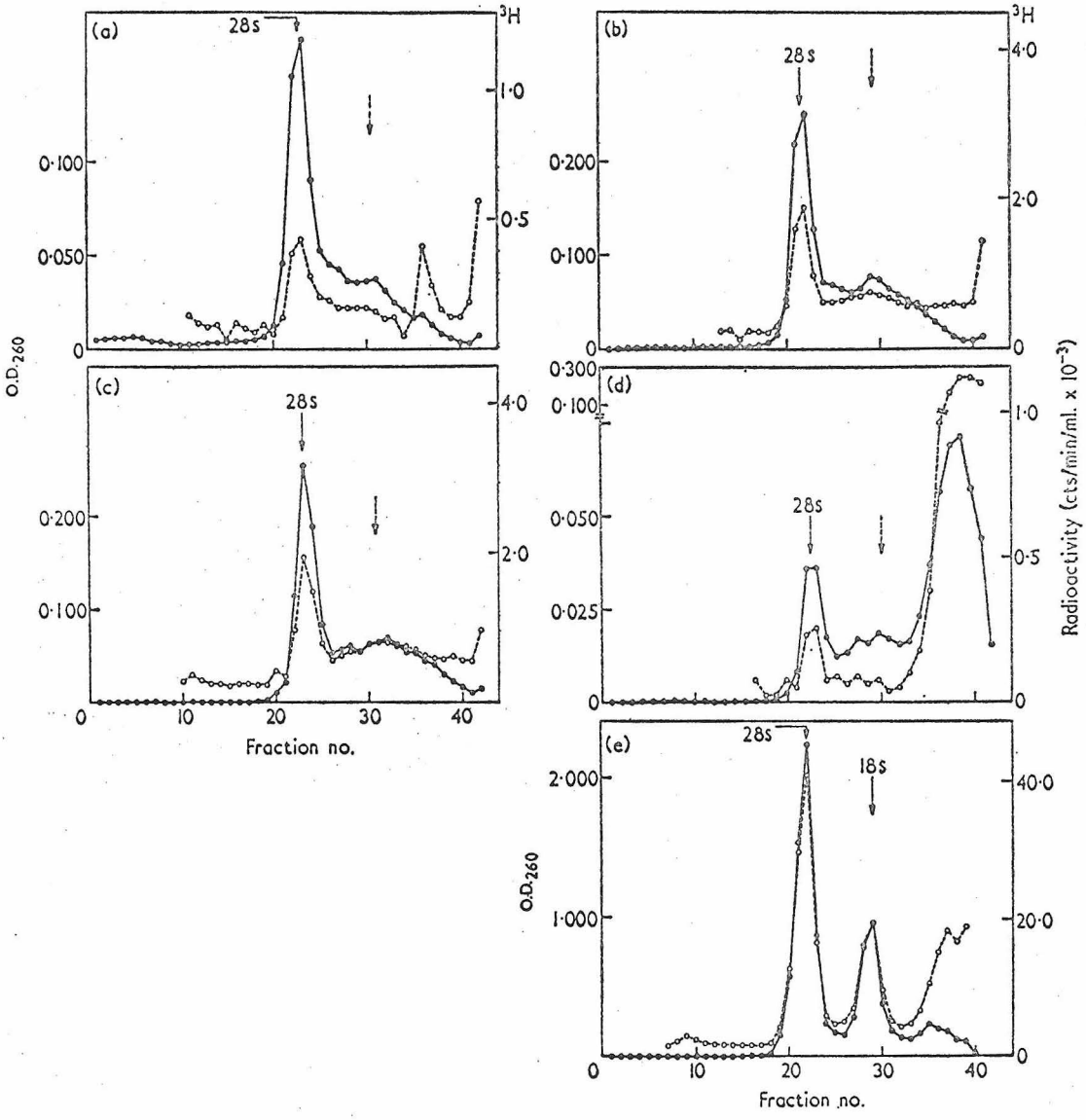

FIG. 7. Sedimentation pattern of RNA extracted from the various components released by NaDOC from the EDTA-treated membrane fraction. (Only the O.D.$_{260}$ and the 24-hr labeling profiles are shown here for the sake of simplicity.)

RNA was released by sodium dodecyl sulfate from the components of the NaDOC lysate of the EDTA-treated membrane fraction shown in Fig. 6(b), and run on sucrose gradients in sodium dodecyl sulfate buffer, as specified in Materials and Methods (e): the patterns (a) to (d) correspond respectively, to sections I to IV in the gradient of Fig. 6(b). The dashed arrow indicates the presumptive position of 18 s RNA. In (e) the sedimentation pattern of free polysomal RNA extracted from the same cells and run on a sucrose gradient under the same conditions is shown to provide a position marker for the two major rRNA species.

