Electrophoretic and Centrifugation Behaviour of Mitochondrial Ribonucleic Acid from Walker 256 Carcinosarcoma

By NESTOR F. GONZÁLEZ-CADAVID and JOSÉ LUIS PÉREZ

Departamento de Biología Celular, Facultad de Ciencias, Universidad Central de Venezuela, Apartado 10098, Caracas, Venezuela

(Received 7 June 1974)

To investigate the possibility that mitochondrial transcription could be altered in tumours we started by characterizing the RNA obtained from mitochondria, isolated from Walker carcinosarcoma and purified by a procedure devised to compensate for the lower size and density of these organelles in 10-day tumours. The RNA was extracted by the 'hot phenol' technique and analysed by electrophoresis in 2.7 and 2.5% polyacrylamide gels at different running times, identifying the usual cytoplasmic contaminants 28 and 18S peaks plus the other five major peaks at 40, 20.5, 16.3, 15.4, and 4S_e. The 28 and 18S_e peaks were not eliminated by digitonin treatment of the mitochondria, indicating that they arise from cytoplasmic ribosomes tightly associated with the mitochondria. From its sensitivity to DNAase (deoxyribonuclease), resistance to RNAase (ribonuclease) and coincidence with external marker DNA, the 40S_e peak was identified as containing mainly DNA. Sucrose-gradient centrifugation for different periods showed a major component at 16.2S, the 28 and 18S cytoplasmic RNA species, peaks at 13.8, 6.4 and 4S and a small 19.5S peak. By polyacrylamide-gel electrophoresis of the purified RNA classes separated by one or two cycles of centrifugation, the following correlations were established: 20.5S_e = 19.5S; 16.3S_e = 16.2S; 15.4S_e = 13.8S. The 6.4S RNA ran as a mixture of 4 and 4.7S_e species. When the 20.5S_e and 15.4S_e RNA species were centrifuged, they behaved as 16.2S and 13.8S respectively, thus suggesting that the 16.2S (16.3S_e) arises by cleavage from the 19.5S (20.5S_e), the 13.8S (15.4S_e) being the other RNA from mitochondrial ribosomes.

Mitochondria from malignant cells show abnormal functional and morphological features, which were initially assumed to be linked to respiratory alterations, mainly the high aerobic glycolysis present in rapidly proliferating tissues (see e.g., Mintz *et al.*, 1967; Arcos, 1971; Sordahl & Schwartz, 1971). None of the described alterations, however, has been conclusively demonstrated as specific for cancer cells, few are generally found in different kinds of tumours, and even many of the functional impairments are simply derived from either a special fragility and lability of neoplastic mitochondria, or the release of damaging factors during disruption of these cells. Most authors would agree therefore with the view that mitochondrial pathology in cancer is the result of subcellular disarrangement arising during malignant proliferation, rather than playing any causal or triggering role in carcinogenesis (see, e.g., Mehard *et al.*, 1971; Feo *et al.*, 1973; Weinhouse, 1972).

The possibility remains, however, that mitochondria could be the location of some steps in the process of cell transformation caused by certain carcinogens, by either helping their action on the genome, or by

becoming targets which, through interference in the mitochondrial–cytoplasmic co-ordination of protein and nucleic acid synthesis, favour the uncontrolled replication characteristic of neoplastic growth. Mitochondrial DNA has received much attention in this respect, and, although the initial findings of large proportions of unicircular dimers and higher oligomers in leukaemic leukocytes correlating with the severity and evolution of the disease (Clayton & Vinograd, 1967, 1969) were later assumed to be non-characteristic of neoplasia (Paoletti *et al.*, 1972; see also Borst, 1972; Paoletti & Riou, 1973), their significance has not yet been disproved (Smith & Vinograd, 1973). Other authors have described recently, in mitochondria from cells transformed by Rous sarcoma virus, the presence and biosynthesis of viro-somes, detecting Gs antigens and virus reverse transcriptase, which, together with the sensitivity of virus replication and malignant transformation to chloramphenicol and ethidium bromide, has suggested that mitochondria play a significant role in the replication of this oncogenic virus and its cell-transforming action (Kára *et al.*, 1972; Richert & Hare, 1972;

Küntzel *et al.*, 1973). Also some plant RNA viruses appear to multiply within mitochondria (see Paoletti & Riou, 1973).

Considering the import of mRNA of cytoplasmic origin to the mitochondria (Avadhani *et al.*, 1973; Kyriakidis & Georgatsos, 1973; Gaitskhoki *et al.*, 1973) and the as yet not discarded possibility of a reverse flow of mRNA to be translated on cytoplasmic ribosomes [see, however, Hirsch & Penman (1973)], the search for specific alterations in mitochondrial transcription in neoplasia might help to define whether or not the cytoplasmic-mitochondrial interactions intervene in the control of cell replication. Although most of the information available on the different RNA species of eukaryotic mitochondria has been collected from cultured cell lines derived from originally malignant tissues, this is generally accepted as a 'normal' model (see, e.g., Borst & Grivell, 1971; Borst, 1972; Wintersberger, 1972). It is not unlikely therefore that tumours *in situ* could show significant differences in this respect as compared with dividing cells outside the host milieu, or normal tissues. In the present work we have approached this question by defining the electrophoretic features of mitochondrial RNA in the Walker carcinoma of the rat and correlating them with its behaviour in sucrose-gradient centrifugation, as the initial basis for further identification of the products of neoplastic mitochondrial transcription *in vivo* with specific inhibitors and molecular hybridization.

Materials and Methods

Materials

Chemicals. [32 P]Phosphoric acid in 0.01M-HCl solution (carrier-free) was obtained from the Commissariat à l'Energie Atomique, Gif-sur-Ivette, France, and neutralized to pH 7.4 with 0.1M-NaOH. Electrophoretically purified RNA-free DNAase* I, pancreatic RNAase type II-A, RNAase T₁; cytochrome *c* (type III), NADPH (type I), D-glucose 6-phosphate, disodium salt, IDP and Na₂SO₄ were all purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Digitonin, diethyl pyrocarbonate (diethyl oxydiformate) and bentonite were from Fisher Scientific Co., Springfield, N.J., U.S.A. Naphthalene-1,5-disulphonic acid, disodium salt pract. was acquired from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. The reagents used for polyacrylamide-gel electrophoresis and radioactivity determinations were as specified by González-Cadavid & Sáez de Córdova (1974). All other materials were from BDH Chemicals Ltd., Poole, Dorset, U.K., AnalaR grade whenever available, except for the suc-

rose used for RNA gradient centrifugation (AristaR grade). Double-glass-distilled water was used throughout this work.

Animals. Male Sprague-Dawley rats (180±5g body wt.) were obtained from a closed colony bred at the Instituto de Medicina Experimental, Universidad Central, Caracas, Venezuela. Subcutaneous transplantation of Walker 256 carcinosarcoma was as specified by González-Cadavid & Herrera Quijada (1974), except that rats were killed after 10 days of inoculation.

