# Base Sequence Differences between the Ribosomal and "Ribosomal Precursor" Ribonucleic Acids from Ehrlich Ascites Cells*

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ABSTRACT: There exist in the nuclei of animal cells two species of high molecular weight ribonucleic acid (RNA) which are characterized by having sedimentation coefficients of approximately 45 and 35 S and which are believed to serve as precursors for ribosomal RNA ("ribosomal precursor" RNA). We have isolated this RNA, together with the 28S and 18S ribosomal RNA (rRNA), from ascites cells and purified each of the four species by zonal centrifugation followed by chromatography on methylated albumin kieselguhr (MAK) columns. The nucleotide composition and sequence in each of these species have been investigated by means of alkaline hydrolysis and by comparing the nucleotide products resulting from enzymic digestion with pancreatic ribonuclease (RNase). No difference could be detected between the nucleotide compositions and sequences of the 45S and 35S species. Both, however, differed slightly in composition and sequence from 28S rRNA and differed widely from 18S rRNA. The significance of these results is discussed in connection with the hypothesis that the 45S and 35S species represent ribosomal precursor RNA.

When animal cells are exposed for short periods of time to radioactive RNA precursors, there appear in the nuclei two species of labeled RNA with sedimentation coefficients of approximately 45 and 35 S (Scherrer and Darnell, 1962).[1] A number of lines of evidence (see Discussion) have led to the hypothesis that these species are composed primarily of ribosomal precursor RNA. This RNA is believed to sediment more rapidly than rRNA because of its larger size, different configuration, or close association with other material, and it is believed to be converted into the 28S and 18S units of rRNA by some mechanism which is not yet understood.

A direct proof of this precursor hypothesis would be facilitated if the four species (45S, 35S, 28S, and 18S RNA) could all be isolated in a pure form. It should then be possible to carry out a detailed comparison between the physical and chemical properties of each of the RNAs and in this way determine the exact relationships, if any, between each of the species. This sort of approach is only slowly being implemented because of the difficulties in obtaining significant amounts of purified 45S and 35S RNAs.

This paper describes the isolation and purification of these RNAs from Ehrlich ascites cells using sucrose density gradient centrifugation followed by chromatography on MAK[2] columns. The purified species were degraded with pancreatic RNase and the products were separated in a two-dimensional system of paper electrophoresis followed by paper chromatography. An analysis of these nucleotide maps provides data relevant to the structure and function of the presumed ribosomal precursor RNA species.

## Materials and Methods

*Preparation of Labeled RNA.* Details have been described concerning the procedures for labeling ascites cells, cell fractionation into nuclear and cytoplasmic components, RNA isolation from each fraction, sedimentation analysis of the RNA, and base ratio determinations (Roberts *et al.*, 1966). For each experiment, 0.1 ml of $^{32}P$ solution (carrier free, 0.15 M in NaCl, and containing 2 mc of $^{32}PO_4^{3-}$/ml) was added to each of seven petri dishes containing the suspended cells.

*MAK Chromatography.* Identical fractions from a number of sedimentation runs were pooled and dialyzed for 16 hr at 4° against two 2000-ml portions of 0.5 M NaCl buffered with 0.1 M potassium phosphate (pH 6.8)–0.001 M EDTA. All dialysis tubing had been previously washed by boiling for 5 min in 0.5%

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[1] The following sedimentation coefficients are used in this paper: the two species of rapidly labeled nRNA, 45 and 35 S; the two species of rRNA, 28 and 18 S. These S values have not been determined exactly but are approximations which are used for the purpose of identification. For the sake of uniformity these values are used in work referred to from other authors, even though the authors may have assigned slightly different sedimentation constants to these species.

[2] Abbreviation used: MAK, methylated albumin kieselguhr.

