proportion of alkylation on the ring. This fact, as well as other differences in reaction conditions, may account for the great variation of values for N-3 methylation of U and T reported by different investigators (Singer, 1975a).

While methylation is, by a factor of 10–30, more efficient than ethylation in alkylating the ring of nucleotides at pH 6.8, phosphate alkylation is less affected by the nature of the alkyl group, and the formation of ethyl esters is thus the by far predominant event. This is in line with our earlier finding that ethylation of DNA or RNA leads to increased alkylation of phosphodiesters (Singer and Fraenkel-Conrat, 1975; Sun and Singer, 1975). We thus propose that ethylating agents have a similar affinity for tertiary phosphates as they are now shown to have for the secondary phosphates of nucleotides. Preliminary experiments on the alkylation of poly(U) (Kuśmierek and Singer, unpublished) also support this, since phosphotriesters are formed in poly(U) when treated at pH 6.8 with Me_2SO_4 and more with Et_2SO_4, and the relative amounts of ethyl and methyl triesters are similar to the ethyl and methyl diesters in Table II.

Acknowledgment

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DNA-Dependent Protein Methylase Activity in Bull Seminal Plasma†

Bertrum Sheid* and Lauri Pedrinan

ABSTRACT: The existence of a DNA-dependent protein methylase activity without any concomitant DNA methylase activity was demonstrated in bull seminal plasma. The enzyme utilized *S*-adenosyl-L-methionine as a methyl donor, and endogenous seminal plasma protein as the substrate. There was no demonstrable enzyme activity when the seminal plasma was preheated at 100° for 10 min, or when the enzyme reaction mixture was incubated at 4°. The protein methylase required a heterologous DNA source, had optimal activity at pH 8.1, and was enhanced in the presence of Mg^{2+}, NH_4^+, and reduced glutathione. After the methylated protein product was separated from DNA by extraction with 0.2 *M* HCl, the incorporated radioactivity was shown to be totally solubilized by incubating the protein either with Pronase or 1 *M* NaOH, while RNase and DNase had no effect. Approximately 70% of the enzymatically synthesized amino acids in the protein product were tentatively identified as O-methylated amino acid ethers by virtue of their elution from a Dowex 50 H^+ column with 0.2 *M* pyridine, and their stability to acid and base hydrolysis. The partially purified methylated product was shown by Sephadex G-50 chromatography to consist of three distinct radioactive proteins with molecular weights of approximately 21,000, 15,000, and 10,000.

The enzymatic methylation of specific amino acid residues in preformed protein macromolecules with *S*-adenosyl-L-methionine as the active biological methyl donor is a well-recognized biological phenomena (Paik and Kim, 1971). However, the physiological consequences of this type of enzymatic restructuring of proteins after their initial synthesis remains an enigma.

Burdon et al. (1967) and Burdon (1971) demonstrated

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that the protein methylase activity in an enzyme extract derived from Krebs 2 ascites cell nuclear chromatin was inversely proportional to the activity of an endogenous DNA methylase. In a similar nuclear preparation from rat spleen, Kalousek and Morris (1968) showed that the DNA and protein methylases were independent enzymes. However, the protein methylase activity was stimulated by an exogenous source of salmon testes DNA, and was able to methylate endogenous nuclear protein.

In the present study, data are presented which demonstrate that although bull seminal plasma does not contain any DNA methylase activity, it does possess a DNA-dependent protein methylase activity with properties similar to the protein methylase described by the latter investigators.

