Fractionation of Transfer Ribonucleic Acid on a Methylated Albumin–Silicic Acid Column. I. Preparation of the Column*

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ABSTRACT: A method is described for the fractionation of transfer ribonucleic acid of *Escherichia coli* on a methylated albumin–silicic acid column. The column has a 100-fold greater adsorptive capacity and a higher resolution for transfer ribonucleic acid than a methylated albumin kieselguhr column of the same dimensions. In order to achieve separation, a lower limit as well as an upper limit is observed for the amount of transfer ribonucleic acid that can be placed on the column. Additional conditions are presented for ensuring optimal and reproducible separation. Two species of phenylalanyl transfer ribonucleic acid are resolved on chromatography of normal *E. coli* transfer ribonucleic acid. Four species of phenylalanyl transfer ribonucleic acid can be resolved on chromatography of transfer ribonucleic acid extracted from a relaxed methionine-requiring mutant of *E. coli* grown in a medium deficient in methionine. The elution profile of lysyl, valyl, and methionyl transfer ribonucleic acid is also presented.

N-Carbobenzyloxyphenylalanyl transfer ribonucleic acid elutes from the methylated albumin–silicic acid column at a considerably higher salt concentration than phenylalanyl transfer ribonucleic acid or any of the other transfer ribonucleic acid chains. This property can be used for the purification of phenylalanine transfer ribonucleic acid and other transfer ribonucleic acids. Upon elution from the methylated albumin–silicic acid column the *N*-carbobenzyloxyphenylalanyl transfer ribonucleic acid is digested with Pronase to liberate free phenylalanine transfer ribonucleic acid. Phenylalanine acceptance measurements show that the liberated phenylalanine transfer ribonucleic acid is about 50% pure.

The MAK[1] column developed by Mandell and Hershey (1960) and Sueoka and Yamane (1962) is one of few techniques available for the rapid chromatography of nucleic acids. A major drawback of the column is its limited capacity. This disadvantage is particularly pronounced in the case of the fractionation of aminoacyl-tRNA, since only a small fraction of the total RNA is specific for any given amino acid. The necessity to develop a rapid and simple fractionation procedure for the resolution of aminoacyl-tRNAs prompted us to further develop the MASA column suggested by Okamoto and Kawade (1963) and to adopt it specifically for tRNA chromatography. The preparation of the column and some observations on the chromatographic properties of normal and methyl-deficient[2] aminoacyl-tRNA are presented in this communication. A preliminary report of these observations has been made (Littauer *et al.*, 1966).

Materials and Methods

Chemicals. Silicic acid (325 mesh) and ammonium sulfate were obtained from the Fischer Scientific Co. Kieselguhr was the product of British Drug House, Ltd.; labeled amino acids were obtained from Schwarz Bio-Research, Inc.; specific activities were 1.5 and 0.32 mCi/μmole for L-[^{3}H]- and L-[^{14}C]phenylalanine, 0.20 and 0.208 mCi/μmole for L-[^{3}H]- and L-[^{14}C]lysine, 0.15 and 0.027 mCi/μmole for L-[^{3}H]- and L-[^{14}C]methionine, 0.29 and 0.185 mCi/μmole for L-[^{3}H]- and L-[^{14}C]valine. [^{14}C]Amino acid mixture was purchased from New England Nuclear Corp. and contained a mixture of 15 L-amino acids at approximately 40 mCi/matom of carbon. One millicurie of this mixture contained: alanine (80 μCi), arginine (70 μCi), aspartic acid (80 μCi), glutamic acid (125 μCi), glycine (40 μCi), histidine (15 μCi), isoleucine (50 μCi), leucine (140 μCi), lysine (60 μCi), phenylalanine (80 μCi), proline (50 μCi), serine (40 μCi), threonine (50 μCi), tyrosine (40 μCi), and valine (80 μCi). Streptomycin sulfate, GSH, and unlabeled amino acids were products of the California Foundation for Biochemical Research. Analytical grade phenol was purchased from Mallinckrodt Laboratories and used without redistillation. Superbrite glass beads (200-μ average diameter) were obtained from the Minnesota Mining and Manufacturing Co. ATP, as the crystalline sodium salt, was purchased from Sigma Chemical Co. Mem-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: MAK, methylated albumin kieselguhr; MASA, methylated albumin–silicic acid.

[2] In this paper methionine-starved tRNA refers to the tRNA extracted from a relaxed methionine-requiring mutant of *E. coli* (A-19, K_{12}W6 or G-15) grown in a medium deficient in methionine. Methyl-deficient tRNA refers only to those species which contain few or no methyl groups. Methionine-starved tRNA is an approximately equal mixture of methyl-deficient tRNA and fully methylated normal tRNA.

brane filters (MF 50) with pore size 0.6 μ were purchased from Membranfiltergesellschaft, Göttingen. Levigated alumina was purchased from the Norton Co. Electrophoretically purified DNase was obtained from Worthington Biochemical Corp.

Growth of Escherichia coli Cells and Preparation of tRNA. *E. coli* MRE 600 was obtained from Dr. H. E. Wade. *E. coli* A-19 RC^{rel} RNase 1^- Met^- was obtained from Dr. S. Spiegelman. *E. coli* $K_{12}W6RC^{rel}$ Met^- was obtained from Dr. Esther Lederberg and *E. coli* G-15 RC^{rel} Met^- His^- biotin$^-$ from Dr. G. Stent. Normal cultures were grown in 12 l. of minimal medium (Hershey and Chase, 1952) containing 0.01 M phosphate supplemented with 15 μg/ml of L-methionine at 37° with forced aeration. Cells were harvested in the middle of the logarithmic growth phase and the tRNA isolated immediately, according to a modification (Katchalski *et al.*, 1966) of the procedure of Zubay (1962). The tRNA was then incubated at 37° in Tris-HCl (pH 8.8, 0.2 M) for 5 hr. Methionine-starved cultures were grown in a medium supplemented with 4 μg/ml of L-methionine. After growth had stopped, the culture was further incubated with continued aeration for an additional 3.5 hr. Starved cultures yielded 50–100% more tRNA than normal cultures.

E. coli strain B tRNA was obtained from Schwarz BioResearch, Inc.

Total *E. coli* RNA was the kind gift of Mr. Meir Perl and prepared by the method of Hayashi and Spiegelman (1961) as modified by Littauer *et al.* (1964a).

Preparation of Phenylalanyl-tRNA Synthetase. A partially purified phenylalanyl-tRNA synthetase was prepared by grinding 10 g of *E. coli* A-19 cells with 25 g of alumina and extracting the paste with 30 ml of 0.01 M Tris-HCl buffer (pH 7.8) containing 0.01 M $MgCl_2$ and 1 μg/ml of DNase. The suspension was centrifuged 15 min at 15,000*g* and the supernatant was centrifuged for 4 hr at 105,000*g*. To 15 ml of the upper two-thirds of the resulting supernatant, 1.5 ml of 10% streptomycin sulfate solution (pH 7.0) was added. This was stirred for 20 min at 4° and centrifuged for 10 min at 15,000*g*, and the pellet was discarded. This material, called streptomycin supernatant, when used as a source of crude phenylalanyl-tRNA synthetase activity, was dialyzed overnight against 0.01 M Tris-HCl (pH 7.4) containing 0.01 M $MgCl_2$ and stored in liquid air.

