Stimulation of Soluble Ribonucleic Acid Methylase Activity by Polyamines*

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ABSTRACT: The effect of polyamines on the activity of soluble ribonucleic acid (sRNA) methylases from rat organs was examined. Addition of spermine, spermidine, or putrescine increased severalfold the specific activity and extent of methylation.

The stimulatory effect of polyamines persisted when rat liver methylases were purified 25–35-fold. Concentrations of spermine and spermidine found to be optimal for stimulation of extent of methylation are comparable with levels of these polyamines reported to be present in rat liver. Treatment of enzyme extracts with 1 mM ethylenediaminetetraacetate (EDTA) resulted in complete loss of methylase activity which could be restored by addition of polyamines to the reaction mixture. Magnesium acetate (0.5–30 mM) permitted only limited stimulation of methylation. These results are consistent with the hypothesis that methylase preparations contain Mg ions which permit a low level of methylase activity, and EDTA treatment results in removal of Mg from the enzymes. Rat liver methylase extracts did not methylate homologous sRNA in the presence of polyamines, indicating that the specificity of the methylation reaction is retained and that rat liver sRNA is methylated *in vivo* to the extent reached *in vitro* by addition of polyamines. It is suggested that alterations in cellular polyamine levels may play a role in regulating the level of sRNA methylation under various growth conditions.

The occurrence of methylated bases in tRNA has been well documented (Borek and Srinivasan, 1966); however, the significance of specific methylation patterns for the function of tRNAs is as yet poorly understood. In 1962, Berquist and Matthews presented evidence that sRNA from tumors contained a higher percentage of methylated bases than sRNA from nonneoplastic tissues, an observation which has been confirmed by Burdon (1966). More recently, Holland *et al.* (1967) have demonstrated chromatographic differences in tyrosyl-tRNA from fibroblasts as compared with normal tissues, and Shugart *et al.* (1968) have reported that several species of undermethylated tRNA have lower aminoacylation activity than comparable normally methylated tRNAs. The correlation between alterations in tRNA methylation and alterations in cell growth lend support to the hypothesis that tRNA methylation is involved in regulatory mechanisms in the cell (Srinivasan and Borek, 1963).

On the assumption that differences in the pattern of tRNA methylation should be reflected in variations in the methylase composition of tissues, several laboratories have investigated methylase activity of organ extracts. Studies such as those of Tsutsui *et al.* (1966) have indicated that, in comparison with nonneoplastic tissues, crude extracts from tumors have more sRNA methylase activity and a greater capacity to methylate a limiting amount of sRNA (extent of methylation). However, the validity of the observations involving *in vitro* studies has been called into question by recent reports indicating the critical effect that ionic environment plays in measurements of sRNA methylase activity. Reports from several laboratories have indicated that dialyzed crude organ extracts have low sRNA methylase activity which can be stimulated four- to six-fold by addition of ammonium salts at concentrations of 0.3–0.4 M (Rodeh *et al.*, 1967; Kaye and Leboy, 1968; Baguley and Staehelin, 1968).

These observations suggest that measurements of specific activity and extent of methylation observed with crude extracts might be influenced by ions present in the homogenate. Tissues from various sources may have altered ion compositions which could produce both differences in *in vitro* methylase measurements and altered methylation patterns *in vivo*. However, the high concentrations of ammonium salts required for optimal activity make it unlikely that ammonium ions are functioning to stimulate methylase activity in the cell. It was therefore of interest to examine other compounds which might produce the same stimulatory effect on sRNA methylase activity at more physiological concentrations. Studies on the DNA-dependent RNA polymerase reaction indicated that low concentrations of the polyamine spermidine could partially replace the requirement for high concentrations of ammonium or potassium salts (So *et al.*, 1967; Fuchs *et al.*, 1967). In this paper, the influence of various polyamines on rat organ tRNA methylase extracts is explored.

Materials and Methods

Normal and methyl-deficient sRNA was prepared from *E. coli* K12 (Hfr C) by the method of Hurwitz *et al.* (1964). Rat liver sRNA was obtained by preparing the pH 5 precipitate as described by Cantoni and Richards (1966), followed by phenol extraction and cold ethanol precipitation as for the bacterial sRNA samples. Soluble RNA from *E. coli* B was purchased from Calbiochem. Unless otherwise specified, methyl-deficient sRNA was used as substrate.