--○--○--, O.D.$_{260}$; —◎——◎—, 24-hr [^{3}H]RNA cts/min.

in the 14 to 20 s region of the sedimentation patterns of RNA from the various NaDOC lysis products of the EDTA-treated membrane fraction (Fig. 7) did not show a clear 18 s RNA component.

4. Discussion

The electron microscopic examination of sections of HeLa cells has confirmed the observations made by others (Epstein, 1961; Journey & Goldstein, 1961; Fuse *et al.*, 1963) concerning the presence in these cells of tubules, vesicles and cisternae of rough endoplasmic reticulum. In HeLa cells, as in other rapidly multiplying cells, the membrane-bound ribosomes represent a relatively minor part of total ribosomes: a rough visual estimate of their proportion in the electron microscope pictures would be between 10 and 20%.

In the present work, most of the elements of rough endoplasmic reticulum were recovered, together with the bulk of mitochondria, in the 8100 *g* membrane fraction. Attempts to resolve rough endoplasmic reticulum from mitochondria either by differential centrifugation or by sedimentation-velocity or buoyant-density fractionation in sucrose gradient gave unsatisfactory results. It was found that even a partial separation of the endoplasmic reticulum involved drastic losses of these structures, in addition to introducing a disturbing selection factor. Since mitochondria are known to contain very small amounts of RNA (Truman & Korner, 1962; Elaev, 1966, Kroon, 1966; O'Brien & Kalf, 1967*a*,*b*) and were therefore expected to contribute a relatively small proportion of the population of membrane-associated ribosomes, it was considered justifiable for the purposes of the present work to utilize the components of the 8100 *g* membrane fraction with a buoyant density in sucrose gradient of 1·16 to 1·20 g/ml. (which include both rough endoplasmic reticulum and mitochondria).

The results obtained in this work on the response of the membrane-associated ribosomes to RNase and EDTA treatment have confirmed the validity of this approach. Thus, under conditions of pancreatic RNase digestion which left the intramitochondrial heterogeneous RNA mostly intact, the RNA of the membrane-associated ribosomes appeared to be degraded to acid-soluble products to approximately the same extent (55 to 60%) as rRNA of free polysomes. Furthermore, exposure to EDTA released from the membrane components into the supernatant fraction almost all the small ribosomal subunits. Under the same conditions, the intramitochondrial heterogeneous RNA was not removed, and judging from its sensitivity to externally added RNase, there was no apparent damage to the mitochondrial membranes which could have resulted in loss of intramitochondrial ribosomal subunits. On the other hand, the amount of residual 28 s RNA in the EDTA-treated membrane fraction was that expected for membrane-stuck 50 s subunits from ribosomes of rough endoplasmic reticulum (see below). These results, therefore, point to the association with endoplasmic reticulum of the bulk of the ribosomes of the membrane fraction. The analysis carried out in this work was not sensitive enough to detect a relatively small amount of intramitochondrial ribosomes. Experiments carried out with purified mitochondria will be necessary to investigate the existence of intramitochondrial ribosomes, which is suggested by the analogy with lower eukaryotic cells (Kuntzel & Noll, 1967; Rifkin *et al.*, 1967; Dure *et al.*, 1967; Rogers *et al.*, 1967; Wintersberger, 1967; Clark-Walker & Linnane, 1967), by the protein synthetic capacity of mitochondria *in vitro* (Roodyn *et al.*, 1961,1962; Craddock & Simpson, 1961; Truman & Korner, 1962; Kroon,

1963*a*,*b*,*c*; Wheeldon & Lehninger, 1966) and by the apparent presence in rat liver mitochondria of specific tRNA species (Buck & Nass, 1968; Smith & Marcker, 1968).

