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.

synthesizing adaptive enzymes and constitutive protein after restoration of methionine (Borek *et al.*, 1956). When protein synthesis is resumed there is concomitant resumption of RNA synthesis.

For several years the amino acid–independent RNA synthesis by *E. coli* K_{12}W-6 has been the unique exception to the control of nucleic acid synthesis by amino acids (Pardee and Prestidge, 1956). However, Stent and Brenner (1961) have prepared multiple auxotrophic derivatives of this unusual mutant by conjugation with auxotrophic organisms subject to normal control. Many of the recombinants synthesize RNA, but not DNA or protein, when deprived of one or more of their essential amino acids, which indicates that there is a transferable genetic deficiency for the control of RNA synthesis in *E. coli* K_{12}W-6.

In this paper we report the nature of the RNA synthesized during methionine starvation of *E. coli* K_{12}W-6 and the nature of the RNA synthesized during histidine starvation of a histidineless methionineless derivative of *E. coli* K_{12}W-6. Particular attention has been given to the methylated base content of the RNA which accumulates in the presence and in the absence of methionine. In addition, some data on the methylated base content of the RNA synthesized in the presence of chloramphenicol and puromycin are presented. Two preliminary communications have appeared (Mandel and Borek, 1961, 1962).

Materials and Methods

Organisms.—The organism which has been designated as *E. coli* K_{12}W-6 by Dr. Lederberg is a derivative of the one bearing the code number 58-161 isolated by Tatum (1945). For a detailed discussion of the genealogy of this organism see Alföldi *et al.* (1962).

A histidineless, methionineless derivative (G-15) which retains the relaxed control over RNA synthesis was kindly supplied to us by Dr. Gunther Stent of the University of California.

Organisms were grown to logarithmic growth phase in media containing 30 μg/ml of the appropriate DL-amino acid as described in the accompanying paper. The deprivation of amino acids was performed as described previously (Borek *et al.*, 1955).

Chemicals and Assays.—All common laboratory chemicals used were reagent-grade commercial products. Phosphoenolpyruvic acid, phosphoenolpyruvic acid kinase, adenosine 5′-triphosphate, guanosine 5′-triphosphate, glutathione, and C^{14}-L-leucine were obtained from the California Corporation for Biochemical Research. C^{14}-methyl-labeled methionine was purchased from Volk Radiochemical Co., P^{32} was obtained as inorganic sodium triphosphate from E. R. Squibb. All other C^{14}-labeled compounds were obtained from Schwarz Bioresearch, Inc. Pancreatic deoxyribonuclease was purchased from the Worthington Biochemical Corporation. Chloramphenicol was a gift of the Parke-Davis Co., and puromycin was a gift of the Lederle Laboratories.

Intracellular RNA was determined as follows: a sample containing approximately 6×10^9 bacterial cells was extracted with 2 ml of 5% trichloroacetic acid at 3° for 30 minutes. The mixture was centrifuged and the precipitate was extracted with 1 ml of 5% trichloroacetic acid for 15 minutes at 100°. The RNA in the hot trichloroacetic acid–soluble fraction was assayed by the orcinol method (Mejbaum, 1939).

Determination of Radioactivity.—Samples of C^{14}- and P^{32}-labeled compounds were plated on stainless steel planchets and dried under heat lamps. Radioactivities were determined at infinite thinness. A Geiger-Muller end-window counter was used in all experiments except for the experiments with "messenger" RNA, in which a gas flow counter was used.

Isolation of Methylated Ribosides.—The methods for the preparation and hydrolysis of RNA, and the techniques for the separation and identification of the methylated ribosides, have been described in the accompanying paper (Mandel and Borek, 1963).

Isolation of Pseudouridylic Acid.—The RNA obtained from 1 g wet weight of bacteria was hydrolyzed to nucleotides (Reich, in preparation). 2′3′-Pseudouridylic acid was isolated by column chromatography on Dowex-1 (formate) according to the method of Cohn (1960).[3]

Preparation of Crude Cell-Free Extracts.—Pellets of microorganisms were ground with three times their weight of alumina powder in an ice-chilled mortar. Then 0.1 mg of deoxyribonuclease was added to depolymerize DNA. The extract was diluted with 10 ml of TM buffer and was clarified by two centrifugations at 18,000 × *g* at 3° for 10 minutes each.

