Synthesis of Ribonucleic Acid by Isolated Rat Liver Mitochondria

By S. FUKAMACHI, B. BARTOEV and K. B. FREEMAN

*Department of Biochemistry, McMaster University,
Hamilton, Ont., Canada*

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Rat liver mitochondria isolated in sucrose–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) incorporated [³H]UTP into RNA for 1 h. Incorporation was inhibited 50% by 1 μg of actinomycin D/ml, 1 μg of acriflavine/ml and 0.5 μg of ethidium bromide/ml but was insensitive to rifampicin, rifamycin SV, streptovaricin and deoxyribonuclease. After the first 10 min of incubation, the synthesis was insensitive to ribonuclease. RNA synthesis by mitochondria isolated in sucrose–EDTA was insensitive to actinomycin D and sensitive to ribonuclease during the first 10 min of the incubation but thereafter the sensitivities were the same as for mitochondria isolated in sucrose–TES. In a hypo-osmotic medium the relative extent of incorporation of the four ribonucleoside triphosphates into RNA was CTP > UTP = ATP ≫ GTP. In an iso-osmotic medium the incorporation of CTP and GTP decreased. All four nucleotides were incorporated into RNA in a DNA-dependent process, as indicated by the inhibition by actinomycin D. In addition, CTP and ATP were incorporated into the CCA end of mitochondrial tRNA. ATP was also incorporated into an unidentified acid-insoluble compound, which hydrolysed in alkali to a product that was not ATP, ADP or 5'- or 2(3')-AMP. Atractyloside inhibited the incorporation of ATP into RNA with 50% inhibition at 2–3 nmol/mg of protein. The [³H]UTP-labelled RNA had peaks of 16S and 13S characteristic of mitochondrial rRNA. In addition a peak at 20–21S was observed as well as heterogeneous RNA sedimenting throughout the gradient. The synthesis of all these species was inhibited by actinomycin D, indicating that rat liver mitochondrial DNA codes for mitochondrial rRNA as well as other as yet unidentified species.

The synthesis of RNA by isolated rat liver mitochondria was first reported by Wintersberger (1964) and Neubert & Helge (1965) and subsequently characterized by Neubert *et al.* (1965), Saccone *et al.* (1967) and Saccone *et al.* (1969). These studies showed that isolated rat liver mitochondria synthesize RNA in a DNA-dependent reaction but that synthesis continued for 10–20 min only (Neubert *et al.*, 1966; Saccone *et al.*, 1968), compared with 1 h for mitochondrial protein synthesis (Haldar & Freeman, 1969). Although all nucleotides were incorporated into RNA there was disagreement on the extent of incorporation of ATP. It was reported as being equal to that of the other nucleotides (Saccone *et al.*, 1969) or much greater (Saccone *et al.*, 1967, 1968), whereas Neubert (1968) was unable to follow its incorporation because of high zero-time counts. The inhibition of ATP incorporation by atractyloside (Saccone *et al.*, 1967), a specific inhibitor of ADP and ATP exchange across the mitochondrial inner membrane (Winkler & Lehninger, 1968), showed the mitochondrial site of the incorporation but the uncertainty over the true amount of ATP incorporation leaves this observation in doubt. In early studies the synthesis of species of RNA characteristic of mitochondria was not found

(Neubert *et al.*, 1968; Saccone *et al.*, 1969), although most of the RNA was synthesized from the heavy strand of mitochondrial DNA (Aaij *et al.*, 1970). Some symmetrical transcription also occurred but its significance is unclear (Aaij *et al.*, 1970). We have reported the synthesis of mitochondrial rRNA by isolated rat liver mitochondria as characterized by gel electrophoresis (Fukamachi *et al.*, 1970) and now characterize the type of RNA synthesized further by sucrose-density-gradient centrifugation. In addition the incorporation of the four ribonucleoside triphosphates and the effect of atractyloside have been re-examined and some of the previous discrepancies explained.

Materials and Methods

Isolation of rat liver mitochondria and nuclei

The mitochondrial fraction was prepared from livers of 125–150 g hooded rats with either 0.25 M-sucrose–2 mM-EDTA, pH 7.4 (sucrose–EDTA), or 0.25 M-sucrose–0.5 mM-TES, * pH 7.4 (sucrose–TES),

* Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

as the isolation medium. The isolation media were sterilized and the pH was readjusted afterwards if necessary. Livers were homogenized with a Potter Teflon homogenizer, and nuclei and cell debris were removed by two centrifugations at 1000g for 10min. Mitochondria were recovered by centrifugation at 6000g for 8min, the pellet was rinsed and the mitochondria were resuspended and recovered by centrifugation. After a further wash, the pellet was resuspended in a small volume of the isolation medium. Further washing of the mitochondrial fraction did not remove any more visible material and did not affect the characteristics of the incorporation. Additional low-speed centrifugation to remove possible remaining nuclei did not affect the incorporation either.

Rat liver nuclei were isolated by the method of Widnell & Tata (1964).

