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SYNTHESIS OF RIBONUCLEIC ACIDS DURING METHIONINE AND VITAMIN B_{12} STARVATION IN ESCHERICHIA COLI 113-3

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SUBMITTED TO THE GRADUATE FACULTY

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HENRY HSIEN-LI HUANG

Oklahoma City, Oklahoma

1970

SYNTHESIS OF RIBONUCLEIC ACIDS DURING METHIONINE AND VITAMIN B_{12}

STARVATION IN ESCHERICHIA COLI 113-3

APPROVED BY

B Connor Johnson

Paul B. McCay

Mary P. Carpenter

Sherril D. Christian

Albert M. Chandler

DISSERTATION COMMITTEE

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SYNTHESIS OF RIBONUCLEIC ACIDS DURING METHIONINE AND VITAMIN B_{12} STARVATION IN ESCHERICHIA COLI 113-3

CHAPTER I

INTRODUCTION

It is known that ribonucleic acids contain methylated purine and pyrimidine bases in addition to the four main bases of their primary structure (1, 2). The methylated derivatives of purines and pyrimidines found in transfer ribonucleic acids to date are 1-methyladenine, 2-methyladenine, N^6-methyladenine, N^6-dimethyladenine, 1-methylguanine, N^2-methylguanine, N^2-dimethylguanine, 7-methylguanine, 1-methylhypoxanthine, 5-methylcytosine and thymine (3).

The problem of methylation of tRNA and rRNA has been studied extensively in methionine requiring strains of _E. coli_. The effect of methionine on the rate of RNA synthesis occurs at a step involving methylation. The methyl groups of these methylated bases are derived from methionine, with S-adenosylmethionine the active methylating agent (4, 5).

It has been shown that the amino acylation activities of normal and methyl-deficient tRNA are not significantly different in _E. coli_ strains requiring methionine (6, 7, 8). An undermethylated acyl-tRNA specific for phenylalanine has been found in _E. coli_ G-15 ($RC^{rel}Meth^-Hist^-Biotin^-$) and a similar undermethylated tRNA specific for leucine has been

found in E. coli 58-161 ($RC^{rel}Meth^-$). A new undermethylated valyl-tRNA has been found in this investigation. These undermethylated species have been separated chromatographically from their normal counterparts (9, 10, 11).

This investigation was undertaken to study the possible role of vitamin B_{12} and methionine in the methylation of RNA and hence a role in protein synthesis. The regulation of specific protein synthesis may provide a possible explanation for birth defects resulting from vitamin B_{12} or methionine deficiency.

The work has indicated a possible role in such specific protein regulation because of an effect on the methylation of tRNA. A new single undermethylated species of valyl-tRNA has been found and others may exist. This was found using a strain of E. coli, 113-3, which requires vitamin B_{12} or methionine. This study suggests that birth defects resulting from the absence of vitamin B_{12} or methionine may be due to abnormal binding or coding properties of the undermethylated tRNA. The possibility of regulation of specific protein synthesis by tRNA and interference in normal protein synthesis by abnormal tRNA adds a new mechanism to those previously proposed for the control of development.

In bacteria two classes of mutants with regard to type of regulation of protein and RNA synthesis have been extensively studied. Stringent control by stringent mutants is defined as the mechanism by which an amino acid-requiring strain ceases any net RNA synthesis when it is deprived of its required amino acid. Relaxed control by relaxed mutants, the second class, is defined as the mechanism by which an amino acid-requiring strain continues to synthesize net RNA when it is deprived of

its required amino acid. Neither type of mutant synthesizes protein in the absence of the required amino acid.

In stringent mutants of *E. coli* the normal formation of both RNA and protein is dependent on the availability of the required amino acid. Thus, a stringent auxotrophic mutant requiring an amino acid for growth ceases synthesizing both RNA and protein when the medium is deprived of the required exogenous amino acid (12, 13, 14, 15, 16, 17, 18). Nevertheless, it has been observed that in polyauxotrophic stringent mutants of *E. coli* a small amount of RNA is synthesized in the absence of the essential amino acid (19,20). Most of this small amount of RNA is mRNA.

In relaxed mutants of *E. coli* the non-availability of a required amino acid does not affect the normal formation of both RNA and protein. Thus, an auxotrophic mutant with relaxed control requiring an amino acid for growth continues to synthesize RNA but ceases to synthesize protein when the medium is deprived of the required exogenous amino acid, e.g., when methionine requiring strains with relaxed control are grown in the absence of methionine, synthesis of RNA continues but synthesis of protein ceases (8, 21, 22, 23). RNA from such cells is undermethylated (3). In the presence of exogenous methionine, RNA with a normal complement of methylated bases and protein are synthesized (4, 24). Such undermethylated RNA can be methylated *in vitro* by homologous enzymes and methylated RNA can be supermethylated by heterologous enzymes. Genetic analysis indicates that the RC locus of this mutant may be a regulator gene or an operator gene controlling RNA synthesis (25). When relaxed strains requiring amino acids other than methionine are grown in the absence of

their essential amino acids, RNA synthesis continues and protein synthesis ceases, but the new RNA synthesized is not undermethylated (9).

Two different types of regulation have been proposed for the regulation of RNA synthesis in stringent strains. First, regulation of RNA synthesis in E. coli may be coordinate, i.e., in some stringent strains the synthesis of all types of RNA are reduced when the required amino acid is lacking (26, 27, 28, 29). Second, regulation of RNA synthesis in E. coli may be non-coordinate, i.e., in other stringent strains rRNA and tRNA synthesis are reduced while mRNA synthesis is not reduced when the required amino acid is lacking (18, 19, 20, 30, 31, 32).

Each type of regulation for stringent strains is also believed to be applicable to the relaxed strains of E. coli. Thus, synthesis of all classes of RNA may be regulated coordinately (rates of synthesis of all three types of RNA change equally) or non-coordinately (the rate of synthesis of one or more of the three types of RNA changes with respect to the other) when an essential amino acid is lacking. At the time the present investigation was performed, there were no experimental data to indicate the type of regulation in a relaxed strain.

Since stringent mutants do synthesize mRNA in the absence of required amino acids, it has been suggested that both types of regulation can not exist in bacteria with stringent control. Synthesis of RNA in stringent strains of E. coli appears more likely to be non-coordinate, because the synthesis of different classes of bacterial RNA are depressed differently during essential amino acid starvation (18, 19, 20, 32, 33, 34). When the stringent strains are deprived of an essential amino acid, tRNA and rRNA synthesis either stops or is reduced, but mRNA synthesis

continues at a normal rate or slightly lower than normal rate (18).

The problem of non-coordinate RNA synthesis is thus still open for investigation. Although non-coordinate synthesis of RNA appears to be probable in stringent strains, it is necessary to investigate relaxed strains before a final conclusion can be made concerning non-coordinate RNA synthesis in the absence of essential amino acids.

Borek *et al.*, (21) discovered in 1955 that *E. coli* 58-161 possesses relaxed control and since that time several other relaxed strains, such as K_{10}, K_{10-13}, and G-15, have been found. These strains have been used to investigate relaxed control, but the problem of non-coordinate RNA synthesis has not been studied. *E. coli* 113-3, a methionine or vitamin B_{12} requiring strain, has been used for this investigation. The present work indicates that *E. coli* 113-3 is a relaxed strain and that the synthesis of RNA with respect to protein and of the three types of RNA in this strain is non-coordinate.

The rates of RNA, DNA, and protein syntheses as well as cell multiplication during vitamin B_{12} and methionine starvation are reported. Non-coordinate RNA synthesis has been investigated by means of the coordination ratio based on the concept that mRNA synthesis is not influenced by methionine or vitamin B_{12}. The coordination ratio indicates that tRNA and rRNA synthesis are regulated non-coordinately relative to each other, to total RNA, and to protein and the increment in RNA synthesis during vitamin B_{12} and methionine starvation is mainly tRNA and less rRNA.

CHAPTER II

MATERIALS AND METHODS

Materials

Microorganism

A culture of *E. coli* mutant 113-3, requiring either vitamin B_{12} or methionine, was kindly provided by Dr. Bernard D. Davis, Harvard Medical School.

Chemicals

L-valine-^{14}C (U) (270 mc/mM) was obtained from Amersham/Searle, Des Plaines, Ill. DL-valine-T (G) (480 mc/mM) was obtained from Nuclear-Chicago, Des Plaines, Ill. S-adenosyl-L-methionine (me-T) was obtained from International Chemical and Nuclear Corporation, City of Industry, California. Highly purified DNase was obtained from Sigma Chemical Co., St. Louis, Missouri. *E. coli* B tRNA was purchased from Calbiochem., Los Angeles, California. Alumina A 305 (bacteriological grade) was purchased from Alcoa Corporation, Bauxite, Arkansas. Aliquot 336 (methyl tricaprylyl ammonium chloride) was purchased from General Mills, Kankakee, Illinois. Freon-214 (tetrachlorotetrafluoropropane, b. p. 114°C) was purchased from E. I. DuPont DeNemours and Co., Inc., Wilmington, Delaware. Chromosorb W (acid washed, mesh size 100/120) was purchased from Johns-

Manville Products Corporation, New York. All other chemicals used were reagent grade.

Methods

Method of Cultivation of *E. coli* Mutant 113-3

E. coli 113-3 was grown according to the method of Hurwitz, Gold and Anders (35). Fifteen to twenty plates of bacteria cultured overnight on Difco Bacto nutrient agar were transferred to either a glucose-salts-methionine or a glucose-salts-vitamin B_{12} medium which contained the following ingredients in grams per liter: NH_4Cl, 1.0; KH_2PO_4, 3.0; $Na_2HPO_4 \cdot 7H_2O$, 11.3; $FeSO_4 \cdot 7H_2O$, 0.0005; $CaCl_2 \cdot 2H_2O$, 0.055; $MgSO_4 \cdot 7H_2O$, 0.49; glucose, 6.0; methionine, 0.05 or vitamin B_{12}, 0.000004.

For investigation of the effect of methionine, the cells were grown with vigorous aeration at 37°C for 18 hours in the presence of methionine and then were harvested with a Sharples super-centrifuge. Transfer ribonucleic acid isolated from such a preparation is defined as normal, i.e., tRNA which contains a full complement of methyl groups. When methionine deficient tRNA was needed, the cells were obtained by the above procedure except that the cells were grown for 20 hours. The harvested cells were washed five times with 150 ml glucose-salts medium without methionine and grown again aerobically at 37°C for either 3 hours or 6 hours as indicated in a methionine deficient medium. Transfer ribonucleic acid isolated from such a preparation is defined as methyl-deficient tRNA.

For investigation of the effect of vitamin B_{12}, the cells were grown aerobically at 37°C for 11 hours in medium containing vitamin B_{12}

and then harvested using a Sharples super-centrifuge. Transfer ribonucleic acid isolated from such a preparation is defined as normal tRNA. In addition cells were grown in the presence of vitamin B_{12} for 13 hours and washed ten times with 150 ml of sterilized water or glucose-salts medium without vitamin B_{12}. They were grown again aerobically at 37°C in medium without vitamin B_{12} for either 3 hours or 6 hours as indicated. The tRNA isolated from these cells was defined as deficient tRNA. All the prepared cells were stored at -18°C.

Preparation of RNA, DNA and Protein

RNA, DNA and protein were isolated from the harvested cells according to the procedure of Hurwitz and Gold (36). For the study of the growth of mutant 113-3 and its relation to RNA and protein synthesis during methionine starvation, the culture grown in the presence of methionine was centrifuged, washed to remove methionine, resuspended in the same volume of glucose-salts medium without methionine and incubated aerobically at 37°C. At the times indicated, 30 ml of culture were withdrawn and absorbance at 620 mμ was determined. Then the samples were centrifuged and the sedimented cells washed. The pellets of cells were suspended in 2.0 ml of 5% trichloroacetic acid, boiled for 20 minutes and centrifuged. The acid soluble material was used to measure RNA while the acid insoluble material, which could be brought into solution with 0.2 N NaOH, was used to determine protein.

For studies of RNA, DNA and protein synthesis during vitamin B_{12} starvation, the cells were grown in the presence of vitamin B_{12}, washed to remove vitamin B_{12}, and resuspended in the glucose-salts medium without vitamin B_{12} (300 mg wet weight of bacteria per 100 ml of glucose-

salts medium). Four micrograms of vitamin B_{12} per 100 ml medium were added to one side-arm flask while no vitamin B_{12} was added to the other. They were incubated at 37°C on a shaking machine. Two 4.0 ml portions of the culture were removed at the times indicated and turbidity at 620 mμ was measured. These cultures were centrifuged, washed with 10 ml of deionized water, recentrifuged and resuspended in 4.0 ml of deionized water. One milliliter of each sample was diluted to 2 ml with a final concentration of 5% trichloroacetic acid and then heated at 100°C for 20 minutes, cooled and centrifuged. Five-tenths milliliter of the acid-soluble material was used to measure RNA while 0.4 ml of the acid-soluble material was used to measure DNA. The acid-insoluble material, which could be brought into solution with 0.2 N NaOH, was used to measure protein.

Preparation of tRNA

Transfer ribonucleic acid was prepared from the frozen cells by the supernatant method with phenol according to Hurwitz, Gold and Anders (35) except that the buffer contained 5 mM Tris, pH 7.4, and 0.1 mM magnesium acetate (37, 38). The cells were ground in the cold with a mortar and pestle with 2.5 times by weight of alumina A 305 (bacteriological grade). After the cells were disrupted, the paste was suspended in a buffer solution (1 gram bacterial wet weight per 5 ml of buffer) which contained 5 mM Tris buffer, pH 7.4, 0.1 mM magnesium acetate and 5 μg of pancreatic DNase per ml of buffer. The suspension was centrifuged at 30,000 x g for 15 minutes. The precipitate was discarded and the supernatant fraction centrifuged for 3 hours at 78,000 x g in the No. 30 rotor of a Spinco ultracentrifuge (model L). The supernatant solution was decanted, incubated at 38°C for 30 minutes, and then stirred with an equal

volume of 80% redistilled phenol for 60 minutes. The aqueous phase was removed by pipet and repeatedly extracted with equal volume of ether until no traces of phenol remained. The aqueous layer was made 2% with respect to potassium acetate buffer, pH 4.5, and 2 volumes of absolute ethanol were added. The solution was allowed to stand at 0°C for several hours and the precipitate was collected by centrifugation. The prepared tRNA was dissolved in 20 ml of 0.2 M glycine buffer at pH 10.2 and incubated at 37°C for 2 hours to discharge any bound amino acid. The solution was made 2% with respect to potassium acetate buffer, pH 4.5, and 2 volumes of absolute ethanol were added. After 3 hours the precipitate was recovered by centrifugation, dissolved in the buffer containing 5 mM Tris, pH 7.5, dialyzed overnight and stored at -18°C.

