On exposure of the substance to 1 N sodium hydroxide at 25°, inorganic phosphate was formed rapidly.

The free tetrulose diphosphoric acid showed $[\alpha]_{589}^{25}$ −1.4° (*c* 0.5, water) and $[\alpha]_{400}^{25}$ −11.7° (*c* 0.5, water).

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The Biosynthesis of Methylated Bases in Ribonucleic Acid*

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The source of the methyl group for the methylated derivatives of the purines and pyrimidines in bacterial ribonucleic acid was investigated. L-Methionine provides the methyl group for 2-methyl adenine, N^6-methyl adenine, 5-methyl cytosine, 5-methyl uracil (thymine), and the methylated derivatives of guanine. When cultures of a methionine auxotroph were grown on medium containing C^{14}-methyl-labeled methionine, the specific radioactivity of the methionine used and the specific radioactivity of the methylated components ultimately isolated from the ribonucleic acid were equivalent. This finding demonstrates a direct transfer of the methyl group from methionine without dilution by the one-carbon-fragment pool. The synthesis of 5-methyl uracil (thymine) of RNA is thus different from that of thymine of DNA, the methyl group of which stems from the one-carbon-fragment pool.

Soluble RNA contains in addition to the four major bases several minor components (Dunn, 1959; Dunn *et al.*, 1960). Among these are methylated derivatives of both the purines and the pyrimidines. The methylated derivatives which have been identified to date are: 1-methyl adenine, 2-methyl adenine, N^6-methyl adenine, N^6-dimethyl adenine, 1-methyl guanine, N^2-methyl guanine, N^2-dimethyl guanine, 5-methyl cytosine, and 5-methyl uracil (thymine). These compounds are present in RNA from a variety of sources at levels ranging from 0.02 to 10% of the uracil content (Dunn and Smith, 1958; Dunn, 1959, 1960, 1961).

While their distribution has been well documented, largely owing to the excellent analytical procedures of Dunn and his collaborators, nothing has been known until recently about the origin of these compounds.

We report here evidence demonstrating that methionine provides the methyl group for the methylated purines and pyrimidines in RNA by direct transmethylation. (A preliminary communication has appeared [Mandel and Borek, 1961].)

Materials and Methods

Organisms.—*E. coli* K_{12} W-6, a methionine-requiring auxotroph, and *E. coli* K_{12} (wild type) were used. They were grown on the medium of Gray and Tatum (1944) supplemented with methionine when necessary.

Bacterial cultures were prepared by inoculating 1 liter of medium with 10^8 cells from a culture which had been grown from a loopful of bacteria obtained from an agar slant. L-Methionine-methyl-C^{14}, diluted with nonlabeled carrier, was added so that 8–10 μg/ml of methionine was provided to the auxotroph. The prototroph received 2 μg/ml of radioactive methionine. The cultures were incubated aerobically at 37° overnight on limiting glucose (0.3 mg/ml) so that growth was limited at 3×10^8 cells/ml. On the following morning cultures were supplemented with 3 mg/ml of glucose and growth was permitted to a cell density of approximately 8×10^8 cells/ml. The cultures were then chilled and harvested by centrifugation at 5000 *g* for 20 minutes at 5°.

Chemicals.—All common laboratory chemicals used were reagent-grade commercial products. All chromatographic standards, including nucleotides, nucleosides, and free bases, were obtained from the California Corporation for Biochemical Research. Thymine riboside was the generous gift of Dr. G. B. Brown of the Sloan Kettering Institute. C^{14}-Methyl-labeled methionine was purchased from the Volk Radiochemical Company. Crude prostatic phosphomonoesterase was obtained from Dr. E. Gray of this department. It was suspended in 0.1 M sodium acetate buffer, *p*H 5, at a concentration of 2 mg/ml. Pancreatic ribonuclease was purchased from the Worthington Biochemical Corporation.

