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Physiological and Biochemical Studies on the Function of 5-Methyluridine in the Transfer Ribonucleic Acid of *Escherichia coli*

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Matched pairs of transductant strains differing by the presence or absence of 5-methyluridine (ribothymidine) (m^5U) in their transfer ribonucleic acid (tRNA) were used to study the function of this modified nucleoside in *Escherichia coli*. Ordinary measurements of growth rate in different media revealed no effect of the loss of m^5U in tRNA. A gene located close to *trmA* (the structural cistron for the methyltransferase that produces m^5U in tRNA), however, was found to reduce the growth rate significantly, depending on the medium and the temperature of cultivation. Measurement of codon recognition, macromolecular composition, tRNA binding to the ribosome, and the rate of protein chain elongation in vivo indicated no disadvantage caused by the lack of m^5U. The regulation of *ilv* and *his* operons seemed also to be unaffected by the absence of m^5U in the tRNA. In a mixed population experiment, however, cells possessing m^5U in their tRNA seemed to have a distinct advantage over cells lacking this modified nucleoside. This experiment provides the first indication of the overall value of m^5U in tRNA.

The sequence of several transfer ribonucleic acid (tRNA) chains has now been determined. All have been found to contain different modified nucleosides, such as methylated nucleosides, thiolated nucleosides, pseudouridine, dihydrouridine, and cytokinin-containing nucleosides (5). Methylated nucleosides are among the most abundant of these modified nucleosides. On the average, they constitute about 2.7 residues on each tRNA chain (27, 39; this paper). The most common methylated nucleoside is 5-methyluridine (m^5U), which has been suggested to occur in one copy per tRNA chain (52). Furthermore, m^5U has been found in the same position in most of the tRNA chains hitherto sequenced, namely, as part of the common sequence G-m^5U-ψ-C (5). The biosynthesis of methylated nucleosides is now fairly well understood; the methylation takes place at the polynucleotide level and is catalyzed by a set of enzymes, the tRNA methyltransferases, using *S*-adenosyl-L-methionine or a tetrahydrofolate derivative as the methyl donor (3, 18, 43, 44). It can be foreseen that the enzyme responsible for this interesting feature of the tRNA structure will be fully characterized before long (see reviews, references 43 and 44).

Although the different kinds of modification of tRNA have been known for along time, the function of the various modified nucleosides is poorly understood. Few tools have been available for investigating the function of most of the modified nucleosides. Generally methyl-deficient tRNA has been used in efforts to shed some light on the function of the methylated nucleosides, and some effects of general methyl deficiency have been demonstrated on amino acid charging, ribosome binding, and codon specificity (see reviews, references 32, 36, 43, and 44). Such studies cannot identify which particular methylated nucleoside is responsible for any particular effect. The function of each individual methylated nucleoside must therefore be studied by other techniques. When mutants lacking m^5U in their tRNA (*trmA* mutants) were isolated (8), a specific tool for investigating the function of m^5U became available, and it became possible to perform physiological experiments to reveal possible effects in vivo of the absence of the m^5U in the tRNA. Results from such experiments are presented in this paper.

Since the mutants described above were heavily mutagenized they were considered un-

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suitable for comparative physiological experiments. The construction of strains isogenic except for the *trmA* allele required knowledge about the genetic location of the *trmA* gene. By transduction experiments described in the preceding paper (7) this gene was located close and clockwise to the *argH* gene (65% co-transduction) at 79 min on the *Escherichia coli* chromosomal map (48). It was then possible by transduction to construct matched pairs of Trm$^+$ and Trm$^-$ cells, and such pairs have been used in all experiments to be reported here. In brief, our results indicate that the loss of m^{5}U from tRNA does not detectably affect the overt growth rate of *E. coli* cells, nor does it affect codon recognition, cell macromolecule composition, tRNA binding to ribosomes, regulation of *ilv* and *his* operons, or the rate of protein chain elongation. Nevertheless, Trm$^-$ cells are at a selective disadvantage when grown together with their Trm$^+$ counterparts. Preliminary reports of some of these results have already been presented (9, 10).

MATERIALS AND METHODS

Abbreviations. Abbreviations used in this paper are those recommended by the Commission on Biochemical Nomenclature (CBN-1970): ψ, pseudouridine; m^{5}U, 5-methyluridine (ribothymidine); m$_2^6$Ade, N^6-dimethyladenine; m^{7}Gua, 7-methylguanine; m^{5}Ura, 5-methyluracil; and so forth. In addition, the tRNA methyltransferase producing m^{5}U is called tRNA (m^{5}U)methyltransferase (EC 2.1.1.35), and *trm* and Trm$^-$ designate the genotype and phenotype, respectively, of tRNA methylation-defective mutants. TGYE medium is a rich medium containing tryptone, glucose, and yeast extract. IPTG is isopropyl-β-D-thiogalactoside.

Strains of bacteria and phages. Derivatives of *E. coli* K-12 have been used in this work. Strain AB1932 (obtained from D. G. Fraenkel) is *metA*, *argH* and λ lysogenic (22); GB1 strains are derivatives of strain AB1932; strain LMUR (obtained from P. Sypherd) is auxotrophic for uracil and lysine, *metB*, *relA*, ribonuclease I$^-$ and λ^- (47; G. Skogman, personal communication); strain G11 is *ilv*, *metB*, *relA*, Hfr, and λ lysogenic (45). Trm$^+$ and Trm$^-$ transductants used are derivatives of strains AB1932, LMUR, and G11, and their relevant properties are summarized in Table 1. For some experiments involving suppression, the LMUR derivatives used were made λ lysogenic.

The following 23 amber mutants of phage T4 were used: T4 *amHL* 628, H11, *am16*, B17, *am20*, *amB21*, *am24*, *am52*, *am54*, *am58*, *am65*, *am81*, *am85*, A459, E757, *amN55*, *amB269*, *amE10*, *am4535d8*, *r67am134*, *amA453*, *am130*, and *am116*; the following 12 ochre mutants of phage T4 (all in the rII region) were also used: N12, N17, X20, N21, N24, N29, AB53, N55, SD160, UV199, 360, and UV375. Two T4 UGA mutants (X655 and X665) (also in the rII region) were used. All mutants were obtained from I. Tessman and R. L. Sommerville, Purdue University.

Media and method of cultivation. The minimal medium used was solution P of Fraenkel and Neidhardt (23), supplemented with $(NH_4)_2SO_4$ (0.2%) and a carbon source (0.2%). The L-epimers of the amino acids were added to a final concentration of 50 μg/ml. The rich medium (TGYE) employed was composed of 10 g of tryptone, 5 g of yeast extract, and 2 g of glucose per liter. Rotary shakers were used to grow the cells aerobically, at the temperature indicated, in Erlen-

TABLE 1. *Characteristics of different transductants of E. coli*[a]

