STEPWISE DEGRADATION OF P^{32}- LABELED RAT LIVER RIBOSOMAL RIBONUCLEIC ACIDS WITH SNAKE VENOM PHOSPHODIESTERASE

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The distribution of nucleotides along ribosomal RNA (rRNA) chains is not known. Stepwise exonucleolytic degradation of RNA may provide information on the overall distribution of nucleotides along the polymer chain (see Burton, 1965). Previous studies with snake venom phosphodiesterase (SVD) provided evidence in favor of a non-random distribution of nucleotides along rRNA chains, the RNA segment near the 3'-end having a higher content of A and U, and a lower content of G and C residues, when compared with the segment near the 5'-end (Hadjiolov et al., 1966, 1967).

In the present work, rat liver rRNA, labeled for 1, 2 and 4 hours with phosphate-P^{32} in vivo, was stepwise degraded with purified SVD. It was found that the rRNA segments degraded initially by this enzyme have a markedly lower GC/AU ratio than those attacked at later stages of hydrolysis. The labeling of subsequently degraded rRNA segments (contributed by all four constituent nucleotides) decreases with progress of SVD action.

Materials and Methods

Male albino rats (120 to 160 g) were used. The livers were homogenized in Medium A (0. 25 M sucrose, 0. 005 M $MgCl_2$, 0. 05 M KCl, 0. 025 M Tris-HCl, pH 7. 6) and nuclei sedimented at 600 g for 5 min. Sodium dodecyl sulfate was added to the cytoplasmic fraction (final conc. 0. 5 %) and rRNA isolated as previously described (Hadjiolov et al., 1967). Agar gel electrophoresis was carried out according to Tsanev et al. (1966a). Snake venom phosphodiesterase (worthington Biochem. Corp., N. J.) was purified on Dowex 50 W, H^+ form, by the method of Keller (1964). Enzymatic hydrolysis of rRNA was performed in a pH-stat titration assembly at pH 8. 5 and 40° (Hadjiolov et al., 1967). At a

defined degree of rRNA hydrolysis, the sample was precipitated at -20° for 20 min. with 2 vol. of 95 % EtOH containing 2 % potassium acetate and centrifuged at 600 g for 5 min. The supernatant, containing the released 5'-mononucleotides, was concentrated in vacuo and the nucleotide composition determined according to Katz and Comb (1963). The precipitate of undigested rRNA was dissolved in distilled water, adjusted to pH 8.5 and hydrolyzed further. The molar ratio of the 5'-nucleotides liberated at 0-10, 10-20, 20-40 and 40-100 percent hydrolysis of rRNA was determined. Control experiments with pure AMP, GMP, CMP and UMP processed under the above conditions showed 95 to 98 % recovery. For radioactivity determinations, the UMP fraction was freed from P_i^{32} contaminants by chromatography on Dowex 1, formate form. The radioactivity of the nucleotide fractions was determined directly in solution with a Vakutronik VA-Z-431 jacket counter.

Results

As shown by agar gel electrophoresis, the bulk of the rRNA used is composed of 28 S and 18 S RNA components. The rRNA preparations analyzed resist 4 hours or more incubation at 40° and pH 8.5 without changes in their electrophoretic profile and are thus free from endonuclease contaminants.

The molar ratios of the 5'-nucleotides released during the stepwise degradation of rRNA with SVD are presented in Figure 1.

As can be seen, a gradual decrease of the molar ratio of pA and pU, and an increase of the molar ratio of pG and pC is established with progress of SVD action. These results confirm previous findings in which the total amount of 5'-mononucleotides released at different degrees of rRNA hydrolysis was determined by stopping the reaction with $HClO_4$ (Hadjiolov et al., 1966, 1967). Here, the use of EtOH precipitation of undigested RNA, gives information on the composition of the rRNA segments hydrolyzed at subsequent stages of SVD action. As expected, in this case, the variations in the molar ratios of the liberated nucleotides are more pronounced. It should be noted, that the GC/AU ratio of 1.11 for the nucleotides released at 0-10 % hydrolysis is lower than that of 18 S rRNA, while the value of 2.82 for the product liberated at 40 to 100 % hydrolysis, is markedly higher than the GC/AU ratio of 1.70 to 1.95 reported for 28 S rRNA (see review by Hadjiolov, 1967).