Paper Chromatography and Radioautography.—All chromatograms were run in the descending direction on Whatman No. 1 filter paper. Solvent systems designated *A*, *B*, *C*, and *D* were used routinely in all the paper chromatographic separations:

A. Isopropanol 70%, water 30%, 5% ammonia in the vapor phase (Markham and Smith, 1952).

B. *n*-Butanol 77%, water 11%, 23 N formic acid 12% (Markham and Smith, 1949).

C. *n*-Butanol 86%, water 14%, 5% ammonia in the vapor phase (Markham and Smith, 1949).

D. Isobutyric acid 62.5%, 0.5 M ammonium hydroxide 37.5% (Magasanik *et al.*, 1950).

Radioautograms were prepared by exposing Kodak no-screen type x-ray film for 2 weeks to papers bearing

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radioactive compounds. Standard photographic procedures were used to develop the radioautograms.

Spectrophotometry.—Ultraviolet-absorbing compounds were eluted from the paper in a suitable volume of 0.1 N HCl, and spectra were measured against appropriate paper blanks in a Beckman DU Spectrophotometer. In several cases spectra were also measured on the same solution after the addition of 5 M KOH to give a final concentration of 0.1 M OH^-. The spectral data of Littlefield and Dunn (1958), Smith and Dunn (1959), and Dunn (1960) were used in determining concentrations.

Determination of Radioactivity.—Samples of C^{14}-labeled compounds were plated on stainless steel planchets and dried under heat lamps. Radioactivities were determined at infinite thinness in a Geiger-Muller end-window counter.

Preparation and Hydrolysis of RNA.—Methylated ribotides and ribosides were isolated from RNA by modifications of the methods of Littlefield and Dunn (1958). The pellets of microorganisms (1–3 g wet weight) were extracted with 100 ml of 60% ethanol, *p*H 5, at 25° for 18 hours to remove soluble nucleotides and nucleosides. After centrifugation at 5000 *g* for 20 minutes, the pellets were washed twice with 100 ml of 60% ethanol, *p*H 5, and once each with 40 ml of 90% ethanol, 40 ml of 95% ethanol, and finally 40 ml of acetone. The pellets were dried for 18 hours in a desiccator over $CaCl_2$, and the nucleic acids were then extracted with 10 ml of 0.1 M sodium citrate, *p*H 7.2, at 100° for 2 hours. The mixtures were centrifuged at 12,000 × *g* for 10 minutes at 25° and the sediment was reextracted with 5 ml of citrate solution for 1 hour at 100°. The mixtures were centrifuged as above and the supernatant fluids were combined. An equal volume of a 1% Dupanol C (Dupont) solution was added to the extracts for deproteinization. The mixtures were incubated 30 minutes at 25° and then held at 3° for 18 hours. They were then centrifuged at 12,000 *g* for 10 minutes at 3°. The precipitated Dupanol-protein complex was discarded. To precipitate the RNA and DNA the *p*H of the solution was adjusted to *p*H 4 with acetic acid and ethanol was added to yield a final concentration of 67%. A few drops of 1 M $MgSO_4$ were added to aid precipitation. The precipitation from citrate solution was repeated twice and the precipitates were washed twice with 60% ethanol.

Samples containing 25 to 100 mg of RNA were hydrolyzed to 2′- and 3′-mononucleotides with 1 to 8 ml of 1 M KOH at 30° for 18 hours. The mixtures were centrifuged to remove alkali-insoluble matter and the supernatant fluids were neutralized with 70% $HClO_4$. Glacial acetic acid was then added to *p*H 4, and ethanol was added to 67% concentration to precipitate the DNA and any additional protein. The insoluble matter was removed by centrifugation. The solutions were concentrated to dryness in a rotary evaporator, and the residues were taken up in 3 ml of water and cooled for 1 hour at 3° to precipitate more $KClO_4$. The aqueous solutions of nucleotides were separated from the residue by centrifugation and were concentrated to 0.5–1.0 ml.

