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Studies on the Mechanism of Biological Methylation of Nucleic Acids*

BURTON E. TROPP†, JOHN H. LAW, AND JOHN M. HAYES

From the James Bryant Conant Laboratory, Harvard University, and the Department of Chemistry, Massachusetts Institute of Technology, Cambridge

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Escherichia coli K-12 W-6 cells were grown in the presence of L-methionine-methyl-d_3. The methylated ribosides, thymine riboside and 6-methylaminopurine riboside, isolated from the soluble ribonucleic acid of these cells were analyzed by mass spectrometry and shown in each case to contain three atoms of deuterium. Thus transmethylation both to the 6-amino group of adenosine and to the 5 position of uridine involves the transfer of an intact methyl group.

Among the most interesting of the several new biological reactions discovered in recent years are those which give rise to the methylated bases of nucleic acids. Mandel and Borek (1963) and Fleissner and Borek (1962, 1963) have shown that this enzymatic process involves a transfer of the methyl carbon of *S*-adenosyl-L-methionine to various bases of intact macromolecular ribonucleic acid, especially the type known as soluble RNA (s-RNA). Similar reactions involving deoxyribonucleic acid were discovered by Gold *et al.* (1963). Since the methylation of s-RNA results in the formation of both carbon-carbon bonds and carbon-nitrogen bonds one might expect to find that two different reaction mechanisms were involved. For example, Keller *et al.* (1949) and DuVigneaud *et al.* (1956) found that methylations of nitrogen involved transfer of an intact methyl group with all three protons, while in the carbon-alkylation reactions leading to tuberculostearic acid and ergosterol, Jauréguiberry *et al.* (1964, 1965) have shown that only two of the three protons of the methionine methyl group were transferred to the products. Therefore it was of interest to study a system where alkylation reactions involving the transfer of methyl groups to both nitrogen and carbon were occurring in the same organism.

We have investigated the formation of methylated bases in s-RNA by the use of methionine-methyl-D_3. The results described in this paper show that the methylation of the 5 position of uridine, unlike the methylation of oleic acid or fungal sterols (Jauréguiberry *et al.*, 1964, 1965), involves the transfer of an intact methyl group with three protons, and thus is more closely related to the methylation of nitrogen and other atoms with nonbonding electrons.

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Materials

L-Methionine-methyl-D_3 was prepared by the method of Melville *et al.* (1947), using methyl iodide-D_3 (Volk Radiochemical Co., Chicago, Ill.) and *S*-benzyl-L-homocysteine (Cyclo Chemical Corp., Los Angeles, Calif.). The twice-recrystallized product gave a single spot corresponding to methionine on paper chromatography (Schlenk and DePalma, 1957). Analysis[1] gave 27.30 atom % excess deuterium (100% of theory for $C_5H_8D_3SN$), and mass spectrometry (Biemann, 1962) confirmed that the sample consisted nearly exclusively of the trideuterated species.

L-[methyl-^{14}C]Methionine was purchased from New England Nuclear Corp., Boston, Mass. Adenosine and 6-methylaminopurine were purchased from California Corp. for Biochemical Research, Los Angeles, Calif.; adenine and thymine were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; and 6-methylaminopurine riboside was the product of Cyclo Chemical Corp., Los Angeles, Calif. Uridine and thymidine were purchased from Schwartz Bioresearch, Inc., Orangeburg, N.Y. Alkaline phosphatase (*E. coli*) and other enzymes were products of Worthington Biochemical Corp., Freehold, N.J.

Experimental Procedure

Escherichia coli, strain K 12 W-6, a methionineless auxotroph, was a gift of Dr. Ernest Borek. The cells were cultured in 40-liter batches in a Biogen (American Sterilizer Co., Erie, Pa.) at 37°, with good aeration. The synthetic medium (Law *et al.*, 1963) was supplemented with 20 mg of L-methionine/liter. Nonisotopic methionine, [methyl-^{14}C]methionine, and methionine-methyl-D_3 were employed for different batches. Near the end of the logarithmic phase of growth the cells

[1] Analysis for deuterium was performed by the falling-drop method by Mr. Josef Nemeth, Urbana, Ill.