Materials and Methods

Ejaculated semen was collected over liquid nitrogen from bulls used for artificial insemination projects. No preservatives or antibiotics were added. The product was generously provided by the Eastern Artificial Insemination Corp., Ithaca, N.Y. *S*-Adenosyl-L-methionine-*methyl*-^3H (specific activity 5 Ci/mmol) was purchased from the New England Nuclear Corporation. Chromatographically pure DNase, RNase (preheated at 80° for 10 min), and the amino acid standards were products of the Sigma Chemical Corp. Salmon testes DNA and calf thymus DNA were purchased from the Worthington Biochemical Corp., while DNA from *Bacillus subtilis, Escherichia coli B, E. coli* K12W6, and bull sperm were isolated by a modification (Garrett et al., 1973) of the method described by Marmur (1961). The protein standards, ovalbumin, chymotrypsinogen, and RNase A, were purchased from Pharmacia Fine Chemicals, Inc. The insulin trimer was purchased from the Sigma Chemical Corp., while the insulin monomer was prepared by heating the trimer in the presence of mercaptoethanol. Eastman Kodak Sheet No. 6061 impregnated with silica gel G was obtained from the Eastman Kodak Co., while Polygram Cel 300 (0.1 mm), a product of the Macherey-Nagel Co., was purchased from Brinkman Instruments.

Equipment used in these experiments was: an International Centrifuge Model PR-2, a Beckman Model DU 2400 spectrophotometer, a Beckman liquid scintillation system LS-100C, and an LKB Ultrorac 7000 fraction collector.

Preparation of Seminal Plasma. Whole semen was centrifuged at 850 *g* at 4° for 20 min in an International Centrifuge. The seminal plasma was removed with a Pasteur pipet and recentrifuged at 850*g* for 15 min at 4°.

DNA-Dependent Protein Methylase Assay. The assay mixture employed to achieve maximal enzyme activity was composed of 25 m*M* Tris, 2 m*M* $MgCl_2$, 20 m*M* NH_4OAc, 3.7 m*M* of freshly prepared neutralized reduced glutathione, 1 mg of seminal plasma protein (as determined by the method of Lowry et al. (1951), 1 mg of salmon testes DNA, and 1 μCi of *S*-adenosyl-L-methionine-*methyl*-^3H in a total volume of 4 ml at a final pH of 8.1. Concurrent blanks were assayed which consisted of either the enzyme assay mixture without any added DNA, or the entire reaction mixture in which the seminal plasma was preheated for 10 min at 100°. After 1 hr at 37°, 0.5 ml of 3 *M* hydroxylamine (pH 7.2) was added to all of the tubes, which were then reincubated at 37° for 15 min (Sheid et al., 1968). The enzyme reactions were terminated by rendering the assay mixtures 0.2 *M* with respect to HCl and 5% with respect to Cl_3CCOOH. After 15 min at 4°, the precipitates were vigorously washed with successive portions of 95% ethanol, ethanol–ether (3:1), two times, and ether. After being dried in a desiccator, the precipitates were dissolved in 88% formic acid at 60°. The digest was then added to 10 ml of a toluene-based scintillating fluid and counted in a liquid scintillation system. An alternative method employed was to place the acid precipitates directly on filter paper, and then wash with 5% Cl_3CCOOH, 95% ethanol, ethanol–ether (3:1), and ethanol. The dried filter papers were placed directly into the scintillating fluid and counted.

Isolation of DNA and the Methylated Proteins. In order to isolate the exogenous DNA after the completion of the enzyme reaction, four separate but identical enzyme assays were performed using the optimal conditions for determining DNA-dependent protein methylase activity. After incubation for 1 hr at 37°, the enzyme assay mixtures were combined and the reaction was terminated with an equal volume of water-saturated phenol. The DNA was isolated and purified by a modification of the method of Marmur (1961) which encompassed a Pronase digestion and an extra phenol extraction step (Garrett et al., 1973).

In order to isolate the methylated proteins after the enzyme reaction, 20 separate but identical enzyme assays were performed. The reactions were terminated with HCl and Cl_3CCOOH, washed, and then dried as described previously. The basic proteins in the individual assay tubes were extracted from DNA with 0.2 *M* HCl and then pooled. After all traces of HCl were removed by repeated flash evaporation, the methylated proteins were solubilized from the residue with 0.05 *M* acetate buffer (pH 5.2).

