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Modification-Deficient Transfer Ribonucleic Acids from Relaxed Control *Escherichia coli:* Structures of the Major Undermodified Phenylalanine and Leucine Transfer RNAs Produced during Leucine Starvation†

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ABSTRACT: The structures of the major, chromatographically unique phenylalanine and leucine tRNAs produced during leucine starvation of a relaxed control (rel⁻) mutant of *E. coli* have been determined. The results demonstrate that the unique species are modification-deficient forms of the major, normally occurring isoacceptor species. The unique $tRNA^{Phe}$ differs from the fully modified species at nucleotide positions 16, 37, 39, 47, and 55 from the 5′ terminus. The unique species contains uridine (U) in place of dihydrouridine-16 (D16), isopentenyladenosine in place of 2-thiomethyl-N^6-(δ^2-isopentenyl)adenosine-37, a mixture of U and pseudouridine (ψ) in position 39, a mixture of U and 3-(3-amino-3-carboxypropyl)uridine at position 47, and a mixture of U and ψ at position 55. The chromatographically normal isoacceptor from amino acid starved cells is deficient in D16 and ψ55, indicating that that species is a mixture of mature and undermodified tRNAs. The unique $tRNA^{Leu}$ isoacceptor consists of two subspecies which are undermodified forms of the major, normally occurring isoacceptor, $tRNA^{Leu}_1$. Both unique subspecies lack the D and ψ residues which occur at positions 16 and 39 from the 5′ terminus; one subspecies also lacks D17. Compared with the $tRNA^{Leu}_1$ from wild-type strains of *E. coli* B and K12, both $tRNA^{Leu}_1$ from nonstarved cells and the unique, rel⁻ $tRNA^{Leu}$ are deficient in the modified guanosine which normally occurs adjacent to the anticodon and the pseudouridine in the GTψC sequence of the ψ loop. Both the unique $tRNA^{Phe}$ and the unique $tRNA^{Leu}$ lack dihydrouridine residues which occur in the 5′ half of the D loop and pseudouridines which occur in the 3′ half of the anticodon loop and adjoining stem. Taken together, these findings suggest that the same enzymes are responsible for the formation of these particular modified bases in both tRNAs. The results further suggest that several, perhaps most, of the tRNAs from cells cultured under conditions in which RNA and protein synthesis are uncoupled will be similarly deficient in dihydrouridine and pseudouridine and other minor nucleosides which occur less frequently. Because both modification-deficient rel⁻ tRNAs have dihydrouridine at position 20 and pseudouridine in the ψ loop (and at position 41 in the unique $tRNA^{Leu}$), the results support the view that there are multiple D- and ψ-forming enzymes in *E. coli,* some of which may turn over rapidly or are selectively inactivated when protein synthesis is blocked. The results are discussed with a view toward understanding the structural basis for the altered biological activity of the unique $tRNA^{Phe}$ species and the order of events in the posttranscriptional modification of newly synthesized tRNA.

Various perturbations of cellular metabolism result in the appearance of chromatographically unique species of tRNA. In *Escherichia coli,* these conditions include: deprivation of essential amino acids (Fleissner, 1967; Capra and Peterkofsky, 1968; Stern et al., 1970; Waters et al., 1973; Fournier and Peterkofsky, 1975; Kitchingman and Fournier, 1974, 1975; Juarez et al., 1975; Katze and Mosteller, 1976; review by Littauer and Inouye, 1973), or iron (Wettstein and Stent, 1968; Rosenberg and Gefter, 1969; Juarez et al., 1975), culturing in the presence of antibiotics (Waters, 1969; Mann and Huang, 1973; Kitchingman and Fournier, 1975) or amino acid analogues (Chase et al., 1974), deviation from standard culturing temperatures (Chase et al., 1974), and reduced rates of aeration (Wettstein and Stent, 1968). The biochemical basis for the formation of these species is known in some instances. Starvation of relaxed control (rel⁻) mutants of *E. coli* for methionine, cysteine, or threonine results in the production of tRNA deficient in methylated nucleosides (Mandel and Borek, 1963), sulfur-containing nucleosides (Harris et al., 1969), or *N*-(purin-6-ylcarbamoyl)threonine (Powers and Peterkofsky, 1972), respectively; tRNA from methionine starved rel⁻ cells is also deficient in 3-(3-amino-3-carboxypropyl)uridine (Nishimura et al., 1974). Production of the undermodified species in these cases is a direct result of the removal of a substrate required for base modification. Removal of iron from the culture medium results in the formation of tRNA deficient in the methylthio moiety of 2-methylthio-N^6-(δ^2-isopentenyl)adenosine (ms^2i^6A) as iron is a necessary cofactor for one of the enzymes involved in the conversion of i^6A to ms^2i^6A (Rosenberg and Gefter, 1969).

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Chromatographically unique species of tRNA also accumulate in relaxed control (rel$^-$) cells starved of amino acids which are not known to be directly involved in the base modification process (Waters et al., 1973; Chase et al., 1974; Fournier and Peterkofsky, 1975; Kitchingman and Fournier, 1975; Fournier et al., 1976). The formation of unique tRNAs under this condition or as a result of treatment with certain antibiotics appears to be related to the cessation of protein synthesis. This view is supported by the recent finding (Huang and Mann, 1974) that the two chromatographically unique species of $tRNA^{Phe}$ present in cells treated with chloramphenicol are undermodified forms of the normal $tRNA^{Phe}$. Both unique species were deficient in dihydrouridine, pseudouridine and 2-thiomethyl-N^6-(δ^2-isopentenyl)adenosine (ms^2i^6A). Others have determined that bulk tRNA from rel$^-$ cells starved of essential amino acids or rel$^+$ tRNA prepared from cells treated with chloramphenicol is deficient in dihydrouridine and perhaps 4-thiouridine (Jacobson and Hedgcoth, 1970; Waters et al., 1973).

