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Transfer Ribonucleic Acids in *Escherichia coli*. Multiplicity and Variation*

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ABSTRACT: Gradient partition chromatography of transfer ribonucleic acid from *Escherichia coli*, strain B, reveals 56 transfer ribonucleic acid chains for 20 amino acids. Acceptor profiles, determined with partially purified aminoacyl transfer ribonucleic acid synthetases, demonstrate three transfer ribonucleic acids for asparagine, cysteine, glycine, histidine, and threonine; four transfer ribonucleic acids for proline, and five transfer ribonucleic acids for tryptophan. Each of the tryptophan transfer ribonucleic acid can exist in an active and an inactive conformation as measured by response to chloroquine in the charging medium. Although asparagine transfer ribonucleic acids and aspartic acid transfer ribonucleic acid emerge in the same region of the profile, asparagine and aspartic acid do not share common acceptors, as shown by studies involving periodate oxidation. The profiles of leucine and tyrosine acceptor are essentially constant for different lots of transfer ribonucleic acid prepared from commercial cells, whereas the acceptor profiles for eight other amino acids vary markedly. The variability is not explainable by differential extraction or artifacts in resolution.

Various techniques of separation (RajBhandary and Stuart, 1966) have revealed multiple tRNAs (isoacceptors) for most of the amino acids. As techniques have improved and multiplied, so has the recorded multiplicity of tRNA expanded. The general validity of this multiplicity is accepted. However, the reasons for multiplicity are incompletely understood. Even less understood are reasons for variable amounts of isoacceptors and for changes in those amounts. To establish the minimum number of tRNAs and the isoacceptor constancy in *Escherichia coli*, we examined four lots of tRNA by gradient partition chromatography. For 7 amino acids not previously studied (Muench and Berg, 1966a) 24 tRNAs were found, bringing the number of tRNAs for 20 amino acids to at least 56. At least two tRNAs have been resolved for each amino acid, five for leucine and tryptophan. Relative amounts of isoacceptors varied widely in some cases and were constant in others.

Experimental Procedures

Materials. E. coli strain B cells were purchased from Grain Processing Corp., Muscatine, Iowa.

Lots 1 and 4 of tRNA were prepared in our laboratory as described (Muench and Berg, 1966a) from two different batches of the commercial cells stated to be grown in minimal[1] medium and harvested in exponential phase at three-fourths maximal growth. Lots 2 and 3 were prepared by Schwarz BioResearch (6701 and 6603, respectively) by the method of Gutcho (1968) from cells also supplied by Grain Processing Corp. but stated to be grown in enriched[1] medium and harvested in exponential phase at three-fourths maximal growth. Lot 2 was derived from several batches of cells, whereas lot 3 was derived from one batch (S. Gutcho, personal communication).

Pure tyrosyl-tRNA synthetase (Calendar and Berg, 1966) was a gift from Dr. R. Calendar. Other aminoacyl-tRNA synthetases were prepared by batch fractionation

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[1] According to the Grain Processing Corp., "minimal" medium consists of 1.0% glucose, 1.0% yeast extract, 2.08% K_2HPO_4, and 1.62% KH_2PO_4; "enriched" medium consists of 4.0% peptone casamino acids, 1% glucose, 0.5% yeast extract, 0.64% K_2HPO_4, 0.04% $(NH_4)_2HPO_4$, 0.003% KCl, 0.001% each of $MgSO_4$, $CaCl_2$, and $ZnSO_4$, and 0.008% $FeCl_3$.

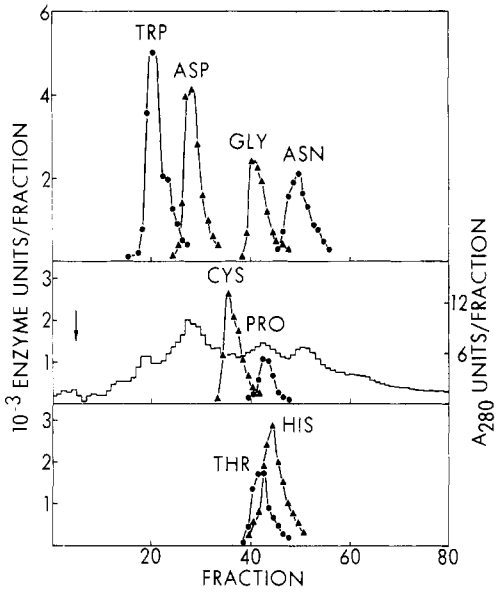

FIGURE 1: Purification of eight aminoacyl-tRNA synthetases on DE-52 cellulose. The peaks indicated by the relevant amino acid are from a single procedure, with the A_{280} profile shown in the middle panel. The start of the gradient is indicated by an arrow. Each fraction was 20 ml.

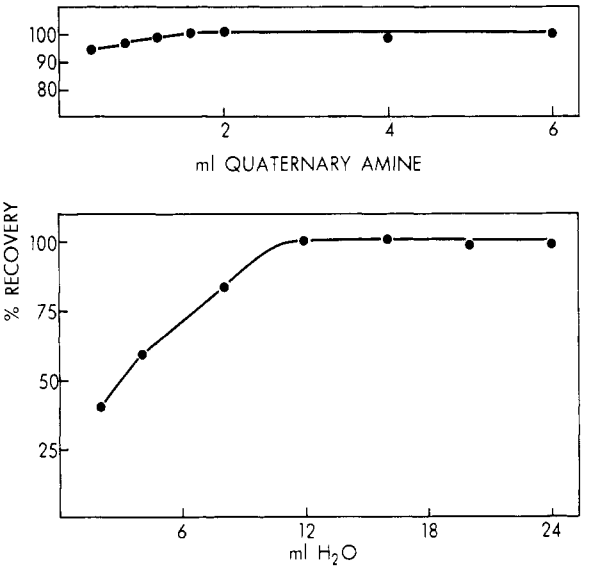

FIGURE 2: Recovery of tRNA from partition fractions as a function of quaternary amine or H_2O added. tRNA (16 A_{260} units) added to 60 ml of upper phase was isolated as described in the text. H_2O (20 ml) was used for the experiment in the upper panel, and 0.10 M cetyltrimethylammonium chloride (2 ml) was used for the experiment in the lower panel.

