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Thermal Stability of Ribosomal Ribonucleic Acid from Baby Hamster Kidney Cells

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1. RNA has been prepared from baby hamster kidney cells by extraction with a phenol–EDTA mixture and further purified by passing through a column of Sephadex G-25 that had been equilibrated with water. 2. Aging of the total RNA extracts at 4° or heating at 95° followed by rapid cooling caused a conversion of 28s RNA into material sedimenting in sucrose gradients at approx. 18s. 3. When heated RNA was re-extracted with phenol the sedimentation profile was not returned to that of the unheated RNA. 4. The 28s and 18s RNA fractions were collected separately from sucrose gradients by precipitation with 2vol. of ethanol and passed through a Sephadex G-25 column equilibrated with water. 5. Heat treatment of purified 28s RNA at 95° caused the sedimentation coefficient to increase to approx. 40s, whereas similar treatment of 18s RNA caused no significant increase. If the RNA was heated before the Sephadex G-25 treatment the sedimentation coefficient of the 28s and 18s RNA decreased to approx. 12s and 8s. 6. Heating mixtures of purified 28s and 18s RNA at 95° caused some aggregation of 18s material with the 28s fraction.

It is now well established that ribosomal RNA from mammalian cells is composed of two fractions, commonly termed the 28s and 18s RNA. A number of reports from different Laboratories on the base composition of the 28s and 18s fractions (Montagnier & Bellamy, 1964; Salzman, Shatkin & Sebring, 1964; Brown & Martin, 1965; Henshaw, Revel & Hiatt, 1965) have demonstrated that the 28s RNA is not a dimer of 18s sub-units.

Although the evidence so far produced points to the continuity of RNA strands from both mammalian and bacterial ribosomal RNA fractions (Boedtker, Moller & Klemperer, 1962; Spirin, 1964; Midgley, 1965*a*,*b*), recent reports demonstrate that the sedimentation profile of ribosomal RNA in sucrose gradients can be readily altered by different ionic conditions (Hayes, Guerin & Hayes, 1963; Asano, 1963; Watson, 1964) or by heat treatment (Kirby, 1965; Henshaw *et al.* 1965). Alterations in the sedimentation profile of RNA corresponding to an increase or decrease in sedimentation rate may be caused by one or more of the following three factors: (1) cleavage of the polynucleotide chains, (2) changes in secondary structure or (3) formation of complexes.

In the first instance, Midgley (1965*c*) has suggested that cleavage of 23s RNA from *Escherichia coli* ribosomes occurs when RNA is incubated at 37° in alkaline medium (about pH9). Under these conditions the 23s RNA is readily converted into a fraction that sediments at the 16s position. Midgley (1965*c*) suggested that the 23s RNA is a dimer of two 16s sub-units combined by an alkali-labile bond. Petermann & Pavlovec (1963) have also found evidence that 28s RNA from rat-liver ribosomes can be converted into 18s material by heating at 90° in certain ionic conditions.

Secondly, a change in configuration induced by heat treatment of rat-liver ribosomal RNA has been observed by Kirby (1965). Heat treatment at 70° in 10mM-acetate, pH5·2, followed by rapid cooling causes a loss of material sedimenting at the 28s position in sucrose gradients with a corresponding increase in the 18s fraction. However, the profile of the heated RNA could be returned to normal by carrying out the sedimentation in sucrose gradients in 200mM-acetate. These experiments are interpreted as meaning that a reversible change in secondary structure occurs under these heating conditions.

A third characteristic that may alter the sedimentation profile is aggregation or formation of complexes between RNA molecules. Numerous reports have described the sedimentation behaviour of rapidly labelled RNA and the evidence suggests that complexes can occur between messenger and ribosomal RNA strands in the presence of multivalent metal ions (Bautz, 1963; Montagnier &

Sanders, 1963). Matus, Ralph & Mandel (1964) have reported a type of complex-formation between high-molecular-weight RNA that appears to depend on the RNA base composition, particularly with RNA molecules possessing a small degree of hydrogen-bonded secondary structure, rather than on the presence of Mg^{2+} ions. Complex-formation is also known to occur between the ribosomal RNA sub-units of both rat liver (Petermann & Pavlovec, 1963) and *E. coli* (Marcot-Queiroy & Monier, 1965) at certain Mg^{2+} ion concentrations, and D. H. Hayes, F. Hayes & M. F. Guerin (personal communication) have found that complexes between 23s and 16s RNA from *E. coli* are formed to a limited extent in solutions of high ionic strength and at low temperatures.