On the basis of the amount of rRNA extracted from the 8100 *g* membrane fraction and from the small proportion of slower sedimenting mitochondria and endoplasmic reticulum present in the 15,800 *g* pellet, it could be estimated that from 10 to 15% (and possibly as many as 20%) of the total ribosomes in HeLa cells are associated with endoplasmic reticulum. Preferential losses of membrane-associated ribosomes in the low-speed centrifugations (Blobel & Potter, 1967*a*) would, however, lead to an under-estimate of their relative amount in the living cell. It should be pointed out that the concentration of Mg^{2+} during the cell homogenization and subsequent fractionation steps was kept to the minimum compatible with stability of polysomes, namely, $1{\cdot}5\times10^{-4}$ M, in order to discourage any aggregation of free ribosomes and polysomes with membrane components. The observation that the ribosomes of the membrane fraction from HeLa cells behave, as concerns response to EDTA treatment, similarly to ribosomes of the rough endoplasmic reticulum from liver and differently from free ribosomes (see below) supports the conclusion that the ribosomes isolated with this fraction are associated with membrane elements in the intact cell. 65 to 70% of these ribosomes are recovered in the form of polysomes after NaDOC treatment of the membrane fraction and are presumably engaged in protein synthesis *in vivo*: the polysomes released by the detergent show the same RNase and EDTA sensitivity as free polysomes. Unlike the situation described for rat liver (Blobel & Potter, 1966; Howell *et al.*, 1964), in HeLa cells exposure to NaDOC of the rough endoplasmic reticulum structures sedimenting with mitochondria does not lead to an extensive breakdown of membrane-bound polysomes by nuclease action. However, the possibility of a partial degradation of polysomal mRNA after treatment with NaDOC cannot be excluded: as a matter of fact, the release of lysosomal RNase by the action of detergents has been recently described (Penman, Vesco & Penman, 1968). Therefore, the amount of polysomes recovered from the membrane fraction by NaDOC treatment can only provide a minimum estimate of the fraction of membrane-bound ribosomes which are associated with mRNA in the living cell. As was mentioned above, the rRNA of endoplasmic reticulum-bound ribosomes showed the same sensitivity to RNase digestion, under the conditions used in the present work, as rRNA of free polysomes. The apparent discrepancy between this result and the previously reported relative RNase resistance of membrane-bound ribosomes from liver (Blobel & Potter, 1967*b*) is presumably due to the different conditions of digestion used here: particularly significant in this regard may be the higher ionic strength and the presence of a chelating agent in the medium (which would probably cause detachment of ribosomes from the membranes and removal of adventitious ribosomal proteins (Warner & Péne, 1966)) and the much higher (at least 15 times) RNase to RNA ratio.

As discussed earlier, treatment with EDTA (35 to 70 μmoles/g cell equivalent of membrane fraction) releases essentially all the 30 S subunits and about 70% of the 50 S subunits of the endoplasmic reticulum-bound ribosomes. The behavior of these ribosomes upon exposure to EDTA appears, therefore, to be similar to that described for ribosomes of the rough endoplasmic reticulum from rat liver (Sabatini *et al.*, 1966). It is not known which factor(s) are involved in the EDTA-resistant sticking of the 50 S subunits to the membrane. There seems to be a functional relationship between strong attachment and activity in protein synthesis of membrane-bound

ribosomes (Sabatini *et al.*, 1966), although the growing polypeptide chain does not seem to be itself the "hook" (Redman & Sabatini, 1966). It is possible that conformational changes induced in the ribosome, or in the membrane or in both, by the formation of the active complex cause a better fit between a specific receptor on the membrane and a binding site on the ribosome. NaDOC liberates from the EDTA-treated membrane fraction only 35 to 40% of the membrane-stuck subunits as free 50 S particles; the remainder are contained in structures sedimenting faster than 50 S (up to more than 130 S). The nature of these heavier structures is uncertain. The virtual absence of small ribosomal subunits and the resistance to EDTA argues against their being polysomes. Their presence may reflect the tendency of some of the EDTA-resistant 50 S subunits (perhaps those with more complete polypeptide chains) to stick to residues of membrane elements or to aggregate with each other, possibly by interacting nascent chains. It should be mentioned in this connection that, in the case of rough microsomes from rat liver, the particulate material released by NaDOC after EDTA treatment (which consists mainly of a 50 S component and of 80 to 90 S heterogeneous material) contains a major portion of the newly synthesized protein and/or nascent polypeptides labeled *in vivo* during a one-minute [^{3}H]leucine pulse (Sabatini *et al.*, 1966).

As concerns the mRNA of membrane-bound polysomes, one would expect it to be released by EDTA together with the 30 S subribosomal particles, in view of the involvement of the small subunits in the binding of messenger (see review by Attardi, 1967) and in view of the evidence indicating that the ribosomes are attached to the membrane through their 50 S subunits and behave as fixed points, with the mRNA being the moving component of the active complex (Sabatini *et al.*, 1966; Blobel & Potter, 1967*b*). It is likely that the relatively minor portion of labeled heterogeneous RNA of the membrane fraction which is found in the supernatant fraction after EDTA treatment consists, at least in part, of this mRNA.

