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In Vitro Enzymatic Assay of RNA Methylation

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by

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ABSTRACT

IN VITRO ENZYMATIC ASSAY OF RNA METHYLATION

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Old Dominion University, 1981
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An assay procedure for in vitro enzymatic methylation of mammalian ribosomal RNA has been developed in this study. The assay procedure, utilized for the comparison of normal and neoplastic methylase activities (using mouse liver and Ehrlich ascites cells as sources of enzyme), is a modification of previously published methods (52,53). A 100,000 x g supernatant (S100) enzyme preparation was incubated with 28S-5.8S rRNA and tritium-labeled S-adenosyl-L-methionine. The RNA was extracted, applied to DEAE cellulose paper, washed, and the radioactivity counted. The neoplastic cell methylase preparation was more active in methylating both exogenous neoplastic and normal 28S-5.8S rRNA than the normal enzyme preparation. However, based on DEAE-Sephadex chromatograms of ribonuclease T_2 digests of tritium-labeled RNA from neoplastic cell methylase assays, the in vitro methylation is almost exclusively restricted to the purine and pyrimidine bases. Although this in vitro base methylation may not represent the cellular situation, which is almost exclusively 2'-O-ribose methylation, the higher neoplastic cell methylase activity correlates well with previously published studies and indicates that this assay procedure is a useful tool for further study of normal and neoplastic cell methylase activities.

DEDICATION

The author would like to dedicate this thesis to her husband

Shelley Paul Gallup, Jr.

for his love and devotion, and his steadfastness through it all.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

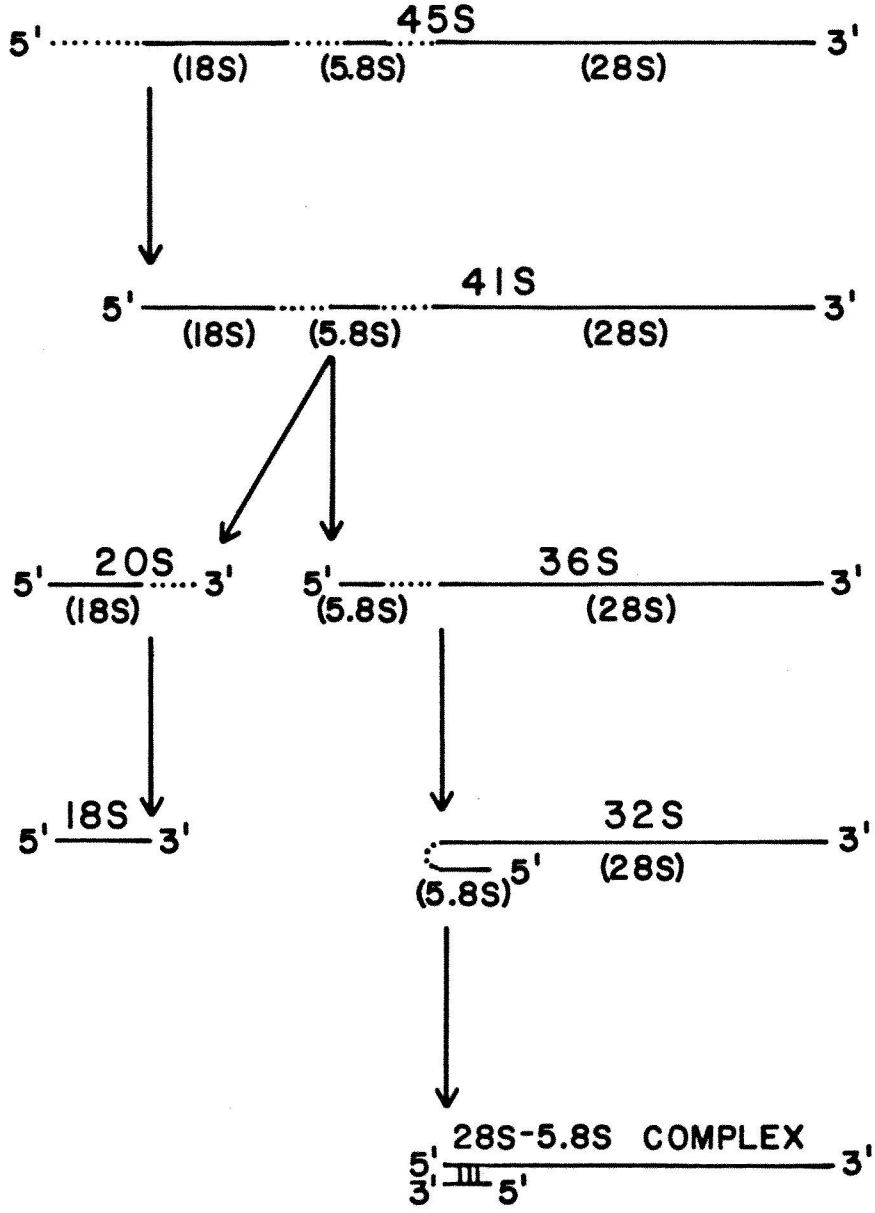
The post-transcriptional processing of eucaryotic ribosomal RNA (rRNA) to form mature functioning molecules active in the synthesis of proteins is accomplished by three specific enzymatic processes: cleavage of precursor sequences, methylation of ribose and base moieties, and conversion of uridine to pseudouridine. This thesis will be concerned primarily with enzymatic methylation of rRNA, especially in regard to differences in normal and neoplastic cells.

The bulk of cytoplasmic rRNA is transcribed in the nucleolus as a 45S rRNA precursor which is cleaved and modified to yield the final product found in cytoplasmic ribosomes (1). The 45S rRNA contains the 18S, 5.8S, and 28S rRNA species along with spacer regions which are removed by specific enzymatic cleavages (2,3). Intermediate sized precursors are formed during the processing of the 45S rRNA precursor to the mature ribosomal species (Figure 1). A complex of 28S rRNA hydrogen-bonded to 5.8S rRNA (3) is found in the 60S ribosomal subunit along with 5S rRNA, an rRNA species transcribed from extranucleolar chromatin. The 18S rRNA species is located in the 40S ribosomal subunit (1).

Coordinated with, but occurring independently of transcription (4), are the nucleotide modification events. These events consist primarily of methylations on 2'-O-ribose and base moieties. Approximately 114 methyl groups occur in mature 18S, 5.8S, and 28S rRNA, with ribose methyl groups accounting for about 95% of the total (5). Almost all of

Figure 1

Schematic diagram for processing of 45S rRNA precursor.


45S
5'
3'
(18S)
(5.8S)
(28S)
41S
5'
3'
(18S)
(5.8S)
(28S)
20S
5'
3'
(18S)
36S
5'
3'
(5.8S)
(28S)
18S
5'
3'
32S
3'
(5.8S)
5'
(28S)
28S-5.8S COMPLEX
5'
3'
3'
5'

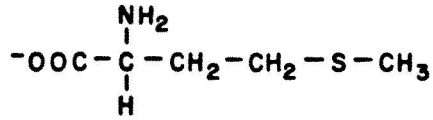
the ribose methylation occur very early on the 45S rRNA precursor and are conserved in the mature products. Later modifications include five base methylations, one occurring in the cytoplasm on 18S rRNA (1), and one ribose methylation occurring in the cytoplasm on 5.8S rRNA (6). Some of the methylation sites are fractionally methylated and occur four times in 18S rRNA, three times in 28S rRNA (5), and once in 5.8S rRNA (7). Thus, the methylations may be described as ribose or base, early or late, and whole or partial.

The importance of early ribose methylation in relation to rRNA maturation and ribosome formation has been extensively studied using many different cell types and experimental approaches. One of the earliest approaches used methionine starvation of HeLa cells (8). Methionine is an essential amino acid and is necessary to form S-adenosylmethionine (SAM), the methyl donor in methyltransfer reactions (Figure 2). During methionine starvation, the methylation of the 45S precursor is severely limited; however, cleavage is able to proceed up through the formation of 32S precursor. No 28S rRNA or 60S ribosomal subunits are produced, although a limited amount of 18S rRNA is processed and extensive degradation of unmethylated species occurs.

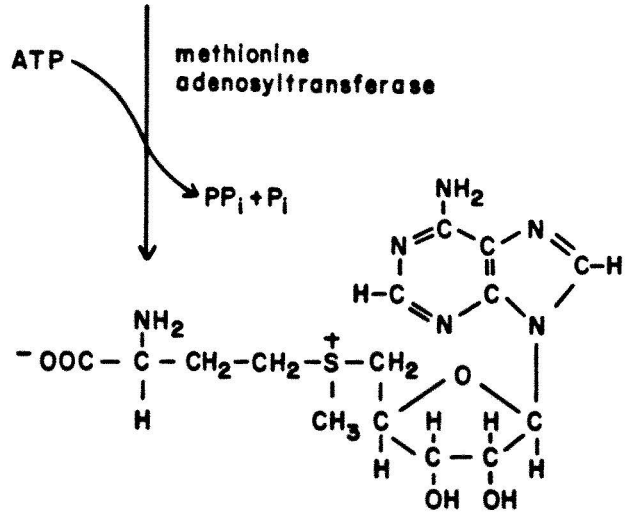
In contrast to the severe disruption of rRNA processing and ribosome formation caused by methionine starvation, histidine starvation in Ehrlich ascites cells (9) results in a 50% reduction in rRNA methylation. However, ribosomes containing hypomethylated rRNA are formed, though more slowly and no unusual degradation of rRNA was observed. Isolated nucleoli from growing cells and histidine starved cells methylate newly synthesized rRNA to an equal extent when placed in media containing labeled SAM; therefore, inhibition of methylases was ruled

Figure 2

Metabolism of S-adenosyl-L-methionine. The enzyme methionine adenosyltransferase catalyzes the reaction of L-methionine with adenosine triphosphate, yielding S-adenosylmethionine, the active sulfonium form of methionine, and the phosphates from ATP, inorganic phosphate and inorganic pyrophosphate. Specific methyltransferases transfer the methyl group of SAM to an appropriate acceptor, in this case an rRNA molecule, yielding a methylated rRNA molecule and S-adenosylhomocysteine.

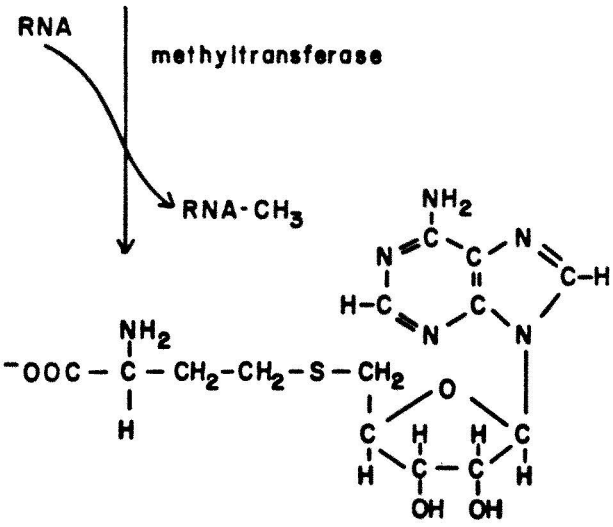


L-METHIONINE



S-ADENOSYL-L-METHIONINE

(SAM)



S-ADENOSYL-L-HOMOCYSTEINE

(SAH)

out as the cause of the hypomethylation. A more probable cause was the decreased levels of ATP and SAM in the histidine starved cells. ATP is necessary for the biosynthesis (10) of SAM by methionine adenosyltransferase (Figure 2). After ATP is dephospholated, 5'-deoxadenosine is transferred to the sulfur in methionine. Decreased levels of substrates (methionine and ATP) necessary for the catalysis of SAM by methionine adenosyltransferase probably affect levels of SAM, which in turn exert a regulatory function in post-transcriptional steps in ribosome formation. The disruption in rRNA processing imposed by methionine and histidine starvation may be due to a failure to normally methylate the primary transcript. The extent of the disruption is less severe in histidine starvation, indicating that the effects of methylation on rRNA processing are a series of graded control points, affecting the extent and rate of post-transcriptional processing.

Administration of methionine analogs and various drugs leads to similar disruption in post-transcriptional processing. The effect of ethionine, a methionine analog, on rat liver is as drastic as methionine starvation of HeLa cells (11). After maturation to the 32S precursor, the rRNA is completely degraded and ribosome synthesis is totally blocked. It was postulated that ethionine lowers the concentration of SAM in the liver, thereby preventing ribosomal precursor methylation. This is similar to results obtained when HeLa cells were deprived of methionine for one hour. Different results were obtained when cycloleucine, a competitive inhibitor of the enzyme methionine adenosyltransferase, was administered to Chinese hamster ovary (CHO) cells (12). First, even through methylation is severely inhibited (95%), cleavage of the precursor did proceed in a stepwise fashion resulting in normal rRNA

products with no extensive degradation. In fact, the life times of the various intermediates are increased resulting in an accumulation of these undermethylated forms in the nucleus. Second, the inhibition of rRNA processing of ribosomes is partially inhibited at several stages in the nucleus and not selectively inhibited at a particular stage. Again, this supports the theory that the degree of methylation and certainly the specificity of methylation modulates the general efficiency of rRNA precursor processing.