We have been studying the effects of leucine starvation on the production of chromatographically unique species of tRNA in rel$^-$ strains of *E. coli*. Evidence has been presented which indicates that several of the unique species, speeifically the major, unique species of $tRNA^{Phe}$ and $tRNA^{Leu}$, are undermodified forms of the major, normally occurring isoacceptors (Kitchingman and Fournier, 1975; Kitchingman et al., 1976). Strong evidence of a precursor-product relationship was provided by results from ^{32}P-label-chase experiments in which direct conversion of the unique species to chromatographically normal forms was observed (Kitchingman et al., 1976; Kitchingman and Fournier, 1976b). It was also determined that the unique species of $tRNA^{Phe}$ is functionally inferior to the normal species with regard to the rate at which it is aminoacylated in vitro and in its ability to support poly(U)[1]-directed protein synthesis (Kitchingman et al., 1976). In order to better understand the mechanism of formation of the unique species of rel$^-$ tRNA and to correlate the functional deficiencies of the unique $tRNA^{Phe}$ with specific structural changes, we have determined the structures of the major rel$^-$ $tRNA^{Phe}$ and $tRNA^{Leu}$ by comparative fingerprint and base compositional analyses. The results from these analyses demonstrate that the rel$^-$ species are, in fact, hypomodified forms of the major, normally occurring isoacceptor species. The unique species lack specific dihydrouridine and pseudouridine residues and are partially deficient in other minor nucleosides. A summary of this work was presented at the 1976 meeting of the American Society of Biological Chemists, San Francisco, Calif. (Kitchingman and Fournier, 1976a).

Experimental Section

Materials

The materials used in these experiments were obtained from the following sources: [^{32}P]orthophosphate (carrier-free) from International Chemical and Nuclear Corp. or New England Nuclear Corp.; [^3H]phenylalanine (24 Ci/mmol), [^3H]leucine (52 Ci/mmol), and [^{35}S]H_2SO_4 (carrier-free) from the New England Nuclear Corp.; benzoylated DEAE-cellulose (BD-cellulose, 50–100 mesh) from Schwarz/Mann (before use, the sorbent was prewashed in buffer that was 0.01 M sodium acetate (pH 4.5)–0.01 M magnesium chloride–2 M sodium chloride–20% ethanol); DEAE-cellulose (Whatman DE-52) from Reeve-Angel (prewashed with 0.5 N HCl and 0.5 N NaOH); poly(ethylenimine)-impregnated cellulose thin-layer chromatograms (Polygram CEL 300 PEI, 20 × 40 cm) from Brinkmann Instruments; cellulose acetate strips (3 × 100 cm) from Schleicher and Schuell, Inc.; cellulose thin-layer chromatography sheets (No. 6064, 20 × 20 cm), acrylamide, bisacrylamide, RP-54 x-ray film, and the electrophoresis indicator dyes, xylene cyanol F. F. (blue), acid fuchsin (red), and methyl orange, from Eastman Kodak; Adogen 464 [methyltrialkyl- (C_8–C_{10}) ammonium chloride] and Adogen 464 (dimethyldicocoammonium chloride) from Ashland Chemical Co., and Plaskon (CTFE-2300 (poly(chlorotrifluoroethylene)powder) from Allied Chemical Co. RPC-5 and RPC-7 sorbents were prepared by method C of Pearson et al. (1971). Ribonuclease T_1 and ribonuclease CB were purchased from Calbiochem; ribonuclease A (pancreatic RNase-RASE), snake venom phosphodiesterase, and alkaline phosphatase (*E. coli*) were obtained from Worthington Biochemical Co.; all other chemicals were reagent grade.

Methods

Preparation of ^{32}P- and ^{35}S-Labeled tRNA. E. coli strain CP79 (arg$^-$, his$^-$, leu$^-$, thr$^-$, thi$^-$, rel$^-$) was cultured in a low phosphate medium (LP) and the RNA labeled with [^{32}P]-orthophosphate either during logarithmic growth or during starvation for leucine as described previously (Kitchingman et al., 1976).

For ^{35}S-labeling of tRNA, cells were grown in LP medium in which the sulfate concentration was reduced to 1 mM and the level of leucine was 5 μg/mL. $H_2{}^{35}SO_4$ (5 mCi) was added when the culture was inoculated and an additional 5 mCi was added after the onset of leucine starvation. Cells were harvested after 6 h of leucine starvation.

Purification of Bulk tRNA. Bulk tRNA from ^{32}P- or ^{35}S-labeled cells was prepared through the DEAE-cellulose step as described previously (Fournier and Peterkofsky, 1975). The RNA was then fractionated by electrophoresis through a slab (10 × 20 × 0.3 cm) of 10% polyacrylamide gel (9.5% acrylamide–0.5% bisacrylamide) according to the method described by DeWachter and Fiers (1971). Under the conditions used for electrophoresis, $tRNA^{Leu}$ migrates slower than the remainder of the tRNAs and can be detected as a separate band fairly well resolved from the bulk of the 4S RNA (Dube et al., 1970). The $tRNA^{Leu}$ and main 4S bands (the latter containing the $tRNA^{Phe}$) were excised with a scalpel using the autoradiograph as a template. The tRNA was twice extracted from the gel slices by diffusion with 2 mL of 2 × SSC (SSC is 0.15 M sodium chloride–0.015 M sodium citrate, pH 7.0) at 37 °C for a total of 24 h. The RNA in the combined eluates was freed of residual acrylamide by adsorption to a small (pasteur pipet) DEAE column equilibrated with 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–0.3 M NaCl and desorption with the same buffer made 1 M in NaCl. The tRNA was precipitated with 2.5 volumes of 95% ethanol after the addition of 0.1 volume of 20% potassium acetate, pH 4.5, and unlabeled carrier tRNA prepared from cells cultured under the same conditions.

Aminoacylation of tRNA. ^{32}P- or ^{35}S-labeled $tRNA^{Phe}$ and ^{32}P-labeled $tRNA^{Leu}$ were aminoacylated essentially as described previously (Fournier and Peterkofsky, 1975) using partially purified preparations of phenylalanine and leucine tRNA synthetase prepared by DEAE-cellulose chromatog-

[1] Abbreviations used: poly(U), poly(uridylic acid); DEAE, diethylaminoethyl; LP medium, low phosphate medium; SSC, 0.15 M NaCl–0.015 M sodium citrate (pH 7.0); Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetate; PEI, polethylenimine; CAM, chloramphenicol.

raphy as described previously (Kitchingman et al., 1976). Unlabeled tRNA to be added as an internal marker was aminoacylated with [^{3}H]phenylalanine (24 Ci/mmol) or [^{3}H]-leucine (52 Ci/mmol) under identical conditions except that the amino acid concentration was 5 μM. Sufficient carrier RNA (approximately 10 A_{260}) was added to yield approximately 3×10^6 cpm of [^{3}H]Phe- or Leu-tRNA. Aminoacylated [^{32}P]- (or [^{35}S]-) tRNA and carrier tRNA were prepared for RPC-5 chromatography by phenol extraction and ethanol precipitation as described previously (Kitchingman and Fournier, 1975).