The observation that the intramitochondrial heterogeneous RNA labeled in a short pulse (which is homologous to mitochondrial DNA (Attardi & Attardi, 1968)) is not removed by EDTA treatment (Table 3) indicates that a portion, possibly the majority, of the heterogeneous RNA remaining in the EDTA-treated membrane fraction is represented by mitochondrial RNA. However, direct evidence obtained with purified subcellular components is needed to establish both the fate, after exposure to EDTA, of the mRNA of endoplasmic reticulum-bound polysomes and the contribution of non-mitochondrial RNA components to the EDTA-resistant fraction.

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ADDITIONAL RESULTS

(a) Isolation of the ribosomal RNA components from free and membrane-bound ribosomes. The two ribosomal subunits were isolated from free and membrane-bound ribosomes of HeLa cells labeled, respectively, with [^{14}C]-uridine and [^{3}H]-uridine; ribosomal RNA was extracted from the subunits and centrifuged in sucrose gradient. Sedimentation analysis of a mixture of the major ribosomal RNA species (from 50 S subunits) and, respectively, of the minor ribosomal RNA species (from 30 S subunits) from the two sources failed to show any difference in sedimentation properties between the homologous rRNA components (Figure 1 a and b). Furthermore, the residual "28 S" rRNA from the EDTA-treated mitochondrial fraction also showed identical sedimentation properties to the 28 S rRNA of free polysomes (Figure 1c).

(b) Kinetics of labeling of the rRNA components from free and membrane-bound ribosomes. HeLa cells were labeled for various times with [5-^{3}H]-uridine (25, 45, 60, 90, 120, 180 min.), and RNA was extracted from the mitochondrial fraction and free polysomes and centrifuged in sucrose gradient (Figure 2). The O. D.$_{260}$ profile shows the two ribosomal RNA components. After 25 min. labeling with [^{3}H]-uridine, all the radioactivity in free polysomal RNA is in the form of heterogeneous RNA (presumably messenger RNA) distributed rather uniformly in the gradient between 6 and more than 45 S (Figure 2a). The RNA of the mitochondrial fraction, after a 25 min. pulse, is 3 to 4 times more labeled, per O. D.$_{260}$ unit of rRNA, than

the free polysomal RNA, reflecting mainly the in situ synthesis of mit-RNA (Figure 2b). One can see in this sedimentation pattern a prominent peak centered around 16-17 S, a smaller broad peak at about 32-33 S, and heavier RNA components accumulated against the cushion of 64% sucrose at the bottom of the tube. (The initial labeling of the 18 S rRNA of the membrane-bound ribosomes can be unmasked after a 30 min. pulse with [5-^{3}H]-uridine if it is carried out in the presence of ethidium bromide to selectively block the transcription of RNA on mit-DNA (see Part I, Chapter 4)). After a 45 min. pulse, the 18 S rRNA component is clearly labeled in the sedimentation patterns from both the free polysomal (Figure 2c) and mitochondrial fractions (where it is superimposed on the broad 16-17 S peak) (Figure 2d); there is no or very little label in either pattern in correspondence with the 28 S O. D. $_{260}$ peak, but a well-defined peak sedimenting slightly ahead of the 28 S (which could mask the initial labeling of 28 S RNA) is again observed in the sedimentation pattern of mitochondria-associated RNA. By 60 min, labeled 28 S RNA can be seen in the free polysomal RNA (Figure 2e), but its specific activity is about one-third of that of the 28 S RNA of the mitochondrial fraction (Figure 2f). At 90 min., the specific activity of the 28 S RNA of free polysomes has become much closer to that of the membrane-associated 28 S RNA (Figure 2g and h) although it tends to remain lower until at least 180 min (Figure 2i and j).

Table 1 summarizes the kinetics of labeling of the two 28 S rRNA components and the ratio of the specific activity of the 28 S RNA from

membrane components to that of the 28 S RNA from free polysomes after different labeling times. The latter is very high after a 60 min. pulse but drops rapidly between 60 and 90 min. labeling time; this drop occurs when the flow of 28 S rRNA into the cytoplasm has become very rapid and is close to the steady state rate.

Concerning the 18 S rRNA, under the conditions of isolation employed here, quantitation is difficult for the RNA of the mitochondrial fraction due to the labeled RNA of mitochondrial origin (16 S and 12 S) which overlaps the 18 S RNA peak. For a quantitative study of the kinetics of labeling of the 18 S rRNA component of membrane-associated ribosomes, it will be necessary to extract RNA from ribosomal subunits released by EDTA from the mitochondrial fraction.