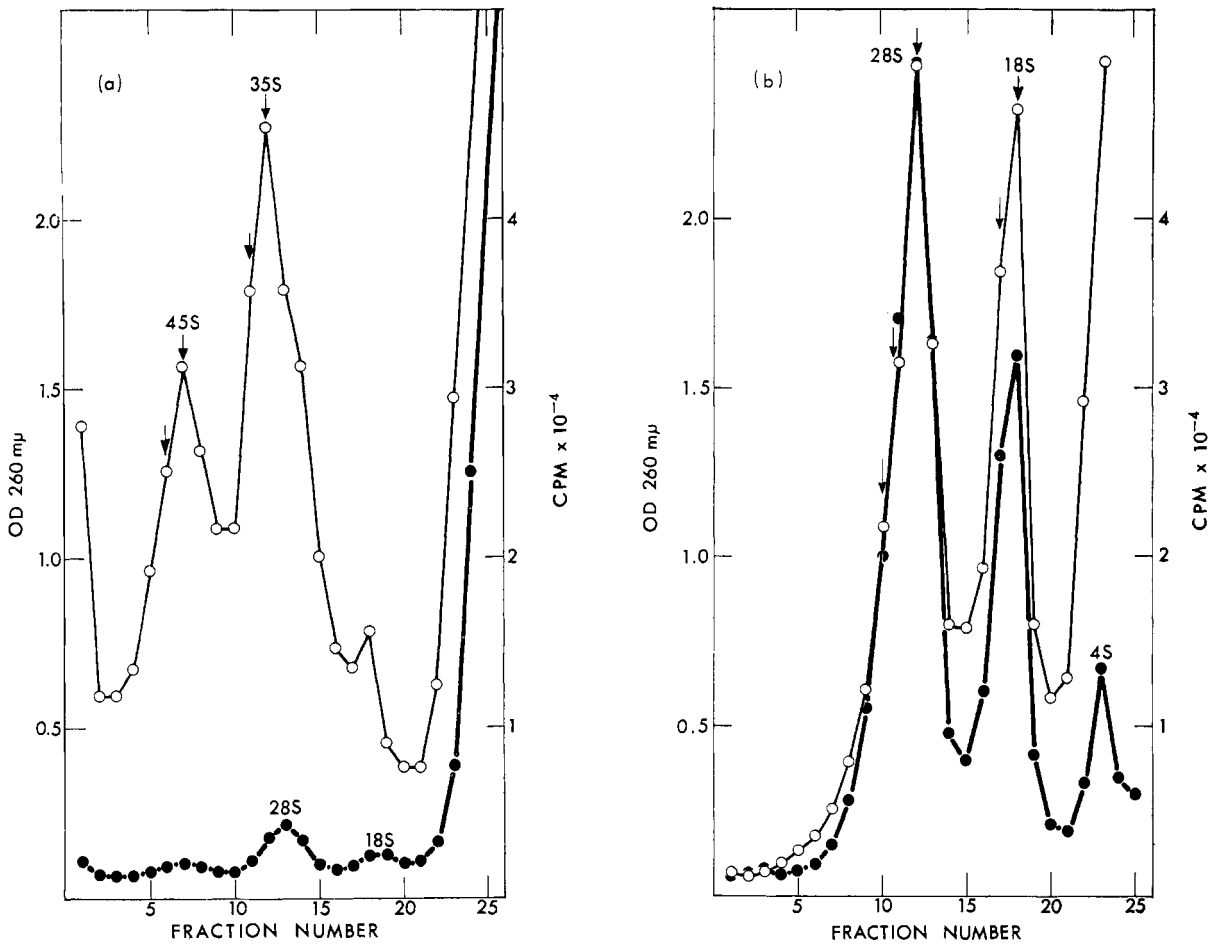


FIGURE 1: Sedimentation patterns of (a) nuclear and (b) cytoplasmic RNA. Ascites cells were labeled with $^{32}P$ for 6 hr. (●) Optical density at 260 mμ; (○) counts per minute.

sodium dodecyl sulfate–0.01 M EDTA, boiling for 5 min in distilled water, and extensive rinsing with distilled water. To the dialyzed sample (approximately 5 ml) was added sufficient carrier rRNA to bring the total amount of RNA to 0.3 mg. The sample was then warmed to 37° and applied to an MAK column for chromatography.

Column chromatography was carried out on MAK columns (8 × 1.3 cm) which had been prepared according to the method of Mandell and Hershey (1960). Columns were washed at 37° with buffered 0.5 M NaCl prior to use; the washing was for 1 hr in the case of columns to be used for the chromatography of 18S and 28S RNA, and for 14 hr in the case of columns to be used for 35S and 45S RNA. The RNA was eluted at 37° by stepwise increases in the concentration of NaCl, the buffer (0.1 M potassium phosphate–0.001 M EDTA, pH 6.8) being held constant for the first three steps and then being changed to pH 10.0 phosphate buffer for the fourth step. The columns were finally stripped with 2.0 M sodium carbonate. Ten 5-ml fractions were collected for each elution step with the flow rate of the elution, as for the original washing, being adjusted to approximately 0.8 ml/min.

*Mapping.* The fractions from the MAK columns which were to be hydrolyzed with pancreatic RNase and mapped were dialyzed overnight at 4° against 2000 ml of distilled water. The dialyzed solution was then concentrated to 1 ml by rotary evaporation at 37°. This concentrated RNA solution contained some salt and a small amount of low molecular weight mtaerial which interfered with the electrophoresis step of the mapping procedure. These substances were separated from the RNA by passing the solution through a Sephadex G-50 column and eluting with water, the RNA being eluted well ahead of the salt and interfering material. To the RNA was then added 0.07 ml of 0.1 M potassium phosphate (pH 7.1) and 2.5 mg of carrier RNA (Torula RNA from Calbiochem, Los Angeles, Calif.) and the solution was concentrated to 0.3–0.4 ml by blowing a jet of air across it at 37°. Enzymic digestion was then carried out by the addition of 0.025 ml of pancreatic RNase (3 mg/ml) and incubating the solution at 37° for 20 hr. The entire enzymic digest was mapped according to the procedure of Rushizky and Knight (1960a) by paper electrophoresis at pH 2.7 in one dimension followed by chromatography with buffered *t*-butyl alcohol in the other. The mono-,