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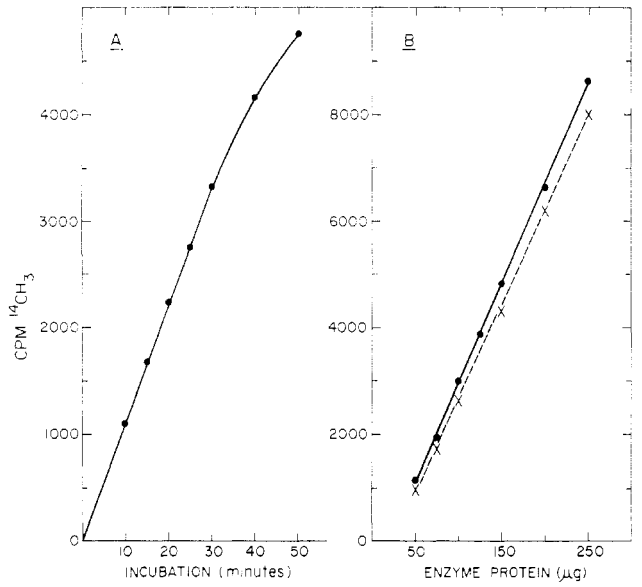

FIGURE 1: Effect of incubation time and enzyme concentration on methylation of *E. coli* B sRNA under conditions for determining specific activity. Source of enzyme was an EDTA-treated ammonium sulfate fraction. A. Time course for incorporation of [^{14}C]methyl groups in the presence of 30 mM putrescine and 100 μg of enzyme protein. B. Effect of enzyme concentration on the rate of incorporation of [^{14}C]methyl groups into sRNA: (●—●) with 30 mM putrescine; (× - - - ×) with 10 mM spermidine.

TABLE I: Summary of Partial Purification Procedures.

Enzyme Fraction	Total Protein (mg)	Total Units[a]	Specific Activity (mμmoles of CH_3/30 min per mg of Protein)
Crude liver extract	770	139	0.18
Ammonium sulfate fraction	300	108	0.36
Alumina C γ eluate	36	46	1.28
DEAE-Sephadex peak	5.6	30	5.35

[a] One unit of enzyme is that amount which transfers 1 mμmole of CH_3 to sRNA in 30 min. Methylase activity was measured in the presence of 30 mM putrescine.

[*methyl*-^{14}C]-*S*-Adenosylmethionine was obtained from Volk Radiochemical with a stated specific activity ranging from 40 to 48 μCi per μmole. This corresponded to 65–85 cpm/μμmole in a Packard liquid scintillation spectrometer. Putrescine dihydrochloride, spermine tetrahydrochloride, and ammonium sulfate were purchased from Mann Research Laboratories, and spermidine trihydrochloride was obtained from Sigma Chemical Co. Alumina C γ gel (17.5 mg of solids/ml) and dithiothreitol were purchased from Calbiochem.

An initial survey of rat organ extract activity was carried out using female Osborne–Mendel rats; other studies involving liver preparations employed adult male Holtzman rats. No differences were observed between the two strains.

Preparation of Enzyme Extracts. CRUDE EXTRACTS. Dialyzed high-speed supernatant extracts of rat organs were prepared as described previously (Kaye and Leboy, 1968).

AMMONIUM SULFATE FRACTIONS. That portion of the high-speed supernatant extract which precipitated between 30 and 48% saturated ammonium sulfate was obtained by addition of solid ammonium sulfate. The resulting precipitate was resuspended in 0.01 M Tris, pH 8.15, containing 0.005 M dithiothreitol and the residual ammonium ion removed either by dialysis or by ultrafiltration using Diaflo UM 20E ultrafilters (Amicon Corp.). EDTA treatment was carried out by ultrafiltration as follows: extracts were placed in an ultrafiltration cell with 25 volumes of Tris–dithiothreitol buffer containing 1 mM EDTA, concentrated to original volume, and the EDTA diluted at least 50-fold by adding fresh Tris–dithiothreitol buffer and reconcentrating several times.

ALUMINA C γ ELUATES. The 30–48% ammonium sulfate fraction (10 ml) was diluted with 100 ml of 0.05 M triethanolamine buffer, pH 8.8, and sufficient alumina C γ gel was added to provide 7 mg of alumina solids/mg of protein. The suspension was stirred for 15 min, centrifuged, the precipitate was washed twice with 100-ml portions of 0.2 M potassium phosphate buffer, pH 6.5, and the washings were discarded. The precipitate was then extracted twice with 50 ml of 0.5 M ammonium sulfate, pH 7.5, and the ammonium sulfate eluates were combined. This solution was adjusted to 60% saturation by addition of solid ammonium sulfate to concentrate the preparation. The precipitate was resuspended in a small volume of Tris–dithiothreitol buffer and residual ammonium sulfate was removed by dialysis or ultrafiltration.

DEAE-SEPHADEX CHROMATOGRAPHY. DEAE column chromatography was carried out under conditions similar to those employed by Baguley and Staehelin (1968). A 1.5 × 22 cm column of DEAE-Sephadex (Pharmacia Corp.) was washed with 0.1 M Tris (pH 8.4 at 4°) containing 1 mM EDTA and 0.01 M mercaptoethanol (buffer E). The alumina C γ extract was concentrated by ultrafiltration to a protein concentration of 15–25 mg/ml, and 1.5–2.0 ml of this extract in 0.1 M Tris was applied to the column. Buffer E (30 ml) was added, followed by a continuous gradient of 0–0.3 M NaCl in buffer E (180 ml). Fractions (4 ml) were collected and checked for absorbance at 280 mμ and methylase activity. All of the activity was eluted between 0.17 and 0.27 M NaCl. The active fractions were pooled and placed in an ultrafiltration cell for concentration and replacement of the eluting buffer by Tris–dithiothreitol buffer.