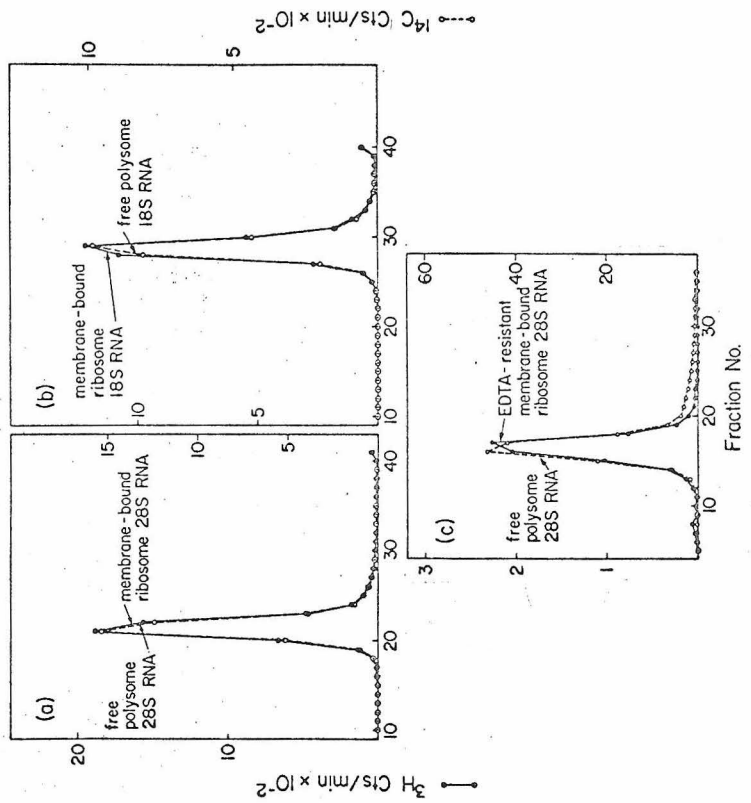
(a)
free polysome 28S RNA
membrane-bound ribosome 28S RNA
(b)
membrane-bound ribosome 18S RNA
free polysome 18S RNA
(c)
free polysome 28S RNA
EDTA-resistant membrane-bound ribosome 28S RNA
³H Cts/min x 10⁻²
¹⁴C Cts/min x 10⁻²
Fraction No.

Figure 1

FIGURE 1. Sedimentation properties of ribosomal RNA from the mitochondrial and free-monomer-polysome fractions of HeLa cells labeled with [5-^{3}H]-uridine or [2-^{14}C]-uridine, respectively, for 48 hr.. (a, b) Two 800 ml. spinners of HeLa cells at 1.4 x 10^{5} cells/ml. were labeled with [5-^{3}H]-uridine (2.5 μC/ml.) or [2-^{14}C]-uridine (0.125 μC/ml.) for 48 hr.. The mitochondrial fraction was prepared from the ^{3}H-labeled cells as described in Materials and Methods of the preceding publication, and treated with DNase (20 μg/ml.) for 20 min. at 3°C. EDTA was then added to 0.03 M, and the suspension, after remaining in the cold for 10 min, was centrifuged at 11,000 g for 10 min.. The supernatant, containing ribosomal subunits, was layered on a 15 to 30% sucrose gradient in 0.05 M tris buffer, pH 7.1, 0.025 M KCl, and 0.001 M EDTA and centrifuged in the Spinco SW 25.2 rotor at 25,000 rev./min. for 17 hr. at 3°C.

The free ribosome-polysome fraction was prepared from the ^{14}C-labeled cells. The first 8100 g supernatant was treated with DNase (20 μg/ml.) for 20 min. at 3°C and then centrifuged in the Spinco Type 65 rotor at 102,000 g for 90 min at 3°C. The resulting pellet was resuspended in 0.25 M sucrose in 0.01 M tris buffer, pH 7.1, 0.01 M EDTA, and centrifuged as above.

The fractions corresponding to the major (50 S) and minor (30 S) derived ribosomal subunits in the two gradients were pooled separately for RNA extraction and precipitated with 2 volumes ethanol in the presence of 0.1 M NaCl and 0.5% SDS. After centrifugation of the

mixtures, each pellet was resuspended in SDS buffer and centrifuged on a 15 to 30% sucrose gradient in SDS buffer in the Spinco SW 25.1 rotor at 25,000 rev./min. for 14.5 hr. at 20°C.

(a) Aliquots of the peak tubes from the sedimentation patterns of ^{14}C-28 S RNA from 50 S subunits of free monomers-polysomes and ^{3}H-RNA from membrane-associated 50 S subunits were mixed and run on a 15 to 30% sucrose gradient in SDS buffer in the SW 25.1 rotor at 20,000 rev./min. for 16 hr. at 20° C.