Incubations

Mitochondria were incubated in a modification of the medium of Parsons & Simpson (1967). The basic medium contained 4mM-KCl, 7mM-MgCl₂, 5mM-potassium phosphate, 7.5mM-sodium pyruvate, 7.5mM-sodium succinate, 0.45mM-sodium malate and the unlabelled ribonucleoside triphosphates (each 0.1mM). The final incubation mixture also contained 33.4mM-sucrose (hypo-osmotic medium) or 183.4mM-sucrose (iso-osmotic medium) and 0.27mM-EDTA or 0.07mM-TES and was at pH 7.4. The hypo-osmotic medium had an osmolality of 128mosmol/litre as measured on an osmometer (Advanced Instruments Inc., Newton Highlands, Mass., U.S.A.) and the iso-osmotic medium had an osmolality of 296mosmol/litre. [5-³H]UTP was added to 3.3μCi/ml for standard incubations or to 8.3μCi/ml for alkaline hydrolysis. All components of the media were sterilized except for the nucleoside triphosphates. The concentration of mitochondrial protein was 3mg/ml, being diluted 7.5-fold from the stock suspension. Mitochondria were incubated at 30°C and were shaken at 80–140 cycles/min.

Nuclei were incubated as described by Weiss (1960).

Isolation of RNA for total incorporation

After the incubation of mitochondria, 0.6ml was added to 0.2ml of ice-cold saturated tetrasodium pyrophosphate and centrifuged at 6000g for 10min in 12ml thick-walled Pyrex tubes. The supernatant was decanted, the pellet rinsed with 5ml of isolation medium and RNA was isolated by a modification of the method of Penman (1966). The pellet was resuspended in 2ml of 0.1M-NaCl–0.02M-sodium acetate, pH 5.0, containing 2% sodium dodecyl sulphate and 0.1% crude yeast RNA. After shaking at 10°C to obtain a clear solution, 2ml of water-saturated phenol was added, the solution mixed and

the emulsion heated at 60°C for 2min. The solution was mixed again, 2ml of chloroform containing 1% of 3-methylbutan-1-ol was added and the emulsion was mixed and heated to 60°C for 2min. The phases were separated by centrifugation at 1000g for 4min at 20°C, the bottom phase was removed with a Pasteur pipette and the upper phase was re-extracted with 4ml of chloroform–3-methylbutan-1-ol at 60°C for 2min. The aqueous phase was recovered and re-extracted at least once. Trichloroacetic acid was added to the aqueous phase to 5% (w/v) at 0°C. RNA was recovered by centrifugation at 15000g for 10min, rinsed with 5ml of 5% trichloroacetic acid and the pellet dissolved in 0.5ml of a strong base (NCS solubilizer) for counting of radioactivity. For alkaline hydrolysis, the RNA was washed further with aq. 95% (v/v) ethanol containing 2% (w/v) potassium acetate, then with ether and finally dried.

After the incubation of nuclei, 2mg of carrier crude yeast RNA was added, followed by trichloroacetic acid to 5% at 0°C. The precipitate of nucleic acid and protein was removed by centrifugation, washed twice with 5% trichloroacetic acid, dissolved and counted for radioactivity as described for mitochondrial RNA.

Alkaline hydrolysis

RNA was hydrolysed with 0.3M-KOH at 37°C for 18h. K⁺ was removed at 0°C by the addition of 6M-HClO₄ to pH 1.0 and neutralization to pH 7.0 with 1M-KOH. The solution was evaporated and the nucleotides were dissolved in 0.2ml of aq. 0.05M-NH₃ solution. Paper chromatography of 0.125ml samples was in 1M-ammonium acetate saturated with boric acid (adjusted to pH 7.5)–aq. 95% ethanol (2:5, v/v). After chromatography the paper was dried, cut into 1cm×2cm pieces and counted for radioactivity.

Measurement of radioactivity

All samples of RNA were counted by adding 0.5ml of water and 10ml of naphthalene–dioxan scintillation solvent (Haldar & Freeman, 1969). The efficiency of counting was 30%. Paper chromatograms were counted with 2ml of a toluene-based scintillation solution (Haldar & Freeman, 1968).

Isolation of RNA for sucrose-density-gradient centrifugation

RNA was isolated by the use of hot phenol–sodium dodecyl sulphate as described above, except that ribosomal RNA from rat liver was used as carrier and the RNA was precipitated with ethanol after extraction. In addition RNA was isolated with cold phenol–sodium dodecyl sulphate, in which only rRNA is recovered as described by Bartoov *et al.* (1970) or in

which total RNA is recovered as follows: after incubation, mitochondria were centrifuged at 6500g for 10min and washed twice with 5ml of isolation medium (sucrose-EDTA). The pellet was suspended in 2ml of the isolation medium, and 1mg of L-cell rRNA and 0.5mg of *Escherichia coli* tRNA were added as marker and carrier RNA. Mitochondria were disrupted by adding 4ml of 25mM-sodium acetate-0.17M-NaCl-10mM-MgCl₂-2% sodium dodecyl sulphate-0.1% macaloid, pH4.8. RNA was extracted at 4°C with 7ml of 4% aminosalicyclic acid-2% sodium dodecyl sulphate-0.1% macaloid-25mM-sodium acetate, pH4.8, and 14ml of phenol-cresol (Bartoov *et al.*, 1970). Sodium tri-isopropyl-naphthalene sulphonate was added to 2.5% and the mixture was stirred at 4°C for 1h. After recovery of the aqueous phase RNA was re-extracted at 4°C with 1 vol of phenol-cresol mixture, precipitated with 2.5 vol. of aq. 95% ethanol and left overnight at -20°C. RNA was washed twice with 1% NaCl in aq. 75% ethanol, twice with absolute ethanol and dried under vacuum overnight. RNA was dissolved in 0.1M-sodium acetate, pH6.0, and applied to a convex sucrose density gradient.

