Ribosomal-Type Ribonucleic Acid from Rodent Mitochondria

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1. Highly purified mitochondria containing 3.0 μ g of RNA/mg of mitochondrial protein were prepared from rat liver by differential centrifugation. 2. RNA, labelled with [32 P]P_i or [3 H]orotate, was isolated from these mitochondria by a phenol extraction method. The RNA sedimented at 15S and 13S on sucrose density gradients. Its nucleotide composition was 23% uridylate, 30% adenylate, 22% guanylate and 25% cytidylate. 3. RNA from mouse L cells was labelled with [3 H]-uridine in the presence of 0.1 μ g of actinomycin D/ml to suppress the synthesis of cytoplasmic rRNA. The RNA isolated from crude L-cell mitochondria by a cold-phenol-sodium dodecyl sulphate method had components sedimenting at 15S and 12.5S. These components had an electrophoretic mobility on agarose-acrylamide gels of 21 and 12S_E compared with 28 and 18S_E for cytoplasmic rRNA. The nucleotide composition was 26% uridylate, 34% adenylate, 18% guanylate and 22% cytidylate. 4. RNA extracted from crude L-cell mitochondria by a hot-phenol-sodium dodecyl sulphate method had an additional component sedimenting at 21S and having an electrophoretic mobility of 18S_E. It was probably DNA because of its sensitivity to deoxyribonuclease and its insensitivity to ribonuclease and alkali. It was present in nuclear fragments contaminating the crude mitochondrial fraction and could be removed by deoxyribonuclease or isopycnic-gradient centrifugation.

Mitochondria from many species synthesize protein (Haldar & Freeman, 1969; Kuntzel & Noll, 1967; Lamb, Clark-Walker & Linnane, 1968). It is not certain in all cases whether this synthesis occurs on a specific class of mitochondrial ribosomes. Mitochondria from *Neurospora crassa* have been shown to contain ribosomes and rRNA that are distinct from those of the cytoplasm (Rifkin, Wood & Luck, 1967; Kuntzel & Noll, 1967; Dure, Epler & Barnett, 1967). Only a preliminary characterization of ribosomes from yeast mitochondria has been given (Morimoto & Halvorson, 1970) but several groups (Wintersberger, 1967; Rogers, Preston, Titchener & Linnane, 1967; Leon & Mahler, 1968; Fauman, Rabinowitz & Getz, 1969) have reported RNA species of 23 and 16S, the same size as bacterial rRNA.

In mammals the situation has not been resolved although ribosomal-type RNA has been isolated from mammalian mitochondrial fractions. Dubin & Brown (1967) and Dubin (1969) found that mitochondrial RNA from hamster BHK-21 cells had sedimentation coefficients of 27 and 17S and the same base composition as cytoplasmic rRNA. However, radioactively labelled mitochondrial RNA isolated from hamster cells, incubated in the presence of a low concentration of actinomycin D to suppress the synthesis of cytoplasmic rRNA, had a

sedimentation coefficient of 17S and a high content of adenine and uracil (Dubin, 1967, 1969). Vesco & Penman (1969) and Zylber, Vesco & Penman (1969) have found species of RNA from mitochondria of HeLa cells, labelled in the presence of actinomycin D, that sedimented at about 12-19S in sucrose density gradients. Electrophoresis on acrylamide gel separated the RNA into species that migrated at 21 and 12S_E.^{*} These species, as well as 28S and 18S RNA, were found in RNA isolated from mitochondria of HeLa cells labelled in the absence of actinomycin D (Vesco & Penman, 1969; Knight, 1969). In contrast with these studies on mitochondrial RNA, Kroon & Aaij (1968) have reported that mitochondria from regenerating rat liver have a 23S RNA species. Attardi & Attardi (1969) found that the main species of RNA in mitochondrial fractions of HeLa cells that hybridizes with mitochondrial DNA is 12S.

Vesco & Penman (1969) found that the mitochondrial RNA of HeLa cells arose from particles sedimenting at 45S. It is not certain whether these particles are ribosomal in nature. O'Brien & Kalf (1967*a,b*) had previously reported a 55S ribosomal particle from rat liver mitochondria.

^{*} Abbreviation: S_E is the electrophoretic mobility obtained by interpolation with the mobility of 28S and 18S cytoplasmic rRNA as the reference.

In the present paper we examine the mitochondrial RNA from highly purified rat liver mitochondria and find that it sediments at 15 and 13S. This is about the same as the RNA isolated from a crude mitochondrial fraction of L cells labelled in the presence of actinomycin D. The L-cell mitochondrial RNA had a mobility of 21 and 12S_E on electrophoresis. The L-cell mitochondrial fraction also contained a component, probably DNA, with a sedimentation coefficient of 21S and an electrophoretic mobility of 18S_E.

METHODS AND MATERIALS

Isolation of rat liver mitochondria. The mitochondrial fraction was prepared from livers of hooded rats with a technique designed to eliminate as much of the endoplasmic reticulum and cytoplasmic ribosomes as possible. It is similar to the method of O'Brien & Kalf (1967a). Livers were homogenized with a Potter Teflon homogenizer in 0.34 M-sucrose–2 mM-EDTA–2 mM-tris–HCl, pH 7.4 (medium A). Nuclei and cell debris were centrifuged at 1000g for 10 min and washed with 5 vol. of medium A. The combined nuclear supernatant fractions were layered over a 20–35% (w/v) sucrose density gradient in 2 mM-EDTA–2 mM-tris–HCl, pH 7.4, which filled about 66% of each tube for the SW25.1 rotor of the Beckman model L2 ultracentrifuge. A mitochondrial pellet was recovered by centrifugation for 2 h at 25000g_{av.} The pellet was then washed four times with medium A by centrifugation at 6500g for 10 min. The fluffy layer was decanted in all cases and the surface of the pellet washed before resuspension of the mitochondrial fraction.

