# Initiation of Protein Synthesis Without Formylation in a Mutant of *Escherichia coli* That Grows in the Absence of Tetrahydrofolate

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Starting from a *p*-aminobenzoate-requiring strain of *Escherichia coli* (*E. coli* K-12 AB3292), we have isolated mutants that can grow in the absence of *p*-aminobenzoate (and thus tetrahydrofolate). The following lines of evidence suggest that at least one of these mutants is capable of initiating protein synthesis without formylation of methionyl-transfer ribonucleic acid (methionyl-tRNA$^{fMet}$). (i) tRNA isolated (and charged in vivo with [$^{35}$S]methionine) from this mutant grown in a *p*-aminobenzoate-free medium contained less than 0.4% of the total methionine charged to the tRNA as formylmethionine. However, when the mutant was grown in the presence of *p*-aminobenzoate, 40 to 50% of the total [$^{35}$S]methionine was detected as formylmethionine. (ii) Extracts of the mutant grown in the absence of *p*-aminobenzoate contained no formyl-tetrahydrofolate, but such extracts did contain formylatable methionyl-tRNA and a functional transformylase. (iii) Tetrahydrofolate-free extracts of the mutant were capable of supporting protein synthesis with viral RNA (from f2) as messenger, but the resulting synthesized proteins contained no formylmethionine, and methionine residues were detected where formylmethionine residues are normally found. In the presence of formyl-tetrahydrofolate, use of a similar extract resulted in the detection of 30 to 40% of the total polypeptide methionine as formylmethionine. (iv) Initiation of protein synthesis in vitro occurred more readily with formyl-tetrahydrofolate-free extracts of the mutant than with similar extracts prepared from the parent strain. However, in the presence of formyl-tetrahydrofolate, initiation of protein synthesis proceeded equally well with both kinds of extracts. tRNA from this mutant and another spontaneously derived mutant was found to be partially deficient in the modified nucleoside ribothymidine (rT). Analysis of extracts showed that the mutants contained decreased levels of the methylase that results in the formation of ribothymidine. In vivo studies with an independently isolated rT$^-$ strain suggest that the lack of rT in tRNA facilitates the growth of *E. coli* under conditions where protein synthesis is forced to take place without formylation.

Procaryotic organisms like *Escherichia coli* require tetrahydrofolate for the biosynthesis of low-molecular-weight compounds such as methionine, glycine, purines, thymidine, and pantothenic acid as well as for the initiation of protein synthesis. The importance of tetrahydrofolate for the initiation of protein synthesis in procaryotic organisms is due to the fact that these organisms utilize formylmethionyl-transfer ribonucleic acid (tRNA) for initiation (1, 4, 32, 35) and that the synthesis of formylmethionyl-tRNA depends upon the presence of 10-

formyl-tetrahydrofolate as a formyl donor (10). In vitro, however, Eisenstadt and Lengyel (12) have shown that under certain conditions initiation of protein synthesis in *E. coli* is independent of formylation. They found that the addition of trimethoprim (a folate antagonist that severely inhibits the action of dihydrofolate reductase) to a culture of *E. coli* growing exponentially in a rich medium caused the growth rate to decrease, and within 15 min the incorporation of amino acids into proteins had ceased. Extracts of the trimethoprim-treated cells were shown to be deficient in formyl-tetrahydrofolate, and when protein synthesis directed by messenger RNA from the virus f2 was measured in the presence of these extracts, little or

no activity could be detected in the presence of 5 mM $Mg^{2+}$, unless formyl-tetrahydrofolate was added; however, at 10 mM $Mg^{2+}$ protein synthesis proceeded in the absence of added formyl-tetrahydrolfate.

Other bacteria that normally require folate for growth are known to be able to grow in its absence if the products of one-carbon metabolism are supplied (23, 25), and it has been shown that in *Streptococcus faecalis* R grown under these conditions initiation of protein synthesis apparently occurs without formylation (23, 26).

In the present work, our aim was to obtain a mutant of *E. coli* that could grow in the complete absence of folate in a medium containing all of the end products of one-carbon metabolism. Such a mutant would clearly have to initiate protein synthesis in the absence of tetrahydrofolate. Studies on such a mutant would be expected to provide valuable information about the importance of formylation for the initiation of protein synthesis. The work presented below describes the isolation of two such mutants and shows that protein synthesis in at least one of these organisms can be initiated in the absence of formylation of methionyl-tRNA$^{fMet}$. Analysis of tRNA from these mutants indicates that the tRNA is partially deficient in ribothymidine (rT); preliminary results suggest that this deficiency is related to the ability of the mutants to utilize unformylated methionyl-tRNA$^{fMet}$.

## MATERIALS AND METHODS

**General.** Amino acids and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Chemical Co.; sulfathiazole and calcium pantothenate were from Nutritional Biochemicals Corp.; trimethioprim and Pronase were from Calbiochem; thiamine hydrochloride was from Mann Laboratories; *p*-aminobenzoic acid (*p*-AB) was from Fisher Scientific Co.; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was from Aldrich Chemicals; trypsin and chymotrypsin were from Worthington Biochemicals Corp.; and benzoylated diethylaminoethyl-cellulose was from Boehringer Mannheim Corp. 5-Formyltetrahydrofolate (Leucovorin) was obtained from Lederle Laboratories and was converted quantitatively to 5,10-methenyl-tetrahydrofolate by incubation for 12 h at room temperature (in the dark) in 0.1 N HCl that contained 50 mM dithiothreitol. The solution containing the product was stored frozen in the dark until used. The sources of materials for column and thin-layer chromatography and nonradioactive markers of nucleoside diphosphates (pNp) are all described elsewhere (13). pTp was prepared by a procedure similar to that used for pCp and pUp (15) and was kindly provided by J. Ziegenmeyer.

**Chromatographic solvents.** The following solvent systems were used for thin-layer chromatography: 1, isobutyric acid-concentrated NH$_4$OH-water

(557:38:385, vol/vol/vol); 2, *t*-butyl alcohol-concentrated HCl-water (70:15:15, vol/vol/vol); 3, isobutyric acid-concentrated NH$_4$OH-water (66:1:33, vol/vol/vol), pH 3.7; 4, 0.1 M sodium phosphate (pH 6.8; 100 ml), (NH$_4$)$_2$SO$_4$ (60 g), and *n*-propyl alcohol (2 ml).

**Radioactive materials.** L-[$^{35}$S]methionine (260 Ci/nmol), L-[$^3$H]leucine (64 Ci/nmol), L-[$^3$H]methionine (11.6 Ci/nmol), *S*-adenosyl [*methyl*-$^3$H]methionine (6 to 11 Ci/nmol), *S*-adenosyl [*methyl*-$^{14}$C]methionine (60 mCi/mmol), and [$^{32}$P]phosphoric acid (carrier free) were all obtained from New England Nuclear Corp. γ-[$^{32}$P]adenosine 5'-triphosphate was prepared (13) by the procedure of Glynn and Chappell.

**Enzymes.** Sources of ribonuclease (RNase) T$_1$, RNase T$_2$, polynucleotide kinase T$_4$, *E. coli* alkaline phosphatase, and yeast hexokinase are all described elsewhere (13). Crude *E. coli* aminoacyl-tRNA synthetases were prepared essentially as described by Muench and Berg (21). Five grams of cells (wet weight) grown to mid-log phase were used for the preparation of these enzymes. The enzymes, freed of tRNA, were stored in 50% glycerol at −20°C (34) and were also used as a source of tRNA methylases (the activities of different extracts were correlated by comparison of the levels of methionyl-tRNA synthetase present in these extracts.

**Bacterial strains.** *E. coli* AB3292 was obtained from the Coli Genetic Stock Center of Yale University. For convenience, this strain will be referred to throughout this article as strain AB3292. This strain is a derivative of *E. coli* K-12 and carried the following genetic markers: Thi$^-$, Ile$^-$, Val$^-$, Arg$^-$, His$^-$, Pro$^-$, Man$^-$, Lac$^-$, Gal$^-$, T6$^r$, ColK$^r$, Str$^+$, *p*-AB$^-$. *E. coli* SR-1 is a mutant of *E. coli* CA274, which is lacking in tRNA ribothymidine methylase; consequently, tRNA isolated from this strain is totally deficient in the modified nucleoside rT (37). *E. coli* SR-1 and *E. coli* SR-3 (the isogenic rT$^+$ strain) were provided by M. Gefter.

