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Characterization of Rapidly Labelled Rat Liver Ribonucleic Acid Showing High Affinity for Columns of Methylated Albumin on Kieselguhr

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Most of the rapidly labelled RNA from rat liver submitted to column chromatography on methylated albumin on kieselguhr remains tightly bound to the column and can only be recovered by elution with M-ammonia. The tightly bound RNA is composed mainly of DNA-like RNA. The binding capacity is dependent not only on base composition but also on molecular size: the heavier RNA molecules show a greater affinity to the column than do the lower-molecular-weight components. Rapidly labelled mouse liver and *Saccharomyces cerevisiae* RNA show similar behaviour to rat liver RNA on columns of methylated albumin on kieselguhr.

MAK* columns have been extensively used for the separation and characterization of nucleic acids of various origins (Mandell & Hershey, 1960). The method makes use of the variable extractability of the nucleic acids from the column with increasing salt concentrations, is fairly easily used and is highly reproducible. Usually an excellent separation of different classes of RNA is obtained.

In connexion with our studies on the mechanism of action of drugs (Geibel, Hennig, Kunz & Schnieders, 1968) and hormones (Sekeris *et al.* 1969) on RNA metabolism in mouse and rat liver, we have applied this method to characterize the rapidly labelled RNA after a 15min [^{3}H]orotic acid pulse. During these experiments we observed that much of the labelled RNA remains tightly bound to the column even after elution with 2.0M-sodium chloride at pH6.7, and can be recovered only by washing the column with M-ammonia. Similar observations have been made by Ellem & Sheridan (1964) with L-cell RNA and by Lingrel (1967) with rabbit marrow RNA. Ellem & Sheridan (1964) presented evidence that a great part of the RNA eluted from the column with M-ammonia is metabolically unstable and has a DNA-like base composition. The present paper deals with the nature of the rapidly labelled rat liver RNA recovered from MAK columns by elution with M-ammonia.

MATERIALS AND METHODS

Animals and chemicals. Male Wistar BR II rats (130–180g) and NMRI mice were obtained commercially. Baker's yeast was obtained commercially. [5-^{3}H]Orotic acid (6.1Ci/mmol) and [6-^{3}H]uracil (1.0mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Potassium polyvinyl sulphate, bentonite and sodium dodecyl sulphate were purchased from Serva, Heidelberg, Germany, and bovine serum albumin (fraction V, grade B) from Calbiochem, Los Angeles, Calif., U.S.A.

Preparation of labelled RNA from rat liver. [^{3}H]Orotic acid was injected intraperitoneally into rats 15min before they were killed. Crude liver nuclei were obtained after homogenization at 0–4°C with a Potter–Elvehjem homogenizer in 5 vol of 50mM-tris–HCl buffer, pH7.0, containing 25mM-KCl, 10mM-$MgCl_2$ and 0.25M-sucrose and subsequent centrifugation at 0–4°C at 800*g* for 5min. The nuclei were then resuspended in 15–20ml of an extraction buffer containing 50mM sodium acetate buffer, pH5.0, 0.14M-NaCl, 150μg of polyvinyl sulphate/ml and 4mg of bentonite/ml, pretreated as described by Fraenkel-Conrat, Singer & Tsugita (1961).

An equal volume of water-saturated phenol was added and a thermal phenol fractionation was performed by the procedure of Georgiev & Mantieva (1962) and Georgiev, Samarina, Lerman, Smirnov & Severtzov (1963), but modified as follows. The mixture was shaken for 10min at 35°C and immediately cooled to 0°C in an ice-bath. The aqueous layer, containing the ribosomal and ribosomal-precursor RNA (R-RNA), obtained after centrifugation at 8000*g* for 10min was aspirated, sodium dodecyl sulphate (final concn. 0.5%) was added to it and it was re-extracted twice with an equal volume of phenol; the aqueous residue was retained. The interphase layer was further extracted at 55°C and 65°C by adding equal volumes of extraction buffer and water-saturated phenol. The aqueous layer of the 55°C extraction was discarded, but the 65°C aqueous layer, containing RNA having a DNA-like composition (D-RNA), was re-extracted twice with phenol. To remove the phenol, the aqueous phases were washed four times with ether. Then 2 vol. of ethanol was added to each and the suspensions were stored for several hours at −20°C. The method of Schütz, Gallwitz & Sekeris (1968) was used for the extraction of total rapidly labelled RNA.