The experiments discussed so far suggest that agents adversely affecting the synthesis of SAM do not have a discriminating effect on inhibition of rRNA methylation in normal cells, such as liver cells, and neoplastic cells, such as HeLa, Ehrlich ascites, and CHO cells. However, methylase inhibitors, such as the homopolyribonucleotide polyinosinate-polycytidylate (polyI-poly C), preferentially inhibit rRNA methylation in Novikoff ascites hepatoma, a rat tumor, but not in normal rat liver (13). The methylation of the rRNA from ascites tumor cells is immediately and progressively inhibited, with a concomitant decrease in ribosome production, especially the 40S subunits. The impairment of rRNA methylation may be the trigger for the inhibition of macromolecular synthesis that follows at later times. This preferential limitation of tumor cell growth by certain selective methylase inhibitors indicates a difference in tumor cell methylases versus normal cell methylases.

Genetic evidence for the importance of early ribose methylation is provided by the characterization of a temperature sensitive mutant (ts422E) of Syrian hamster cell line BHK 21 (14,15,16). This mutant grows normally at 33^{o}C, but at 39^{o}C growth stops after one cell doubling.

This phenomenon is attributed to a defect in methylation of rRNA, specifically inhibiting 32S precursor (60% undermethylated) conversion to 28S rRNA. No effect is noted on 18S rRNA or 40S ribosome subunits, however, the eventual degradation of the 32S precursor results in no 28S rRNA or 60S ribosome subunits. The temperature dependent defect in ts422E cells has one main phenotypic effect: the inhibition of 32S processing. Whether this defect is due to abnormal methylases or abnormal ribonucleoproteins remains speculative.

Perhaps the most interesting demonstration of the regulatory function of early methylation in post-transcriptional processing of rRNA is provided by foot-and-mouth disease viral (FMDV) infection of BHK cells, strain 21, clone 13 (17,18). Methylation of host cell nuclear rRNA decreases to about 50% at 60 minutes post-infection and continues to decrease thereafter, with a concomitant decrease in the number of BHK ribosomes. In contrast, tRNA methylation is first inhibited and then stimulated prior to production of FMDV proteins and enzymes. These results suggest that FMDV selectively inhibits host rRNA methylation.

In conclusion, although early ribose methylation in rRNA processing is not full understood, these <u>in vivo</u> studies strongly suggest that the function of individual methylations in ribosomal synthesis can be described as a series of complex interrelationships, influenced by rRNA conformation and rRNA association with ribosomal proteins. Individual methylations are also affected by SAM concentrations; these in turn are influenced by concentrations of ATP and methionine, and activity of adenosyl methyltransferase. All of these individual components correlate with the ability of the cell to efficiently synthesize mature, functioning, cytoplasmic ribosomes.

In studies of microbial resistance to antibiotics, individual base methylations of ribosomal nucleic acid may serve as the mechanism of resistance to the antibiotic. The first example of altered rRNA methylation resulting in antibiotic resistance was demonstrated in certain strains of Staphylococcus aureus (19). Erythromycin induced resistance is shown to be due to methylation of adenosine in 23S rRNA resulting in N^6-dimethyladenosine. Inducibly-resistant cells contain 50S ribosomal subunits with a reduced ability to bind erythromycin and three to eight other classes of 50S subunit inhibitors. In contrast to the above example, studies on the mechanism of resistance to the antibiotic kasugamycin in strains of Escherichia coli show that resistance is due to a lack of dimethylation of two adjacent adenosine residues (20). Sensitive strains contain a methylase capable of methylating 16S rRNA of cesium chloride 30S core particles from resistant strains. These results suggest that a mutation in the resistant strain alters the primary structure of the methylase, causing it to become inactive. These two examples illustrate the importance of single base methylations in rRNA.

A eucaryotic microorganism, Streptomyces azureus, illustrates the importance of a single ribose methylation. Ribose methylation of a single adenylic acid residue in 23S rRNA prevents the antibiotic thiostrepton from binding, thereby rendering the organism resistant to its own antibiotic product (21,22). In these three cases of microbial resistance to antibiotic products, a conformational change in the rRNA brought about by the absence or presence of a single base or ribose methyl group may be the factor in determining the capability of the antibiotic to bind to the ribosome.

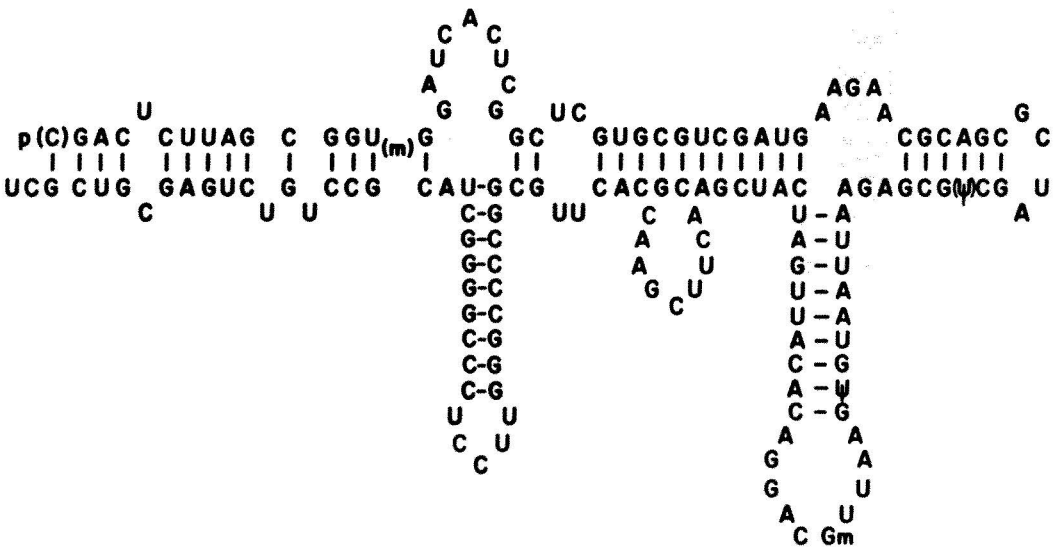
Studies on mammalian 5.8S rRNA suggest that a conformational change is caused by a late cytoplasmic ribose methylation of a single uridylic acid residue (23). Little is known about the function of late methylation of mammalian rRNA. However, partially methylated residues such as the late cytoplasmic ribose methylation of 5.8S rRNA may provide another type of control of the ribosome. Position 14, the partially methylated 2'-O-methyluridylic acid (UmG) in the 157-160 nucleotide sequence of mammalian 5.8S rRNA (Figure 3), has come under intense scrutiny in the investigation of rRNA methylation for two main reasons.

First, 5.8S rRNA is the only rRNA molecule in the 45S precursor that has been sequenced (24,25) and its secondary structure relatively well characterized. A "universal" secondary structure has been proposed (25,26,27) and corroborated (28). Further studies have shown 5.8S rRNA to exist in three major conformational isomers with at least one isomer unmethylated at position 14 (23,29). A more compact configuration has been suggested for the unmethylated isomer which could conceivably affect its spatial relationship with and function in the 60S ribosomal subunit. Position 14 in the 5' end may be involved in the junction complex, which is the site of the interaction of 5.8S rRNA with 28S rRNA and includes both the 3' (30) and 5' regions (31,32) of 5.8S rRNA. Heat release and reassociation data for 5.8S rRNA from yeast ribosomes suggests that the 28S-5.8S rRNA association site is stabilized by protein interaction and is readily available on the ribosome (33). The easy accessibility of 5.8S rRNA in the ribosomes could be the basis for studies on the effect of UmG (position 14) on ribosome function.

The second reason 5.8S rRNA is being intensely investigated is because the degree of methylation at position 14 is very different in

Figure 3

Nucleotide sequence and secondary structure for 5.8S rRNA.


p(C)GAC CUUAG C GGU(m)G GC GUGCGUCGAUG CGCAGC C
UCG CUG GAGUC G CCG CAU-GCG CACGCAGCUAC AGAGCG(ψ)CG U
C U U C-G UU C A U-A A
G-C A C A-U
G-C A U G-U
G-C G C U U-A
G-C C U-A
C-G A-U
C-G C-G
C-G A-U
U U C-G
C U A A
C G A
G U
A U
C Gm

neoplastic and normal cells (34). Very low levels of methylation are found in neoplastic cells such as Novikoff ascites hepatoma (23%), mouse myeloma (18%), and HeLa cells (17%). The highest levels are found in rat and mouse liver (72%) and mouse kidney and spleen (61%). Intermediate levels of methylation are found in rapidly-growing tissues such as pregnant mouse mammary gland (51%), mouse embryo (40%), and regenerating rat liver (31%). The three other post-transcriptional modifications of 5.8S rRNA have the same molar yields in all the cells: one molar yield for pseudouridylic acid at position 71, one molar yield for 2'-O-methylguanylic acid at position 77 (GmC), and about half-molar yield for pseudouridylic acid at position 57. Only UmG (position 14) demonstrates consistently low yields for neoplastic versus normal cell types.

In contrast to low methylation levels of rRNA in neoplastic cells, methylase activity in neoplastic cells is elevated. The activity of rRNA methylase has been compared in isolated nucleoli of Novikoff ascites, tumor cells, and rat liver (35). The tumor enzymes are about three times more efficient in methylating newly synthesized precursor rRNA than the liver methylases. The enzymes also react differently to cellular metabolites. Tumor enzymes are inhibited by catabolic products of RNA such as ApA (a dinucleotide), but are stimulated by anabolic metabolites of RNA such as ATP. The reactions of liver enzymes are completely opposite. These results suggest that the relative amounts of RNA metabolites may play a role in the control of RNA processing and ribosome formation.

Lower levels of methylation and increased activity of methylases in neoplastic cells have been noted for tRNA (37) as well as for rRNA.

This interesting paradox cannot be explained yet; however, studies by Godburn (38) on levels of SAM and SAH (S-adenosylhomocysteine) in normal and neoplastic cells indicate that the ratio of SAM to SAH is inversely related to the level of UmG in 5.8S rRNA. In neoplastic cells, the SAM/SAH ratio is increased, due to higher SAM levels and similar SAH levels, when compared to normal cells. SAH, the product of SAM methyltransferase reactions (Figure 2), acts as a competitive inhibitor of methylases, and generally has a higher affinity for methylases than SAM has (39). This suggests a regulatory function for SAH, but the study by Godburn indicates that some other inhibitor may be involved. These results suggest a very complex regulatory mechanism controlling RNA methylation.

Understanding the regulatory mechanism of RNA methylation requires characterization of the methylases involved. Several tRNA (40) and mRNA (41) methylases have been isolated and characterized, however, no rRNA methylases have been isolated. Understanding the paradox of low methylation levels and high methylase activity in neoplastic cells requires that an enzyme assay be developed.

CHAPTER 2

STATEMENT OF THE PROBLEM

The purpose of this study was to develop an assay for *in vitro* enzymatic methylation of mammalian rRNA. Previous studies of rRNA methylation, for purposes other than identification and quantification, have focused upon total methylation in intact cells (8,9,11,12,13) and isolated nucleoli (9,35), and individual methylations in micro-organisms (19,20,21). Assays for specific tRNA (40) and mRNA (41) methylases have been developed, however, no studies of individual rRNA methylases have been made.

This assay was needed to facilitate our understanding of the complex regulatory mechanisms involved in the processing and functioning of rRNA, particularly the mechanisms involved in the paradox of low methylation levels and high methylase activity in neoplastic cells. Optimum conditions for an rRNA methylase assay were to be determined, including those that allowed for maximum activity of methylase enzyme and minimum contamination of undesirable nucleases. Whether the differences between normal and neoplastic cells extend to the functioning of ribosomes is not known, but every effort should be made to extend our knowledge in this direction because of the intense need for more information about neoplastic cells.

CHAPTER 3

EXPERIMENTAL

A. Materials

Radioactive orthophosphate and S-(methyl-^{3}H)adenosyl-L-methionine (specific activity 5 to 15 Ci/nmole) were purchased from New England Nuclear. Electrophoresis grade N,N'-methylene-bisacrylamide and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were obtained from Eastman Kodak Chemical Company. Cellulose acetate strips from Kalex Scientific Company and 3MM and DE-81 (DEAE) paper from Whatman Company were purchased. Handifluor and Dilufluor scintillants were bought from Mallinckrodt, Scientific Products. The Bio-Rad protein assay kit was obtained from Bio-Rad Laboratory and heparin (grade 1, sodium salt) was obtained from Sigma. Density gradient grade crystalline sucrose (ribonuclease free) was obtained from Schwarz/Mann Company. All other chemicals were of reagent grade and purchased commercially. X-ray film developing privileges were provided by the Public Health Service Radiology Department, Norfolk, Virginia.