To the streptomycin supernatant, 0.43 volume of saturated ammonium sulfate solution was added slowly, with stirring, at 4°. Stirring was continued for 1 hr. The solution was centrifuged for 30 min at 12,000*g* and the pellet discarded. Saturated ammonium sulfate solution (0.4 volume) was added, stirred for 1 hr, and centrifuged for 30 min at 12,000*g*; the resulting pellet was dissolved in 3 ml of 0.5 M sodium phosphate buffer (pH 7.4). To this solution were added: 0.2 volume of 0.1 M ATP, 0.2 volume of 0.1 M L-phenylalanine, 0.14 volume of 0.05 M potassium fluoride, 0.06 volume of 0.5 M dibasic sodium phosphate, and 0.04 volume of 0.5 M $MgCl_2$. This mixture was incubated at 55° for 45 min, then cooled to 4°, and centrifuged for 10 min at 15,000*g*, and the pellet was discarded. To the supernatant, 1.6 volumes of saturated ammonium sulfate solution was slowly added, stirred for 1 hr in the cold, and centrifuged for 30 min at 12,000*g*, and the pellet was dissolved in 2 ml of 0.01 M Tris-HCl (pH 7.4). The solution was then passed through a Sephadex G-50 column (2 × 20 cm) and eluted with 0.01 M Tris-HCl buffer (pH 7.4). The enzyme preparations were pooled from the tubes containing the peak of optical density at 280 mμ and neutralized GSH was added to a final concentration of 0.01 M, distributed among a number of small test tubes, and stored in liquid air for several months.

The final protein concentration was 5–6 mg/ml and the ratio of A_{280}: A_{260} was 1.5–1.65. Two μg of this preparation supplied excess enzyme activity to the standard reaction mixture.

Preparation of Methionyl-tRNA Synthetase. For the preparation of methionyl-tRNA synthetase, 30 g of *E. coli* A-19 was ground with 75 g of alumina and extracted with 90 ml of 0.1 M potassium phosphate buffer (pH 7.0), containing 0.01 M β-mercaptoethanol. The suspension was centrifuged for 25 min at 15,000*g* and the precipitate was discarded. The supernatant fluid was incubated for 4 hr at 37° and then cooled. To 70 ml of this solution, 0.7 ml of 0.1 M EDTA and 14.7 g of solid ammonium sulfate were added. After 10 min the suspension was centrifuged and the precipitate was discarded. To the supernatant 4.4 g of additional ammonium sulfate was added. The precipitate was collected by centrifugation, dissolved immediately in 10 ml of 0.02 M potassium phosphate buffer (pH 7.5) containing 0.01 M β-mercaptoethanol, and dialyzed overnight against the same buffer.

The dialyzed enzyme solution was adsorbed to a DEAE-cellulose column (2 × 8 cm) which had previously been equilibrated with 0.02 M phosphate buffer (pH 7.5). The column was washed with the same buffer until the optical density at 280 mμ fell below 0.002. The enzyme was eluted from the column with 0.10 M potassium phosphate buffer (pH 7.0). Solid ammonium sulfate was added to 60% saturation at 0°. The precipitate was collected by centrifugation for 20 min at 15,000*g* and dissolved in 3 ml of 0.5 M potassium phosphate buffer (pH 7.4). To this solution were added: 0.2 volume of 0.1 M ATP, 0.2 volume of 0.1 M L-methionine, 0.14 volume of 0.05 M potassium fluoride, 0.06 volume of 0.5 M dibasic potassium phosphate, and 0.04 volume of 0.5 M $MgCl_2$. This mixture was incubated at 55° for 45 min, cooled to 4°, and centrifuged for 10 min at 15,000*g*, and the pellet was discarded. The supernatant was dialyzed overnight against 0.01 M Tris-HCl (pH 7.4), distributed among a number of small tubes, and stored in liquid air. The methionyl-tRNA synthetase did not contain any detectable tRNA methylase activity as assayed by the method of Littauer and Milbauer (1965); thus aminoacylation of methionine-starved tRNA could be performed without concomitant methylation.

Preparation of Valyl-tRNA Synthetase. Valyl-tRNA synthetase was prepared by a modification of the procedure of Bergmann *et al.* (1961), as previously described (Katchalski *et al.*, 1966).

Preparation of Aminoacyl-tRNA and Assay of Amino Acid Acceptance. The tRNA preparations were charged with lysine using the reaction mixture described by Stern

and Mehler (1965). Twenty millimicromoles of each of the 19 other [^{12}C]amino acids without lysine was included in the reaction mixture. The lysyl-tRNA synthetase was prepared up to and including the DEAE-cellulose step.

For assay of phenylalanyl-tRNA formation, the reaction mixture (0.1 ml) contained: 0.20 mg of tRNA, 5.5 μmoles of Tris-HCl buffer (pH 7.8), 0.20 μmole of ATP, 0.3 μmole of β-mercaptoethanol, 0.6 μmole of $MgCl_2$, 0.8 mμmole of [^{14}C]- or [^{3}H]phenylalanine, and sufficient phenylalanyl-tRNA synthetase to ensure complete charging in 15 min at 23°. Higher levels of amino acid acylation of tRNA were obtained at 23° than at 37°. In some experiments the reaction mixture contained, in addition to labeled phenylalanine, 20 mμmoles of each of the 19 other [^{12}C]amino acids. The reaction mixture was chilled. Cold 10% trichloroacetic acid (3 ml) was added and kept for 10 min at 0°. The radioactive precipitable material was collected on a membrane filter, washed three times with 10-ml portions of cold 5.0% trichloroacetic acid, dried, and placed in 10 ml of toluene scintillation fluid, and radioactivity was counted in a Packard Tri-Carb scintillation counter.

For the preparation of Phe-tRNA the reaction mixture contained [^{3}H]- or [^{14}C]phenylalanine (0.15 or 0.032 mCi/μmole), and a four-times higher than usual concentration of tRNA and enzyme, After the incubation, the reaction mixture (1.0 ml) was chilled and 1.0 ml of 0.2 M sodium acetate buffer (pH 4.5), 1.5 ml of 70% phenol, and 0.15 ml of chloroform were added, followed by vigorous shaking for 10 min at 4°. The mixture was centrifuged for 10 min at 10,000g at 4° and the aqueous phase was removed and extracted again for 5 min with the phenol–chloroform mixture. The mixture was centrifuged for 10 min and to the aqueous phase 0.2 volume of 5 M NaCl and 2.2 volumes of 95% ethanol were added and stored at −15° for 12 hr. The resulting precipitate was centrifuged for 10 min at 10,000g, the pellet was dissolved in 0.2 ml of 0.2 N sodium acetate buffer (pH 4.5), and the salt–ethanol precipitation was repeated.

The yield of optical density at 260 mμ and the radioactivity was about 70%. The resulting suspensions were stored in ethanol for several months at −15° aliquots were removed as needed and centrifuged, and the precipitate was dissolved in the required buffer. The amount of acid-insoluble radioactivity was determined prior to use. The same procedure was used for the isolation of all aminoacyl-tRNAs. The extent of phenylalanine acylation was 0.6–1.2 mμmoles of amino acid/mg of tRNA. Lysine acylation was 0.3–0.6 mμmole/mg of tRNA.