* Abbreviations: MAK, methylated albumin on kieselguhr; D-RNA, DNA-like RNA, isolated with phenol at 65°C; R-RNA, RNA isolated with phenol at 35°C.

Preparation of labelled RNA from mouse liver and of unlabelled RNA used as carrier for the MAK column chromatography. For the labelling experiments [5-^{3}H]-orotic acid was injected intraperitoneally 15min before the animals were killed. The labelled RNA was prepared essentially by the method of Georgiev & Mantieva (1962). The RNA fractions obtained at 0°C and 65°C were combined and used as such. Usually 6–8mg of unlabelled RNA (Georgiev & Mantieva, 1962) was used as carrier for MAK chromatography.

Sucrose-density-gradient analysis of RNA. For sucrose-density-gradient analysis the precipitated RNA was sedimented by ultracentrifugation for 30min at 120000*g*, dried and dissolved in 2ml of 50mM-sodium acetate buffer, pH5.0, containing 0.14M-NaCl and 1mM-EDTA. The same buffer was used for the preparation of the sucrose density gradient. The sample containing RNA was layered on the top of a 28ml 15–30% (w/v) or 5–40% (w/v) sucrose gradient and ultracentrifuged in a Spinco L2 ultracentrifuge at 20000 rev./min for 14h (SW 25.1 rotor). Fractions (1ml) were collected by puncturing the tube with a hypodermic needle. To measure the radioactivity, a sample of each fraction was precipitated on paper discs (2043b; Schleicher und Schuell paper) with ice-cold 5% (w/v) $HClO_4$ and counted in a Nuclear–Chicago scintillation counter as described by Lang & Sekeris (1964).

From the fraction extracted at 65°C RNA corresponding to sedimentation coefficients of 70S, 45S, 30S and 18S were obtained after sucrose-density-gradient centrifugation by precipitating the appropriate fractions from the gradient with 2 vol. of ethanol. The RNA was collected by ultracentrifugation for 30min at 120000*g*. The 45S ribosomal precursor RNA was obtained in a similar way after sucrose-density-gradient analysis of the 35°C extract. The sedimentation coefficients were determined as described by Staehelin, Wettstein, Oura & Noll (1964).

MAK-column chromatography. The preparation of the methylated albumin and of the column was as described by Mandell & Hershey (1960). The methylated albumin solutions were always prepared immediately before use (Koch & Kubinski, 1964).

MAK columns (2cm×13cm) were equilibrated with 50mM-NaCl in 50mM Sörensen phosphate buffer, pH6.7, under a pressure of 0.5kg/cm^2. The nucleic acids (not exceeding 9mg) were taken up in the same buffer and applied to the column. The column was washed with the same buffer and then eluted with a linear 0.1–2.0M-NaCl gradient in the phosphate buffer, pH6.7, and finally with M-ammonia. The ammonia fractions were immediately neutralized. Column flow was 25ml/h and 10ml fractions were collected. The extinction at 254nm was recorded with a LKB Uvicord spectrophotometer.

Determination of the radioactivity of the fractions. A 1ml sample of each fraction was added to 10ml of Bray's (1960) solution and counted in a Nuclear–Chicago mark I liquid scintillation counter.

