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Characterization of S-Adenosylmethionine: Ribosomal Ribonucleic Acid-Adenine (N^6-) Methyltransferase of *Escherichia coli* Strain B

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This study is concerned with the isolation and characterization of the enzyme, S-adenosylmethionine:ribosomal ribonucleic acid-adenine (N^6-) methyltransferase [rRNA-adenine (N^6-) methylase] of *Escherichia coli* strain B, which is responsible for the formation of N^6-methyladenine moieties in ribosomal ribonucleic acids (rRNA). A 1,500-fold purified preparation of the species-specific methyltransferase methylates a limited number of adenine moieties in heterologous rRNA (*Micrococcus lysodeikticus* and *Bacillus subtilis*) and methyl-deficient homologous rRNA. The site recognition mechanism does not require intact 16 or 23S rRNA. The enzyme does not utilize transfer ribonucleic acid as a methyl acceptor nor does it synthesize 2-methyladenine or N^6-dimethyladenine moieties. Mg^{2+}, spermine, K^+, and Na^+ increase the reaction rate but not the extent of methylation; elevated concentrations of the cations inhibit markedly. The purified preparations utilize 9-β-ribosyl-2,6-diaminopurine (DAPR) as a methyl acceptor with the synthesis of 9-β-ribosyl-6-amino-2-methylaminopurine. A comparison of the two activities demonstrated that one methyltransferase is responsible for the methylation of both DAPR and rRNA. This property provides a sensitive assay procedure unaffected by ribonucleases and independent of any specificity exhibited by rRNA methyl acceptors.

Escherichia coli utilizes S-adenosyl-L-methionine (SAM) as the methyl donor to metabolize 9-β-ribosyl-2,6-diaminopurine (DAPR) and 9-β-(5'-phosphoribosyl-)-2,6-diaminopurine to 9-β-ribosyl-6-amino-2-methylaminopurine (N^2-MeDAPR) and 9-β-(5'-phosphoribosyl-)-6-amino-2-methylaminopurine, respectively (16, 19). Other 2-amino-substituted purine analogues are methylated to a lesser degree; the nature of the substitution at position 6 of the purine methyl acceptor greatly influences the rate of methylation of the 2-amino group (17). The methyltransferase has wide distribution among gram-negative bacteria, though considerable variation exists in the magnitude of this activity; the enzyme has not been identified in other microorganisms or animal cells (18).

The analogy between the methylation of the nonphysiological purine analogues and the ubiquitous presence of methylated purine moieties in nucleic acids prompted a study to purify the methyltransferase activity and to determine whether its physiological role was that of a nucleic acid-purine methylase. If the suggested physiological role were to be established, previous studies utilizing the analogue would suggest that this specific nucleic acid methylase is characteristic of and unique to many, or possibly all, gram-negative bacteria.

MATERIALS AND METHODS

Cell preparation and disruption. *E. coli* strain B was cultured in minimal medium (2) to late log or early stationary phase at 37 C with vigorous aeration. Cells were suspended in eight volumes (v/w) of 10^{-2} M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, containing 10^{-2} M $MgCl_2$ and 10^{-3} M ethylenediaminetetraacetic acid (EDTA), and disrupted by sonic vibration (Branson Sonifier, Branson Instruments, Inc., model no. LS-75) at 0 to 6 C. Unbroken cells and cell debris were sedimented at 20,000 × *g* for 30 min. Alternatively, cells were disrupted by grinding with alumina (2 g/g, wet weight) for 10 min. The resulting paste was suspended in six volumes (v/w) of the above buffer and centrifuged for 15 min at 24,000 × *g*. *Bacillus sub-*

tilis and *E. coli* strain W-6 were washed with 0.9% NaCl and disrupted by grinding with alumina.

Preparation of ribosomes and ribosomal ribonucleic acids. Cell-free extracts of *E. coli* B were centrifuged at 100,000 × *g* for 3 hr to sediment the particulate fraction. The latter fraction was suspended in 10^{-2} M Tris-hydrochloride, *p*H 7.5, containing 10^{-2} M $MgCl_2$, and centrifuged at 24,000 × *g* for 15 min to sediment the lipid-membrane fraction. The resulting supernatant fraction was centrifuged at 100,000 × *g* for 3 hr to sediment the 70*S* ribosomal fraction. The latter was suspended either in the above buffer or, alternatively, in 10^{-2} M Tris-hydrochloride, *p*H 7.5, containing 10^{-4} M $MgCl_2$, to dissociate the 70*S* structures into the 30*S* plus 50*S* subunits. The subunits were sedimented at 100,000 × *g* for 6 hr and suspended in the same buffer. Ribosomes were also prepared from *Micrococcus lysodeikticus* (5), *B. subtilis* [extraction medium contained 1 mM $MgCl_2$ and 0.1 mg of bentonite/ml (12)], and *E. coli* strain W-6 (4). Chloramphenicol particles from *E. coli* B and methionine-starved particles from *E. coli* strain W-6 were prepared as described previously (4). Ribosomal suspensions were adjusted to 0.5% with regard to sodium dodecyl sulfate (SDS) and ribosomal ribonucleic acid (rRNA) was isolated (6) by phenol extraction and precipitation with 0.1 volume of 20% potassium acetate, *p*H 5, plus 2 volumes of absolute ethanol. The crude rRNA was further purified by preferential precipitation (at least twice) with 2 M NaCl (14) or by chromatography on Sephadex G-200 (5); the purified rRNA was precipitated with ethanol, washed with 80% ethanol, 100% ethanol, and anhydrous ether, air-dried, and dissolved in 10^{-2} M Tris-hydrochloride, *p*H 8.

