Biochem. J. (1965) **96**, 266

Isolation and Characterization of Ribosomal Ribonucleic Acid

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(*Received* 7 *October* 1964)

1. Ribosomal RNA has been prepared by extracting tissues with a phenol–cresol mixture, and ribosomal RNA can be selectively precipitated with *m*-cresol. No rapidly labelled RNA was associated with this material. 2. However, if RNA and DNA are extracted with 4-aminosalicylate and phenol–cresol mixture and the nucleic acids precipitated, DNA, glycogen and s-RNA (transfer RNA) can be extracted with 3M-sodium acetate and in this case rapidly labelled RNA remains associated with the ribosomal RNA. 3. The ribosomal RNA is stable in the presence of concentrated salt solution and, although the secondary structure is lost by heating at 70° in 10mM-sodium acetate, it can be re-formed in the presence of 200mM-sodium acetate. 4. The 28s and 18s components have been separated and their base compositions determined.

Although phenol gives a good separation of RNA from mammalian tissues (Kirby, 1956), ribonuclease survives the extraction (Huppert & Pelmont, 1962; Littauer & Sela, 1962) and, on removal of the phenol, degradation of the RNA may occur. A new method for the isolation of ribosomal RNA has been devised in which dialysis is avoided; the product is stable in the presence of 0·5M-salt and has good thermal stability.

MATERIALS

m-Cresol should be colourless and distilled under reduced pressure if necessary. The phenol–cresol mixture was made from phenol (detached crystals, 500g.), *m*-cresol (70ml.), water (55ml.) and 8-hydroxyquinoline (0·5g.).

Sucrose gradients [5–20% (w/v) sucrose] were made up in 10mM-sodium acetate, pH5·2, unless otherwise stated. RNA (1mg.) was dissolved in 100mM-sodium acetate, pH5·2, and layered on top of the gradient.

RNA was labelled by intraperitoneal injection of 200μc of [^{3}H]orotic acid 20min. before the animals were killed (cf. Kidson, Kirby & Ralph, 1963).

METHODS AND RESULTS

Preparation of ribosomal RNA. Method 1. The livers were excised from the rats and dropped immediately into liquid nitrogen. The livers were then broken down in a cooled blender containing cold 0·5% disodium naphthalene-1,5-disulphonate (10vol.) and phenol–cresol mixture (10vol.). This mixture was transferred to a beaker, stirred for 20min. at 20° and then centrifuged at 6000*g* for 20min. at 5°. The aqueous phase was removed, made 5% (w/v) with respect to sodium tri-isopropylnaphthalenesulphonate (Kodak Ltd., Kirkby, Liverpool), 0·5vol. of phenol–cresol mixture was added, and the mixture was stirred for 15min. at 20° and then centrifuged at 8000*g* for 10min. at 5°. The aqueous phase was removed and to each 100ml. were added sodium chloride (3g.) and sodium benzoate (20g.) followed by *m*-cresol (10ml.). The gelatinous precipitate was almost invisible, but was centrifuged off at 500*g* for 10min. at 5°, and the sediment was washed twice with a cold mixture of water (100ml.), sodium chloride (3g.), sodium benzoate (20g.) and *m*-cresol (10ml.), once with a cold mixture of water (25ml.), sodium chloride (1g.) and ethanol (75ml.), once with 75% (v/v) ethanol and twice with ethanol, and then dried in a vacuum desiccator. The yield was about 60mg. from two livers (14g.); the atomic extinction coefficient with respect to phosphorus, $\epsilon_{(P)}$, at 260mμ (Chargaff & Zamenhof, 1948) was 6850 in 150mM-sodium chloride–15mM-trisodium citrate, pH7·0, and 8750 in water. The material was free from DNA and glycogen and contained only traces of s-RNA (transfer RNA) and rapidly labelled RNA. The pattern obtained on centrifugation in a sucrose density gradient is shown in Fig. 1.

A similar result was obtained when either phenolphthalein diphosphate or pyrophosphate (both 150mM, pH6·0) (cf. Kirby, 1961) was used in place of naphthalene-1,5-disulphonate, and the method has worked equally well for the extraction of ribosomal RNA from a transplantable hepatoma and a sarcoma.

