The Nature of Polydisperse Ribonucleic Acid in Plants

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The aggregation of ribosomal RNA species during chromatography on methylated albumin on kieselguhr was decreased from 50 to 15% by the lower salt concentrations made possible by the use of higher pH values. The polydisperse RNA was resolved into two fractions. About 50% was eluted with the rRNA whereas the remainder was bound to the column, and was recovered only by solubilization of the methylated albumin. Both fractions of polydisperse RNA were similar in size range, but the bound fraction was considerably richer in AMP. No D-RNA (DNA-like RNA) peak was resolved under these conditions of column fractionation. However, the properties of the bound RNA were consistent with it containing both D-RNA and TB-RNA (tenaciously bound RNA). The relationship between these two fractions of AMP-rich RNA was considered. The bound RNA and ribosomal RNA responded differently to various treatments. The salt concentration required to elute ribosomal RNA was halved by increasing the pH of the fractionation, but the amount of bound RNA was in fact increased. Denaturation by hot urea decreased the binding of ribosomal RNA to the methylated albumin, but did not facilitate elution of bound RNA. The high affinity between the AMP-rich polydisperse RNA and the methylated albumin does not therefore appear to arise for the secondary structure conferred by the high AMP content.

Most methods of RNA fractionation work on the basis of molecular size and, although applicable for the separation and preparation of RNA species of discrete molecular weight, such as rRNA species, are unsuitable for the isolation of a heterogeneous, or polydisperse, class of RNA. A large percentage of newly synthesized RNA, as studied by the incorporation of radioactive precursors during short periods, is, however, polydisperse in nature. In contrast to the well-defined rRNA species relatively little is known about the properties and functions of this RNA.

MAK† column chromatography differs from methods of fractionation such as sucrose-density-gradient centrifugation and gel electrophoresis in that separation depends on other factors in addition to molecular size. Although the mechanism is not fully understood three factors are thought to influence the binding and elution of nucleic acids from the column. These are size, base composition and secondary structure, and the last two factors have been exploited for the separation of polydisperse RNA from rRNA. Several laboratories have used MAK chromatography to fractionate newly synthesized RNA, and have found a peak of radioactive RNA, heterogeneous

in size and with a base composition resembling that of the DNA, eluted just after the heavy rRNA. It was therefore called D-RNA (Key & Ingle, 1964; Ellem & Sheridan, 1964; Ingle *et al.*, 1965; Billing & Barbirolli, 1970; Johri & Varner, 1970; Key *et al.*, 1972). It was concluded that the synthesis of D-RNA was necessary for several parameters of plant growth (Key & Ingle, 1964) and that it had many of the properties expected of mRNA (Ingle & Key, 1965; Ingle *et al.*, 1965). A second fraction of RNA having somewhat similar properties to D-RNA, but which remained bound to the MAK column after salt elution, has been described (Ellem & Sheridan, 1964; Yoshikawa *et al.*, 1964; Tester & Dure, 1967; Billing & Barbirolli, 1970; Johri & Varner, 1970). This TB-RNA, which accounted for as much as 60% of the newly synthesized RNA (Ellem & Sheridan, 1964), was recovered by solubilizing the methylated albumin (to which the nucleic acid is bound) from the column. Recently D-RNA and TB-RNA have been extensively purified and characterized from soya-bean tissue (Key *et al.*, 1972). Both were polydisperse in size and contained high proportions of AMP and UMP. The TB-RNA was smaller in average size and had a higher AMP content than the D-RNA.

There are, however, certain problems associated with MAK chromatography. One of these is the non-quantitative recovery of RNA, particularly newly synthesized RNA, and another is the high concentration of salt necessary to elute the RNA from the column. There is good evidence for the interaction of

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† Abbreviations: MAK, methylated albumin on kieselguhr; D-RNA, DNA-like RNA; TB-RNA, tenaciously bound RNA.