Isolation of Radioactive Amino Acids. One portion of the acetate buffer containing 0.5 mg of protein was set aside for ion-exchange chromatography. The other fraction of acetate buffer containing 0.9 mg of protein was brought to dryness by flash evaporation. The resultant residue containing the methylated proteins was then dissolved in 6 *M* HCl. The tubes were sealed and the protein was hydrolyzed by heating at 115° for 24 hr. All traces of HCl were then removed by repeated flash evaporation, and the resultant amino acid residue was dissolved in 10 ml of water. The solution was then shaken with 2 g of washed Dowex 50 H^+ form (200–400 mesh) for 1 hr. The bound amino acids were then eluted by shaking vigorously for 45 min with successive portions of 0.2 *M* pyridine, 1 *M* pyridine, and 4 *M* NH_4OH. Approximately 90% of the bound radioactive amino acids were recovered in the 0.2 *M* pyridine fraction.

Molecular Weight Estimation. Protein (0.5 mg) dissolved in 50 m*M*, pH 5.2, acetate buffer was subjected to Sephadex G-50 chromatography in a column 0.75 cm × 120 cm. The column was equilibrated and eluted with 50 m*M*, pH 5.2, buffer; 1-ml fractions were collected at a rate of 0.5 ml/min. The 280 A and radioactivity in each of the fractions were then measured. Protein standards of ovalbumin (mol wt 45,000), chymotrypsinogen (mol wt 25,000), insulin trimer (mol wt 18,000), RNase A (mol wt 13,700), and insulin monomer (mol wt 6,000) were also passed through the same Sephadex G-50 column, eluted with 50 m*M*, pH 5.2, acetate buffer, and collected in 1-ml fractions.

Formation of Methanol by Distillation. After acid hydrolysis of the methylated proteins, the HCl was removed by repeated flash evaporation. The residue was dissolved in 2 ml of 0.3 *M* KOH, after which 1 ml of carrier methanol was added. Distillation was then performed according to the method of Kim and Paik (1971). An alternative procedure was to directly hydrolyze the protein in 0.3 *M* KOH before adding the methanol and distilling.

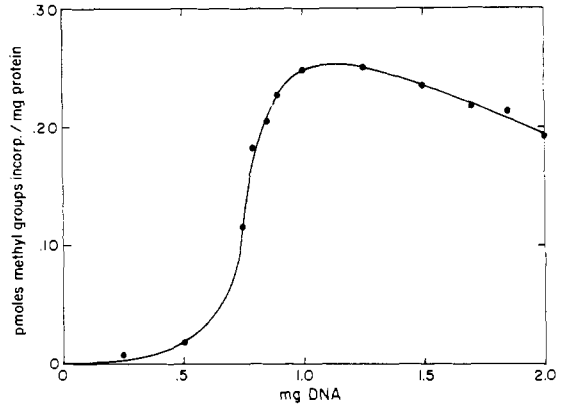

FIGURE 1: Dependence of the protein methylase on exogenously added DNA. Varying amounts of salmon testes DNA were added to the enzyme assay mixture, as detailed under Materials and Methods. Blanks, which were concurrently assayed with the samples, contained preheated enzyme extract (100° for 10 min) with the appropriate amount of DNA.

Table I: DNA-Dependent Protein Methylase Activity in the Presence of DNA from Different Sources.[a]

Source of DNA	Enzyme Activity (pmol of Methyl Groups Incorp. per mg of DNA per mg of Protein
Salmon testes (native)	0.29
Salmon testes (denatured)	0.28
Calf thymus	0.25
Yeast	0.12
E. coli K12W6	0.08
E. coli B	0.04
B. subtilis	0.03
Bull sperm	0

[a] One milligram of DNA was used in all of the assays, which were performed in accordance with optimal enzyme conditions as described under Materials and Methods. Blanks, which consisted of seminal plasma which had been preheated for 10 min at 100°, and the appropriate DNA were assayed concurrently with all of the samples.