Purification of tRNA^{Phe}. The method used to purify tRNA^{Phe} was essentially the same as that developed by Shugart and Stulberg (1974). Aminoacylated tRNA^{Phe} was dissolved in 0.5 mL of 10 mM sodium acetate, (pH 4.5)–10 mM $MgCl_2$–1 mM Na_2 EDTA–0.5 M NaCl and applied to a 0.5 × 27 cm RPC-5 column equilibrated with the same buffer. Conditions for formation of the gradient and operation of the column have been described (Kitchingman and Fournier, 1975; Fournier et al., 1975). Fifty microliters from every second fraction was diluted with 0.4 mL of H_2O and counted in 5 mL of a scintillation cocktail consisting of Liquifluor–toluene–Triton X-100 (84:916:500) in a Beckman LS-250 liquid scintillation spectrometer under double label conditions (either ^{32}P:^{3}H or ^{35}S:^{3}H). The resulting elution profiles have been corrected for spillover. Counting efficiencies under the conditions used were 8% for ^{3}H, 60% for ^{32}P, and 40% for ^{35}S.

The appropriate tRNA^{Phe}-containing fractions from the RPC-5 column were pooled and the NaCl concentration was reduced to 0.4 M by the addition of buffer that was 10 mM sodium acetate–(pH 4.5)–10 mM $MgCl_2$–1 mM Na_2 EDTA. The sample was then applied to a RPC-7 column (0.5 × 27 cm) equilibrated with the same buffer made 0.4 M in NaCl. The column was washed briefly and then developed under the same conditions described for the RPC-5 fractionation except that the limits of the gradient were 0.4 and 0.7 M NaCl. tRNA^{Phe} was located as described above, and the appropriate fractions were pooled, dialyzed extensively against H_2O, then 0.2 M Tris-HCl, pH 8.5 (room temperature) for 2 h to deacylate the tRNA, and against H_2O again. The sample was taken to dryness in vacuo and twice precipitated from 2% potassium acetate, pH 4.5, with 95% ethanol.

Occasionally, the fingerprints of the unique tRNA^{Phe} indicated a need for further purification. This was accomplished by chromatography on BD-cellulose. The pooled fractions from RPC-7 were diluted to a final salt concentration of 0.4 M and the tRNA absorbed to a 4-mL BD-cellulose column equilibrated with 10 mM sodium acetate (pH 4.5)–10 mM $MgCl_2$–0.4 M NaCl. After washing with ~10 mL of the same buffer, the [^{3}H]phenylalanyl[^{32}P]tRNA was eluted with a 75-mL linear gradient from 0.4 to 1.5 M NaCl–15% ethanol. Fractions were initially 2 mL, but owing to the presence of alcohol, decreased to 1.1 mL by the end of the gradient. Phenylalanyl-tRNA was located and prepared for fingerprint analysis as described above.

Purification of tRNA^{Leu}. Following fractionation of the bulk tRNA by electrophoresis, the tRNA^{Leu} was applied to a BD-cellulose column (4 mL volume) equilibrated with buffer that was 10 mM sodium acetate (pH 4.5)–10 mM $MgCl_2$–0.4 M NaCl. After washing with several column volumes of the same buffer, tRNA$^{Leu}_I$ and tRNA$^{Leu}_U$ were eluted together with the same buffer containing 0.8 M NaCl. All column operations were performed at room temperature.

The tRNA^{Leu} was further enriched by RPC-5 chromatography. Aminoacylated tRNA^{Leu} was solubilized in 1 mL of 10 mM sodium acetate (pH 4.5)–10 mM $MgCl_2$–1 mM Na_2-EDTA–0.4 M NaCl and applied to a 0.9 × 69 cm RPC-5 column equilibrated with the same buffer. The tRNA was eluted with a 600-mL linear salt gradient with lower and upper limits of 0.4 and 1.2 M NaCl, respectively. The flow rate was maintained at about 0.75 mL per min by a Milton-Roy Minipump and fractions of 2 mL were collected. Fractions containing tRNA^{Leu} were indentified, pooled, and prepared for sequence analysis as described above.

Fingerprinting and Oligonucleotide Analyses. Separation of RNase T_1 and RNase A digests of purified ^{32}P-labeled tRNAs was accomplished by the two-dimensional electrophoresis chromatography procedure of Griffin (1971) except that the oligonucleotides were transferred from the cellulose acetate strip to the PEI plate by the method of Southern (1974). T_1 RNase oligonucleotides were digested with RNase A and the products fractionated by electrophoresis on DEAE paper (DE-81) at 1500 V for 105 min in pH 3.5 pyridine-acetate buffer without urea (Brownlee, 1972).

One- and two-dimensional base compositional analyses were performed according to Ohashi et al. (1974) using solvent systems 1 and 2. Where indicated, one-dimensional analyses were performed using solvent system 2.

Chromatography of ^{35}S-Labeled Nucleosides. [^{35}S]tRNA was digested to nucleosides (Randerath et al., 1972) and the hydrolysate fractionated by two-dimensional chromatography on 20 × 20 cm cellulose thin-layer sheets. In the first dimension, the solvent was 86% butanol (Markham and Smith, 1949), and, in the second, 2-propanol–1% $(NH_4)_2SO_4$ (2:1; Hall, 1971).

Results

^{32}P Labeling of the Unique and Normal tRNA Species. When [^{32}P]orthophosphate was added to a culture of *E. coli* CP79 after the onset of leucine starvation, only the unique tRNA^{Phe} and tRNA^{Leu} isoacceptor species were labeled; no ^{32}P-labeled normal tRNA^{Phe} or tRNA$^{Leu}_I$ was formed. On the other hand, both the unique and normal species were labeled when $^{32}PO_4^{3-}$ was present in the culture medium before and during leucine starvation. When this latter labeling regimen was used, however, the chromatographically normal ^{32}P-labeled tRNA^{Phe} and tRNA^{Leu} obtained were partially deficient in dihydrouridine and pseudouridine. For this reason, the normal tRNA^{Phe} and tRNA^{Leu} used for sequence analysis was prepared from nonstarved cells harvested in mid-log phase. Because the minor, unique tRNA^{Phe} generally observed in starved cultures of *E. coli* CP79 accounts for only about 2% of the total tRNA^{Phe} (Fournier and Peterkofsky, 1975), it was considered technically unfeasible to perform a rigorous structural analysis on that species; thus, comparative fingerprint and nucleotide analyses were performed with the major unique and normal isoacceptor species only.

