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# Precursor Relationship of Phenylalanine Transfer Ribonucleic Acid from *Escherichia coli* Treated with Chloramphenicol or Starved for Iron, Methionine, or Cysteine

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When treated with chloramphenicol, *Escherichia coli* 15T⁻ produces two new species (IV and V) of transfer ribonucleic acid specific for phenylalanine in addition to the major normal species (II) and two minor normal species (I and III), which are seen as distinct components upon fractionation by chromatography on columns of benzoylated diethylaminoethyl-cellulose. Species IV is produced when cells are grown in iron-deficient medium and is, therefore, probably deficient in the 2-methylthio modification of $N^6$-($\Delta^2$-isopentenyl)adenosine. A new minor species (Va) also appears under those conditions. All of the new components elute earlier than the major normal species. Addition of chloramphenicol to iron-deficient cells leads to the production of species V, and that production is blocked by rifampin, as is the production of species IV. Thus, species IV and V appear to be transcriptional products. Although *E. coli* 15T⁻ appears to be *rel*⁺, starvation for methionine or cysteine leads to the accumulation of species IV (without addition of chloramphenicol); rifampin blocks the accumulation. Species V is still produced on addition of chloramphenicol to starved cultures. Starvation for arginine or tryptophan does not alter the chromatographic profile from the normal case. Treatment with permanganate indicates that species II and IV contain isopentenyladenosine but that species V does not. Species V appears to be deficient in both isopentenyl and methylthio modifications of adenosine and perhaps at least one other modification, because removing the isopentenyl moiety from adenosine does not convert species IV into species V, but converts it into species Va. A precursor relationship among species V, IV, and II is suggested by following the chromatographic profile of phenylalanine transfer ribonucleic acid during recovery of *E. coli* from treatment with chloramphenicol; the various species increase and decrease in a sequential manner.

A single major species of phenylalanyl-transfer ribonucleic acid (Phe-tRNA) is observed upon chromatography of *Escherichia coli* tRNA in a reversed-phase system (16) and on benzoylated diethylaminoethyl-cellulose (BD-cellulose) (A. C. Skjold, Ph.D. thesis, Kansas State University, Manhattan, 1970). Treatment of *E. coli* with chloramphenicol (CP) causes the appearance of extra chromatographic peaks of tRNA specific for phenylalanine ($tRNA^{Phe}$) (16; Skjold, Ph.D. thesis). Waters (16) suggested that the isoaccepting species of $tRNA^{Phe}$ appearing in response to CP are intermediates in the maturation of tRNA. The inhibition of protein synthesis in *E. coli* by CP or by amino acid starvation of a *rel*⁻ strain leads to a decrease in the amount of dihydrouridine and 4-thiouridine in bulk tRNA synthesized under those conditions (8, 17). Additional species of $tRNA^{Phe}$ also are observed in bacteria grown with limited aeration (19) or at low iron concentrations (13, 20). Iron deficiency results in undermodification of the adenosine on the 3′ side of the anticodon in $tRNA^{Tyr}$ and probably other tRNA's responding to codons beginning with U. The missing modification is the 2-methylthio moiety of 2-methylthio-$N^6$-($\Delta^2$-isopentenyl)adenosine ($ms^2i^6A$) (13).

We have extended such observations by attempting to correlate the appearance of additional species of $tRNA^{Phe}$ in cells treated with CP with deficiencies in the formation of minor nucleosides. Correlations have been made with iron-deficient cells, cells starved for cysteine and methionine, and cells treated with CP. Of two additional, clearly defined species of

$tRNA^{Phe}$ from BD-cellulose chromatography, one appears to be deficient in the 2-methylthio modification; the other species appears to be deficient in that modification, in the isopentenyl modification, and in at least one other modification. Studies of $tRNA^{Phe}$ during recovery of *E. coli* from treatment with CP indicate a precursor relationship of the various peaks, in agreement with results recently reported by Waters et al. (17), and a possibly obligatory sequence of modification forming the fully modified $ms^2i^6A$.

## MATERIALS AND METHODS

**Bacterial growth conditions.** *E. coli* $15T^-$ (555-7), from K. G. Lark, was grown in M9-glucose medium (1) supplemented with $CaCl_2$ (15 μg/ml) and the following growth requirements (per milliliter): thymine, 4 μg; arginine, 34 μg; methionine, 30 μg; and tryptophan, 14 μg. *E. coli* $15T^-Cys^-$, isolated in this laboratory by Dolores Juarez, was similarly supplemented and, in addition, received cysteine (30 μg/ml). Conditions for starvation of cells for amino acids are indicated with the appropriate experiment. Experimental cultures were initiated with a 1% inoculum and grown to an absorbancy at 450 nm ($A_{450}$) of 0.1 (approximately $2.5 \times 10^8$ cells/ml).

Iron-free medium was prepared by extraction of solutions with chloroform-8-hydroxyquinolone (15). Distilled water, M9 salt solution, and a solution containing 15% glucose, 3 mM $CaCl_2$, and 30 mM $MgSO_4$ were extracted separately. When extracted solutions were used, $ZnSO_4$, $CuSO_4$, and $MnCl_2$ were added to media to give concentrations of $1.5 \times 10^{-7}$, $1.5 \times 10^{-7}$, and $2 \times 10^{-7}$ M, respectively. $FeCl_3$ was added as necessary to give a final concentration of $7.5 \times 10^{-6}$ M.

**Preparation of tRNA.** tRNA was extracted as described previously (8), with the inclusion of a step to remove amino acids from tRNA. After removal of ribosomal RNA by LiCl treatment and precipitation of tRNA, discharging of aminoacyl-tRNA was done in 0.5 ml of 1.8 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8) for 60 min at 37 C. The concentration of tRNA was calculated on the basis that 1 mg/ml gives an $A_{260}$ of 24 for a 1-cm light path.