The valine reaction mixture contained in 0.10 ml: 10 μmoles of Tris-HCl (pH 7.4), 0.10 μmole of ATP, 0.2 μmole of $MgCl_2$, 0.5 μmole of β-mercaptoethanol, 0.10 mg of tRNA, 2.5 mμmoles of uniformly labeled [^{14}C]- or [^{3}H]valine, 20 mμmoles each of 19 other amino acids without valine, and enzyme. The reaction mixture was incubated for 30 min at 37°. For the preparation of valyl-tRNA the subsequent steps were the same as for the preparation of Phe-tRNA.

The methionine reaction mixture contained in a total volume of 0.10 ml: 3.0 μmoles of Tris-HCl buffer (pH 7.5), 0.20 μmole of ATP, 1.0 μmole of $MgAc_2$, 0.20 mg of tRNA, 0.7 mμmole of labeled methionine, 2 mμmoles of each of 19 [^{12}C]amino acids without methionine, and enzyme. The reaction was incubated for 30 min at 37°. The subsequent steps were the same as for the preparation of the Phe-tRNA. The extent of valine and methionine acylation was 2.0–3.0 and 0.9–1.5 mμmoles of amino acid per mg of tRNA, respectively. The concentration of tRNA was estimated by measuring the absorption at 260 mμ in 0.01 N NaOH. A value of 30 was assumed to equal 1 mg of tRNA (Berg *et al.*, 1961).

Preparation of N-Carbobenzyloxy-[^{14}C]Phe-tRNA. [^{14}C]Phe-tRNA (200 mg) was dissolved in 40 ml of 0.5 M KPO_4 buffer (pH 7.0) in a 250-ml glass-stoppered erlenmeyer flask and stirred on a shaker at 0° for 30 min. At 0, 10, and 20 min, 2.0-ml aliquots of 100% carbobenzyloxy chloride solution were added. The residual carbobenzyloxy chloride was removed by adding 80 ml of ethyl ether and shaken briefly in a separatory funnel. The aqueous layer was removed, leaving behind the ether layer and the interphase. The aqueous layer was then reextracted with ethyl ether until all traces of carbobenzyloxy chloride had been removed. Ethanol (2.1 volumes) and 5 M NaCl (0.2 volume) were added and the precipitate was centrifuged and dissolved in 0.05 M sodium acetate buffer (pH 5.5) containing 0.78 M NaCl. Under these conditions, the amino acid acceptance of tRNA is not impaired. However, if the reaction is run at pH 8.0 over 40% of the amino acid acceptance capacity of the tRNA is destroyed.

Results

Preparation of the MASA Column. The preparation of the MASA column was similar to that described by Okamoto and Kawade (1963). The silicic acid was suspended and decanted 20 times with tap water and twice with distilled water. The suspension was treated with 1 N HCl, washed with water until the pH had reached that of water, and dried in an oven. The powder was stored at room temperature. Methylated albumin was prepared according to the method of Mandell and Hershey (1960). A jacketed column was used (2 × 30 cm) containing a sintered-glass disk.

Buffered solutions used were 0.05 M sodium phosphate (pH 6.8), and 0.05 M sodium acetate (pH 5.5) containing 0.8 and 1.15 M NaCl. Stock solutions of 1.0 M buffer without NaCl were adjusted so as to give the desired pH on dilution to 0.05 M at 20°. The diluted buffers were not readjusted following the addition of NaCl.

To prepare a column (2 × 12 cm), 20 g of silicic acid was suspended in 100 ml of 0.05 M sodium phosphate buffer (pH 6.8), boiled for 2 min, and cooled. The methylated albumin (1 g) was dissolved in 100 ml of water. The methylated albumin solution was added slowly to the silicic acid solution, with stirring, at room temperature. Stirring was continued for 2 hr. The material was then washed several times by decanting and resuspending the protein–silicic acid mixture in 100 ml of 0.05 M sodium acetate buffer (pH 5.5), containing 1.15 M NaCl (terminal buffer). These washings permitted a more rapid flow rate. Glass beads were placed at the

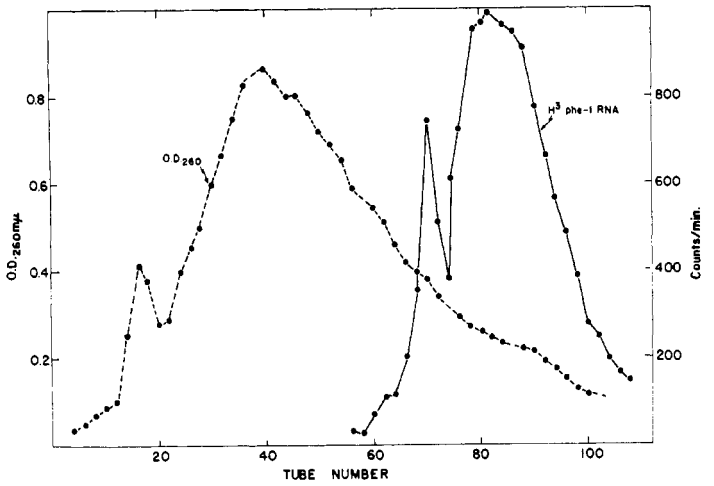

FIGURE 1: MASA column chromatography of Phe-tRNA from normal *E. coli* A-19. tRNA (0.2 mg) was charged with [^{3}H]phenylalanine and mixed with 3 mg of uncharged carrier tRNA together with 10 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl. The solution was placed on the column (2 × 12 cm) which was then washed with 25 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.8 M NaCl. The RNA was eluted with a 400-ml linear gradient formed from 200 ml each of 0.80 and 1.15 M NaCl solution in 0.05 M sodium acetate buffer (pH 5.5). Fractions (3.3 ml) were collected at 16° over a period of 8 hr. The A^{260} and radioactivity were measured as described under Methods.

bottom of the column to a height of 2 cm. The methylated albumin–silicic acid mixture (10 ml) was pipetted onto this bed. The column outlet was opened and the excess buffer was forced out under an air pressure of 2–3 psi, just down to the level of the packed material, and the addition of 10-ml aliquots was repeated as

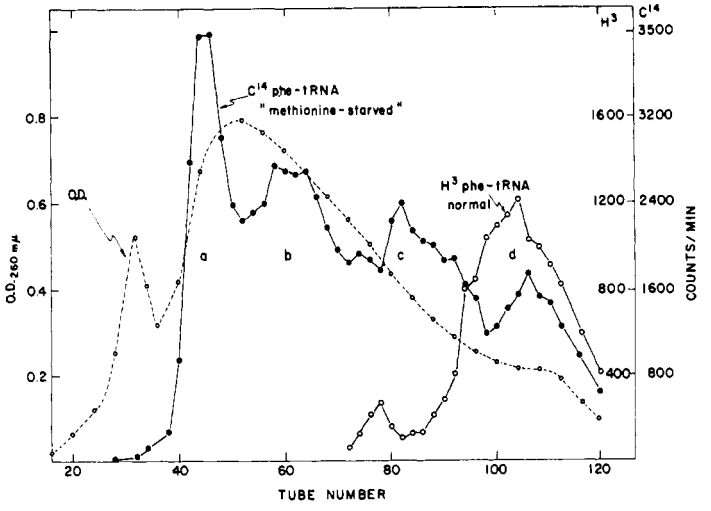

FIGURE 2: MASA column chromatography of normal and methionine-starved Phe-tRNA from *E. coli* A-19. Normal tRNA (0.2 mg) was charged with [^{3}H]phenylalanine and methionine-starved tRNA (0.2 mg) was acylated with [^{14}C]-phenylalanine. The two phenylalanyl-tRNAs were mixed together with 4.5 mg of uncharged methionine-starved *E. coli* A-19 tRNA in 10 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl. This mixture was applied to a MASA column and chromatographed as described under Figure 1.