Purification of ^{32}P-Labeled tRNA^{Phe}. The purification scheme used for the isolation of the unique and normal species of tRNA^{Phe}, designated tRNA$^{Phe}_U$ and tRNA$^{Phe}_N$, respectively, was adapted from the procedure developed by Shugart and Stulberg (1974) for the purification of normal and methyl-deficient tRNA^{Phe}. After separation of the tRNA^{Leu} and tRNA^{Phe} by acrylamide gel electrophoresis, the tRNA^{Phe} isoacceptors were purified by RPC-5, RPC-7, and, where indicated, BD-cellulose column chromatography. The details and results obtained for each purification step are provided in a supplement which accompanies this paper (see paragraph at end of paper regarding supplementary material).

Fingerprinting and Nucleotide Analyses of ^{32}P-Labeled

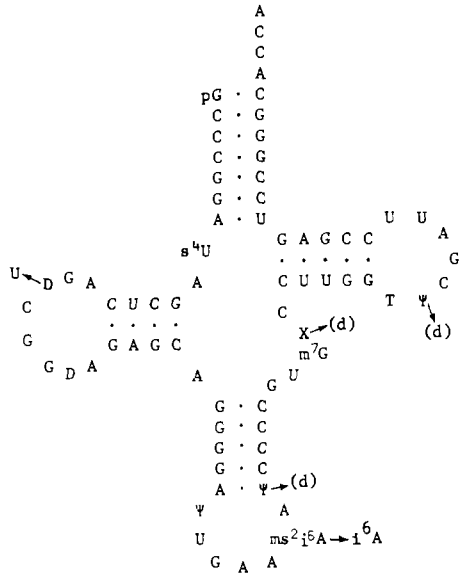

FIGURE 1: Structures of normal $tRNA^{Phe}$ and the major, unique phenylalanine tRNA isoacceptor ($tRNA^{Phe}_U$) from leucine starved rel^- *E. coli*. The arrows indicate the positions at which the major unique rel^- $tRNA^{Phe}$ differs from normal $tRNA^{Phe}$. (d) indicates a partial deficiency. The base X is 3-(3-amino-3-carboxypropyl)uridine (Ohashi et al., 1974).

Unique and Normal $tRNA^{Phe}$. Purified ^{32}P-labeled $tRNA^{Phe}_N$ and $tRNA^{Phe}_U$ were first digested with RNase T_1 and fingerprinted by the two-dimensional electrophoresis–chromatography procedure developed by Griffin (1971). The resulting fingerprints were very similar (see supplementary material), indicating that the sequences of the unique and normal isoacceptors are closely related. Fifteen major and several minor oligonucleotides were derived from each tRNA. Because the minor spots were present in variable, but usually low yield (<0.3 M yield), they were presumed to be contaminants and were not considered further.

The identification of each oligonucleotide of the major unique and normal species of $tRNA^{Phe}$ was made on the following bases: (1) reference to the established T_1 oligonucleotide map of Barrell and Sanger (1969); (2) analysis of the products released by treatment of the T_1 fragments with pancreatic RNase; and (3) determination of the base composition of each T_1 oligonucleotide.

Of the 16 oligonucleotides expected from a RNase T_1 digest of $tRNA^{Phe}_N$, 15 were identified by analysis of the pancreatic RNase and RNase CB digestion products. The only oligonucleotide not identified by this method was the 3′-terminal fragment, $CACCA_{OH}$. This oligonucleotide appeared as a diffuse spot near the solvent front in the second dimension and was tentatively identified by its mobility alone.

Normal $tRNA^{Phe}$ (shown in Figure 1) contains ten modified bases: 3 pseudouridines (ψ), 2 dihydrouridines (D), 1 each of 4-thiouridine (s^4U), 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A), 7-methylguanosine (m^7G), 3-(3-amino-3-carboxypropyl)uridine (4 abu^3U, the "X" base), and ribothymidine (T). All were found in $tRNA^{Phe}_N$ in molar yield.

Two of the modified nucleotides present in normal $tRNA^{Phe}$ were totally absent in $tRNA^{Phe}_U$ and three others were determined to be present in less than molar yields. An unmodified uridine was found in place of the dihydrouridine normally located 16 nucleotides from the 5′ terminus. The second modification which was totally lacking in $tRNA^{Phe}_U$ was the methylthio moiety of ms^2i^6A.

Two fragments from $tRNA^{Phe}_U$ were found which were related to $AAms^2i^6AA\psi CCCCG$ of $tRNA^{Phe}_N$. Both fragments yielded i^6A upon complete digestion (determined from analysis of ^{35}S-labeled tRNA), but one fragment contained U in place of $\psi 39$. Both i^6A-containing fragments were found in submolar yields, but together were present in approximately molar yield in a ratio of 60 (U-containing):40 (ψ-containing).

The two other modified bases found in reduced quantities in $tRNA^{Phe}_U$ were $4abu^3U47$ and $\psi 55$. A 20% reduction in the amount of $4abu^3U$ was observed, consistent with the results obtained from previous studies on the derivatization of this base with phenoxyacetyl-*N*-hydroxysuccinimide (Kitchingman et al., 1975; Fournier et al., 1976). The unique $tRNA^{Phe}$ contained about 60% as much $\psi 55$ as $tRNA^{Phe}_N$.

Analysis of ^{35}S-Labeled $tRNA^{Phe}_N$ and $tRNA^{Phe}_U$. The presence of 4-thiouridine in the unique and normal $tRNA^{Phe}$ isoacceptors could be inferred from the electrophoretic–chromatographic behavior and apparent heterogeneity of certain oligonucleotides in the T_1-RNase fingerprint, but not proven directly. Similarly, the presence of i^6A in $tRNA^{Phe}_U$ rather than ms^2i^6A was inferred on the basis of the major shift in elution position for $tRNA^{Phe}_U$ on RPC-5 chromatography. Both uncertainties were resolved by direct analysis of the sulfer-containing nucleosides derived from unique and normal ^{35}S-labeled $tRNA^{Phe}$. The results (see supplementary material) demonstrated that normal $tRNA^{Phe}$ had two sulfur-containing bases, corresponding to s^4U and ms^2i^6A, while only s^4U was found in the nucleoside digest of $tRNA^{Phe}_U$.