Sucrose-density-gradient centrifugation

Either centrifugation on isokinetic gradients from 15 to 33% (w/v) sucrose-0.1M-sodium acetate, pH6.0 (Noll, 1967; Bartoov *et al.*, 1970), or linear gradients from 5 to 20% (w/v) sucrose, were used. Centrifugation was in the SW41 rotor of the Beckman L2-65B ultracentrifuge. Fractions were collected dropwise, the extinction at 260nm was measured and the radioactivity determined by the addition of 0.5ml of NCS solubilizer and 10ml of dioxan scintillation liquid. For some gradients, RNA was first precipitated with 5% trichloroacetic acid after addition of 0.5mg of crude yeast RNA to separate it from the radioactive precursor extracted with the RNA.

Determination of protein, RNA and DNA

Measurements were by the methods described previously (Freeman, 1965).

Materials

All chemicals were reagent grade where possible. Actinomycin D was a gift of Merck, Sharp and Dohme Ltd., Montreal, P.Q., Canada. Rifampicin and rifamycin SV were gifts from Dr. P. Sensi of Leptit S.p.A. Milan, Italy, and streptovaricin was a gift from Dr. S. Mizuno, National Institute of Health, Tokyo, Japan and from the Toyojoso Co., Tokyo, Japan. Atractyloside was a gift of Dr. A. L. Lehninger. Nucleoside triphosphates were obtained from Boehringer(Mannheim) Corp., New York, U.S.A. Deoxyribonuclease and ribonuclease A, type

IIA, were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. NCS solubilizer, [5-³H]UTP (2-4Ci/mmol), [2-³H]ATP (0.45Ci/mmol), [8-³H]-GTP (11.8Ci/mmol) and [5-³H]CTP (20.4Ci/mmol) were obtained from Amersham/Searle Corp., Toronto, Ont., Canada.

Results

Mitochondria

The mitochondrial fractions isolated in sucrose-EDTA and sucrose-TES contained $0.42 \pm 0.04 \mu\text{g}$ of DNA/mg of protein. These values are comparable

Fig. 1. Effect of nucleases and antibiotics on the incorporation of [³H]UTP into RNA by mitochondria isolated in sucrose-TES

Incubations were at 30°C in the hypo-osmotic medium. The reaction was stopped by pipetting 0.6ml of the incubated mixture into 0.2ml of ice-cold saturated pyrophosphate and RNA was extracted by the hot-phenol-sodium dodecyl sulphate method and counted for radioactivity as described in the Materials and Methods section. o, Control; ×, deoxyribonuclease (83 $\mu\text{g}/\text{ml}$); Δ, ribonuclease (40 $\mu\text{g}/\text{ml}$); □, acriflavine (40 $\mu\text{g}/\text{ml}$); ■, actinomycin D (40 $\mu\text{g}/\text{ml}$); □, ethidium bromide (1 $\mu\text{g}/\text{ml}$).

with 0.2–0.5 μg of DNA/mg of protein (Leffler *et al.*, 1970) for mitochondria from which DNA free of nuclear DNA was obtained. This indicates that there was almost no contamination of the mitochondrial fraction with nuclei. RNA values varied from 6 to 11 $\mu\text{g}/\text{mg}$ of protein.

Mitochondria as the site of RNA synthesis

Two possible sources of RNA synthesis with isolated mitochondria are bacteria and nuclei. Differentiation from synthesis by nuclei was reported earlier (Fukamachi *et al.*, 1970). Bacteria were eliminated as a source of synthesis by using sterile techniques. Bacterial counts on blood-agar plates ranged from 0 to 30 bacteria/0.1 ml of the incubation mixture after incubations for 1 h. The isolated mitochondrial preparations usually contained no bacteria but occasionally counts were obtained of 2 or 3/0.1 ml of mitochondrial suspension. It is likely that [^3H]UTP can be incorporated into bacterial RNA only via the nucleoside because nucleotides do not cross bacterial membranes (Price *et al.*, 1967). Uridine at 1 mg/ml did not inhibit incorporation, indicating that bacteria were not involved.