B. Methods

1. Cell cultures and tissues

Normal rat kidney cells (42) and human osteosarcoma TE-85 clone F5 (43) were maintained in Eagle's minimal essential media (MEM) supplemented with 10% fetal calf serum and 50 μg/ml neomycin in an atmosphere of 5% CO_2 at 37°C. All cultured cells were obtained from

Dr. Ken Somers, Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, Virginia. Carworth CF-1 outbred albino mice were the source of normal mouse liver and Ehrlich ascites tumor cells, and were obtained from Dr. Lloyd Wolfinbarger, Department of Biological Sciences, Old Dominion University, Norfolk, Virginia.

2. ^{32}P-labeling of cultured cells

Cells grown to about 75-85% confluence in 75 cm^2 T-flasks were washed with five milliliters of GKN buffer (0.4 g KCl, 8.0 g NaCl, 1.0 g glucose, 0.005 g phenol red per liter water). The buffer was discarded and ten milliliters of 32P-medium (one mCi ^{32}P per ten milliliters phosphate-free MEM supplemented with 10% dialyzed fetal calf serum and 50 µg/ml neomycin) was added (26). The flasks were incubated at 37°C for 24 hours.

3. Preparation of whole-cell-homogenate

The ^{32}P-medium in each T-flask of labeled cells was discarded and the cells washed with ten milliliters of GKN at 37°C. The buffer was discarded and two milliliters of trypsin-EDTA buffer (GKN buffer plus 0.05% trypsin, 0.05% EDTA, and 50 µg/ml neomycin) at 37°C was added for approximately five minutes to loosen cells from the flask. To neutralize the trypsin five milliliters of MEM (supplemented with 10% fetal calf serum and 50 µg/ml neomycin) was added. After trypsinization the cells were centrifuged in a 15 ml Corex tube at 1,200 x g for two minutes and the supernatant discarded. The cells were then washed three times with ice-cold GKN and suspended in five to ten milliliters of ice-cold homogenate buffer (0.01 M Tris-Cl, 0.001 M mercaptoethanol, pH 7.8). This cell suspension was homogenized either with a small stainless steel Waring blender or with a Teflon pestle homogenizer until

cell breakage was obtained. The homogenate was kept ice-cold until used in the assay.

4. Whole-cell homogenate assay

One to two milliliters of whole-cell-homogenate were incubated in 15 ml glass Corex tubes with various reagents (as listed in Table 1) in a 37° water bath for 15-30 minutes and the tubes were then plunged into an ice-water bath. The RNA was extracted immediately as described below.

5. RNA extraction and precipitation

RNA was extracted from whole cells, cell homogenates, and S100 supernatants using sodium dodecyl sulfate (SDS) and phenol (44). Ten percent SDS was added to whole-cell-homogenate assays to give a final concentration of 0.3% SDS. For S100 enzyme assays and whole cell extractions an equal volume of RNA extraction buffer (0.14 M NaCl, 0.05 M sodium acetate, 0.3% SDS, pH 5.1) was added. One volume of phenol-cresol-8HQ (1,892 ml phenol, 420 ml cresol, 270 ml water, and 2.6 g 8-hydroxyquinoline; the phenol and cresol were freshly distilled) was added followed by vigorous stirring for 30 min at room temperature. The SDS/phenol mixture was centrifuged at 4,600 x g for 30 min. The top aqueous phase containing the RNA was removed and precipitated in a sterile tube using two volumes of 95% ethanol plus 2% potassium acetate at -20°C overnight.

6. Polyacrylamide gel slab electrophoresis

The 5.8S rRNA was isolated using polyacrylamide gel electrophoresis (45). Two flat glass plates (20 x 41 x 0.5 cm) and two Teflon strips (41 x 0.5 cm) were assembled and sealed with tubing and clamps. A ten percent acrylamide mixture of 1:39 bisacrylamide and acrylamide

in borate buffer (ten times concentrated stock buffer: 105 g Tris, 55 g boric acid, and 9.3 g EDTA per liter water) was degassed and poured into the glass mold to four centimeters from top, overlayered with water, and allowed to polymerize. Gel plugs were inserted at sides and to form slots. The gel was pre-electrophoresed before applying samples. In order to dissociate the 28S-5.8S rRNA complex, samples were heated for two minutes at 60°C in 50% formamide and quickly cooled to 0°C. Bromphenol blue dye marker was added and the samples applied. The gel was electrophoresed for 16 hr at 30 mamps and 300-500 volts. The 5.8S rRNA band was located by autoradiography or by staining with methylene blue dye.

RNA was recovered by homogenization of the excised gel in ten milliliters water or RNA extraction buffer with a Teflon pestle homogenizer (25). Most of the polyacrylamide was removed by centrifugation at 27,000 x g for one hour. After precipitation of RNA, the RNA pellet was solubilized in water and fine particles of polyacrylamide were removed by passing RNA through a 0.45 micrometer Millipore filter. The RNA was then precipitated again with ethanol, dissolved in water, and then transferred to a microcup and dried.

7. One dimensional paper electrophoresis

The ^{32}P-labeled 5.8S rRNA from whole-cell-homogenate assays was hydrolyzed using a T_2 ribonuclease and alkaline phosphatase digest (T_2/AP) (46). The RNA was dissolved in ten microliters T_2 (100 units of T_2 per milliliter of 0.05 M acetate buffer, pH 4.5) and incubated at 37°C for one hour. Ten microliters alkaline phosphatase (16 mg enzyme per milliliter of 0.1 M Tris-Cl buffer, pH 8.3) was added and then incubated at 37° for three hours.

After the T_2/AP digest of 5.8S rRNA, one dimensional paper electrophoresis was used to separate and quantitate the ^{32}P-labeled UmpG and GmpC dinucleotides (46). Whatman 3MM paper (18 in x 70 cm) was spotted with digest samples, dye marker (one percent each xylene cyanol FF, acid fuchsin, and methyl orange in distilled water), and unlabeled nucleotide markers. The paper was sprayed thoroughly with acetate buffer (5% acetic acid, pH adjusted to 3.5 with ammonium hydroxide) and placed in a tank of acetate buffer for electrophoresis at 2,000 volts for three hours. The paper was dried and the nucleotide markers localized with U.V. light and the dinucleotides with autoradiography. Radioactive spots were cut out and counted in seven milliliters of Dilufluor in a liquid scintillation counter (Beckman LS-100C). Since GmpC occurs in one molar yield, the ratio of UmpG to GmpC gives the fraction of position 14 that is methylated.

8. Two dimensional ionophoretic fractionation procedure

The ^{32}P-labeled 5.8S rRNA from whole-cell-homogenate assays was hydrolyzed using a pancreatic ribonuclease A (RNase A) digest. The sample was digested in ten microliters RNase A (0.1 mg enzyme per milliliter of 0.01 M Tris buffer, pH 7.4) at 37°C for 45 minutes (47).

After the RNase A digest of 5.8S rRNA, two dimensional paper electrophoresis was used to separate and quantitate the ^{32}P-labeled oligonucleotides GGUp, GGAUp, and GGUmGGAUp (48). Samples were applied ten centimeters from the end of a 47 x 1.5 cm strip of cellulose acetate soaked for 15 min in urea buffer (7 M urea, 5% acetic acid, 0.001 M EDTA, pH 3.5). After applying dye marker, the strips were electrophoresed in 5% acetate buffer (pH adjusted to 3.5 with ammonium hydroxide) at 2,000 volts for three hours for the first dimension.

For the second dimension the sample was transferred to DEAE paper (100 cm long) by capillary action using 3MM paper wicks. After the DEAE paper was dried and sprayed with 7% formic acid, dye marker was applied and the paper electrophoresed in 7% formic acid at 1,500 volts for about 16 hours. After drying the paper, the radioactive oligonucleotide spots were localized by autoradiography, cut out, and quantitated by scintillation counting. The GGUmGGAUp counts were ratioed to the total counts in all three oligonucleotides to obtain the fraction of methylated position 14.

9. SAM purification

Unlabeled SAM used in whole-cell-homogenate assays was purified to remove SAH using a Dowex-1 column prepared by a 4 M NaCl wash followed by a water wash (49). Elution of SAM was with water or 0.01 M NaCl. The purity of SAM was analyzed by high voltage electrophoresis (2,000 volts) on Whatman 3MM paper with 5% acetic acid (adjusted to pH 3.5 using ammonium hydroxide) (50), or by thin-layer chromatography using Baker-flex cellulose F sheets developed with n-butanol:acetic acid: water (12:3:5) (51).

10. Preparation of 100,000 x g enzyme supernatant (S100)

For Ehrlich ascites S100 the mice were sacrificed and the harvested cells were filtered through cheesecloth into ice-cold Dulbecco's phosphate buffered saline without calcium (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, and 0.1 g $MgCl_2$ per liter water). The cells were pelleted at 800 x g for two minutes and the supernatant decanted. To lyse red blood cells, ice-cold hypotonic buffer (0.01 M Tris-HCL, 0.01 M NaCl, 0.0015 M $MgCl_2$, 0.001 M dithiothreitol, pH 8.0) was added and centrifuged immediately. The supernatant fluid was decanted, the

tumor cells were washed with Dulbecco's saline, counted with a hemocytometer, and brought up to a concentration of 6×10^7 cells per milliliter of hypotonic buffer. Alternatively, the cells were weighed and four milliliters of hypotonic buffer added per gram cells. After incubation at $0^{\circ}C$ for ten minutes the cell suspension was homogenized using a Teflon pestle homogenizer (75 strokes) kept ice-cold. The homogenate was centrifuged for two hours at $4^{\circ}C$ using a Beckman SW27 rotor at 25,000 rpm (100,000 x g) or a Beckman 75Ti rotor at 35,000 rpm in a Beckman L5-65 ultracentrifuge.

In order to prepare mouse liver S100, the liver was removed, immediately rinsed in ice-cold Dulbecco's saline, blotted dry and weighed. For each gram of tissue, 4.5 ml hypotonic buffer was added to minced tissue, after which the procedure was like that for Ehrlich ascites S100 (52,53).

11. SAH affinity column chromatography

An SAH affinity column was prepared using CNBr activation of 4B-Sepharose to couple the spacer 3,3'-diaminodipropylamine (54,55), and carbodiimide to couple SAH to the spacer (56). Briefly, 50 ml Sepharose 4-B was washed with distilled water and 0.1 M $NaHCO_3$ buffer (pH 9.5) and filtered to dryness. Fifty milliliters of buffer was added to Sepharose and the temperature ($10-15^{\circ}C$) and the pH (pH 11) was maintained while ~2.5 g CNBr in 15 ml acetonitrile was added. After 20 min, the Sepharose was washed with buffer and water and added to two millimoles of 3,3'-diaminodipropylamine in 50 ml water at pH 9.7, and stirred at $4^{\circ}C$ for 20 hours.

Ten milliliters of Sepharose with coupler was washed with water (pH 4.6), fifty milligrams of SAH in ten milliliters of water was added,

and the pH readjusted to 4.6. Solid carbodiimide powder was added to give a final concentration of 0.1 M. The pH was maintained at 4.6 for 30 minutes after mixing. After washing with water, the amount of SAH in the effluent was determined using the spectrophotometric absorbance at 260 nm and a molar absorptivity coefficient of 14,700 liter $mole^{-1}$ cm^{-1} (49).

The column (1 x 5 cm) was prepared by equilibrating matrix with hypotonic buffer. The S100 supernatant was washed into the column with hypotonic buffer until a minimum absorbance at 260 nm was detected, followed by elution with hypotonic buffer adjusted to pH 5, then by elution with hypotonic buffer adjusted to 1M NaCl. The pH 5 eluate was adjusted to pH 8 and the 1 M NaCl eluate was dialyzed against hypotonic buffer being used in an S100 assay.

12. S100 assay

The 100 µl enzyme assay contained 100-200 µg 28S-5.8S rRNA, 88 µl of S100 supernatant, and 0.001 M ^{3}H-SAM (5-15 Ci/mmol). The S100 enzyme preparation and ^{3}H-SAM were added to ice-cold RNA in 1.5 ml Eppendorf tubes just before incubation at 25°C or 37°C for various times up to 80 minutes. The reaction was stopped using 250 µl of RNA extraction buffer and 350 µl phenol-cresol-8HQ. After mixing for 30 minutes at room temperature, the samples were centrifuged at 3,000 rpm for 15 minutes in a Beckman TJ-6 table-top centrifuge.

Three 75 µl aliquots of the aqueous phase were applied to three pieces (2 x 2 cm) of DEAE paper (52,53) and washed three times in 5% Na_2HPO_4, once in water, twice in 95% ethanol, and allowed to dry before being placed in scintillation vials containing six milliliters Handifluor and 750 µl water. The sealed vials were then vigorously shaken by hand,

allowed to sit approximately eight hours, and were shaken again before counting in the scintillation counter.

13. DEAE-Sephadex chromatography

The ^{3}H-labeled 28S-5.8S rRNA complex from S100 supernatant assays was hydrolyzed (57) using four units T_2 ribonuclease per milligram RNA in 0.01 M sodium acetate buffer at 37°C for 16 hours. The pH was then adjusted to one using HCl and kept at 4°C for 16 hours. The sample was then neutralized and diluted with urea buffer (7 M urea, 0.025 M Tris-HCl, pH 7.5) before applying to column.