Transductants	Donors	Selected markers	*trm* allele	Auxotrophic markers	References
GB1-4-II9	IB4	*metA*$^+$-*thiA*$^+$	+	*argH*	(7)
GB1-4-IB	IB4	*metA*$^+$-*thiA*$^+$	A4	*argH*	(7)
LMUR-4-269	IB4	*metB*$^+$	+	*lys*, *ura*	L. Isaksson, Uppsala, Sweden
LMUR-4-268	IB4	metB$^+$	A4	*lys*, *ura*	L. Isaksson, Uppsala, Sweden
GB1-5-41	IB5	*metA*$^+$-*thiA*$^+$	+	*argH*, Lac$^-$	(7)
GB1-5-41 Lac$^+$ [b]	IB5	*metA*$^+$-*thiA*$^+$		*argH*, Lac$^+$	
GB1-5-39	IB5	*metA*$^+$-*thiA*$^+$	A5	*argH*, Lac$^-$	(7)
GB1-5-39 Lac$^+$ [b]	IB5	*metA*$^+$-*thiA*$^+$	A5	*argH*, Lac$^+$	
LMUR-5-267	IB5	*metB*$^+$	+	*lys*, *ura*	L. Isaksson, Uppsala, Sweden
LMUR-5-266	IB5	*metB*$^+$	A5	*lys*, *ura*	L. Isaksson, Uppsala, Sweden
G11-5-17	IB5	*metB*$^+$	+	*ilv*	L. Isaksson, Uppsala, Sweden
G11-5-18	IB5	*metB*$^+$	A5	*ilv*	L. Isaksson, Uppsala, Sweden
GB1-9-10	IB9	*argH*$^+$-*thiA*$^+$	+	*metA*	This paper
GB1-9-7	IB9	*argH*$^+$-*thiA*$^+$	A9	*metA*	This paper
GB1-1metA$^+$	CP79	*metA*$^+$	+	*thiA*	This paper

[a] Phage Plkc or Plv was grown on indicated donors which are described in the preceding paper (7). They possess the *trmA* allele indicated in the table. The recipient *E. coli* strains AB1932 (*metA*, *thiA*$^+$, *argH*), whose derivatives are designated GB1, LMUR (*lys*, *ura*, *metB*) and G11 (*ilv*, *metB*), were all Trm$^+$ and are further described in Materials and Methods.

[b] The Lac$^+$ phenotype was transduced into GB1-5-41 and GB1-5-39.

meyer flasks filled to one-fifth their capacity. Optical density (OD) at 420 nm, as measured by a Zeiss PMQII spectrophotometer with a path length of 1 cm, was used to monitor growth. When growth rate was to be accurately measured, the cells were pregrown for several generations in the same medium and kept below OD_{420} = 1.0 (2×10^8 cells/ml) to assure that the cells were in balanced growth. The growth rate was determined by the rate of increase in cell mass between OD_{420} = 0.1 to 1.0. At least three measurements were made during each doubling. Growth rates are expressed as the specific growth rate constant, k, calculated by the relation: k = ln2/mass doubling time in hours.

Mixed population experiment. *E. coli* strains GB1-5-41 (*trmA*$^+$, *sloA*$^+$) and GB1-5-39 (*trmA5*, *sloA1*) were grown in separate flasks in glucose minimal medium containing normal (10^{-3} M) Mg^{2+} at 37 C. When cells were in balanced growth at OD_{420} = 0.7 to 0.9, suitable amounts of the two cultures to give 87% Trm$^-$ cells were mixed on a membrane filter (Millipore Corp.). In another experiment, strains GB1-1metA$^+$, (*trmA*$^+$, *sloA1*) and GB1-5-39 (*trmA5*, *sloA1*) were mixed to give 55% Trm$^-$ cells. The cells were washed with 20 ml of saline and transferred to a glucose-minimal medium containing 10^{-5} M Mg^{2+}. Samples were withdrawn at different amounts of doublings and plated on TGYE agar plates after proper dilutions. The Trm property was tested by growing single cell colonies and assaying for methyl group acceptor activity (see preceding paper [7]). Fifty colonies were tested for each sample, except that 100 colonies were tested in the starting sample and the last sample of the GB1-1*metA*$^+$ and GB1-5-39 mixing experiment. The number of doublings of the mixed culture was calculated from the increase in cell mass measured by OD_{420}. All Trm$^+$ colonies at the end of the experiment, as well as a few of both the Trm$^+$ and Trm$^-$ colonies from the beginning of the experiment, were tested either for the *argH* or *thiA* marker. All contained the appropriate marker, thus ruling out a possible contamination during the experiment.

Preparation of enzyme extract used for tRNA methylation. Spheroplasts of cells were used as starting material to reduce the amount of ribonuclease I present (35). Enzyme extracts were prepared as described before (8). The procedure includes disintegration of spheroplasts by the method of French and Milner (24), incubation for 10 min at 37 C with electrophoretically purified deoxyribonuclease (2 μg/ml), fractionation with 0 to 80% ammonium sulfate, and dialysis. The absorbancy at 280 nm-to-absorbancy at 260 nm ratio of such a preparation was usually about 0.6.

Labeling in vivo of RNA with L-[methyl-^{14}C]methionine. The procedure used has been described before (8). The minimal medium was the one described above, supplemented with thiamine (1 μg/ml), L-methionine (30 μg/ml), and adenine (30 μg/ml), and with L-arginine (50 μg/ml) for arginine-requiring strains. L-Methionine and adenine were present to repress the biosynthesis of methionine and purines, respectively. The efficacy of this move was indicated by the fact that ribosomal RNA (rRNA) from *metA* strains had the same activity ([^{14}C]methyl groups/microgram of rRNA) as other prototrophic strains. Furthermore, no radioactivity was found in the adenine spot, indicating that purine biosynthesis was effectively blocked. The labeling medium also contained L-[*methyl*-^{14}C]methionine (final specific activity, 5.7 or 11.3 mCi/mmol). The cells were grown from OD_{420} = 0.015 to 0.020 to OD_{420} = 2.5 to 3.0 (about 5×10^8 to 6×10^8 cells/ml) when they were harvested. Cells were washed once with 5 ml of 5 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0, containing 10 mM magnesium acetate and kept frozen.

Preparation of RNA. RNA from cells labeled in vivo was prepared and fractionated essentially as described before (8). Cells were suspended in 1 ml of the washing buffer described above and lysozyme (200 μg) and electrophoretically purified deoxyribonuclease I (25 μg) were added. The suspension was frozen and thawed three times and then incubated for 10 min at 30 C. Water-saturated phenol (1 ml) and 1% Duponol (0.2 ml) were added, and the mixture was shaken at room temperature for 10 min. The phenol phase was again extracted with 0.5 ml of the buffer. The combined aqueous phases were then extracted with 0.5 ml of fresh phenol. The aqueous phase was then extracted three times with ether. After removal of the ether, the aqueous phase containing RNA was applied to a Sephadex G200 column, operated with 0.05 M triethyl-ammonium acetate buffer (pH 5.2) (12). The fractions containing rRNA (both 23*S* and 16*S* RNA) and those containing tRNA (both 5*S* rRNA and tRNA) were pooled separately and stored frozen.

The tRNA preparations used for experiments on methyl group incorporation into tRNA in vitro were prepared according to Avital and Elson (4). The cells were grown in the indicated medium and washed with 0.9% NaCl. The method involves phenol extraction in the presence of chloroform, stripping of the amino acids (1 h at 37 C in 0.5 M Tris, pH 9.7), and fractionation of rRNA and tRNA with 2 M LiCl in 0.1 M potassium acetate (pH 5.0). The tRNA was dialyzed for 22 h against 6 liters of 10^{-4} M Mg^{2+}. Such tRNA preparations are reported to have a very small amount of high-molecular-weight contaminants (4), and a very small amount of rRNA was detected by Sephadex G100 molecular sieve chromatography.