(b) Aliquots of the peak tubes from the sedimentation pattern of ^{14}C-18 S RNA from 30 S subunits of free monomers-polysomes and of ^{3}H-RNA from membrane-associated 30 S subunits were mixed and centrifuged as above.

(c) One spinner (400 ml.) at 1.2 x 10^{5} cells/ml. was labeled with [5-^{3}H]-uridine (0.3 μC/ml.) for 24 hr.. The mitochondrial fraction was resuspended in 0.25 M sucrose in 0.01 M tris buffer, pH 7.1, with 0.03 M EDTA, left 10 min. in the cold, and centrifuged at 11,000 g for 10 min.. The pellet was washed once with 0.015 M EDTA and then centrifuged on a 30 to 48% sucrose gradient in the Spinco SW 25.1 rotor at 25,000 rev./min. for 22 hr. at 3°C. The fractions corresponding to the mitochondrial band were pooled, and RNA was extracted as above and centrifuged on a 15 to 30% sucrose gradient in SDS buffer in the SW 25.1 rotor at 20,000 rev./min. for 17.5 hr. at 20°C. Aliquots of the two peak fractions corresponding to "28 S" RNA were mixed with ^{14}C-28 S RNA from 50 S subunits of free monomers-polysomes and centrifuged on a 15 to 30% sucrose

gradient in SDS buffer in the SW 25.1 rotor at 20,000 rev./min. for 15 hr. at 20°C.

●——— ^{3}H cts./min.

o------o ^{14}C cts./min.

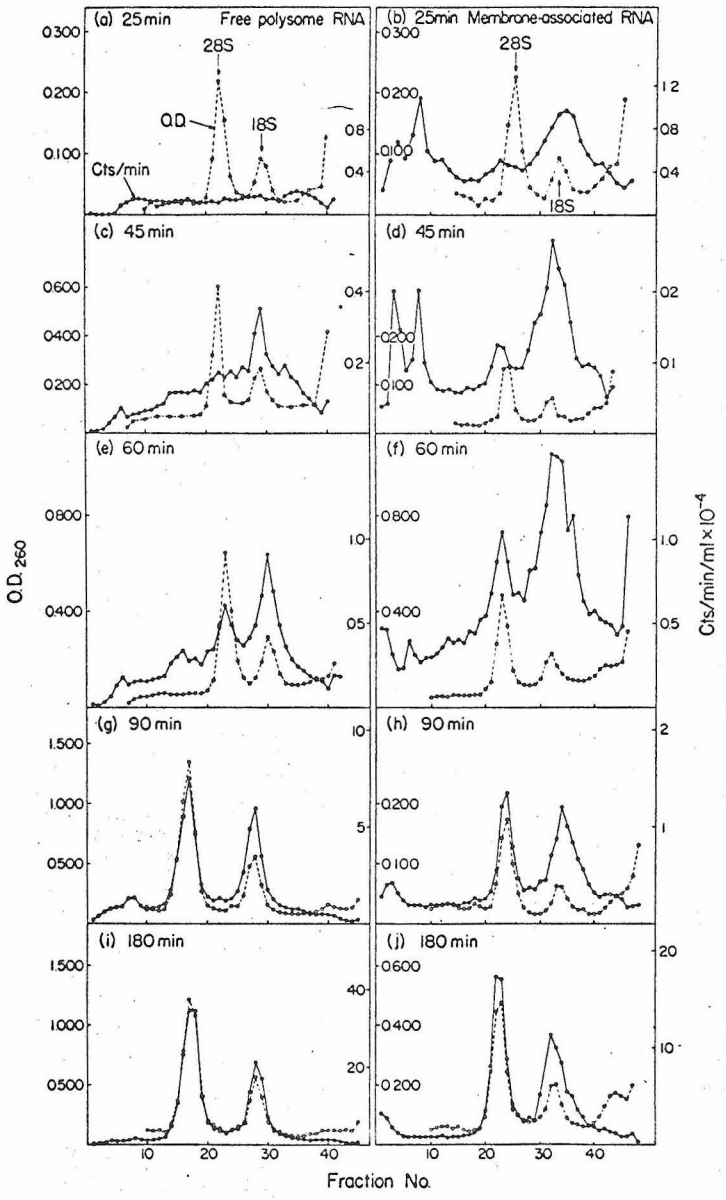
(a) 25 min
Free polysome RNA
28S
O.D.
18S
Cts/min
(b) 25min Membrane-associated RNA
28S
18S
(c) 45 min
(d) 45 min
(e) 60 min
(f) 60 min
(g) 90 min
(h) 90 min
(i) 180 min
(j) 180 min
O.D.260
Cts/min/ml x 10⁻⁴
Fraction No.

