JOURNAL OF BACTERIOLOGY, May 1975, p. 538-548

Vol. 122, No. 2
Printed in U.S.A.

Formation of Chromatographically Unique Species of Transfer Ribonucleic Acid During Amino Acid Starvation of Relaxed-Control *Escherichia coli*

MAURILLE J. FOURNIER[1]* AND ALAN PETERKOFSKY

Laboratory of Biochemical Genetics, National Heart and Lung Institute, Bethesda, Maryland 20014

Received for publication 4 February 1975

Examination of the transfer ribonucleic acid (tRNA) produced by starving, relaxed-control (*rel*$^-$) strains of *Escherichia coli* for required amino acids revealed the occurrence of a number of chromatographically unique subspecies. Leucine starvation results in the formation of new isoacceptor species of leucine-, histidine-, arginine-, valine-, and phenylalanine-specific tRNA and quantitative changes in the column profiles of serine, glycine, and isoleucine tRNA. Evidence that the unique tRNA species are synthesized de novo during amino acid starvation comes from the findings that the major unique leucine isoacceptor species is not formed in stringent control cells or in *rel*$^-$ cells starved for uracil or treated with rifampin. Furthermore, heat treatment of the unique leucine tRNA does not alter its chromatographic behavior, indicating that the species is not an aggregate or nuclease-damaged form of a normal isoacceptor tRNA. The methyl acceptor activities of tRNA from leucine-starved and nonstarved *rel*$^+$ or *rel*$^-$ cells were found to be essentially the same. This result and the finding that the chromatographic behavior of the unique leucine-specific tRNA was not altered after treatment with tRNA methylase suggests that gross methyl deficiency is probably not the biochemical basis for the occurrence of the unique species.

Although it is well known that multiple, chromatographically distinct isoacceptor transfer ribonucleic acids (tRNA's) for a specific amino acid can occur in an organism, the biochemical basis of this multiplicity is understood in only a few cases. Some tRNA isoacceptor species are the products of different cistrons, whereas others may differ only in the extent to which post-transcriptional modification has occurred and, thus, are related as precursor and product (2, 9, 27). Owing to the degeneracy of the genetic code and the finding that some isoacceptor species differ in coding response, it has been generally accepted that isoacceptor tRNA's may play a regulatory role in translation (9). This view has been supported by the discovery that changes in the composition of isoacceptor tRNA species are known to occur under several metabolic conditions, including virus infection, development, neoplastic transformation, and in response to changes in culturing condition (9, 20).

Interest in the biological role of tRNA isoacceptor species has been intensified recently by the findings that tRNA's are involved in cellular processes other than protein synthesis. tRNA isoacceptors are now known to function in cell wall biosynthesis, regulation of amino acid biosynthesis, secondary modification of protein, and the inactivation of tryptophan pyrrolase (9). Furthermore, the biosynthesis of fatty acids and RNA is regulated by amino acids through mechanisms that may include the involvement of tRNA (25).

The study presented here was undertaken to determine the intracellular condition of tRNA during amino acid deprivation. If specific isoacceptor tRNA's are involved in amino acid-mediated repression and end-product inhibition, it seemed reasonable to expect that a comparison of tRNA from amino acid-starved wild-type cells and certain regulatory mutants would reveal compositional differences. We report that changes in the composition of tRNA isoacceptor species do indeed occur in relaxed-control (*rel*$^-$) *Escherichia coli* during conditions of amino acid starvation. Changes in tRNA patterns detected by column chromatography have been observed previously with tRNA from methionine-starved cells. However, in that case the tRNA's produced were methyl-deficient, inasmuch as *S*-adenosyl methionine, the methyl

[1] Present address: Department of Biochemistry, University of Massachusetts, Amherst, Mass. 01002.

donor in the formation of methylated nucleosides, was limiting (11). The effect we report here for leucine deprivation seems more general in nature, in that there is no known direct involvement of leucine in tRNA modification.

(Some of these results were presented at the 1970 Federation of American Societies for Experimental Biology Annual Meeting, Atlantic City, N.J. [Fed. Proc. **29**:468, 1970].)

MATERIALS AND METHODS

Materials. Radioactive amino acids were obtained from either Schwarz/Mann or New England Nuclear. ^{14}C-labeled L-amino acids were uniformly labeled and used at the stated specific activities. ^{3}H-labeled L-amino acids were generally labeled and adjusted to final specific activities of 1 to 5 Ci/mmol before use. Specific activity of the *S*-[^{14}C]adenosyl methionine was 42 mCi/mmol.

Diethylaminoethyl-cellulose (Whatman DE-52) was washed with 0.5 N HCl and 0.5 N NaOH before use. Aliquat 336 (tricaprylylmethyl-ammonium chloride) was obtained from General Mills, Inc.; Chromosorb W (100 to 200 mesh), acid washed and dimethyldichlorosilane treated, was from Johns Manville Products Corp.; Freon 214 (tetrachlorotetrafluoropropane) was from E. I. duPont de Nemours and Co.; Adogen 464 [methyltrialkyl (C_8-C_{10}) ammonium chloride] was from Ashland Chemical Co.; and Plaskon CTFE 2300 (polychlorotrifluoroethylene powder) was from Allied Chemical Co. All other chemicals were of reagent grade.

Bacteria and culture conditions. *E. coli* CP78 (*thi*⁻, *arg*⁻, *his*⁻, *thr*⁻, *leu*⁻, *rel*⁺), CP79 (*thi*⁻, *arg*⁻, *his*⁻, *thr*⁻, *leu*⁻, *rel*⁻), and K-12 strains 58-161 (*met*⁻, *bio*⁻, *rel*⁻) and W-1305 U⁻ were used in the present study. Strain W-1305 U⁻ is a uracil-requiring mutant derived by nitrosoguanidine mutagenesis of strain W-1305 (*met*⁻, *leu*⁻, *rel*⁻) (8). Strains CP78, CP79, and W-1305 U⁻ were a gift of Robert Lazzarini. The cells were grown in the tris(hydroxymethyl)aminomethane-glucose-salts medium of Edlin and Maaloe (T medium; 4) or in the citrate-glucose-salts medium of Vogel and Bonner (21) to which $ZnCl_2$ was added to the same concentration (10^{-5} M) as that in the T medium. Amino acids were present at concentrations of 20 μg/ml and thiamine was present at 2 μg/ml for strains CP78 and CP79. The concentrations of amino acids and uracil for strain W-1305 U⁻ growth were 25 and 10 μg/ml, respectively. Cultures were grown in flasks at 37 C in a rotary-action shaking incubator. Full growth under these conditions corresponds to optical densities at 450 nm of about 1.2 for strains CP78 and CP79 and about 1.5 for strain W-1305 U⁻.