Analysis of the distribution of methylated bases. The RNA was concentrated by *n*-butanol extraction of the buffer as described before (8) and transferred to a small Pyrex tube. The RNA solution was dried in a stream of air, 15 to 25 μl of 1 M HCl was added, and the tube was sealed. The RNA was hydrolyzed at 100 C for 30 min to degrade the RNA to purine bases and pyrimidine nucleotides. Synthetic methylated bases were added as markers, and a portion of the hydrolysate was subjected to two-dimensional thin-layer chromatography in *n*-butanol-water (86:14, vol/vol; ammonia in gas phase; solvent 1) and isopropanol-concentrated HCl (170:41; water to 250; solvent 2). Details of the procedure and its resolving capability have been described before (11). Another portion was used for determination of the total amount of radioactivity applied. On each chromato-

gram between 6,000 to 11,000 counts/min (corresponding to 5.3 to 15.5 μg of tRNA) was applied, and the recoveries were between 85 to 100%. From these data and the known specific activity of RNA, the amount of tRNA applied could then be calculated. The thin-layer plates used were coated with microcrystalline cellulose Avicel without fluorescent indicator. This procedure resolves the six methylated adenines and guanines found in *E. coli*. Since the methylated pyrmidine nucleotides do not migrate in the first solvent, they appear together with all other charged material between the origin and the front of the second solvent. This area was divided into strips, each 0.5 cm wide, in the direction of the migration of the second solvent (see Fig. 1). The positions of the methylated bases added as markers were determined by ultraviolet light. The cellulose in the area of each marker and in the strips mentioned above was scraped off with a spatula and drawn by suction into a scintillation vial as described before (11). The radioactivity was then determined in a scintillation counter.

Assay of methyl group incorporation into tRNA in vitro. The methylation of RNA was measured as the amount of radioactive methyl groups transferred from *S*-[*methyl*-^{14}C]adenosyl-L-methionine to the different RNA preparations tested. The reaction mixture for the assay contained: 0.1 M Tris-hydrochloride, pH 8.0, 2 mM glutathione, 0.1 mM ethylenediaminetetraacetic acid (disodium), 10 mM $MgSO_4$, 20 mM NH_4Cl, 8.6 nmol of *S*-[*methyl*-^{14}C]adenosyl-L-methionine per ml, and varying amounts of RNA and enzyme. The reaction was performed at 37 C, except where otherwise stated, and stopped with 2 ml of 0.01 M $La(NO_3)_3$ in 0.5 M $HClO_4$. The precipitates were washed on a glass-fiber filter (Reeve-Angel no. 934AH) with 10 ml of 5% trichloroacetic acid and 5 ml of cold 67% ethanol. The filters were dried, and the radioactivity was measured in a scintillation counter.

Assay of the delay time for β-galactosidase. The time necessary for the first β-galactosidase activity to appear after induction (the delay time) was measured by a modification of the method described by Coffman et al. (16). Cells, grown in succinate-minimal medium to avoid catabolite repression, were induced by adding IPTG to a final concentration of 5×10^{-4} M, when the OD_{420} of the culture was 0.5. Samples (0.5 ml) were withdrawn with an automatic pipette at 10-s intervals and transferred to 0.5 ml of 0.1 M phosphate buffer (pH 7.0) containing 200 μg of chloramphenicol. The cell suspension was then incubated for 15 min at 37 C to allow maturation of finished β-galactosidase polypeptides. A lysis solution (0.025 ml) containing toluene, 10% sodium dodecyl sulfate, 2-mercaptoethanol, and 0.02 M manganese sulfate (1:1:1:1) was added, and the mixture was agitated vigorously for 15 s. After incubation for 30 min at 37 C it was again agitated, and then 1.0 ml of ortho-nitrophenyl-β-D-galactoside (1 mg/ml) in 0.1 M phosphate buffer (pH 7.0) was added. The reaction mixture was incubated at 28 C until yellow color developed and was then stopped by adding 0.5 ml of 1 M sodium carbonate. Samples were read at 420 nm. Samples from cells containing the basal level of β-galactosidase required overnight incubation. The analytical data were plotted as described by Schleif et al. (39). The amount of enzyme present at t, $E(t)$ minus the basal level of enzyme $E(0)$, is proportional to t^2, or $\sqrt{(E)(t) - E(0)}\alpha t$. Plotting the square root of the difference between the amount of enzyme at t and the basal level allows the use of the early points to determine more precisely the time at which induced enzyme first begins to appear.

Threonine deaminase assay. Cell extracts were prepared essentially as described by Anderson and Neidhardt (2), but the buffer was supplemented with dithiothreitol and L-isoleucine as described by Calhoun et al. (15). The buffer contained: potassium phosphate buffer (0.05 M, pH 7.2), ethylenediaminetetraacetic acid (0.0015 M), L-isoleucine (20 μg/ml), and dithiothreitol (0.5 mM). The threonine deaminase assay and the reaction mixture were as described by Anderson and Neidhardt (2). One unit of enzyme is defined as the amount that forms 1 μmol of α-ketobutyrate per h.

Histidinol phosphate phosphatase assay. Histidinol phosphate phosphatase was measured as described by Ames et al. (1) but using 0.02 ml of 50 mM histidinol phosphate, as suggested by Lewis and Ames (31).

Binding of [^{14}C]phenylalanyl tRNA to ribosomes and its release. Ribosomes were prepared from strain LMUR as described by Kurland (29).The soluble fraction II (SFII) of Wood and Berg (49) was used to aminoacylate tRNA. Spheroplasts from strains GB1-1metA$^+$ or GB1-5-39 were used as a source for SFII and used to aminoacylate tRNA from the respective strain. Binding of [^{14}C]phenylalanyl-tRNA ([^{14}C]Phe-tRNA) was performed as described by Levin and Nirenberg (30) using (per milliliter): ribosomes, 2.5 OD_{260} units; polyuridylic acid [poly(U)], 1 mg; and [^{14}C]Phe-tRNA, 2.5 OD_{260} units. The assay buffer was: 0.1 M Tris-acetate (pH 7.2), 0.02 M $MgCl_2$, 0.05 M KCl. The mixture was incubated at 24 C, and samples were diluted in cold assay buffer. The ribosomes were adsorbed to a membrane filter (type HA, 0.45-μm pore size; Millipore Corp.) and washed with 10 ml of cold assay buffer and 10 ml of 67% ethanol.

For release of [^{14}C]Phe-tRNA, the [^{14}C]Phe-tRNA-poly(U)-ribosome complex prepared at 24 C as described above was diluted 100-fold with assay buffer at the desired temperature. Samples were withdrawn and the ribosomes were immediately adsorbed to a membrane filter (Millipore Corp.) and washed with cold assay buffer as described above.

Other methods. The procedure of Lowry et al. (33) was used to determine protein concentrations, RNA was determined chemically by the orcinol method (40), and the samples were processed as described by Fraenkel and Neidhardt (23). RNA was also determined from the adsorption at 260 nm in a Zeiss spectrophotometer. A solution of 1 mg of tRNA per ml gives an absorption at 260 nm of about 25 (1-cm light path).