**Growth media.** All media described below contained the following salts (per liter): NH$_4$Cl, 1 g; MgSO$_4$·7H$_2$O, 0.25 g; KH$_2$PO$_4$, 4.5 g; Na$_2$·HPO$_4$, 10.5 g; Ca(NO$_3$)$_2$·4H$_2$O, 7 mg; FeSO$_4$·7H$_2$O, 0.25 mg. Medium A contained, in addition to the salts listed above: glucose, 5 g; DL-isoleucine, 0.15 g; DL-valine, 0.3 g; L-arginine, 0.15 g; L-proline, 0.15 g; L-histidine, 0.05 g; and thiamine, 2 mg. The amino acids and vitamins listed above for medium A are nutritional requirements for *E. coli* AB3292. In addition, this organism requires *p*-AB, and when this compound is included in the medium it will be stated when the individual experiments are described.

Medium B contained all of the components listed for medium A plus the following (per liter): glycine, 0.045 g; L-methionine, 0.05 g; L-serine, 0.05 g; adenosine, 0.028 g; thymidine, 4 mg; and calcium pantothenate, 0.4 mg. These compounds are the products known to be made by biosynthetic pathways that, in each case, include at least one enzymatic reaction in which tetrahydrofolate participates as a coenzyme.

Medium C contained all of the components of medium B plus sulfathiazole (0.1 mM) and trimethoprim (0.1 mM).

Since previous experience indicated that amino acids are likely to contain small quantities of p-AB, a procedure was used to free each amino acid from contaminating p-AB. For this purpose, a solution (200 ml of 10 mM) of the amino acid was adjusted to pH 2.5 with 6 N HCl and 0.4 g of Darco G-60 activated charcoal (Atlas Powder Co.) was added. The mixture was heated at 90°C with stirring for 0.5 h, after which the charcoal was removed by filtration. The cooled solution was then adjusted to pH 7.0 with NaOH.

**Isolation of a mutant that grows in the absence of p-AB.** Since we wished to determine if it is possible in *E. coli* to initiate protein synthesis in the absence of the formylation of methionyl-tRNA, we needed to obtain a culture of *E. coli* that we were sure could grow in the complete absence of tetrahydrofolate, the coenzyme needed for formylation. Our approach to this problem was to grow a strain of *E. coli* (K-12, AB3292), known to require p-AB, in a p-AB-free medium that contained all of the end products whose biosynthesis includes one or more enzymatic reactions for which tetrahydrofolate is needed as a coenzyme. Under these conditions, the only need for tetrahydrofolate (and therefore for p-AB, an essential component of this coenzyme) would be for the formylation of methionyl-tRNA. Presumably, then, any bacteria that would grow on such a p-AB-free medium should be mutants able to initiate protein synthesis without formylation.

To increase the possibilities of obtaining such a mutant, strain AB3292 was treated with the mutagen nitrosoguanidine according to the general procedure of Adelberg et al. (2), and bacterial colonies were selected for the ability to grow on a p-AB-free medium that contained the products for whose biosynthesis tetrahydrofolate is needed. Sulfathiazole and trimethoprim were also included in the medium to inhibit the growth of possible revertants that might have acquired the ability to synthesize p-AB. Several such colonies were picked, and one was selected on which further investigations were carried out. This organism will be referred to as strain AB3292-i (to distinguish it from its parent, strain AB3292), since work to be presented below indicates that it differs from strain AB3292 in initiation of protein synthesis.

We also attempted to select for a mutant that may have arisen spontaneously. For this purpose, we followed the selection procedure described above, except that treatment with the mutagen was not included. A mutant was obtained that grew in the absence of p-AB. This mutant will be referred to in the text as strain AB3292-i2.

**Protein synthesis, in vitro.** Extracts of *E. coli* (called "S-30 extracts") were prepared from cells (grown to log phase) by the procedure described by Lodish (20), except that the buffer used contained the following components: 0.01 M Tris-hydrochloride (pH 7.5), 0.01 M MgCl₂, 0.06 M KCl, and 0.006 M mercaptoethanol. In all of the experiments to be reported, messenger RNA used was f2 RNA. The reaction mixtures contained [³H]leucine as the radioactive marker, and incubation conditions were as described previously (19). Assay for protein synthesis was as described elsewhere (31).

Digestion with Pronase of proteins synthesized in vitro in the presence of f2 RNA was carried out by the procedure of Pine et al. (23). Digestion of the synthesized proteins with a mixture of trypsin and chymotrypsin and fingerprinting of the resulting peptides were as described by Lodish (18). RNA from f2 virus was obtained and purified by the procedure of Goldman and Lodish (14), except that the treatment with sodium dodecyl sulfate was omitted.

**Isolation of tRNA.** Cells were grown to mid-log, late-log, or stationary phase in 10 liters of medium and harvested in a Sharples centrifuge. The cells were resuspended in buffer (2 ml/g of cells) containing 1 mM Tris-hydrochloride (pH 7.5) and 10 mM MgCl₂ and then used for the isolation of tRNA essentially as described previously by Holley (17) for yeast. Any aminoacyl residues attached to tRNA through ester linkages were removed by incubation with 1.8 M Tris-hydrochloride (pH 8.5) for 50 min at 37°C (28). The tRNA was then precipitated with ethanol and dissolved in 1 to 2 ml of water, and the solution was dialyzed against 300 to 500 volumes of 5 mM Tris-hydrochloride, pH 7.5. All tRNA samples were stored at −20°C. Yields of tRNA ranged from 60 to 100 absorbancy units at 260 nm per g (wet weight) of cells. Assays for the enzymatic aminoacylation of the tRNA and the formylation of methionyl-tRNA were as described previously (24, 33).

**Purification of tRNA^fMet.** As described previously (33), purification of tRNA^fMet required two steps of column chromatography, first on benzoylated diethylaminoethyl (BD)-cellulose and then on diethylaminoethyl-Sephadex A-50. Approximately 1,000 $A_{260}$ units of crude tRNA were used for these purifications. The final yield of purified tRNA^fMet ranged from 5 to 20 $A_{260}$ units. The tRNA was at least 75% (and in most cases greater than 95%) pure as assayed by methionine acceptor activity.

**Determination of extent of formylation of methionyl-tRNA.** Extent of formylation of methionyl-tRNA was determined by alkaline hydrolysis of the aminoacylated tRNA, followed by the separation of methionine from formyl-methionine by paper electrophoresis (24) or by extraction with ethyl acetate (23).

**In vitro methylation of tRNA.** Incubation mixtures contained (per milliliter): 120 mM Tris-hydrochloride (pH 8.0), 6 mM β-mercaptoethanol, 4 mM MgCl₂, 20 μM S-adenosyl [*methyl*-³H]methionine, 1 to 2 $A_{260}$ units of tRNA, and crude *E. coli* aminoacyl-tRNA synthetases, which were used as a source of methylase activity. Reactions were carried out at 37°C. Samples were removed and assayed for the extent of methylation by precipitation of the methylated tRNA on filter-paper disks with 5% trichloroacetic acid.

**Preparative methylation of tRNA.** Preparative methylation of tRNA was carried out as described above except that S-adenosyl [*methyl*-¹⁴C]methionine was used in place of S-adenosyl [*methyl*-³H]-methionine. Incubation was at 37°C for 90 min in a total volume of 1.5 ml (containing 2 $A_{260}$ units of tRNA). The reactions were terminated by the addition of an equal volume of phenol (equilibrated with 10 mM Tris-hydrochloride, pH 8.0). After thorough mixing, the aqueous and phenolic layers

were separated by centrifugation. The phenolic layer was then re-extracted with 0.5 ml of 10 mM Tris-hydrochloride (pH 8.0), and the first and second aqueous layers were combined. The methylated tRNA was recovered from the aqueous layer by precipitation with ethanol ($-20°C$ for 1 h). The tRNA in the precipitate was dissolved in 1 ml of water, and the solution was dialyzed against 1 liter of buffer containing 1 mM Tris-hydrochloride (pH 8.0) and 0.5 M NaCl at 4°C for 3 h to remove excess $S$-adenosyl-methionine and then an additional 3 h against 1 mM Tris-hydrochloride, pH 8.0 (1 liter). It was then stored frozen at $-20°C$.

**Analysis of products of methylation of strains AB3292-i and AB3292-i2 tRNA (34).** The $methyl$-$^{14}$C-labeled tRNA (30,000 cpm, 1 $A_{260}$ unit) was mixed with 1 $A_{260}$ unit of crude strain AB3292 tRNA, and the solution was evaporated to dryness. The residue was dissolved in 20 mM ammonium acetate, pH 4.4 (50 $\mu$l); RNase $T_2$ (2 units) was added, and the solution was incubated at 37°C for 5 h. The incubation mixture was evaporated to dryness; the residue was dissolved in 0.05 ml of water and the solution was again evaporated to dryness. The latter procedure was repeated twice, and the residue was finally dissolved in water (0.002 ml) and then used for two-dimensional thin-layer chromatography. Chromatography in the first dimension was with solvent 1, and in the second dimension it was with solvent 2. Nucleotidic material was visualized under a Mineralight ultraviolet lamp, and radioactivity was determined by autoradiography.