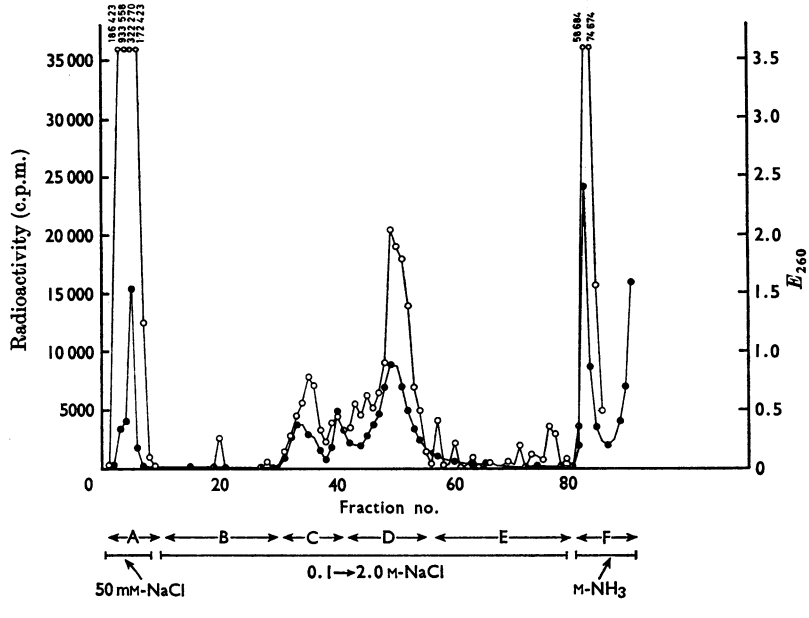

Fig. 1. MAK-column chromatography of rat liver nRNA. Total nRNA was isolated as described in the Materials and Methods section 15min after intraperitoneal injection into rats of 100μCi of [^{3}H]orotic acid/100g body wt. MAK-column chromatography was performed as described in the Materials and Methods section. ○, Radioactivity; ●, E_{260}.

RESULTS

RNA isolated from rat liver 15min after injection of [^{3}H]orotic acid was submitted to MAK-column chromatography (Fig. 1). Apart from the radioactivity found in peak A, which is composed mainly of oligo- and mono-nucleotides, less than half of the labelled RNA is eluted with the sodium chloride gradient, whereas the bulk of the radioactive RNA is recovered from the column after elution with M-ammonia. As found previously, sucrose-density-gradient analysis of total rat liver RNA labelled for 10–15min with orotic acid shows heterogeneous sedimentation profiles with sedimentation values from 8S to over 70S (Fig. 2). This RNA is composed mainly of DNA-like RNA and of smaller amounts of ribosomal and ribosomal precursor RNA (Schütz *et al.* 1968).

To define which kind of RNA is preferentially bound to the MAK column, we further subfractionated the rapidly labelled RNA obtained by phenol extraction at different temperatures (Georgiev & Mantieva, 1962; Georgiev *et al.* 1963) into an RNA fraction obtained at 35°C (R-RNA) and a second fraction obtained at 65°C (D-RNA). The R-RNA consists mainly of ribosomal and ribosomal-precursor RNA, whereas D-RNA is almost exclusively made up of molecules having a DNA-like composition and a heterogeneous molecular size. The sedimentation profiles of the two RNA fractions are shown in Fig. 3.

The two RNA fractions were further submitted to MAK column chromatography. The results are shown in Table 1. Only 22.5% of the labelled R-RNA is eluted with the M-ammonia peak, about 13 and 19% are found on the 4–5S (fraction C) and 18–28S RNA (fraction D) respectively and 39% is eluted in the region between the 28S and the ammonia fraction, which very probably contains the 45S ribosomal-precursor RNA (fraction E). In contrast, 75% of the radioactivity of the D-RNA is eluted with M-ammonia and only small amounts of radioactive RNA are eluted with fractions C, D and E.

To characterize further which part of the D-RNA is preferentially bound to the column, D-RNA of average sedimentation coefficients 18, 30, 45 and 70S were isolated from a sucrose density gradient and submitted to MAK column chromatography. The results are shown in Table 2. It is evident that the binding capacity of the RNA depends on the molecular size, the heavier RNA species being more tightly bound to the column than are the lighter fractions. That binding capacity is dependent not only on molecular size but also on base composition is evident from the results of MAK chromatography of 45S R-RNA and 45S D-RNA (Table 2). More than twice the amount of labelled D-RNA is eluted with M-ammonia as is R-RNA.