Materials. *S*-[*methyl*-^{14}C]adenosyl-L-methionine (52 and 59 mCi/mmol; 93% of the radioactivity in the

methyl group), L-[*methyl*-^{14}C]methionine (60 mCi/mmol, with 95% of the radioactivity in the methyl group), and [^{14}C]phenylalanine (513 mCi/mmol) were purchased from Amersham/Searle. Deoxyribonuclease I from beef pancreas (1× crystallized), crystalline egg white lysozyme, ortho-nitrophenyl-β-D-galactoside, and IPTG were obtained from Sigma Chemical Co., St. Louis, Mo. Electrophoretically purified deoxyribonuclease was purchased from Worthington Biochemical Corp., Freehold, N.J., and all methylated bases were from Cyclo Chemical Co., Los Angeles, Calif. Thin-layer chromatography plates coated with microcrystalline cellulose Avicel without fluorescent indicator were obtained from Brinkmann Instruments Inc., Westbury, N.Y. Glass-fiber filters (no. 934AH) were purchased from H. Reeve Angel & Co., Clifton, N.J., and membrane filters (type HA, 0.45-μm pore size) were obtained from Millipore AB, Gothenburg, Sweden.

RESULTS

The methylation-deficient mutants previously isolated (8) had been heavily mutagenized with ethylmethanesulfonate and could contain more than the *trmA* mutation. Furthermore, the isolation procedure required that the mutants be able to grow. A coexisting unknown mutation could therefore affect the expression of the methylation defect by modifying or suppressing a harmful effect of the loss of m⁵U. For this reason, and because any unknown mutations in the methylation-deficient strains could potentially lead to misleading results, it was necessary to develop pairs of strains that were isogenic except for the *trmA* locus. The best way to construct isogenic pairs is to isolate true spontaneous revertants. There is, however, no selection method for Trm$^+$ revertants. Instead, the Trm$^-$ property was transduced from our original mutants to other strains of *E. coli*. The gene, *trmA*, responsible for the formation of m⁵U in the tRNA was located close to and clockwise from the *argH* gene, at 79 min on the genetic map of *E. coli*, as described in the preceding paper (7). With this knowledge it was possible to construct matched pairs of transductants, one transductant containing the wild-type allele (*trmA*$^+$) and the other the mutated one (*trmA*). Transduction can, of course, lead to the introduction of more than one mutated gene. To decrease the possibility that our results were affected by such a fortuitous event, we took the following steps. (i) We employed several independently constructed transductant pairs; (ii) we paired the *metA*$^+$-*trmA* or *argH*$^+$-*trmA* transductants, not with the recipient strain, but with a transductant that had received either the *metA*$^+$ or *argH*$^+$ allele from the donor; (iii) transductions were carried out at multiplicities of infection of approximately one or less; (iv) we transduced the *trmA* allele from independently isolated mutants; and (v) we introduced the deoxyribonucleic acid segment, including the *trmA*$^+$ gene from the unmutated parent strain CP79, to serve as control for any gene present in CP79 which could interfere in physiological experiments when transferred to a new genetic background.

Characterization of transductants. It was necessary to verify that the tRNA of the Trm$^-$ transductants had an altered level of m⁵U. Transfer RNA from cells labeled in vivo with L-[*methyl*-^{14}C]methionine was analyzed for the distribution of methylated compounds as described above. The results are shown in Table 2. All values are calculated using m²Ade as an internal standard, the average value being determined from nine independent analyses. This value (11 pmol/μg of tRNA) is higher than that reported before (8 pmol/μg of tRNA). This discrepancy might be explained by the fact that smaller fractions (1 ml as compared to 3.5 ml) were collected during the purification of tRNA by Sephadex G200 molecular sieve chromatography, and therefore the contamination by 5*S* rRNA was less in these tRNA preparations than in those reported before (8). Since 5*S* rRNA in *E. coli* does not contain any methylated nucleosides (13), this difference in tRNA purity could in part explain the higher value of the absolute content of m²Ade in the analyses reported here.

The only component which was significantly changed in the *trmA* transductants was methylated uridylic acid (mUp), the same component that was altered in the original mutants. The extent of the deficiency differs in the different Trm$^-$ transductants. Except for the *trmA9* allele, the absolute values agree well with the values obtained in the original mutants, if the amount of "unknown 3" is included in the value of mUp in Table 2. These two compounds probably migrate together in paper chromatography, which was used earlier (8).

Examination of the histogram from the origin to the front of the second solvent (Fig. 1) indicates that cells of strain GB1-5-39 (*trmA5*) do not contain any mUp when grown either at 30 C (results not shown) or at 37 C, indicating that the methyltransferase in this strain is inactive over this temperature range. The low value shown as mUp in Table 2 originates from radioactivity in the area where mUp should migrate, but no indication of a separate compound was formed and the radioactivity could easily be spillover from the two neighboring components, "unknown 2" and "unknown 3."

TABLE 2. *Distribution of methylated compounds in tRNA from transductants of E. coli AB1932 labeled in vivo at 37 C with* L-[*methyl*-^{14}C]*methionine*[a]

Methyl compound	Control strains[b]	Methylated compounds (pmol/μg of tRNA) from transductants:[b]		
		GB1-4-IB	GB1-5-39	GB1-9-7
m_2^6Ade	0.06	0.03	0.06	0.05
m^6Ade	3.0	3.2	3.0	2.8
m^2Ade (standard)	11	11	11	11
Ade + m^1Ade	0.02	0	0	0.04
m_2^2Gua	0.01	0	0.03	0.04
m^2Gua	0.06	0	0.03	0.1
m^7Gua	29	32	31	33
m^1Gua	6.5	6.9	5.1	7.0
Unknown 1	11	12	12	11
mCp	6.7	4.3	7.2	4.5
Unknown 2	4.9	7.8	6.6	4.1
mUp	49	*4.0*	*1.0*	*4.6*
Unknown 3	1.4	3.5	3.5	5.9

[a] Cells were grown at 37 C in medium supplemented with L-[*methyl*-^{14}C]methionine, and tRNA was isolated and hydrolyzed with 1 M HCl; the products were subjected to two-dimensional thin-layer chromatography. Details of the procedure are found in Materials and Methods. The columns headed "Control strains" and "Transductants" give the average value of the four independent analysis of *E. coli* AB1932 and the transductants GB1-4-II9, GB1-5-41, and GB1-9-10. Their characteristics are shown in Table 1. Strains GB1-4-IB and GB1-5-39 were grown at 30 C, and analysis of the methylated constituents was performed as described above. The results were the same as compared to the results shown in the table, which were obtained from cells grown at 37 C. All values have been corrected to two significant figures. The average value for the internal standard m^2Ade determined from nine independent analyses was 11 ± 1.5 pmol/μg of tRNA (P = 0.05). Compounds labeled "unknowns" all migrate only in the second solvent and are defined in Fig. 1. Unknown 3 is usually not distinguished as a separate component in analysis of tRNA from wild type but is easily detected in the Trm$^-$ mutant, especially in *trmA4* and *trmA9*, where it is separated from residual methyluridylic acid (see Fig. 1). In *trmA5* no methyluridylic acid was seen, and the low level shown is the radioactivity found between unknown 2 and unknown 3 in the region where methyluridylic acid normally should migrate. Figures in italics are regarded as significantly different from the value found in the wild type.

[b] *trm* alleles: control, +; GB1-4-IB, A4; GB1-5-39, A5; GB1-9-7, A9.

The original mutant strain IB5 (*trmA5*) does not contain any m^5Ura (8), and therefore it seems reasonable to assume that mUp analyzed by the thin-layer chromatography technique (Table 2) is a true estimate of m^5Up, and that no such compound is present in the tRNA of strain GB1-5-39 (*trmA5*).