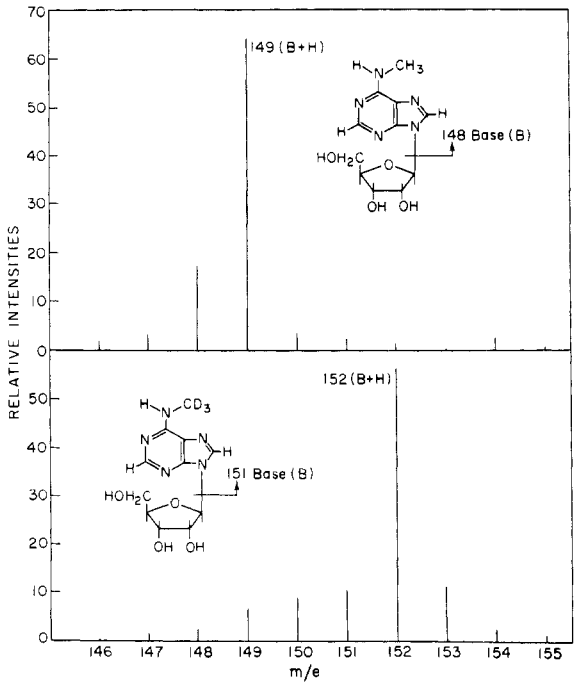


FIG. 1.—Portion of mass spectra of 6-methylaminopurine riboside. Upper chart, nucleoside isolated from s-RNA of cells grown on nonisotopic methionine. Lower chart, nucleoside isolated from s-RNA of cells grown on methionine-methyl-D_3.

were cooled rapidly to 15° and were harvested by centrifugation.

s-RNA was isolated from the cells by the following procedure: Bacterial cells (85 g wet wt) were ground in a mortar at 0° with 170 g of levigated alumina (Norton Co., Worcester, Mass.). When the mixture reached a pasty consistency it was extracted with 85 ml of cold buffer, which contained 8.5 mmoles Tris-HCl, *p*H 8.0, 85 μmoles $MgCl_2$, and 425 μg deoxyribonuclease. The cell debris and alumina were removed by centrifugation for 20 minutes at 20,000 × *g*. The supernatant fluid was centrifuged at 100,000 × *g* for 3 hours in a Spinco Model L ultracentrifuge. The 100,000 × *g* supernatant solution was treated with an equal volume of water-saturated distilled phenol. The mixture was stirred at room temperature for 30 minutes, and the phases were separated by centrifugation. The aqueous phase was reserved and the phenol phase was washed once with distilled water. The aqueous wash was combined with the earlier aqueous phase, and the combined aqueous solutions were again extracted with phenol in the same fashion. The aqueous phase was then made up to 2% with potassium acetate, *p*H 5.0, and the RNA was precipitated by the addition of two volumes of ethanol. After several hours at −20° the precipitate was collected by centrifugation and the supernatant fluid was discarded. The pellet was dissolved in a minimal amount of water and the potassium acetate–ethanol precipitation was repeated. The pellet was again dissolved in a minimal volume of water and the solution was desalted by passage over a column of Sephadex G-25, coarse-bead form (Pharmacia, Uppsala, Sweden).

One preparation of deuterated s-RNA was purified further by the following procedure: After the first precipitation with potassium acetate–ethanol, the RNA pellet was dissolved in water at 4° and sodium chloride was added to a concentration of 1 M. After 3 hours at 4° the precipitate which formed was removed by centrifugation. This precipitate was redissolved in

