Fractionation of Nuclear and Cytoplasmic Ribonucleic Acids from Rat Tissues on the Methylated Albumin–Kieselguhr Column

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1. The conformation of RNA was found to affect its behaviour on methylated albumin–kieselguhr chromatography. The less regular the secondary structure of RNA, the more tightly it binds to the methylated albumin–kieselguhr column. 2. The presence of various denaturing agents (such as urea or perchlorate) in the medium while RNA was adsorbed on the column increased the resolving power of the technique as exemplified by the separation of rat liver rRNA into two distinct peaks. A special procedure for selective adsorption of the cytoplasmic DNA-like RNA on the preparative scale has been developed. Polyribosomal mRNA (rapidly labelled RNA formed in the presence of small doses of actinomycin D) can also be adsorbed selectively by the column. 3. A type of tissue specificity was detected in nuclear RNA from rat liver, kidney, thymus and spleen by using a modified salt and temperature gradient for the chromatographic fractionation (Lichtenstein, Piker & Shapot, 1967; Shapot, Lichtenstein & Piker, 1967). It was also found that cytoplasmic RNA from the different rat tissues contained no tenaciously bound fraction at all, whereas it constituted about 50% of the nuclear RNA. The problem of the possible biological function of the tenaciously bound fraction is discussed.

Chromatography on MAK* columns is a method used widely for fractionation of RNA species from different sources but the method is limited by its inability to resolve the 18S and 28S rRNA components from higher organisms and the non-quantitative recovery of RNA.

Ellem & Sheridan (1964) have, however, shown that a tenaciously bound fraction of RNA from animal cells, which was characterized by a DNA-like nucleotide composition and high metabolic activity, could be eluted from the MAK column either at a high temperature (90°C) or by ammonium hydroxide. It is noteworthy that this tenaciously bound RNA escaped attention until the Ellem & Sheridan (1964) paper appeared.

We introduced a modified elution procedure by using a temperature gradient after the salt gradient was completed, allowing fractionation of tenaciously bound RNA (Lichtenstein, Piker & Shapot, 1967; Shapot, Lichtenstein & Piker, 1967; Piker, Lichtenstein, Shapot & Rodionova, 1968; Lichtenstein, 1970).

In the present study we use this method to investigate RNA fractions from rat tissues.

* Abbreviations: MAK, methylated albumin–kieselguhr; nRNA, nuclear RNA; cpRNA, cytoplasmic RNA.

For simplicity some assumptions have been made concerning the terminology of RNA separated on MAK columns. The terms nRNA and cpRNA refer to nuclear and cytoplasmic RNA that were isolated by the differential thermal extraction procedure (Georgiev & Mantjeva, 1962) applied to both tissue homogenates and isolated nuclear or cytoplasmic fractions. The nRNA is divided into two classes, one that eventually enters into the cytoplasm and the other that does not leave the nucleus and undergoes a rapid turnover. The mRNA is considered to be the fraction of non-ribosomal RNA associated with polyribosomes. In the experiments reported below it was assumed that most of the rapidly labelled RNA recovered in polyribosomes from animals pretreated with small doses of actinomycin D, which preferentially blocks ribosomal RNA synthesis (Georgiev, Samarina, Lerman, Smirnov & Severtzov, 1963), represented mRNA.

After the present work was completed, Billing & Barbiroli (1970) reported conclusions similar to ours regarding the nature of RNA formed with small doses of actinomycin D. They also used MAK column chromatography to characterize the radioactive products.

Some aspects of the RNA fractionation method were examined to find a way of increasing the resolving power of the MAK chromatography.

MATERIALS AND METHODS

Animals. White rats (male and female) weighing 150–200 g were used. The rats were starved overnight before the experiment.

Preparation of nRNA and cpRNA. The nRNA and cpRNA were isolated by the method of Georgiev & Mantjeva (1962) as modified by Greenman, Wick & Kenney (1965). The rat tissues to be studied were removed and briefly homogenized in 3 vol. of 0.14M-NaCl in a blender and an equal volume of 80% (w/v) phenol was added. The phenol had been saturated with 0.14M-NaCl solution and adjusted to pH 6.0 by 1M-KOH. The mixture was shaken for 30 min at 20°C, then centrifuged at 15000g for 15 min at 4°C. The aqueous phase was collected and the phenol was discarded. The interphase and pellet were passed through the same procedure repeatedly. All the aqueous phases were pooled and considered to constitute the cpRNA fraction.

This extraction procedure was repeated, at 65°C and 75°C. In the latter case the aqueous phase contained 0.5% sodium dodecyl sulphate. The aqueous extracts of the 65°C and 75°C extractions were pooled, and constituted the nRNA fraction.

To prevent the enzymic degradation of RNA in some cases the homogenization of the tissue was performed in 40% (w/v) phenol; bentonite (1 mg/ml) prepared by the method of Petermann (1964) was also used.