Isolation of L-cell mitochondria. L cells were grown in suspension in Joklik's modification of minimal essential medium (Eagle, 1959) with 5% foetal calf serum and 1% (v/v) anti-PPLO (mycoplasma) agent. Mitochondria were prepared from cells disrupted with an Ultra-Turrax homogenizer essentially as described by Freeman (1965). About $1.8\text{--}2\times 10^8$ cells were washed twice with 0.3 M-sucrose–2 mM-EDTA–2 mM-tris–HCl, pH 7.4, suspended in 10 ml of this solution and disrupted by homogenization for 90 s (30 s intervals) at 70 V. KCl was added to a final concentration of 0.02 M. Nuclei and cell debris were removed by two centrifugations at 1000g for 15 min. The mitochondrial fraction was centrifuged at 6500g for 15 min and washed three times with 0.3 M-sucrose–2 mM-EDTA–2 mM-tris–HCl, pH 7.4. The crude mitochondrial fraction was purified further by isopycnic-gradient centrifugation (Freeman, 1965) for 3 h at 165000g_{av.} on a 30–60% sucrose density gradient in 2 mM-EDTA–2 mM-tris–HCl, pH 7.4, in the SW41 rotor of the Beckman model L2 ultracentrifuge. The bulk of the fraction was recovered as a band in the centre of the tube and was designated the mitochondrial fraction (Freeman, 1965). In addition nuclei and nuclear fragments sedimented to the bottom of the tube and an unidentified band was observed above the mitochondrial band.

Labelling of RNA. The mitochondrial RNA of rat liver was labelled by intraperitoneal injection of four 150 g rats with a total of 10 mCi of [³²P]P_i or 5 mCi of [5-³H]orotate 24–48 h before the rats were killed. Cytoplasmic RNA of L cells was labelled with 0.2 μCi of [2-¹⁴C]uridine/ml at a

cell concentration of 3.0×10^5 /ml for 20 h. The mitochondrial RNA of L cells was labelled with 10 μCi of [5-³H]uridine/ml in the presence of 0.1 μg of actinomycin D/ml. The conditions of labelling are given for each experiment.

Extraction of RNA. Rat liver cytoplasmic rRNA was isolated by method I of Kirby (1965) and stored as a dried powder at –20°C. Rat liver mitochondrial RNA was also extracted by method I of Kirby (1965) modified in two ways. In the first method, 2 g of rat liver was extracted with the phenol–cresol mixture as described by Kirby (1965). The aqueous layer from this extract was added to 1 vol. of phenol–cresol mixture containing 8% (w/v) of sodium tri-isopropyl-naphthalenesulphonate, stirred and the emulsion added to the rat liver mitochondria of about 120 mg of protein which had been suspended in 2 ml of medium A. Stirring was continued for 1 h at 25°C. The aqueous phase was recovered, re-extracted with 0.5 vol. of phenol–cresol mixture and the RNA recovered as described by Kirby (1965). The yield of RNA was 10 mg. In one experiment, mitochondrial RNA was isolated by this method in the absence of carrier cytoplasmic RNA. In the second modification, the mitochondrial pellet of about 120 mg of protein was made up to 2 ml in ice-cold medium A and the mitochondria were then disrupted by the addition at 25°C of 5 ml of a solution of 2% sodium dodecyl sulphate, 0.1% macaloid, 25 mM-sodium acetate, 1% NaCl, 10 mM-MgCl₂ and 5 mg of rat liver cytoplasmic rRNA, all at pH 4.8 (medium B). After 1 min, 1 vol. of phenol–cresol mixture, containing 5% (w/v) sodium tri-isopropyl-naphthalenesulphonate, was added and the mixture stirred for 1 h at 25°C. The aqueous layer was re-extracted once with 1 vol. and once with 0.5 vol. of phenol–cresol mixture and the RNA recovered by the method of Kirby (1965).

The RNA from mitochondria from 2×10^8 L cells was extracted essentially as in the second method described above. The mitochondria (4 mg of protein) were suspended in 2 ml of ice-cold medium A, disrupted by the addition of 5 ml of medium B without cytoplasmic rRNA, immediately added to 1 vol. of phenol–cresol mixture containing 5% of sodium tri-isopropyl-naphthalenesulphonate and stirred for 1 h at 4°C or 25°C. Previously 0.3 ml of a freshly prepared post-nuclear supernatant from 1.1×10^7 L cells was added to the phenol–cresol mixture to provide carrier RNA. The only other essential difference from that described above was in the initial precipitation of RNA. It was necessary to add *m*-cresol until turbidity occurred. After 1 h at 0°C, the wash solution used in method I of Kirby (1965) was added dropwise until the turbidity disappeared. On centrifugation, a gelatinous pellet of RNA was recovered. This was washed and dried as described by Kirby (1965). This method is referred to below as the cold-phenol–sodium dodecyl sulphate technique.