Assays

Ribonucleic acid was measured by the orcinol method (39). Four millimolar $CuCl_2 \cdot H_2O$ in concentrated HCl (density 1.19) was prepared by dissolving 0.68196 gm of $CuCl_2 \cdot H_2O$ in 1000 ml of concentrated HCl. Two-hundred milligrams of orcinol were dissolved in concentrated HCl; 10 ml of the $CuCl_2 \cdot H_2O$ solution were added, and the volume was made up to 100 ml with concentrated HCl. To 5 ml of the solution to be tested, 5 ml of the reagent prepared as described were added. The test tubes were shaken well and heated at 100°C for 40 minutes; then they were cooled under running water. The color was extracted with 5 ml of isoamyl alcohol and after centrifugation was read on a Spectronic 20 (Bausch and Lomb Optical Co., Rochester, N. Y.) at 675 mμ against a blank obtained by treating distilled water in the same manner as the samples. A standard curve was prepared by the same method using solutions of known concentra-

tion of E. coli B tRNA.

Protein was determined by the method of Lowry et al., (40). The reagents were prepared as follows:

Double strength reagent A: 4% Na_2CO_3 in 0.2 N NaOH.

Reagent B: 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate.

Reagent C: 50 ml of reagent A and 2 ml of reagent B. This reagent was discarded after 1 day.

Reagent E: the Folin-Ciocalteu reagent (Fisher Scientific Co.) diluted to 1 N in acid.

To 2 ml of the sample to be tested, 2 ml of reagent C was added, mixed well, and allowed to stand for more than 10 minutes at room temperature. Four-tenths milliliter of reagent E was added rapidly and mixed well. After 30 minutes the optical densities were read using a Spectronic 20 at 750 mμ. The protein concentration was calculated by comparison with a standard curve prepared in the same manner with solutions of known concentration of bovine serum albumin.

Deoxyribonucleic acid was determined by the method of Burton (41). Diphenylamine reagent was prepared by dissolving 1.5 g of recrystallized diphenylamine in 100 ml of redistilled acetic acid and adding 1.5 ml of concentrated H_2SO_4. One-tenth milliliter of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of diphenylamine reagent. To 0.2 ml of sample to be tested, 1 ml of diphenylamine reagent containing acetaldehyde was added. The color was developed by incubating at 30°C for 18 hours and was read on a Spectronic 20 at 600 mμ. The DNA concentration was calculated by comparison with a standard curve prepared in the same manner with solutions of known concentration of E. coli B DNA.

For determinations of radioactivity, samples were dissolved in Bray's solution and counted in a liquid scintillation counter.

The Uptake of [^{3}H] Uracil into RNA
in vivo by *E. coli* 113-3

The incorporation of [^{3}H] uracil into RNA was determined by counting radioactivity in a Tri-Carb liquid scintillation counter by a modification of the method of Edlin, Stent, Baker and Yanofsky (18). Bacteria were grown in the presence of vitamin B_{12} (4 µg/liter), harvested, washed to remove vitamin B_{12} and resuspended in 100 ml of glucose-salts medium and in 100 ml of glucose-salts medium supplemented with 4 µg vitamin B_{12} (300 mg wet weight of bacteria per 100 ml of medium). In one experiment 1.74 µg (0.072 mc/mg) of [^{3}H] uracil were added, while in the other experiment in addition to 3.48 µg (0.072 mc/mg) of [^{3}H] uracil, 1 mg of unlabeled uracil was added (10 µg uracil/ml). They were grown in side-arm flasks on a shaking machine at 37°C. At each time indicated two 2.5 ml portions of the culture were removed and turbidity measured. Five-tenths milliliter of culture was suspended in 2.0 ml of 5% trichloroacetic acid and the bacteria were collected on Millipore filters (HA Millipore filter funnel) and washed thoroughly with 50 ml of 2.5% trichloroacetic acid. The filters were dried and the amount of [^{3}H] radioactivity in each sample was determined by counting with a Tri-Carb liquid scintillation counter.

Preparation of Enzymes

Crude aminoacyl-tRNA synthetases were prepared according to the method of Stulberg, Novelli and Kelmers (42). Transfer ribonucleic acid methylating enzymes were prepared from the supernatant solution according

to Hurwitz et al., (35). Log phase cells of the 113-3 mutant, which had been grown in a medium containing 4 μg/liter of vitamin B_{12} and 50 mg/liter of methionine, were used as a source of synthetases and methylating enzymes.

All purification procedures in the preparation of crude aminoacyl-tRNA synthetases were carried out at 4°C. The procedure routinely used was the following: 10 grams (wet weight) of E. coli 113-3 were ground with a mortar and pestle with 25 grams of alumina A 305. The paste was suspended in 50 ml of 0.01 M Tris-HCl buffer, pH 7.4, containing 0.01 M magnesium acetate and 0.001 M 2-mercaptoethanol and the suspension was centrifuged at 35,000 x g for 40 minutes. The supernatant solution was decanted and then further centrifuged at 78,000 x g in the No. 30 rotor of a Spinco ultracentrifuge for 3 hours to remove particulate matter. The supernatant solution was dialyzed overnight against 2 liters of the same buffer. One-tenth volume of 10% cold streptomycin sulfate solution was added, and after having been stirred for 3 hours, the precipitate of nucleic acids was removed by centrifugation. The supernatant solution was adjusted to pH 7.5 with KOH and maintained at that pH during the addition of solid ammonium sulfate to 65% saturation. After having been stirred for half an hour, the precipitate was collected by centrifugation. The precipitate was redissolved in 35 ml of buffer and dialyzed overnight against 6 liters of buffer. One-fourth volume of cold glycerol was added to the enzyme solution and it was stored at -18°C. The enzymes were stable for at least one month.

The preparation of tRNA methylating enzymes was also carried out at 4°C in the following steps. Ten grams (wet weight) of E. coli 113-3

were ground with a mortar and pestle with 25 grams of alumina A 305. The disrupted cells were extracted with 50 ml of 0.01 M Tris buffer, pH 7.5, containing 0.01 M magnesium chloride, 0.005 M 2-mercaptoethanol and 0.001 M Versene. The suspension was centrifuged at 35,000 x g for 40 minutes and then at 78,000 x g for 3 hours. The supernatant solution was adjusted to a concentration of 1% streptomycin by the addition of 6% cold streptomycin sulfate. After having been stirred at 4°C for 20 minutes, the solution was centrifuged to remove nucleic acids. The supernatant solution was brought to 60% saturation with ammonium sulfate by addition of the solid salt. The precipitate was dissolved and dialyzed against the buffer. The enzymes were used immediately after being prepared.

Preparation of Aminoacyl-tRNA

Transfer ribonucleic acid was assayed for amino acid-accepting activity by a slight modification of the methods of Mans and Novelli (43), Bollum (44), Nishimura and Novelli (45) and Stulberg _et al._, (42). The incubation mixture varied in size depending on the experiment. Each 1.0 ml of incubation mixture contained: Tris-HCl buffer, pH 7.4, 100 μmoles; magnesium acetate, 40 μmoles; potassium chloride, 5 μmoles; ATP, 20 μmoles; CTP, 0.5 μmole; 2-mercaptoethanol, 1 μmole; tRNA, 0.5 mg; [^{14}C] or [^{3}H]-amino acids, 40 mμmoles ([^{14}C], 10.8 μc; [^{3}H], 19.2 μc); 40 mμmoles of each of the 19 remaining non-radioactive amino acids; and enzyme, 0.5 mg. After 25 minutes of incubation at 37°C, an equal volume of water-saturated phenol was added. The mixture was shaken vigorously at 0°C and then centrifuged for 10 minutes at 2000 x g. The aqueous layer was removed and the phenol was shaken again with 1/3-1/2 volume of

water. The water phase was extracted 2-4 times with ether to remove the phenol and then 0.1 volume of 20% potassium acetate, pH 4.5, and 2.5 volumes of chilled ethanol were added. The precipitate was left for several hours at -18°C, centrifuged and dissolved in 2-5 ml of chromatography solution A.

In vitro Enzymic Methylation of tRNA

Methylation of tRNA was performed according to the modification of Hurwitz *et al.*, (35) and Capra and Peterkofsky (46). The reaction mixture contained the following: Tris-HCl buffer, pH 8.2, 5 μmoles; magnesium acetate, 1 μmole; ammonium acetate, 20 μmoles; 2-mercaptoethanol, 0.5 μmole; Me-[^{3}H]-S-adenosylmethionine, 0.5 mμmole (0.5 μc); tRNA, 100 μg; and enzyme, 60 μg, in a final volume of 200 μl. The mixture was incubated for 90 minutes at 37°C and then tRNA was precipitated with 5% trichloroacetic acid at 0°C. The precipitate was filtered on a Millipore HA membrane and washed with 30 ml of 5% trichloroacetic acid. The filter disc was then transferred to a counting vial, neutralized with 0.5 ml of 1.5 M ammonia and dissolved in 15 ml of Bray's solution. The radioactivity was measured in a liquid scintillation counter. Control samples contained all compounds with the exception of tRNA or protein or Me-[^{3}H]-S-adenosylmethionine. These compounds were added to the cooled reaction mixtures just before the precipitation of tRNA with trichloroacetic acid.

Separation of Aminoacyl-tRNAs by Reverse Phase Chromatography

The columns were prepared according to Kelmers and Weiss (47). Aliquot 336 (methyl tricaprylyl ammonium chloride) in Freon 214 (tetrachlorotetrafluoropropane) was supported as a thin film on hydrophobic

diatomaceous earth. This organic phase was prepared by dissolving Aliquot 336 in Freon 214 on a 5% by volume basis and was washed successively with two volumes each of 1 M NaOH, 1 M HCl and 0.5 M NaCl to remove any water soluble contaminants. The organic phase was removed and dried over silica gel. The organic phase (336 ml) was slowly dropped into 600 grams of Chromosorb W with mixing on a shaking machine for several days to provide for even distribution of the organic phase on the solid support. Before being applied to the column, it was made into a slurry with initial elution solution A. The column (200 x 1 cm dimension) was filled with initial elution solution A and the slurry was poured into the column to settle while maintaining maximum possible aqueous flow through the column. Equilibrium was obtained by running the initial elution solution A through the column before applying the sample. The [^{3}H]-aminoacyl-tRNA (usually 3 mg) or the mixture of [^{14}C] and [^{3}H]-aminoacyl-tRNA (usually 100,000 cpm of each) was applied to the column. Elution was effected with a concave 0.30 - 0.65 M sodium chloride gradient containing 0.01 M sodium acetate buffer, pH 4.5, 0.01 M magnesium acetate and 0.001 M EDTA at room temperature (48, 49). A nine-chamber gradient generator was employed to produce the concave gradient. The composition of solutions used in generating the gradient is shown below:

Chamber No.	Solution A (ml)	Solution B (ml)
1	140	0
2	128	12
3	124	16
4	117	23

Chamber No.	Solution A (ml)	Solution B (ml)
5	106	34
6	93	47
7	79	61
8	52	88
9	0	140

Solution A: 0.30 M sodium chloride containing 0.01 M sodium acetate buffer, pH 4.5, 0.01 M magnesium acetate and 0.001 M EDTA.

Solution B: 0.65 M sodium chloride containing 0.01 M sodium acetate buffer, pH 4.5, 0.01 M magnesium acetate and 0.001 M EDTA.

Five to six milliliter fractions were collected at the rate of one tube every 20-25 minutes. A sample of 0.5 ml of each fraction was then mixed with 15 ml of Bray's solution and the radioactivity was measured in the liquid scintillation counter.

CHAPTER III

RESULTS

Characteristics of the E. coli Strain 113-3

The *E. coli* mutant strain 113-3, which has an absolute requirement for either vitamin B_{12} or methionine (50), was used. This strain was obtained by selection from ultraviolet irradiation of *E. coli* strain W by Davis (51). The organism was grown in glucose-salts medium and in glucose-salts medium supplemented with 0.05 mg/ml of L-methionine at 37°C with shaking. The turbidity was measured at intervals of one hour. Figure 1 presents the growth characteristics. Adequate growth was obtained with 23 hours of incubation at 37°C on a shaking machine, the initial stationary phase continuing for a period of nearly 12 hours.

When the culture was grown for 18 hours, centrifuged, washed to remove L-methionine and resuspended in the original glucose-salts medium, the cell turbidity did not change during a 22 hour period. This is in contrast to previous reports that there was an increase in turbidity during methionine starvation (21, 22). Borek *et al.*, (21) explained the increase in turbidity as due to an increase in cell size or a change in the light scattering properties of the starved cells since they found no detectable change in viable cell number throughout a six hour methionine starvation.

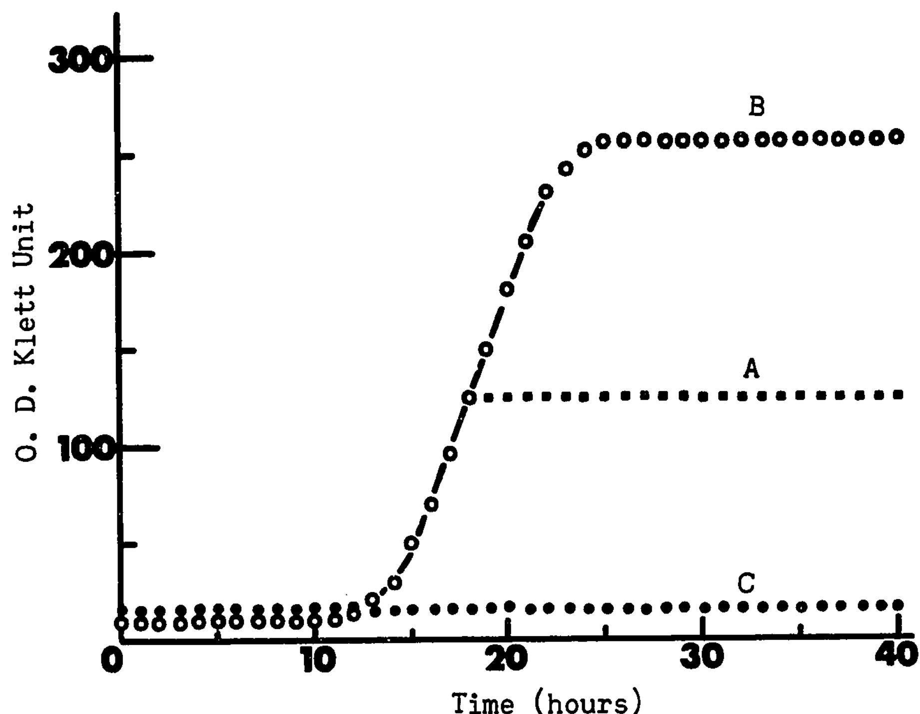


Figure 1. The growth of E. coli 113-3 during methionine supplementation and during methionine starvation.