Separation of Soluble and Ribosomal RNA.—RNA was separated into soluble and ribosomal fractions by centrifugation of crude cell free extracts at 100,000 × *g* for 2 hours at 5° in the Spinco preparative ultracentrifuge. The upper four fifths of the supernatant fluid was removed with a pipet, the remainder of the supernatant fluid was discarded, and the ribosomal RNA pellet was rinsed with TM to remove any adhering soluble RNA. The pellet was then suspended in 8 ml of TM. Trichloroacetic acid was added to each fraction to 5% concentration. The mixtures were centrifuged and the supernatant fluids discarded. The precipitates of RNA were washed once with 5% trichloroacetic acid. Hydrolysis to nucleotides was performed according to the method of Neidhardt and Magasanik (1960).

Phenol Extraction of RNA.—RNA was purified by phenol extraction by a modification of the Kirby procedure (Kirby, 1956). An equal volume of 90% phenol was added to the crude cell-free extract. The mixture was shaken vigorously for 30 minutes at 25° and was centrifuged at 15,000 × *g* at 3° for 10 minutes. The upper aqueous layer was removed carefully with a pipet. To the solution one tenth of its volume of 20% potassium acetate solution was added, followed

[2] Kellenberger *et al.* (1962) have recently claimed DNA synthesis in the absence of methionine in an organism which accumulates RNA. Unfortunately their conclusion is negated by their data. They report that "starved aliquots increased by a factor of 1.6 (60% of the cells divided during the 90 min. period)." Obviously since there was cell division no unbalanced synthesis could have been achieved. In our first publication (Borek *et al.*, 1955) we defined the requirements for a valid conclusion for unbalanced growth. In a study of DNA synthesis during methionine starvation we pointed out the caution which must be exercised in drawing conclusions: "No unequivocal interpretation can be offered for the 17 per cent increase in total DNA during the first hour of starvation. It may represent increased DNA per cell, or it may be the result of a correspondingly increased cellular population—from the utilization of residual intracellular methionine—during the first 15 to 20 minutes of incubation in the methionine deficient medium. The magnitude of the increase in DNA is not quite double the magnitude of the error of cell counting under the best conditions, and, unfortunately, cell counting during the first 20 minutes after the resuspension of a centrifuged bacterial pellet yields erratic results due to clumping of cells."

[3] We are indebted to Dr. E. Reich of the Rockefeller Institute for the isolation of pseudouridylic acid.

by 2 volumes of ethanol. The mixture was held at −15° for 30 minutes to precipitate RNA, which was harvested by centrifugation at 1000 × *g* for 15 minutes at −15°. The RNA was taken up in 1 ml of 0.01 M NaCl and the solution was dialyzed for 18 hours against 2 liters of distilled water at 3°.

Sucrose Gradient Centrifugation.—Phenol-purified RNA in 1-mg lots was sedimented at 25,000 rpm through a linear 20%-5% sucrose gradient for 14 hours in the Spinco SW 25 rotor (Martin and Ames, 1961). Each fraction collected was diluted to 2.6 ml with water, the absorption at 260 mμ was determined, and 1-ml portions were plated on planchets for counting.

"Messenger" RNA Assays.—Assays for "messenger" RNA were performed with Dr. D. Nathans of the Rockefeller Institute under conditions described later in the text.

Results

Inability of E. coli K_{12}W-6 to Synthesize Methylated Bases in the Absence of Methionine.—Preliminary investigations of the composition of the RNA accumulated during methionine starvation of *E. coli* K_{12}W-6 revealed no change in the relative amounts of the adenine, guanine, cytosine, or uracil (Borek *et al.*, 1957). Studies on the amounts of the methylated bases in the accumulated RNA, however, revealed profound changes (Mandel and Borek, 1961).

From the RNA of the mutant grown in the presence of methionine, N^6-methyl adenosine, 2-methyl adenosine, and thymine riboside were isolated and their amounts relative to uridine were determined. Similar determinations on RNA extracted from organisms which had been starved of methionine for 3 hours—with a consequent increase of the RNA—revealed that the relative amounts of the minor components decreased (Table I).