Time-course of incorporation

The time-course of incorporation of [^3H]UTP by mitochondria isolated in sucrose–TES and incubated in the hypo-osmotic medium is shown in Fig. 1. There was a lag in incorporation but, after 10 min, incorporation continued at a linear rate for the next 50 min. Both acriflavine and actinomycin D at 40 $\mu\text{g}/\text{ml}$ strongly inhibited incorporation whereas ethidium bromide at 1 $\mu\text{g}/\text{ml}$ required 10–20 min to act. Ribonuclease inhibited at early times of incorporation but not in the last 40 min. Deoxyribonuclease had no effect. In separate experiments it was found that, in the iso-osmotic medium, actinomycin D inhibited 40% at 10 min, 78% at 20 min and 96% at 1 h and ribonuclease had the same effect as in the hypo-osmotic medium. After preincubation of the mitochondria for 10 min in 20 mM-potassium phosphate, pH 7.4 at 20°C, a 79 and 44% increase in incorporation at 10 and 20 min respectively was observed but thereafter incorporation levelled off. Although this increase in initial activity could be from an increase in the permeability of the mitochondria to nucleotides (Neubert *et al.*, 1968), it is equally possible that it could arise from a depletion of the endogenous nucleotide pool since mitochondria must be centrifuged after the preincubation.

The time-course of incorporation of [^3H]UTP by mitochondria isolated in sucrose–EDTA and incubated in the hypo-osmotic medium is shown in Fig. 2. It is similar to that shown in Fig. 1 except that, in the first 10 min, there was no lag in incorporation and

Fig. 2. Effect of nucleases and antibiotics on the incorporation of [^3H]UTP into RNA by mitochondria isolated in sucrose–EDTA

Mitochondria were incubated at 30°C in the hypo-osmotic medium and RNA was recovered and counted for radioactivity as described in Fig. 1. $\circ$, Control; $\times$, deoxyribonuclease (83 $\mu\text{g}/\text{ml}$); ∇ , ribonuclease (40 $\mu\text{g}/\text{ml}$); Δ , acriflavine (40 $\mu\text{g}/\text{ml}$); $\bullet$, actinomycin D (40 $\mu\text{g}/\text{ml}$); $\bullet$, ethidium bromide (0.33 $\mu\text{g}/\text{ml}$); $\square$, ethidium bromide (1 $\mu\text{g}/\text{ml}$).

the incorporation was insensitive to actinomycin D and acriflavine. Similar results were obtained with the iso-osmotic medium or with mitochondria isolated in sucrose–EGTA. The reason for the difference in incorporation in the first 10 min between mitochondria isolated in sucrose–TES or sucrose–EDTA is not clear. The lower sensitivity to actinomycin D and acriflavine in the first 10 min of incorporation could be explained if mitochondria isolated in sucrose–EDTA were less permeable to the antibiotics. Preincubation of the mitochondria with or without the antibiotics for 10 or 20 min at 30°C before adding the isotope and antibiotics if necessary led to a slightly increased inhibition, but not as great as with mitochondria isolated in sucrose–TES.

Fig. 3. Effect of concentration of antibiotics on the incorporation of [3H]UTP into RNA by mitochondria isolated in sucrose-TES

Mitochondria were incubated at 30°C for 20min in the hypo-osmotic medium and RNA was recovered and counted for radioactivity as described in Fig. 1. Δ , Rifampicin; $\blacksquare$, rifamycin SV; $\square$, streptovaricin; $\blacktriangle$, acriflavine; $\circ$, actinomycin D; $\blacksquare$, ethidium bromide.

Effect of inhibitors at different concentrations

The effect of several antibiotics on [3H]UTP incorporation by mitochondria isolated in sucrose-TES and incubated in the hypo-osmotic medium is shown in Fig. 3. Both acriflavine and actinomycin D inhibited 50% at about 1 μ g/ml and ethidium bromide at 0.5 μ g/ml. The antibiotics rifampicin, rifamycin SV and streptovaricin did not inhibit.

Incorporation of the four ribonucleoside triphosphates

The incorporation of the four ribonucleoside triphosphates into RNA is shown in Table 1. Base-labelled ribonucleoside triphosphates, when incorporated into internal internucleotide linkages of RNA, are recovered as 2(3')-nucleotides after alkaline hydrolysis. Nucleotides, added at the 3'-hydroxyl terminal end of RNA with no subsequent addition, are recovered as nucleosides. Based on the recovery of 3H-labelled 2'(3')-nucleotides, the relative extent of incorporation was in the order CTP>ATP=UTP>GTP. For [3H]UTP, [3H]CTP and [3H]GTP an unidentified compound that was probably in part residual precursor was found near the origin of the paper chromatogram. For [3H]ATP the unidentified compound, which ran near ADP, was the major product. Its nature and origin are considered below. The low incorporation of [3H]GTP was not due to its hydrolysis, since paper chromatography of the medium after a 20min incubation with [3H]UTP and [3H]GTP showed no difference in the rate of hydrolysis to the nucleoside diphosphate, nucleoside monophosphate and the nucleoside.

The amount of [3H]uridine recovered did not increase with time from 10 to 60 min and, in three experiments, for mitochondria isolated in sucrose-TES an

Table 1. Incorporation of ribonucleoside triphosphates into RNA

Mitochondria were isolated either in sucrose-TES or in sucrose-EDTA. All incubations were in the hypo-osmotic medium at 30°C for 20min. The concentration of the 3H-labelled nucleoside triphosphate was 8.3 μ M and that of three unlabelled nucleoside triphosphates was 100 μ M. About 3.4mg of mitochondrial protein/ml was present in each incubation. The RNA was isolated by the hot-phenol-sodium dodecyl sulphate method, hydrolysed with 0.3M-KOH for 18h at 37°C and the hydrolysate separated by paper chromatography as described in the Materials and Methods section.