The present paper reports attempts to prepare thermally stable ribosomal RNA from baby hamster kidney cells. Comparison of sucrose-gradient sedimentation profiles of both thermally stable and unstable RNA preparations suggests that both specific aggregation and specific chain cleavage may occur under appropriate conditions.

MATERIALS AND METHODS

Culture conditions. Baby hamster kidney cells, designated BHK 21 (clone no. 13), were isolated in the Institute of Virology, Glasgow, and have been described by Macpherson & Stoker (1962). The cells were grown as monolayers in Roux bottles in Eagle's medium containing 10% (v/v) of ox serum, 0·2% (w/v) of tryptose–phosphate broth and antibiotics (penicillin, streptomycin and polymyxin at 100 units/ml. each). When the bottles contained approx. 10^8 cells, the sheets were rinsed three times with cold phosphate-buffered saline (Dulbecco & Vogt, 1954) and scraped off the glass with a rubber 'policeman'. The tissue was collected by centrifugation and stored at −20° until required.

Preparation of RNA. The cells from four Roux bottles were shaken at room temperature for 5min. with 10ml. of phenol saturated with 0·01M-EDTA and 10ml. of 0·01M-EDTA. The aqueous layer was separated by centrifugation and extracted three times with phenol saturated with 0·01M-EDTA. The RNA was precipitated from the final aqueous layer by adding 2vol. of ethanol and stored at −20° overnight.

The precipitates were dissolved in sterile water and applied in a small volume (2–5ml.) to a Sephadex G-25 column (35cm.×2cm.) that had been equilibrated with sterile water. The RNA was eluted in approx. 30ml. This procedure gave a concentration suitable for direct application to sucrose gradients. The RNA was stored in 3ml. samples at −20°.

Sucrose-gradient centrifugation and preparation of 28s and 18s RNA. Centrifugation was carried out in a Spinco model L ultracentrifuge with an SW25 head. All sucrose solutions were autoclaved for 10min. at 15lb./in.^{2} to inactivate any contaminating nucleases present in the reagents. Sucrose gradients were prepared from 12·5ml. each of 25% and 5% (w/v) sucrose in 0·1M-sodium acetate, pH5·0. Then 3ml. samples of RNA were applied and centrifugation was carried out at 2° for 14hr. at 20000–25000 rev./min. At the end of this period 1ml. fractions were collected from the bottom of the tube and their E_{260} values measured.

The three fractions with the highest E_{260} values in each of the 28s and 18s peaks were precipitated with 2vol. of ethanol at −20°. The precipitates (28s or 18s RNA) were collected by centrifugation and dissolved in 2ml. of water. Then 1ml. of each sample was passed through a short Sephadex G-25 column (1cm.×10cm.). These fractions are termed 'salt-free' 28s or 18s RNA. Sedimentation values were calculated from the relative position in sucrose gradients by the Martin & Ames (1961) approximation.

Heat treatment of RNA. Samples of RNA were heated at 95° for 5min. and then cooled rapidly by pouring into an ice-cold vessel. Under these conditions cooling from 95° to 8° was accomplished in approx. 10sec.

RESULTS

Effect of aging on total RNA extracts. To investigate the stability of RNA preparations in 'salt-free' solutions, total RNA extracts were stored at 4° for up to 20 days and samples examined at intervals by sucrose-gradient analysis. The profiles shown in Fig. 1 demonstrate that there is a gradual