TABLE I: Chromatography of RNA on MAK Columns.[a]

| | Fractions | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| | | | 18S RNA | | |
| Salt concentration (M) | NaCl (0.5) | NaCl (0.8) | NaCl (1.0) | NaCl (1.0), pH 10 | $Na_2CO_3$ (2.0) |
| Per cent eluted | Trace | Trace | 61 | 14 | 10 |
| Base ratios | | | C, 26.0; A, 21.4; G, 30.3; U, 22.3 | C, 25.1; A, 22.5; G, 29.5; U, 22.9 | C, 24.1; A, 24.0; G, 25.9; U, 26.1 |
| | | | 28S RNA | | |
| Salt concentration (M) | NaCl (0.5) | NaCl (0.8) | NaCl (1.0) | NaCl (1.0), pH 10 | $Na_2CO_3$ (2.0) |
| Per cent eluted | Trace | Trace | 68 | 14 | 7 |
| Base ratios | | | C, 30.1; A, 16.2; G, 35.1; U, 18.7 | C, 27.8; A, 19.7; G, 30.4; U, 22.0 | C, 27.2; A, 21.0; G, 26.9; U, 24.9 |
| | | | 35S RNA | | |
| Salt concentration (M) | NaCl (0.5) | NaCl (0.7) | NaCl (1.2) | NaCl (1.2), pH 10 | $Na_2CO_3$ (2.0) |
| Per cent eluted | Trace | Trace | 57 | 20 | 21 |
| Base ratios | | | C, 30.3; A, 14.5; G, 34.5; U, 20.6 | C, 28.9; A, 16.6; G, 32.3; U, 22.3 | C, 25.8; A, 19.4; G, 28.2; U, 26.6 |
| | | | 45S RNA | | |
| Salt concentration (M) | NaCl (0.5) | NaCl (0.7) | NaCl (1.2) | NaCl (1.2), pH 10 | $Na_2CO_3$ (2.0) |
| Per cent eluted | Trace | Trace | 50 | 9 | 31 |
| Base ratios | | | C, 30.6; A, 14.4; G, 33.4; U, 21.7 | C, 28.2; A, 16.6; G, 32.3; U, 22.9 | C, 27.9; A, 18.6; G, 28.0; U, 25.6 |

[a] Each column fraction was obtained by stepwise elution with 50 ml of the salt solution indicated. The base compositions listed under fraction 3 represent averages of at least five determinations on separate samples of RNA.

di-, tri-, and tetranucleotides were located under ultraviolet light from the absorbancies resulting from hydrolysis of the marker RNA. These spots were then cut out and the radioactivity was counted directly in a scintillation counter. The identity of each compound was established by comparing its map position with those of the compounds mapped by Rushizky and Knight. These identifications were corroborated by cutting out and eluting each spot, hydrolyzing it with alkali, and determining its base composition.

## Results

*Separation of Labeled RNAs by Zonal Centrifugation.* Ascites cells were exposed to $^{32}P$ for various periods of time in order to determine the maximum time of labeling which would still permit the isolation of 45S and 35S RNA as distinct peaks upon zonal centrifugation. A long labeling period was desirable since it would ensure the equilibration of the $^{32}P$ pool within the cell, minimize in each fraction the relative amounts of contaminating radioactive RNA with high adenine plus uracil base ratios ("DNA-like" RNA), and give good yields of the labeled RNA species to be studied. A labeling time of 6 hr was selected as being optimal for this. The sedimentation patterns of the RNA isolated from the nuclear and cytoplasmic fractions of ascites cells labeled for this period of time are shown in Figure 1.

The optical density profiles in Figure 1 show the usual 28S, 18S, and 4S peaks in the cytoplasmic RNA (cRNA) and 45S, 28S, and 18S RNA in the nuclear fraction. The relative amounts of radioactivity in the 45S, 35S, 28S, and 4S species are about what one would expect when these sedimentation patterns are compared with those of RNA isolated from ascites cells labeled for different periods of time (Roberts and Newman, 1966). The slowly sedimenting optical density and radioactivity observed in the sedimentation profiles of nuclear RNA (nRNA) is due primarily to degraded DNA.

*Purification of RNA by Chromatography on MAK Columns.* Fractions of 45S, 35S, 28S, and 18S RNA, shown by the arrows in Figure 1, were collected from sucrose gradient sedimentation runs. It is known that all of the RNA isolated in this manner will be contaminated with varying amounts of "DNA-like" RNA (Latham and Darnell, 1965; Houssais and Attardi, 1966; Soeiro *et al.*, 1966; Roberts and Newman, 1966). The use of MAK columns for the removal of this contaminating RNA was suggested by the success of Koch and Kubinski (1964) and Yoshikawa-Fukada *et al.* (1965) in fractionating similar species of animal-cell RNA by MAK column chromatography.

Initial attempts to purify the RNA fractions using

this method proved disappointing due to the poor recoveries of radioactive nRNA. Often less than one-half of the added RNA could be eluted by conventional salt gradients, the remainder requiring alkali to strip it from the column. This difficulty in eluting high molecular weight animal cell RNA from MAK columns has been observed by others (Ellem and Sheridan, 1964; Ellem, 1966). For our studies, comparing RNAs isolated in such a manner would have questionable value since it would be difficult to prove that we were not comparing minority species.

