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Methylation of Sugars and Bases in Ribosomal and Rapidly Labeled Ribonucleates from Normal and Puromycin-Treated L Cells*

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ABSTRACT: Pulse-labeled ribonucleates were prepared from cells grown for 15–150 min in media containing [*methyl*-^{14}C]methionine, [^{14}C]cytidine, or [^{3}H]cytidine. Ribosomal ribonucleates were prepared from cells grown for 24 hr in media containing the same precursors. All ribonucleate specimens were repeatedly precipitated from 2.5 M NaCl, in order to remove low molecular weight ribonucleates, and were then subjected to both sedimentation and chemical analyses. Pulse-labeled specimens contained radioactive ribonucleates that sedimented faster than 28S ribosomal ribonucleate. With cytidine precursors, most of the radioactivity was confined to cytidine constituents of the ribonucleates even after 24 hr. When [*methyl*-^{14}C]-methionine was used as a precursor for the methyl substituents in the ribonucleates, a large proportion of radioactivity entered the carbon skeletons of adenosine and guanosine, even after the shortest period of labeling. The distribution of $O^{2'}$-methylribose among sequences of the type NmpN (where m denotes $O^{2'}$ methylation) was similar for *all* preparations, both pulse-labeled and ribosomal ribonucleates, but the amount of $O^{2'}$-methylribose, as a per cent of total sugars, increased with time of pulse labeling. Three methylated bases found in ribosomal ribonucleates were also detected in pulse-labeled ribonucleates. A comparison was made of pulse-labeled ribonucleates from cells grown for the same period of time in the presence, and in the absence, of puromycin. Ribonucleates from puromycin-treated cells contained a larger proportion of fast-sedimenting ribonucleates (>28 S) than did ribonucleates from normal cells.

However, the $O^{2'}$-methylribose content of ribonucleates from puromycin-treated cells was only slightly lower than that of ribonucleates from normal cells, and the pattern of NmpN sequences was nearly identical in the two types of ribonucleates. Significantly, ribonucleates from puromycin-treated cells contained much less N^6,N^6-dimethyladenine, and had a different pattern of carbon skeleton labeling, than did [*methyl*-^{14}C]methionine-labeled ribonucleates from normal cells. Accumulated data on the end groups and $O^{2'}$-methylribosyl constituents of pulse-labeled and ribosomal ribonucleates from L cells have been discussed in terms of the possibility that the fast-sedimenting material (>28 S) in ribonucleates from pulse-labeled cells may arise by secondary structural interactions within, or among, newly synthesized polynucleotides.

We have recently reported sedimentation and chemical analyses for several high molecular weight RNA specimens that were prepared from L cells, which had been incubated with four tritiated nucleoside precursors for periods of 15 min, 30 min, 90 min, and 24 hr (Lane and Tamaoki, 1967; Tamaoki and Lane, 1967a,b). Low molecular weight RNA was removed from the preparations by precipitation of the RNA from 2.5 M sodium chloride solution.

After a 15-min labeling period, 75% of the rapidly

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labeled RNA sedimented ahead of 28S rRNA in a sucrose density gradient, and the remainder of the RNA showed no definite correspondence with the sedimentation profile of the ultraviolet-absorbing rRNA components. After a 90-min labeling period, only 20% of the rapidly labeled RNA sedimented ahead of 28S rRNA in a sucrose density gradient, and the remainder of the RNA displayed substantial correspondence with the sedimentation profile of the rRNA components.

By chemical analysis, it was found that the pattern of 5′-linked (3′-hydroxyl) termini for *all* rapidly labeled RNA preparations was remarkably similar to that of rRNA from cells that had been incubated with tritiated nucleosides for 24 hr. It was also observed that $O^{2'}$-methylribose-containing nucleosides accounted for about 1% of the constituent nucleosides in 24-hr-labeled rRNA, but for a smaller fraction of the total nucleoside constituents in rapidly labeled RNA: only 0.25% after a 15-min labeling period, and 0.7% after a 90-min labeling period. For all RNA specimens, rRNA and rapidly labeled RNA, the $O^{2'}$-methylribose was found to be widely distributed, in similar proportions, among most of the 16 possible alkali-stable dinucleotide sequences of the type NmpNp.[1]

The present report records the results of further experiments designed to measure, more precisely, the distribution of NmpN sequences in the rRNA and rapidly labeled RNA preparations from L cells. For this purpose, L cells have been incubated for periods of 15 min, 30 min, 90 min, and 24 hr in a medium containing [*methyl*-^{14}C]methionine, which serves as the *in vivo* donor of methyl groups for $O^{2'}$ methylation of RNA. Additional experiments were designed in order to learn if the sedimentation profile of rapidly labeled RNA could be correlated with $O^{2'}$-methylribose content, or with the quantitative and/or qualitative disposition of $O^{2'}$-methylribose among different sequences of the type NmpN. Because puromycin arrests the changes of sedimentation profile that accompany synthesis of high molecular weight RNA in normal cells (Tamaoki and Mueller, 1963; Holland, 1963; Soeiro *et al.*, 1968), an analytical comparison was made of RNA samples prepared from cells incubated, in the presence and absence of puromycin, with [*methyl*-^{14}C]methionine, [^{14}C]-cytidine, or [^{3}H]cytidine.

In the course of these experiments, data have been accumulated on the methyl-substituted bases in rRNA and rapidly labeled RNA from L cells, and on the incorporation of radioactivity from [*methyl*-^{14}C]methionine into the carbon skeletons of the major purine nucleoside constituents of rRNA and rapidly labeled RNA.

Materials and Methods

Preparation of RNA Labeled with [methyl-^{14}C]Methionine 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 with [*methyl*-^{14}C]methionine (1 μCi/ml of culture, 52 mCi/mmole) for the periods indicated in each experiment.

