Secondary Methylation of Ribosomal Ribonucleic Acid in HeLa Cells*

Ernest F. Zimmerman†

ABSTRACT: Although earlier studies have shown that methylation of ribosomal ribonucleic acid occurs on 45S ribosomal ribonucleic acid precursor as it is being transcribed, dimethylaminopurine is found in 18S ribosomal ribonucleic acid and not in 45S ribosomal ribonucleic acid. In the present study, when HeLa cells were exposed to [^3H_3C]methionine for 22 hr, radioactive dimethylaminopurine was again found in cytoplasmic 18S ribosomal ribonucleic acid and not in 28S ribosomal ribonucleic acid. After a 60-min pulse, dimethylaminopurine was observed in 18S ribosomal ribonucleic acid while none was found in 35S ribosomal ribonucleic acid. When labeled methionine was added to cells for 30 min, in the presence of actinomycin which prevented transcription and hence methylation of 45S ribonucleic acid, dimethylaminopurine appeared in 18S ribonucleic acid. It was shown that dimethylaminopurine associated with 18S ribosomal ribonucleic acid did not arise from contamination in sucrose gradients, since little or no dimethylaminopurine was found in regions heavier or lighter than 18S ribosomal ribonucleic acid. The level of dimethylaminopurine in ribonucleic acid was higher in the nucleus (15-min pulse) than in the cytoplasm; and, the amount of dimethylaminopurine in the nucleolus exceeded that in the nucleoplasm by 60%. At later times, the level of dimethylaminopurine in the nucleolus and nucleoplasm did not increase, while dimethylaminopurine in the cytoplasm did increase. These findings indicate that dimethylaminopurine was formed in the nucleolus. It is concluded that a secondary methylation of ribosomal ribonucleic acid occurs in the nucleolus at about the time of cleavage of 45S ribonucleic acid precursor, with dimethylaminopurine appearing in 18S ribosomal ribonucleic acid shortly thereafter. Although it is estimated that one dimethylaminopurine base is present in each 18S ribosomal ribonucleic acid chain, dimethylaminopurine was not found at either the 3′-OH or 5′-PO_4 end of the chain.

Previous study of the kinetics of rRNA methylation revealed that 45S ribosomal precursor RNA is the first molecule in the maturation process of rRNA to be methylated (Zimmerman and Holler, 1966, 1967; Greenberg and Penman, 1966). We offered evidence for the process (eq 1 and 2) of maturation of rRNA. This supported the previous conclusions of Scherrer *et al.* (1963) and Penman (1966). It was also found that methylation of 45S rRNA occurred at the time of transcription from DNA in accord with the results of Greenberg and Penman (1966). Thus, a conformational change of two 45S rRNA molecules induced by methylation could not explain the appearance of 35S and 18S RNA. It was concluded that 35S and 18S RNA resulted from cleavage of a single 45S RNA molecule.

$$45\ S \longrightarrow 35\ S + 18\ S \quad (1)$$

$$35\ S \longrightarrow 28\ S \quad (2)$$

When an analysis of the methylated products in 45S rRNA was performed, 2′-*O*-methylcytidylic acid and MAP[1] were identified. Two methylated purine bases were also observed which had the same chromatographic mobility in 1-butanol–NH_3 as guanine and adenine. The unknown methylated purines and ribose methylated nucleotides were also reported by Brown and Attardi (1965) to be present in 28S and 18S rRNA of HeLa cells. However, they did not report the existence of MAP, but rather found DMAP in 18S rRNA. Since 45S rRNA is the precursor of 18S rRNA and since methylation of 45S rRNA occurred at the time of its transcription, then DMAP would be expected to be present in both 45S and 18S rRNA. The purpose of this investigation was to locate the time and site of methylation of RNA, whereby the DMAP residue in the RNA is formed. It was found that a secondary methylation of rRNA takes place, in which the DMAP residue is formed after the initial methylation process of 45S rRNA in the nucleolus and is found in 18S rRNA. The possible physiological significance of the appearance of DMAP after the major methylation of 45S rRNA precursor is discussed.

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† Present address: Institute for Developmental Research, Children's Hospital Research Foundation, University of Cincinnati, Cincinnati, Ohio 45229.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: MAP, 6-methylaminopurine; DMAP, 6-dimethylaminopurine; DMAPR, 6-dimethylaminopurine riboside; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Experimental Section

Materials

[*methyl*-^{3}H]Methionine was purchased from Nuclear-Chicago Corp.; 6-dimethylaminopurine, 6-dimethylaminopurine riboside, and 6-methylaminopurine from Calbiochem; bacterial alkaline phosphatase and deoxyribonuclease from Worthington Biochemicals Corp.; snake venom phosphodiesterase from Sigma Chemical Co. Actinomycin D was kindly supplied by the Cancer Chemotherapy National Service Center; Sarkosyl NL30 (sodium lauryl sarcosine) from Geigy Industrial Chemicals, Ardsley, N. Y.; BRIJ-58 from Atlas Chemical Industries, Inc., Wilmington, Del.

Methods

Cell Propagation. Monolayer cultures of HeLa S3 growing exponentially (Zimmerman and Greenberg, 1965) were used as the source of cells for all experiments. The cells were grown in medium (Eagle, 1959) containing 10% calf serum.

Incorporation of Radioactive Precursor. LONG-TERM PULSE experiments were carried out in monolayer cultures by decanting the medium of exponentially growing cells at 37° and replacing it with one containing 10% dialyzed calf serum and radioactive methionine (3–9 mg/l.). The cells were allowed to grow for one to two generations (24–48 hr). The cells were harvested by decanting the medium, scraping the cells in cold Earle's saline, centrifuging in the cold at 600*g* for 5 min, and washing the cell pellet with Earle's saline.

SHORT-TERM PULSE experiments were carried out by decanting the medium from monolayer cells growing exponentially and immediately replacing the medium with one containing dialyzed calf serum and lacking methionine. At 37° the cells were scraped, centrifuged at 1000*g* for 5 min, resuspended to a concentration of 10^7 cells/ml, and high specific activity [^3H_3C]methionine (1610–4330 μCi/μmole) was added. At appropriate times, aliquot portions of the cell suspension were pipetted into cold Earle's saline to stop the reaction. The cells were centrifuged and washed as described above.

