The Chain Termini and Alkali-Stable Dinucleotide Sequences in Rapidly Labeled Ribonucleates from L Cells*

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ABSTRACT: When rRNA (ribosomal ribonucleates) or sRNA (soluble ribonucleates) are hydrolyzed in alkali, the resulting hydrolysis products can be resolved into four separate classes: nucleosides (N), nucleoside 2′- and 3′-phosphates (Np), nucleoside 2′(3′),5′-diphosphates (pNp), and alkali-stable dinucleotides (NxpNp, where x denotes 2′-*O*-methylation). The N and pNp compounds derive from 5′-linked and 3′-linked termini, respectively, while Np and NxpNp compounds derive from internal positions of polynucleotide chains: (3′-linked terminus) pNpNpNpN·····pNpNpNpN (5′-linked terminus). In the present study, the distribution of radioactivity among these different classes of compounds in alkali hydrolysates of L cell rRNA, has been compared with corresponding data for rapidly labeled RNA from L cells. Three rapidly labeled RNA specimens were prepared from L cells that had been exposed to four tritiated nucleosides (adenosine (A), guanosine (G), cytidine (C), and uridine (U)) for 15, 30, and 90 min, while rRNA was prepared from cells exposed to the tritiated nucleosides for 24 hr. All RNA preparations were repeatedly precipitated from 2.5 M sodium chloride solution at 0°, in order to remove sRNA.

The rapidly labeled RNA preparations were polydisperse and characterized by different amounts (20–75%) of material sedimenting faster than 28 S, whereas the 24-hr preparation displayed an essentially bimodal sedimentation profile, with peaks at 16 and 28 S. In the case of nucleosides, *all* preparations were remarkably similar with respect to the proportionate amount of radioactivity in a given nucleoside (N) relative to its homologous nucleotide (Np), *i.e.*, A/Ap, G/Gp, C/Cp, and U/Up radioactivity ratios were similar for all preparations. By contrast, in the case of pNp and NxpNp compounds, the proportionate amounts of radioactivity in rapidly labeled RNA were much lower than for rRNA, after 15 min, but approached the proportions for rRNA, after 90 min. These empirical similarities and differences are based on the primary experimental measurements, no assumptions having been made with respect to the relative mean specific activities at terminal and nonterminal positions in the polynucleotide chains of the different preparations. As such the relations can be considered as characteristic analytical parameters, which distinguish rRNA from rapidly labeled RNA. By invoking assumptions, the primary data can be used to assess molar percentages of the different components in the RNA preparations. These data are discussed in terms of the degree to which they might reflect features of the primary structure of the polynucleotide chains, and also in terms of the extent to which they might reflect differences of mean specific activity between the termini of the polynucleotide chains in the different RNA specimens.

In the first paper of this series we reported that it is possible, by using an alkali hydrolysis technique, to characterize terminal residues and 2′-*O*-methylribose-containing dinucleotide sequences in 16S and 28S ribosomal RNA[1] isolated from L cells incubated with four tritiated nucleosides (Lane and Tamaoki, 1967). This report describes a study designed to obtain similar analytical data for rapidly labeled RNA preparations. As in the previous work with ribosomal RNA, it was hoped that analyses of this kind might provide information about primary structure that could, in turn, be useful for evaluating the sedimentation profile of rapidly labeled RNA. With a variety of animal cells, rapidly labeled RNA has been shown to exhibit a polydisperse sedimentation profile, which is characterized by a large proportion of fast sedimenting material (>28 S) (Scherrer and Darnell, 1962; Tamaoki and Mueller, 1962; Perry, 1962, 1967; Hiatt, 1962; Georgiev *et al.*, 1963; Harel *et al.*, 1963; Seed and Goldberg, 1963; Yoshikawa *et al.*, 1964; Rake and Graham, 1964; Mach and Vassalli, 1965; Steel *et al.*, 1965; Scherrer *et al.*, 1966; Brown and Gurdon, 1966; Tsanev *et al.*, 1966; Attardi *et al.*, 1966; Georgiev, 1967).

The present experiments employed three pulse-labeled RNA preparations, which were prepared from L cells

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[1] Abbreviations used: 15-min RNA, 2.5 M NaCl-insoluble RNA prepared from L cells incubated for 15 min with four tritiated nucleosides; N, ribonucleoside; Np, ribonucleoside 2′(3′)-monophosphate; pNp, ribonucleoside 2′(3′),5′-diphosphate; NxpNp, alkali-stable dinucleotide in which N is any of the four principal ribonucleosides of RNA and Nx is the 2′-*O*-methyl derivative of any of the four major ribonucleosides.

TABLE I: Nucleosides Released from 5′-Linked Termini by Alkali Hydrolysis of Pulse-Labeled and rRNA.