Curve A - E. coli 113-3 were grown in glucose-salts medium supplemented with 50 mg per liter of L-methionine for 18 hours at 37°C with aeration. Bacterial cells were then collected by centrifugation, washed with glucose-salts medium (without L-methionine) five times, resuspended in glucose-salts medium to the original volume and again incubated at 37°C with aeration for an additional 22 hours. Optical density was measured with a Klett-Summerson photoclectric colorimeter.

Curve B - E. coli 113-3 were grown in glucose-salts medium containing 50 mg methionine per liter for 40 hours.

Curve C - E. coli 113-3 were suspended in glucose-salts medium without methionine or vitamin B_{12} for 40 hours.

We have confirmed the previous report that the strain requires 20 μg of methionine per ml for optimum growth on this medium (50). The results are presented in Figure 2. Transfer ribonucleic acid (defined as normal tRNA) isolated from organisms grown with at least 20 μg per ml of L-methionine is expected to contain a full complement of methyl groups while tRNA (defined as deficient tRNA) isolated from those grown with less than 20 μg per ml of L-methionine should contain some undermethylated tRNA. In the latter case methylated tRNA should be formed in the early log phase growth, but undermethylated tRNA should be gradually produced beginning during late log phase growth (35, 36). Methyl-deficient tRNA can also be produced from "normal" bacteria after transfer into and incubation in a medium without methionine (21).

Since this strain requires either vitamin B_{12} or methionine for full growth, it was of interest to investigate the effects of vitamin B_{12} on its growth. Equal portions of bacteria were grown in glucose-salts medium only, in glucose-salts medium supplemented with 4 μg per liter of vitamin B_{12}, and in glucose-salts medium with 50 mg per liter of L-methionine. Growth curves for these three conditions are presented in Figure 3. The initial stationary phase in the presence of vitamin B_{12} is shorter than that in the presence of methionine while the log phase remains the same. The growth curve for bacteria in the presence of both methionine and vitamin B_{12} is the same as that for bacteria grown with vitamin B_{12} only. The cells do not show any multiplication in glucose-salts medium alone.

In certain bacteria, especially *E. coli* 113-3, the pathway for methionine biosynthesis from its precursor, homocysteine, is believed to

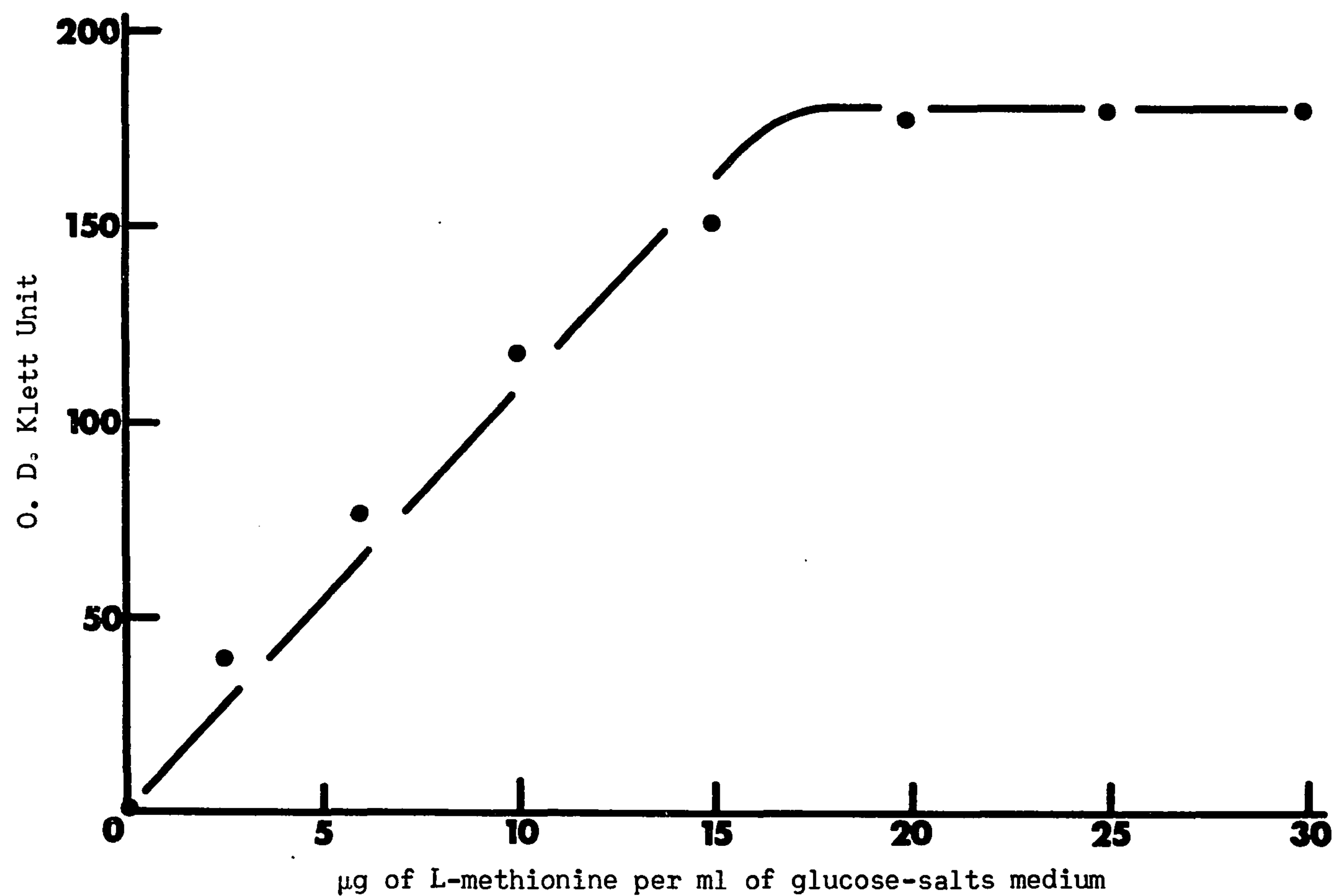


Figure 2. Quantitative determination of the amount of methionine required for growth of E. coli 113-3.

Bacteria were grown in cultures of glucose-salts medium supplemented with various amounts of L-methionine as indicated at 37°C with aeration for 18 hours. Optical densities of the cultures were determined as in Figure 1.

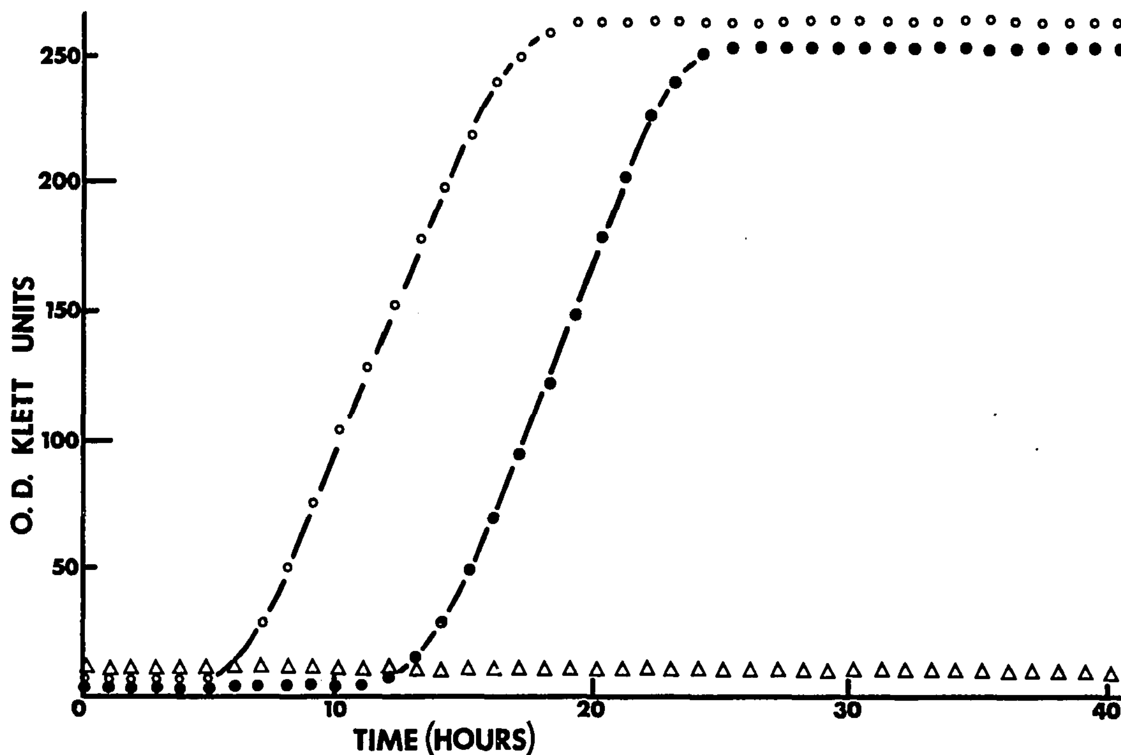


Figure 3. The growth characteristics of E. coli 113-3 with respect to vitamin B_{12} and methionine.

Bacterial cells were transferred into 100 ml of glucose-salts medium; -△-△-, glucose-salts medium only; -o-o-, glucose-salts medium supplemented with 8 μg of vitamin B_{12} per liter of medium; -•-•-, glucose-salts medium supplemented with 50 mg of methionine per liter of medium. The cells were grown at 37°C on a shaking machine and turbidity was measured at intervals of one hour with a Klett colorimeter.

involve methyl vitamin B_{12}, which is derived from vitamin B_{12} and methyl tetrahydrofolate and serves as an immediate methyl donor for homocysteine (52, 53, 54, 55, 56, 57, 58). It has been proposed (59, 60, 61) that enzyme-bound methyl vitamin B_{12} is the true intermediate in that the enzyme-bound vitamin B_{12} is methylated by reaction with N^5-methyl tetrahydrofolate. The methyl group is then transferred to homocysteine. The methyl group of methyl tetrahydrofolate is derived from carbon dioxide, which is obtained as a product of glucose breakdown by means of the Krebs cycle or as a component of air. In addition, methyl tetrahydrofolate is derived from some other sources, such as the β-carbon of serine and formate (62). It is also found that methanol can serve as a methyl donor for vitamin B_{12} in the formation of methyl vitamin B_{12} (63). The possible metabolic pathways for methyl vitamin B_{12} are summarized in Figure 4.

In the absence of exogenous methionine, vitamin B_{12} is necessary for synthesis of methionine in this strain. In this case the initial stationary phase in the presence of methionine should be shorter than that in the presence of vitamin B_{12}. However, the opposite is the case in this experiment. Alternately, the assumption may be made that vitamin B_{12} and methionine differ in their abilities to penetrate the cell wall and that the cells can utilize vitamin B_{12} faster than methionine.

In order to determine the quantity of vitamin B_{12} necessary for optimum growth, equal portions of bacteria were grown in glucose-salts medium supplemented with different amounts of vitamin B_{12}. Figure 5 shows the results. It is apparent that this strain requires at least 1.0 μg of vitamin B_{12} per liter of glucose-salts medium. A tremendous difference between the quantitative requirements for vitamin B_{12} and

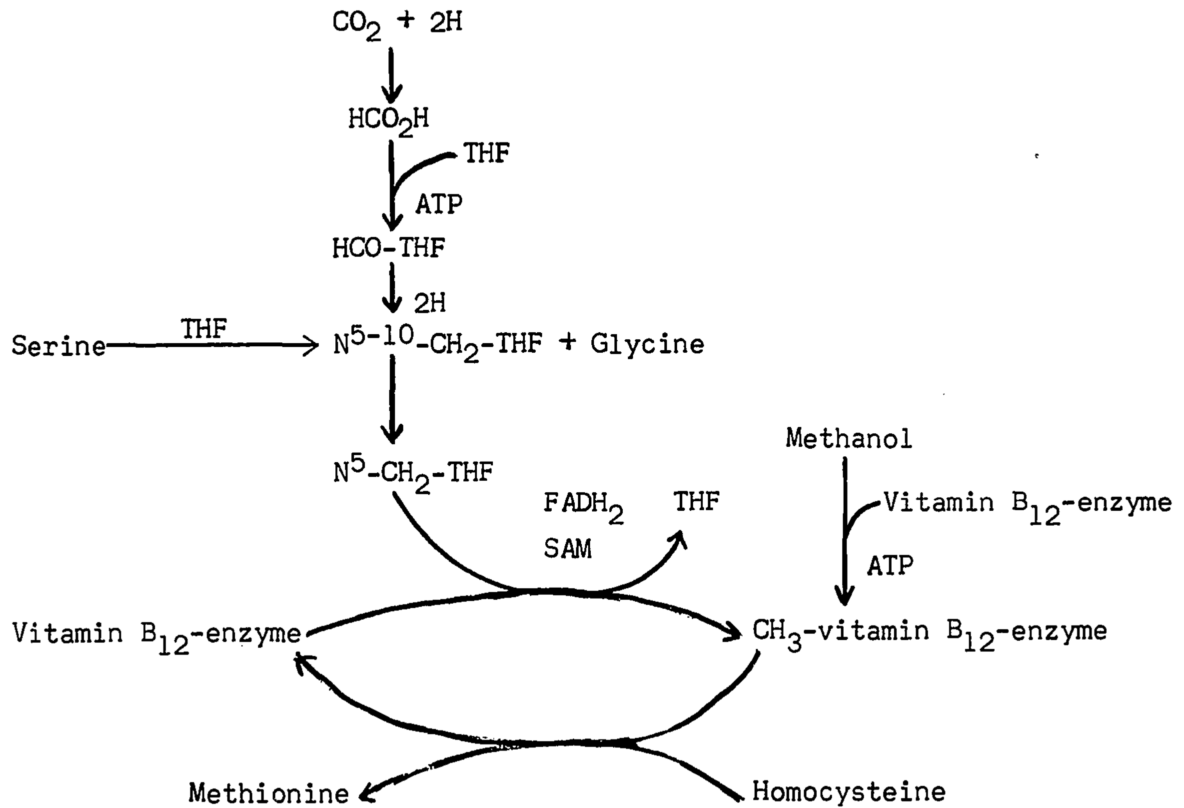


Figure 4. Biosynthetic pathways in methyl-vitamin B_{12} formation.

Abbreviations used: THF, tetrahydrofolate; SAM, S-adenosylmethionine; ATP, adenosine triphosphate; $FADH_2$, reduced flavine adenine dinucleotide.