rRNA molecules (Möller, 1964; Marcot-Querioz & Monier, 1965) and of rRNA with rapidly labelled polydisperse RNA (Asano, 1965) at these high salt concentrations. Ingle & Key (1968) confirmed that the abnormally high ratio of heavy to light rRNA observed in MAK-column fractionations was caused by aggregation of rRNA components, with the consequent elution of a large proportion of the light rRNA in the heavy-rRNA region. The results of Marcot-Querioz & Monier (1965) suggest that this aggregation could be decreased if K^+ was used instead of Na^+ , and if the salt concentration required to elute the RNA could be decreased. Such conditions would necessarily involve a decrease in the affinity between RNA and the methylated albumin, and may therefore also be beneficial in improving the recovery of newly synthesized RNA from the column. The present paper describes conditions that enable the rRNA to be eluted from a MAK column at much lower salt concentrations, with subsequent decrease in the extent of aggregation. Under these conditions the polydisperse RNA was fractionated into two classes, which were not equivalent to D-RNA and TB-RNA.

Experimental

Materials

Growth and incubation of seedlings. Intact unblemished pea seeds (*Pisum sativum* var. Meteor) were sterilized in 2% sodium hypochlorite for 10 min and thoroughly washed in sterile water. Seeds were germinated under sterile conditions in a large germination dish (about 200 seeds/dish), being held on the surface of water by Netlon (5 mm mesh). After 72 h seedlings with roots 3.5–4.5 cm long were transferred to a smaller container for incubation in radioactive precursor. The linear growth rate of the roots was unaffected by this transfer. High-specific-radioactivity orthophosphate (50–100 Ci/mg of P; The Radiochemical Centre, Amersham, Bucks., U.K.) was evaporated to dryness, re-dissolved in water, sterilized by autoclaving and then added to the incubation medium (0.4 μ M-phosphate, pH 6.7) at a concentration of 1.5 mCi or 4.0 mCi/25 ml.

Methods

Preparation of nucleic acid. Root tips (1 cm long) were excised and homogenized in a detergent medium in a cold mortar and the nucleic acid was extracted by the method of Parish & Kirby (1966) as previously described (Jackson & Ingle, 1973). The nucleic acid from the final aqueous phase was precipitated with 2 vol. of ethanol and stored overnight at 0°C. The precipitate was washed several times in 80% (v/v) ethanol containing 0.2% sodium dodecyl sulphate, dissolved in 0.5% sodium dodecyl sulphate–0.15 M-

sodium acetate (final concn. approx. 1 mg/ml) and then precipitated with ethanol.

Total and acid-precipitable radioactivity. For total radioactivity a 10 μ l sample of the relevant solution was dried on a glass-fibre disc (Whatman GF/A; 1 cm diam.). Acid-precipitable radioactivity (i.e. that incorporated into nucleic acid) was determined by diluting samples with an equal volume of cold 10% (w/v) trichloroacetic acid. The solution was left at 0°C for 1 h and then the precipitate was collected and thoroughly washed with cold 5% (w/v) trichloroacetic acid on a membrane filter (3 cm diam.; grade 0.45; Oxoid Ltd., London S.E.1, U.K.). Both glass-fibre and membrane filters were counted for radioactivity in an Inter-technique SL 30 Scintillation Counter with a toluene-based scintillator: 5% (w/v) 2,5-diphenyloxazole, 0.3% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene.

MAK column chromatography. Columns were prepared as described by Mandell & Hershey (1960) with the modifications that the columns were much smaller in size (containing only 1.8 g of kieselguhr compared with 24 g) and the kieselguhr (Hyflo Supercel; Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) was coated with about 20 times the concentration of methylated albumin [fraction V (Sigma Chemical Co., St. Louis, Mo., U.S.A.), methylated for 5 days at 37°C as described by Mandell & Hershey (1960)]. Columns were packed and eluted at various pH values, the fractionation being designated pHx-pHy where x was the pH at which the column was packed and y the pH of elution. The amount of total nucleic acid fractionated was 100–200 μ g. Details of the salt gradients are given in relevant parts of the text. The elution profile was recorded continuously at 258 nm with an LKB Uvicord II detector (0.5 cm path length) and fractions were collected at timed intervals. The E_{260} of the fractions in the rRNA region of the column was determined with a Unicam SP. 500 spectrophotometer.