Except where indicated, amino acid starvation of the bacteria was effected by growth in medium supplemented with a limiting amount of amino acid. Concentrations were selected that would result in termination of growth at about the mid-point of the logarithmic phase. For strains CP78 and CP79 these concentrations are: leucine or arginine, 5 μg/ml; and histidine, 1 μg/ml. Nonstarved control cultures were harvested at the same turbidity as that at which the starved culture ceased to grow.

Preparation of tRNA. Cells collected by centrifugation were suspended in about 10 volumes of a buffer consisting of 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.0) and 0.01 M $MgCl_2$, and then extracted with an equal volume of redistilled 90% phenol. The aqueous phase was reextracted two times with phenol, and the RNA was precipitated by addition of 0.10 volume of 20% potassium acetate (pH 4.5) and 2 volumes of cold 95% ethanol. After a period of several-hours to overnight at −20 C, the precipitate was collected by centrifugation, solubilized in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.5), and incubated for 3 h at room temperature to discharge the aminoacylated tRNA. The sample was then dialyzed against 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.0) and applied to a small column (5- to 10-ml bed volume) of diethylaminoethyl-cellulose equilibrated with the same buffer containing 0.3 M sodium chloride. After the column was washed with this buffer, the tRNA was eluted with buffer containing 1 M NaCl and precipitated with 2.5 volumes of cold ethanol. Where indicated, tRNA was heat denatured by incubation at 80 C for 3 to 5 min in 0.015 M sodium citrate/0.15 M sodium chloride (pH 7.0) at a concentration less than 1 mg/ml. Methyl-deficient tRNA was prepared as described previously (3).

Preparation of aminoacyl-tRNA synthetases and tRNA methylase. Crude preparations of synthetases free from tRNA and ribonuclease activity were prepared by the method of Muench and Berg (12), except that the cells were disrupted by grinding with alumina in a mortar with pestle (13) and the final preparations were adjusted to 50% glycerol and stored at −20 C; tRNA methylase was prepared as described previously (3).

Aminoacylation procedure. tRNA was charged with specific radioactive amino acids by the method of Rubin et al. (16) with the following modifications. A mixture of 19 unlabeled amino acids (20 common minus the labeled species) was included at a final concentration of 5×10^{-5} M to minimize the effect of contaminating radioactive amino acids and attachment of the labeled amino acid to a noncognate tRNA. The concentrations of enzyme and radioactive amino acid required for complete acylation in 15 min at 37 C were determined in a preliminary experiment. The final concentrations of amino acid adopted were between 10^{-4} and 5×10^{-6} M and were close to or greater than reported K_m values. The kinetics of acylation was monitored by sampling the reaction mixture at intervals. The samples were precipitated with 5% cold trichloroacetic acid and collected on glass-fiber or cellulose-nitrate filters (Millipore Corp., Bedford, Mass.). After the filters were dried at 100 C the extent of charging was determined by liquid scintillation counting in toluene-Liquifluor (24:1, New England Nuclear). Samples to be chromatographed were extracted with an equal volume of 90% phenol after addition of 0.1 volume of 20% potassium acetate, pH 4.7. The aminoacyl-tRNA was then dialyzed against the initial column buffer. Samples to

be cochromatographed were combined just prior to dialysis.

In vitro methylation of tRNA. In vitro methylation reactions were carried out by the method of Capra and Peterkofsky (3).

Chromatographic analysis of tRNA. Radioactive aminoacyl-tRNA's were analyzed by reversed-phase chromatography on RPC-2 (24) or RPC-5 (15) columns. The RPC-2 column was 1 by 240 cm or 0.5 by 115 cm and was loaded with 100 to 200 absorbancy units (260 nm) of RNA containing between 2×10^5 and 10^6 counts of ^{14}C and ^3H per min at an isotope ratio of about 1:3. The column was maintained at 24 C, and the RNA was eluted with a linear NaCl gradient in buffer containing 0.01 M sodium acetate (pH 4.5) and 0.01 M $MgCl_2$. The initial buffer (against which the samples were dialyzed prior to application) was 0.35 M in NaCl and the terminal buffer was 0.65 M in NaCl. Total volume of the gradient was 4.4 liters for the large column and 700 ml for the small column. The flow rate was controlled between 0.5 and 1 ml/min. Fractions of 10 ml (large column) or 2 or 3 ml (small column) were collected.

The RPC-5 column fractionations were accomplished with a high-pressure analytical column (0.9 by 69 cm; Beckman Instruments). Samples containing 5 to 10 absorbancy units (260 nm) of RNA and between 5×10^4 and 2×10^5 counts/min (^3H to ^{14}C ratio about 3:1) were applied in a buffer that was 0.01 M sodium acetate (pH 4.5), 1 mM ethylenediaminetetraacetic acid, 0.01 M $MgCl_2$, and 0.40 M sodium chloride. The tRNA's were eluted with a linear NaCl gradient with limits of 0.40 and 1.2 M. Total volume of the gradient was 600 ml. The flow rate was 1 ml/min and the pressure was between 400 and 500 lb/in^2 (Milton Roy Minipump). Fractions of 2 ml were collected. Fractions to be analyzed for radioactivity were chilled, adjusted to 5% trichloroacetic acid, and then filtered through cellulose-nitrate or glass-fiber disks (Millipore Corp.). Dried filters were counted as described above. Counting efficiencies in the double-label experiments were 46 and 25% for ^{14}C and ^3H, respectively. The resulting profiles have been normalized.

RESULTS

For our comparative study, a pair of *E. coli* amino acid auxotrophs was selected that are isogenic except for the *rel* locus, which is involved in the control of RNA synthesis (17). One strain, CP78, is under stringent or wild-type control (*rel*$^+$), whereas the other, CP79, is a relaxed-control mutant (*rel*$^-$). Unlike the wild-type strains, which exhibit stringent control over RNA synthesis, relaxed mutants continue to produce RNA in the absence of a required amino acid (17). Figure 1 shows the reversed-phase column profiles of leucine tRNA derived from leucine-starved cultures of stringent- and relaxed-control leucine auxotrophs. A comparison of the two patterns reveals both qualitative and quantitative differences. The most dramatic difference is the occurrence of a unique leucine isoacceptor species in the CP79 tRNA (fractions 115 to 140) which is absent in the tRNA derived from the stringent CP78 cells. In this particular preparation, the unique subspecies accounted for over 20% of the total leucine acceptor activity. In other preparations derived from cells starved for longer periods this species accounted for up to 50% of the leucine tRNA. The unique species was not present in nonstarved cells of either genotype, and the leucine tRNA profiles from the nonstarved stringent and relaxed cells (not shown here) were virtually identical to that for the leucine-starved stringent cells shown in Fig. 1.

