Low Molecular Weight Methylated Ribonucleic Acid Species from Chinese Hamster Ovary Cells. I. Isolation and Characterization*

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With the Technical Assistance of J. L. Hanners

ABSTRACT: Preparative polyacrylamide gel electrophoresis has been used to isolate four methylated, low molecular weight ribonucleic acid molecules from Chinese hamster cells. They have been shown to be distinct from transfer ribonucleic acid and 5S ribosomal ribonucleic acid and are found in a fraction enriched with respect to nuclei. Investigation of the products of alkaline hydrolysis of three of these supports the conclusion that they are unique ribonucleic acid species, distinguishable from other known methylated ribonucleic acids or from their possible breakdown products.

Until recently, RNA was considered to belong to one of three categories: transfer, ribosomal, or messenger. Development of more sophisticated analytical techniques has permitted the detection of a number of new RNA species, among which are RNAs of relatively low molecular weight (5–8 S). Such small RNAs have been found in or associated with ribosomes (Rosset and Monier, 1963), microsomal membranes (Gardner and Hoagland, 1968), smooth endoplasmic reticulum (King and Fitschen, 1968), nuclei (Dingman and Peacock, 1968), and nucleoli (Nakamura *et al.*, 1968). In the course of an investigation of the small RNAs isolated from exponentially growing Chinese hamster cells, we observed that 5–6% of the radioactivity from L-[methyl-^{14}C]methionine-labeled 2–10S RNA was found in the 5S RNA region (*cf.* Figure 2a; Galibert *et al.*, 1967) of a Sephadex G-100 chromatogram. Since it has been reported that 5S rRNA is unmethylated (Watson and Ralph, 1966; Comb and Zehavi-Willner, 1967), a more extensive investigation was initiated. This paper reports the isolation of four methylated, low molecular weight RNAs which are distinguishable from tRNA or 5S rRNA. These small methylated RNA species are found in a cell fraction enriched with respect to nuclei and are perhaps nucleolar. Through the use of preparative gel electrophoresis, three of these RNAs have been sufficiently resolved that further studies on the methyl distribution within each were possible.

The methyls in RNA are attached either to the heterocyclic base moiety or to the 2′-oxygen of ribose. Since sugar methylation renders the adjacent phosphodiester bond refractory to alkaline hydrolysis (Lane and Butler, 1959; Smith and Dunn, 1959), valuable data on the distribution of methyls within a RNA molecule can be readily obtained by DEAE–urea fractionation of methylmethionine-labeled, alkali-generated fragments. In such a separation scheme base-methylated species are liberated as mononucleotides with a charge of −2, an *O*-methyl ribotide manifests itself as a dinucleotide diphosphate with a charge of −3, and two adjacent *O*-methyl ribotides form a trinucleotide with a charge of −4. The separation of such components by virtue of these differences in charge by chromatography on DEAE-cellulose at pH values near neutrality in the presence of 7.0 M urea is well documented (Tomlinson and Tener, 1963; Singh and Lane, 1964; Nichols and Lane, 1967). When methyl-labeled RNA from Chinese hamster cells was degraded and fractionated in this manner, it was noted that (in addition to the predicted materials) a small amount of labeled material behaved as though it had no charge. Appropriate experiments have shown that such material is not a 3′-end group, nor does it arise from dephosphorylation of mononucleotides. Such a species (hitherto unreported) has been found in substantial amount in each of the three methylated, presumably nuclear, RNA species discussed in this paper. Furthermore, it will be shown that these "nuclear" RNA species are unique in that they lack significant amounts of methylated mononucleotides but have an abundance of di- and trinucleotides in their alkali digestion products.

Experimental Procedures

Cell Culture. The line of Chinese hamster cells used in these experiments and its growth characteristics have been described (Petersen and Anderson, 1964; Enger

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et al., 1968). The cells are aneuploid and have a modal chromosome number of 21. Under the growth conditions employed during isotope administration, their doubling time was 18 ± 1 hr. Ham's F-10 medium was used as described (Enger *et al.*, 1968) except that half the usual amount of methionine was used in the synthetic portion of the medium. Calf serum (which makes up 15% of the medium) was not dialyzed to remove endogenous methionine. Cell concentrations were kept between 9×10^4 and 5×10^5 per ml at all times in order to maintain exponential growth.

Cell Fractionation. Cells were centrifuged from the growth medium and washed with cold 0.25 M sucrose, and finally with 0.25 M sucrose containing 5 mg of bentonite/10^8 cells. Washed cells were suspended at approximately 5×10^7 ml in 0.01 M sodium cacodylate (pH 7.4) containing 0.13 M NaCl and 1.5×10^{-3} M $MgCl_2$ and were shell frozen in Dry Ice–ethanol. The frozen suspension was rapidly thawed in a 20° water bath and then brought to 4°. The suspension was made 1% in Nonidet P-40 (Borun *et al.*, 1967) by addition of a 10% solution and agitated vigorously on a Vortex mixer for 15 sec. Cell breakage was 100% complete at this stage as measured by microscopic observation or by the absence of electrical resistivity in a Coulter particle counter. After standing at 4° for 10–15 min, sodium deoxycholate as a 5% solution was added to a final concentration of 0.5%. The suspension was then agitated on a Vortex mixer, allowed to stand at 4° for 15 min, agitated for an additional 15 sec, and centrifuged for 10 min at 800*g*. The crude nuclear pellet was suspended in pH 7.5 buffer, and the Nonidet–deoxycholate treatment was repeated. Such treatment released 65–90% of the total RNA and less than 4% of the DNA into the pooled 800*g* supernatants which were layered over 5.0 ml of 45% sucrose in 0.05 M sodium cacodylate (pH 7.4)–1.5 mM $MgCl_2$ and centrifuged for 4 hr at 30,000 rpm in a Spinco No. 30 rotor. The pellet was rinsed twice with sodium cacodylate buffer and phenol–sodium dodecyl sulfate extracted to prepare rRNA. A supernatant from a centrifugation performed without the presence of a layer of 45% sucrose was extracted with phenol–sodium dodecyl sulfate to obtain 105,000*g* supernatant RNA.

RNA Extraction. Phenol–sodium dodecyl sulfate extraction of RNA at 4° was performed as described (Saponara and Enger, 1964). Hot phenol–sodium dodecyl sulfate extractions (Saponara and Enger, 1964; Enger *et al.*, 1968) were performed at 58° rather than at 60°.

Zone Sedimentation. Analytical or preparative sucrose gradient centrifugation was performed as described (Saponara and Enger, 1964).

Preparative Gel Electrophoresis. A Canalco preparative disc electrophoresis unit with the PD-2–320 upper column was used. Gels of 8% final monomer concentration were poured to a height of 6 cm. Polyacrylamide gels were prepared in 0.05 M Tris-acetate buffer (pH 7.0) and contained 7.8% (w/v) acrylamide (Eastman 5521), 0.2% *N,N'*-methylenebisacrylamide (Eastman 8383), 0.1% ammonium persulfate (Baker and Adamson), and 0.1% dimethylaminopropionitrile (Matheson Coleman and Bell 7269). Tris-acetate (0.05 M, pH 7.0) was employed as eluent and electrolyte. The current was maintained at 37.5 mA by a Canalco Model 1400 constant rate power supply. In the course of the 6.5 hr required for separation, the voltage increased exponentially from 280 to 1050 V. The gel column was maintained at 15°. Fractions of 2 ml were collected at a flow rate of 1.0 ml/min maintained by use of a Sigmamotor pump. Aliquots of 0.2–1.0 ml were counted in a Packard Tri-Carb scintillation spectrometer after the addition of 10 ml of Bray's scintillation fluid (Bray, 1960). The RNA samples subjected to electrophoresis were ethanol precipitates of the 2–10S region of sucrose gradients. The sample, which contained about 1.0 mg of RNA dissolved in 0.5 ml of buffer containing 10% (w/v) sucrose, was layered on top of the gel.