on DEAE-cellulose as described by Muench and Berg (1966c). Except in the case of asparagine, no difference in acceptor profiles resulted from the use of more purified enzymes. However, the enzymes used in almost every case were further purified from the DEAE-cellulose fraction. All steps in the enzyme purifications were performed at 0–2°. DEAE-cellulose fraction containing 350 mg of protein was applied to a 31 × 1.5 cm DE-52 (microcrystalline DEAE-cellulose) column equilibrated with 0.02 M potassium phosphate buffer (pH 6.9), 0.02 M 2-mercaptoethanol, 0.001 M $MgCl_2$, and 10% glycerol. After 100 ml of the initial buffer the column was developed at a rate of 40 ml/hr by a linear gradient from 0.02 to 0.25 M potassium phosphate buffer (pH 6.9) in a total volume of 1600 ml of 0.02 M 2-mercaptoethanol, 0.001 M $MgCl_2$, and 10% glycerol. A typical elution pattern is shown in Figure 1. The A_{280}[2] pattern is a reproducible feature which permits easy location of specific enzyme peaks. Peak fractions were pooled and concentrated by dialysis against the initial buffer containing 10–30% polyethylene glycol (mol wt 6000) then stored at −15° in 50% glycerol (Muench and Berg, 1966c). Histidyl-tRNA synthetase was stabilized prior to dialysis by addition of crystalline bovine serum albumin to a final concentration of 2 mg/ml. The procedure provided all enzymes 6–20-fold purified over extracts. The final aminoacyl-tRNA synthetases from Figure 1, for example, had specific activities in units per milligram as follows for the amino acids indicated: asparagine, 140; aspartic acid, 320; cysteine, 360; glycine, 250; histidine, 400; proline, 85; threonine, 180; and tryptophan, 430; when 1 unit formed 1 mμmole of aminoacyl-tRNA in 10 min under standard assay conditions. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as the standard.

A DEAE-cellulose batch fraction containing 400–3100 units/ml of aminoacyl-tRNA synthetases contained 17 units/ml of tRNA adenylyltransferase (Preiss *et al.*, 1961) with 1 unit equal to addition of 1 mμmole of AMP residue in 10 min. The enzyme emerged from the DE-52 column ahead of tryptophanyl-tRNA synthetase (Figure 1) and was not present in any of the purified aminoacyl-tRNA synthetases.

DE-52 chromatography completely separated aspartyl- from asparaginyl-tRNA synthetase, as shown in Figure 1. However, certain preparations of asparaginyl-tRNA synthetase remained contaminated with asparaginase. The contaminant was removed by further chromatography as follows. Asparaginyl-tRNA synthetase (6500 units) (43 mg of protein) was applied to a 19 × 1 cm DE-52 column equilibrated with 0.05 M Tris-HCl buffer (pH 8.1), 0.001 M $MgCl_2$, and 10% glycerol. The enzyme was eluted at 10 ml/hr by a linear gradient between 0.30 and 0.50 M Tris-HCl buffer (pH 8.1) in a total volume of 280 ml of 0.001 M $MgCl_2$ and 10% glycerol. Recovery was 70–100% and specific activity 470 units/mg in the pooled peak fractions, which were concentrated and stored as described above. Five units of this final preparation converted no detectable (less than 0.06%) L-[^{14}C]asparagine into L-[^{14}C]aspartic acid in 30 min.

All radioactive amino acids, purchased from New England Nuclear Corp. or Nuclear-Chicago, were adjusted to specific activities of 4–8 × 10^6 cpm per μmole by dilution with the corresponding L-[^{12}C]amino acids

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: MAK, methylated albumin kieselguhr; A_{260}, absorbance at 260 mμ, determined in 0.005 M KH_2PO_4–0.005 M K_2HPO_4; A_{250} absorbance at 280 mμ.

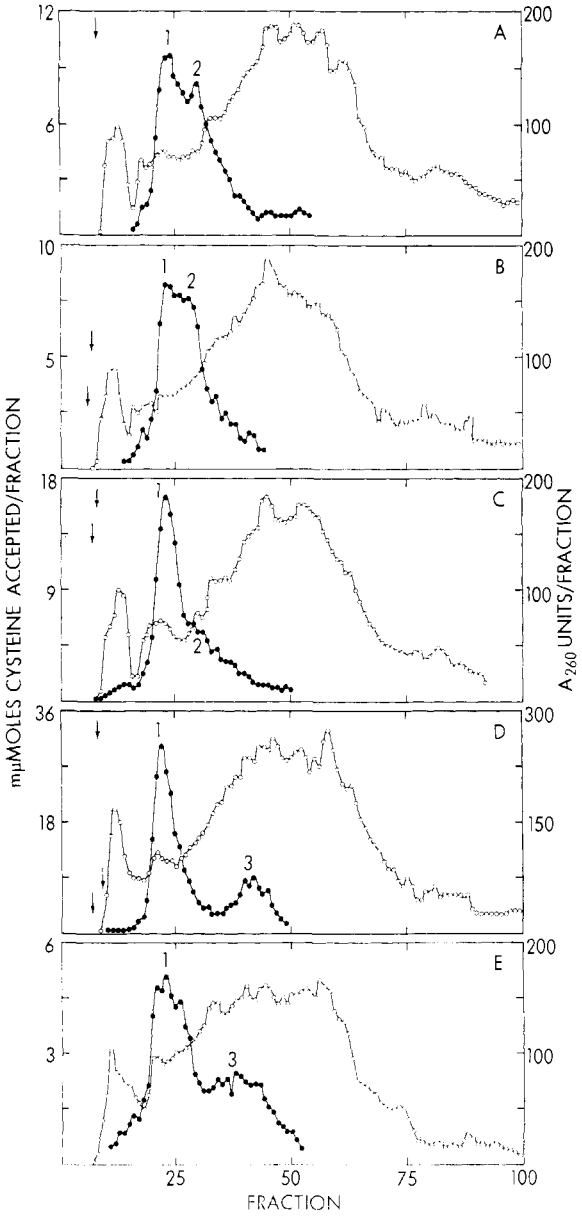

FIGURE 3: Profiles of A_{260} (O) and $tRNA^{Cys}$ (●). Inputs were 9960 A_{260} units of tRNA lot 1 for column A, 8920 A_{260} units of tRNA lot 1 for B, 9950 A_{260} units of tRNA lot 2 for C, 15,500 A_{260} units of tRNA lot 3 for D, and 10,400 A_{260} units of tRNA lot 4 for E. All columns were 5.5 cm^2 in cross section. Column lengths were A, 216 cm; B, 205 cm; C, 213 cm; D, 216 cm; and E, 213 cm. The arrows indicate positions and relative concentrations of the bromocresol green markers. Recoveries of A_{260} units (see text) and $tRNA^{Cys}$ were, respectively, column A, 85 and 82%; B, 83 and 67%; C, 74 and 103%; D, 76 and 89%; and E, 75 and 71%.