Methods

Isolation of the mitochondrial fraction. Several procedures were tested while designing a technique specifically suited for the Walker carcinoma. All were carried out at 2–4°C, in the SS-34 rotor of the Sorvall RC2-B centrifuge, except for the microsomal fraction which was prepared in the 50 rotor of the Spinco L2 65 B ultracentrifuge and other cases as indicated. The tumour pieces were forced through a tissue press, made to the design of Porterfield (1960) and purchased from Climpex Ltd., London N.W.7, U.K., and then homogenized in the appropriate medium in a Potter-Elvehjem homogenizer, with a Teflon pestle (diam. 25mm) with 0.15–0.22mm clearance from the glass mortar. A portion of the homogenate corresponding to 10g of tumour was processed for the enzyme assays. Procedure no. 1 was based on the method of Roodyn *et al.* (1961), and procedure no. 2 was as described by O'Brien & Kalf (1967), both for liver mitochondria. Procedure no. 3 was as described by Li *et al.* (1965) for Walker carcinoma. Procedure no. 4, the method finally adopted, was developed in the present work. The tumour was homogenized, in 2 vol./g of tumour, of 0.25M-sucrose in a buffer designated C containing 1mM-EDTA, 2mM-Tris-HCl, pH 7.4, at 2°C and 0.5% of naphthalene-1,5-disulphonate. This last compound was used as an RNAase inhibitor but was omitted when enzymes were assayed. Twenty up-and-down strokes at 3000–4000 rev./min were applied and the suspension was made up to 4.5 vol./g of tumour. The nuclear fraction was sedimented at 600g for 10 min and the supernatant kept while each pellet was rehomogenized in 0.7 vol. of medium with ten up-and-down strokes. After being made up to 1.2 vol., the nuclear suspension from each 10g of tumour was layered on to a linear gradient prepared with 10.4 ml of 0.5M-sucrose and 9.6 ml of 1.0M-sucrose, both in buffer C. Centrifugation was performed at 3700g for 40 min in the SW 25.1 rotor of the Spinco L2-65B ultracentrifuge. The upper 27 ml of each gradient was sucked out, leaving behind the nuclear pellet, diluted with 1 vol. of buffer C, and pooled with the first postnuclear supernatant. The mitochondrial fraction was sedimented at 12000g for 10 min, resuspended with a Vortex mixer in 2.5 vol. of

* Abbreviations: DNAase, deoxyribonuclease; RNAase, ribonuclease.

medium and centrifuged again at 12000g for 10 min, repeating the washing steps twice more. The mitochondrial fraction from each 10g of tumour was then suspended in a final volume of 1.6ml with homogenization medium and layered on to a gradient prepared with 6.2ml of 1.128M-sucrose and 5.8ml of 1.562M-sucrose, both in buffer C. Isopycnic centrifugation was performed at 201800g for 30min in the SW 40 rotor of the Spinco L2-65B ultracentrifuge. For enzyme analysis the gradient was pumped out from the bottom of the tube with a peristaltic pump at 1ml/min and collected in six 2ml and one 1.6ml fractions, which were numbered from 1 (bottom) to 7 (top), discarding the pellet. In one instance the isopycnic centrifugation was carried out by layering 3.2ml of the crude mitochondrial suspension on to 28ml of the linear gradient and centrifuging at 62500g for 120min in the SW 25.1 rotor. Six 4.6ml and one 3.6ml fractions were collected from the bottom. The intermediate, microsomal, and cell sap fractions were obtained from the post-mitochondrial fraction (not including the washing steps) at 15000g for 20min and 165000g for 40min respectively. The sample taken was usually equivalent to 1g of tumour.

In certain cases of isolation of mitochondrial RNA, where more tissue was required, the amount of tumour processed was raised to multiples of 10g. The maximum capacity of the SW 25.1 rotor is therefore 30g of tumour, but with the SW 27 rotor it is possible to process in a single run 60g which would then be adequate for filling the six positions of the SW 40 rotor. The procedure for fraction collection after isopycnic centrifugation was simplified by suction with a pipette provided with a pipette filler. The top 2.1ml was discarded, the next 4.0ml only being collected (some of fraction 4, all of 5, and most of 6), and the remainder being left behind. The pool was diluted with 2.5 vol. of buffer C, made up to 45ml with homogenization medium, and the '4-6' mitochondrial fraction was sedimented at 18000g for 10min.

In one experiment the mitochondria were treated with digitonin as described by Malkin (1971), at a digitonin/protein ratio of 1/100 and a protein concentration of 25mg/ml, to remove any residual contamination by endoplasmic-reticulum fragments. The mitochondria were recovered by centrifugation at 15000g for 10min and washed under identical conditions.

Enzyme assays. They were performed the same day of fractionation on samples from the tumour homogenate and the suspensions of the mitochondrial sub-fractions as obtained from the gradient. The other fractions were homogenized in the fractionation medium used in each case. Protein was estimated by the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard. Cytochrome *c* oxidase was assayed by following the oxidation at 30°C

of 0.037mM-cytochrome *c*, reduced by the method of Yonetani & Ray (1965), in 1mM-EDTA-30mM-phosphate buffer, pH 7.4, after treating the mitochondrial suspension with 0.01% Triton X-100.

The activity is expressed as the first-order velocity constant/min per mg of protein ($k \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$). NADPH-cytochrome oxidase was assayed by the method of Sottocasa *et al.* (1967) and the activity is expressed as nmol of cytochrome *c* reduced/min per mg of protein. Glucose 6-phosphatase was assayed by the procedure of de Duve *et al.* (1955) and the activity is expressed as nmol of P_i released/min per mg of protein.

RNA isolation. The 'hot phenol' method of Penman (Penman, 1966; Wagner *et al.*, 1967) was used because it favours the recovery of mRNA (Abe *et al.*, 1972) and has been successfully applied to the isolation of RNA from HeLa-cell mitochondria. The fresh pellets of the '4-6' mitochondrial fraction (from 10-30g of tumour) and the microsomal fraction (from samples equivalent to 1-2g of tumour) were suspended in 2ml of a medium containing 2% sodium dodecyl sulphate, 0.1M-NaCl, 0.01M-Tris-HCl, pH 7.4, at 20°C, 0.001M-EDTA and 0.125% bentonite. The bentonite was prepared by the method of Watts & Mathias (1967) and suspended in 0.01M-sodium acetate buffer, pH 6.0, to a final concentration of 6%. In certain instances, the medium described in the original procedure was used, which differs in the absence of bentonite and a lower sodium dodecyl sulphate concentration (0.5%). In both cases, 0.04ml of 50% diethyl pyrocarbonate in ethanol was added. The RNA was precipitated from the aqueous phase with 2.5 vol. of ethanol at -20°C, and the mixture stored as such at -20°C.