Isolation of Cytoplasmic RNA. After harvesting and washing of cells with Earle's saline, the cell pellet was resuspended in 2 ml of buffer containing 0.01 M Tris (pH 7.4), 0.01 M NaCl, 0.0015 M $MgCl_2$ (RSB), and 0.5% BRIJ-58. After 10-min incubation at 0°, cells were homogenized with ten strokes of a tight-fitting pestle in a Dounce homogenizer and centrifuged at 1500*g* for 3 min. The supernatant fraction was recentrifuged at 15,000*g* for 20 min, and the 15,000*g* supernatant fraction was centrifuged at 150,000*g* for 60 min. The ribosome pellet was extracted with 1.5 ml of 0.5% Sarkosyl, 0.01 M sodium acetate (pH 5.1), 0.05 M NaCl, 10^{-4} M $MgCl_2$, and 10 μg of bentonite/ml. The solution was layered on 25 ml of a 5–20% sucrose gradient containing the above buffer lacking bentonite and Sarkosyl and centrifuged for 16 hr at 22,000 rpm in a SW 25.1 Spinco rotor at 0–5°. Absorbancy at 260 mμ was monitored automatically through a flow cell in a Gilford recording spectrophotometer. Drops were collected at 0°. Aliquot portions were made to 5% trichloroacetic acid to precipitate the RNA. Precipitates were collected on Whatman glass filters and dried by infrared heat. Radioactivity was measured in a Packard scintillation counter using a toluene–PPO–POPOP mixture.

Isolation of Total RNA. Total RNA was extracted from the cell pellets employing the detergent Sarkosyl and phenol at 60° as previously described (Zimmerman and Holler, 1967). The RNA pellet was dissolved in buffer containing 0.01 M sodium acetate (pH 5.1), 10^{-4} M $MgCl_2$, 0.05 M NaCl, 0.5% Sarkosyl, and 10 μg of bentonite/ml. The solution was layered on a 5–20% linear gradient of sucrose containing the above buffer lacking Sarkosyl and bentonite. The extracts were centrifuged as indicated in the legends of the figures. Aliquot portions from each tube in the gradient were assayed for the radioactivity in RNA, as described above.

Isolation of DMAP from RNA. The appropriate fractions of RNA isolated by sucrose gradient centrifugation were pooled. RNA was precipitated with two volumes of ethanol at −20° for 30–60 min and centrifuged at 10,000 rpm for 20 min in a Servall swinging-bucket rotor. HCl (2 ml of 1 N) was added to each RNA pellet and heated at 95° for 30 min to release purine bases and pyrimidine nucleotides. The HCl was removed by blowing air over the samples at 40°, and the samples were solubilized with 0.1 ml of 1 N NH_4OH and chromatographed (descending) on Whatman No. 3MM paper in 1-butanol (86%)–NH_3 (5%) using MAP and DMAP as markers. Radioactivity of the chromatogram was monitored by cutting out 1 × 5 cm paper strips and counting in a toluene–PPO–POPOP mixture directly. Compounds which were localized by this procedure were (in some cases) rechromatographed in isopropyl alcohol (68%)–HCl (17%) or 1-butanol (77%)–formic acid (10%) by decanting off the scintillation mixture, drying, eluting with distilled water, and evaporating to dryness as described above.

In some experiments where only the radioactivity of DMAP was monitored, the marker DMAP was added to the sample, located by use of an ultraviolet lamp, and radioactivity in DMAP was measured.

Isolation of 18S rRNA from Cytoplasmic 40S Ribosomal Precursor Particles and Polysomes. Washed cell pellets were resuspended in 3 ml of RSB-BRIJ and incubated for 10 min at 0°. Cells were homogenized with ten strokes of a tight-fitting pestle in a Dounce homogenizer and nuclei were removed by centrifugation at 600*g* for 5 min. The supernatant fraction was centrifuged at 30,000*g* for 30 min and the 30,000*g* supernatant fraction was layered on 30 ml of a 15–30% sucrose gradient in RSB and centrifuged at 18,000 rpm for 16 hr. Absorbancy of the ribosome particles was monitored and the 40S ribosomal precursor particles were pooled. Nonradioactive ribosomes from 6.5 × 10^7 cells dissolved in 2 ml of 0.01 M sodium acetate (pH 5.1) with 0.5% Sarkosyl and 10 μg/ml of bentonite were added to the 40S ribosome particles as a carrier. The labeled polysomes were collected by dissolving the pellet in 4 ml of the above buffer and adding those particles from the gradient that were heavier than 78S. RNA from each fraction was precipitated with ethanol, dissolved in 0.3 ml of buffer containing 0.1 M sodium acetate (pH 5.1) and

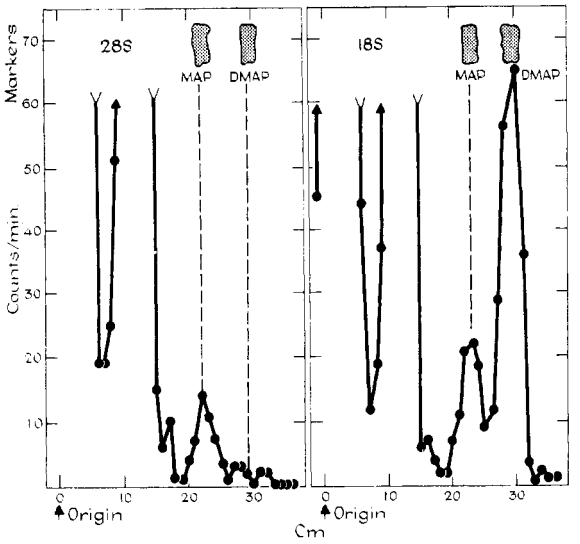

FIGURE 1: Chromatographic separation of products from methyl-labeled cytoplasmic 28S and 18S rRNA hydrolyzed by HCl. HeLa cells were labeled with a long-term pulse. The medium from 5×10^7 cells (8.4×10^6 cells/70 ml) was decanted at 37° and replaced with warmed medium containing dialyzed calf serum and [^3H_3C]methionine (16.7 μCi/μmole, 9-mg/l. final concentration). Cells were incubated at 37° for 22 hr. RNA from cytoplasmic ribosomes was extracted as described in Methods. The RNA was layered on 25 ml of a 5–20% sucrose gradient and centrifuged for 16 hr at 22,000 rpm. Aliquot portions from the gradient containing 28S and 18S rRNA were each pooled, and RNA was precipitated with ethanol and hydrolyzed with HCl. The hydrolysates were chromatographed in 1-butanol–NH_3 and radioactivity on the chromatograms was measured as described in Methods.