above. When all of the methylated albumin–silicic acid mixture had been applied, additional buffer was added to wash excess protein from the walls of the column. Kieselguhr (2 g) was suspended in 10 ml of the 0.2 M NaCl (pH 6.8)–phosphate buffer, boiled for 2 min, cooled, and added to the top of the column bed as a protective covering. The column was then washed with terminal buffer until the optical density at 260 mμ fell below 0.02 (about 1 l.). The column was washed with 500 ml of sodium acetate buffer (0.05 M, pH 5.5) containing 0.8 M NaCl (initial buffer). The column was then ready for use.

tRNA (3–80 mg) was dissolved in 10 ml of initial buffer, mixed with 2 ml of water, and applied to the column. The column was washed with 50 ml of initial buffer and the tRNA was then eluted with a linear gradient containing 200 ml of initial buffer and 200 ml of terminal buffer. Fractions (120) of 3.3 ml each were collected at a flow rate of 1 ml/min using a hydrostatic pressure of 1 m between gradient reservoir and column outlet. The column was run at 16° and required 8–16 hr for completing. Each receiving tube contained 0.2 ml of 1 M sodium acetate buffer (pH 4.0) to minimize aminoacyl-tRNA hydrolysis before the tubes could be assayed. Optical density at 260 mμ was determined, aliquots of the fractions were removed, 1 drop of carrier RNA (5 mg/ml) was added, and the RNA was then precipitated with equal volumes of 10% cold trichloroacetic acid and filtered onto membrane filters. The filters were washed three times with 10 ml each of 2.5% trichloroacetic acid and dried. The membrane filters were placed in 10 ml of toluene scintillation fluid and radioactivity was counted in a Packard Tri-Carb scintillation counter.

The optical density at 260 mμ usually emerged between fractions 15 and 100. In most RNA preparations, a narrow peak of absorbance appeared preceding the main peak in an amount characteristic for each preparation of tRNA. In some cases it was entirely absent.

Recovery of Biological Activity. It has been reported that recovery of amino acid acceptor activity from methylated albumin columns is unsatisfactory (Okamoto and Kawade, 1963). However, this difficulty, due to methylated albumin eluted from the column, can be circumvented by Pronase treatment of the column fractions (Revel and Littauer, 1965). Proteolysis of the methylated albumin was accompanied by hydrolysis of aminoacyl-tRNA bonds. Upon reacylation of the recovered material, all of the original amino acid acceptor activity was obtained. However, only 60–70% of transfer ability to polypeptide was regained after this treatment.

It was shown (U. Z. Littauer and Y. Galenter, 1966, unpublished data) that extensive dialysis against water followed by lyophilization causes inactivation of tRNA. Consequently tRNA fractions were dialyzed against 10^{-3} M NaCl. Fractions were then lyophylized to a small volume and precipitated with 0.2 volume of 5 M NaCl and 2.5 volumes of 96% ethanol at 0°. The material was allowed to precipitate for several hours at −15° and centrifuged, and the pellet was dissolved in 1.0 ml of 0.01 M Tris-HCl (pH 7.4) and incubated at 37° for 5 hr with 0.7 ml of Pronase solution. To reduce possible traces of

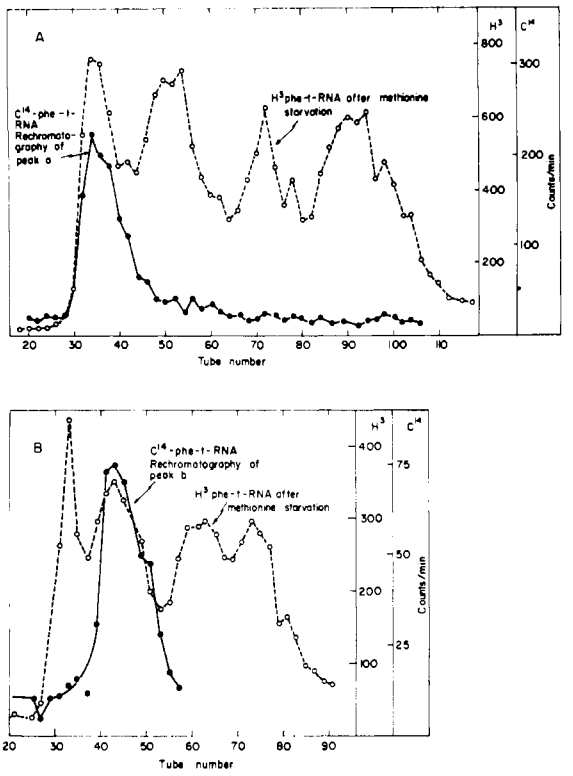

FIGURE 3: MASA column rechromatography of methyl-deficient Phe-tRNA peaks. The pooled tubes of the peak a region obtained similarly to that described under Figure 2 were mixed with an equal volume of 0.05 M sodium acetate buffer (pH 5.5) containing 3 mg of *E. coli* strain B tRNA and 0.1 mg of methionine-starved *E. coli* A-19 [^{3}H]Phe-tRNA. This mixture was applied to a MASA column and chromatographed as described under Figure 1 (A). The pooled tubes of peak b region obtained similarly to that described under Figure 2 were chromatographed under identical conditions as for fraction a (B).

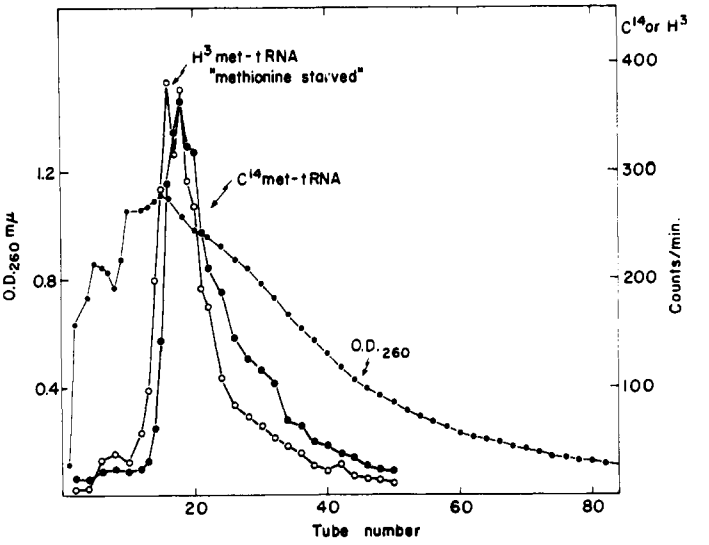

FIGURE 4: MASA column chromatography of normal and methionine-starved *E. coli* A-19 methionyl-tRNA. Normal [^{14}C]methionyl-tRNA (0.17 mg) and methionine-starved [^{3}H]methionyl-tRNA (0.7 mg) were mixed together with 5 mg of uncharged *E. coli* A-19 normal tRNA in 10 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl. This mixture was placed on a MASA column and chromatographed as described under Figure 1.

