Effect of Ethionine on the Ribonucleic Acid, Deoxyribonucleic Acid, and Protein Content of Escherichia coli

ROBERT C. SMITH AND W. D. SALMON

Department of Animal Science, Agricultural Experiment Station, Auburn University, Auburn, Alabama

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Abstract

Smith, Robert C. (Auburn University, Auburn, Ala.), and W. D. Salmon. Effect of ethionine on the ribonucleic acid, deoxyribonucleic acid, and protein content of Escherichia coli. J. Bacteriol. 89:657–692. 1965.—The addition of ethionine to cultures of Escherichia coli K-12 W6, a methionine-requiring auxotroph, led to inhibition of the rate of increase in optical density when the ratio of ethionine to methionine was 200:1. When the ratio was 600:1, the increase in optical density became linear. When ethionine was substituted for methionine in the medium, the optical density of the culture increased, and there was a parallel increase in protein content. There was no cell division in these cultures. The rate of synthesis of ribonucleic acid (RNA) in a culture containing ethionine was similar to that of a culture deprived of methionine, but the synthesis of deoxyribonucleic acid in a culture with ethionine was about twice that of a culture deprived of methionine. No detectable radioactivity from ethionine-ethyl-1-C14 was incorporated into RNA. Ethionine-ethyl-1-C14 was readily incorporated into the protein fraction.

When amino acid auxotrophs of bacteria are deprived of the required amino acid, they generally stop synthesizing ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) within a short time. Borek, Ryan, and Rockenbach (1955) reported the first exception to this general finding. They found that Escherichia coli K-12 W6, a methionine-requiring auxotroph, continued to synthesize RNA even after methionine was withdrawn from the medium. During 3 hr of methionine deprivation, the RNA content of the bacteria increased 80% and the DNA content increased about 25%. Stent and Brenner (1961) demonstrated that the dependence of RNA synthesis on amino acids is under the control of a genetic locus that is distinct from the genes governing the amino acid requirement itself.

Some amino acid auxotrophs can increase their cell mass if inoculated into media in which the required amino acid is replaced by an analogue (Richmond, 1962). For example, 7-azatryptophan can support increases of 100% in optical density, protein, and RNA of a culture of E. coli that requires tryptophan (Pardee, Shore, and Pestidge, 1956).

In the present study, the optical density, RNA, DNA, and protein content of a culture of E. coli K-12 W6 incubated in a medium containing l-ethionine in place of l-methionine were followed.

Materials and Methods

Organism and growth conditions. The methionine-requiring cultures of E. coli K-12 W6 were grown in a synthetic medium (pH 7.5) containing (in 1 liter of water): tris(hydroxymethyl)-amino-methane (Tris), 12 g; KCl, 2 g; NH4Cl, 2 g; MgCl2·6H2O, 0.5 g; NaHPO4·2H2O, 50 mg; Na2SO4, 20 mg; concentrated HCl, 6.9 ml; and glucose, 2 g. L-Methionine and L-ethionine were added to the medium at the concentrations given in the text. The bacteria were incubated in Erlenmeyer flasks shaken in a water bath maintained at 37°C. Growth was followed by optical-density readings (450 nm, 1-cm path) against a Tris medium blank in a Beckman DU spectrophotometer. Exponential growth was reached and maintained for at least three generations before an experiment was begun. To remove the methionine supplement, the bacteria were harvested by filtration on a membrane filter (Millipore Filter Corp. Bedford, Mass.) and were rinsed with Tris medium without glucose before they were resuspended in fresh, prewarmed medium. In one experiment, the bacteria were harvested by centrifugation in the cold, washed
twice with Tris medium without glucose, and resuspended in fresh, prewarmed medium. Viable counts were made by dilution of the cultures in Tris medium without glucose at room temperature, and portions containing 300 to 500 viable cells were mixed with 5 ml of Tris-agar (45 C) containing 0.2% glucose and 10 µg/ml of methionine. This mixture was poured over a layer of Tris-agar supplemented as above, and incubated at 37 C.

Chemical assays. Samples (24 ml) of the culture were added to cold 50% trichloroacetic acid (6 ml) and kept in an ice bath overnight. The samples were centrifuged; the resultant pellets were suspended in 5 ml of 5% trichloroacetic acid and heated at 100 C for 30 min. The hydrolysates were cooled and centrifuged, and the supernatant fluids were decanted. Portions of the supernatant fluid were used to assay for RNA by an oriocin method (Brown, 1946) and for DNA by a diphenylamine method (Burton, 1956). The pellet from the hot trichloroacetic acid extract was dissolved in 1 N NaOH at 90 C for 30 min, and protein was determined by the method of Lowry et al. (1951).