Taken together, the results show that the unique species lacks one dihydrouridine residue (D16), is deficient in $\psi 39$, $\psi 55$, and $4abu^3U47$, and contains i^6A in place of ms^2i^6A. The structure of the unique $tRNA^{Phe}$ is shown in Figure 1.

Purification of ^{32}P-Labeled Unique and Normal $tRNA^{Leu}$. The method used to purify the unique and normal species was based on that developed by Dube et al. (1970) for the isolation of normal $tRNA^{Leu}_I$. Full details can be found in the supplementary material to this paper.

Fractionation of the unique $tRNA^{Leu}$ by RPC-5 chromatography showed that species to be heterogeneous. This heterogeneity, observed earlier (Kitchingman and Fournier, 1975), is best observed with samples of highly enriched $tRNA^{Leu}_U$. For sequence analysis, the unique $tRNA^{Leu}$ was divided into two subfractions designated UI and UII, respectively. The sequencing results revealed that the early (UI) and late (UII) eluting subspecies differ only in the number of dihydrouridine residues in the dihydrouridine loop.

Comparative Fingerprint and Nucleotide Compositional Analyses. The comparative analysis of the $tRNA^{Leu}_I$ species was performed as described above for the unique and normal $tRNA^{Phe}$. The autoradiograms of the T_1 RNase fingerprints of $tRNA^{Leu}_I$ and $tRNA^{Leu}_U$ were virtually identical, indicating that the primary structures of these isoacceptors, like the unique and normal species of $tRNA^{Phe}$, are very closely related.

$tRNA^{Leu}_I$, shown in Figure 2, contains 87 nucleotides, 9 of which are modified, and yields 16 oligonucleotides upon digestion with RNase T_1 (Dube et al., 1970; Blank and Söll, 1971). The 9 minor nucleosides include: 3 pseudouridines, 3 dihydrouridines, one each of 2′-*O*-methylguanosine (Gm), ribothymidine, and an unidentified derivative of guanosine (G*). The 9 modified nucleosides occur in just 5 T_1 oligonucleotides.

Of the 16 oligonucleotides expected from a T_1 RNase digest of $[^{32}P]tRNA^{Leu}_I$, all were positively identified except for the

fragments having the sequence G*ψG, which could not be located, and the 3′-terminal oligonucleotide, $CACCA_{OH}$; the latter fragment was identified solely on the basis of its position in the fingerprint. The absence of the G*ψG fragment probably results from the lack of formation of G* rendering the fragment susceptible to further cleavage by RNase T_1 to yield G and ψG. The only site in $tRNA^{Leu}_I$ from which ψG could be generated is within the sequence G*ψG. From the absence of G*ψG and the occurrence of ψG, it can be tentatively concluded that most, and perhaps all, of G38 was not modified in $tRNA^{Leu}_I$. This may be due to a peculiarity of the bacterial strain used, or to the particular culturing conditions used. The other seven modified bases in $tRNA^{Leu}_I$ were contained in the expected T_1 fragments. All were identified directly and determined to occur in molar yields.

Analysis of the RNase T_1 oligonucleotides derived from $tRNA^{Leu}_U$ confirmed that this tRNA is, indeed, a modification-deficient form of $tRNA^{Leu}_I$. Certain of the modified nucleotides which occur in $tRNA^{Leu}_I$ were not present in $tRNA^{Leu}_U$, and mononucleotide analyses of the T_1 fragments from $tRNA^{Leu}_U$ did not reveal the occurrence of any new modifications.

Of the three dihydrouridine residues present in the D loop of $tRNA^{Leu}_I$ (D16, D17, and D20), only D20 was found in $tRNA^{Leu}_U$, the others being replaced by uridine. Dihydrouridine 20 and Gm18 were both present in molar yields.

No fragment corresponding to G*ψG was found in $tRNA^{Leu}_U$, indicating that G* was absent in the unique species as well as in the normal form. The absence of ψ in the spot containing UG and ψG indicates that ψ39 is completely lacking in $tRNA^{Leu}_U$. Thus, neither of the modified bases in the anticodon loop was formed during leucine starvation of this rel⁻ strain.

Pseudouridine-41 was found in molar yield and both T65 and ψ66 were found in the appropriate T_1 oligonucleotide. However, the presence of some uridine in the fragment containing T65 and ψ66 indicated that one of these two modified nucleotides was present in less than molar amounts. Quantitation of the content of individual nucleotides showed that approximately 40% of the $tRNA^{Leu}_U$ molecules contained U in place of ψ66.

As indicated earlier, the unique $tRNA^{Leu}$ is heterogeneous. Analysis of various subfractions of $[^{32}P]tRNA^{Leu}_U$ recovered from the RPC-5 column showed that the early and late eluting subspecies differed only in their content of dihydrourdine. The heterogenity was shown by further analysis to be present in the T_1 oligonucleotide AADDGmG. When the RPC-5 profile of $tRNA^{Leu}_U$ was divided into early, middle, and late fractions, the U:D ratios of the $tRNA^{Leu}_U$ in these pooled fractions were: 3:1, 7:1, and 18:1, respectively. Analysis of the pancreatic RNase products of AA[U,D]GmG showed D17 to be absent in all subspecies and the content of D16 to be variable.

It can be concluded from the fingerprint and compositional analyses that the major, chromatographically unique species of leucine tRNA consists of undermodified subspecies of $tRNA^{Leu}_I$. One unique subspecies ($tRNA^{Leu}_{UI}$) lacks D17 and ψ39 and is deficient in ψ66. The other subspecies, $tRNA^{Leu}_{UII}$, has the same deficiencies and, in addition, lacks D16. Both the unique and normal rel⁻ isoacceptors differ from the $tRNA^{Leu}_I$ sequences determined by others. Neither species contains G*38 and the normal $tRNA^{Leu}_I$ from *E. coli* CP79 is also partially dificient in ψ39 and ψ66. All other oligonucleotides in $tRNA^{Leu}_U$ and $tRNA^{Leu}_I$ were identical, firmly establishing that $tRNA^{Leu}_U$ is a hypomodified form of $tRNA^{Leu}_I$. The structures of the rel⁻ leucine tRNAs are shown in Figure 2.