The nRNA and cpRNA fractions were further deproteinized by shaking them twice with phenol at 20°C after addition of sodium dodecyl sulphate to a final concentration of 0.5%. RNA was precipitated by the addition of potassium acetate to a concentration of 2% and 2 vol. of 95% ethanol. Precipitation was repeated several times in the presence of 5 mM-EDTA. In some cases RNA was precipitated by 2.5M-NaCl. The final preparation was dissolved in 0.14M-NaCl–5 mM-triethanolamine–HCl, pH 6.7, and stored at –78°C.

In some cases rat tissues were homogenized in 0.25M-sucrose–5 mM-MgCl₂–50 mM-KCl–25 mM-tris–HCl buffer, pH 7.6, in a Potter–Elvehjem homogenizer. The homogenate was filtered through several layers of cheesecloth and the nuclear fraction was centrifuged at 1000g for 5 min, then washed twice with the same buffer. The crude nuclear fraction and the cytoplasmic post-mitochondrial supernatant were extracted with phenol as described above to give nRNA and cpRNA fractions respectively.

Preparation of polyribosomes and polyribosomal RNA. Polyribosome preparations were obtained by the method of Leytin & Lerman (1969). RNA from the polyribosomes was extracted by phenol deproteinization in the presence of 0.5% sodium dodecyl sulphate.

Preparation of labelled RNA samples. nRNA was pulse-labelled with [¹⁴C]orotic acid (200 μ Ci/rat; introduced 40 min before death).

Polyribosomal mRNA and cytoplasmic DNA-like RNA were labelled selectively, because synthesis of rRNA was suppressed by actinomycin D (50 μ g/rat) (Georgiev *et al.* 1963). [¹⁴C]Orotic acid was introduced intraperitone-

ally (40 μ Ci/rat) 1 h after actinomycin injection and 3 h before death.

MAK chromatography. The MAK column was prepared as described by Sueoka & Cheng (1962). Adsorption of RNA (100 μ g/ml) on to the MAK was performed in 0.6M-NaCl–5 mM-triethanolamine–HCl buffer, pH 6.7, at room temperature. A linear salt gradient (0.6–1.5M-NaCl) in the 5 mM-triethanolamine–HCl buffer, pH 6.7, was applied at 37°C to elute the ‘salt’ RNA fraction. When the salt-gradient elution was completed the salt concentration was kept constant and the temperature was increased from 37°C to 98°C (1.3°C/min). Under these conditions a ‘temperature’ fraction analogous with the tenaciously bound RNA was eluted (Lichtenstein *et al.* 1967; Shapot *et al.* 1967; Piker *et al.* 1968; Lichtenstein, 1970). All the modifications of the standard procedure used are noted in the text. The elution profile was registered automatically with a Uvicord LKB (0.5 cm light-path, E_{253}). Fractions (2.5 ml) were collected for measurement of radioactivity and, in some cases, E_{260} .

Sucrose-density-gradient ultracentrifugation. Ultracentrifugation of RNA preparations was performed in linear (5–20%) sucrose density gradients containing 0.1M-NaCl–0.01M-sodium acetate–1 mM-EDTA buffer, pH 5.1, on a Spinco model L 2 centrifuge, with a SW 50 rotor at 37000 rev./min for 200 min at 4°C. The S values were calculated by the method of Martin & Ames (1961). Tubes were pierced and three-drop fractions were collected (two drops for determination of radioactivity and one drop for u.v. extinction measurements).

Radioactivity measurements. Radioactivity was counted in a Nuclear–Chicago scintillation counter. Bovine serum albumin was added to fractions of RNA as a carrier and RNA precipitates formed in 5% (w/v) trichloroacetic acid were collected on Millipore filters.

Determination of RNA nucleotide compositions. The nucleotide composition of RNA fractions was determined by high-voltage electrophoresis (70 V/cm) on Whatman no. 1 paper of the hydrolysates obtained by incubation of RNA with 0.3M-KOH at 37°C for 18 h, and u.v.-extinction measurements of the separated (2′-3′)-nucleotides.

RESULTS

Some aspects of the method. Experiments were conducted to confirm that the resolving power of MAK chromatography is based on the differences in the magnitude of the ‘reacting’ surface of the RNA molecules subjected to fractionation. In solution a single-stranded polynucleotide chain forms a coil with many phosphate groups hidden inside. The various denaturing agents present in the medium unfold the RNA coil and thus increase the RNA ‘reacting’ surface. In our view, the unfolding would cause a tighter binding of the RNA to the MAK thus raising the ‘temperature’/‘salt’ fraction ratio.

