Liver Ribosomal Ribonucleic Acid Structural Studies. Characterization of Fragments from Partial Nuclease Digestion*

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ABSTRACT: Fragments of high molecular weight ribonucleic acid (RNA) from rabbit liver ribosomes were obtained by different degrees of digestion with T_1 or pancreatic ribonuclease and then separated by Sephadex G-200 chromatography. Guanylic acid (Gp) and cytidylic acid (Cp) rich segments were isolated from 28S and 18S RNA digests; these regions most resist nuclease attack. From the 28S RNA, the following fragments were isolated. (a) The largest fragments from digests using T_1 or pancreatic ribonuclease were isolated and analyzed. When 37% of the RNA was digested with T_1 ribonuclease to less than 2S fragments, resistant segments were obtained that comprise about 13% of the RNA, contain 82% Gp+Cp, and have a sedimentation value of $s_{20,w} = 6.8$ S. The largest fragments from pancreatic ribonuclease digests where 13% of the RNA was digested to less than 2 S are about 7S chains, contain 81% Gp+Cp, and comprise about 7% of the 28S RNA. Resistant segments from both enzymatic digests have more uracil residues than adenine residues and the Gp content nearly equals that of Cp. (b) Under conditions of more extensive degradation with pancreatic or T_1 ribonuclease where approximately 54% of the RNA is degraded to less than 2S fragments, approximately 40% of the 28S RNA can be isolated as small fragments of ~2.5 S. These segments are also rich in Gp+Cp but have nearly equal Gp:Cp and Ap:Up ratios. (c) Fragments relatively rich in Ap+Up have been obtained by mild T_1 ribonuclease digestion. These comprise about 25% of the RNA and are about 4.5S and 5.5S chains, and their nucleotide composition approaches that of deoxyribonucleic acid (DNA)-like RNA. The results show that the 28S RNA contains regions of high and low Gp+Cp:Ap+Up and that the high Gp+Cp regions have specific Gp:Cp and Ap:Up ratios.

Through an analysis of polynucleotide fragments isolated from partial T_1 ribonuclease digests, rabbit liver rRNA was found to have segments rich in Gp[1] and Cp (Delihas and Bertman, 1966). Hadjiolov *et al.* (1967) find that the 3′ ends of rat liver 18S and 28S RNAs have a higher pA+pU content relative to the 5′ ends. Both these studies show an unbalanced distribution of nucleotides along the chains of mammalian liver rRNA.

The study here is a further analysis of fragments from partial T_1 and pancreatic RNase digests of 28S and 18S RNAs. Enzymic digestion was studied at different stages and the resulting fragments were isolated and characterized by sedimentation rate and nucleotide composition. Resistant fragments with high Gp+Cp were isolated from pancreatic and T_1 RNase digests and were found in 18S and 28S RNA. Regions most susceptible to enzymatic cleavage were found to be those relatively rich in Ap and Up.

Experimental Procedure

Preparation of rRNA. Rabbit livers were obtained from Pel-Freez Biologicals, Rogers, Ark. Livers were from mature animals weighing 4–5 kg. The breed is predominantly New Zealand White. Liver tissue (200 g) was homogenized near 0° with a Waring Blendor in 500 ml of 0.01 M Tris-HCl (pH 7.5), 0.005 M $MgCl_2$, 0.25 M sucrose, and 0.2% bentonite. After centrifugation at 8000 rpm (10,400*g*) for 15 min the supernatant was passed through gauze for clarification and treated with 0.5% sodium deoxycholate at 0° for 20 min (Korner, 1961). Ribosomes were prepared by centrifugation at 40,000 rpm for 2 hr using the Spinco Model L 40 rotor. To extract RNA, ribosome pellets from four centrifugations were homogenized for 1 min at 22° with a Virtis "45" homogenizer at low speed. The homogenizing solution was 100 ml of 0.01 M Tris-HCl (pH 7.5), 0.1% bentonite, and an equal volume of 88% phenol (Mallinckrodt)–0.1% quinolinol. Only fresh or redistilled phenol was used for extractions. After four phenol extractions, RNA in the final aqueous phase was precipitated with two volumes of ethanol, washed with ether, and dried under vacuum in the cold, yielding ~30 mg of rRNA/100 g of liver (wet weight).