At the end of incubation, the reaction was terminated by pouring the culture over crushed ice prepared from culture medium. The cells were harvested by centrifugation, washed with saline, and lysed, at a concentration of 15×10^6 cells/ml, in a solution of 0.2% sodium dodecyl sulfate–0.5 M NaCl–0.02 M trisodium citrate. Bentonite (powder, Fisher Scientific Co.) was added to the lysate to give a concentration of 0.2%, and the mixture was shaken with an equal volume of water-saturated phenol at room temperature. The aqueous layer was separated by centrifugation and reextracted with phenol twice more. The final extract was shaken with ether to remove residual phenol, and the dissolved ether was removed *in vacuo*. RNA was precipitated from the extract by the addition of either three volumes of 95% ethanol or 1.5 volumes of 2-propanol. The precipitate was dissolved in 0.01 M Tris buffer (pH 7.4) and reprecipitated with 2.5 M NaCl at 0°. Sodium chloride precipitation was repeated twice more. The final precipitate was dried by successive washing in 67% ethanol, 95% ethanol, and ether, and then stored at −30°.

The fractionation of 28S and 16S rRNA components was achieved by sucrose density gradient centrifugation as described previously (Lane and Tamaoki, 1967).

Preparation of RNA Labeled with [^{3}H]- or [^{14}C]Cytidine from L Cells. The method was the same as that used for preparation of RNA labeled with [*methyl*-^{14}C]-methionine, except that L cells were incubated with [^{3}H]cytidine or [^{14}C]cytidine at 1 μCi/ml of culture.

Sucrose Density Gradient Centrifugation. RNA, dissolved in 0.01 M Tris buffer (pH 7.4), was placed on a 5–20% sucrose gradient prepared from either Tris buffer or 0.001 M Mg^{2+}–0.01 M Tris buffer (pH 7.4), and was then centrifuged at 35,000 rpm for 3.5 (in the case of Tris buffer) or 2.5 hr (in the case of Tris–Mg^{2+} buffer) at 2–4° in the SW39 rotor in a Spinco Model L or L4 centrifuge. The absorbance at 260 mμ was monitored continuously from the top of the gradient as described previously (Tamaoki and Miyazawa, 1966). The effluent

[1] Abbreviations for nucleosides and dinucleoside phosphates conform with the recommendations of the Office of Biochemical Nomenclature (Director, W. E. Cohn, Oak Ridge National Laboratory, Oak Ridge, Tenn.). The symbol N is used as a general abbreviation for ribonucleosides, and the symbols A, G, C, U, and I are used as abbreviations for adenosine, guanosine, cytidine, uridine, and inosine, respectively. Methyl substitution of the *base* in a nucleoside is indicated by placing m at the left of the symbol for the nucleoside (mN), whereas methyl substitution of the *sugar* in a nucleoside is indicated by placing m at the right of the symbol for the nucleoside (Nm). The position of methyl substitution in a base is indicated by a superscript at the right of the symbol m, and if there is more than one methyl substituent at this position, the number of methyl substituents is indicated by a subscript at the right of the symbol m. Where methyl substitution occurs at an endocyclic atom, the position of methyl substitution is designated in terms of the position of attachment of the methyl substituent to the base; where methyl substitution occurs at an exocyclic N atom, the position of methyl substitution is designated in terms of the position of attachment of the exocyclic N atom to the base. Thus, 1-methyladenosine is abbreviated as m^1A, N^6,N^6-dimethyladenosine (6-dimethylaminopurine ribonucleoside) is abbreviated as m_2^6A, $O^{2'}$-methyladenosine is abbreviated as Am, and alkali-stable dinucleoside phosphates are generally abbreviated as NmpN.

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 Fiegelson, 1957) and the radioactivity was counted in a Nuclear-Chicago Mark I liquid scintillation counter.

Materials. [*methyl*-^{14}C]Methionine was purchased from Schwarz BioResearch Inc., Orangeburg, N. Y. (specific activity 52 mCi/mmole). [^{3}H]Cytidine (2.34 Ci/mmole) was purchased from New England Nuclear Corp., Boston, Mass. [^{14}C]Cytidine (27.2 mCi/mmole) was purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y. Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. *E. coli* alkaline phosphatase was purchased as a chromatographically purified enzyme (activity 32 units/mg) from Worthington Biochemicals Corp., Freehold, N. J. Nucleosides containing methyl substituents in their N-heterocycles were purchased from Cyclo Corp., Los Angeles, Calif., m^1A, m^6A, m_2^6A, m^1G, m^2G, m_2^2G, m^7G, m^3C, m^5C, m^5U, and m^1I. Alkali-stable NmpNp compounds were prepared from wheat embryo rRNA (Singh and Lane, 1964a).

Hydrolysis of RNA and Analysis of the Hydrolysates for Methyl-Substituted Components. A simplified system of paper chromatographic resolution has been developed as a replacement for the column chromatographic technique used in earlier studies (Singh and Lane, 1964a; Gray and Lane, 1967; Nichols and Lane, 1967; Tamaoki and Lane, 1967a,b). In earlier studies of nonradioactive RNA specimens, the use of column chromatographic techniques was essential since, for spectral analysis of NmpNp compounds, it was necessary to fractionate the hydrolysis products from 50 to 500 mg of RNA. With radioactive RNA specimens, of sufficiently high specific activity, the analyses for NmpNp compounds can be effected with much smaller quantities of RNA (1–5 mg), for which the capacity of paper chromatographic methods is adequate.

RNA (1–5 mg), dissolved in 225 μl of water, was mixed with 25 μl of 10 M NaOH, and the resulting hydrolysate (1 M NaOH) was incubated at room temperature for 90 hr. The hydrolysate was neutralized by addition of 100 μl of 10% acetic acid and evaporated to dryness, and the dry residue was mixed with 200 μl of carrier NmpNp compounds (1 μmole total, in the proportions found in wheat embryo rRNA), 10 μl of *Escherichia coli* alkaline phosphatase, and 50 μl of 1 M ammonium formate buffer (pH 9.2). The solution was incubated for 3 hr at 37° in order to dephosphorylate the Np and NmpNp compounds to yield N and NmpN compounds. After incubation, the digest was diluted to 100 ml, adjusted to pH 4, and desalted by passage through 200 mg of charcoal as described previously (Nichols and Lane, 1967), prior to application to Whatman No. 1 paper for descending paper chromatography.