After the gradient elution the column was washed with 20 ml of water to remove the salt, and any nucleic acid remaining on the column was then recovered by solubilizing the protein with 2% sodium dodecyl sulphate. The column was finally washed with 20 ml of aq. 2 M- NH_3 to ensure the complete removal of protein, and samples of the kieselguhr, suspended in a small volume of liquid, were counted for radioactivity in a Beckman Low-Beta gas-flow planchet counter. Radioactivity in the salt gradient, sodium dodecyl sulphate and NH_3 fractions was determined by Čerenkov counting (aqueous solution, 60% gain). The radioactivity in the NH_3 fractions and on the kieselguhr was negligible.

Fractions of the sodium dodecyl sulphate eluate containing the radioactive peak were pooled, 100 μ g of carrier RNA (pea root total nucleic acid) was added and the nucleic acid was recovered by ethanol

precipitation after the normal phenol deproteinization (Jackson & Ingle, 1973). Fractions of the MAK salt gradient were pooled, the salt concentration was adjusted to 1.0M and 0.3 μ l of diethyl pyrocarbonate/ml was added. The nucleic acid was recovered by centrifugation at 130000g for 16h.

Purification of the 32 P-labelled RNA. As a consequence of the high input of radioisotope necessary to obtain nucleic acid of sufficiently high specific radioactivity for further analysis, only a small percentage (1–5%) of the 32 P radioactivity in the final nucleic acid preparation was actually incorporated into RNA and DNA, as determined by acid precipitation and membrane filtration. It was therefore necessary to establish that the radioactivity in the various fractions from the column, and particularly that bound to the column, was nucleic acid. Acid precipitation and membrane filtration of the fractions from the salt gradient of the column indicated that even after the column had been washed with 40ml of salt containing 0.05M-potassium phosphate buffer there was significant (30%) contamination by non-RNA radioactivity. The extent of contamination in the TB-RNA could not be directly determined by acid precipitation, since sodium dodecyl sulphate, used to elute this fraction from the column, interfered with the precipitation. It was therefore necessary to remove the contaminating radioactivity from the nucleic acid preparation. Repeated re-precipitations from 0.15M-sodium acetate containing 0.5% sodium dodecyl sulphate, with thorough washing of each pellet in 80% ethanol containing 0.2% sodium dodecyl sulphate, was unsuccessful. Five such re-precipitations increased the percentage of 32 P-labelled RNA from about 1% to only 5–10% of the radioactivity. A preparation containing only 5–10% contamination was obtained by high-speed centrifugation (130000g for 16h) from 1M-KCl, followed by a single re-precipitation. Polyacrylamide-gel electrophoresis, MAK chromatography and specific-radioactivity measurements before and after centrifugation showed that the nucleic acid was quantitatively recovered with essentially no degradation.

By using such purified preparations it was possible to estimate the extent of contaminating radioactivity in fractionations of crude preparations. About 30% of the TB-RNA radioactivity was due to contamination, i.e. very similar to the general degree of contamination in the salt-gradient fractions.

Polyacrylamide-gel electrophoresis. RNA was fractionated on 2.2% gels at 50V (6mA/tube) for the times stated (Loening, 1967). Gels were scanned by using a Joyce-Loebl Polyfrac with Servoscribe recorder, frozen rapidly in powdered solid CO_2 and cut into 0.5mm slices with a Mickle gel slicer. Each slice was dried on 16mm film and the radioactivity was determined by using a Geiger tube connected to an IDL scaler system.

Base compositions. Relevant slices were removed from the film after counting, and hydrolysed together with 250 μ g of carrier RNA in 10% (v/v) piperidine–10mM-EDTA at 60°C for 48h. The piperidine was removed by evaporation and the RNA mononucleotides were recovered from the slices with two washes of 2ml of pyridine buffer (0.75% pyridine, 7.5% acetic acid, pH4.0). The buffer containing the nucleotides was concentrated by evaporation and the nucleotides were separated by high-voltage paper electrophoresis (1200V for 2h) in the pyridine buffer. The chromatograms were dried, the spots detected under u.v. and the areas containing the nucleotides cut out and counted for radioactivity. The base composition was expressed as mol/100mol, based on the 32 P distribution.

Results

Effect of pH on the elution of rRNA from the MAK column

The salt concentration required to elute rRNA from the column depended on both the pH at which the column was packed and the pH of elution. The higher the pH of either solution, the lower the salt concentration necessary for elution (Table 1). At the higher pH values the rRNA did not bind to the column, but was washed straight through, whereas at the lowest pH (4.4) no rRNA was eluted from the column at concentrations up to 2.2M-KCl. The rRNA was eluted by 0.6M-KCl from a pH7.8–8.9 fractionation, just half the salt concentration necessary for elution from the normal pH6.7–pH6.7 column.