It was found that factors such as low ionic strength, perchlorate, urea and high temperature, which were applied during the RNA adsorption to the column, increased the ratio (Table 1). Fig. 1 shows that 95% of the cpRNA is eluted by the

Table 1. *Influence of conditions under which cpRNA was adsorbed on the MAK column*

The MAK column with a working volume of 8 ml was prepared by mixing 0.6 ml of aq. 1% bovine serum methylated albumin with 2 g of Celite 545 (60–80 mesh) in 15 ml of 0.6 M-NaCl–5 mm-triethanolamine-HCl buffer, pH 6.7, at room temperature. The Celite suspension was previously boiled for 5 min. The column was washed first with the same solution and secondly with that in which cpRNA was later adsorbed on the MAK. All solutions were prepared in 5 mm-triethanolamine-HCl buffer, pH 6.7. The cpRNA, about 20 E_{260} units, in 10 ml of the appropriate solution was passed through the column under the conditions shown. The column was washed by the initial solution of the salt gradient to remove any u.v.-absorbing material. Then the temperature of the column was adjusted to 37°C, at which the linear salt gradient (total volume of 60 ml) was always performed. When the salt-gradient elution was completed the salt concentration was kept constant and the temperature was increased from 37°C to 98°C. The u.v.-absorbing material eluted by the salt and temperature gradients represent the 'salt' fraction and 'temperature' fraction respectively. The 'temperature'/'salt' fraction ratio was calculated to demonstrate the action of some denaturing agents.

Conditions of adsorption of cpRNA on MAK	Salt gradient used at 37°C before 37°C–98°C temperature gradient at final salt concentration	Ratio of 'temperature' fraction/'salt' fraction
(1) 0.6 M-NaCl, 20°C (standard conditions)	0.6–1.5 M-NaCl	0.03
(2) 0.1 M-NaCl, 20°C	0.6–1.5 M-NaCl	0.15
(3) 0.1 M-KCl, 20°C	0.6–1.5 M-KCl	1.30
(4) 0.1 M-NaClO ₄ , 20°C	0.1–1.0 M-NaClO ₄	16.10
(5) 0.6 M-NaCl, 8 M-urea, 37°C	0.6–1.5 M-NaCl	17.20
(6) 0.6 M-NaCl, 70°C	0.6–1.5 M-NaCl	23.10

Fig. 1. Effect of perchlorate on MAK chromatography of rat liver cpRNA. Adsorption of cpRNA on the column was performed in 0.1 M-NaClO₄–5 mm-triethanolamine-HCl buffer, pH 6.7, at 20°C. The salt gradient was 0.1–1.0 M-NaClO₄ at 37°C. The temperature gradient was 37°C–98°C in 1.0 M-NaClO₄ (when 98°C was reached the temperature was kept constant). For detailed conditions of the chromatographic procedure see the legend to Table 1. —, % transmission at 253 nm; ---, temperature.

temperature gradient in the presence of perchlorate, whereas under standard conditions cpRNA can be eluted almost completely by the salt gradient (Fig. 3b). It is well known that the perchlorate is a strong denaturing agent that destroys hydrophobic bonds (Hamaguchi & Geiduschek, 1962).

It is noteworthy that the substitution of Na⁺ by K⁺ caused the bulk of the fraction eluted by the salt gradient to be eluted during the temperature gradient. This phenomenon can be explained by the fact that K⁺ is a poorer phosphate group screening agent than Na⁺.

A series of experiments was performed to study the binding of RNA to the MAK column under different conditions. A fixed concentration of cpRNA was passed through the column while the salt concentration of the medium was gradually decreased. The RNA binds to the adsorbent only when the salt concentration is decreased to a certain critical value, designated by us as a concentration of binding (C_b). The extent of the binding was determined by measuring the extinction of the solution after passage through the column. The C_b values of the same RNA species obtained in various salt solutions are a measure of the effect of various cations (differing in their capacity of screening the phosphate groups of RNA) and some anions differing from each other in their affinity for the MAK (Fig. 2a). The results confirmed that Na⁺ is a more effective screening agent than K⁺, as shown above. Additionally, the anions can be arranged in a sequence in their order of increasing affinity for the MAK column:

The affinity of RNA for the MAK increased with temperature. The higher the concentration of the competing anion (Cl[−]) the higher the temperature required for adsorption of the RNA (Fig. 2b).

Fig. 2. (a) Adsorption of polynucleotides on the MAK column, depending on ionic strength and ionic composition of the medium. Nucleic acids at constant concentration ($10\text{--}15\text{ }\mu\text{g/ml}$) were passed through the MAK column in the salt solution, the concentration of which was diminishing linearly. The decrease in the extinction of the solution flowing out of the column reflects the extent of adsorption of nucleic acids on column reflects the extent of adsorption of cpRNA on the adsorbent. $\circ$, 0.95M-NaCl ; $\bullet$, 1.0M-NaCl ; Δ , of rat liver cpRNA in NaCl solution at 20°C (C_b , 0.95); $\bullet$, adsorption of rat liver cpRNA in KCl solution at 20°C (C_b , 1.0); Δ , adsorption of rat liver cpRNA in sodium acetate solution at 20°C (C_b , 0.90); $\blacksquare$, adsorption of rat liver cpRNA in NaClO_4 solution at 20°C (C_b , 0.28); $\square$, adsorption of rat liver cpRNA in NaClO_4 solution at 42°C (C_b , 0.40); $\blacktriangle$, adsorption of denatured DNA of rat liver in NaCl solution at 20°C (C_b , 0.95). (b) Effect of ionic strength and temperature on the cpRNA adsorption on MAK. The cpRNA ($15\text{ }\mu\text{g/ml}$) was passed through the column in various solutions of constant ionic strength while the temperature was increased gradually. The decrease in extinction of the solution flowing out of the column reflects the extent of adsorption of cpRNA on the adsorbent. $\circ$, 0.95M-NaCl ; $\bullet$, 1.0M-NaCl ; Δ , 1.1M-NaCl ; $\square$, 1.25M-NaCl .