Chromatography. SAMPLE PREPARATION. RNA fractions from preparative gel electrophoresis were recovered by ethanol precipitation after the addition of unlabeled carrier yeast RNA. Samples were hydrolyzed by incubation at 37° in 0.3 N KOH for 18 hr or at 25° in 1.0 N KOH for 90 hr (Gray and Lane, 1967). No differences in ^{14}C-labeled digestion products were detected using the two procedures. They were neutralized to a neutral red end point with perchloric acid and were frozen at −15° for several hours to aid in the removal of potassium perchlorate. Solid reagent grade urea was added to the sample to bring the molarity to 7.0 prior to DEAE–urea chromatography.

DEAE–UREA. DEAE cellulose (DE-52), having a capacity of 1.0 mequiv/g, was purchased from Whatman. Prior to use it was washed by decantation with 1 M acetic acid, water, 1 M ammonia, water, 95% ethanol, water, and was finally equilibrated with 0.01 M ammonium acetate (pH 7.0) (molarity expressed with respect to acetic acid). Prior to use, the cellulose was sucked dry on a sintered-glass funnel and equilibrated with 0.01 M ammonium acetate which was made 7.0 M in urea. A column (1.0 × 75 cm) was packed with the aid of a Sigma motor pump at approximately three times the flow rate at which the column was to be operated. A linear gradient (400 ml) proceeding from 0.01 to 0.5 M ammonium acetate (and 7.0 M in urea) was used as eluent. Fractions of approximately 3–4 ml were collected every 15 min. Absorbancy at 254 mμ was followed with an Isco Model UA flow monitor. Aliquots of 1.0 ml were transferred to glass scintillation vials and taken to dryness under a heat lamp to remove ammonium acetate. Samples were counted in a Packard Tri-Carb scintillation spectrometer after the addition of 1.0 ml of water and 10 ml of Bray's scintillation fluid (Bray, 1960). The same procedures for packing, monitoring, and counting were used in all column chromatography described.

SEPARATION OF DINUCLEOTIDES. The dinucleotide region of DEAE–urea columns was pooled, and the urea was removed by adsorption of the nucleotides to charcoal. Adsorbed material was recovered by elution with 50% ethanol containing 10% concentrated ammonia (v/v). After removal of the solvent in a lyophilizer, the sample was dephosphorylated with *E. coli* alkaline phosphatase (EC 3.1.3.1) purchased from

Worthington Biochemical (BAPC 66B). The sample in 1.0 ml of ammonium acetate (pH 8.6) was incubated with 0.05 mg of alkaline phosphatase at 37° for 5 hr. Salt was removed by lyophilization, and the sample was applied to a DEAE column equilibrated with 0.02 M ammonium acetate (pH 4.2). The column was developed with 400 ml of a linear gradient proceeding from 0.02 to 0.25 M ammonium acetate (pH 4.2). A dinucleotide mixture used as a reference marker was obtained from commercial yeast RNA. Sigma grade VI Torula RNA (1 g) was hydrolyzed in 100 ml of 0.3 N KOH as described. After treatment with alkaline phosphatase, the mixture was applied to a DEAE-cellulose column equilibrated with 0.01 M ammonium acetate (pH 7.0). The column was washed with the initial buffer until all nucleosides had been removed. This fraction was discarded. A linear gradient proceeding from 0.01 to 1.0 M ammonium acetate was then applied. The eluted material containing di- and trinucleotides was lyophilized.

PHOSPHOCELLULOSE. Phosphocellulose (P-11) (capacity 7.4 mequiv/g) was purchased from Whatman and washed as was DEAE except that a 1.0 M formic acid step preceded the ethanol wash. The cellulose was equilibrated with 0.01 M ammonium formate (pH 3.85) (molarity expressed with respect to ammonia). Elution was performed with 400 ml of a linear gradient of ammonium formate (pH 3.85) from 0.01 to 0.30 M. The common nucleosides elute in the order uridine, guanosine, adenosine, and cytidine.

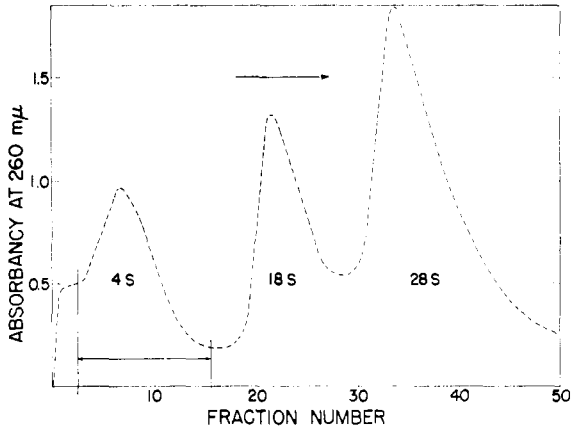

FIGURE 1: Zonal centrifugation of Chinese hamster cell RNA through a gradient of sucrose. The preparation and sedimentation of RNA were performed as described in the Experimental Procedures. The direction of sedimentation is indicated by the arrow. The portion of the gradient taken for further separation by electrophoresis is shown. In this paper the term 2–10S RNA is used to refer to this portion of a sucrose gradient. Absorbancy at 260 mμ (- - -).

Results

Isolation. All the electrophoretic separations presented here were performed on the low molecular weight RNA (2–10 S) obtained by sucrose density gradient centrifugation of phenol-extracted RNA (Figure 1). RNA isolated from whole cells labeled with [^{32}P]- and L-[methyl-^{3}H]methionine gave the electrophoretic pattern shown in Figure 2. It should be noted that the methyl-^{3}H-labeled species in fractions 120–130 did not migrate with the bulk RNA in this region. For this reason the mass species, represented in Figure 2 by either absorbancy at 260 mμ or ^{32}P counts, are identified by letters A–H from left to right and the methylated species by Roman numerals I–VII. With the aid of data presented in Figures 2–4, previously described species (peaks A, B, E, and F) are identified.

Peak A or I (Figure 2, fractions 60–70) is degraded DNA. This region of the electrophoregram is found to be occupied only when the RNA sample has been subjected to DNase treatment. RNA samples extracted from cells labeled with [^{3}H]thymidine and incubated with DNase have ^{3}H label only in the region of peak A. A fraction of the sample shown in Figure 2, subjected to electrophoresis without prior DNase treatment, gave a pattern indistinguishable from Figure 2 in the fractions from 90 to 175. The last two pieces of data

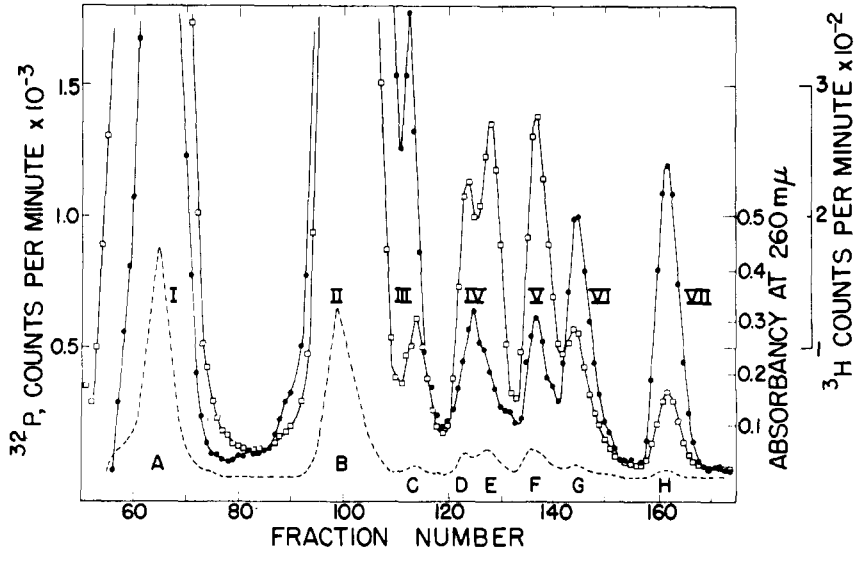

FIGURE 2: Preparative polyacrylamide gel electrophoresis of the 2–10S fraction of total cellular RNA. Cells were exposed to 1 μCi/ml of L-[methyl-^{3}H]methionine (Schwarz Bio-Research, 3.1 Ci/mmole) and 0.4 μCi/ml of ^{32}P (Abbott, 24 mCi/mg of phosphate) for three generations. RNA was extracted at 58° as described and was incubated in 5 × 10^{-3} M $MgCl_2$ with 20 μg of deoxyribonuclease I (EC 3.1.4.5, Worthington DPFF) for 30 min at 4°. Absorbancy at 260 mμ (- - -), counts per minute from ^{3}H (●-●-●), and counts per minute from ^{32}P (□-□-□).