Uracil-8-C14 experiments. Cultures were grown with seration in Tris medium supplemented with 10 µg/ml of methionine and 10 µg/ml of uracil. At an optical density of 0.5, the bacteria were filtered, washed, and resuspended in fresh Tris medium containing 10 µg/ml of uracil and about 0.05 µC/ml of uracil-8-C14. Samples (2 ml) of the culture were removed and added to 6 ml of cold 10% trichloroacetic acid. After all the samples were extracted at least 30 min, they were centrifuged, and the pellets were washed with cold 95% ethanol and centrifuged. The pellets were suspened in 1 ml of 0.3 N KOH and incubated for 18 hr at 37 C. The tubes were cooled, and cold 10% trichloroacetic acid was added to precipitate the DNA and protein; these were removed by centrifugation. Samples (0.2 ml) of the supernatant fluids were removed and counted as a measure of the RNA synthesized. The residues were washed once with cold 5% trichloroacetic acid and then hydrolyzed in 8% trichloroacetic acid at 100 C for 30 min. The tubes were centrifuged, and samples (0.2 ml) of the supernatant fluid were removed for counting as a measure of the DNA synthesized. All counting was done in a Packard Tricarb scintillation counter.

Fractionation of radioactive RNA. The soluble RNA fraction from cells grown in the presence of either methionine-methyl-C14 or ethionine-ethyl-l-C14 was separated by the procedure of Brawerman, Hufnagel, and Chargaff (1962). Although this fraction was not tested for its ability to accept amino acids, it was presumed to contain soluble RNA because the radioactivity of methionine-methyl-C14 remained in this fraction. Mandel and Borek (1965) showed that the major purine and pyrimidine bases of the RNA from bacteria grown with methionine-methyl-C14 were not radioactive, but the methylated purine and pyrimidine bases, which are found predominantly in the soluble RNA fraction (Dunn, 1959), were highly radioactive. The RNA was hydrolyzed in 0.3 N KOH at 37 C for 18 hr. Perchloric acid (PCA) was added to the hydrolysate to a final concentration of 0.5 N PCA. The precipitate was removed by centrifugation, and the supernatant solution was neutralized to pH 7.0 with 1 N KOH. The potassium perchlorate was removed by centrifugation, and the supernatant solution evaporated to dryness. The dried residue was dissolved in a minimal volume of water and spotted on sheets (28 by 28 cm) of Whatman no. 1 filter paper for two-dimensional chromatography. The first solvent system was isopropanol-HCl-water (170:41:39), and the second system was isobutyric acid-ammonium hydroxide-water (06: 1:33). After chromatography, the chromatogram was dried and a radioautogram was prepared with Kodak "no screen" X-ray film. The ultraviolet-absorbing spots were eluted with 0.1 N HCl for 18 hr, and the spectra were determined. The four nucleotides, adenosine monophosphate (AMP), cytidine monophosphate (CMP), guanosine monophosphate (GMP), and uridine monophosphate (UMP), were identified by comparison of their ultraviolet spectra and their chromatographic properties with those of authentic nucleotides.

Incorporation of ethionine-ethyl-l-C14. The total incorporation of the radioactivity of ethionine-ethyl-l-C14 into the cells was measured by the rapid filtering technique of Brittin, Roberts, and French (1955). Samples (2 ml) of the culture were filtered on 25-mm Millipore filters and washed three times with 5 ml of Tris medium. The filters were air-dried at 60 C for 0.5 hr and suspended upright in a vial containing 10 ml of scintillation mixture: 2,5-diphenyloxazole, 5 g per liter; 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene, 300 mg per liter in toluene. The samples were then counted.

The incorporation of the radioactivity into protein was measured by adding 0.1 ml of the protein dissolved in 1 N NaOH to 1 ml of 1 M Hyamine (Packard Instrument Co., La Grange, Ill.) in a counting vial; 10 ml of scintillation mixture was added, the contents of the vial were mixed, and the samples were counted.