**Preparation of aminoacyl-tRNA ligases.** Aminoacyl-tRNA ligases were prepared as described previously (14).

**Aminoacylation of tRNA.** The reaction mixture for charging tRNA with phenylalanine contained in 1 ml: Tris-hydrochloride (pH 7.4), 0.1 M; magnesium acetate, 20 mM; adenosine 5'-triphosphate, 2 mM; L-[$^{14}$C]phenylalanine (455 mCi/mmol; Schwarz/Mann), 2.5 μCi, or L-[$^3$H]phenylalanine (20 Ci/mmol; Schwarz/Mann), 12.5 μCi; 2-mercaptoethanol, 24 mM; 19 unlabeled 5 μM amino acids not including phenylalanine; tRNA, 100 μg; and 175 μg of protein from the aminoacyl-tRNA ligase preparation. Incubation was for 30 min at 37 C. At intervals, 10-μliter portions were transferred onto Whatman 3 MM disks (2.4 cm) which were dropped into ice-cold 10% trichloroacetic acid. Disks were prepared for the determination of radioactivity by the procedure of Barnett and Jacobson (2). In all cases, a maximal level of charging was reached in about 5 min. The reaction was stopped by the addition of 1 volume of water-saturated phenol. After centrifugation, the phenol phase was washed with 0.5 volume of 40 mM potassium acetate buffer (pH 5) containing 4 mM magnesium acetate; both aqueous phases were combined. After addition of 0.1 volume of 20% potassium acetate (pH 5) and 100 μg of tRNA as carrier, tRNA was precipitated by addition of 3 volumes of 95% ethanol. After two additional precipitations from acetate buffer, the precipitate was washed with ethanol and then with ethanol-ether (50:50), dried in vacuo, and dissolved in 0.3 ml of starting buffer for BD-cellulose chromatography.

**BD-cellulose chromatography.** BD-cellulose was prepared by the method of Gillam et al. (4). Before chromatography, the total amount of tRNA was adjusted to 3 mg with *E. coli* tRNA and was applied to a column (0.9 by 120 cm) of BD-cellulose equilibrated with starting buffer (10 mM sodium acetate buffer [pH 5] containing 0.65 M NaCl and 10 mM $MgCl_2$). Then, after addition of starting buffer (60 ml), a linear gradient (200 ml) from 0.65 M NaCl to 1.05 M NaCl and 12% methoxyethanol was started; the gradient was buffered at pH 5 with 10 mM sodium acetate containing 10 mM $MgCl_2$. A final wash consisted of 60 ml of acetate buffer containing 15% methoxyethanol, 1.5 M NaCl, and 10 mM $MgCl_2$. The eluate was monitored at 254 nm, and 2-ml fractions were collected. Flow rates were 0.5 to 1 ml/min. After addition of 100 μg of yeast RNA to each fraction, RNA was precipitated in cold 10% trichloroacetic acid. The precipitate was collected by filtration on glass-fiber disks (Whatman GF/C), washed once with 2 ml of cold 5% trichloroacetic acid, dried, and transferred to vials for the determination of radioactivity by scintillation spectroscopy.

**Permanganate treatment of tRNA.** tRNA was treated with $KMnO_4$ by the method of Kline et al. (9). A 1.0-mg amount of tRNA in 0.4 ml of water was treated for 15 min at 25 C with 0.25 ml of 0.01% $KMnO_4$. The remaining permanganate was destroyed by adding 20 μg of $Na_2SO_3$. tRNA was precipitated with 0.1 ml of a 1% solution of hexadecyltrimethylammonium bromide and was washed twice with 0.5 ml of 0.1 M sodium acetate (pH 5) in 70% ethanol, once with 95% ethanol, and twice with acetone. tRNA was dried in vacuo and dissolved in 0.5 ml of water.

## RESULTS

**CP Phe-tRNA.** The Phe-tRNA population of *E. coli* $15T^-$ starved for arginine and then treated with CP was determined with Phe-tRNA from exponential-phase cells as a control. A major species of Phe-tRNA (II) and two minor species (I and III) are observed in the control (Fig. 1). Two new species (IV and V) of Phe-tRNA appear after CP treatment. The new species are not artifacts: the CP Phe-tRNA profile is not affected upon exposure of CP

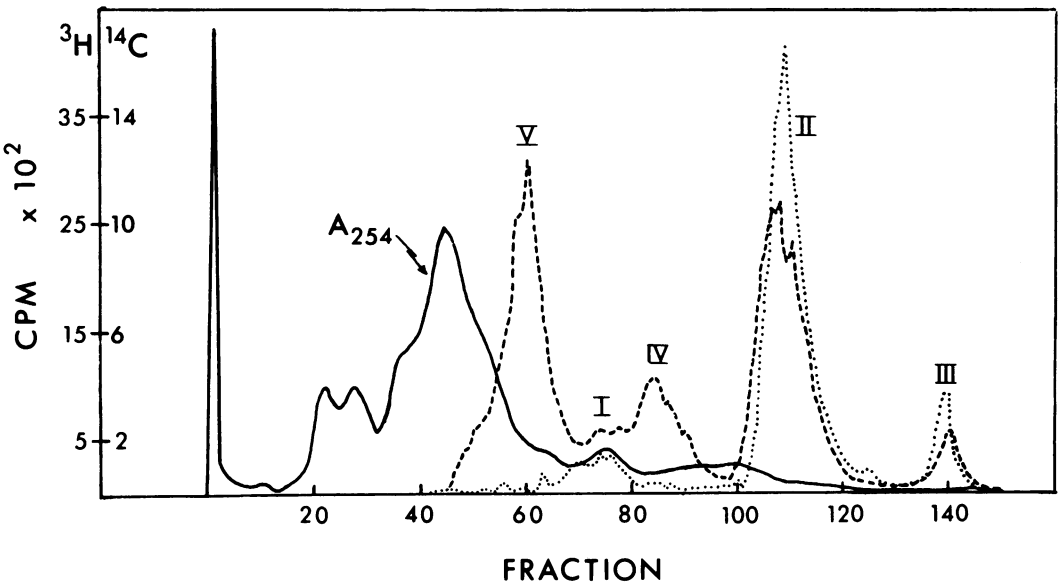


FIG. 1. *BD-cellulose chromatography of Phe-tRNA from E. coli 15T⁻ treated with CP. One culture was grown to an $A_{450}$ of 0.1 and harvested (----, [$^{14}C$]Phe-tRNA). A second culture was grown with only enough arginine (11 μg/ml) to reach an $A_{450}$ of 0.1, and after 3 h of starvation the cells were treated with CP (200 μg/ml) for 4 h before harvesting (· · · ·, [$^3H$]Phe-tRNA).*

tRNA to denaturing conditions (10), and when isolated and recharged they chromatograph as in the initial profile (data not shown). Also, *E. coli* strains B and K-12 similarly treated with CP have only a new species IV.

**Iron deficiency experiments.** The appearance of new species of Phe-tRNA in iron-deficient medium was observed by Wettstein and Stent (20). Rosenberg and Gefter (13) then showed that iron deficiency leads to the absence of the methylthio moiety from $ms^2i^6A$ of $tRNA^{Tyr}$ and to altered chromatographic profiles of tRNA's normally containing the modification. Iron-deficient growth conditions clearly produce a change in the chromatographic profile of Phe-tRNA from *E. coli* 15T⁻; species IV becomes the major species (as compared with species II as the major one in the control), and another new species, Va, eluting just after the position of species V, is observed (Fig. 2a). The accumulation of the large amount of species IV under iron-deficient conditions suggests that the 2-methylthio moiety is missing from $tRNA^{Phe}$ from CP-treated cells and iron-deficient cells. To determine whether iron-deficient cells could synthesize species V, we treated an iron-deficient culture with CP. Species V does accumulate under such conditions (Fig. 2b). The possibility that species V was a degradation product of species IV was explored by performing a similar experiment with rifampin added to the culture 15 min before CP addition. Species V is not produced under these conditions (Fig. 2b); therefore, it is probably not a degradation product. The results also suggest that both species IV and V are deficient in the methylthio modification.