The solvent used for chromatographic development was freshly prepared by mixing 50 volumes of 1-butanol, 35 volumes of water, and 17 volumes of 95% ethanol (Markam and Smith, 1949). After development for 15 hr, *all* of the NmpN compounds were resolved as a group from the nucleosides, which migrated ahead of the NmpN compounds as shown in Figure 1. By hydrolyzing 1 mg of RNA in 25 μl (rather than 250 μl) of 1 M NaOH, and by treating the hydrolysis products with 5 μl (rather than 10 μl) of alkaline phosphatase in a solution containing 20 μl (rather than 50 μl) of buffer, it was found that the evaporated digest, *without desalting*, could be directly applied to papers, without the detrimental effects observed when digests containing larger amounts of salt were chromatographed in the 1-butanol–ethanol–water solvent system. Recovery of materials was quantitative, and for this reason the abbreviated procedure was particularly useful in the analysis of samples having low specific activity.

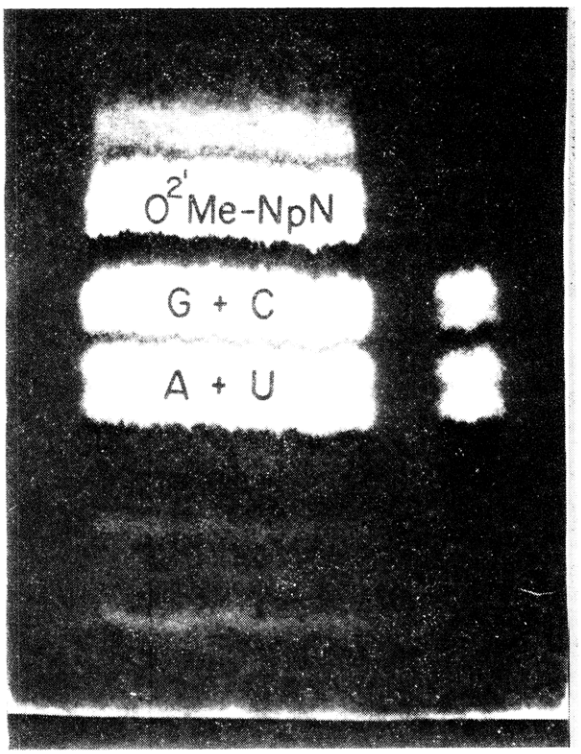

FIGURE 1: Ultraviolet contact photograph of a paper chromatogram showing the resolution of $O^{2'}$-Me-NpN compounds from nucleosides. The $O^{2'}$-MeNpN compounds (*ca.* 1 μmole) were added, as carriers, to an hydrolysate of L cell RNA that contained about 5 μmoles of nucleosides. The chromatogram was developed for 15 hr with the 1-butanol–ethanol–water solvent system (see text).

The chromatograms which had been developed with the 1-butanol–ethanol–water solvent system were sectioned into two parts: section A, containing material between the origin and the leading edge of the NmpN band (*cf.* Figure 1); section B, containing material between the rear edge of the G + C band and the solvent front (*cf.* Figure 1). Section A contained *all* of the methyl-substituted *sugar* derivatives, while section B contained, in addition to the four major nucleosides, *all* of the methyl-substituted *base* derivatives. Each of sections A and B was cut into small pieces for elution with 100 ml of distilled water. The eluates were evaporated to dryness, and the dry residues were dissolved in a small volume of water. The NmpN compounds from section A were separated by the two-dimensional paper chromatographic technique described earlier (Singh and Lane, 1964a). The nucleosides from section B were

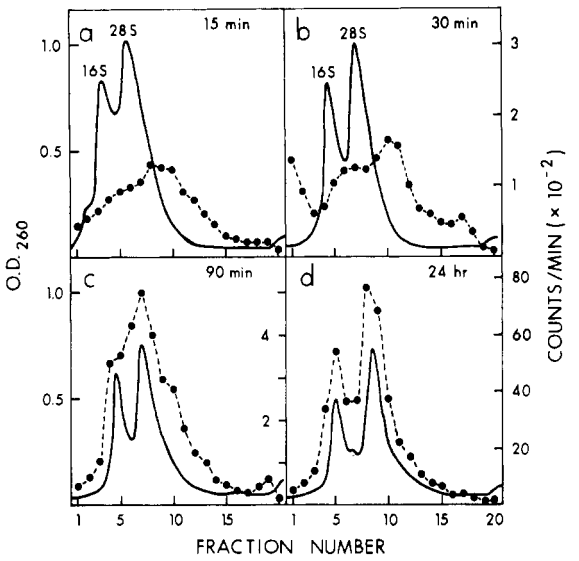

FIGURE 2: Sedimentation patterns of RNA prepared from L cells incubated with [*methyl*-^{14}C]methionine for (a) 15 min, (b) 30 min, (c) 90 min, and (d) 24 hr. (——) OD_{260}; (●– – –●) counts per minute. The direction of sedimentation is from left to right.

also resolved in two dimensions by the same paper chromatographic technique, under conditions previously described for resolution of the corresponding 5′-nucleotides (Hudson *et al.*, 1965). The solution of nucleosides was supplemented with 0.2 μmole of each of m^1A, m^6A, m_2^6A, m^1G, m^2G, m_2^2G, m^7G, m^3C, m^5C, m^5U, and m^1I, just prior to two-dimensional paper chromatography.

All areas of two-dimensional paper chromatograms were excized for radioactivity measurements, and more than 90% of the total radioactivity was recovered from the chromatograms. A small proportion of the total radioactivity (less than 10% of the radioactivity in the NmpN compounds) was present in the form of NmpN-mpN compounds, most of which were resolved from the NmpN compounds by two-dimensional paper chromatography (Singh and Lane, 1964b; Lane, 1965).