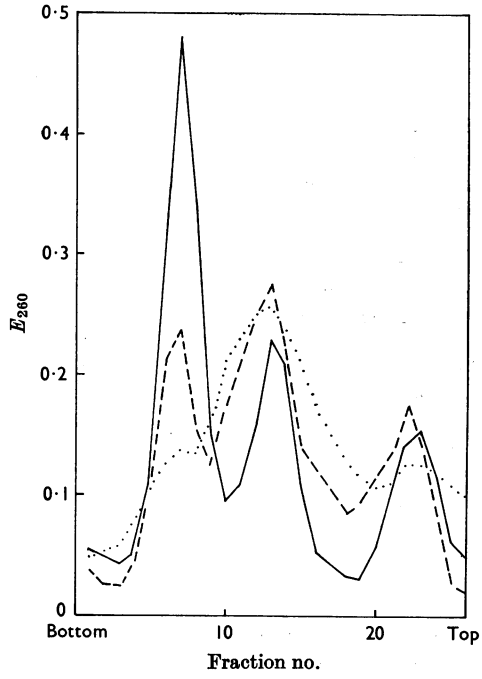

Fig. 1. Sucrose-gradient centrifugation of total RNA extracts after storage at 4° for various periods. The sucrose gradients (5–25% sucrose in 0·1M-acetate, pH5·0) were run for 14hr. at 4° in the SW25 rotor of the Spinco model L centrifuge at 23000 rev./min. ——, 0 days; – – –, 5 days; ····, 20 days.

conversion of RNA sedimenting at the 28s position into a fraction sedimenting at approximately the 16–18s region of the gradients. Although this conversion is time-dependent, there is no significant alteration in the proportion of 260mμ-absorbing material remaining at the top of the gradient. This result suggests that the alteration in sedimentation behaviour of the ribosomal RNA is not caused by random nuclease action. If degradation is occurring it is apparently highly specific.

Heat treatment of total RNA extracts. The effect on the sedimentation profile caused by heating total RNA extracts in 'salt-free' solution for various times at 95° is shown in Fig. 2. Here again there is a gradual loss of 28s RNA with an increase predominantly at the 18s position. After 10min. at 95° all the 28s has disappeared and there is an appreciable appearance of degradation products. As heating for 5min. showed little alteration to the sedimentation profile other than increase in the 18s fraction, conditions were therefore standardized at heating for 5min. at 95° in the following experiments. The thermal stability of the RNA was found to vary considerably from preparation to preparation and, as shown below, depends on the purity of the RNA extract.

Phenol extraction of heated RNA. To determine whether the alteration of 28s to 18s RNA was caused by a change in configuration or by chain cleavage, heated RNA was treated with phenol as in the initial extraction procedure described in the Materials and Methods section. It seemed possible that the RNA strands, if remaining continuous, would be returned to their initial configuration after a second phenol treatment. However, Fig. 3 demonstrates that re-extraction with phenol does not reverse the effect of heating the RNA in 'salt-free' solutions. This result supports the conclusion that the 28s fraction may be degraded to 18s sub-units under these heating conditions.

Heat treatment of purified 28s RNA. It was possible that components, other than nucleic acids, that may remain at the top of the sucrose gradient are the cause of this apparently specific degradation of the 28s RNA. The 28s RNA was therefore further purified by sedimentation through a sucrose gradient and the 28s fractions were then precipitated by adding 2vol. of ethanol. The ethanol precipitate was dissolved in sterile water and either heated at 95° directly or passed through a column of Sephadex G-25 before heating. In Fig. 4 the sedimentation profiles of heated 28s RNA, with or without Sephadex G-25 treatment, are seen to

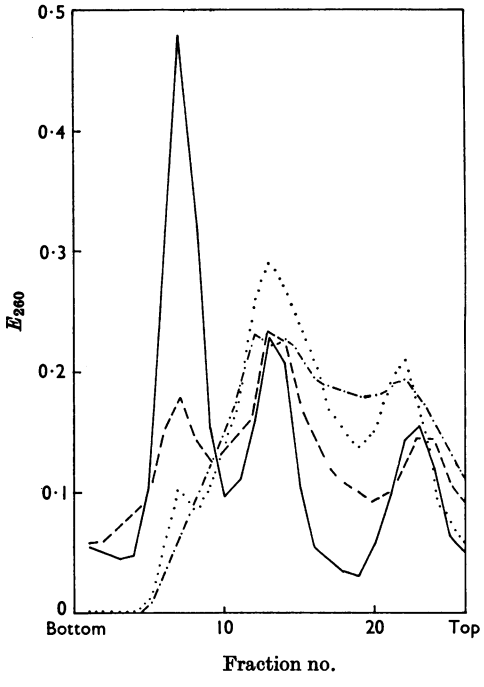

Fig. 2. Sucrose-gradient centrifugation of total RNA extracts after heating at 95° for various times followed by rapid cooling. The sucrose gradients (5–25% sucrose in 0·1M-acetate, pH5·0) were run for 14hr. at 4° in the SW25 rotor of the Spinco model L centrifuge at 23000 rev./min. ——, Unheated; – – – –, heated for 2·5min.; ····, heated for 5min.; –·–·, heated for 10min.