RNAs (28 and 18 S) were separated by zone centrifugation; 120 OD_{260} of rRNA in 2 ml of 0.01 M Tris-HCl (pH 7.15)–0.1 M NaCl was layered over a 51-ml

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[1] Abbreviations used: p on the right side of the nucleoside symbols signifies 2′(3′)-phosphate; p on the left side of the nucleoside symbols signifies 5′-phosphate.

linear gradient of sucrose 7–35%, which was also in Tris-HCl–NaCl, and centrifuged at 4° for ~20 hr. Centrifuge tubes were punctured from the bottom and the contents pumped into a fraction collector. Fractions of 1.0–1.5 ml were collected.

Partial Digestions. Ribonuclease T_1 was bought from Worthington Biochemical Corp. and pancreatic ribonuclease A (type XII-A) from Sigma. Digestion of RNA was in 1–2 ml of 0.1 M Tris-HCl (pH 7.5), with 0.02 M EDTA. Temperature, duration of digestion, and enzyme quantities are given in the text. Digestion was stopped by addition of phenol (previously saturated with Tris-HCl). After two extractions, phenol was removed with ether and the ether was removed with nitrogen bubbling. Recovery of RNA was ~75% without phenol-phase washing and 90% with washes. Losses were assumed to be random since most of the loss could be accounted for by volume loss.

Sephadex Chromatography. RNA fragments were separated with Sephadex G-200. The column procedure was as previously described (Delihas and Staehelin, 1966). Distribution coefficients, K_d, were calculated from $K_d = (V_e - V_0)/V_i$, V_e = elution volume, V_0 = void volume, and V_i = inner volume.

Sedimentation Measurements. The Spinco Model E ultracentrifuge, equipped with ultraviolet optics, monochromator, and photoelectric scanner, was used to study sedimentation. Sedimentation rates were calculated from the linear plot of ln x *vs.* t, where x is the distance of the midpoint of the boundary to the center of rotation and t is the time. Sedimentation rates given as $s_{20,w}$ in the text were measured values corrected to 20° and water as the solvent. All other quoted s values were obtained from a calibration curve of $s_{20,w}$ *vs.* distribution coefficient, K_d, in Sephadex G-200.

Nucleotide Analyses. Samples were digested in 0.03–0.05 ml of 0.3 N KOH for about 18 hr at 37°. After neutralization, nucleotides were separated by thin layer electrophoresis (Tometsko and Delihas, 1967). Elution from thin layer plates was with buffer at pH 7.0. The following extinctions in M^{-1} cm^{-1} at 260 mμ (pH 7.0) were used to determine nucleotide concentrations: Up, 10×10^3; Gp, 11.4×10^3; Ap, 15×10^3; Cp, 7.6×10^3. Nucleotide compositions shown in the text are major and minor nucleotides grouped together. Values are given in moles/100 moles of nucleotide plus and minus average deviation of two experiments. Where no deviations are given, values represent one determination.

Results

RNA from Rabbit Liver Ribosomes. Approximately 90% of RNA from ribosome preparations was high molecular weight RNA (Figure 1). The low molecular weight material consisted of a 5S component and tRNA.

RNAs (28 and 18 S) were separated by sucrose gradient zone centrifugation (Figure 2a). Fractions were pooled as shown in Figure 2a for sedimentation and nucleotide analysis and for subsequent studies

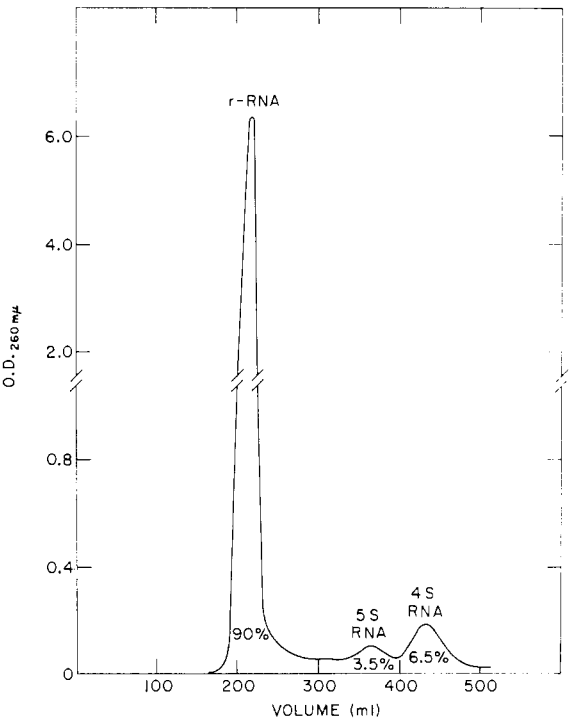

FIGURE 1: Sephadex G-200 column chromatography of RNA from rabbit liver ribosomes. Column volume, 650 ml; elution with 0.01 M potassium acetate (pH 7.0) at 22°. rRNA appears at the void volume of the column; 5S RNA at $K_d = 0.40$ and 4S RNA at $K_d = 0.56$.