Preparations	Hydrolysis Condn	Nucleotides (cpm × 10^{-6})				Nucleosides (cpm)				Nucleosides as Mole % of Homologous Nucleotides	
		Ap	Gp	Cp	Up	A	G	C	U	A/Ap	G/Gp
15-min RNA	0.25 M NaOH, 37°, 18 hr	1.39	2.09	1.55	0.894	2596	718	370	1391	0.19	0.034
30-min RNA	1.0 M NaOH, 25°, 24 hr	2.54	6.42	3.04	2.61	4870	1988	659	3897	0.19	0.031
90-min RNA	0.25 M NaOH, 37°, 18 hr	1.64	3.67	3.44	1.27	3028	1330	554	1943	0.18	0.036
24-hr RNA 1	1.0 M NaOH, 25°, 24 hr	3.30	4.88	8.84	3.93	4690	1250	1027	5542	0.14	0.026
24-hr RNA 2	0.25 M NaOH, 37°, 18 hr	5.98	5.50	9.48	5.08	7470	1522	710	8466	0.12	0.028

[a] These values were calculated from the values of nucleosides as mole per cent of homologous nucleotides (A/Ap, G/Gp, C/Cp, and U/Up) using the nucleotide composition data shown in Table II. For 15-, 30- and 90-min RNA preparations, the nucleotide composition of 30-min [^{32}P]RNA was used, and for 24-hr RNA preparations the nucleo-

exposed to four tritiated nucleosides for 15, 30, and 90 min. The sets of analytical data obtained for these RNA preparations were compared with the corresponding data obtained for ribosomal RNA of 24-hr labeled L cells.

When the RNA specimens were subjected to alkali hydrolysis, it was observed that characteristic proportions of the radioactivity in each of the four major nucleotides (Ap, Gp, Cp, Up) could be recovered in the homologous nucleosides (A, G, C, U) released from 5′-linked termini. The proportions (A/Ap, G/Gp, C/Cp, U/Up) found for pulse-labeled RNA preparations were remarkably similar to those for ribosomal RNA and displayed preponderant proportions of adenosine and uridine.

On the other hand, at 3′-linked termini, the pulse-labeled RNA preparations differed from ribosomal RNA. Following alkali hydrolysis, smaller proportions of the radioactivity in each of the four major nucleotides (Ap, Gp, Cp, Up) could be recovered in the homologous nucleoside 2′(3′),5′-diphosphates (pAp, pGp, pCp, pUp) released from 3′-linked termini of pulse-labeled RNA. The proportions (pAp/Ap, pGp/Gp, pCp/Cp, pUp/Up) were much smaller for 15- and 30-min preparations of pulse-labeled RNA, but approached those for ribosomal RNA, after 90 min.

Most of the 16 possible alkali-stable, 2′-*O*-methylribose-containing dinucleotides of the type NxpNp, have been recovered from alkali hydrolysates of both pulse-labeled RNA and ribosomal RNA. The proportionate radioactivities in the alkali-stable sequences of pulse-labeled RNA were markedly lower than those for ribosomal RNA, after 15 min, but approached the proportions in ribosomal RNA after 90 min.

The extent to which these differences may be related to a progressive change in specific activity of nucleotide residues, between the two ends of newly synthesized RNA molecules, and more general implications of such a change for chain-length assessments of pulse-labeled RNA, will be considered in the Discussion of the paper.

Materials and Methods

The methods employed in this study were essentially the same as those reported earlier (Lane and Tamaoki, 1967) and are briefly outlined below.

Preparation of Pulse-Labeled [^{3}H]RNA and Ribosomal [^{3}H]RNA from L Cells. L cells were grown in spinner flasks as described previously (Tamaoki, 1966). Cells at the stage of exponential growth (14 × 10^4 cells/ml) were incubated for 15, 30, and 90 min or 24 hr with [^{3}H]adenosine (8.76 c/mmole), [^{3}H]guanosine (2.0 c/mmole), [^{3}H]cytidine (2.34 c/mmole), and [^{3}H]uridine (9.34 c/mmole) at 0.8 μc/ml of culture medium each. RNA was extracted by shaking with phenol in the presence of 0.2% sodium dodecyl sulfate–0.2% bentonite, and precipitated once with two volumes of ethanol and then three times with 2.5 M NaCl. The NaCl precipitation procedure is known to remove DNA and low molecular weight RNA (4S and 5S RNA) (Crestfield *et al.*, 1955; Comb and Zehavi-Willner, 1967). The final precipitate was dried by successive washing in 67% ethanol, 95% ethanol, and ether.

In order to obtain sufficiently high total radioactivity suitable for end-group analysis, larger numbers of cells were employed for shorter term labeling: 640 × 10^6, 440 × 10^6, 150 × 10^6, and 90 × 10^6 cells were employed for 15-, 30-, and 90-min, and 24-hr labeling, respectively.

Determination of Chain Termini. RNA preparations were hydrolyzed in 1 M NaOH at 25.0° for 24 hr or in 0.25 M NaOH at 37.0° for 18 hr at a ribonucleate concentration of about 2 mg/100 μl of hydrolysate. A full description of the methods used for analysis of alkali hydrolysates of RNA has been published (Lane and Tamaoki, 1967).