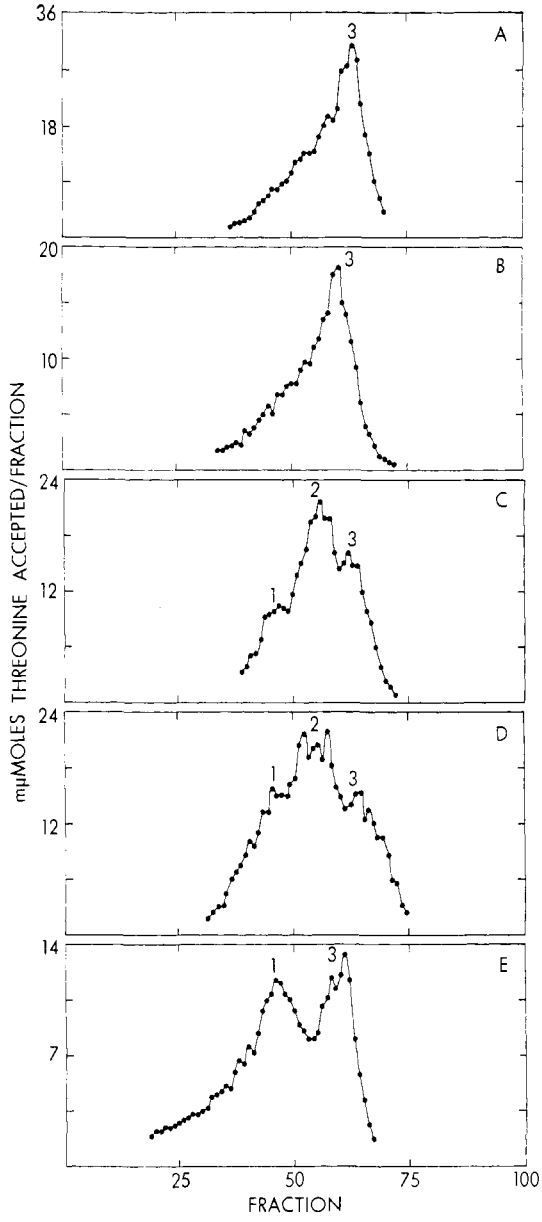

FIGURE 4: Profiles of $tRNA^{Thr}$ for the column runs described in the legend to Figure 3. Recoveries were for column A, 97%; B, 85%; C, 90%; D, 69%; and E, 82%.

from Sigma Chemical Co. or Mann Research Laboratories.

L-[U-^{14}C]Asparagine was freed of any L-[U-^{14}C]aspartic acid by passage in 0.1 M acetic acid over a column of Dowex 1 (Hirs *et al.*, 1954). DL-[1-^{14}C]Cysteine and DL-[3-^{14}C]cysteine were prepared by addition of a twofold molar excess of dithiothreitol (Cleland, 1964) to DL-[1-^{14}C]cystine or DL-[3-^{14}C]cystine in 0.1 M Tris-HCl buffer (pH 8.0). L-[U-^{14}C]Histidine was purified by elution from a column of Dowex 50 in a linear gradient from 1 to 4 N HCl. After flash evaporation of the peak fractions the labeled histidine was passed over a Dowex 1 column in 0.1 M acetic acid (Hirs *et al.*, 1954). L-[3-^{14}C]-Tryptophan was passed over a Dowex 1 column in 0.5 M acetic acid in order to remove radioactive contaminants which produce a high blank in the standard glass filter assay. Amino acid concentrations were determined by the method of Moore and Stein (1954).

Chloroquine dihydrochloride was purchased as a sterile solution of Aralen (50 mg/ml) from Winthrop Laboratories. DE-52 cellulose was purchased from H. Reeve Angel and Co. Cetyltrimethylammonium chloride was the product, Arquad 16–50, of Armour Industrial Chemical Co. 2-Ethylisothionicotinamide was provided by Ives Laboratories as ethionamide hydrochlo-

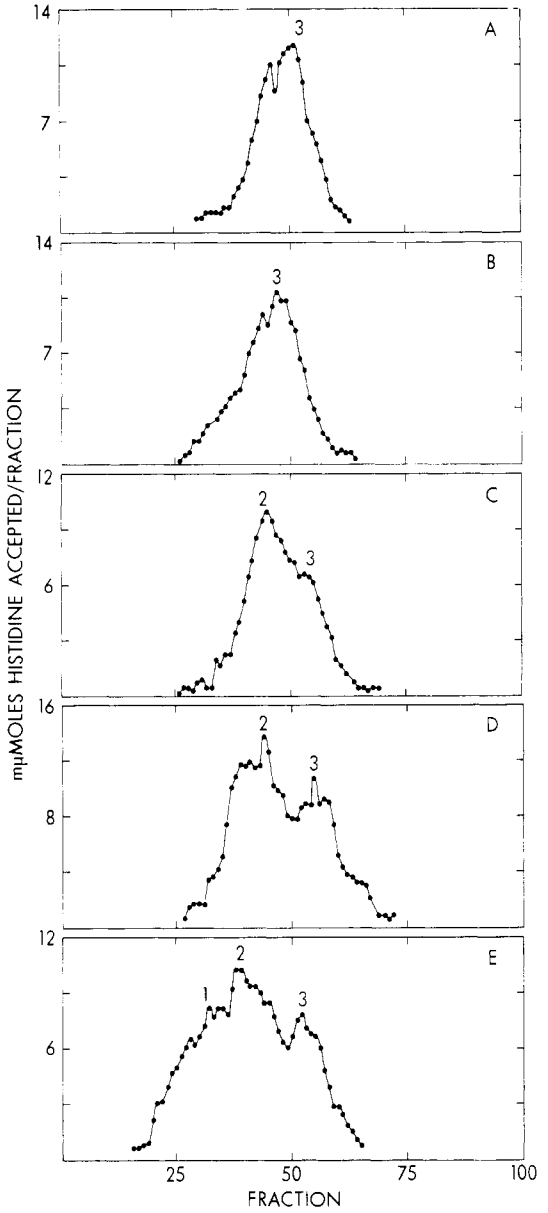

FIGURE 5: Profiles of $tRNA^{His}$ for the column runs described in the legend to Figure 3. Recoveries were for column A, 85%; B, 88%; C, 93%; D, 105%; E, 89%.