**Methionine and cysteine starvation experiments.** The possibility that species IV and V are deficient in the methylthio modification prompted a study of the effects of starvation for cysteine and methionine on tRNA profiles. Cysteine is the sulfur donor (3) and methionine is the methyl donor (5) in the biosynthesis of the methylthio moiety. Cells starved for cysteine (Fig. 3a) or methionine (Fig. 3b) accumulate species IV. In both cases, adding rifampin at the beginning of starvation blocks the appearance of species IV; the data again support the view that the species is not a product of degradation. In contrast, tRNA from cells starved for arginine or tryptophan give Phe-tRNA profiles like the tRNA from exponential cells.

Adding CP to cultures starved for cysteine or methionine causes the appearance of species V (Fig. 3c). Since both starvations show about the same changes, the results again are compatible with the absence of the methylthio moiety from $tRNA^{Phe}$ in both species IV and V, although conceivably only the methyl group might be missing in the case of starvation for methionine.

**Permanganate treatment of tRNA.** Gentle oxidation of tRNA with $KMnO_4$ (with a molar ratio of tRNA/$KMnO_4$ of about 1) causes the specific cleavage of the isopentenyl moiety from $ms^2i^6A$ (9, 12). When tRNA from exponentially growing cells is treated with $KMnO_4$, species II shifts to the species I position (Fig. 4a). When tRNA, predominantly species IV, from iron-deficient cells is subjected to oxidation, species IV is eluted at the position corresponding to species Va (Fig. 4a). The results suggest that both species IV and II contain the isopentenyl group before treatment. In contrast, species V from CP tRNA appears resistant to $KMnO_4$ oxidation (Fig. 4b), at least as indicated by a lack of a shift in chromatographic position of the species. In addition to the deficiency in the methylthio modification, species V is probably deficient in the isopentenyl modification.

**Precursor relationship of species of CP Phe-tRNA.** The sum of the results suggests a precursor relationship of species of Phe-tRNA from CP-treated cells. To test the hypothesis of a precursor sequence of V to IV to II, *E. coli*

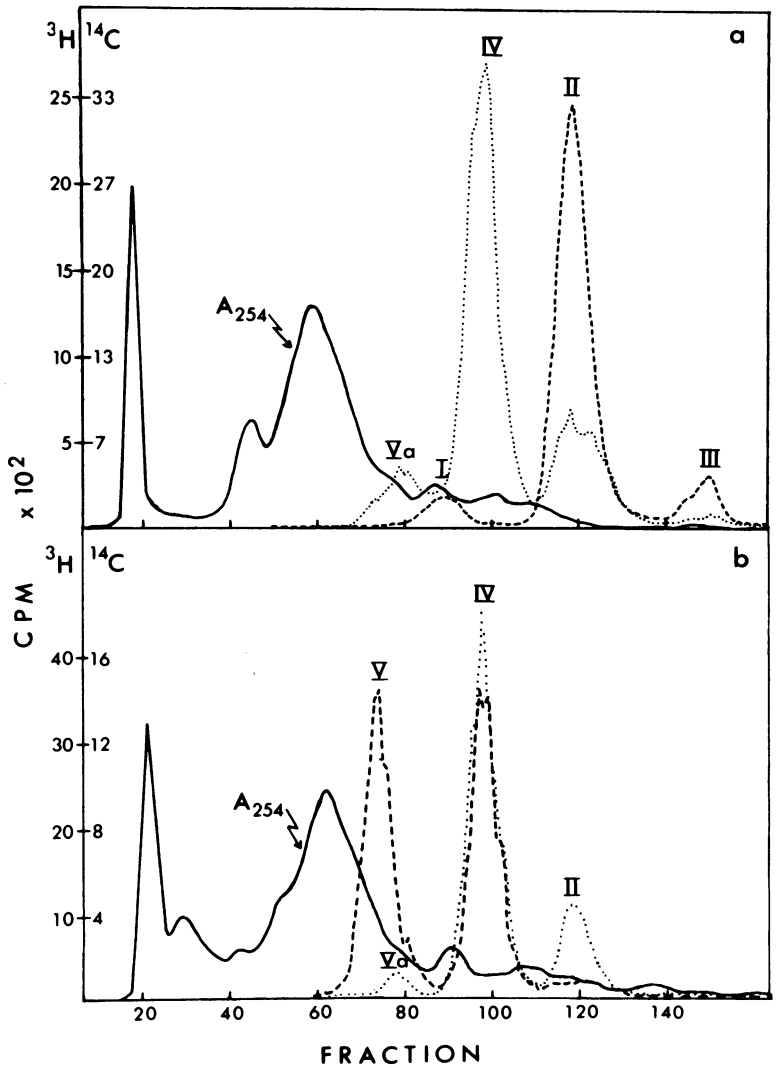


FIG. 2. *BD-cellulose chromatography of Phe-tRNA from E. coli 15T⁻ grown in iron-deficient medium with or without CP. (a) Cells were grown in iron-deficient medium (· · · ·, [³H]Phe-tRNA) or in the same medium supplemented with iron (----, [¹⁴C]Phe-tRNA) to an $A_{450}$ of 0.1. (b) Cells were grown in iron-deficient medium to an $A_{450}$ of 0.1, at which point one culture was treated with CP (200 μg/ml) for 2 h (----, [¹⁴C]Phe-tRNA), and a second culture was treated for 10 min with rifampin (150 μg/ml) and then with CP for 2 h (· · · ·, [³H]Phe-tRNA).*