		10 ⁻¹⁵ × Incorporation (mol/mg of protein)							
Isolation medium ...		Sucrose-TES				Sucrose-EDTA			
Precursor ...		UTP	ATP	CTP	GTP	UTP	ATP	CTP	GTP
Product									
2'(3')-Nucleotide		123	144	238	31.2	110	141	178	27.5
Nucleoside		2.7	75	157	1.8	10.6	54.8	102	1.1
Unidentified		19.2	301	66.0	34.8	17.6	273	22	41.8

Table 2. *Effect of actinomycin D and the osmolarity of the medium on the incorporation of ribonucleoside triphosphates into RNA*

Mitochondria isolated either in sucrose-TES or in sucrose-EDTA were incubated for 20min in the hypo-osmotic medium and in the iso-osmotic medium in the presence and absence of actinomycin D (40 µg/ml) with [³H]UTP, [³H]ATP, [³H]CTP or [³H]GTP as the precursor in each incubation.

		Incorporation (% of total hypo-osmotic counts)			
Isolation medium ...		Sucrose-TES		Sucrose-EDTA	
Precursor	Medium	Nucleotide	Nucleoside	Nucleotide	Nucleoside
UTP	Hypo-osmotic	98	2	86	14
	+actinomycin D	6	2	23	13
	Iso-osmotic	59	2	95	10
ATP	+actinomycin D	20	2	39	11
	Hypo-osmotic	69	31	73	27
	+actinomycin D	22	32	25	24
CTP	Iso-osmotic	110	35	137	38
	+actinomycin D	41	38	34	35
	Hypo-osmotic	59	41	60	40
GTP	+actinomycin D	31	39	31	43
	Iso-osmotic	6	1	23	5
	+actinomycin D	2	1	6	7
GTP	Hypo-osmotic	97	3	93	7
	+actinomycin D	5	6	18	7
	Iso-osmotic	15	2	67	9
	+actinomycin D	2	5	7	7

average of 4% and 0.7% [³H]uridine was obtained at 20min and 60min. This gives an average chain length of 140 synthesized in 60min. More [³H]-uridine was observed in the RNA synthesized by mitochondria isolated in sucrose-EDTA (Table 1), indicating a shorter average chain length.

The effect of the osmolarity of the incubation medium and actinomycin D at 40 µg/ml is shown in Table 2. For mitochondria isolated in sucrose-TES the incorporation of [³H]GTP and [³H]CTP was less in the iso-osmotic medium but that of [³H]ATP was greater. The effect of osmolarity was less marked for mitochondria isolated in sucrose-EDTA. Actinomycin D inhibited similarly in both media except for the incorporation of [³H]UTP described above. The incorporation of [³H]UTP or [³H]GTP was strongly inhibited by actinomycin D but not that of [³H]CTP or [³H]ATP, as judged by the recovery of ³H-labelled 2'(3')-nucleotides. The presence of an actinomycin D-resistant incorporation of [³H]CTP, the high incorporation of [³H]CTP compared with the other nucleotides (Table 1) and large recovery of [³H]-cytidine and [³H]adenosine (Table 1) are suggestive of turnover of the CCA end of tRNA. In addition [³H]CTP incorporated in 20min turned over 58% with a further incubation of 40min in the presence of a 'chase' of non-radioactive CTP, but [³H]UTP

incorporated into RNA in 20min did not turn over when non-radioactive UTP was added.

Incorporation of [³H]ATP into other acid-insoluble compounds

As described above [³H]ATP was incorporated into an acid-insoluble material, which on hydrolysis gave rise to a compound that ran close to ADP on paper chromatograms with one solvent system. From Table 1 it can be seen that the acid-insoluble material accounted for 60-75% of the total acid-insoluble incorporation after lipid extraction. After phenol extraction and acid-precipitation only, it accounted for 80-90% of the counts. The acid-insoluble material had about the same lability to alkali as RNA but was much more stable to acid. In 5% trichloroacetic acid at 70°C the rate constant of its hydrolysis was $4.8 \times 10^{-3} \text{ min}^{-1}$ compared with 0.2 min^{-1} for RNA.

After alkaline hydrolysis the [³H]ATP-labelled material became acid-soluble and the radioactivity was recovered in three spots, 2'(3')-[³H]adenylate and [³H]adenosine from RNA and an unidentified compound running near ADP, which was thought to arise from the acid-insoluble material described above. Paper chromatography in several solvents

Table 3. *Effect of atractyloside on incorporation of [³H]ATP*

Mitochondria were isolated in sucrose-EDTA and incubated as described in Table 1 with [³H]ATP as the precursor, and the effect of atractyloside was examined. The RNA was isolated, hydrolysed and the hydrolysate chromatographed as described in Table 1.