Method 2. Livers, which had been dropped into

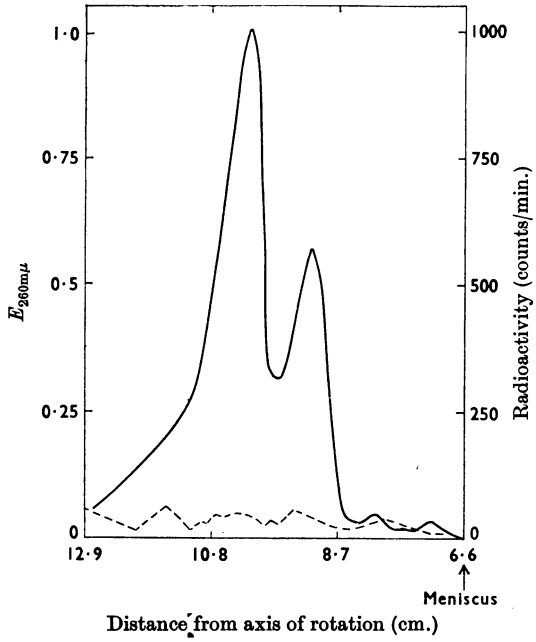


Fig. 1. Centrifugation pattern in sucrose density gradient (5–20% sucrose in 10mM-sodium acetate, pH5·2) of rat-liver ribosomal RNA prepared by method 1 (see the text). Centrifugation was at 21500rev./min. for 16hr. in the Spinco model L centrifuge (SW25.1 rotor). ——, $E_{260m\mu}$; ----, radioactivity.

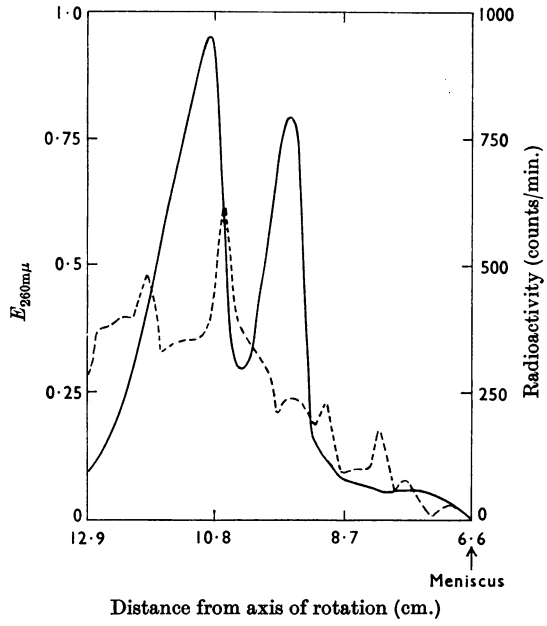
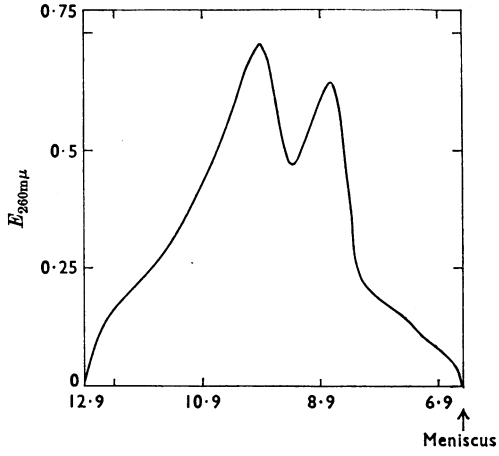


Fig. 2. Centrifugation pattern in sucrose density gradient (5–20% sucrose in 10mM-sodium acetate, pH5·2) of rat-liver ribosomal RNA prepared by method 2 (see the text). Centrifugation was at 23500rev./min. for 16hr. in the Spinco model L centrifuge (SW25.1 rotor). ——, $E_{260m\mu}$; ----, radioactivity.

liquid nitrogen, were broken down in a blender with a mixture of 6% (w/v) sodium 4-aminosalicylate and phenol–cresol mixture (15vol. of each solution). The mixture was stirred for 20min. at 20° and then centrifuged at 6000*g* for 30min. at 5°, and if any emulsion remained in the upper phase this was removed and centrifuged again. Sodium chloride (3g.) was added to each 100ml. of the clear top layer, which was then re-extracted with 0·5vol. of phenol–cresol mixture for 10min. at 20° and then centrifuged at 8000*g* for 10min. at 5°. The aqueous phase was removed and mixed with 2vol. of ethanol–*m*-cresol (9:1, v/v), and the mixture allowed to stand for 30–60min. at 2°. The precipitate was centrifuged off and extracted twice with cold 3M-sodium acetate, pH6·0 (25ml. each time). This removed DNA, s-RNA and glycogen. The ribosomal RNA was then washed once with a cold mixture made in the proportions water (25ml.), sodium chloride (1g.) and ethanol (75ml.), once with 75% (v/v) ethanol and twice with ethanol, and dried in a vacuum desiccator. The yield was about 80mg. from two livers. Rapidly labelled RNA remained associated with ribosomal RNA as shown in the sucrose-density-gradient diagram (Fig. 2).