Determination of Nucleotide Compositions. Nucleo-

TABLE I (*Continued*)

Nucleosides as Mole % of Homologous Nucleotides		Nucleosides as Mole % of Total Nucleotides[a]					Nucleosides as Mole % of Total Nucleosides				
C/Cp	U/Up	A/Np	G/Np	C/Np	U/Np	N/Np	A/N	G/N	C/N	U/N	Mean Chain Lengths
0.024	0.15	0.034	0.011	0.0065	0.035	0.087	39	13	7	40	$0.087^{-1} \times 100 = 1.1 \times 10^3$
0.022	0.15	0.034	0.010	0.0059	0.035	0.085	40	12	7	41	$0.085^{-1} \times 100 = 1.2 \times 10^3$
0.016	0.15	0.032	0.011	0.0044	0.035	0.082	39	13	5	43	$0.082^{-1} \times 100 = 1.2 \times 10^3$
0.012	0.14	0.027	0.0087	0.0035	0.025	0.064	42	14	5	39	$0.064^{-1} \times 100 = 1.6 \times 10^3$
0.0075	0.17	0.024	0.0093	0.0022	0.030	0.066	36	14	3	45	$0.066^{-1} \times 100 = 1.5 \times 10^3$

tide composition data of 24-hr [^{32}P]RNA was used. Example: mole % A/Np of 15-min RNA = mole % A/Ap × mole % Ap/Np × 1/100 = 0.19 × 18.0 × 1/100 = 0.034.

tide compositions of rapidly labeled RNA and ribosomal RNA were determined using ^{32}P-labeled preparations. Rapidly labeled [^{32}P]RNA was prepared from a culture incubated for 30 min with ^{32}P (33 mc/mg of P, Charles and Frosst and Co., Montreal, Quebec) at 4 μc/ml of culture medium, and ^{32}P-labeled ribosomal RNA from a culture incubated for 24 hr with the same amount of ^{32}P. The method for preparation of RNA was the same as that employed for ^{3}H-labeled RNA.

Sucrose Density Gradient Centrifugation. RNA dissolved in 0.001 M Mg^{2+}–0.01 M Tris-HCl buffer (pH 7.4) was placed on a 5 to 20% sucrose gradient made up with the same buffer, and then centrifuged at 35,000 rpm for 2 hr at 2 to 4° in the SW39 rotor of a Spinco Model L centrifuge. The optical density at 260 mμ was monitored continuously from the top of the gradient as described previously (Tamaoki and Miyazawa, 1966). The effluent was then collected in 0.25-ml fractions. Each fraction was mixed with 0.5 ml of water and 10 ml of "Polyether 611" scintillation fluid (Davidson and Feigelson, 1957) and then counted in a Nuclear-Chicago Mark I liquid scintillation counter.

Results

Sedimentation Characteristics of Pulse-Labeled RNA and Ribosomal RNA. The sedimentation patterns of RNA preparations obtained from L cells labeled with four tritiated nucleosides for 15, 30, and 90 min, and also for 24 hr, are shown in Figure 1. The 15-min RNA[1] (Figure 1a) sedimented over a wide range of the gradient with a major peak of radioactivity at 41 S, as estimated by comparison with the ultraviolet-absorbing 28S peak (Martin and Ames, 1961). Approximately 75% of the radioactive material sedimented faster than the 28S

TABLE II: The Nucleotide Composition of Pulse-Labeled [^{32}P]RNA and [^{32}P]rRNA from L Cells.

Preparations	Adenylate	Guanylate	Cytidylate	Uridylate
30-min [^{32}P]RNA	18.0	31.8	26.9	23.4
24-hr [^{32}P]RNA	19.4	33.3	29.3	17.9

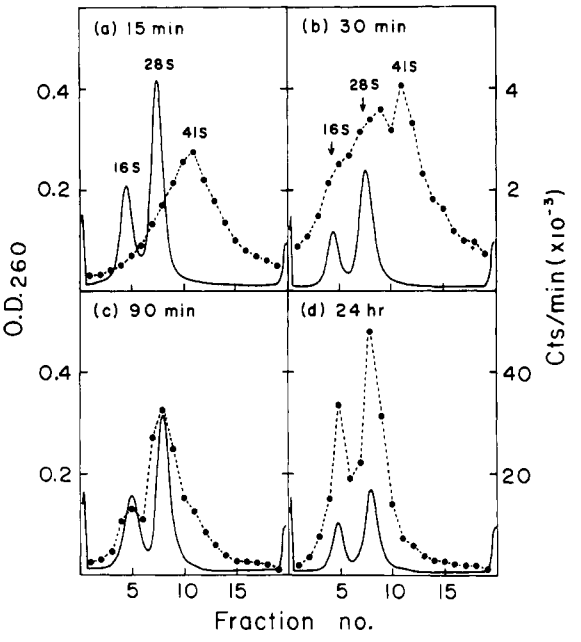

FIGURE 1: Sedimentation patterns of RNA prepared from L cells incubated with four tritiated nucleosides for 15, 30, and 90 min and 24 hr. (a) 15-min RNA; (b) 30-min RNA; (c) 90-min RNA; and (d) 24-hr RNA. (———) OD_{260}; (●- - - -●) cpm. The direction of sedimentation is from left to right.