Medium	Incorporation (% of control)			
	Total	Unidentified compound	2'(3')-Adenylate	Adenosine
Iso-osmotic	100	58.0	34.4	7.5
Iso-osmotic+atractyloside				
(3.04 μ M: 0.6 nmol/mg of protein)	77.0	44.7	25.5	6.8
(15.2 μ M: 3 nmol/mg of protein)	37.9	23.1	11.3	3.5
(76 μ M: 15 nmol/mg of protein)	19.0	13.0	3.8	2.2
(216 μ M: 94.5 nmol/mg of protein)	8.4	2.9	4.1	1.4
Hypo-osmotic	100	55.2	27.4	17.3
Hypo-osmotic+atractyloside				
(15.2 μ M: 3 nmol/mg of protein)	28.3	15.8	5.3	7.2

showed that the unidentified compound was not ATP, ADP or 5'- or 2'(3')-AMP. Further characterization was not attempted.

Effect of atractyloside

It has been shown that atractyloside inhibits the transport of adenine nucleotides across the inner mitochondrial membrane (Winkler & Lehninger, 1968; Vignais & Vignais, 1970). Inhibition of [³H]-ATP incorporation into RNA by atractyloside would strongly indicate that the synthesis was proceeding within the inner mitochondrial membrane. Because [³H]ATP was incorporated into acid-insoluble compounds in addition to RNA, the effect of atractyloside on RNA synthesis had to be followed by alkaline hydrolysis and paper chromatography (Table 3). The incorporation into RNA, recovered as 2'(3')-[³H]-adenylate, was inhibited 50% at about 2 nmol of atractyloside/mg of protein for mitochondria isolated in sucrose-EDTA and incubated in the iso-osmotic medium. The same inhibition was observed in the hypo-osmotic medium (Table 3). For mitochondria isolated in sucrose-TES 50% inhibition was at 3 nmol of atractyloside/mg of protein. The incorporation into the unidentified compound and adenosine was slightly less sensitive.

The specificity of this inhibition, is shown by the effect of atractyloside on [³H]UTP incorporation (Fig. 4). At low concentrations of ATP, atractyloside inhibited [³H]UTP incorporation, but as the concentration of ATP was raised the inhibition was eliminated. In addition, as the ATP concentration was raised the total incorporation decreased slightly.

Nature of the RNA synthesized

Previously we had shown with electrophoretic analysis that isolated rat liver mitochondria syn-

Fig. 4. *Effect of ATP and atractyloside on the incorporation of [³H]UTP into RNA*

Mitochondria were isolated in sucrose-EDTA, incubated at 30°C for 20 min in the hypo-osmotic medium and RNA was recovered and counted as described for Fig. 1. ○, Control; ●, atractyloside (28 μ M or 7.5 nmol/mg of protein).

thesize mitochondrial rRNA as well as a heterodisperse RNA (Fukamachi *et al.*, 1970). This is shown further in Figs. 5-7, where the product of [³H]UTP incorporation was examined by sucrose-density-gradient centrifugation. In Fig. 5, where RNA was extracted by a method that yields high-molecular-weight RNA only, peaks at 16S and 13S characteristic of mitochondrial rRNA are clearly seen. In addition there is a very small peak at 21S. The bulk of the synthesized RNA gives a heterodisperse

pattern including RNA sedimenting at greater than 28S. Extraction with hot phenol-sodium dodecyl sulphate gave a similar pattern (Fig. 6), although the 13S component was present only as a shoulder on the 16S peak. Some 4S RNA was observed. A similar pattern of incorporation of [3 H]UTP into RNA was obtained with a cold-phenol-sodium dodecyl sulphate extraction method, as shown in Fig. 7. The sensitivity of the synthesis to actinomycin D is also shown in Fig. 7. A peak at 20–21S was always observed (Figs. 5–7) regardless of the method of extracting the RNA.

In contrast with [3 H]UTP incorporation, radioactivity from [3 H]CTP was recovered in a product that sedimented at about 4S, corresponding to *E. coli* tRNA (Fig. 8). Its synthesis was not inhibited by actinomycin D.

Discussion

Although we had previously shown that isolated rat liver mitochondria synthesized mitochondrial

rRNA (Fukamachi *et al.*, 1970) the characteristics of the process were not presented. The site of incorporation is within the inner mitochondrial membrane. This is most clearly shown by the specific inhibition of [3 H]ATP incorporation by atracyloside, which inhibits ADP and ATP exchange only across the inner mitochondrial membrane (Winkler & Lehninger, 1968), and by the inhibition of [3 H]UTP incorporation by acriflavine, actinomycin D and ethidium bromide, since mitochondrial DNA is within the inner mitochondrial membrane (Nass *et al.*, 1965). Kalf & Faust (1969) showed that isolated inner membrane preparations (digitonin particles) synthesize RNA.

Inhibition by actinomycin D, acriflavin and ethidium bromide demonstrates not only the site of synthesis but also that the synthesis is DNA-dependent, in agreement with previous results (Neubert *et al.*, 1965; Saccone *et al.*, 1969; Fukamachi *et al.*, 1970). With mitochondria isolated in sucrose-EDTA, but not in sucrose-TES, an actinomycin D-insensitive incorporation of [3 H]UTP occurred in the first

Fig. 5. Sedimentation on a linear sucrose density gradient of RNA synthesized with [3 H]UTP after a 1 h incubation in the hypo-osmotic medium and extracted by the cold-phenol-sodium dodecyl sulphate method

Mitochondria were isolated in sucrose-EDTA and incubated with [3 H]UTP for 1 h at 30°C in the hypo-osmotic medium. RNA was extracted by the cold-phenol-sodium dodecyl sulphate method, in which high-molecular-weight RNA only is recovered. The RNA was centrifuged on a 5–20% linear sucrose gradient for 12 h at 108 000g at 4°C. The E_{260} was read and the radioactivity counted as described in the Methods and Materials section. ■, Cytoplasmic ribosomal RNA; ○, mitochondrial RNA.