Because the tRNA's in these experiments were charged with aminoacyl-tRNA synthetases from the homologous sources, it was necessary to perform heterologous aminoacylations to rule on possible differences in the synthetases. Analyses with aminoacyl-tRNA synthetases from amino acid-starved and nonstarved cells of either strain gave the same result, showing the differences to be attributable to the tRNA and not the enzymes. Thus, we conclude that the unique leucine tRNA was produced in the *rel*$^-$ cell in response to amino acid deprivation and did not occur in readily detectable amounts in these cells under normal amino acid-supplemented growth conditions.

New synthesis or alteration of preexisting tRNA? Given the observation that a unique leucine tRNA is formed in relaxed-control cells during amino acid starvation, the question of the origin of this tRNA arises. Is this species formed by an enzymatic alteration of a preexisting tRNA or is it a product of new synthesis? To test the possibility that the chromatographically unique tRNA may differ from the normal isoacceptor species only in quarternary structure, that is, may be a dimer or higher aggregate, the tRNA preparations from starved and nonstarved *rel*$^-$ cells were heat treated under conditions known to dissociate tRNA aggregates (10) and analyzed by reversed-phase column chromatography. The elution position and relative amount of unique leucine tRNA were unchanged by this treatment (results not shown here), indicating that the species is monomeric in form. This result also indicates that the unique species is not a nuclease-damaged tRNA since such a molecule would be dissociated into its component fragments upon denaturation and would probably not accept amino acids (14).

To determine whether RNA synthesis is required for the production of the unique species we examined the leucine-specific tRNAs ($tRNA^{Leu}$) from two relaxed auxotrophs starved

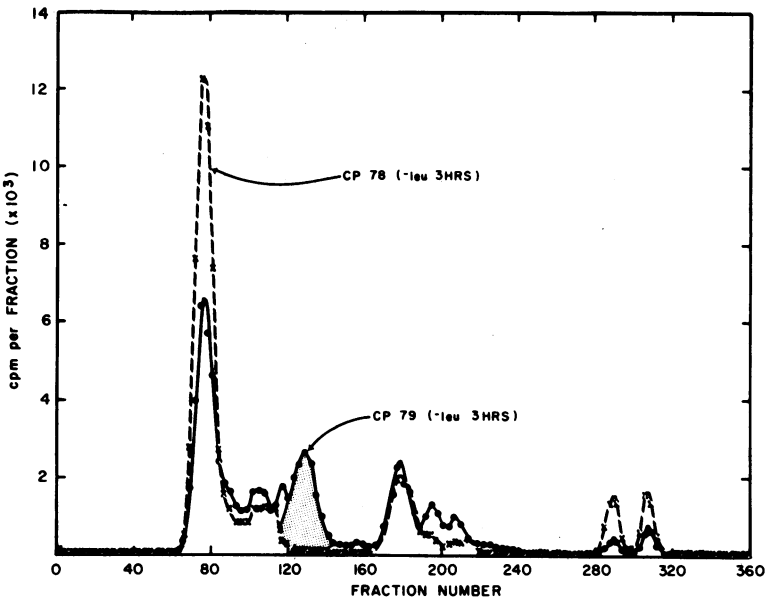

FIG. 1. *Effect of leucine starvation on the leucine tRNA from rel⁺ and rel⁻ E. coli. tRNA prepared from mid-log-phase cultures of E. coli CP78 (rel⁺) and CP79 (rel⁻) deprived of leucine for 3 h was aminoacylated with [³H]leucine (CP 78) or [¹⁴C]leucine (CP 79), mixed, and cochromatographed on a large RPC-2 column. Symbols: ×, CP 78 tRNA^{Leu}; ●, CP 79 tRNA^{Leu}.*

for leucine under conditions that prohibit RNA synthesis. In the first experiment, the leucine tRNA column profiles were compared for tRNA derived from cells starved for leucine or leucine plus uracil. The cells were *E. coli* W-1305 U⁻ (*met*⁻, *leu*⁻, *ura*⁻, *rel*⁻). If the unique tRNA^{Leu} is newly synthesized in response to amino acid starvation, simultaneous starvation for leucine and uracil should prevent its appearance. The results of this experiment are depicted in Fig. 2. The profiles in the upper panel are of leucine tRNA from leucine-starved and nonstarved *E. coli* W-1305 U⁻. It can be seen that the unique leucine tRNA was produced in this *rel*⁻ strain also (fractions 120 to 140), demonstrating that the phenomenon is not limited to *E. coli* CP79. The profiles in Fig. 2B compare the leucine tRNA's from cultures deprived of leucine and leucine and uracil. It is evident that the unique leucine tRNA was absent in the cells deprived of both leucine and uracil, indicating that RNA synthesis is required for its formation.

Additional evidence that the new species was not derived from one of the normally occurring, preexisting species comes from an experiment in which RNA synthesis was blocked at the onset of leucine deprivation by addition of the inhibitor rifampin (Fig. 3). It should be pointed out that this analysis was performed on an RPC-5 column not RPC-2 as were the previous analyses; however, the elution profiles for leucine tRNA are comparable for the two columns. As before, the unique species was produced in the leucine-starved *rel*⁻ culture but not in the starved cells treated with rifampin. Inasmuch as the formation of the unique tRNA^{Leu} did not occur in *rel*⁺ cells during leucine starvation or in *rel*⁻ cells under conditions that prevent RNA synthesis, we conclude that the isoacceptor species was synthesized de novo in response to amino acid starvation.

