Studies on the Enzymatic Methylation of Soluble RNA. I. Methylation of the s-RNA Polymer*

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We have previously reported an enzyme activity in extracts of *E. coli* responsible for methylation of s-RNA at the polynucleotide level. The experimental basis of this conclusion is here reported in detail. *In vivo* experiments are described in which incorporation of labeled methyl groups into RNA takes place in the absence of RNA synthesis. Experiments with a soluble enzyme extract contribute three additional pieces of evidence for methylation of the RNA polymer. (1) Incorporation of labeled methyl groups from methionine into RNA bases shows a requirement for a specific methyl-deficient s-RNA as a substrate. (2) In the same system no label is incorporated into RNA from UDP-C^{14}. (3) The amount of methyl group incorporation is proportional to the substrate RNA added over a 10-fold range. A procedure for the purification of the enzyme activity is presented: The purified enzyme fraction is virtually free of nucleic acid and transfers methyl groups from *S*-adenosyl-methionine to methyl-deficient RNA, forming the methylated bases which are typically present in transfer RNA. The methylation reaction has a *p*H optimum of 8 and a requirement for magnesium ions. The enzyme activity is protected by sulfhydryl compounds. Certain observations on the possible roles of methylated bases in transfer RNA are presented.

Soluble RNA[1] is characterized by the presence of methylated purines and pyrimidines. These minor bases have been found in the soluble RNA fraction of many species, but their origin has until recently been obscure. It has been shown in our laboratory that in *E. coli* the direct precursor of the methyl groups in these compounds is the methyl group of methionine (Mandel and Borek, 1961b) and that a soluble enzyme exists in *E. coli* extracts which catalyzes the transfer of methyl groups from methionine to the intact s-RNA molecule (Borek *et al.*, 1962; Fleissner and Borek, 1962). The RNA substrate which was used for this demonstration is a unique, soluble (4 *S*) RNA which accumulates in the mutant *E. coli* K-12 W-6 when it is starved of its essential nutrient, methionine (Borek *et al.*, 1955). It has been demonstrated in this laboratory that the soluble RNA synthesized by this mutant under conditions of methionine starvation lacks the normal methylated bases (Mandel and Borek, 1961a). The methylation reaction is apparently a finishing touch in the normal course of synthesis of s-RNA. In this paper we will present additional evidence that the methyl receptor is macromolecular RNA and show that the direct methylating agent is *S*-adenosyl-methionine. Certain characteristics of a partially purified enzyme system will be described. Confirmation of the methylation of methyl-deficient s-RNA has been published from the laboratories of Boman (Svensson *et al.*, 1963) and Starr (1963a).

MATERIALS AND METHODS

E. coli strains K-12 W-6 and K-12 S were grown on a mineral salts and glucose medium (Gray and Tatum, 1944), with DL-methionine added at a concentration of 30 μg per ml for the methionine-requiring W-6 strain. The organism was starved of methionine as previously described (Borek *et al.*, 1955), the usual period of starvation being 3 hours. The accumulation of RNA during starvation was checked by measuring the increment in optical density at 260 mμ in an aliquot of washed cells disintegrated by sonic vibration, or by the orcinol color reaction (Mejbaum, 1939) on a hot TCA extract of cells previously washed with cold TCA. The relative increment in RNA for a given number of cells is easily calculated, since the viable count does not change during methionine deprivation (Borek *et al.*, 1955). Ordinarily RNA increments of 60–80% were observed during a 3-hour starvation.

For the simultaneous labeling with $P^{32}O_4$ and methionine-methyl-C^{14} *E. coli* K-12 W-6 was grown in 1-liter lots to logarithmic growth phase and the cells were harvested when the population reached 5×10^8 cells/ml. The cells were washed free of methionine and divided into two parts, one to serve as a control culture and the other to undergo methionine starvation. The control culture was resuspended immediately in normal salts-glucose medium supplemented with L-methionine-methyl-C^{14} at a concentration of 25 μc/500 ml of a specific activity of 1.4 μc/μmole plus 0.5 mc of $P^{32}O_4$/500 ml. These cells were allowed to resume logarithmic growth for a specified time, were then chilled in an ice bath, and were harvested by centrifugation. The other culture was washed again to remove all traces of methionine and then allowed to starve of methionine in a salts-glucose medium for 3 hours. The starved culture was centrifuged and resuspended in the same radioactive medium to which the control culture had been exposed. The cells were allowed to "recover" in this medium for the same period of time as the duration of the exposure of the control culture to the same medium, and were then harvested.

RNA was extracted from both cell samples by the procedure of Littlefield and Dunn (1958). The final RNA-DNA precipitate was dialyzed against 0.01 M sodium phosphate buffer, *p*H 6.8, and against distilled water. After lyophilization the residue was hydrolyzed in 2 ml of 0.3 N KOH at 37° for 16 hours. The DNA

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[1] The following abbrevations are used in this paper: RNA, ribonucleic acid; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate; UDP, uridine diphosphate; PEP, phosphoenolpyruvate; GSH, glutathione.

and protein were precipitated by acidifying with $HClO_4$ and adding two volumes of ethanol. After centrifugation the supernatant fluid was neutralized with KOH and the precipitate of $KClO_4$ was removed. The solution of RNA nucleotides was concentrated to dryness under nitrogen and the residue was redissolved in 1 ml of water. After cooling in an ice bath and centrifugation to remove insoluble material, the solution was again concentrated under nitrogen and was chromatographed as a band in solvent system A according to the method of Littlefield and Dunn. The regions of the major nucleotides and the area up to, but not including, the band of nucleosides were eluted, and aliquots were removed for determination of C^{14}-to-P^{32} ratios in the RNA. This was done by counting the planchets in a GM end-window counter with and without a shield consisting of two layers of Parafilm over the end of the GM tube. This shielding reduced the counts due to P^{32} by 35%, and completely eliminated counts due to C^{14}. By appropriate calculation the activities of C^{14} and P^{32} in each sample could be estimated.