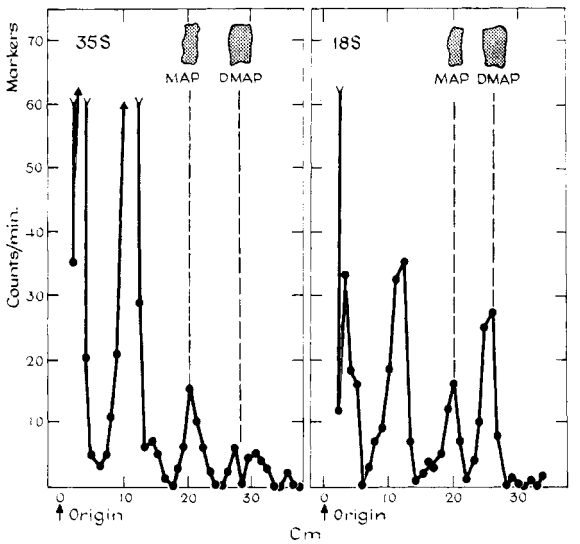

FIGURE 2: Chromatographic separation of products from methyl-labeled 35S and 18S rRNA hydrolyzed by HCl. HeLa cells were labeled with a short-term pulse. To a suspension of 9×10^7 cells was added 304 μCi of [^3H_3C]-methionine (1610 μCi/μmole), and the cells were incubated for 60 min at 37°. Total RNA was extracted as described in Methods and 0.6 ml of the RNA solution was layered on 30 ml of a 5–20% sucrose gradient and centrifuged at 23,000 rpm for 17 hr. The methyl-labeled products from HCl-hydrolyzed 35S and 18S RNA were separated and measured as in Figure 1.

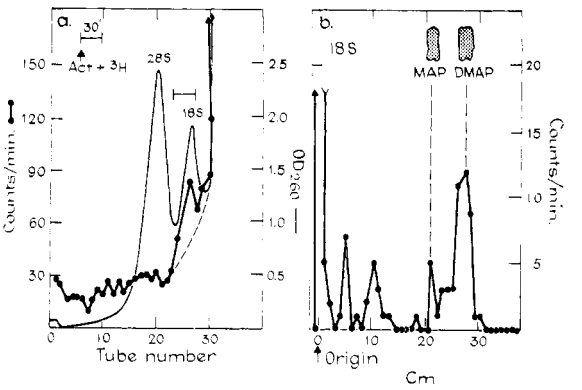

FIGURE 3: Appearance of DMAP in 18S rRNA in the absence of RNA synthesis. (a) HeLa cells were labeled with a short-term pulse. To a suspension culture (5×10^7 cells) were added simultaneously 92 μCi of [^3H_3C]methionine (1610 μCi/μmole) and actinomycin D (Act) (5 μg/ml), and the culture was incubated for 30 min at 37°. Total RNA was extracted as in Methods and 0.6 ml of the RNA solution was layered on 30 ml of a 5–20% sucrose gradient and centrifuged at 22,000 rpm for 16 hr. Four drops per tube were assayed for radioactivity, while 26 drops/tube were saved and the 18S rRNA was pooled (see bracket). (b) Chromatographic separation of the methyl-labeled products from 18S rRNA as in Figure 1.

0.05 M NaCl, 10^{-4} M MgCl, 0.5% Sarkosyl, and 10 μg/ml of bentonite. RNA (18S) was separated from 28S RNA by centrifuging in a Spinco SW 50 rotor at 50,000 rpm for 3 hr.

Separation of Nucleoli, Nucleoplasm, and Cytoplasm. The separation of nucleoli, nucleoplasm, and cytoplasm was essentially carried out by the method of Penman (1966) and Penman *et al.* (1966) in the following manner. Washed cell pellets (1×10^8 cells each) were resuspended in 5 ml of RSB-BRIJ and incubated for 10 min at 0°. Cells were homogenized as previously described and nuclei were removed by centrifugation at 600*g* for 5 min. The supernatant fraction was saved and was considered to represent the cytoplasm. The nuclei were washed with 2 ml of RSB and the wash was saved. The nuclei were then resuspended in 2 ml of RSB to which 0.3 ml of a detergent mixture (10% sodium deoxycholate–10% Tween 40, 1:2) was added. The nuclear suspension was vigorously stirred on a Vortex mixer for 3 sec and the nuclei were centrifuged. This wash was recombined with the previous wash, both of which are referred to as nuclear wash. The nuclear pellet was then resuspended in 2 ml of high salt buffer (0.5 M NaCl, 0.05 M $MgCl_2$, and 0.01 M Tris at pH 7.4) and incubated at 37° for 2 min in the presence of 100 μg of DNase. After the suspension was rapidly cooled to 0°, it was centrifuged at 10,000*g* for 5 min. The supernatant fraction was considered to be nucleoplasm and the pellet nucleoli. The nucleoli were resuspended in 4 ml of 0.01 M Tris (pH 7.4), 0.1 M NaCl, 0.001 M EDTA, 0.5% Sarkosyl, and 10 μg of bentonite/ml.

Results

Kinetics of Appearance of DMAP in RNA. In order to confirm the observation of Brown and Attardi (1965)

TABLE I: Chromatographic Identification of DMAP in 18S rRNA.[a]

Treatment	Expt Peak	Marker		Solvent
		Method A[b]		
1. HCl hydrolysis	0.77	DMAP	0.77[c]	1-Butanol–NH_3
2. Elution of peak from 1	0.48	DMAP	0.49	Isopropyl alcohol–HCl
3. Elution of peak from 1	0.38	DMAP	0.39	1-Butanol–formic acid
		Method B[d]		
1. HCl hydrolysis	0.76	DMAP	0.77[c]	1-Butanol–NH_3
2. Alkaline phosphatase	0.57	DMAPR	0.57	1-Butanol–NH_3

[a] Cells were labeled with a long-term pulse and RNA was extracted with Sarkosyl and phenol; 18S rRNA was separated by sucrose gradient centrifugation, pooled, and precipitated with ethanol as described in Methods. Values for R_F of experimental peak and reference markers are presented. [b] HCl hydrolysis. [c] R_F of DMAP is greater than that reported previously (0.66) (Zimmerman and Holler, 1967) since solvent was allowed to run off serrated edges on bottom of paper in order to move DMAP further down the paper. [d] Alkaline hydrolysis, DEAE-cellulose chromatography. RNA was hydrolyzed in 0.3 M KOH and compounds separated on a DEAE-cellulose column (Figure 8). Aliquot portions from column were appropriately pooled, and subjected to HCl hydrolysis and alkaline phosphatase treatment (Figure 9).