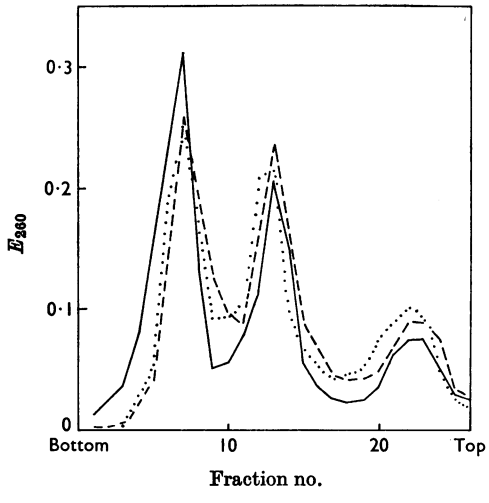

Fig. 3. Sucrose-gradient centrifugation of total RNA extracts after heating at 95° for 5min. followed by rapid cooling and re-extraction with phenol. The sucrose gradients (5–25% sucrose in 0·1M-acetate, pH5·0) were run for 14hr. at 4° in the SW25 rotor of the Spinco model L centrifuge at 23000 rev./min. ——, Unheated; – – – –, heated at 95°; ····, heated at 95° and phenol-extracted.

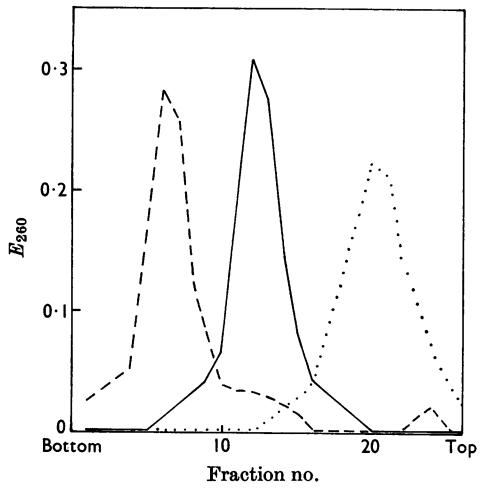

Fig. 4. Sucrose-gradient centrifugation of 28s RNA. The sucrose gradients (5–25% sucrose in 0·1 M-acetate, pH 5·0) were run for 14 hr. at 4° in the SW25 rotor of the Spinco model L centrifuge at 20000 rev./min. ——, 28s RNA filtered through Sephadex G-25 but not heated; ----, 28s RNA filtered through Sephadex G-25 and then heated at 95° for 5 min.; ····, 28s RNA not passed through Sephadex G-25 but heated at 95° for 5 min.

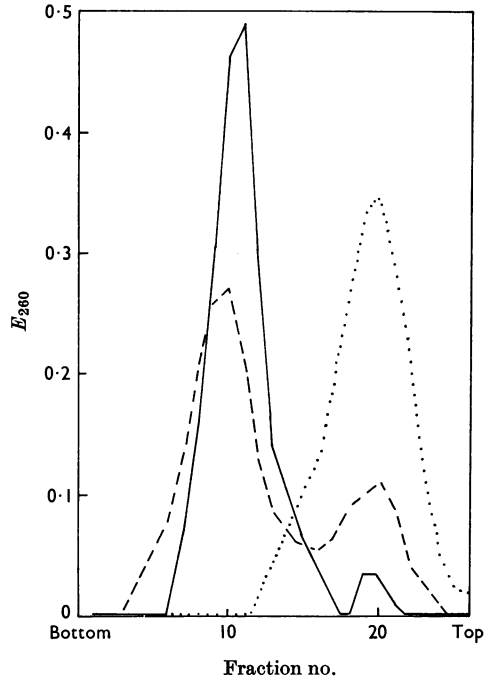

Fig. 5. Sucrose-gradient centrifugation of 18s RNA. The sucrose gradients (5–25% sucrose in 0·1 M-acetate, pH 5·0) were run for 14 hr. at 4° in the SW25 rotor of the Spinco model L centrifuge at 24000 rev./min. ——, 18s RNA filtered through Sephadex G-25 but not heated; ----, 18s RNA filtered through Sephadex G-25 and then heated at 95° for 5 min.; ····, 18s RNA not passed through Sephadex but heated at 95° for 5 min.

contrast markedly. Unless the RNA has been purified by Sephadex G-25 treatment before heating there is degradation of the RNA. On the other hand, if the 28s RNA is heated after passing through a Sephadex column there is an increase in the sedimentation coefficient to approx. 40s.