For the isolation of ribothymidylic acid from the nucleotide mixture two-dimensional chromatography in solvent systems B and C was used (Price *et al.*, 1963). In this chromatography the best results were obtained when traces of salts had been removed by adsorbing the nucleotides on Norit; this was especially true if the chromatography in solvent A were omitted. The removal of salts as described by Tsuboi and Price (1959) consisted of adsorbing the nucleotides to Norit (100 mg for 3 mg RNA nucleotides) from a solution of 5% $HClO_4$ in the cold. The charcoal was collected on a Celite filter and was washed with cold water. Elution of the nucleotides was effected with a solution of ethanol, water, and ammonia (60:38:2) at 37°. The eluate could be concentrated and applied directly to the filter paper (Whatman 3 MM paper). Best results were obtained with ascending chromatography for 24 hours in solvent B, followed by 48 hours descending in solvent C.

C^{14}-to-P^{32} ratios in the isolated ribothymidylic acid were obtained using a Packard Tri-Carb scintillation counter, by counting the two isotopes sequentially at different voltage settings. The nucleotides could be dissolved in a three-component system of toluene, ethanol, and water (30:12.5:1).

Substrate RNA for the *in vitro* methylation experiments was prepared from *E. coli* K-12 W-6 after 3 hours of methionine starvation. The washed cells were ground in the cold with twice their weight of wet alumina and extracted with a buffer containing 0.01 M Tris, *p*H 8.0, and 0.01 M $MgCl_2$ and pancreatic deoxyribonuclease (5 μg/ml). Where large quantities of cells were extracted, they could be disrupted in a Waring Blendor with glass beads (Chamberlin and Berg, 1962). The extract was centrifuged at 20,000 $\times$ *g* for 20 minutes and at 100,000 $\times$ *g* for 3 hours. The clear supernatant liquid was shaken with an equal volume of water-saturated, redistilled phenol for 15 minutes at room temperature, and the emulsion was broken by brief centrifugation. The upper, aqueous layer was removed, NaCl was added to 0.1 M, and the solution was mixed with two volumes of cold ethanol to precipitate the soluble RNA. The precipitate was dissolved in a small volume of 0.01 M Tris, *p*H 7.8, and dialyzed against the same buffer and against water to remove traces of phenol. The dialyzed solution was lyophilized, the RNA was redissolved in water to a concentration of 20 mg/ml, and the solution was stored frozen. The ratio of optical density at 260 mμ to that at 280 mμ was almost exactly 2 in these solutions. These RNA preparations did not lose activity in the enzyme systems after a dozen or more cycles of freezing and thawing. The RNA displayed a single, symmetrical 4 *S* peak in a sucrose gradient centrifugation.

For the preparation of enzyme extracts cells were treated as above, including the centrifugation at 100,000 $\times$ *g* (for only 2 hours in this case), but in the early experiments the extracting buffer contained 0.2 M $MgCl_2$ and 0.015 M GSH in addition to the usual Tris and deoxyribonuclease. The high Mg^{++} concentration and the GSH were added to provide optimal conditions for the methionine-activating enzyme in the incubations, with the assumption that the enzyme had characteristics similar to the enzyme from rabbit liver studied by Cantoni (1955). In later experiments, where the assay mixture contained *S*-adenosyl-methionine, the enzyme extracts were prepared with a buffer containing 0.01 M Tris, *p*H 8, 0.01 M $MgCl_2$, and 0.005 M β-mercaptoethanol ("standard buffer"). Deoxyribonuclease was added at 5 μg/ml during extraction.

The assay procedure generally involved incubation of the methyl-deficient soluble RNA with a radioactive methyl donor in the presence of the enzyme. "Standard buffer" at *p*H 8 was generally used for incubations, since this *p*H had been shown to be optimal (cf. Results) for methyl group transfer. Thus a typical incubation mixture would contain 0.01 μmole of *S*-adenosyl-methionine-methyl-C^{14}, 0.5 mg of s-RNA, and 2 mg of "enzyme" protein with standard buffer in a final volume of 1 ml. In the earliest experiments, when methionine-methyl-C^{14} was used, an ATP-generating system was included, and in place of adenosyl-methionine the incubation mixture contained 3 μmoles of phosphoenolpyruvate, 4 μg of pyruvate kinase, 0.4 μmole ATP, and 0.04 μmole methionine-methyl-C^{14}. Incubations were usually performed for 30 minutes at 30°, after which the samples were cooled in an ice bath and 0.5 ml of 20% TCA was added to precipitate RNA and protein. The acid-insoluble fraction was washed in one of two ways, depending on what the incubation mixture had contained. If the incubation contained C^{14}-adenosyl-methionine and a more purified enzyme fraction, the suspended TCA precipitate was poured onto a millipore filter (1.2 μ) and the precipitate was washed with three 5-ml portions of cold 5% TCA. If the incubation contained methionine-C^{14} and a crude enzyme preparation, a more extensive washing procedure was employed, adapted from a procedure used by Kornberg to assay DNA-glucosyl-transferase of bacteriophage-infected *E. coli* (Kornberg *et al.*, 1961). Prior to the initial precipitation with 20% TCA, 0.5 ml of 3 M NH_2OH-HCl at *p*H 7 was added and incubation at 30° was continued for 5 minutes. The samples were then cooled and 0.25 ml of 6 N HCl was added, followed by 0.7 ml of 20% TCA. After precipitation was completed, 7.5 ml of cold water was added, and the precipitate was centrifuged. The precipitate was suspended in 1 ml of 0.2 M Tris, *p*H 10, yielding a final *p*H of 9–9.5. The suspension was heated to 50° for 10 minutes, then cooled, and 0.05 ml of 6 N HCl plus 0.4 ml of 20% TCA was added. After a delay of several minutes to complete the precipitation, 4.5 ml of cold water was added and the suspension was centrifuged. The precipitate was washed with 10 ml of 66% ethanol and was then suspended in 1 ml of 2 N NH_4OH at 50° for 10 minutes. This suspension was centrifuged and the supernatant fluid was poured directly into stainless steel planchets, which were then dried. In the simpler washing procedure using the millipore filters, the filter disks were mounted in steel planchets with rubber cement before counting.