DEAE-Sephadex column chromatography (58) was used to separate the mono-, di-, and trinucleotides in T_2 digests of ^{3}H-methyl labeled 28S-5.8S complex. The diluted sample was loaded on a column (0.7 x 20 cm) equilibrated with urea buffer and eluted with a 200 ml linear gradient of 0-0.3 M NaCl formed in urea buffer. Two milliliter fractions were collected. The absorbance at 260 nm was measured and 0.5 ml aliquots were counted in 0.5 ml water and six milliliters Handifluor scintillant.

The specific activity of pooled fractions of mononucleotides and dinucleotides was determined by ratioing the total ^{3}H-cmp to the total mass of RNA. The concentration of RNA was determined spectrophotometrically using an absorbtivity, $A^{1\%}_{260\ nm}$, 1 cm=200.

14. Sucrose density gradients

RNA was fractionated using 5-25% linear sucrose density gradients, prepared with a Technicon Auto-analyzer proportional pump. The pump tubing and ultracentrifuge tubes were treated with diethyl pyrocarbonate to inactivate any nucleases. The 36 milliliter linear gradient was formed (59) using 25% (w/w) sucrose solution continuously diluted

with buffer (0.1 M NaCl, 0.01 M sodium acetate, 0.001 M EDTA, pH 5.1). One milliliter of RNA (5 mg/ml) in buffer was layered on the gradient and centrifuged at 25,000 rpm for 16 hours at 4°C using a Beckman SW27 rotor in a Beckman L5-65 ultracentrifuge. Fractions were collected using an Isco-model 185 density gradient fractionator while monitoring at 254 nm. The desired RNA fraction was diluted with water and precipitated with ethanol.

15. Protein determination

Protein was determined using the Bio-Rad protein assay (60, 61) with bovine serum albumin used as the standard.

CHAPTER 4

RESULTS

A. Methylation assay using whole-cell-homogenate as enzyme source.

Several experiments were performed using a whole-cell-homogenate as the source of methylase and ^{32}P-labeled rRNA substrate. Table 1 gives a summary of conditions and data for one assay. The level of UmG methylation determined without incubation at 37°C (temperature controls, samples one and two) established the basis for comparison of the effect of various reagents with 37°C incubation. SAM was added (samples three and six through nine) at a concentration determined after consulting several articles on tRNA methylation assays. EDTA was added (samples four and six through nine) to destabilize ribosomes, thereby "opening up" rRNA sites for methylation. Heparin was added (samples five through nine) to inhibit nuclease activity. SAH was added (samples eight through ten) to determine the effect of a competitive inhibitor of methylases, at a concentration consistent with the finding that SAH has a higher affinity for methylases than SAM has (39). Duplicates were performed in some cases to determine the degree of variability inherent in the technical aspects of the methods as performed.

As the data shows, very little reproducibility was shown between duplicate samples as analyzed by the method of Ryan (46). One problem experienced with this particular assay was the remainder of a rather large amount of radioactivity at the origin during electrophoresis. This could explain the inconsistency within duplicates.

Table 1

Assay of 5.8S rRNA Methylation Using Whole-Cell-Homogenate

Sample	1 mM SAM	5 mM EDTA	50 ug/ml Heparin	0.1 mM SAH	% UmG Ryan	% UmG Sanger
1	-	-	-	-	54.7	44.0
2	-	-	-	-	60.3	44.0
3	+	-	-	-	60.5	40.6
4	-	+	-	-	71.9	35.1
5	-	-	+	-	68.0	40.1
6	+	+	+	-	82.5	34.7
7	+	+	+	-	60.8	38.2
8	+	+	+	+	79.1	35.8
9	+	+	+	+	64.0	*
10	-	-	-	+	65.7	46.6

Assay of methylation at position 14 in NRK 5.8S rRNA. Six flasks of NRK cells (passage 74) were labeled with one milliCurie phosphate per flask for 24 hours. SAM, SAH, heparin, and EDTA were added to one milliliter of whole-cell-homogenate as indicated. RNA in samples one and two was extracted immediately (temperature control). Other samples were incubated at 37°C for 15 minutes before RNA extraction. The percent methylation was analyzed using the one dimensional paper electrophoresis method of Ryan (46) and the two dimensional paper electrophoresis method of Sanger (48) as described in the methods.

*The results were not obtained due to technical problems.

As analyzed by the method of Sanger (48), duplicates appear to be fairly consistent in value. However, the results do not show an increase in methylation above the temperature control when SAM is added. In fact, a decrease in methylation occurs in all the samples except sample ten, where only SAH was added to the homogenate.

B. Methylation assay using SAH-affinity-column eluates as enzyme source.

Several experiments were performed using SAH-affinity-column chromatography in an attempt to purify methylase enzymes in an S100 supernatant preparation. In Figure 4, an SAH-affinity column chromatogram is shown of an S100 supernatant of Ehrlich ascites cells. The pH 5 eluate and the 1 M NaCl eluate were adjusted to pH 8 and dialyzed against hypotonic buffer, respectively, before being used as the methylase enzyme source in an assay (Table 2) for 28S-5.8S rRNA methylation. Comparison of the samples containing exogenous rRNA (samples one, four, and seven) with the rRNA controls (samples two, five, and eight) and the temperature controls (three, six, and nine) indicate that most of the activity may be either methylation of protein or entrapment of tritium-labeled SAM in protein-RNA aggregates on the DEAE paper. Since the washing procedure may not distinguish between protein and RNA attachment to DEAE paper, a series of experiments (not reported here) was made to determine the best procedure for counting only radioactive RNA from methylation assays. The best procedure entails an RNA extraction step before washing on DEAE paper, as explained in the methods.

C. Methylation assay using S100 supernatants as enzyme source.

The revised washing procedure as outlined in the methods made possible a very low zero-time control (100-300 cpm) and an increase in

Figure 4

SAH-affinity column chromatography of S100 supernatant of Ehrlich ascites cells. After a three milliliter sample of S100 supernatant was applied, the column (1 x 5 cm) was washed with column buffer and eluted with pH 5 or 1 M NaCl column buffer as in methods. Absorbance was monitored at 280 nm. The eluates were then used as the enzyme source in an assay of RNA methylation (Table 2).

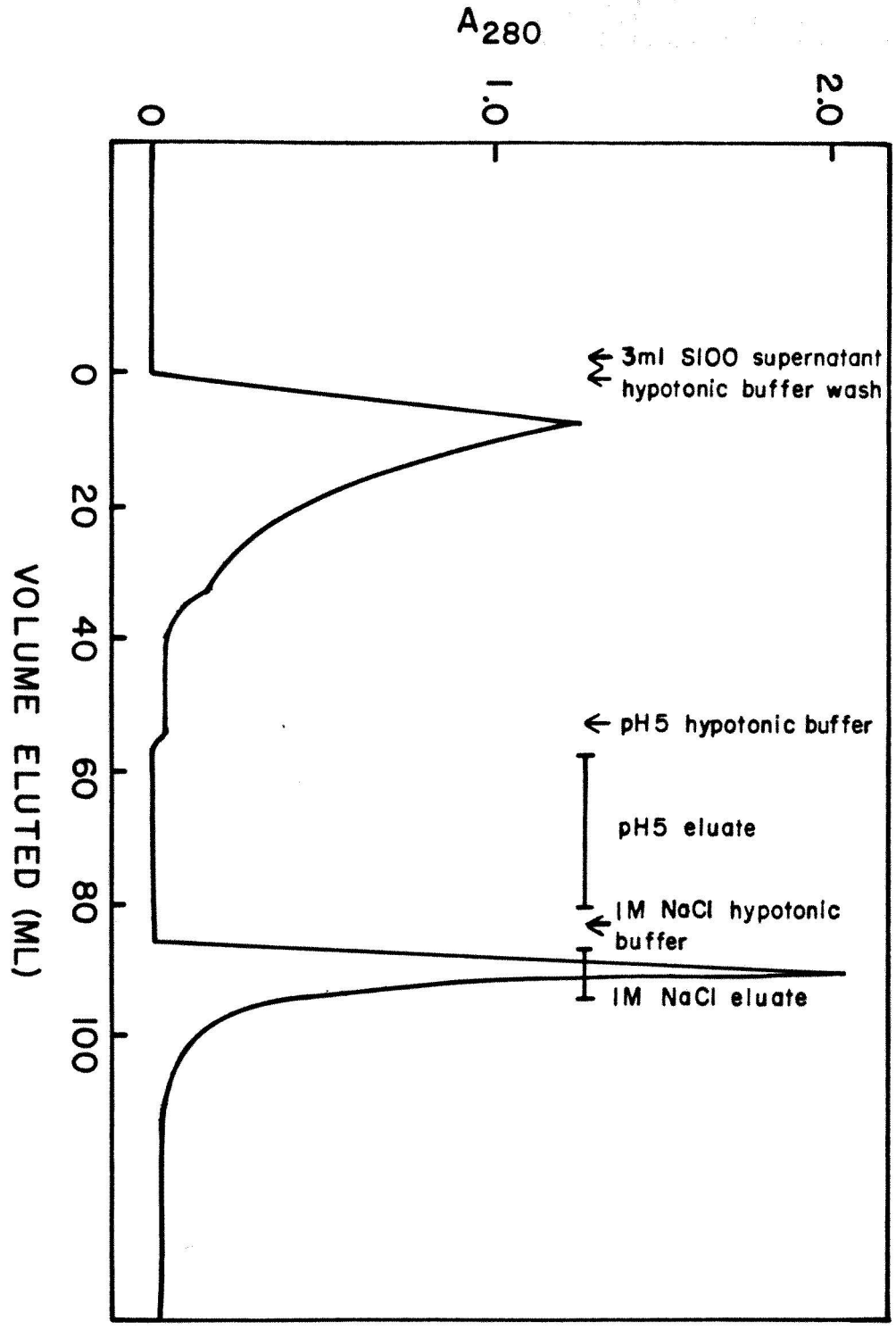

A280
0
1.0
2.0
VOLUME ELUTED (ML)
0
20
40
60
80
100
3ml S100 supernatant
hypotonic buffer wash
pH5 hypotonic buffer
pH5 eluate
IM NaCl hypotonic
buffer
IM NaCl eluate

Table 2

Assay of RNA Methylation Using
SAH-Column Enzyme

Sample Number	RNA 100 ug 5.8S-28S	Incubation 37°C, 30 min	^{3}H-cpm	Enzyme Source
1	+	+	12,467	S100 supernatant
2	-	+	12,106	
3	+	-	10,001	
4	+	+	1,131	pH 5.0 elution of SAH column
5	-	+	842	
6	+	-	1,698	
7	+	+	5,368	1 M NaCl elution of SAH column
8	-	+	4,959	
9	+	-	4,937	

The assay contained enzyme (88 µl), RNA (100 µg of Ehrlich Ascites 28S-5.8S rRNA) or hypotonic buffer, and tritium-labeled SAM (1 µM) in a total volume of 100 µl. After incubation, a 75 µl aliquot of sample was applied to DEAE cellulose paper (2 x 2 cm) and washed three times in five percent Na_2HPO_4, once in distilled water, twice in ethanol:ether (1:1), and once in ether. Aliquots of samples not incubated at 37°C were immediately applied to DEAE paper and washed. The paper was dried and counted in Dilufluor. Protein was determined by the Bio-Rad protein assay procedure (61). Samples 1-3, 4-6, and 7-9 contained 392, 5, and 104 µg protein per assay, respectively.

methylation of exogenous RNA above that for endogenous RNA. In Figure 5, a time study of Ehrlich ascites (EA) S100 supernatant incubated with exogenous 28S-5.8S rRNA shows both an increase in methylation with time and an increase in methylation above that of endogenous RNA. A similar time study in Figure 6 using mouse liver S100 supernatant and heterogenous EA 28S-5.8S rRNA shows a different methylation curve over a period of time. Methylation increases with time up to about 15 minutes, and declines to about half the peak value at 60 minutes. Also the peak value for the mouse liver S100 supernatant is about four times less than the peak value for the EA S100 supernatant, although the mouse liver preparation has about three times more protein. Methylation of endogenous RNA in the mouse liver S100 supernatant exhibits the same general curve of methylation over time as does exogenous RNA.

The results of a more comprehensive study involving longer incubation times and heterogenous and homogenous mixtures for RNA and enzyme is presented in Figure 7. It appears that the type of exogenous RNA does not matter to the enzyme; the curve produced when exogenous RNA is added to an S100 supernatant is indicative more of which type of enzyme source is used than whether the exogenous RNA is homogenous or heterogenous. Even the endogenous RNA methylation curves are indicative of the enzyme source: for EA S100 the curve continues to increase at 80 minutes; for mouse liver S100 the curve peaks and is declining at 80 minutes. Again, there appears to be less methylation occurring in the mouse liver S100 supernatant samples compared to EA S100 supernatant samples. This could be due to a decrease in methylase activity or an increase in nuclease activity in liver S100 compared to Ehrlich ascites S100.