Assays. The ability of the enzyme preparations to utilize purine analogues as methyl acceptors was determined by measuring the transfer of the labeled methyl group of SAM to DAPR to yield N^2-MeDAPR. The standard incubation mixture contained 17 μmoles of Tris-hydrochloride (*p*H 8.0), 5 μmoles of β-mercaptoethanol, 25 μg of bovine serum albumin, 0.25 μmole of DAPR, either 3.9 nmoles of SAM-*methyl*-^{14}C or 0.2 nmole of SAM-*methyl*-^{3}H, and enzyme in a final volume of 0.3 ml. The mixture was incubated for 15 min at 37 C. The reaction was terminated by the addition of 0.7 ml of 0.82 N HCl containing 1.4 μmoles of 6-amino-2-methylaminopurine and 1.74 μmoles of adenine. Purine-ribosyl derivatives were hydrolyzed for 30 min at 100 C, and the free purines were selectively precipitated by the addition of 0.5 ml of 40% $AgNO_3$ and 2 ml of concentrated NH_4OH. After standing for at least 30 min, the silver-purine complex was harvested by centrifugation, washed three times with 4 ml of distilled water, suspended in 1 ml of 0.2 N HCl, and heated at 100 C for 4 min to regenerate the free purine bases, and the ^{3}H or ^{14}C content of the supernatant solution was determined. The rate of DAPR methylation was directly proportional to enzyme concentration provided less than 50 to 60% of the methyl donor had been utilized; however, the transfer of methyl groups went essentially to completion in the presence of excess enzyme. One unit of DAPR methyltransferase activity was defined as the amount of enzyme that transferred 1 pmole of methyl group from SAM to DAPR. Specific activity was defined as units of enzyme per milligram of protein.

The methylation of RNA was determined by measuring the transfer of the labeled methyl group of SAM into an alcohol-insoluble product (referred to as total RNA methylase assay). The standard reaction mixture (0.3 ml) contained 17 μmoles of Tris-hydrochloride (*p*H 8.0), 5 μmoles of β-mercaptoethanol, 25 μg of bovine serum albumin, 300 μg of *M. lysodeikticus* rRNA, either 7.8 nmoles of SAM-*methyl*-^{14}C or 1.2 nmoles of SAM-*methyl*-^{3}H, and enzyme. The assay mixtures were incubated at 37 C for 20 min, after which 0.1 volume of 20% potassium acetate, *p*H 5.0, and 2 volumes of absolute ethanol were added. The mixture remained at least 15 min at 0 C before the rRNA was recovered by centrifugation and washed three times with 4 ml of cold 67% ethanol, containing 0.1 M sodium acetate, *p*H 5.1, and once with 2 ml of absolute ethanol. The RNA was prepared for counting either by dissolving the RNA in 10^{-2} M Tris-hydrochloride, *p*H 8, or by hydrolyzing it in 0.2 N HCl for 10 min at 100 C. In addition to methylpurine, this procedure also detects the formation of 2′-O-methylribose and methylpyrimidine derivatives. Therefore, an assay that detected only methylpurines in RNA was developed by combining the two previously described methods. The incubation mixture was that of the total RNA methylase assay; the reaction was terminated by the addition of 0.7 ml of 0.82 N HCl containing 3.15 μmoles of N^6-methyladenine plus 1.74 μmoles of adenine. The RNA was hydrolyzed for 60 min at 100 C; the silver-purine complex was prepared and extracted as previously described. To reduce the background activity due to residual SAM, the purines in the 0.2 N HCl extract were precipitated again, washed with water, extracted with 0.2 N HCl, and then counted. The activity of the RNA-pyrimidine plus 2′-O-ribose methylases was determined as the difference between the total RNA methylase and the RNA-purine methylase activities. Since the chromatographic analysis of the methylated bases in rRNA (*see below*) demonstrated that N^6-methyladenine was the only methylpurine synthesized by the *E. coli* system, determination of methylpurine synthesis via the silver-purine complex method may be considered as equivalent to a measure of N^6-methyladenine synthesis.

Ribonuclease was assayed by a modification of a published method (13): reaction mixtures (0.25 ml) containing 17 μmoles of Tris-hydrochloride (*p*H 8.0), 5 μmoles of β-mercaptoethanol, and 200 μg of *E. coli* B rRNA were incubated for 45 min at 37 C. The incubation was terminated by the addition of 0.9 ml of 3% $HClO_4$. By definition, a unit of ribonuclease liberated a quantity of acid-soluble nucleotides that gave an optical density of 0.01 at 260 nm.

Protein was determined by the Lowry method (8) with crystalline bovine serum albumin used as the standard. RNA was determined as acid-precipitable ribose by the orcinol reaction (20), with *E. coli* B transfer ribonucleic acid (tRNA) used as the standard.

Identification of methylated moieties in enzymatically methylated rRNA. Components of the standard reaction mixture were increased proportionally to contain at least 2 mg of rRNA and incubated under standard conditions. Alternatively, the incubation time or enzyme volumes were selected to allow maximal methylation to detect the presence of minor methyltransferase activities. RNA was isolated by phenol extraction and ethanol precipitation. Approximately 1 mg of RNA plus reference compounds [2-methyladenine, 1-methyladenine, N^6-methyladenine, 1-methylguanine, N^2-methylguanine, 7-methylguanine (0.045 μmole of each), N^6-dimethyladenine (0.060 μmole), and N^2-dimethylguanine (0.020 μmole)] were made 1 N with respect to HCl and hydrolyzed for 1 hr at 100 C in glass-stoppered tubes. The hydrolysates were dried in vacuo over concentrated H_2SO_4 and solid NaOH, and the residue was dissolved in 0.05 ml of water. Samples were applied to Whatman no. 1 paper under a stream of warm air and separated by two-dimensional descending chromatography (7): first dimension, *n*-butanol-water (86 ml:14 ml) with NH_3 in the vapor phase, for 25 to 30 hr; second dimension, isopropanol-concentrated HCl (170 ml:40 ml) with water to 250 ml, for 35 to 40 hr. The bases were observed by their ultraviolet absorption or fluorescence and identified by comparison with standard chromatograms.

Detection of ^{3}H- and ^{14}C-labeled components. Radioactivity (^{14}C and ^{3}H) in aqueous solutions was determined by the addition of 0.8 ml of sample to 10 ml of a toluene-Omnifluor-BBS-3 solubilizer scintillation mixture. Radioactive areas (^{14}C) on paper chromatograms were excised in 1 by 3 cm strips and counted directly in 15 ml of the toluene-Omnifluor fluid; ^{3}H-labeled components were eluted from the paper with 3 ml of 0.2 N HCl and then counted. Samples were counted in Packard model 3320 or Nuclear-Chicago model 720 scintillation systems.