eliminate the possibility that any of the material in fractions 90–175 derives from DNA. Peak B or II (Figure 2, fractions 90–108) contains tRNA. RNA isolated from a 105,000*g* supernatant has 88.8% of its mass and 96.1% of its L-[methyl-^{14}C]methionine label in fractions 90–118 (Figure 3). The total fraction of ^{14}C in the region from tube 119–175 in Figure 3 is 1.45% compared with 5.7% in this region for Figure 2. RNA labeled with L-[2-^{14}C]methionine is found exclusively in peak, B, indicating the absence of methionyl-tRNA species from other regions of the electrophoregram.

Peaks D, E, and F are rRNA since they, along with tRNA, are the main fractions found associated with isolated ribosomes (Figure 4). We tentatively identify peak E as 5S rRNA, since it is the only one of the three that is extracted from ribosomes with 4° phenol. Phenol extraction at 4° is known to release 5S RNA from ribosomes (Forget and Weissman, 1967a). As discussed below, peak F may represent 28S-associated, hot phenol-released 7S RNA.

We shall now present evidence that the methylated species III, IV, VI, and VII, which are found neither in the 105,000*g* supernatant of cells disrupted with Nonidet–deoxycholate nor in the ribosomes, are concentrated in a particulate fraction sedimenting in 10 min at 800*g*. This low-speed pellet is a nuclear fraction, since it contains at least 96% of the total cellular DNA. It should be noted, however, that in our preparations 10–35% of the total cellular RNA is still associated with this fraction. Figure 5 shows that in such a nuclear pellet the fraction of methylated low molecular weight RNA larger than tRNA has been enriched to 34%, a sixfold increase from the 5.7% found in Figure 2. A clear demonstration of the nonidentity of the ribosomal species D, E, and F with the nuclear methylated species III, IV, VI, and VII is shown in Figure 6. Nuclear RNA (labeled with L-[methyl-^{3}H]methionine) and rRNA

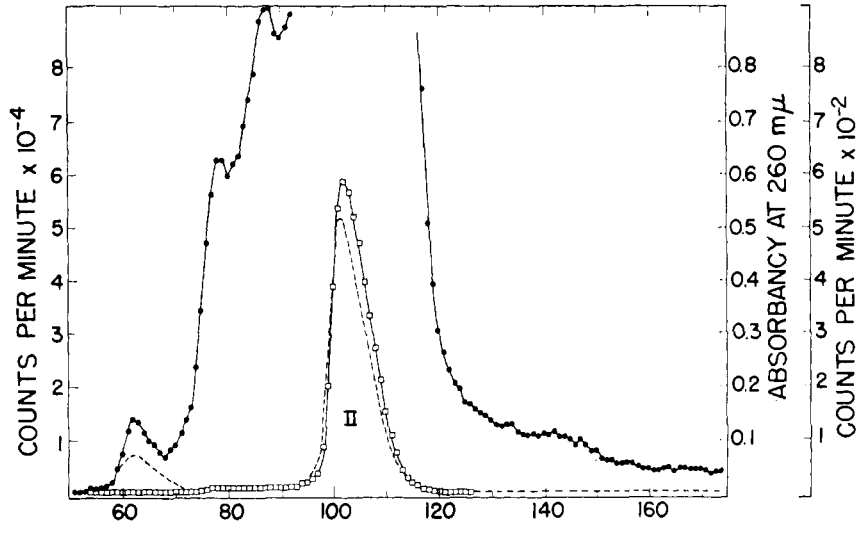

FIGURE 3: Preparative polyacrylamide gel electrophoresis of 105,000*g* supernatant RNA. Cells were exposed to 0.4 μCi/ml of L-[methyl-^{14}C]methionine (Schwarz BioResearch, 52 mCi/mmole) for 50 hr. The 105,000*g* supernatant fraction, obtained as described under the Experimental Procedures, was subjected to a phenol extraction at 58°. Absorbancy at 260 mμ (- - -), cpm × 10^{-4} (□-□-□), and cpm × 10^{-2} (●-●-●).

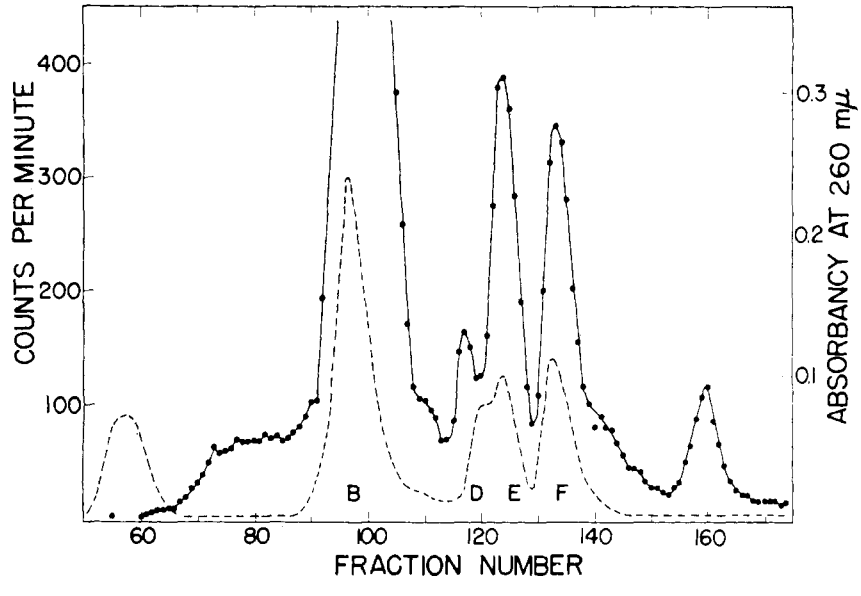

FIGURE 4: Preparative polyacrylamide gel electrophoresis of the 2–10S fraction from rRNA. Cells were exposed to 1.25 μCi/ml of L-[methyl-^{3}H]methionine for 50 hr. RNA from ribosomes, obtained as described under the Experimental Procedures, was extracted with phenol at 58°. The sample was treated with DNase. Absorbancy at 260 mμ (- - -) and counts per minute (●-●-●).