Figure 5

Assay of RNA methylation using Ehrlich ascites S100 supernatant. Enzyme (88 µl), RNA (200 µg of Ehrlich ascites 28S-5.8S rRNA) or buffer, and tritium-labeled SAM (1 µM) were incubated at 37°C in duplicate in a total volume of 100 microliters for times indicated. RNA was extracted, applied to DEAE paper, washed, and counted as in methods. Counts are corrected for zero-time control. Each sample contained 21 µg protein as measured by the Bio-Rad protein assay procedure (61). Exogenous RNA (○—○); no exogenous RNA (△—△).

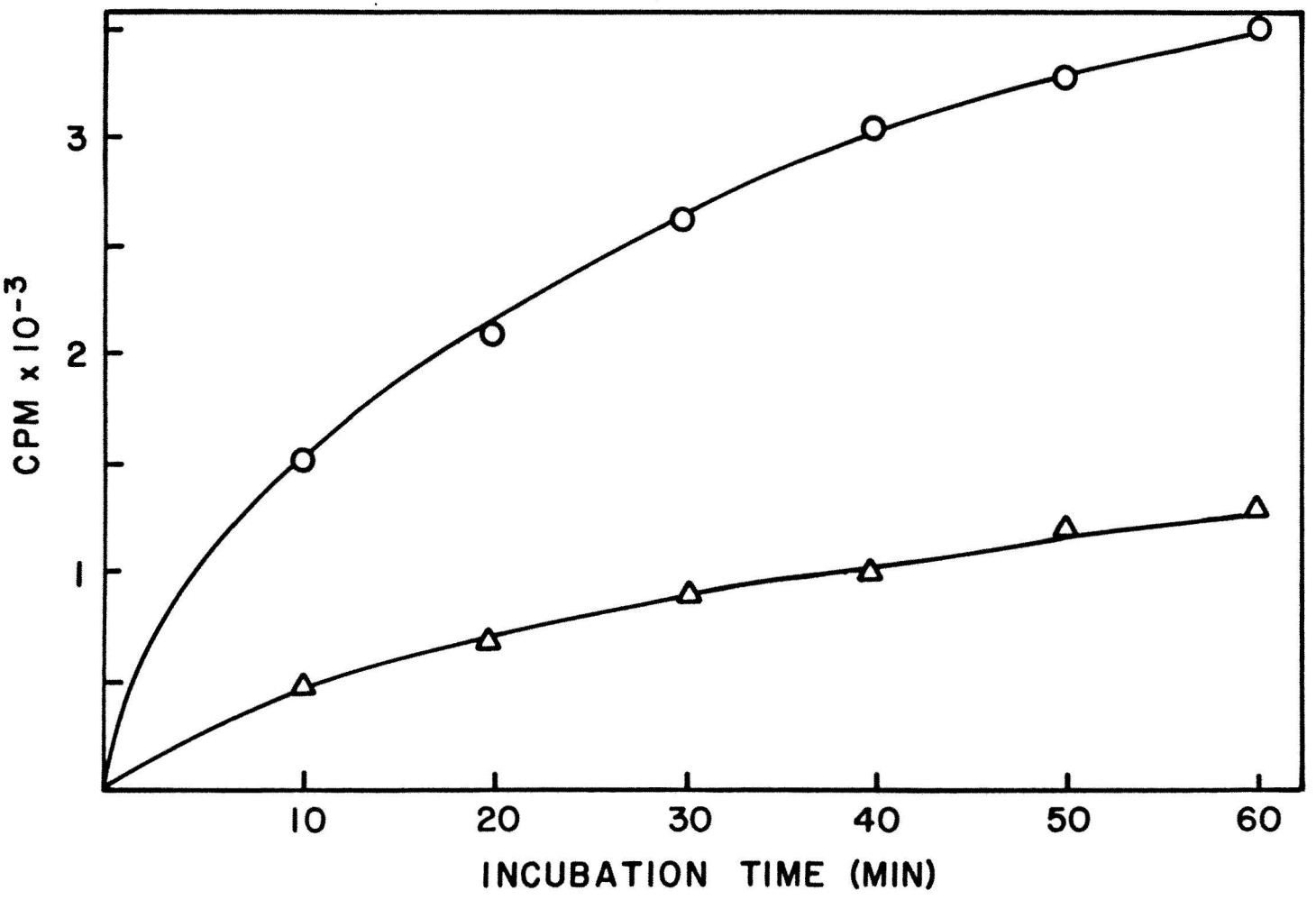

CPM x 10-3
3
2
1
10
20
30
40
50
60
INCUBATION TIME (MIN)

Figure 6

Assay of RNA methylation using mouse liver S100 supernatant. Aliquots of 100 µl were taken out of a two milliliter incubation sample at specified times and transferred to Eppendorf tubes containing 500 µl of SDS/phenol mixture. Each aliquot contained enzyme (88 µl of mouse liver S100 supernatant), RNA (200 µg of Ehrlich ascites 28S-5.8S rRNA) or hypotonic buffer, and tritium-labeled SAM (1 µM). A 225 µl aliquot of the aqueous layer was transferred to DEAE paper (2 x 2 cm) and washed three times in five percent sodium phosphate (dibasic) and once in water. Counts are corrected for zero-time control. Each sample contained 62 µg protein as measured by the Bio-Rad protein assay procedure (61). Exogenous RNA (○—○); no exogenous RNA (□—□).

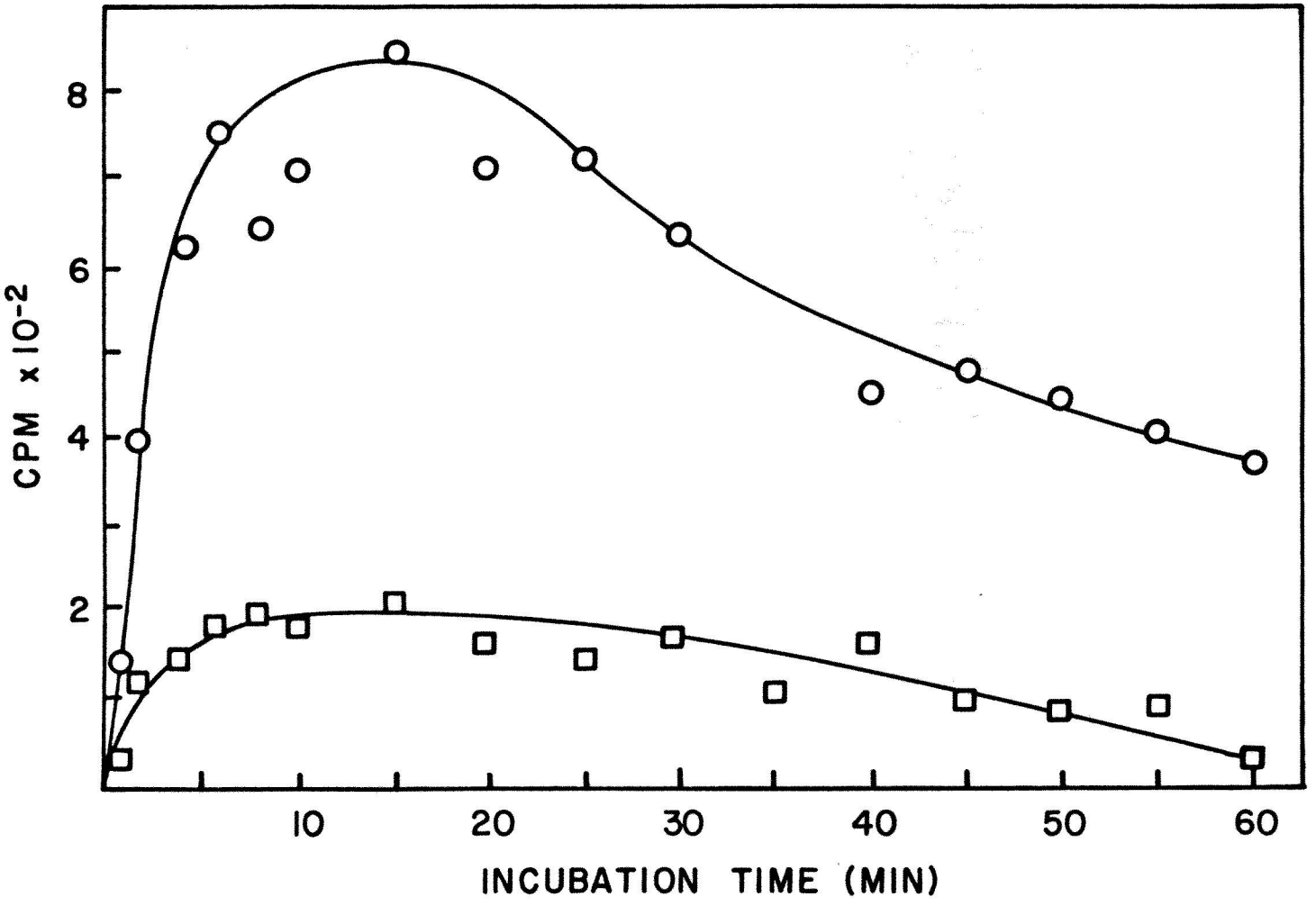
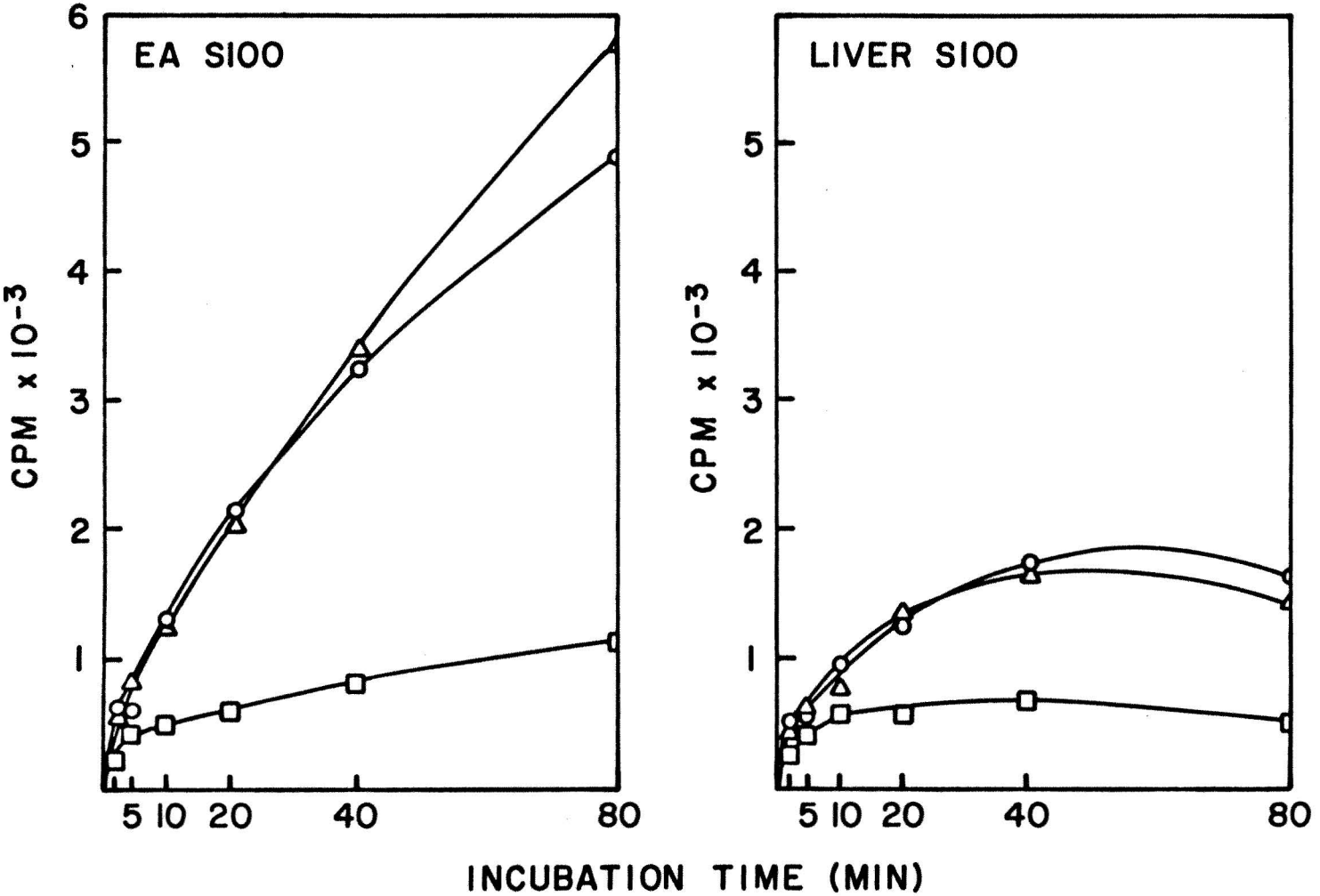

CPM x 10-2
8
6
4
2
10
20
30
40
50
60
INCUBATION TIME (MIN)

Figure 7

Assay of RNA methylation using Ehrlich ascites or mouse liver S100 supernatants as enzyme source. Enzyme (277 μg Ehrlich ascites S100 protein or 235 μg mouse liver S100 protein), RNA (200 μg of 28S-5.8S rRNA from Ehrlich ascites cells or mouse liver) or buffer, and tritium-labeled SAM were incubated at 37°C in duplicate in a total volume of 100 microliters for indicated times. RNA was extracted, applied to DEAE paper, washed, and counted as in methods. Counts are corrected for zero-time control. Protein was determined by the Bio-Rad protein assay procedure (61). Exogenous Ehrlich ascites RNA (○—○); exogenous liver RNA (△—△); no exogenous RNA (□—□).