Optimum salt gradients were derived for each pH fractionation to give good resolution of the rRNA components, and the scans were examined for the extent of aggregation. Fractionation at higher pH values decreased the observed ratio of heavy to light rRNA from 4:1, indicating aggregation of about 40% of the 0.7×10^6 mol.wt. rRNA (pH6.7–pH6.7), to 2.4:1, 15% aggregation (pH6.7–pH8.9; Fig. 1). RNA from the rRNA region of a pH7.8–pH8.9 MAK fractionation (heavy to light rRNA ratio 2.2:1) was recovered by high-speed centrifugation and analysed by gel electrophoresis (Figs. 2 and 3). The amount of the light rRNA (mol.wt. 0.7×10^6) present in fractions taken from the heavy rRNA region of the MAK fractionation was greatly decreased compared with that from a pH6.7–pH6.7 column, but was not completely eliminated. Approx. 8% of the rRNA present in regions (d), (e) and (f), which should be completely free from the tail of the light rRNA peak, was 0.7×10^6 mol.wt. rRNA. This indicated that approx. 20% of the 0.7×10^6 mol.wt. rRNA was aggregated in some way, compared with 48% aggregation at pH6.7–pH6.7.

Table 1. *Effect of pH on MAK fractionation of RNA*

The nominal pH values of the phosphate buffers (0.05M) are given, with the true pH (after addition of the salt) shown in parentheses. The columns were packed in 0.1M-KCl at the various pH values and 150 μ g of pea total nucleic acid was fractionated with a 100ml linear gradient of 0.4–1.4M-KCl at the various pH values. The concentration of KCl at which the heavy rRNA (mol.wt. 1.3×10^6) was eluted was used as a measure of the fractionation. Each value represents an average of at least two fractionations. pH9.0 was obtained with Tris-HCl buffer (0.05M).

Column pH	Elution pH	...	Concn. of KCl (M) required to elute 1.3×10^6 mol.wt. rRNA				
			4.4 (4.2→4.1)	6.7 (6.4→6.2)	7.8 (7.4→7.3)	8.9 (8.5→8.4)	9.0 (9.1→9.2)
4.4			Bound	1.20	1.15	0.95	0.70
6.7			Bound	1.22	1.17	0.83	0.73
7.8			Bound	1.08	0.90	0.63	0.62
8.9			Bound	0.69	0.60	0.53	0.52
9.0			Bound	0.53	0.47	Wash	Wash

Fig. 1. *Effect of pH on the aggregation of rRNA during MAK fractionation*

Pea root nucleic acid (150 μ g) was fractionated by MAK chromatography with a 100ml linear gradient. Columns are designated pH x -pH y , where x is the pH at which the column was packed and y the pH of elution. The values in parentheses refer to the salt concentrations at which the DNA and heavy and light rRNA species were eluted. (a) pH6.7-pH6.7. Gradient 0.4–1.2M-NaCl. Ratio of heavy to light rRNA was 4:1. (b) pH6.7-pH8.9. Gradient 0.4–1.4M-KCl. Ratio of heavy to light rRNA was 2.4:1.

Characterization of the salt-eluted RNA

The analysis of the MAK column fractions (Figs. 2 and 3) was extended to the newly synthesized RNA, based on the distribution of radioactivity. The amount of each class of newly synthesized RNA, the 2.5×10^6 mol.wt. precursor rRNA, the 1.4×10^6 mol.wt. precursor plus 1.3×10^6 mol.wt. rRNA, the 0.7×10^6 mol.wt. rRNA and the polydisperse RNA was estimated from the area of each component on the gel fractionation, and expressed as a percentage of the total radioactivity (Fig. 3; Table 2). The occurrence of 0.7×10^6 mol.wt. rRNA in the heavy rRNA region of the MAK column (regions *d*, *e* and *f*) has been considered in terms of the E_{265} profile, and this was confirmed in terms of the radioactivity distribution. The 2.5×10^6 mol.wt. precursor rRNA was confined to the back region of the heavy rRNA (regions *d*, *e* and *f*, Fig. 3). Each region from the MAK column contained, in addition to the rRNA, a large percentage of polydisperse RNA. Region (*a*), the light rRNA from the MAK column, contained about 55% of 0.7×10^6 mol.wt. rRNA and 45% of polydisperse RNA, ranging in size from about 2×10^6 daltons down to 10^5 daltons. Region (*f*) contained 28% of polydisperse RNA, with a size range from 4×10^6 to 0.5×10^6 daltons. Analysis by gel electrophoresis of the total newly synthesized RNA used in these experiments (Fig. 4) showed 53% of polydisperse RNA (Table 2), indicating that