that 18S rRNA contained DMAP, exponentially growing HeLa cells were labeled with [*methyl*-^{3}H]methionine for 22 hr. The RNA in cytoplasmic ribosomes was extracted with Sarkosyl at 0° and isolated by sucrose gradient centrifugation. RNA (28 S and 18 S) from the gradients were each pooled, ethanol precipitated, and hydrolyzed with 1 N HCl. The hydrolysate was chromatographed in 1-butanol–NH_3, where the pyrimidine nucleotides remained at the origin and the purine bases were separated. Figure 1 shows that a radioactive peak in 18S rRNA was present having the same R_F as DMAP (0.77), which was 2.6% of the total radioactivity on the chromatogram (average of three experiments) and corresponded to one or less DMAP mol-

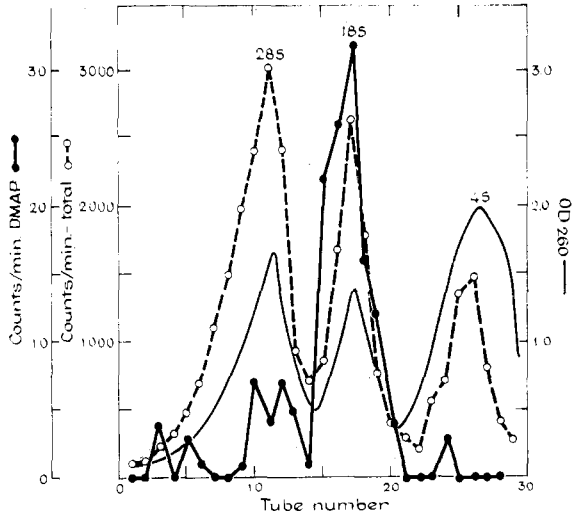

FIGURE 4: Distribution of DMAP in various species of RNA isolated by sucrose gradient centrifugation. HeLa cells were labeled with a long-term pulse. The medium (from 5.5 × 10^7 cells/70 ml) was decanted and replaced with warmed medium containing dialyzed calf serum and [^3H_3C]-methionine (13.7 μCi/μmole, 9-mg/l. final concentration). Cells were incubated at 37° for 23 hr. Total RNA was extracted and layered on 30 ml of a 5–20% sucrose gradient and centrifuged at 25,000 rpm for 18 hr. Four drops per tube were assayed for total radioactivity while 40 drops/tube were saved for assay of DMAP. Sufficient quantity of non-radioactive HeLa RNA was added to each tube to make 5 OD_{260} units. RNA was precipitated with ethanol and assayed for DMAP.

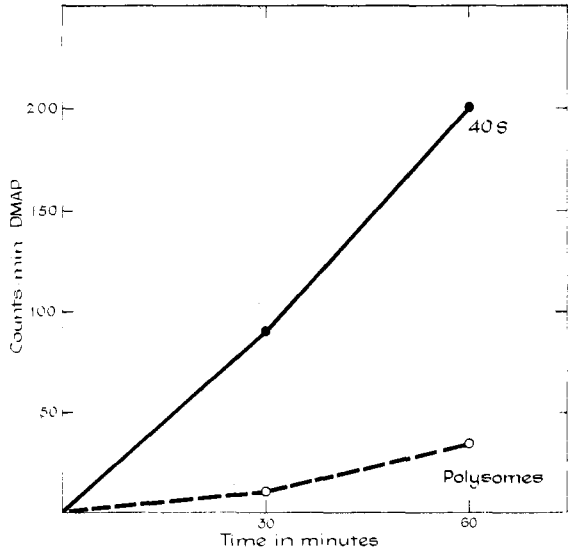

FIGURE 5: Kinetics of formation of DMAP in 18S rRNA of cytoplasmic 40S ribosomal precursor particles and polysomes. HeLa cells were labeled with a short-term pulse. To a suspension culture of 2 × 10^8 cells was added 608 μCi of [^3H_3C]methionine (1610 μCi/μmole) and incubated at 37°. At 30 and 60 min, equal portions of the cell suspension were harvested and 18S rRNA from 40S ribosomal precursor particles and polysomes isolated as described in Methods. rRNA (18S) was then assayed for DMAP.

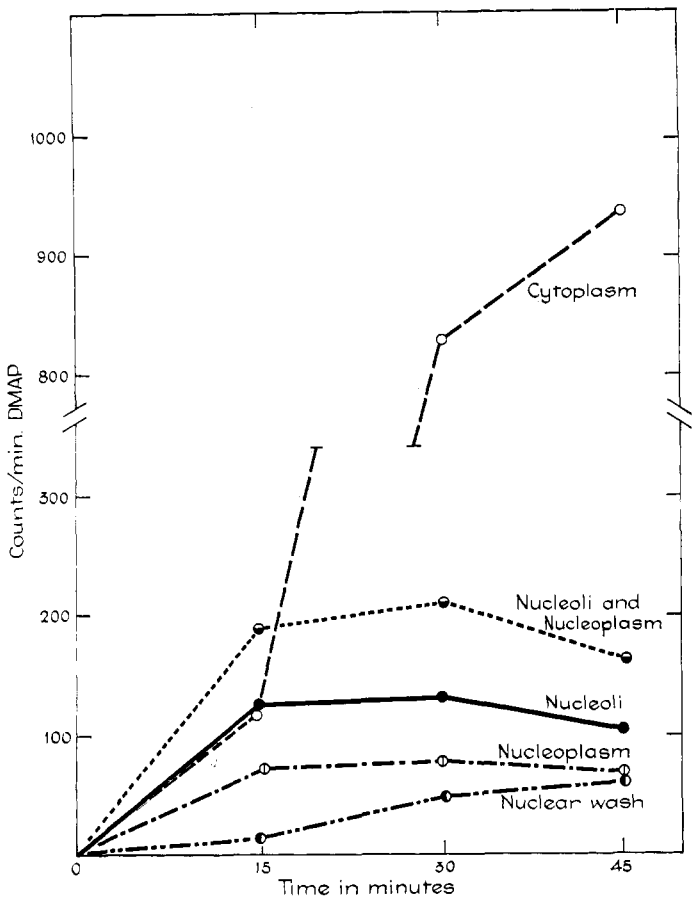

FIGURE 6: Kinetics of formation of DMAP in nucleoli, nucleoplasm, and cytoplasm. HeLa cells were labeled with a short-term pulse. [^3H_3C]Methionine (500 μCi) (4330 μCi/μmole) was added to a suspension culture of 3.1 $\times$ 10^8 cells and incubated at 37°. At indicated times, 10-ml aliquot portions of cell suspension were removed. Nucleoli, nucleoplasm, nuclear wash, and cytoplasm of each sample were fractionated as described in Methods and assayed for DMAP.

ecule per 18S rRNA chain.[2] No comparable peak was found in 28S rRNA, which is in accord with the findings of Brown and Attardi (1965). However, radioactive peaks which had the same R_F value as MAP (0.63) were found in both 28S and 18S rRNA. This would be consistent with the finding that MAP was found in 45S rRNA, which is precursor to both 28S and 18S rRNA. MAP was not reported to be present in either 28S or 18S rRNA by Brown and Attardi (1965). Radioactive peaks were also found at the origin, and at R_F values identical with adenine and guanine, which would agree with the 2'-*O*-methyl nucleotides and the two unknown methylated purine bases found in 45S rRNA (Zimmerman and Holler, 1967; Wagner *et al.*, 1967).