Cytoplasmic RNA of L cells labelled with [2-¹⁴C]uridine was isolated from a post-mitochondrial supernatant fraction. The RNA was extracted first with an equal volume of phenol–cresol mixture, re-extracted with 0.5 vol. of phenol–cresol mixture containing 5% (w/v) of sodium tri-isopropyl-naphthalenesulphonate and the RNA precipitated, washed and dried as in the extraction of L-cell mitochondrial RNA.

RNA was also extracted from mitochondrial fractions

from 2×10^8 L cells by the method of Penman (1966) in the presence of post-nuclear supernatant from 2×10^7 cells. Precipitation of the RNA with alcohol was overnight at -20°C . This method involves a hot-phenol-sodium dodecyl sulphate extraction and will be so designated. The method used to extract RNA is indicated in the appropriate legend.

Sucrose-density-gradient centrifugation of RNA. Sucrose density gradients [15–33% (w/v) sucrose in 0.1 M-sodium acetate, pH 6.0] were prepared as described by Noll (1967). This is a convex gradient that gives isokinetic sedimentation. Centrifugation was for 11 h at 2°C at 180000 g_{av} . in the SB283 head of the International B 60 ultracentrifuge. Alternatively linear density gradients [15–30% (w/v) sucrose] were used as described by Gilbert (1963), with centrifugation for 16 h at 15°C at 75000 g_{av} . The gradients were fractionated by puncturing the bottom of the tube and 0.2 ml fractions were collected dropwise. The E_{260} of each fraction was read and the radioactivity determined.

Measurement of radioactivity. Fractions of ^{32}P -labelled RNA were dried on Whatman 3MM paper, and their radioactivities counted in a toluene scintillation solution (Haldar & Freeman, 1968). Fractions of ^3H - or ^{14}C -labelled RNA were digested overnight with 0.4 ml of water and 0.5 ml of NCS solubilizer, a strong base, and the radioactivity was determined in 12 ml of naphthalene-dioxan scintillation solvent (Haldar & Freeman, 1969). Dual-labelled samples (^{14}C and ^3H) were counted on two channels of the Nuclear-Chicago mark 1 counter. Corrections for ^{14}C counts in the ^3H channel were made by the addition of an internal standard ($[^{14}\text{C}]$ methanol), re-counting and computer calculation.

Electrophoresis of RNA. Electrophoresis was by a modification of the methods of Watanabe, Prevec & Graham (1967) and Peacock & Dingman (1968). A solution containing 4.4% (w/v) of acrylamide, 0.24% (w/v) of methylenebisacrylamide, 0.05% (w/v) of $\text{NNN}'\text{N}'$ -tetramethylenediamine and 1% (w/v) of sodium dodecyl sulphate in $2\times$ concentrated reservoir buffer, was kept at 0°C for 0.5 h. Ammonium persulphate was added to 0.1% and the solution was mixed with an equal volume of a boiling solution of 0.5% (w/v) agarose. This was poured into plugged electrophoresis tubes, allowed to solidify for 3 h and used immediately. The plug was removed, the tube inverted and a sample of 30 μg of RNA in 0.01 M-sodium acetate, pH 6.0, was applied to the surface of the gel. The RNA was either $[5\text{-}^3\text{H}]$ uridine-labelled mitochondrial RNA with unlabelled carrier, sometimes with $[2\text{-}^{14}\text{C}]$ uridine-labelled cytoplasmic rRNA added as a marker, or the marker alone. Electrophoresis was for 45 min at 200 V (30 V/cm) at 20 mA/tube. The reservoir buffer contained 108 g of tris, 9.3 g of EDTA and 55 g of boric acid in 10 litres of water at a final pH of 8.3. Sodium dodecyl sulphate was added to the buffer to 0.25% before electrophoresis.

The logarithm of the sedimentation rate or molecular weight of 28 and 18 S cytoplasmic rRNA and 4 S RNA was linearly related to the distance moved (Loening, 1968). The mobilities interpolated for mitochondrial RNA are designated S_E . For counting of radioactivity, gels were sliced in 1 mm widths with a machine designed in our laboratory. Slices were digested overnight with 0.5 ml of water and 0.5 ml of NCS solubilizer. Then 10 ml of naphthalene-dioxan scintillation fluid was added and

the vials were shaken for 20 min and their radioactivities counted.

Nucleotide composition. The nucleotide composition of rat liver mitochondrial RNA and mitochondrial RNA of L cells, extracted by the cold-phenol-sodium dodecyl sulphate method, was determined. About 0.5 mg of RNA labelled with $[^{32}\text{P}]\text{P}_i$ was digested in 0.2 ml of 0.3 M-KOH for 18 h at 37°C . K^+ was removed at 0°C by the addition of 2 M- HClO_4 to pH 7.0. Nucleotides were separated by chromatography (Lane, 1963) or by electrophoresis on Whatman 3MM paper at pH 3.5 in 0.05 M-ammonium formate buffer for 2 h at 3500 V. The results were identical and only those obtained by electrophoresis are given. The nucleotides were located by u.v. light, cut out and their radioactivities counted with the toluene-based scintillation solvent. No radioactivity was found between the spots. In one experiment unlabelled mitochondrial RNA, isolated in the absence of carrier cytoplasmic RNA, was analysed for nucleotide composition by the method of Katz & Comb (1963).