EA S100
LIVER S100
CPM x 10-3
1
2
3
4
5
6
5 10 20 40 80
INCUBATION TIME (MIN)

D. Effect of heparin and decreased temperature on methylation assays.

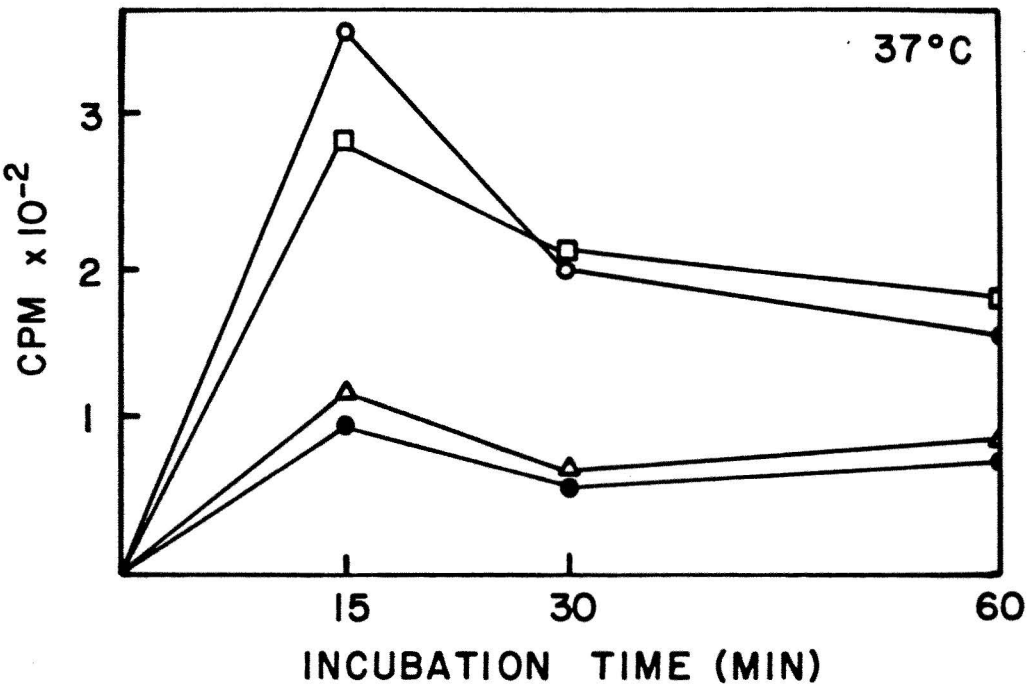
In an attempt to determine whether the decline in methylation over longer incubation periods for mouse liver S100 is due to nuclease activity, a study was made of the effect of heparin and decreased temperature on RNA methylation. Heparin acts as a competitive inhibitor of ribonucleases (62) and decreased temperature may retard nuclease activity more than methylase activity (63). In Figure 8, heparin does not cause a significant increase in RNA methylation whether enzyme is incubated with or without exogenous RNA at either temperature. However, the 25°C incubation curves peak at 30 minutes versus 15 minutes at 37°C, and have not declined as much at 60 minutes. Since the levels of methylation at 15 minutes are very similar at both temperatures, lowered temperature does not appear to affect methylase activity, but could be decreasing the effect of nuclease activity which appears over a longer incubation period.

E. Chromatography of T_2 digests of tritium-labeled RNA

DEAE-Sephadex chromatograms of ribonuclease T_2 hydrolysates of tritium-labeled RNA from Ehrlich ascites S100 supernatant samples incubated without exogenous rRNA (Figure 9) and with exogenous rRNA (Figure 10) are presented. Of the total tritium-labeled oligonucleotides, approximately 56% and 93% were recovered in the mononucleotide peak of Figures 9 (fractions 22-34) and 10 (fractions 30-39), respectively. Dinucleotides comprise 14% and 2% of the total tritium radioactivity in Figures 9 (fractions 45-55) and 10 (fractions 51-61), respectively. Mononucleosides contain 30% and 5% of the radioactivity in Figures 9 (fractions 3-21) and 10 (fractions 3-23), respectively.

Figure 8

The effect of heparin and decreased temperature on RNA methylation using mouse liver S100 supernatant. Total volume of each assay was 510 µl. Aliquots of 102 µl were removed at 0, 15, 30, and 60 minutes incubation (at 37°C or 25°C) and transferred to Eppendorf tubes containing 500 µl of SDS/phenol mixture. Each aliquot contained enzyme (88 µl of mouse liver S100 supernatant), RNA (200 µg of Ehrlich ascites 28S-5.8S rRNA) or hypotonic buffer, heparin (25 µg/ml) or buffer, and tritium-labeled SAM (1 µM). RNA was extracted, applied to DEAE paper, washed, and counted as in methods. Counts are corrected for zero-time control. Each aliquot contained 308 µg protein as determined by the Bio-Rad protein assay procedure (61). Exogenous RNA with heparin (○—○); exogenous RNA without heparin (□—□); heparin with no exogenous RNA (△—△); and no heparin or exogenous RNA (●—●).


37°C
CPM x 10⁻²
3
2
1
15
30
60
INCUBATION TIME (MIN)

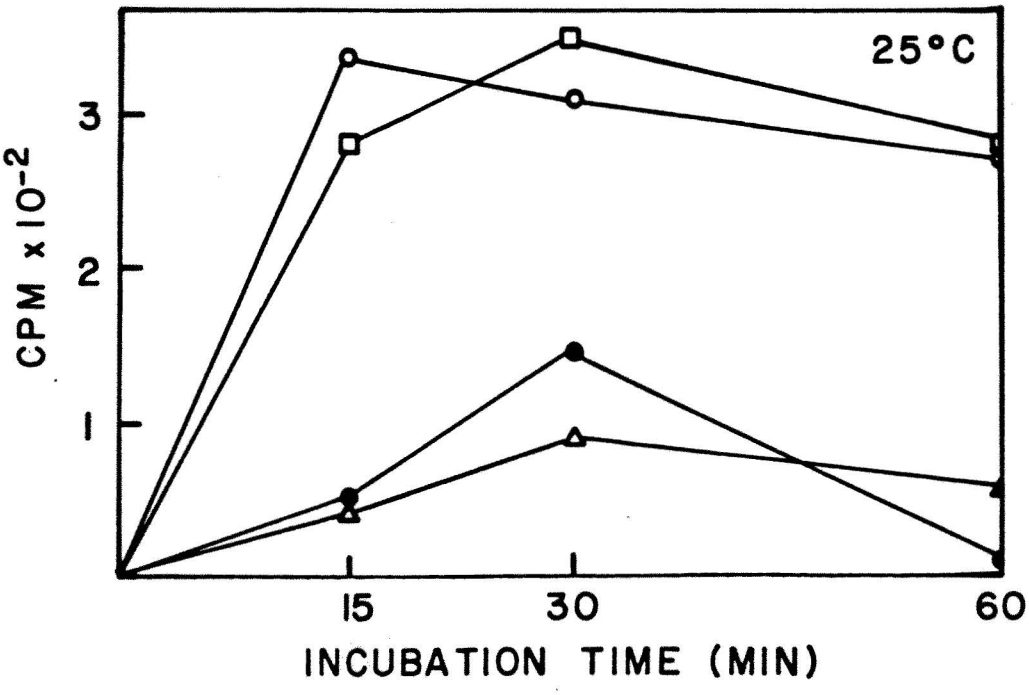

25°C
CPM x 10⁻²
3
2
1
15
30
60
INCUBATION TIME (MIN)

Figure 9

DEAE-Sephadex chromatography of ribonuclease T_2 hydrolysate of tritium-labeled endogenous RNA in Ehrlich ascites S100 supernatant assay. The sample containing tritium-labeled SAM (1 μM) in a total volume of one milliliter was incubated at 37°C for one hour. The RNA was extracted, approximately 5.8 mg of unlabeled RNA from Ehrlich ascites S100 supernatant was added as a marker, and then the sample was digested with RNase T_2. Column chromatography was performed as in the methods. The absorbance at 260 nm (◇—◇) and the radioactivity (○—○) for each fraction was plotted.

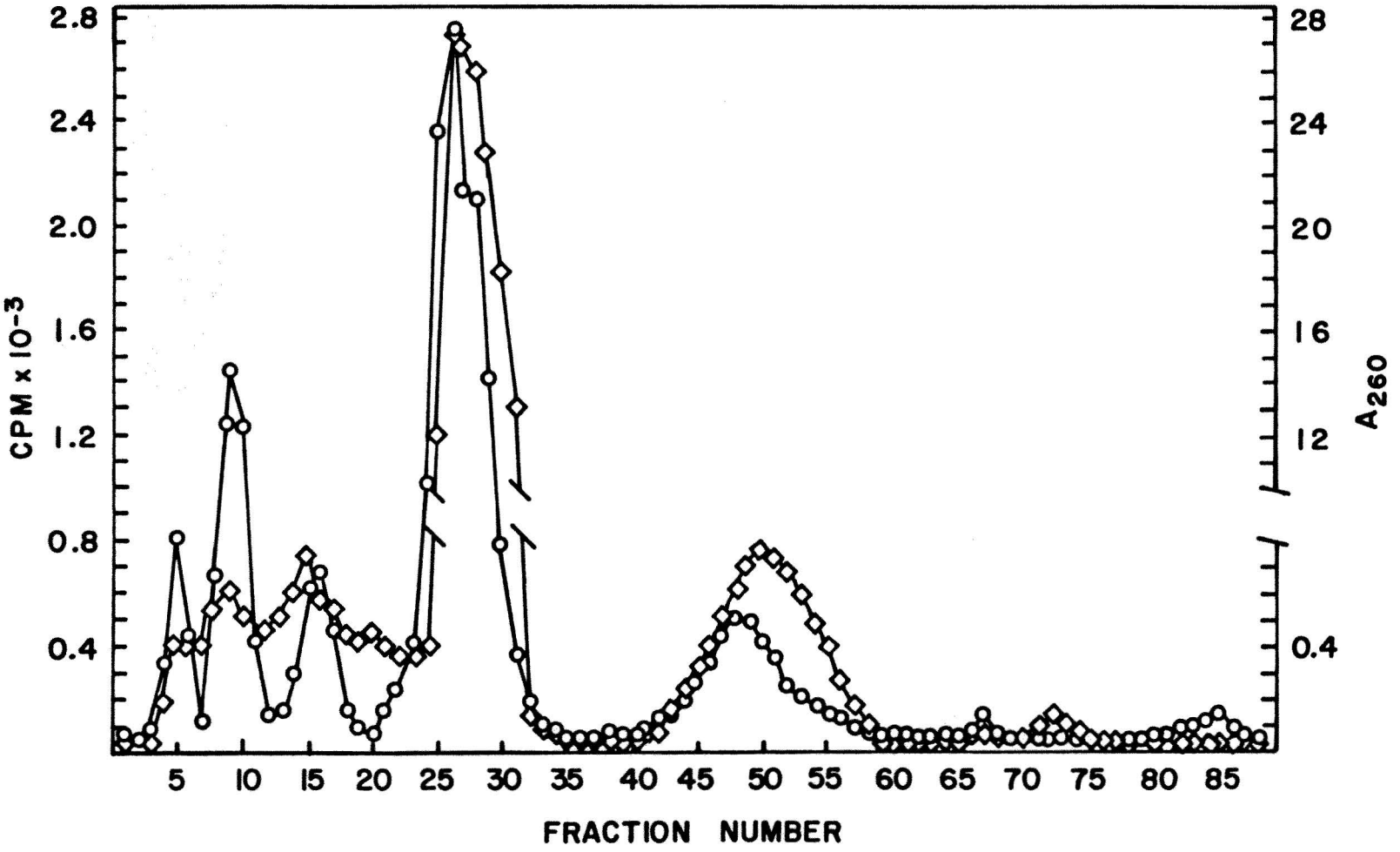
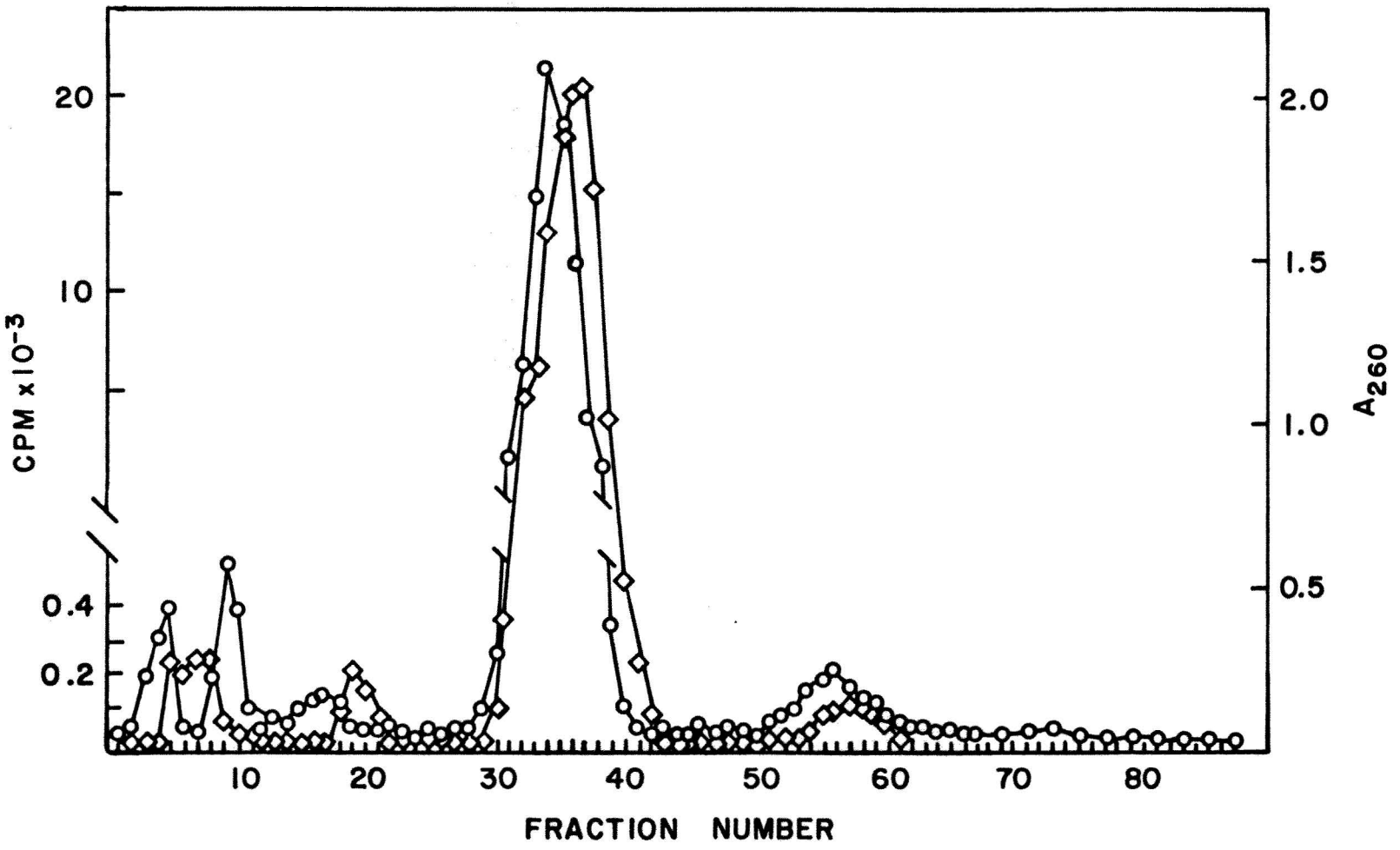