Results

Enzymatic Requirements for Endogenous DNA. Figure 1 shows that when employing 1 mg of seminal plasma protein, increasing amounts of salmon testes DNA resulted in an increased incorporation of methyl groups from *S*-adenosyl-L-methionine into the plasma proteins. The addition of 0.3 mg of exogenous DNA was necessary to observe the enzyme reaction, while 1 mg of DNA was employed to obtain the optimal activity of 0.26 pmol of methyl groups incorporated per mg of protein. Without the presence of exogenous DNA, 0.04 pmol of methyl groups was incorporated into the 1 mg of seminal plasma protein. However, this incorporation was the same even after preheating the enzyme extract at 100° for 10 min, or when the enzyme reaction was incubated at 4° in lieu of 37°. Therefore, this latter activity without the presence of DNA was assumed to be due to nonspecific absorption of *S*-adenosyl-L-methionine to protein, which persisted even after washing. It was also demonstrated that there was essentially no enzyme activity when seminal plasma, preheated at 100° for 10 min, was added to the complete reaction mixture which included the exogenous DNA. In ensuing experiments, the radioactivity incorporated into the plasma proteins, employing the complete enzyme assay mixture with preheated seminal plasma, was utilized as the enzyme blank value. These values were subsequently subtracted from the results obtained when unheated seminal plasma was used with the complete reaction mixture.

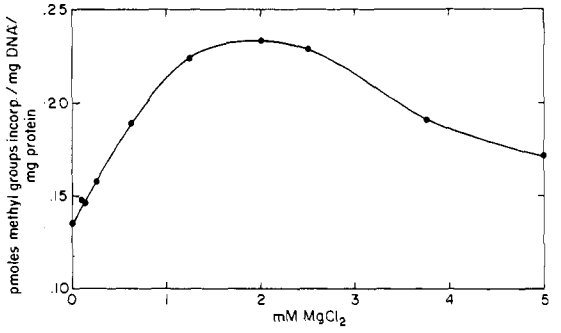

FIGURE 2: Stimulation of DNA-dependent protein methylase activity by magnesium. Varying amounts of $MgCl_2$ were added to the standard enzyme assay mixtures and their blanks, as described under Materials and Methods.

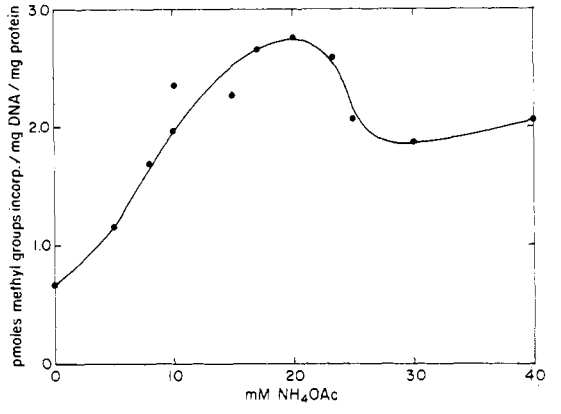

FIGURE 3: Stimulation of DNA-dependent protein methylase by ammonium. Different concentrations of ammonium acetate were added to the enzyme assay mixtures and their blanks. Optimal enzyme conditions were employed as described under Materials and Methods.

Table I illustrates the fact that salmon testes (native and denatured) DNA and calf thymus DNA served as the most efficient cofactors which were tested. DNA from yeast and various bacteria was less efficient, while homologous DNA from bull sperm was unable to participate at all in the enzyme reaction.