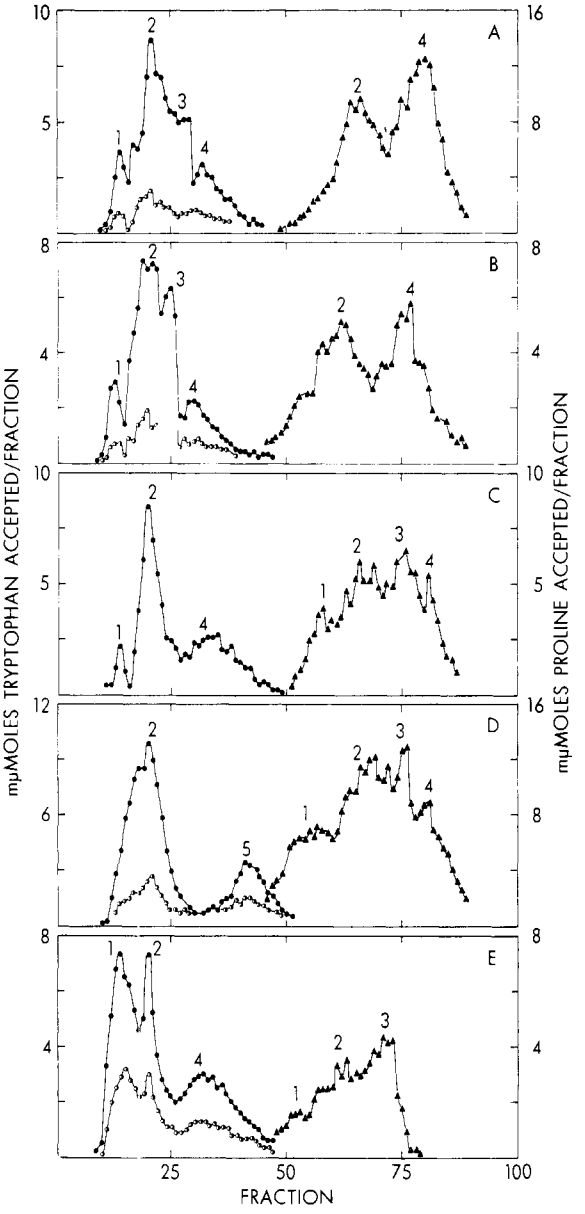

FIGURE 6: Profiles of $tRNA^{Pro}$ (▲) and $tRNA^{Trp}$ assayed with 2.5 mM chloroquine (●) or without chloroquine (◑) for the column runs described in the legend to Figure 3. Recoveries of $tRNA^{Pro}$ were for column A, 145%; B, 63%; C, 108%; D, 74%; and E, 89%. Recoveries of $tRNA^{Trp}$ were for column A, 64%; B, 95%; C, 67%; D, 62%; and E, 73%.

ride. Dithiothreitol was purchased from Calbiochem. Other materials were purchased from sources previously named (Muench and Berg, 1966a–c).

Methods. Asparaginase was determined by measuring disappearance of L-[U-^{14}C]asparagine and appearance of L-[U-^{14}C]aspartic acid after incubation of L-[U-^{14}C]asparagine in the standard tRNA charging assay mixture with a measured amount of partially purified asparaginyl-tRNA synthetase. After termination of the reaction by addition of one-tenth volume of 1 M acetic acid, an aliquot was placed over a 8 cm × 0.2 cm^2 Dowex 1 column equilibrated with 0.1 M acetic acid. Stepwise column development ensued with 5 ml of 0.1 M acetic acid, 5 ml of 0.5 M acetic acid, and 5 ml of 1.0 N HCl; five 1-ml fractions were collected during each step. An aliquot of each was dried on a GF/C disk, placed in scintillator solution, and counted. L-[U-^{14}C]-Asparagine emerged in the 0.1 M acetic acid; L-[U-^{14}C]-aspartic acid emerged in the 1.0 N HCl (Hirs *et al.*, 1954).

Aminoacyl-tRNA synthetases and specific tRNA acceptors were assayed as previously described (Muench and Berg, 1966a,c) with these modifications. (1) Because both phosphate and cacodylate buffers inhibit the prolyl-tRNA synthetase (Mehler and Jesensky, 1966), L-[U-^{14}C]prolyl-tRNA formation was measured in an assay mixture composed of 50 μmoles of Tris-HCl buffer (pH 7.2), 0.50 μmole of ATP, 2.5 μmoles of $MgCl_2$, 0.50 μmole of reduced glutathione, and 0.10 μmole of L-[U-^{14}C]proline in a final volume of 0.5 ml. (2) Chloro-

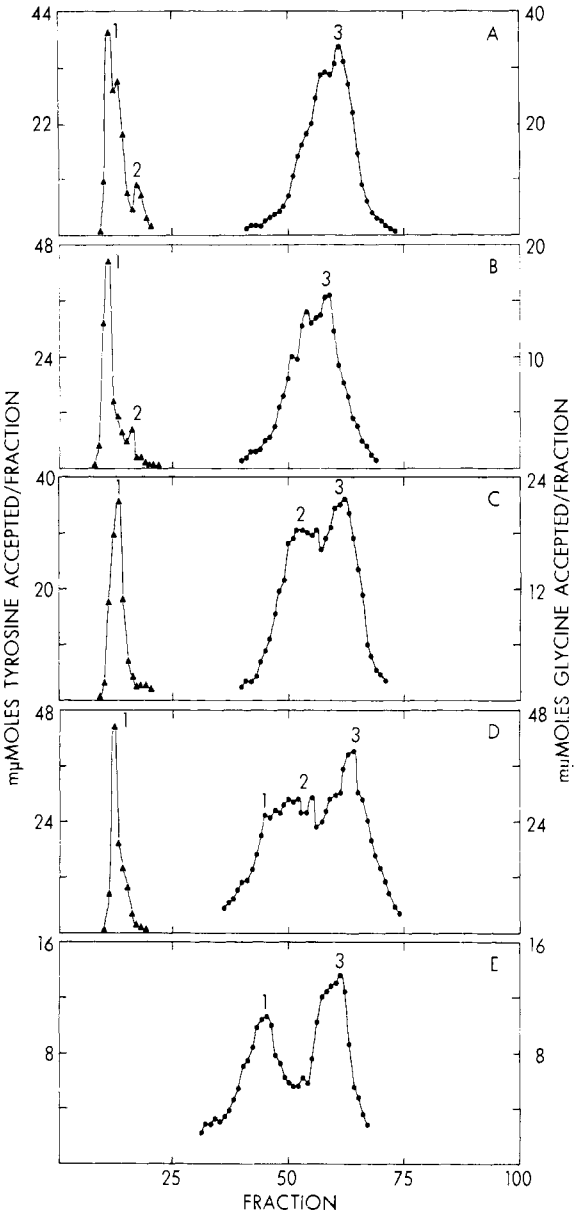

FIGURE 7: Profiles of $tRNA^{Tyr}$ (▲) and $tRNA^{Gly}$ (●) for the column runs described in the legend to Figure 3. Recoveries of $tRNA^{Tyr}$ were for column A, 107%; B, 93%; C, —,[3] and D, 71%. Recoveries of $tRNA^{Gly}$ were for column A, 153%; B, 109%; C, 109%; D, 117%; and E, 61%.