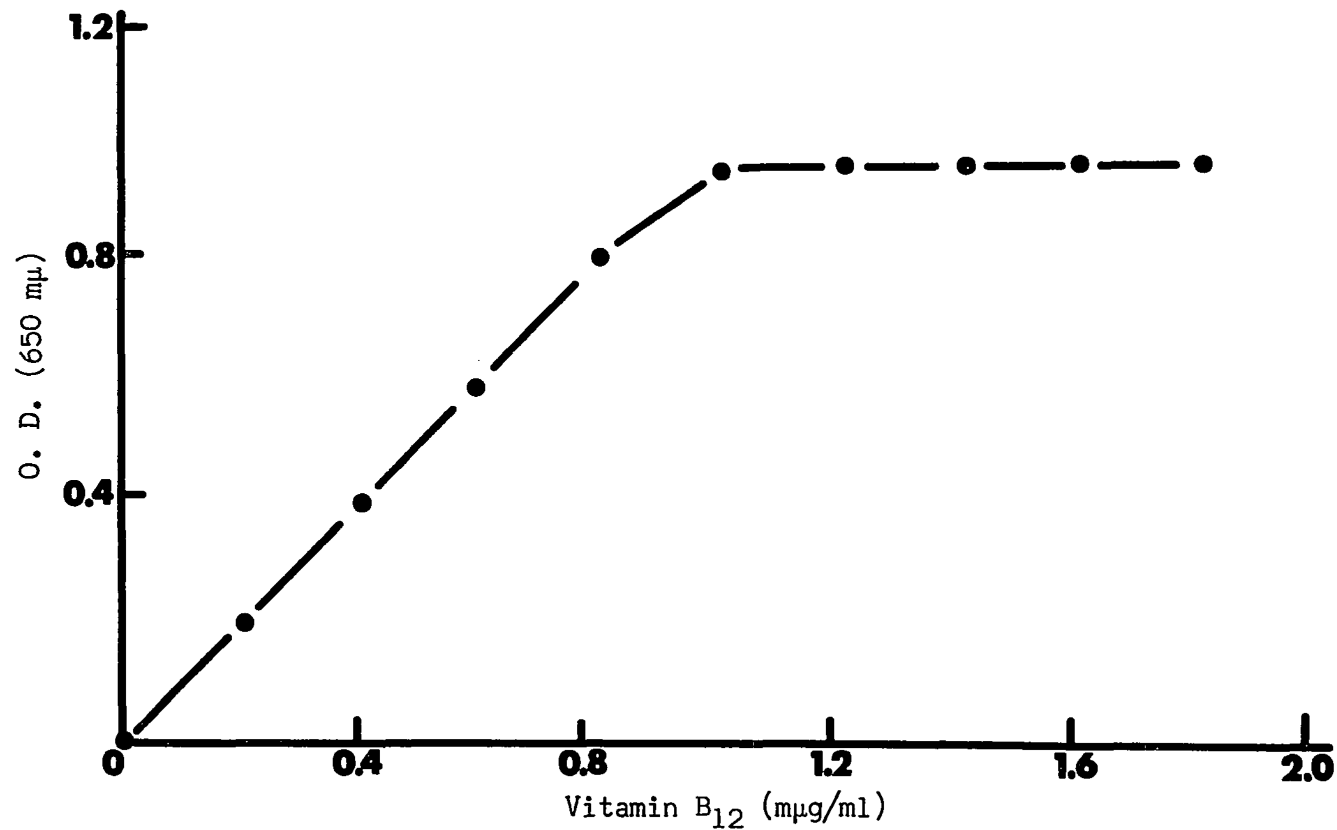


Figure 5. Growth of *E. coli* 113-3 in glucose-salts medium supplemented with increasing amounts of vitamin B_{12}.

Bacteria were transferred into test tubes with 10 ml glucose-salts medium supplemented with different amounts of vitamin B_{12} as indicated and grown for 13 hours at 37°C on a shaking machine. The turbidity was measured at 650 mμ with a Beckman DB spectrophotometer.

methionine is apparent. This difference reflects the catalytic role of vitamin B_{12}. Hatch et al., (52) have reported that vitamin B_{12} is associated with a protein moiety as a tightly bound prosthetic group forming the B_{12} enzyme, which is concerned with the transfer of the methyl group to form methionine in E. coli 113-3. Since this strain is believed to have a genetic block in methyl vitamin B_{12} biosynthesis which results in a secondary phenotypic block at a step between homocysteine and methionine, it is considered that 1.0 µg of vitamin B_{12} per liter of glucose-salts medium is sufficient for this reaction and that enough methionine can thereby be produced to meet cell requirements.

In order to investigate their growth properties in the absence of vitamin B_{12}, bacteria were grown aerobically in the presence of vitamin B_{12} for 13 hours, harvested, washed and resuspended in glucose-salts medium with and without vitamin B_{12}. Equal aliquots of cells in the culture were removed at intervals of one hour and the turbidity measured. Figure 6 shows that in the presence of vitamin B_{12} the cells multiply normally until the culture becomes unfavorable for growth while in the absence of vitamin B_{12} the turbidity increases slowly for about two hours but thereafter remains constant. The initial stationary phase resulting from transfer from agar to glucose-salts medium (Fig. 3) is not present when the cells have been grown previously under normal conditions. When the bacteria are transferred from agar to glucose-salts medium supplemented with vitamin B_{12} (or methionine), the bacteria are probably in a metabolically inactive form. Once the bacteria have been grown in a favorable medium they appear to be metabolically active.

As compared with the observation that there was no increase in

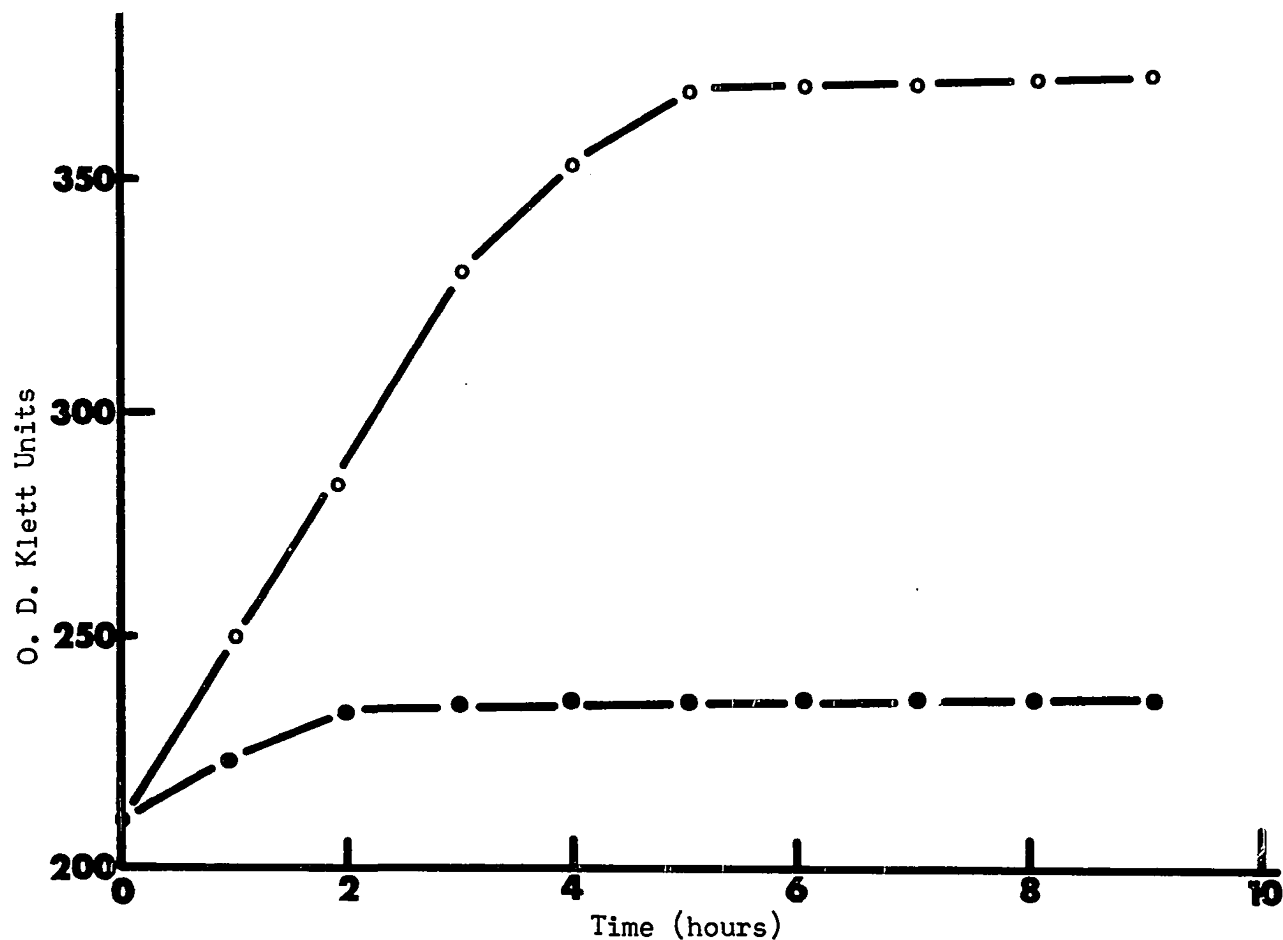


Figure 6. The growth of E. coli 113-3 in the presence and absence of vitamin B_{12}.

Bacteria (300 mg) grown in the presence of vitamin B_{12} for 13 hours and washed to remove vitamin B_{12} were transferred into side-arm flasks with 100 ml of glucose-salts medium (-•-•-) or with 100 ml of glucose-salts medium supplemented with 4 µg of vitamin B_{12} (-o-o-). They were grown at 37°C on a shaking machine and turbidity was measured at intervals of one hour.

the turbidity during methionine starvation, a small increase in turbidity is observed for about two hours when vitamin B_{12} is deprived. Because vitamin B_{12} has a very strong binding ability, the small increase in turbidity may be attributed to the trace amount of vitamin B_{12} adhering to the cell wall after washing. Alternately, this may be due to the presence of vitamin B_{12} enzyme produced in the normal condition which allows the formation of some methionine. Thus, it requires about two hours for complete vitamin B_{12} depletion.

Synthesis of RNA and tRNA during Methionine Starvation

In order to investigate the synthesis of RNA under conditions of methionine starvation, normal bacteria obtained from 18 hours growth in the presence of excess methionine were washed, resuspended in 1/3 of the original volume (about 300 ml) of medium without methionine, and incubated. Equal volumes of this suspension were withdrawn at one hour intervals and the growth of the bacteria estimated. RNA and protein were isolated from each sample and the amounts of RNA and protein synthesized during methionine starvation were determined. The data are presented in Table 1. It can be seen that neither the turbidity nor the amount of protein showed any increase during methionine starvation while the amount of RNA increased continually for at least 6-8 hours.

An RNA ratio was computed by dividing the total amount of RNA present after methionine starvation by the amount of RNA present before starvation. The RNA ratio for three hours methionine starvation is 1.21, which is equal to an RNA increment of 21%, whereas the RNA ratio for six hours methionine starvation is 1.475, equal to an RNA increment of 47.5%.

TABLE 1

SYNTHESIS OF RNA AND PROTEIN DURING METHIONINE STARVATION

Starvation Time (hrs)	Bacterial growth (O.D.Klett Unit)	Amount of Protein (μg)	Amount of RNA (μg)	RNA Ratio[a]
0	378	28.0	19.0	1.000
1	380	26.2	20.4	1.072
2	380	26.2	21.5	1.132
3	379	26.0	23.0	1.210
4	380	26.7	24.3	1.280
5	378	26.3	24.6	1.295
6	378	26.0	28.0	1.475
7	380	26.1	28.0	1.475
8	378	26.0	29.0	1.525
9	378	26.3	28.3	1.490

[a]RNA ratio is defined as the amount of RNA after methionine starvation divided by the amount of RNA before starvation.

The organisms were grown for 18 hours in glucose-salts medium supplemented with 50 mg/liter of L-methionine. The bacteria were collected by centrifugation, washed to remove methionine, resuspended in about 1/3 of the original volume of the glucose-salts medium lacking L-methionine, and incubated again with aeration at 37°C. A 30 ml portion of the culture was removed each hour and turbidity at 620 mμ was measured. Each 30 ml portion was centrifuged, washed and suspended in 20 ml of sterilized water. Five-tenths ml was diluted to 2 ml with trichloroacetic acid to give a final concentration of 5%, heated at 100°C for 20 minutes, cooled and centrifuged. One-tenth ml of the acid-soluble supernatant solution was used to determine RNA. One-tenth ml of the acid-insoluble precipitate was used to determine protein as described in materials and methods.

It is also observed that synthesis of RNA essentially stops after six hours methionine starvation. These results are similar to those reported for _E. coli_ 58-161 (21, 22).

Since these results showed that synthesis of RNA is relaxed (continued synthesis of RNA in the absence of methionine) significantly in _E. coli_ 113-3, it was of interest to investigate what class of RNA is increased. It has been proved that the methylated bases present in RNA in small amounts are mainly localized in the soluble RNA fraction and methionine is the origin of the methyl groups of these minor components. Accordingly tRNA synthesis was investigated. A large quantity of normal bacteria was prepared from 10 liter cultures of glucose-salts medium supplemented with excess methionine (50 mg per liter). They were harvested with a Sharples super-centrifuge and washed. The yield of these normal bacteria was from 1.5 to 2.3 gm wet weight per liter. The bacteria were divided into three portions as shown in Table 2. Two portions were further incubated in the methionine-free medium to produce three hour and six hour methionine deficient bacteria. The tRNA from each sample was prepared from the supernatant fraction of the ruptured cells and the amount of tRNA determined. Table 2 shows the results. It is apparent that the amount of tRNA per gram of bacterial wet weight increased from 3.2 to 10.70 during three hours methionine starvation and to 13.60 during six hours methionine starvation.

It appears that both _E. coli_ 113-3 and _E. coli_ 58-161 contain about the same amount of tRNA when grown under normal conditions. Peterkofsky and Capra (64) reported 1.13 mg tRNA per gram wet cells when extracted in 10 mM magnesium ion. This agrees very well with the present

TABLE 2

SYNTHESIS OF tRNA DURING METHIONINE STARVATION

Starvation Time (hours)	Bacterial Wet Weight Before Starvation (gms)	Bacterial Wet Weight After Starvation (gms)	tRNA Extracted (mgs)	mg tRNA / gm Bacterial Wet Wt.	tRNA Ratio[a]
0	9.13	-----	30.4	3.32	1.00
3	21.50	19.40	208.0	10.70	3.22
6	31.00	27.50	374.0	13.60	4.10

[a]The tRNA ratio is defined as the amount of tRNA after methionine starvation divided by the amount of tRNA before starvation.