It is reasonable to surmise that the C_b value depends not only on the nature of the anions and cations but also on the affinity of the RNA molecules themselves for the MAK column. Indeed tRNA and rRNA cease to bind to the column in 0.5M- and 0.95M-NaCl respectively. Thus the various RNA species were found to have different C_b values in otherwise identical conditions. Because of this, conditions for the selective adsorption of the different types of RNA could be found. To study the C_b values of the 'salt' fraction and the 'temperature' fraction in various conditions rat liver cpRNA that had lost the 'temperature' fraction (see below) was used as 'salt' fraction material. 'Temperature' fraction RNA was obtained from rat liver nRNA by elution with $1.5\text{M-sodium chloride}$ at 90°C after the salt gradient was completed. Adsorption of the 'temperature' fraction appears to be almost quantitative in $0.6\text{M-sodium chloride-}3\text{M-urea-}5\text{mM-triethanolamine-hydrochloric acid}$ buffer, pH 6.7 , at 20°C but the 'salt' fraction is not adsorbed at all under the same conditions. Thus the technique permits a selective adsorption of the 'temperature' fraction.

Nuclear-cytoplasmic interrelationships revealed by chromatography of the RNA species. Fig. 3(a) shows the chromatographic profile of rat liver nRNA as well as its rapidly labelled fraction. The 'salt' fraction of nRNA consists in general of nuclear rRNA species as confirmed by the nucleotide composition results (Table 2) whereas the curve of radioactivity not coincident with the rRNA peak is thought to demonstrate the unresolved q_1 and q_2

peaks (Yoshikawa, Fukada & Kawade, 1964). However, the larger part of the light-absorbing and radioactive material is in the 'temperature' fraction. The nucleotide composition of the 'temperature' fraction is close to that of the DNA-like fraction (Table 2). It is noteworthy that in most studies that use MAK chromatography tenaciously bound RNA is eluted as a single peak by sodium dodecyl sulphate solutions (Ellem, 1966; Muramatsu, Hodnett & Busch, 1966; Roberts & Quinlivan, 1969; Billing & Barbiroli, 1970). However, the temperature-gradient procedure reveals the heterogeneity of the 'temperature' fraction itself as well as its diversity in regard to incorporation of radioactive precursor. The minor component of the 'temperature' fraction, peak T_3 , eluted from 80°C to 98°C (fractions 38–44), appears to have the greatest specific radioactivity under the conditions of the pulse-labelling experiment.

Fig. 3(b) shows the chromatographic profile of the total cpRNA (curve of extinction) and cytoplasmic DNA-like RNA (curve of radioactivity) since this preparation of cpRNA was labelled after treatment of animals with small doses of actinomycin D (see the Materials and Methods section). The cpRNA seems to have a significantly more restricted chromatographic profile as compared with nRNA. Both cpRNA and cytoplasmic DNA-like RNA are concentrated within the 'salt' fraction. Chromatographic profiles of the cpRNA samples of other tissues (rat spleen, kidney, thymus) have the same character. In all cases cpRNA contains practically no 'temperature' fraction. The minor component

Fig. 3. (a) Chromatography of rat liver nRNA on MAK by using salt and temperature gradients. Adsorption of nRNA on the column, prepared as shown in the legend to Table 1, was performed in 0.6M-NaCl at 20°C. Elution: salt gradient, 0.6–1.5M-NaCl at 37°C. Temperature gradient, 37°C–98°C in 1.5M-NaCl (increment about 1.3°C/min). When the temperature gradient was used degassing of the water was necessary before preparation of the eluent. Fractions (2.5ml) were collected. The arrow indicates the start of the temperature gradient. —, E_{260} ; ---, radioactivity. Rat liver nRNA was pulse-labelled with [^{14}C]orotic acid (200 $\mu\text{Ci}/\text{rat}$) injected intraperitoneally 40 min before death. (b) Chromatography of rat liver cpRNA on MAK by using salt and temperature gradients. For conditions of chromatography see (a). —, E_{260} ; ---, radioactivity. Rat liver cpRNA was labelled with [^{14}C]orotic acid (100 $\mu\text{Ci}/\text{rat}$) introduced 2 h before death. Animals were pretreated with small doses of actinomycin D (see the Materials and Methods section).