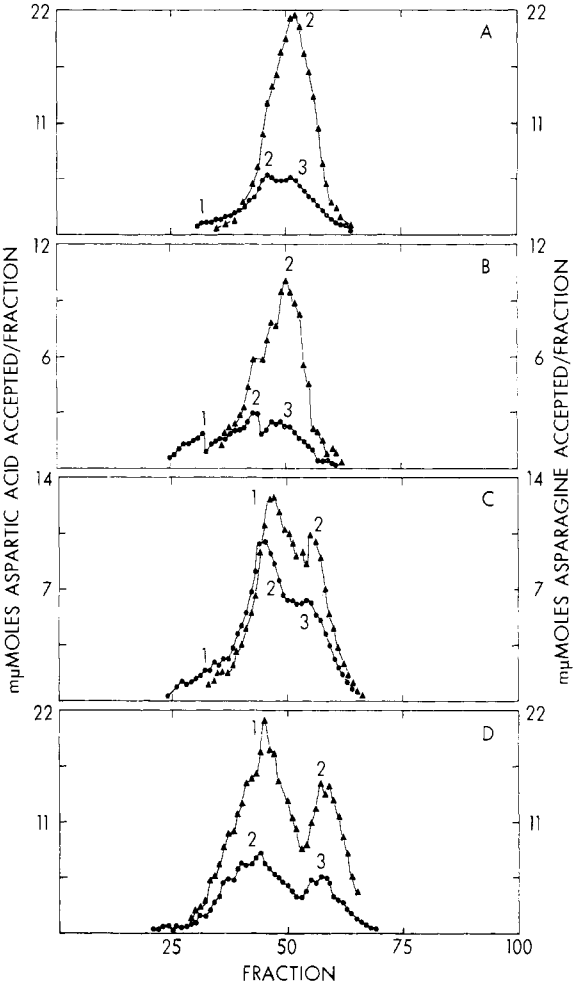

FIGURE 8: Profiles of $tRNA^{Asp}$ (▲) and $tRNA^{Asn}$ (●) for four of the column runs described in the legend to Figure 3. Recoveries of $tRNA^{Asp}$ were for column A, 203%;[4] B, 97%; C, 133%; and D, 102%. Recoveries of $tRNA^{Asn}$ were for column A, 104%; B, 75%; C, 81%; and D, 87%.

quine dihydrochloride (2.5 mM) was present in certain assay mixtures for L-[3-^{14}C]tryptophanyl-tRNA (Muench, 1966).

tRNA adenylyltransferase was measured essentially as described by Preiss *et al.* (1961), but in the medium used to measure aminoacyl-tRNA synthetases and with the GF/C filter method (Muench and Berg, 1966a,c). For substrate the 3′-terminal AMP and CMP was removed from tRNA with snake venom phosphodiesterase as described by Zubay and Takanami (1964).

The partition column was prepared and operated as previously described (Muench and Berg, 1966a) with these modifications. An aqueous solution containing 4000–5000 A_{260} units of tRNA/ml was mixed at 23° with five volumes of initial upper phase containing a trace of bromocresol green, and sediment was removed by brief centrifugation. (This sediment comprises about 5% of the total A_{260} units in tRNA preparations and will not redissolve in water.) The clear supernatant solution was placed into the column matrix, 5.5 × 205–216 cm. The bromocresol green served as a marker, emerging immediately before the first tRNAs.

Development was achieved at 15 ml/hr with 700 ml of initial upper phase, followed by a linear triethylamine gradient established by 2000-ml volumes of the initial and final upper phases, followed by final upper phase un-

[3] Where recovery is not given, the unfractionated tRNA actually placed over the designated column was unavailable and a substitute preparation was assayed in parallel with the column fractions.

[4] The 203% recovery was found again upon repeat assay. A mixing experiment revealed no inhibitor in the unfractionated tRNA. This and other high recoveries may have resulted from differential denaturation of the unfractionated *vs.* the fractionated tRNA.

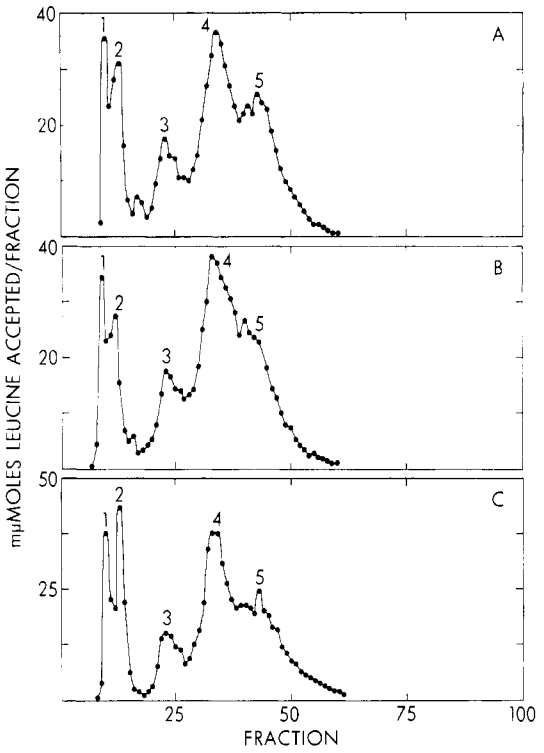

FIGURE 9: Profiles of $tRNA^{Leu}$ for three of the column runs described in Figure 3. Recoveries were for column A, —;[3] B, 101%; and C, 92%.

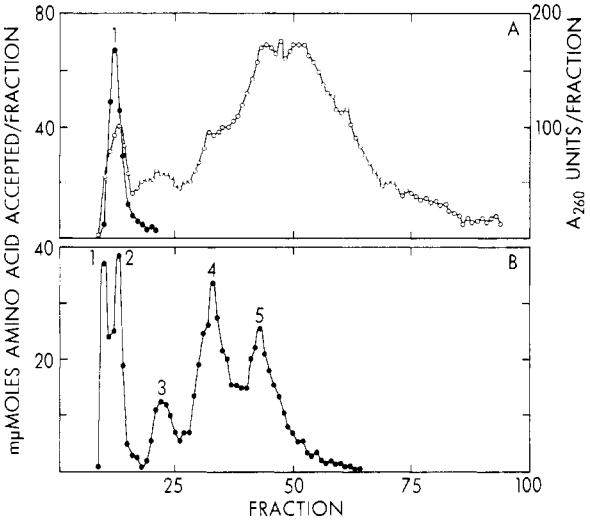

FIGURE 10: Profiles of A_{260} (○), $tRNA^{Tyr}$ (●) (panel A), and $tRNA^{Leu}$ (●) (panel B) resolved from 8200 A_{260} units of tRNA lot 3 on a 5.5 cm^2 × 213 cm column developed at 30 ml/hr. Recovery of A_{260} was 83%; of $tRNA^{Tyr}$, 168%; and of $tRNA^{Leu}$, 88%.

til recovery of tRNA was complete. Non-tRNA material comprising 10–20% of the A_{260} units in the input was eluted by the lower phase of the initial solvent system. Washing the column with the lower phase, then the initial upper phase, prepared it for subsequent runs.

To obtain flow rates up to 60 ml/hr, effluent was collected at the level of the column top by use of 1.5-mm polypropylene tubing.