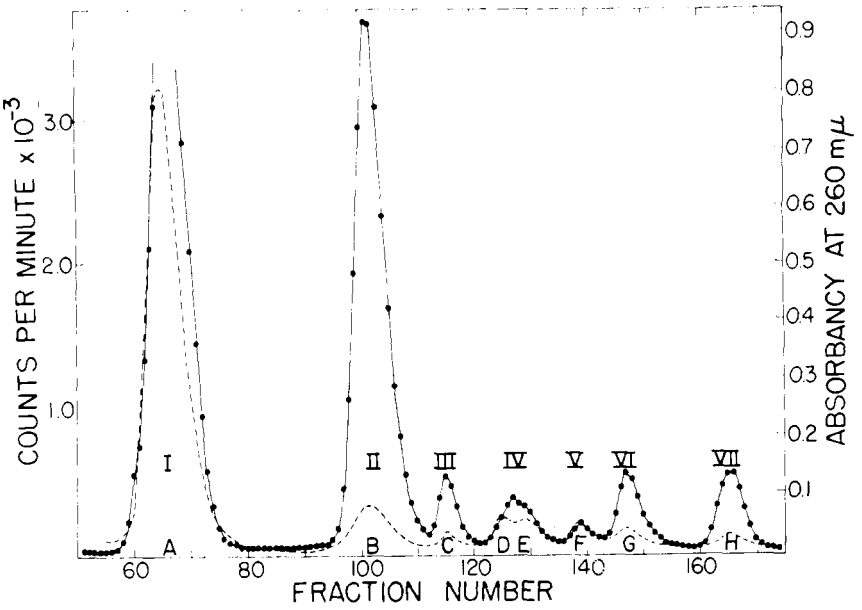

FIGURE 5: Preparative polyacrylamide gel electrophoresis of the 2–10S fraction from nuclear RNA. Cells were exposed to 0.4 μCi/ml of L - [methyl - ^{14}C]methionine for 50 hr. Nuclei isolated as described were subjected to phenol extraction at 58°. Absorbancy at 260 mμ (- - -) and counts per minute (●-●-●).

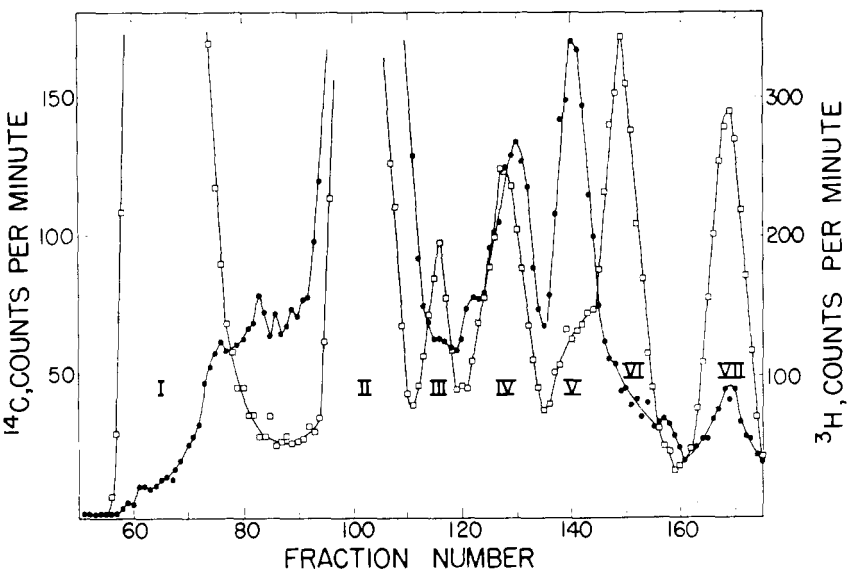

FIGURE 6: Preparative polyacrylamide gel electrophoresis of the 2–10S fraction from rRNA and nuclear RNA. Ribosomal RNA labeled with L-[methyl - ^{14}C]methionine was extracted from a portion of the cells used in Figure 5. The nuclear RNA of cells labeled with 0.5 μCi/ml of L-[methyl-^{3}H]methionine for 48 hr was extracted with phenol at 58°. The preparations were mixed, treated with DNase, and subjected to electrophoresis. Counts per minute from ^{3}H (□-□-□) and counts per minute from ^{14}C (●-●-●).

(labeled with L-[methyl-^{14}C]methionine) were extracted with phenol at 58°, pooled, and subjected to electrophoresis.

All of the above data were obtained by extracting RNA with phenol at 58°. The possibility that heat-induced artifacts accounted for some of the results was investigated by preparing RNA with phenol at 4°. Figure 7 is a composite comparing the mass distribution of 2–10S RNA extracted from Chinese hamster cells using hot or cold phenol procedures. The absence of peaks D and F in the cold phenol extract should be noted. Unpublished studies performed in collaboration with R. A. Walters indicate that peak D may represent another form of peak E, which appears to be 5 S, and that peak F may represent 28S-associated 7 S. That is, the relative amount of peak D plus E in a hot phenol extract is equal to that of peak E alone in a replicate but cold phenol prepared ^{32}P-labeled sample. Also, 58° phenol–sodium dodecyl sulfate treatment of cold phenol extracted 28S RNA liberates peak F. The amount liberated represents approximately 5% of the mass of 28 S as judged by relative ^{32}P contents.

When the RNA from nuclei was extracted with phenol at 4°, the pattern shown in Figure 8 was obtained. Comparison of this pattern with that for 58° extracted nuclei (Figure 6, ^{3}H) shows the absence of both absorbancy and counts in the region of peak F (tubes 135–140). Aside from this difference, the presence of methylated species III, IV, VI, and VII is seen in both preparations and in similar amounts relative to one another.

Because relatively large amounts of RNA, including 18S RNA, were found in our nuclear fractions (*cf.* Penman, 1966), there was reasonable doubt as to the *nuclear* origin of the low molecular weight, methylated

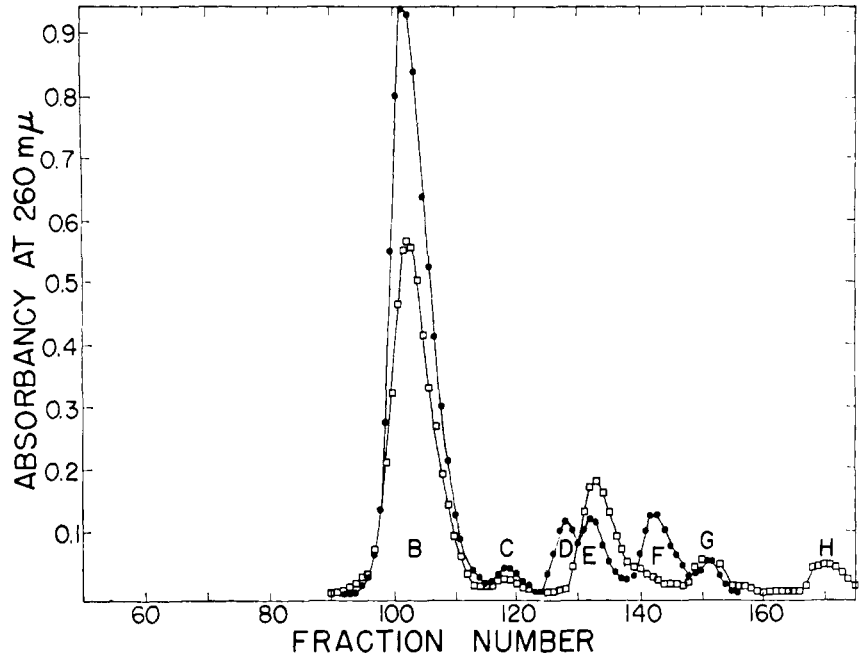

FIGURE 7: Preparative polyacrylamide gel electrophoresis of the 2–10S fraction from total cellular RNA. Cells in exponential growth were extracted with phenol at 58° (●-●-●) or with phenol at 4° (□-□-□). The graph is a composite of two separate electrophoretic separations.