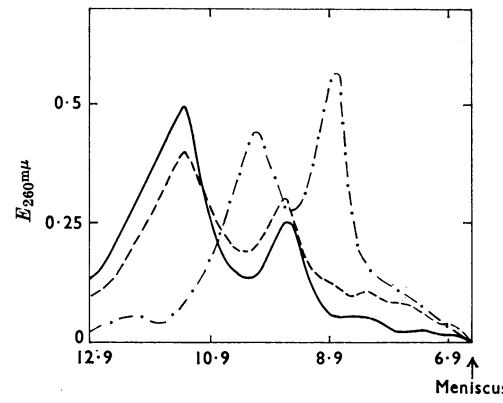
Separation of glycogen from RNA as the tetraethylammonium salt. Crude RNA (50–100mg.) was dissolved in 5ml. of 10mM-tetraethylammonium bromide, and 20ml. of 0·2M-tetraethylammonium bromide in 50% (v/v) ethanol was added; the addition of a further 5ml. of ethanol precipitated the polysaccharide, which was removed by centrifuging at 8000*g* for 5min. at 5°. The supernatant liquid was poured off, 25ml. of ethanol added and then 0·5g. of sodium benzoate in water (2ml.) and ethanol (4ml.). RNA was precipitated, centrifuged off, washed and dried as usual. The triethylammonium salt of RNA can be used equally well for the separation, by using triethylammonium acetate. The sucrose-density-gradient pattern of the RNA so separated is shown in Fig. 3.

Effect of heating ribosomal RNA. RNA (method 1 preparation) (3·3mg.) was dissolved in 10mM-sodium acetate, pH5·2 (1·5ml.). A 1ml. sample was removed and heated at 70° for 10min. and then cooled to 20°. The solution was divided into two; 0·5ml. of 10mM-sodium acetate, pH5·2, was added to one half (sample 1) and 0·5ml. of 200mM-sodium acetate, pH5·2, was added to the other half (sample 2) and also to the original unheated solution (sample 3). Sample 1 was layered on a 5–20% sucrose



Distance from axis of rotation (cm.)

Fig. 3. Centrifugation pattern in sucrose density gradient (5–20% sucrose in 10mM-sodium acetate, pH5·2) of rat-liver ribosomal RNA, prepared through the tetraethylammonium salt (see the text). Centrifugation was at 24000rev./min. for 16hr. in the Spinco model L centrifuge (SW25.1 rotor).



Distance from axis of rotation (cm.)

Fig. 4. Centrifugation patterns in sucrose density gradients (5–20% sucrose) of rat-liver ribosomal RNA prepared by method 1 (see the text). All curves are extinction readings at 260mμ: ——, RNA, unheated, with gradient made up in 200mM-sodium acetate, pH5·2; ·—·—, RNA, heated at 70° for 10min., with gradient made up in 10mM-sodium acetate, pH5·2; – – – –, RNA, heated at 70° for 10min., with gradient made up in 200mM-sodium acetate, pH5·2. Centrifugation was at 21500rev./min. for 16hr. in the Spinco model L centrifuge (SW25.1 rotor).

gradient in 10mM-sodium acetate, pH5·2, and samples 2 and 3 were layered on sucrose gradients in 200mM-sodium acetate, pH5·2. The curves obtained after centrifugation are shown in Fig. 4.

Separation of the 28s *and* 18s *components of ribosomal RNA.* Ribosomal RNA (9mg.) was centrifuged in three sucrose density gradients at 24000rev./min. for 16hr. The tubes containing each peak were combined and made 5% with respect to sodium acetate; the RNA was precipitated with 2vol. of acetone and centrifuged off, washed and dried. The yield was about 4mg. of 28s RNA and 2mg. of 18s RNA.

The base compositions were: 28s RNA, G:A:C:U 33·0:17·8:32·2:17·0; 18s RNA, G:A:C:U 32·4:19·8:29·8:18·0. The compositions were determined by acid hydrolysis of RNA (Markham & Smith, 1949), followed by separation on paper with methanol–ethanol–11·6N-hydrochloric acid–water (50:25:6:19, by vol.); C and U have been corrected by 5% for loss on hydrolysis. The result should be compared with that of Munro (1964), who obtained RNA from a preparation of rat-liver ribosomes and found: for 28s RNA, G:A:C:U 32·9:18·3:29·8:19·0; for 19s RNA, G:A:C:U 30·3:22·4:27·8:19·6.