A new procedure for tRNA isolation adapted from Mirzabekov *et al.* (1964) was performed in Kimax No. 29033-F 125-ml separatory funnels with Teflon stopcocks. The organic solvents were removed from each 60-ml fraction by two extractions with 40 ml of diethyl ether containing 0.01% 2-mercaptoethanol. The lower phase, turbid after one extraction, cleared after the second. H_2O (20 ml) was added, followed by 0.10 M hexadecyltrimethylammonium chloride (4 ml) (64 g of Arquad 16–50 brought to 1000 ml with H_2O) and 20 ml of the ether–mercaptoethanol.

The funnels were warmed in a 37° water bath for 30 min, then allowed to cool to 23°. If the lower phase was not completely clear, the warming step was repeated. The tRNA was now present as a film at the interface. The upper and lower phases were withdrawn, and the tRNA was dissolved in 1 M NaCl, precipitated with two volumes of ethanol, harvested by centrifugation, and finally dissolved in water for assay.

The variation of the tRNA recovery with volumes of H_2O and cetyltrimethylammonium chloride is shown in Figure 2. Recovery did not vary with the range of concentration of triethylamine present in the upper phase, nor with concentration of tRNA up to 1100 A_{260} units/60-ml fraction. Cetyltrimethylammonium chloride up to 10^{-4} M in the usual charging mixture did not interfere with aminoacylation of tRNA.

An IBM 7040 computer was used in the calculation of recoveries of A_{260} units and acceptor activity, the specific acceptor activity of each fraction, and in the graphical representations in Figures 3–10.

Results

Initial attempts to define the number of resolvable tRNAs for the seven amino acids not previously studied (asparagine, cysteine, glycine, histidine, proline, threonine, and tryptophan) revealed different acceptor profiles in two different lots of tRNA, as shown in column runs A and E (Figures 3–7). The profiles in run E were not altered by the presence of the other 19 L-[^{12}C] amino acids, each 0.1 mM. The A_{260} profiles (Figure 3) were structured and highly similar to one another and to those previously published (Muench and Berg, 1966a) enabling location of specific tRNAs by relationship to the A_{260} pattern. Further differences were found in acceptor profiles of two other lots of tRNA (columns C and D, Figures 3–9). The profiles of $tRNA^{Asp}$ (Figure 8) also varied for different lots, whereas those for $tRNA^{Leu}$ (Figures 9 and 10) and $tRNA^{Tyr}$ (Figure 7) were nearly constant.

When the tRNA resolved in column run A (Figures 3–9) was restudied on a different column after 6 months in storage at −20°, the patterns shown in column run B (Figures 3–9) were obtained. In every case the acceptor profile matched that found originally, although in run B each profile was shifted several fractions to the left, presumably because the column in run B was shorter than that in run A. Since a single preparation of tRNA gave identical acceptor profiles on separate columns, the differences noted in acceptor profiles for runs

A–E (Figures 3–8) were taken to reflect actual differences between lots of tRNA.

Closer examination of the profiles revealed a consistent picture for each of the varying acceptors. For each amino acid at least one tRNA peak appeared in a constant location within the acceptor profiles. Other peaks were missing from certain lots but were present or even predominant in other lots. Therefore, the total number of tRNAs resolvable for certain amino acids was evident only after examination of all four profiles.

In all cases the tRNA chains specific for a given amino acid emerged in a continuous profile, no one tRNA being totally separated from all the others of the same specificity.

tRNA^{Cys}. As shown in Figure 3 there were three peaks of tRNA^{Cys}. Whereas tRNA$_1^{Cys}$ was present in all lots, tRNA$_2^{Cys}$ and tRNA$_3^{Cys}$ were detectable in only two lots each.

tRNA^{Thr}. The threonine acceptor profiles revealed three tRNA^{Thr}s, seen in Figure 4. In runs C and D all three were evident, with tRNA$_2^{Thr}$ predominant and tRNA$_1^{Thr}$ and tRNA$_3^{Thr}$ appearing only as shoulders. In runs A and B peak 3 predominated, and peaks 1 and 2 were represented only by an unusually long leading edge to the acceptor profile. In run E, tRNA$_2^{Thr}$ was not evident, and nearly equal quantities to tRNA$_1^{Thr}$ and tRNA$_3^{Thr}$ provided a profile with two peaks.

tRNA^{His}. The histidine acceptor profile revealed one consistent peak, tRNA$_3^{His}$, present in all lots of tRNA (Figure 5). Three lots (runs C–E) contained tRNA$_2^{His}$ in addition, and one lot (run E) contained a shoulder designated tRNA$_1^{His}$.

An attempt to delineate the peaks more clearly with 2-ethylisothionicotinamide, an antituberculous drug which inactivates one of two tRNA^{His}s in beef liver (Wevers and Ralph, 1966), was fruitless. The drug was without effect on the extent of charging any of the tRNA^{His}s of *E. coli*, at concentrations of either 0.10 or 2.0 mM in either the charging medium used herein or that used by Wevers and Ralph (Loftfield and Eigner, 1963).

tRNA^{Trp}. Acceptor activity for tryptophan emerged in a profile of five peaks, only four of which were clearly seen in any single lot of tRNA (Figure 6). In three lots (runs A–D) tRNA$_2^{Trp}$ predominated. In one lot (run E) the usually minor peak tRNA$_1^{Trp}$ predominated. The peak tRNA$_5^{Trp}$ was seen in only one lot (run D).

Each tRNA^{Trp} consisted of tRNA^{Trp} in both active and inactive states (Gartland and Sueoka, 1966; Muench, 1966) as shown by the response to presence of chloroquine during the charging incubation (runs A, B, D, E). Therefore, the two conformations for tRNA^{Trp} which separate upon MAK chromatography (Sueoka and Yamane, 1962) did not contribute to the multiplicity of tRNA^{Trp} peaks seen in partition chromatography.

tRNA^{Pro}. Also depicted in Figure 6 is tRNA^{Pro}, which eluted in what appeared to be four poorly differentiated peaks in runs C and D. In runs A and B tRNA$_2^{Pro}$ and tRNA$_4^{Pro}$ were prominent, and the others were not clearly distinguishable, although tRNA$_1^{Pro}$ contributed to the leading edge of the profile. In run E, tRNA$_4^{Pro}$ was absent entirely, and tRNA$_1^{Pro}$, tRNA$_2^{Pro}$, and tRNA$_3^{Pro}$ were poorly defined.