A summary of the purification procedures is presented in Table I. All preparations, stored in Tris–dithiothreitol buffer, were relatively stable through the stage of alumina C γ extraction, losing less than 10% of their activity after 1 week at −15°. The DEAE-Sephadex eluate, however, lost approximately 10% activity in 24 hr at either 0 or −15°. The nature of the sRNA methylase complement recovered during the purification steps was checked by determining the extent of methylation reached with the methylating preparations and by examining the distribution of [^{14}C]methyl in nucleotides of substrate RNA after alkaline hydrolysis and high-voltage electrophoresis (Kaye and Leboy, 1968). By both of these

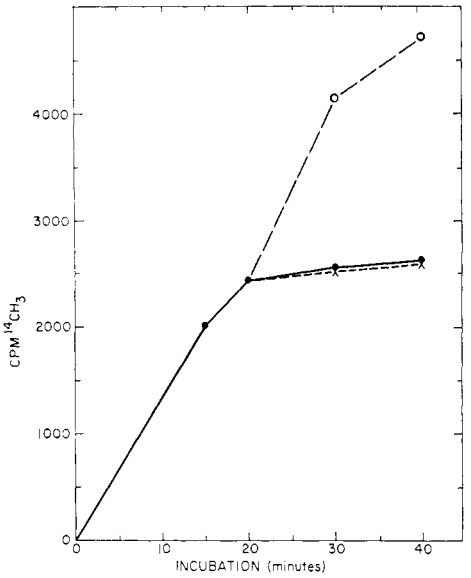

FIGURE 2: Effect of additional enzyme and sRNA on extent of methylation of *E. coli* B sRNA. Incubation was started at zero time in the presence of 2 μg of sRNA and 1 mg of protein from an EDTA-treated ammonium sulfate fraction. After 20 min, additions were made of 1 mg of enzyme protein (×- - -×) or 2 μg of sRNA (O- - -O). Reaction was carried out in the presence of 30 mM putrescine.

criteria, little or no loss of a specific methylase was detected during the earlier steps. DEAE-Sephadex chromatography frequently resulted in about 10% decrease in extent of methylation and significant diminution of cytosine methylase activity.

Methylase Assays. The standard incubation mixture for determining enzyme specific activity consisted of 0.03 M triethanolamine buffer (pH 8.9), 4 mM mercaptoethanol, 21 μM [*meinyl*-^{14}C]-*S*-Adenosylmethionine, 50 μg of sRNA, and 100–200 μg of protein from the enzyme extract in a final volume of 125 μl. After incubation at 37° for 30 min, the tubes were immersed in ice and the RNA was precipitated with approximately 5 ml of cold 5% trichloroacetic acid. The samples were collected on membrane filters (MF Millipore, 1.2 μ pore size), washed three times with 2% trichloroacetic acid, dried, and the radioactivity was measured in a Packard liquid scintillation spectrometer. In each experiment, control mixtures lacking sRNA were included and the values obtained, representing methylation of protein, were subtracted from the values found in the experimental assays. Using the standard conditions described, incorporation of [^{14}C]CH_3 into RNA proceeded linearly for approximately 40 min (Figure 1A), and the methylation of RNA was proportional to the amount of enzyme extract added (Figure 1B).

Measurement of the extent of methylation was carried out as previously described (Kaye and Leboy, 1968). sRNA was added at 2–4 μg/250 μl of reaction mixture and sufficient enzyme extract was added to ensure that the enzyme concentration was not rate limiting. When using crude extracts of ammonium sulfate fractions, 0.8–1.0 mg of protein/250 μl was added; with the more highly purified DEAE-Sephadex eluate, 50–100 μg of protein was sufficient. Under these conditions, [^{14}C]CH_3 incorporation was a function of RNA concentration (Figure 2).

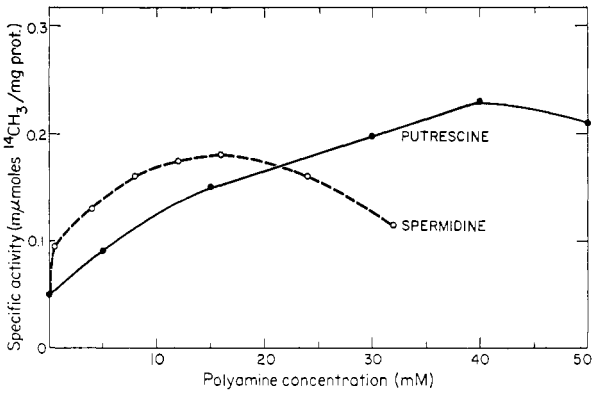

FIGURE 3: Polyamine stimulation of sRNA methylase activity in a dialyzed crude rat liver extract.