Are non-leucine tRNA's affected by leucine starvation? Having established that qualitative and quantitative changes occur in the leucine tRNA from relaxed-control cells as a result of leucine starvation, it is important to determine whether other tRNA's are also affected by leucine deprivation and, furthermore, whether the effect on leucine tRNA is leucine specific. To gain insight into the first question, other tRNA's from leucine-starved cells were analyzed by reversed-phase column chromatography and compared with tRNA from nonstarved control cultures (Fig. 4). Panels A through D show that new isoacceptor tRNA's for histidine (fractions 150 to 190), arginine (fractions 130 to 155), valine (fractions 128 to 136), and phenylalanine (fractions 102 to 111 and 123 to 135) were formed. Quantitative changes, that is, changes in the ratios of nor-

mally occurring isoacceptor tRNA's were observed in these profiles and also in those depicted in panels E through G. These latter panels show the column profiles for isoleucine-, serine-, and glycine-specific tRNA's. Panel H shows the proline tRNA patterns to be virtually identical for leucine-starved and nonstarved cells. This apparent identity was observed on both RPC-2 and RPC-5 columns. Thus, except for proline tRNA, qualitative or quantitative changes have been observed for every tRNA tested. It is possible that such changes also occur with proline tRNA but are simply not detected because of the resolving power of the fractionation methods used here.

Is the formation of unique tRNA's leucine specific? To determine whether the qualitative changes in tRNA depicted in Fig. 4 occur in relaxed-control cells deprived of amino acids other than leucine, tRNA's from arginine-starved *E. coli* CP79 were analyzed. The results (not presented here) show that unique leucine, arginine, valine, and phenylalanine tRNAs were formed under these conditions as well. Indeed, the unique isoacceptor species formed by arginine-deprived cells migrated with those produced during leucine starvation, suggesting that the species formed under the two conditions may be identical (G. R. Kitchingman and M. J. Fournier, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, p. 207, p. 175; manuscript in preparation).

Effects similar to those described here have also been observed with tRNA isolated from chloramphenicol-treated *rel*$^+$ *E. coli* (22), suggesting that these effects can occur under any condition leading to an inhibition of protein synthesis. Contrary to this view, we find that no unique leucine or histidine tRNA is formed in histidine-starved cells. The respective profiles are virtually indistinguishable from those derived from nonstarved cultures. Knowing that RNA synthesis is a prerequisite for the formation of the unique species, we have determined that *E. coli* CP79 does indeed continue to produce RNA in the absence of histidine as in the case during leucine or arginine starvation. Indeed, the rates of incorporation of [^{3}H]uracil into RNA were very similar for both leucine- and histidine-starved cells. Furthermore, polyacrylamide gel profiles of ^{32}P-labeled RNA from leucine- and histidine-starved cultures revealed that tRNA was produced under both conditions and that the differences in the amounts of RNA synthesized under the two conditions were not large (G. Kitchingman and M. Fournier, unpublished data). The studies reported here cannot distinguish whether the qualitative and quantitative changes observed occur only upon starvation for certain amino acids or whether the effect is widespread and histidine starvation is an unusual case.

Are the unique tRNA's undermodified precursors? One hypothesis that can be advanced to explain the biochemical basis of the formation of the unique tRNA's is that these species are newly synthesized tRNA's that have not undergone all of the post-transcriptional modifications associated with the maturation of precursor tRNA's (1, 19). Thus, the unique tRNA's may be undermodified precursors to the normally occurring species. This view is supported by the reversed-phase chromatography results presented in Fig. 1 through 4. Careful inspection of the patterns for leucine-, phenylalanine-, isoleucine-, and glycine-specific tRNA reveals that the appearance of the major unique tRNA was matched by a decrease of the same magnitude in the relative amount of the major, normally occurring species.

Of the more than 25 different modified bases identified thus far for *E. coli* tRNA, approximately 50% are methylated (2). On the average, there are about three methylated nucleosides per chain of *E. coli* tRNA (2). To test the possibility that the unique leucine tRNA produced during leucine starvation is a methyl-deficient form of a normally occurring species the column profiles of methyl-deficient $tRNA^{Leu}$ and $tRNA^{Leu}$ from leucine-starved cells were compared. Methyl-deficient tRNA can be prepared by methionine starvation of *rel*$^-$ *E. coli* (11). For this experiment, *E. coli* W-1305 U$^-$ was used. The elution profiles obtained are shown in Fig. 5. As expected, the pattern for $tRNA^{Leu}$ from the methionine-starved cells differs from the nonstarved control profiles shown earlier, in that a number of quantitative and qualitative differences exist. Altogether, at least 10 peaks can be seen, whereas the "normal" tRNA usually contained 6 or 7 subspecies. The important point is that one of the isoacceptor species from the methionine-starved cells (fractions 130 to 150) was eluted in the region characteristic of the unique tRNA from the leucine-starved cells. However, because of the multiplicity of isoacceptors in that region, this experiment does not provide definitive evidence for the identity of the unique isoacceptors produced by leucine and methionine starvation.

To test the hypothesis that the tRNA from leucine-starved cultures may be methyl poor, the methyl acceptor activity of this tRNA was compared with those for tRNAs from non-