Chemical and enzymic determinations. Protein, RNA and DNA were measured and cytochrome oxidase and malate dehydrogenase (EC 1.1.1.37) assayed as described previously (Freeman, 1965). Glucose 6-phosphatase (EC 3.1.3.9) was assayed as described by Roodyn, Reis & Work (1961) and acid phosphatase (EC 3.1.3.2) was measured by the method of Gianetto & de Duve (1955). In each case, a unit of enzyme activity is defined as the release of 1 μg of P_i /min under the conditions of assay. P_i was measured by the method of Chen, Toribara & Warner (1956).

Materials. All chemicals were reagent-grade where possible. Actinomycin D was a gift of Merck, Sharp and Dohme Ltd., Montreal, Que., Canada. Sucrose for the gradient centrifugation was ribonuclease-free and was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Acrylamide was the highest purity grade from Distillation Products, Rochester, N.Y., U.S.A. Methylenebisacrylamide was purified as described by Loening (1967). Agarose was obtained from Bausch and Lomb Co. Ltd., Toronto, Ont., Canada; not every batch was suitable. Lot 962506 was used for all experiments reported here. Joklik's modification of minimal essential medium, foetal calf serum and anti-PPLO agent were obtained from Grand Island Biological Co., Grand Island, N.Y., U.S.A. Macaloid was a gift of the Inerto Co., Las Vegas, Nev., U.S.A. Deoxyribonuclease, electrophoretically purified from ribonuclease, was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. and ribonuclease A type IIA was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. NCS solubilizer was obtained from Amersham/Searle Corp., Toronto, Ont., Canada. Carrier-free $[^{32}\text{P}]\text{P}_i$ was obtained from Atomic Energy of Canada Ltd., Chalk River, Ont., Canada, and $[^{14}\text{C}]$ methanol (50 mCi/mmol), $[5\text{-}^3\text{H}]$ orotic acid (4 Ci/mmol), $[5\text{-}^3\text{H}]$ uridine (2–5 Ci/mmol), $[6\text{-}^{14}\text{C}]$ orotic acid (50 mCi/mmol) and $[2\text{-}^{14}\text{C}]$ uridine (50 mCi/mmol) were obtained from Amersham/Searle Corp., Toronto, Ont., Canada.

RESULTS

Rat liver mitochondria. A typical purification of rat liver mitochondria is illustrated in Table 1.

Table 1. *Distribution of enzymic activity during purification of rat liver mitochondria*

Mitochondria were purified and enzymes were assayed as described in the Methods and Materials section. The mitochondria (gradient) and mitochondrial supernatant fractions refer to the pellet and supernatant obtained on sucrose-density-gradient centrifugation. The final fraction (mitochondria) is the pellet obtained on washing the mitochondrial pellet from the gradient four times. The enzymic activity in the nuclear supernatant is taken as 100%.

Fraction	Cytochrome oxidase		Malate dehydrogenase		Acid phosphatase		Glucose 6-phosphatase	
	(units/mg of protein)	(%)	(units/mg of protein)	(%)	(units/mg of protein)	(%)	(units/mg of protein)	(%)
Nuclear supernatant	1.842	100.0	1.908	100.0	0.210	100.0	1.410	100.0
Mitochondria (gradient)	2.980	71.5	2.016	48.2	0.373	78.5	0.873	27.4
Mitochondrial supernatant	0.149	11.0	0.634	48.1	0.061	41.0	0.716	71.4
Mitochondria (4 times washed)	2.990	20.7	2.060	15.6	0.015	1.0	0.157	1.6

Fig. 1. Sucrose-density-gradient centrifugation of RNA from rat liver mitochondria labelled with [^3H]-orotic acid. RNA of four rats was labelled by intraperitoneal injection of a total of 5mCi of [^3H]orotate 24h before the rats were killed. RNA was extracted from the purified mitochondria with 2g of carrier rat liver by the first modification of the method of Kirby (1965) as described in the Methods and Materials section. RNA (800 μg) was centrifuged on an isokinetic sucrose density gradient and the E_{260} and the radioactivity of each fraction were determined as outlined in the Methods and Materials section. $\square$, E_{260} of cytoplasmic rRNA; $\bullet$, radioactivity of mitochondrial RNA.

Centrifugation through the 20–35% sucrose density gradient freed the mitochondria from some of the endoplasmic reticulum as judged by the recovery of glucose 6-phosphatase. After extensive washing of the mitochondrial fraction about 21% of the cytochrome oxidase activity and 16% of malate dehydrogenase activity was recovered in the purified mitochondria. Only about 1% of the acid phosphatase

and glucose 6-phosphatase activity remained, indicating the removal of the lysosomes and endoplasmic reticulum respectively from the mitochondria. These activities were recovered in the wash solutions. The activities of acid phosphatase and glucose 6-phosphatase in the mitochondrial fraction, when followed in successive washings, reached a minimum. Electron microscopy by

negative staining (Parsons, 1967) of the purified mitochondrial fraction showed only mitochondria, very often lacking outer membrane, and no endoplasmic reticulum.

The purified mitochondrial fraction from rat liver had $3.0\mu\text{g}$ of RNA/mg of protein and $0.24\mu\text{g}$ of DNA/mg of protein. The former value compares with one of $6.6\mu\text{g}$ of RNA/mg of protein found by O'Brien & Kalf (1967a) and $0.54\mu\text{g}$ of DNA/mg of protein found by Schneider & Kuff (1965). Two factors were important in the preparation of a rat liver mitochondrial fraction with a small amount of RNA. The first was the removal of as much as possible of the endoplasmic reticulum and ribosomes before the mitochondria were sedimented for the first time. This was achieved by layering the nuclear supernatant over a sucrose density gradient so that on centrifugation the mitochondria but not much of the endoplasmic reticulum and ribosomes sedimented. Secondly, successive washings with a sucrose-EDTA solution was very effective in lowering the RNA content whereas isopycnic gradient centrifugation was not. This was also observed by O'Brien & Kalf (1967a).