Eventually it was found that both washing the MAK columns extensively before use and running the salt elutions at slightly higher pH values increased the recovery from the columns. Using this procedure we were routinely able to elute between 50 and 80% of 45S and 35S RNA from the columns at neutral pH values and at moderate salt concentrations. A stepwise gradient was used to permit the recovery of RNA fractions in small volumes of effluent.

Table I summarizes the data from typical MAK chromatography runs on each of the four RNA species. The percentages eluted in each fraction and the base compositions in fractions 4 and 5 were somewhat variable, whereas the base compositions in fraction 3 were usually constant within experimental error. It is known that animal cell RNA which has a DNA-like base composition is bound more firmly to MAK columns than is rRNA (Ellem, 1966). This being the case, our results can be interpreted in terms of a homogeneous species of ribosomal or ribosomal precursor RNA being eluted in fraction 3 and variable amounts of this RNA and DNA-like RNA being eluted in fractions 4 and 5.

The degree of homogeneity of each species of RNA in fraction 3 is difficult to establish, but some information can be obtained by base ratio comparisons and by sedimentation analysis. The base ratios of the isolated 28S and 18S RNAs can be compared with those determined by optical density measurements or by long-term labeling of rRNA. Theoretical base compositions may be determined indirectly for 45S and 35S RNA by correcting the base ratios of the impure 45S and 35S RNA isolated by zonal centrifugation for the contribution of the DNA-like RNA contaminating each species. Roberts and Newman (1966) have used low concentrations of actinomycin D to selectively inhibit the synthesis of ribosomal precursor RNA in ascites cells. This permits a calculation to be made concerning the amounts and base ratios of the DNA-like RNA which sediments at 45 and 35 S. If one assumes that the DNA-like RNA synthesized in the presence of low concentrations of actinomycin D is normal in terms of amount and base composition, then one can subtract its contribution to the over-all base ratios and obtain corrected base compositions for 45S and 35S RNA. A comparison of all these base compositions is given in Table II.

The base compositions of the 28S and 18S RNAs isolated in fraction 3 can be seen to be what would be expected for these purified species. This indicates

TABLE II: Base Compositions of RNA.

| Source | C | A | G | U |
|---|---|---|---|---|
| 18 S (optical density)[a] | 26.0 | 21.1 | 31.6 | 21.2 |
| 18 S (labeled 24 hr)[a] | 26.1 | 22.1 | 29.8 | 22.1 |
| 18 S (MAK fraction 3) | 26.0 | 21.4 | 30.3 | 22.3 |
| 28 S (optical density)[a] | 29.4 | 16.7 | 36.1 | 17.8 |
| 28 S (labeled 24 hr)[a] | 28.4 | 16.8 | 36.6 | 19.2 |
| 28 S (MAK fraction 3) | 30.1 | 16.2 | 35.1 | 18.7 |
| 35 S | 28.6 | 16.8 | 32.4 | 22.2 |
| 35 S (corrected for DNA-like RNA)[a] | 29.6 | 15.3 | 34.2 | 21.0 |
| 35 S (MAK fraction 3) | 30.3 | 14.5 | 34.5 | 20.6 |
| 45 S | 28.4 | 17.0 | 31.8 | 22.8 |
| 45 S (corrected for DNA-like RNA)[a] | 30.0 | 14.5 | 34.4 | 21.0 |
| 45 S (MAK fraction 3) | 30.6 | 14.4 | 33.4 | 21.7 |
| 18 S + 28 S (optical density)[b] | 28.3 | 18.2 | 34.6 | 18.9 |

[a] From results of Roberts and Newman (1966). [b] Calculated assuming the molecular weight of 28S RNA to be twice that of 18S RNA.

that the 6-hr labeling period used in these experiments was sufficient to equilibrate the $^{32}P$ pool within the cells. The compositions of 45S and 35S DNA, unpurified, are indeed similar to what one might expect for precursors of 28S and 18S RNA. However, the percentage of adenylic acid in both the 45S and 35S RNA is quite low and if one assumes part of these base compositions to be contributed by contaminating DNA-like RNA (high in adenine and uracil) then the adenylic acid content in the purified species should be lower still. An examination of the 35S and 45S RNA isolated in fraction 3 shows this to be the case. The percentage of adenylic acid in both species is considerably lower than what would be expected for a precursor of 28S plus 18S RNA and even somewhat lower than might be expected for precursors of 28S RNA alone. Also, it can be seen that the over-all base compositions of the 45S and 35S RNAs are almost identical, suggesting that these species may be very similar in their primary polynucleotide structure.

A number of the base compositions given in Table II have been corroborated by recent experiments in which ribosomal precursor RNA was separated from nuclear DNA-like RNA by means of a different MAK column fractionation procedure (W. K. Roberts and L. D'Ari, unpublished data). These experiments showed 45S and 35S RNA purified in this manner to have a base composition which was almost identical with those shown in Table II for the purified 45S and 35S species, and the DNA-like RNA to have a similar base composition to the DNA-like RNA determined