Hydrolysis by ribonuclease was carried out on samples containing 30 mg. of RNA in 5 ml of 0.02 M phosphate buffer, *p*H 7.6. Five mg of ribonuclease was added, and the mixtures were incubated for 18 hours at 25°. After centrifugation to remove any insoluble material, the supernatant fluids were adjusted to *p*H 4 with acetic acid and 2 volumes of ethanol were added to precipitate DNA, protein, and unhydrolyzed RNA. The supernatant solutions were concentrated to 0.5 ml.

For conversion to nucleosides, 1-ml samples containing approximately 30 mg of nucleotides were incubated for 5 hours at 37° with 2 ml of 0.1 M sodium acetate buffer, *p*H 5, containing 0.1 ml of the prostatic phosphomonoesterase solution. At the end of the incubation, the mixtures were centrifuged to remove insoluble material and the supernatant fluids were concentrated to 0.5 ml.

To convert nucleosides or DNA to free bases, samples were hydrolyzed with 1 to 3 ml of 7.5 M $HClO_4$ at 100° for 1 hour. The insoluble material was removed by centrifugation, the hydrolysates were neutralized, and the solutions were chromatographed.

Results

Methionine as a Methyl Donor for Ribosylthymine and Methyl Adenines.—*E. coli* K_{12} W-6 was grown to logarithmic growth phase in 1 liter of medium containing 50 μc of C^{14}-methyl-labeled methionine (specific radioactivity 10^5 cpm/μmole). In a similar experiment *E. coli* K_{12} (wild type) was grown to a cell density of 8×10^8 cells/ml on medium containing 25 μc of C^{14}-methyl-labeled methionine per liter (specific radioactivity 2×10^5 cpm/μmole). In both cases, the nucleic acids were extracted and the RNA degraded to 2′- and 3′-ribonucleotides with alkali. Samples of nucleotides were chromatographed on large scale for 18 hours in solvent A. The area of the paper containing the cytidylic, adenylic, and uridylic acids, together with the area ahead, up to the band of the trace of ribosides, was eluted in water for 6 hours at 37°. After being concentrated to a small volume the nucleotides were dephosphorylated with the prostatic phosphomonoesterase. Samples representing 1.5–2.0 mg of nucleosides were chromatographed in solvent *B* for 40 hours. The adenosine, uridine, and methylated ribosides migrating ahead were chromatographed in the second dimension for 24 hours in solvent *C*. The pattern of the chromatogram thus obtained was almost identical to the one illustrated by Littlefield and Dunn (1958), with the exception that no N^6-dimethyl adenosine was detected. A radioautogram of a typical two-dimensional chromatogram is illustrated in Fig 1. The specific radioactivities of uridine, adenosine, 2-methyl adenosine, N^6-methyl adenosine, and thymine riboside were determined.

From the data in Table I it is apparent that when auxotrophic and prototrophic *E. coli* are grown on C^{14}-methyl-labeled methionine, the major purines and pyrimidines are not radioactive, while the methylated derivatives are highly radioactive. In the case of the auxotroph, the specific radioactivity of the three methylated nucleosides approximates that of the L-methionine used in the experiment (10^5 cpm/μmole).

Table I

Origin of the Methyl Group for Thymine and Methyl Adenines in RNA

Compound	Specific Radioactivity (cpm/μmole) *E. coli* K_{12} W-6	*E. coli* K_{12}
Methionine-methyl-C^{14} provided in growth medium	100,000	200,000
2-Methyl adenosine	90,000	46,000
N^6-Methyl adenosine	110,000	62,000
Thymine riboside	72,000	37,000
Uridine	100	100
Adenosine	200	100