Heat treatment of purified 18s *RNA.* Purified 18s RNA prepared from sucrose gradients as described for the 28s fraction were also heated at 95° for 5 min. before or after Sephadex G-25 treatment. Fig. 5 shows that after filtration through Sephadex G-25 the 18s RNA is apparently stable to heating, whereas degradation occurs when heating is carried out on RNA preparations that had not been passed through a Sephadex G-25 column. However, in contrast with the stability of the 28s fraction, heat treatment of the 18s RNA invariably resulted in the formation of some slowly sedimenting material.

Heat treatment of mixtures of 28s *and* 18s *RNA.* Fractions of both 18s and 28s RNA were purified independently by one cycle of sucrose-gradient sedimentation and subsequent filtration through Sephadex G-25. The fractions were then mixed, heated at 95° for 5 min. and studied by sucrose-gradient sedimentation. The sedimentation profiles shown in Fig. 6 demonstrate that heating causes a much greater alteration in the position of the 28s RNA compared with that of the 18s RNA. The amount of RNA sedimenting at 40s after heating mixtures of 28s and 18s RNA is about 10% more than that found when the 28s fraction is heated alone. This increase can only be accounted for by the formation of complexes between 28s RNA and some 18s RNA.

DISCUSSION

It is now accepted that the rate of sedimentation of RNA in sucrose gradients is influenced considerably by the ionic composition of the gradient. Thus the position of the 28s and 18s fractions can be drastically altered by the presence or absence of Mg^{2+} ions in the gradient medium. Consequently, in the present experiments all the sedimentations were carried out under the same ionic conditions, i.e. 0·1 M-sodium acetate, pH 5·0, so that any alterations in sedimentation behaviour of the RNA must have been caused by changes taking place before sedimentation.

When 28s or 18s is heated in 'salt-free' solutions

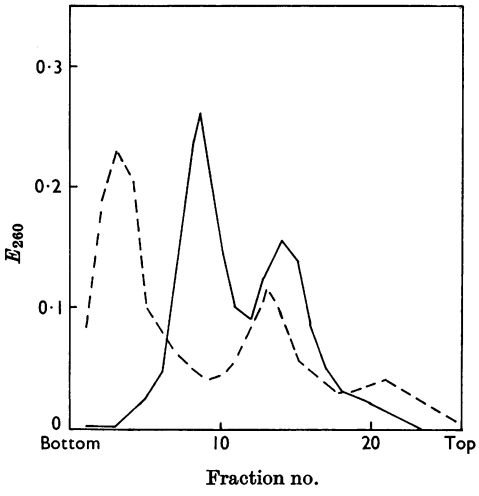

Fig. 6. Sucrose-gradient centrifugation of a mixture of 28s and 18s RNA. The sucrose gradients (5–25% sucrose in 0·1 M-acetate, pH 5·0) were run for 14 hr. at 4° in the SW 25 rotor of the Spinco model L centrifuge at 20000 rev./min. ——, 28s and 18s RNA filtered through Sephadex G-25, the fractions being mixed but not heated; – – – –, 28s and 18s RNA filtered through Sephadex G-25, the fractions being mixed and then heated at 95° for 5 min.