Table I

Decrease in the Methylated Base Content of the Total RNA Synthesized During Methionine Starvation

Compound	Change from Normal Organism
Thymine riboside	−45%
2-Methyl adenosine	−40%
N^6-Methyl adenosine	−50%

For independent confirmation of these observations we undertook to determine whether labeled precursors of methylated purines and pyrimidines are incorporated into the RNA which accumulates during methionine starvation.

In studies on the possible synthesis of thymine riboside, 4 liters of the mutant were grown on medium containing methionine and 25 μc/liter of uniformly labeled DL-aspartic acid-C^{14}. When the culture reached a cell density of 7 × 10^8 cells/ml it was divided in half. Ribonucleosides were isolated from one half. The other portion was washed free of methionine and free of radioactive aspartic acid and was incubated in 2 liters of methionineless medium for 3 hours at 37°. Ribonucleosides were isolated from this portion also. The specific radioactivity of the uridine and thymine riboside was determined. (The purine nucleosides were, of course, not radioactive.)

From the data in Table II it is apparent that during starvation there is a decrease in the specific radioactivity of the uridine due to its synthesis from non-

Table II

Change in the Specific Radioactivity of Pyrimidine Nucleosides during Methionine Starvation

Compound	Decrease
Uridine	42%
Thymine riboside	1%

radioactive precursors in the starvation medium. On the other hand, there is essentially no decrease in the specific radioactivity of the thymine riboside during starvation, demonstrating that it was not synthesized in the absence of methionine.

For confirmation of the exclusion of the methylated purines from the RNA synthesized in the absence of methionine studies on the metabolism of adenine-8-C^{14} during growth and starvation were undertaken.

Two liters of the mutant were grown in medium containing methionine and 50 μc/liter of adenine-8-C^{14}. The RNA was degraded to nucleosides and the specific radioactivity of the purine ribosides was determined. Organisms were also grown in nonradioactive medium and were then resuspended in 2 liters of medium containing 25 μc/liter of adenine-8-C^{14} but lacking methionine. In a second, identical experiment the microorganisms were resuspended in methionineless medium containing 50 μc/liter of adenine-8-C^{14}. In both cases the cultures were incubated for 3 hours at 37°. Ribonucleosides were isolated from the RNA and the specific radioactivity of the purine ribonucleosides was determined.

The data in Table III show that when adenine-8-C^{14} is added to the growing culture of *E. coli* K_{12}W-6, the RNA yields adenosine and its methylated derivatives with the same specific radioactivity. However, when adenine is supplied to organisms in the absence of methionine the methylated adenosines isolated from the RNA have only a fraction of the radioactivity (16% or less) of the adenosine. Therefore in the absence of methionine the synthesis of methylated purines in the accumulating RNA is minimal.

Table III

Specific Radioactivities of the Purine Ribonucleosides Isolated from RNA Synthesized During Normal Growth and During Starvation

Compound	50 μc/liter Adenine-8-C^{14} Added to Growing Organisms	25 μc/liter Adenine-8-C^{14} Added to Starving Organisms	50 μc/liter Adenine-8-C^{14} Added to Starving Organisms
Adenosine	48,500	16,000	37,000
2-Methyl adenosine	48,000	1,000	6,000
N^6-Methyl adenosine	46,500	1,000	1,000

Ability of E. coli K_{12}W-6 to Synthesize Pseudouridylic Acid in the Absence of Methionine.—A 500-ml culture of *E. coli* K_{12}W-6 was grown to a cell density of 8 × 10^8 cells/ml, was washed free of methionine, and was resuspended in 800 ml of medium lacking methionine. At 20-minute intervals 4-μc portions of uracil-2-C^{14} were added for 2.5 hours (28 μc total). Uridylic and pseudouridylic acids were isolated by column chromatography according to the method of Cohn (1960), and their specific radioactivities were determined.

From the data presented in Table IV, it is evident that the minor component pseudouridylic acid is synthesized and incorporated into the RNA accumulated during methionine starvation of *E. coli* K_{12}W-6.