nucleases, the Pronase solution was preincubated for 90 min at 37° (2 mg/ml in 0.01 M Tris-HCl buffer, pH 7.4) before use.[3]

At the end of the incubation the solution was cooled to 4°. Phenol (0.7 volume of 70%) and chloroform (0.07 volume) were added and shaken for 30 min at 4°. The mixture was centrifuged and the phenol phase was saved. To the aqueous phase, 0.4 volume of 70% phenol and 0.04 volume of chloroform was added, shaken for 30 min at 4°, and centrifuged. The two phenol phases were combined, shaken briefly with 0.5 volume of 0.01 M Tris-HCl buffer (pH 7.4), and centrifuged, and the aqueous phase was combined with the other aqueous fraction. NaCl (0.2 volume of 5 M) and ethanol (2.2 volumes of 96%) were added and stored at −15° overnight. The solution was centrifuged and the pellet was dissolved in 1 ml of water (or less, depending upon the RNA concentration). The salt–ethanol precipitation was repeated after 1 hr at −15°. The suspension was centrifuged and the pellet was dissolved in a small volume of water. The material was then ready for reacylation. Recovery was about 70%.

Reuse of the Column. To reuse, the column was washed with 0.05 M sodium acetate buffer (pH 5.5) containing 2.0 M NaCl until the A_{260} fell below 0.02 and then washed with 500 ml of initial buffer. After repeated use, the optical density profile became increasingly constricted, with more material being eluted at lower salt concentrations. This did not affect the resolution of aminoacylated-tRNA species. The protective keiselguhr layer at the top of the column became tarnished with prolonged use and was easily replaced. It was also observed that with continued use the flow rate decreased as the uppermost part of the kieselguhr layer became matted with a fibrous material. The flow rate of the column was restored by resuspending a portion of that layer.

Resolution of Phenylalanyl-tRNAs. The resolution of two Phe-tRNA species from a normal preparation of *E. coli* tRNA is shown in Figure 1. Both of these peaks emerged after the bulk of the tRNA had been eluted from the column. There was a minor peak which was variable in its amount and a major peak which eluted at higher NaCl concentrations. A similar pattern was obtained by Revel and Littauer (1965) on MAK column chromatography. The nature of the differences between these two species will be disscussed in a future publication.

Figure 2 shows the resolution of four distinct species of the [^{14}C]Phe-tRNA from a methionine-starved preparation of the tRNA. Only three components were obtained from a methionine-starved preparation on the MAK column (Revel and Littauer, 1965, 1966). It should be recalled that methionine-starved tRNA is an approximately equal mixture of normal and methyl-de-

[3] When the Pronase solution was not preincubated, incubation with tRNA caused a 10–25% reduction in the amino acid acceptor capacity of the tRNA.

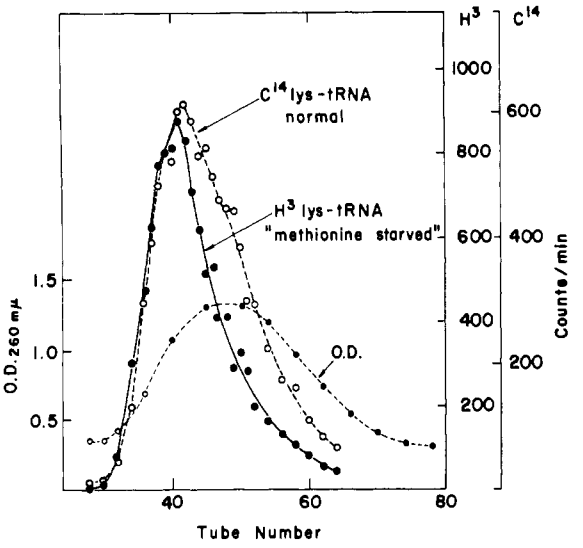

FIGURE 5: MASA column chromatography of normal and methionine-starved *E. coli* G-15 lysyl-tRNA. Normal [^{14}C]lysyl-tRNA (0.15 mg) and methionine-starved [^{3}H]-lysyl-tRNA (6.0 mg) were mixed together with 5 mg of uncharged *E. coli* strain B tRNA in 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl. This mixture was placed on a MASA column and chromatographed as described under Figure 1.

ficient tRNA. Peak a and possibly peak b correspond to methyl-deficient Phe-tRNA, while peak d corresponds to a fully methylated "normal" Phe-tRNA. The identity of peak c has not yet been well established. An identical pattern was obtained with tRNA extracted from methionine-starved *E. coli* A-19 or *E. coli* K_{12}W6 cells. The biological and chemical properties of these four components of phenylalanyl-tRNA will be presented in a following publication.

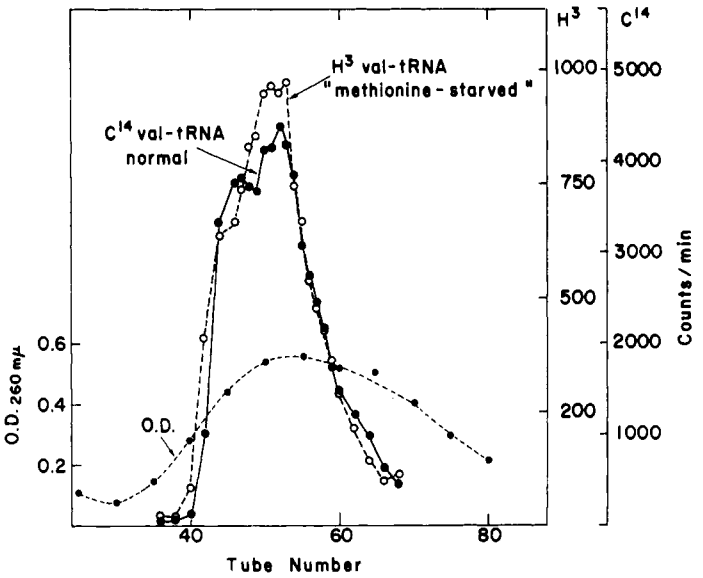

FIGURE 6: MASA column chromatography of normal and methionine-starved *E. coli* G-15 valyl-tRNA. Normal [^{14}C]valyl-tRNA (0.12 mg) and methionine-starved [^{3}H]-valyl-tRNA (0.4 mg) were mixed together with 4 mg of uncharged *E. coli* strain B tRNA in 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl. This mixture was placed on a MASA column and chromatographed as described under Figure 1.

When methionine-starved tRNA was charged with [^{14}C]phenylalanine in the presence of 19 amino acids and applied to a MASA column together with [^{3}H]phenylalanyl-tRNA which had been acylated in the absence of other amino acids, the two patterns were identical. tRNA was acylated with phenylalanine in the absence of 19 unlabeled amino acids in subsequent preparations.