2.8
2.4
2.0
1.6
1.2
0.8
0.4
CPM x 10-3
28
24
20
16
12
0.4
A260
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
FRACTION NUMBER

Figure 10

DEAE-Sephadex chromatography of T_2 hydrolysate of endogenous and exogenous tritium-labeled RNA in Ehrlich ascites S100 supernatant assay. The sample containing tritium-labeled SAM (1 μM) and exogenous RNA (two milligrams of Ehrlich ascites 28S-5.8S rRNA) in a total volume of one milliliter was incubated at 37°C for one hour. The RNA was extracted, digested, and chromatographed as described in Figure 9. Absorbance at 260 nm (◇—◇); ^{3}H-cpm (○—○).


20
10
0.4
0.2
CPM x10⁻³
2.0
1.5
1.0
0.5
A260
10
20
30
40
50
60
70
80
FRACTION NUMBER

F. Comparison of methylation of endogenous RNA in S40 and S100 supernatants.

A comparison was made of RNA methylase activity of Ehrlich ascites S40 (40,000 x g) and S100 (100,000 x g) supernatants using only endogenous RNA as the substrate. Figure 11 shows that S40 supernatant samples incubated with tritium-labeled SAM have higher levels of endogenous RNA methylation than do S100 supernatant samples. In Figure 12, sucrose density gradient profiles of RNA show that the S40 supernatant has the 28S and 18S peaks indicative of ribosomal RNA, and the 4S peak indicative of transfer RNA. The S100 supernatant has only the 4S peak expected in the more rigorous centrifugation. Although the data is not shown, the RNA peaks by A_{260} were nearly identical for both 15 and 80 minute incubation times. Both supernatants have methylation only in the 4S peak, demonstrating that methylation occurred only on "free" RNA substrate, i.e., tRNA, and not on RNA in ribosomes. There was more radioactivity incorporated in the 80 minute incubation period (data not shown), and more radioactivity incorporated in the samples incubated without heparin.

Figure 11

Assay of endogenous RNA methylation using S40 (○—○) and S100 (△—△) supernatants. After homogenization of Ehrlich ascites cells in hypotonic buffer, an S40 supernatant was obtained after centrifugation at 18,000 rpm for one hour. Half of this supernatant was further centrifuged at 25,000 rpm for two hours to obtain an S100 supernatant. The S40 and S100 supernatants were assayed in duplicates with tritium-labeled SAM (1 μM) in a total volume of 100 μl. Incubation was at 37°C for times indicated. RNA was extracted, washed, and counted as in the methods. Counts are corrected for zero-time control. Protein was not determined due to lack of enzyme preparation.

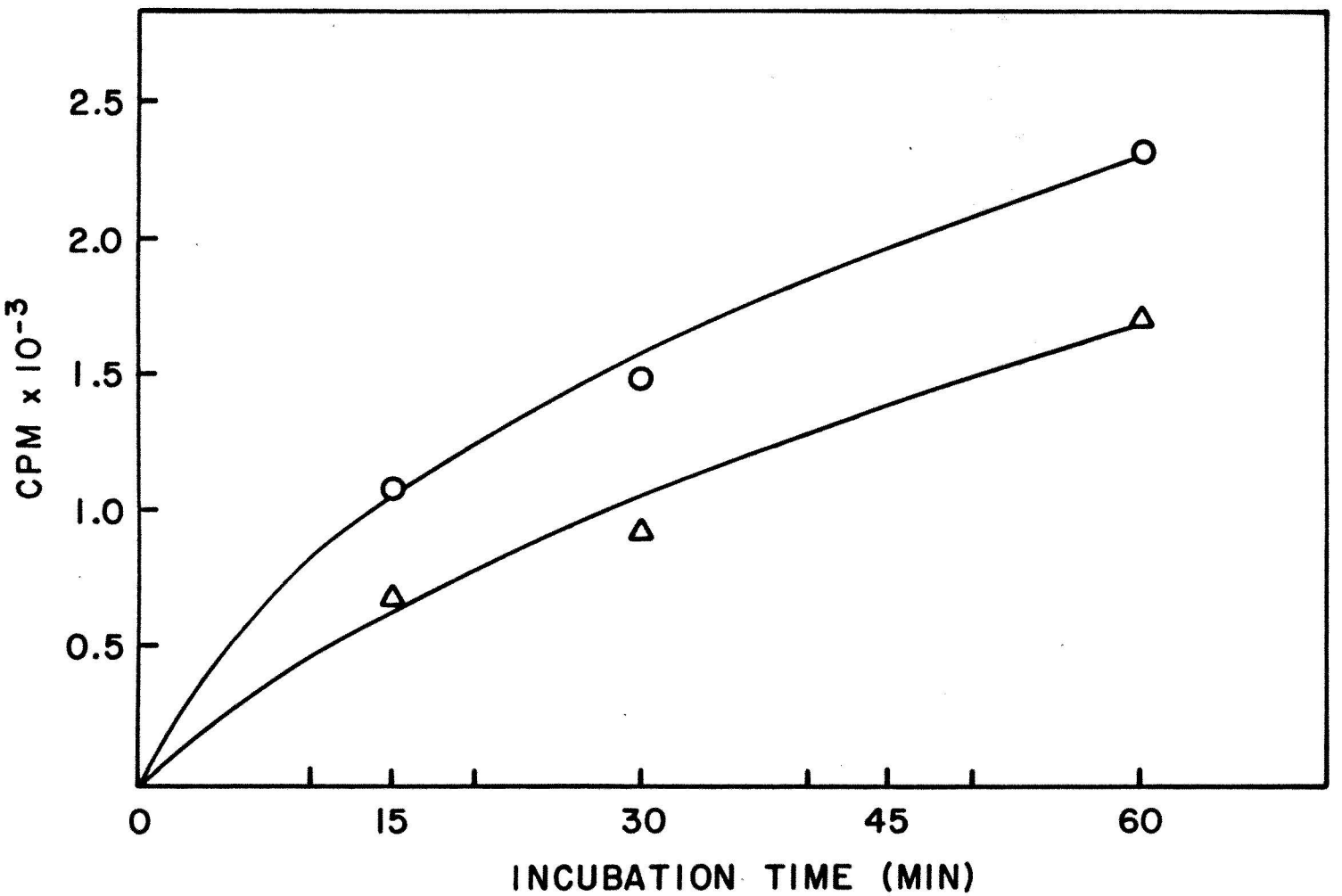
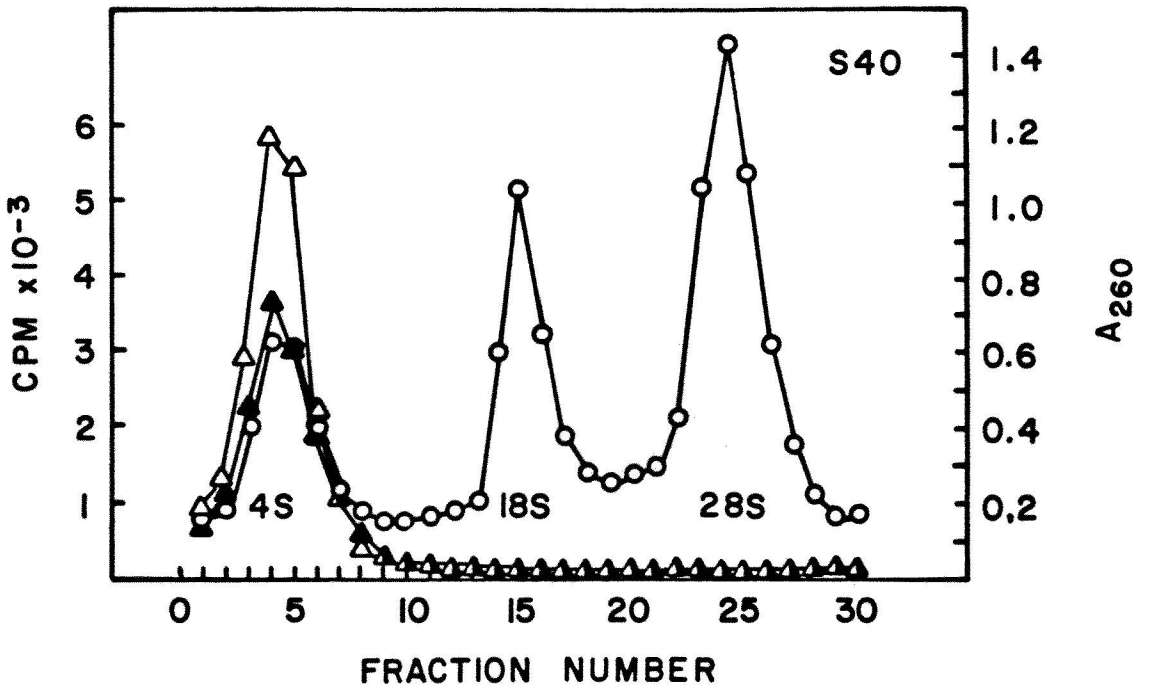
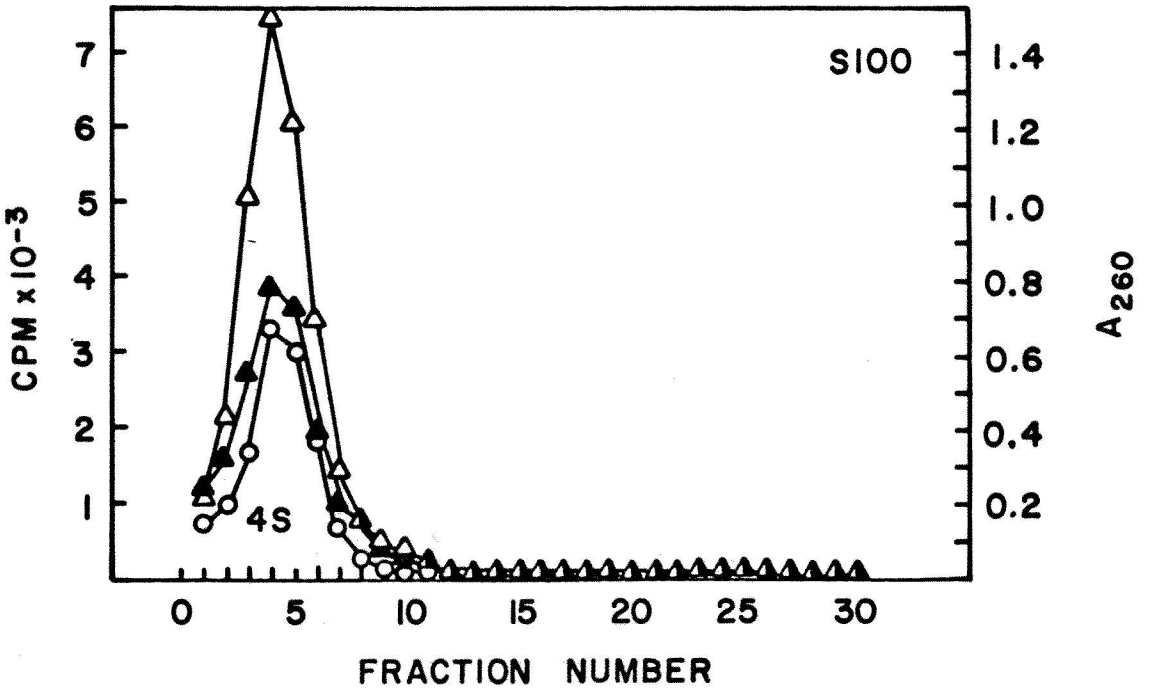