TABLE IV

SPECIFIC RADIOACTIVITIES OF URIDYLIC AND PSEUDOURIDYLIC ACIDS IN RNA SYNTHESIZED DURING METHIONINE STARVATION

Compound	Specific Radioactivity (cpm/μmole)
Uridylic acid	32,000
Pseudouridylic acid	31,000

Ability of a Histidineless, Methionineless Derivative of E. coli K_{12}W-6 to Synthesize Methylated Bases During Histidine Starvation.—A 1-liter culture of a histidineless methionineless derivative of *E. coli* K_{12}W-6 (G-15) was grown on medium containing 30 mg/liter each of DL-histidine and DL-methionine. When the cell density reached 8×10^8 cells/ml the culture was harvested and the bacteria were washed with the medium without the amino acids. The microorganisms were then incubated at 37° in 1200 ml of a histidineless medium containing 7.5 mg L-methionine and glucose. Ten minutes after the onset of starvation 50 μc (1.5 mg) of C^{14}-methyl-labeled L-methionine was added; this achieved a specific radioactivity of approximately 10^5 cpm per μmole. After 2 hours of incubation the RNA content had increased 70% while the viable cell number had increased only 10%. The RNA isolated from the bacteria was degraded to nucleosides. The specific radioactivity of thymine riboside, 2-methyl adenosine, and N^6-methyl adenosine was determined. The major base nucleosides were not radioactive.

Since the specific radioactivity of the methionine was 10^5 cpm/μmole and the intracellular RNA increased 70%, it can be calculated that approximately 4×10^4 cpm/μmole would be the expected value for the specific radioactivity of the total methylated bases. The specific radioactivities of the isolated methylated bases (Table V) indicate that normal incorporation into the newly formed RNA occurred during the period of deprivation of histidine.

Ability of E. coli K_{12}W-6 to Synthesize Methylated Bases in the Presence of Chloramphenicol or Puromycin.—The antibiotics chloramphenicol (Gale and Folkes, 1953) and puromycin (Takeda *et al.*, 1960) alter cellular metabolism, causing bacteria to accumulate both RNA and DNA, while suppressing protein synthesis. To determine whether methylated bases are synthesized in the presence of either of the two antibiotics the following experiments were performed.

One liter of *E. coli* K_{12}W-6 was grown on medium containing nonradioactive methionine. When the culture reached a cell density of 6×10^8 cells/ml, 20 μg/ml of chloramphenicol and 25 μc of adenine-8-C^{14} were added. The culture was incubated for 2 more hours. To another liter culture of the mutant at a cell density of 6×10^8 cells/ml, 1 mg/ml of puromycin and 25 μc adenine-8-C^{14} were added and the culture was incubated for 2 hours at 37°.

TABLE V

SPECIFIC RADIOACTIVITIES OF THE METHYLATED RIBONUCLEOSIDES ISOLATED FROM THE RNA SYNTHESIZED BY *E. coli* G15 DURING HISTIDINE STARVATION

Compound	Specific Radioactivity (cpm/μmole)
Thymine riboside	45,000
2-Methyl adenosine	47,000
N^6-Methyl adenosine	43,000

In both experiments the RNA content increased approximately 70% after the addition of the antibiotic, but the viable cell number increased only 15%. The RNA obtained from the organisms in the two experiments was degraded to ribonucleosides. Adenosine and 2-methyl adenosine were isolated by paper chromatography and their specific radioactivities were determined.

It is apparent from the data in Table VI that the RNA accumulated by *E. coli* K_{12}W-6 in the presence of either chloramphicol or puromycin contains the 2-methyl derivative of adenine.

TABLE VI

SPECIFIC RADIOACTIVITY OF ADENOSINE AND 2-METHYL ADENOSINE IN RNA ACCUMULATED DURING EXPOSURE TO ANTIBIOTICS

Compound	Specific Radioactivity (cpm/μmole)	
	20 mg Chloramphenicol and 25 μc Adenine-8-C^{14}/liter	1 g Puromycin and 25 μc Adenine-8-C^{14}/liter
Adenosine	47,000	52,000
2-Methyl adenosine	53,000	58,000