**In vitro labeling of fragments present in an RNase $T_1$ digest of tRNA$^{Met}$.** This was carried out essentially as described in a recent publication (13). Separation of the 5'-end group-labeled oligonucleotides by two-dimensional electrophoresis (27) and the 5'-end group analyses on these oligonucleotides were exactly as described in reference 13.

**Miscellaneous procedures.** Diethylaminoethyl-cellulose and diethylaminoethyl-Sephadex A-50 were prepared for use in chromatography as described earlier (24, 31). BD-cellulose, before use, was washed with buffer containing 0.01 M Tris-hydrochloride (pH 7.5), 0.01 M MgCl$_2$, 1.1 M NaCl, and 10% ethanol followed by a second wash with a solution containing 0.01 M Tris-hydrochloride (pH 7.5), 10 mM MgCl$_2$, and 0.4 M NaCl.

Radioactivity was determined with a Packard scintillation counter. Optical density readings were made with a Zeiss PMQ II spectrophotometer.

## RESULTS

**Growth experiments with strain AB3292.** The growth response of mutant AB3292 to $p$-AB was determined with the following results. No growth, as measured by turbidity, was evident at concentrations of $p$-AB below 10 pM. At concentrations from 10 to 100 pM, growth was directly proportional to $p$-AB concentration. The amount of $p$-AB required depends on the nature of the growth medium and the method used to prepare the inoculum. The growth data were obtained with the use of medium B, which con-

tained all of the known products of biosynthetic pathways in which a folate coenzyme is involved. The medium was inoculated with cells that had been starved for $p$-AB. Other experiments have shown that when inoculum cells are not starved for $p$-AB the growth response to $p$-AB is erratic and that significant growth occurs in the absence of $p$-AB, probably because the cells had stored $p$-AB during growth on the $p$-AB-rich medium.

The concentrations of the antimetabolites sulfathiazole and trimethoprim needed to inhibit completely the growth of strain AB3292 on medium B in the presence of a constant amount of $p$-AB have been determined to be approximately 500 and 300 times, respectively, the concentration of $p$-AB present in the medium.

**Properties of a mutant that grows in the absence of $p$-AB.** The isolation of the mutant, strain AB3292-i, selected for growth in the complete absence of $p$-AB, is described in Materials and Methods. When this mutant is grown in the absence of $p$-AB, it should require the end products of one-carbon metabolism, since tetrahydrofolate is required for the synthesis of these products. We have tested methionine and pantothenic acid as representatives of these end products and found that both are required. In addition, we have determined, as expected, that neither is needed when $p$-AB is supplied but that the organism still requires isoleucine and valine. The latter observation establishes that the mutant is a derivative of strain AB3292 and not merely a contaminant that might have been selected.

We have also shown that if the mutant is grown in the presence of $p$-AB and then transferred to a $p$-AB-free medium growth still occurs; under the same conditions the parent, strain AB3292, will not grow after the $p$-AB is exhausted.

Other growth characteristics are that the mutant grows significantly faster in the presence of $p$-AB (doubling time of 1.4 h) than in its absence (1.5 to 2.5 h) and the doubling time of the mutant, even in the presence of $p$-AB, is greater than that of the parent, strain AB3292 (0.8 h). Another observation is that maximum growth of the mutant in the absence of $p$-AB (achieved after 24 h of incubation) is never as great as that observed for the parent in the presence of $p$-AB. The growth medium was modified in a number of ways to try to increase the cell yield. Increasing the concentrations of the compounds included in the supplement (i.e., methionine, adenosine, thymidine, glycine, and pantothenic acid) had no effect. The possibility was considered that growth in the absence of a folate coenzyme might interfere

with aerobic metabolism and thus increase the requirement for glucose. However, a higher concentration of glucose did not increase the cell yield. Addition of aromatic amino acids also had no effect. It was also established that the lower yield of cells was not due to the presence of either sulfathiazole or trimethoprim, since deletion of these from the medium had no effect on maximum growth. No evidence could be obtained for the production of a toxin that might limit growth, since the spent growth medium could support further growth when supplemented with additional glucose and reinoculated. The reason for limited growth of strain AB3292-i in the absence of $p$-AB remains unexplained.

**Lack of formylation of methionyl-tRNA$^{fMet}$ in vivo by strain AB3292-i.** Strain AB3292-i tRNA that had been charged in vivo with L-[$^{35}$S]methionine (see Table 1) was isolated and analyzed for the presence of formylmethionine.

TABLE 1. *Extent of formylation of methionyl-tRNA charged in vivo*[a]

| Organism | Growth con- ditions | Radioactivity (cpm) present as: | |
| --- | --- | --- | --- |
| | | Methi- onine | Formylme- thionine |
| *E. coli* AB3292 | Plus $p$-AB | 875 | 855 |
| *E. coli* AB3292-i | Plus $p$-AB | 2,205 | 1,645 |
| *E. coli* AB3292-i | Minus $p$-AB | 3,650 | 15 |

[a] Strain AB3292 was grown on medium B plus $p$-AB (10 nM). Strain AB3292-i was grown in either medium B plus $p$-AB (10 nM) or on medium C (no $p$-AB). In each case, approximately 10 ml of a culture grown to stationary phase was used to inoculate 100 ml of fresh medium. These cultures were incubated at 37°C until mid-log phase of growth was achieved (40 to 50 Klett units). The cells were collected by centrifugation, washed with sterile saline to remove excess methionine, and resuspended in 100 ml of either methionine-free medium B plus $p$-AB (AB3292 or AB3292-i) or methionine-free medium C (AB3292-i). The cultures were incubated in the methionine-free media for 1 to 2 h, after which L-[$^{35}$S]methionine, 10 μCi (260 mCi/mmol), was added to each culture and incubation was continued for 3 min. Cold (4°C) phenol (100 ml), saturated with 0.05 M sodium acetate buffer (pH 5.0), was added to each culture, and the mixture was shaken at room temperature for 10 min. The aqueous and phenolic layers were separated by centrifugation at 2,500 rpm for 5 min. The phenol was extracted with 50 ml of 0.05 M sodium acetate buffer, pH 5.0. The combined aqueous layers were extracted four times with 100-ml portions of cold ether. To the resulting aqueous material was added enough solid NaCl to make the solution 1 M in NaCl. The tRNA was precipitated by the addition of 2 volumes of cold (−20°C) ethanol. The RNA was recovered by centrifugation and dissolved in 5 to 10 ml of acetate buffer, pH 5.0. The RNA was reprecipitated with cold ethanol and dissolved in 5 ml of acetate buffer. This solution was dialyzed against 100 volumes of 0.02 M acetate buffer to remove excess [$^{35}$S]methionine, salts, and ethanol. The extent of formylation of the methionyl-tRNA was determined by the ethyl acetate extraction method described in Materials and Methods.

The results (Table 1) show that when either strain AB3292 or AB3292-i was grown in the presence of $p$-AB, from 40 to 50% of the methionine charged to tRNA in vivo was recovered as formylmethionine. However, when strain AB3292-i was grown in the absence of $p$-AB, virtually none of the labeled methionine was recovered as formylmethionine.

**Formylation of methionyl-RNA in vitro.** The following procedure was devised to test for the presence of tetrahydrofolate in extracts of strain AB3292-i. The organism was grown in the presence (medium B plus 10 nM $p$-AB) or absence (medium C) of $p$-AB to log phase, and cell extracts were prepared as described by Lodish (20) in buffer containing 30 mM mercaptoethanol to prevent the oxidation of tetrahydrofolate. The extracts were equivalent to S-30 preparations used for in vitro protein synthesis (20) and thus should contain tRNA, aminoacyl-tRNA synthetases, the methionyl-tRNA formylase, and endogenous tetrahydrolfate. The tRNA present in the extract was aminoacylated with L-[$^{35}$S]methionine (as described in Table 2) in the presence and absence of added formyl-tetrahydrofolate, and the charged tRNA was isolated. The amount of the radioactive methionine present in the tRNA as formylmethionine is shown in Table 2. When strain AB3292-i was grown in the absence of $p$-AB, the results in Table 2 show that no formylation of methionyl-tRNA occurred, an indication that no endogenous formyl-tetrahydrofolate was present in the extract. However, when the mutant was grown in the presence of $p$-AB, formylation occurred without the addition of formyl-tetrahydrofolate. This indicates that the latter compound was present in the extract. Addition of formyl-tetrahydrofolate to either extract resulted in the recovery of 46% of the labeled methionine as formylmethionine. The latter observation indicates that the mutant contains a formylatable methionyl-tRNA and a functional transformylase even when it is grown in the absence of $p$-AB.