2.5
2.0
1.5
1.0
0.5
CPM x 10-3
0
15
30
45
60
INCUBATION TIME (MIN)

Figure 12

Sucrose density gradient profile of RNA from S40 and S100 supernatants. A large scale assay using two milliliters of S40 or S100 supernatant was incubated with tritium-labeled SAM (1 μM), heparin (50 μg/ml) or buffer, at 37°C for 15 minutes. The RNA was extracted with SDS/phenol, precipitated with ethanol twice, and separated using 5-25% sucrose gradients as outlined in the Methods. One milliliter fractions were collected, absorbance at 260 nm was measured, and radioactivity in 100 μl aliquots from fractions was counted. RNA with heparin by CPM (▲—▲); RNA without heparin by CPM (△—△); RNA by A_{260} (○—○).


S40
CPM x10-3
A260
4S
18S
28S
FRACTION NUMBER


S100
CPM x10-3
A260
4S
FRACTION NUMBER

CHAPTER 5

DISCUSSION

The first approach toward development of an *in vitro* assay for rRNA methylase activity took advantage of materials and methods commonly used in this laboratory (Table 1). Previous studies suggest that the methylation of position 14 in 5.8S rRNA occurs in the cytoplasm (23); however, a whole-cell-homogenate instead of a cytosolic homogenate was used initially as the source of methylase. Conceivably, any and all types of reactions associated with SAM could occur in this type of assay. Substrate specificity was obtained by isolating 5.8S rRNA by polyacrylamide gel electrophoresis following the enzyme incubation. The RNA from each sample was extracted from the polyacrylamide gel and equally divided for analysis by two methods. The two analysis methods utilize different rationales for detection of methylation of position 14 in 5.8S rRNA, and, therefore, can test the plausibility of the data. The Ryan method (46) compares the methylation of position 14 (UmG) which is partially methylated, with that of position 77 (GmC) which is wholly methylated, to arrive at a percent methylation. The Sanger method (48) utilizes the sequence surrounding position 14 as cleaved by RNase A to determine the percent methylation.

As seen in Table 1, the two methods did not give comparable results. In general, comparison of the incubated samples with the temperature controls show a higher percent methylation in the incubated samples as analyzed by Ryan's method, but a lower percent methylation as

analyzed by Sanger's method. This anomaly may reflect the possible contamination of the 5.8S rRNA by other RNA species and degradative fragments of other RNA species during the extraction and purification procedures. The low molecular weight nuclear (LMWN) RNA species contain 2'-O-methylations and could pose a problem with Ryan's method, resulting in false higher levels of methylation of 5.8S rRNA (46). Incomplete digestion of RNA by T_2/AP as suggested in the results may also be a contributory factor. Two potential problems, namely, insufficient transfer of the seven-nucleotide-long sequence (GGUmGGAUp) to the DEAE paper (the second dimension), and 5S rRNA contamination, can lead to a lower estimate (46) of the percent methylation of 5.8S rRNA as analyzed by Sanger's method. The problems with the data, i.e., the wide range of values within duplicates (as analyzed by Ryan's method), the decrease in methylation with the addition of SAM (as analyzed by Sanger's method), and the lack of correlating values between the two analysis methods, makes examination of the effects of different reagents very difficult and probably futile. Another major problem with this approach is the long time span (one to four weeks) required for analysis by Ryan's or Sanger's method before the final data for each experiment is obtained. Since these problems were also noted in the other experiments (not presented here) using whole-cell-homogenates as the source of both methylase and RNA substrate, this procedure was discontinued.

The next methodology utilized a different enzyme and RNA substrate preparation and a different method of analysis of methylation (Table 2). Preparation of the enzyme utilized a two-part procedure. The first step was fractionation of a whole-cell-homogenate by centrifugation at approximately 100,000 x g for two hours (52,53)

to remove nuclei, mitochrondria, lysosomes, and ribosomes, thereby obtaining a post-ribosomal supernatant (S100 supernatant). The next step employed a SAH-affinity chromatography column (Figure 4) to purify methylases from the S100 supernatant (54,55,56). SAH has a strong inhibitory effect on methylase systems (49,50) and acts as a bio-specific ligand for binding methylases in an affinity chromatography system.

The assay conditions and quantitation of methylation were adapted from methylase assays involved in the post-transcriptional processing of the 5'-end of mRNA (52,53). Eluates from the affinity column were monitored for methylase activity against purified Ehrlich ascites 28S-5.8S rRNA as the substrate, with tritium-labeled SAM as the methyl donor (Table 2). The data indicates that some activity above the temperature controls was achieved. The pH 5 elution, which ostensibly contains the methylase fraction, has about seven times as many counts per minute per microgram protein as does the S100 supernatant sample, and about four times as many counts per minute per microgram protein as does the 1 M NaCl elution samples. Although the SAH-affinity column methodology seems promising in view of the fact that several laboratories have used it successfully, certain technical aspects of the assay procedure had to be resolved at this stage of the study.

A series of experiments not presented here resolved the problems with protein carry-over into the radioactivity counting process which resulted in high temperature controls or zero-time controls. A RNA extraction step immediately after incubation was used not only to extract RNA and remove protein, but also to stop the enzymatic reaction. Aliquots of the aqueous layer containing the RNA were applied to DEAE

paper and washed several times. The washing procedure was found to be extremely effective in removing unreacted tritium-labeled SAM. The efficiency of the radioactivity counting procedure was greatly improved and stabilized when a small amount of water was added to the scintillation vials containing the Handifluor and DEAE paper, and also if the sealed vials were then vigorously shaken before counting in the scintillation counter. The most important step in achieving zero-time controls was to initiate the RNA extraction procedure immediately after adding the tritium-labeled SAM.

After the assay conditions and radioactivity counting procedures were established, the only enzyme preparations studied were mainly high-speed supernatant fractions of Ehrlich ascites cells or mouse liver. It was assumed that the endogenous RNA in the S100 samples in Figures 5, 6, and 7 represented tRNA and therefore would consist primarily of base methylation, since this type of methylation is most frequently found in tRNA (1). Figure 12 confirms that only tRNA is endogenous in S100 supernatants. It was hoped that the additional incorporation of radioactivity present in samples containing exogenous RNA in Figures 5 and 6 represented ribose methylation since this type of methylation is predominant in rRNA (1). Conceivably this could result in less methylation when mouse liver 28S-5.8S rRNA was used as an RNA substrate instead of Enrlich ascites 28S-5.8S rRNA, since mouse liver has a higher degree of methylation. However, Figure 7 shows that methylation of exogenous RNA by the S100 supernatants is almost identical for either type of enzyme, i.e., the normal mouse liver enzyme preparation does not discriminate in methylation of liver rRNA and Ehrlich ascites rRNA, nor does the neoplastic Ehrlich ascites enzyme preparation distinguish between the two

exogenous rRNA substrates. This indicates that the methylation of the exogenous rRNA may not represent the cellular situation.

The apparent elevation of methylase activity in Ehrlich ascites cell assays when compared with liver cell assays could be due to (a) the neoplastic cell enzymes actually being more active than the normal cell enzymes, (b) the normal cell ribonucleases being more active than the neoplastic ribonucleases, (c) a combination of the preceding two possibilities, or (d) the presence of a demethylase in liver cells. In Figure 8, the second possibility was examined using heparin, a nuclease inhibitor, and decreased incubation temperature to determine whether nuclease activity could be inhibited in assays using normal methylases. The effect of heparin at both temperatures was negligible, and could have been due to levels of heparin being too low to be effective as a ribonuclease inhibitor. The amount of methylation at 25°C versus 37°C was comparable at 15 minutes and even higher at 60 minutes. The decline noted in total methylation at 60-80 minutes in assays of normal methylases could be due to higher ribonuclease activity or a demethylase. However, even at shorter time intervals the assays of neoplastic methylases show greater methylation than do the normal methylases (Figures 5, 6, and 7). This phenomenon may be due to greater enzyme activity in neoplastic methylases and has been noted in Novikoff ascites tumor and rat liver nucleoli assays (35) and in tRNA assays (37).

Hydrolysis of RNA by ribonuclease T_2 and separation of oligonucleotides by DEAE-Sephadex chromatography (Figures 9 and 10) was performed in order to determine the percentage of ribose or base methylation occurring in the methylase assays. Hydrolysis with T_2 leaves phosphodiester linkages adjacent to a 2'-O-methylated-ribose uncleaved,

resulting in a dinucleotide. Two adjacent 2'-O-methylated ribose nucleotides result in a trinucleotide and so on. A base-methylated nucleotide does not affect the cleavage by T_2 and hence results in a mononucleotide along with other nucleotides not methylated at either a base or ribose position. The oligonucleotides are separated by DEAE-Sephadex chromatography according to chain length (57,58). The dispersion of tritiated methyl groups into base or ribose positions is somewhat different for endogenous (Figure 9) versus exogenous RNA (Figure 10). The high proportion of nucleosides is unusual and no explanation is immediately apparent. The amount of radioactivity in tri- and tetranucleotide peaks in either figure is less than one percent of the total radioactivity. The most significant point is the preponderance of base methyl groups, especially in the chromatogram of exogenous 28S-5.8S rRNA in Figure 10. Again, this indicates that the predominant in vitro methylation observed may not represent the cellular situation.

Since the exogenous purified 28S-5.8S rRNA added to the methylase assays represents a conceivably "unnatural" substrate to the methylases, an attempt was made to offer a more "natural" or intact substrate for methylation. Accordingly, an S40 supernatant, which contains ribosomes, was prepared from Ehrlich ascites cells and compared with an S100 supernatant prepared from the same homogenate (Figure 11). The activity of the S40 supernatant appears to be somewhat greater than that of the S100 supernatant. To determine if the increased methylation was due to the presence of RNA in ribosomes, the RNA was extracted after a 15-minute incubation and separated by sucrose density gradient fractionation (Figure 12). All of the methylation in both enzyme preparations occurred in the 4S or tRNA fraction. This indicates that

rRNA in the intact ribosomes is unavailable even for the base methylation in the in vitro assays. Although only one A_{260} RNA profile is shown for each assay system, the RNA profile by A_{260} was virtually identical whether heparin was used or not, showing very little apparent degradation in tRNA or ribosomes after 15 minutes or even after 80 minutes (not shown). Also, there actually appears to be less methylation occurring when heparin is present than when absent. Since heparin is a competitive ribonuclease inhibitor, it could conceivably be interacting in some way with methylases.

In summary, an in vitro assay for rRNA methylation using tritium labeled SAM as the methyl donor and exogenous 28S-5.8S rRNA has been developed. With the RNA substrates and enzyme preparations used the in vitro methylation of rRNA does not appear to represent the cellular situation, in which the methylation is predominantly ribose. All of the high-speed supernatant preparations contained tRNA, in which the in vitro methylation may be closer to the cellular situation, in which the methylation is predominantly base. Ribosomal RNA substrates tested in the established assay method include Ehrlich ascites 28S-5.8S rRNA (Figures 5-8 and 10), mouse liver 28S-5.8S rRNA (Figure 7), and Ehrlich ascites ribosomes (Figures 11 and 12). Enzyme preparations used in the assay method include Ehrlich ascites S100 supernatant (Figures 5, 7, and 9-12), mouse liver S100 supernatant (Figures 6-8), and Ehrlich ascites S40 supernatant (Figures 11 and 12). Other substrates that could be tested include 60S core particles (20) and purified 5.8S rRNA. Other methylase preparations include chromatography on DEAE-cellulose (40), SAH-affinity column chromatography (55,56), phosphocellulose affinity-elution chromatography using purified 5.8S rRNA to affinity-elute (40),

and ammonium sulfate fractionation (40). In conclusion, this assay is a useful tool for comparing total in vitro activity of normal and neoplastic RNA methylases.

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