When isolation of ribothymidylic acid or ribothymidine

TABLE I

C^{14}/P^{32} RATIOS IN RNA NUCLEOTIDES

In vivo labeling of logarithmic and methionine-starved *E. coli* K-12 W-6. For details see text.

Exposure to Label (min)	Origin of RNA Sample	C^{14} (cpm)	P^{32} (cpm)	C^{14}/P^{32}	C^{14}/P^{32} in Starved Cells / C^{14}/P^{32} in Log Cells
15	Starved cells	390	520	0.76	7.9
	Log cells	270	2900	0.097	
30	Starved cells	660	1200	0.54	7.3
	Log cells	415	5600	0.076	

was intended, the ethanol-washed TCA precipitate was incubated with 0.3 N KOH for 16 hours at 37°. $HClO_4$ was then added to the chilled solution to give a net excess of 5% of the acid. The precipitate was centrifuged and washed with 5% $HClO_4$. The pooled extract and washings were treated with Norit, and ribothymidylic acid was isolated as already described. To obtain ribothymidine the eluate of the nucleotides from the charcoal was concentrated to dryness under a stream of nitrogen. The residue was dissolved in 0.1 N sodium acetate, *p*H 5, and to this solution was added one-tenth of its volume of 2% solution of crude prostatic monoesterase (Markham and Smith, 1952b). The nucleotides were incubated with the phosphatase for 6 hours at 37°. At the end of the incubation, the whole incubation mixture was concentrated and chromatographed. Two mg of nucleosides were applied in a volume of 0.1 ml to a spot 1 cm in diameter on Whatman No. 1 paper. The nucleosides were chromatographed, first in solvent D for 40 hours and then for 20 hours in solvent E, both descending (Littlefield and Dunn, 1958). The clearly resolved ribothymidine spot was identified by its mobility and by its spectral characteristics (Fox *et al.*, 1956).

The first step in removing indigenous RNA from the enzyme extracts was to adjust the *p*H to 5 at 10° with 1 N acetic acid. The precipitate was removed by centrifugation (at 10,000 × *g* for 10 minutes). To the supernatant fluid was added 0.5 ml of 2% protamine for each 100 ml of enzyme solution (containing 750 mg of protein). The solution was stirred in the cold for 20 minutes, and the precipitate was centrifuged off. The supernatant solution, still at *p*H 5, was now fractionated with ammonium sulfate. First, 21 g was added for each 100 ml of enzyme extract, and the solution was stirred for 20 minutes in the cold. The suspension was then centrifuged and the precipitate discarded. Next, 13 g was added for each 100 ml of solution, and the precipitate was collected as before and was saved. This precipitate, representing the fraction precipitating out between approximately 35% and 55% ammonium sulfate saturation, was taken up in one twenty-fifth of the original volume of standard buffer and was dialyzed for 6 hours against the same buffer. Alternatively, the solution of the ammonium sulfate precipitate was passed through a column of G-75 Sephadex equilibrated with standard buffer and the initial faintly yellow protein band was collected in a volume double that applied to the column. For the determination of the requirements of sulfhydryl group and Mg^{++} the Sephadex column was equilibrated with 0.01 M Tris-HCl, *p*H 8. After the ammonium sulfate precipitation, the inclusion of mercaptoethanol was found to be unnecessary if the enzyme preparation was kept concentrated, e.g, at 20 mg protein/ml. Under these conditions the enzyme activity was unchanged after 2 days of storage at 5°. If such preparations were diluted 20-fold and assayed at once, there was no requirement for mercaptoethanol in the assay. Nucleic acid content, as determined by the ratio of optical densities at 280 and 260 mμ (Warburg and Christian, 1941), was 2% in this fraction and 1% in the dialyzed enzyme preparation.

Solvent systems.—The following solvent systems were used: (A) 70% isopropanol with 7 ml of concentrated NH_4OH added separately in the bottom of a 20-liter tank (Markham and Smith, 1952a); (B) five parts isobutyric acid and 3 parts 0.5 M NH_4OH (Magasanik *et al.*, 1950); (C) tertiary amyl alcohol, 88% formic acid, and water, 6:1:3 (Hanes and Isherwood, 1949); (D) *n*-butanol, 88% formic acid, and water, 77:12:11 (Markham and Smith, 1949); (E) 86% *n*-butanol with 5% NH_4OH added to the solvent in the bottom of the tank (Markham and Smith, 1949).

Chemicals.—All chemicals not otherwise mentioned were reagent grade or of highest purity obtainable. Methionine-methyl-C^{14} was obtained from the California Corporation for Biochemical Research. *S*-Adenosyl-methionine-methyl-C^{14} was obtained from Tracerlab, Waltham, Mass. A sample of this compound was also obtained as a gift from Drs. J. D. Mann and G. L. Cantoni, and unlabeled adenosyl-methionine used to dilute the labeled compound in some experiments was a gift of Dr. J. H. Law. $P^{32}O_4$ was obtained from E. R. Squibb. PEP and pyruvate kinase were from the California Corporation for Biochemical Research and deoxyribonuclease was from Worthington Biochemical Corporation. Sephadex was from Pharmacia, Uppsala. UDP-C^{14} was from Schwarz Bioresearch, Inc.

RESULTS

In vivo Experiments.—During studies of the recovery of *E. coli* K-12 W-6 from methionine starvation, we had observed that re-exposure of the culture to methionine resulted in a rapid uptake of label from methionine-methyl-C^{14} into the RNA fraction during the first 45 minutes (Fleissner and Borek, unpublished experiments). Earlier observations (Borek and Ryan, 1958) had shown that in the same period there appeared to be little RNA synthesis. This suggested that the non-methylated species of s-RNA which had accumulated during methionine starvation (Mandel and Borek, 1961a, 1963) might now be receiving its normal complement of methyl groups by a methyl transfer directly to the polynucleotide. To test this hypothesis further, experiments were designed to measure methyl group incorporation and new nucleotide bond formation in the RNA-enriched organisms. Recovering cells (previously starved of methionine) were suspended in a medium containing both methionine-methyl-C^{14} and $P^{32}O_4$. Control cultures in logarithmic growth phase with similar density of cells were exposed to the same ratio of labeled compounds. It was postulated that, if the recovering cells were adding methyl groups to preformed RNA molecules, the ratio of $C^{14}H_3$ to $P^{32}O_4$ incorporation in the RNA of these cells would be much

TABLE II

C^{14}/P^{32} RATIOS IN RIBOTHYMIDYLATE

In vivo labeling of the minor base nucleotide isolated from RNA samples in Table I. For details see text.