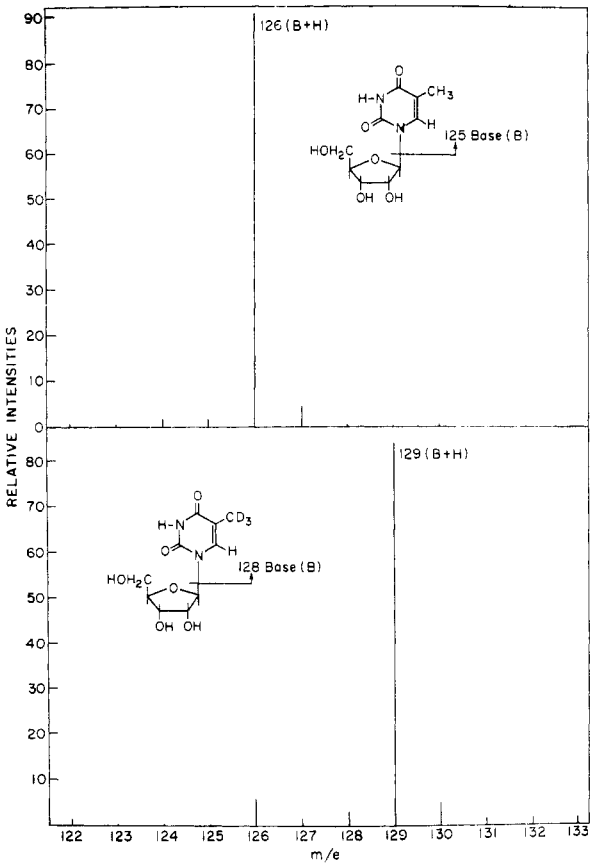


FIG. 2.—Portion of mass spectra of thymine riboside. Upper chart, nucleoside isolated from s-RNA of cells grown on nonisotopic methionine. Lower chart, nucleoside isolated from s-RNA of cells grown on methionine-methyl-D_3.

water and reprecipitated with sodium chloride. The combined sodium chloride supernatant solutions were mixed with two volumes of ethanol and allowed to stand at 4° for several hours. The precipitated s-RNA was collected by centrifugation and dissolved in cold 0.1 M Tris-HCl, *p*H 7.5, and the solution was passed over a colum of DEAE-cellulose, as described by Holley *et al.* (1961). s-RNA was precipitated from the column eluate with ethanol and dissolved in water, and the solution was passed over a Sephadex G-25 column as described earlier.

The deuterated s-RNA was examined in a Spinco Model E ultracentrifuge. The bulk of the material (85%) had a sedimentation coefficient of 3.8 S, while a smaller portion had a larger sedimentation coefficient. The deuterated s-RNA was also assayed for amino acid–accepting activity, using a modification of the procedure of Von Ehrenstein and Lipmann (1961), and was found to have acceptor activity.

The s-RNA (*ca.* 100 mg) was hydrolyzed in 100 ml of 0.3 M KOH at 37° for 18 hours. The *p*H was lowered to 8.5 by the addition of $HClO_4$, the solution was reduced to half of its volume, and the precipitate of $KClO_4$ was filtered out. The filtrate was applied to a column of Dowex-1 × 8 (formate), (200–400 mesh, 4 × 8 cm) (Cohn, 1950; Spahr and Tissières, 1959; Cantoni *et al.*, 1962). The nucleotides were eluted in three fractions, the "cytidine nucleotide" fraction, with 1 liter of 0.1 M formic acid, the "adenosine nucleotide" fraction with 0.5 liter of 1 M formic acid, and the "uridine and guanosine nucleotide" fraction with 2 liters of 5 M formic acid. Each fraction was taken to dryness *in vacuo*. The "uridine and guanosine nucleo-

tide" fraction was resolved into a "uridine nucleotide" fraction and a "guanosine nucleotide" fraction by the method of Katz and Comb (1963), using a column of Dowex-50 × 4 (H^+), (200–400 mesh, 2.25 × 30 cm).

Experiments with s-RNA from cells grown on [methyl-^{14}C]methionine showed that the desired 6-methylaminopurine nucleotide could be found in the "adenosine nucleotide" fraction, and that the thymine ribotide could be found in the "uridine nucleotide" fraction. These could be converted to the nucleosides and then isolated in pure form by paper chromatography.

The nucleotides were dissolved in 100 ml of 0.2 M NH_4HCO_3, *p*H 8.0. Alkaline phosphatase, 0.5 mg, was added and the mixtures were incubated for several hours at room temperature. The solutions were then reduced to dryness *in vacuo*. The residue was dissolved in 10 ml of 1 N HCl. The "uridine" fraction was desalted by passing over a column of 1 × 8 cm of Dowex-1 × 8 (formate) packed on top of 1 × 8 cm of Dowex-50 × 4 (H^+). The "adenosine" fraction was desalted by passing onto a column 2.25 × 16 cm of Dowex-50 × 4 (H^+). After thorough washing of the column with distilled water, the "adenosine" fraction was eluted with 350 ml of 1.5 M NH_4OH. The eluates which contained nucleosides were reduced to dryness.