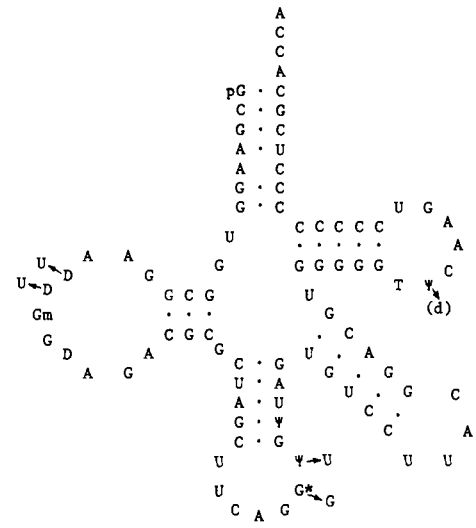

FIGURE 2: Structures of $tRNA^{Leu}_I$ and the major, unique leucine tRNA ($tRNA^{Leu}_U$) from leucine-starved rel⁻ *E. coli*. The sequence shown is of the $tRNA^{Leu}_I$ which occurs in wild-type strains of *E. coli* B (Dube et al., 1970) and *E. coli* K 12 (Blank and Söll, 1971). $tRNA^{Leu}_I$ from nonstarved cultures of *E. coli* CP79 is partially, perhaps completely, deficient in G*38 and partially deficient in ψ39. The arrows indicate the positions at which the unique $tRNA^{Leu}$ (subspecies $tRNA^{Leu}_{UII}$) differs from fully modified $tRNA^{Leu}_I$. (d) indicates a partial deficiency. Subspecies UI of $tRNA^{Leu}_U$ differs from subspecies UII only at position 16; $tRNA^{Leu}_{UII}$ contains an unmodified uridine at this position while $tRNA^{Leu}_{UI}$ contains D16.

Discussion

The results demonstrate that the major, unique phenylalanine and leucine tRNAs produced in a rel⁻ strain of *E. coli* starved of leucine are undermodified forms of the major, normally occurring isoacceptor species. The unique $tRNA^{Phe}$ contains uridine in place of dihydrouridine-16 and i^6A in place of ms^2i^6A37. Reduced levels of ψ39, $4abu^3U47$, and ψ55 were also found in the unique species, being replaced by uridine to extents of 50% (ψ39), 20% ($4abu^3U$), and 40% (ψ55), respectively. The chromatographically normal $tRNA^{Phe}$ from starved cells is also deficient in D16 and ψ55, indicating that that species is a mixture of fully modified and modification-deficient subspecies.

The unique $tRNA^{Leu}$ which accumulates during leucine starvation consists of two major subspecies, $tRNA^{Leu}_{UI}$ and $tRNA^{Leu}_{UII}$. Both subspecies contain unmodified uridines in place of dihydrouridine-17 and pseudouridine-39; unique subspecies $tRNA^{Leu}_{UII}$ also lacks D16. While $tRNA^{Leu}_I$ from wild-type strains of *E. coli* B and K12 contains a modified guanosine adjacent to the anticodon in position 38, this minor nucleoside could not be detected in either the unique or normal species isolated from the strain used here (*E. coli* CP79). Relative to fully modified $tRNA^{Leu}_I$, the unique and normal species were also partially deficient in ψ66.

Taken together, the results indicate a common biochemical basis for the occurrence of undermodified tRNAs in rel⁻ *E. coli*. As suggested earlier (Fournier and Peterkofsky, 1975), certain of the tRNA base modifying enzymes may be selectively inactivated or rapidly degraded in the absence of continuing protein synthesis. Accordingly, modification-deficient tRNA will be synthesized under conditions where RNA and protein synthesis are partially or completely uncoupled.

Inasmuch as both the unique $tRNA^{Phe}$ and $tRNA^{Leu}$ species lack the dihydrouridine residues which normally occur in the 5′ half of the D loop and are deficient in the pseudouridines located at position 39 in the 3′ half of the anticodon loop, the results suggest that the same D- and ψ-forming enzymes are

responsible for the formation of these particular residues in both tRNAs. Because these residues occur frequently in other tRNAs in the same relative positions (Sodd, 1976), it seems likely then that several, perhaps the majority, of the other rel$^-$ tRNAs will be similarly deficient. This suggestion is supported by the finding that qualitative or quantitative changes were observed in the isoacceptor patterns for seven of eight rel$^-$ tRNAs analyzed when preparations of tRNA from leucine-starved cultures of rel$^-$ and rel$^+$ *E. coli* were compared (Fournier and Peterkofsky, 1975).

While the apparent loss of modification activity may be due to rapid turnover rates and, thus, rapid decay in the absence of protein synthesis, there is now evidence that not all physiological conditions which cause protein and RNA synthesis to be uncoupled have precisely the same effect on tRNA modification. Specifically, the structure of the unique rel$^-$ $tRNA^{Phe}$ is similar, but not identical with that of the major, unique species of $tRNA^{Phe}$ ($tRNA^{Phe}_{II}$) from chloramphenicol-treated (CAM) cells (Huang and Mann, 1974). It was determined that CAM-$tRNA^{Phe}_{II}$ lacked D16, ψ32, and ψ39, contained i^6A in place of ms^2i^6A, and was deficient in ψ55. Thus, both the rel$^-$ and CAM species lack D16, are deficient in ψ55, and have i^6A in place of ms^2i^6A. However, we have consistently found ψ32 to be present in rel$^-$ $tRNA^{Phe}_U$, while CAM-$tRNA^{Phe}_{II}$ apparently has an unmodified uridine at this position. Although ψ39 is completely lacking in CAM-$tRNA^{Phe}_{II}$, a mixture of ψ and U was found in this position in the rel$^-$ species. It is not known if CAM-$tRNA^{Phe}_{II}$ contains s^4U, m^7G, or $4abu^3U$, as the contents of these nucleotides were not determined. Thus, while the major, unique species of $tRNA^{Phe}$ from leucine-starved and chloramphenicol-treated cells are chromatographically similar, they do differ in the degree to which they are undermodified.