Exposure to Label (min)	Origin of Ribothymidylate	C^{14} (cpm)	P^{32} (cpm)	C^{14}/P^{32}	$\frac{C^{14}/P^{32} \text{ in Starved Cells}}{C^{14}/P^{32} \text{ in Log Cells}}$
15	Starved cells	1110	0.4	2800	250
	Log cells	320	30	11	
30	Starved cells	1600	5	320	27
	Log cells	260	22	12	

higher than in the RNA of the control cells, where polynucleotide formation and methyl-group incorporation were going on simultaneously. The results of the experiments were in accord with this prediction (see Table I). A purified RNA preparation from recovering cells had seven to eight times as many atoms of C^{14} per atom of P^{32} as a similar RNA preparation from cells in logarithmic growth. The ratio was highest for the shortest period of exposure to label (15 minutes).

To ascertain that both labels were incorporated into a minor base nucleotide, ribothymidylic acid was isolated both from the organisms in logarithmic growth phase which had been exposed to the double labeling and from the recovering cells exposed to the same isotopic milieu. While to save space in the presentation of our data we focus on ribothymidylate synthesis in this paper, it should be clear that the other minor bases are also synthesized in a manner analogous to the methylation of uracil. Evidence for this is presented in the *in vitro* enzyme studies (see Fig. 3). It was anticipated that the difference between the $C^{14}H_3$-to-$P^{32}O_4$ ratio in the normal and recovering cells would be more marked in the isolated nucleotide than in the total RNA since in the recovering cells some of the $P^{32}O_4$ might have been incorporated into RNA fractions other than the transfer RNA. It is evident from the data in Table II that this prediction was borne out. After 15 minutes of exposure to the mixed label, the ribothymidylic acid isolated from recovering cells had 250 times as many atoms of C^{14} per atom of P^{32} as the same nucleotide from cells in logarithmic growth. When the period of incubation with the isotopes was 30 minutes, the number of C^{14} atoms per atom of P^{32} was 27 times higher in the recovering cells than in the logarithmic cells. It should be noted that the RNA was hydrolyzed in alkali, resulting in the transfer of the phosphate group to a different nucleotide from the one on which it had presumably entered the RNA molecule during polymerization. The conclusion, however, remains the same: in the recovering cells the methyl group entered a nucleotide which was part of a previously polymerized polynucleotide sequence. If the C^{14}-to-P^{32} ratios for cells in logarithmic growth in Table I are compared to the ratios in Table II, it may be seen that the ratios in Table I are 0.6–0.9% of those in Table II. Since the P^{32} should appear in both major and minor base nucleotides, while the C^{14} should be restricted to the methylated base nucleotides, these figures would show that 0.6–0.9% of total RNA bases are methylated. The lower of these values, obtained for the 30-minute labeling time, is in good agreement with the figure of 0.5% calculated from the direct analysis of the methylated base content of total RNA in *E. coli* and *A. aerogenes* (Littlefield and Dunn, 1958; Smith and Dunn, 1959; Dunn, 1960). Nihei and Cantoni (1962) and Lagerkvist and Berg (1962) have found that the great majority of the methylated bases appear to occur at positions removed from the ends of the transfer RNA strands. Thus, incorporation of a large number of preformed methylated bases would require new synthesis of the major portion of the transfer RNA molecules, yet such extensive RNA synthesis is ruled out by our experiments as well as by earlier work (Borek and Ryan, 1958). The sole mechanism which would apparently elude our hypothesis is that the RNA accumulated during methionine starvation was degraded and resynthesized during the recovery period and the nucleotides which had been in the accumulated RNA were reincorporated into new RNA. This would allow for methylation of the bases while they were in a monomeric, phosphorylated condition. To explore this possibility we labeled differentially the bases of the accumulated RNA and the bases of the nucleotide pool during recovery. These experiments indicate no equilibration of the nucleotide pool, at any level of phosphorylation, with the soluble RNA during the first 45 minutes of recovery (Fleissner and Mandel, unpublished results).

In vitro Experiments.—The results of the *in vivo* experiments encouraged us to look in a cell-free system for an enzymatic activity which would accomplish methylation of the s-RNA macromolecule. The natural choice of a substrate was the soluble RNA accumulated during methionine starvation by the methionine-requiring mutant. It was expected that the actual methyl donor would be *S*-adenosyl-methionine; therefore, in the crude extract incubations ATP and an ATP-generating system were included. In Materials and Methods components of typical enzyme incubations are given. The inclusion of GSH and a high concentration of Mg^{++} was based on the requirements of the mammalian methionine-activating enzyme as reported by Cantoni. For rapid assays of the presence or absence of enzyme activity the C^{14} was counted in the acid-insoluble residue after extraction of the incubation mixture with cold TCA and 66% ethanol. One interfering complication was the radioactivity which could be incorporated owing to the attachment of methionine to the appropriate s-RNA in the presence of the ATP-generating system. It should be pointed out that the enzyme extract which had been obtained from organisms in logarithmic growth phase contained considerable amounts of normally methylated s-RNA, capable of activating amino acids. We sought to eliminate this incorporation by the usual mild alkaline treatment: *p*H 10 at 30° for 1 hour. However, treatment even with 1 N sodium carbonate in the presence of large amounts of C^{12}-methionine failed to reduce greatly the incorporation of C^{14} from methionine into an acid-insoluble form by enzyme controls. One expedient which proved effective in reducing this incorporation by the enzyme extract alone (in the absence of exogenous RNA) was incubation in 1 M hydroxylamine for 5 minutes at 30° (Berg and Ofengand, 1958). This procedure and copious washes of the precipitate as detailed under Methods reduced effectively the background radioactivity from this source. With this treatment a 10-fold stimulation of