TABLE III: Nucleoside Diphosphates Released from 3′-Linked Termini by Alkali Hydrolysis of Pulse-Labeled and rRNA.

Preparations	Hydrolysis Condn	Nucleoside Monophosphates (cpm × 10^{-6})				Nucleoside Diphosphates (cpm)			
		Ap	Gp	Cp	Up	pAp	pGp	pCp	pUp
15-min RNA	0.25 M NaOH, 37°, 18 hr	1.39	2.06	1.55	0.894	397	1692	306	247
30-min RNA	1.0 M NaOH, 25°, 24 hr	2.54	6.42	3.04	2.61	978	2167	515	733
90-min RNA	0.25 M NaOH, 37°, 18 hr	1.64	3.67	3.44	1.27	3437[b]		2391	1234
24-hr RNA 1	1.0 M NaOH, 25°, 24 hr	3.30	4.88	8.84	3.93	837	3250	8407	4760
24-hr RNA 2	0.25 M NaOH, 37°, 18 hr	5.98	5.50	9.48	5.08	2687	3833	8680	6167

[a] For method of calculation, see Table I. [b] Owing to incomplete separation of pAp and pGp in this analysis, it was necessary to combine the quantities of these compounds. [c] The pNp compounds were reincubated in 1 M NaOH at room temperature for 48 hr in order to effect the conversion of contaminating normal oligonucleotides to Np prior

component, and the remaining 25% sedimented more slowly, without showing definite correspondence with the ultraviolet absorption profile of the ribosomal RNA.

The 30-min RNA (Figure 1b) also exhibited a major peak of 41 S, but the relative amount of radioactive material sedimenting faster than the 28S component decreased to 60%, while that sedimenting more slowly increased to 40%.

After 90-min labeling (Figure 1c), 80% of the radioactive material sedimented with the ultraviolet-absorbing components, while the remaining 20% was found in the region of >28S.

After labeling for 24 hr (Figure 1d), the radioactive pattern coincided with that of the optical density of ribosomal RNA.

Terminal Groups of Pulse-Labeled RNA and Ribosomal RNA. Before describing the present results, it may be appropriate to summarize the earlier findings concerning the chain termini of the 16S and 28S components of L cell ribosomal RNA (Lane and Tamaoki, 1967).

Hydrolysis of 16S and 28S RNA in alkali releases nucleoside 2′(3′),5′-diphosphates and nucleosides in nearly equimolar amounts. Consequently the following structure has been assigned to these RNA components.

(3′-linked end or 5′-phosphomonoester end) pNpNpN- - - - - - - -

pNpNpN(5′-linked end or 3′-hydroxyl end)

With 16S RNA, uridine and adenosine are the major nucleoside constituents at 3′-linked and 5′-linked termini, respectively. With 28S RNA, cytidine and uridine are the principal nucleoside constituents at 3′-linked and 5′-linked termini, respectively. The mean chain lengths estimated from the amounts of nucleosides recovered from 5′-linked termini after alkali hydrolysis are 1.4 and 2.0 × 10^3 nucleotide residues for 16S and 28S RNA, respectively.

The chain-length estimate for 16S RNA is consonant with what might be expected from the particle weight found for the slower sedimenting component of ribosomal RNA from other animal cells. The chain-length estimate for 28S RNA, on the other hand, is substantially lower than expected from the particle weight estimates for the faster sedimenting component of ribosomal RNA from other animal cells. Possible reasons for the discrepancy between the chain-length assessment from alkali hydrolysis and the particle weight by physicochemical techniques, in the case of 28S RNA, include polydispersity of chain length, aggregation, or atypical nonphosphodiester bonds, and have been considered in an earlier report (Lane and Tamaoki, 1967).

5′-LINKED CHAIN TERMINI. Hydrolysis of pulse-labeled RNA preparations in alkali, as was the case with ribosomal RNA, was found to release nucleosides from 5′-linked termini. If it is assumed that the specific activity for a given nucleotide is the same at both terminal and nonterminal positions in the ribonucleate chain, then the proportion of a given nucleotide that is present at 5′-linked termini is given by the ratio of the amount of radioactivity found in a given nucleoside relative to that found in the homologous nucleotide (A/Ap, G/Gp, C/Cp, U/Up) following alkali hydrolysis. For each nucleotide, this ratio was calculated and recorded in Table I. It is seen that there is a marked similarity in these figures among the pulse-labeled RNA preparations studied. These ratios are in turn similar to those of 24-hr RNA, also shown in Table I, although there is a tendency for some figures to decrease, particularly that of A/Ap, after 24-hr labeling.