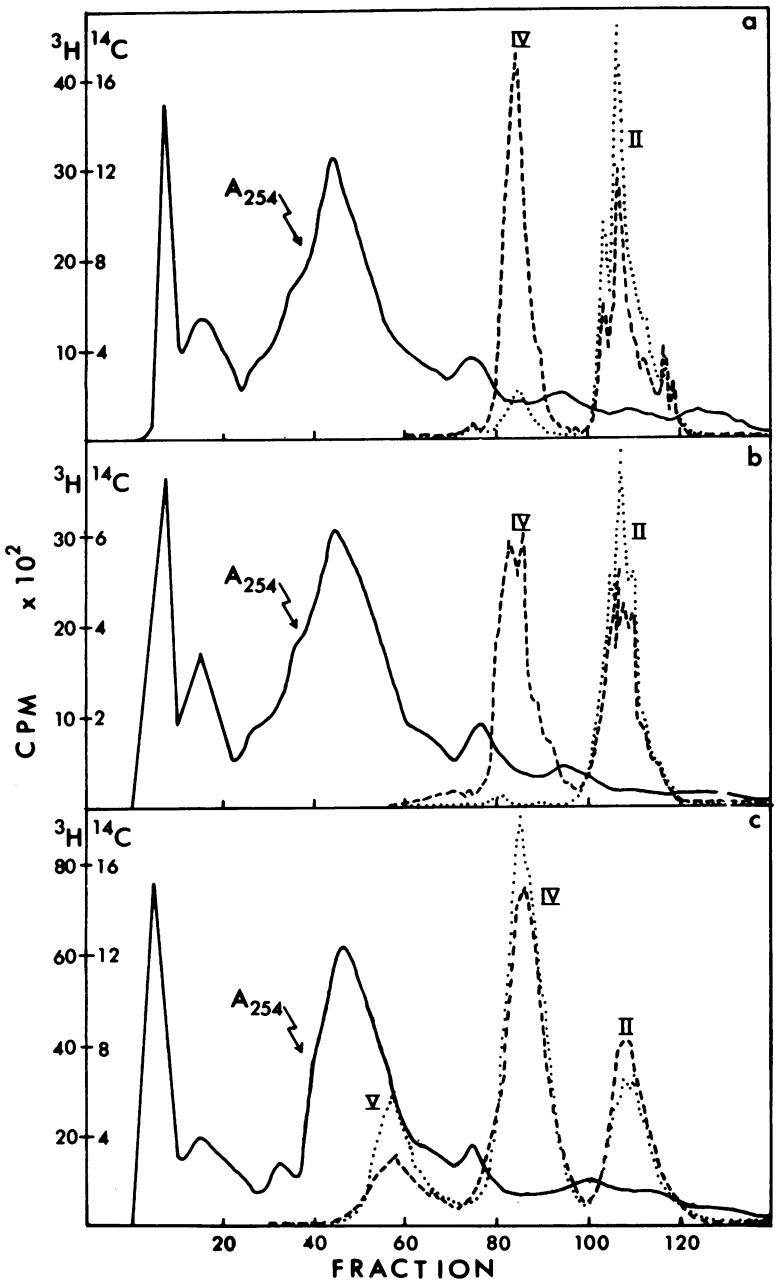


FIG. 3. *BD-cellose chromatography of Phe-tRNA from E. coli 15T⁻ starved for cysteine or methionine and treated with rifampin. (a) Two cultures of E. coli 15T⁻Cys⁻ were grown with only enough cysteine (2 μg/ml) to reach an $A_{450}$ of 0.1, at which point one culture received rifampin (150 μg/ml) (· · · ·, [³H]Phe-tRNA) and was harvested after 2.5 h, and the other culture was starved for 2.5 h (----, [¹⁴C]Phe-tRNA). (b) Two cultures of E. coli 15T⁻ were grown with only enough methionine (2 μg/ml) to reach an $A_{450}$ of 0.1, at which point one culture was treated with rifampin (150 μg/ml) for 2.5 h (· · · ·, [³H]Phe-tRNA), and the second culture was starved for 2.5 h (----, [¹⁴C]Phe-tRNA). (c) Cultures of E. coli 15T⁻ and E. coli 15T⁻Cys⁻ were starved as detailed above for 2.5 h for methionine (----, [³H]Phe-tRNA) and cysteine (· · · ·, [¹⁴C]Phe-tRNA), respectively, and then treated with CP (200 μg/ml) for 4 h.*

15T⁻ was starved for arginine and then treated with CP. The cells were allowed to recover from CP treatment after removal of CP, and at various intervals equal portions of recovering cells were taken from the main culture. One portion was harvested immediately; the other portion was incubated for 40 min with rifampin to inhibit further synthesis of RNA. The protocol provided a monitor of Phe-tRNA during RNA synthesis and, with rifampin, the processing of existing Phe-tRNA in the absence of new synthesis. Cells also were harvested immediately before and during CP treatment to permit observation of the appearance of the Phe-tRNA species. The growth curve and details of the protocol are illustrated in Fig. 5.

tRNA from cells collected immediately before addition of CP show insignificant amounts of species V and IV (Fig. 6). Species IV and V are apparent after 60 min of CP treatment, and species V continues to increase during the next 60 min, whereas species IV increases only