**Protein synthesis in vitro.** The following experiment was designed to determine whether or not initiation of protein synthesis could proceed without formylation in extracts of strain AB3292-i. Cells were grown in the presence (medium B plus 10 nM $p$-AB) or absence (medium C) of $p$-AB, and extracts were prepared. These extracts were used in f2 RNA-directed protein synthesis with [$^{35}$S]methionine as the radioactive label. The [$^{35}$S]methionine-labeled protein was then digested with Pronase. Further details of these experiments are described in Materials and Methods and in Table 3. The results (Table 3) show that when protein syn-

TABLE 2. *In vitro formylation of methionyl-tRNA with extracts of E. coli AB3292-i* [a]

| Growth conditions | Addition to extract | Formylmethio-nine detected (cpm) | Methionine de-tected (cpm) | Portion (%) of $^{35}$S present as formyl-methionine |
|---|---|---|---|---|
| Minus p-AB | None | 17 | 5,539 | 0.27 |
| Minus p-AB | f-folate-H$_4$[b] | 1,987 | 2,278 | 46 |
| Plus p-AB | None | 773 | 3,397 | 16 |
| Plus p-AB | f-folate-H$_4$ | 1,814 | 2,122 | 46 |

[a] Reaction mixtures (0.1 ml each) were prepared to contain: 0.05 M Tris-hydrochloride (pH 7.8), 2.5 mM adenosine 5'-triphosphate, 5 mM MgCl$_2$, 0.01 ml of extract (prepared as described in the text), and 1 μCi of L-[$^{35}$S]methionine (260 Ci/mmol). As shown, *dl*-formyl-tetrahydrofolate, 75 μM, was also added to some mixtures. Incubation was at 37°C for 5 min. The reaction was terminated by the addition of 0.1 ml of cold phenol, and the charged tRNA was isolated and analyzed for [$^{35}$S]methionine and formyl-[$^{35}$S]methionine as described in Table 3.

[b] f-folate-H$_4$, Formyl-tetrahydrofolate.

TABLE 3. *Formylmethionine in f2 proteins synthesized in vitro with extracts of E. coli AB3292 and AB3292-i* [a]

| Strain of E. coli | Growth condi-tion | Amt (cpm) of $^{35}$S-labeled compounds in protein di-gest as:[b] | |
|---|---|---|---|
| | | Methio-nine | Formylme-thionine |
| AB3292-i | Minus p-AB | 4,841 | 0 |
| AB3292-i | Plus p-AB | 3,469 | 2,425 |
| AB3292 | Plus p-AB | 3,659 | 1,676 |

[a] Reaction mixtures (0.1 ml each with 9 mM MgCl$_2$) for protein synthesis directed by f2 messenger RNA were prepared as described in Materials and Methods. To each mixture was added 1 μCi of L-[$^{35}$S]methionine (260 Ci/μmol). The protein isolated (see Materials and Methods) contained 50,000 to 100,000 cpm of radioactive methionine. The protein was digested with Pronase (23), and a portion was used to determine the amounts of methionine and formylmethionine present by electrophoretic separation of these two compounds (14).

[b] Although the results shown were obtained with a concentration of 9 mM MgCl$_2$ in the reaction mixtures, similar results were also obtained when the concentration of MgCl$_2$ was at 5 or 7 mM.

thesis was carried out in the presence of an extract of strain AB3292-i grown in the absence of p-AB no formylmethionine could be detected in the protein. However, when similar experiments were performed with extracts of either strain AB3292 or AB3292-i grown in the presence of p-AB, 30 to 40% of the [$^{35}$S]methionine incorporated into f2 proteins was recovered as formylmethionine. Thus, the mutant grown in the absence of p-AB contains no tetrahydrofolate. These results also indicate that no other *N*-acylmethionyl-tRNA is used to initiate protein synthesis since the method (paper electrophoresis) used to show the presence of formylmethionine would also have separated other acylmethionine compounds from methionine,

unless such compounds were particularly unstable.

In a more direct approach to determine whether or not protein synthesis can be initiated by methionyl-tRNA instead of formylmethionyl-tRNA, an extract of strain AB3292-i (grown in the absence of p-AB) was incubated with [$^{35}$S]methionyl-tRNA$^{fMet}$ and f2 RNA, and the resulting f2 proteins were then digested with a mixture of trypsin and chymotrypsin and fingerprinted (18, 31). The results showed a major radioactive band, which corresponded to the N-terminal methionine-containing peptide of the coat protein, but no evidence was obtained for any of the corresponding formylmethionine-containing peptide. When a similar experiment was performed with an extract prepared from strain AB3292-i that had been grown in the presence of p-AB, the major radioactive band corresponded to that expected for the formylmethionine-containing peptide of the coat protein. Smaller amounts of the deformylated form of this peptide and of the formylmethionine-containing peptide derived from the f2 RNA polymerase were also detected. Thus, under the conditions used in the above experiments: (i) initiation of f2 protein synthesis in the absence of tetrahydrofolate occurs without detectable formylation of methionyl-tRNA; (ii) initiation without tetrahydrofolate seems to occur with methionyl-tRNA instead of formylmethionyl-tRNA; (iii) despite the fact that initiation can occur without formylation, the viral coat protein is the major in vitro product (as it is during initiation with formylmethionyl-tRNA); and (iv) when extracts of the mutant (strain AB3292-i) grown in the presence of p-AB are used, initiation of protein synthesis occurs with formylmethionyl-tRNA.

Evidence that the mutant, strain AB3292-i, has acquired the ability to initiate protein synthesis without formylation of methionyl-

tRNA$^{fMet}$ is derived from a comparison of protein synthesis between folate-free extracts prepared from strains AB3292 and AB3292-i. Protein synthesis was studied at two Mg$^{2+}$ concentrations (5 and 7 mM) with f2 RNA as messenger and [$^3$H]leucine as the radioactive label. Figure 1 shows the results of these experiments. At 25°C and in the presence of 5 mM Mg$^{2+}$, protein synthesis supported by a tetrahydrofolate-free extract of strain AB3292-i is three to five times greater than that observed in the presence of an extract of strain AB3292 treated with trimethoprim (see Fig. 1A). The experiment was repeated at 37°C (Fig. 1A) with virtually the same results. At the optimal concentration of Mg$^{2+}$ (7 mM) for protein synthesis, the rate of protein synthesis at 25°C is substantially greater with a tetrahydrofolate-free extract of strain AB3292-i than that observed in the presence of the formyl-tetrahydrofolate-free extract of strain AB3292 (Fig. 1B).

An experiment similar to that described in Fig. 1 was performed, except that formyl-tetrahydrofolate was added to the reaction mixtures. At both optimal (7 mM) and suboptimal (5 mM) concentrations of Mg$^{2+}$ for protein synthesis, no substantial differences were observed between the abilities of extracts of strains AB3292 and AB3292-i to support protein synthesis when formyl-tetrahydrofolate was present, at an incubation temperature of either 25 or 37°C. These results indicate that protein synthesis in the absence of formylation is more efficient in the mutant (strain AB3292-i) than in the parent strain (AB3292), but there is virtually no difference when formyl-tetrahydrofolate is present.

**Partial deficiency of the modified nucleo-**side rT in tRNA isolated from strains AB3292-i and AB3292-i2. A possible explanation for the relative ease with which initiation of protein synthesis without formylation occurs in strain AB3292-i is that the tRNA$^{fMet}$ from this organism may differ from that of the parent strain, AB3292, and this difference might allow efficient initiation with methionyl-tRNA$^{fMet}$ in place of formylmethionyl-tRNA$^{fMet}$. Such an explanation would be similar to that proposed by Rabinowitz and co-workers (8, 26) for the initiator tRNA isolated from *S. faecalis* grown in the absence of folate. These authors have shown that in contrast to the tRNA$^{fMet}$ isolated from *S faecalis* grown in the presence of folate (plus-folate), unformylated methionyl-tRNA$^{fMet}$ from *S. faecalis* grown in the absence of folate (minus-folate) can initiate protein synthesis without formylation. Comparison of the sequences of plus-folate and minus-folate *S. faecalis* tRNA$^{fMet}$ led to the finding that they differed in a single nucleotide residue; the modified nucleoside rT present in the sequence G-T-$\Psi$-C- in loop IV of the plus-folate tRNA$^{fMet}$ is lacking and is replaced by an unmodified U in the minus-folate tRNA$^{fMet}$. In view of this, we have investigated whether tRNA$^{fMet}$ isolated from strain AB3292-i also differs from the parent strain, AB3292. Our results suggest that bulk tRNA and tRNA$^{fMet}$ from the mutant strain contain much reduced levels of the modified nucleoside rT compared to strain AB3292.