Because both the unique leucine and phenylalanine tRNAs have D residues at position 20 and ψ in the ψ loop (and at position 41 in $tRNA^{Leu}_U$) but are lacking other D and ψ residues, the sequencing results reported here provide additional support to the view that there are multiple D- and ψ-forming enzymes in *E. coli*. It has already been established that this is the case for pseudouridine biosynthesis in *Salmonella tymphimurium* and thus, most likely, *E. coli* (Singer et al., 1972; Allaudeen et al., 1972). Analysis of the histidine tRNA and $tRNA^{Leu}_I$ from histidine regulatory mutants of *S. tymphimurium* which harbor a defective *his* T gene has shown these tRNAs to be devoid of the pseudouridines which normally occur in the 3′ half of the anticodon loop and adjoining stem. However, these mutants are able to form the ψ in the GTψC sequence of the ψ loop (Singer et al., 1972).

A comparison of the structures of *his* T tRNAs known (i.e., $tRNA^{His}$ (Singer et al., 1972); $tRNA^{Leu}_I$ (Allaudeen et al., 1972)) or suspected (i.e., $tRNA^{Tyr}_I$, $tRNA^{Tyr}_{II}$ (Singer et al., 1972)) to be lacking in ψ in the anticodon region reveals that the pseudouridines affected occur two, three, or four residues from the anticodon in the 3′ portion of the anticodon loop and adjoining stem. This finding strongly suggests that ψ39 in $tRNA^{Phe}$ is also formed by the *his* T gene product. In support of this view is the finding that $tRNA^{Phe}$ from *his* T mutants elutes considerably later on RPC-5 chromatography than $tRNA^{Phe}$ from wild-type cells (Webb and Fournier, unpublished results; C. Turnbough, personal communication). The finding that both ψ32 and ψ39 are absent in CAM-$tRNA^{Phe}_{II}$ while rel$^-$ $tRNA^{Phe}_U$ is deficient only in ψ39 suggests that there may be differential effects of leucine starvation and CAM treatment on the *his* T gene product or, alternatively, on the *his* T gene product and a different ψ-forming enzyme which is responsible for the formation of ψ32.

Inasmuch as the product of the *his* T gene appears to be required for the formation of both ψ39 and ψ41 in $tRNA^{Leu}_I$ (Allaudeen et al., 1972), it is of interest that the unique rel$^-$ $tRNA^{Leu}$ lacks only one of these modifications. The formation of ψ41, but not ψ39, in rel$^-$ cells could result from: (1) differential effects of starvation on the activity of a single ψ-forming enzyme; (2) the occurrence of two different site-specific ψ-forming enzymes with a common polypeptide derived from the *his* T gene; or (3) improper conformation of the tRNA. In this last regard, certain of the tRNA base modification reactions may normally occur early in the maturation of precursor tRNAs. Once other modifications have occurred, it may be difficult or impossible to effect a modification that usually occurs as an early event.

Results from ^{32}P-label-chase experiments presented elsewhere showed that subspecies of both the unique $tRNA^{Phe}$ and $tRNA^{Leu}$ can be converted to chromatographically normal forms in vivo, but only slowly, and that in the case of $tRNA^{Phe}$, the conversion may occur by more than one pathway (Kitchingman et al., 1976; Kitchingman and Fournier, 1976b). The finding that the unique species can be converted only slowly to a normal form suggested the possibility that the rel$^-$ tRNAs may be poor substrates for certain of the base modifying enzymes. At the time, it was suggested that some of the modification reactions may normally occur in a sequential fashion and that violation of the sequence may result in the formation of tRNA species that are inferior substrates. In this regard, the formation of D16 in $tRNA^{Phe}$ and D16 and D17 in $tRNA^{Leu}_I$ may normally occur early. This suggestion is supported by the finding that dihydrouridine residues can be found in the D loops of several cellular and phage T4 monomeric and multimeric precursor tRNAs (Barrell et al., 1974; Sakano et al., 1974; Chang and Carbon, 1975). Perhaps these modifications occur exclusively at the precursor level and mature-size tRNA or partially modified 4S tRNA is not a good substrate for the dihydrouridine-forming enzymes.

In earlier work it was determined that $tRNA^{Phe}_U$ from leucine-starved rel$^-$ cells was aminoacylated at a slower rate than $tRNA^{Phe}_N$ and was less efficient in promoting poly(U)-directed protein synthesis (Kitchingman et al., 1976). The modified residues which are absent in $tRNA^{Phe}_U$ may be directly involved in the recognition of this tRNA by its synthetase and by protein synthesis factors or, by their absence, effect a conformational change which compromises these interactions. Recent theories on the nature of the interaction of tRNA with aminoacyl-tRNA synthetase suggest that the dihydrouridine stem and portions of the D loop and the anticodon loop and stem serve as synthetase recognition sites (Kisselev and Favorova, 1974; Söll and Schimmel, 1974; Rich and Schimmel, in preparation). The two modifications which are completely missing (D16 and the *S*-methyl moiety of ms^2i^6A37) and a third, partially deficient residue (ψ39) are located in these regions. It is possible that the absence of dihydrouridine 16 alone is responsible for the slower rate of aminoacylation, as the absence of the methylthio moiety of ms^2i^6A in *E. coli* tyrosine tRNA appears not to affect its recognition by tyrosyl-tRNA synthetase (Gefter and Russell, 1969). Neither does the absence of the two pseudouridine residues in the anticodon region of $tRNA^{His}$ from *Salmonella typhimurium* have an effect on its interaction with histidyl-tRNA synthetase (Brenner et al., 1972).

It seems likely that the lack of the methylthio moiety of ms^2i^6A is responsible for the decreased efficiency of $tRNA^{Phe}_U$ in supporting poly(U)-directed protein synthesis. Gefter and

Russell (1969) demonstrated that a methylthio-deficient form of $tRNA^{Tyr}$ isolated from $\phi 80su^{+}_{III}$ phage-infected *E. coli* was only 60% as efficient as fully modified $tRNA^{Tyr}$ in suppressing an amber mutation in an in vitro translation assay. In vitro ribosome binding studies subsequently revealed that the reduced efficiency of the modification-deficient $tRNA^{Tyr}$ stemmed from a reduction in the affinity of the tRNA for its ribosome-bound codon. The unique, rel⁻ $tRNA^{Phe}$ was also only 60% as efficient as normal $tRNA^{Phe}$ in supporting poly(U)-directed protein synthesis (Kitchingman et al., 1976).