approximately half of the polydisperse RNA had been resolved from the rRNA by the MAK fractionation.

The base composition of the RNA from each of the MAK-column regions was determined (Table 3). From the distribution of rRNA species in each fraction (Table 2) and the base compositions of the rRNA species (Table 3) the approximate composition of the polydisperse RNA was calculated (Table 3). The values, though only approximate, indicated that the polydisperse RNA eluted from the MAK column by the salt gradient had a significantly higher GMP/AMP ratio than that polydisperse RNA bound to the column (0.68; Table 3).

Effect of pH on the bound RNA

The effect of pH on the percentage of the RNA that remained bound to the MAK column after the salt elution was investigated by using RNA preparations containing very little contaminating radioactivity. Although rRNA was eluted more readily at the higher pH values, the percentage of nucleic acid that remained bound to the column increased slightly with pH. The base composition of the bound RNA was similar regardless of the pH of elution (Table 4). The percentage of nucleic acid that was bound did not bear any relationship to the salt concentration at

Fig. 2. Analysis of the rRNA region from a MAK fractionation

Nucleic acid prepared from roots incubated in 4mCi of [32 P] P_i for 1 h was fractionated by MAK chromatography on a pH7.8–pH8.9 column with a 135ml linear gradient from 0.35 to 1.2M-KCl. RNA was recovered from the regions indicated for further analysis (see Fig. 3). The continuous line represents the E_{258} and the histogram the radioactivity as c.p.m./1.4ml fraction.

Fig. 3. Polyacrylamide-gel analysis of rRNA from a MAK column

Nucleic acid was fractionated by MAK chromatography (Fig. 2), and RNA recovered from regions (a)–(f) by centrifugation ($130000g$ for 16h) was analysed by gel electrophoresis (2.2% gel, 50V, 3h). The results are shown in the correspondingly lettered parts of the figure. The continuous line represents the E_{265} and the histogram the radioactivity as c.p.m./0.5mm gel slice. The stable rRNA species (1.3×10^6 and 0.7×10^6 mol.wt.) and the precursor rRNA species (2.5×10^6 and 1.4×10^6 mol.wt.) are indicated in (c) and (f) respectively.

which rRNA was eluted. Thus the bound RNA did not respond to pH in the same way as rRNA.

The bound RNA from a pH7.8–8.9 column was recovered with sodium dodecyl sulphate, and the size distribution determined by gel electrophoresis. The RNA was heterogeneous in size, with discernible peaks of molecular weights 1.9×10^6 – 2.3×10^6 , 1.3×10^6 and 0.7×10^6 – 0.9×10^6 above a very high polydisperse background (Fig. 5). The base compositions of the different size classes were determined (Table 5). There

was a decrease in the GMP/AMP ratio, from 0.8 to 0.6, with decrease in size of the polydisperse RNA. The bound polydisperse RNA was considerably richer in AMP than the polydisperse RNA that was eluted from the MAK column with the rRNA. At no point along the gel was the base composition rRNA-like, indicating that very little rRNA remained bound to the MAK column after the salt gradient. The quantitative elution of rRNA by the gradient was confirmed by fractionation of nucleic acid pre-

Table 2. *Distribution of newly synthesized RNA on a MAK column*

Nucleic acid from roots incubated in [³²P]P_i (4mCi/25ml) was fractionated by MAK chromatography (Fig. 2) and the RNA from regions (a)–(f) was recovered and analysed by gel electrophoresis (Fig. 3).