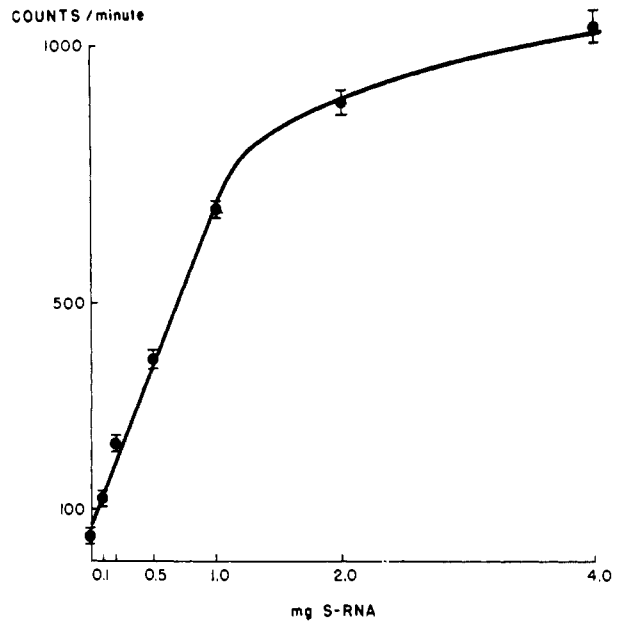

FIG. 1.—The substrate dependence of RNA methylase. The s-RNA is a mixture of the methylated and nonmethylated species.

incorporation of methyl groups could be observed when s-RNA from the methionine-starved organisms was included in the incubation. Another way of eliminating most of incorporation by controls was to subject the enzyme extract to precipitation at *p*H 5. This treatment left the RNA-methylating activity in the supernatant fluid. If the enzyme was then adjusted to *p*H 8, it could be shown that the stimulation of incorporation by substrate RNA was proportional to the concentration of the latter over a 10-fold range, above which the incorporation leveled off (Fig. 1).

In order to confirm that the stimulation of incorporation by the methyl-deficient s-RNA was indeed due to the formation of methylated bases, ribothymidylic acid was isolated from three different experiments. The first incubation lacked any additional substrate RNA, the second contained additional normal s-RNA, and the third contained the methyl-deficient RNA. The ribothymidylic acid was isolated by two-dimensional chromatography (Price *et al.*, 1963) and identified by its spectrum. The radioactivity data on the three samples are given in Table III.

Similar results could be obtained with an enzyme extract from the prototrophic *E. coli* K-12, and in the work which follows *E. coli* K-12 and *E. coli* K-12 W-6 were used interchangeably as enzyme sources.

The mechanism which we propose for these results, that methylation of the RNA polymer occurred in the

TABLE III

DEPENDENCE OF METHYLATION ON RNA FROM METHIONINE-STARVED *E. coli* K12 W-6

Concentrations of components in the cell-free incubation are described in Materials and Methods.

	1	2	3
Standard buffer	+	+	+
ATP and generating system	+	+	+
Methionine-methyl-C^{14}	+	+	+
Enzyme extract	+	+	+
s-RNA ("starved")	0	0	+
s-RNA ("log")	0	+	0
cpm/10^{-8} Mole ribothymidylate	7	5	820

TABLE IV

NONINCORPORATION OF C^{14} FROM UDP-C^{14} INTO RNA IN CELL-FREE EXTRACTS CAPABLE OF METHYLATING RNA

Radioactivity in nucleotides isolated from RNA at the end of a cell-free incubation. For conditions of incubation see Materials and Methods.

	1	2
Standard buffer	+	+
ATP and generating system	+	+
Enzyme extract	+	+
s-RNA ("starved")	+	+
C^{12}-Methionine	+	+
Methionine-methyl-C^{14}	0	+
UDP-C^{14}	+	0
cpm/10^{-8} Mole uridylate	<0.5	
cpm/10^{-8} Mole ribothymidylate	<0.5	930

cell-free extracts in the presence of the nonmethylated RNA substrate, might be challenged with an argument already invoked in connection with the *in vivo* system. It might be argued that the formation of methylated bases is dependent on the presence of nonmethylated RNA in the incubation because this is precisely the RNA which is broken down and resynthesized after a small fraction of the monomeric intermediates had received the radioactive methyl group. The *in vivo* experiments with P^{32} had indicated that such intermediates would have to remain phosphorylated. Now a more direct test of such specific degradation and resynthesis was possible. In the cell-free system we could introduce a labeled phosphorylated intermediate which would necessarily be a participant in such a cycle of depolymerization and resynthesis. The compound which we chose was UDP-C^{14} with the label in carbon 2 of the ring. Incubations were carried out with the 100,000 × *g* supernatant cell fraction as enzyme source and nonmethylated s-RNA with the addition in one set of UDP-C^{14} and C^{12}-methionine and in the other methionine-methyl-C^{14}. In the first instance uridylic acid and ribothymidylic acid were isolated from alkaline degradation of the RNA, and in the second instance ribothymidylic acid was isolated. As shown in Table IV both of the nucleotides isolated from the UDP-C^{14} incubations were nonradioactive, while the ribothymidylic acid from the methionine-methyl-C^{14} incubation had its usual high radioactivity. Thus at least in the case of thymine there appears to be no alternative but to conclude that the formation of the base in RNA occurred by transfer of a methyl group from methionine to a uracil which had been previously integrated into the polymer.