Results and Discussion

The Effect of Polyamines on Crude Organ Extracts. The effect of spermidine and putrescine (1,4-diaminobutane) on the specific activity of sRNA methylases in dialyzed rat liver extracts is shown in Figure 3. Putrescine, at optimal concentrations of 30–40 mM, resulted in elevation of methylase activity to levels comparable with those observed with 0.36 M ammonium acetate. Addition of spermidine to the reaction mixture at a final concentration of 10 mM usually resulted in specific activity values slightly lower than those observed with putrescine. Attempts to equal the level of activity seen with putrescine resulted in a decline in activity which coincided with the formation of a visible precipitate. The polyamine spermine, while also showing stimulation of methylation, formed a precipitate at even lower concentrations; 0.6 mM spermine produced stimulation to approximately 60% of the levels found with putrescine, and higher concentrations caused precipitation. The observed precipitation is presumably due to formation of insoluble complexes of the polyamines with both added substrate sRNA and nucleic acid present in the crude high-speed supernatant extracts. Such complexes are known to form with polyamines, but not with the diamine putrescine (Razin and Rozansky, 1959).

A survey of methylase activity in dialyzed crude extracts from various rat organs was carried out in the presence of 30 mM putrescine, 10 mM spermidine, or 0.36 M ammonium acetate. In all cases, activity five- to ninefold higher than that found with no added ions was observed (Table II). Proof that the radioactive label in sRNA was due to methylation rather than to possible formation of aminoacylated sRNA was obtained by several types of evidence. (1) Incubations were carried out in the presence of a mixture of 18 unlabeled amino acids (0.1 μmole of each amino acid/ml). (2) The methylation of stripped and nonstripped *E. coli* B sRNA were compared. (3) After incubation, Tris buffer (pH 10) was added to a final concentration of 0.1 M and the tubes were placed at 37° for an additional 15 min. None of these procedures affected the incorporation of radioactivity into sRNA. In addition, alkaline hydrolysis of the sRNA substrate and electrophoresis of the nucleotides indicated that a majority of the counts were present in the four nucleotides.

Kerr (1969) has recently reported the presence of inhibitors in adult organ methylase extracts, and has attributed the

TABLE II: Rate of Methylation of *E. coli* sRNA by Dialyzed High-Speed Supernatant Extracts.[a]

Organ	Specific Activity (mμmoles of CH_3 Incorporated/mg of Protein per 30 min)			
	No Additions	Spermidine (10 mM)	Putrescine (30 mM)	Ammonium Acetate (0.36 M)
Liver	0.03	0.16	0.19	0.18
Lung	0.03	0.15	0.18	0.17
Spleen	0.04	0.32	0.35	0.35
Thymus	0.06	0.56	0.60	0.59

[a] All values represent averages of determinations performed on at least three preparations of organ extracts from female Osborne–Mendel rats.

TABLE III: Addition of EDTA to Methylase Reaction Mixture.

Ammonium Sulfate Fraction	EDTA (mM)	Methylase Activity[a]	
		No Additions	+30 mM Putrescine
Preparation 1[b]		15	62
	0.1	15	67
	0.3	5	72
	0.5	<1	88
	1.0	<1	92
Preparation 2[b]		14	72
	0.1	7	81
	0.3	<1	93
	0.5	<1	93

[a] μμmoles of [^{14}C]CH_3 incorporated/30 min per 200 μg of enzyme protein; 1 μμmole of CH_3 was equivalent to 80 cpm. [b] Preparation 1 was derived from rat liver homogenized in extraction buffer containing 0.01 M $MgCl_2$ (Kaye and Leboy, 1968). Homogenizing buffer used for preparation 2 contained no Mg.

effect of ammonium ion to a partial inactivation of these inhibitors. In order to explore the possibility that the stimulatory effects of polyamines might be due to their interaction with an inhibitor present in crude preparations, the influence of polyamines on the activity of partially purified methylase extracts was examined.

Effect of Polyamines on Ammonium Sulfate Fractions. A doubling of the specific activity was achieved by selecting that fraction of crude liver extract which was precipitated between 30 and 48% saturated ammonium sulfate. This preparation, after dialysis to remove ammonium ion, showed a degree of stimulation by polyamines similar to that observed with the unfractionated crude extract. The specific activity ranged from 0.06 to 0.08 mμmole of CH_3 incorporated per mg of enzyme protein in the absence of added ions, and 0.35 to 0.37 mμmole per mg of protein in the presence of 30 mM putrescine.