In order to prove that the radioactive peak which had the same R_F value as DMAP was, in fact, DMAP, chromatography in other solvent systems was employed. When the radioactive peak corresponding to the DMAP marker was eluted from the chromatogram with water and rechromatographed, the radioactive peaks had R_F values similar to marker DMAP in isopropyl alcohol–HCl and 1-butanol–formic acid (Table IA). In addition, methyl-labeled 18S rRNA was hydrolyzed in alkali, chromatographed on a column of DEAE-cellulose, and the appropriate fractions were pooled. Radioactive peaks were found in the eluate corresponding to DMAP marker (R_F 0.77) after HCl hydrolysis and to DMAPR (R_F 0.57) after alkaline phosphatase digestion (Table IB). In view of the above studies and those of Brown and Attardi (1965), it is concluded that DMAP is present in 18S rRNA.

Next, the time of appearance of DMAP in 18S rRNA was investigated. The cleavage products of 45S rRNA (35S and 18S rRNA) prominently appear in sucrose gradients after HeLa cells were incubated for 60 min with appropriate radioactive precursors, *e.g.*, uridine (Scherrer and Darnell, 1962) and methionine (Zimmerman and Holler, 1967). Therefore, HeLa cells were pulse labeled with [^3H_3C]methionine for 60 min and 18S rRNA was assayed for DMAP. Figure 2 indicates that DMAP was found in 18S rRNA, while none was found in 35S rRNA, as expected since 35S rRNA is precursor to 28S rRNA. The same methylated products of rRNA labeled for 22 hr (Figure 1) and 60 min (Figure 2) were observed.

To test more rigorously whether DMAP was formed during the period of time after transcription of the 45S rRNA to the time of its cleavage, [^3H_3C]methionine and actinomycin D were simultaneously added to HeLa cells for 30 min. It is observed that when the synthesis of 45S rRNA is blocked by actinomycin, a small peak of radioactivity appears in 18S rRNA (Figure 3a). Note that methylation of 45S RNA does not occur to any appreciable extent (see Figure 8 of Zimmerman and Holler (1967) for comparison with control cells not treated with actinomycin). When samples from the gradient containing 18S rRNA were pooled (see bracket), acid hydrolyzed, and chromatographed, a radioactive peak moving with marker DMAP can be seen (Figure 3b). Therefore, DMAP has been formed in 18S rRNA during the 30-min period in which cleavage of 45S rRNA has occurred. The proportion of DMAP to total methylated products in the chromatogram increased from 2.6% when the methylation process was in the steady state (20–24-hr pulse) to 31% in the absence of RNA synthesis. If the radioactivity below the dotted line (Figure 3a) is taken as tRNA contamination, then 18S rRNA is 38% of the sample. Thus, the appearance of the other methylated products can be attributed to contamination of the 18S rRNA peak in the sucrose gradient with tRNA and the only methylation that occurs after transcription to the time of cleavage of 45S rRNA is the formation of DMAP.

[2] If a molecular weight of 600,000 is assumed for 18S rRNA, then the chain contains 1700 nucleotide residues. Since Brown and Attardi (1965) first showed that 1 out of 48 nucleotide residues of 18S rRNA is methylated (35 methylated residues/chain), then 2.6% DMAP/total methylation represents 0.46 DMAP base/18S rRNA chain, if 2 methyl groups are added to adenine. However, this figure may be underestimated if any labeled DMAP is bound to the origin of the chromatogram, or if any labeled methionine is contaminating the 18S rRNA preparation. The latter appears likely because varying amounts of radioactivity are not absorbed on charcoal after HCl hydrolysis (Figure 9; E. F. Zimmerman, unpublished data).

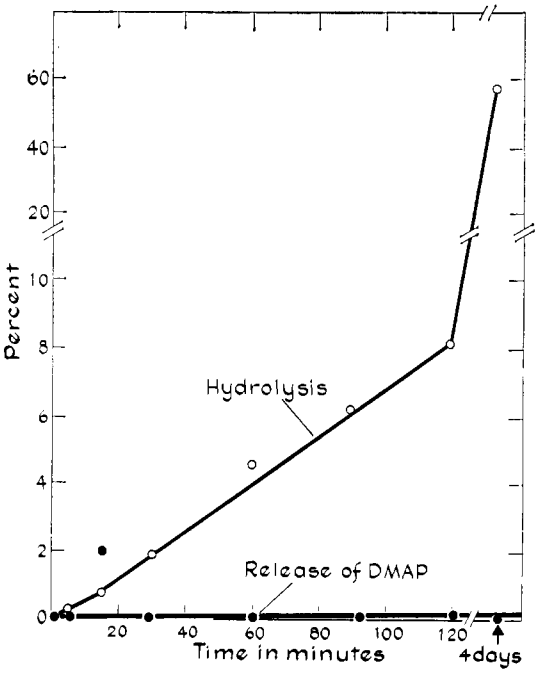

FIGURE 7: Rate of release of DMAP from methyl-labeled 18S rRNA after digestion with snake venom phosphodiesterase. Methylated 18S rRNA was extracted and isolated as previously described from HeLa cells exposed to a long-term pulse of [^3H_3C]methionine. Labeled RNA (1 mg) was incubated at 37° in 5 ml of 0.1 M Tris (pH 8.5), 0.01 M $MgCl_2$, and 100 μg of snake venom phosphodiesterase. At indicated times, 0.5-ml aliquot portions were added to cold 0.5 ml of 1 N perchloric acid, and centrifuged at 10,000 rpm for 30 min to remove unhydrolyzed RNA. Hydrolysis of RNA was measured by the release of nucleotides into the supernatant fraction which absorbed at 260 mμ. DMAP release into the supernatant fraction was assayed as follows. Acidified supernatant fractions were passed through charcoal columns. Nucleotides were eluted with 10 ml of ethanol (50%)–NH_3 (10%) and after NH_3 was removed by evaporation, nucleotides were hydrolyzed with HCl and assayed for DMAP as described in Methods.