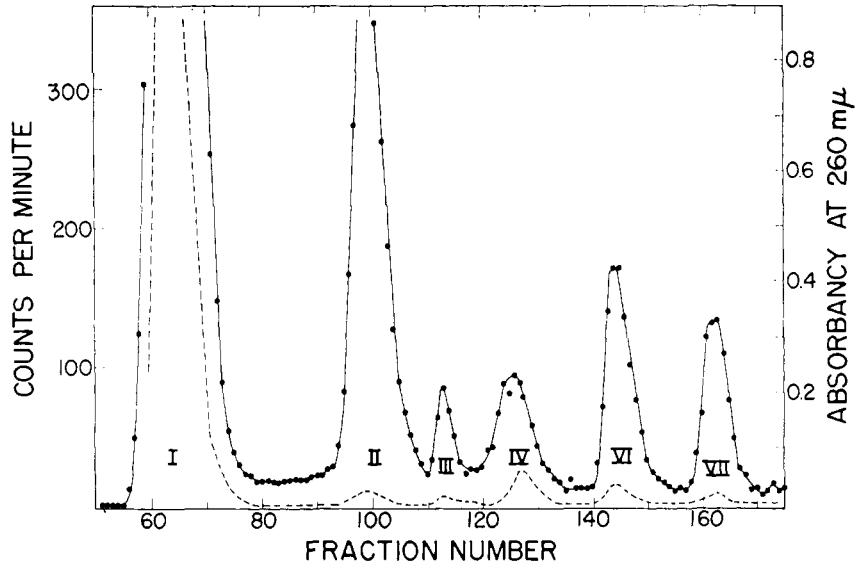

FIGURE 8: Preparative polyacrylamide gel electrophoresis of the 2–10S fraction from nuclear RNA isolated with phenol at 4°. Cells were labeled with L - [methyl - ^{3}H]-methionine as described in Figure 6. RNA was obtained from a nuclear fraction treated with DNase and subjected to electrophoresis. Absorbancy at 260 mμ (- - -) and counts per minute (O-O-O).

RNAs obtained. Therefore, nuclei prepared as described under Experimental Procedures were further treated with RSB–Tween–deoxycholate in low ionic strength buffer as described by Penman (1966). They were then digested with DNase in high salt buffer (Penman *et al.*, 1968). The digestion products were subjected to 900*g* for 30 min. The pellet, which contained less than 7% of the total cellular RNA and which appeared under the microscope to be comprised of nucleoli, was subjected to phenol extraction and the RNA therefrom to electrophoretic analysis, as were the supernatant fractions from the above treatments. More than 90% of the cell's methylated species III, IV, VI, and VII were found in the RNA prepared from the nuclear digest pellet. The pattern of methylated species resembles that of Figure 8 except that the methyl label in peaks I and II is diminished to the extent that they are of approximately the same magnitude (in terms of methyl label content) as peak VI. Thus, although we do not consider the nuclear or nucleolar location of peaks III, IV, VI, and VII to be unequivocally established, it is clear that we may separate these RNAs from most of the other cellular nucleic acids using the cell fractionation, sucrose gradient, and zone electrophoretic protocol outlined above.

Analysis of Methylation Patterns. Figures 9–11 show, respectively, the patterns given on DEAE-urea by alkali-degraded 4S, 18S, and 28S RNAs labeled with L-[methyl-^{14}C]methionine. The RNA fractions were obtained from sucrose gradients. Sephadex G-100 column chromatography was used to isolate 4S tRNA from the 2–10S sucrose gradient fraction. We shall refer to the species in fractions 10–20 of the DEAE-

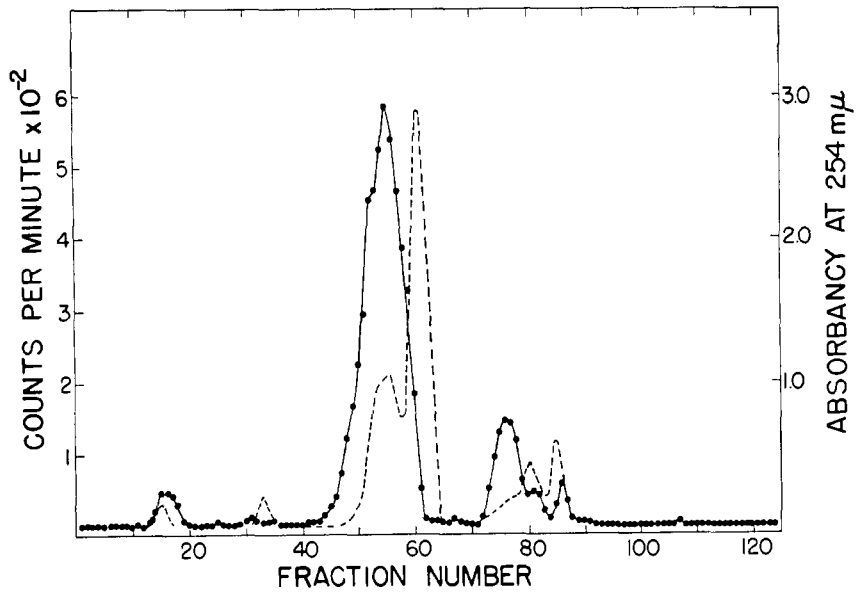

FIGURE 9: DEAE–urea chromatography of an alkaline digest of tRNA. Cells were exposed to 0.5 μCi/ml of L-[methyl-^{14}C]-methionine for 21 hr. The 2–10S region of a sucrose gradient of phenol-extracted RNA was further fractionated on Sephadex G-100. The tRNA region was pooled, precipitated with two volumes of ethanol, and hydrolyzed with 1 N KOH at 23° for 90 hr. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●).

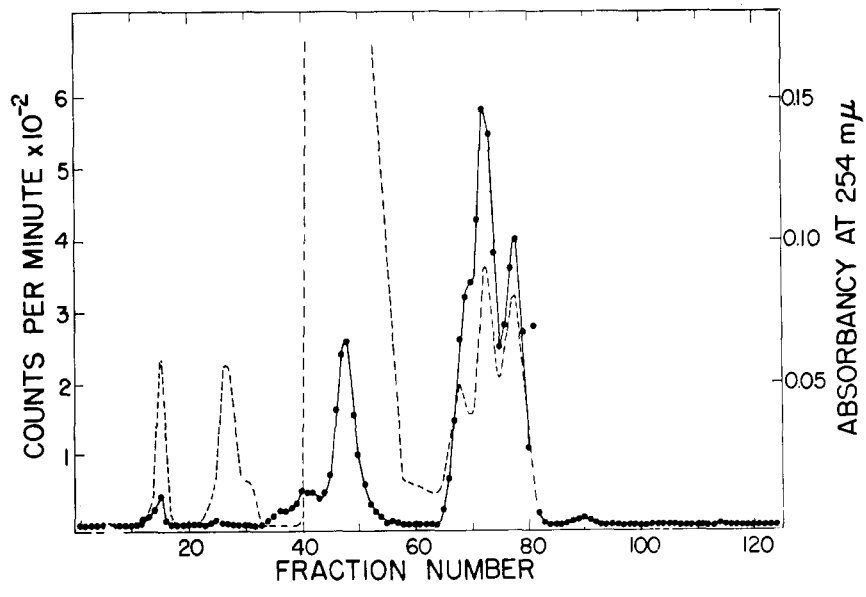

FIGURE 10: DEAE–urea chromatography of an alkaline digest of 18S RNA. Cells were labeled and RNA was fractionated as described in the legend to Figure 9. The Sephadex G-100 step was eliminated. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●).

urea chromatograms as the noncharged material. Ultraviolet absorption in this region is from added adenosine. The second absorbancy peak is from added 3′,5′-cyclic adenylic acid, which has a charge of −1 at pH 7.0. Fractions 40–60 comprise that region of the gradient where mononucleotides elute. This is shown by the following facts: (i) the bulk of the ultraviolet absorbancy of the RNA sample elutes in this region after alkaline digestion, (ii) AMP elutes in this region, and (iii) treatment with alkaline phosphatase renders these compounds nonadsorbable to DEAE-cellulose at either pH 7.0 or 4.2.

Radioactivity in fractions 65–90 is from dinucleotides. This is shown by the following facts: (i) fractionation of RNA fragments produced by pancreatic ribonuclease under our conditions of DEAE–urea chromatography gives resolution of oligomers out to the heptamer peak, and the pancreatic ribonuclease dinucleotides elute in the region we have ascribed to alkali-stable, O-methylated dinucleotides; (ii) such dinucleotides move with similar mobility to ADP; and (iii) treatment of these dinucleotides with alkaline phosphatase does not remove all phosphate as evidenced by the fact that they still behave as anions on DEAE-cellulose at pH 4.2. In addition, 28S RNA has some material larger than dinucleotides (fractions 90–120 of Figure 11). Note that such species are totally absent in tRNA and 18S RNA. Experiments on isolated 18S and 28S RNA labeled with ^{32}P have shown that the fraction of ^{32}P in the dinucleotide region is consistent with the number of methyl groups found in dinucleotides. It has been shown that the majority of the label in the dinucleotide region is due to 2′-O-methylation of ribose, since treatment with snake venom and alkaline phosphatase (Hall, 1964) gives rise to four methyl-labeled species (accounting for 94.4% of the total ^{14}C counts per minute) which elute slightly before their respective parent nucleosides on phosphocellulose.