Fig. 6. Sedimentation on an isokinetic sucrose density gradient of RNA synthesized with [3 H]UTP after a 1 h incubation in the hypo-osmotic medium and extracted by the hot-phenol-sodium dodecyl sulphate method

Mitochondria were isolated in sucrose-EDTA and incubated with [3 H]UTP for 1 h at 30°C in the hypo-osmotic medium. RNA was extracted by the hot-phenol-sodium dodecyl sulphate method and centrifuged on a 15–33% isokinetic sucrose density gradient for 13 h at 192 000g at 4°C. The E_{260} was read and the radioactivity counted as described in the Methods and Materials section. ■, Cytoplasmic ribosomal RNA; ○, mitochondrial RNA.

Fig. 7. Sedimentation on an isokinetic sucrose density gradient of RNA synthesized with $[^3\text{H}]\text{UTP}$ after a 1 h incubation in the hypo-osmotic medium with and without actinomycin D

Mitochondria were isolated in sucrose-EDTA and incubated with $[^3\text{H}]\text{UTP}$ for 1 h at 30°C in the hypo-osmotic medium. Actinomycin D was added to $40\text{ }\mu\text{g/ml}$ in one case. RNA was extracted by the cold-phenol-sodium dodecyl sulphate method, in which total RNA is recovered. The RNA was centrifuged as described in Fig. 6. The E_{260} was read and the radioactivity counted as described in the Methods and Materials section. ■, Cytoplasmic rRNA and *E. coli* tRNA; ○, mitochondrial RNA synthesized in the absence of actinomycin D; ●, mitochondrial RNA synthesized in the presence of actinomycin D.

10 min of incubation. It was sensitive to ribonuclease and because of the high recovery of $[^3\text{H}]\text{uridine}$ might be incorporation into short oligonucleotide chains or terminal addition. The site of this synthesis is not known. Less inhibition of $[^3\text{H}]\text{UTP}$ incorporation by actinomycin D was seen with mitochondria isolated in sucrose-TES and incubated in the iso-osmotic medium. Inhibition by actinomycin D was not observed previously with short incubations (5 or 10 min) in iso-osmotic medium except with swollen mitochondria and higher concentrations of the antibiotic (Neubert *et al.*, 1965; Saccone *et al.*, 1969). These results are consistent if intact mitochondria are slowly permeable to actinomycin D but swollen mitochondria, including those incubated in hypo-osmotic media in which the matrix space increases (Stoner & Sirak, 1969), are permeable to the antibiotic. The higher incorporation of $[^3\text{H}]\text{UTP}$, $[^3\text{H}]\text{CTP}$ and $[^3\text{H}]\text{GTP}$, but not $[^3\text{H}]\text{ATP}$, in the hypo-osmotic medium further indicates that mitochondria became more permeable under those conditions.

Fig. 8. Sedimentation on an isokinetic sucrose density gradient of RNA synthesized with $[^3\text{H}]\text{CTP}$ for 20 min with and without actinomycin D

Mitochondria were isolated in sucrose-EDTA and incubated with $[^3\text{H}]\text{CTP}$ for 20 min in the hypo-osmotic medium. Actinomycin D was added to $40\text{ }\mu\text{g/ml}$ in one case. The RNA was extracted and characterized as described in Fig. 7. ■, Cytoplasmic rRNA and *E. coli* tRNA; ○, mitochondrial RNA synthesized in the absence of actinomycin D; ●, mitochondrial RNA synthesized in the presence of actinomycin D.

ATP is readily exchanged across the mitochondrial membrane, but, since UTP, CTP and GTP are taken up or exchanged at a low rate (Duée & Vignais, 1969), an increase in permeability would increase their incorporation. Mitochondria isolated in sucrose-EDTA are probably more permeable to nucleotides because the osmolarity of the medium had a smaller effect on the incorporation of $[^3\text{H}]\text{GTP}$ and $[^3\text{H}]\text{CTP}$.

Rifampicin did not inhibit RNA synthesis although this might be expected in view of the suggested origin of mitochondria from bacteria (Roodyn & Wilkie, 1968). Some recent studies with partially purified mitochondrial RNA polymerase from yeast have not found an inhibition by rifampicin (Wintersberger, 1970; Tsai *et al.*, 1971), but in most studies this enzyme from many species including mammals was inhibited (Küntzel & Schäfer, 1971; Reid & Parsons, 1971; Scragg, 1971; Horgen & Griffin, 1971). Therefore, if the latter observations are correct, although the mitochondria were permeable to actinomycin D in the hypo-osmotic medium, lack of inhibition by rifampicin, rifamycin SV and streptovaricin might indicate that under these conditions the mitochondria

were impermeable to these antibiotics. Alternatively it might mean that few RNA chains are initiated because this is the step inhibited by rifampicin in bacteria (Wehrli *et al.*, 1968; Sippel & Hartmann, 1968; Mizuno *et al.*, 1968; DiMauro *et al.*, 1969).