Methodology

It should be commented that the paper chromatographic resolution obtained with the 1-butanol–ethanol–water solvent system is especially suitable for fractionating the dephosphorylated alkali hydrolysis products from [*methyl*-^{14}C]RNA specimens. If the system is used for other purposes, then there are a number of considerations that deserve special attention. (i) The nucleosides m^1A, m^7G, and m^3C, unlike other methylated mononucleoside derivatives, are found to migrate in the area occupied by NmpN compounds in the 1-butanol–ethanol–water solvent system, presumably because they bear a positive electrical charge at pH values in the neighborhood of 5–7. However, m^1A and m^7G do not survive alkali hydrolysis (Brookes and Lawley, 1960, 1961), and m^3C is not a component of animal cell rRNA (Hall, 1963). Therefore, discrete separation of methyl-substituted *sugar* derivatives (NmpN), from methyl-substituted *base* derivatives, is possible in the case of products derived from an alkali hydrolysate of RNA. (ii) With [*methyl*-^{14}C]RNA preparations, it is not important to extend the time of hydrolysis in 1 M NaOH beyond 24 hr, because those normal oligonucleotides which are hydrolyzed very slowly, (*e.g.*, ApAp, ApCp; Lane and Allen, 1961) are not significantly radioactive and, as a consequence, do not interfere with measurements of NmpN. With [*cytidine*-^{14}C]RNA preparations, it is imperative to extend the time of hydrolysis in 1 M NaOH beyond 24 hr, because slowly hydrolyzed normal oligonucleotides, particularly ApCp in this case, are radioactively labeled, and if present, would cause erroneously high estimates of the NmpN compounds. For instance, [^{14}C]cytidine-labeled ApC would migrate with the NmpN compounds during chromatography in the 1-butanol–ethanol–water solvent system, and has been found to migrate with GmpC during the subsequent two-dimensional paper chromatographic separation of the NmpN compounds (Singh and Lane, 1964a). (iii) With [*methyl*-^{14}C]RNA hydrolysates, more than 90% of the radioactivity from section A of the 1-butanol–ethanol–water chromatograms is found to co-chromatograph with the carrier NmpN compounds during two-dimensional paper chromatography. However, with [*cytidine*-^{14}C]RNA hydrolysates, as much as 50% of the radioactivity from section A is cleanly resolved from the NmpN carrier compounds during the subsequent two-dimensional paper chromatography. Most of the additional radioactivity moves as fairly compact areas just ahead of cytidine and uridine in both dimensions, and as a consequence is well removed from the NmpN compounds. This material has not been further characterized, but it seems that it may be an alkali-conversion product of Cp, and to a lesser degree of Up, formed during the prolonged 90-hr hydrolysis in 1 M alkali (see Röttger and Fritz, 1962).

Results

Analysis of [methyl-^{14}C]rRNA Prepared from L Cells That Had Been Incubated with [methyl-^{14}C]-Methionine for 24 hr. The data in Table I summarize the results obtained from analyses of alkali hydrolysates of bulk rRNA (Figure 2d), 16S RNA, and 28S RNA specimens that were prepared from L cells incubated for 24 hr with [*methyl*-^{14}C]methionine. From the analytical data for bulk rRNA, it can be seen that about 35% of the total ^{14}C radioactivity was in the carbon skeletons of adenosine and guanosine. This ^{14}C radioactivity in the carbon skeletons of adenosine and guanosine contributes to the radioactivity measurements upon which the sedimentation profile is based, but, since the methyl-substituted components comprise only about 1% of the constituent nucleosides in L cell rRNA, this labeling of carbon skeletons accounts for less than 1% of the ^{14}C radioactivity found in methyl-substituted components, and makes no significant contribution to the analyses for NmpN sequences in Table I. Weighted proportions of the NmpN sequences in bulk rRNA (last column in Table I), *calcu-*

TABLE I: The Distribution of Radioactivity among Different Components of [*methyl*-^{14}C]16S RNA, -28S RNA, and -Bulk rRNA from L Cells.

Component	16S RNA cpm	16S RNA %[b]	28S RNA cpm	28S RNA %[b]	Bulk rRNA cpm	Bulk rRNA %[c]	16S + 28S RNA[a] %[b]	
Normal nucleosides								
A	14,620		16,236		102,207	35		
G					100,491			
Base-methylated nucleosides								
m^6A					11,236	5		
m_2^6A					10,363			
m^5C					8,605			
Sugar-methylated nucleosides								
AmpA	1,439	12	691	6	28,986	60	8	8
AmpG + GmpA	1,139	10	2,010	18	50,000		14	15
AmpC	730	6	866	8	24,390		7	8
AmpU	1,430	12	965	9	32,787		9	10
GmpG	1,417	12	1,582	14	46,512		13	14
GmpC + CmpG	1,097	9	925	8	27,777		8	9
GmpU + UmpG	1,144	10	1,106	10	39,215		11	10
CmpA	388	3	583	5	16,129		5	5
CmpC	745	6	761	7	23,810		7	7
CmpU + UmpC	1,041	9	1,106	9	37,736		11	9
UmpA	573	5	280	3	13,423		4	4
UmpU	492	4	130	1	9,803		3	2

[a] The weighted mean proportions were calculated from the measured proportions for 16S RNA and 28S RNA, assuming a mixture of the two types of RNA is comprised of two parts of 28S RNA and one part of 16S RNA, by weight. The 16S and 28S RNA preparations were prepared from cells incubated with [*methyl*-^{14}C]methionine (Calbiochem), which had one-fifth of the specific activity of the [*methyl*-^{14}C]methionine (Schwarz BioResearch) used to prepare the bulk rRNA, in a separate experiment. [b] Radioactivity in an individual NmpN compound, expressed as a percentage of the total radioactivity found in all of the NmpN compounds. [c] The percentage distribution of total ^{14}C radioactivity among the carbon skeletons of the *normal* nucleosides and the *methyl* substitutents of the methylated nucleosides.

lated for a 67% weight percentage of 28S RNA and a 33% weight percentage of 16S RNA, show good agreement with the *measured* proportions for bulk rRNA (second column from end in Table I), which was shown by sucrose density gradient centrifugation to be composed of 67% 28S RNA and 33% 16S RNA.