Rat liver mitochondrial RNA. There are two major problems that must be overcome in the

isolation of RNA from rat liver mitochondria. The first is their low content of RNA and the second is the presence of three ribonucleases in the mitochondrial fraction (Rahman, 1966). The first problem can be solved by labelling with either $[5\text{-}^3\text{H}]\text{orotic acid}$ or $[^{32}\text{P}]\text{P}_i$ and isolating the RNA in the presence of cytoplasmic carrier rRNA. To overcome the second problem the RNA must be isolated by a method that removes or inactivates the ribonucleases. Success in this can be judged only by the nature of the RNA isolated. A modification of the phenol extraction method (Kirby, 1965) in which rRNA is separated from low-molecular-weight RNA was used in the present study. The results of typical extractions are shown in Figs. 1 ($[5\text{-}^3\text{H}]\text{orotic acid}$ -labelled RNA) and 2 ($[^{32}\text{P}]\text{P}_i$ -labelled RNA). There were two major radioactive peaks which sedimented at an average of 15.3 and 12.8S as calculated by the method of Noll (1967), 28S and 18S cytoplasmic rRNA being used as the reference standards. Because of the limits of accuracy values of 15 and 13S for the mitochondrial RNA will be used in subsequent discussion. There was always some radioactive RNA with higher sedimentation values, including peaks at 28 and 23S, which were probably cytoplasmic contaminants.

Fig. 2. Sucrose-density-gradient centrifugation of RNA from rat liver mitochondria labelled with $[^{32}\text{P}]\text{P}_i$. RNA of four rats was labelled by intraperitoneal injection of a total of 10 mCi of $[^{32}\text{P}]\text{P}_i$ 48 h before the rats were killed. RNA was extracted from the purified mitochondria by the second modification of the method of Kirby (1965) as described in the Methods and Materials section. Sucrose-density-gradient centrifugation of $400\mu\text{g}$ of RNA was as described in Fig. 1. $\square$, E_{260} of cytoplasmic rRNA; $\bullet$, radioactivity of mitochondrial RNA.

In some experiments when a small amount of degradation of the mitochondrial RNA was apparent, there was a peak at 9S. In all experiments, as shown in Figs. 1 and 2, some mitochondrial 4S RNA but no cytoplasmic 4S RNA was observed. No 4S RNA would be expected by the method of Kirby (1965), in which rRNA is selectively recovered. If the 15S and 13S RNA is mitochondrial rRNA then the sedimentation values are less than those of rRNA from eucaryotic and procaryotic organism. It is possible therefore that they represent degradation products of higher-molecular-weight components. This possibility has not been completely excluded but it has been found that once isolated and separated by sucrose-density-gradient centrifugation the RNA is stable. Purified RNA components larger than 16S were not degraded to 15S and 13S RNA nor were the latter degraded to smaller components when recentrifuged on sucrose density gradients.

Several attempts were made to analyse rat liver mitochondrial RNA by electrophoresis in gels of acrylamide-agarose, but without success. The cytoplasmic rRNA entered the gel and separated into 28S_E and 18S_E species but the mitochondrial RNA stayed on the top of the gel. This result is unexpected, because slower-sedimenting RNA migrates more rapidly on electrophoresis. The nucleotide composition of rat liver mitochondrial RNA is discussed below.

In one experiment an attempt was made to isolate unlabelled mitochondrial RNA without carrier cytoplasmic rRNA. No distinct species of RNA, as judged by sedimentation were obtained

but the nucleotide composition of the RNA was determined and the results are given below.

L-cell mitochondrial RNA extracted by the cold-phenol-sodium dodecyl sulphate method. The RNA isolated from a crude mitochondrial fraction of L cells sediments at the same rate as carrier cytoplasmic RNA except for a small amount of RNA sedimenting from 11 to 18S. RNA extracted by the cold-phenol-sodium dodecyl sulphate method at 25°C from a crude mitochondrial fraction of L cells labelled with [³H]uridine in the presence of 0.1 µg of actinomycin D/ml to suppress the synthesis of cytoplasmic rRNA (Penman, Vesco & Penman, 1968) sedimented at about 15 and 12.5S, as shown in Fig. 3. RNA extracted at 4°C, as shown in Fig. 4, or at 25°C, showed major peaks at 21.0 and 12.3S_E on electrophoresis and a minor peak at 18S_E. Because of the limits of accuracy values of 21 and 12S_E will be used for the mitochondrial RNA. Electrophoresis of fractions from gradients showed that the 15S RNA ran at 21S_E and the 13S RNA at 12S_E. The ratio of the 21S_E to the 12S_E component averaged 1.8. This calculation did not include the area just preceding the 21S_E and 12S_E peaks. If cells were labelled in the presence of 1.0 µg of ethidium bromide/ml the synthesis of the 21S_E and 12S_E mitochondrial RNA was completely inhibited but that of the 18S_E component was not (Fig. 4). The 18S_E component could be cytoplasmic 18S RNA whose synthesis was not completely suppressed by actinomycin D (Kay & Cooper, 1969). However, RNA extracted by the hot-phenol-sodium dodecyl sulphate method contained a larger component that ran at 18–19S_E on electrophoresis