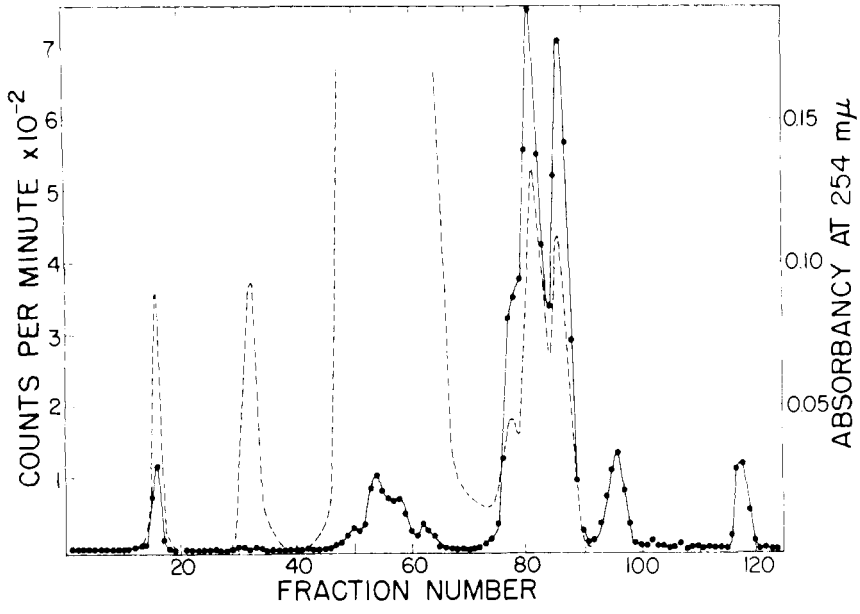

FIGURE 11: DEAE-urea chromatography of an alkaline digest of 28S RNA. Cells were labeled and RNA was fractionated as described in the legend to Figure 9. The 28S region of a sucrose gradient was taken for analysis without the Sephadex G-100 step. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●).

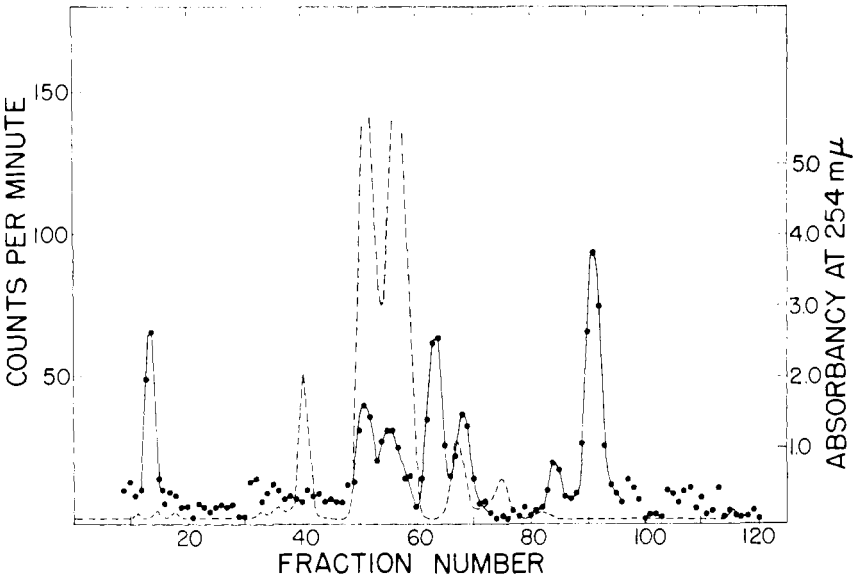

FIGURE 12: DEAE-urea chromatography of an alkaline digest of the nuclear RNA fraction IV. Tubes 122–130 of the electrophoretic separation shown in Figure 8 were pooled, hydrolyzed with 0.3 N KOH, and chromatographed as described in the Experimental Procedures. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●). The absorbancy peaks are: tube 40, added 3′,5′-AMP; tubes 48–60, mononucleotides from the RNA preparation; tube 67, added 5′-ADP; and tube 75, added 5′-ATP. The absorbancy peaks in Figures 13 and 14 are also from the above constituents.

Figures 12–14 show DEAE-urea chromatographic patterns of the alkali digests of RNA species IV, VI, and VII. These species were isolated from the nuclear fraction of methylmethionine-labeled cells using the cold phenol–sodium dodecyl sulfate procedure (*cf.* Figure 8). A distinctive characteristic of alkali digestion products of these RNAs is the large fraction of methyl label seen in the noncharged species and in the fragments larger than dinucleotides. The *noncharged* species present in the *alkali* digests of each of these "nuclear" RNAs is a single substance. This is shown in Figure 15, where the noncharged material from IV, VI, and VII has been pooled and rerun on phosphocellulose along with the added nucleosides uridine, guanosine, adenosine, and cytidine. Digestion of RNA species IV, VI, and VII with *snake venom* and *alkaline phosphatase*, followed by chromatography on phosphocellulose at pH 7.0, demonstrates the presence of a *positively* charged species which comigrates with added 7-methylinosine (Figure 16, fractions 50–53). When this substance was treated with 1 N NH_3 at room temperature, followed by 3-hr incubation with 1 N HCl at room temperature, column chromatography on phosphocellulose at pH 3.85 demonstrated the appearance of a new compound which comigrated with the noncharged compound released by alkali digestion. Digestion of the tRNA fraction with snake venom and alkaline phosphatase demonstrated the presence of 1-methyladenosine and 7-methylguanosine. After similar treatment 18S RNA gave rise mainly to 7-methylguanosine and 28S to 1-methyladenosine. The positively charged species present in fractions IV, VI, and VII appears to be absent from tRNA and the rRNAs.

We shall now present evidence that each of these nuclear RNA species is chemically different from the others. This evidence is based on dissimilarities in the

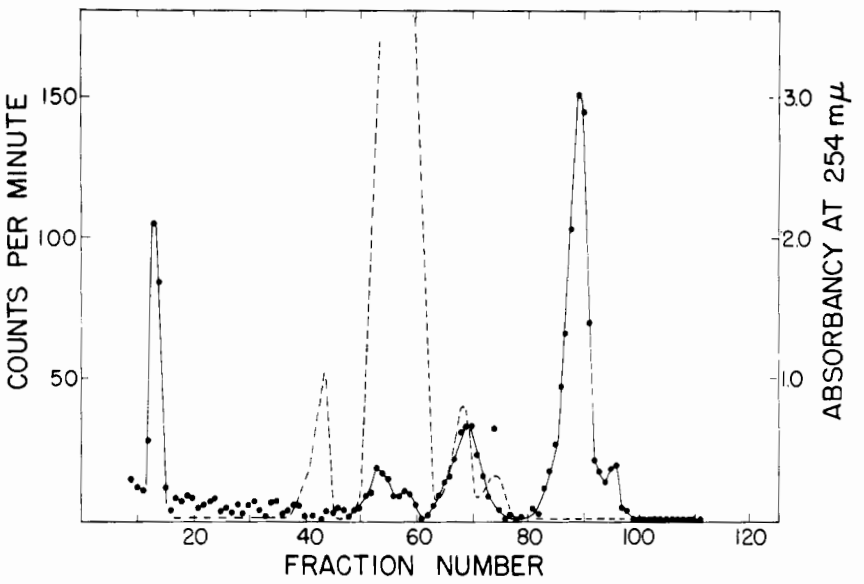

FIGURE 13: DEAE–urea chromatography of an alkaline digest of the nuclear RNA fraction VI. Tubes 140–150 from the electrophoretic separation shown in Figure 8 were pooled and treated as described in the legend to Figure 12. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●).