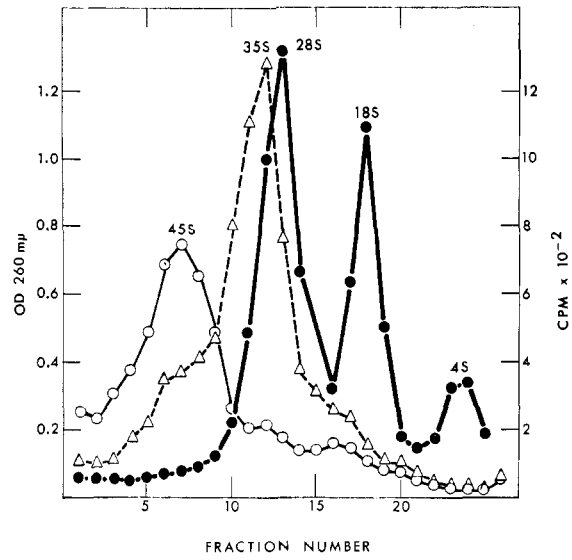


FIGURE 2: Sedimentation patterns of the 45S and 35S RNAs eluted from MAK columns. A portion of fraction 3 from the chromatography of each species was combined with marker RNA and phenol was extracted to remove contaminating methylated albumin. The RNA was then precipitated, dissolved in buffer, and centrifuged through a surcrose gradient in the usual manner. (●) Optical density at 260 mμ of marker RNA; (○) counts per minute of 45S RNA; (△) counts per minute of 35S RNA.

by the actinomycin studies. It is also of relevance that the base ratios shown for 45S and 35S RNA in Table II are very similar to those determined for the 45S and 35S RNAs which were isolated from the nucleoli of rat liver (Muramatsu *et al.*, 1966a) and various tumor cells including Ehrlich ascites cells (Smith *et al.*, 1967; Nakamura *et al.*, 1967).

Portions of the 45S, 35S, 28S, and 18S RNA fractions from sedimentation runs were periodically examined by recentrifugation before the fractions were purified further. These sedimentation patterns revealed that the 28S and 18S fractions each were contaminated with about 5% of the other ribosomal RNA species, whereas the cross-contamination between 45S and 35S RNA varied between 7 and 18%. It can be calculated from Table II that this amount of contamination would not affect significantly the base compositions of any of these species. Except for this contamination, no RNA was observed other than the RNA species being centrifuged.

Following dialysis, Sephadex treatment, and chromatography on MAK columns, the possibility of degradation of the RNA in each fraction again was checked by zonal centrifugation. The sedimentation patterns of the 45S and 35S RNAs isolated in fraction 3 are shown in Figure 2. It can be seen that a minor fraction from each species has undergone partial degradation during the purification. However, since the degradation products are still of high molecular weight they should accompany the undegraded molecules during the purification procedure and should not alter the base composition of the species.

*Mapping of Products Resulting from Hydrolysis of RNA with RNase.* The determination of base ratios is a common method of comparing chemically various RNA species. A more sensitive method, however, and one which gives information on nucleotide sequence as well as over-all composition, is a comparison of the products formed after hydrolysis of the RNAs with a base-specific nuclease. For example, Rushizky and Knight (1960b) have used digestion with pancreatic RNase to compare the RNAs in several strains of tobacco mosaic virus. We have used this method of Rushizky and Knight to examine more closely the possible relationships between 18S and 28S rRNA and 35S and 45S ribosomal precursor RNA.

To test this procedure, radioactive rRNA was hydrolyzed with pancreatic RNase and the products were mapped by electrophoresis at pH 2.7 in one dimension followed by paper chromatography in buffered *t*-butyl alcohol in the other dimension. An autoradiogram of the resulting map is shown in Figure 3. The distribution of these radioactive spots is almost identical with the distribution of nucleotide products found by Rushizky and Knight (1960b). Two exceptions are spot 33, in which we were unable to clearly separate $A_3U$[3] from the much larger GGC spot and therefore analyzed the two together, and spot 12. Spot 12 was not observed in the digests of tobacco mosaic virus RNA and has been identified as pseudouridylic acid by its mobility during paper electrophoresis and paper chromatography (Davis and Allen, 1957) and by its elution with pseudouridylic acid from a Dowex 1 ion-exchange column (Cohn, 1960).

Samples of 18S, 28S, 35S, and 45S RNA which had been purified by MAK chromatography (fraction 3) were combined with carrier RNA, hydrolyzed with pancreatic RNase, and mapped as above. Each spot was located by the ultraviolet absorbancy resulting from the hydrolyzed marker RNA and then cut out and the radioactivity was counted. Approximately 75% of the radioactivity from each RNA species was accounted for in the 18 spots investigated, the bulk of the remainder being found near the origin. The results of these experiments are tabulated in Table III.

The data in Table III can be used to test directly the feasibility of the hypothesis that 45S and 35S RNA represent precursors of rRNA. The most detailed version of this hypothesis pictures 45S RNA being cleaved to 18S RNA and 35S RNA with the 35S RNA subsequently undergoing a transition to 28S RNA (Penman, 1966). Evidence has also been presented (Penman, 1966) that 18S and 28S rRNA are produced in nearly equal molar amounts and that the conversion of 35S RNA and 28S RNA takes place with the loss

[3] The nucleotide abbreviations used are the same as those used by Rushizky and Knight (1960a).