The transductant from strain IB4 (GB1-4-IB, *trmA4*) had the same level of mUp as the original mutant both at 37 and 30 C. Figure 1 indicates that strain GB1-4-IB contains low but definite levels of mUp, and therefore m^5U, and that the defective enzyme in this organism does not show any variation in activity between 30 and 37 C.

On the other hand, the transductant from IB9 (GB1-9-7 *trmA9*) exhibited a different level of mUp from that exhibited by the original mutant. About three times as much m^5Ura was found in the original mutant (8) as the level of mUp in Table 2, indicating perhaps that this mutation is expressed differently in different cellular environments. This observation shows the importance of the in vivo measurements presented here; one cannot rely on the analyses in the original mutants and apply them directly to the transductants.

Next, the ability of tRNA from the transductants to accept methyl groups in vitro was determined. Cells were grown in TGYE medium

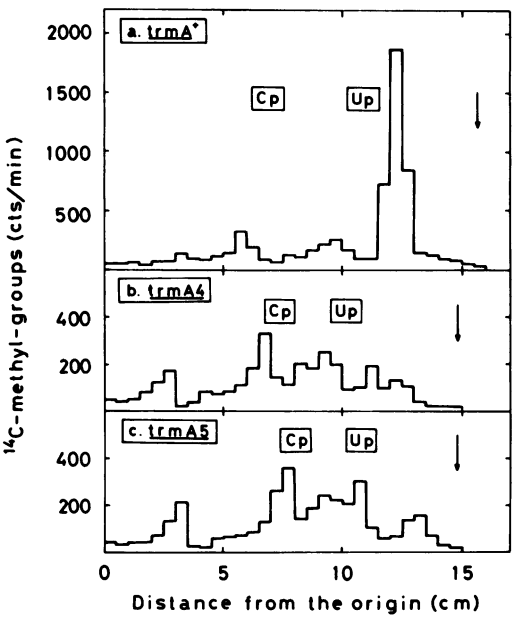

FIG. 1. *Histograms of nucleotide region from two-dimensional thin-layer chromatography of HCl-hydrolyzed tRNA labeled in vivo at 37 C. (Similar histograms were also obtained from tRNA labeled in vivo at 30 C.) The histograms show the separation of radioactive compounds migrating only in the second direction. The positions of cytidylic acid (Cp) and uridylic acid (Up) were determined by ultraviolet absorbance. Unknown 1 in Table 2 migrates after Cp (i.e., 9 to 10 cm in [b]); methylated cytidylic acid (mCp) migrates immediately ahead of Cp. Unknown 3 migrates faster than mUp (i.e., 12 to 13.5 cm in [b]), which migrates immediately ahead of Up. The component that migrated to about 3 cm is part of m^7Gua. (a) tRNA from E. coli strain GB1-5-41 (trmA+); (b) tRNA from E. coli strain GB1-4-IB (trmA4); (c) tRNA from E. coli strain GB1-5-39 (trmA5).*

at the indicated temperatures, and tRNA was purified with 2 M LiCl. Methyl group acceptance was determined, and the results are shown in Table 3. Measurements of the time course of the reaction did not reveal any ribonuclease activity in the enzyme extract used, and since the incorporation was proportional to the concentration of tRNA over a three- to sixfold range, it is likely that the plateau was reached in each case and that ribonuclease was not interfering. Although different preparations of tRNA (from different experiments) varied in the extent of the incorporation of methyl groups (twofold), approximately the same level of incorporation in vitro was obtained as with the original mutants.

From Table 3 it can be seen that about 50% of the methyl groups missing in vivo can be added in vitro. We determined whether this discrepancy was caused by the difference in media in the two different analyses, but no difference was found between tRNA prepared from strain GB1-4-IB grown in the medium used for the labeling in vivo (results not shown) or in TGYE medium. The inability of enzyme preparations to add all of the missing methyl groups to the tRNA is unclear and warrants further investigation. An interesting possibility is that the native substrate for the tRNA(m⁵U)methyltransferase is some precursor molecule and not a tRNA lacking only m⁵U.

Enzyme extracts from the Trm⁻ transductants were unable to methylate undermethylated tRNA when either tRNA from the same strain or from the other Trm⁻ transductant was used. This result suggests that the same enzyme is mutated in strain GB1-4-IB and strain GB1-5-39, in agreement with the genetic data. Similar results were obtained with the original mutants.

TABLE 3. *Comparison of two trmA transductants for methylated constituents of tRNA formed in vitro with wild-type enzymes and in vivo during exponential growth*

Source of tRNA	Temp of cultivation (C)	*trm* allele	mUp formed (pmol/μg of tRNA)[a]		
			In vivo	In vitro	In vitro + in vivo
GB1-5-41	37	+	49	3-5	53
GB1-4-IB	37	A4	4.0	21	25
GB1-4-IB	30	A4	4.4	19	23
GB1-4-IB	25	A4	—	17	—
GB1-5-39	37	A5	1.0	14	15
GB1-5-39	30	A5	0.6	17-25	25

[a] Data in the column "In vivo" are taken from Table 2. Data in the column "In vitro" represent extent of methylation of tRNA extracted from cells grown in TGYE at indicated temperatures. A time curve at 37 C was done for four different amounts of tRNA, and the average values at 60, 90, and 120 min (plateau level) were corrected for incorporation in enzyme alone. The four different amounts of tRNA were plotted versus the incorporation of methyl groups, and picomoles per microgram of tRNA was calculated from the straight line obtained by connecting the four points with the origin.

The results presented in this section demonstrated that the only methylated compound deficient or absent in the tRNA of the transductants is mUp, that this deficiency is not modified by the growth medium or the temperature of cultivation, and that the same enzyme [most likely the tRNA(m⁵U)methyltransferase] is mutated in the different transductants. These transductants were therefore considered appropriate to use in studying the physiological consequences of m⁵U deficiency.

Growth rate characteristics of matched pairs of transductants. Growth rates were determined in several media at 37 C. Strains lacking m⁵U (*trmA5*) had a growth rate the same as or lower than that of the corresponding Trm⁺ strain (Table 4). The same growth rate characteristics were obtained when the *trmA4* allele was brought into *E. coli* strains AB1932 (i.e., GB1 derivatives) and LMUR (results not shown). These results suggested that the growth rate differences seen, especially in GB1 Trm⁺ and Trm⁻ (both *trmA4* and *trmA5*) derivatives, were caused by the lack of m⁵U. However, it is possible that in the region close to *trmA* there is a gene, *sloA*, the allelic state (*sloA1*) of which causes slow growth in strain CP79 (parent of the original mutants) when transferred to strain AB1932. The *sloA1* allele could have been transferred together with the *trmA* gene to one of the transductants while the control *trmA*⁺ transductant retained the *sloA*⁺ allele. A possible influence of *sloA1* could be avoided if the *trmA* cells as well as *trmA*⁺ cells contained the same allelic state of *sloA*. With our improved mapping of the *trmA* gene, described in the preceding paper (7), we were able to construct derivatives of strain AB1932 (*argH*, *metA*) which had received the deoxyribonucleic acid segment between *argH* and *metA* from the parental strain CP79 (*argH*⁺, *thiA*, *metA*⁺). Two *argH*⁺ transductants and two *metA*⁺ transductants which had all become Thi⁻ and received the *metA*⁺ and *argH*⁺ genes, respectively, from CP79 were constructed. Growth characteristics for one of these strains (GB1-1metA⁺; *argH*⁺, *trmA*⁺, *sloA1*, *thiA*, *metA*⁺) are shown in Table 4. Similar results were obtained with all four constructed derivatives,