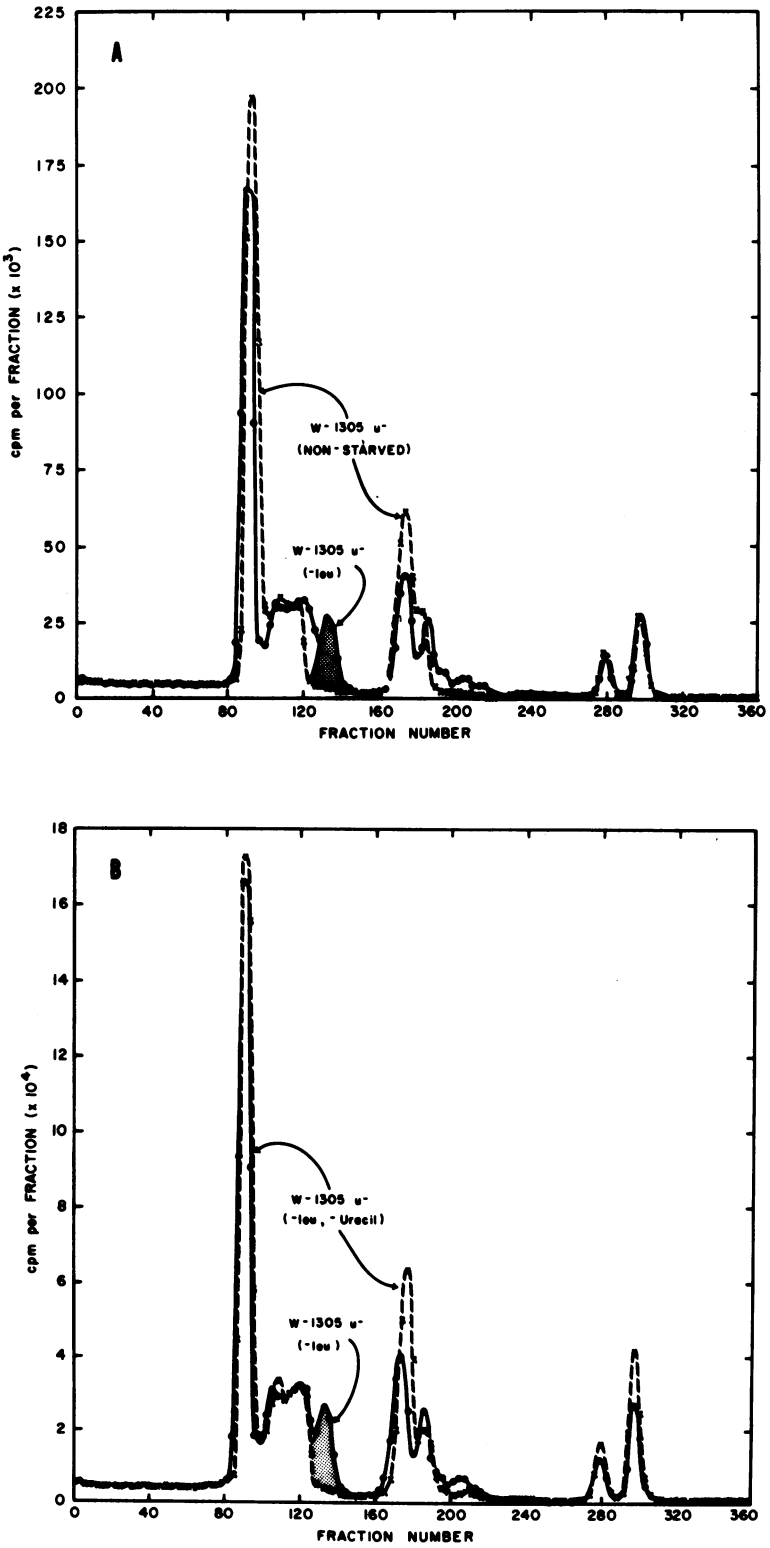

FIG. 2. *Effect of uracil starvation on the formation of the unique leucine isoacceptor tRNA. (A) Comparison of leucine tRNAs from nonstarved and leucine-starved E. coli W-1305U⁻. Starvation time, 3 h. Fractionation was effected on a large RPC-2 column. (×) [³H]leucine, nonstarved; (●) [¹⁴C]leucine, leucine starved. (B) Leucine tRNAs from E. coli W-1305U⁻ deprived of leucine or leucine and uracil for 3 h. Large RPC-2 column. (×) [³H]leucine starved; (●) [¹⁴C]starved of leucine and uracil.*

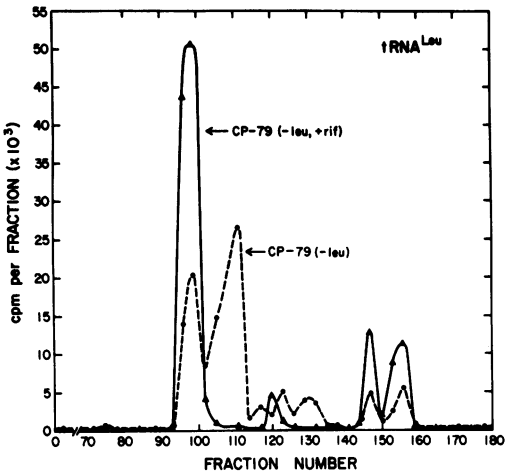

FIG. 3. *Effect of rifampin on the production of unique leucine tRNA in rel⁻ E. coli. Cells from a mid-log culture of E. coli CP 79 were isolated, washed, and incubated for 6 h in media lacking leucine or lacking leucine but containing rifampin (40 μg/ml). Symbols: ●, [³H]leucyl-tRNA, leucine starved; ▲, [¹⁴C]leucyl-tRNA, leucine starved plus rifampin. Fractionation was by RPC-5 chromatography.*

starved and methionine-starved cells (Table 1). As expected, the methyl-deficient tRNA from methionine-starved *rel⁻* cells (strain W-1305 U⁻) was a good substrate for the tRNA methylase preparation used and accepted about 0.5 methyl groups per chain on tRNA. The tRNA's from the nonstarved or leucine-starved cells on the other hand, exhibited very low methyl acceptor activities, which were only about 1 to 6% of the methyl-deficient RNA. Virtually identical results were obtained with methylase prepared from *rel⁻* cells. The small differences observed cannot be considered statistically significant since they are well within experimental error. Although it can be concluded that the unfractionated tRNA from leucine-starved cells is not grossly deficient in methylated bases, the unique species may be. Since the proportion of total tRNA that is "unique" is not known and may be small, it is possible that differences may occur that cannot be detected by the incorporation assay used here.

Inasmuch as the unique species may have been methylated in vitro with an attendant reversion of its chromatographic behavior to that of a normal isoacceptor species, the elution profile of methylase-treated $tRNA^{Leu}$ from starved cells was examined. The results of the analysis (not shown here) indicated that incubation of the unique leucine tRNA with methylase had no effect on its chromatographic properties. The methylase-treated and untreated $tRNA^{Leu}$ from leucine-starved cells gave identical column profiles.

The recent discovery of a threonine-containing adenine nucleotide, *N*-[9-(β-D-ribofuranosyl) purin-6-ylcarbamoyl] threonine, in *E. coli* tRNA (18) raises the question of the possible occurrence of other amino acid-containing nucleosides in tRNA. If leucine is a precursor for a leucine-containing base, then leucine starvation could block its formation and lead to the accumulation of modification-deficient tRNA's which are chromatographically unique. To test this possibility an attempt was made to incorporate radioactive leucine into tRNA in vivo at a site other than the amino acid acceptor site.

tRNA prepared from *E. coli* CP79 cells grown in medium containing [³H]leucine was found to contain only enough radioactivity after discharging of amino acid bound to the $-CpCpA_{OH}$ terminus to account for less than 0.2% labeling on a molar basis. Unless the leucine was incorporated into a base through a bond that was labile under the mild alkaline conditions used for deacylation, the amount of incorporation obtained is clearly insufficient to support the view that hypomodification of such a base is the chemical basis for the phenomenon reported here.