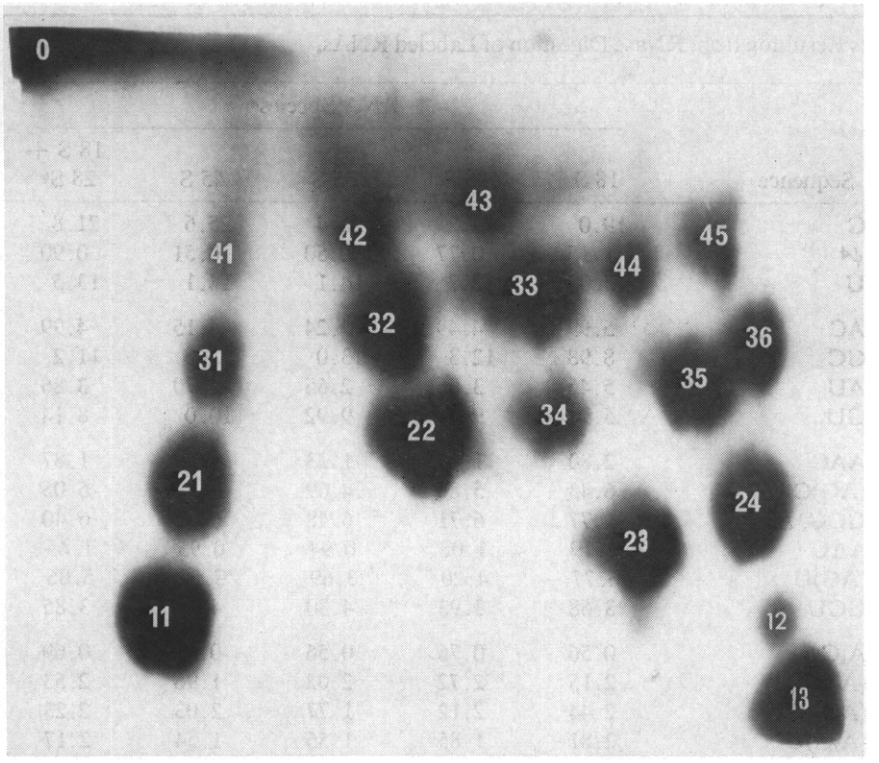


FIGURE 3: Map of the products resulting from pancreatic RNase hydrolysis of a mixture of the 28S and 18S radioactive RNAs. The digestion mixture was spotted at the origin (○) and the nucleotides were separated by electrophoresis from left to right followed by chromatography from top to bottom. The spots were detected by autoradiography.

of relatively little material. The conservation of most of the 45S RNA during its supposed conversion to rRNA has been implied by actinomycin chase experiments (Scherrer *et al.*, 1963), similarities in base ratios (Scherrer *et al.*, 1963; Houssais and Attardi, 1966; Soeiro *et al.*, 1966), and by the fact that the presence of a large amount of extraneous RNA sedimenting at 45 S would make suspect much of the other evidence linking 45S RNA to rRNA.

A careful examination of the data in Table III makes a quantitative conversion of 45S RNA to 28S and 18S RNA appear impossible. Based upon the relative amounts of absorbancy at 260 mμ which can be determined for each species of rRNA following sedimentation analysis of cRNA, 28S RNA can be assumed to have approximately twice the molecular weight of 18S RNA. This permits a sequence composition to be calculated for a theoretical precursor containing one piece of 28S RNA and one piece of 18S RNA. A comparison of these values with those of 45S RNA reveals that 45S RNA contains only about 70% of the base sequences in spots 23, 31, 34, 35, and 44 that would be expected for a theoretical precursor of 28S and 18S RNA. This would mean that at most 70% of the 45S RNA could be undergoing conversion to equal molar amounts of 28S and 18S RNA.

These data do not preclude the conversion of 35S RNA to 28S RNA, but do suggest that this conversion cannot involve the conservation of the entire 35S molecule. Many small differences between the sequence compositions of 28S and 35S RNA are observed, but most of these are within the range of possible experimental error. However, a number of these sequences (spots 32, 36, 41, and 42) do seem to differ by significant amounts. This suggests that a transformation of 35S RNA to 28S RNA may occur by the excision of a piece of RNA from the 35S material.

The differences in the relative amounts of pseudouridylic acid in each of the species is interesting but probably of little value in evaluating the precursor hypothesis. This is because the synthesis of pseudouridylic acid may well take place *via* intramolecular rearrangements and there is no knowledge concerning the stage(s) in the synthesis of RNA molecules where these rearrangements might be occurring.