The resolution of the four peaks of Phe-tRNA from a methionine-starved preparation of tRNA was not a column artifact. Fractions a and b from a column (Figure 3) were collected, diluted with an equal volume of 0.05 M sodium acetate buffer (pH 5.5), and applied directly to another column, together with 0.1 mg of unfractionated methionine-starved [^{3}H]Phe-tRNA and 3 mg of unacylated normal tRNA. Figure 3A,B demonstrates that the first and second peaks of Phe-tRNA were eluted at the same position upon rechromatography.

It was shown previously (Revel and Littauer, 1965, 1966) that a MAK column is able to resolve methionine-starved Phe-tRNA into only three peaks. Since the MAK column chromatography was run at pH 6.8, it was of interest to examine whether the appearance of the four Phe-tRNA peaks on MASA column chromatography was due to the lower pH (pH 5.5). To test this possibility, a MAK column was run at pH 5.5 using 0.05 M sodium acetate buffer and the usual NaCl gradient of 0.2–1.1 M NaCl. This column had the same pattern as obtained from a column run at pH 6.8.

Resolution of Other Aminoacyl-tRNAs. The elution profiles of methionyl-, lysyl-, and valyl-tRNA from methionine-starved or normal tRNA on MASA column are shown in Figures 4–6, respectively. Normal methionyl-tRNA was eluted as a single peak from both the MAK column (Sueoka and Yamane, 1962) and from the MASA column. Methionine-starved tRNA contained a possible additional peak of methionyl-tRNA which preceded the normal species on elution from the MASA column (Figure 4). Normal and methionine-starved lysyl-tRNA emerged as single components from the MASA column (Figure 5). Similar elution patterns were also obtained for normal or methionine-starved valyl-tRNA (Figure 6).

Conditions for Optimal Resolution. A number of conditions for successful fractionation of tRNA on both MAK and MASA columns were observed. A temperature of 16° was used for all columns. Resolution was impaired as the temperature increased, particularly above 23°. The pH optimum for separation of species of aminoacyl-tRNA differed for each amino acid. Species of methionine-starved phenylalanyl-tRNA did not resolve as well at pH 5.0 as at pH 5.5.

Both a lower as well as an upper limit existed for the tRNA which could be placed on the MAK and the MASA column. For optimal resolution, 1–2 mg was used on the MAK column and 3–80 on the MASA column. Figure 7 compares the chromatographic profile when an optimal amount, 1.12 mg, and an insufficient amount, 0.15 mg, of labeled methionine-starved Phe-tRNA were placed on a MAK column. The two major components of methionine-starved Phe-tRNA failed to

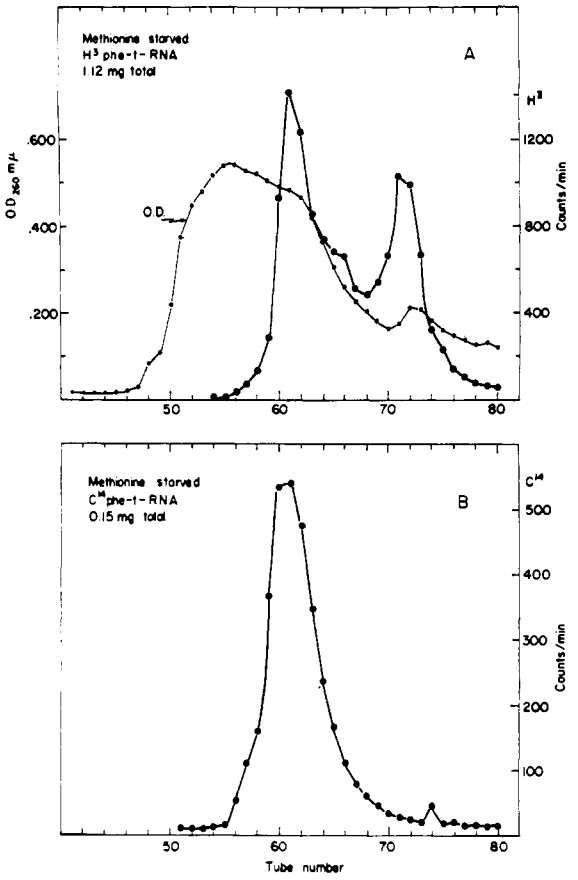

FIGURE 7: MAK column chromatography of Phe-tRNA from methionine-starved *E. coli* G-15; loss of resolution at low RNA concentrations. (A) A solution of methionine-starved [^{3}H]Phe-tRNA (0.12 mg) was mixed with 1.0 mg of uncharged methionine-starved tRNA and applied to a MAK column (2 × 10 cm) in 20 ml of 0.05 M sodium phosphate (pH 6.8) buffer containing 0.2 M NaCl. The column was washed with 50 ml of the same buffer and the RNA was eluted with a linear gradient of 190 ml of 0.2 M NaCl and 190 ml of 1.1 M in NaCl, both in 0.05 M sodium phosphate buffer (pH 6.8). The flow rate was 0.7 ml/min at 16°. In each fraction (2.5 ml) the A_{260} and trichloroacetic acid precipitable radioactivity were determined. (B) Methionine-starved [^{14}C]Phe-tRNA (0.15 mg) was added in the absence of additional carrier tRNA onto a MAK column as described under part A.

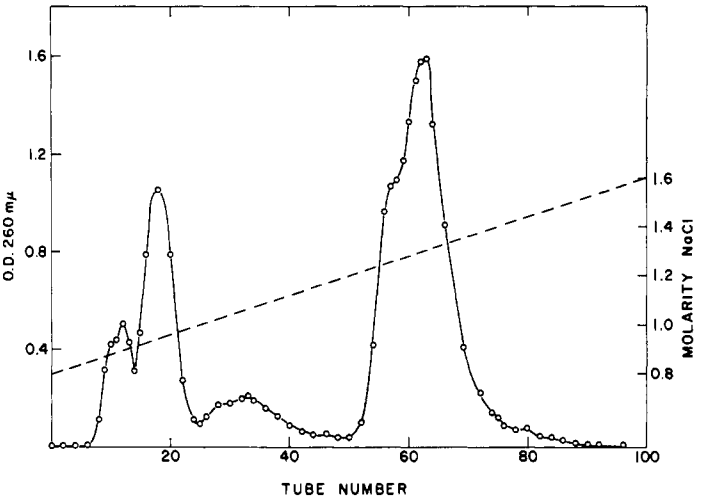

FIGURE 8: MASA column chromatography of total *E. coli* RNA. *E. coli* MRE 600 cells grown to the middle of the logarithmic growth phase and stored as a frozen cell paste were the source of total cell RNA extracted according to a modification of the method of Hayashi and Spiegelman (1961) with phenol and SDS. One hundred A_{260} units were applied to the column which was then washed with 50 ml of 0.05 M sodium acetate buffer (pH 5.5), 0.8 M in NaCl. The RNA was eluted with a gradient formed from 200 ml each of 0.8 and 1.6 M NaCl in 0.05 M sodium acetate buffer (pH 5.5). Fractions (4 ml) were collected. The column was run at room temperature.

be resolved when a lower concentration of RNA was used on the column. A similar observation was made with MASA columns when less than 2 mg of methionine-starved Phe-tRNA was placed on the column. The four components of Phe-tRNA were not as well resolved. Therefore, whenever the chromatography of a small amount of labeled aminoacyl-tRNA was required, non-labeled carrier tRNA was added, to enhance the resolution on the column. The upper limit for optimal resolution of tRNAs on these columns was below their capacity to retain tRNA. Resolution was inferior when 3 mg was placed on the MAK column. The upper limit for the MASA column was 150 mg.