The proportions of these terminal groups as mole per cent of the total nucleotides (A/Np, G/Np, C/Np, U/Np) were calculated using the nucleotide compositions of 30-min [^{32}P]RNA and 24-hr [^{32}P]RNA shown in Table II. It is seen that in all RNA preparations,

TABLE III (*Continued*)

Nucleoside Diphosphates as Mole % of Homologous Nucleoside Monophosphates				Nucleoside Diphosphates as Mole % of Total Nucleotides[a]				
pAp/Ap	pGp/Gp	pCp/Cp	pUp/Up	pAp/Np	pGp/Np	pCp/Np	pUp/Np	pNp/Np
0.027	0.082	0.020	0.028	0.0049	0.026	0.0054	0.0066	0.043
0.039	0.034	0.017	0.028	0.0070	0.011	0.0046	0.0066	0.029[c]
0.065[b]		0.070	0.097	0.032[b]		0.018	0.023	0.073
0.025	0.067	0.095	0.12	0.0049	0.022	0.028	0.021	0.076[c]
0.045	0.070	0.092	0.12	0.0087	0.023	0.027	0.022	0.081

to analysis. The Np compounds formed by reincubation in alkali were removed prior to two-dimensional chromatographic separation of the individual pNp compounds (Lane and Tamaoki, 1967).

adenosine and uridine comprise the bulk of the 5′-linked termini, each accounting for approximately 40% of the total nucleosides recovered from 5′-linked termini.

The mean chain length of each RNA preparation can be calculated from the total amount of nucleosides recovered and was found to be 1.1–1.2 $\times 10^3$ nucleotide residues for the pulse-labeled RNA preparations and 1.5–1.6 $\times 10^3$ nucleotide residues for the 24-hr RNA preparations.

It should be noted that the above values depend upon the nucleotide composition with which mole per cent of A/Ap, G/Gp, C/Cp, and U/Up are converted to A/Np, G/Np, C/Np, and U/Np, respectively. It seems reasonable to assume that the over-all nucleotide compositions of 15-, 30-, and 90-min [^{3}H]RNA would not differ greatly from that of 30-min [^{32}P]RNA. As shown in Table II, the nucleotide composition of 30-min [^{32}P]RNA is similar to that for the 24-hr [^{32}P]RNA except for the higher value of Up. Recalculation of the figures for pulse-labeled RNA preparations using the nucleotide composition for the 24-hr [^{32}P]RNA does not result in important changes in the relative amounts of the 5′-linked terminal residues and the chain-length estimates.

It should also be noted that the data for the 5′-linked termini of the 24-hr RNA in Table I are in reasonable agreement with the values expected from end-group analyses of the individual 16S and 28S components reported previously (Lane and Tamaoki, 1967).

3′-LINKED CHAIN TERMINI. Analytical data for 3′-linked termini of RNA preparations are shown in Table III. As in the case of 5′-linked termini (Table I), the end groups recovered from 3′-linked termini, in the form of nucleoside diphosphates, are expressed as mole per cent of homologous nucleoside monophosphates (pAp/Ap, pGp/Gp, pCp/Cp, pUp/Up) and also as mole per cent of total nucleotides (pAp/Np, pGp/Np, pCp/Np, pUp/Np). For technical reasons which have been discussed in detail in the previous paper (Lane and Tamaoki, 1967), the data for 3′-linked termini are much less reliable than those obtained for 5′-linked termini, and the figures shown in Table III should be regarded as maximal values. It is apparent, however, that there is a marked difference between pulse-labeled RNA and ribosomal RNA.

In the case of 24-hr RNA, the amount of pNp compounds (0.08 mole %) can be regarded, with due allowance for experimental precision, as being equimolar with the quantity of nucleosides (0.065 mole %) since the somewhat higher estimate for pNp compounds is ascribable to technical factors concerned with the experimental measurements (Lane and Tamaoki, 1967).

The hydrolysis of the 24-hr RNA preparations was effected under conditions most suitable for pNp analysis: prolonged incubation in 1 M NaOH at room temperature (24-hr RNA 1 in Table III) (see Table III legend and Lane, 1965), and also under conditions where nucleoside tetraphosphates (pppNp) have been shown to be stable; 0.25 M NaOH at 37° for 18 hr (24-hr RNA 2 in Table III) (Maitra *et al.*, 1965; Bremer *et al.*, 1965). The similarity of the results obtained under these two sets of conditions indicates that the pNp compounds do not arise by decomposition of pppNp compounds.

In the case of 15- and 30-min RNA, significantly smaller amounts of pNp compounds than of nucleosides were found in alkali hydrolysates (0.04 *vs.* 0.08 mole % for 15-min RNA and 0.03 *vs.* 0.08 mole % for 30-min RNA). In the analysis of pNp of the 30-min RNA preparation, the pNp fraction eluted from the DEAE-cellulose column was reincubated, prior to paper chromatographic analysis, in alkali for 48 hr at room temperature to hydrolyze contaminating oligonucleotides. Failure to remove the oligonucleotides in this way results in a high-background radioactivity just behind the area occupied by the slowest moving pNp compounds (pAp and pGp) on two-dimensional paper chromatograms (Lane, 1965). With the 15-min

RNA preparation, this reincubation step was omitted, and as a consequence, the pNp compounds of the 15-min RNA preparation were more seriously overestimated than those of the 30-min RNA preparation. The analysis of pNp for 15-min RNA has little quantitative significance, and has been included simply for purposes of illustration.