Agar gel electrophoresis of P^{32}-labeled rRNA showed, in accordance with data of Tsanev et al., (1966a), that the radioactivity profile coincides with the

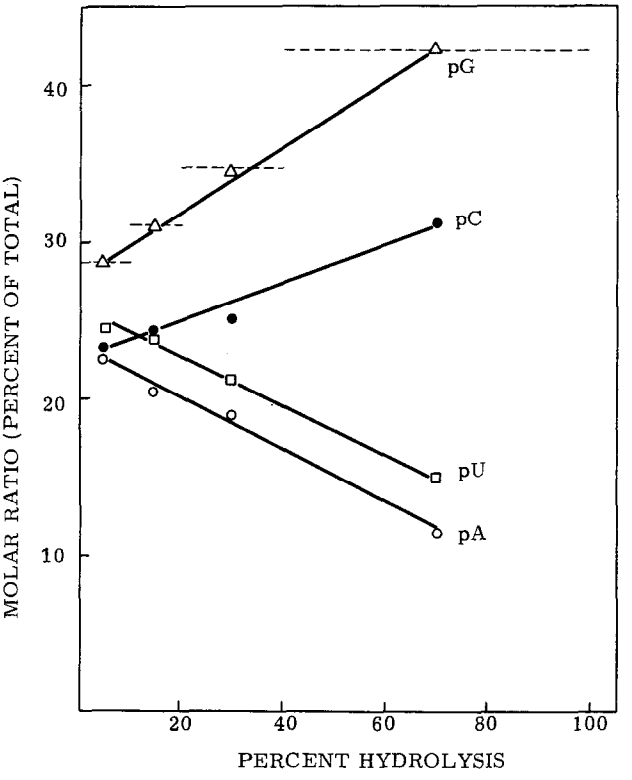

Figure 1. Changes in the molar ratios of 5'-mononucleotides released by stepwise hydrolysis of rat liver rRNA with snake venom phosphodiesterase. The experimental points represent the molar ratios of 5'-nucleotides which constitute the rRNA segments degraded at 0-10, 10-20, 20-40 and 40-100 % hydrolysis of rRNA (see dotted line for pG). Average values of 10 independant experiments are presented in the figure.

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28 S and 18 S rRNA peaks. The labeling of 18 S RNA is higher than that of 28 S RNA. The 18 S / 28 S ratio of the specific radioactivities of rRNA's decreases with extending the time of labeling (Henshaw et al., 1965; Hadjiolov, 1966; Tsanev et al., 1966a). The P^{32}-labeled rRNA was stepwise degraded with SVD and the specific radioactivity of the released 5'-mononucleotides determined. The activity of the rRNA segments degraded at subsequent stages of hydrolysis was calculated according to the formula

$$\text{s. a. RNA} = \frac{a_5A_5 + u_5U_5 + g_5G_5 + c_5C_5}{100}$$

where: s. a. RNA is the specific radioactivity of a given rRNA segment expressed

in counts/min per micromole of average nucleotide; a_5, u_5, g_5, c_5 - the molar ratio; A_5, U_5, G_5, C_5 - the specific activities of the respective 5'-mononucleotides. The results of these experiments are presented in Table 1.

The observed differences in the labeling of RNA-derived 5'-mononucleotides reflect variations in the labeling of ∝-phosphates in precursor nucleoside-5'-triphosphates at the nuclear sites of rRNA synthesis. The higher labeling of pA may be correlated with the role of adenine nucleotides as primary acceptors of P_i in oxidative phosphorylation. The markedly lower initial

Table 1. Specific radioactivities of the 5'-mononucleotides released during stepwise hydrolysis of P^{32}-labeled rRNA with snake venom phosphodiesterase.

Time of labeling*	Percent hydrolysis of rRNA	Agent	Specific activity (counts/min/micromole nucleotide)					
			Nucleotide				Segment of rRNA	Total rRNA
			A	U	G	C		
1 hr	0- 10	SVD	4794	3602	820	1482	2564	--
(N=3)**	10- 20	"	2477	985	479	689	1060	--
	20- 40	"	1691	432	409	627	671	--
	40-100	"	1270	254	194	486	416	746***
	0-100	OH^-	909	832	536	656	--	694****
2 hrs	0- 10	SVD	19634	10645	2274	5249	8978	--
(N=4)	10- 20	"	12348	4538	1460	3248	4859	--
	20- 40	"	9505	2321	1468	2554	3448	--
	40-100	"	5178	1745	823	1813	1764	3368
	0-100	OH^-	4902	3170	2804	3313	--	3132
4 hrs	0- 10	SVD	11074	2126	2950	3783	4788	--
(N=2)	10- 20	"	7442	1179	2116	2152	2994	--
	20- 40	"	5643	757	1834	1909	2347	--
	40-100	"	5240	712	1297	1763	1802	2329
	0-100	OH^-	2454	2104	2169	1813	--	2103

* The following doses of phosphate-P^{32} were administered intraperitoneally: 1 and 2 hrs - 1.0 mC; 4 hrs - 0.4 mC per 100 g of body weight.