The addition of EDTA to the reaction mixture at a final concentration of 0.5 mM had a marked effect on the methylation reaction in the absence of polyamines; no significant incorporation of CH_3 groups into sRNA could be detected under these conditions. However, in the presence of 30 mM putrescine a stimulatory effect of EDTA was noted (Table III). These observations suggested that the enzyme extract might contain a divalent cation which could be chelated by EDTA, thereby altering the ionic environment. Therefore, enzyme preparations were diluted with buffer containing 1 mM EDTA, the EDTA was removed, and these EDTA-washed preparations were tested for their methylase activity in the presence and absence of added putrescine. The results observed were identical with those obtained by addition of EDTA to the reaction mixture; no detectable methylase activity was found in the absence of added ions and the activity of the extract in the presence of putrescine was higher than that found prior to EDTA treatment (Table IV). Using an EDTA-washed enzyme fraction, no further effect of EDTA in the reaction mixture was detectable.

Sheid *et al.* (1968) have reported that the presence of 2×10^{-4} M EDTA during preparation of liver homogenates for DNA methylase activity is essential in order to avoid inhibitory effects of excess enzyme. However, unlike the sRNA methylase system, the addition of EDTA to the reaction mixture did not affect the activity of DNA methylase extracts.

When varying concentrations of spermine, spermidine, and putrescine were added to methylase assay tubes containing EDTA-washed ammonium sulfate fractions and excess sRNA substrate, curves similar to those obtained with the crude extract resulted (Figure 4A). Putrescine showed optimal stimulation at 30–50 mM concentrations. Spermidine and spermine, in the presence of 20 or 50 μg of sRNA substrate, caused increased specific activity values until polyamine was added in high enough concentration to cause precipitation.

Measurements of the maximal extent of methylation permitted by methylase enzymes are carried out in the presence of small amounts of sRNA and an excess of enzyme such that sRNA is rate limiting in the assay. When ammonium sulfate fractions are used, even large amounts of extract contribute little nucleic acid. Therefore, under these conditions, no detectable precipitate forms upon addition of spermine or spermidine at concentrations which cause precipitation at higher RNA concentrations. The effect of putrescine, spermidine, and spermine on extent of methylation attained with an EDTA-washed ammonium sulfate fraction is shown in Figure 4B. The concentrations of amines necessary for optimal stimulation were significantly lower than had been suggested by experiments carried out at the higher RNA levels used to measure specific activity. In the presence of 0.5 mM spermidine or 10 mM putrescine, 20–21 mμmoles of CH_3 was incorporated per mg of methyl-deficient sRNA; at 0.05–0.1 mM spermine the extent of methylation was 18–19 mμmoles of methyl/mg of sRNA.

The distribution of [^{14}C]methyl in nucleotides of substrate RNA was investigated by alkaline hydrolysis and high-voltage electrophoresis. Unlike the results obtained in the presence of 0.36 M NH_4^+ (Kaye and Leboy, 1968) no marked elevation of methylated cytidylic acid compounds was observed after incubation with polyamines. The pattern of methylation,

TABLE IV: Washing of Enzyme Extracts in Buffer Containing 1 mM EDTA.

Enzyme Extract	Amount of Enzyme in Reaction Mixture (μg of Protein)	EDTA Exposure	Methylase Activity (μμmoles of CH_3 Incorporated/30 min)[a]	
			No Additions	+30 mM Putrescine
Ammonium sulfate fraction	200	−	15	62
Ammonium sulfate fraction	200	+	<1	104
Ammonium sulfate fraction	200	+	<1[b]	104[b]
DEAE peak	20	+	<1	107

[a] A value of <1 indicates less than 80 cpm incorporated into sRNA. [b] Incubation mixture contained 0.3 mM EDTA.

as viewed by this technique, indicated no gross differences between RNA samples methylated in the presence of the various polyamines. However, this procedure would not necessarily detect differences in specific methylated bases; therefore, patterns of methylation are being further investigated after hydrolysis of substrate RNA to the nucleoside level.

Polyamine Effects on DEAE-Sephadex Eluates. The methylase preparations eluting from a DEAE-Sephadex column between 0.17 and 0.27 M NaCl had specific activities 25 to 35 times those obtained with crude high-speed supernatants from rat liver. These eluates showed negligible protein methylase activity at up to 200 μg of enzyme protein/assay tube. Their response to addition of polyamines was similar to that observed with ammonium sulfate fractions (Figure 4A), and optimal concentrations for measuring extent of methylation were identical with those previously obtained with less purified extract (Figure 4B). The extents of methylation attained at optimal amine concentrations were slightly lower than obtained using a less purified fraction, suggesting the loss of one or more specific methylase. These preparations, eluted in buffer containing 1 mM EDTA (see Methods), showed no detectable methylase activity in the absence of added ions (Table IV and Figure 4).

The observation that stimulation of activity by added polyamines persists during several stages of purification suggests that the effect of positively charged groups is not solely one of inactivating inhibitors which may be present. The possibility that polyamines might be acting to prevent degradation of the substrate was further investigated by preincubating enzyme preparations with sRNA before addition of polyamines. The standard reaction mixture for determining extent of methylation in the absence of added ions was incubated for 20 min, after which putrescine was added and the incubation continued for an additional 20 min. The extent of methylation obtained was identical with that found without preincubation, indicating no significant nuclease activity. Therefore, the sRNA methylation reaction appears to have a requirement for polyamine, or ions which act in a similar fashion.