When indicated, DNAase treatment of RNA was done in a buffer containing 10mM-Bicine, 0.15M-NaCl, 10mM-MgCl₂, with RNAase-free DNAase (20µg/ml) for 15min at 22°C. RNAase treatment of RNA was carried out in the same buffer, with a mixture of pancreatic RNAase II-A (50µg/ml) and RNAase T₁ (30µg/ml) (previously heated at 90°C for 10min), for 30min at 37°C. In both instances, the added enzymes were eliminated by repeating the phenol-chloroform extractions and ethanol precipitation.

Polyacrylamide-gel electrophoresis of RNA. The procedure of Weinberg *et al.* (1967) was followed; the RNA suspensions were centrifuged at 22000g for 10min and suspended in 75µl (mitochondrial RNA) or 300µl (microsomal RNA) of buffer E, containing 0.04M-Tris, 0.02M-sodium acetate, 2mM-EDTA, 0.2% sodium dodecyl sulphate and 30% glycerol, all at pH 7.4 (20°C). Samples (75µl) were layered on cylindrical gels (0.6cm × 10cm) containing 2.5 or 2.7% (w/v) purified acrylamide, 0.25% ethylenediacrylate, 0.08% NNN'-tetramethylethylenediamine, 0.08% (NH₄)₂S₂O₈, 10% glycerol, 0.04M-Tris,

0.02M-sodium acetate, 2mM-EDTA, all at pH 7.4 (20°C). Electrophoresis was performed at 5mA/gel, for periods from 2 up to 6h, in a buffer similar to buffer E except that glycerol was at 10% concentration

The gels were scanned for absorbance in a 6.5mm-light-path cuvette with a Joyce-Loebl u.v. scanner (265nm filter) coupled to a Bryans type 27000 chart recorder, the 1V×6 attenuation range most often being used. Under these conditions a 2.5h run of 1 E unit (50µg) of RNA isolated from the cell sap of the Walker tumour gave a single narrow peak with a maximum height of 0.32 on graph paper normalized from 0 to 1. The amount of RNA in each peak can be roughly calculated by assuming that it is directly proportional to the height of the peak. The baseline was reasonably constant when recrystallized acrylamide was used and the gel was washed for 30min in fresh electrophoresis buffer immediately after the run. However, in many instances it increased steadily towards the gel bottom, owing to a high background of heterogeneous RNA, and an artificial baseline had to be drawn for calculating the peak heights. Overlapping of adjacent peaks and widening of some regions, particularly in running periods longer than 3h, are obvious drawbacks, but at constant running times, the peak heights can be taken as relative estimates for comparative purpose. For the calculation of unknown S_e values, microsomal and cell-sap RNA species from Walker carcinoma were isolated and run in separate gels. A straight line was obtained when the logarithms of the S values of the marker peaks were plotted against the distance migrated, as done by Groot *et al.* (1970)

After the densitometry, some gels were frozen in a hexane-solid CO₂ bath as described by Weinberg *et al.* (1967), and cut into 2mm slices with an electronically controlled gel slicer (Metaloglass Inc., Boston, Mass., U.S.A.). Slices were collected in scintillation vials, hydrolysed with 0.4ml of concentrated aq. NH₃ for 1–2h, and used for the determinations of radioactivity. In some instances the slices corresponding to the area under each peak were pooled, and not submitted to hydrolysis but counted directly. Then they were suspended in 8ml of buffer E without glycerol containing 0.15M-NaCl and 1% diethyl pyrocarbonate and disintegrated by passage through a 13mm-length 16-gauge syringe needle. The RNA was eluted overnight with gentle shaking of the suspensions at 22°C. The gel was sedimented by centrifugation at 20000 g for 10min in graduated conical tubes and the top 6ml was sucked out, precipitated with 15ml of ethanol at –20°C and stored for further analysis.

Sucrose-gradient centrifugation of RNA. The procedure used by Penman (1966) was followed. The RNA pellets were suspended in 1.0ml of 0.5% sodium dodecyl sulphate, 0.1M-NaCl, 0.005M-Tris-HCl,

pH 7.3, and layered on to a 12ml gradient of 15–30% (w/v) sucrose containing 0.5% sodium dodecyl sulphate, 0.1M-NaCl and 0.005M-Tris-HCl, pH 7.3. Centrifugation was performed in the SW40 rotor of the Spinco L2-65B ultracentrifuge at 18°C for times and g as specified in each experiment. The E_{260} was recorded in a Beckman DU-2 spectrophotometer with a 10 inch (25.4 cm) logarithmic recorder, by pumping the gradient from the bottom of the tube at 0.8ml/min through a flow cell (10mm light-path). When necessary, 0.25ml fractions were collected and those belonging to each RNA peak were pooled, either immediately or after radioactivity determinations. Ethanol (2.5vol.) was added at –20°C and the suspension stored.

For calculations of unknown S values, microsomal and cell-sap RNA species from Walker carcinoma were centrifuged in separate tubes. The plot of S values of the marker peaks against effluent volumes from the flow cell was not strictly linear, since the gradients were not isokinetic.

Determination of radioactivity. The ³²P contained in the fractions obtained after polyacrylamide-gel electrophoresis and sucrose-gradient centrifugations was generally determined by counting directly the Cerenkov radiation in a 3385 Packard liquid-scintillation counter at 42% efficiency, irrespective of whether the gel slices were digested or not with aq. NH₃. All results were corrected to d.p.m. according to the respective efficiencies and for radioactive decay.

Results

Mitochondrial purification

The application to the Walker carcinoma of a conventional method used for the isolation of liver mitochondria (Roodyn *et al.*, 1961) was inadequate because of the high proportion of mitochondria trapped in the nuclear fraction and the poor specific activity of cytochrome oxidase (Table 1). Glucose 6-phosphatase was very low in the microsomal fraction (38 units as compared with 310 in liver), but still showed 25% contamination of mitochondria by fragments of the endoplasmic reticulum. An even poorer recovery was obtained by a method designed for an extreme purification of liver mitochondria (O'Brien & Kalf, 1967) for the preparation of mitochondrial ribosomes. NADPH-cytochrome *c* reductase was no better than glucose 6-phosphatase as a microsomal marker, since its specific activity was also 10-fold lower than in liver. When a procedure applied by Li *et al.* (1965) to Walker carcinoma was tested, only 3% of the cytochrome oxidase was recovered in the mitochondrial fraction, as compared with 41% obtained by these authors. Our extremely low yield is in agreement with the recovery by the other two methods, considering that in the procedure of Li *et al.* (1965)

Table 1. *Recovery of mitochondria from Walker carcinoma by the use of different procedures*

Each procedure was applied in a separate experiment to 10 g of tumour as described in the text. Results are averages of duplicate experiments except in procedure no. 3. In tumour homogenates the average specific activity of cytochrome oxidase was $3.7 k \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and the average protein content was 119 mg/g of tumour.