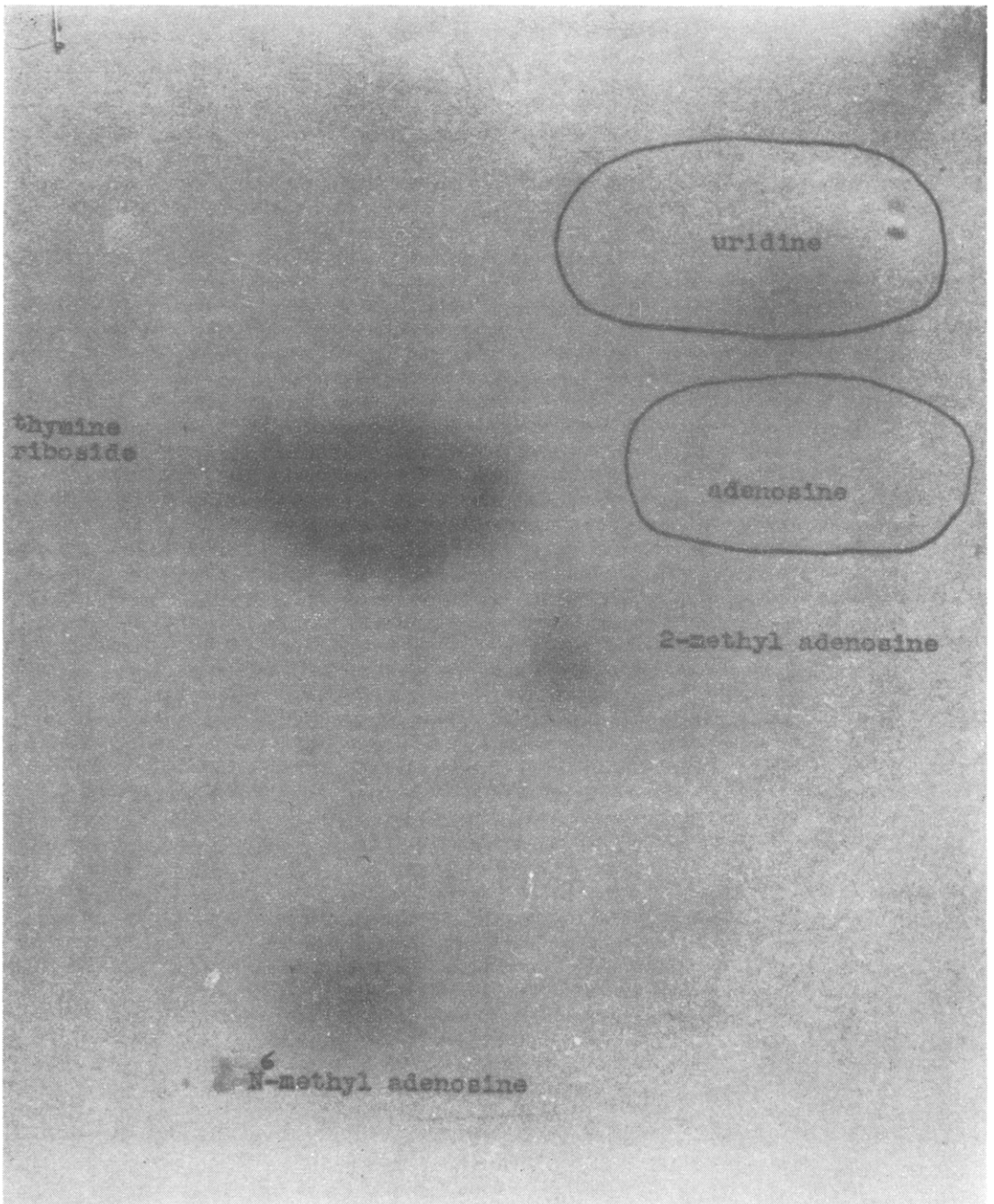

FIG. 1.—Radioautogram of methyladenosines and thymine riboside. (The radioactive spot between uridine and adenosine has been tentatively identified as a methylated guanine [E. Fleissner, unpublished results].)

It can therefore be concluded that in the synthesis of these three methylated bases in RNA there is a direct transfer of the methyl group from methionine without dilution by the one-carbon-fragment pool which is synthesized by these organisms.