with partial nuclease digestions. Figure 2b shows scanner tracings of the two rRNAs using the Model E analytical ultracentrifuge. Nucleotide compositions of the two rRNAs are also given in Figure 2.

Partial Digestion of 28S and 18S RNAs with RNase T_1. RNA (28 S) was digested at different temperatures with RNase T_1 and the resulting RNA fragments were separated with Sephadex G-200 (Figure 3). Nucleotide compositions of pooled fractions are given in Table I and sedimentation analyses of fraction 1 (the largest fragments of each digest) are shown in the insert of Figure 3. Under moderate conditions of digestion as shown in Figure 3a, ~30% of the fragmented RNA ($s_{20,w}$ = 9.3 S) has a slightly higher Gp+Cp/Ap+Up ratio than the average for intact 28S RNA. This can readily be seen from the specific nucleotide ratio

$$\left[\frac{Gp+Cp}{Ap+Up}\right]_{sp}.$$

defined as

$$\left(\frac{Gp+Cp}{Ap+Up}\right)_{fragment} \Big/ \left(\frac{Gp+Cp}{Ap+Up}\right)_{intact\ RNA}$$

The parts of the 28S RNA that are initially degraded to smaller fragments (less than 6 S) are relatively rich in

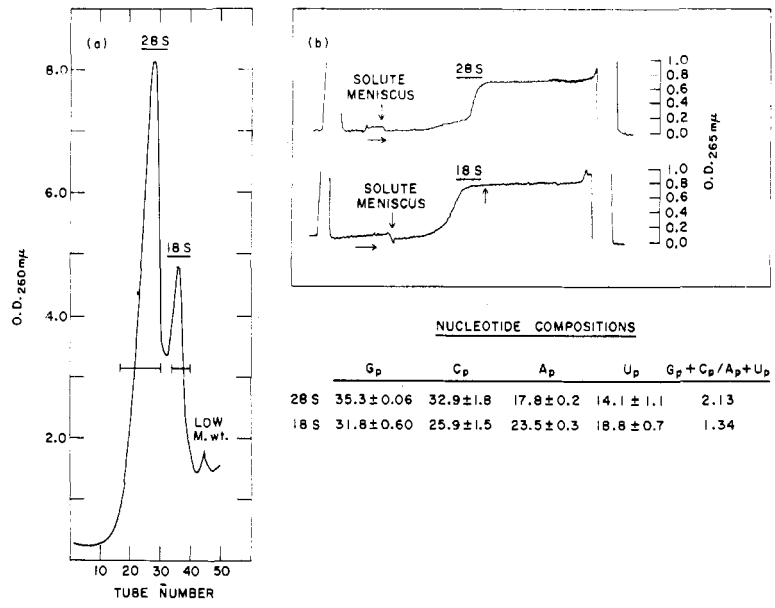

FIGURE 2: rRNA fractionation and ultraviolet absorption patterns. (a) rRNA fractionation by zone centrifugation. Samples layered over a linear sucrose gradient 7–35% in 0.1 M NaCl–0.01 M Tris-HCl (pH 7.15). Centrifugation at 20,000 rpm at 4° for 19 hr in the SW 25.2 Spinco rotor. (b) Ultraviolet absorption patterns using Spinco photoelectric scanner system with the Model E ultracentrifuge. Sedimentation is from left to right. Conditions: solvent, 0.01 M Tris-HCl (pH 7.15)–0.2 M NaCl; temperature, 24°. Top: 36,000 rpm, scan time 28 min after full speed attained. Bottom: 48,000 rpm, scan time 17 min after full speed attained. Measured values are $s_{20,w}$ = 28.1 S and 16.9 S for 28S and 18S RNAs, respectively.