Characteristics of the Enzyme Reaction. The optimal enzyme activity was achieved at pH 8.1. There was no enzyme activity below pH 5, or above pH 11. Figure 2 illustrates the fact that the enzyme reaction was stimulated approximately 70% in the presence of 2.5 m*M* Mg^{2+}, while Figure 3 shows that 20 m*M* NH_4^+ stimulated the enzyme activity by about 400%. The protein methylase activity was also enhanced by approximately 70% in the presence of 3.7 m*M* reduced glutathione. As illustrated in Figures 2 and 3, increasing the amounts of Mg^{2+} and NH_4^+ past their peak values resulted in declining enzyme activity. It was also demonstrated that the enzyme activity was enhanced by adding increasing amounts of seminal plasma protein. The activity first became apparent when 0.3 mg of protein was used, then increased sixfold when 0.5 mg of protein was employed. The enzyme activity then remained constant in the presence of 0.6–2.0 mg of endogenous seminal plasma protein.

Identification of Protein as the Methylated Substrate. DNA which was isolated from the reaction mixture and

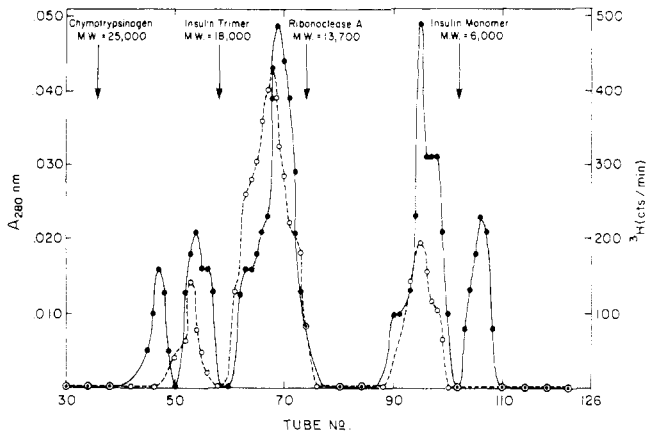

FIGURE 4: Isolation and molecular weight estimation of enzymatically methylated seminal plasma proteins. (●) 280 A used to measure protein content; (○) recovery of radioactive ^3H (cpm). Each tube contains 1 ml of eluent. After the enzyme assay was terminated, the plasma proteins were extracted from the DNA with 0.2 *M* HCl. After all traces of HCl were removed by repeated flash evaporation, the methylated proteins were solubilized from the residue in 0.1 *M*, pH 5.2, acetate buffer, 0.5 mg of the isolated protein was passed through a Sephadex G-50 column (0.75 cm × 120 cm) which was equilibrated and eluted with 50 m*M*, pH 5.2, acetate buffer at a rate of 1 ml/2 min. Purified protein standards of ovalbumin, chymotrypsinogen, RNase A, and the monomer and trimer of insulin were also passed through the same Sephadex G-50 column, and eluted in the exact manner as the plasma proteins. The fractions in which the individual protein standards were eluted are indicated by the appropriate arrows.

then purified did not contain any of the incorporated radioactive methyl groups. The radioactivity was found to reside exclusively with the seminal plasma proteins which were separated from the DNA by extraction with 0.2 *M* HCl. After the HCl was removed by flash evaporation, the protein from the residue was found to be solubilized by 0.1 *M*, pH 5.2, acetate buffer. These procedures afforded a 15–20-fold purification of the enzymatically methylated seminal plasma proteins.

In initial experiments it was demonstrated that DNase was able to solubilize the radioactive protein which was bound to the DNA in the crude enzyme assay mixture. This solubilization was subsequently proven to be due to the fact that after the DNA was digested by DNase, the methylated protein became soluble in the acid media used to precipitate the remaining proteins. In these same initial experiments, Pronase was only able to partially hydrolyze (50%) the modified protein. This was latter demonstrated to be due to the protection of the methylated protein afforded by the bound DNA. 1 *M* NaOH was able to totally hydrolyze the methylated protein, even in the presence of complexed DNA, while RNase had no effect. After being separated from DNA by extraction with 0.2 *M* HCl, the radioactivity in the methylated proteins was completely solubilized after incubation with either Pronase, 0.5 *M* NaOH, 0.3 *M* KOH, or 6 *M* HCl, while DNase had no effect.