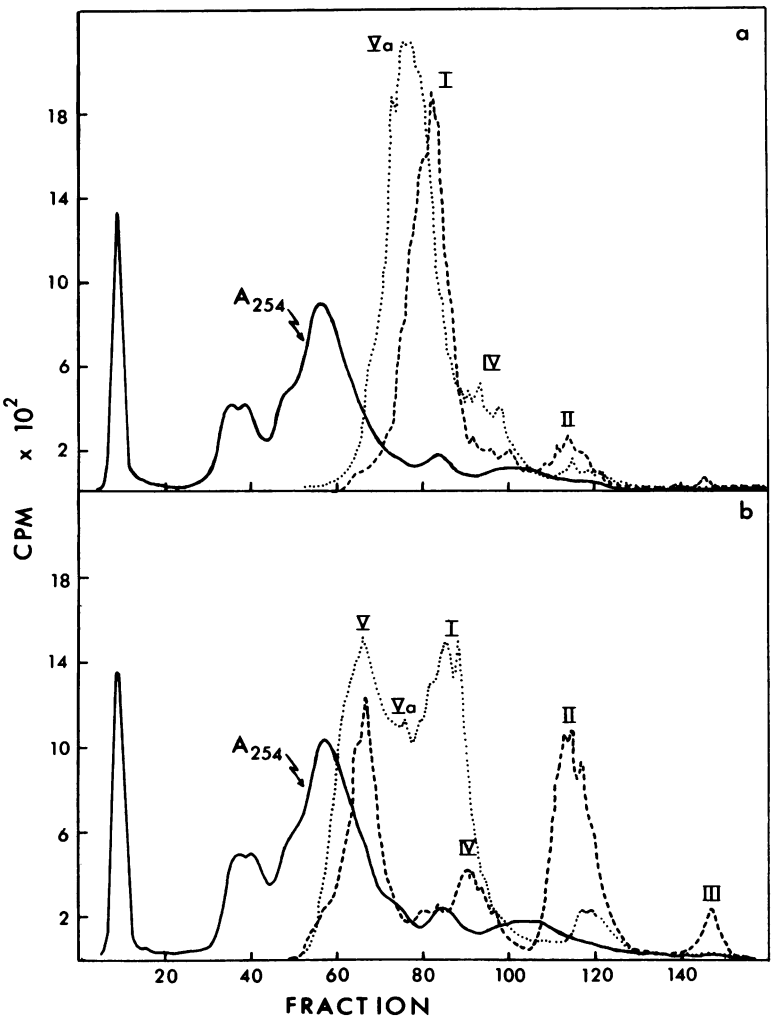


FIG. 4. *BD-cellulose chromatography of Phe-tRNA from permanganate-treated tRNA from cells grown in normal medium, iron-deficient medium, and arginine-deficient medium plus CP. (a) Permanganate-treated Phe-tRNA from cells grown in normal (----, [³H]Phe-tRNA) or iron-deficient media (· · · ·, [³H]Phe-tRNA). (b) Phe-tRNA from cells starved for arginine and treated with CP as in Fig. 1: permanganate treated (· · · ·, [¹⁴C]Phe-tRNA); nontreated (----, [¹⁴C]Phe-tRNA). Both graphs (a) and (b) are compilations of separate chromatographic experiments. Both tRNA's in (a) were cochromatographed, individually, with the arginine-starved, CP-treated tRNA of (b), which permitted marking of species. The permanganate-treated tRNA of (b) also was cochromatographed with the permanganate-treated, exponential tRNA of (a), which permitted matching corresponding species according to position of elution.*

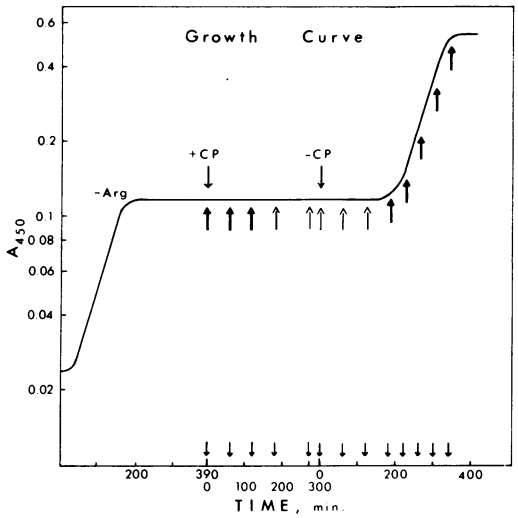


FIG. 5. *Recovery of E. coli 15T⁻ from treatment with CP. Cells were starved for arginine for 210 min after exhaustion of a limiting amount of arginine in the medium, treated with CP (200 μg/ml) for 260 min, washed free of CP by centrifugation, and suspended in complete medium without CP. (Washing and resuspension involved 40 min. The -CP arrow indicates the time of resuspension.) Cells resumed growing 180 min after resuspension and were monitored until the beginning of stationary phase. Samples (100 ml) were taken at the points indicated by the arrows below the curve. Arrows above the curve represent the points of addition and withdrawal of CP. After suspension of the cells in medium without CP, duplicate samples (100 ml) were taken at all subsequent points. One of the duplicate samples was harvested immediately; the other was treated with rifampin and harvested when the next set of duplicate samples was obtained. The thin arrows represent points at which no change in the profile of Phe-tRNA occurred with respect to the 120-min CP point. For clarity, the arrows above the abscissa indicate actual times at which each sample was taken.*

slightly (Fig. 6). The greater amount of species V accumulating after 60 min probably results from a reduction in the conversion of V to IV. After 120 min of CP treatment, the relative amounts of the species show no further significant change until 180 min after resuspension of the cells in medium without CP.

Changes in the Phe-tRNA profile occur around 180 min into the recovery process, which is also about the time of reinitiation of growth. It is clear from the Phe-tRNA profiles of portions of the recovery culture not treated with rifampin (Fig. 7a, c, e, g, and i) that species V decreases and species IV increases with increasing time. By 300 min of recovery time, species V is no longer found, and species II is slightly larger than species IV (Fig. 7g). After an additional 40 min, species II predominates (Fig. 7i). The results are consistent with a precursor relationship of the species. However, the following two factors need to be considered to satisfactorily explain the data. (i) A considerable amount of $tRNA^{Phe}$ must be synthesized during the recovery period. (ii) The enzymatic activities responsible for the conversion of one species into another are not recovered coordinately. Specifically, the accumulation of species IV (Fig. 7c) is not accompanied by a corresponding decrease in the amount of species V. Thus, species IV could accumulate for two reasons: new $tRNA^{Phe}$ is being transcribed, and the pathway from species V to species IV is more efficient than the pathway from IV to II.

Profiles from portions of the recovering culture treated with rifampin indicate changes occurring between sequential recovery points when RNA synthesis is inhibited. At 180 min of recovery time, the capacity to convert species V into IV is similar to the capacity to convert species IV into II (Fig. 7a and b). Thus, synthesis of new tRNA and an uncoordinated enzymatic recovery in the next 40 min would explain the disproportion in the peaks observed in Fig. 7c.

Data from the next rifampin point (Fig. 7d), treatment for 40 min with rifampin at 220 min of recovery time, confirm that at 220 min sufficient enzymatic activity is present to convert species V into IV, but the pathway on to species II still not functioning as efficiently. Similarities of profiles from recovery points with the corresponding profiles from rifampin-treated recovery points (Fig. 7d and e; f and g; h and i) suggest that the cells are able to process the greater amount of tRNA synthesized during recovery because of the concomitant recovery of enzymatic activity in generally proportionate amounts. In the period from 260 to 300 min, there is a significant increase in the capacity to convert species IV to II (Fig. 7f and g), but the capacity apparently never reaches a completely normal level since some species IV persist in the recovering culture, whereas inhibition of RNA synthesis by rifampin permits almost complete conversion of IV to II (Fig. 7h and i). It should be noted that the cells are in a late exponential phase of growth by 340 min.