Region	Distribution (% of total radioactivity)			
	2.5 × 10 ⁶ mol.wt. precursor rRNA	1.4 × 10 ⁶ mol.wt. precursor rRNA plus 1.3 × 10 ⁶ mol.wt. rRNA	0.7 × 10 ⁶ mol.wt. rRNA	Polydisperse RNA
(a)	0	0	53.6	46.4
(b)	4.8	25.2	25.0	44.9
(c)	3.4	37.4	13.4	46.0
(d)	13.1	47.0	6.5	36.0
(e)	18.1	46.8	4.0	30.9
(f)	22.1	46.0	3.8	28.3
Total nucleic acid preparation	10.0	26.6	9.8	53.0

Fig. 4. *Gel electrophoresis of the total nucleic acid preparation used in the MAK column analysis*

A sample of the nucleic acid used for the MAK fractionation (Fig. 2) was fractionated by polyacrylamide-gel electrophoresis (2.2% gel, 50 V, 3 h). The continuous line represents the E₂₆₅ and the histogram the radioactivity as c.p.m./0.5mm gel slice. The precursor rRNA species (2.5 × 10⁶ and 1.4 × 10⁶ mol.wt.) and the stable rRNA species (1.3 × 10⁶ and 0.7 × 10⁶ mol.wt.) are indicated.

pared from roots labelled for 24h, after which time most of the radioactivity incorporated was in the stable rRNA species. Only 1% of the radioactivity in such a preparation remained bound after the normal salt elution.

Effect of secondary structure on the binding of RNA to MAK

RNA may remain bound to MAK because its high AMP content confers on it a secondary structure that

Table 3. *Base compositions of RNA recovered from the rRNA region of the MAK column*

RNA was recovered from regions (a)–(f) of the MAK column shown in Fig. 2. The base compositions are single determinations for each fraction from one MAK column, which was replicated with very similar results. The bound RNA, recovered by sodium dodecyl sulphate, was from the same column. Values for the rRNA species were determined from gel fractionations of RNA prepared from 24h-labelled roots, and the precursor rRNA from 1h-labelled tissue (Fig. 4). The approximate base composition of the polydisperse RNA from each MAK fraction, calculated from the total composition and the percentage distribution of the newly synthesized RNA (Table 2), is shown in parentheses.

RNA	Composition (mol/100mol)				GMP/AMP ratio
	CMP	AMP	GMP	UMP	
MAK fraction (a)	21.0 (20.8)	27.6 (30.0)	27.8 (27.2)	23.5 (21.7)	1.01 (0.91)
MAK fraction (b)	19.8 (19.6)	26.8 (27.8)	27.2 (23.8)	26.4 (28.4)	1.02 (0.86)
MAK fraction (c)	20.2 (19.5)	25.8 (25.8)	31.1 (31.2)	22.7 (23.1)	1.21 (1.21)
MAK fraction (d)	20.8 (21.8)	24.8 (21.6)	29.8 (27.6)	24.6 (29.3)	1.20 (1.28)
MAK fraction (e)	19.9 (18.2)	28.2 (32.0)	28.8 (25.0)	23.1 (26.0)	1.02 (0.78)
MAK fraction (f)	20.3 (19.3)	25.4 (25.6)	29.0 (27.4)	25.5 (33.9)	1.14 (1.07)
Bound RNA	18.4	36.4	24.6	21.0	0.68
1.3 × 10 ⁶ mol.wt. rRNA	21.7	25.1	32.3	21.4	1.28
0.7 × 10 ⁶ mol.wt. rRNA	20.9	25.7	28.3	25.2	1.10
1.4 × 10 ⁶ mol.wt. precursor rRNA	20.2	27.6	31.5	20.8	1.14
2.5 × 10 ⁶ mol.wt. precursor rRNA	20.1	27.5	29.2	23.2	1.06

Table 4. *Effect of pH on bound RNA*

Nucleic acid prepared from roots incubated in [³²P]P_i (1.5mCi/25ml) for 1h was fractionated by MAK chromatography at various pH values, as described in the Experimental section.