The high specific radioactivity of thymine riboside indicates that the methyl group for this base also stems from methionine. This was unexpected, for the synthesis of the thymine of DNA is known to proceed by the methylation of deoxyuridine 5′-monophosphate by a one-carbon fragment which does not stem from methionine.

To establish unequivocally this dichotomy in the path of synthesis of the same base in RNA and DNA, confirmatory evidence was sought. The DNA which survived the alkaline hydrolysis in the above experiments was isolated and hydrolyzed with perchloric acid to the level of free bases, and aliquots of the hydrolysate were chromatographed for 18 hours in solvent *C*. Thymine, the fastest-moving base, was isolated and its radioactivity was determined. It is apparent from the data in Table II that the transfer of label from methionine to the thymine of DNA is negligible.

TABLE II
SOURCE OF THE METHYL GROUP FOR RIBOSYLTHYMINE

Compound	Specific Radioactivity (cpm/μmole)
DNA thymine	200
RNA thymine riboside	37,000
Thymine riboside from above diluted 30-fold with non-radioactive synthetic thymine riboside	1,300
Thymine degraded from the diluted thymine riboside above	1,300

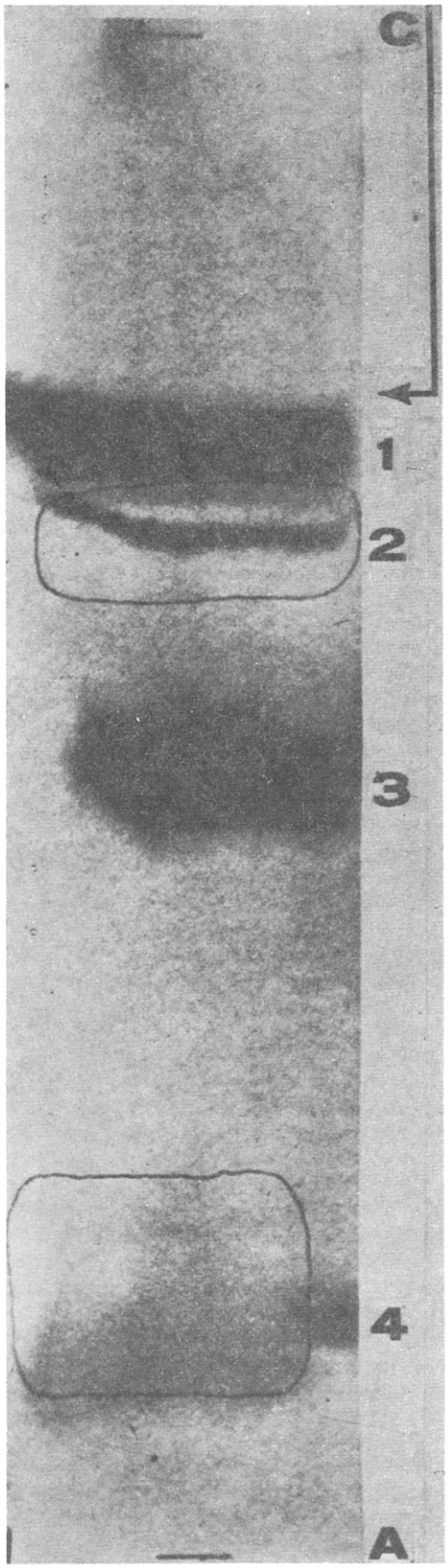

FIG. 2.—Radioautogram of methylated guanine nucleotides. *C*, *A*, cathode, anode; arrow, origin; *1*, cytidylic acid, 5-methyl cytidylic acid (?); *2*, adenylic acid, methyl adenylic acids; *3*, methylated guanylic acids; *4*, uridylic acid, ribothymidylic acid.