As seen in Table I, $O^{2'}$ methylation of sugars accounts for about 90% of the total methyl groups in L cell rRNA, and the remaining 10% of the methyl groups is present in bases. The amount of radioactivity found in each of m^6A, m_2^6A, and m^5C has been entered in Table I. Quite possibly, m^6A derives from m^1A by internal rearrangement during the alkali-catalyzed hydrolysis of RNA (Brookes and Lawley, 1960).

Analysis of [*methyl*-^{14}C]*RNA Prepared from L Cells That Had Been Incubated with* [*methyl*-^{14}C]*Methionine for 15, 30, and 90 min.* RNA from L cells incubated with [*methyl*-^{14}C]methionine for 15, 30, and 90 min exhibited sedimentation profiles of the type shown in Figure 2a–c, respectively. Chemical analyses of these RNA specimens are summarized by the data in Table II. It can be seen that 80% of the total ^{14}C radioactivity was incorporated into the carbon skeletons of adenosine and guanosine after 15 min, and that 65% of the total radioactivity was in carbon skeletons after 90 min. It seems particularly noteworthy that there was extensive incorporation into carbon skeletons of the L cell RNA, even after these brief periods of incubation with [*methyl*-^{14}C]methionine. It is also apparent from the data in Table II that the relative proportions of different NmpN sequences in *all* of these pulse-labeled RNA preparations are similar to the proportions found for the 24-hr-labeled rRNA. Any differences between the proportions found for rRNA and rapidly labeled RNA can probably be attributed, in large part, to a decreased analytical precision in analyses of rapidly labeled RNA specimens, where smaller amounts of radioactivity were available for the measurements.

A high proportion of carbon skeleton labeling has special relevance to the interpretation of sedimentation distributions of ^{14}C radioactivity, and for this reason,

TABLE II: The Distribution of Radioactivity among Different Components of Rapidly Labeled [*methyl*-^{14}C]RNA from L Cells.[a]

Component	15-min RNA		30-min RNA		90-min RNA		24-hr RNA	
	cpm	%[b]	cpm	%[b]	cpm	%[b]	cpm	%[b]
Normal nucleosides								
A + G	2,041		1,482		7,559		202,698	
Sugar-methylated nucleosides								
AmpA	56	10	83	7	279	8	28,986	8
AmpG + GmpA	81	15	178	15	519	14	50,000	14
AmpC	32	6	90	8	258	7	24,390	7
AmpU	51	9	116	10	351	10	32,787	9
GmpG	67	12	158	13	500	14	46,512	13
GmpC + CmpC	42	8	105	9	264	7	27,777	8
GmpU + UmpG	62	11	123	10	434	12	39,215	11
CmpA	22	4	60	5	178	5	16,129	5
CmpC	40	7	90	8	239	7	23,810	7
CmpU + UmpC	67	12	127	11	360	10	37,736	11
UmpA	17	3	44	4	143	4	13,423	4
UmpU	19	3	29	2	98	3	9,804	3

[a] The 15- and 30-min RNA preparations were prepared from cells incubated with [*methyl*-^{14}C]methionine (Calbiochem), which had one-fifth of the specific activity of the [*methyl*-^{14}C]methionine (Schwarz BioResearch) used to prepare the 90-min and 24-hr RNA preparations. Radioactivity entering the carbon skeletons of purine nucleosides accounts for about 80% of the total radioactivity in the 15-min RNA sample. Since the methylated components comprise only about 0.5% of the component nucleotides in a 15-min RNA specimen (using tritiated nucleoside precursors), the carbon skeletons of the methylated components contain only about 0.4% of the total radioactivity in a 15-min RNA sample. Since the methylated components contain about 20% of the total radioactivity in 15-min RNA, and about 0.4% of the total radioactivity is in their carbon skeletons, the radioactivity in the carbon skeletons of methylated components is (0.4/20.4) × 100 = *ca.* 2% of their *methyl*-^{14}C radioactivity. Since a correction for carbon skeleton labeling would be smaller than the experimental precision of ^{14}C-radioactivity measurements on the methylated components of 15-min RNA, no correction has been introduced. Correction for carbon skeleton labeling of methylated components would be even smaller for any other of the RNA specimens, and such a correction has been deemed negligible (see text, also). [b] Radioactivity in an individual NmpN compound, expressed as a percentage of the total radioactivity found in all of the NmpN compounds.

a preparation of [*methyl*-^{14}C]RNA from cells labeled for 30 min was resolved by sucrose density gradient centrifugation into two fractions. One fraction contained material of S > 28, and the other contained material of S < 28. The proportion of total radioactivity that was found in the carbon skeletons of each fraction was similar to that found for the parent, unfractionated RNA. There were only small quantitative differences between the two fractions with respect to the proportions of NmpN sequences.

The Effect of Puromycin, in the Incubation Medium, on the Pattern of RNA Methylation, When L Cells are Incubated for 90 min with [methyl-^{14}C]Methionine. The sedimentation patterns of RNA prepared from L cells incubated with [*methyl*-^{14}C]methionine for 90 min in the presence and absence of puromycin are shown in Figure 3. It is apparent that RNA from puromycin-treated cells has a lower mean specific activity and contains a larger proportion of rapidly sedimenting radioactive component than the RNA from cells cultured in the absence of puromycin. In spite of these differences, the data in Table III show that the pattern of NmpN sequences is very similar for the RNA specimens from both puromycin-treated and normal cells. The proportions found for nucleosides bearing methyl substituents in their bases were also generally similar for the RNA from puromycin-treated and normal cells. One exception, which appears to be significant, is that RNA from puromycin-treated cells contained proportionately less m_2^6A than RNA from normal cells. Another significant difference between RNA from puromycin-treated and normal cells can be seen in the degree to which the carbon skeletons of adenosine and guanosine are labeled with ^{14}C. Thus, in contrast with RNA from normal cells, the RNA from puromycin-treated cells displayed a proportionately greater labeling of adenosine than of guanosine.