Examination of the Macromolecular Nature of the RNA Synthesized During Methionine Starvation of E. coli K_{12}W-6.—A culture of the mutant was raised to a population of 9×10^8 cells/ml in 800 ml of medium containing 15 μc of uracil-2-C^{14}. The culture was then divided in half. One portion was frozen immediately. The other portion was washed free of methionine and of radioactive uracil and was incubated in nonradioactive, methionineless medium for 3 hours. Crude cell-free extracts were prepared from the bacterial pellets of the starved and normal cultures, and the RNA was separated into soluble and ribosomal fractions. The RNA of each fraction was hydrolyzed to nucleotides with alkali. Aliquots of the hydrolysates were chromatographed on Whatman No. 1 paper in the descending direction in a solvent consisting of 65% isopropanol, 18% water, and 17% concentrated HCl (Wyatt, 1951). Uridylic acid was isolated from each sample and its specific radioactivity determined.

In a second experiment 800 ml of *E. coli* K_{12}W-6 was grown in nonradioactive medium and was starved of methionine in 1 liter of medium to which 2 μc portions of uracil-2-C^{14} were added every 30 minutes for 2.5 hours. Uridylic acid in the soluble and ribosomal RNA was isolated by the methods described above.

From the data in Table VII, it is evident that when uracil-2-C^{14} is incorporated into growing organisms there is subsequent dilution of the specific radioactivity of the uridylic acid in soluble and ribosomal RNA fractions during methionine starvation. When the isotope is added to starving organisms (column 2), radioactive uridylic acid can be isolated from both major fractions. Therefore, both species of RNA are synthesized during methionine deprivation of *E. coli* K_{12}W-6.

To determine the pattern of accumulation of RNA during the early minutes of starvation and compare it to that of a 3-hour starvation, the following experiment was performed.

A culture of *E. coli* K_{12}W-6 was raised on nonradioactive medium and then transferred to 2 liters of medium devoid of methionine. Ten minutes later, 25 μc of uracil was added. Three equal portions of

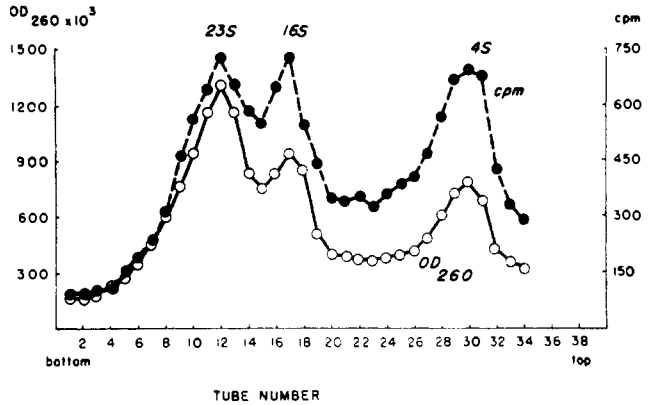

FIG. 1.—Sedimentation profile of RNA obtained from *E. coli* K_{12}W-6 after methionine starvation in P^{32} medium.

TABLE VII

SPECIFIC RADIOACTIVITY OF URIDYLIC ACID IN SOLUBLE AND RIBOSOMAL RNA

Source of Uridylic Acid	Specific Radioactivity (cpm/μmole)	
	Uracil-2-C^{14} Added to Growing Organisms	Uracil-2-C^{14} Added to Starving Organisms
Ribosomal RNA (nonstarved)	19,000	—
Soluble RNA (nonstarved)	19,000	—
Ribosomal RNA (starved)	11,000	13,000
Soluble RNA (starved)	7,000	23,000

the culture were removed from the incubation flask 5 minutes, 15 minutes, and 35 minutes after addition of the uracil. The specific radioactivity of the uridylic acid which was isolated from the soluble and ribosomal RNA fractions was determined.

From the data in Table VIII it is apparent that the specific radioactivity of uridylic acid in soluble RNA is approximately twice that of ribosomal RNA any time after the onset of starvation. Moreover, after 3 hours of such a starvation this ratio remains very close to 2 (Table VII, column 2). Consequently there is no preferential synthesis of any one major fraction of RNA any time during the starvation.

The nature of the accumulating RNA was also studied by sedimentation analyses on sucrose gradients.