Fraction	Percentage distribution referred to sum of fractions							
	Procedure no. 1 [Roodyn <i>et al.</i> (1961) modified]		Procedure no. 2 (O'Brien & Kalf, 1967)		Procedure no. 3 (Li <i>et al.</i> , 1965)		Procedure no. 4 (Present work)	
	Protein	Cytochrome oxidase	Protein	Cytochrome oxidase	Protein	Cytochrome oxidase	Protein	Cytochrome oxidase
Nuclear	44.3	77.8	—	—	43.8	62.9	40.7	49.1
Mitochondrial	2.4	9.3	0.2	0.6	0.9	2.9	1.7	11.6
Intermediate	2.8	8.9	5.0	5.7	2.8	13.6	6.0	20.3
Microsomal	6.2	4.0	7.9	2.3	20.2	20.6	13.7	19.0
Cell sap	44.3	0	—	—	32.3	0	37.9	0
Specific activity of cytochrome oxidase ($k \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein)								
Mitochondrial	—	13.5	—	10.6	—	15.6	—	21.4
Intermediate	—	11.2	—	5.0	—	22.5	—	10.6

the mitochondria are sedimented and washed at lower speeds (3300g), but the discrepancy with the original results might be ascribed to differences in mitochondrial size in strains of the same tumour.

The abnormal distribution of cytochrome oxidase in the Walker carcinosarcoma was assumed to be due to three main factors: (a) incomplete disruption of the plasma membrane; (b) trapping of mitochondria within the nuclear fraction; (c) lower size and density of the tumour mitochondria as compared with liver mitochondria. The first two problems were partially counteracted by increasing the homogenization period and re-extracting the nuclear fraction by sedimentation centrifugation through a density gradient. The last factor was the most important, since even after 20000g for 20 min 15% of the cytochrome oxidase remained unsedimented. A compromise had to be attained, however, between recovery and purity, and 12000g for 10 min was shown to be the most adequate centrifugation. Under these conditions, although yield was still poor (15–20% of the total cytochrome oxidase activity), the mitochondrial fraction was less contaminated, as judged by the specific activity of cytochrome oxidase, and owing to the re-extraction steps was more representative of the whole mitochondrial population.

The purity was increased by the introduction of an isopycnic centrifugation through a sucrose gradient of lower density than usual for liver mitochondria, which in 30 min at 201 800g equilibrated in the bottom layers around the normal mean density of 1.18 (Table 2). The tumour mitochondria were concentrated mainly in the top layers, particularly around a mean density of 1.15. As a result, whereas the recovery of mitochondria in fractions '4+5+6' was for carcinoma nearly 80% of the sum of all gradient fractions (except

the pellet), that for liver was only 14%. The specific activity of cytochrome oxidase was in the case of tumour 1.5-fold over that of the crude mitochondria. The assays of NADPH-cytochrome *c* reductase (not tabulated here) showed that contamination with fragments of the endoplasmic reticulum increased progressively from top to bottom of the gradient. A 30 min centrifugation was enough to achieve isopycnic equilibration at 201 800g, since the extension to 90 min did not significantly change the pattern of distribution of protein or cytochrome oxidase. When a larger-capacity rotor (SW25.1) was used at maximum speed (63 600g) for 2 h, the results were similar. The small size, low density, fragility and difficulty in removing microsomal contamination are general features of tumour mitochondria (Mintz *et al.*, 1967; Galeotti *et al.*, 1971; White & Tewari, 1973).

Electrophoretic mobilities of the RNA species present in the mitochondrial fraction

Fig. 1(a) shows the electrophoretic profile obtained in 2.7% (w/v) polyacrylamide gel after a 2.5 h run (in a buffer containing sodium dodecyl sulphate) of mitochondrial RNA isolated from the tumour of a rat injected with ^{32}P and killed 3 h later. Seven peaks are clearly distinguished and, by comparison with a standard microsomal RNA marker, the second, fourth and sixth peaks (labelled with arrows), could be identified as 28, 18 and 4S RNA species respectively. The third and fifth peaks did not coincide with any cytoplasmic RNA and were assumed to be mitochondrial RNA species with roughly 20 and 15–16 S_e values. The first peak, very prominent in this run, will be discussed below, and the seventh peak, running faster than 4S RNA, will not be considered in the

Table 2. *Subfractionation of the 'crude' mitochondrial fraction from the Walker carcinoma by sucrose-gradient centrifugation under different conditions*

In one experiment, the mitochondrial fraction was isolated simultaneously from 10g of tumour and 10g of liver, by applying procedure no. 4, and then submitted to sucrose-gradient centrifugation in the SW 40 rotor exactly as described in the text. In another experiment, the mitochondrial fraction was isolated from 20g of tumour and one-third of the suspension was fractionated as indicated in the SW 40 rotor, whereas the remaining two-thirds were fractionated in the SW 25.1 rotor. Protein yield in fractions '4+5+6' obtained by the standard procedure (SW 40 rotor, 30 min) was 0.65 mg/g of tumour (average of four experiments).

Mitochondrial subfraction no.	Percentage distribution referred to sum of subfractions									
	Liver		Walker carcinoma							
	201 800g × 30 min		201 800g × 30 min		201 800g × 90 min		63 600g × 120 min			
	Protein	Cytochrome oxidase	Protein	Cytochrome oxidase	Protein	Cytochrome oxidase	Protein	Cytochrome oxidase	Protein	Cytochrome oxidase
1	19.3	23.8	11.2	3.3	15.9	8.1	15.6	5.7	—	—
2	24.0	33.1	15.5	6.2	13.1	7.5	11.8	5.5	—	—
3	26.0	26.6	13.3	7.7	11.0	5.3	11.3	9.1	—	—
4	8.4	4.4	12.0	14.9	10.6	13.2	10.3	14.0	—	—
5	7.2	5.7	13.3	19.8	9.7	13.4	11.7	12.1	—	—
6	9.9	3.9	26.7	44.7	24.8	45.2	24.4	48.0	—	—
7	5.4	2.3	8.0	3.4	14.9	7.3	14.9	5.6	—	—
'4+5+6'	25.5	14.0	52.0	79.4	45.1	71.8	46.9	74.1	—	—
Specific activity of cytochrome oxidase ($k \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein)										
6	—	13.0	—	33.5	—	37.1	—	40.3	—	—
'4+5+6'	—	18.0	—	29.7	—	32.4	—	32.4	—	—

Fig. 1. Polyacrylamide-gel electrophoresis of RNA isolated from the mitochondrial fraction by the use of two different procedures