The effect of puromycin on the incorporation of [^{3}H]-cytidine into RNA, for periods of 30, 60, and 90 min, is shown in Figure 4. It is evident from this figure

TABLE III: The Distribution of Radioactivity among Different Components of Rapidly Labeled (90 min) [*methyl*-^{14}C]RNA Prepared from L Cells That Were Incubated Either in the Presence or Absence of Puromycin.[a]

Component	−Puromycin cpm	−Puromycin %[b]	+Puromycin cpm	+Puromycin %[b]
Normal nucleosides				
A	3,875		1,739	
G	3,684		694	
Base-methylated nucleosides				
m^6A	145		74	
m_2^6A	102		20	
m^5C	105		46	
Sugar-methylated nucleosides				
AmpA	279	8	95	8
AmpG + GmpA	519	14	188	14
AmpC	258	7	88	7
AmpU	351	10	127	9
GmpG	500	14	190	13
GmpC + CmpG	264	7	108	8
GmpU + UmpG	434	12	177	11
CmpA	178	5	61	5
CmpC	239	7	89	7
CmpU + UmpC	360	10	158	11
UmpA	143	4	45	4
UmpU	98	3	32	3

[a] The figures for the nucleosides and for the dinucleoside phosphates are not directly comparable, since the recoveries of the two types of compound may not have been precisely the same when eluates from the 1-butanol–ethanol–water separation were recovered for two-dimensional paper chromatographic resolution of the individual nucleosides from section B and the individual dinucleoside phosphates from section A (see text). Thus, the recovery of nucleosides may be as much as 15% different from the recovery of dinucleoside phosphates, but the relative recoveries of individual nucleosides are probably very similar, as are the recoveries of individual dinucleoside phosphates. [b] Radioactivity in an individual NmpN compound, expressed as a percentage of the total radioactivity found in all of the NmpN compounds.

that the incorporation of [^{3}H]cytidine into RNA is greatly suppressed in the presence of puromycin. A comparison of the labeling patterns illustrated in Figures 3b and 4f shows that the [^{3}H]cytidine-labeled RNA (Figure 4f) contains a larger proportion of rapidly sedimenting radioactive material than does the [^{14}C]-methionine-labeled RNA (Figure 3b). This difference could reflect an incorporation of methyl groups from [^{14}C]methionine into polynucleotides, which had been

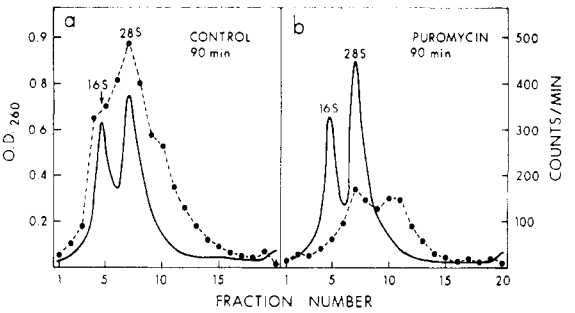

FIGURE 3: Sedimentation patterns of RNA prepared from L cells incubated with [*methyl*-^{14}C]methionine for 90 min in the presence or absence of puromycin. (a) RNA from untreated cells. (b) RNA from puromycin-treated cells. Puromycin (60 μg/ml) was added to the culture 30 min prior to the addition of [*methyl*-^{14}C]methionine. (——) OD_{260}; (●– – –●) counts per minute. The direction of sedimentation is from left to right.

synthesized before uptake of [^{14}C]methionine by the L cells (Saponara and Enger, 1966).

The Effect of Puromycin on the Extent of RNA Methylation When L Cells Are Incubated for 90 and 150 min with [^{14}C]*Cytidine.* Having established that the pattern of NmpN sequences in RNA from puromycin-treated and normal cells was similar, it was of further interest to determine if the extent of $O^{2'}$ methylation of newly synthesized RNA was also similar for puro-

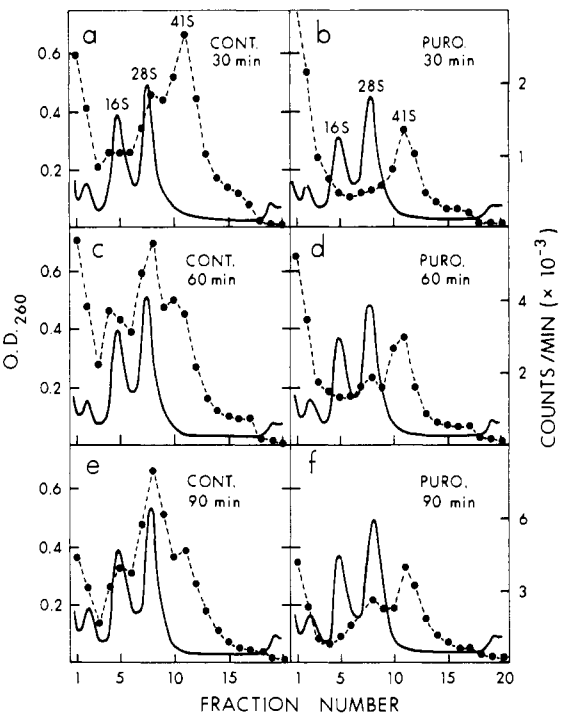

FIGURE 4: Sedimentation patterns of RNA prepared from L cells incubated with [^{3}H]cytidine in the presence or absence of puromycin. Puromycin (60 μg/ml) was added to the culture 30 min prior to the addition of [^{3}H]cytidine. (a) Control, 30 min labeled; (b) puromycin treated, 30 min labeled; (c) control, 60 min labeled; (d) puromycin treated, 60 min labeled; (e) control, 90 min labeled; and (f) puromycin treated, 90 min labeled. (——) OD_{260}; (●– – –●) counts per minute. The direction of sedimentation is from left to right.