TABLE 4. *Comparison of growth rates in different media at 37 C for transductants of different strains of E. coli possessing or lacking m⁵U in their tRNA*

Transductants	*trm* allele	Change in *k*[a]							
		TGYE		Glu-min		Gly-min		Suc-min	
		k	Change in *k* (%)	*k*	Change in *k* (%)	*k*	Change in *k* (%)	*k*	Change in *k* (%)
GB1-5-41	+	1.14		0.73		0.58		0.50	
GB1-5-39	A5	0.95	−20	0.70	−4	0.56	−4	0.44	−14
GB1-1metA⁺	+	0.97	−18					0.43	−16
LMUR-5-267	+	1.60						0.72	
LMUR-5-266	A5	1.54	−4					0.60	−20
G11-5-17	+	1.54						0.53	
G11-5-18	A5	1.54	0					0.53	0

[a] The strains used are described in Table 1. Growth rates are expressed as *k*, the specific growth rate constant. Glu-min, Suc-min, and Gly-min are glucose-, succinate-, and glycerol-minimal medium, respectively. The change in *k* is expressed as $[k_{\text{strain indicated}} - (k_{\max}/k_{\text{strain indicated}})] \times 100$. Changes of 7% or less were considered insignificant. The same results were also obtained when the *trmA4* derivatives of strains AB1932 and LMUR, described in Table 1, were used.

showing that the growth rate difference seen between GB1-5-41 (Trm⁺) and GB1-5-39 (Trm⁻) is not caused by the allelic state of the *trmA* gene, but by the allelic state of *sloA*. This growth rate difference in TGYE medium is temperature dependent, since no growth rate differences were observed at 30 C or below (results not shown). The *sloA* gene should be located in the region *argH-thiA*, since strain GB1-5-39 (*argH*, *trmA5*, *thiA*⁺, *metA*⁺) exhibited the same growth rate in TGYE medium at 37 C as did an *argH*⁺ transductant which had become *trmA5* but still retained the recipient allele of the *thi* (*thiA*⁺) and *metA* genes (results not shown).

These growth studies thus failed to demonstrate any change in growth rate that could be attributed to the absence of m⁵U in the tRNA.

Coding response and macromolecular synthesis in Trm⁺/Trm⁻ transductants. To test if a lack of m⁵U introduced coding errors, 23 amber, 12 ochre, and two UGA mutants of phage T4 were tested for ability to grow on the five matched pairs of transductants of strains AB1932 (i.e., GB1 derivatives), LMUR, and G11 (Table 1). No differences in the suppression pattern were observed, as judged by spotting the phages on a lawn of Trm⁺ or Trm⁻ cells. Burst sizes for one T4 amber mutant were similar in Trm⁺ and Trm⁻ derivatives of strains LMUR and G11 (L. Isaksson, personal communication). These results indicate that neither the methyl group in m⁵U per se in the tRNA, nor any structural feature of tRNA that might depend on this group, is important for proper codon recognition.

We next studied the ability of phenylalanyl-tRNA (Phe-tRNA) to bind to the ribosome in the presence of poly(U). No difference was observed in the kinetics of the formation of the Phe-tRNA-poly(U)-ribosome complex when Phe-tRNA originated from GB1-1metA⁺ (*trmA*⁺, *sloA1*) or GB1-5-39 (*trmA5*, *sloA1*). Furthermore, Fig. 2 shows that the stability of the Phe-tRNA-poly(U)-ribosome complex at 42 or 30 C (data not shown) is not affected by the absence of m⁵U in the tRNA. Thus, the lack of the methyl group in the sequence G-m⁵U-ψ-C in $tRNA^{Phe}$ does not influence the binding of this tRNA to the ribosome.

We grew strains GB1-1metA⁺ (*trmA*⁺, *sloA1*) and GB1-5-39 (*trmA5*, *sloA1*) in media containing valine, isoleucine, and leucine or without

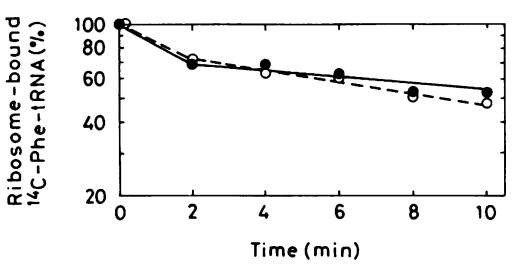

FIG. 2. *Release of [¹⁴C]Phe-tRNA from ribosomes. [¹⁴C]Phe-tRNA-poly(U)-ribosome complex was formed at 24 C, and at time zero the assay mixture was diluted 100-fold in assay buffer at 42 C. Samples were withdrawn at indicated times, and the amount of [¹⁴C]phe-tRNA bound to ribosomes was determined. Symbols: ●, [¹⁴C]phe-tRNA from GB1-1metA⁺, 100% was 6,500 counts/min; ○, [¹⁴C]phe-tRNA from GB1-5-39 (trmA5), 100% was 2,900 counts/min. Notice the logarithmic scale.*

these amino acids at 37 C. No difference in specific activity of threonine deaminase, the first enzyme in isoleucine biosynthesis, was observed that could be related to the *trmA5* mutation.

When the same strains were grown in media with or without histidine no difference in the specific activity of the histidine biosynthetic enzyme, histidinol phosphate phosphatase, was observed which could be related to the *trmA* allele. Thus, tested in this manner the *trmA* mutation does not grossly affect the regulation of the *ilv* or *his* operons, despite the implication of tRNA in these processes.

Before it can be concluded that similarity in growth reflects a similarity in efficiency of translation, it must be shown that the Trm⁻ cells have not adjusted their protein-synthesizing machinery to compensate for some harmful effect of the lack of m⁵U in tRNA. Therefore, RNA-protein ratios and cell size were determined as described by Fraenkel and Neidhardt (23) for cells grown either in TGYE or in glucose-minimal medium. No significant difference was detected between the Trm⁺ and Trm⁻ cells (the RNA-protein ratio was 0.76 to 0.78 in TGYE medium and 0.40 to 0.41 in minimal medium; the average cell size in TGYE medium was inferred from the number of cells per milliliter of culture at OD_{420} = 1, 1.6×10^8 to 1.9×10^8). Furthermore, cell extracts prepared from Trm⁺ and Trm⁻ cells in TGYE medium at 37 C were analyzed by centrifugation through a 5 to 20% sucrose gradient under conditions which separate the 50*S* and 30*S* ribosomal subunits and 4*S* RNA. In both cell types the 4*S* material was 22% of the total RNA (measured as absorbancy at 260 nm), indicating that Trm⁻ cells are not unusually rich in tRNA.