Chemicals, reagents, and cells. Chemicals and reagents were obtained from the following sources: deoxyribonucleic acid (DNA) of salmon sperm and calf thymus, tRNA from *B. subtilis*, yeast, rat liver, and *E. coli* strains B, W, and K_{12}, and *E. coli* strain W-6 (a Met^-, Rel^- derivative of strain K-12, methionine-limited) frozen cell paste (General Biochemicals); bovine liver rRNA (Mann Research Laboratories); 2-methyladenine, 1-methyladenine, N^6-methyladenine, N^6-dimethyladenine, 1-methylguanine, N^2-methylguanine, N^2-dimethylguanine, 7-methylguanine, 2,6-diaminopurine riboside, 2-aminopurine riboside, 6-amino-2-methylaminopurine, and 6-amino-2-methylaminopurine riboside (Cyclo Chemical Co.); *S*-adenosyl-L-methionine-*methyl*-^{14}*C*, 40 to 50 mCi/mmole, and *S*-adenosyl-L-methionine-*methyl*-^{3}*H*, 1 Ci/mmole, (International Chemical and Nuclear Corp.); alumina C-gamma gel (A-grade, aged), calcium phosphate gel (A-grade, aged), and adenine (Calbiochem); polyriboguanylic acid, polyriboadenylic acid, *M. lysodeikticus* ATCC 4698 lyophilized cells, *M. lysodeikticus* DNA, and *B. subtilis* ATCC 6633 frozen cell paste (Miles Laboratories); egg white lysozyme and deoxyribonuclease (Worthington Enzymes); pancreatic ribonuclease and alumina-type 305 (Sigma Chemical Co.); Sephadex G-200, and CM Sephadex C-50 (Pharmacia Chemicals); Omnifluor scintillator (New England Nuclear Corp.); Bio-Solv solubilizer, formula BBS-3 (Beckman Instruments, Inc.). Hydroxyapatite was prepared by the method of Tiselius et al. (23).

RESULTS

Subcellular localization of the methyltransferase. At least 90 to 95% of the methyltransferase (DAPR as methyl acceptor) sedimented (100,000 × *g*) with ribosomal structures in buffers containing 10^{-4} to 10^{-2} M Mg^{2+} regardless of the method of cell disruption employed. The quantity of methyltransferase recovered in the 100,000 × *g* particulate fraction progressively decreased to zero as the $MgCl_2$ concentration was either increased from 10^{-2} to 0.1 M or decreased from 10^{-4} M to zero. Under the former conditions, RNA remained in the particulate fraction, whereas, under the latter, the RNA paralleled the distribution of the methyltransferase. Once dissociated from the RNA (0.1 M Mg^{2+}), the soluble methyltransferase reassociated with particulate fractions when the concentration of Mg^{2+} was reduced to 10^{-2} M by either dialysis or dilution. The methyltransferase, associated with the 70*S* fraction, sedimented as two components in the 50 to 70*S* range (Fig. 1A), whereas the activity associated with the 30*S* plus 50*S* subunit fraction migrated as a single component between the 30 and 50*S* subunits (Fig. 1B). Thus, the methyltransferase was not specifically associated with either the 70, 50, or 30*S* units; the nonspecific nature of these associations was emphasized by demonstrating that the purified methyltransferase binds extensively to free rRNA (to be published separately).

To determine whether the observed sedimentation of the methyltransferase was dependent upon intact ribosomes or subunit particles, the ribosomes were extensively degraded in vivo by culturing cells in a magnesium-deficient medium for 20 hr (10). Samples of cells were harvested at intervals, disrupted in 10^{-2} M Tris-hydrochloride, *p*H 7.5, containing 10^{-2} M $MgCl_2$ and 10^{-3} M EDTA, and the extracts were centrifuged for 3 hr at 100,000 × *g*. Despite extensive catabolism of the ribosomes (loss of 85% of the sedimentable RNA), the methyltransferase, which decreased by 25%, was consistently recovered in the high-speed pellet; only a low and relatively constant amount persisted in the 100,000 × *g* supernatant fraction.

Approximately 90% of the DNA-purine methylase activity (salmon sperm and calf thymus DNA) and the tRNA methylase ac-

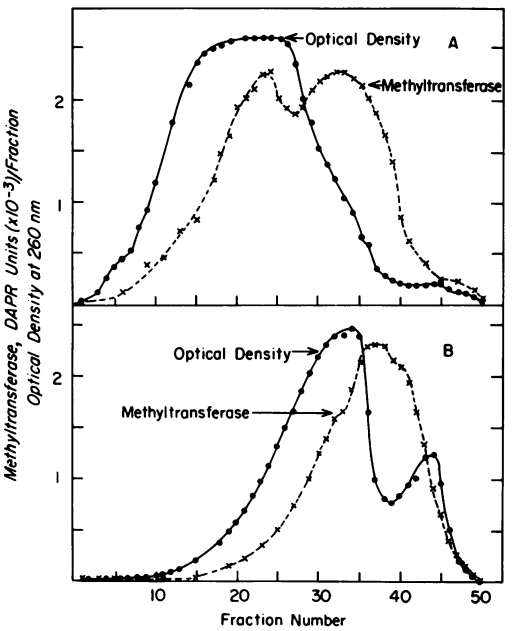

FIG. 1. *Association of methyltransferase with ribosomal units. (A) One milliliter of the 70S ribosomal fraction was layered onto 34 ml of a 5 to 20% linear sucrose gradient in 10^{-2} M Tris-hydrochloride, pH 7.5, containing 10^{-2} M $MgCl_2$, and centrifuged for 6 hr at 24,000 rev/min in an SW-27 rotor of a Spinco L2-65 ultracentrifuge. (B) One milliliter of the 30S plus 50S ribosomal subunit preparation was layered onto a 34-ml gradient prepared in 10^{-2} M Tris-hydrochloride, pH 7.5, containing 10^{-4} M magnesium acetate and centrifuged as described. The tubes were capped and pierced at the bottom; the fluid was forced out the bottom by pumping water onto top of gradient. Determination of methyltransferase (DAPR and ^{14}C-SAM) did not require removal of the sucrose.*

tivity (*B. subtilis* tRNA) was localized in the 100,000 × *g* supernatant fraction (10^{-2} M Mg^{2+}). By contrast, 90% of the rRNA-purine methylase activity (*M. lysodeikticus* and *B. subtilis* rRNA) was associated with the ribosomal fraction and dissociated from that fraction in the presence of 0.1 M $MgCl_2$. Thus, of the three families of nucleic acid methylases, only the methylation of rRNA-purine could be correlated with DAPR methylation.

Purification of the methyltransferase. The following procedure allowed a 1,500-fold purification of the methyltransferase with regard to its activity toward DAPR and with an overall yield of 20% (Table 1). Enzyme solutions were maintained at 0 to 4 C throughout the purification procedure and at −20 C for extended periods of storage.