Conditions of the charging reaction played a role in the resolution of tRNA species. Aminoacylation of tRNA with phenylalanine reached a plateau after 15 min in the usual reaction mixture. When streptomycin supernatant was used as the source of phenylalanyl-tRNA synthetase, a decrease in amino acid acceptor ability was observed with incubations longer than 15 min. This was attributed to nuclease activity plus hydrolysis of the aminoacyl-ester bond. This decrease was not observed with the partially purified enzyme preparation. However, inferior chromatographic profiles of methionine-starved Phe-tRNA were obtained when the acylation reaction with the partially purified enzyme preparation was permitted to continue for 40 min. This suggested that nuclease activity may have been present even though there was no decrease in the amount of Phe-tRNA after the reaction had reached a plateau. On the other hand, it may be that changes in configuration had occurred during the course of the incubation which caused the observed chromatographic shift (Gartland and Sueoka, 1966; Lindahl *et al.*, 1966).

The tRNA samples were applied to the column in a solution higher than 0.4 M in NaCl. A large difference between the salt concentration of the sample and the concentration of the initial gradient buffer caused erratic chromatographic patterns. A similar observation has been made on MAK columns (Mandel and Hershey, 1960) and on benzoylated–naphthoylated DEAE-cellulose columns (Sedat *et al.*, 1967).

It was observed that the methylated albumin columns cannot be scaled down without changing the chromatographic properties of the column. When dimensions of the column, size of gradient, and amount of material placed on the column were reduced to one-tenth, the label contained in the Phe-tRNA or the lysyl-tRNA coincided with the optimal density pattern. When only the gradient was increased to the standard volume (400

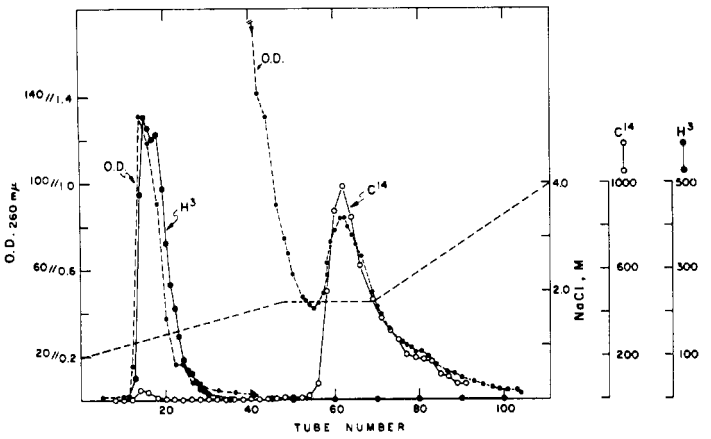

FIGURE 9: MASA chromatography of *N*-carbobenzyloxy-[^{14}C]phenylalanyl-tRNA and [^{3}H]phenylalanyl-tRNA. A new MASA column was used for these experiments. The column was washed with 4 M NaCl containing 0.05 M sodium acetate buffer (pH 5.5) and then equilibrated with 0.78 M NaCl containing 0.05 M sodium acetate buffer (pH 5.5). *E. coli* A-19 tRNA was first purified by passing it through a MASA column (2 × 12 cm) to remove traces of high molecular weight RNA that are strongly held to the column. *E. coli* A-19 tRNA (200 mg) was dissolved in 6 ml of water and mixed with 10 ml of sodium acetate buffer (0.05 M, pH 5.5), containing 0.78 M NaCl and applied on a MASA column. The tRNA was eluted from the column with 0.05 M sodium acetate buffer (pH 5.5) containing 1.8 M NaCl and isolated by ethanol precipitation. The isolated tRNA was then charged with [^{14}C]phenylalanine. Before use, the phenylalanine tRNA synthetase preparation was freed of any amino acids by Sephadex G-50 gel filtration. The [^{14}C]-Phe-tRNA was precipitated with ethanol, dissolved in 40 ml of 0.5 M KPO_4 buffer (pH 7.0), and treated with carbobenzyloxy chloride. *N*-Carbobenzyloxy-[^{14}C]phenylalanyl-tRNA (84,000 cpm) (91 mg) was mixed with 2 mg of [^{3}H]phenylalanyl-tRNA (27,000 cpm) in 13 ml of 0.78 M NaCl containing 0.05 M sodium acetate buffer (pH 5.5). This solution was placed on a new MASA column (2 × 12 cm). The RNA was eluted with a linear gradient formed from 60 ml each of 0.78 and 1.78 M NaCl solutions in 0.05 M sodium acetate buffer (pH 5.5). The column was further eluted with 50 ml of 1.78 M NaCl–sodium acetate and then a linear gradient of 60 ml each of 1.78 and 4.0 M NaCl in sodium acetate buffer. Fractions (2.5 ml) were collected at 16°.

ml), aminoacylated-tRNA species still failed to be resolved.

Chromatography of Total E. coli RNA. Total *E. coli* RNA was fractionated on a MASA column. One hundred A_{260} units were placed on the column and eluted with a linear gradient formed by 200 ml each of 0.8 and 1.6 M NaCl in 0.05 M sodium acetate buffer (pH 5.5). The ultraviolet absorption profile is shown in Figure 8. The column separated 4S from rRNA but gave only partial separation of the 16S from the 23S component. Additional unidentified fractions were also present.

Chromatography with Other Basic Protein Columns. Are the properties of methylated albumin unique or can other basic proteins be used for the fractionation of tRNA species? The use of histone columns (Brown and Watson, 1953) and protamine kieselguhr columns (Legault-Demare *et al.*, 1964; Hayashi, 1966; Brown and Littna, 1966) have been reported. A protamine–silicic acid column was prepared. It had a high capacity for tRNA, but no resolution of methyl-deficient from normal Phe-tRNA was achieved.

Chromatography of N-Carbobenzyloxyphenylalanyl-tRNA. N-Carbobenzyloxyphenylalanyl-tRNA was more strongly held to MASA than Phe-tRNA or any of the other tRNA chains. The elution of this compound was achieved at a relatively high sodium chloride concentration of 1.78 M. This property ws exploited for the purification of $tRNA^{Phe}$. *N*-Carbobenzyloxy-^{14}-C]phenylalanyl-tRNA (91 mg) was mixed together with a small amount (2 mg) of [^{3}H]phenylalanyl-tRNA and applied onto a MASA column, which was then eluted with a steep sodium chloride gradient formed from 0.78 to 1.78 M. Figure 9 shows that the [^{3}H]Phe-tRNA and the bulk of tRNA are eluted already with 1 M NaCl. At the end of this gradient the *N*-carbobenzyloxy-[^{14}C]phenylalanyl-tRNA was still held to the column. On further elution with 1.78 M sodium chloride a small peak adsorbing at 260 mμ appeared together with the ^{14}C-labeled derivative. Moreover, the labeled material almost coincided with the optical density profile. The $tRNA^{Phe}$ was liberated from the *N*-carbobenzyloxyphenylalanyl-tRNA by digestion with Pronase and its ability to accept various amino acids was measured. Table I shows that the $tRNA^{Phe}$ was purified considerably by this method; its capacity to accept phenylalanine was increased 16-fold while its ability to accept other amino acids was markedly decreased.