A rat carrying a 12-day tumour was injected with 0.8 mCi of ^{32}P and 3 h later killed. The mitochondrial fraction from 17 g of tumour was purified, lysed with 2 ml of a buffer containing 0.5% sodium dodecyl sulphate, 0.01 M-Tris-HCl, pH 7.4, (20°C), and divided into two 1 ml samples. One (a) was made to 2% sodium dodecyl sulphate, 0.1 M-NaCl, 0.01 M-Tris-HCl, pH 7.4 (20°C), 0.001 M-EDTA and 0.125% bentonite, and the other (b) was made to 1.25% sodium dodecyl sulphate, 0.5% sodium deoxycholate, 0.5% sodium cholate, 0.03 M-Tris-HCl, pH 6.8 (20°C), 0.0125 M-KCl, 0.005 M-EDTA and 0.125% bentonite, based on the method of Attardi & Attardi (1971). RNA was extracted as described in the text and samples equivalent to 3.8 g of tumour were run in 2.7% polyacrylamide gels for 2.5 h and the absorbance was recorded at an attenuation 2-fold lower than the usual 1 V $\times$ 6 setting. —, E_{265} ; O, radioactivity. The arrows indicate the positions of 28S, 18S and 4S RNA in the densitometric tracing as determined in separate gels with microsomal RNA. From left to right the peaks are 40S, 28S, 20S, 18S, 16S, 15S and 4S_e (last peak not considered).

present work. When the disruption of mitochondrial membranes was performed with a mixture of 1.25% dodecyl sulphate–0.5% deoxycholate–0.5% cholate, as used by Attardi & Attardi (1971), instead of with 2% dodecyl sulphate alone, the profile was not altered (Fig. 1b). No differences were found either in the distribution of ^{32}P along the gel. Notice the bifid appearance of the 4–5S_e region. In another experiment, unlabelled RNA from carcinoma mitochondria was extracted as in Fig. 1(a) but without bentonite. Although the profile obtained was similar to the one with bentonite, this RNAase inhibitor was used as a routine as a further precaution against degradation.

When the concentration of polyacrylamide was lowered to 2.5%, a 4 h run showed that the fifth peak could be resolved into two peaks (Fig. 2a). By progressively increasing the running time to 6 h (Fig. 2c) it was possible to separate the two peaks rather well. However, a 5 h run (Fig. 2b) was better, and this was chosen as the running time for most of the remaining experiments. The 4S_e peak showed a distinct trailing shoulder at 3 h, but the whole peak disappeared in a 4 h run (Fig. 2a).

Under these conditions, 2.5% polyacrylamide, 5 mA/gel, a number of experiments were carried out at different running times and the electrophoretic mobilities were calculated. Table 3 shows that, with

only one exception, there was a good agreement between the results obtained at 3, 4, 5 and 6 h. The high-molecular-weight peak (the first peak) running before the 28S RNA had an S_e of 40, whereas the peak between 28S and 18S RNA (the third peak) was roughly a '20S_e' RNA. The zone resolved into two peaks in runs longer than 4 h was identified as containing a 16 and a 15S_e RNA. Finally, the three small peaks after the 15S_e were calculated as having approximate S_e values of 13, 12 and 11 respectively.

The arbitrary value reached by the height of the 20S_e peak obtained from the purified mitochondria from a fixed weight of tumour was constant in a series of experiments. By using the assumptions detailed in the Materials and Methods section it was possible to calculate a yield for this peak of about 180 μg /100 g of tumour, or 2.5–3 μg /mg of mitochondrial protein. The heights of the presumably cytoplasmic peaks were in a relatively fixed proportion to the 20S_e peak, roughly 2.9-, 1.5- and 1.2-fold for the 28, 18 and 4S peaks respectively. The heights of the other two main peaks, 16 and 15S_e, were about 40% lower than that of the 20S_e peak, but probably the corresponding amounts were somewhat larger, since the width of the peaks increases with the S_e values. The true amounts of the three remaining RNA species were lower than one-fifth of the 20S_e RNA, since the

Fig. 2. Effect of running time on the separation of mitochondrial RNA species by polyacrylamide-gel electrophoresis

The mitochondria from 54 g of tumour (10 days) obtained from three rats were lysed with 2 ml of a buffer containing 2% sodium dodecyl sulphate, 0.1 M-NaCl, 0.01 M-Tris-HCl, pH 7.4, at 20°C, 0.001 M-EDTA and 0.125% bentonite, and the RNA was extracted. Samples equivalent to 13 g of tumour were run in 2.5% polyacrylamide gels for 4 h (a), 5 h (b) and 6 h (c). The recorder attenuation was set at the usual value in (a) but was 1.5-fold lower in (b) and (c). ----, Baseline used for calculating the height of the peaks. From left to right the peaks are 40, 28, 18, 16 and 15S_e.

Table 3. Electrophoretic mobilities of the main RNA species present in the mitochondrial fraction

The S_e values were calculated from the positions of cytoplasmic 28, 18 and 4S RNA species run in separate gels, and are averages of the number of experiments indicated in parentheses in each case. The peak heights were estimated as described in the text and the reference value obtained for the 20S_e RNA (0.350) corresponds to 30 g of tumour at the standard 1V×6 attenuation of the recorder. The apparent molecular weights are only rough approximations calculated by assuming that a linear relationship exists between log (molecular weight) and distance migrated (Loening, 1968).

Designation	Time of run (h) ...	Height of peak compared with 20S _e peak	S _e values				General average*	10 ⁻⁶ × Apparent molecular weights
			3	4	5	6		
		5 (8)	(1)	(4)	(8)	(2)	(12)	(12)
40S _e		0.93	48	39.5	40.5	43.0	40.0	3.10
28S _e		2.87	—	—	—	—	—	1.75
20S _e		1.00	21.6	20.0	21.0	20.6	20.5	0.91
18S _e		1.53	—	—	—	—	—	0.70
16S _e		0.57	16.2	16.6	16.0	16.7	16.3	0.55
15S _e		0.53	15.0	15.3	15.4	15.8	15.4	0.49
4S _e		1.23	—	—	—	—	—	0.025

* Only the intermediate runs (4 and 5 h) were included.

estimates appearing in Table 3 are high owing to the ascending baseline. Finally, the consistently observed trailing shoulder on the 4S_e peak in short runs, had a calculated S_e value of 4.7 and the height was one-sixth of the 4S_e RNA. Table 3 shows also the approximate molecular weights only as rough estimates to be compared with those calculated from the sedimentation values. The 20S_e peak behaves as if it has nearly double the molecular weight of the 15S_e peak.

Assessment of contamination by DNA and cytoplasmic RNA

The persistence of 28 and 18S_e peaks in keeping an approximately constant height ratio to the 20S_e RNA,

the extensive mitochondrial purification notwithstanding, posed the question of whether they were truly mitochondrial constituents rather than contaminants. To remove ribosomes attached to endoplasmic-reticulum fragments, we treated the purified mitochondria with digitonin, as did Malkin in liver (Malkin, 1971). The height ratios to the 20S_e peak (Fig. 3a) decreased from 2.9 to 1.4 for the 28S_e peak (second peak) and from 1.5 to 0.9 for the 18S_e peak (fourth peak), but the considerable amount that was insensitive to digitonin treatment suggested that at least some of these RNA molecules were firmly attached to the mitochondria.