there is an increase in the sedimentation rate. This increase is much greater with 28s RNA than with 18s RNA. Heat treatment of a mixture of both 28s and 18s RNA results in an increase in the proportion of the RNA sedimenting at 40s relative to that sedimenting at 28s in the unheated mixture. The interpretation of this phenomenon depends on the purity and thermal stability of the RNA samples. In the first instance the removal of trace nucleases from the 28s or 18s RNA fractions has been effected by sedimentation in sucrose gradients. In similar experiments in which ribonuclease (0·02 μg.) was added to sucrose gradients followed by sedimentation, fractions of the gradient corresponding to S values greater than 14s did not contain sufficient contaminating nucleases to alter the sedimentation profile of RNA of baby hamster kidney cells. One may conclude therefore that sucrose-gradient sedimentation is an efficient means of removing trace amounts of ribonuclease from ribosomal RNA fractions. If it is assumed that thermal cleavage of the polynucleotide chains does not occur under these conditions (Spirin, 1964) and that the purified samples are free from nucleases, then the increase in sedimentation coefficient to 40s after heating purified 28s RNA is probably caused by aggregation rather than by a change in secondary structure. On the other hand, if chain breakage is occurring, either by thermal or enzymic action, then the sedimentation profiles obtained can only be explained by assuming a highly specific and complete association of the smaller fragments produced, as no residual low-molecular-weight components are found and the 40s fraction is a sharp peak, similar in character to the profile obtained for unheated 28s RNA. It has been shown (Brown & Martin, 1965) that the base compositions of the 28s and 18s RNA of baby hamster kidney cells are significantly different (28s RNA: adenine, 16·4; cytosine, 29·6; guanosine, 34·3; uracil, 17·7; 18s RNA: adenine, 22·1; cytosine, 25·9; guanosine, 30·5; uracil, 21·5). If the formation of complexes between RNA strands is caused by hydrogen-bonding between complementary bases, then it would be expected that RNA with a high guanosine + cytosine content would have a greater tendency to form complexes than molecules with a low guanosine + cytosine content. Matus *et al.* (1964) have shown that this type of complex-formation can occur between turnip yellow-mosaic virus RNA and Chinese-cabbage RNA or tobacco-leaf RNA, or between tobacco mosaic virus RNA and Chinese cabbage-leaf RNA. These aggregates appear to be non-specific and dependent on the guanosine + cytosine contents of the RNA species.

Under the heating conditions used in the present experiments it is probable that the secondary and tertiary structure of the 28s molecules is disrupted and single-stranded open chains are produced, as would occur in the model for ribosomal RNA proposed by Spirin (1964). The ribosomal RNA at these elevated temperatures will possess little, if any, hydrogen-bonded structure, and presumably during the subsequent treatment re-formation of hydrogen bonds between different molecules takes place. In a study of the interaction between homopoly-ribonucleotides and bacterial RNA in solutions of high ionic strength, D. H. Hayes, M. Grunberg-Manago & M. F. Guerin (personal communication) have demonstrated the importance of high ionic strength in promoting complex-formation. However, in other cases of association between ribosomal and pulse-labelled RNA, considerably lower salt concentrations are required (Staehelin, Wettstein, Oura & Noll, 1964; Montagnier & Sanders, 1963; Matus *et al.* 1964).

The behaviour of 28s RNA from baby hamster kidney cells indicates that interaction between different RNA molecules such as virus RNA or messenger RNA and ribosomal RNA may be an important factor in determining the sedimentation profile after heat treatment of RNA.

The work of Midgley (1965c) with *E. coli* ribosomal RNA and of Petermann & Pavlovec (1963) with rat-liver RNA suggests that under appropriate conditions the 28s fraction is degraded to sub-units.

Midgley (1965c) has shown that incubation of 23s RNA in alkaline medium causes an alteration of the sedimentation profile with a decrease in the proportion of 23s RNA and a corresponding increase in the proportion of the 16s RNA. In the present experiments aging or heat treatment of total RNA extracts causes similar alterations in the sedimentation behaviour of the 28s RNA. The absence of any significant increase in the RNA sedimenting at the top of the gradient, at the 4s position, indicates that the apparent degradation is not random breakdown but can be accounted for by specific cleavage of the 28s chains. To decide whether the alteration in sedimentation behaviour after heating total RNA extracts is caused by change in secondary structure or by specific cleavage of the polynucleotide chain, heated samples were again extracted with phenol in an attempt to bring the sedimentation pattern back to normal. The fact that the sedimentation profile of heated RNA is unaffected by phenol extraction supports the hypothesis that chain cleavage is occurring rather than alteration in secondary structure. It is suspected that some component associated with the slowly sedimenting material may catalyse this cleavage of the 28s RNA. This behaviour is in contrast with the thermal stability of 28s RNA after purification by Sephadex treatment and points to the importance of using highly purified preparations in experiments involving heat treatment of RNA.

The present results seem to be in agreement with the hypothesis (Midgley, 1965c) that the 28s and 18s strands are continuous polynucleotide chains. However, the 28s RNA seems to be composed of sub-units joined by 'labile' bonds. The base compositional data indicate that the 28s RNA of mammalian cells cannot be composed of dimers of 18s molecules as such. It is more likely that the sub-units of the 28s fraction have a composition different from that of the 'native' 18s RNA.

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