Table 2. Nucleotide composition of nRNA and cpRNA fractions from rat liver

Source of material	Fraction	Amount of nucleotide (%)				Ratio (G + C)/(A + U)
		CMP	AMP	GMP	UMP	
nRNA	'Salt'	31.1	17.3	32.4	19.2	1.74
	T ₁	28.6	23.2	22.5	25.7	1.04
	T ₂ + T ₃	24.8	29.3	23.3	22.6	0.93
cpRNA	'Salt'	29.2	19.6	35.5	15.7	1.83
	T	30.5	19.7	32.3	17.5	1.70
	ct	21.8	24.8	31.8	21.6	1.15

in the temperature-gradient elution represents a small contamination (3–5% of the whole extinction) with the rRNA from the 'salt' fraction. This has been confirmed by rechromatography of this 'temperature' fraction material, when it elutes in the salt gradient, and also by its base composition (Table 2). The extent of contamination is variable and depends on the conditions of adsorption and elution of RNA samples on the column. As shown in Table 1, the 'salt' fraction can under certain conditions be eluted during the temperature gradient thus contaminating the latter. Also, the 'salt' fraction is eluted non-quantitatively if the salt-gradient elution is performed at a temperature lower than 37°C (A. V. Lichtenstein & V. S. Shapot, unpublished work). In our procedure this contamination was relatively small and it does not influence the profile of the nRNA.

The minor components that can be seen at the beginning of the salt-gradient elution of nRNA and cpRNA (Figs. 3a and 3b) represent the DNA impurity and some low-molecular-weight nucleic acids respectively. They were not taken into consideration in the present study.

Isolation of the cytoplasmic DNA-like RNA. Experiments described above suggest that both components of rRNA, along with cytoplasmic mRNA, emerge as a single peak in the salt part of the chromatogram. However, it was reasonable to expect that the use of denaturing agents may increase the resolving power of the technique.

Rat liver cpRNA was adsorbed on the column under the conditions favouring its partial unfolding and then eluted with a solution of constant salt concentration while the temperature was raised gradually (temperature gradient). This technique

Fig. 4. Chromatography of rat liver polyribosomal RNA on the MAK column by using denaturing agent. Adsorption of RNA was performed in 0.01M-NaClO_4 at 20°C . The column was washed out with 1.5M-NaClO_4 at 20°C , the temperature gradient was performed from 20°C – 98°C in 1.5M-NaClO_4 . —, E_{260} ; ---, radioactivity. Rat liver polyribosomal RNA was labelled under the same conditions as indicated in the legend to Fig. 3(b).

ensured the separation of rat liver rRNA into two components corresponding to the 18S and 28S rRNA (Fig. 4). This was confirmed by separating 18S and 28S rRNA by sucrose-density-gradient ultracentrifugation and subjecting each to the procedure described above. The results (Fig. 5) show that the first and second peaks obtained by this fractionation represent 18S and 28S rRNA respectively. This result has not been achieved previously with animal rRNA. Further, this technique permits much better fractionation of labelled polyribosomal mRNA (Fig. 4) as compared with both the standard MAK procedure and sucrose-density gradient ultracentrifugation (Figs. 3b and 7).

A technique of selective adsorption described below was exploited to separate the cytoplasmic RNA with DNA-like nucleotide composition. A large amount of cpRNA, 25–40mg ($100\mu\text{g/ml}$), pH 6.7, in 0.45M -sodium chloride– 3.0M -urea– 0.02M -EDTA was passed through the column (working volume 18cm^3) at 20°C . Most cpRNA ($>95\%$) passed straight through the column and a small fraction was adsorbed and gradually saturated the adsorbent. After adsorption was completed the column was washed with 60ml of the same solution, then with 0.6M -sodium chloride at 20°C and 37°C in succession and finally with 1.5M -sodium chloride at 37°C to remove rRNA contamination. Then the temperature in the column jacket was increased stepwise to 98°C and the RNA fraction of interest was eluted with 1.5M -sodium chloride as

Fig. 5. Chromatography of 18S and 28S components of rat liver rRNA on the MAK column by using denaturing agent. The 18S and 28S rRNA components were separated by linear (5–20%) sucrose-density-gradient ultracentrifugation. Centrifugation was performed in a Spinco model L2 centrifuge with the SW-50 rotor at 37000 rev./min for 200 min at 4°C . Fractions corresponding to 18S and 28S rRNA were collected, precipitated with 2 vol. of ethanol, then dissolved in 0.01M-NaClO_4 – 5mM -triethanolamine-HCl buffer, pH 6.7, and chromatographed on MAK. Conditions of chromatography were as for Fig. 4. (a) 18S rRNA; (b) 28S rRNA. —, % Transmission; ---, temperature.

soon as possible to prevent its thermal degradation. It was precipitated with 2 vol. of ethanol and treated with 5mM -EDTA. This fraction, designated by us as ct fraction, comprises about 1.5% of the total cpRNA. Fig. 6 shows the results of the sucrose-density-gradient ultracentrifugation of the ct fraction. It appears to be heterogeneous with maxima of extinction at 253nm corresponding to 17S and 23S. Table 2 shows that its nucleotide composition is close to that of DNA-like RNA, differing significantly from that of the total cpRNA in this respect.