Fig. 3. Sucrose-density-gradient centrifugation of RNA from L-cell mitochondria extracted by the cold-phenol-sodium dodecyl sulphate method. RNA of 2×10^8 cells was labelled with $10 \mu\text{Ci}$ of [³H]uridine/ml for 4 h at a cell density of 5×10^6 /ml in the presence of $0.1 \mu\text{g}$ of actinomycin D/ml. RNA was extracted by the cold-phenol-sodium dodecyl sulphate method at 25°C as described in the Methods and Materials section. RNA ($200 \mu\text{g}$) was centrifuged on a linear sucrose density gradient for 16 h at $75000g_{\text{av}}$ at 15°C. □, E_{260} of cytoplasmic rRNA; ●, radioactivity of mitochondrial RNA.

Fig. 4. Gel electrophoresis of RNA from L-cell mitochondria extracted by the cold-phenol-sodium dodecyl sulphate method. RNA was labelled and extracted from mitochondria of 1.8×10^8 L cells as described in Fig. 3, except that the extraction was at 4°C . In one case $1 \mu\text{g}$ of ethidium bromide/ml was added to the culture. Electrophoresis was as described in the Methods and Materials section. Cytoplasmic rRNA labelled with $[2\text{-}^{14}\text{C}]$ uridine was added as a marker to the sample of mitochondrial RNA labelled in the absence of ethidium bromide. The mitochondrial RNA labelled in the presence of ethidium bromide was subjected to electrophoresis separately. □, Cytoplasmic RNA; ●, mitochondrial RNA; △, mitochondrial RNA from cells labelled in the presence of ethidium bromide.

and its possible nature and origin is considered below.

L-cell mitochondrial RNA extracted by the hot-phenol-sodium dodecyl sulphate method. Although in some experiments the RNA extracted from crude mitochondria of L cells labelled in the presence of $0.1 \mu\text{g}$ of actinomycin D/ml by the hot-phenol-sodium dodecyl sulphate method sedimented mainly at about 15 and 12.5S, it was more usual to obtain a large heterodisperse component that sedimented with a peak at 21S in addition to the 15S and 12.5S RNA, as shown in Fig. 5. The 12.5S RNA usually appeared as a shoulder on the 15S RNA peak. There was also a greater amount of heterodisperse RNA throughout the sucrose density gradient compared with mitochondria extracted by the cold-phenol-sodium dodecyl sulphate method (Fig. 3). The cytoplasmic carrier RNA was more degraded when extracted by the hot-phenol-sodium dodecyl sulphate method. As shown in Fig. 5, 4S mitochondrial RNA was recovered by the hot extraction and ethanol precipitation. Electrophoresis of another sample of RNA extracted by the hot-phenol-sodium dodecyl sulphate method is shown in Fig. 6. Control experiments showed that the component running at 18S_E was present in the 16–26S region of sucrose density gradients. The 21S_E mitochondrial RNA represents a small peak compared with the 18S_E component. When the mitochondrial fraction was purified further by

isopycnic-gradient centrifugation and the RNA extracted by the hot-phenol-sodium dodecyl sulphate method the main components were at 21 and 12S_E in a ratio of 2.2:1 (s.d. ± 0.2) and only a small amount of the 18S_E component remained (Fig. 7). The RNA extracted from the nuclear pellet obtained on isopycnic-gradient centrifugation showed the 18S_E component (Fig. 7). The 18S_E component was present in the crude nuclear pellet ($1000g$ for 10 min) and it is concluded that its presence in crude mitochondrial fractions is due to nuclei and nuclear fragments. This is further supported by its removal from the crude mitochondrial fraction by an incubation at 4°C for 4 h with $10 \mu\text{g}$ of deoxyribonuclease/ml. It has also been found that if after disruption of the cells potassium chloride is added to a final concentration of 0.05M rather than 0.02M, the amount of nuclear fragments in the mitochondrial fraction is greatly decreased.

The 18S_E (21S) component was found to be resistant to ribonuclease and 0.3M-potassium hydroxide but was degraded by deoxyribonuclease. These properties suggest that it is DNA. Further, its synthesis was resistant to ethidium bromide ($1.0 \mu\text{g}/\text{ml}$).

Nucleotide composition. The nucleotide composition of RNA from rat liver and mouse L-cell cytoplasm and mitochondria is shown in Table 2. The mitochondrial RNA from both tissues had more

Fig. 5. Sucrose-density-gradient centrifugation of L-cell mitochondrial RNA extracted by the hot-phenol-sodium dodecyl sulphate method. RNA of 1.8×10^8 cells was labelled with $10 \mu\text{Ci}$ of $[5\text{-}^3\text{H}]\text{uridine/ml}$ for 4 h at a cell density of $6 \times 10^6/\text{ml}$ in the presence of $0.1 \mu\text{g}$ of actinomycin D/ml. RNA was extracted from the crude mitochondrial fraction by the hot-phenol-sodium dodecyl sulphate method as described in the Methods and Materials section. RNA was centrifuged on a linear sucrose density gradient for 16 h at $75000g_{av.}$ at 15°C . $\square$, E_{260} of cytoplasmic RNA; $\bullet$, radioactivity of mitochondrial RNA.