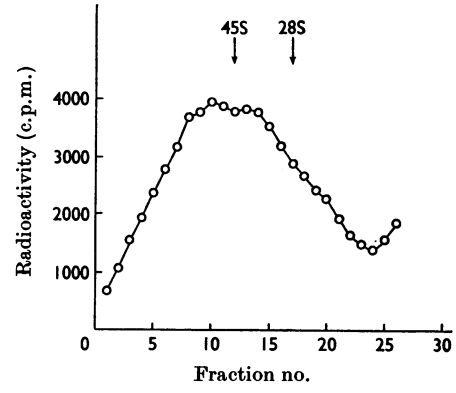

Fig. 2. Sucrose-density-gradient centrifugation of total nRNA. Total nRNA was isolated as described in the Materials and Methods section 15min after intraperitoneal injection into rats of 100μCi of [^{3}H]orotic acid/100g body wt. Centrifugation was at 20000rev./min for 12h. A 5–40% (w/v) linear sucrose gradient was used.

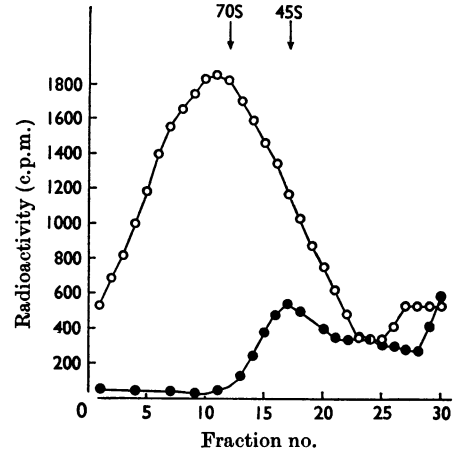

Fig. 3. Sucrose-density-gradient centrifugation of R-RNA and D-RNA. The two RNA fractions were isolated from liver nuclei of rats injected intraperitoneally with 100μCi of [^{3}H]orotic acid/100g body wt. 15min before they were killed. A 15–35% (w/v) linear sucrose gradient was used, and centrifugation was at 20000 rev./min in a SW 25.1 rotor for 14h. ○, D-RNA; ●, R-RNA.

We have also compared the behaviour on MAK columns of rapidly labelled RNA isolated from mouse liver and from the yeast *Saccharomyces cerevisiae*. The chromatograms are similar in appearance to those of rat liver RNA, showing also the presence of a great amount of rapidly labelled

RNA tightly bound to the column and eluted only with M-ammonia.

DISCUSSION

The results of MAK-column chromatography of rapidly labelled RNA from rat and mouse liver as well as that from *Saccharomyces cerevisiae* show that a great part of the labelled RNA remains tightly bound to the column and can only be recovered with M-ammonia and not by eluting with a sodium chloride gradient. These results agree with the findings of Ellem & Sheridan (1964) and Ellem (1966) for L-cell RNA, of Lingrel (1967) for bone-marrow RNA, of Roberts & Quinlivan (1969) for Ehrlich ascites-cell RNA and of Billing, Inglis & Smellie (1969) for rat liver RNA. The present results point to the caution needed in interpreting MAK-column chromatographs in which the alkali elution step has not been taken. Under such conditions much of the labelled RNA is lost. With regard to the nature of the RNA bound to the column, it is evident that most of this RNA is composed of DNA-like species, only a small part being of the R-RNA type, as already found by the above-named authors for RNA isolated from other organisms. This conclusion is supported by the behaviour of R-RNA and D-RNA during MAK chromatography. In addition, the amount of tightly bound RNA decreases after longer labelling periods during which the ratios of D-RNA to R-RNA decrease (W. Kunz & B. Schnieders, unpublished work). Both the base composition and the molecular size play a role in the binding capacity of the RNA to the MAK column. Thus the heavy 70S D-RNA binds to a much greater extent than the 18S D-RNA. After partial degradation of the heavy D-RNA caused by storage, the amount of tightly bound RNA progressively decreases. That both molecular size and base composition determine the binding capacity is illustrated by comparing the behaviour of the 45S ribosomal-precursor RNA with that of the 45S D-RNA; more than 60% of the 45S D-RNA is tightly bound on the MAK column compared with only 33% of the 45S R-RNA.