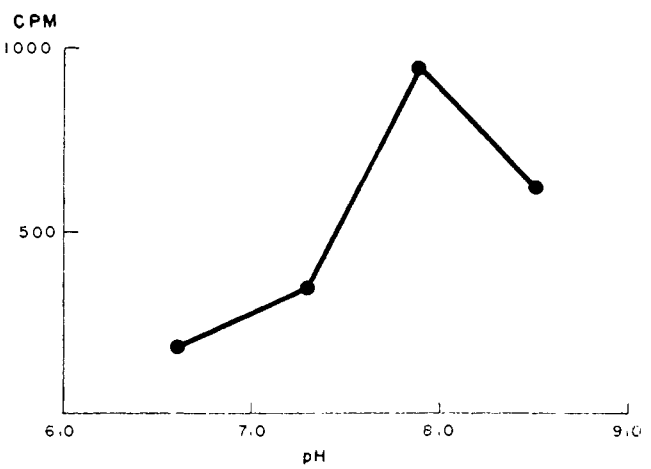

FIG. 2.—The *p*H dependence of uracil RNA methylase.

The *p*H optimum of the methylation reaction was established by performing incubations at varied *p*H levels and measuring the specific activity of the thymine formed in the RNA. As can be seen from Figure 2, the optimum *p*H for the methylation of RNA-uracil was somewhat alkaline. The methylation of RNA in this experiment is complicated by the necessity for at least two enzymes participating in the reaction: an RNA-uracil-methylase and a methionine-activating enzyme. (However, we have found the same *p*H optimum for methyl-group incorporation into whole s-RNA using the more purified enzyme described below with *S*-adenosyl-methionine as a substrate.)

Final proof for methylation of the RNA polymer was obtained by experiments with an enzyme preparation freed of virtually all indigenous RNA, which showed an absolute requirement for methyl-deficient RNA as a substrate in a methylation reaction in which *S*-adenosyl-methionine served as the methyl donor. It was found that most of the indigenous RNA could be removed from the enzyme extracts by a sequence of three treatments during which the enzyme activity remained in soluble form: (1) adjustment of the *p*H to 5 with 1 N acetic acid, (2) addition of protamine at *p*H 5, and (3) addition of $(NH_4)_2SO_4$ to 35% saturation. Details are given under Materials and Methods. After the enzyme activity had been segregated by the addition of ammonium sulfate to 55% saturation, solution in dilute Tris buffer, and dialysis or passage through Sephadex G-75, the protein solution contained 1–2% residual nucleic acid. This preparation could transfer C^{14}-methyl groups from *S*-adenosyl-methionine to non-methylated RNA, but incorporation of label into the acid-insoluble fraction was negligible when no RNA was added or when ribosomal RNA from normal cells was added. When soluble RNA from normal cells was included, the incorporation was 2.5% of that observed with s-RNA from "starved" cells (Table V).

TABLE V

METHYLATION OF DIFFERENT RNA FRACTIONS FROM *E. coli* BY AN *E. coli* ENZYME PREPARATION

For conditions of incubation see Materials and Methods. In this experiment the initial TCA precipitate was heated to 70° for 30 minutes in 0.2 M Tris-HCl, *p*H 9, and was then reprecipitated with cold TCA and washed on a millipore filter with TCA before counting.

	1	2	3
Standard buffer	+	+	+
Enzyme preparation	+	+	+
S-Adenosyl-methionine-methyl-C^{14}	+	+	+
"Starved" s-RNA	+	0	0
"Log" s-RNA	0	+	0
Ribosomal s-RNA	0	0	+
Sample 1 (cpm)	1925	50	3
Sample 2 (cpm)	1850	47	4

The enzyme preparation required Mg^{++} ions: in the absence of the metal only 20% the full activity was observed. A mixture of nucleosides prepared from RNA labeled by the enzyme system was chromatographed. Ribothymidine was isolated and its specific activity (7 $\mu c/\mu mole$) was found to be 50% of the specific activity of the *S*-adenosyl-methionine-methyl-C^{14} (14 $\mu c/\mu mole$) used in the enzyme incubation. The radioactive thymine formed must have been diluted by an equal amount of nonlabeled thymine preexisting in the RNA added to the incubation. This is what one would expect, since the substrate RNA

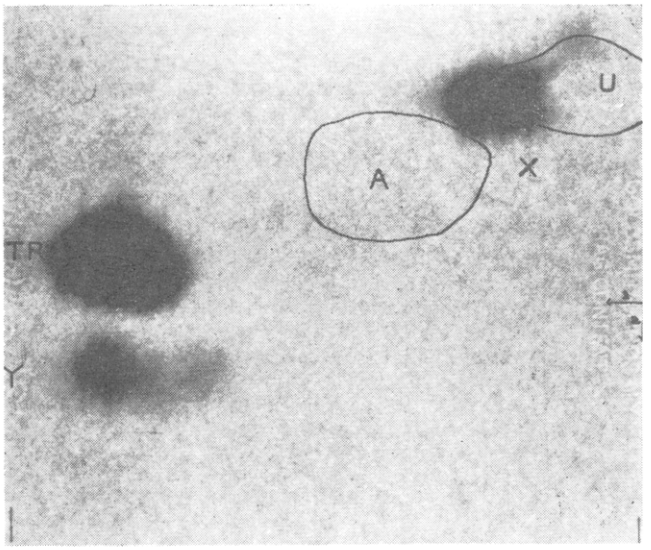

FIG. 3.—Radioautogram of nucleosides isolated from s-RNA methylated *in vitro*. TR is thymine riboside, Y is a mixture of methylated adenosines, and X is a methylated guanosine. A and U are adenosine and uridine. For details of chromatography see text.

consists of about 50% normal, methylated s RNA. The latter represents, of course, the normal RNA which is in K-12 W-6 prior to methionine starvation and which is still present at the end of starvation, when the s-RNA content of organism has doubled. We conclude that there are a limited number of sites available for uracil methylation in the polymer and that when these methyl-deficient sites have received methyl groups, methylation stops. It was found that, in addition to ribothymidine, compounds with the chromatographic, electrophoretic, and spectral characteristics of methylated adenosines and guanosines had been formed as well by this enzyme preparation (Fig. 3). We are now engaged in isolating sufficient quantities of these compounds to permit their precise identification. This information will be reported in a subsequent paper, together with evidence that different enzyme fractions are involved in the formation of various methylated bases in the RNA.