A culture of *E. coli* K_{12}W-6 was grown in medium containing half the normal amount of KH_2PO_4 and K_2HPO_4, and 200 μc of $P^{32}O_4^{\equiv}$. Five-tenths ml samples of the RNA purified by phenol extraction were sedimentated through the linear sucrose gradient and

TABLE VIII

SPECIFIC RADIOACTIVITY OF URIDYLIC ACID ISOLATED FROM SOLUBLE RNA AND RIBOSOMAL RNA DURING EARLY MINUTES OF METHIONINE STARVATION

Time After Addition of Uracil-2-C^{14} (min)	Specific Radioactivity (cpm/μmole)	
	Soluble RNA	Ribosomal RNA
5	4,000	2,000
15	12,000	5,000
35	26,000	13,000

fractions were analyzed by the techniques described earlier.

From the optical density and radioactivity profiles in Figure 1 it is apparent that the accumulated RNA is a mixture of the three major species of RNA present in a normal bacterial cell (Kurland, 1960; Tissières, 1959). However, the soluble fraction, which normally contains the bulk of the methylated bases (Dunn, 1959; Dunn *et al.*, 1960) is devoid of these minor components.

The synthesis of "messenger" RNA during methionine starvation was studied by assaying for it in a cell-free, protein synthesizing system. RNA purified by phenol extraction was obtained from *E. coli* K_{12}W-6 grown to logarithmic growth phase in 1 liter of medium. A similar preparation of RNA was obtained from 1 liter of organisms which had accumulated 80% RNA during a 2.5-hour starvation of methionine.

Samples of phenol-purified RNA prepared from normal or from methionine-starved organisms were added to a constant amount of a defined incubation mixture (Table IX, column 2). Water was added to each assay sample to 0.5 ml and the final mixtures (Table X) were incubated for 90 minutes at 35°. Then 0.2 ml of 0.1 M C^{12}-L-leucine solution was added to dilute any C^{14}-L-leucine which might adhere to the precipitate formed by the addition of 4 ml of 5% trichloroacetic acid. The mixture was heated 15 minutes at 90° and was centrifuged. The supernatant fluid was discarded and the precipitate was washed three times with 3 ml each of 5% trichloroacetic acid and then once with 2 ml of ethanol-ether (1:1). The final precipitate was dissolved in approximately 1 ml of concentrated formic acid and was plated for counting.

TABLE IX

MIXTURES FOR THE "MESSENGER" RNA ASSAYS

Ingredient	(1) Vol. for 10 ml Preincubation Mixture[a] (ml)	(2) Vol. for One Incubation Assay (ml)
0.10 M Adenosine 5'-triphosphate	0.30	0.015
0.05 M Phosphoenolpyruvic acid	1.90	0.100
10 mg/ml Phosphoenolpyruvic acid kinase	0.03	0.015
0.05 M Guanosine 5'-triphosphate	0.01	0.001
0.02 M Glutathione	0.40	0.025
0.20 M $MgCl_2$	0.25	0.004
1.0 M KCl	0.30	0.015
1.0 M Tris buffer, *p*H 7.8	0.50	0.025
0.4 mg/ml Deoxyribonuclease	0.25	—
0.002 M Each, all C^{12}-L-amino acids	0.50	—
0.002 M Each, all C^{12}-L-amino acids, except L-leucine	—	0.010
50 μc (5 μmoles)/ml L-leucine-C^{14}	—	0.005
1.3 mg/ml *E. coli* B soluble RNA[b]	3.82	—
28 mg/ml *E. coli* B ribosomal RNA[b]	1.65	—
Preincubation mixture	—	0.100
Total volume	10.00	0.315

[a] The preincubation mixture was incubated at 30° for 1 hour. It was then dialyzed against water for 18 hours at 3° and frozen until ready for use. [b] *E. coli* B soluble and ribosomal RNA were prepared by ultracentrifuging crude cell free extracts at 100,000 × *g* for 2 hours. The pellet of ribosomal RNA which was obtained was washed once with the Tris-$MgCl_2$ buffer and then centrifuged at 20,000 × *g* to remove any insoluble material.