Separation of radioactive protein. The residue from the hot 5% trichloroacetic acid hydrolysate of bacteria grown in the presence of either methionine-methyl-C14 or ethionine-ethyl-l-C14 was dissolved in 1 N NaOH and dialyzed in running tap water until the pH was 7.0 or less. The protein was dialyzed in distilled water for 24 hr, evaporated to dryness, and hydrolyzed in 6 N HCl for 24 hr at 110 C under reflux. The hydrolysate was evaporated to dryness several times and chromatographed in the following solvent systems: (i) tertiary butanol-methyl ethyl ketone-water-diethylamine (40:40:20:4), (ii) butanol-acetic acid-water (60:15:25), (iii) phenol-water (80:20), (iv) methanol-water- pyridine (80:20:4), and (v) ethanol-water (77:23).

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Source of chemicals. L-Ethionine, L-ethionine-ethyl-1-C\textsubscript{14}, and L-methionine-methyl-C\textsubscript{14} were purchased from Calbiochem. L-Methionine was purchased from Mann Research Laboratories, New York, N.Y. Dl-Ethionine sulfoxide, Dl-ethionine sulfone, Dl-methionine sulfone, and Dl-methionine sulfoxide were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Uracil-2-C\textsubscript{14} was purchased from Volk Radiochemical Co., Chicago, Ill.

Results

Effect of ethionine on optical density. The effect of ethionine on the optical density of cultures containing 2 \( \mu \)g/ml of methionine and variable amounts of ethionine is shown in Fig. 1. The data have been plotted on both logarithmic and arithmetic scales to demonstrate the linear increase in optical density. The minimal concentration of ethionine to give inhibition was 400 \( \mu \)g/ml. Greater concentrations of ethionine caused more inhibition of the rate of increase in optical density. Linear growth resulted when the concentration of ethionine was 1,200 \( \mu \)g/ml or higher. At 8 hr, the optical densities of all the cultures except the two with ethionine at concentrations of 1,600 \( \mu \)g/ml and 2,000 \( \mu \)g/ml had surpassed the optical density of the control culture, and were about 50% higher. These results suggested that cells of \textit{E. coli} K-12 W6 could increase their optical density by using ethionine once the culture had exhausted the methionine in the medium.

The increase in optical density of cultures grown with ethionine and with neither methionine nor ethionine is given in Fig. 2. The optical density of the culture with no amino acid increased about 30% during 3 hr of incubation. The viable counts of this culture remained at the same level. The optical density of the culture containing ethionine doubled, although the viable counts remained constant. Borek et al. (1955) reported a 30% increase in optical density of cultures deprived of methionine, although they found no detectable change in the number of viable cells.

The incorporation of radioactivity from ethionine-ethyl-1-C\textsubscript{14} into the cells of \textit{E. coli} K-12 W6 incubated in Tris medium containing 1 \( \mu \)g/ml of ethionine and about 0.01 \( \mu \)c/ml of ethionine-ethyl-1-C\textsubscript{14} is shown in Fig. 3. About

Fig. 1. Effect of concentration of ethionine on the optical density of cultures of \textit{Escherichia coli} K-12 W6. Bacteria in the exponential phase of growth were filtered, washed, and resuspended in Tris medium without glucose. Samples (8 ml) of the suspension were used to inoculate 58 ml of Tris medium containing 2 \( \mu \)g/ml of methionine plus ethionine at the following levels (\( \mu \)g/ml): \( \bullet \), 0; \( \bigcirc \), 200; \( \Delta \), 400; \( \triangle \), 800; \( \blacksquare \), 1,000; \( \square \), 1,600; \( \updownarrow \), 2,000.

Fig. 2. Increase in optical density of cultures of \textit{Escherichia coli} K-12 W6 incubated in medium containing ethionine. Bacteria were filtered, washed, and resuspended in Tris medium without glucose. Samples (8 ml) of the suspension were used to inoculate 58 ml of Tris medium containing: \( \bullet \), no amino acid addition; \( \bigcirc \), 10 \( \mu \)g/ml of ethionine. The viable counts of these cultures are also given: \( \Delta \), no amino acid addition; \( \triangle \), 10 \( \mu \)g/ml of ethionine.

Fig. 3. Uptake of the radioactivity of ethionine-ethyl-1-C\textsubscript{14} when \textit{Escherichia coli} K-12 W6 was incubated in Tris medium containing ethionine.
80% of this radioactivity was found in the protein fraction.