Properties of the Radioactive Amino Acids. Following acid hydrolysis of the methylated protein in 6 *M* HCl, approximately 70% of the radioactivity from the resultant amino acids was found to bind to Dowex 50 H^+. The resin was eluted with successive 20-ml portions of 0.2 *M* pyridine, 1 *M* pyridine, and 4 *M* NH_4OH. Essentially all of the bound radioactivity was recovered in the 0.2 *M* pyridine wash. Distillation of either acid- or base-hydrolyzed methylated proteins in the presence of carrier methanol resulted in the recovery of approximately one-third of the radioactive counts.

Molecular Weight Estimation of the Methylated Protein Substrates. Five different protein peaks were resolved by passing the partially purified methylated protein through a Sephadex G-50 column, ss depicted in Figure 4. Radioactive methyl groups appeared in three of the protein peaks, which were calculated to have molecular weights of 21,000, 15,000, and 10,000. The molecular weights were derived from a graph which was a plot of the molecular weights of the standard proteins vs. the tube number in which the standards were eluted. The same Sephadex G-50 column was used for eluting the standard proteins and the methylated seminal plasma proteins.

Discussion

It has been demonstrated in the present study that bull seminal plasma contains a DNA-dependent protein methylase which is capable of methylating endogenous basic seminal plasma proteins. In preliminary experiments it was shown that apparently there was no DNA-dependent protein methylase activity in sperm cell nuclei. However, it is possible that the enzyme is present in sperm nuclei, but not detectable because other nonsubstrate basic proteins may exist in these fractions which inhibit the protein methylase activity by competing for the DNA binding sites. Therefore, one cannot eliminate the possibility that the methylase enzyme found in the seminal plasma is a leakage product from intact sperm cells. The enzyme activity observed in these experiments may very well be similar to the DNA-stimulated protein methylase activity observed in rat spleen nuclei by Kalousek and Morris (1968).

The protein methylase activity was enhanced in the presence of NH_4^+, Mg^{2+}, and reduced glutathione. The fact that DNA from different sources vary in their ability to act as a cofactor for the protein methylase indicates that there exists specificity. The rather large amount of DNA necessary to observe the enzyme reaction (0.3 mg of DNA/mg of plasma protein) may be due to either the presence of other basic proteins which inhibit the enzyme reaction by competing for the DNA binding site, as stated previously, or to the possibility that a more specific DNA is required as the cofactor.

Approximately 70% of the enzymatically synthesized amino acid residues in the methylated proteins were tentatively identified as O-methylated amino acid ethers. This conclusion may be inferred from the fact that the methylated amino acids were able to bind to Dowex 50 H^+, and then be eluted with 0.2 *M* pyridine. The O-methylated amino acids, which are Lewis bases, may bind to the Dowex resin by virtue of hydrophobic binding, and then can be readily displaced by 0.2 *M* pyridine. Since there was essentially no radioactivity recovered after washing the Dowex 50 with 1 *M* pyridine and 4 *N* NH_4OH following the 0.2 *M* pyridine wash, one can eliminate the synthesis of the methylated derivatives of imidazoles, lysines, and arginines as products of the enzymatic reaction (Haverberg et al., 1974; Kakimota and Akazawa, 1970).

The possible enzymatic synthesis of aspartyl and glutamyl methyl esters was eliminated, based on the fact that distillation of methanol after either base or acid hydrolysis of the methylated protein yielded the same amount of radioactivity (approximately one-third of the total). It is well known that acid hydrolysis destroys ester bonds, while ethers are usually HCl resistant. The only possible enzymatically synthesized methylated amino acid derivatives, which are HCl-resistant and can theoretically form distilla-

ble methanol after acid hydrolysis (by an α,β elimination mechanism), are *O*-methylserine and *O*-methylthreonine. Therefore, these two *O*-methylated amino acids may account for one-third of the amino acid ethers which are enzymatically synthesized in these experiments. Confirmation of these data awaits further resolution and analysis of sufficient quantities of the O-methylated amino acids.