Although ATP was incorporated into RNA it was also incorporated to a greater extent into an acid-insoluble compound that was relatively acid-stable and hydrolysed in alkali to a low-molecular-weight compound that was not ATP, ADP or 5'- or 2'(3')-AMP. This might explain the high incorporation of ATP compared with UTP and GTP reported by Saccone *et al.* (1967, 1968). Since ATP was not incorporated into RNA only, earlier studies that showed atractyloside inhibition of ATP incorporation into acid-insoluble material (Saccone *et al.*, 1967, 1968) were repeated. About 2 and 3 nmol of atractyloside/mg of protein gave 50% inhibition of RNA synthesis as measured by recovery of 2'(3')-adenylate. Further, ATP had to be present otherwise atractyloside inhibited [³H]UTP incorporation. Mitra & Bernstein (1970) found that 1 nmol of atractyloside/mg of protein inhibited [³H]dATP incorporation into DNA by isolated rat liver mitochondria by 50% and that ATP prevented atractyloside inhibition of [³H]-TTP incorporation into DNA.

A number of observations are consistent with the turnover of the CCA end of mitochondrial tRNA. These are the high recovery of [³H]cytidine and [³H]adenine from the 3'-terminal fragment of RNA, a high actinomycin D-insensitive fraction of 2'(3')- [³H]cytidylate obtained on alkaline hydrolysis of the RNA product and the turnover of incorporated radioactivity from [³H]CTP but not from [³H]UTP. The RNA synthesized with [³H]CTP as the precursor ran in the tRNA region in a sucrose density gradient and its synthesis was actinomycin D-insensitive. The turnover was of tRNA in mitochondria because the osmolarity of the medium affected the recovery of the [³H]CTP products and atractyloside inhibited the incorporation of [³H]ATP. Saccone *et al.* (1969) previously suggested a turnover of the terminal adenosine of mitochondrial tRNA but not that of the cytidylate. Turnover of the CCA end of tRNA is catalysed by a specific tRNA pyrophosphorylase that has been isolated from *E. coli* (Preiss *et al.*, 1961) and rat liver (Daniel & Littauer, 1963). It is likely that rat liver mitochondria have a similar enzyme.

[³H]UTP was incorporated both into 16S and 13S RNA characteristic of mitochondrial ribosomal RNA (Bartoov *et al.*, 1970), a peak at 20–21S and a heterodisperse RNA found throughout the gradient including RNA sedimenting at greater than 28S. All these species of RNA were synthesized on a DNA template, as shown by inhibition by actinomycin D. The size of the RNA synthesized, as indicated by alkaline hydrolysis, was 140 nucleotides, which is much smaller than that shown by sedimentation

analysis. This could be explained if isolated mitochondria only complete chains initiated *in vivo* or if there is synthesis of a large number of short chains or chain termini in addition to the high-molecular-weight RNA. Saccone *et al.* (1969) also observed that the RNA synthesized by isolated rat liver mitochondria was present in sizes from 9S to greater than 23S. The present study confirms our earlier conclusion (which utilized the greater resolving power of acrylamide gel electrophoresis; Fukamachi *et al.*, 1970) that mitochondrial DNA codes for mitochondrial rRNA. This is in agreement with studies by other approaches (Bartoov *et al.*, 1970; Zylber *et al.*, 1969; Aloni & Attardi, 1971a). However, only a small proportion of the RNA formed by isolated mitochondria was mitochondrial rRNA. The time of synthesis was 1 h at 30°C, which with a Q_{10} of 2–3 is equivalent to 20–30 min at 37°C. This compares with the usually much longer times of up to 24 h in which mainly mitochondrial rRNA is labelled *in vivo* (Bartoov *et al.*, 1970; Zylber *et al.*, 1969; Dubin & Czaplicki, 1970). The bulk of the RNA therefore could be significant products of the mitochondrial DNA, perhaps of a messenger type or a rRNA precursor. At present mitochondrial mRNA or rRNA precursors have not been demonstrated. Since mitochondria appear to synthesize a number of proteins of varying molecular weights (Coote & Work, 1971), mRNA could be synthesized from mitochondrial DNA templates. The size of some of the RNA seen on the gradients is very large and if they represent mRNA then they must either be cut down in size for translation or they are polycistronic mRNA species. Aloni & Attardi (1971b) and Dubin & Czaplicki (1970) have reported high-molecular-weight RNA synthesized from mitochondrial DNA in whole mammalian cells. Dubin & Czaplicki (1970) also reported a 21S species of mitochondrial RNA. In addition Aloni & Attardi (1971b) report that much of the high-molecular-weight RNA might be synthesized from the light DNA strand. They also concluded that the synthesis might be symmetrical, that is from both strands of DNA. Aaij *et al.* (1970) also found that isolated rat liver mitochondria synthesized RNA from the light DNA strand and that there was symmetrical synthesis. The significance of this is still unclear.

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