Data from recovery cultures and the rifampin-treated cultures are consistent with the concept that changes observed in the proportion of the species are the result of the transformation of one species into another and not the result of a separate transcription of each spe-

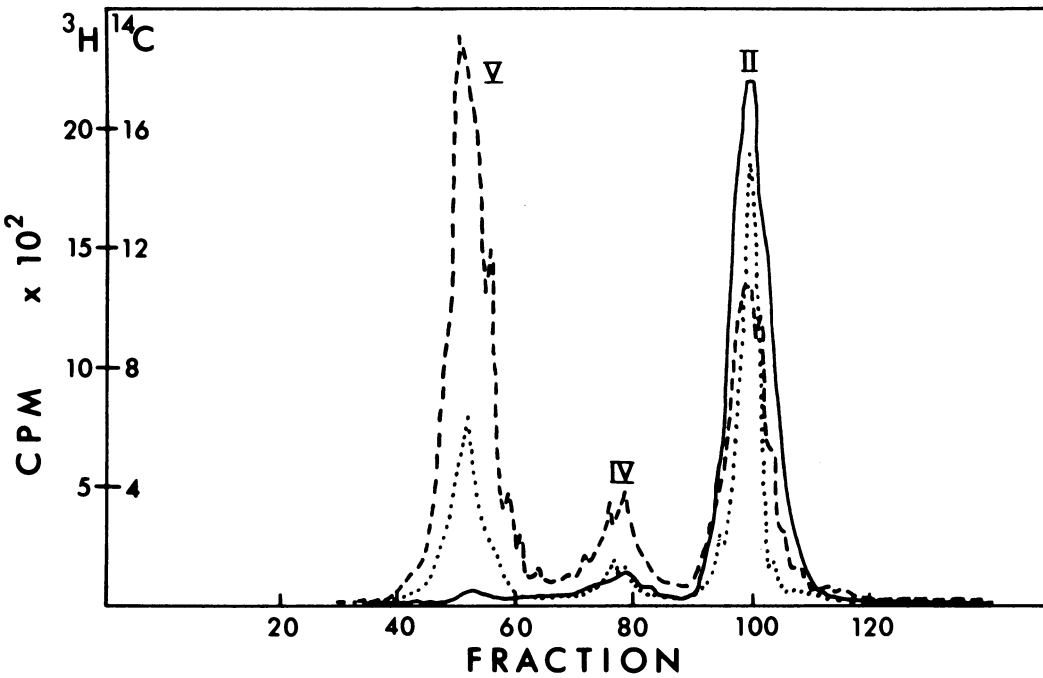


FIG. 6. *BD-cellulose chromatography of Phe-tRNA from cells starved for arginine for 210 min or starved for arginine for 210 min followed by CP treatment as described in the legend to Fig. 5. This is a composite from chromatograms run with suitable controls. Starvation (——, [³H]Phe-tRNA); starvation and 60 min of CP treatment (· · · ·, [¹⁴C]Phe-tRNA); starvation and 120 min of CP treatment (----, [³H]Phe-tRNA).*

cies. The sequential nature of changes in the amounts of each species can be seen in Fig. 8 in which the quantitative relationship is outlined.

## DISCUSSION

tRNA from exponentially growing *E. coli* 15T⁻ has three isoaccepting species of $tRNA^{Phe}$, I, II, and III, as shown by chromatography on BD-cellulose. Species II represents 90% or more of the $tRNA^{Phe}$ population; I and II are not always seen. Treating cells with CP after 3 h of starvation for arginine causes the appearance of the two new species of $tRNA^{Phe}$, IV and V. Cells were starved for arginine prior to treatment with CP to enhance any possible effects resulting from inactivation of unstable modification enzymes that would not be resynthesized.

The new species of $tRNA^{Phe}$ could arise in cells subjected to metabolic stress by three different mechanisms. (i) The new species and species II could be derivatives of a single transcription product with different degrees of modification. (ii) Each of the major species observed, II, IV, and V, could be distinct transcription products. (iii) IV and V could be products, e.g., nicked molecules, of the action of nuclease. The last possibility is excluded because exposing CP tRNA to denaturing conditions does not affect chromatographic properties or acceptor capacities of $tRNA^{Phe}$ species and because the appearance of IV and V is blocked by rifampin, an inhibitor of the initiation step in RNA synthesis. Data reported in this paper are consistent with species II, IV, and V as $tRNA^{Phe}$ species derived from a common transcription product.

Species II from exponential cultures is assumed to be functional, fully modified $tRNA^{Phe}$, and when treated with $KMnO_4$ it chromatographs in the position of species I. If species I and oxidized species II are identical structurally as well as chromatographically, species I would be missing only the isopentenyl modification, and the occasional presence of small amounts of species I could mean that the addition of the isopentenyl moiety forming $ms^2i^6A$ is rate limiting in the processing of $tRNA^{Phe}$, especially if the normal or major route is the addition of the isopentenyl moiety followed by thiolation and methylation. It should also be noted that permanganate oxidation does not quantitatively remove the isopentenyl moiety (9, 12).

Most of the Phe-tRNA from cells grown in iron-deficient medium chromatographs in the position of species IV; therefore, species IV is