When a portion of this RNA was treated with DNAase no significant change occurred in the 28 to 15S_e RNA species (Fig. 3b, second to fifth peaks), but

Fig. 3. Effect of digitonin, RNAase and DNAase on the polyacrylamide-gel electrophoretic profile of mitochondrial RNA

Two rats carrying a 10-day tumour were injected with 0.5 mCi of ^{32}P and were killed 3 h later. The mitochondria from 30 g of tumour were purified by the usual procedure and then treated with digitonin as described. The RNA was extracted as in Fig. 2; the aqueous layer was divided into three samples and then precipitated with ethanol. One of the sediments was digested with DNAase and the other with RNAase. Samples were run in 2.5% polyacrylamide gels for 5 h. (a) Control sample; (b) DNAase-treated sample; (c) RNAase-treated sample; (d) DNA from Walker tumour mitochondria, 3 h run. The recorder attenuation was 1.5-fold lower than the usual setting. In (b) and (c) the RNA input was only half of the control (a). Radioactivity determinations were performed directly on the gel slices (without digestion with aq. NH_3) by counting Čerenkov radiation. —, E_{265} ; ○, radioactivity. The broken arrow shows the mitochondrial 40S_e peak. The gel slices corresponding respectively to the 20 and 15S_e radioactive peaks from (a) and (b) were pooled and the RNA was eluted and stored at -20°C as described in the text. From left to right the absorbance peaks in (a) and (b) are 40, 28, 20, 18 and 15S_e. The 16S_e RNA appears only as a shoulder of the 15S_e peak.

the slowest peak was considerably decreased, suggesting that it was mainly DNA. This was confirmed by RNAase digestion, which left only the high-molecular-weight peak, and eliminated completely all other peaks (Fig. 3c). Additional proof was that mitochondrial DNA from Walker carcinoma migrated in a separate shorter run with an electrophoretic mobility equivalent to the one expected for the so-called 40S_e peak (Fig. 3d).

Sedimentation coefficients of the RNA species present in the mitochondrial fraction

Short centrifugations at 201 800g in 15–30% (w/v) sucrose gradients containing sodium dodecyl sulphate were insufficient for resolving peaks other than the 28S cytoplasmic RNA (Fig. 4a). A broad zone partially overlapped with the 18S RNA and a low-molecular-weight zone was evident near the top. An 8.5h centrifugation (b) allowed some resolution of two peaks running behind the 18S RNA, but only at

12.5h (c) was it possible to separate both peaks and even see a third one running ahead of the 18S RNA, and a small shoulder on the trailing edge of the slowly migrating peak. In this run the low-molecular-weight RNA was divided into two peaks, one coinciding with the 4S cytoplasmic RNA and the other running ahead, but in most other experiments the 4S zone appeared only as a trailing shoulder of the main peak. A considerable background of heterogeneous RNA was observed below the peaks. In this last experiment the RNA was labelled *in vivo* with ^{32}P for 3 h and the pattern of radioactivity coincided with the absorbance, except for the low-molecular-weight highly labelled material (insert of Fig. 4c).

The sedimentation coefficients for the three heavier mitochondrial RNA species, other than 28 and 18S, were calculated from these and other runs as being roughly 20, 16 and 14S (Table 4). With these values as new standards, the low-molecular-weight RNA species could be defined with more precision in 12.5h runs, as having S values of 6.4 and 4.

Fig. 4. Effect of running time on the separation of mitochondrial RNA species by sucrose-gradient centrifugation

RNA was extracted from the purified mitochondria from 25 g of tumour (12 days) and samples equivalent to 8 g of tissue were centrifuged in 15–30% (w/v) sucrose gradients containing 0.5% sodium dodecyl sulphate as described in the text, at 201 800g for 6.5 (a), and 8.5 h (b). In another experiment (c), a rat bearing a 7-day tumour was injected with 1 mCi of ^{32}P and killed 3 h later. The RNA was extracted from the purified mitochondria from 12 g of tumour and centrifuged as above, but for 12.5 h. —, E_{260} ; ○, radioactivity. Notice the different scale in the insert in (c). The pellet had 1820 d.p.m. Direction of sedimentation was from left to right. The arrows indicate the positions of cell-sap 4S RNA from rat liver and microsomal 18 and 28S RNA from Walker tumour. From left to right the peaks in (c) are 4, 6.4, 14, 16 and 18S RNA.

Table 4. Sedimentation coefficients of the main RNA species present in the mitochondrial fraction

The S values were calculated from the positions of cytoplasmic 28S, 18S and 4S RNA species centrifuged in separate tubes, and are averages of the number of experiments indicated in parentheses in each case. The average height of the 16S peak was 1.55 E_{260} units for the RNA obtained from 30 g of tumour. The molecular weights are only rough approximations and were determined from a curve relating known molecular weights of eukaryotic cytoplasmic RNA species to S values [data compiled by Burdon (1971) were used]. The 10–11S RNA was often a zone rather than a definite peak.

Designation	Height of peaks as compared with 16S	Sedimentation coefficients (S)					General average (9)	$10^{-6} \times$ Approx. molecular weights
	$2.53 \times 10^6 \text{ g-h}$ (6)	$1.00 \times 10^6 \text{ g-h}$ (1)	$1.31 \times 10^6 \text{ g-h}$ (1)	$1.72 \times 10^6 \text{ g-h}$ (1)	$2.59 \times 10^6 \text{ g-h}$ (6)			
28S	0.93	—	—	—	—	—	—	1.75
20S	>0.70	—	—	—	19.5	19.5	—	0.85
18S	0.78	—	—	—	—	—	—	0.70
16S	1	16.2	16.1	16.3	16.2	16.2	16.2	0.58
14S	0.83	13.8	13.8	13.8	13.8	13.8	13.8	0.39
6S	1.23	—	—	—	6.4	6.4	6.4	0.036

Correspondence between the sedimentation coefficients and electrophoretic mobilities

Several differences were noticed between the centrifugation and the electrophoretic patterns. First, the main peak other than those at 4, 18 or 28S was 20S_e in polyacrylamide-gel electrophoresis, but the presumably equivalent peak in sucrose-gradient centrifugation, the 20S peak, was smaller than the 14 and 16S peaks and quite often disappeared, overlapped by the 18S RNA. Second, the 16S peak was higher than the 18 or 28S peaks (Table 4), whereas in electrophoresis the 16S_e or the 15S_e was less than one-quarter as high as the 28S_e peak. Third, the low-molecular-weight RNA ran as the 4S from liver cell

sap in polyacrylamide-gel electrophoresis but sedimented mainly as 6.4S RNA in sucrose-gradient centrifugation. Although some of these discrepancies could be ascribed to the larger amount of heterogeneous RNA in sucrose-gradient centrifugation than in polyacrylamide-gel electrophoresis, other factors were probably involved.