Table 1. *MAK-column chromatography of R-RNA and D-RNA from rat liver*

The two RNA fractions were isolated from liver nuclei of rats injected intraperitoneally with 100 μCi of [^{3}H]-orotic acid/100g body wt. 15min before they were killed. Conditions of MAK-column chromatography were as described in the Materials and Methods section. The radioactivity of fraction A, consisting mainly of oligo- and mono-nucleotides, has been omitted from the table.

	% of radioactivity recovered	
Fraction	R-RNA	D-RNA
B	6.3	3.7
C	12.9	5.7
D	19.0	7.1
E	39.3	8.3
F	22.5	75.1

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REFERENCES

Billing, R. J., Inglis, A. M. & Smellie, R. M. S. (1969). *Biochem. J.* **113**, 571.
Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
Ellem, K. A. O. (1966). *J. molec. Biol.* **20**, 283.
Ellem, K. A. O. & Sheridan, J. W. (1964). *Biochem. biophys. Res. Commun.* **16**, 505.
Fraenkel-Conrat, H., Singer, B. & Tsugita, A. (1961). *Virology*, **15**, 541.

Table 2. *MAK-column chromatography of* 18S, 30S, 45S *and* 70S *D-RNA and of* 45S *R-RNA from rat liver nuclei*

The RNA fractions were obtained as described in the Materials and Methods section 15min after intraperitoneal injection into rats of 100 μCi of [^{3}H]orotic acid/100g body wt.

	% of radioactivity recovered				
	D-RNA				R-RNA
	18S	30S	45S	70S	45S
B	7.3	6.0	3.4	2.1	7.9
C	8.8	5.5	3.7	2.3	4.5
D	20.8	13.7	17.3	10.3	20.3
E	22.9	17.7	9.2	4.0	38.9
F	40.2	57.1	66.1	81.3	33.4

Geibel, U., Hennig, W., Kunz, W. & Schnieders, B. (1968). *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **260**, 118.

Georgiev, G. P. & Mantieva, V. L. (1962). *Biochim. biophys. Acta*, **61**, 153.

Georgiev, G. P., Samarina, O. P., Lerman, M. T., Smirnov, M. N. & Severtzov, A. N. (1963). *Nature, Lond.*, **28**, 1291.

Koch, G. & Kubinski, H. (1964). *Z. Naturf.* **19**b, 683.

Lang, N. & Sekeris, C. E. (1964). *Hoppe-Seyler's Z. physiol. Chem.* **338**, 238.

Lingrel, J. B. (1967). *Biochim. biophys. Acta*, **142**, 75.

Mandell, J. D. & Hershey, J. A. (1960). *Biochemistry, Easton*, **1**, 66.

Roberts, W. K. & Quinlivan, V. D. (1969). *Biochemistry, Easton*, **8**, 288.

Schütz, G., Gallwitz, D. & Sekeris, C. E. (1968). *Eur. J. Biochem.* **4**, 149.

Sekeris, C. E., Homoki, J., Beato, M., Lukacs, I., Gallwitz, D. & Seifart, K. H. (1969). In *Advances in the Biosciences*, vol. 2, p. 222. Ed. by Raspé, G. London. Pergamon Press Ltd.; Braunschweig: Vieweg.

Staehelin, T., Wettstein, O., Oura, H. & Noll, H. (1964). *Nature, Lond.*, **210**, 264.