## Discussion

In order to reconcile our results with the ribosomal precursor RNA hypothesis we can postulate that included in the structures of the 45S RNA and 35S RNA are significant amounts of nonribosomal RNA. This RNA would be responsible for the differences observed in base compositions and sequence analyses between the ribosomal precursor and rRNAs and would be eliminated from the precursor molecules during their conversion to rRNA. In the case of 35S RNA we can calculate from Table III that in order to

TABLE III: Products Resulting from RNase Digestion of Labeled RNAs.

| Map No. | Sequence | RNA Species[a] 18 S | 28 S | 35 S | 45 S | 18 S + 28 S[b] | Std Dev[c] |
|---|---|---|---|---|---|---|---|
| 11 | C | 19.0 | 23.2 | 26.4 | 25.6 | 21.8 | 1.0 |
| 12 | $\psi$[d] | 1.15 | 0.77 | 0.50 | 0.51 | 0.90 | 0.12 |
| 13 | U | 15.0 | 12.7 | 14.1 | 14.1 | 13.5 | 0.6 |
| 21 | AC | 5.99 | 4.49 | 4.24 | 4.15 | 4.99 | 0.21 |
| 22 | GC | 8.98 | 12.3 | 13.0 | 12.6 | 11.2 | 0.4 |
| 23 | AU | 5.45 | 3.06 | 2.65 | 2.70 | 3.86 | 0.17 |
| 24 | GU | 5.98 | 9.22 | 9.92 | 10.0 | 8.14 | 0.40 |
| 31 | AAC | 2.80 | 1.41 | 1.23 | 1.29 | 1.87 | 0.11 |
| 32 | (AG)C | 6.43 | 5.86 | 4.69 | 4.62 | 6.05 | 0.22 |
| 33 | GGC, $A_3U$ | 5.77 | 6.71 | 6.48 | 6.38 | 6.40 | 0.25 |
| 34 | AAU | 2.19 | 1.08 | 0.94 | 0.95 | 1.45 | 0.10 |
| 35 | (AG)U | 6.77 | 4.20 | 3.69 | 3.59 | 5.06 | 0.21 |
| 36 | GGU | 3.68 | 3.93 | 4.80 | 4.97 | 3.85 | 0.17 |
| 41 | $A_3C$ | 0.56 | 0.76 | 0.56 | 0.54 | 0.69 | 0.08 |
| 42 | $(A_2G)C$ | 2.15 | 2.72 | 2.02 | 1.96 | 2.53 | 0.15 |
| 43 | $(AG_2)C$ | 2.44 | 2.12 | 1.77 | 2.03 | 2.23 | 0.15 |
| 44 | $(A_2G)U$ | 2.81 | 1.85 | 1.56 | 1.54 | 2.17 | 0.16 |
| 45 | $(AG_2)U$ | 2.29 | 2.46 | 2.09 | 2.10 | 2.40 | 0.12 |

[a] Figures give the percentage of the total radioactivity from each species found in a given sequence. Each number represents an average of at least five determinations on separate samples of RNA. [b] Calculated from the 18S and 28S columns. These values are for equal molar amounts of 18S and 28S RNA and assume that the molecular weighth of 28S RNA is twice that of 18S RNA. [c] Average of the standard deviations calculated for the spots in each of the four RNA species. [d] $\psi$, pseudouridylic acid.

fit our data no more than about 20% of the molecule need be lost during its transformation to 28S RNA. The loss of this amount of material would not seriously conflict with any of the evidence which supports the hypothesis that this transformation takes place.

In the case of the postulated conversion of 45S RNA to 28S and 18S rRNA a more difficult problem arises. Our results indicate that if such a transformation were taking place at least 30% of the 45S RNA must be nonribosomal RNA. The percentage probably would be larger than this, since the contaminating nonribosomal RNA would undoubtedly include some of the limiting base sequences of compounds 23, 31, 34, 35, and 44 in its structure. The existence of such a large amount of RNA with an unknown structure and function would not only be surprising but it would raise serious questions concerning the validity of much of the evidence that 45S RNA is ribosomal precursor RNA. Also, the fact that we could detect no base sequence differences between 45S and 35S RNA would be a very fortuitous observation, if this transformation involved the loss from the 45S molecule of 18S and nonribosomal RNA.

We feel that a more attractive hypothesis would be one in which the primary structures of the 45S and 35S RNAs are very similar. This would imply that 45S RNA is transformed into 35S RNA, perhaps by a change in the conformation of the molecule, and then the 35S RNA is converted into 28S rRNA. The 18S ribosomal RNA would be synthesized independently.

It should be mentioned that although the above discussion concerning nonribosomal RNA has implied that such RNA would be an integral part of the 45S and 35S RNA molecules, this need not be the case. The nonribosomal RNA could also consist of separate molecules which because of their similar size and high G–C base composition remain as a persistent contamination of the ribosomal precursor RNA species.

As noted earlier, each of the four RNA species studied was contaminated slightly with the ribosomal precursor or rRNA adjacent to it in a sedimentation run. It can be calculated from Tables II and III that the amount of contamination observed would not affect significantly the base compositions or sequences which were found. However, contamination with other species of RNA is always possible, not only of the 45S and 35S RNAs but also of the 28S and 18S rRNAs. For example, 28S RNA has been isolated from the nucleoli of liver cells (Muramatsu *et al.*, 1966a) and several lines of tumor cells (Smith *et al.*,

1967; Nakamura *et al.*, 1967) and shown to be virtually identical in base composition with the 45S and 35S RNA from these species. This could mean that a direct conversion between ribosomal precursor RNA and 28S nucleolar RNA takes place and that the 28S RNA which we have isolated from the cytoplasm of ascites cells is contaminated and therefore different in base composition from the nucleolar species. However, the 28S RNA isolated from the cytoplasm of liver cells (Muramatsu *et al.*, 1966b) also had a base composition which appeared significantly different from that of the corresponding nucleolar species (Muramatsu *et al.*, 1966a). An interpretation of the nucleolar base ratio results is complicated further by the fact that nucleolar 18S RNA also had a base composition which was virtually identical with that of 45S and 35S RNA (Nakamura *et al.*, 1967), suggesting that the 28S and 18S nucleolar species might be degradation products of the 45S and 35S species.