The demonstrated stability of pppNp compounds after 18 hr in 0.25 M NaOH at 37° (Maitra *et al.*, 1965) suggests that it would be preferable in future studies to increase the time of incubation under these conditions in order to effect a complete hydrolysis of normal oligonucleotide material and thereby reduce the background of radioactivity impinging on the pAp and pGp areas of the chromatograms. It is perhaps noteworthy that, in the case of analysis of the 30-min RNA preparation in which contaminating oligonucleotides were removed by reincubation in alkali, no significant amount of radioactivity was detected in areas of the two-dimensional paper chromatograms where the pppNp compounds might have been found. The present experiments, however, were not specifically designed to examine the fate of pppNp compounds, and the failure to detect these compounds in this study cannot be taken as final evidence that pppNp compounds were absent from the alkali hydrolysates of the RNA preparations.

Alkali-Stable, 2'-O-Methylribose-Containing Dinucleotide Sequences. The proportions of different alkali-stable dinucleotide sequences in pulse-labeled RNA and 24-hr RNA preparations are presented in Table IV. These data are intended to be illustrative rather than quantitative since the accuracy of the measurements is limited by the sizeable normalization factors required to correct for unequal specific activities of the constituent nucleosides in the dinucleotides. The significant feature of the data is that most of the sixteen alkali-stable sequences of the type NxpN are present in pulse-labeled as well as ribosomal RNA, albeit in markedly smaller amounts, particularly in 15-min RNA.

Discussion

The four (radioactive) RNA preparations employed in this study were distinct from one another in their sedimentation properties (Figure 1). Yet, these same preparations exhibited remarkable similarities at 5'-linked termini. Characteristic proportions of the radioactivity in each of the major nucleotides (A/Ap, G/Gp, C/Cp, U/Up), could be recovered in the homologous nucleosides from 5'-linked termini. The proportions were similar for all preparations, although there was a decrease in some of the ratios, particularly A/Ap in ribosomal RNA. By assuming that the mean specific activity at terminal and nonterminal positions of polynucleotide chains is the same, these proportions (A/Ap, G/Gp, C/Cp, U/Up) can be taken to represent the moles of nucleosides at 5'-linked termini/100 moles of homologous nucleotides. These latter proportions can then be used, in concert with nucleotide composition data, to assess the proportions of different 5'-linked termini, and to arrive at an estimate of number-average chain length for the RNA specimens. On this basis, the nearly equimolar amounts of adenosine and uridine were found to cumulatively account for 80% of the 5'-linked termini in both pulse-labeled and 24-hr RNA, and the number-average chain lengths for the polynucleotides in each preparation, were in the range $1.1–1.6 \times 10^3$ nucleotide residues.

These interpretations of the data, in molecular terms, are subject to the reservations implicit in the assumption stated above, and warrant further discussion. A progressive increment of specific activity between the sites of chain initiation (3'-linked termini) and termination (5'-linked termini) would be expected to exert a less profound effect on the proportions of different 5'-linked termini, than on the estimates of polynucleotide chain length. Thus, it seems not unlikely that, for a given RNA preparation, there may be similar relative increments of specific activity for each of the four major nucleotides, and consequently, the proportions found for each nucleoside at 5'-linked termini, could reflect the molar proportions in which they are actually present. Although a remarkable series of compensatory changes could lead to the uniformity found for A/Ap, G/Gp, C/Cp, and U/Up, in different preparations, it seems more probable that the relative proportions of 5'-linked termini, are in fact, similar for all preparations. On the other hand, the increment of specific activity between initiation and termination sites could lead to reduced values for chain-length estimates based on 5'-linked termini. The magnitude of the relative increment of specific activity between 3'-linked and 5'-linked termini would be expected to be greatest when the rate of change of specific activities in the precursor pools was maximal, most likely during the early stages of uptake of the tritiated nucleosides by the L cells. Thus, it would be advisable to obtain further independent evidence bearing on the polynucleotide chain lengths for the different preparations, particularly for the shorter pulse times. Bramwell and Harris (1967) have suggested that the mean polynucleotide lengths in rapidly labeled RNA preparations may be similar to those for rRNA, and this would be consonant with the present findings. It should be noted that the present estimates, being number-average values, could be different from weight-average estimates for these same preparations, and this will be an important consideration with rapidly labeled RNA specimens, just as it appears that it may be with rRNA specimens (Lane and Tamaoki, 1967).

Finally, in regard to the 5'-linked termini, it should be noted that the basic pattern of similarity between pulse-labeled RNA and rRNA is empirical, and was not reliant on any interpretation of the measurements in molecular terms. In this context, the findings can be considered as important analytical parameters that serve to characterize the different preparations.

It has been suggested, based on kinetic and "pulse-chase" experiments that rapidly labeled RNA contains ribosomal precursor (Perry, 1962; Scherrer *et al.*, 1963; Rake and Graham, 1964), and unstable, DNA-like

TABLE IV: Alkali-Stable, 2′-*O*-Methylribose-Containing Dinucleotide Sequences in Pulse-Labeled RNA and rRNA.