The nucleoside mixtures were resolved by chromatography on Whatman 3MM paper, using the solvent mixtures of Littlefield and Dunn (1958). The various nucleosides were identified by comparison of their mobilities on paper with authentic standard substances and with reported R_F values. The positions of the methylated nucleosides were confirmed in experiments with ^{14}C-labeled compounds. After elution from the paper, the purified nucleosides were examined with a recording ultraviolet spectrophotometer. The spectra in acidic, basic, and neutral solutions were in good agreement with those reported for these compounds (Littlefield and Dunn, 1958).

For the determination of the deuterium content of the bases, the nucleosides were examined with a Bendix Time-of-Flight mass spectrometer (Biemann and McCloskey, 1962). Aqueous solutions of nucleosides (containing approximately 100 μg pyrimidine nucleosides or 25 μg of purine nucleosides) were reduced to a volume of 0.1 ml and transferred to glass sample containers. After removal of the solvent the samples were introduced directly into the ion source of the instrument, and the spectra were recorded.[2]

Results and Discussion

Figures 1 and 2 show the comparison of a portion of the mass spectra obtained for thymine riboside and 6-methylaminopurine riboside isolated from the s-RNA of cells grown on nonisotopic methionine and on methionine-methyl-D_3. The results show clearly that in each case three protons have been transferred with the methyl carbon, and there are only small peaks corresponding to the D_2, D_1, or D_0 species.

Figures 3 and 4 show the spectra of adenosine and of uridine isolated from deuterated s-RNA. These spectra are identical to those obtained with commercial samples of these compounds. This demonstrates that

[2] The mass spectra observed in these experiments differed from those reported by Biemann and McCloskey (1962). The operating conditions were different and probably greater pyrolysis of the nucleosides was achieved in these experiments. Nucleosides isolated from RNA and commercial nucleoside samples gave similar spectra under the conditions employed.

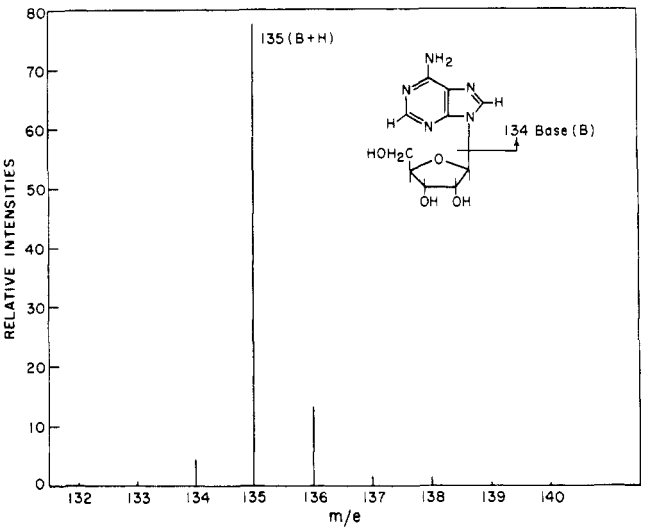
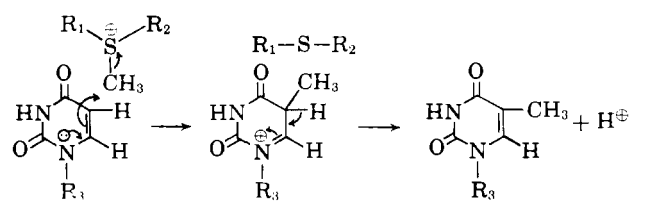


FIG. 3.—Portion of mass spectrum of adenosine isolated from s-RNA of cells grown on methionine-methyl-D_3.

the deuterium atoms found in the methylated bases are not randomly distributed throughout the nucleoside molecule, for if this were the case deuterium would surely be found in the nonmethylated nucleosides also.

The clear results obtained in these experiments provide a strong confirmation of the conclusions reached by DuVigneaud *et al.* (1956). The use of a methionine auxotroph to eliminate endogenous material, and mass spectrometric analysis of the products give a much more sensitive measure of the deuterium content of the transmethylated products than could be obtained with earlier methods. The small peaks which correspond to D_2, D_1, and D_0 species of thymine; for example, have intensities of less than 5% of the intensity of the peak corresponding to the trideuterated species. This rules out the possibility that a significant number of methyl groups have lost deuterium through exchange with the medium or through oxidation to other 1-carbon compounds.