TABLE I: Nucleotide Composition of 28S RNA Fragments from Partial T_1 RNase Digest.[a]

Expt	Fraction	% Total RNA	Nucleotide Composition (moles/100 moles) Cp	Gp	Ap	Up	$\frac{Gp+Cp}{Ap+Up}$	$\left[\frac{Gp+Cp}{Ap+Up}\right]_{sp}$
a (see Figure 3a)	1	30	36.1 ± 0.0	34.1 ± 0.5	15.2 ± 0.2	14.6 ± 0.2	2.36	1.11
	2	42	31.6 ± 0.2	34.3 ± 0.8	17.5 ± 0.4	16.7 ± 0.1	1.93	0.91
	3	10	27.8 ± 0.1	31.2 ± 0.2	22.1 ± 0.05	19.1 ± 0.2	1.44	0.68
	4	12	25.2 ± 0.5	31.5 ± 0.7	20.6 ± 0.7	20.7 ± 1.1	1.31	0.62
b (see Figure 3b)	1	13	38.4	43.2	7.7	10.7	4.44	2.08
	2	9	38.1	40.3	9.6	12.1	3.63	1.70
	3	13	36.7 ± 0.0	36.5 ± 0.1	13.3 ± 0.0	13.5 ± 0.2	2.73	1.29
	4	14	30.5	36.5	17.7	15.6	2.00	0.94
	5	14	29.6 ± 0.7	27.2 ± 0.6	22.7 ± 0.8	20.5 ± 0.4	1.31	0.61

[a] Fragments shown in Figure 3.

Ap and Up and have a nucleotide composition approaching that of DNA-like RNA (see Table I, expt a, fractions 3 and 4). Thus the regions of 28S RNA that are most susceptible to degradation by T_1 RNase are those having a Gp+Cp/Ap+Up ratio considerably less than that of the intact RNA.

Under more extensive degradation, as shown in Figure 3b, ~13% of the 28S RNA remains as fragments with $s_{20,w}$ = 6.8 S and 37% is degraded to small molecules less than 2 S. The resistant 6.8S fragments had 82% Gp+Cp with adenine residues amounting to only 7.7% (Table I, expt b, fraction 1). The segments of 28S RNA that are difficult to degrade are those that are predominantly composed of Gp and Cp and have the smallest quantity of Ap.

With the same conditions of digestion described in Figure 3b, 45% of the 18S RNA was degraded to fragments less than 2 S, and the rest of the RNA