Influence of Mg. Polyamines can partially replace Mg^{2+} in the synthesis of bacterial RNA and the association of 30S and 50S ribosomal subunits (Cohen and Raina, 1967). The reported relationship between the effects of Mg ions and those of polyamines, taken in conjunction with the effect of EDTA on methylase enzyme extracts, suggested that the methylase preparations might have contained Mg^{2+} bound to the enzyme and that these Mg ions might permit a low level of methylation. Therefore, magnesium acetate in varying concentrations was added to *in vitro* methylation systems containing either the EDTA-washed ammonium sulfate fraction or the untreated ammonium sulfate fraction. In the absence of other ions, addition of magnesium acetate at 2.5–12.5 mM resulted in limited stimulation of methylase activity (Figure 5, closed circles). The addition of increasing levels of magnesium salt to a reaction mixture containing 30 mM putrescine caused inhibition of methylation (Figure 5, open circles). Under conditions where extent of methylation was measured, magnesium acetate, over a concentration range of 0.5–30 mM, again showed limited ability to stimulate methylation. At the apparent optimum of 10 mM magnesium, the extent of methylation was less than 30% of that reached with added amines (Table V).

In view of these observations, it seems likely that EDTA treatment elevated putrescine-stimulated methylation levels and reduced activity in the absence of added amines by

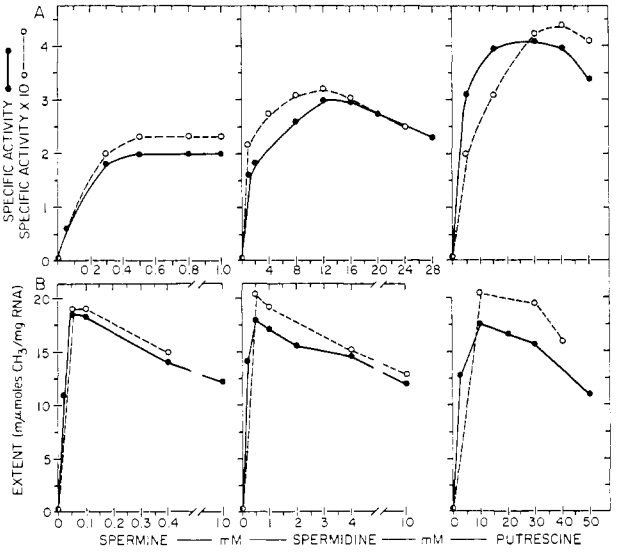

FIGURE 4: Effect of polyamines on partially purified rat liver methylase extracts: A, specific activity; B, extent of methylation; (○---○) EDTA-treated ammonium sulfate fraction (●---●) DEAE-Sephadex peak.

TABLE V: Extent of Methylation of RNA Substrates.[a]

Substrate	mμmoles of CH_3 Incorporated/mg of RNA				
	No Additions	Magnesium Acetate (10 mM)	Spermine (0.08 mM)	Spermidine (0.5 mM)	Putrescine (10 mM)
Rat liver sRNA	<0.4	<0.4	<0.4	<0.4	<0.4
E. coli K12 methyl-deficient sRNA		5.3	18.6	20.6	20.2
E. coli K12 normal sRNA		2.8	8.7	9.4	9.6

[a] Each reaction tube contained 4 μg of sRNA and EDTA-washed ammonium sulfate extract equivalent to 1 mg of protein in a final volume of 0.25 ml. [*methyl*-^{14}C]-*S*-Adenosylmethionine had an activity of 65 cpm/μμmole; therefore <0.4 mμmole of CH_3/mg of RNA indicates less than 100 cpm incorporated/4 μg of sRNA.

removing magnesium bound to the enzyme preparation. Indeed, when extracts were prepared in homogenizing buffer from which $MgCl_2$ had been omitted, lower concentrations of EDTA were required to affect the methylase activity (Table III, preparation 2). However, a significant amount of cation still appeared to be bound to the enzyme preparation. This suggested the possibility that the methylating enzymes *in vivo* might contain bound Mg^{2+}, and that the sRNA might normally be methylated just to the extent permitted by magnesium. If this were the case, the activity of the amines would represent overmethylation of the RNA substrate. Therefore, rat liver sRNA was prepared and the effect of added polyamines on methylation of this substrate was examined. Even in the presence of excess sRNA, spermine, spermidine, and putrescine caused no detectable methylation of rat liver sRNA by the homologous methylase preparations (Tables V and VI).