The DNA-like ct fraction of cpRNA may be

Fig. 6. Sucrose-density-gradient ultracentrifugation (5-20%) of the ct fraction of rat liver cpRNA. Centrifugation was performed under the conditions given in the legend to Fig. 5. —, Total cpRNA used as a marker; ---, ct fraction.

Fig. 7. Sucrose-density-gradient ultracentrifugation (5-20%) of the rat liver polyribosomal RNA. For conditions of labelling see the Materials and Methods section. Conditions of sedimentation were as for Fig. 6. —, E_{260} ; ---, radioactivity.

mRNA. If this is true, this fraction should also be found in the polyribosome preparation. To verify this supposition an additional experiment was performed.

Total polyribosomal RNA, according to current views, includes only two distinct types of high-molecular-weight molecules, mRNA and rRNA. Because rRNA synthesis is extremely sensitive to actinomycin D it is possible to selectively label polyribosomal mRNA (Georgiev *et al.* 1963; Perry, 1963; Penman, Vesco & Penman, 1968). Rat liver polyribosomal RNA was labelled after the rats had been treated with a small dose of actinomycin D to inhibit the rRNA synthesis (see the Materials and Methods section). Fig. 7 shows almost complete absence of label from the peaks corresponding to rRNA components, indicating that the label belonged mainly to mRNA, since insignificant amounts of [^{14}C]orotic acid were incorporated into the low-molecular-weight RNA. Fig. 8 shows the adsorption of the polyribosomal RNA to the MAK column, where the curve of the extinction reflects the binding of the total RNA and the curve of the radioactivity reflects the binding of the mRNA in general.

The proportion of the total RNA adsorbed on the column (revealed as the difference between the E_{260} of the fluid entering and leaving the column) is insignificant (not exceeding 2-3%) whereas the proportion of the mRNA adsorbed (found as the difference between the radioactivity of the solution entering and leaving the column) is many-fold higher (approx. 50%). Hence polyribosomal mRNA is selectively adsorbed by this procedure.

Correspondingly, the specific radioactivity of the adsorbed RNA fraction, which could be eluted at

the increased temperature, was found to be many-fold higher than that of the total polyribosomal RNA. The stepwise increase of temperature during the adsorption increases the extent of the binding of the mRNA but not of the total RNA.

The fact that not all mRNA is adsorbed (about 50% of the whole radioactivity only) might be explained by the partial degradation of the polyribosomal RNA during the time-consuming isolation procedure. As can be seen (Fig. 7), polyribosomal RNA is slightly degraded, but both peaks of rRNA seem to be equal.

DISCUSSION

The experiments described above show that the various denaturing agents and conditions differing in their mode of action (urea, perchlorate, low ionic strength, elevated temperature) that were used during the process of the adsorption of the RNA on the MAK column exerted the same effect: they increased the tightness of binding of RNA to the adsorbent and thus changed its chromatographic elution profile.

This result appears to support the view that the above effect was caused by the unfolding of the polynucleotide chain and the consequent increase in the number of its phosphate groups able to interact with the MAK, i.e. its 'reacting' surface.

We believe that even in the absence of denaturing agents the MAK itself might produce some destabilizing effect on the secondary structure of the RNA molecule by attracting RNA phosphate

Fig. 8. Adsorption of the total polyribosomal RNA and polyribosomal mRNA on the MAK column. Polyribosomal RNA was passed through the column (fractions 10–65) in 0.45 M-NaCl–3.0 M-urea–0.02 M-EDTA, pH 6.7, at the temperatures indicated. The stepwise increase of temperature is indicated by the arrows. Then the column was washed out by 1.5 M-NaCl solution at 37°C (fractions 65–90). The temperature gradient was performed from 37°C–98°C in 1.5 M-NaCl (fractions 90–115). The polyribosomal RNA had been labelled under conditions ensuring the inhibition of rRNA synthesis (see the Materials and Methods section). ○, E_{260} of the solution entering the column; ●, E_{260} of the solution flowing out of the column; △, radioactivity of the solution entering the column; ▲, radioactivity of the solution flowing out of the column.

groups with its positive charged amino groups [besides electrostatic bonds other kinds of interaction may perhaps be involved (Ellem, 1966; Ellem & Rhode, 1969)]. The unfolding effect of the MAK column on the secondary structure of 5S RNA has been reported by Raue & Gruber (1970).

It is clear that an AU-type RNA would bind to the column more tenaciously than a GC-type RNA since the former is expected to be more sensitive to the unfolding effect of the adsorbent than the latter. In other words, the unfolding action of the MAK reveals the differences in the nucleotide composition of the RNA species fractionated.