Rate-limiting quantities of tRNA from Trm⁺ and Trm⁻ cells have been compared in protein synthesis in vitro using poly(U) or f2 RNA as messenger, and no effects attributable to lack of m⁵U in tRNA were observed (46). However, these experiments were performed in vitro under conditions most probably far from the conditions existing in vivo. Therefore, we considered it valuable to know the rate of protein synthesis, i.e., the rate of polypeptide chain extension, in vivo. This was done by measuring the appearance of the first β-galactosidase molecule after induction in Trm⁺ and Trm⁻ cells. Plotting the experimental data according to Schleif et al. (39) allows a very precise determination of the time at which induced β-galactosidase first began to appear. Figure 3 shows this time to be slightly after 1.1 min both in

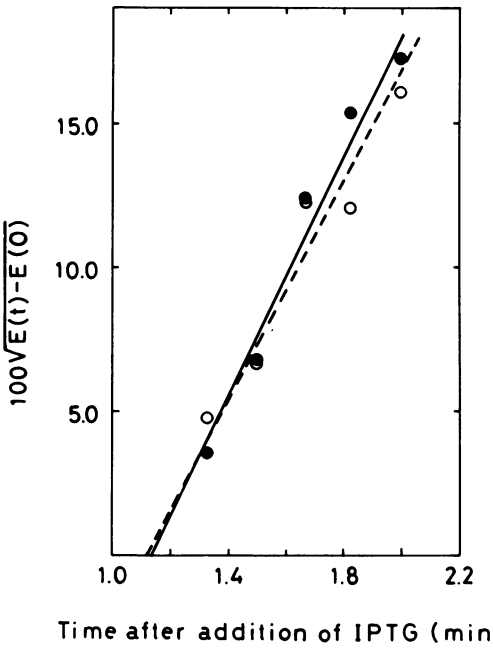

FIG. 3. *Kinetics of induction of β-galactosidase in trmA⁺ and trmA5 cells. Strains GB1-5-41 Lac⁺ (●) and GB1-5-39 Lac⁺ (○) were grown in succinate-minimal medium at 37 C. At time zero IPTG was added, and samples were withdrawn every 10 s, and the activity of β-galactosidase was measured. The square root of the amount of enzyme is plotted versus time. Details are found in Materials and Methods.*

Trm⁺ and Trm⁻ cells, which agrees well with the reported value of slightly before 1.2 min (39). This indicates that the rate of ribosome function in vivo is also similar in Trm⁻ and Trm⁺ cells.

In summary, these results fail to establish any major translational aberration in Trm⁻ cells. Within the limits of the methods used, no change in the reading of the three chain termination codons could be demonstrated. The normal levels of tRNA and ribosomes and the normal rate of polypeptide chain elongation were found in Trm⁻ cells. These findings would suggest that the m⁵U deficiency does not seriously handicap a cell despite the normal occurrence of this modified base in most if not all tRNA species in *E. coli*. These results do not rule out the possibility that some subtle but important secondary adjustment has been made by the cells to compensate for the altered tRNA structure.

Mixed population experiments in magnesium-limiting media. Growth in mixed populations can be a sensitive way to detect a differen-

tial selective advantage between two cell types. An experiment was performed in which Trm$^+$ (GB1-5-41; *trmA*$^+$, *solA*$^+$) and Trm$^-$ (GB1-5-39; *trmA5*, *sloA1*) cells were grown in competition. The Trm$^+$ and Trm$^-$ cells were grown separately in glucose-minimal medium. When the cultures were in balanced growth they were mixed, and then the cells were collected by filtration and transferred to a glucose-minimal medium containing Mg^{2+} at 10^{-5} instead of the usual 10^{-3} M. (The Mg^{2+} concentration was lowered with the thought that this might enhance any structural consequences of m^{5}U deficiency, since Mg^{2+} affects tRNA conformation in vitro; given the active transport of this ion [41], however, it is uncertain what the in vivo concentration really is under these conditions.) The mixed culture was incubated, and samples were removed at various times to determine the relative amounts of Trm$^+$ and Trm$^-$ cells. No attempt was made to keep the cells in logarithmic growth; the culture was incubated overnight twice and was therefore twice in stationary phase for a prolonged period of time. Figure 4 clearly shows that, as the mixture grew, the amount of Trm$^-$ cells in the population diminished; after 30 doublings the proportion of Trm$^-$ was only 0.08. The Trm$^-$ strain used here, GB1-5-39, also contains the *sloA1* allele, which the Trm$^+$ strain did not. Therefore, a similar experiment was performed by mixing the Trm$^+$ strain, GB1-1metA (*trmA*$^+$, *sloA1*), with GB1-5-39 (*trmA5*, *sloA1*). Figure 4 shows that, as the mixture grew, the Trm$^-$ cells in the population diminished, but not as fast as in the preceding experiment. After 30 doublings the proportion of Trm$^-$ was only 0.5 that at the start. Thus, cells possessing m^{5}U in the tRNA have a selective advantage.

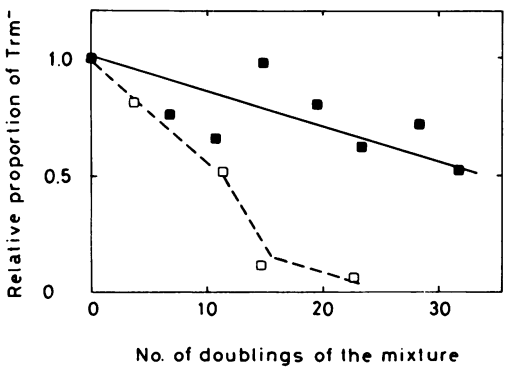

FIG. 4. *Mixed population experiment. E. coli strains GB1-5-41 (trmA*$^+$, *sloA*$^+$*) and GB1-5-39 (trmA5, sloA1)* (□) *or E. coli strains GB1-1metA*$^+$ *(trmA*$^+$, *sloA1) and GB1-5-39 (trmA5, sloA1)* (■) *were mixed and transferred to minimal medium containing a low concentration of* Mg^{2+}. *At the indicated amount of doublings the proportion of Trm*$^-$ *cells was determined as described in Materials and Methods.*

Some experiments have been performed to learn in which growth phase there is a disadvantage to the cell of possessing either the *sloA1* allele or the *trmA5* allele. A direct measurement of growth rate did not answer the question, and therefore we explored the effect of Mg^{2+} limitation further by growing each strain separately in a Mg^{2+} limiting chemostat to learn whether there was a great difference in the ability of GB1-5-41 (*trmA*$^+$ *sloA*$^+$) and GB1-5-39 (*trmA5*, *sloA1*) cells to grow under these conditions. At several flow rates it was found that the steady-state level of Trm$^+$ and Trm$^-$ cells was indistinguishable. This means that the concentration of Mg^{2+} required for growth at these flow rates was the same for both types of cells. Furthermore, in a mixed population experiment with strain GB1-1metA (*trmA*$^+$, *sloA1*) and GB1-5-39 (*trmA5*, *sloA1*) no change in the fraction of Trm$^-$ was observed when the mixture was transferred to a new fresh medium after one cycle of growth in 10^{-5} M Mg. However, one can confidently state only that the Trm$^-$ cells had the same capacity to grow logarithmically as Trm$^+$. This result and the result obtained with the chemostat experiment support the idea that the major effect seen in the mixed population experiment was the result of differences in the capacity to survive during stationary phase or in the length of the lag period when cells are transferred to new media.

The mixed population experiments provide the first experimental evidence of a beneficial biological effect of m^{5}U in tRNA.

DISCUSSION

Previous to these experiments, tRNA, which was generally methyl deficient, had been used to study the effect of methylation on codon recognition, the translation process, and amino acid acceptance (32, 36, 43, 44). Therefore, conclusions about the function of a specific methylated nucleoside can only be made with difficulty. The tRNA^{Phe} of *E. coli* contains three methylated nucleosides: m^{5}U, m^{7}G, and ms^{2}i^{6}A (6), and thus one might conclude that these three methylated bases have no function in the reaction studied. However, it is possible that a loss of two (or more) methyl groups would mask the effect of the one which is important in the reaction studied. Furthermore, different methylated nucleosides may have dif-

ferent functions, and even the same methylated base may play a different role in different tRNA chains. It is therefore useful to work with a mutant lacking only one methylated nucleoside and to compare it with an otherwise isogenic wild-type strain. Another important advantage with such sets of almost isogenic strains is that the effect of a specific methylated nucleoside can be analyzed under conditions existing in vivo.