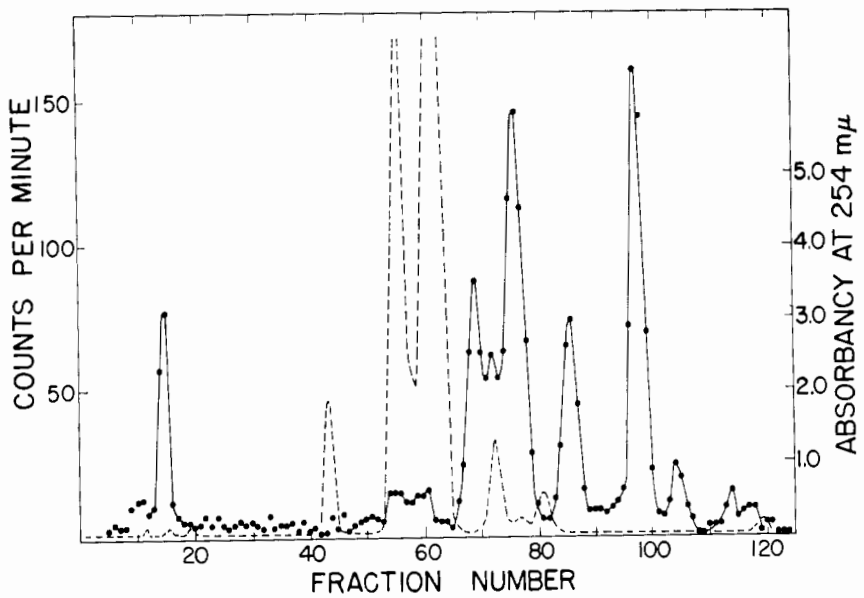

FIGURE 14: DEAE–urea chromatography of an alkaline digest of the nuclear RNA fraction VII. Tubes 157–165 from the electrophoretic separation shown in Figure 8 were pooled and treated as described in the legend to Figure 12. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●).

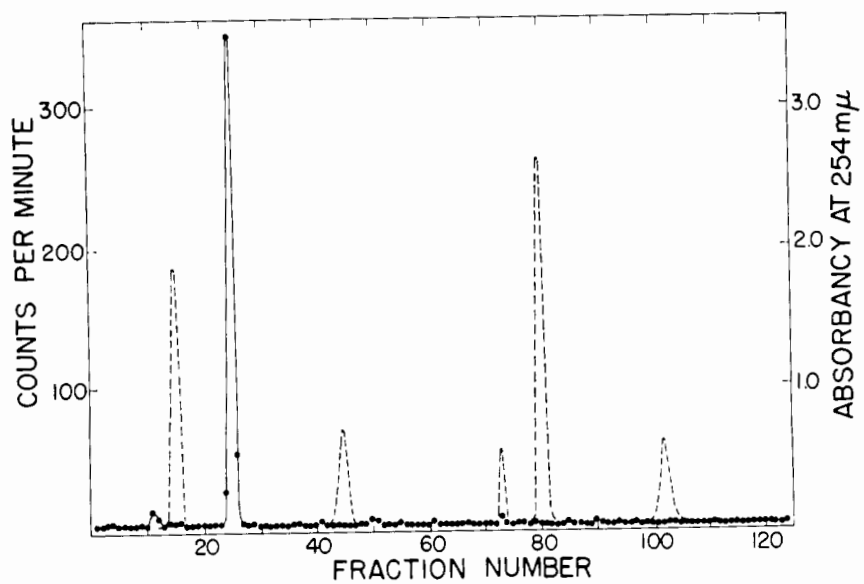

FIGURE 15: Phosphocellulose column chromatography of the noncharged species from the alkaline digests of nuclear RNA fractions IV, VI, and VII. Tubes 10–20 from the DEAE–urea columns depicted in Figures 12–14 were pooled, mixed with uridine, guanosine, adenosine, and cytidine, and fractionated on phosphocellulose as described in the Experimental Procedures. The material eluting at tube 73 is N^6,N^6-dimethyladenosine, an impurity in the nucleoside mixture. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●).

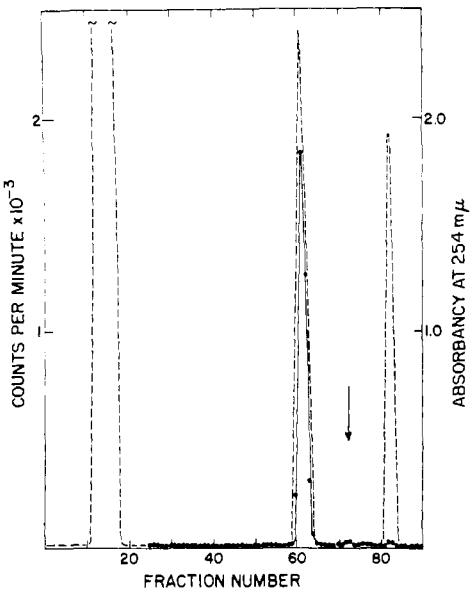

FIGURE 16: Phosphocellulose column chromatography of the snake venom and alkaline phosphatase digestion products of small RNA larger than tRNA. The RNA preparation was obtained by hot-phenol extraction of cells labeled with [methyl-^{14}C]methionine. An electrophoretic cut similar to one which would have been obtained from fractions 125–165 of Figure 2 was hydrolyzed and chromatographed on phosphocellulose at pH 7.0. The gradient proceeded linearly from 0.01 to 1.0 M ammonium acetate. The void volume (tubes 10–25) contained 75,625 cpm. The positively charged species in tubes 59–65 contained 11,541 cpm or 13.2% of the total label. It should be noted that this may not represent a quantitative yield, since the venom digestion was performed at pH 8.6 and the substance is known to be labile to alkali. Similar digestion and chromatography of the pooled species IV, VI, and VII isolated with cold-phenol from a nuclear fraction showed this species to account for 17.5% of the total methyl label. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●). The absorbancy peaks are from added 7-methylinosine (peak at tube 61) and 7-methylguanosine (peak at tube 82). The arrow shows the approximate position of elution of 1-methyladenosine. Absorbancy in the void volume is from digestion products of added carrier RNA.

dinucleotides present in each. The dinucleotide regions from chromatograms of alkali digests of IV, VI, and VII were mixed separately with the dinucleotides of commercial yeast RNA and treated with alkaline phosphatase, and the resulting dinucleoside monophosphates were fractionated on DEAE-cellulose at pH 4.2. The absorbancy markers from a digest of yeast RNA enabled comparison of the three separate runs. The dinucleotide pattern of fraction VII is shown in Figure 17. Fractions IV and VI had different dinucleotides. Dinucleotides A and B were present in fraction IV, while fraction VI contained dinucleotides A and D.

Discussion

Our finding of methylated small RNAs other than tRNA in the 5S region of sucrose gradients requires discussion, since reports of total nucleotide sequences for the 5S species from *E. coli* (Brownlee *et al.*, 1967) and KB cells (Forget and Weissman, 1967b) show these species to be homogeneous and nonmethylated. However, it is becoming apparent that the composition of the small RNA fraction is extremely sensitive to the conditions of isolation and that species of small RNA other than transfer or ribosomal 5S may be present. Hindley (1967), for example, using *E. coli* MRE 600 (the same strain used by Brownlee *et al.*, 1967) reports the presence of two homogeneous RNA species in addition to 4 and 5 S. Many recent reports suggest that the small RNAs from mammalian cells are even more complex (Knight and Darnell, 1967; Forget and Weissman, 1967a). For example, Figure 5A of Knight and Darnell (1967) shows at least five methylated nuclear RNAs separated from tRNA by gel electrophoresis.