DISCUSSION

The results presented demonstrate that a number of qualitative and quantitative changes occur in the tRNA's from relaxed-control *E. coli* during starvation for a required amino acid. These changes, reflected in altered chromatographic patterns, are not due to differences in the aminoacyl tRNA synthetases, since tRNA's acylated with synthetases from starved or nonstarved cells of either the relaxed or stringent genotype give the same profiles. It also seems clear that the qualitative differences observed are not due to lack of acylation in vitro of a modified aminoacylated species of the sort described by Yegian and Stent (26). In an examination of the intracellular condition of *E. coli* tRNA, those investigators discovered that some leucine tRNA was protected from deacylation presumably because the tRNA was esterified in vivo with a substance other than the cognate amino acid. The protected species were enzymatically deacylated, however, and reacylated in a conventional in vitro aminoacylation reaction. Under our conditions any such tRNA species would become labeled and, thus, be detectable in the column assay.

Because heat treatment of the unique leucine tRNA isoacceptor species prior to acylation did not result in a loss of activity or change in

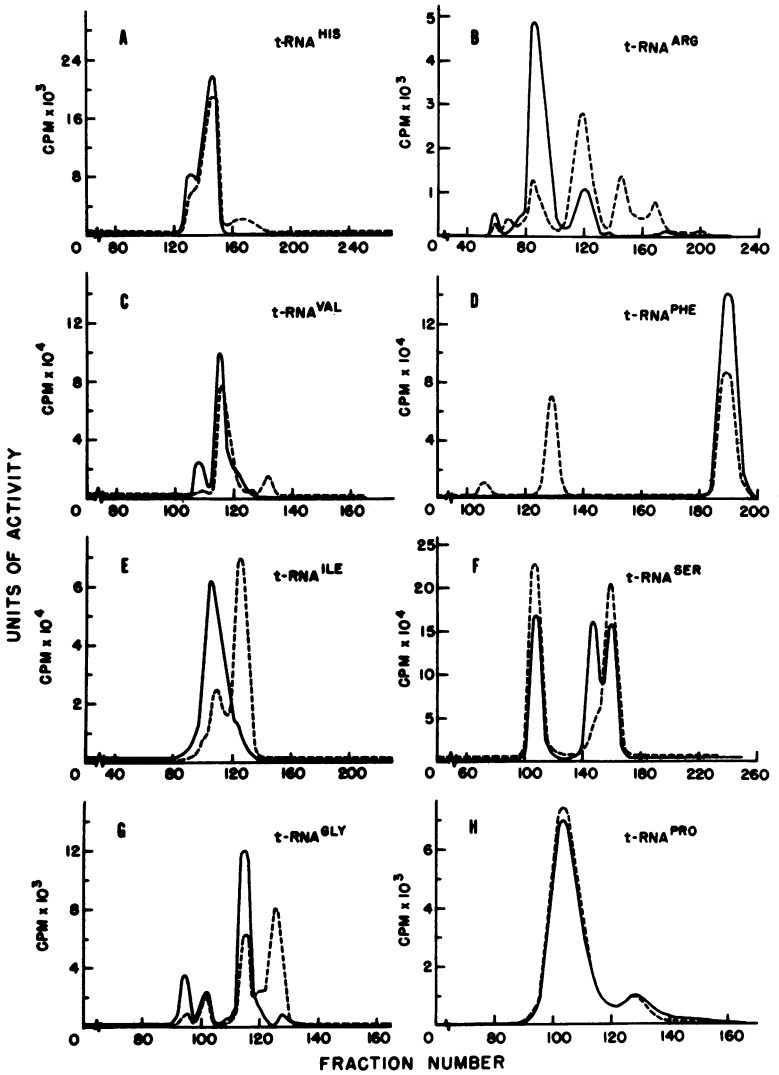

FIG. 4. *Effect of leucine starvation on tRNA from relaxed-control E. coli. Column profiles of aminoacyl-tRNA from leucine-starved E. coli CP 79 are compared with tRNA from nonstarved cultures or cultures of E. coli CP 78 (rel⁺) starved for leucine. Cells were starved from 4 to 6 h. Except where indicated, all analyses were performed as described in Materials and Methods. (A) Histidine tRNA: (——) CP 78 (– leucine), ³H; (– – –) CP 79 (– leucine), ¹⁴C; large RPC-2 column. (B) Arginine tRNA: (——) CP 78 (– leucine), ³H; (– – –) CP 79 (– leucine), ¹⁴C; large RPC-2 column. (C) Valine tRNA: (——) CP 78 (– leucine), ³H; (– – –) CP 79 (– leucine), ¹⁴C; RPC-5 chromatography. (D) Phenylalanine tRNA: (——) CP 79 (nonstarved), ¹⁴C; (– – –) CP 79 (– leucine), ³H; RPC-5 chromatography. (E) Isoleucine tRNA: (——) CP 78 (– leucine), ³H; (– – –) CP 79 (– leucine), ¹⁴C; small RPC-2 column with 800-ml gradient and 3-ml fractions. (F) Serine tRNA: (——) CP 78 (– leucine), ¹⁴C; (– – –) CP 79 (– leucine), ³H; small RPC-2 column, 3-ml fractions. (G) Glycine tRNA: (——) CP 79 (– leucine), ¹⁴C; (– – –) CP 78 (– leucine), ³H; RPC-5 chromatography. (H) Proline tRNA: (——) CP 79 (– leucine), ¹⁴C; (– – –) CP 78 (– leucine), ³H; small RPC-2 column, 3-ml fractions.*

column elution position, it can be concluded that this species is not simply an aggregate (10) or nuclease-damaged form of a normally occurring subspecies that can still be aminoacylated. In this last regard, Nishimura and Novelli (14) have demonstrated that ribonuclease-damaged tRNA may retain acceptor activity. This activity is abolished, however, if the molecule is dissociated under conditions where reassociation of the fragments cannot occur.