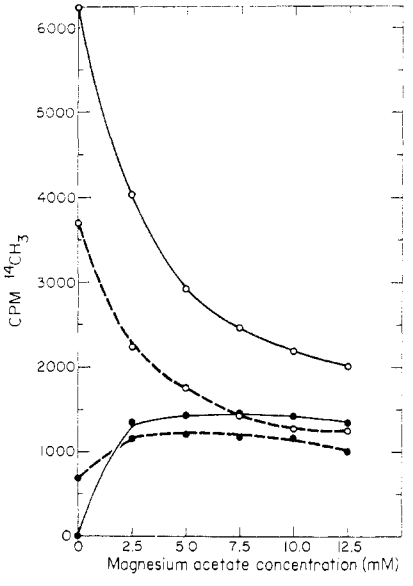

FIGURE 5: Effect of magnesium acetate on [^{14}C]methyl incorporation into *E. coli* B sRNA. Aliquots containing 200 μg of protein from an EDTA-treated ammonium sulfate fraction (——) or an untreated ammonium sulfate fraction (- - - -) were added to reaction mixtures containing 30 mM putrescine (○) or no added polyamine (●).

The results obtained using rat liver enzyme preparations and homologous sRNA as substrate indicate that fidelity of the methylation reaction is preserved in the presence of polyamines. One may therefore conclude that the sRNA methylation occurring *in vivo* has the characteristics obtained in the *in vitro* system by addition of amines, rather than those found by addition of magnesium, or omission of ions entirely. The observation that methylase preparations retain some activity after dialysis, and that EDTA treatment of the enzyme is necessary before an absolute ion requirement may be seen, suggests that stimulatory ions interact with the methylating enzymes rather than with the RNA. However, the possibility that polyamines may be affecting the methylation reaction by an influence on the substrate RNA cannot at present be excluded.

Although NH_4^+ or K^+ stimulate methylases *in vitro* to levels similar to those obtained with polyamines (Kaye and Leboy, 1968), the high concentrations required make it unlikely that any one of these ions serves to elevate methylase activity *in vivo*. It is conceivable that the activation of cellular methylases may be accomplished by several different monovalent and divalent cations acting cooperatively. However, a simpler hypothesis is that cellular polyamines stimulate sRNA methylases.

TABLE VI: Specific Activity Using Various RNA Substrates.[a]

Substrate	μμmoles of CH_3 Incorporated/30 min per 100 μg of Protein		
	No Additions	Spermidine (10 mM)	Putrescine (30 mM)
Rat liver sRNA	<1[b]	<1	<1
E. coli K12 methyl-deficient sRNA	<1	42	52
E. coli B sRNA	<1	32	40

[a] The enzyme was an EDTA-washed ammonium sulfate fraction. [b] A value of <1 indicates less than 80 cpm incorporated.

The diamine putrescine is found in low concentrations in animal tissues (Tabor and Tabor, 1964), although it is thought to be an intermediate in the formation of spermidine in mammals (Pegg and Williams-Ashman, 1969). The triamine spermidine and the tetraamine spermine have been shown to be widely distributed in animal tissues (Rosenthal and Tabor, 1956). The polyamine content of adult rat liver has been determined in several laboratories, with reports indicating spermine present at 0.5–1.1 μmoles/g wet weight of liver, and similar values for spermidine (Tabor and Tabor, 1964; Neish and Key, 1967; Jänne *et al.*, 1964; Dykstra and Herbst, 1965). Therefore, the spermine and spermidine concentrations used to obtain maximal extents of methylation fall well within the range of reported physiological concentrations of these compounds.

Recent investigations have indicated that intracellular polyamine concentrations vary under altered growth conditions. By comparison with values obtained for adult rat liver, the spermidine:spermine ratio is elevated in newborn animals (Rosenthal and Tabor, 1956; Jänne *et al.*, 1964), regenerating liver (Dykstra and Herbst, 1965), and animals with transplantable tumors (Neish and Key, 1967). Further evidence for changes in polyamine content has been obtained by comparing rapidly growing tissues with respect to ornithine decarboxylase levels, since this enzyme leads to the formation of putrescine and spermidine (Russell and Snyder, 1968).

Several of those growth conditions which alter cellular polyamine content have also been reported to influence the methylation of sRNA. Tumor cell RNA possesses a high degree of methylation relative to that found in analyses of nonneoplastic cell RNA (Berquist and Matthews, 1962; Burdon, 1966). Increased specific activity has been found using crude methylase extracts from tumors (Hancock, 1967; McFarlane and Shaw, 1967; Baguley and Staehelin, 1968) and from newborn animals (Hancock *et al.*, 1967; Kaye and Leboy, 1968). Several laboratories have also reported increased extents of methylation and altered patterns of methylated bases when undialyzed tumor extracts have been used as the source of sRNA methylases (Tsutsui *et al.*, 1966; Mittelman *et al.*, 1967; McFarlane and Shaw, 1967; Gantt and Evans, 1969). In contrast to these reports, experiments carried out with dialyzed tumor extracts and optimal concentrations of ammonium ion have indicated that under these conditions tumors and normal organ extracts produce similar extents and patterns of methylation (Kaye and Leboy, 1968; Baguley and Staehelin, 1968). The present observations suggest the possibility that variations in cellular polyamine content may influence the extent and/or pattern of methylation of tRNA, and that results obtained using undialyzed crude extracts may reflect differences in polyamine levels in homogenates from different sources. Therefore, sRNA methylase activity of rapidly growing tissues is being reexamined to determine the effect of added polyamines, and patterns of methylation produced in the presence of various polyamines are under investigation.