Fig. 6. Gel electrophoresis of RNA from L-cell mitochondria extracted by the hot-phenol-sodium dodecyl sulphate method. RNA was labelled and extracted from the mitochondrial fraction as described in Fig. 5 and subjected to electrophoresis as described in Fig. 4. The cytoplasmic rRNA was subjected to electrophoresis in a parallel tube. $\square$, Cytoplasmic RNA; $\bullet$, mitochondrial RNA.

Fig. 7. Gel electrophoresis of RNA isolated by the hot-phenol-sodium dodecyl sulphate method from the mitochondrial and the nuclear fractions separated by isopycnic-gradient centrifugation. The crude mitochondrial fraction from which the RNA was extracted and subjected to electrophoresis in Fig. 6 was purified by isopycnic-gradient centrifugation (see the Methods and Materials section) and RNA extracted by the hot-phenol-sodium dodecyl sulphate method from the resulting mitochondrial band and nuclear pellet. Electrophoresis was as described in Fig. 4. ●, Mitochondrial RNA; ○, nuclear component.

Table 2. Nucleotide composition of RNA from rat liver and mouse L-cell cytoplasm and mitochondria

The nucleotide composition for rat liver cytoplasmic RNA was obtained from Kirby (1965) and that of mouse L-cell cytoplasmic RNA from Lane & Tamaoki (1967). The nucleotide compositions of RNA of the mitochondrial fractions were obtained as described in the Methods and Materials section by alkaline digestion of either ³²P-labelled RNA or unlabelled RNA.

Source	Nucleotide			
	UMP	AMP	GMP	CMP
Rat liver cytoplasm 18 S	18.0	19.8	32.4	29.8
28 S	17.0	17.8	33.0	32.2
Mouse L cell 18 S	19.3	23.0	29.8	27.5
28 S	16.0	17.5	35.3	30.8
Rat liver mitochondria ³² P-labelled	22.9 ± 0.9	30.5 ± 1.3	21.6 ± 2.6	24.9 ± 1.5
Unlabelled	22.4	29.2	24.3	24.0
Mouse L-cell mitochondria ³² P-labelled	26.3	34.1	17.4	22.2

adenylate and uridylate and less guanylate and cytidylate than cytoplasmic ribosomal RNA. The determination of nucleotide composition by ³²P-labelling was satisfactory because the result with direct nucleotide analysis was identical (Table 2). The nucleotide composition distinguishes the mitochondrial RNA as a distinctive class. The difference in nucleotide composition between

mitochondrial and cytoplasmic RNA is not as great with rat liver, perhaps reflecting a greater amount of contamination with cytoplasmic rRNA. The composition of the L-cell mitochondrial RNA might be affected by the presence of heterodisperse RNA (Vesco & Penman, 1969).
Molecular weight. Molecular weights of the 21S_E and 12S_E RNA from L-cells were 0.96 × 10⁶ and

0.30×10^6 respectively as determined by the method of Loening (1968), the electrophoretic mobility of mouse liver cytoplasmic rRNA being used as reference standard. This result must be treated with some caution because there would appear to be a difference in the gross conformation of mitochondrial and cytoplasmic RNA. The 15S RNA ran at 21S_E. This suggests a more open conformation, which slows this RNA both on sedimentation and on electrophoresis. If this is so, the molecular-weight determination, which must depend on similar conformations of the standard and unknown RNA, will be in error. From these molecular weights and a ratio of 2.2 for the 21S_E to the 12S_E components (hot-phenol-sodium dodecyl sulphate) the molar ratio of these components is 0.73:1.

DISCUSSION

Characterization of possible high-molecular-weight RNA components from mammalian mitochondria has been difficult because mitochondria isolated by the usual centrifugal techniques are contaminated with cytoplasmic ribosomes. Two methods were used to circumvent this problem, and in both the mitochondrial RNA was identified by radioactive labelling. In the first method, rat liver mitochondria were purified by an improved centrifugation procedure. The RNA content was lower than any previous reported (O'Brien & Kalf, 1967*a*). Because in this approach RNA is isolated from pure mitochondria, the nature of mitochondrial RNA is directly determined. Alternatively, as first used by Dubin (1967), the synthesis of cytoplasmic rRNA was suppressed in tissue-culture cells with a low concentration of actinomycin D (Penman *et al.* 1968). Mitochondrial RNA continues to be synthesized under these conditions because either mitochondria are impermeable to the antibiotic (Neubert & Helge, 1965; Dubin, 1967) or the amount of DNA coding for the RNA is smaller than that for cytoplasmic rRNA (Perry & Kelley, 1969). This approach has two disadvantages. The actinomycin D might affect the synthesis of the presumed mitochondrial RNA and the mitochondrial fraction might contain other cellular components containing RNA whose synthesis is resistant to actinomycin D.

RNA isolated from mitochondria of rat liver and actinomycin D-treated L cells showed distinctive species sedimenting at about 15 and 13S on sucrose-density-gradient centrifugation. On electrophoresis the RNA of L-cell mitochondria migrated at 21 and 12S_E. The nucleotide composition of the mitochondrial RNA from the two sources was slightly different because of possible contamination of the rat liver mitochondrial RNA with cytoplasmic

rRNA and the L-cell mitochondrial RNA with heterodisperse cytoplasmic RNA (Vesco & Penman, 1969).