The only other acceptor as late in the chromatogram as tRNA$_4^{Pro}$ is tRNA^{Ala} (Muench and Berg, 1966a). Recently Yarus and Berg (1967) have shown that material in this region competes with tRNA^{Ile} in binding to isoleucyl-tRNA synthetase and that the material is not tRNA^{Ala}. Altered tRNAs incapable of charging amino acids but still capable of interacting with their aminoacyl-tRNA synthetases may exist in this region.

tRNA^{Gly}. Glycine acceptor emerged as three peaks, only one of which, tRNA$_3^{Gly}$, was detectable in all four lots of tRNA (Figure 7), appearing with tRNA$_1^{Gly}$ in run E and with tRNA$_2^{Gly}$ in run C. In run D all three were present, tRNA$_1^{Gly}$ and tRNA$_2^{Gly}$ appearing as an unresolved plateau before the tRNA$_3^{Gly}$ peak.

tRNA^{Tyr}. The relatively constant tRNA^{Tyr} profile, previously described in other tRNA preparations (Muench and Berg, 1966a), provided a useful comparison with the more variable species (Figure 7). In every case tRNA$_1^{Tyr}$ dominated the profile, but in one lot (runs A and B) a minor tRNA$_2^{Tyr}$ was definable. Pearson and Kelmers (1966) have described a tRNA$_2^{Tyr}$ seen in hydroxylapatite chromatography.

tRNA^{Asn} and tRNA^{Asp}. The asparagine acceptor profile consisted of three peaks, and the aspartic acid acceptor profile consisted of two peaks, as shown in Figure 8. Although the profiles for tRNA^{Asp} and tRNA^{Asn} were clearly differentiated by use of the purified activating enzymes, tRNA$_2^{Asn}$ and tRNA$_1^{Asp}$ overlapped, as did tRNA$_3^{Asn}$ and tRNA$_2^{Asp}$. The independent variation of the two acceptor profiles indicated separate acceptors for the two amino acids. The possibility that the two amino acids had major common acceptors among the tRNAs within the profile was ruled out by the findings in Table I, obtained by periodate oxidation studies. tRNA^{Asp} was unable to accept asparagine, and tRNA^{Asn} was unable to accept aspartic acid at significant levels.

tRNA^{Leu}. In marked contrast to the variable patterns of other tRNAs was the constancy of tRNA^{Leu}, shown for 3 of the lots of tRNA in Figures 9 and 10. The familiar five-peak pattern, identical with that described for other lots of tRNA (Muench and Berg, 1966a), served as an internal control for the other acceptor profiles.

Recovery and Purification. The recovery of A_{260} units listed in each figure legend does not include material eluting when the columns were washed with lower phase during regeneration. This material had no acceptor activity for the 20 coded amino acids. When it was included, over-all recovery of A_{260} units was 95–100%.

The variation of recovery of acceptor activity probably resulted from chemical hydrolysis of aminoacyl-tRNAs during the assay procedure, as pointed out by Gillam *et al.* (1967). The variation in recovery played no role in the variability of acceptor profiles, as can be seen by comparison of recoveries and profiles in Figures 3–8.

Purification varied with position in the chromatogram. Thus the peak fractions of tRNA$_1^{Cys}$ or tRNA$_4^{Pro}$ had specific activities 10–14 times greater than unfractionated tRNA, without correction for multiple peaks,

TABLE I: Specificity of $tRNA^{Asp}$ and $tRNA^{Asn}$.[a]

	$tRNA^{Asp}$		$tRNA^{Asn}$	
	pmoles of Amino Acid/A_{260} unit	%	pmoles of Amino Acid/A_{260} unit	%
Original charge	25 Asp	100	12 Asn	100
Remaining after oxidation	23 Asp	92	12 Asn	100
Remaining after discharge	<0.3 Asp	<2	<1.3 Asn	<11
Recharge	21 Asp	84	8.1 Asn	68
Recharge	3 Asn	12	<0.3 Asp	<3
Recharge	<1 Tyr	<4	<1 Tyr	<9

[a] tRNA (94 A_{260} units) (Schwarz, lot 6703) was charged with labeled aspartic acid or asparagine as in Methods. The charged tRNA was isolated from the assay mixture by precipitation with potassium acetate buffer (pH 4.8) and ethanol, and dissolved in 0.005 M $NaIO_4$–0.1 M potassium acetate buffer (pH 4.8). After 30 min at 23°, the tRNA was collected by addition of ethanol and centrifugation. The oxidation step was repeated until the supernatant contained excess IO_4^-, as measured by A_{232} before and after addition of excess ethylene glycol. The oxidized tRNA pellet was dissolved in 0.01 M ethylene glycol and precipitated with salt–ethanol. The charged amino acids were removed enzymatically and the tRNA was isolated from the reaction mixture on G-25 Sephadex columns (Muench and Berg, 1966b). The tRNA was then assayed for ability to charge asparagine, aspartic acid, and tyrosine. All values were obtained from acid-insoluble counts retained on GF/C filters as in Methods.

whereas those tRNAs eluting in the central region of the chromatogram were less well purified.

Effect of Flow Rate on Resolution. The ability of the column to resolve tRNAs at increased rates of flow is shown in Figure 10. The five peaks of $tRNA^{Leu}$ and the single $tRNA^{Tyr}$ peak were resolved as well at 30 ml/hr (Figure 10) as at 15 ml/hr (Figures 7 and 9). At 60 ml/hr $tRNA^{Leu}$ was less well resolved, emerging as two peaks, one in the position of $tRNA_{1,2}^{Leu}$ and one in the position of $tRNA_{3-5}^{Leu}$. A purification at 60 ml/hr can be used to separate $tRNA_{1,2}^{Leu}$ from $tRNA_{3-5}^{Leu}$ preliminary to finer resolution at lower flow rates.

Discussion

As summarized in Table II, gradient partition column chromatography reveals at least 56 tRNAs for 20 amino acids, although no single lot of tRNA contains each of these tRNAs in sufficient amount to be detected against the background of isoacceptors with similar chromatographic mobility. The figure is conservative, because tRNAs for only 10 amino acids have been examined in detail. Moreover peaks not resolved by this method are detectable by other methods, as, for example, $tRNA_3^{Ala}$ is resolved by hydroxylapatite chromatography (Muench and Berg, 1966b).

The validity of multiple acceptor peaks in gradient partition chromatography has been demonstrated by several criteria (Muench and Berg, 1966a). First, peaks rechromatograph in original positions. Second, tRNA recovered from the partition solvent is free of aggregates. Finally, tRNA prepared as described is free of hidden internucleotide breaks and is at least 95% intact in terms of its 3′-terminal adenylate residue. Although a single missing 3′-terminal adenylate residue can cause separation of tRNAs otherwise identical (RajBhandary *et al.*, 1966), the differences in acceptor profiles seen in the present work cannot be so explained, because the differences were seen when aminoacyl-tRNA synthetases free of tRNA adenylyltransferase were used for assay.