TABLE IV: The Distribution of Radioactivity among Different Components of Rapidly Labeled (90 and 150 min) [*cytidine*-^{14}C]RNA Prepared from L Cells That Had Been Incubated Either in the Presence or Absence of Puromycin.

Conditions	Normal Nucleosides (N) (cpm)	Sugar-Methylated Nucleosides (NmpN) (cpm)	% of the Total Radioactivity Present in NmpN	Mole % $O^{2'}$-Methylribose[a] in the RNA
−Puromycin				
90-min RNA	448,665	4,899	1.09	0.68
150-min RNA	724,646	8,810	1.21	0.76
+Puromycin				
90-min RNA	180,614	1,668	0.92	0.58
150-min RNA	185,129	1,703	0.92	0.58

[a] Mole % $O^{2'}$-methylribose in RNA ($O^{2'}$-methylribose content), as a percentage of the total sugars, has been calculated from the values for % of the total radioactivity in NmpN (see text), *i.e.*, mole % $O^{2'}$-methylribose in RNA = $^1/_2 \times {}^{10}/_8 \times$ (% total radioactivity in NmpN). The specific activity of [*cytidine*-^{14}C]RNA from normal cells was 95.8×10^4 cpm/mg after 90 min, and 153.5×10^4 cpm/mg after 150 min. The specific activity of [*cytidine*-^{14}C]RNA from puromycin-treated cells was 45.5×10^4 cpm/mg after 90 min, and 62.5×10^4 cpm/mg after 150 min. In the case of RNA from both types of cell, 85% of the radioactivity was confined to cytidine, most of the remainder being in uridine, after correction for 10% deamination of cytidine during the 90-hr hydrolysis period in 1 M NaOH. The abbreviated technique described in the Methods section of this paper was used in processing the hydrolysates, in order to ensure uniform and quantitative recoveries for all components in the neutralized alkali hydrolysates.

mycin-treated and normal cells. In earlier investigations (Lane and Tamaoki, 1967; Tamaoki and Lane, 1967a), we measured the extent of $O^{2'}$ methylation of L cell rRNA and rapidly labeled RNA, by using RNA preparations labeled with four tritiated nucleosides. It was found in the case of rRNA that the proportion of total cytidine in sequences of the type NmpN was 1.6%, a value comparable with the proportion of total nucleosides present in the same sequences (2.0%). For this reason, and also because there is only limited equilibration of precursor cytidine with other nucleoside precursors, the extent of $O^{2'}$ methylation of RNA has been approximated in these present studies by using [^{14}C]-cytidine as the sole radioactively labeled precursor for RNA synthesis. The ^{14}C isotope rather than the ^{3}H isotope of cytidine was used in order to permit direct radioactivity measurements to be made with paper areas excized from paper chromatograms. The efficiency of measuring ^{14}C radioactivity is nearly tenfold greater than the efficiency of measuring ^{3}H radioactivity in this way. Since $O^{2'}$-methylribose comprises one-half of the constituent sugars in NmpN sequences, and since the proportion of total RNA cytidine (1.6%) is 80% of that of total RNA nucleosides (2.0%) in the NmpN sequences of rRNA, the $O^{2'}$-methylribose content of RNA (mole per cent) can be calculated by multiplying the proportion (per cent) of total radioactivity in the NmpN sequences of [*cytidine*-^{14}C]RNA specimens by $^1/_2 \times {}^{10}/_8$ (see Table IV).

Sedimentation patterns of the RNA preparations used in the study are shown for 90- and 150-min labeling periods in Figure 5, and chemical analyses of these RNA specimens from puromycin-treated and normal cells have been summarized in Table IV. The extent of $O^{2'}$ methylation after 90 min was about 0.68% with RNA from normal cells and about 0.58% with RNA from puromycin-treated cells. After labeling for 150 min, the extent of $O^{2'}$ methylation increased to 0.76% for normal cell RNA, but the value for RNA from puromycin-treated cells remained the same (0.58%). Notably, incorporation of [^{14}C]cytidine into the RNA from puromycin-treated cells was only 40–50% as great as incorporation into the RNA from normal cells.

Discussion

The results of the present experiments with [*methyl*-^{14}C]methionine-labeled RNA from normal cells have confirmed and extended the conclusions from our earlier studies in which preliminary estimates were obtained for the proportions of different NmpN sequences in RNA preparations labeled with four tritiated nucleosides (Lane and Tamaoki, 1967; Tamaoki and Lane, 1967a). Particularly significant is the similarity of the pattern of NmpN sequences in *all* rapidly labeled [*methyl*-^{14}C]RNA specimens with the corresponding pattern in 24-hr-labeled [*methyl*-^{14}C]rRNA. This similarity was anticipated from the results of our earlier experiments (Tamaoki and Lane, 1967a), but the earlier findings were not definitive because only limited precision is possible in experiments in which four tritiated nucleosides, of different specific activities, are incorporated into ribonucleates, for which only approximate nucleotide compositions are measurable. The approximate data obtained in the earlier investigation of [^{3}H-nucleoside]RNA corroborate the present data obtained with [*methyl*-^{14}C]RNA, and conse-

quently, independent support has been provided for the assumption that the mean specific activity of *methyl*-^{14}C from methionine is the same in all of the NmpN sequences, as has been assumed in the present study, where proportionate radioactivities have been taken as being the same as molar proportions of NmpN sequences.