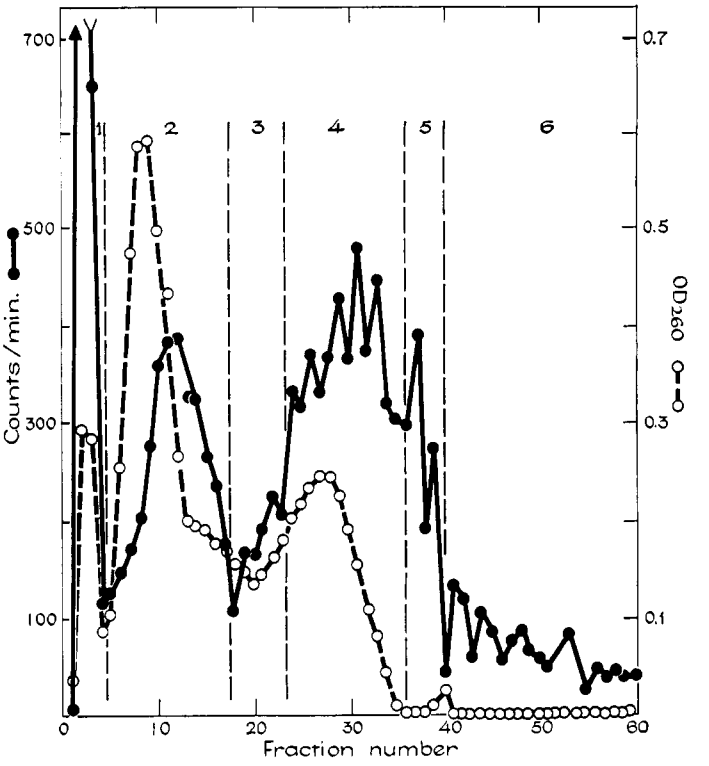

FIGURE 8: DEAE-cellulose chromatography of methyl-labeled 18S rRNA after KOH hydrolysis. [^3H]RNA was incubated at 37° for 18 hr in 0.3 ml of 0.3 M KOH. The solution was neutralized with Dowex 50 (H^+) and the Dowex was removed by centrifugation, washed twice with 0.1 ml of distilled water, and all aqueous fractions were combined. DMAPR (0.5 μmole) marker was added and solution was applied to a DEAE-cellulose column (1 × 22 cm, equilibrated in distilled water) and eluted in the cold with 1200 ml of a linear gradient of 0–0.3 M ammonium carbonate (pH 8.7) (Bell *et al.*, 1963). Fractions (10 ml) were collected, absorbancy at 260 and 280 mμ was measured, and radioactivity in 0.2-ml aliquot portions was measured (in 0.8 ml of distilled water plus 10 ml of Bray's solution). Only the first 600 ml of eluate from the column is presented since radioactivity and absorbancy thereafter were negligible.

In order to rule out the possibility that the labeled DMAP in sucrose gradients represented methylation of mRNA or tRNA rather than 18S rRNA, methyl-labeled RNA (23-hr exposure to [^3H_3C]methionine) was isolated by sucrose gradient centrifugation. Aliquot portions from each tube in the gradient were assayed for total incorporation of the methyl label and for incorporation of radioactivity into DMAP. It was observed that although 28S, 18S, and 4S RNA contained radioactivity in total methylated RNA, only 18S rRNA contained a significant amount of labeled DMAP (Figure 4). There was a small amount of labeled DMAP in the region of 28S rRNA, which may represent aggregation of 18S rRNA. No radioactivity was found in regions of the gradient lighter than 18 S. Since mRNA is found as a broad heterogeneous peak from 12 to 20 S (Latham and Darnell, 1965) and tRNA has a sedimentation value of 4 S, it is concluded that DMAP is formed only in 18S rRNA and does not arise from contamination of the 18S rRNA in the gradient with DMAP-containing mRNA or tRNA.

Site of Formation of DMAP in the Cell. The site in the cell where methylation of RNA which forms DMAP was next investigated. Since it is known that methylation of small molecular weight substrates can occur in rat liver microsomes (Bremer and Greenberg, 1961), the possibility was explored that DMAP was formed in 18S rRNA when it was in a ribonucleoprotein of the cytoplasmic polysomes. HeLa cells were pulsed with [^3H_3C]methionine for 30 and 60 min and cytoplasmic 40S ribosome particles and polysomes were isolated by sucrose gradient centrifugation. RNA (18 S) of each fraction was extracted with Sarkosyl, isolated as described in Methods, and DMAP was assayed. Figure 5 shows that 40S particles, which are one of the precursors to cytoplasmic polysomes (Girard *et al.*, 1965; McConkey and Hopkins, 1965) contained tenfold more radioactive DMAP than did the polysomes at 30 min, and about sixfold more at 60 min. Since the 40S ribosome precursor particles contained labeled DMAP before the polysomes, the cytoplasmic polysomes are not the site where DMAP is formed.

The above result would imply that DMAP is formed

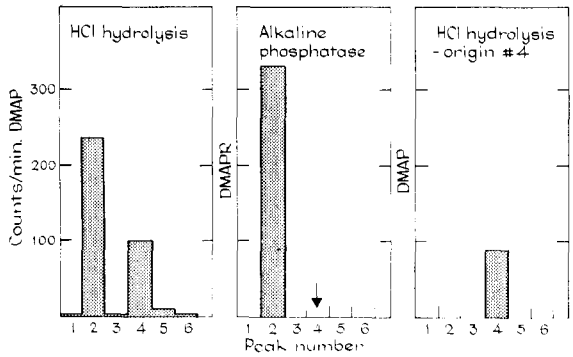

FIGURE 9: Analysis of DMAP and DMAPR in pooled fractions from Figure 8. Fractions between dotted lines were pooled, acidified with 1 N HCl (pH 1–2), and adsorbed onto charcoal columns to remove salts. Ethanol (50%)–NH_3 (10%) (10 ml) was used to elute radioactive compounds, which were taken to 1 ml at 40° with air stream. Five-tenths milliliter was subjected to HCl hydrolysis and chromatographed in 1-butanol–NH_3 as previously described (left panel). The remainder was reduced to 0.1 ml to which 0.1 ml of 0.1 M glycine buffer (pH 9.2) and 50 μg of bacterial alkaline phosphatase were added. Reaction was carried out at 37° for 60 min. Reaction mixture was applied directly to paper with marker DMAPR and chromatographed in 1-butanol–NH_3 (middle panel). Radioactivity in all 1-cm paper strips of chromatogram was counted. Paper strips representing the origin of the chromatogram from peak 4 after alkaline phosphatase treatment (see arrow) were removed from the toluene scintillation fluid, dried, and eluted with 3 ml of 0.1 N NH_4OH. NH_3 was removed by evaporation. The sample was hydrolyzed in HCl, chromatographed, and assayed for DMAP as described in Methods (right panel).