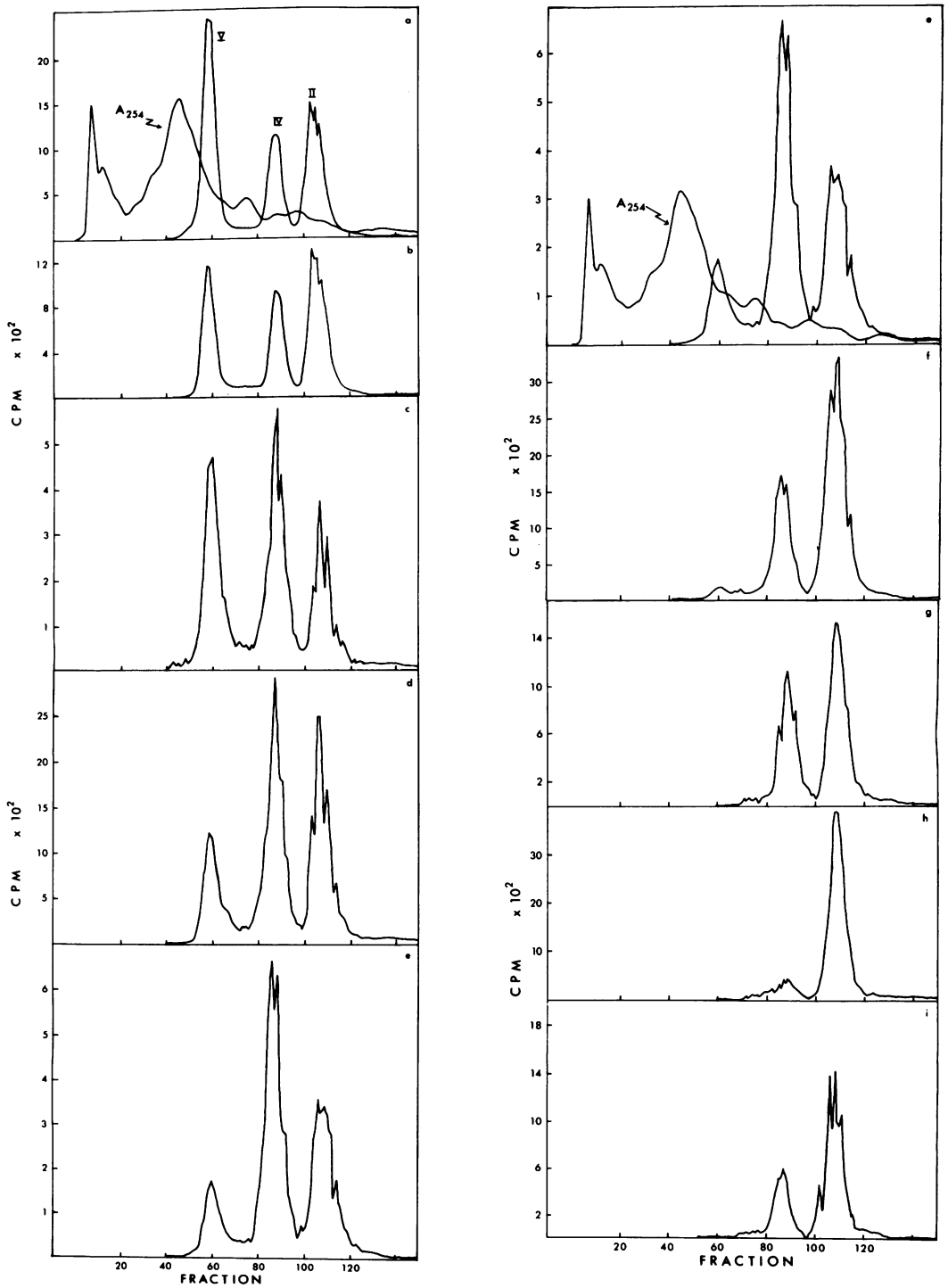


FIG. 7. *BD-cellulose chromatography of Phe-tRNA from cells recovering from CP treatment. The profiles represent samples taken every 40 min after the cells resumed growth as described in the legend to Fig. 5. (a) 180 min of recovery, [$^{14}C$]Phe-tRNA; (b) 180 min of recovery and then 40 min of rifampin treatment, [$^{3}H$]Phe-tRNA; (c) 220 min of recovery, [$^{14}C$]Phe-tRNA; (d) 220 min of recovery and then 40 min of rifampin treatment, [$^{3}H$]Phe-tRNA; (e) 260 min of recovery, [$^{3}H$]Phe-tRNA; (f) 260 min of recovery and then 40 min of rifampin treatment, [$^{3}H$]Phe-tRNA; (g) 300 min of recovery, [$^{14}C$]Phe-tRNA; (h) 300 min of recovery and then 40 min of rifampin treatment, [$^{3}H$]Phe-tRNA; (i) 340 min of recovery, [$^{14}C$]Phe-tRNA.*

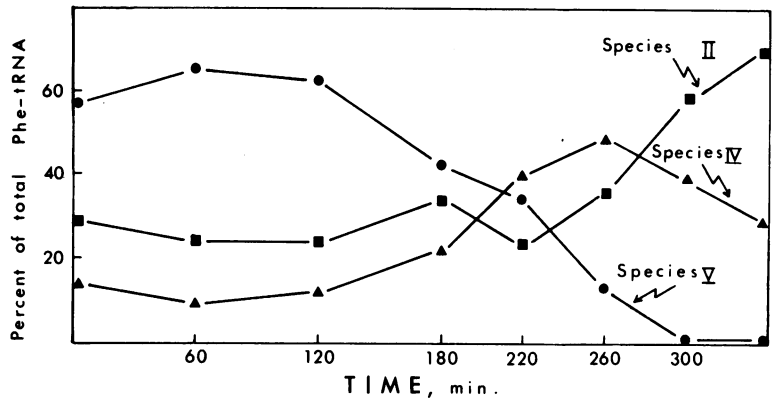


FIG. 8. *Distribution of species II, IV, and V throughout the recovery period. Points represent the percentage of Phe-tRNA found as species II, IV, and V after BD-cellulose chromatography of tRNA from cells harvested at the time indicated during recovery from CP treatment.*

probably deficient in the 2-methylthio modification. Further support for that possibility is based on the accumulation of species IV when cells are starved for cysteine or methionine. Also, it was reported that CP tRNA (*E. coli* B) has less than the normal amount of $ms^2i^6A$ which apparently is replaced by $i^6A$ [$N^6$-($\Delta^2$-isopentenyl)adenosine] (17). tRNA from methionine-starved *E. coli* (*rel*$^-$) has appreciable quantities of $i^6A$ and possibly $s^2i^6A$ (2-thio-$N^6$-($\Delta^2$-isopentenyl)adenosine) but only a small amount of the fully modified nucleoside ( P. F. Agris, D. J. Armstrong, K. P. Schäfer, and D. Söll, manuscript in preparation). Independent observations on $tRNA^{Phe}$ from methionine-starved *E. coli* (*rel*$^-$) also indicate the absence of $s^2i^6A$ and the presence of $i^6A$ (7). Therefore, either thiolation is not a stable modification without subsequent methylation, or thiolation depends on the potential for methylation, so that species IV appears in the same chromatographic position for tRNA from cysteine- or methionine-starved cells.

Although *E. coli* 15T$^-$ appears to be a *rel*$^+$ strain (Hedgcoth, unpublished data), the small amount of continuing RNA synthesis (less than 10% of the exponential rate) when cells are starved for cysteine or methionine for 2.5 h includes the synthesis of appreciable amounts of $tRNA^{Phe}$ species IV (Fig. 3). When starved for arginine or tryptophan, *E. coli* 15T$^-$ either does not synthesize as much $tRNA^{Phe}$ as when starved for cysteine or methionine or, more likely, full modification forming the 2-methylthio moiety of the modified adenosine occurs so that only species II is seen.

Species V appears only when protein synthesis is inhibited by CP, and it seems to be the least modified of the observed $tRNA^{Phe}$ species on the basis of several observations. (i) *E. coli* 15T$^-$ has the capacity for synthesizing or accumulating species V even when only species IV and II are observed, since subsequently treating cells with CP causes species V to accumulate. Such accumulation after inhibiting protein synthesis is thought to result from a lack of some modification that was taking place prior to inhibition. Also, since species V accumulates after adding CP to an iron-deficient culture and to cysteine- or methionine-starved cultures, it must be deficient in the methylthio moiety. (ii) Species V is probably deficient in the isopentenyl moiety since oxidation by $KMnO_4$ causes no change in the chromatographic profile. (iii) Species V elutes earlier than species Va, which is produced along with species IV under iron-deficient conditions and by oxidation of species IV with $KMnO_4$; species Va, therefore, is probably deficient in the methylthio and isopentenyl modifications, and species V is deficient in those modifications and at least one additional modification to give it chromatographic distinction from species Va. Species Va could be an intermediate in the conversion of species V to IV. (iv) Data from the experiment monitoring $tRNA^{Phe}$ profiles during recovery from CP treatment are consistent with species V as a precursor of IV.