Components	15-min RNA[d]			90-min RNA			24-hr RNA		
	Measured Radioactivity (cpm)[a]	Weighted Radioactivity (cpm)[b]	Mole %[c]	Measured Radioactivity (cpm)[a]	Weighted Radioactivity (cpm)[b]	Mole %[c]	Measured Radioactivity (cpm)[a]	Weighted Radioactivity (cpm)[b]	Mole %[c]
Ap	1.39×10^6	1.39×10^6		1.64×10^6	1.64×10^6		5.98×10^6	5.98×10^6	
Gp	2.09×10^6	2.38×10^6		3.67×10^6	2.82×10^6		5.50×10^6	10.2×10^6	
Cp	1.55×10^6	2.09×10^6		3.44×10^6	2.47×10^6		9.48×10^6	9.03×10^6	
Up	0.89×10^6	1.27×10^6		1.27×10^6	1.51×10^6		5.08×10^6	5.52×10^6	
	5.92×10^6	7.14×10^6		10.02×10^6	8.44×10^6		26.0×10^6	30.7×10^6	
AxpA	2,220	1,110	6	9,298	4,649	8	47,019	23,510	8
AxpG + GxpA	5,805	3,088	18	21,875	9,511	17	65,968	42,836	14
AxpC	2,320	1,333	8	10,614	4,441	8	48,140	23,483	8
AxpU	2,825	1,662	10	11,207	6,091	11	64,555	33,622	11
GxpG	3,945	2,241	13	16,636	6,398	11	24,118	22,331	7[e]
GxpC + CxpG	2,500	1,543	9	8,172	3,186	6	51,795	32,575	11[e]
GxpU + UxpG	1,610	1,019	6	9,276	4,334	8	31,607	21,649	7
CxpA	1,195	687	4	7,910	3,310	6	35,217	17,179	6
CxpC	1,740	1,176	7	11,956	4,301	8	56,428	26,870	9
CxpU + UxpC	2,770	1,924	11	13,432	6,023	11	75,682	38,417	13
UxpA	1,120	659	4	4,576	2,487	4	25,096	13,070	4
UxpU	1,408	1,006	6	3,070	1,827	3	18,448	10,026	3
	29,458	17,448		128,022	56,558		544,073	305,568	
Mole % Nx	$100 \times 17{,}448/7.14 \times 10^6 = 0.25$			$100 \times 56{,}558/8.44 \times 10^6 = 0.67$			$100 \times 305{,}568/30.7 \times 10^6 = 1.0$		

[a] Total radioactivity after correction for carrier recovery. [b] Radioactivity in each component has been weighted in accord with the specific activity of the component nucleoside(s). The specific activity for Ap(γ_{Ap}) was arbitrarily assigned a value of 1.00, and the specific activities of Gp, Cp, and Up were calculated relative to that for Ap using the nucleotide composition data of 24-hr RNA. The specific activities of the dinucleoside phosphates were obtained by adding the specific activities of the constituent nucleosides, assuming the specific activities of the nucleotides are the same throughout the ribonucleate chains. The specific activities for the nucleotides were: for 15-min RNA, $\gamma_{Gp} = 0.88$, $\gamma_{Cp} = 0.74$, $\gamma_{Up} = 0.70$; for 90-min RNA, $\gamma_{Gp} = 1.30$, $\gamma_{Cp} = 1.39$, $\gamma_{Up} = 0.84$; and for 24-hr RNA, $\gamma_{Gp} = 0.54$, $\gamma_{Cp} = 1.05$, $\gamma_{Up} = 0.92$. Thus, for example, $\gamma_{AxpC} = 1.74$ and 2.05 for 15-min and 24-hr RNA, respectively. The weighted values for radioactivity in each compound were then used to calculate the molar proportions of the alkali-stable sequences. [c] Relative amounts of the different alkali-stable sequences expressed as mole per cent of the total alkali-stable sequences. [d] Aside from the uncertainty inherent in the assumption of a mean specific activity for a given nucleotide throughout the chains, it should be noted that the values for 15-min RNA are subject to the additional uncertainty imposed by the smaller amounts of radioactivity available for the experimental measurements. [e] Technical problems concerning the estimation of carrier recoveries, in this particular analysis, are probably responsible for the underestimation of GxpG and the overestimation of Gxp C + CxpG.

RNA, and that the relative amounts of these species depend upon the time of labeling (Harris, 1963; Rake and Graham, 1964; Warner *et al.*, 1966; Perry, 1967). The existence of such different species is not revealed by pronounced quantitative or qualitative changes at 5′-linked termini. It should be noted that end-group methods, being number-average techniques, accentuate the contribution from smaller polymers and can be relatively insensitive to small weight percentages of larger polymers. This would be particularly relevant if any DNA-like RNA in the preparations examined in this work, had a much larger mean chain length than ribosomal precursor RNA. It is possible however that DNA-like RNA was eliminated by precipitation from sodium chloride solution, or by other preparative steps, used in the present study.