DISCUSSION

The existence of an enzyme which effects methylation of macromolecular RNA focuses attention on the possible biological function of such an enzyme and of its product, methylated s-RNA. This question is important for at least two reasons. First of all the product of the enzyme is transfer RNA, with its key role in protein synthesis. Moreover, this enzymatic activity exists in a variety of organisms, including not only coliform bacteria but also yeast (Svensson *et al.*, 1963), pea seedlings (Birnstiel *et al.*, 1963), spinach leaves, and rat liver (Srinivasan and Borek, 1963). The need for an enzyme which transfers the methyl group to the completed macromolecule is rooted in the origin of s-RNA. If the primary sequence of bases in the nonmethylated transfer RNA is determined by a DNA template or by an RNA template which is a direct gene product, it would be difficult to code for the diversity of methylated bases during the synthesis of the primary sequence.

There are several more or less specific roles which may be postulated for the methyl groups in soluble RNA. These roles may be conveniently grouped under three headings. (1) Methylation produces a

specific effect upon the *intra*molecular structure of the transfer RNA molecule. (2) Methylation affects the *inter*molecular binding of transfer RNA to other macromolecules. (3) The addition of the methyl group renders the transfer RNA resistant to ribonucleases. In the discussion which follows reference will be made to methylated bases which appear either in *E. coli* or in higher organisms.

The positions of the methyl groups in the methylated purines (on the 1-,[2] 2-, and 6-amino positions in adenine and in the 1- and 2-amino positions in guanine) suggest an effect on hydrogen bonding by the bases concerned. Adenine and guanine doubly methylated on the extracyclic amino group and 1-methyl-guanine would lose one hydrogen bond each for pairing with their respective complementary bases, uracil and cytosine. In the case of 1-methyl-guanine, it is possible that a pairing with cytosine according to the usual model would be sterically hindered. Several lines of evidence, including hypochromicity, X-ray diffraction, and optical rotation studies (Tissières, 1959; Brown and Zubay, 1960) indicate considerable regions of helical structure in transfer RNA. In a single-stranded molecule this would imply that the polynucleotide chain is folded back upon itself in a "hairpin" structure, the two halves of the molecule being wound around each other in a double-helical configuration (Spencer *et al.*, 1962). The ability of transfer RNA to renature after heating in dilute solutions (Brown and Zubay, 1960) is consistent with this single-strand model, and suggests that the structure is thermodynamically highly favored. It is possible that the methylated purines with their tendency to weaken or prevent hydrogen bonding help to determine which portions of the s-RNA molecule lie outside the helical region and form the "bend" of the hairpin. Some other mechanisms must be postulated for the methylated pyrimidines, 5-methyl-uracil (thymine) and 5-methyl-cytosine. An explanation is offered by an observation by Fuller (1961) that the methyl group of thymine and the 2′-hydroxyl group of ribose are sterically incompatible in a double-helical model of RNA. Should this be the case, then the methylated pyrimidines might also help to determine points of discontinuity in the helical structure. If we assume that a given intramolecular helix is intrinsically favored by the major base sequence of the s-RNA and the optimal base-pairing allowed by that sequence, in other words, that the s-RNA has already assumed its helical configuration prior to methylation, then the only uracil moieties accessible to the methylating enzymes might be those outside the helical regions, since the others would be masked by the ribose ring of the neighboring nucleotide in the helix. This hypothesis would then provide a function for the pyrimidine methyl group in giving extra stability to the secondary structure. It would also enable us to explain the surprising specificity of the methylation reaction, which selects, for example, one uracil out of the many in a transfer RNA molecule.

There are several objections to this hypothesis, both empirical and theoretical. Shugar has observed that polyribothymidylic acid is capable of forming a structure with a high degree of hypochromicity, either by itself or together with polyriboadenylic acid (Shugar and Szer, 1962). If these structures are true helices, according to the criteria which were mentioned above, then there would not seem to be an incompatibility between the methylation of the 5-position in the pyrimidine ring and the formation of helical structure in ribonucleic acid. The second logical objection is that if the specificity of the methylation reaction depends on which of the bases are sufficiently exposed to be accessible to the enzyme, this implies that the s-RNA molecule has considerable stability prior to methylation. It is then unnecessary that the specificity of helical folding be determined by the methylation reaction, for the specificity of the methylation would be determined by a preexisting secondary structure. Actually, the alternatives may not be so sharply defined. Thus, as we have suggested, the methylation may confer *extra* stability on a preexisting polynucleotide structure. The methylated purines, however, present a more involved problem. For example, if the secondary structure of a nonmethylated s-RNA leaves one or two guanine residues free of intramolecular hydrogen bonds, these residues might be targets for a methylating enzyme. Even though there is at least one enzyme specifically responsible for methylation of guanine in the polymer (as we shall show in a later communication), this enzyme would have to methylate the 1-position in some cases and the 2-amino group in others. However, it is possible that there is more than one enzyme responsible for methylating guanine at different positions. In any case the explanation for this intrabase specificity is not likely to be the steric availability of sites uncovered by the secondary structure.

A second reason for methylation might be to contribute to the "transfer" function of the s-RNA. In this function as it is presently conceived the s-RNA forms a transient, reversible attachment to a portion of the ribosome. The point of attachment is thought to be an RNA molecule which is encoded with the information for the assembly of a specific protein. In the course of this attachment the s-RNA may bind not only to the template RNA but also to adjacent s-RNA or protein molecules. If the binding involves formation of hydrogen bonds, then methylation could affect the strength of these bonds just as it could affect the internal hydrogen bonds described above. If the interaction is hydrophobic or involves interaction of π-orbitals of the RNA bases, then the methyl groups could affect these bonds also, by adding additional hydrophobic surfaces or by affecting the π-bond structure in certain s-RNA bases. Until a specific mechanism is proposed for the participation of s-RNA in protein synthesis at the ribosome, these must remain highly speculative suggestions.