Other evidence can be found which supports the hypothesis that 28S but not 18S rRNA is derived from ribosomal precursor RNA (see Steele and Busch, 1966). However, since this does represent an important modification of the usual precursor hypothesis, it is necessary to examine briefly and critically the evidence which has led to the idea that both species of rRNA are synthesized *via* large precursor molecules.

The evidence which supports this idea is of several types. The kinetics of labeling of the RNA in animal cells favors a precursor interpretation (Scherrer and Darnell, 1962; Hiatt, 1962; Rake and Graham, 1964), as does the kinetics of methylation of the various RNA species (Greenberg and Penman, 1966; Zimmerman and Holler, 1967); the base composition of rRNA and ribosomal precursor RNA are similar (Scherrer *et al.*, 1963; Houssais and Attardi, 1966; Soeiro *et al.*, 1966); and all species seem to be synthesized in or around the nucleoli (Perry, 1962; Brown and Gurdon, 1964) and be inhibited in their synthesis to a similar extent by actinomycin (Perry, 1962; Georgiev *et al.*, 1963; Roberts and Newman, 1966). The results of these experiments all are suggestive of a precursor relationship between 45S and 35S RNA and rRNA. However, it should be recognized that these results are from a number of different experiments which in themselves must be regarded as necessary but not sufficient pieces of evidence that such a precursor relationship does exist.

The two types of experiments which would seem to serve as direct evidence for the ribosomal precursor hypothesis are those involving DNA–RNA hybridization (Perry *et al.*, 1964; Yoshikawa-Fukada, 1966) and actinomycin chase experiments (Scherrer *et al.*, 1963; Penman, 1966; Warner *et al.*, 1966). In the hybridization experiments it was shown that rRNA competed with 45S and 35S RNA in hybridization with DNA, presumably because the base sequences along large regions of the rRNA molecules are identical with those in the presumptive ribosomal precursor RNA. Such experiments are difficult to interpret, however, both because of technical difficulties in doing the experiments and because theoretically it is not clear how many nucleotides or nucleotide regions must be complementary in molecules of this size before hybridization can take place. For example, Attardi *et al.* (1965) have reported that in hybridization experiments between rRNA and DNA from HeLa cells the 28S and 18S rRNAs competed up to 60% with each other. Yet these species have base compositions which are markedly different, resembling each other less than the base compositions of 45S and 35S RNA resemble the composition of either species of rRNA.

The actinomycin experiments were carried out by pulse labeling the 45S ribosomal precursor RNA, stopping further RNA synthesis by the addition of actinomycin D, and during the ensuing chase observing an apparent conversion of radioactive 45S RNA to radioactive rRNA. In the original experiments it was reported that the majority of the rapidly labeled RNA in the nuclei of HeLa cells is stable, being conserved by its conversion to rRNA (Scherrer *et al.*, 1963). This observation was disputed by Harris (1964) who claimed that most of the rapidly labeled RNA in HeLa cell nuclei is converted instead into acid-soluble products. There now appears to be substantial agreement that the majority (about 65%) of the pulse labeled nRNA is in fact not conserved when chased for a sufficient period of time in the presence of actinomycin D (Warner *et al.*, 1966; Penman, 1966). We have obtained results which are very similar to those of Warner *et al.* and Penman with ascites cells which were labeled with $^{32}P$ for up to 2 hr and then chased in the presence of actinomycin (W. K. Roberts and J. F. E. Newman, unpublished data). It is undoubtedly true that part of the nRNA which is broken down in the presence of actinomycin is DNA-like RNA. However, it is not clear that all of the labile nRNA is of this type, and because of this the *efficient* conversion of 45S and 35S RNA to rRNA in the presence of actinomycin D must still be questioned (Yoshikawa-Fukada, 1966).

The interpretation of the actinomycin results is complicated further by the fact that it is difficult to prove that actinomycin D immediately stops the synthesis of *all* species of RNA, particularly if this synthesis is taking place during the concomitant degradation of other labile RNA species. Also, it is known that actinomycin can affect activities other than RNA synthesis, such as the transport of rRNA from the nucleus into the cytoplasm (Harris, 1964; Penman, 1966), and it is possible that the fate of 45S and 35S RNA in the presence of actinomycin is aberrant.

We feel that although the existing information which concerns the relationships between ribosomal precursor and rRNA favors the hypothesis that both the 45S and 35S RNAs serve as precursors of 28S RNA only, it does not rule out the possibility that 45S RNA serves as a precursor for both 28S and 18S rRNA with a large amount of nonribosomal, non-DNA-like RNA being contained within the 45S fraction, either as a contaminant or as an integral part of the 45S molecule. Distinguishing between these and other

alternatives regarding the exact structures, transformations, and perhaps also the function of ribosomal precursor RNA must await the application of more refined methods of RNA separation and analysis.

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