(i) Preparation and extraction of the ribosomal fraction. *E. coli* B (80 g wet weight) was suspended in 640 ml of 10^{-2} M Tris-hydrochloride, *p*H 7.5, containing 10^{-3} M EDTA and 10^{-2} M $MgCl_2$, disrupted by ultrasonic vibration (Fraction 1), and centrifuged for 3 hr at 78,000 × *g*. The ribosomal-membrane pellet was suspended with the aid of a mechanical homogenizer in 300 ml of 10^{-2} M Tris-hydrochloride, *p*H 7.5, containing 0.1 M $MgCl_2$ and centrifuged at 78,000 × *g* for 3 hr. The supernatant fraction (Fraction 2) was retained.

(ii) Ammonium sulfate fractionation. A 47 to 75% ammonium sulfate fraction of Fraction 2 was obtained by the successive addition of 0.332 g and 0.198 g of solid $(NH_4)_2SO_4$ per ml. The resulting fraction was dissolved in 100 ml of 10^{-2} M Tris-hydrochloride, *p*H 7.5, containing 10^{-3} M EDTA, and dialyzed against 1 liter of the same buffer for a maximum of 8 to 10 hr. The dialyzed fraction (Fraction 3) was used immediately for further fractionation.

(iii) Alumina gel fractionation. Alumina gel (equivalent to 1 mg dry weight/mg of protein) was added to Fraction 3, stirred slowly for 10 min, harvested by centrifugation, and washed once by suspension in 25 ml of 3×10^{-2} M potassium phosphate, *p*H 7.4. The methyltransferase was eluted with 7 ml of 0.17 M potassium phosphate, *p*H 7.4. This elution process was repeated twice; the combined supernatant fractions were referred to as Fraction 4.

(iv) Calcium phosphate gel fractionation. Fraction 4 was diluted 10-fold with water prior to the addition of calcium phosphate gel (3.4 mg dry weight/mg of protein). The suspension was stirred for 10 min and centrifuged, and the gel was washed once by suspension in 25 ml of 2×10^{-2} M potassium phosphate, *p*H 7.4. The enzyme was eluted by suspending the washed gel in 7 ml of 0.10 M potassium phosphate, *p*H 7.4. The elution procedure was repeated twice;

TABLE 1. *Purification of methyltransferase*

Fraction no. and procedure	Methyltransferase[a]		
	Units ($\times 10^{-6}$)	Per cent yield	Specific activity ($\times 10^{-3}$)
1. Crude extract[b]	21.82	100	2
2. Magnesium extract	16.19	74	25
3. $(NH_4)_2SO_4$	11.38	52	53
4. Alumina gel	8.68	40	124
5. Calcium phosphate gel	5.97	27	497
6. CM Sephadex	4.76	22	2935

[a] Assays contained DAPR and ^{14}C-SAM.
[b] Starting weight of *E. coli* B was 80 g wet weight.

the combined supernatant fractions were referred to as Fraction 5.

(v) Chromatography on CM Sephadex. Fraction 5 was further purified on columns of CM Sephadex either as described in the legend to Fig. 2 or, alternatively, by applying Fraction 5 directly to columns equilibrated with 0.1 M potassium phosphate, *p*H 7.4. In the latter case, the columns were washed successively with one void volume of 0.1 M, one void volume of 0.15 M, and three void volumes of 0.2 M potassium phosphate, *p*H 7.4. With both methods, the flow rate was 0.4 ml/min, and the methyltransferase (Fraction 6) was recovered within the second void volume of 0.2 M potassium phosphate, *p*H 7.4.

Elution profiles of methyltransferases from CM Sephadex and hydroxyapatite. The elution profiles of Fraction 5 from CM Sephadex (Fig. 2) revealed that Protein Fraction IV (equivalent to Fraction 6) contained 18% of the applied protein, 2% of the ribonuclease, and at least 97% of the DAPR-rRNA-purine methyltransferase. The rRNA-pyrimidine methylases could be detected in Protein Fraction IV only by employing a quantity of enzyme that represented saturation concentrations in regard to the rRNA-purine methyltransferase. Protein Fraction I contained rRNA-pyrimidine and rRNA-purine methylases; however, the high content of ribonuclease precluded quantitative determinations. Several modifications in the chromatographic procedure verified the identical nature of the elution profile of the activities toward DAPR and rRNA-purine in Protein Fraction IV.

To determine whether more than one methyltransferase (DAPR as methyl acceptor) was eluted from the ribosomes with 0.1 M $MgCl_2$ even though only one was recovered during subsequent steps, Fraction 2 was dialyzed against three changes of 0.1 M potassium phosphate, *p*H 7.4, and then subjected to the CM Sephadex (C-50) chromatographic procedure. Only one symmetrical enzyme peak was observed in the elution profile.

Protein Fraction IV (Fig. 2) was diluted to 2 $\times$ 10^{-2} M potassium phosphate, *p*H 7.4, with water, concentrated to the original sample volume by ultrafiltration (PM-10 membrane,