In preliminary experiments, we examined whether or not bulk tRNA from strains AB3292-i and 3292-i2 contained any rT. The tRNA's isolated from these strains were digested with RNase T$_2$, and the resulting nucleo-
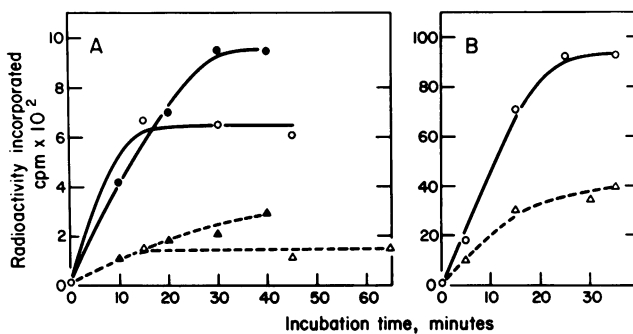


FIG. 1. *Protein synthesis with folate-free extracts of E. coli AB3292 and AB3292-i. Reaction mixtures were prepared, as described in Materials and Methods, to contain MgCl$_2$ at either 5 or 7 mM. Radioactivity was supplied as [$^3$H]leucine (2 μCi, 64 Ci/mmol). An extract of either strain AB3292-i or AB3292 (treated with trimethoprim as decribed in the text) was added, and incubation was at either 25 or 37°C. Portions of 0.1 ml each were withdrawn at various times and assayed for incorporation of $^3$H into protein. (A) 5 mM MgCl$_2$: (○) extracts of strain AB3292-i and incubation at 25°C; (●) extract of strain AB3292-i and incubation at 37°C; (△) extract of strain AB3292 and incubation at 25°C; (▲) extract of strain AB3292 and incubation at 37°C. (B) 7 mM MgCl$_2$, with all incubations at 25°C: (○) extract of strain AB3929-i; (△) extract of strain AB3292.*

side 3'-phosphates were separated by two-dimensional thin-layer chromatography (22, 34). The presence in such digests of material corresponding in chromatographic characteristics to rTp suggested that these tRNA's were not totally deficient in rT.

Since the material (corresponding in $R_f$ values to rTp) from tRNA's isolated from the mutant strains was considerably less than that observed in a parallel experiment with tRNA from the parent strain AB3292, we investigated the possibility that the tRNA's from the mutants may be partially but not totally deficient in rT. As an assay, we measured the ability of the bulk tRNA from the mutant to be methylated in vitro by crude extracts from the parent strain. Figure 2 compares the kinetics of incorporation of methyl groups from S-adenosyl [methyl-³H]methionine into tRNA's isolated from strains AB3292 and AB3292-i. No significant incorporation of methyl group into strain AB3292 tRNA occurred over that observed in the absence of any added tRNA. In contrast, methylation of strain AB3292-i tRNA occurred readily, and the final extent of methylation was 30 to 60% of that obtained with tRNA from E. coli SR-1 (a strain totally deficient in rT, not shown). Fig. 2 further shows that tRNA isolated from strain AB3292-i grown in the presence of p-AB is methylated at the same rate and to the same extent as tRNA isolated from strain AB3292-i grown in the absence of p-AB. Thus, the undermethylation of tRNA from strain AB3292-i is independent of the presence or absence of folate or of the antimetabolites trimethoprim and sulfathiazole 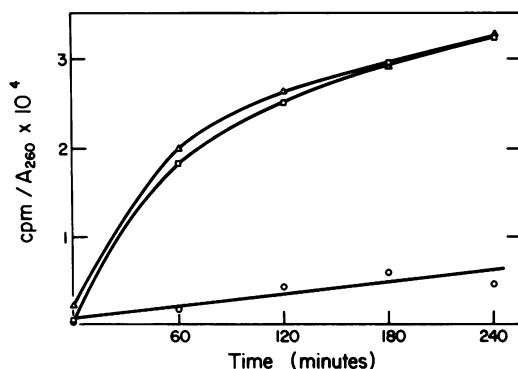in the growth medium. Other studies (not shown) further indicate that strain AB3292-i tRNA is undermethylated irrespective of whether tRNA's are isolated from cells harvested at mid-log, late-log, or stationary phase.

To characterize the product of in vitro methylation, strain AB3292-i tRNA was methylated with S-adenosyl [methyl-¹⁴C]methionine as the methyl donor. The methyl-¹⁴C-labeled tRNA was isolated and digested with RNase $T_2$, and the methyl-¹⁴C-labeled nucleoside-3' phosphate was analyzed by two-dimensional thin-layer chromatography (34). Figure 3 shows the results of such an experiment. Only a single radioactive spot was seen, and this corresponded exactly to a marker of rTp present in the digest of strain AB3292 tRNA added as carrier. Thus, in vitro methylation of strain AB3292-i tRNA resulted in the production of only rT.

**Partial deficiency of rT in tRNA^fMet from strains AB3292-i and AB3292-i2.** To determine whether or not the partial deficiency in rT observed in crude tRNA is also true of initiator
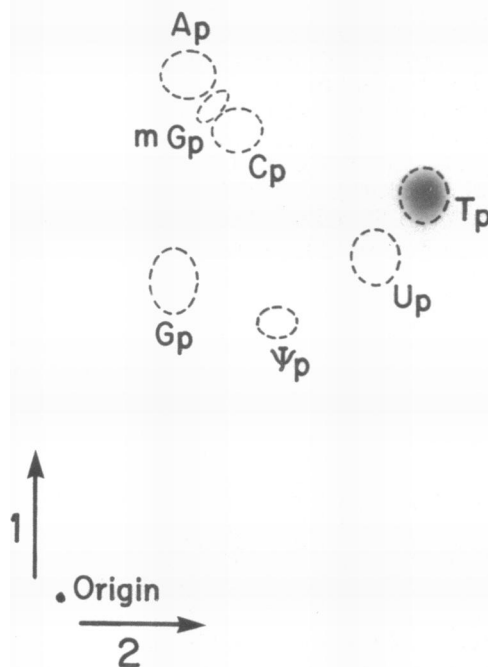


FIG. 2. *Methylation, as a function of time, of tRNA from E. coli AB3292-i and AB3292 in the presence of crude extract prepared from E. coli AB3292. Symbols: (○), tRNA prepared from strain AB3292; (□), tRNA prepared from strain AB3292-i grown in the presence of p-AB; (△), tRNA prepared from strain AB3292-i grown in the absence of p-AB.*



FIG. 3. *Two-dimensional thin-layer chromatography of an RNase $T_2$ digest of methyl-¹⁴C-labeled E. coli AB3292-i tRNA methylated in vitro with S-adenosyl [methyl-¹⁴C]methionine. Dotted circles indicate the location of the various nucleoside 3' phosphates as seen under an ultraviolet lamp.*

tRNA, analyses were carried out on purified tRNA$^{fMet}$ obtained from strains AB3292 and AB3292-i. To minimize the amounts of tRNA$^{fMet}$ necessary for such experiments, the procedure used for these analyses differs from that used above and involves in vitro labeling of oligonucleotides present in RNase $T_1$ digests of the tRNA's (30) with $^{32}P$ at the 5'-end. Since in an RNase $T_1$ digest of E. coli tRNA$^{fMet}$ rT is present as part of the sequence T-Ψ-C-A-A-A-U-C-C-G- or U-Ψ-C-A-A-A-U-C-C-G- if the tRNA lacks rT (11), in vitro labeling of this fragment yields [$^{32}P$]T (or U)-Ψ-C-A-A-A-U-C-C-G. These two related fragments (which differ only by the presence of rT or U at the 5'-end) do not separate from each other but can be resolved from the other oligonucleotides present in RNase $T_1$ digests of tRNA$^{fMet}$ by two-dimensional electrophoresis (27). Isolation and treatment of the above $^{32}P$-labeled fragments with RNase $T_2$ yielded [$^{32}P$]Tp and [$^{32}P$]Up (13, 30, 34). Separation of these nucleoside 3',5'-diphosphates and quantitative radioactive analyses allowed an accurate estimate of the level of modification of U to rT in tRNA$^{fMet}$ in strains AB3292 and AB3292-i.