For the confirmation of the identity of the isolated thymine riboside, a sample of the chemically synthesized product (Fox *et al.*, 1956) was added in excess to an aliquot of ribonucleosides isolated from the RNA of *E. coli* K_{12} grown on radioactive methionine and the mixture was chromatographed in two dimensions in solvents *B* and *C*. Thymine riboside was isolated and the specific radioactivity of an aliquot was determined. It was found that the specific radioactivity of the nucleoside isolated from bacterial RNA had diminished in proportion to the amount of authentic sample of nonlabeled synthetic product added. To determine that the radioactivity resides in the base moiety, the nucleoside was degraded with $HClO_4$ and the specific radioactivity of the free base was determined.

It is apparent from the data in Table II that a dichotomy in the pathway of synthesis of thymine from the two sources indeed exists.

Methionine as a Methyl Donor for Methyl Guanines.—Two liters of *E. coli* K_{12} W-6 were grown in a medium containing 20 μc/liter of C^{14}-methyl-labeled methionine (specific radioactivity 40,000 cpm/μmole). The nucleic acids were extracted, and the RNA was degraded to nucleotides with alkali and the methylated derivatives of guanylic acid were isolated by the method of Smith and Dunn (1959).

Nucleotides were chromatographed for 18 hours in solvent *A*. Guanylic acid was eluted and its specific radioactivity was determined. The area of the papers containing the adenylic, uridylic, and cytidylic acids was eluted with water. Aliquots of the solution which had been concentrated to about 1 ml were applied to Whatman 3 MM filter paper strips (6.4 × 46 cm), which were then subjected to electrophoresis for 3.5 hours in 0.05 M phosphate buffer, *p*H 2.1, in a pressure plate, tap-water-cooled electrophoresis apparatus at 16 v/cm length of paper.

A radioautogram of the electropherogram obtained is illustrated in Figure 2. The area of the electropherogram containing the methylated guanine nucleotides was eluted and the specific radioactivity of an aliquot was measured by determining the radioactivity of spectrophotometrically determined amounts. Since the spectral characteristics of 1-methyl guanylic acid and N^2-methyl guanylic are very similar and easily masked by minor contaminants, it was not possible to determine conclusively whether either one or both compounds were present. N^2-Dimethyl guanylic acid, which has a more characteristic ultraviolet absorption spectrum, was not detected. The rest of the methyl guanine nucleotides were converted to nucleosides with prostatic phosphomonoesterase. Aliquots of the nucleosides were chromatographed in solvent *C*. However, no adequate separation could be achieved because the presence of salt and other contaminants which affect the R_F values of the methylated guanosines made their separation by paper chromatography difficult. It is apparent from the data in Table III that methionine is the source of the methyl groups for the methylated guanines of bacterial RNA. An independent confirmation of such a pathway in ascites cells was provided by Biswas *et al.* (1961).

Methionine as a Methyl Donor for 5-Methyl Cytosine.—*E. coli* K_{12}W-6 was grown in 1 liter of medium con-

TABLE III
ORIGIN OF THE METHYL GROUP FOR METHYLGUANYLIC ACIDS

Compound	Specific Radioactivity (cpm/μmole)
Methionine-methyl-C^{14} provided in growth medium	40,000
Methyl guanylic acids	30,000
Guanylic acid	350