**RNA, DNA, and protein synthesis in medium containing ethionine.** The uptake of radioactivity from uracil-2-C\(^{14}\) into the alkali-soluble (RNA) and hot trichloroacetic acid-soluble (DNA) portions of cultures containing either 10 \(\mu\)g/ml ethionine or no amino acid addition is given in Fig. 4. The uptake of radioactivity into the alkali-soluble portion of the cultures was approximately the same for the first 120 min. The total radioactivity incorporated in a culture with ethionine averaged about 10% higher than in a culture with no amino acid addition. The radioactivity in the alkali-soluble portion accounted for about 90% of the total radioactivity in the RNA and DNA fractions. The incorporation of radioactivity into the trichloroacetic acid-soluble portion of the cells grown with ethionine was much greater than the incorporation in the culture with no amino acid addition; the total radioactivity incorporated was about 70% higher in the culture with ethionine. Since the incorporation of uracil-2-C\(^{14}\) into the nucleic acids could be due to the turnover of the nucleic acids during starvation (Mandelstam, 1969), the nucleic acids in these cultures were determined by chemical methods to show net synthesis. The synthesis of RNA, DNA, and protein by cultures of *E. coli* K-12 W6 incubated in Tris medium containing either ethionine or no amino acid addition is shown in Fig. 5. The results for RNA and DNA, which are similar to those found with uracil-2-C\(^{14}\), show that the amount of bacterial RNA increased at approximately the same rate in both cultures and that the amount of DNA increased at a faster rate in the culture containing ethionine. The level of protein in the culture that had no amino acid addition did not change. The level of protein in the culture with ethionine increased at approximately the same rate as the optical density of the culture.

**Incorporation of the radioactivity of methionine-methyl-C\(^{14}\) and ethionine-ethyl-1-C\(^{14}\) into RNA and protein.** Bacteria in the exponential phase of growth were centrifuged, washed, and resuspended in Tris medium. This suspension was used to inoculate 400 ml of Tris medium containing either 1 \(\mu\)g/ml of methionine and about 0.01 \(\mu\)g/ml of methionine-methyl-C\(^{14}\) or 1 \(\mu\)g/ml of ethionine and about 0.01 \(\mu\)g/ml of ethionine-ethyl-1-C\(^{14}\). In both cases, the RNA was extracted and degraded to the 2',3'-ribonucleotides with alkali. A radioautogram of the two-dimensional chromatogram of the RNA hydrolysate from the culture grown with methionine-methyl-C\(^{14}\) is illustrated in Fig. 6. The positions of the

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**Fig. 4. Incorporation of uracil-2-C\(^{14}\) into the alkali-soluble (A) and hot trichloroacetic acid-soluble (B) portions of *Escherichia coli* K-12 W6. Bacteria were filtered, washed, and resuspended in Tris medium without glucose. This suspension was used to inoculate 100 ml of Tris medium plus uracil-2-C\(^{14}\) containing: ○, no amino acid addition; □, 10 \(\mu\)g/ml of ethionine.**

**Fig. 5. RNA, DNA, and protein levels of *Escherichia coli* K-12 W6 incubated in cultures containing either no amino acid addition or 10 \(\mu\)g/ml of \(L\)-ethionine. (A) RNA content of cultures containing: ○, no amino acid addition; □, 10 \(\mu\)g/ml of ethionine. (B) DNA content of cultures containing: ○, no amino acid addition; □, 10 \(\mu\)g/ml of ethionine. (C) Protein content of cultures containing: △, no amino acid addition; ▲, 10 \(\mu\)g/ml of ethionine. The optical densities of the cultures are given for comparison: ○, no amino acid addition; □, 10 \(\mu\)g/ml of ethionine.**
ultraviolet-absorbing spots corresponding to AMP, CMP, GMP, and UMP are shown. Six radioactive spots were found. They did not coincide with the positions of the major purine and pyrimidine bases, which suggested that they were probably the methylated bases. The nucleic acid base of spot 5 was identified as thymine. A radioautogram prepared from the culture grown with ethionine-ethyl-1-C14 did not contain any radioactive spots. A second experiment was conducted in which a culture was deprived of methionine for 2 hr to allow "nonmethylated" RNA to accumulate. Ethionine-ethyl-1-C14 was then added to the culture, and it was incubated for 3 hr. The RNA separated from this culture was also unlabeled. If the ethyl group of ethionine is incorporated into RNA under these conditions, it is less than 0.1% the amount of methionine-methyl-C14 incorporated.