TABLE X
"MESSENGER" RNA INCUBATION ASSAYS

	Assay No.								
	1	2	3	4	5	6	7	8	9
Incubation mixture (ml)	0.315	0.315	0.315	0.315	0.315	0.315	0.315	0.315	0.315
Phenol-purified RNA from normal *E. coli* K_{12} W-6 (ml)	0	0.05	0.10	0.15	0.20	—	—	—	—
Phenol-purified RNA from starved *E. coli* K_{12} W-6 (ml)	0	—	—	—	—	0.01	0.02	0.06	0.08
μg phenol-purified RNA added	0	100	200	300	400	50	100	300	400
Water to 0.515 ml	0.20	0.15	0.10	0.05	0	0.19	0.18	0.14	0.12
cpm/planchet	600	780	910	1075	1355	845	1105	1900	2650
cpm-blank (600)	—	180	310	475	755	245	505	1300	2050
Specific activity in cpm/μg of added phenol-purified RNA	—	1 8	1.6	1.6	1.9	4.9	5.1	4.4	5.1

In Table X the contents of each incubation assay are presented. When no RNA was added, 600 cpm was obtained in the hot trichloroacetic acid–insoluble product of the reaction; this is the value of the blank. All cpm above 600 represent the amounts of C^{14}-leucine incorporated into the product by the presence of the added phenol-purified RNA acting as the "messenger." From the data in the last row of Table X, it may be concluded that the RNA prepared from starved organisms has an amino acid–incorporating activity approximately three times greater than that of a similar amount of RNA from normal cells. Therefore, an RNA fraction which is functional in stimulating amino acid incorporation into protein in an *in vitro* system is synthesized when *E. coli* K_{12}W-6 is deprived of methionine.

DISCUSSION

When *E. coli* K_{12}W-6 is deprived of methionine, the RNA which it accumulates contains no methylated bases. We have presented three different lines of evidence to demonstrate the inability of this organism to incorporate methylated derivatives of both purines and pyrimidines into the RNA synthesized in the absence of methionine.

When a multiple auxotrophic derivative of strain *E. coli* K_{12}W-6 is deprived of one or more of its essential amino acids, RNA continues to be synthesized but protein and DNA synthesis ceases. Such a mutant, requiring both histidine and methionine, when deprived of histidine but supplied with methionine methyl-C^{14} accumulates RNA which contains radioactive methylated bases.

There are other environmental factors which alter cellular metabolism and yield unbalanced RNA synthesis in microorganisms. For example, in the presence of chloramphenicol or puromycin bacteria accumulate large amounts of both RNA and DNA while protein synthesis is minimal. Under these conditions the newly synthesized RNA contains methylated components provided methionine is present.

While the methylated bases are not synthesized during methionine starvation of *E. coli* K_{12}W-6, pseudouridylic acid is incorporated into the accumulating RNA. This finding indicates that the nucleotide chain of soluble RNA produced under these conditions is normal and only the peripheral methyl groups are lacking.

The synthesis of nonmethylated soluble RNA must be the sequential result of two separate deficiencies in the organisms: first, the lack of normal amino acid control over RNA synthesis and, second, the failure of methylation due to the absence of a methyl source.

The synthesis of a nonmethylated *S*-RNA is a unique case of synthesis in a normal organism of a component with so profound a structural change. The availability of nonmethylated species of the *S*-RNA will afford an opportunity to study the possible function of the presence of the methylated bases. Work in this area is in progress in our laboratory.

Stent and Brenner (1961) have made the important observation that the RNA accumulation which we have originally observed on methionine starvation is the result of a genetic deletion which produced a loss of control over RNA synthesis by other amino acids as well. To determine the nature of genetic control over RNA synthesis it seemed important to determine the kinds of RNA which accumulate.

Data from experiments in which the synthesis of soluble RNA and ribosomal RNA during the starvation of methionine were determined revealed that both species had been synthesized.

Taking into account the specific radioactivities in the two newly formed species and their normal abundance it appears that approximately 40% of the accumulating RNA is soluble RNA while 60% of the accumulating RNA is ribosomal RNA.

Sucrose gradient sedimentation analyses of the accumulated RNA revealed that three species of RNA are synthesized in the absence of methionine. Two of these species (23 S and 16 S) correspond to RNA of ribosomal size and the third one (4 S) corresponds to the soluble RNA fraction of the normal bacterial cell.