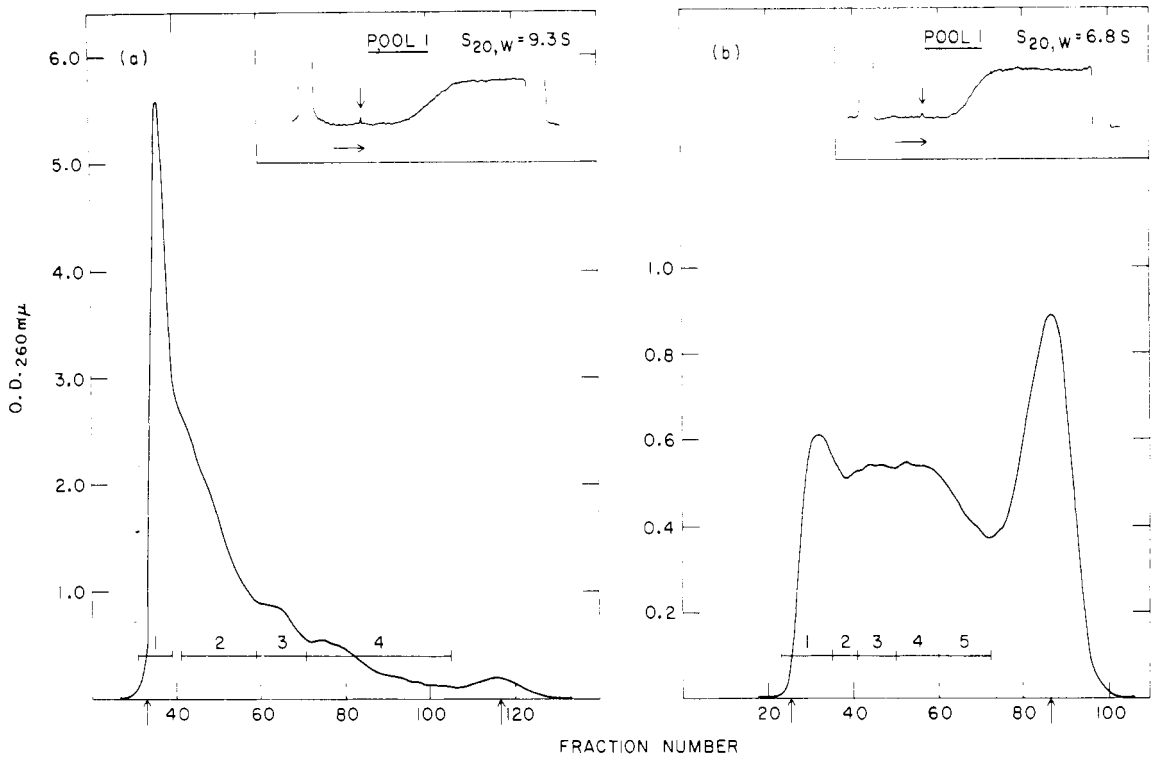

FIGURE 3: Sephadex G-200 separation of 28S RNA fragments produced by partial RNase T_1 digestion. Arrows pointing upward on abscissa represent void and total volumes of the column. (a) Digestion at 15° for 5 min in 0.1 M Tris-HCl–0.02 M EDTA (pH 7.5); enzyme/RNA = 10 units/OD_{260}. Insert: ultraviolet absorption patterns in analytical centrifuge, 44,000 rpm, scan time 50 min after full speed. (b) Digestion at 30° for 15 min, 0.1 M Tris-HCl–0.02 M EDTA (pH 7.5); 10 units/OD_{260}. Insert: 48,000 rpm, 45 min after full speed.

was degraded predominantly to 5S and 3.5S fragments (Figure 4). Nucleotide analyses (Table II) show that the 18S RNA is also cleaved in a specific manner. The enzyme appears to attack the 18S RNA in a fashion similar to the 28S RNA, *i.e.*, the distribution of nucleotides among the various fragments relative to that of the intact RNA molecule resembles that of the 28S RNA segments. This is readily seen in Figure 5 where the specific nucleotide ratio

$$\left(\frac{Gp+Cp}{Ap+Up}\right)_{fragment} \Big/ \left(\frac{Gp+Cp}{Ap+Up}\right)_{intact\ RNA}$$

is plotted against the distribution coefficient in Sephadex G-200. At least 25% of the fragments produced under the conditions of digestion have a Gp+Cp content higher than the average.

Partial Digestion of 28S RNA with Pancreatic RNase. Since the specificity of pancreatic RNase differs from that of RNase T_1, experiments were done to determine whether high Gp+Cp regions of the 28S RNA were also obtainable with pancreatic RNase. RNA (28 S) was digested with different amounts of pancreatic RNase at 0° for 5 min in 0.1 M Tris-HCl–0.02 M EDTA (pH 7.5). In Figure 6a, 13% of the RNA was degraded to chains less than 2 S with 7% broken down to 7S fragments. The largest fragments were high Gp+Cp segments resisting degradation (Table III, expt a, fraction 1). The relationship of Gp+Cp/Ap+Up to polynucleotide size is surprisingly similar to that of the T_1 RNase digests. Figure 5 shows that the relationship of the specific nucleotide ratio $(Gp+Cp/Ap+Up)_{sp}$ to the distribution coefficient K_d follows that of the T_1 RNase fragments. It appears that the high Gp+Cp regions are the last to be degraded by both enzymes.

Under conditions of more extensive degradation by pancreatic RNase (Figure 6b), ~6% of the RNA with sedimentation value of about 5.5 S contains 84% Gp+Cp. About 40% of the 28S RNA (fraction 2) appeared to resist degradation and is found largely in the form of small fragments of ~2.5 S. The specific nucleotide ratio is greater than 1.0 for these small segments and they have nearly equal Gp:Cp and Ap:Up ratios (Table III, expt b, fraction 2). Similar results were obtained with T_1 RNase when the RNA was degraded to the same extent, as shown in Figure 6b.