after the 40S ribosome particles leave the nucleus or during synthesis in the nucleus (Vaughn *et al.*, 1967). In order to test the two possibilities, HeLa cells were pulsed for 15, 30, and 45 min with methyl-labeled methionine, and the cells were fractionated into cytoplasm, nuclear wash, nucleoli, and nucleoplasm. It was observed that in 15-min nucleoli plus nucleoplasm (representing the total nuclear fraction) contained more labeled DMAP than did the cytoplasm (Figure 6). In 30 and 45 min, radioactivity in DMAP was sharply increased in the cytoplasm while radioactive DMAP did not increase in the nuclear fraction. Therefore, DMAP is synthesized in the nucleus. When radioactive DMAP in the nucleoli and nucleoplasm were compared, it was found that there was about 60% more DMAP in the nucleolar fraction than in the nucleoplasm at each time. It would therefore appear that the site of formation of DMAP is in the nucleolus where 45S rRNA synthesis and cleavage occur.

Position of DMAP at Ends of 18S rRNA Chain. Since it appeared that there was only one DMAP molecule per 18S rRNA chain, the following hypothesis was considered. A nucleotide on the 45S rRNA chain is methylated (DMAP nucleotide) which could act as a "signal" for a nucleolar phosphodiesterase to cleave the 45S molecule into 35 and 18 S at the position of the DMAP nucleotide. The DMAP remains on the 18S chain. If correct, the hypothesis would predict that DMAP would be situated at either the 3′-OH or 5′-PO_4 end of the 18S rRNA chain. In order to test whether DMAP was positioned on the 3′-OH end, methyl-labeled 18S rRNA was hydrolyzed with snake venom phosphodiesterase, since this exonuclease hydrolyzes nucleic acids at the 3′-OH terminus and continues to release nucleotides in a stepwise fashion proceeding toward the 5′-PO_4 end of the molecule (Razzell and Khorana, 1959). Therefore, if DMAP was at the 3′-OH end of the 18S chain, then all of the DMAP would be released in the first few minutes of the incubation. The results are seen in Figure 7 and show that DMAP is not located at or even near the 3′-OH end of 18S rRNA. In fact, the reaction was even allowed to go 4 days, which produced 60% hydrolysis. No DMAP was released.

Another experiment to support the above result and to test whether DMAP was located at the 5′-PO_4 end of 18S rRNA was then carried out. [*methyl*-^{3}H]rRNA (18S) was hydrolyzed with 0.3 N KOH at 37° overnight, producing a nucleoside derived from the 3′-OH end, a nucleoside 3′,5′-diphosphate derived from the 5′-PO_4 end, 3′(2′)-nucleotides, and dinucleotides where 2′-*O*-methyl nucleotides exist in the chain. These components were separated by chromatography on DEAE-cellulose. In addition, DMAPR was added to the hydrolysate to serve as a marker for the nucleosides which are not retained by the column and appear first after elution. Figure 8 shows the OD_{260} and radioactivity profile. Unlabeled DMAPR appears with a large radioactive peak in fractions 2 and 3. The four 3′(2′)-nucleotides were not completely separated. Absorbance ratios of 280:260 mμ and subsequent HCl hydrolysis or alkaline phosphatase treatment (Figure 9) and chromatography in butanol–NH_3 of the compounds pooled between dotted lines (Figure 8) revealed that 3′(2′)-AMP and 3′(2′)-CMP were in the large peak at fraction 9; 3′(2′)-UMP was present in the shoulder between fraction 12 and 20 and also extending into the next large peak (fraction 26); 3′(2′)-GMP comprised most of the broad peak at fraction 26. Analysis of the radioactivity profile revealed that the methylated compounds in the peak at fraction 11 (pooled peak 2) were the 3′(2′)-methylated nucleotides, including MAP nucleotide. The broad peak of radioactivity at fraction 31 (pooled peak 4) was composed of dinucleotides due to the presence of 2′-*O*-methyl nucleotides. These dinucleotides extended into pooled peaks 5 and 6. The location of DMAP in the pooled peaks (between the dotted lines of Figure 8) is presented in Figure 9. When half of each pooled peak was hydrolyzed in HCl and separated by paper chromatography, it is observed that the radioactivity found in peak 1 was not associated with DMAP. Thus, no DMAPR was present. The large amount of radioactivity in peak 1 did not even adsorb onto charcoal and probably represents contaminating labeled methionine. However, about two-thirds of the DMAP was found in peak 2 where the mononucleotides reside and about one-third was in peak 4 where the dinucleotides (2′-*O*-methyl-NpNp) and the terminal nucleoside diphosphate (pNp) reside. If DMAP was present as the terminal nucleoside diphosphate rather than in a dinucleotide, treatment of the other half of the pooled peak with alkaline phosphatase should remove both PO_4 groups and labeled DMAPR should be found. When DMAPR was assayed

after alkaline phosphatase treatment, it is observed that none was present in peak 4 (arrow). Therefore the DMAP present in peak 4 was probably in the dinucleotide, representing an internal position in the chain. The dinucleotide possessing a 3′,5′-phosphodiester linkage would be resistant to alkaline phosphatase and, since it carries a negative charge, it should be at the origin of the chromatogram after 1-butanol–NH_3 chromatography. This was confirmed by eluting the material of peak 4 off the origin. After HCl hydrolysis and rechromatography, all of the expected DMAP base was found. Therefore, it is concluded that DMAP is not located at the 5′-PO_4 end or the 3′-OH end of the chain.

Discussion

We have previously demonstrated that methylation of rRNA of the HeLa cells occurs on the 45S rRNA precursor as it is being transcribed on the DNA, presumably in the nucleolus. An analysis of the methylated products of 45S rRNA revealed that about 80% of the methyl groups was in pyrimidine nucleotides, and 2′-*O*-methylcytidine was identified. Two unidentified methylated purines and MAP were also isolated. However, DMAP was not seen in 45S rRNA, while it was reported to be located in 18S rRNA by Brown and Attardi (1965). This apparent paradox was clarified when it was observed that a secondary methylation of rRNA occurred. The salient features are: (1) DMAP is located in 18S rRNA and not in 28S or 35S rRNA; (2) DMAP appears in 18S rRNA at about the time of cleavage of 45S rRNA rather than at the time of synthesis (no significant amount of DMAP was observed in 45S rRNA); (3) DMAP is synthesized in the nucleolus, where cleavage of 45S rRNA occurs (both 45S and 35S rRNA are found there) (Penman, 1966); (4) the amount of DMAP present corresponds to about one DMAP molecule per 18S rRNA chain.