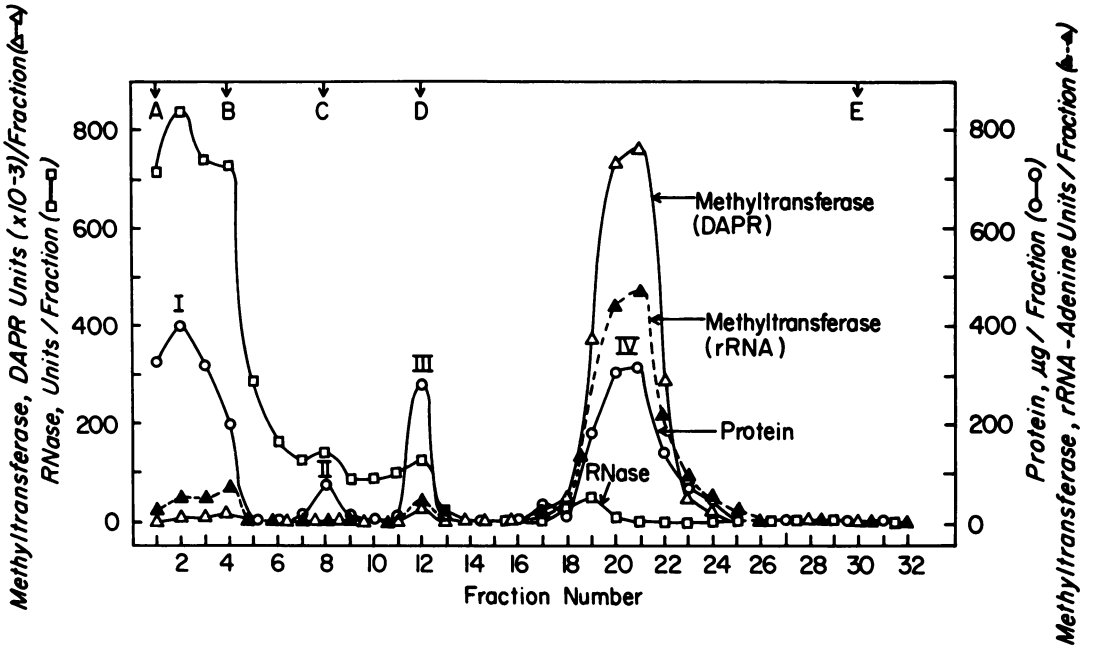

FIG. 2. *Chromatography of Fraction 5 on CM Sephadex. Approximately 4.5 $\times$ 10^6 units (DAPR) of Fraction 5 were adjusted to 5 $\times$ 10^{-2} M potassium phosphate, pH 7.0, with 1 M potassium phosphate, pH 5.5, and water, and applied to a column (1.25 by 20 cm) of CM Sephadex C-50 equilibrated with 5 $\times$ 10^{-2} M potassium phosphate, pH 7.0. The column was eluted in succession with (A) 30 ml of 5 $\times$ 10^{-2} M potassium phosphate, pH 7.0 (10-ml fractions); (B) 20 ml of 5 $\times$ 10^{-2} M potassium phosphate, pH 7.4 (4-ml fractions); (C) 30 ml of 0.1 M potassium phosphate, pH 7.4 (7.5-ml fractions); (D) 40 ml of 0.2 M potassium phosphate, pH 7.4 (2.4-ml fractions); and (E) 20 ml of 0.2 M potassium phosphate, pH 8.0 (10-ml fractions). Methyltransferase assays were carried out either with DAPR and ^{14}C-SAM or with M. lysodeikticus rRNA, ^{3}H-SAM, and 0.6 mM spermine.*

Amicon Corp.), applied to a column of hydroxyapatite (0.9 by 25 cm), and eluted with 0.1 M potassium phosphate, pH 7.4. Throughout the elution there was a correspondence between the methylation of DAPR and rRNA-purine. The recovery was at least 95% of that applied to the column. The eluted enzyme decayed approximately 60% per day at −20 C with respect to both methyl acceptors; the addition of albumin (5 mg/ml) greatly decreased the rate of inactivation.

Enzyme stability. No differential decay of methyltransferase activity toward either rRNA or DAPR was observed. The activity of whole cells stored at −20 C was stable for a minimum of 9 months. Fractions 1, 2, 4, and 5 were relatively stable at 2 C for at least 2 weeks, whereas Fraction 3 lost 90% of its activity. For maximum stability, Fractions 5 and 6 were stored at −20 C; at this temperature both were stable for at least 9 months.

The methylation of both DAPR and rRNA was similarly affected when Fraction 6 was heated at 50 C before assay of activity. In the case of newly prepared enzyme preparations, a 40% increase in activity occurred during the first 6 min at 50 C, followed by a slow but steady decay of the activity (32 to 38% of the initial activity remained after 18 min). However, preparations which had been stored at −20 C for several months did not exhibit the initial activation phenomena but only a steady inactivation. The heat activation phenomena observed with fresh preparations reflect, at least in part, the preferential inactivation of the contaminating ribonuclease. In subsequent studies concerning characterization of the methyltransferase, Fraction 6 was either heated at 50 C for 5 min in 7×10^{-2} M Tris-hydrochloride, pH 8, containing albumin and β-mercaptoethanol (refer to assay procedure), or stored at −20 C for several months to minimize ribonuclease activity.

The addition of 5 mM β-mercaptoethanol to enzyme preparations had neither beneficial nor detrimental effects on the stability of the methyltransferase at 2 C but was responsible for its rapid (overnight) and complete inactivation toward both methyl acceptors at −20 C. However, β-mercaptoethanol was included in the incubation mixtures as it increased methylation by 10 to 15%.

Substrate specificities and reaction products. Fraction 6 methylated *B. subtilis* rRNA, *M. lysodeikticus* rRNA, and methyl-deficient *E. coli* B rRNA; methyl-deficient *E. coli* strain W-6 rRNA was a comparatively poor methyl acceptor (Table 2). Bovine liver rRNA and *E. coli* B rRNA were unreactive. *B. subtilis* rRNA was the preferred methyl acceptor with respect to both rate and maximum extent of methylation. Assuming an average molecular weight of 8.75×10^5 for *M. lysodeikticus* rRNA, the apparent K_m and V_{max} (in the absence of spermine or Mg^{2+}) were calculated from Lineweaver-Burk plots to be 1.2×10^{-7} M and 2.8×10^{-12} moles/min, respectively. By comparison, the K_m and V_{max} for DAPR were calculated to be 3.6×10^{-5} M and 1×10^{-10} moles/min, respectively. DNA (calf thymus, *M. lysodeikticus*, and salmon sperm), tRNA (*E. coli* strains B, W, and K_{12}, *B. subtilis*, yeast, and rat liver), polyguanylic acid, and polyadenylic acid were not methylated to an extent greater than that of the homologous nucleic acid species from *E. coli* B. The inclusion of ribonuclease (10 μg) in the incubation mixture eliminated all apparent activity toward *M. lysodeikticus* rRNA, whereas the addition of proteins (deoxyribonuclease, 10 μg; or bovine serum albumin, 25 μg) stimulated methylation 10 to 30%. Methylation of DAPR was similarly stimulated by the above proteins, including ribonuclease.

Base analysis of *M. lysodeikticus* rRNA methylated by Fraction 6 revealed that at least 93% of the methyl groups incorporated into the rRNA were associated with N^6-methyladenine and 4% with two methylpyrimidine nucleotides tentatively identified as ribothymidylic acid and 5-methylcytidylic acid. In the case of *B. subtilis* rRNA, 98 to 99% of the incorporated methyl groups were found in N^6-methyladenine. Similar analyses demonstrated that the methylated purine synthesized by the rRNA-purine methyltransferase(s) in Protein Fraction I (Fig. 2) and Fractions 2 and 5 was likewise N^6-methyladenine.