Organisms were grown in glucose-salts medium supplemented with 50 mg/liter of L-methionine for 18 hours, then harvested with a Sharples super-centrifuge, washed and suspended in water. This suspension was divided into 3 parts and each centrifuged. All these bacterial pellets were weighed to determine the wet weight before starvation and then two of the pellets were resuspended in glucose-salts medium without methionine (about 2 gm of bacterial wet weight per liter) and incubated at 37°C with aeration for 3 hours and 6 hours. The zero hour starvation cells were not incubated again. The tRNA was prepared and determined as described in materials and methods.

finding of 3.32 on the basis of the reports that extraction with 0.1 mM magnesium ion gives threefold better yields (37, 38).

The cell turbidity does not change during a period of nine hours methionine starvation and the bacterial wet weight (Table 2, column 3) indicates only a small loss in cell wet weight after three hours and six hours of starvation. This loss may be due to incomplete recovery of cells during harvesting with the Sharples super-centrifuge. It is clear that the cells do not multiply during starvation while tRNA increases from a ratio of 1 to 3.22 (222% increment) after 3 hours and to 4.10 (310% increment) after 6 hours methionine starvation. Actual synthesis of tRNA is presumably higher than the results presented here indicate because degradative, as well as synthetic, reactions probably occur during the starvation period (9). Even if degradation ceased during methionine starvation, no increase in tRNA would be seen without synthesis.

Synthesis of RNA During Vitamin B_{12} Starvation

In order to compare [^{3}H] uracil incorporation into RNA in the presence and in the absence of vitamin B_{12}, bacteria were grown with both an excess and a limited supply of [^{3}H] uracil. At each time indicated equal portions of this culture were withdrawn and the [^{3}H] radioactivity incorporated into RNA was determined. Figures 7 and 8 show that the incorporation of [^{3}H] uracil into RNA in the absence of vitamin B_{12} is similar to that in the presence of vitamin B_{12} although the level of net RNA synthesis is lower in the absence of vitamin B_{12}. The degradation of RNA is apparent when the amount of [^{3}H] uracil is limited. Under such conditions RNA is degraded completely in about four hours in the presence of vitamin B_{12} while in the absence of vitamin B_{12} the degradation is slow

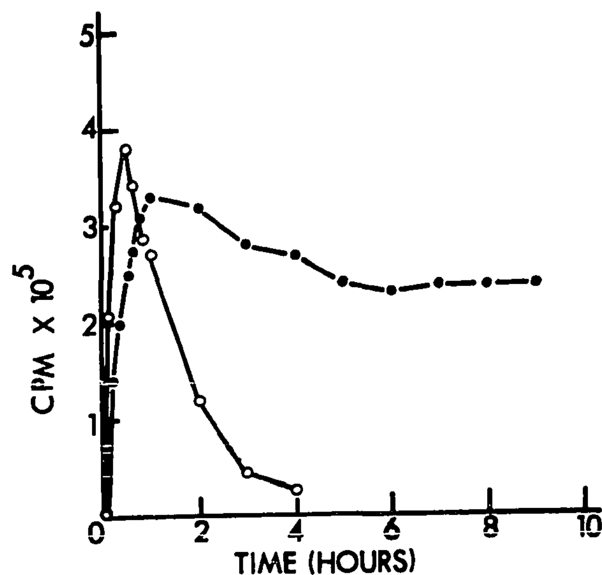


Figure 7. RNA synthesis as determined by the uptake of [^{3}H] uracil into RNA in vivo from a limited supply of uracil.

Bacterial cells (300 mg bacterial wet weight per 100 ml glucose-salts medium) which had been grown in the presence of an excess of vitamin B_{12} (4 μg/liter) and then washed thoroughly were transferred to two side-arm flasks. One flask contained 100 ml of glucose-salts medium (-•-•-) and the other contained 100 ml of glucose-salts medium supplemented with 4 μg vitamin B_{12} (-o-o-). To each flask was added 1.74 μg of [^{3}H] uracil (0.072 mc/mg). The flasks were incubated at 37°C on a shaking machine. At each time indicated 2.5 ml samples were withdrawn from each flask. Five-tenths milliliter of each sample was washed with 50 ml of 2.5% trichloroacetic acid to remove unincorporated radioactivity and counted in a Tri-Carb liquid scintillation counter.

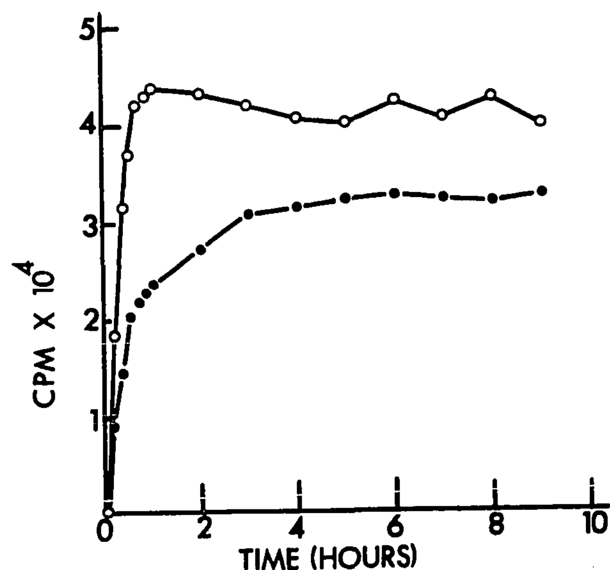


Figure 8. RNA synthesis as determined by the uptake of [^{3}H] uracil into RNA *in vivo* from an unlimited supply of uracil.

Bacterial cells (300 mg bacterial wet weight per 100 ml glucose-salts medium) which had been grown in the presence of an excess of vitamin B_{12} (4 μg/liter) and then washed thoroughly were transferred to two side-arm flasks. One flask contained 100 ml of glucose-salts medium (-•-•-) and the other contained 100 ml of glucose-salts medium supplemented with 4 μg vitamin B_{12} (-o-o-). To each flask were added 3.48 μg of [^{3}H] uracil and 1.0 mg of unlabeled uracil, making a total of 1003.48 μg of [^{3}H] uracil (0.25 μc/mg). The flasks were incubated at 37°C on a shaking machine. At each time indicated 2.5 ml samples were withdrawn from each flask. Five-tenths milliliter of each sample was washed with 50 ml of 2.5% trichloroacetic acid to remove unincorporated radioactivity and counted in a Tri-Carb liquid scintillation counter.

and is balanced by synthesis after six hours. When [^{3}H] uracil is in excess, its net incorporation into RNA increases during the first hour as in the case of a limited amount of [^{3}H] uracil. In the presence of vitamin B_{12} the dynamic process is balanced after one hour while in the absence of vitamin B_{12}, net incorporation slowly increases for almost six hours. However, when growth in the presence of vitamin B_{12} is taken into consideration and pulse-labeled RNA is calculated on the basis of bacterial wet weight, the degradation process again becomes evident, as can be seen in Figures 9 and 10.

Further evidence for the synthesis of RNA during vitamin B_{12} starvation was obtained directly. Bacteria were re-grown in the absence of vitamin B_{12} and equal portions of the culture were removed at intervals and analyzed for RNA, DNA and protein. Figure 11 presents the synthesis of RNA in the presence and absence of vitamin B_{12} as determined by the orcinol method. Net synthesis of RNA in the presence of vitamin B_{12} ceases at about four hours which corresponds to the cessation of bacterial growth, but the synthesis of RNA during vitamin B_{12} starvation continues after bacterial growth ceases (Figure 6). The synthesis of RNA during vitamin B_{12} starvation is about 50% of that in the presence of vitamin B_{12}; however, there is no significant growth of bacteria in the absence of vitamin B_{12} and hence much more RNA synthesis per cell (Figure 12).

To provide a better understanding of RNA synthesis during vitamin B_{12} starvation, the synthesis of RNA was determined on the basis of milligrams RNA per gram bacterial wet weight and the RNA ratio was calculated. This ratio was computed by dividing the total amount of RNA

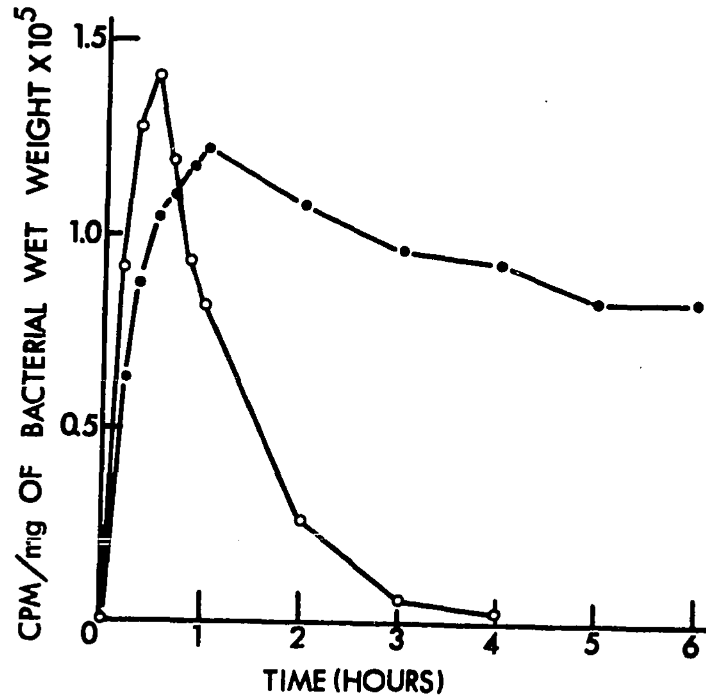


Figure 9. RNA synthesis per mg of bacterial wet weight as determined by the uptake of [^{3}H] uracil into RNA _in vivo_ from a limited supply of uracil.

The experiment is the same as that described in Figure 7. Every time a 2.5 ml sample was withdrawn, the turbidity was measured with a Klett-Summerson colorimeter. The Klett reading was compared to a standard curve to estimate the wet weight of cells present. [^{3}H] uracil uptake per mg of bacterial wet weight was then calculated. -o-o-, [^{3}H] uracil uptake in the presence of vitamin B_{12}; -•-•-, [^{3}H] uracil uptake in the absence of vitamin B_{12}.

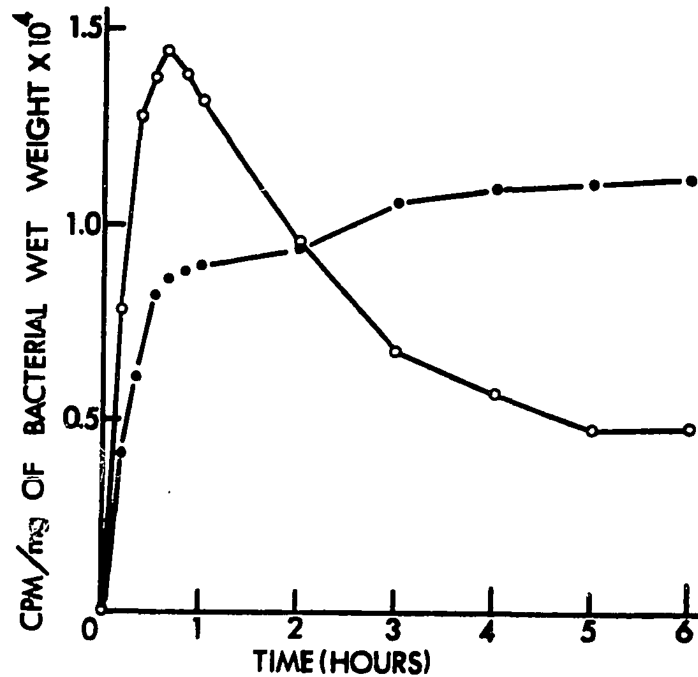
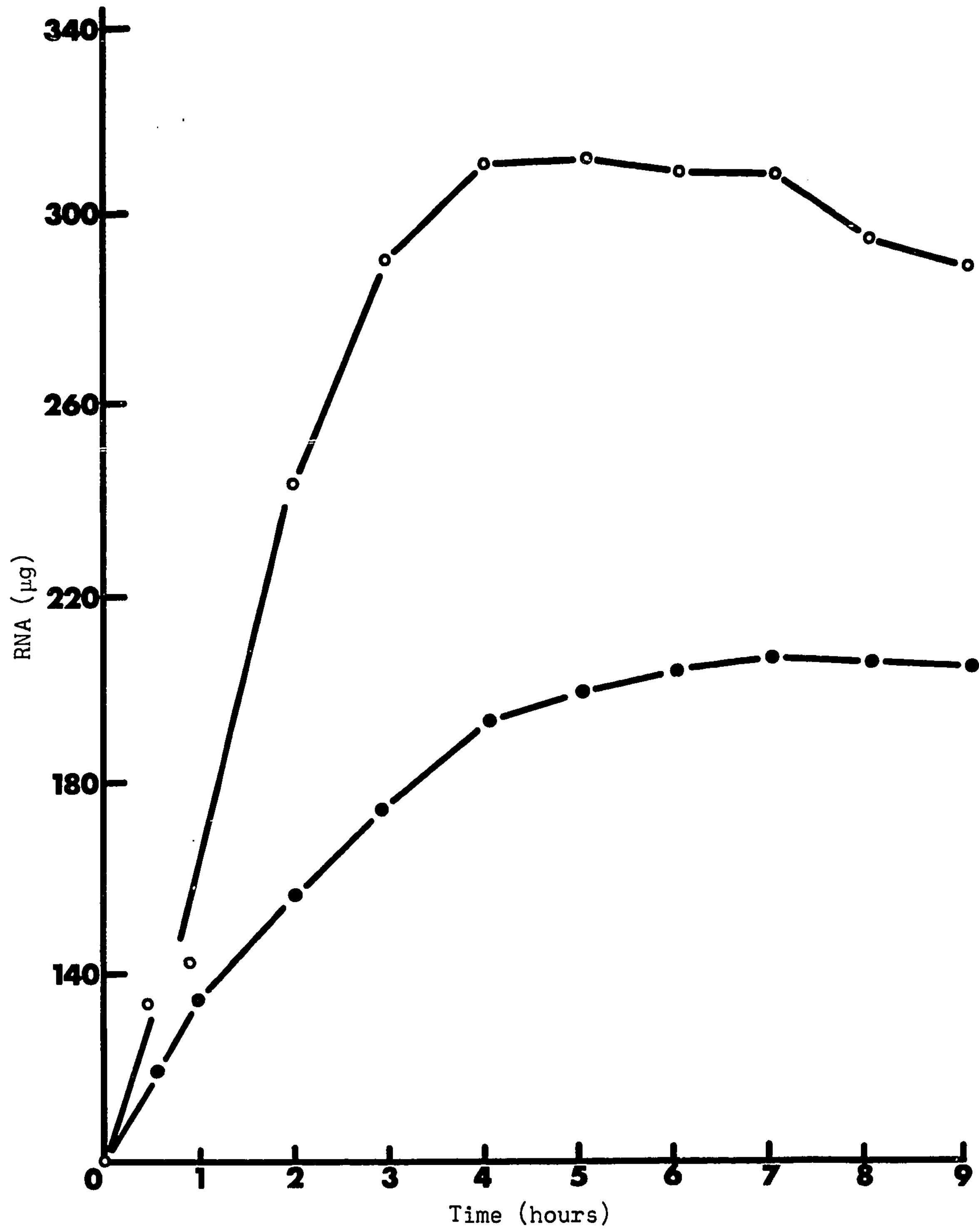


Figure 10. RNA synthesis per mg of bacterial wet weight as determined by the uptake of [^{3}H] uracil into RNA in vivo from an unlimited supply of uracil.