Functional assays with $tRNA^{Leu}_{U}$ indicate that it is charged at the same rate as $tRNA^{Leu}_{I}$ and occurs on ribosomes in vivo with the same frequency as $tRNA^{Leu}_{I}$ (Kitchingman and Fournier, 1975, and unpublished results); the activity of $tRNA^{Leu}_{U}$ in a cell-free protein synthesizing system has not yet been determined. Because both the unique leucine and phenylalanine tRNAs lack the dihydrouridine residues which occur in the 5′ half of the D loop and are lacking or are deficient in $\psi 39$, the reduced rate of aminoacylation observed with the rel⁻ $tRNA^{Phe}$ may result from one of the other deficiencies or possibly a combination of deficiencies. Alternatively, as these tRNAs are from different structural classes (Sodd, 1976), the nature of the aminoacyl-tRNA synthetase–RNA interaction may be different for the two tRNAs.

It should be borne in mind that the absence of a detectable difference in biological activity in an in vitro assay may be a negative result at best. That the reaction conditions can be critical in such comparative analyses was made clear in preliminary attempts to characterize the aminoacylation of rel⁻ $tRNA^{Phe}$. Comparing the RPC-5 elution patterns of partially and fully aminoacylated $tRNA^{Phe}$ from leucine-starved cells (a mixture of unique and normal isoacceptors) showed that the unique species was charged at about one-fifth or less of the rate of the normal species (Kitchingman et al., 1976, and unpublished results). However, when assays to determine the kinetic properties were performed in the classic manner with enzyme limiting and at a lower temperature (25 vs. 37 °C), the unique species was charged at a rate that was only about 20–30% lower than that observed for normal $tRNA^{Phe}$. In this case, the functional inferiority of the unique species was very apparent at high concentrations of enzyme when assayed at 37 °C, yet barely evident with limiting enzyme at 25 °C. Thus, the presence or absence of a functional difference in some comparative analyses may provide information which only obtains for a specific assay condition.

While the effects of inhibiting protein synthesis on the posttranscriptional modication of tRNA may be general in nature, the possibility that certain of the modifications (or modification deficiencies) may be important in responding to certain conditions of physiological stress should not be overlooked. Of the modifications blocked by amino acid starvation or treatment with chloramphenicol, thus far only the ψ in the 3′ half of the anticodon loop can be suggested to have a possible regulatory role. This suggestion is based on the knowledge that cells harboring the *his* T mutation are defective in their ability to regulate histidine (Lewis and Ames, 1972) and isoleucine-valine and leucine (Rizzino et al., 1974; Cortese et al., 1974) biosynthesis. In each case it has been established that the cognate tRNAs are involved in the regulatory process and that the tRNAs from the regulatory-deficient cells lack ψ residues in the 3′ half of the anticodon loop/stem region. While there is no evidence that phenylalanine tRNA has such a regulatory role, it has been reported that $tRNA^{Phe}$, like $tRNA^{His}$, does bind to the allosteric first enzyme (Kovach et al., 1970) in the biosynthetic pathway for its amino acid (Duda et al., 1968).

With the knowledge that leucine-specific tRNA is involved in the regulation of leucine biosynthesis (Hatfield and Burns, 1970; Rizzino et al., 1974; Cortese et al., 1974) and serves as a donor in the addition of aminoacyl residues to the NH_2 terminus of specific classes of protein acceptors (Leibowitz and Soffer, 1971; and see review by Soffer, 1974), the possibility that the role of the missing minor nucleotides may not be related to protein biosynthesis must be given serious consideration. A number of findings suggest that $tRNA^{Leu}_{I}$ itself may have a unique role in the cell: (1) It is by far the most abundant tRNA in the cell. $tRNA^{Leu}_{I}$ normally accounts for 70–80% of the total leucine tRNA which, in turn, is two- to threefold more abundant than other acceptor species (von Ehrenstein, 1970). (2) Transcription of the genes for $tRNA^{Leu}_{I}$ is regulated in a manner different from that of the bulk of the other tRNA genes. $tRNA^{Leu}_{I}$, unlike other tRNAs, continues to accumulate in a culture of rel⁺ *E. coli* treated with the antibiotic trimethoprim (Ikemura and Dahlberg, 1973). Although the precise mechanism of action of this drug is not known, cells treated with the agent are effectively starved of methionine; the $tRNA^{Leu}_{I}$ and other RNAs which accumulate in its presence are methyl deficient. (3) $tRNA^{Leu}_{I}$ is synthesized as a large multimeric precursor from a transcriptional unit that contains a cluster of at least five contiguous cistrons for $tRNA^{Leu}_{I}$ (Schedl et al., 1974; Ilgen et al., 1976). (4) Although $tRNA^{Leu}_{I}$ is by far the predominant $tRNA^{Leu}$ isoacceptor in the cell, analyses of ribosome-bound tRNA have shown that the ratio of $tRNA^{Leu}_{I}$ to the other leucine isoacceptors is significantly lower on the ribosome than in the cytoplasm (Wettstein and Stent, 1968; Kano-Sueoka and Sueoka, 1969; Kitchingman and Fournier, 1975). This finding suggests that there may be considerably more $tRNA^{Leu}_{I}$ in the cell than is required for the translation process alone.

The finding that the rel⁻ leucine and phenylalanine tRNAs lack certain dihydrouridine residues suggests the possibility that these and other rel⁻ tRNAs might be useful in studying the biosynthesis of dihydrouridine. Progress on this important problem has been limited (Söll, 1971) in large part, for lack of a suitable tRNA substrate. Thus, it may be possible now to purify and characterize a dihydrouridine-forming enzyme and to study the mechanism and regulation of the biosynthesis of this ubiquitous nucleotide. These studies, currently in progress in our laboratory, should provide more insight into the important questions of the biosynthesis and role of modified nucleotides in tRNA.

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Supplementary Material Available

Full details of the results obtained in the purification and structural analyses of the undermodified and normal phenylalanine and leucine isoacceptor tRNAs (ten figures and two tables; 24 pages). Ordering information is given on any current masthead page.

Added in Proof

In vitro assays of pseudouridylate synthetase activity in extracts from rel⁻ cells have revealed the enzyme levels to be the same in leucine-starved and nonstarved cells.

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