TABLE 2. *Methylation of rRNA*

Source of rRNA	Methylation of rRNA-adenine[a] (pmoles)
Bacillus subtilis	19.80
Micrococcus lysodeikticus	10.30
Escherichia coli strain W-6 (methyl-deficient)[b]	0.43
E. coli B	0.0
E. coli B (methyl-deficient)[c]	1.68
Bovine liver	0.0

[a] The assay samples, containing 300 μg of RNA, 0.6 mM spermine, 600 pmoles of ^{3}H-SAM, and Fraction 6, were incubated for 30 min at 37 C.

[b] Isolated from methionine-starved particles.

[c] Isolated from chloramphenicol particles.

The rRNA substrates were methylated by Fraction 6 and the isolated products were subjected to centrifugation through sucrose gradients (Fig. 3). The progressive shift in the optical density patterns of the rRNA indicated that the contaminating ribonuclease was an endonuclease producing primarily large fragments. The sedimentation pattern of the unmethylated rRNA substrate revealed that the rRNA, particularly the 23*S* fraction, had been subjected to partial degradation (11) during the isolation procedure. Nevertheless, the distribution of N^6-methyladenine suggested that both the 23*S* and 16*S* *M. lysodeikticus* rRNA molecules, as well as the large fragments (possibly derived from the 23*S* rRNA), served as methyl acceptors. In the case of *B. subtilis* rRNA, it appeared that only the 23*S* rRNA and the fragments were methylated.

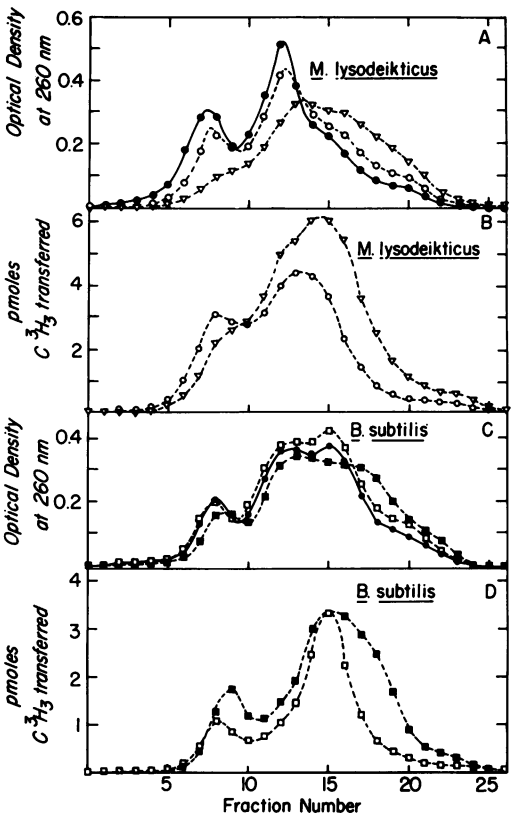

FIG. 3. *Separation of methylated-rRNA reaction products by centrifugation through sucrose gradients. (A, B) M. lysodeikticus rRNA (0.3 mg) was methylated in the presence of ^{3}H-SAM, 0.6 mM spermine, and Fraction 6. The rRNA-methyl-^{3}H was isolated, dissolved in 0.5 ml of 5% sucrose (ribonuclease-free) containing 10^{-2} M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, and 0.5% SDS, layered on top of a 13-ml linear sucrose gradient (5 to 20%) containing 10^{-2} M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, and 0.5% SDS, and centrifuged at 40,000 rev/min for 5.25 hr at 19 C in the SW-40 rotor of a Beckman L2-65B ultracentrifuge. Fractions (0.5 ml) were collected and assayed by the silver-purine method for tritiated N^6-methyladenine. (C, D) The above procedure was repeated with B. subtilis. Symbols: (●) unmethylated control; (○) 20-min incubation with 0.2 ml of Fraction 6; (▽) 60-min incubation with 0.2 ml of Fraction 6; (□) 20-min incubation with 0.025 ml of Fraction 6; and (■) 20-min incubation with 0.05 ml of Fraction 6.*

Properties of the methyltransferase. The *p*H activity profile of the methyltransferase in Tris-hydrochloride, *p*H 7 to 9, was essentially constant within the range of *p*H 7.5 to 8.2 and decreased rapidly at either higher or lower *p*H. The methyltransferase (Fraction 6) did not exhibit an absolute requirement for divalent cations as demonstrated by the slight stimulation of rRNA methylation in the presence of EDTA (Table 3). Nevertheless, both Mg^{2+} and spermine stimulated methylation; the effects were not additive. The velocity-enzyme concentration curve was sigmoidal in the absence of either Mg^{2+} or spermine and hyperbolic in

TABLE 3. *Effect of EDTA, spermine, and magnesium ions on the methylation of rRNA*

Expt	Additions[a]	Methylation of rRNA-adenine (pmoles)
1	None	7.54
	EDTA (1 mM)	7.87
	EDTA (5–10 mM)	9.27
	Spermine (0.2 mM)	21.50
	Spermine (0.4 mM)	29.00
	Spermine (0.6 mM)[b]	30.87
	Spermine (0.6 mM) + EDTA (10 mM)	28.40
	$MgCl_2$ (2.5 mM)	39.30
	$MgCl_2$ (5 mM)	42.50
	$MgCl_2$ (10 mM)	36.50
	$MgCl_2$ (5 mM) + EDTA (0.5 mM)	40.60
2	None	7.37
	Spermine (0.6 mM)	21.11
	$MgCl_2$ (5 mM)	32.11
	Spermine (0.6 mM) + $MgCl_2$ (5 mM)	23.88
3	None	16.90[c]
	Spermine (0.6 mM)	45.20[c]

[a] In addition to *M. lysodeikticus* rRNA, ^{3}H-SAM, and Fraction 6.

[b] The rRNA precipitated when spermine concentration was greater than 0.6 mM.