The experiment is the same as that described in Figure 8. Every time a 2.5 ml sample was withdrawn, the turbidity was measured with a Klett-Summerson colorimeter. The Klett reading was compared to a standard curve to estimate the wet weight of cells present. [^{3}H] uracil uptake per mg of bacterial wet weight was then calculated. -o-o-, [^{3}H] uracil uptake in the presence of vitamin B_{12}; -•-•-, [^{3}H] uracil uptake in the absence of vitamin B_{12}.

Figure 11. Synthesis of RNA during normal growth and vitamin B_{12} starvation in *E. coli* 113-3.

Bacterial cells grown in the presence of vitamin B_{12} (4 μg per liter of glucose-salts medium) were washed to remove vitamin B_{12} and transferred to two side-arm flasks with 100 ml of glucose-salts medium (about 300 mg of bacteria per 100 ml of medium). To one flask was added 4 μg of vitamin B_{12} while to the other no vitamin B_{12} was added. They were incubated at 37°C on a shaking machine. At 0.5 hour, one hour, and at one hour intervals thereafter, 4.0 ml samples were withdrawn, centrifuged, washed with 10 ml of deionized water, recentrifuged and resuspended in 4 ml of deionized water. One milliliter of each sample prepared in this way was diluted with 1 ml of 10% trichloroacetic acid and then heated at 100°C for 20 minutes, cooled and centrifuged. Five-tenths milliliter of the acid-soluble material was used to determine RNA as described in materials and methods. Results are reported on the basis of 1 ml of culture. -.-.-, RNA synthesis in the absence of vitamin B_{12}; -o-o-, RNA synthesis in the presence of vitamin B_{12}.


340
300
260
220
180
140
0
1
2
3
4
5
6
7
8
9
RNA (μg)
Time (hours)

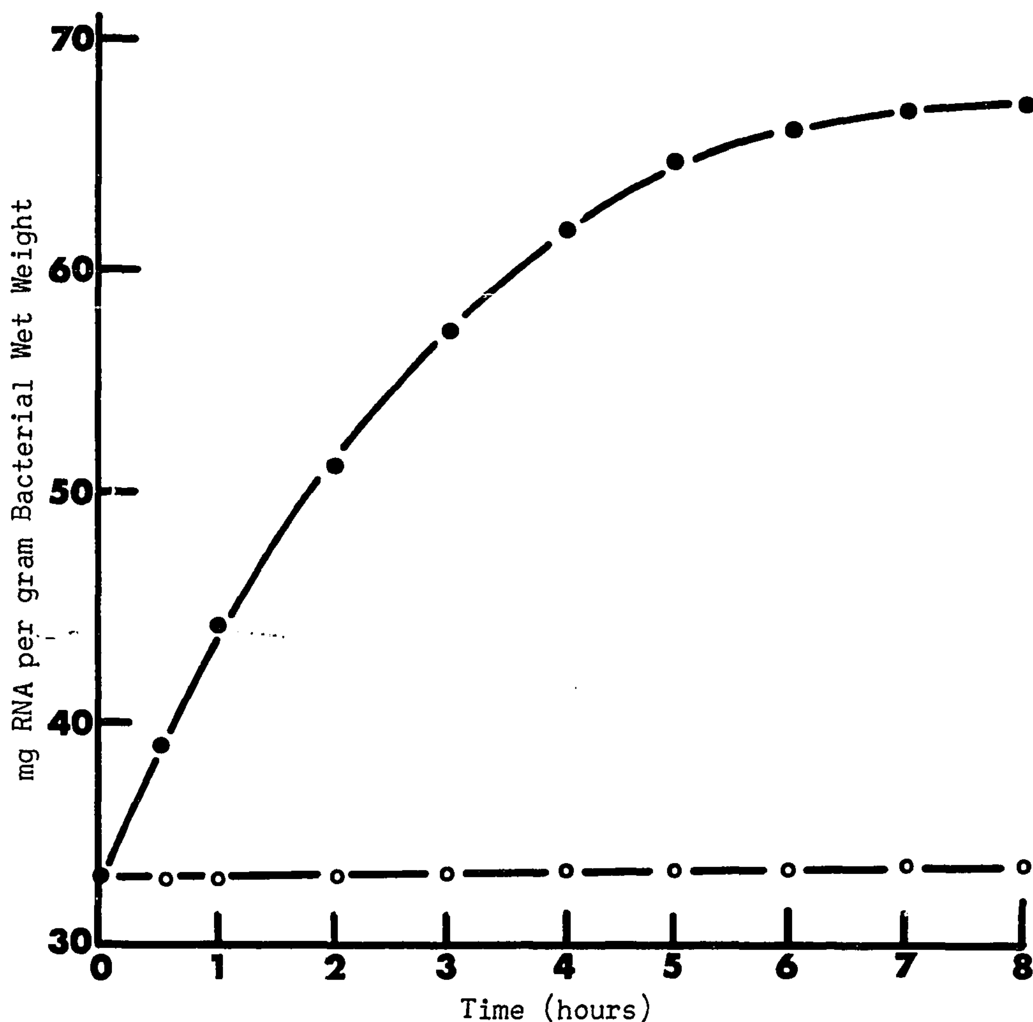


Figure 12. Synthesis of RNA expressed as mg RNA per gram bacterial wet weight during normal growth and vitamin B_{12} starvation.

The experiment is the same as that described in Figure 11. Every time a 4.0 ml sample was withdrawn, the turbidity was measured with a Klett-Summerson colorimeter. The Klett reading was compared to a standard curve to estimate the wet weight of cells present. RNA synthesis per gram bacterial wet weight was then calculated. -•-•-, RNA synthesis in the absence of vitamin B_{12}; -o-o-, RNA synthesis in the presence of vitamin B_{12}.

present after vitamin B_{12} starvation by the amount of RNA present before starvation. The results are presented in Table 3. It is clear that RNA is synthesized by *E. coli* 113-3 during vitamin B_{12} starvation. The RNA ratio for three hours starvation is 1.74, which is equal to an RNA increment of 74%, and the RNA ratio for six hours starvation is 2.06, equal to an RNA increment of more than 100%.

DNA and protein synthesis in the presence and absence of vitamin B_{12} were also investigated in the same experiment. The results are presented in Figures 13 and 14. In the presence of vitamin B_{12} the synthesis of DNA and protein proceeds for about four hours, corresponding to the period of bacterial growth. In the absence of vitamin B_{12}, DNA synthesis continues for six hours, corresponding to the period of RNA synthesis, but protein synthesis does not continue.

Synthesis of tRNA During Vitamin B_{12} Starvation

Transfer ribonucleic acid synthesis during vitamin B_{12} starvation was investigated for purposes of comparison with the results reported under conditions of methionine starvation. Bacteria grown in the presence of excess vitamin B_{12} were washed thoroughly and divided into three portions as indicated in Table 4. Two of the portions were grown again in the absence of vitamin B_{12} for three and six hours. An increase in bacterial wet weight is observed in accordance with the increase in turbidity shown in Figure 6.

Using the same isolation procedure the bacteria provide almost the same amount of tRNA per gram of bacterial wet weight, i.e., in the presence of vitamin B_{12} about 3.20 mgs of tRNA were extracted from one gram wet weight of bacteria while in the presence of methionine about

TABLE 3

SYNTHESIS OF RNA DURING VITAMIN B_{12} STARVATION

I	II	III	IV
Starvation Time (hours)	Amount of RNA (μgs/4 ml)	mgs RNA / gm Bacterial Wet Wt.	RNA Ratio
0	400	32.9	1.00
0.5	480	39.2	1.20
1	540	44.0	1.35
2	630	51.5	1.57
3	700	57.2	1.74
4	772	63.0	1.91
5	800	65.3	1.98
6	812	66.4	2.01
7	828	67.6	2.06
8	824	67.1	2.04
9	828	67.6	2.06

The details of this procedure have been described in materials and methods. The RNA ratio is defined as the amount of RNA after vitamin B_{12} starvation divided by the amount of RNA before starvation. Column III is computed from the amount of RNA in column II divided by the bacterial wet weight in 4 ml of sample (12.25 mg). The bacterial wet weight is considered constant during vitamin B_{12} starvation.

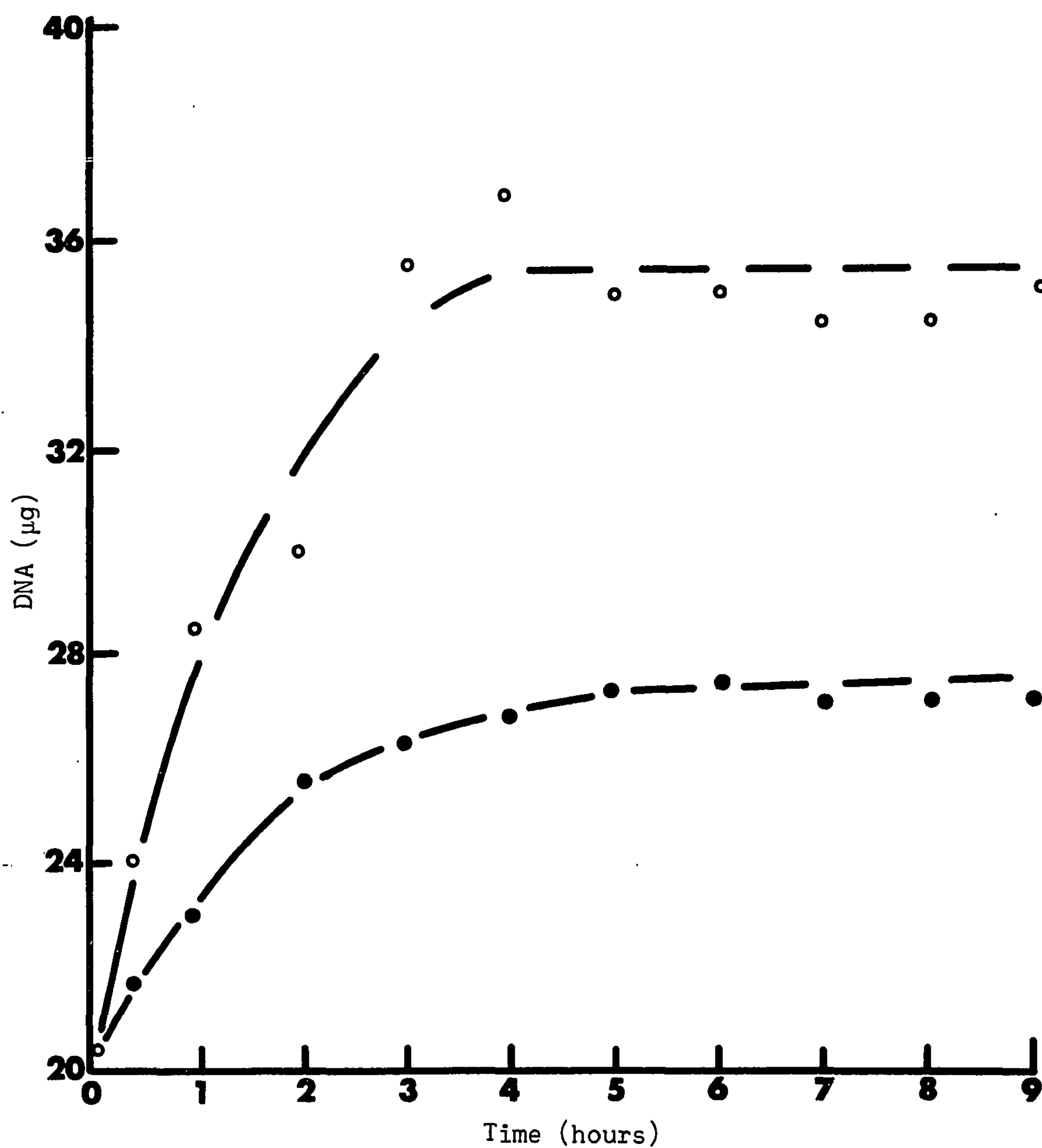


Figure 13. DNA synthesis during normal growth and vitamin B_{12} starvation.

The experiment is the same as that described in Figure 11 except that 0.4 ml of the acid-soluble material was used to determine DNA as described in materials and methods. Results are reported on the basis of 1 ml of culture. -•-•-, DNA synthesis in the absence of vitamin B_{12}; -o-o-, DNA synthesis in the presence of vitamin B_{12}.