It is reasonable to assume that mammalian semen requires an efficient biological mechanism to prevent the integration of foreign DNA into the spermatozoa. Since the modification-restriction mechanism of bacteria to rapidly destroy exogenous DNA (Meselson et al., 1972) has never been demonstrated in any mammalian tissue, it becomes tempting to speculate at this juncture that specific semen proteins bind to exogenous DNA via their basic amino acids and, in so doing, alter their conformation so as to expose amino acid residues which can subsequently be O-methylated. The O-methylated amino acids may then function as recognition sites for restriction nucleases which inactivate the foreign DNA.

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Methylated Messenger RNA in Mouse Kidney†

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ABSTRACT: Polyadenylated messenger RNA from mouse kidney labeled in vivo exhibited a pattern of methylation distinct from that of rRNA and tRNA. After mice were given L-[*methyl*-^{3}H]methionine, 4% of the polyribosomal RNA label was bound to oligo(dT)-cellulose; 20–24% of orotate- or adenine-labeled polyribosomal RNA eluted in the poly(A)$^+$ RNA fraction under similar conditions. [^{3}H]Methyl radioactivity was not incorporated into low molecular weight (5–5.8 S) rRNA, indicating the extent of nonmethylpurine ring labeling was negligible. [^{3}H]Methyl-labeled poly(A)$^+$ RNA sedimented heterogeneously in sodium dodecyl sulfate containing gradients similarly to poly(A)$^+$ mRNA labeled with [^{3}H]orotic acid. Based on an average molecular length of 2970 nucleotides, renal mRNA was estimated to contain 8.6 methyl moieties per molecule. Analysis of alkaline-hydrolyzed RNA samples by DEAE-Sephadex-urea chromatography provided estimates of the relative amounts of base and ribose methylation. Although 83% of the [^{3}H]methyl radioactivity in rRNA was in the 2′-*O*-methylnucleotide fraction, no methylated dinucleotides were found in mRNA. In poly(A)$^+$ mRNA 60% of the [^{3}H]methyl label was in the mononucleotide fraction; the remainder eluted between the trinucleotide and tetranucleotide markers and had a net negative charge between −4 and −5. The larger structure, not yet characterized, could result from two or three consecutive 2′-*O*-ribose methylations, and is estimated to contain 2.6 methyl residues. Alternatively, the oligonucleotide could be a 5′-terminal methylated nucleotide species containing 5′-phosphate(s) in addition to the 3′-phosphate moiety resulting from alkaline hydrolysis. Either structure could have a role in the processing or translation of mRNA in mammalian cells.

Stable rRNA and tRNA in eukaryotic cells originate in the nucleus as larger precursor molecules that must be modified before appearing in the cytoplasm (Darnell, 1968; Weinberg and Penman, 1970; Burdon and Clason, 1969; Bernhardt and Darnell, 1969). Maturation of both rRNA and tRNA involves post-transcriptional methylation of specific nucleotides in the nuclear precursor molecule (Greenberg and Penman, 1966; Vaughan et al., 1967; Choe and Taylor, 1972), with the methylated regions being conserved in the mature cytoplasmic species (Weinberg et al., 1967; Vaughan et al., 1967; Maden et al., 1972; Maden and Salim, 1974).

Although the mRNAs of certain viruses contain methylated sequences (Wei and Moss, 1974; Shatkin, 1974; Furuichi, 1974; Rhodes et al., 1974), mammalian mRNAs have generally been considered nonmethylated (Greenberg and Penman, 1966; Perry and Kelley, 1970). The presence of

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