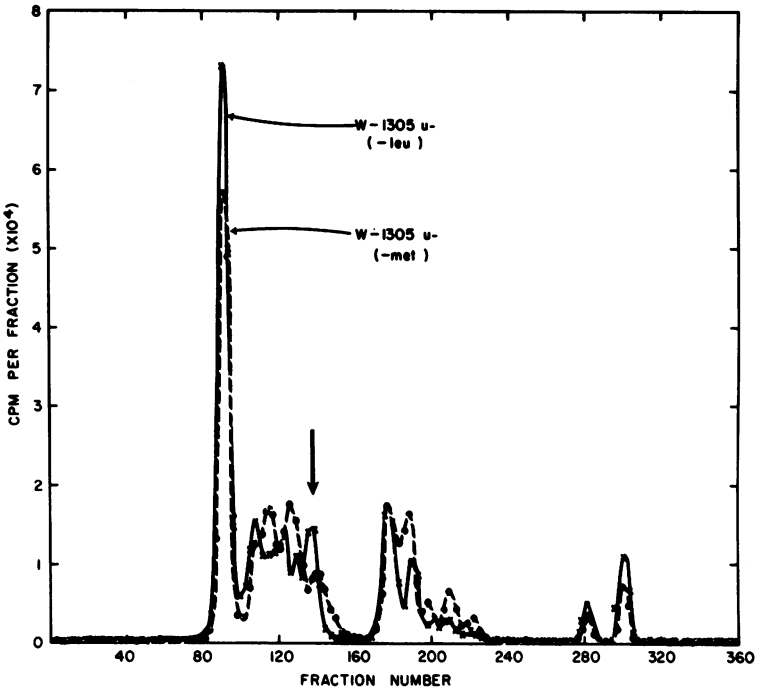

FIG. 5. *Comparison of the leucine isoacceptor tRNAs from leucine- and methionine-starved E. coli W-1305U⁻. A mid-log-phase culture was centrifuged, washed, divided into two parts, and suspended in media lacking either leucine or methionine. The cultures were incubated for 3 h. Symbols:* ×, *W-1305U⁻ (– leucine),* ^{14}C; ●, *W-1305U⁻ (– methionine),* ^{3}H. *Large RPC-2 column.*

TABLE 1. *Methyl acceptor activity of rel⁺ and rel⁻ tRNA's*[a]

Source of tRNA	pmol of CH_3/ pmol of tRNA
E. coli K12-58-161 *rel⁺* (NS)	0.035
E. coli K12-58-161 *rel⁻* (NS)	0.020
E. coli CP78 *rel⁺* (NS)	0.017
E. coli CP78 *rel⁺* (–Leu)	0.018
E. coli CP79 *rel⁻* (–Leu)	0.005
E. coli W-1305 U⁻ *rel⁻* (NS)	0.007
E. coli W-1305 U⁻ *rel⁻* (–Leu)	0.015
E. coli W-1305 U⁻ *rel⁻* (–Met)	0.460

[a] The methyl acceptor activity of 1 absorbancy unit (260 nm) for tRNA from nonstarved (mid-log) or amino acid-starved (3 to 4 h) *rel⁺* and *rel⁻* strains of *E. coli* was determined as described in the text. NS, Nonstarved; –Leu, starved for leucine; –Met, starved for methionine.

It seems clear that the unique species are newly synthesized and that hypermodification of an existing species is not the basis for their appearance. Evidence supporting this view comes from the findings that the unique species are not produced in amino acid-starved stringent control cells nor in relaxed-control strains deprived of uracil or treated with rifampin.

Since seven of the eight tRNA's examined show qualitative and/or quantitative changes, it is likely that the effect is general and not restricted to a specific class of tRNA's. Codon class-specific effects are known to occur (19). Specifically, the tRNA's in the uracil (Ura) and adenine (Ade) codon classes (which respond to triplets in which the first base is uracil or adenine) are known to contain a specific hypermodified base adjacent to the anticodon on the 3′ side. In the Ura codon class this base is 6-(Δ^2-isopentenyl) adenosine, and in the Ade codon class it is the threonine-containing nucleoside *N*-[9-(β-D-ribofuranosyl) purin-6-ylcarbamoyl] threonine. The tRNA's examined by us include species from all four codon classes. Those tRNA's that have chromatographically unique subspecies respond to codons starting with Ura (tRNA^{Phe}), cytosine (tRNA^{His} and tRNA^{Arg}), Ade (tRNA^{Arg}), and guanine (Gua) (tRNA^{Val}). The tRNAs for which quantitative changes are observed are in the Ade (tRNA^{Ile} and tRNA^{Ser}), Ura (tRNA^{Ser}), and Gua (tRNA^{Gly}) codon classes.

We have asked whether the unique leucine tRNA is an undermodified precursor tRNA

deficient in methylated nucleotides or a hypothetical leucine-derived nucleotide. No positive evidence favoring either possibility was found. The methyl acceptor activities of tRNA from amino acid-starved *rel*⁻ cells did not differ significantly from those for tRNA from nonstarved *rel*⁺ or *rel*⁻ cells or from amino acid-starved *rel*⁺ cells. In addition, the chromatographic position of the unique leucine tRNA was not altered after incubation with a tRNA methylase preparation. The methylation results must be considered negative, however, since the formation of certain methylated bases may proceed poorly in vitro. Methylase preparations such as that used in this study are known to be very active with regard to the ability to form ribothymidine (5-methyluridine [5]), suggesting that the RNA is not deficient in that base.

In an in vivo incorporation experiment designed to test the notion that a leucine-containing base may be involved, less than 0.2% of the tRNA was labeled with radioactive leucine. Inasmuch as the unique leucine tRNA alone represented better than 20% of the leucine acceptor activity in a tRNA preparation that was about 5% leucine specific (making the unique leucine tRNA species about 1% of the total tRNA), this incorporation is too low to support the view that the absence of such a minor base is the basis of the changes observed.

Recent work by others indicates that preparations of unfractionated tRNA from chloramphenicol-treated *E. coli* and *Salmonella typhimurium* and amino acid-starved *rel*⁻ *E. coli* are probably deficient in the minor bases 5,6-dihydrouridine and 4-thiouridine (7, 23). Although such deficiencies may occur in the unique species observed by us, preliminary base compositional analyses of the bulk tRNA from leucine-starved *rel*⁻ cells have not revealed any consistent deficiencies (Kitchingman and Fournier, unpublished data).

The unique leucine and arginine tRNA's formed during starvation of *rel*⁻ cells for leucine or arginine do not accumulate upon histidine deprivation, although RNA is synthesized under this condition. The intracellular condition of histidine-starved cells may be quite different from that during leucine or arginine starvation, or the effect may be a strain peculiarity. If chloramphenical treatment and amino acid starvation have the same effect(s) on tRNA, the biochemical basis of the changes may be identical and general in nature. If such is the case, the most attractive hypothesis to explain these results is that undermodified tRNA's are produced under this condition owing to the absence of specific tRNA base-modifying enzymes. Perhaps these modifying enzymes are short-lived and must be synthesized continually to assure complete maturation of precursor tRNA's.