Why should the HeLa cell methylate just one base in a nucleic acid? Although no physiologic function is known for methylation of nucleic acids, the above-mentioned observations suggested the following possibility. A nucleotide on the 45S rRNA chain is methylated (DMAP nucleotide), which could act as a signal for a nucleolar phosphodiesterase to cleave the 45S molecule into 35S and 18S rRNA at or near the location of the DMAP nucleotide. The DMAP remains on the 18S rRNA chain. That the DMAP in the chain could contain such information is suggested by the fact that the nucleotide cannot form hydrogen bonds, since poly DMAP does not complex with poly U (Griffin *et al.*, 1964). If DMAP in the 45S rRNA molecule acts as such a signal, then of course, a specific DMAP methylase must methylate 45S rRNA in a specific site on the chain. Also, one would suspect that DMAP would be located on or very near either end of the 18S rRNA chain. Experiments were carried out to determine if DMAP was, in fact, located on the 5′-PO_4 or the 3′-OH end of the chain. The results indicated that this was not the case. Why DMAP was found as a mononucleotide and a dinucleotide after alkaline hydrolysis is not known. Possibly the DMAP nucleotide is adjacent to a 2′-*O*-methyl nucleotide and one-third was still hydrolyzed, or it is adjacent to a nonmethylated nucleotide and hydrolysis was incomplete, or there are two or more DMAP nucleotides present in the chain and the per cent DMAP found was low. It is still possible that DMAP is not on but near the terminal end of the chain and confers information to a phosphodiesterase. However, it has been recently shown by Weinberg *et al.* (1967) that there may be more complex interconversions of the rRNA precursors than hitherto believed. These workers found RNA molecules having *s* values of 45, 41, 36, 32 (equivalent to 35S RNA described here), 20, and 18 S. If there are multiple cleavages of the 45S RNA, then it is hard to imagine that DMAP could possess an informational role in the cleavage of one of these intermediates.

Since DMAP was found in the nucleolus and no 18 S is found there (Penman *et al.*, 1966), it appears likely that DMAP appears first in one of the cleavage products of 45S RNA. With the methods used in this study, if DMAP was on either the 41S or 36S RNA, then it would probably be detected in my 45S or 35S RNA fraction. This was not found. Therefore, it is more likely that DMAP is present in 20S nucleolar RNA, which is converted into 18S rRNA (Weinberg *et al.*, 1967).

An alternate explanation for the appearance of DMAP in 18S rRNA might be considered. Since there is evidence that 45S RNA and subsequent cleavage products are associated with proteins as ribonucleoprotein particles (Tomaoki, 1966; Warner and Soeiro, 1967), then only one site on one of these ribonucleoproteins might be exposed to DMAP methylase. Thus the ribosomal proteins would be covering up most potential methylation sites. This would be in accord with the finding that most methylation of 45S RNA occurs at the time of its transcription from the DNA (Zimmerman and Holler, 1967; Greenberg and Penman, 1966), since the nascent RNA growing chain would be exposed to the methylating enzymes. After the ribosomal proteins associate with the RNA, then the only site left on one of the ribonucleoproteins would be exposed to DMAP methylase and DMAP nucleotide would be formed. This ribonucleoprotein which underwent a secondary methylation would then mature into the 40S ribosomal particle containing 18S rRNA.

It is not known whether DMAP was synthesized by the addition of one methyl group on MAP formed at the time of transcription or by the addition of two methyl groups on adenine.

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Association of Complementary Oligoribonucleotides in Aqueous Solution*

S. R. Jaskunas, Charles R. Cantor,† and I. Tinoco, Jr.

ABSTRACT: Oligoribonucleotide complexes are plausible models for codon–anticodon interactions and the double-strand regions in transfer ribonucleic acid. Several pairs of antiparallel complementary oligoribonucleotides have been mixed in a 1:1 mole ratio under conditions favorable for intermolecular association. The conditions were 0.01 M total residue concentration, 0.01 M $MgCl_2$, or 0.5 M NaCl, pH 7, and 1°. Optical rotatory dispersion has been used to detect the existence of a complex. Interaction between GpGpC and GpCpC has been observed.

The complex probably contains two molecules of GpGpC for every molecule of GpCpC. However, there are more than three trinucleoside disphosphates per complex. Other pairs of complementary oligo ribonucleotides were mixed under similar conditions, but no interaction was observed. These include ApC and GpU, ApCpU and ApGpU, ApGpC and GpCpU, and ApApApA and UpUpUpU. Self-aggregation has been observed with ApGpC and GpGpC. Aggregates of the latter compound contain up to 40 trinucleoside diphosphates/complex. Our results suggest that the ribosome or transfer ribonucleic acid structure must help, in some way, to stabilize complexes between the anticodon and messenger ribonucleic acid. Calculations of the stability of triple-strand regions in transfer ribonucleic acid like $(GpGpC)_2$:GpCpC indicate that such structures could exist.

A base-pairing arrangement resembling a cloverleaf has been suggested as a possible secondary structure for tRNA molecules (Holley *et al.*, 1965). In this model the polynucleotide chain folds back upon itself forming loops that are held together by A–U and G–C base pairs. Each molecule contains several loops, separated by short double-stranded regions.

The existence of base pairs in tRNA is supported by several different types of evidence (Englander and Englander, 1965; Felsenfeld and Sandeen, 1962; Cantor *et al.*, 1966). It is also known that the base pairs are organized into more than one distinct helical region (Fresco *et al.*, 1963; Felsenfeld and Cantoni, 1964). Recent small-angle X-ray (Lake and Beeman, 1967) and chemical studies (Brostoff and Ingram, 1967; Nelson *et al.*, 1967) are in general consistent with the cloverleaf model for tRNA. Perhaps the best evidence for this cloverleaf pattern of base pairs is the fact that this model can be constructed for every one of the tRNAs of known

* From the Department of Chemistry and the Chemical Biodynamics Laboratory, University of California, Berkeley, California 94720. *Received March 20, 1968.* This work was supported in part by Research Grant GM 10840 from the National Institutes of Health, and the U. S. Atomic Energy Commission.

† Present address: Department of Chemistry, Columbia University, New York, N. Y.