Reduced amounts of radioactivity in the pNp compounds derived from 3′-linked termini of pulse-labeled RNA provide an additional analytical parameter, which in this case, distinguishes pulse-labeled RNA from rRNA. The reduced proportion of radioactivity in the pNp compounds from pulse-labeled RNA could reflect an indirect route for the metabolic events that produce 5′-phosphomonoester termini, or a progressive increase of specific activity between 3′-linked and 5′-linked termini. Even if there is a very rapid rate of nucleotide polymerization,[2] the reduced proportion of radioactivity in pNp compounds could arise if 3′-linked termini are incorporated from a separate pool of compounds, which equilibrate relatively slowly with exogenous radioactivity.

In agreement with others (Saponara and Enger, 1966; Greenberg and Penman, 1966; Zimmerman and Holler, 1967), the present studies show that there are appreciable amounts of 2′-*O*-methylribosyl residues in rapidly labeled RNA. Because alkali-stable, 2′-*O*-methylribose-containing dinucleotide sequences are widely distributed in the polynucleotide chains of rRNA (McLennan and Lane, 1967), the proportions found in the present study are probably not profoundly affected by a progressive change of specific activity in nucleotides between the chain termini. Further studies, now underway, using [^{14}C]methyl of methionine as a precursor for the 2′-*O*-methyl groups in RNA, should produce additional evidence to test the conclusion from this present study, that there is a progressive increase of 2′-*O*-methylribose content with increased time of pulse labeling.

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[2] Assuming that "45S RNA" comprises 1% of the total RNA in an L cell and that "45S RNA" is turned over in roughly 1 hr in exponentially growing cells (Rake and Graham, 1964), then a molecular complement of 1500 nucleotides could be polymerized in about 10 sec, even if the molecules were being synthesized uniformly on as many as 1000 different cistrons.

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A New Heterocyclic Ring System, 8-Acetonyl-4-oxo-4,5-dihydroimidazo[2,1-*b*]purine, Resulting from the Reaction of Guanine and Deoxyribose*

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ABSTRACT: The white fluorescent material produced on acid hydrolysis of deoxyribonucleic acid is also formed on heating guanine with deoxyribose or furfuryl alcohol in dilute acid.

Complete structural elucidation, establishing the fluorescent compound as 8-acetonyl-4-oxo-4,5-dihydroimidazo[2,1-*b*]purine (I), was accomplished in the following steps. (1) Degradation of I with alkali gave guanine. (2) A survey of purines and pyrimidines demonstrated that a group such as N(R)C(NHR)=N or =NC-(NHR)=N was essential for production of fluorescent material upon reaction with furfuryl alcohol. (3) Bromine–water degradation of I produced an unstable intermediate which gave, after neutralization, guanine and presumed 2,4-dioxopentanal. The latter was converted to 1,2,4-pentanetriol by borohydride reduction. (4) The reduction product of I, 8-(2′-hydroxypropyl)-4-oxo-4,5-dihydroimidazo[2,1-*b*]purine (II), when subjected to bromine–water degradation gave a stable intermediate, 2-(3′,5′-dideoxy-2′ketopentofuranosyl)amino-6-oxo-1,6-dihydropurine (V) with an ultraviolet spectrum similar to that of 2-methylamino-6-oxo-1,6-dihydropurine-(N^2-methylguanine). Borohydride reduction of V gave an unstable product which decomposed to guanine and 2,4-dihydroxypentanal. (5) Methylation of II by dimethyl sulfate gave a dimethylated derivative (IV) which after bromine–water degradation, borohydride reduction, and neutralization gave 1,7-dimethylguanine and 2,4-dihydroxypentanal. (6) Nuclear magnetic resonance, infrared, and titration measurements confirmed the identity of specific structural features. Reaction sequences are proposed to explain the formation of I and its degradation by bromine–water.

Levy and Snellbaker (1954) originally detected a white fluorescent spot on paper chromatography of dilute acid hydrolysates of DNA and attributed this to "....a hitherto undescribed constituent of DNA." Frick (1956) and Dunn (1955) described the formation of what appears to be the same compound. Dunn presented evidence pointing to guanine as a precursor of the white fluorescent compound, and concluded that the white fluorescent compound is formed by a reaction between guanine and a degradation product of 2-deoxy-D-ribose in the presence of phosphoric acid. Simultaneous work by Hoard (1957) also demonstrated that guanine is a precursor of the white fluorescent compound; however, no requirement for phosphoric acid could be found. Hoard observed that the white fluorescent compound was formed when 2′-deoxyguanylic acid, 2′-deoxyguanosine, an equimolar mixture of 2-deoxy-D-ribose, and guanine, or a mixture of guanine and furfuryl alcohol was submitted to the same conditions used by Levy and Snellbaker for the production of the white fluorescent compound from DNA. None of the other usual purine or pyrimidine derivatives was active in the formation of the white fluorescent compound. It appears, then, that the white fluorescent compound is an artifact formed from guanine and an active fragment arising from 2-deoxy-D-ribose. Although Hoard's data were insufficient to arrive at

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