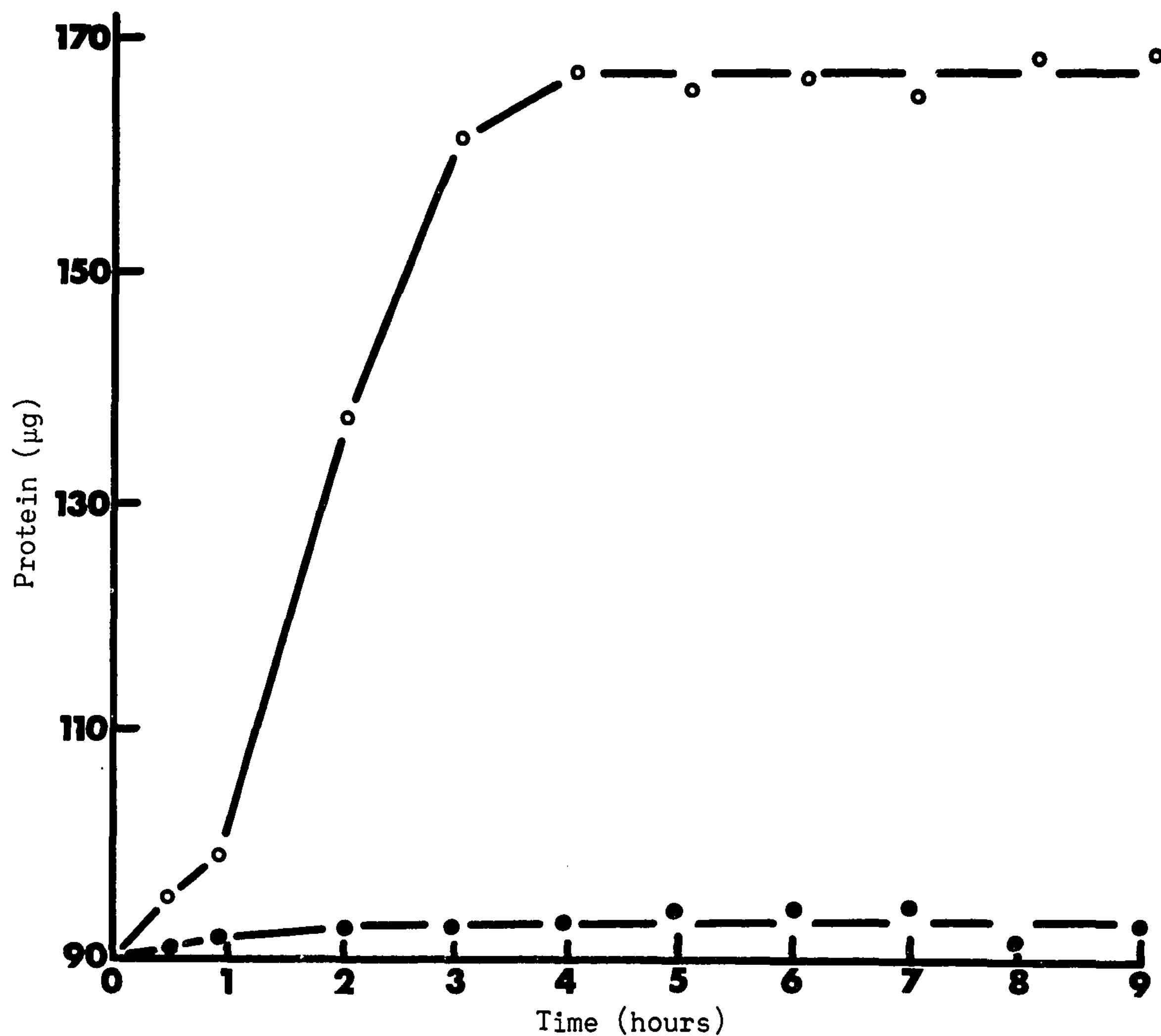


Figure 14. Protein synthesis during normal growth and vitamin B_{12} starvation.

The experiment is the same as that described in Figure 11 except that the acid-insoluble material was suspended in 3 ml of 0.2 N NaOH and 1.0 ml of each of these solutions was used to determine protein as described in materials and methods. Results are reported on the basis of 1 ml of culture. -•-•-, protein synthesis in the absence of vitamin B_{12}; -o-o-, protein synthesis in the presence of vitamin B_{12}.

TABLE 4

SYNTHESIS OF tRNA DURING VITAMIN B_{12} STARVATION

I	II		III	IV	V
Starvation Time (hours)	Bacterial Wet Weight		tRNA Extracted (mgs)	$\frac{\text{mgs tRNA}}{\text{gm Bacterial Wet Wt.}}$	tRNA Ratio[a]
	Before Starvation (gms)	After Starvation (gms)			
0	10.256	------	32.8	3.20	1
3	12.120	14.483	107.5	7.43	2.32
6	13.320	16.155	155.5	9.62	3.00

[a]The tRNA ratio is defined as the amount of tRNA after vitamin B_{12} starvation divided by the amount of tRNA before starvation.

The details of this tRNA preparation have been described in materials and methods.

3.32 mgs were extracted. From Table 4 it is clear that the amount of tRNA per gram of bacterial wet weight increased more than twofold during three hours starvation and threefold during six hours starvation.

Synthesis of rRNA During Vitamin B_{12} Starvation

It has been demonstrated that about 80 to 90% of the total RNA of the cell is rRNA and 10 to 20% is tRNA present in the "soluble" or "non-sedimentable" fraction of the cell (25, 65, 66). It is considered that the percentage of mRNA is small and can be neglected in order to calculate the amount of rRNA by subtracting tRNA from total RNA.

To investigate the synthesis of rRNA during vitamin B_{12} starvation, the amounts of rRNA synthesized were compared on the basis of milligrams RNA per gram bacterial wet weight (Table 5). The considerable synthesis of rRNA during vitamin B_{12} starvation is apparent. An increase of 67% in 3 hours and 91% in 6 hours starvation occurred. Such rRNA synthesis has also been reported during methionine or other essential amino acid starvation (8, 18, 22, 25). It is possible that the new rRNA synthesized during methionine starvation was undermethylated.

Synthesis of Specific tRNA During Methionine Starvation

Further confirmation of the synthesis of tRNA during methionine starvation can be achieved by resolution of specific sub-species of tRNA with reverse phase chromatography. This chromatographic technique has been used extensively during the study of specific aminoacyl-tRNAs (42, 47) as well as in the isolation of new species of undermethylated phenylalanyl-tRNA and leucyl-tRNA (9, 10, 11). Transfer ribonucleic acid prepared in the preceding experiments was subjected to [^{3}H]-amino acid-tRNA

TABLE 5

SYNTHESIS OF rRNA DURING VITAMIN B_{12} STARVATION

I	II	III	IV	V
Starvation Time (hours)	mgs RNA / gm Bacterial Wet Wt.	mgs tRNA / gm Bacterial Wet Wt.	mgs rRNA / gm Bacterial Wet Wt.	rRNA Ratio
0	32.9	3.20	29.70	1.00
3	57.2	7.43	49.77	1.67
6	66.4	9.62	56.78	1.91

This table was calculated from column III of Table 3 and column IV of Table 4. Milligrams rRNA per gram bacterial wet weight were obtained by subtracting milligrams tRNA per gram bacterial wet weight from milligrams total RNA per gram bacterial wet weight. This calculation is based on the assumption that the amounts of mRNA and other components which will give color reaction with orcinol are negligible.

acylation and the elution profiles from reverse phase chromatography of the tRNA samples from zero, three and six hours starvation were examined. The changes in elution profile of valyl-tRNA as a result of methionine starvation for these periods are presented in Figure 15. The results in Figure 15[A] are in agreement with the findings of Kelmers and Weiss (47) that valyl-tRNA from normally grown bacteria was resolvable into two peaks. However, the elution profiles of valyl-tRNA after three and six hours of methionine starvation indicated a profound change with the appearance of a third component which eluted earlier than the two original components. This new peak is higher after six hours methionine starvation than after three, while it is absent in the zero hour cells.

For further confirmation of the relative elution positions of each peak from normal and methionine starved organisms, normal tRNA was acylated with [^{14}C]-valine while deficient tRNA was acylated with [^{3}H]-valine. Figure 16 shows the elution profiles of normal and three and six hour methionine depleted tRNA using this double label. In agreement with the experiment employing a single label, the accumulation of peak I can be seen. The shape of each peak in the double label experiment is the same as the corresponding peak in the single label experiment and the coincidence of each elution position indicates that the appearance of the first peak component is not a chromatographic artifact.

Synthesis of Undermethylated tRNA During Methionine Starvation

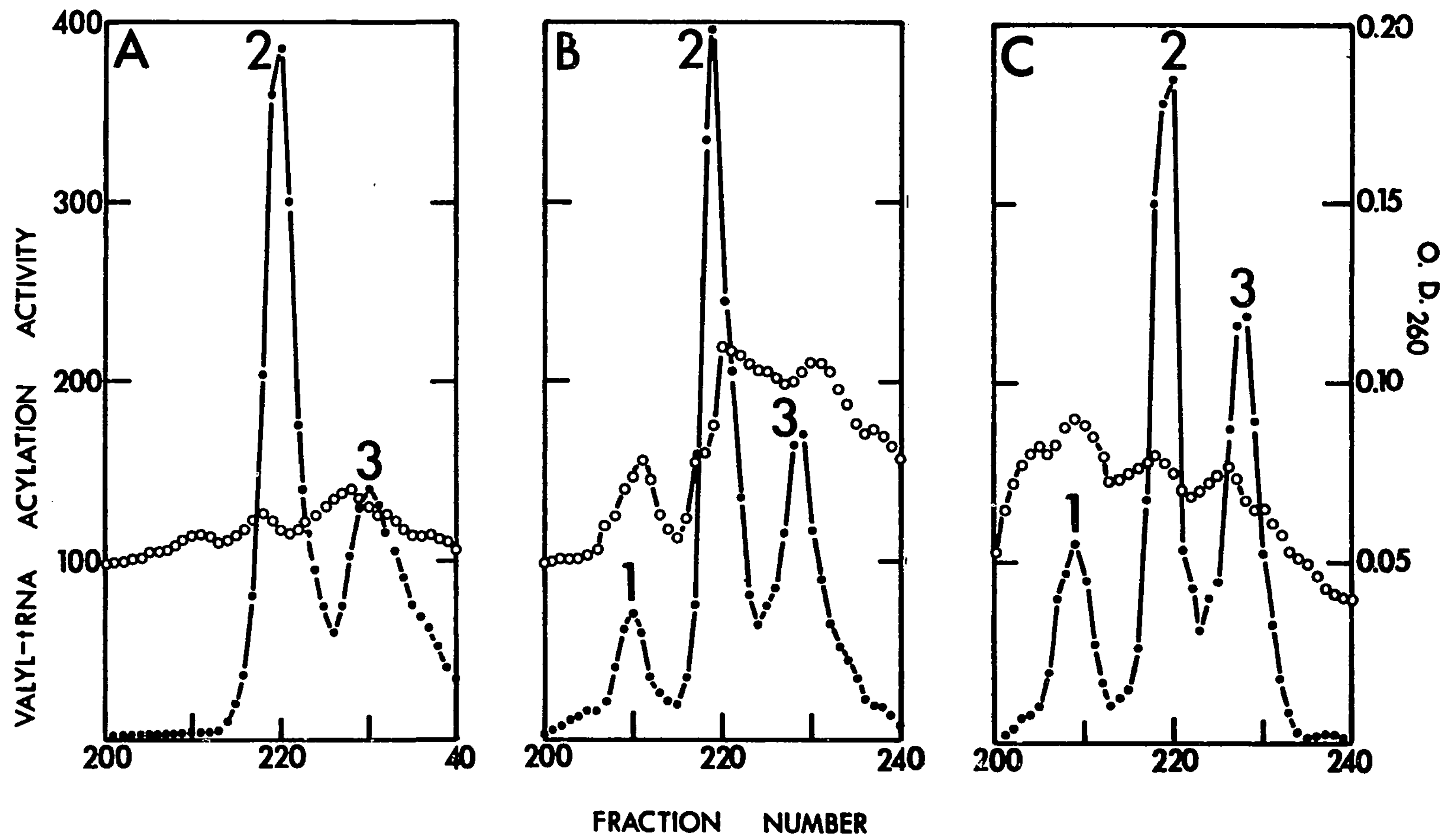
One of the best techniques to study the degree of methylation is to investigate the capacity of the prepared tRNA to be methylated by S-adenosylmethionine in the presence of methylating enzymes (35). Normal

Figure 15. Changes in the elution pattern of [^{3}H]-valyl-tRNA during methionine starvation.

Transfer ribonucleic acid was prepared from methionine starved cells after three different periods of incubation. The tRNA samples (3 mg) were charged with [^{3}H]-valine as described in materials and methods and were subjected to the column fractionation. Radioactivity of each 0.5 ml fraction was measured in a liquid scintillation counter. Absorbance at 260 mμ for each tube was also determined.

A - Normal [^{3}H]-valyl-tRNA
B - Three-hour methionine starved [^{3}H]-valyl-tRNA
C - Six-hour methionine starved [^{3}H]-valyl-tRNA

-o-o-, absorbance at 260; -•-•-, radioactivity (cpm).


A
B
C
1
2
3
400
300
200
100
200
220
40
240
0.20
0.15
0.10
0.05
VALYL-tRNA ACYLATION ACTIVITY
O. D. 260
FRACTION NUMBER

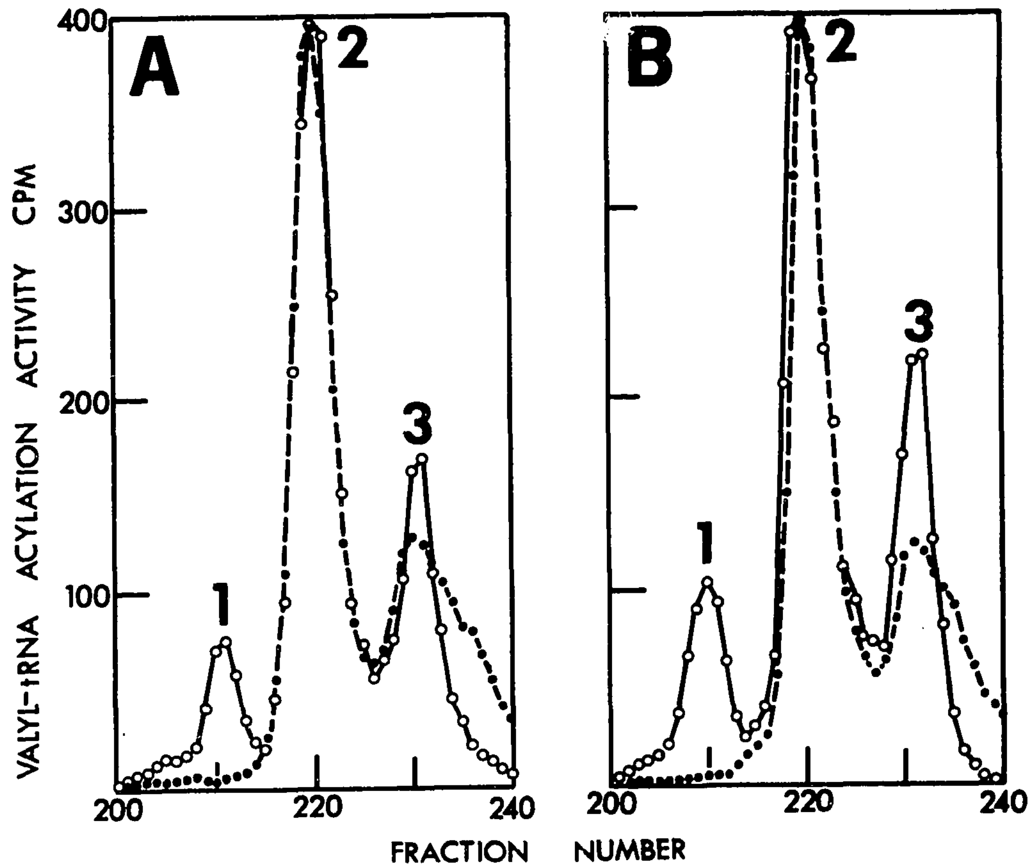


Figure 16. Elution pattern of normal [^{14}C]-valyl-tRNA and deficient [^{3}H]-valyl-tRNA by reverse phase chromatography.