Figure 2

FIGURE 2. Sedimentation patterns of RNA from the mitochondrial fraction and from free polysomes of HeLa cells labeled for various times with [5-^{3}H]-uridine. [5-^{3}H]-uridine (3.3 μC/ml.) was added to each of two spinners containing 900 ml. HeLa cells at 1.4×10^5 cells/ml.. At the following times 300 ml. samples were removed from the spinners and poured over 50 ml. frozen and crushed NKM (0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$): 25, 45, 60, 90, 120, and 180 min.. The mitochondrial and free ribosomal-polysomal fractions were prepared as described in Materials and Methods in the preceding publication. The mitochondrial fraction was centrifuged on a 30 to 48% sucrose gradient in 0.01 M tris buffer, pH 7.1, in the Spinco SW 25.1 rotor at 25,000 rev./min. for 20 hr. at 3°C. Free polysomes were separated from monomers, ribosomal subunits, and soluble components by centrifuging 3 to 5 ml. of the 15,800 g supernatant through a sucrose gradient consisting, from the meniscus to the bottom, of 6 ml. 23% sucrose, 12 ml 23 to 55% sucrose gradient, and 6 ml. 55% sucrose, all in 0.01 M tris buffer, pH 7.1, 0.00015 M $MgCl_2$, in the SW 25.1 rotor at 25,000 rev./min. for 5 hr. at 3°C.

RNA was extracted from the mitochondrial band and from the free polysomes by SDS and centrifuged on a 15 to 30% sucrose gradient (over 3 ml. 64% sucrose) in SDS buffer (0.01 M tris buffer, pH 7.0, 0.1 M NaCl, 0.001 M EDTA, 0.5% SDS) in the SW 25.1 rotor at 22,000 rev./min. for 16 hr. at 20°C.

0------0 O. D.$_{260}$ ●——● ^{3}H cts./min.

TABLE 1

Specific Activity of 28 S rRNA from membrane-bound ribosomes and free polysomes from HeLa cells labeled for various times with [5-^{3}H]-uridine

Labeling time (min.)	Cts. /min. /ml. /O. D.$_{260}$		
	Membrane-bound 28 S r RNA	Free polysomal 28 S r RNA	Membrane-bound 28 S / Free polysomal 28 S
60	12,000	3,930	3.05
90	67,200	54,700	1.23
120	139,000	121,000	1.15
180	353,000	322,000	1.10

The data shown in this table are derived from the RNA sedimentation patterns of Figure 2 and two additional sedimentation patterns of RNA labeled during a 120 min pulse with [5-^{3}H]-uridine. The O. D.$_{260}$ in 28 S rRNA was estimated by drawing a baseline under the two rRNA components; the baseline for radioactivity in 28 S RNA was drawn from the furrow between the 18 S and 28 S peaks to the advancing edge of the 28 S peak.

ADDITIONAL DISCUSSION

The results presented in the preceding publication have shown that 10-15% of the ribosomes in HeLa cells can be isolated from the mitochondrial fraction which contains, in addition to mitochondria, at least 85-90% of the elements of rough endoplasmic reticulum found in the HeLa cell cytoplasm. In this work considerable attention was devoted to obtaining evidence as to what, if any, portion of the ribosomes of the mitochondrial fraction is located in mitochondria. Due to the difficulty of obtaining mitochondria free from contaminating E. R., either by differential centrifugation or by buoyant density or sedimentation velocity fractionation in sucrose gradients, the sensitivity to pancreatic RNase of the membrane-associated ribosomes and their response to EDTA treatment were used to distinguish extramitochondrial from intramitochondrial ribosomes. Both types of analysis led to the conclusion that the great majority of the ribosomes of the mitochondrial fraction are bound to the membranes of the rough E. R. although, as discussed below, the presence of a small amount of intramitochondrial ribosomes has not been excluded.

The sensitivity of the rRNA of the membrane-associated ribosomes to RNase digestion <u>in situ</u> under the conditions used here (which leave the great majority of pulse-labeled mit-RNA intact) is very similar to that of the rRNA of free polysomes. If as many as 10% of the ribosomes of the mitochondrial fraction were intramitochondrial, in order to account for the observed effects of the RNase, the unlikely assumption would have to be made that the sensitivity to

RNase of the rRNA of membrane-bound ribosomes is significantly higher than that of rRNA of free polysomes.