It has been reported that 15–20% of the total microsomal RNA of rat liver is of low molecular weight and is distinct from tRNA or 5S RNA (Gardner and Hoagland, 1968). Since this type of RNA is rendered nonparticulate by treatment with deoxycholate, it should be present in our 105,000*g* supernatant (Figure 3). Its

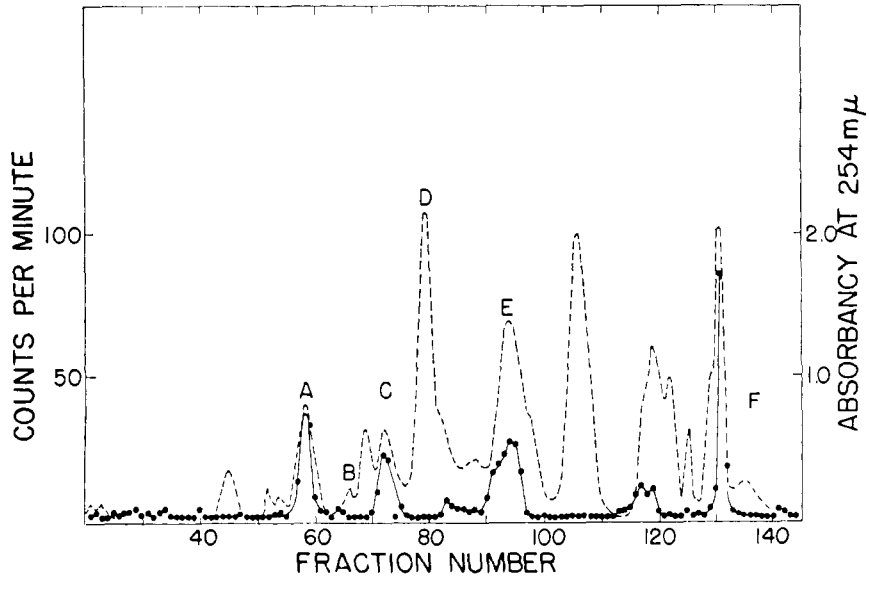

FIGURE 17: Separation of the alkali-stable dinucleotides from the nuclear RNA fraction VII. Tubes 65–90 from the DEAE-urea separation shown in Figure 14 were pooled and treated as described in the Experimental Procedures. The letters B and D refer to species found as alkali-stable dinucleotides in RNA IV and RNA VI, respectively. Note that the data points on the trailing edge of dinucleotide F have not been connected. Absorbancy at 254 mμ (- - -) and counts per minute (●-●-●).

TABLE I: Distribution of Mass and Relative Methylation of Low Molecular Weight RNA Species.

Fraction[a]	% of Mass[b]			Extent of Methylation Rel to B			
	Absorbancy (%)		^{32}P (%)				
	Cold[c]	Hot[d]	Hot[d]	1[e]	2	3	Av
B	74.6	75.7	75.7	1.00	1.00	1.00	1.00
C	3.0	2.6	2.8	0.49	0.46	0.47	0.47
D	Absent	4.9	3.6				
E	14.7	6.2	6.2				
F	Absent	7.9	6.8	0.08	0.07	0.09	0.08
G	3.6	2.4	3.2	0.29	0.29	0.31	0.30
H	3.2		1.8	0.60	0.54	0.66	0.60

[a] Fraction letters refer to the component shown in Figure 2. [b] Calculated from Figures 2 and 7. [c] RNA extracted with phenol at 4°. [d] RNA extracted with phenol at 58°. [e] Calculated from electrophoretic separations performed on the 2–10S fractions of three independently prepared total RNA samples.

molecular weight (22,500) would seem to preclude its separation from tRNA in our system. In this connection, it should be stressed that our identification of peak B with tRNA does not imply that we have any data on the purity of this fraction. The data of Figure 3 show that a 105,000*g* supernatant fraction which contains 90% of the total tRNA moves largely as a single peak on electrophoresis. Such a preparation has less than one-fourth the amount of methyl label found in the region of species III–VII compared to the amount present when low molecular weight RNA from whole cells is electrophoresed.

The differences we have reported between hot and cold phenol extraction are very similar to the observations of Forget and Weissman (1967a) for KB cell RNA. Those authors interpreted part of the effect as heat-induced aggregation of 5S RNA, citing as evidence similar pancreatic ribonuclease oligonucleotide maps generated from the two. Perhaps a similar effect obtains in the case of our peaks D and E. Another explanation for the additional species in hot phenol-prepared RNAs may be found in the work of Pene *et al.*, (1968). These authors have presented evidence for a 7S RNA, noncovalently bound to 28S rRNA, which is released by phenol at 60°. Since our peak F is released from 28S with such a hot phenol treatment, it is likely that it represents a 7S species similar to that of Pene.

Our observation that the bulk of the methylated species observed is concentrated in a nuclear pellet fraction from which at least 93% of the total cellular RNA has been removed argues against the possibility that these species result from generalized degradation of RNA during isolation. In addition, species IV, VI, and VII differ qualitatively and quantitatively from 4S, 18S, or 28S RNA with respect to their methylated alkaline degradation products. Three of them give methyl distribution patterns which are very similar. After alkali digestion, all have a large fraction of their total methyl label in noncharged material and in di- and trinucleotides. To our knowledge, this is the first report of the existence of noncharged-methylated species in alkaline hydrolysates of RNA. The nucleotide which gives rise to this degradation product has been shown to be unique to "nuclear" RNA and to bear a positive charge at pH 7.0. The positively charged nucleoside derived therefrom is separable from 1-methyladenosine and 7-methylguanosine, which have been identified as constituents of RNA (Dunn, 1961; Dunn *et al.*, 1963). Its lability to 1 N ammonia at room temperature is similar to that shown for 7-methylguanosine (Haines *et al.*, 1962). Although the nucleotide migrates with 7-methylinosine under the conditions of Figure 16, it can be distinguished from it since acid hydrolysis does not produce 7-methylhypoxanthine. The product formed does not adsorb to phosphocellulose at pH 3.85, which indicates that it probably does not have exocyclic nitrogens. It is readily distinguished from 7-methylxanthosine, since this compound does not adsorb to phosphocellulose at pH 7.0, presumably due to its unusual phenolic ionization (xanthosine has a p*K* of 5.75). We believe that the nucleotide may be a derivative of either 7-methylinosine or 7-methylxanthosine. The identity and chemical properties of this RNA constituent will be the subject of a subsequent paper. In addition, two of the small RNAs (VI and VII) show an extreme paucity of methylated mononucleotides. Some counts in the mononucleotide fraction in alkaline digests of RNA IV seem to be present, but this may result from contamination since the region of the electrophoregram from which this RNA species is taken is complex. Some of the methyl label in mononucleotides may be due to the reversion of methyl from methionine into the formyl pool and reincorporation into the carbon skeletons of adenosine and guanosine (Tamaoki and Lane, 1968). Whether the small amount of methyl label found in peaks E and F (Figure 4), which is accounted for by the presence of a single methyl per molecule, is due to such labeling requires further investigation. Analyses of the methyl-labeled mononucleosides from total RNA on phosphocellulose at

pH 3.85 show less than 1% of the total label migrating with adenosine. This fact makes such a possibility unlikely.

The degree of methylation of the various electrophoretically resolved, low molecular weight RNAs of Chinese hamster cells is presented in Table I. The specific methylation of species C, D, and H are 47, 30, and 60%, respectively, of the tRNA containing fraction B.

In contrast to the internal similarity in the kinds and numbers of methylated species in the alkaline digests of these small "nuclear" RNAs is the extreme dissimilarity when they are compared with tRNA and the rRNAs. Alkaline degradation of either tRNA or 18S rRNA results in no fragments larger than dinucleotide. Taken together with their "nuclear" location, our data on methyl distribution make it likely that the RNAs reported upon in this paper are *bona fide*, discrete, intracellular species.

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