Figure 4 shows the fingerprints of 5'-$^{32}P$-labeled oligonucleotides obtained from an RNase

$T_1$ digest of strains AB3292 and AB3292-i tRNA$^{fMet}$. Except for the different relative locations of spot 2, which was observed only when this particular preparation of strain AB3292-i tRNA$^{fMet}$ was used, no other significant differences between the two fingerprints are evident. (This difference in the relative locations of spot 2 is most likely due to the fact that the tRNA$^{fMet}$ used lacks the 3'-terminal A residue. Thus, spot 2, which is [$^{32}P$]C-A-A-C-C-A in Fig. 4a, is now [$^{32}P$]C-A-A-C-C in spot 2 of Fig. 4b.) Spot 15 corresponds to [$^{32}P$]T (or U)-Ψ-C-A-A-A-U-C-C-G, spot 14 to [$^{32}P$]C$_m$-U-C-A-U-A-A-C-C-C-G, and spot 8 to [$^{32}P$]m$^7$G-U-C-G, and these three oligonucleotides account for all the methylated nucleotides present in E. coli tRNA$^{fMet}$ (11). The radioactive oligonucleotides present in spots 15 and 14 were eluted and digested with RNase $T_2$, and the resulting $^{32}P$-labeled nucleotides were separated by thin-layer chromatography. Figure 5 shows the results of chromatography of such a digest in solvent 3, and Fig. 6 shows that in solvent 4. The results can be summarized as follows.

(i) Analysis of material in spot 15 of Fig. 4 shows that tRNA$^{fMet}$ isolated from strain AB3292 yielded almost exclusively [$^{32}P$]rTp and very little [$^{32}P$]Up (Fig. 5 and 6, spot 15a). In
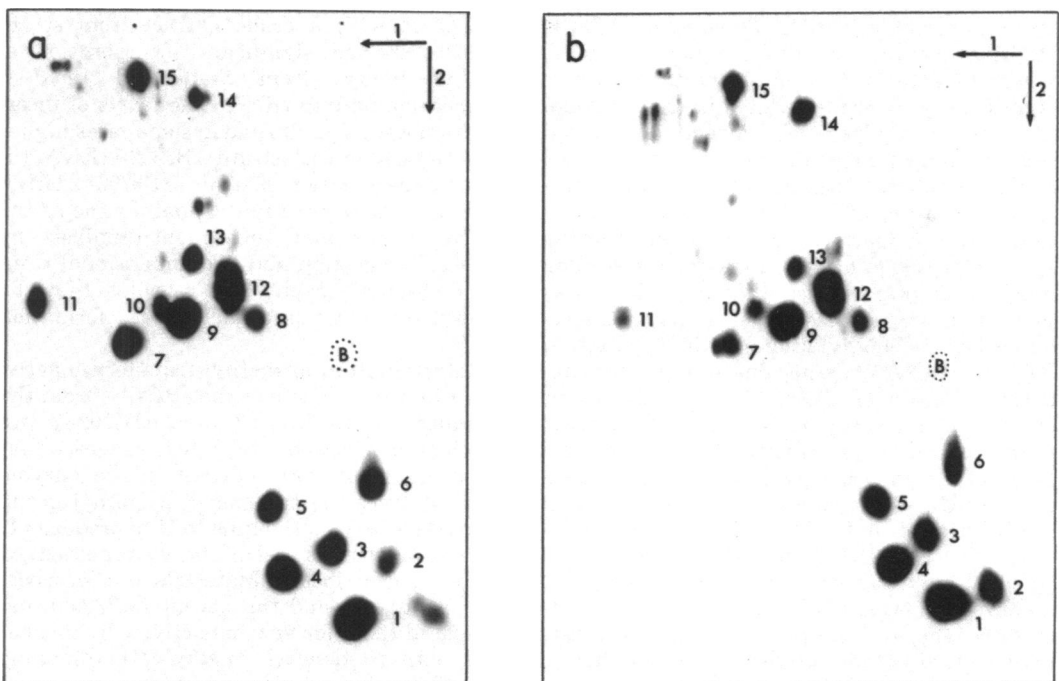


FIG. 4. Autoradiograph of 5'-$^{32}P$-labeled oligonucleotides obtained from an RNase $T_1$ digest of E. coli AB3292 and AB3292-i tRNA$^{fMet}$. The areas labeled with a circled B represent the areas of migration of the blue dye marker. (a) Strain AB3292; (b) strain AB3292-i.
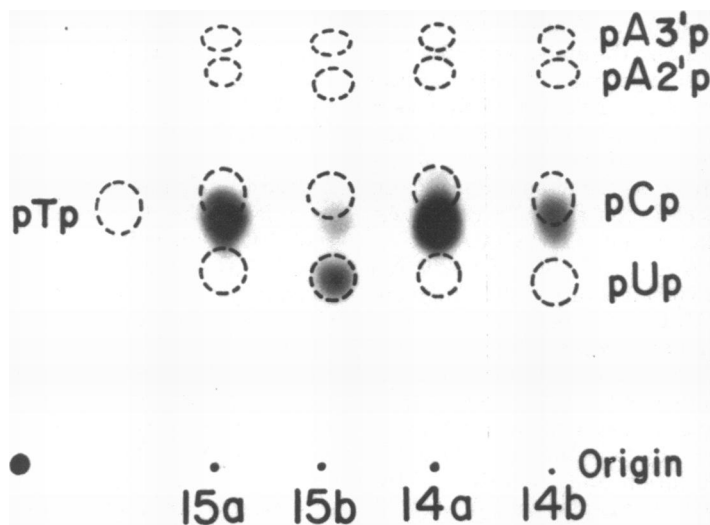
FIG. 5. *Autoradiography of nucleoside 3',5'-diphosphates obtained by digestion of material from spots 15 and 14 of Fig. 4 with RNase $T_2$. Thin-layer chromatography was in solvent 3. Dotted circles indicate the location of markers of synthetic pAp, pCp, pUp, and pTp, which are isomeric mixtures of 2',5'- and 3',5'-diphosphates. This solvent system separates the two isomeric adenosine diphosphates but not the others. a and b are radioactive fragments derived from tRNA from strains AB3292 and AB3292-i, respectively.*

contrast, tRNA$^{fMet}$ from strain AB3292-i yielded a mixture of [$^{32}$P]rTp and [$^{32}$P]Up (spot 15b). Approximately 60% of the total radioactivity was present as [$^{32}$P]Up. Thus, strain AB3292 tRNA$^{fMet}$ contains its full complement of rT, whereas the tRNA$^{fMet}$ from the mutant strain is significantly undermethylated in this position. (ii) Similar analysis of radioactive material present in spot 14 of Fig. 4 shows that in all cases the radioactive product has mobility similar to that expected for [$^{32}$P]C$_m$-Up. Determination of the radioactivity of the material in the spots corresponding to ultraviolet-absorbing markers of pCp showed that no [$^{32}$P]C$_p$ was present in any of these digests. Thus, the reduced level of methylation of U to rT in strain AB3292-i tRNA$^{fMet}$ is not due to a general undermethylation of tRNA$^{fMet}$ in these mutant strains. Further support for this is also derived from the finding (not shown) that the relative levels of [$^{32}$P]m$^7$G-U-C-G (spot 8 of Fig. 4) present in RNase T$_1$ digests of tRNA$^{fMet}$ from strains AB3292 and AB3292-i are also the same.

The fact that tRNA from strain AB3292-i is deficient in rT is consistent with the theory that undermodification of tRNA at this position facilitates the initiation of protein synthesis without formylation. To determine whether or not this deficiency also occurs in other mutants selected for their growth in the absence of folate, we analyzed the tRNA from strain AB3292-

i2, a spontaneous mutant derived from strain AB3292. The following results were obtained. (i) In vitro incubation of bulk tRNA from strain AB3292-i2 with a crude extract from strain AB3292 showed significant incorporation of methyl groups from S-adenosyl [*methyl-*$^3$H]methionine into tRNA. The levels of incorporation were equal to and in some cases higher than those seen with strain AB3292-i tRNA. (ii) In vitro methylation of strain AB3292-i2 tRNA with S-adenosyl [*methyl-*$^{14}$C]methionine as the methyl donor and subsequent analysis by RNase T$_2$ digestion and two-dimensional thin-layer chromatography (34) revealed that the methyl group was used solely for the formation of rT.