To investigate this question the central portions of the peaks from a 12.5 h run were collected, pooled, concentrated by ethanol precipitation, and analysed by polyacrylamide-gel electrophoresis. Fig. 5(a) shows that the pellet was essentially pure 28S RNA, and that the 18–20S fraction contained both types of RNA plus a substantial amount of 28S RNA. The

Fig. 5. Electrophoretic mobilities of the RNA peaks obtained in sucrose-gradient centrifugation

The RNA was obtained from the purified mitochondria isolated from 27 g of tumour (two rats bearing 10-day tumours) and fractionated by sucrose-gradient centrifugation in the presence of 0.5% sodium dodecyl sulphate, at 201 800g for 12.5 h. Material from the absorbance peaks was collected, the RNA was precipitated as described in the text and run in 2.7% polyacrylamide gels for 5 h (a, b, c), or 2.5 h (d, e, f). Input was 0.9–1.2 E units, except in (a) 1.8 units and (d) 0.5 unit. Recording of the densitometric tracing was carried out at the standard 1 V × 6 attenuation setting (b) or at a setting 1.5-fold lower (other fractions). (a) 18–21 S fraction, ---- shows a superimposed curve corresponding to the pellet; (b) 16 S fraction; (c) 14 S fraction; (d) intermediate fraction between 13 and 8 S; (e) 6.4 S fraction; (f) 4 S shoulder of the 6.4 S fraction, ---- shows a superimposed curve corresponding to a standard 4 S RNA from rat liver cytosol. From left to right solid arrows indicate the positions of 28 and 18 S RNA from the microsomal fraction from Walker carcinoma and 4 S RNA from rat liver cytosol. The broken arrows show the 40, 20, 16, 15 and 4.7 S_e RNA species from the whole mitochondrial fraction as determined in a separate gel.

16 S fraction (Fig. 5b) migrated exactly as the 16 S_e RNA, but was heavily contaminated by 18 S RNA. Although the 14 S fraction (Fig. 5c) showed a shoulder corresponding to 15 S_e RNA, the main portion had an S_e value of 14.0. The heterogeneous zone spreading from 13 to 8 S in the gradient (Fig. 5d) had one main distinct peak at 11 S_e. The 6.4 S fraction (Fig. 5e) migrated as 4 S_e with considerable contamination with 4.7 S RNA, and the trailing shoulder of the 6.4 S fraction (Fig. 5f) coincided exactly with the 4 S_e value, with only a 4.7 S_e shoulder.

These results were confirmed by repeating the experiment but using for polyacrylamide-gel electrophoresis the re-purified 18, 16 and 14 S fractions obtained after a second sucrose-gradient centrifugation of the central portions of the peaks from the previous centrifugation. The RNA species moved in the gradient to the positions expected, and when they

were submitted to electrophoresis (results not shown) the S_e obtained coincided with the experiment of Fig. 5, with lower cross-contamination, the 14 S fraction having again a 14 S_e peak.

The fact that the main 20 S_e electrophoretic peak was considerably decreased after sucrose-gradient centrifugation, whereas the 16 S_e RNA equivalent to the 16 S RNA was converted into the main peak, suggested that the 16 S fraction arose from the 20 S_e fraction presumably through a cleavage process occurring in the high-ionic-strength conditions of sucrose-gradient centrifugation which caused a decrease in size. To test this hypothesis, both the 20 and the 15 S_e peaks obtained by polyacrylamide-gel electrophoresis were eluted, and then centrifuged as usual. The radioactivity profile clearly showed that the 20 S_e RNA sedimented mainly as 16 S RNA (Fig. 6a). Except for the broad shoulder in the 10–14 S region, practically

Fig. 6. Size decrease of the 20 and 15S_e RNA after sucrose-gradient centrifugation

The 20 and 15S_e RNA species labelled with ³²P were obtained, in the experiment described in Fig. 3, by polyacrylamide-gel electrophoresis followed by elution and ethanol precipitation. They were then analysed by sucrose-gradient centrifugation at 201 800g for 8.5 h and the fractions were collected and counted for radioactivity. (a) 20S_e RNA; (b) 15S_e RNA. —, *E*₂₆₅ (not recorded in a); ○, radioactivity. From left to right solid arrows indicate the positions of 18 and 28S RNA from the microsomal fraction and the broken arrows show the 6.4, 14, 16 and 20S RNA from the mitochondrial fractions, as determined in separate runs; 60 and 70% respectively of the radioactivity originally present in the 20 and 15S_e peaks in the gel were recovered after the whole procedure.

no counts were recovered in the low-molecular-weight zones. The 15S_e RNA sedimented as a peak of 13.8S (Fig. 6b).

Discussion

Our experimental evidence demonstrates that in the purified mitochondrial fraction from the Walker carcinosarcoma seven main peaks and three minor species can be identified by polyacrylamide-gel electrophoresis. Of the main peaks, the higher molecular weight corresponds to DNA, and the 28, 18 and 4S_e peaks coincide with the migration of their cytoplasmic counterparts thus leaving only three species as exclusively mitochondrial. The values for their electrophoretic mobilities and the equivalent sedimentation coefficients were respectively 20.5S_e and 19.5S, 16.3S_e and 16.2S, and 15.4S_e and 13.8S. The differences for each RNA species between the S and S_e values may represent conformational changes due to the influence of the lower ionic strength used in polyacrylamide-gel electrophoresis (Groot *et al.*, 1970; Mitra *et al.*, 1972).

The existence of three mitochondrial RNA peaks in the 14–21S/S_e region is somewhat surprising, considering that all prokaryotic and eukaryotic mitochondrial RNA so far described consists of only two species of this size, corresponding to the large and small ribosomal subunits (see reviews by Borst & Grivell, 1971; Borst, 1972; Wintersberger, 1972). Our results suggest, however, that only the 20.5S_e (19.5S) and the 15.4S_e (13.8S) peaks are the native RNA species, whereas the 16.3S_e (16.2S) RNA seems to arise by cleavage from the larger RNA. There are

some hints suggesting that the 15.4S_e RNA would also suffer a slight decrease in size, and not simply a change of conformation to yield the 13.8S species, since the electrophoresis of this last peak obtained by sucrose-gradient centrifugation did not give back a 15.4S_e, but a 14S_e peak.

Rounding off the S_e values to 20 and 15 respectively, they are nearly identical with the values reported by Groot *et al.* (1970) and Bartoov *et al.* (1970) for rat liver mitochondrial RNA, and something similar occurs with our 15 and 14S values when compared with the 16 and 13S peaks obtained by the same authors. Groot *et al.* (1970) and Mitra *et al.* (1972) showed that the discrepancy between the S and S_e values of the larger RNA in rat liver were due simply to conformational changes occurring during electrophoresis, and which were evident by carrying out the runs at different temperatures and ionic strengths. In the case of Walker carcinoma this explanation cannot account either for the presence of both 20 and 16S_e peaks instead of only one of them as should result from a conformational change, or for the electrophoretic migration as 16S_e of the 16S peak obtained by gradient centrifugation. Therefore, if this RNA arises by cleavage of the larger species, it is likely that this process involves a dissociation rather than a simple degradation, because the ratio 16S_e/21S_e was relatively constant in many different preparations, and no signs of considerable RNAase action were noticed.