In experiments to be described later it was observed that restoration of protein synthesis in a leucine-starved culture of *E. coli* CP79 did not result in a rapid disappearance of the unique tRNA's. The major unique $tRNA^{Leu}$ and $tRNA^{Phe}$ persisted for several hours after resuspension of a starved culture in fresh, fully supplemented medium (Kitchingman and Fournier, manuscript in preparation), whereas all or most of the starved cells recovered when leucine was restored to the medium, the results suggested the possibility that the unique tRNA's are not normal intermediates in the biosynthesis of tRNA.

Another hypothesis that cannot be ruled out at this time is that the unique tRNA's are the products of tRNA genes not normally expressed. These "silent" genes may be derepressed during amino acid starvation. Inasmuch as there is no precedence for this second model, we tend to favor the first.

ACKNOWLEDGMENTS

M.J.F. was a postdoctoral fellow of the American Cancer Society (grant PG-588).

We wish to thank B. P. Doctor, in whose laboratory the early experiments were performed, for his continued interest in the work and for many stimulating discussions. We also wish to thank G. R. Kitchingman for technical assistance with some of these experiments.

LITERATURE CITED

1. Altman, S., and H. D. Robertson. 1973. RNA precursor molecules and ribonucleases in *E. coli*. Mol. Cell. Biochem. **1**:83-93.
2. Barrell, B. G., and B. F. C. Clark. 1974. Handbook of nucleic acid sequences. Joynson-Bruvvers Ltd., Oxford, England.
3. Capra, J. D., and A. Peterkofsky. 1968. Effect of *in vitro* methylation on the chromatographic and coding properties of methyl-deficient leucine transfer RNA. J. Mol. Biol. **38**:591-607.
4. Edlin, G., and O. Maaloe. 1966. Synthesis and breakdown of messenger RNA without protein synthesis. J. Mol. Biol. **15**:428-434.
5. Fleissner, E., and E. Borek. 1962. A new enzyme of RNA synthesis: RNA methylase. Proc. Natl. Acad. Sci. U.S.A. **48**:1199-1203.
6. Hall, R. H. 1971. The modified nucleosides in nucleic acids. Columbia University Press, New York.
7. Jacobson, M., and C. Hedgcoth. 1970. Levels of 5,6-dihydrouridine in relaxed and chloramphenicol transfer ribonucleic acid. Biochemistry **9**:2513-2519.
8. Lazzarini, R. A., and A. Peterkofsky. 1965. The characterization of a new species of leucyl-sRNA formed during methionine deprivation of *Escherichia coli* with relaxed control. Proc. Natl. Acad. Sci. U.S.A. **53**:549-556.
9. Littauer, U. Z., and H. Inouye. 1973. Regulation of tRNA. Annu. Rev. Biochem. **42**:439-470.
10. Loehr, J. S., and E. B. Keller. 1968. Dimers of alanine transfer RNA with acceptor activity. Proc. Natl. Acad.

Sci. U.S.A. **61**:1115–1122.
11. Mandel, L. R., and E. Borek. 1961. Variability in the structure of ribonucleic acid. Biochem. Biophys. Res. Commun. **4**:14–18.
12. Muench, K. H., and P. Berg. 1966. Preparation of aminoacyl ribonucleic acid synthetases from *Escherichia coli*, p. 375–383. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research, vol. 1. Harper and Row, New York.
13. Nirenberg, M. W. 1963. Cell-free protein synthesis directed by messenger RNA, p. 17–23. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 6. Academic Press Inc., New York.
14. Nishimura, S., and G. D. Novelli. 1965. Dissociation of amino acid acceptor function of sRNA from its transfer function. Proc. Natl. Acad. Sci. U.S.A. **53**:178–184.
15. Pearson, R. L., J. F. Weiss, and A. D. Kelmers. 1971. Improved separation of transfer RNAs on polychlorotrifluoroethylene-supported reversed-phase chromatography columns. Biochim. Biophys. Acta **228**:770–774.
16. Rubin, I. B., A. D. Kelmers, and G. Goldstein. 1967. The determination of transfer ribonucleic acid by aminoacylation. Anal. Biochem. **20**:533–544.
17. Ryan, A. M., and E. Borek. 1971. The relaxed control phenomenon, p. 193–228. *In* J. N. Davidson and W. E. Cohn (ed.), Progress in nucleic acid research and molecular biology, vol. 11. Academic Press Inc., New York.
18. Schweizer, M. P., G. B. Chheda, L. Baczynskyj, and R. H. Hall. 1969. Aminoacyl nucleosides. VII. (N-purin-6-ylcarbamoyl) threonine. A new component of transfer ribonucleic acid. Biochemistry **8**:3283–3289.
19. Söll, D. 1971. Enzymatic modification of transfer RNA. Science **173**:293–299.
20. Sueoka, N., and T. Kano-Sueoka. 1970. Transfer RNA and cell differentiation, p. 23–55. *In* J. N. Davidson and W. E. Cohn (ed.) Progress in nucleic acid research and molecular biology, vol. 10. Academic Press Inc., New York.
21. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithinase of *Escherichia coli:* partial purification and some properties. J. Biol. Chem. **218**:97–106.
22. Waters, L. C. 1969. Altered chromatographic properties of tRNA from chloramphenicol-treated *Escherichia coli*. Biochem. Biophys. Res. Commun. **37**:296–304.
23. Waters, L. C., L. Shugart, W. Yang, and A. N. Best. 1973. Some physical and biological properties of 4-thiouridine- and dihydrouridine-deficient tRNA from chloramphenicol-treated *Escherichia coli*. Arch. Biochem. Biophys. **156**:780–793.
24. Weiss, J. F., and A. D. Kelmers, 1967. A new chromatographic system for increased resolution of transfer ribonucleic acids. Biochemistry **6**:2507–2513.
25. Williams, L. S., and M. Freundlich. 1969. Role of valine transfer RNA in control of RNA synthesis in *Escherichia coli*. Biochim. Biophys. Acta **179**:515–517.
26. Yegian, C. D., and G. S. Stent. 1969. An unusual condition of leucine transfer RNA appearing during leucine starvation of *Escherichia coli*. J. Mol. Biol. **39**:45–58.
27. Zachau, H. G. 1969. Transfer ribonucleic acids. Angew. Chem. Int. Ed. Engl. **8**:711–727.