The effect of polyamines on the activity of sRNA methylases adds another item to a growing list of reactions influenced by these molecules. Polyamines have been reported to affect rRNA synthesis in sea urchins (Barros and Guidice, 1968), *in vitro* RNA polymerase activity (Abraham, 1968; Petersen *et al.*, 1968), and binding of aminoacyl-tRNA to ribosomes (Tanner, 1967; Takeda, 1969). These observations, when correlated with the reported alterations in polyamine levels under varying growth conditions, make it increasingly plausible that polyamines participate in the regulation of macromolecular syntheses.

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Exonuclease (Phosphodiesterase) from the Testes of Chinook Salmon (*Oncorhynchus tschawytscha*)*

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ABSTRACT: An exonuclease, present in the cytoplasmic fraction of partially mature *Oncorhynchus tschawytscha* testes, has been purified 1000-fold. The enzyme, which has optimal activity at pH 5.5, liberates nucleoside 3′-phosphates from the 5′ termini of oligonucleotides. No other enzymes capable of hydrolyzing phosphate esters were detectable in the purified exonuclease.

The stability of the enzyme and its minimal requirements suggest that it could be a useful tool in nucleic acid biochemistry.

As part of studies on the sexually maturing salmonid (Schmidt *et al.*, 1965; Ingles *et al.*, 1966; Dixon and Smith, 1968; Wilson and Smith, 1968) we are investigating the relationship of the enzymes of nucleic acid metabolism to spermatogenesis. This report is concerned with the purification and catalytic properties of an exonuclease which acts on the 5′-hydroxyl termini of oligonucleotides to release nucleoside 3′-phosphates. The enzyme is therefore analogous in its action to spleen phosphodiesterase and can be classified as a phosphodiesterase of type II (Razzell, 1967). The exonuclease activity is found in association with all of the subcellular fractions obtained by differential centrifugation of disrupted testis cells from *Oncorhynchus tschawytscha*. The enzyme described here was isolated from the soluble fraction. It shows some minor differences from spleen phosphodiesterase in its chromatographic properties and requirements for optimum activity. It is like the spleen enzyme in its ability to synthesize larger oligonucleotides from a dinucleoside phosphate at high substrate concentration.

Materials and Methods

Collection of Testes. Chinook salmon (*Oncorhynchus tschawytscha*) were caught in June and July during their spawning migration along the Fraser River, British Columbia. The fish had an average weight of 6000 g with testes approximately 2% of the body weight. Histological examination (Robertson, 1958) indicated that spermatogonia and spermatocytes were the predominant cell types. The testes were frozen immediately in solid carbon dioxide and were subsequently stored at −80°.

Enzyme Substrates. Thymidine 3′-*p*-nitrophenyl phosphate was synthesized using the procedure of Borden and Smith (1966). Both this substrate and thymidine 5′-*p*-nitrophenyl phosphate are available from Raylo Chemicals Limited, Edmonton, Alberta. Thymidine 3′- and thymidine 5′-2,4-dinitrophenyl phosphates were synthesized by the procedure of von Tigerstrom and Smith (1969). Thymidine 3′-phenyl phosphate was synthesized by reaction of 5′-*O*-monomethoxytritylthymidine with phenyl phosphorodichloridate in pyridine (R. G. von Tigerstrom and M. Smith, unpublished procedure). Thymidine 3′- and thymidine 5′-phosphorofluoridates were synthesized using the procedure of Witmann (1963) except that the nucleotides were purified by chromatography on DEAE-cellulose. The synthesis of dTpdT[1] was by the method of Gilham and Khorana (1958). Adenosine 2′,3′-cyclic phosphate and adenosine 3′,5′-cyclic phosphate were synthesized as described by Smith and Khorana (1963). The dinucleotide pApA was isolated from the embryos of *Euchaeta japonica* (Hepner and Smith, 1967). The oligonucleotides ApA, CpA, UpA, and ApApA were purchased from Miles Laboratories. Other nucleotides and polynucleotides were conventional commercial products.

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[1] Abbreviations used are: ApA, adenylyl-(5′→3′)-adenosine; pApA, adenylyl-(5′→3′)-adenosine 5′-phosphate; CpA, adenylyl-(5′→3′)-cytidine; UpA, adenylyl-(5′→3′)-uridine; ApApA, adenylyl-(5′→3′)-adenylyl-(5′→3′)-adenosine; dTpdT, deoxythymidylyl-(5′→3′)-deoxythymidine.