The finding that the enzymatic alkylation of the 5 position of uridine by *S*-adenosylmethionine is a true transmethylation permits some speculation about the mechanism of this reaction. While it is not possible to formulate a mechanism with confidence on the basis of information now available, attention should be drawn to the points of similarity in other known reactions of uracil derivatives. In the enzymatic condensation of ribose phosphate and uracil to form 5-ribosyluracil-5′-monophosphate, the electrophilic ribose carbonyl group is added to the uracil 5 position (Heinrikson and Goldwasser, 1964). The chemical bromination and nitration of pyrimidine derivatives, in which the attacking species is probably electrophilic, occurs readily in the 5 position (Michelson, 1963). It seems likely that electrons of nitrogen-1 would participate in these reactions to facilitate the attack of electrophilic reagents at position 5, as shown below. Ample analogy for non-



enzymatic reactions of this type may be found in the literature (Stork *et al.*, 1954; Opitz and Mildenberger, 1961; Opitz *et al.*, 1961). A mechanism such as this

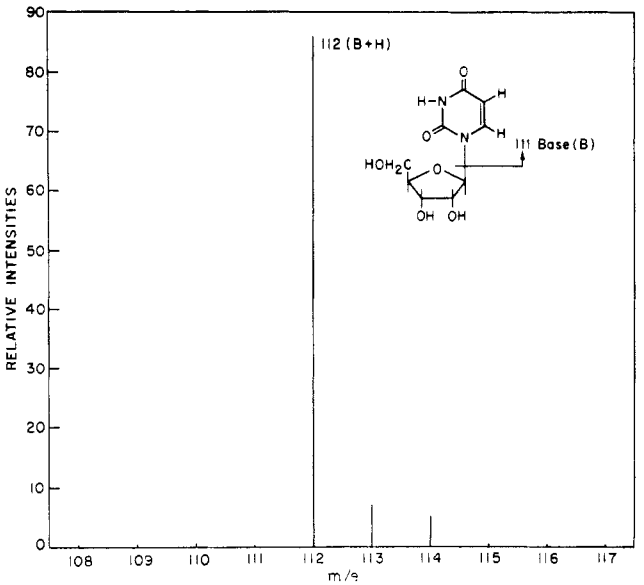


FIG. 4.—Portion of mass spectrum of uridine isolated from s-RNA of cells grown on methionine-methyl-D_3.

may also apply to other substitution reactions which take place at the 5 position of pyrimidine nucleotides, such as the reactions catalyzed by thymidylate synthetase and deoxycytidylate hydroxymethylase. Certainly no similar mechanism can be involved in the alkylation of the double bond of oleic acid in the enzymatic formation of tuberculostearic acid.

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Pseudouridylic Kinase Activity in *Escherichia coli**

SAI-HANG D. KO, MURRAY RABINOWITZ,† AND IRVING H. GOLDBERG‡

From the Departments of Medicine and Biochemistry, and the Argonne Cancer Research Hospital,§ The University of Chicago, Chicago, Ill.

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An enzyme fraction that catalyzes the synthesis of the pseudouridine diphosphate and pseudouridine triphosphate from pseudouridylic acid has been isolated from *Escherichia coli*. Separation of pseudouridylic from uridylic kinase activity was not observed. The rate of the kinase reaction with pseudouridylic acid was 1–2% of that with uridylic acid.

Pseudouridylic acid (5-ribosyluridylic acid) is an important component of transfer RNA (Davis *et al.*, 1959; Dunn, 1959; Otaka *et al.*, 1959; Dunn *et al.*, 1960), but the mechanism of its incorporation into RNA is still unknown. We have previously shown that pseudouridine (ψU) chemically phosphorylated in its 5′ position is converted to its nucleoside triphosphate by an enzyme system present in partially purified yeast extracts (Goldberg and Rabinowitz, 1961a). Further-

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‡ Recipient of Faculty Research Associate Award of the American Cancer Society. Present address: Department of Medicine, Harvard Medical School and Beth Israel Hospital, Boston, Mass.

§ Operated by the University of Chicago for the U. S. Atomic Energy Commission.