Estimation of Chain Lengths of Largest Fragments from Partial Digests. Data on the largest fragments from various partial digests of 28S RNA are summarized in Table IV. Sedimentation values, percentage of the total RNA in each fragment, the Gp+Cp/Ap+Up ratios, and approximate chain lengths are shown. Rough estimates of molecular weight and therefore

TABLE II: Nucleotide Composition of 18S Fragments from Partial T_1 RNase Digest.[a]

Fraction	% Total RNA	Nucleotide Composition (moles/100 moles) Cp	Gp	Ap	Up	$\frac{Gp+Cp}{Ap+Up}$	$\left[\frac{Gp+Cp}{Ap+Up}\right]_{sp}$
1	4.5	42.6	32.0	11.2	14.2	2.94	2.20
2	18	35.7 ± 0.2	32.0 ± 1.1	15.1 ± 0.3	17.1 ± 1.0	2.10	1.56
3	29	32.3 ± 0.1	26.0 ± 0.1	22.1 ± 0.05	19.6 ± 1.1	1.40	1.05
4	10	33.5	15.5	27.0	24.0	0.97	0.72

[a] Fragments shown in Figure 4.

TABLE III: Nucleotide Composition of 28S RNA Fragments from Partial Pancreatic RNase Digest.[a]

Expt	Fraction	% Total RNA	Nucleotide Composition (moles/100 moles) Cp	Gp	Ap	Up	$\frac{Gp+Cp}{Ap+Up}$	$\left[\frac{Gp+Cp}{Ap+Up}\right]_{sp}$
a (see Figure 6a)	1	7	39.5	41.8	6.2	12.5	4.34	2.03
	2	18	34.7	38.1	13.3	14.0	2.67	1.25
	3	25	34.3	33.4	16.2	16.1	2.08	0.98
	4	22	28.6	33.1	20.4	17.9	1.61	0.75
b (see Figure 6b)	1	5.6	41.4	42.6	4.8	11.2	5.24	2.46
	2	41	37.2	36.7	13.8	12.3	2.83	1.33

[a] Fragments shown in Figure 6.

chain length of RNA fragments can be made from sedimentation values of RNA polynucleotides (Hall and Doty, 1958; Boedtker, 1960). In this paper, estimations of chain lengths have been made from a plot of $s_{20,w}$ values and molecular weight determinations from recent data (Hamilton, 1967; Comb and Zehavi-Willner, 1967; Stanley and Bock, 1965; Petermann and Pavlovec, 1966; Kurland, 1960; Brown, 1963). Molecular weights were obtained from the relationship $s_{20,w} = 0.025(\text{mol wt}^{0.492})$. Chain-lengths were estimated by assuming an average nucleotide molecular weight to be 322. These estimations should be considered approximate in view of the dependence of sedimentation rate on the shape of molecules and thus the environmental conditions of centrifugation. The chain lengths given in Table IV are those estimated from sedimentation values. It can be seen from Table IV that the 6.8S fragments having Gp+Cp/Ap+Up = 4.44 are approximately 270 in chain length.

TABLE IV: Estimation of Fragment Chain Length.[a]

Enzyme Employed	$s_{20,w}$ (S)	$\frac{Gp+Cp}{Ap+Up}$	% of 28S RNA	Approx Chain Length from Sedimentation Values
	28	2.13		
T_1	9.3	2.36	30	520
T_1	9.3	2.48	20	520
T_1	7.6	3.27		340
T_1	6.8	4.44	13	270

[a] Data from the largest fragments of different digests.

Discussion

The experiments described show that 28S and 18S RNA molecules contain segments with a Gp+Cp content higher than the average Gp+Cp composition of the intact molecules. That the high Gp+Cp regions of the 18S RNA originate from cross-contamination from the 28S RNA is unlikely. Ultracentrifuge ultraviolet photoelectric scanner patterns of the 18S RNA showed that no more than 5% contamination with 28S RNA can be present in the 18S RNA prepared from sucrose gradient separations (the arrow pointing upward in Figure 2b shows the position where the 28S RNA boundary would be if present in the 18S RNA preparations). In view of the labile nature of the large rRNA component (Midgley, 1965; Martin, 1966),