On the other hand, a high-molecular-weight RNA possesses a correspondingly greater number of reacting groups on its surface and therefore binds to the column more tenaciously than a low-molecular-weight one.

The concept that the 'reacting' surface of the RNA molecule is the basis for chromatographic separation not only facilitates the understanding of the principles of the fractionation of nucleic acids on the column noted by Sueoka & Cheng (1962) but also allows the choice of conditions that increase the resolution of this method. One can predict, for example, that the proper use of denaturing agents would intensify the differences in the 'reacting' surface of the RNA species differing

in their molecular weight and nucleotide composition. There is some evidence supporting this view.

First, as is well known, rRNA components of animal origin, unlike those of bacterial origin, could not be resolved on MAK. It is thought to be because of the lower $(G+C)/(A+U)$ ratio in 18S rRNA as compared with 28S rRNA, in contrast with bacterial rRNA components, which have a similar nucleotide composition (Spahr & Tissieres, 1959; Midgley, 1962; Lerner, Bell & Darnell, 1963; Zimmerman, Heeter & Darnell, 1963; Ellem, 1966; Muramatsu *et al.* 1966; Higashi, Gotoh, Matsuhisa, Tateishi & Sakamoto, 1970). So animal rRNA components, when chromatographed, elute in almost the same position (Ellem, 1966), the effect of the different molecular weights being compensated by the effect of the nucleotide composition. As was expected, the partial unfolding of RNA samples during their adsorption does allow separation of rRNA components from each other as well as to fractionate the polyribosomal mRNA. Thus the procedure proposed is simple, rapid and reproducible and could be effectively used for the analytical fractionation but it is not so suitable for preparative use since rigorous heating can affect the primary and secondary structures of the polynucleotides chromatographed. Moreover,

since this technique involves a denaturation step of the RNA samples it is expected to be more sensitive to the hidden breakage of the polynucleotide chain than other conventional methods.

Secondly, exploiting the idea mentioned above, we were able to elaborate a technique for selective adsorption of the polyribosomal and cytoplasmic high-molecular-weight AU-type RNA on the MAK column. This technique made use of low concentrations of urea, in which the bulk of the ribosomal RNA passes through the column while the DNA-like RNA is bound.

One can suppose that the denaturing agents used could affect not only the RNA molecule fractionated but also the polypeptide chain of the methylated albumin itself, thus resulting in the increase of the 'reactive' surface of the latter as well. Indeed, both adsorption of methylated albumin on Celite in the presence of 0.05M-sodium perchlorate and adsorption of a large amount of methylated albumin on Celite gave a similar effect of the increasing capacity of the column and the tightness of RNA binding to MAK. However, methylated albumin adsorbed on Celite seems to be not sensitive to the subsequent action of denaturing agents though this point needs further investigation (A. V. Lichtenstein & V. S. Shapot, unpublished work).

Comparative studies of nRNA species and cpRNA species from different rat tissues on the column revealed significant differences between them; there was a lack of the 'temperature' fraction in cpRNA preparations whereas the 'temperature' fraction comprised about 50% of nRNA preparations. The RNA of the 'temperature' fraction has a DNA-like nucleotide composition and incorporates the radioactive precursor rapidly, especially in the T₃ peak.

The fact that the 'temperature' fraction is practically undetectable in cpRNA preparations suggests that they contain no significant nuclear contaminants. This observation might be considered as an argument in favour of the view that the 'temperature' fraction represents the RNA species rapidly degraded within the nucleus, which never enters the cytoplasm. However, there are some results (Spiegelman, 1963; Ellem, 1966; Arion & Georgiev, 1967) suggesting that the nuclear precursors of mRNA are polycistronic and split to the fragments corresponding to separate cistrons while they are being transported into the cytoplasm.

If this was so, nuclear 'heavy' mRNA species might represent a part of the 'temperature' fraction of nRNA. But after entering the cytoplasm they would be revealed in the 'salt' fraction because of their decreased molecular weight. However, the nuclear 'temperature' fraction may consist of a mixture of polycistronic mRNA and a 'true

nuclear' RNA with as yet unknown functions (Harris, 1963; Houssais & Attardi, 1966; Church & McCarthy, 1967; Arion & Georgiev, 1967; Soeiro, Vaughan, Warner & Darnell, 1968).

Recently attempts have been made to fractionate cytoplasmic or polyribosomal RNA species on MAK and compare them with the nuclear ones (Miller, Dhont, de Vreese & van Nimmen, 1968; Roberts & Quinlivan, 1969). These authors have found a tenaciously bound RNA fraction in the cytoplasm. However, we could not find a similar fraction of cpRNA by using MAK chromatography under standard conditions. A small RNA fraction found in the temperature-gradient elution part of the chromatogram has been shown to be an rRNA contaminant. Only the modified conditions (the use of denaturing agents) allowed us to separate DNA-like cytoplasmic RNA and rRNA on the column.