The third possible function for methylation is protection of the s-RNA molecule from nucleases. If there exist specific nucleases which break down short-lived messenger RNA, it would be plausible that the s-RNA would be protected from these enzymes, since its role in protein synthesis is catalytic. It has been observed that bacterial "messenger" RNA is broken down in cell-free systems (Tissières and Watson, 1962) and that synthetic polynucleotides are unstable in the presence of a 100,000 $\times$ *g E. coli* supernatant (Barondes and Nirenberg, 1962), whereas transfer RNA retains full activity after long incubation with the same cell fractions (Tissières, 1959; Tissières and Watson, 1962). There are observations that purified transfer RNA is somewhat resistant to some ribonucleases (for example, Cantoni *et al.*, 1962). But these observations could be explained by the highly ordered secondary structure of s-RNA or by the presence of pseudouridine in the molecule. Experiments in our laboratory have indicated that s-RNA without the usual methyl groups is not significantly more labile toward some ribonucleases.

The methylated bases of s-RNA do not appear to be involved in the coding for specific amino acids, for it has been shown by both Boman and his collaborators (Svensson *et al.*, 1963) and by Starr (1963b) that the

[2] In rat liver s-RNA

mixture of methylated and nonmethylated s-RNA which accumulates in *E. coli* K-12 W-6 on methionine starvation accepts amino acids as well as the fully methylated s-RNA.

Added in Proof

Since the preparation of this manuscript confirmation of the *in vitro* methylation of methyl-deficient RNA and of the multiplicity of RNA methylases has been published from Dr. Hurwitz's laboratory (Gold and Hurwitz, 1963, *Fed. Proc. 22*). Dr. Uriel Littauer has performed exacting studies on amino acid acceptance and transfer by methyl-deficient RNA (Cold Spring Harbor Symposium, 1963) and Dr. D. G. Comb made a highly important contribution by demonstrating the transient existence of a methylatable RNA in the nucleus of the water mold (*Fed. Proc. 22*)

References

Barondes, S. H., and Nirenberg, M. W. (1962), *Science 138*, 810.

Berg, P., and Ofengand, E. J. (1958), *Proc. Nat. Acad. Sci. U.S. 44*, 78.

Birnstiel, M., Fleissner, E., and Borek, E. (1963), *in preparation.*

Borek, E., Mandel, L. R., and Fleissner, E. (1962), *Fed. Proc. 21*, 379.

Borek, E., and Ryan, A. (1958), *J. Bacteriol. 75*, 72.

Borek, E., Ryan, A., and Rockenbach, J. (1955), *J. Bacteriol. 69*, 460.

Brown, G. L., and Zubay, G. (1960), *J. Mol. Biol. 2*, 287.

Cantoni, G. L. (1955), *in* Methods in Enzymology, Vol. II, Colowick, S. P., and Kaplan, N. O., eds., New York, Academic, p. 254.

Cantoni, G. L., Gelborn, H. V., Luborsky, S. W., Richards, H. H., and Singer, M. F. (1962), *Biochem. Biophys. Acta 61*, 354.

Chamberlin, M., and Berg, P. (1962), *Proc. Nat. Acad. Sci. U.S. 48*, 81.

Dunn, D. B. (1960), *Biochim. Biophys. Acta 38*, 176.

Fleissner, E., and Borek, E. (1962), *Proc. Nat. Acad. Sci. U.S. 48*, 1199.

Fox. J. J., Yung, N., Davoll, J., and Brown, G. B. (1956), *J. Am. Chem. Soc. 78*, 2117.

Fuller, W. (1961), *J. Mol. Biol. 3*, 175.

Gray, C. M., and Tatum, E. L. (1944), *Proc. Nat. Acad. Sci. U.S. 30*, 404.

Hanes, C. S., and Isherwood, F. A. (1949), *Nature 164*, 1107.

Kornberg, S. R., Zimmerman, S. B., and Kornberg, A. (1961), *J. Biol. Chem. 236*, 1487.

Lagerkvist, U., and Berg, P. (1962), *J. Mol. Biol. 5*, 139.

Littlefield, J. W., and Dunn, D. B. (1958), *Biochem. J. 70*, 642.

Magasanik, B., Vischer, E., Doniger, R., Elson, D., and Chargaff, E. (1950), *J. Biol. Chem. 186*, 37.

Mandel, L. R., and Borek, E. (1961a), *Biochem. Biophys. Res. Commun. 4*, 14.

Mandel, L. R., and Borek, E. (1961b), *Biochem. Biophys. Res. Commun. 6*, 138.

Mandel, L. R., and Borek, E. (1963), *Biochemistry, 2*, 555, 560.

Markham, R., and Smith, J. D. (1949), *Biochem. J. 45*, 294.

Markham, R., and Smith, J. D. (1952a), *Biochem. J. 52*, 552.

Markham, R., and Smith, J. D. (1952b), *Biochem. J. 52*, 558.

Mejbaum, W. (1939), *Z. Physiol. Chem. 258*, 117.

Nihei, T., and Cantoni, G. L. (1962), *Biochim. Biophys. Acta 61*, 463.

Price, T. D., Hinds, H. A., and Brown, R. S. (1963), *J. Biol. Chem. 238*, 311.

Shugar, D., and Szer, W. (1962), *J. Mol. Biol. 5*, 580.

Smith, J. D., and Dunn, D. B. (1959), *Biochem. J. 72*, 294.

Spencer, M., Fuller, W., Wilkins, M. H. F., and Brown, G. L. (1962), *Nature 194*, 1014.

Srinivasan, P. R., and Borek, E. (1963), *Proc. Nat. Acad. Sci. U.S. 49*, 529.

Starr, J. L. (1963a), *Biochem. Biophys. Res. Commun. 10*, 175.

Starr, J. L. (1963b), *Biochem. Biophys. Res. Commun. 10*, 181.

Svensson, I., Boman, H. G., Eriksson, K., and Kjellin, K. (1963), *Acta Chem. Scand.*, in press.

Tissières, A. (1959), *J. Mol. Biol. 1*, 365.

Tissières, A., and Watson, J. D. (1962), *Proc. Nat. Acad. Sci. U.S. 48*, 1061.

Tsuboi, K. K., and Price, T. D. (1959), *Arch. Biochem. Biophys. 81*, 223.

Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.