Discussion

It was shown by Mandel and Borek (1961, 1963) that methyl-deficient tRNA is synthesized by a relaxed methionine-requiring mutant of *E. coli* (K_{12}W6) grown in the absence of methionine. The tRNA prepared from such methionine-starved cultures is an approximately equal mixture of methyl-deficient tRNA and fully methylated tRNA which was present in the cell prior to methionine deprivation. A major problem in the study of the function of methylated bases in tRNA has been to obtain a method for the isolation of methyl-deficient tRNA free of normal tRNA. Attempts to use MAK columns for this purpose have been made. Chromatography of methionine-starved leucyl-tRNA (Peterkofsky, 1964) or Phe-tRNA (Revel and Littauer, 1965) on MAK columns reveal that, in addition to the normal components, new, easily separable, components appear. It was also demonstrated that the new components arise only following methionine deprivation. On the other hand, tRNA extracted from cells deprived of an amino acid other than methionine did not contain this new tRNA component (Lazzarini and Peterkofsky, 1965; Revel and Littauer, 1965; Littauer and Revel, 1966). In the case of Phe-tRNA, it was shown that the new peak appearing on methionine starvation is deficient in methylated bases (Revel and Littauer, 1965; Fleissner, 1967).

A limitation of the MAK column is its small capacity for tRNA. For that reason it was decided to develop a system which would have a larger capacity for tRNA while retaining the ability to separate methyl-deficient from normal species. Initially it was assumed that a more basic protein, such as protamine, would be more

TABLE I: Amino Acid Acceptance of Purified $tRNA^{Phe}$.[a]

Sample	Amino Acid Acceptance Act. (mμmoles/mg of RNA)				
	Phenylalanine	Valine	Methionine[b]	Lysine	Amino Acid Mixture (cpm/mg of RNA)[c]
Initial tRNA	1.1	2.9	0.96	0.55	3900
Purified $tRNA^{Phe}$	18	0.04	0.03	0.11	300

[a] Tubes 60–63 from the column shown in Figure 9 were pooled and dialyzed for 2 hr against 0.001 M NaCl at 0°. The solution was then concentrated by lyophilization to 3.0 ml. To liberate tRNA from the *N*-carbobenzyloxyphenylalanyl-tRNA this solution was mixed with 0.8 ml of 1 M Tris-HCl buffer (pH 8.8) and 0.012 ml of Pronase solution (10 mg/ml), and water to a final volume of 4.0 ml. (To reduce possible traces of ribonuclease the Pronase solution was preincubated for 90° in 0.01 M Tris 7.4 at 37°; Revel and Littauer, 1965). The mixture was incubated for 2 hr at 37°, treated with phenol and $NaClO_4$ according to a previously published procedure (Katchalski *et al.*, 1966), and then dialyzed for 24 hr at 4° against three changes of 1 l. of 0.005 M NaCl. [b] Assay with L-[^{14}C]methionine, 187 mCi/mmole. [c] Assay with 0.005 ml of crude aminoacyl-tRNA synthetase (Muench and Berg, 1966) and a mixture of 15 L-[^{14}C]-amino acids mixed together with 50 μmoles/ml of unlabeled L-phenylalanine.

effective than methylated albumin in achieving the desired resolution. A protamine–silicic acid column had an increased capacity for tRNA, but was unable to resolve methyl-deficinet from normal Phe-tRNA. Adaptation of the MASA column to tRNA chromatography showed that both increased capacity and separation could be achieved with this column.

It appears that the MASA column has a better ability to resolve the various components of a given aminoacyl-tRNA than the MAK column. This fact is particularly pronounced in the case of methionine-starved Phe-tRNA, where four peaks were resolved after chromatography on a MASA column (Figure 2). At least one of the two new peaks appearing on methionine starvation is actually methyl deficient (Revel and Littauer, 1965). This has been confirmed recently by Fleissner (1967) with fractions obtained from countercurrent distribution. However, it is possible that in addition to the effect of methyl groups on the position of the Phe-tRNA on MASA columns, other factors might contribute to the differences in the elution pattern between normal and methionine-starved tRNA.

In the case of methionyl-tRNA (Figure 4), a small difference was found in the elution pattern of normal and methionine-starved tRNA. The altered chromatographic behavior alone does not necessarily imply that the new peak is methyl deficient. Further experiments would be required to establish this point. The increased resolving power of the MASA column has been confirmed recently by Leder and Bursztyn (1966) who used this chromatographic procedure to separate *E. coli N*-formylmethionyl-tRNA from the nonformylated methionyl-tRNA species. Methylated albumin columns separate nucleic acids on the basis of GC content, size, and hydrogen-bond content (Mandell and Hershey, 1960; Sueoka and Cheng, 1962). However, there is evidence that conformation also plays a role in the column resolution (Littauer and Stern, 1967). The principle of separation of tRNA on the methylated albumin columns can be inferred, in part, from empirical observations which were made to obtain successful chromatography. A lower limit exists for the amount of tRNA which could be placed on the column. This suggests that a displacement phenomenon is occurring, since minimal amounts of tRNA must occupy sites on the column bed.

It was found that *N*-carbobenzyloxyphenylalanyl-tRNA is bound more strongly to the MASA column than Phe-tRNA (Figure 9). This is probably due to the aromatic nature of the carbobenzyloxy group and is similar to the effect of linking *N*-phenoxyacetyl groups to aminoacyl-tRNA (Gillam *et al.*, 1967). This finding was used as a means of obtaining a highly purified $tRNA^{Phe}$ (Table I). The phenylalanine acceptance values show that this preparation has a purity of about 50%, assuming a molecular weight of 27,000 for $tRNA^{Phe}$. It was observed that the ester link between *N*-carbobenzyloxyphenylalanine and tRNA is more resistant to alkaline hydrolysis than the ester bond in Phe-tRNA; in this respect the blocking of the amino group of phenylalanine with a carbobenzyloxy group has the same effect on the stability of the ester linkage as the introduction of a polypeptide group (Simon *et al.*, 1964; Littauer *et al.*, 1964b). However, $tRNA^{Phe}$ could be liberated from the *N*-carbobenzyloxyphenylalanyl-tRNA by digestion with Pronase (Katchalski *et al.*, 1966). The major difficulty in this method is avoiding RNase contamination during the incubation with Pronase. Trace amounts of RNase bound to tRNA or introduced with the Pronase digestion were found to cause considerable inactivation of the purified $tRNA^{Phe}$. Therefore, the purity of the recovered $tRNA^{Phe}$ is probably somewhat higher than that indicated from the phenylalanine acceptance values. Preliminary experiments indicated that *N*-carbobenzyloxyvalyl-tRNA is also separated from the bulk of the

tRNA on MASA column chromatography. Thus, it is possible to adopt this method for the purification of tRNA species other than $tRNA^{Phe}$.

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