[c] *B. subtilis* rRNA replaced *M. lysodeikticus* rRNA.

their presence (Fig. 4A). By contrast, monovalent K^+ stimulated methylation only to a comparatively minor degree. The apparent maximal extents of methylation, as obtained by increasing the concentration of enzyme (Fig. 4A), were substantially exceeded by extending the incubation time (Fig. 4B). Under these conditions, methylation ceased as a result of a depletion of the methyl acceptor sites rather than a lack of SAM or inactivation of the methylase. The maximal number of methyl groups introduced into rRNA was the same both in the absence and presence of spermine or Mg^{2+}. At elevated concentrations Mg^{2+}, K^+, and Na^+ inhibited methylation (Fig. 5); K^+ and Na^+ exhibited their inhibitory properties even in the presence of stimulatory concentrations of Mg^{2+}. The ability of cations to inhibit appeared related to the ionic strength of the incubation mixture as well as to the nature of the ion. Thus, the failure to obtain true maximal incorporation by increasing the enzyme concentration (Fig. 4A) was due to the progressively more severe inhibition exerted by K^+, the concentration of which increased in proportion to the volume of the enzyme added.

Spermine (0.6 to 3 mM) produced a 20 to 25% stimulation of the methylation of DAPR regardless of the enzyme concentration. The velocity (DAPR)-enzyme concentration curve was hyperbolic both in the presence and absence of spermine.

Diluting the *M. lysodeikticus* rRNA in the incubation mixture with 300 μg of inactive rRNA (*E. coli* B) reduced the rate of methylation (in the presence of spermine) by 10 to 25%; dilution with 300 and 600 μg of methyl-deficient *E. coli* strain W-6 rRNA (poor methyl acceptor) reduced methylation by 44 and 73%, respectively.

Macromolecular inhibitor of the methyltransferase. Preparations of *M. lysodeikticus* rRNA which had not been purified by precipitation with 2 M NaCl contained a noncompetitive inhibitor (albumin omitted from assay mixture) which inhibited the same quantity of enzyme whether rRNA or DAPR was the methyl acceptor. By contrast, *M. lysodeikticus* DNA, *E. coli* B tRNA, and crude *E. coli* B rRNA produced either no effect or a slight stimulation of the methylation of both DAPR and purified *M. lysodeikticus* rRNA. Protein

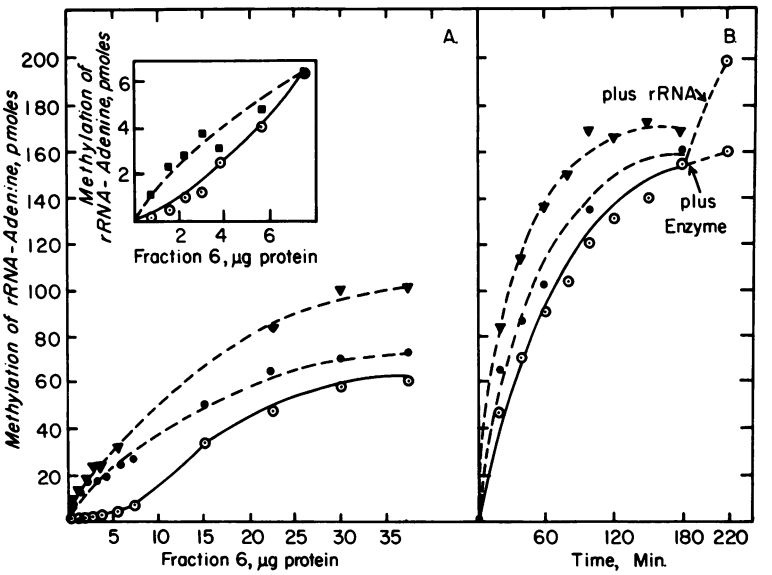

FIG. 4. *Effect of time and cations on methylation of rRNA-adenine. (A) Incubation mixtures contained 300 μg of M. lysodeikticus rRNA, ^{3}H-SAM, Fraction 6 in 0.2 M potassium phosphate, pH 7.4 (0.3 mg protein/ml), and concentrations of cations as indicated. Mixtures were incubated for 20 min. Symbols: (○) no additions; (●) plus 0.6 mM spermine; (▼) plus 5 mM $MgCl_2$; and (■) plus potassium phosphate, pH 7.4, to yield a final K^+ concentration of 16.6 mM (inset). (B) Incubation mixtures contained 300 μg of M. lysodeikticus rRNA, ^{3}H-SAM, Fraction 6 (22.5 μg of protein), and cations as indicated. The mixtures were incubated for the indicated time periods. Symbols: (○) no additions; (●) plus 0.6 mM spermine; and (▼) plus 5 mM $MgCl_2$. To verify that the methyltransferase and ^{3}H-SAM were still functional at 180 min, selected samples received either additional methyltransferase (22.5 μg of protein) or M. lysodeikticus rRNA (300 μg). These samples were incubated for an additional 40 min.*

such as bovine serum albumin, ribonuclease (DAPR as methyl acceptor), and deoxyribonuclease completely prevented the inhibition by crude *M. lysodeikticus* rRNA. The inhibitory component was removed from the rRNA by treatment with 2 M NaCl and was accounted for in the supernatant fraction after removal of the NaCl by dialysis. By contrast, it could not be separated from the rRNA by either dialysis or by chromatography on Sephadex G-200.

The addition of 25 μg of potassium polyvinylsulfate or 1 μmole of adenine (18) to the standard assay system inhibited by 100% and 40%, respectively, the methylation of both rRNA and DAPR.

DISCUSSION

The present investigation reveals that a 1,500-fold purified methyltransferase preparation from *E. coli* B methylates, in addition to DAPR, a limited number of adenine bases in rRNA, but not DNA or tRNA, to produce exclusively N⁶-methyladenine moieties. A comparison of the methyltransferase activities toward rRNA-adenine and DAPR indicates that both exhibit equivalent (i) association with ribosomal structures, (ii) fractionation properties including identical elution profiles on CM Sephadex and hydroxyapatite, (iii) stability during storage at 2 C and −20 C, (iv) inactivation by β-mercaptoethanol at −20 C, (v) inactivation at 50 C, (vi) inhibition by adenine, polyvinylsulfate, and an unidentified macromolecular inhibitor from *M. lysodeikticus*, and (vii) stimulation by Mg^{2+} and spermine. These results indicate that one enzyme, *S*-adenosylmethionine:rRNA-adenine (N⁶-) methyltransferase [rRNA-adenine (N⁶-) methylase] is responsible for the synthesis of N⁶-methyladenine moieties in rRNA and for the methylation of nonphysiological 2-amino-substituted purine analogues. The assay procedure involving the methylation of DAPR is a valuable tool since it is sensitive, applicable to crude and purified preparations, insensitive to ribonuclease, and may circumvent the characteristic species specificity common to nucleic acid methyltransferases.