While methylthio and isopentenyl modifications appear to influence strongly the chromatographic behavior of $tRNA^{Phe}$ and thus are modifications more easily studied indirectly, other modifications could also be missing in $tRNA^{Phe}$ from metabolically stressed cells. Results from this laboratory (8) and Waters et al. (17) indicate that *E. coli*, in which RNA synthesis proceeds while protein synthesis is inhibited either by chloramphenicol or by star-

vation or a *rel*⁻ strain for a required amino acid, produces tRNA deficient in dihydrouridine and 4-thiouridine. It should be noted that the degree of undermodification of tRNA from inhibited cells differs with different strains. We have not observed species V in strains other than 15T⁻ (Juarez and Hedgcoth, unpublished data); when strains B and K-12 are inhibited by CP, species IV accumulates without species V appearing.

There are two possible pathways for the conversion of the methylthio- and isopentenyl-deficient peak V $tRNA^{Phe}$ to the fully modified species II; (A37 represents nucleotide 37, an adenylate residue, in the primary sequence of $tRNA^{Phe}$)

(1) A37(species V) → $i^6$A37(species IV) → $s^2i^6$A37 → $ms^2i^6$A37(species II)

(2) A37(species V) → $s^2$A37 → $ms^2$A37(species I?) → $ms^2i^6$A37(species II)

The first pathway was suggested by Harada et al. (6) and is supported by our data from the recovery experiment. No intermediates are observed between species IV and V or between II and IV even after 220 min of recovery, at which point species II visibly accumulates and thiolation and methylation must be occurring. Therefore, pathway (1) is not only functional but is possibly a preferred pathway in normal cells.

After the work reported here was completed, Mann and Huang (11) proposed an equivalent scheme based on their observations of $tRNA^{Phe}$ from CP-treated *E. coli*. Our work complements and extends their work by correlating various observations on iron-deficient cells, cells starved for cysteine and methionine, and cells treated with chloramphenicol.

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## LITERATURE CITED

1. Anderson, E. H. 1946. Growth requirements of virus-resistant mutants of *Escherichia coli* strain "B." Proc. Nat. Acad. Sci. U.S.A. **32**:120-128.
2. Barnett, W. E., and K. B. Jacobson. 1964. Evidence for degeneracy and ambiguity in interspecies aminoacyl-sRNA formation. Proc. Nat. Acad. Sci. U.S.A. **51**:642-647.
3. Gefter, M. L. 1969. The *in vitro* synthesis of 2'-O-methylguanosine and 2-methylthio-$N^6$-($\gamma$,$\gamma$-dimethylallyl)adenosine in transfer RNA of *Escherichia coli*. Biochem. Biophys. Res. Commun. **36**:435-441.
4. Gillam, I., S. Millward, D. Blew, M. von Tigerstrom, E. Wimmer, and G. M. Tener. 1967. The separation of soluble ribonucleic acids on benzoylated diethylaminoethylcellulose. Biochemistry **6**:3043-3056.
5. Goodman, H. M., J. Abelson, A. Landy, S. Brenner, and J. D. Smith. 1968. Amber suppression: a nucleotide change in the anticodon of a tyrosine transfer RNA. Nature (London) **217**:1019-1024.
6. Harada, F., H. J. Gross, F. Kimura, S. H. Chang, S. Nishimura, and U. L. RajBhandary. 1968. 2-Methylthio-$N^6$-($\Delta^2$-isopentenyl)adenosine: a component of *E. coli* tyrosine transfer RNA. Biochem. Biophys. Res. Commun. **33**:299-306.
7. Isham, K. R., and M. P. Stulberg. 1974. Modified nucleosides in undermethylated phenylalanine transfer RNA from *Escherichia coli*. Biochim. Biophys. Acta **340**:177-182.
8. Jacobson, M., and C. Hedgcoth. 1970. Levels of 5,6-dihydrouridine in relaxed and chloramphenicol transfer ribonucleic acid. Biochemistry **9**:2513-2519.
9. Kline, L. K., F. Fittler, and R. H. Hall. 1969. $N^6$-($\Delta^2$-isopentenyl)adenosine. Biosynthesis in transfer ribonucleic acid *in vitro*. Biochemistry **8**:4361-4371.
10. Lindahl, T., A. Adams, and J. R. Fresco. 1966. Renaturation of transfer ribonucleic acids through site binding of magnesium. Proc. Nat. Acad. Sci. U.S.A. **55**:941-948.
11. Mann, M. B., and P. C. Huang. 1973. Behavior of chloramphenicol-induced phenylalanine transfer ribonucleic acid during recovery from chloramphenicol treatment in *Escherichia coli*. Biochemistry **12**:5289-5294.
12. Robins, M. J., R. H. Hall, and R. Thedford. 1967. $N^6$-($\Delta^2$-isopentenyl)adenosine. A component of the transfer ribonucleic acid of yeast and of mammalian tissue, methods of isolation, and characterization. Biochemistry **6**:1837-1848.
13. Rosenberg, A. H., and M. L. Gefter. 1969. An iron-dependent modification of several transfer RNA species in *Escherichia coli*. J. Mol. Biol. **46**:581-584.
14. Skjold, A. C., H. Juarez, and C. Hedgcoth. 1973. Relationships among deoxyribonucleic acid, ribonucleic acid, and specific transfer ribonucleic acids in *Escherichia coli* 15T⁻ at various growth rates. J. Bacteriol. **115**:177-187.
15. Waring, W. S., and C. H. Werkman. 1942. Growth of bacteria in an iron-free medium. Arch. Biochem. **1**:303-310.
16. Waters, L. C. 1969. Altered chromatographic properties of tRNA from chloramphenicol-treated *Escherichia coli*. Biochem. Biophys. Res. Commun. **37**:296-304.
17. Waters, L. C., L. Shugart, W. K. 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.
18. Weiss, J. F., and A. D. Kelmers. 1967. A new chromatographic system for increased resolution of transfer ribonucleic acids. Biochemistry **6**:2507-2513.
19. Wettstein, F. O. 1966. Differential *in vivo* aminoacylation and utilization of homologous species of *E. coli* transfer RNA. Cold Spring Harbor Symp. Quant. Biol. **31**:595-599.
20. Wettstein, F. O., and G. S. Stent. 1968. Physiologically induced changes in the property of phenylalanine tRNA in *Escherichia coli*. J. Mol. Biol. **38**:25-40.