**Determination of methyl-transferase activity in mutants.** The fact that tRNA's from the mutants, strains AB3292-i and AB3292-i2, are deficient in ribothymidylic acid suggests that these organisms are deficient in the enzyme that catalyzes the transfer of a methyl group from S-adenosylmethionine to U to produce rT. To investigate this possibility, we prepared extracts (as described in Materials and Methods) of the mutants and the parent (AB3292) and compared them for enzyme activity by incubation with S-adenosyl [*methyl-*$^3$H]methionine and rT-deficient tRNA prepared from *E. coli* SR-1 and measurement of incorporation of radioactivity into the tRNA. The results given in
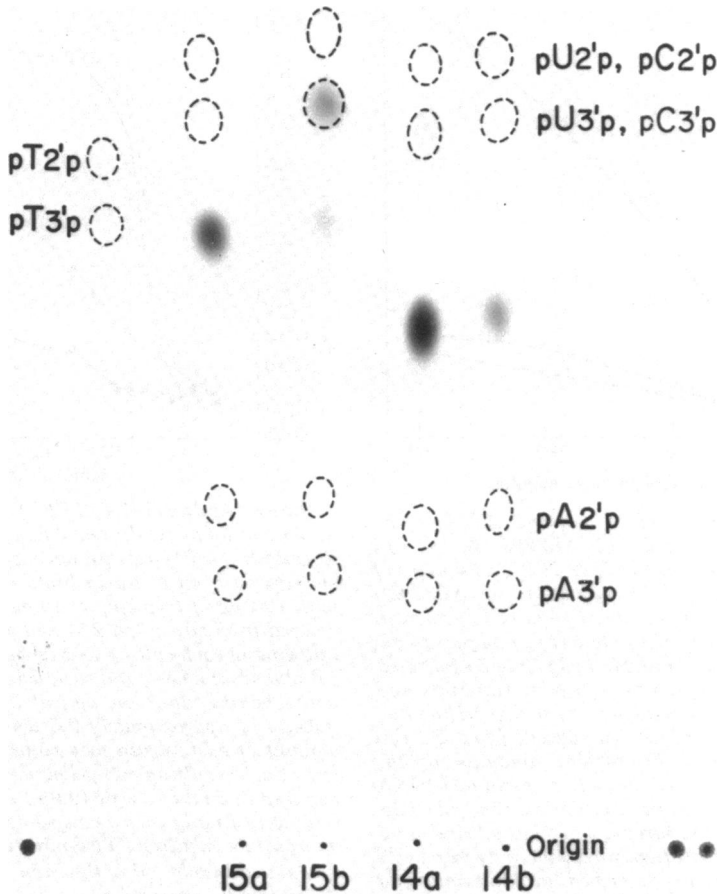
FIG. 6. *Autoradiography of the same materials described in Fig. 5, with solvent 4 as the developing agent. This solvent separates the 2',5' and 3',5' isomers of all the diphosphates. The relative mobility of 2',5' versus the 3',5' isomers is reversed as compared to solvent 3.*

Fig. 7 show that the enzyme activities of the mutant extracts were significantly lower than the activity present in the parent (strain AB3292). This low level of activity in the mutant extracts was apparent whether the cells were grown in the presence or absence of $p$-AB (data not shown for cells grown in the presence of $p$-AB). Thus, it appears that the deficiency of rT in the mutant tRNA can be explained as a mutation that causes less enzyme activity to be evident.

There are several possible explanations for the low levels of enzyme activity in the mutants. One possibility is that the mutant enzymes are more labile at physiological temperatures. To test this, the extracts were incubated at 37°C for up to 60 min before enzyme activity was measured. We found that such treatment resulted in no decrease of activity. Another pos-

sibility is that mutant extracts contain an inhibitor not present in the parent extracts. However, this possibility also seems unlikely since we found that the inclusion of mutant extracts in reaction mixtures did not affect the enzyme activity of the parent extract. Thus, although we do not yet know why the activities are low in the mutants, it seems likely that the mutations result in the production of either lesser amounts of the enzymes or altered enzymes that function with less efficiency.

**Growth experiments with *E. coli* SR-1.** Since the results presented in the preceding sections indicate that tRNA from *E. coli* mutant strains 3292-i and 3292-i2 is deficient in rT, a possible explanation for the ability of these mutants to grow in the absence of folate is that the presence of U in place of rT in the tRNA$^{fMet}$ allows initiation of protein synthesis without
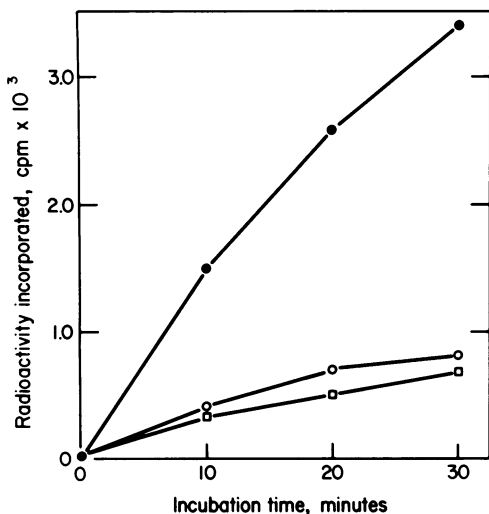
FIG. 7. Methylation of rT-deficient tRNA in the presence of extracts of E. coli AB3292 (●), E. coli AB3292-i (○), or E. coli AB3292-i2 (□) as sources of enzymes. Extracts of the mutants (strains AB3292-i and AB3292-i2) were prepared from cells grown in the absence of p-AB. Extracts were prepared as described in Materials and Methods. Incubation mixtures were prepared (as described in Materials and Methods) to contain S-adenosyl [methyl-³H]methionine, rT-deficient tRNA (prepared from E. coli SR-1), and extract. Equivalent amounts of the three extracts were used (based on methionyl-tRNA synthetase activity). At the appropriate time, portions of the incubation mixtures were removed and analyzed for the incorporation of radioactivity into acid-insoluble material as described in Materials and Methods.

FIG. 8. Ability of E. coli SR-1 (rT⁻) to grow in the absence of folate. Cultures of E. coli SR-1 (rT⁻) and E. coli SR-3 (rT⁺) were grown overnight at 37°C with shaking in 5 ml of Luria broth (LB) supplemented with 100 μg of tryptophan per ml (both strains are derived from strain CA 274 and are thus trp⁻). One milliliter of each culture was inoculated into 20 ml of LB plus tryptophan, and each was incubated at 37°C with shaking until an optical density at 600 nm ($OD_{600}$) of approximately 0.5 was reached. A 5-ml amount of each strain was collected by centrifugation. Each resulting cell pellet was washed by resuspending in 5 ml of sterile (0.9%) saline. The washed cells were finally then resuspended in 2.5 ml of the appropriate medium. Two-milliliter amounts were inoculated into 50 ml of the same medium (in which the cells were suspended), and the cultures were incubated at 37°C with shaking. At various intervals of time, the $OD_{600}$ was determined as a measure of growth. The media used in these experiments were medium B plus 10 nM p-AB and medium C. The contents of these media are described in Materials and Methods. The difference in the two media is that medium C contains the antimetabolites (sulfathiazole and trimethoprim), whereas medium B does not. Thus, growth on medium C would be interpreted as growth in the absence of folate. Results are shown for the growth of E. coli SR-1 (rT⁻) on medium B plus p-AB (△) and on medium C (▲) and for the growth of E. coli SR-3 (rT⁻) on medium B plus p-AB (○) and on medium C (●).

formylation. If this is a valid explanation, the mutant known to be completely devoid of rT, E. coli SR-1, would also be expected to initiate protein synthesis without formylation and, thus, would be able to grow in the absence of tetrahydrofolate. To test this possibility, growth experiments were carried out with this organism and the corresponding rT⁺ strain (Fig. 8). The results presented in Fig. 8 show (i) that both strains grow equally well in the presence of p-AB (and the absence of antimetabolites) and (ii) that the rT⁻ strain grows less well in the presence of the antimetabolites (and thus in the absence of tetrahydrofolate) than in their absence, but still the growth of this strain is much greater than that of the rT⁺ strain in the presence of the antimetabolites. The small amount of growth of the rT⁺ strain in the presence of the antimetabolites can be observed even when an end product such as pantothenic acid is omitted from the growth medium (data not shown). This growth probably resulted from
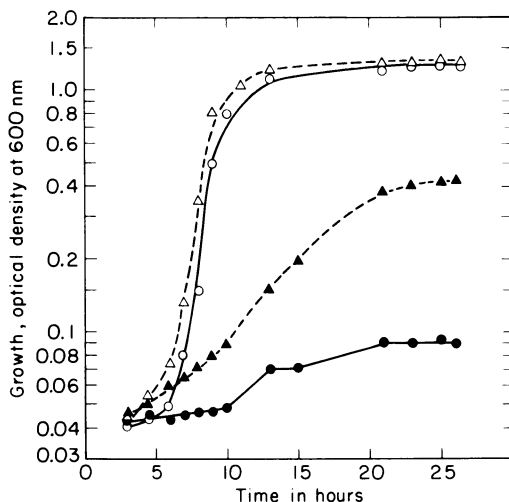
the presence of small quantities of tetrahydrofolate in the cells either carried over from the cells used as inoculum or because of a low level of synthesis of tetrahydrofolate in the presence of the antimetabolites. These results are consistent with the hypothesis that the absence of rT in tRNA^fMet will allow initiation of protein synthesis without formylation of the methionine of methionyl tRNA^fMet.