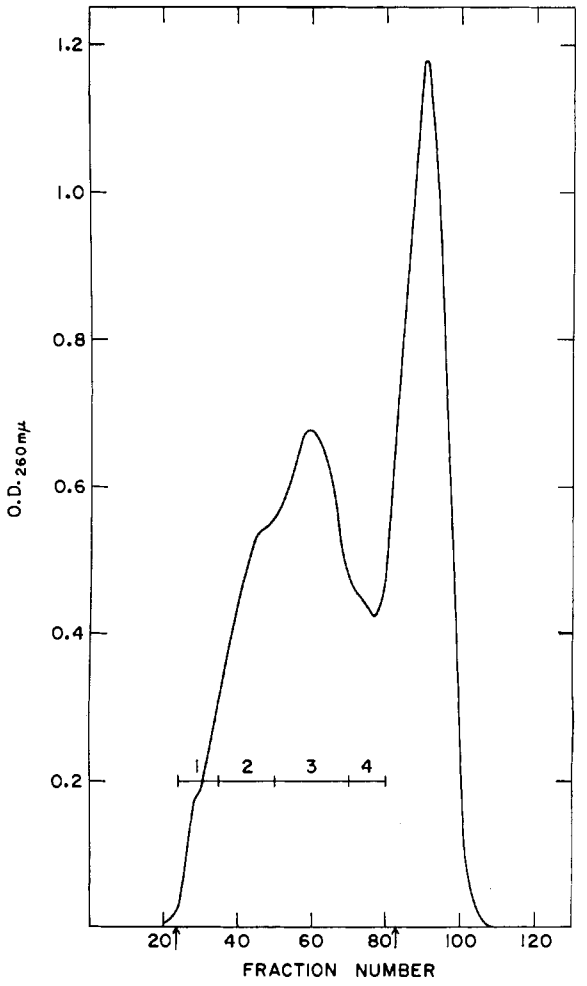

FIGURE 4: Sephadex G-200 separation of 18S RNA fragments from partial RNase T_1 digestion. Arrows on abscissa represent void and total volumes of the column. Digestion at 30° for 15 min, 0.1 M Tris-HCl–0.02 M EDTA (pH 7.5); 10 units/OD_{260}.

however, we cannot exclude the possibility of some 28S RNA degradation into the 18S RNA fraction before or during sucrose gradient centrifugation, although this appears unlikely in these experiments.

The present studies with 28S RNA fragments show that at least 10% of the RNA contains more than 80% Gp+Cp and more than twice the average Gp+Cp/Ap+Up value for the intact RNA. These high Gp+Cp regions are also characterized by a very low Ap content and Gp:Cp nearly equal to 1. The chain-length estimates of the largest fragments isolated from various digests (see Table IV) indicate that fragments with Gp+Cp:Ap+Up = 4.44 are ~270 in chain length. By a study of fragments from mild digestion, regions of 28S RNA rich in Ap+Up and having a nucleotide composition approaching that of DNA-like RNA were isolated. They consist primarily of 4.5S and 5.5S fragments and represent about 25% of the RNA.

Hadjiolov *et al.* (1967) have shown that the 3′ ends

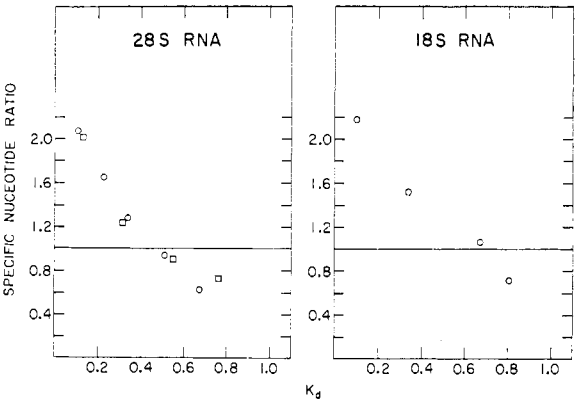

FIGURE 5: Relationship of specific nucleotide ratio, $(Gp + Cp/Ap + Up)_{sp}$, and distribution coefficient, K_d, in Sephadex G-200. Specific nucleotide ratio defined as

$$\left(\frac{Gp + Cp}{Ap + Up}\right)_{fragment} \Big/ \left(\frac{Gp + Cp}{Ap + Up}\right)_{intact\ RNA}$$

(○) T_1 RNase products; (□) pancreatic RNase products.

of 18S and probably 28S rat liver RNA are rich in adenine and uracil residues. Assuming rat and rabbit liver rRNAs have similar structures, one can exclude the 3′ end of the 28S RNA as the source of the high Gp+Cp segments and consider the 3′ end as a possible source of the Ap+Up-rich fragments.