The 4S_e (4S) RNA is a normal constituent of other kinds of mitochondria, but it is probably contaminated with tRNA from the cytosol. The nature of the 4.7S_e shoulder and the correspondence of both RNA

species identified by gel electrophoresis to the low-molecular-weight peaks obtained by sucrose-gradient centrifugation must be clarified by further studies.

The 28S and 18S RNA species present in the mitochondrial fraction do not seem to be ordinary products of cytoplasmic contamination since they keep a relatively constant ratio to the standard mitochondrial peaks and are resistant to digitonin treatment. This might reflect a tighter association of 80S ribosomes with the mitochondria or alternatively might be due to their presence inside the organelle (see Kellems & Butow, 1972; Keyhami, 1973).

Tumour transplantation and animal treatments were efficiently performed by Miss Emilia Pérez Ayuso, and the rats were provided by the Instituto de Medicina Experimental (UCV) to whom our thanks are given. This work was supported by a grant from the Consejo de Desarrollo Científico y Humanístico, U.C.V.

References

- Abe, S., Fujisawa, T., Satake, M. & Ogata, K. (1972) *J. Biochem. (Tokyo)* **72**, 561–570
- Arcos, J. C. (1971) *J. Theor. Biol.* **30**, 533–543
- Attardi, B. & Attardi, G. (1971) *J. Mol. Biol.* **55**, 231–249
- Avadhani, N. G., Battula, N. & Rutman, R. J. (1973) *Biochemistry* **12**, 4122–4129
- Bartoov, B., Mitra, R. S. & Freeman, K. B. (1970) *Biochem. J.* **120**, 455–466
- Borst, P. (1972) *Annu. Rev. Biochem.* **41**, 333–376
- Borst, P. & Grivell, L. A. (1971) *FEBS Lett.* **13**, 73–88
- Burdon, R. H. (1971) *Progr. Nucl. Acid Res. Mol. Biol.* **11**, 33–79
- Clayton, D. A. & Vinograd, J. (1967) *Nature (London)* **216**, 652–657
- Clayton, D. A. & Vinograd, J. (1969) *Proc. Nat. Acad. Sci. U.S.* **62**, 1077–1084
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Feo, F., Canuto, R. A. & Garcea, R. (1973) *Eur. J. Cancer* **9**, 203–214
- Gaitskhoki, V. S., Kisselev, O. I. & Neifakh, S. A. (1973) *FEBS Lett.* **31**, 93–96
- Galeotti, T., Cittadini, A., Dionisi, O., Russo, M. & Terranova, T. (1971) *Biochim. Biophys. Acta* **253**, 303–313
- González-Cadavid, N. F. & Herrera Quijada, F. (1974) *Biochem. J.* **138**, 129–141
- González-Cadavid, N. F. & Sáez de Cordova, C. (1974) *Biochem. J.* **140**, 157–167
- Groot, P. H. E., Aaij, C. & Borst, P. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1321–1327
- Hirsch, M. & Penman, S. (1973) *J. Mol. Biol.* **80**, 379–391
- Kára, J., Dvorák, M. & Cerná, H. (1972) *FEBS Lett.* **25**, 33–37
- Kellems, R. & Butow, R. A. (1972) *J. Biol. Chem.* **247**, 8043–8050
- Keyhami, E. (1973) *J. Cell Biol.* **58**, 480–484
- Küntzel, H., Barath, Z., Ali, I., Kind, J. & Althaus, H. H. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 1574–1578
- Kyriakidis, D. & Georgatos, J. G. (1973) *Biochem. Biophys. Res. Commun.* **53**, 1167–1172
- Li, Y. T., Li, S. C. & Shetlar, M. R. (1965) *Cancer Res.* **25**, 1225–1231
- Loening, U. E. (1968) *J. Mol. Biol.* **37**, 355–365
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Malkin, L. I. (1971) *Biochemistry* **10**, 4752–4756
- Mehard, C. W., Packer, L. & Abraham, S. (1971) *Cancer Res.* **31**, 2148–2160
- Mintz, H. A., Yawn, D. H., Safer, B., Bresnick, E., Liebelt, A. G., Blailock, Z. R., Rabin, E. R. & Schwartz, A. (1967) *J. Cell Biol.* **34**, 513–523
- Mitra, R. S., Bartoov, B., Monahan, J. & Freeman, K. B. (1972) *Biochem. J.* **128**, 1033–1041
- O'Brien, T. W. & Kalf, G. F. (1967) *J. Biol. Chem.* **242**, 2172–2179
- Paoletti, C. A. & Riou, G. (1973) *Progr. Mol. & Sub-Cell. Biol.* **3**, 203–248
- Paoletti, C., Riou, G. & Pairault, J. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 847–850
- Penman, S. (1966) *J. Mol. Biol.* **17**, 117–130
- Porterfield, J. S. (1960) *Bull. W.H.O.* **22**, 373–380
- Richert, N. J. & Hare, J. D. (1972) *Biochem. Biophys. Res. Commun.* **46**, 5–10
- Roodyn, D. B., Reiss, P. J. & Work, T. S. (1961) *Biochem. J.* **80**, 9–21
- Smith, C. A. & Vinograd, J. (1973) *Cancer Res.* **33**, 1065–1070
- Smith, L. (1955) in *Methods of Biochemical Analysis*, (Glick, D., ed.) vol. 2, pp. 427–434, Interscience Publishers, New York
- Sordahl, L. A. & Schwartz, A. (1971) in *Methods in Cancer Research* (Busch, H., ed.), vol. 6, pp. 159–186, Academic Press, New York
- Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32**, 415–438
- Wagner, K., Katz, L. & Penman, S. (1967) *Biochem. Biophys. Res. Commun.* **28**, 152–159
- Watts, R. L. & Mathias, A. P. (1967) *Biochim. Biophys. Acta* **145**, 828–831
- Weinberg, R. A., Loening, U., Willems, M. & Penman, S. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 1088–1095
- Weinhouse, S. (1972) *Cancer Res.* **32**, 2007–2016
- White, M. T. & Tewari, K. K. (1973) *Cancer Res.* **33**, 1645–1653
- Wintersberger, E. (1972) in *Mitochondria: Biogenesis and Bioenergetics. Biomembranes: Molecular Arrangements and Transport Mechanisms* (van der Berg, G. S., Borst, P. & Slater, E. C., eds.), pp. 21–33, North-Holland, Amsterdam
- Yonetani, T. & Ray, G. S. (1965) *J. Biol. Chem.* **240**, 3392–3398