## DISCUSSION

The work presented in this paper describes the isolation and preliminary characterization of two mutants of *E. coli* able to initiate protein synthesis without formylation. The mutants were isolated by exposing a *p*-AB⁻ strain, *E. coli* AB3292, to selective media containing the end products of folate metabolism plus two inhibitors of folate synthesis, sulfathiazole and trimethoprim. Detailed studies carried out on one of the mutants (strain AB3292-i) indicate that, in this mutant, no endogenous formyltetrahydrofolate is present and protein synthesis both in vivo and in vitro occurs without any detectable formylation of methionyl-tRNA$^{fMet}$.

The isolation of mutants that grow in the presence of trimethoprim and sulfanilamide has also been described by Danchin (7). Cells selected on the basis of their ability to grow in the presence of trimethoprim and sulfanilamide were found to be enriched with respect to spectinomycin and streptomycin resistance. Conversely, mutants selected for spectinomycin or streptomycin resistance were shown to be capable of growth in the presence of trimethoprim and sulfanilamide. On the basis of these findings, Danchin concluded that a ribosomal mutation resulting in either spectinomycin or streptomycin resistance also increases the ability of *E. coli* to initiate protein synthesis in the absence of formylation. *E. coli* AB3292 is streptomycin resistant; nevertheless, it possesses an absolute requirement for *p*-AB even when supplied with the end products of folate metabolism. If one assumes that the resistance to streptomcyin observed with strain AB3292 is the result of a ribosomal mutation, this finding would suggest that the alteration of the ribosome resulting in streptomycin resistance does not by itself enable *E. coli* to initiate protein synthesis without formylation. The results with strain AB3292 do not, however, rule out the possibility that the mutation to streptomycin resistance can, in combination with other mutations, facilitate the initiation of protein synthesis in the absence of formylation.

Analysis of tRNA from strains AB3292-i and AB3292-i2 reveals that these tRNA's are undermethylated with respect to the parent tRNA. The following results suggest that the mutation(s) responsible for the reduction in methylation alters the tRNA only at the rT position. (i) Methylation of crude tRNA from strains AB3292-i and AB3292-i2 in vitro by extracts of *E. coli* leads to the production of rT and no other nucleoside. (ii) Of the three methylated nucleosides (rT, m⁷G, and 2'OMeC) present in *E. coli* tRNA$^{fMet}$, only the levels of rT are reduced in the initiator tRNA from strain AB3292-i.

The undermethylation of tRNA from strain AB3292-i and AB3292-i2 tRNA has been shown to result from a deficiency in the methylase, resulting in the synthesis of rT. Crude extracts isolated from the two mutants were found to contain less than 20% of the methylase activity normally observed in extracts from the parent strain (Fig. 7). At the present time, it is not known whether the decrease in the levels of the methylase activity is due to a mutation in the structural gene for the enzyme or in a regulatory gene responsible for controlling the levels of enzyme within the cell.

The results of experiments with an independently isolated rT⁻ strain of *E. coli* (SR-1) suggest that the deficiency in rT plays a role in the ability of strains AB3292-i and AB3292-i2 to initiate protein synthesis without formylation in vivo. *E. coli* SR-1, a strain lacking methylase activity for the product of rT, was found to grow significantly better than the isogenic rT⁺ strain (SR-3) under conditions where initiation was forced to take place with unformylated methionyl-tRNA (Fig. 8). Two features distinguish the growth characteristics of *E. coli* SR-1 under these conditions from those of strains AB3292-i and AB3292-i2. (i) The generation time of the rT⁻ strain (5 h) is more than twice as long as that of the two initiation mutants (about 2 h). (ii) Strain SR-1 reaches stationary phase at a lower cell density than either strain AB3292-i or AB3292-i2. These differences may reflect a requirement for an additional mutation (or mutations) before initiation without formylation can be carried out at optimal efficiency. It is also noteworthy that both strains AB3292-i and AB3292-i2 are only partially deficient in rT, even though the lack of rT has been shown to have little or no effect on the ability of the tRNA to function in protein synthesis (4). It is therefore possible that a small amount of rT⁺ tRNA is required for the efficient growth of *E. coli* in the absence of endogenous folate.

The findings described in this paper are consistent with the hypothesis proposed by Samuel and Rabinowitz (based on their studies with *S. faecalis*) that undermodification at the T position facilitates initiation with unformylated methionyl-tRNA$^{fMet}$ (26). The results obtained with the two initiation mutants from *E. coli* differ in several respects from those reported for *S. faecalis*, however. In *S. faecalis*, tRNA from cells grown in the absence of folate (and thus formylation) is totally deficient in rT. tRNA from *E. coli* AB3292-i and AB3292-i2 is only partially deficient in rT. Furthermore, modifi-

cation of *S. faecalis* tRNA is directly influenced by the availability of folate; the tRNA lacks rT only when folate metabolism is prevented. In strains AB3292-i and AB3292-i2, on the other hand, the extent of methylation is independent of the presence or absence of *p*-AB (and consequently folate) in the growth medium. Thus, whereas *S. faecalis* tRNA is rT⁻ only under those conditions where the cell is forced to initiate protein synthesis without formylation, the mutation responsible for rT deficiency in strains AB3292-i and AB3292-i2 is expressed whether or not the tRNA is formylated. The rT methylase from *S. faecalis* has recently been shown to require a folate coenzyme as a methyl donor (9). Thus, both the rT⁻ state of the tRNA and the requirement for an unformylated initiator tRNA are induced by folate deprivation. In *E. coli*, *S*-adenosylmethionine (and not a folate derivative) serves as the methyl donor for the rT methylase reaction (5). This fact, plus the finding that tRNA from strains AB3292-i and AB3292-i2 is undermethylated under all growth conditions, suggests that the partial deficiency in rT is not caused directly by folate inhibition but is a manifestation of a specific mutation that allows the strains to bypass the requirement for formylation. When combined with the results of the growth studies with *E. coli* SR-1 and SR-3, these findings strongly implicate a rT-deficient tRNA in initiation without formylation in *E. coli*.

Since both mutant strains, AB3292-i and AB3292-i2, grow more efficiently than strain SR-1 under conditions where formylation is inhibited, the mutant cells may contain an additional factor which facilitates initiation without formylation. It is possible, for example, that the mutants have an increased intracellular concentration of some component involved in the initiation process. Using wild-type *E. coli*, Harvey found that the concentrations of ribosomal subunits and tRNA^fMet were increased by severalfold under conditions of folate deprivation (16). Comparisons of tRNA isolated from strains AB3292 and AB3292-i (grown in the absence of *p*-AB) have shown that neither the level of tRNA nor the relative proportion of tRNA^fMet is increased in the mutant (unpublished data). Thus, strain AB3292-i does not respond to the requirement for initiation without formylation by increasing the intracellular concentration of the initiator tRNA. However, preliminary results indicate that the levels of initiation factor 2 present in strain AB3292-i are three- to fourfold higher than those present in strain AB3292. The increase in initiation factor 2 concentration is not restricted to strain

AB3292-i growing in the absence of *p*-AB, but is also observed when the cells are grown in the presence of *p*-AB. Thus, like the partial deficiency in rT, the high levels of initiation factor 2 are not induced by the growth conditions.

The results obtained with the initiation mutants of *E. coli* and the findings reported in *S. faecalis* provide two examples in which an alteration at the rT position of the tRNA is associated with the ability to initiate protein synthesis without formylation. It is also worth noting that eukaryotic cytoplasmic initiator tRNA's, which normally participate in protein synthesis with formylation, contain the sequence AUC or AU*C rather than rTΨC in this region (30). Further characterization of the components from *E. coli* AB3292-i and AB3292-i2 involved in initiation, particularly the ribosomes and initiation factors, is currently in progress.

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