Fractionation conditions	Concn. of salt required to elute 1.3 × 10 ⁶ mol.wt. rRNA (M)	Bound RNA (% of total newly synthesized RNA)	Base composition of bound RNA (mol/100mol)				GMP/AMP ratio
			CMP	AMP	GMP	UMP	
pH 6.7–pH 6.7	1.22	24	18.7	38.1	26.2	17.0	0.68
pH 7.8–pH 7.8	0.98	27	19.5	39.4	23.1	19.0	0.58
pH 7.8–pH 8.9	0.83	34	18.4	36.4	24.6	21.0	0.68

is essentially 'melted' at room temperature. If this were the case removal of the secondary structure of rRNA by the presence of 8M-urea should lead to the binding of rRNA to the column, in addition to the normal bound RNA. Since the fractionation involves a protein column it was necessary to determine the effect of urea on the MAK column. Columns were

equilibrated for 2h with 8M-urea, and then the urea was washed out of the column and the column used for a routine (pH 6.7–pH 6.7) fractionation of nucleic acid. The fractionation after urea treatment was normal, indicating that the urea had little effect on the methylated albumin under these conditions. When nucleic acid was heated to 60°C in 8M-urea, and then

Fig. 5. Fractionation of bound RNA by polyacrylamide-gel electrophoresis

The bound RNA was recovered from the MAK fractionation shown in Fig. 2, and fractionated with unlabelled carrier nucleic acid by gel electrophoresis (2.2% gel, 50V, 3h). RNA from the regions marked 1–10 was hydrolysed and the base compositions were determined (Table 5). The continuous line represents the E_{265} (carrier nucleic acid) and the histogram the radioactivity as c.p.m./0.5mm gel slice (the bound RNA).

Table 5. Base compositions of different size classes of bound RNA

Bound RNA was recovered with sodium dodecyl sulphate from the MAK column shown in Fig. 2 and fractionated by gel electrophoresis (Fig. 5). The RNA was hydrolysed from relevant regions of the gel (1–10), and base compositions (single determinations) were obtained by high-voltage paper electrophoresis. The base composition is expressed as the percentage of the ^{32}P occurring in each of the four ribonucleotides.

Region of the gel	Composition (mol/100mol)				GMP/AMP ratio
	CMP	AMP	GMP	UMP	
1	16.3	33.6	26.4	22.2	0.83
2	17.5	31.2	26.4	25.0	0.85
3	19.6	30.3	26.6	23.1	0.88
4	21.3	32.8	25.3	20.5	0.77
5	19.3	33.5	26.4	20.8	0.79
6	18.9	33.1	25.5	22.5	0.77
7	17.1	35.7	23.7	23.5	0.66
8	16.9	35.4	22.6	24.9	0.64
9	19.2	35.7	22.1	23.0	0.62
10	17.9	37.8	21.1	22.1	0.59

Fig. 6. Fractionation of newly synthesized nucleic acid in the presence of urea

Nucleic acid was prepared from roots incubated in [³²P]P_i (1.5 mCi/25 ml) for 1 h. (a) 20 µg of nucleic acid was fractionated by gel electrophoresis (2.2% gel, 50 V, 3 h). (b) 20 µg of nucleic acid was fractionated by gel electrophoresis in the presence of 8 M-urea at 50°C (2.0% gel, 50 V, 3 h). The continuous line represents the E₂₆₅ and the histogram the radioactivity as c.p.m./0.5 mm gel slice. (c) 150 µg of nucleic acid was heated to 60°C in 8 M-urea, and fractionated on a pH 6.7–pH 6.7 MAK column with a 100 ml linear gradient from 0.3 to 1.4 M-KCl in the presence of 8 M-urea. The continuous line represents the E₂₅₈ and the histogram the radioactivity as c.p.m./1.4 ml fraction.

fractionated on an MAK column in the presence of 8 M-urea, the RNA was eluted at a very low salt concentration, approx. 0.45 M (Fig. 6). Polyacrylamide-gel electrophoresis in the presence of 8 M-urea at 50°C confirmed that most of the rRNA was still of high molecular weight under these conditions, and that this rapid elution was not due to the breakdown of RNA into very small molecules when the secondary structure was removed (Fig. 6).

Although the rRNA eluted rapidly from the urea–MAK column, 33% of the newly synthesized RNA remained bound to the column after the gradient elution.

Discussion

The detailed analysis by gel electrophoresis of the products of MAK-column fractionation indicated that two fractions of polydisperse RNA were resolved. One, rather less than half of the total polydisperse RNA, was eluted by the salt gradient

together with the rRNA species, and the other remained bound to the MAK. The size distribution of RNA within these two fractions was similar, but the base compositions differed in that the bound RNA was considerably richer in AMP.