In our opinion, this contradiction might be explained by some deviations in the procedure of fractionation. For instance, Roberts & Quinlivan (1969) introduced certain factors (relatively high pH, 9.0, intermediate washing of the MAK column with water) that may cause some denaturing effect. On the other hand, cpRNA species from tissues studied might not be similar in this respect (Billing, Inglis & Smellie, 1969; Billing, Barbiroli, Inglis & Smellie, 1969).

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REFERENCES

- Arion, V. J. & Georgiev, G. P. (1967). *Dokl. Akad. Nauk SSSR*, **172**, 716.
- Billing, R. J. & Barbiroli, B. (1970). *Biochim. biophys. Acta*, **211**, 434.
- Billing, R. J., Barbiroli, B., Inglis, A. M. & Smellie, R. M. S. (1969). *Biochem. J.* **114**, 43P.
- Billing, R. J., Inglis, A. M. & Smellie, R. M. S. (1969). *Biochem. J.* **113**, 571.
- Church, R. & McCarthy, B. J. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 1548.
- Ellem, K. A. O. (1966). *J. molec. Biol.* **20**, 283.
- Ellem, K. A. O. & Rhode, S. L. (1969). *Biochim. biophys. Acta*, **174**, 117.
- Ellem, K. A. O. & Sheridan, J. W. (1964). *Biochem. biophys. Res. Commun.* **16**, 505.
- Georgiev, G. P. & Mantjeva, V. L. (1962). *Biochim. biophys. Acta*, **61**, 153.
- Georgiev, G. P., Samarina, O. P., Lerman, M. I., Smirnov, M. N. & Severtzov, A. N. (1963). *Nature, Lond.*, **200**, 1291.
- Greenman, D., Wick, W. D. & Kenney, F. T. (1965). *J. biol. Chem.* **240**, 4420.
- Hamaguchi, K. & Geiduschek, P. (1962). *J. Am. chem. Soc.* **84**, 1329.
- Harris, H. (1963). *Proc. R. Soc. B*, **158**, 79.

- Higashi, K., Gotoh, S., Matsuhisa, T., Tateishi, N. & Sakamoto, Y. (1970). *Biochim. biophys. Acta*, **217**, 408.
- Houssais, J. F. & Attardi, G. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 616.
- Lerner, A. M., Bell, E. & Darnell, J. E. (1963). *Science, N.Y.*, **141**, 1187.
- Leytin, V. L. & Lerman, M. I. (1969). *Biokhimiya*, **34**, 839.
- Lichtenstein, A. V. (1970). *Dokl. Akad. Nauk SSSR*, **193**, 936.
- Lichtenstein, A. V., Piker, E. G. & Shapot, V. S. (1967). *Biochim. biophys. Acta*, **138**, 441.
- Martin, R. G. & Ames, B. N. (1961). *J. biol. Chem.* **236**, 1372.
- Midgley, J. E. M. (1962). *Biochim. biophys. Acta*, **61**, 513.
- Miller, A. O. A., Dhont, E., de Vreese, A. M. & van Nimmen, L. (1968). *Biochem. biophys. Res. Commun.* **33**, 702.
- Muramatsu, M., Hodnett, J. L. & Busch, H. (1966). *J. biol. Chem.* **241**, 1544.
- Penman, S., Vesco, C. & Penman, M. (1968). *J. molec. Biol.* **34**, 49.
- Perry, R. P. (1963). *Expl Cell Res.* **29**, 400.
- Petermann, M. L. (1964). *The Physical and Chemical Properties of Ribosomes*, p. 451. Amsterdam, London and New York: Elsevier Publishing Co.
- Piker, E. G., Lichtenstein, A. V., Shapot, V. S. & Rodionova, N. P. (1968). *Vest. Akad. med. Nauk SSSR*, **3**, 26.
- Raue, H. A. & Gruber, M. (1970). *FEBS Lett.* **8**, 45.
- Roberts, W. K. & Quinlivan, V. D. (1969). *Biochemistry, Easton*, **8**, 288.
- Shapot, V. S., Lichtenstein, A. V. & Piker, E. G. (1967). *Proc. int. Symp. Biochemistry of Ribosomes and Messenger RNA, Schloss Reinhardsbrunn*, p. 92.
- Soeiro, R., Vaughan, M. N., Warner, J. R. & Darnell, J. E. (1968). *J. Cell Biol.* **39**, 112.
- Spahr, P. F. & Tissieres, A. (1959). *J. molec. Biol.* **1**, 237.
- Spiegelman, S. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.* **22**, 36.
- Sueoka, N. & Cheng, T. Y. (1962). *J. molec. Biol.* **4**, 161.
- Yoshikawa, M., Fukada, T. & Kawade, Y. (1964). *Biochem. biophys. Res. Commun.* **15**, 22.
- Zimmerman, E. F., Heeter, M. & Darnell, J. E. (1963). *Virology*, **19**, 400.