The RNA extracted from the mitochondrial fraction is clearly distinguished as a species distinct from cytoplasmic rRNA by its sedimentation rate, electrophoretic mobility and nucleotide composition. These characteristics do not establish whether the RNA is a normal constituent of mitochondria or is a contaminant. The RNA species obtained from the crude mitochondrial fraction of actinomycin D-treated L cells are similar to those from actinomycin D-treated hamster (BHK-21) cells (Dubin, 1967) and human (HeLa) cells (Vesco & Penman, 1969). In both these cases only a broad peak of RNA centred at about 17S was observed on sucrose-density-gradient centrifugation but a separation into 21S_E and 12S_E species was seen on electrophoresis of HeLa-cell mitochondrial RNA (Vesco & Penman, 1969) and that of hamster cells (Dubin & Monteneourt, 1970). D. T. Dubin (personal communication) has found that the mitochondrial RNA of L cells has a lower mobility on electrophoresis than the mitochondrial RNA of hamster cells. These components could be an artifact of drug treatment because they are only clearly seen under these conditions, but Vesco & Penman (1969) and Knight (1969) did identify them in control cell cultures. A more serious problem is that the mitochondrial fractions are not pure. Contamination from other cellular fractions, viruses or mycoplasma is possible. RNA species rich in A and U have been identified in tissue-culture cells infected with mycoplasma and their sedimentation rate has been reported as 14–20S (Levine, Burleigh, Boone & Eagle, 1967; Levine, Thomas, McGregor, Hayflick & Eagle, 1968) and 16 and 23S (Markov *et al.* 1969). In our experiments with L cells this was probably not the case because there was no disturbance of RNA synthesis as reported by Levine *et al.* (1968) and because the cells were grown in the presence of an anti-mycoplasma drug. More important, the finding of distinctive RNA species in highly purified rat liver mitochondria is strong evidence that the 15S and 13S (21S_E and 12S_E) RNA are truly mitochondrial. Their presence in tissue-culture cells is therefore probably not due to mycoplasma, an artifact of drug treatment or contamination of the mitochondrial fraction. The inhibition of the synthesis of the RNA by ethidium bromide confirms the results of Zylber *et al.* (1969) and suggests that mitochondrial rRNA is coded for by mitochondrial DNA as has been found for *Neurospora* (Wood & Luck, 1969) and yeast (Wintersberger & Viehhauser, 1968).

The results reported here and by Dubin (1967, 1969) and Vesco & Penman (1969) are not in agreement with two other reports on the nature of mam-

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

The significance of the 21S (18S_E) component is not known. There is evidence of 10S and 24S (alkaline sucrose gradient) intermediates (Kidwell & Mueller, 1969) in the synthesis of DNA in HeLa cells. Vesco & Penman (1969) reported a 26S_E (18S) component in mitochondrial RNA of HeLa cells. Its sedimentation and electrophoretic properties are different from the 21S component seen here.

If the 15S and 13S (21S_E and 12S_E) species are indeed mitochondrial, what is their role? As calculated in the results, the 21S_E and 12S_E components are present in an approximate molar ratio 0.73:1. However, the molecular weight of the RNA species is in doubt because of the anomalous retardation of the 15S species to 21S_E on electrophoresis. Until accurate values of the molecular weight are obtained the ratio 0.73:1 must be treated with caution. The ratio, however, is close to 1:1, which suggests they could arise from a mitochondrial ribosome. Values of 1:3 for HeLa cells (Vesco & Penman, 1969) and 1:1 for hamster cells (Dubin & Monteneourt, 1970) have been reported. There has as yet been no definitive characterization of mitochondrial ribosomes from higher species. However, Haldar, Freeman & Work (1967) and Rabinowitz, Sinclair, DeSalle, Haselkorn & Swift (1965) have reported mitochondrial ribosomes in mouse ascites cells and chick embryo cells respectively, demonstrated by electron microscopy, that are smaller than cytoplasmic ribosomes. O'Brien & Kalf (1967a,b) and Ashwell & Work (1970) have found a 55S ribosomal particle in rat liver mitochondria, but again this seems smaller than one would expect, compared with other ribosomes, and it could be a subunit. Vesco & Penman (1969) and ourselves (B. Bartoov,

R. S. Mitra & K. B. Freeman, unpublished work) have found mitochondrial RNA in the 45S region of deoxycholate extracts of mitochondria.

All mitochondrial rRNA is A+U-rich whereas rRNA from most species is G+C-rich (Amaldi, 1969). But if the mitochondrial RNA seen here and by others in mammalian cells is from ribosomes then it is very much smaller than any previously reported rRNA (Kuntzel & Noll, 1967; Stutz & Noll, 1967; Loening & Ingle, 1967; Taylor & Storek, 1964). It is also smaller than the mitochondrial rRNA from *Neurospora* (Rifkin *et al.* 1967; Kuntzel & Noll, 1967; Dure *et al.* 1967), yeast (Wintersberger, 1967; Rogers *et al.* 1967; Leon & Mahler, 1968) and *Tetrahymena pyriformis* (Suyama & Eyer, 1968) but the same size as that from *Xenopus laevis* (Dawid, 1969). There has been a progressive phylogenetic decrease in the size of mitochondrial DNA. In *Neurospora* the DNA has a molecular weight of 66×10^6 (Wood & Luck, 1969) but in *Xenopus* (Wolstenholme & Dawid, 1968) and in L cells (Nass, 1969) it is 9×10^6 . It is possible, then, that the rRNA and the ribosomes of the higher organism might be smaller, as is the DNA. This contrasts with an increase in molecular weight of cytoplasmic rRNA in the higher vertebrates (Loening, 1968).

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