The D-RNA peak observed on MAK fractionation by other workers (Ingle *et al.*, 1965; Ellem & Sheridan, 1964; Johri & Varner, 1970) was not seen with any column during the course of our experiments. There were two possible reasons. The amount of D-RNA in the intact root tips could be very low compared with the more commonly used excised tissues (Ingle & Key, 1965), or the modifications to the MAK column could have altered the fractionation of rapidly labelled components, even though the fractionation of rRNA was not affected. To distinguish between these possibilities a sample of RNA from carrot disc tissue (kindly prepared by Dr. C. J. Leaver), known to contain a high proportion of D-RNA, was fractionated by MAK chromatography. No D-RNA peak was resolved, so it was concluded

that the column modifications had affected the fractionation. The D-RNA may therefore be eluted with the rRNA in the salt gradient or, under these fractionation conditions, may be bound to the column with the TB-RNA. The base composition of the salt-eluted polydisperse RNA was not typical of D-RNA, in that its GMP/AMP ratio was approx. 1 compared with values of 0.6 for D-RNA (Ingle *et al.*, 1965). A comparison of the properties of the bound RNA observed in these experiments with those of purified D-RNA and TB-RNA described by Key *et al.* (1972) suggested that the bound RNA contained both D-RNA and TB-RNA. D-RNA varied in size from 10S to greater than 60S, and had a base composition, independent of size, with a GMP/AMP ratio of 0.74–0.79. TB-RNA had a narrower size range, around 16–18S, and a GMP/AMP ratio of 0.45–0.50. The size distribution (Fig. 5) and the size-dependent base composition (Table 5) of bound RNA would be consistent with it being a mixture of D-RNA and TB-RNA.

It has been suggested, on the basis of column fractionation, size range, base composition and stability, that D-RNA and TB-RNA represent two distinct types of rapidly labelled AMP-rich RNA (Key *et al.*, 1972). It is possible, however, that the purification procedure employed was selecting the two extremes of a heterogeneous population, since the purified D-RNA and TB-RNA comprised only 60 and 50% respectively of the initial crude fractions. This possibility is supported by the complete lack of resolution of D-RNA and TB-RNA observed in these experiments, despite the range of MAK-column-fractionation conditions employed (i.e. low to high pH values and urea treatment).

The bound RNA appeared quite different from rRNA in many of its properties. It must be emphasized that under these conditions of MAK fractionation the bound RNA did not include any significant amount of rRNA, in contrast with the reports of Ellem & Sheridan (1964) and Tester & Dure (1967). Although the affinity between rRNA and methylated albumin was considerably decreased at the higher pH values, such that the salt concentration required to elute rRNA was halved, elution of the bound RNA was not facilitated; in fact the percentage of bound RNA increased slightly (Table 4). This increase in bound RNA may have been the result of the decreased aggregation under the high pH conditions. The aggregation of the 0.7×10^6 mol.wt. rRNA was decreased from approx. 50 to 15%, but was not completely eliminated. The decrease in aggregation probably reflected the loss of rRNA–rRNA complexes, whereas the remaining 15% was possibly due to rRNA–polydisperse RNA interaction. This latter type of aggregation, which was less affected by the higher pH, probably influenced the binding of polydisperse RNA to the

methylated albumin. Denaturation by hot urea decreased the affinity between rRNA and the MAK column, but again this treatment did not significantly decrease the interaction between the bound polydisperse RNA and MAK. This observation contrasts with that of Lichtenstein & Shapot (1971), who reported that treatment with urea increased the binding of rRNA to MAK.

The reason for the very high affinity between a proportion of the polydisperse RNA and the methylated albumin still remains unknown. The binding appears essentially irreversible, in that the recovery of the bound RNA depends on treatment, such as with detergent or alkali, that solubilizes the methylated albumin from the kieselguhr. Guanidine thiocyanate, which has been reported to elute TB-RNA from the MAK column (Ellem & Rhode, 1968), also removes protein from the column. The bound RNA differs from the other RNA species in being rich in AMP, and its binding properties appear to be related directly to this, rather than indirectly via the secondary structure conferred by such a base composition.

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