Although labeling with [*methyl*-^{14}C]methionine affords a convenient measure of the relative proportions of different $O^{2'}$-methylribose-containing NmpN sequences, it does not provide a measure of the amount of $O^{2'}$-methylribose as a per cent of the total sugars in an RNA specimen. In our earlier studies, using [^{3}H-nucleoside]RNA, it was possible to assess the amount of $O^{2'}$-methylribose as a per cent of the total sugars in RNA preparations. It was shown that $O^{2'}$-methylribose comprises 1.0% of the sugars in 16S plus 28S RNA from L cells (Tamaoki and Lane, 1967a). The amounts of $O^{2'}$-methylribose in rapidly labeled RNA preparations were smaller, being only 0.25% after 15 min, but increasing to 0.7% after 90 min (Tamaoki and Lane, 1967a). Similarly, in the present experiments, the increase in the labeling time from 90 to 150 min was found to be accompanied by increased $O^{2'}$-methylribose content and a decreased proportion of rapidly sedimenting RNA (Figure 5a,c).

A comparison of rapidly labeled RNA from puromycin-treated and normal cells for the same period of pulse labeling shows that a smaller proportion of rapidly sedimenting material in the RNA from normal cells is associated with a somewhat higher $O^{2'}$-methylribose content (Table IV). After labeling for 150 min, the difference in the $O^{2'}$-methylribose contents, between "normal" and "puromycin" RNA, became more pronounced, since there was no further increase in the degree of methylation of "puromycin" RNA. The cause of this arrested $O^{2'}$-methylribose content, in the presence of puromycin, is not clear at present. However, it may be said, with RNA samples from cells labeled for 90 min, that the large difference in sedimentation profiles between "normal" and "puromycin" RNA is not reflected by a correspondingly large difference in the degree of $O^{2'}$ methylation in the RNA samples.

Any small quantitative differences between the RNA specimens from normal and puromycin-treated samples, with respect to $O^{2'}$-methylribose content, are overshadowed by the striking similarity between their patterns of $O^{2'}$ methylation (Table III). A discrete difference between RNA from puromycin-treated and normal cells has been observed in the case of one of the base-methylated components, m_2^6A, which was found in smaller proportion in the RNA from puromycin-treated cells than in RNA from normal cells. This may indicate that methylation of adenine, to yield N^6,N^6-dimethyladenine, occurs relatively late in the processing of precursor rRNA in L cells.

The results of our studies show broad general agreement with studies of the $O^{2'}$ methylation of rRNA and nucleolar precursor rRNA (45S RNA) from Hela cells (Vaughan *et al.*, 1967). The most significant difference between L cell and Hela cell RNA appears in relation to the sequence AmpC. This sequence, which

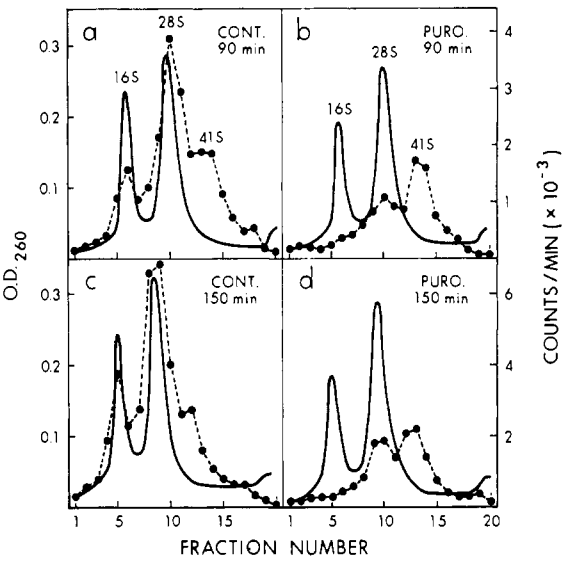

FIGURE 5: Sedimentation patterns of RNA prepared from L cells incubated with [^{14}C]cytidine in the presence or absence of puromycin. Puromycin (60 μg/ml) was added to the culture 30 min prior to the additon of [^{14}C]cytidine. (a) Control, 90 min labeled; (b) puromycin treated, 90 min labeled; (c) control, 150 min labeled; and (d) puromycin treated, 150 min labeled. (——) OD_{260}; (●– – –●) counts per minute. The direction of sedimentation is from left to right.

we have found in 16S RNA, 28S RNA, and *all* rapidly labeled RNA preparations from L cells, was not found in either of the rRNA components from Hela cells (Wagner *et al.*, 1967).

We have shown (Tamaoki and Lane, 1967a,b) that the pattern of 5′-linked (3′-hydroxyl) termini of pulse-labeled RNA is similar to that of bulk rRNA from L cells. Additionally, the mean chain length of the pulse-labeled RNA, estimated from end-group data, was also found to be similar to that of bulk rRNA. These data suggest that pulse-labeled RNA sediments faster than rRNA because the polynucleotides in pulse-labeled RNA tend to aggregate or to have a more compact conformation than the polynucleotides in rRNA (Muramatsu *et al.*, 1966; Bramwell and Harris, 1967). It is possible that $O^{2'}$ methylation might influence inter- or intramolecular interactions by abolishing a hydrogen bond between the 2′-hydroxyl function of ribose and the internucleoside phosphate–oxygen (Brahms and Sadron, 1966). Such an abolition of hydrogen bonds might directly induce localized expansions in the secondary structure of a polynucleotide, or indirectly potentiate tertiary structural changes that may accompany encapsulation of rRNA during ribosome formation.

In the present study of L cells, it has been found that an increased $O^{2'}$-methylribose content in rapidly labeled RNA preparations is not accompanied by significant changes in the *relative* amounts of different $O^{2'}$-methylribose-containing NmpN sequences. Similar observations with Hela cell RNA have been interpreted to indicate that undermethylated segments in a giant-size polynucleotide are deleted by selective chain scissions (Weinberg *et al.*, 1967; Vaughn *et al.*, 1967). However, it should be noted that the same observations would be equally compatible with the view that a

mixed population of methylated and undermethylated (or unmethylated) polynucleotides, similar in *mean* chain length to rRNA, is being depleted with respect to its *proportion* of undermethylated (or unmethylated) polynucleotides.

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