** N - number of independent experiments. Average values are presented. Only the changes which were observed in all independent experiments of a given set are considered in the text.

*** Average values calculated from data for the separate rRNA segments.

**** The GC/AU ratios calculated from P^{32}-distribution among the 3'-nucleotides obtained on alkaline hydrolysis are: 1 hr - 1.12; 2 hrs - 1.27; 4 hrs - 1.47 .

(1 and 2 hrs) labeling of pG suggests a delayed metabolism of guanine nucleotides in nuclei. Delayed labeling of pG in nuclear RNA of Ehrlich ascites tumor cells has been reported (Hadjiolov et al., 1965).

Table 1 shows also that at all labeling times the specific activity of the 5'-nucleotides decreases with progress of rRNA hydrolysis by SVD. The derived specific radioactivity for RNA reveals that the rRNA segments degraded initially are more highly labeled than those hydrolyzed at later stages of SVD action. Contribution by all four 5'-nucleotides shows that our results reflect the synthesis of rRNA molecules de novo rather than addition of a single nucleotide to preexisting RNA chains. It should be noted also that the calculated specific activity of rRNA segments is obviously independent of nearest-neighbor relationships of the constituent nucleotides. As shown in Figure 2, non-equal labeling of subsequently degraded rRNA segments is the most pronounced at 1 hour labeling. A marked tendency toward equalization of the specific activities of rRNA segments with values for total rRNA is established with extension of labeling time.

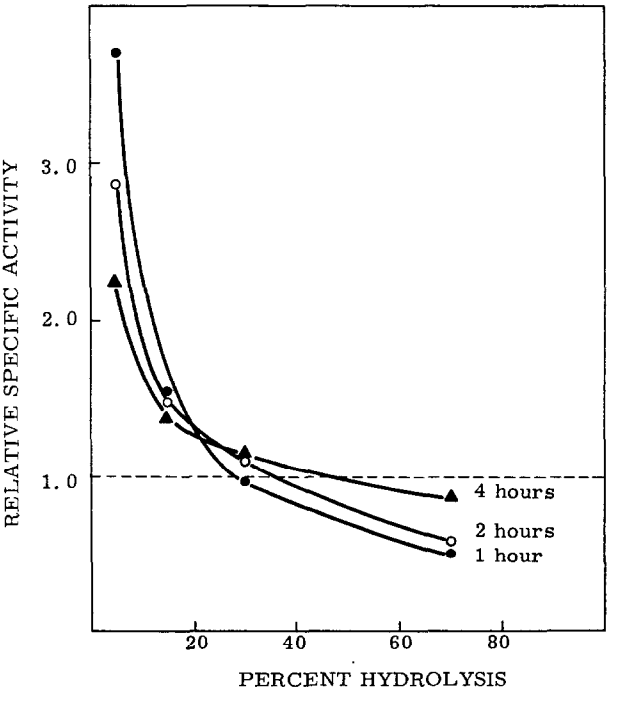

Figure 2. Specific radioactivity of rRNA segments related to the specific radioactivity of total rRNA taken as 1.0 . The experimental points represent the specific activity of the respective rRNA segments as indicated in Figure 1.

Discussion

As discussed previously (Hadjiolov et al., 1967), a major interference by endonuclease contaminants is highly unlikely. The absence of randomization of the label in subsequently degraded rRNA segments is in keeping with this conclusion. Two alternative explanations of our results may be envisaged.

1/ The rRNA studied is a mixture of rRNA and a hypothetical "DNA-like" RNA, the latter being characterized by a higher turnover rate (Henshaw et al., 1965; Tsanev et al., 1966b). In such case our results indicate that the "DNA-like" RNA: (a) is homogeneous in size and coincides with the ribosomal RNA components ; (b) constitutes a substantial amount of rRNA (about 10 to 12 %) ; (c) is degraded preferentially by snake venom phosphodiesterase.

2/ The distribution of nucleotides along rRNA chains is non-random. In such case our results show that: (a) the GC/AU ratio near the 3'-end of rRNA (18 S or 28 S, or both) is lower than near the 5'-end ; (b) the synthesis of rRNA proceeds from the 5'-end toward the 3'-end of the rRNA precursor molecule ; (c) uneven labeling along the rRNA chain is observed up to 4 hours labeling in vivo.

The observed GC/AU ratio of 2.82 for the last rRNA segment degraded by snake venom phosphodiesterase is incompatible with the first assumption since it shows directly that the overall distribution of nucleotides in rRNA is non-random. Recently, Delihas and Bertman (1966) isolated from partial T_1 ribonuclease digests of animal rRNA a high molecular weight fragment with a GC/AU ratio of 3.1 to 3.6. These results give independent evidence for the non-random distribution of nucleotides in rRNA.

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