DISCUSSION

The addition of *m*-cresol to the phenol has two advantages: (1) the mixture can be added to the cold naphthalenedisulphonate solution without crystallization of the phenol; (2) phenol–cresol mixtures are better deproteinizing agents than phenol alone (K. S. Kirby, unpublished work). The two-stage extraction is essential for the preparation of stable RNA. The first stage leaves DNA at the interface, the second removes most of the protein still present in the aqueous phase and any residual protein is not precipitated by *m*-cresol. Tri-isopropylnaphthalenesulphonate is a suitable detergent, as it can be used with phenol in a greater concentration than dodecyl sulphate without forming a single-phase system. *m*-Cresol forms a two-phase system with water, but forms a single-phase system with 20% sodium benzoate (sodium chloride is present to maintain ionic strength required to prevent loss of secondary structure of the RNA in the presence of *m*-cresol). Under these conditions the addition of 0·1–0·15vol. of *m*-cresol precipitates only ribosomal RNA; the addition of 0·4vol. precipitates ribosomal RNA and glycogen, and s-RNA can be precipitated from the remaining solution if the tri-isopropylnaphthalenesulphonate is first removed as the potassium salt.

The second method of preparation depends on the release of all nucleic acids into the aqueous phase by 4-aminosalicylate and phenol (Kirby, 1957), but, as some protein is solubilized into this phase, a second extraction with sodium chloride solution is necessary to remove this protein into the phenol phase.

The total nucleic acids are precipitated by ethanol, and then DNA, s-RNA and glycogen are removed by extraction with 3M-sodium acetate.

The main difference in the methods is that the rapidly labelled RNA remains with the DNA in method 1 and with the ribosomal RNA in method 2 (cf. Figs. 1 and 2). These results indicate that this RNA is not free but is associated with material, possibly protein, that is not removed by naphthalenedisulphonate but is removed by 4-aminosalicylate.

It is emphasized that bentonite has been used neither in the isolation nor in the sucrose-density-gradient centrifugations of the RNA, and that RNA prepared in this way is stable in the presence of 0·5M-salt in the absence of bentonite. Bentonite is a very effective inhibitor of ribonuclease (Brownhill, Jones & Stacey, 1959; Fraenkel-Conrat, Singer & Tsugita, 1961; Huppert & Pelmont, 1962; Littauer & Sela, 1962).

Secondary structure of RNA. The evidence for secondary (or helical) structure of ribosomal RNA has been reviewed by Spirin (1963). The sucrose-density-gradient patterns and $\epsilon_{(P)}$ values in sodium chloride and water show that RNA prepared by the present method has good secondary structure. The ease with which some of this is lost is shown by the alteration in pattern on sucrose-density-gradient centrifugation of the material recovered by solubilizing the tetraethylammonium salt of RNA in 50% ethanol (cf. Fig. 3). This loss of secondary structure is intensified on heating the RNA in 10mM-sodium acetate at 70° for 10min.; little further change takes place up to 30min. The effect is largely reversed by centrifuging the heated RNA in 200mM-sodium acetate (Fig. 4). The result supports the suggestion that ribosomal ribonucleic acids are each a single chain (cf. Spirin, 1963); it indicates that the increase of the 18s peak after heating is due not to the 28s unit being converted into one of size 18s, but to loss of secondary structure on heating by breaking A–U and G–C linkages. These bonds reassociate on cooling and centrifuging in more concentrated salt solution. The 18s unit is relatively less affected; but, since the overall base compositions of the two components are almost identical, the difference may lie in the nucleotide sequences of each. There are considerable variations in base compositions of segments derived from ribosomal RNA (Kirby, 1962), and the adenine content varies from 6 to 36%. If there were segments of higher AU content in the 28s unit, then it would be expected to lose its secondary structure more readily than the 18s unit. It should be possible to test this by separating the two species, degrading them to 4s sub-units and studying the variations in base segments by counter-current distribution.

The choice of salt for studying loss and re-formation of secondary structure of RNA is important. For example, heating in 0·3M-sodium acetate results in aggregation of the RNA, which then gives a main peak of about 40s; 150mM-sodium chloride and 15mM-sodium citrate were also unsatisfactory, although this mixture was suitable for ribosomal RNA from *E. coli*. However, 150mM-pyrophosphate was almost as suitable as 200mM-sodium acetate, indicating that Mg^{2+} ions were not necessary for the re-formation of secondary structure.

Improvements in the preparation of ribosomal RNA have been reported by Ralph & Bellamy (1964) and by Cox & Arnstein (1963). The present methods have the advantages that ribosomal RNA can be selectively separated, no dialysis is necessary, thermal stability is good and rapidly labelled RNA can be left behind or associated with the ribosomal RNA, facilitating studies on the biosynthesis of the RNA.

It has been assumed that there are two units of ribosomal RNA, 28s and 18s. Not all the 28s RNA had disappeared on heating at 70°. It is possible therefore that each 'unit' may consist of several molecular species. It is hoped that further fractionation will clarify this point.

I thank Professor A. Haddow, F.R.S., for his interest and Mr E. A. Cook for technical assistance. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the Public Health Service Research Grant no. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service.

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