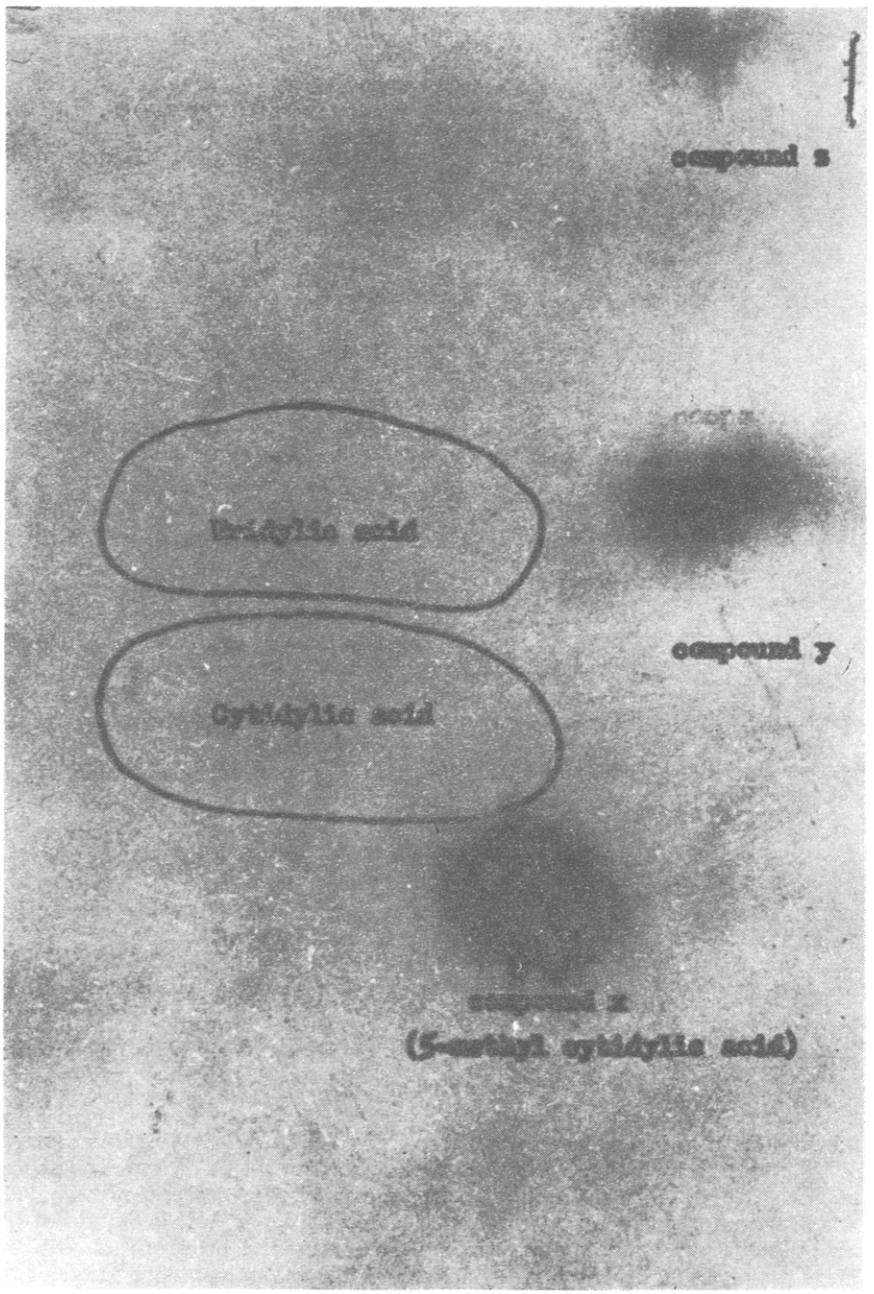

FIG. 3.—Radioautogram of 5-methyl ribosylcytidylic acid.

taining 15 μc of C^{14}-methyl-labeled methionine (specific radioactivity 40,000 cpm/μmole). The nucleic acids were extracted and RNA was hydrolyzed with pancreatic ribonuclease. For the isolation of 5-methyl ribosyl cytidylic acid by the method of Weisner (1962) the ribonuclease hydrolysate was chromatographed on large scale for 18 hours in solvent *A*. The band of uridylic and cytidylic acids was eluted together with the area 10 cm ahead. Aliquots of the concentrated solution were chromatographed in two dimensions, first for 72 hours in solvent *A*, and then for 24 hours in solvent *D*. A radioautogram of the resultant chromatogram is illustrated in Fig 3.