The synthesis of "messenger" RNA during methionine starvation was also assayed in a cell-free protein-synthesizing system. It was demonstrated that the accumulated RNA prepared from cells starved of methionine had a specific activity approximately three times greater than the RNA obtained from normal organisms, indicating an enrichment with a fraction of RNA which is active in stimulating amino acid incorporation.

It is apparent, therefore, that all the recognized functional components of RNA accumulate during amino acid starvation. This finding at first sight might indicate that the control of all RNA synthesis is invested in a single genetic locus. However, it is also possible that the synthesis of all RNA fractions is interdependent and genetic control thus need be exercised only over one of them. Relaxation of control over one might therefore yield the whole spectrum of ribonucleic acids.

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Studies of Fluorinated Pyrimidines. XVIII. The Degradation of 5-Fluoro-2′-deoxyuridine and Related Compounds by Nucleoside Phosphorylase*

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The degradation of 5-fluoro-2′-deoxyuridine and a variety of other nucleosides to the corresponding pyrimidine bases by the high-speed (105,000 × *g*) supernatant fraction of Ehrlich ascites cells has been investigated. Both 5-fluoro-2′-deoxyuridine and 5-fluorouridine were degraded rapidly by this preparation at *p*H optima of 6.4 and 7.4, respectively. A number of other pyrimidine nucleosides, in particular 2′-deoxyuridine and the 5-halogen-substituted derivatives of 2′-deoxyuridine, were also degraded to the free bases at varying rates under the same conditions. 3′-Monoacetyl-5-fluoro-2′-deoxyuridine and 3′,5′-diacetyl-5-fluoro-2′-deoxyuridine were not degraded by the high-speed supernatant fraction of Ehrlich ascites cells or of human, rat, or mouse liver. However, powerful deacetylase activity was found in a particulate fraction from liver; only very weak deacetylase activity was present in Ehrlich ascites cells. The phosphorylase activity of Ehrlich ascites cells towards 5-fluoro-2′-deoxyuridine was inhibited by several compounds, in particular by 5-fluorouridine and uridine. Uridine was found to be a competitive inhibitor of the reaction.

Previous work from this laboratory has clearly demonstrated that the inhibition of thymidylate synthetase and, hence, of DNA biosynthesis, is the mechanism by which 5-fluorouracil (FU) inhibits the growth of neoplastic tissue (Bosch *et al.*, 1958; Harbers *et al.*, 1959; Hartmann and Heidelberger, 1961). The actual inhibitor of thymidylate synthetase has been shown to be 5 - fluoro - 2′ - deoxyuridine - 5′ - monophosphate (FUDRP) (Cohen *et al.*, 1958; Hartmann and Heidelberger, 1961) and, further, the formation of this inhibitory nucleotide from FU has been found to follow the same pathway as that of deoxyuridylic acid from uracil (Chaudhuri *et al.*, 1958; Harbers *et al.*, 1959; Sköld, 1960a,b). Thus, the first steps in the formation of FUDRP are the formation of 5-fluoro-2′-deoxyuridine (FUDR) or 5-fluorouridine (FUR) from FU and deoxyribose-1-phosphate or ribose-1-phosphate, respectively, reactions which are catalyzed by deoxyuridine phosphorylase or uridine phosphorylase (Sköld, 1960b). In view of this, FUDR should be a better precursor of FUDRP than FU, particularly if the main pathway of synthesis of the deoxyribonucleotide is *via* FUR and 5-fluorouridine-5′-monophosphate (FURP) (Chaudhuri *et al.*, 1958). The hypothesis was confirmed when it was found, first, that FUDR was a more effective carcinostatic agent than FU against the Ehrlich ascites carcinoma and sarcoma-180 *in vivo* (Heidelberger *et al.*, 1958); second, that the deoxyribonucleoside inhibited the incorporation of formate-C^{14} into the DNA-thymine of Ehrlich ascites cells *in vitro* to a greater extent than did FU (Bosch *et al.*, 1958); and, third, that FUDR, in the presence of ATP, was phosphorylated to FUDRP by a high-speed (105,000 × *g*) supernatant fraction from Ehrlich ascites cells, whereas FU did not give rise to FUDRP under the same conditions (Hartmann and Heidelberger, 1961). However, it was found that, *in vivo*, the effectiveness of FUDR was not as much greater than that of FU as might be

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