By present concepts, rRNA secondary structure consists of polynucleotide chains containing both single- and double-stranded regions where the molecule is folded upon itself to produce helical regions with base-pair hydrogen bonding (see Fresco *et al.* (1960) and Spirin (1964) for detailed review). Support for this

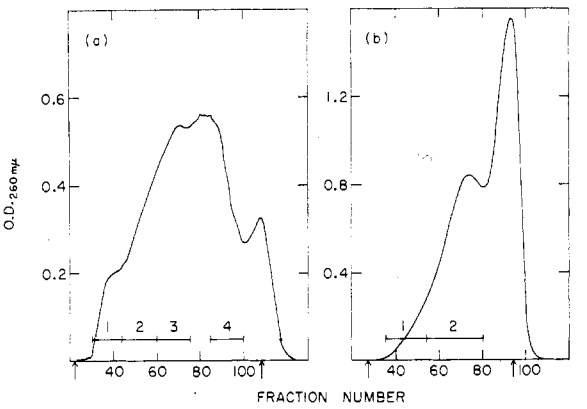

FIGURE 6: Sephadex G-200 separation of 28S RNA fragments produced by partial pancreatic RNase digestion. Arrows on abscissa represent void and total volumes of the column. (a) Digestion at 0° for 5 min, 0.1 M Tris-HCl (pH 7.5)–0.02 M EDTA; enzyme/RNA, 0.028 $\mu g/OD_{260}$. (b) Digestion similar to a except that enzyme/RNA is 1.45 $\mu g/OD_{260}$.

model comes from thermal denaturation studies (Doty *et al.*, 1959), spectrophotometric pH–titration studies (Cox and Arnstein, 1963), and, at least in the case of yeast RNA, the discovery of crystallizable fragments (Spencer and Poole, 1965).

One explanation for the resistance of high Gp+Cp segments to degradation by pancreatic and RNase T_1 is that these segments have extensive base-pair hydrogen bonding. The largest fragments isolated from several digests of 28S RNA are mostly Gp+Cp, where Gp:Cp nearly equals 1. The small fragments of ~2.5 S obtained under somewhat extensive degradation of 28 S RNA (Figure 6b; Table III, expt b, fraction 2) have near equal complementarity of base residues and may represent helical regions that resist enzymatic cleavage. Digestions, however, were performed under conditions unfavorable to a stable secondary structure, *i.e.*, they were carried out in Tris-HCl, with EDTA, without added salt. If these segments are largely helical regions, they must remain stable under these conditions.

Resistance to enzymatic attack may also be due to sites not readily available for cleavage because of polynucleotide complexing other than possible hydrogen bonding. For example, there may be hydrophobic interactions between guanine residues in guanine-rich fragments.

Resistance of RNA fragments to nuclease attack has been observed by others. Yeast RNA fragments, containing 50% guanine residues, that have not been completely deproteinated, are resistant to RNase T_1 (Itagaki *et al.*, 1965). Sober *et al.* (1966) found virus RNA fragments with high Gp and Cp to be protected by polylysine–polynucleotide interactions. In the studies described here, it is possible that the high Gp+Cp fragments have protein tightly bound and thereby offer resistance to nuclease attack, but changes in resistance after additional phenol extractions have not been observed.

Although there are striking similarities in the nucleotide compositions of T_1 and pancreatic RNase produced fragments, there are differences in the amounts of polynucleotide with given sizes in the intermediate digests. A comparison of Figure 3b, Table I, expt b and Figure 6a, Table III, expt a shows this to be so. Thirteen per cent of the 28S RNA is degraded to fragments less than 2 S with pancreatic RNase and only 7% of the RNA remains in the very high Gp+Cp fragments. With T_1 RNase, approximately three times as much (37%) is cleaved to very small fragments and about twice as much (13%) is present as large high Gp+Cp segments. Thus the high Gp+Cp regions are more susceptible to pancreatic RNase than to T_1 RNase, relative to degradation of the rest of the RNA to very small fragments.

The high Gp+Cp fragments separated by Sephadex G-200 may be heterogeneous. Studies using other separation techniques are needed to clarify this point.

Resistant fragments with a nucleotide composition similar to those described in this paper for rabbit liver rRNA have been obtained from chick embryo liver rRNA (unpublished data). Segments with over 80% Gp+Cp where the Up content is greater than that of Ap have been isolated from partial pancreatic RNase digests of chick embryo liver rRNA. Thus the high Gp+Cp regions may be common to liver rRNAs of many animal species.

Acknowledgments

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