Another possible explanation for multiplicity in acceptor peaks is that each represents a different conformation of tRNA rather then a different primary structure (Lindahl *et al.*, 1966; Gartland and Sueoka, 1966; Muench, 1966). That has proved to be the case in MAK chromatography of $tRNA^{Trp}$ from *E. coli* (Sueoka and Yamane, 1962; Gartland and Sueoka, 1966; Muench, 1966). However, since both the active and inactive conformations of all five $tRNA^{Trp}$ peaks migrate together on gradient partition columns, in at least that instance multiple conformations cannot be invoked to explain multiple peaks. During partition chromatography in the organic solvent the five $tRNA^{Trp}$ peaks may actually each exist in a single intermediate conformation, which changes to either of the two activity states in the transition to aqueous medium during isolation of the tRNA from the partition solvent.

The role of multiple tRNAs for each amino acid is not understood. There is no uniform correlation between multiplicity of tRNAs and degeneracy of the genetic code. Certain isoacceptor tRNAs have different coding properties *in vitro* in response to both synthetic (Weisblum *et al.*, 1962; Von Ehrenstein and Dais, 1963; Bennett *et al.*, 1963) and natural mRNA (Weisblum *et al.*, 1965, 1967; Gonano, 1967). However, in yeast one $tRNA^{Phe}$ may translate more then one codon, and two $tRNA^{Gly}$s may translate the same codon (Söll *et al.*, 1966). In yeast two $tRNA^{Ser}$s have the same probable anticodon, IGA, according to complete primary structure determinations (Zachau *et al.*, 1966). Presumably both $tRNA^{Ser}$s would translate the same codon.

At least one aminoacyl-tRNA of a group specific for

one amino acid may function as a corepressor of the enzymes synthesizing that amino acid (Eidlic and Neidhardt, 1965; Neidhardt, 1966; Freundlich, 1967). Thus histidyl-tRNA in *E. coli* is a probable corepressor of the histidine synthetic enzymes, as shown by studies with α-methylhistidine, an inhibitor of histidyl-tRNA synthetase (Schlesinger and Magasanik, 1964). Similarly, the data of Silbert *et al.* (1966) on HisR mutants in *Salmonella typhimurium* suggest that histidyl-tRNA acts as a core pressor of the histidine operon. The HisR mutants, derepressed for enzymes of the histidine biosynthetic pathway, have only one-half the normal level of $tRNA^{His}$, suggesting total absence of one of two varieties. However, attempts to resolve two varieties of $tRNA^{His}$ in *S. typhimurium* by MAK, DEAE-Sephadex, hydroxylapatite, and gel filtration columns or in *E. coli* by countercurrent distribution have not been successful (Silbert *et al.*, 1966). Since gradient partition chromatography has resolved three (*E. coli*) $tRNA^{His}$s, it may be applicable to further study of the HisR mutants. Two (*E. coli*) $tRNA^{His}$s have been resolved by reversed-phase partition chromatography (Weiss and Kelmers, 1967) and by hydroxylapatite chromatography (Harding *et al.*, 1966).

Corollary to the problem of multiplicity is that of variation in relative levels of isoacceptors. Changes in kinds or amounts of tRNA in bacteria in response to various environmental stimuli have been reported. Lazzarini and Peterkofsky (1965) demonstrated that during methionine starvation *E. coli* 58–161 (RC^{rel} Met^-) synthesizes a different $tRNA^{Leu}$, detectable on MAK columns, and they presented evidence that this $tRNA^{Leu}$ was methyl deficient. The work suggests that multiple tRNAs for a single amino acid can be interconverted by modifications of certain bases, for example, by the tRNA methylases. Kaneko and Doi (1966) observed changes in relative amounts of two $tRNA^{Val}$s distinguished by MAK columns during sporulation of *Bacillus subtilis*, but opposite findings were reported by Heyman *et al.* (1967).

The same technique revealed differences in the number of $tRNA^{Ser}$s in *B. subtilis* as a function of growth medium (Goehler *et al.*, 1966). Lazzarini and Santangelo (1967) noted a new $tRNA^{Lys}$ in sporulating *B. subtilis*, but only when sporulation occurred on certain media.

Sueoka and Kano-Sueoka (1964) first observed appearance of a different $tRNA^{Leu}$ distinguishable by MAK chromatography in T2 phage-infected *E. coli*. The change was specific for T-even phages, but occurred in a variety of *E. coli* strains and in *Shigella dysenteriae* (Kano-Sueoka and Sueoka, 1966). Others (Waters and Novelli, 1967) confirmed the finding and reported new $tRNA^{Leu}$ appearing later in infection by use of reversed-phase partition columns (Kelmers *et al.*, 1965). Pollack (1966) was unable to see changes in *E. coli* $tRNA^{Leu}$ after phage T2 and T4 infection.

The present work has revealed marked variation among different lots of tRNA in levels of isoacceptors for eight amino acids. In contrast isoacceptors for leucine and tyrosine were relatively constant. The differences observed in elution profiles of specific tRNAs isolated from different batches of commercially grown *E. coli* B may have resulted from undefined variations in physiologic state either existing when harvest was begun, or arising from harvest and washing procedures. All batches were stated to be harvested at three-fourths of maximum turbidity during exponential growth. However, such variables as batch size and attendant cooling rate during harvest are not known. Although the bacteria were stated to be grown on two different media, variation between tRNAs from two batches of cells grown on the same medium was as great as variation between tRNAs from cells grown on different media. In the past, we have found marked variation in levels of whole tRNA and aminoacyl-tRNA synthetases in various batches of commercially grown cells.

TABLE II: tRNAs in *E. coli* by Gradient Partition Chromatography.[a]

Amino Acid	No. of Peaks
Alanine	2
Arginine	2
Asparagine	3
Aspartic acid	2
Cysteine	3
Glutamic acid	2
Glutamine	2
Glycine	3
Histidine	3
Isoleucine	2
Leucine	5
Lysine	2
Methionine	2
Phenylalanine	3
Proline	4
Serine	4
Threonine	3
Tryptophan	5
Tyrosine	2
Valine	2
20 amino acids	56

[a] Data are from the present and from previous work (Muench and Berg, 1966a).

The possibility of differential removal or inactivation of tRNA during purification from cells is unlikely for several reasons. Since the five-peak $tRNA^{Leu}$ profile is an almost constant feature in gradient partition chromatography, $tRNA^{Leu}$s would have to be exempt from such loss. Secondly, the differential loss of $tRNA_1^{Thr}$, for example, in one preparation of tRNA and $tRNA_2^{Thr}$ in another is unlikely. Finally, there are no regular differences in acceptor profiles for the tRNAs prepared by the two different methods.

Column artifact is an unlikely explanation for the differences in acceptor profiles because of the reproducibility of acceptor profiles of duplicate runs on different columns, and because of the constancy of $tRNA^{Leu}$ and

$tRNA^{Tyr}$ control profiles. In addition, variable tRNA profiles differ independently of others in the same region of the chromatogram.

The present observations cannot define the cause for the variation in isoacceptor levels. The results emphasize that rigid physiologic controls must precede invoking specific reasons for such variation.

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