The protein separated from bacteria grown with either methionine-methyl-C14 or ethionine-ethyl-1-C14 was hydrolyzed and chromatographed. Radioautograms of the chromatograms of the protein hydrolysates from the cultures grown with methionine had five radioactive compounds. Three of these were identified as methionine, methionine sulfoxide, and methionine sulfone by chromatography with the authentic compounds in five solvent systems. The other compounds, which had less radioactivity, were not identified. The protein hydrolysate from the culture incubated with ethionine-ethyl-1-C14 contained two radioactive compounds. These were identified as ethionine and ethionine sulfoxide by chromatography with the authentic compounds. To determine whether the ethionine sulfoxide could be formed during the acid hydrolysis of the protein, ethionine-ethyl-1-C14 was added to nonradioactive protein and the mixture was hydrolyzed in acid. Two products, ethionine and ethionine sulfoxide, were isolated from the protein hydrolysate, which demonstrated that part of the ethionine could be oxidized to ethionine sulfoxide during acid hydrolysis.

**Discussion**

The addition of ethionine to exponentially growing cells of *E. coli* K-12 W6, a methionine-requiring auxotroph, led to inhibition of the rate of increase in optical density when the ratio of the amount of ethionine to methionine was 200:1. When the ratio was 600:1, the increase in optical density became linear. After 8 hr of incubation, the optical densities of all cultures with ethionine reached or exceeded the final optical density of a culture incubated for 8 hr with 2 μg/ml of methionine and no ethionine. This could be because either the cells use some of the ethionine in the medium continuously as they grow or the cells use the ethionine for protein synthesis only after the methionine is exhausted. Prototrophic bacteria apparently do not overcome ethionine inhibition on prolonged incubation (Loveless, Spoer, and Weisman, 1954). This difference between wild-type bacteria and methionine-requiring bacteria could be due to the ability of the mutant to utilize the methionine in the medium, although at a very slow rate. In the case of the wild-type bacteria, which are synthesizing methionine, ethionine may completely stop methionine biosynthesis by a feedback mechanism. Rowbury and Woods (1961) showed that ethionine can repress the synthesis of the enzymes involved in methionine biosynthesis.

In the cultures where ethionine was completely substituted for methionine in the growth medium of *E. coli* K-12 W6, the observed increase in optical density was accompanied by a parallel increase in the protein content of the bacteria. The proteins synthesized in the presence of ethionine should contain primarily ethionine, because the only methionine available would be that contained in the pool of amino acids present at the start of the experiment plus the methionine returned to the pool from the turnover of protein. The fact that no cell division was observed during this period of protein synthesis might indicate that ethionine affects cell division much more than protein synthesis. Ethionine apparently had no effect on the rate of synthesis
of RNA in a culture deprived of methionine. The rate of DNA synthesis of a culture grown with ethionine was about twice that of a culture deprived of methionine. Gros and Gros (1958) reported a similar study with E. coli ML3280, a methionine-requiring strain that does not accumulate RNA when it is deprived of methionine. They reported that the rate of synthesis of protein was not markedly affected when ethionine replaced methionine in the growth medium, although the rate of synthesis of RNA and DNA was markedly decreased. In the present study, the rate of synthesis of DNA was slightly greater than the rate of synthesis of protein in cultures where methionine was replaced by ethionine.

Farber and Magee (1960) and Stekol, Mody, and Perry (1960) reported that the carbon of the ethyl group of ethionine-ethyl-1-C14 injected into rats was incorporated into the RNA of rat liver. Natori (1963) found that the specific activity of the soluble RNA from the livers of rats injected with ethionine-ethyl-1-C14 was higher than that of the microsomal RNA. In the present study, we were unable to detect the incorporation of radioactivity from ethionine-ethyl-1-C14 into the nucleotides of the soluble RNA fraction of E. coli K-12 W6 when methionine was replaced by ethionine. Because ethionine was readily incorporated into protein, the ethionine was probably activated with adenosine triphosphate and transferred to soluble RNA to form a soluble RNA-ethionine complex (Glenn, 1961). The fact that the ethyl group was not incorporated into soluble RNA suggests that a greater specificity is involved in the methylation of soluble RNA by methionine than in the incorporation of methionine into protein. A comparison of the total uptake of labeled methionine and ethionine with the radioactivity incorporated into the soluble RNA fraction showed that the incorporation of ethionine-ethyl-1-C14 into soluble RNA was less than 0.10% of the incorporation of methionine-methyl-C14.

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LITERATURE CITED