Three milligrams of normal and deficient tRNA were charged with [^{14}C]-valine and [^{3}H]-valine separately as described in materials and methods. Equal amounts of radioactivity (100,000 cmp) from each sample were mixed. The mixture of [^{14}C] and [^{3}H] valyl-tRNA was applied to the column. Five-tenths milliliter of each fraction tube was measured in a liquid scintillation counter.

A - Double label of normal and three hour starvation tRNA elution profiles; -o-o-, three hour starvation [^{3}H]-valyl-tRNA; --.----.--, normal [^{14}C]-valyl-tRNA.

B - Double label of normal and six hour starvation tRNA elution profiles; -o-o-, six hour starvation [^{3}H]-valyl-tRNA; --.---.--, normal [^{14}C]-valyl-tRNA.

and six hour deficient tRNA were subjected to [^{3}H]-Me-S-adenosylmethionine for methylation. The extent of incorporation of radioactivity into the tRNA is given in Table 6. The data indicate that the tRNA synthesized during starvation is undermethylated. Thus, it appears probable that the newly synthesized valyl-tRNA peak I is a methyl-deficient species. The ability of this new species of valyl-tRNA accumulated during methionine starvation to perform normally in protein biosynthesis is open to question.

The Coordination Ratio of RNA Synthesis

The coordination ratio is a convenient way to evaluate if the synthesis of total RNA and tRNA is coordinate during methionine starvation. The term is defined as the tRNA ratio divided by the total RNA ratio. The tRNA ratio was defined as the amount of tRNA present after methionine starvation divided by the amount before methionine starvation, and the total RNA ratio as the amount of all classes of RNA present after methionine starvation divided by the amount present before starvation. If tRNA synthesis is coordinate with all other RNA synthesis, the coordination ratio will be one, but will not be equal to one if RNA synthesis is non-coordinate. A coordination ratio larger than one indicates synthesis of one particular type of RNA (tRNA in this case) at a rate in excess of the total, whereas a ratio less than one would indicate the opposite.

The coordination ratios for total and tRNA synthesis in *E. coli* 113-3 during methionine starvation are presented in Table 7. It is clear that the coordination ratio of tRNA to total RNA is considerably increased from 1.0 to 2.68 and to 2.78 during three and six hours of methionine

TABLE 6

THE CAPACITIES OF NORMAL AND DEFICIENT tRNAs FOR METHYLATION

Experiment	Activity (cpm/100 μg tRNA) tRNA from normal cells	Activity (cpm/100 μg tRNA) tRNA from deficient cells
1	200	792
2	70	670

Transfer ribonucleic acid from normal and six hour methionine starved cells was prepared as described in materials and methods. The methyl acceptance of the tRNA was carried out with [^{3}H]-Me-S-adenosylmethionine as described in materials and methods.

TABLE 7

THE COORDINATION RATIO OF tRNA TO TOTAL RNA IN *E. coli* 113-3
DURING METHIONINE STARVATION

Starvation Time	RNA Ratio[a]	tRNA Ratio[b]	Coordination Ratio[c]
0	1.000	1.00	1.00
3	1.210	3.22	2.68
6	1.475	4.10	2.78

[a]RNA ratio is defined as the amount of RNA after methionine starvation divided by the amount of RNA before starvation.

[b]tRNA ratio is defined as the amount of tRNA after methionine starvation divided by the amount of tRNA before starvation.

[c]The coordination ratio of tRNA to total RNA is computed by dividing the tRNA ratio by the RNA ratio at each starvation time.

starvation.

The coordination ratio during vitamin B_{12} starvation is presented in Table 8. The coordination ratio for tRNA is increased from 1.0 to 1.33 and 1.49 during three and six hours of vitamin B_{12} starvation while the coordination ratio for rRNA is unchanged. It is, therefore, concluded that the increase in tRNA is proportionally greater than the increase in rRNA.

The coordination ratio for rRNA is close to one at all the times. Since rRNA constitutes 90% of the total RNA, the changes in rRNA ratio during starvation are similar to the changes of total RNA ratio and thus coordination ratios for rRNA during starvation remain close to one. This, however, does not mean that RNA synthesis is coordinate. Since tRNA constitutes only 10% of the total RNA, a small increase in tRNA during starvation will change the tRNA ratio significantly while the total RNA ratio remains almost unchanged.

TABLE 8

THE COORDINATION RATIO OF E. coli 113-3 DURING VITAMIN B_{12} STARVATION

I	II	III	IV	V	VI
Starvation Time (hours)	RNA Ratio	tRNA Ratio	rRNA Ratio	Coordination Ratio with respect to tRNA	Coordination Ratio with respect to rRNA
0	1.00	1.00	1.00	1.00	1.00
3	1.74	2.32	1.67	1.33	0.96
6	2.01	3.00	1.91	1.49	0.95

This table was calculated from the last columns of Tables 3, 4 and 5. The coordination ratio is defined as the tRNA ratio or rRNA ratio divided by the total RNA ratio.

CHAPTER IV

DISCUSSION

The work presented here suggests that RNA synthesis in *E. coli* 113-3 is relaxed during methionine and vitamin B_{12} starvation and that the synthesis of different classes of RNA in this strain is non-coordinate. It has been demonstrated that *E. coli* 58-161 synthesizes different RNAs non-coordinately during amino acid deprivation. Borek *et al.*, (21) and Wild *et al.*, (22) have reported an increase of up to 100% in RNA while Peterkofsky and Capra (64) have reported an increase of up to 500% in tRNA during methionine starvation. These figures indicate a coordination ratio of tRNA to total RNA greater than unity and therefore non-coordinate synthesis of these two RNA species. Neidhardt and Eidlic (8), using *E. coli* K_{10}, an RC^{rel} mutant, also found a greater increase in tRNA than in total RNA under conditions of amino acid deprivation. The data presented here indicate that in the *E. coli* mutant 113-3 the synthesis of tRNA and rRNA increases during methionine deprivation. This is in agreement with the studies of other investigators using other types of mutants and different approaches (18, 19, 20, 30, 31, 32, 33, 67, 68).

We have also demonstrated that the synthesis of RNA during vitamin B_{12} starvation is relaxed as is that during methionine starvation in both this strain and *E. coli* 58-161. Transfer ribonucleic acid and rRNA

continue to be synthesized, but synthesis of protein and cell multiplication cease during vitamin B_{12} starvation. It is therefore further confirmed that E. coli 113-3 possesses the RC^{rel} allele which is mainly concerned with the regulation of tRNA and rRNA synthesis. When regulation of RNA synthesis is non-coordinate, the effect of the RC^{rel} mutation is not a cessation in mRNA synthesis, which is nearly normal during starvation (18). Our results from [^{3}H] uracil incorporation into RNA support this concept for E. coli 113-3. In contrast to the stringent strains, tRNA and rRNA syntheses are not drastically depressed. The synthesis of RNA during vitamin B_{12} starvation continues for about six hours and the relative increase in tRNA is greater than that of rRNA. All these results indicate that RNA synthesis in E. coli 113-3 is non-coordinate.

Argument against this non-coordinate concept is based on the fact that there is no cessation of mRNA, tRNA and rRNA syntheses during starvation in relaxed strains. Thus it seems necessary to consider the proposition that RNA synthesis in relaxed strains could be coordinate.

It appears that the rate of mRNA synthesis is not affected by starvation in either stringent or relaxed strains (18, 30, 31, 33). Accordingly the pulse-labeled RNA synthesis during vitamin B_{12} starvation is not controlled by the single RC locus alone. This single RC locus controls only the synthesis of rRNA and tRNA, i.e., the regulation of RNA synthesis is non-coordinate.

It should be pointed out that the amount of RNA synthesized during vitamin B_{12} starvation in this relaxed strain is about 50% of that found during exponential growth (Figure 11) while the increase in RNA during starvation is about 100% (Figure 12). Similar results have been

reported in _E. coli_ 58-161, a methionine-requiring strain (21, 22). This increase of 100% in RNA is mainly a result of the synthesis of tRNA and rRNA. On the other hand, it has been shown that a small amount of RNA (about 5 to 20% of that observed during exponential growth) is formed in the absence of an essential amino acid in stringent strains and that this RNA made during starvation is largely mRNA (18, 19, 20).

Thus, during starvation stringent strains synthesize up to 20% of the RNA synthesized during exponential growth and relaxed strains about 50% of that amount. It seems that the borderline between stringent and relaxed control is not well defined. Obviously the distinction between the stringent and relaxed control mechanisms depends on the synthesis of tRNA and rRNA controlled by the RC locus of the genome and this RC locus does not affect the synthesis of mRNA in either stringent or relaxed control.

Borek _et al_., (21) have reported a 30% increase in turbidity of cell suspensions during methionine starvation in _E. coli_ 58-161. This is attributed to either an increase in cell size or a change in the light scattering properties of the starved cells since there is no detectable change in the number of viable cells. In agreement with this result, there is an increase in turbidity during vitamin B_{12} starvation in E. _coli_ 113-3, but this phenomenon does not occur during methionine starvation. These results can be explained on the basis of the washing procedure to remove methionine and vitamin B_{12}. In my experiments the cells were washed five times with 150 ml of medium without methionine for methionine starvation and for vitamin B_{12} starvation they were washed ten times with the same amount of medium without vitamin B_{12}, while Borek

et al., washed the cells only once in their investigation. It is possible that trace amounts of methionine are still present if washing is not repeated. On the other hand, due to strong binding of vitamin B_{12} with the cell before starvation, trace amounts of methionine can be formed. These trace amounts of methionine in both strains will be enough to allow for increase in cell size but not cell division. Accordingly, the light scattering properties of the enlarged cells are changed.

When E. coli 113-3 cells are washed five times, methionine appears to be completely removed. This can be seen from the investigation during methionine starvation. First, there is no increase in turbidity. Second, the wet weight of the cells does not increase. Third, protein is not synthesized. These results indicate that the cells neither divide nor enlarge under such conditions. In contrast to methionine starvation the culture does increase in turbidity and wet weight but there is little protein synthesis during vitamin B_{12} starvation. This is also true for E. coli 58-161 as reported by Borek et al., (21).

It has been demonstrated that heterogeneity exists for many aminoacyl-tRNAs and that the heterogeneity is reproducible and is related to different codons for the same amino acid (69, 70). There are some types of tRNA which, as a result of modification, have multiple sub-species on chromatography after aminoacylation with specific amino acids. There are several ways in which one can obtain such sub-species in addition to the native species. Phage infected E. coli cells show an alteration in leucyl-tRNA elution profiles with methylated albumin-kieselguhr and reverse phase column chromatography (71, 72). Undermethylation of tRNA may also provide a source of new sub-species peaks which are dis-

tinct from the normal varieties. A new valyl-tRNA is reported here from methionine depleted E. coli 113-3. New undermethylated phenylalanyl-tRNA and leucyl-tRNA have been reported from E. coli 58-161 and G-15 (9, 10, 11). A marked difference in the coding response as a result of these heterogeneities has been found by ribosome binding assays (64, 73, 74).

Evidence to support the view that this heterogeneity is not a chromatographic artifact is based on the following observations. First, in a double auxotroph E. coli W-1305 (RC^{rel} leu^- met^-), new sub-species of leucyl-tRNA are not observed during leucine starvation while during methionine starvation, a new sub-species was found indicating that the new sub-species synthesized was a methyl-deficient tRNA (9). Second, new sub-species of leucyl-tRNA do not appear when the stringent auxotroph E. coli 58-161 (RC^{str} met^- $biotin^-$) is deprived of methionine (9). Third, for a double auxotroph of E. coli (RC^{rel} his^- met^-) tRNA synthesized during histidine starvation in the presence of methionine is methylated indicating that new undermethylated tRNA is not formed during histidine starvation (4).

Buck and Nass (75) have reported heterogeneity between mitochondrial and cytoplasmic aminoacyl-tRNAs and the presence of some aminoacyl-tRNAs specifically of mitochondrial origin in rat liver. They therefore believe that the specific roles of tRNAs in protein biosynthesis in mitochondria and in cytoplasm are distinguishable.

Finally, the observation of Bernhardt and Darnell (76) indicates a pre-tRNA which is transformed into tRNA by methylation. This precursor of tRNA also has been partially purified by Burdon and Clason (77). Recently, Peterkofsky and Capra (46) reported that the chromatographic pro-

file of abnormal leucyl-tRNA synthesized during six hours methionine starvation is converted to a profile which resembles that of normal leucyl-tRNA by *in vitro* methylation. Accordingly, the suggestion can be made that these new sub-species of valyl-tRNA, phenylalanyl-tRNA and leucyl-tRNA can be considered as examples of pre-tRNA.

CHAPTER V

SUMMARY

Escherichia coli mutant 113-3, derived from strain W, requires either vitamin B_{12} or methionine for growth.

Reverse phase chromatography revealed two peaks for valyl-tRNA under normal conditions, while under conditions of methionine deprivation an additional peak was found. These observations indicate the synthesis of a new species of undermethylated tRNA which is charged by valyl-tRNA synthetase. The possibility is discussed that this new undermethylated sub-species of valyl-tRNA synthesized in the absence of methionine is a pre-tRNA.

Ribonucleic acids are synthesized without concomitant protein synthesis and cell multiplication when a culture of this mutant is incubated in the absence of methionine. The amounts of total RNA and tRNA synthesized during methionine starvation depended on the duration of incubation. The coordination ratio of tRNA to total RNA indicated that synthesis of tRNA is regulated non-coordinately with reference to total RNA during methionine starvation. It is proposed that all classes of RNA are regulated non-coordinately in the absence of methionine in this strain.

In the absence of vitamin B_{12} in glucose-salts medium, cell

multiplication and protein synthesis stop, but RNA and DNA syntheses continue. Quantitative determinations show a significant increase in the amount of tRNA and rRNA during vitamin B_{12} starvation, but the degree of increment of tRNA is greater than that of rRNA. The coordination ratio indicates that the different classes of RNA are regulated non-coordinately in the absence of vitamin B_{12} in this strain in that the RC^{rel} mutation mainly concerns the regulation of tRNA and rRNA syntheses with either vitamin B_{12} or methionine.

The incorporation of [^3H] uracil into RNA during vitamin B_{12} starvation is similar to that during exponential growth in the presence of vitamin B_{12} although the overall level of RNA synthesis during vitamin B_{12} starvation is lower than that during exponential growth. With a limited amount of [^3H] uracil present in the medium, the [^3H] radioactivity incorporated into RNA decreases to zero in about four hours during exponential growth, but in the absence of vitamin B_{12} the decrease is very slow. When [^3H] uracil is present in the medium in excess, the degradative and synthetic processes are balanced during exponential growth, while in the absence of vitamin B_{12} the synthetic process exceeds the degradative process.

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