The analysis performed in vivo of the distribution of methylated compounds in the tRNA of the transductants clearly indicated that the only methylated nucleoside which was affected by the mutation was m⁵U. These results agree with data obtained by Yang et al. (50), which showed that the modifications of ψ and Gm present in $tRNA^{Tyr}$ were unaffected by the absence of m⁵U in a Trm⁻ transductant. In *Streptococcus faecalis* and *Bacillus subtilis*, m⁵U in tRNA is synthesized probably by a tetrahydrofolic acid-dependent tRNA(m⁵U)-methyltransferase (3, 18). Yang et al. (50) used [^{32}P]PO_4 labeling when they established the absence of m⁵Up in Trm⁻ mutants. Therefore, it is unlikely that the Trm⁻ mutants are using an alternative metabolic route to synthesize m⁵U in the tRNA, and thus our measurement by labeling in vivo with L-[*methyl*-^{14}C]methionine probably reflects the actual level of m⁵U in the tRNA of the Trm⁻ mutants. The average value of methyl groups per tRNA chain using the data in Table 2 is 3.0, as compared to reported values of 2.3 (27) and 2.7 (34). Our value of 1.1 m⁵U/tRNA chain is also higher than has been suggested, that is, 1.0 (52), and also higher than the value of 0.8 m⁵U/tRNA chain reported before (8). Part of the discrepancy between our own two analyses (1.1 and 0.8) might be accounted for by a cleaner tRNA preparation than used before.

The sequence G-m⁵U-ψ-C seems to be a common feature of loop IV of all tRNAs and has therefore been thought to be involved in some function common to all different tRNA molecules, such as ribosomal binding (52). The oligonucleotide G-m⁵U-ψ-C has been shown to possess a specific inhibitory effect on the ribosomal binding of tRNA, thus indicating that this part of the tRNA molecule may participate in binding (37). Furthermore, this common sequence in the tRNA is protected by 30*S* ribosomes when the ribosome-formylmethionyl-tRNA initiation complex was digested with RNase T_1 (19). It has been suggested that this part of the tRNA is able to bind to 5*S* RNA present in the 50*S* subunit of the ribosome (19, 21). The tetranucleotide m⁵Up-ψ-p-Cp-Gp inhibits the elongation factor EF-Tu-directed aminoacyl-tRNA binding, EF-Tu-linked guanosine triphosphatase hydrolysis, and ribosome-dependent synthesis of magic spot (38). However, tRNA from our mutants, which has uridine instead of m⁵U in this sequence, permits a normal rate of protein synthesis in vitro using poly(U) or f2 RNA as messenger (46). This result agrees with those obtained with $tRNA^{Ile}$ from *Mycoplasma*, which also lacks m⁵U in its tRNA (28). Furthermore, the tRNA from Trm⁻ mutants binds as well to the ribosome as tRNA from wild-type cells (Fig. 2) and is able to stimulate the *rel*-factor-dependent synthesis of guanosine tetra- and pentaphosphate (Kjeldgaard, personal communication).

S. faecalis seems to be able to initiate the synthesis of protein by a m⁵U-deficient unformylated $tRNA_f^{Met}$ (17). In a preliminary experiment we have found that trimethoprim decreases to the same extent the growth rate of GB1-1metA⁺ (*trmA*⁺, *sloA1*) and GB1-5-39 (*trmA5*, *sloA1*) in a medium supplemented with purines, pyrimidines, thymidine, all 20 amino acids, and a vitamin mixture (unpublished data). Under these conditions initiation of protein synthesis seems to occur with unformylated $tRNA_f^{Met}$ (25). Thus, our results indicate that a m⁵U deficiency in $tRNA_f^{Met}$ from *E. coli* does not compensate for formylation of methionyl-$tRNA_f^{Met}$, as has been suggested for *S. faecalis* (17). It should, however, be remembered that, since m⁵U is probably found in all tRNA in *E. coli*, the major function ought to be something common to all tRNA chains and not only affect the initiator tRNA.

The suppression pattern for the three-chain termination codons was found to be the same in Trm⁺ and Trm⁻ cells, suggesting that m⁵U is not important in proper codon recognition. The rate of protein synthesis was estimated in vivo and found to be the same in Trm⁺ and Trm⁻ cells (Fig. 3). The same results were also obtained by Yang et al. (50). No major change in the production of ribosomes or tRNA occurred in the Trm⁻ cells. Taken together these results would indicate that neither the methyl group in m⁵U per se, nor any structural feature of tRNA that might depend on this group, is important for proper codon recognition or for the efficiency of translation.

Valyl-tRNA has been implicated in the regulation of the leucine-valine pathway, since the *ilv* operon becomes derepressed at high temperature when the cells harbor a temperature-sensitive valyl-tRNA ligase (20). Further-

more, it has been suggested that the first enzyme in this pathway, threonine-deaminase, binds valyl-isoleucyl-, and leucyl-tRNA and, in doing so, acts as a repressor complex for the operon (14, 26). Histidyl $tRNA^{His}$ has been shown to be involved in regulation of the histidine operon, and, in fact, one mutation, *hisT*, which causes derepression of the *his* operon, also lacks two pseudouridine residues in the anticodon stem of the $tRNA^{His}$ (42). However, the regulation of the *ilv* and *his* operons seemed to be unaffected by the specific loss of m^5U in the tRNA.

The state of methylation of tRNA has been suggested to be of importance in the amino acid activation reaction, but the results are contradictory (44). When tRNA from our mutants completely lacking m^5U was tested in the amino acid-charging reaction for seven amino acids at different pH and magnesium concentrations, no effect of the methylated nucleoside could be demonstrated (46). These results are also in agreement with the results obtained with $tRNA^{Ile}$ from *Mycoplasma* (28) and with $tRNA^{Tyr}$ (50). All these data indicate that m^5U in tRNA is not important in the amino acid-charging reaction in vitro.

The physiological experiments performed with our isogenic pairs of Trm^+ and Trm^- cells have not established the specific reaction(s) for which presence of m^5U in tRNA is important. They have, however, demonstrated that cells possessing the methyl group have an advantage over the mutant under some conditions. This is even more significant when it is recalled that Zamenhof and Eichhorn (51) have shown that on adequate media "defective" mutants, auxotrophs and others, have a selective advantage over parental strains. Thus, the mixed population experiments at low magnesium concentration are good demonstrations of the overall value of m^5U in tRNA and clearly show a selective biological advantage of methylated tRNA (Fig. 4). Since *E. coli* probably has evolved under strong evolution pressure to compete during feast and famine cycles and under anaerobic conditions, the selective advantage of m^5U in tRNA might be less its ability to permit faster growth in rich medium under aerobic conditions than its ability to confer stability and quick responsiveness on cells under suboptimal conditions. Whatever the actual function of m^5U is, the existence of isogenic strains differing only in the level of this methylated nucleoside in the tRNA should make it possible to elucidate the function of m^5U the most abundant of the methylated nucleosides in the tRNA of most organisms.

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