Although the methyltransferase is localized in the ribosomal fraction (in contrast to the soluble or membrane fractions) of cell extracts prepared in buffers of low ionic strength containing 10^{-4} to 10^{-2} M Mg^{2+}, the authors favor the view that the observed association may in part represent an artifact of cell disruption rather than a specific functional relationship. The cationic methylase may form ionic bonds (dissociable at higher ionic strength) with a

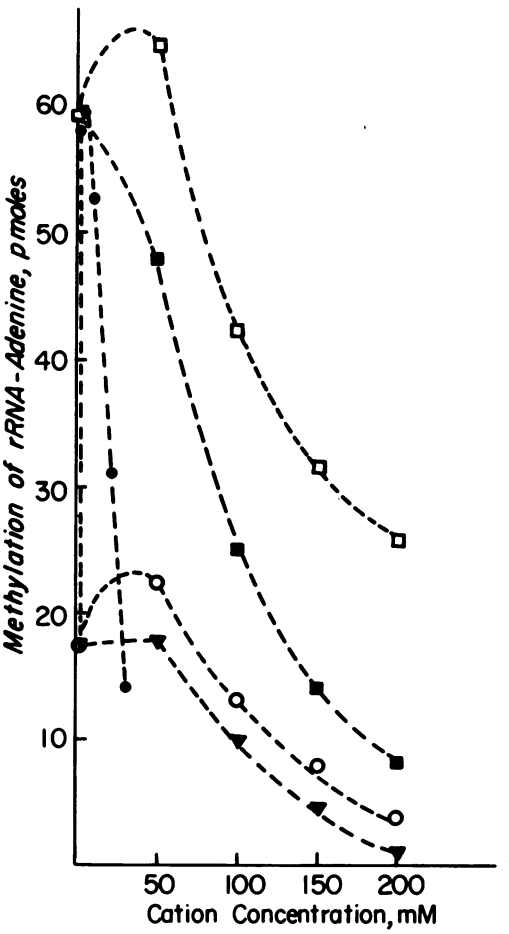

FIG. 5. *Effect of cations on methylation of rRNA. Incubation mixtures, containing 0.05 ml of Fraction 5 (1 mg of protein/ml), were supplemented with concentrations of ions as indicated. Symbols:* (○) *KCl,* (▼) *NaCl,* (●) $MgCl_2$, (□) $MgCl_2$ *(5 mM) plus KCl, and* (■) $MgCl_2$ *(5 mM) plus NaCl.*

variety of sedimentable ribonucleoproteins containing exposed rRNA segments (anionic), such as normal ribosomal units, partially degraded or damaged particles, or precursor particles (24). However, the ability to bind to ribonucleoproteins is a property unique to the rRNA methylase since neither tRNA or DNA methylases sedimented with the ribosomal fractions. Electrostatic binding to partially dissociated, sedimentable particles (22) could explain the observed sedimentation of the methylase during in vivo degradation of ribosomes by Mg^{2+} starvation.

The species and strain specificity of the rRNA-adenine (N⁶-) methylase is demonstrated by its (i) extensive methylation of

rRNA from *M. lysodeikticus* and *B. subtilis*, (ii) the comparatively poor methylation of rRNA from *E. coli* strain W-6, and (iii) inability to utilize homologous rRNA from strain B as a methyl acceptor unless the rRNA is methyl-deficient. The difference in the degree to which inactive and relatively inactive RNA molecules inhibit methylation of *M. lysodeikticus* rRNA indicates that the methylase does not indiscriminately bind to RNA molecules lacking active recognition sites for methylation.

The rRNA-adenine (N^6-) methylase of *E. coli* B recognizes a number of sites in the *M. lysodeikticus* rRNA including those in 23*S* rRNA, 16*S* rRNA, and relatively large fragments presumably resulting from ribonuclease hydrolysis of one or both units. Methylation of *B. subtilis* rRNA is more restricted, in that the 16*S* rRNA may not be methylated. In both cases, methylation of the large fragments indicates that the site recognition mechanism does not require intact 23 or 16*S* rRNA units.

The kinetic constants (K_m and V_{max}) support the concept that rRNA is a physiologically significant methyl acceptor. The sigmoidal relationship between the rate of rRNA methylation and enzyme concentrations is eliminated in the presence of Mg^{2+} or spermine. These ions exert only a modest stimulation of DAPR methylation. These observations may be explained on the basis that Mg^{2+} and spermine facilitate the formation of either the enzyme-rRNA complex or the favored conformation of the rRNA rather than an activation of the enzyme per se. The reaction rate is thereby increased without altering the maximal number of recognition sites on the rRNA. These conclusions are substantiated by reports that Mg^{2+} and spermine (i) bind to RNA and alter the secondary structure of the rRNA (1, 21) and (ii) satisfy the absolute requirement of rat liver tRNA methylase for di- and polyvalent cations (9, 15). The ability of mono- and divalent cations to inhibit at elevated concentrations may be related to the ability of ions to dissociate the enzyme-rRNA complex (analogous to the dissociation of the enzyme-ribosomal complex).

The probability for the existence of more than one rRNA-adenine (N^6-) methylase is implicit in the report (3) that 23*S* rRNA of *E. coli* strain W-6 has two distinct sequences containing N^6-methyladenine. Although the present studies fail to offer evidence for the subfractionation of the rRNA-adenine (DAPR) methylase, fractionation of Fraction 5 on CM Sephadex offers tentative evidence for the existence of a second, less cationic, rRNA-adenine (N^6-) methylase which apparently does not react with DAPR. Definitive evidence for the existence of the second rRNA-adenine methylase depends upon its separation from ribonuclease which all but obscures its existence.

A comparison of rRNA-adenine (N^6-) methylase from *E. coli* strain B with rRNA-adenine II methylase from strain W (5), utilizing *M. lysodeikticus* rRNA as the heterologous methyl acceptor, reveals that both are species- and strain-specific, associated with the ribosomal fraction, and responsible for the synthesis of N^6-methyladenine moieties. In marked contrast to the preparation from strain W, the methylase from strain B synthesizes neither N^6-dimethyladenine nor 2-methyladenine moieties, nor does it efficiently methylate either tRNA or methyl-deficient rRNA from *E. coli* strain W-6.

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