As concerns EDTA treatment, investigations carried out on rough microsomes from rat liver have shown that if these are treated with sufficiently high concentrations of EDTA, essentially all the small ribosomal subunits and a limiting amount of 50-60% of the large ribosomal subunits are released (4). A situation similar to that observed for the microsomes of rat liver appears to hold for the membrane-associated ribosomes of HeLa cells; EDTA treatment of the mitochondrial fraction results in the release of about 70% of the large ribosomal subunits and 85-90% of the small subunits. (The latter figure, revised since the preceding publication, has been estimated from the amount of O. D.$_{260}$ in 18 S rRNA in the sedimentation profile of RNA extracted from the EDTA-treated mitochondrial fraction and centrifuged on long sucrose gradients in the SW 25.3 or SW 27 Spinco rotors (see Part I, Chapters 3, 4)). This residual 18 S RNA not removed by EDTA may set an upper limit for the amount of intramitochondrial ribosomes (representing, at most, therefore, 1-2% of the total cytoplasmic ribosomes); however, these hypothetical intramitochondrial ribosomes, containing 18 S and 28 S RNA, because of the delay in appearance of label in their rRNA components, the general similarity in base composition and methylation pattern of the EDTA-resistant 28 S rRNA component to the 28 S rRNA of free polysomes, and the failure of the EDTA-resistant 28 S and 18 S rRNA components to hybridize with mit-DNA (Part I, Chapter 3)

appear to be of nuclear origin. In addition, the sedimentation properties of the two rRNA species remaining in the mitochondrial fraction after EDTA treatment are identical to those of the two free polysomal rRNA's (for 28 S see Additional Results, Figure 1c; for 18 S see Part I, Chapter 3).

In Neurospora and yeast the occurrence of specific mitochondrial ribosomes containing rRNA components with sedimentation properties and base composition different from those of the homologous cytoplasmic species (5, 6, 7, 8, 9, 10) and which hybridize with mit-DNA (11, 12) has been reported; furthermore, polysome-like structures have been isolated from the mitochondria of Neurospora (6) and yeast (10), and clusters of ribosomes have been observed in the electron microscope in yeast mitochondria (10). In higher organisms the existence of mitochondrial ribosomes can be inferred from the protein synthetic capacity of mitochondria in vitro (13, 14, 15, 16) including HeLa cells (M. Lederman, personal communication) and from the presence in mitochondria from rat liver (17, 18) and HeLa cells (19) of specific tRNA species, but there is as of yet no definitive evidence as to the nature of these ribosomes. It is likely that the early observations of 78-80 S ribosomes in mitochondrial preparations from animal cells (20, 21, 22) were due, at least in part, to contamination by elements of rough E. R. because of the difficulty in separating the two types of organelles as described here; however, it is not ruled out that animal cell mitochondria contain a small number of ribosomes of nuclear origin similar to the cytoplasmic species.

A 55 S particle, which is labeled by incubation of intact mitochondria in vitro with [^{14}C]-leucine for 5 min., has been isolated by O'Brien and Kalf (23) from rat liver mitochondria extensively washed with medium lacking Mg^{++}. Although it has not rigorously been excluded that this particle represents membrane-stuck large ribosomal subunits derived from dissociation of membrane-bound ribosomes in the absence of Mg^{++}, the possibility that it represents some sort of specific mitochondrial particle has been supported by recent evidence of Ashwell and Work (24). The latter authors have confirmed the isolation from rat liver of a 50-55 S particle which is preferentially labeled during a short incubation of mitochondria in vitro with a labeled amino acid; furthermore, they have also shown that this labeling can be chased from the 50-55 S peak by puromycin, suggesting that it is in nascent polypeptide chains, and that it is sensitive to chloramphenicol, which inhibits selectively protein synthesis in mitochondria (25, 26) and insensitive to cycloheximide, which inhibits, on the contrary, protein synthesis on cytoplasmic ribosomes (27, 28). These particles could correspond to the intramitochondrial RNA-containing particles, smaller than cytoplasmic ribosomes (their reported diameter varies from 80 to 150 Å as compared to 150 to 200 Å for bacterial ribosomes (29, 30) and 180 to 230 Å for cytoplasmic ribosomes of higher organisms (29, 31, 32)) which electron microscopic examination has revealed in cells from various higher organisms (32, 33, 34, 35) and which may be homologous to small granules observed also in the mitochondria from HeLa cells

in the present work. However, the final identification of the 55 S particle will depend on the characterization of the RNA it contains.

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