TABLE IV

ORIGIN OF THE METHYL GROUP FOR 5-METHYL RIBOSYLCYTIDYLIC ACID

Compound	Specific Radioactivity (cpm/μmole)
Methionine-methyl-C^{14} provided in growth medium	40,000
5-Methyl ribosylcytidylic acid	35,000
Cytidylic acid	500
Uridylic acid	200

One of the three radioactive spots labeled "*x*" was identified spectrophotometrically as 5-methyl cytidylic acid by virtue of its unique absorption spectrum (optical density maximum at 287 mμ at *p*H 1, Fox *et al.*, 1959). Radioactive spots *y* and *z* gave no distinct spectra and have not as yet been identified. From the data in Table IV it is evident the methionine provides the methyl group for 5-methyl ribosylcytidylic acid in bacterial RNA.

DISCUSSION

Several methylated derivatives of purines and pyrimidines are present in RNA, the bulk of them in the soluble RNA fraction. Of the nine known naturally occurring methylated bases, bacterial RNA lacks only

N^2-dimethyl guanine and 1-methyl adenine. (Recent studies have shown that 1-methyl adenine is restricted to plant and mammalian RNA [Dunn, 1961].)

We have found that L-methionine provides the methyl group for all of these compounds in both wild-type and methionine-requiring bacteria.

Data obtained with the latter revealed that the methyl groups for all of the methylated bases have the same specific radioactivity as the donor methionine, which indicates the origin of all of them from the same methyl pool.

The synthesis of thymine of RNA in this manner is of particular significance, since the same base intended for incorporation into DNA is produced by an entirely different pathway from deoxyuridine 5'-monophosphate and N^5,N^{10}-methylene tetrahydrofolic acid (Friedkin and Kornberg, 1956).

The origin of all methyl groups in soluble RNA from the same pool and the synthesis under certain conditions of soluble RNA without these minor components (see accompanying article) pointed to the possibility that methylation occurs after the synthesis of the polynucleotide. The existence of an enzyme system which can achieve methylation of soluble RNA at the polynucleotide level has recently been demonstrated (Fleissner and Borek, 1962).

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The Nature of the RNA Synthesized During Conditions of Unbalanced Growth in *E. coli* K_{12}W-6*

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When the methionine-requiring auxotroph *E. coli* K_{12}W-6 is deprived of its essential nutrient it can double its intracellular RNA content while its protein and DNA remain constant. Study of the composition of the RNA accumulated during methionine starvation revealed that while it contains the four main base components and pseudouridylic acid, it is devoid of the naturally occurring methylated components. Multiple auxotrophs derived from *E. coli* K_{12}W-6 also synthesize large amounts of RNA, but no DNA or protein, upon deprivation of one or more essential amino acids. When a histidineless methionineless mutant is starved of its essential histidine, but is supplied with methionine, the accumulated RNA does contain the methylated bases. Moreover, other agents which cause bacteria to accumulate nucleic acids while suppressing protein synthesis cause production of newly synthesized RNA which contains methylated bases. Sucrose gradient sedimentation analyses of phenol-purified RNA obtained from cultures of *E. coli* K_{12}W-6 which had been incubated in the absence of methionine revealed that the RNA synthesized during methionine starvation is a mixture of the three macromolecular species of RNA present in normal bacteria. In addition, evidence is presented which reveals the accumulation of a functional "messenger" RNA fraction.

When the methionine-requiring auxotroph *E. coli* K_{12}W-6 is incubated in medium devoid of methionine, its intracellular RNA[1] content almost doubles within 3 hours, while its DNA and protein content remain virtually constant (Borek *et al.*, 1955[2]). During this period there is no cell division, but the cells grow to approximately twice their normal size. When methionine is restored to cultures of these organisms, the accumulated RNA is not excreted from the recovering bacterial cells (Borek and Ryan, 1958). Moreover, the RNA-enriched organisms are uniquely slow in

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[1] The following abbreviations have been used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; TM, 10^{-2} M Tris buffer containing 10^{-2} M $MgCl_2$, pH 7.4.