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Fractionation of Transfer Ribonucleic Acid by Gradient Partition Chromatography on Sephadex Columns*

Karl H. Muench† and Paul Berg

ABSTRACT: t-RNA (transfer ribonucleic acid) from *Escherichia coli* may be resolved on partition columns composed of Sephadex G-25 fine beads and a biphasic solvent mixture. The mixture is composed of potassium phosphate pH 6.88 buffer, ethoxyethanol, butoxyethanol, mercaptoethanol, and triethylamine. Linear variation in triethylamine concentration results in an exponential variation of the partition coefficient of t-RNA in the mixture, allowing t-RNA dissolved in aqueous phase immobilized on the Sephadex beads to be extracted by mobile phase containing a gradient of triethylamine. Resolution is sufficient to provide individual amino acid specific t-RNA's of 25–40% purity, as much as 24-fold enriched, in one passage over the column.

Of 13 specific t-RNA's examined, at least 9 are heterogeneous, and 5 separable varieties of t-RNA accept leucine. Results are reproducible for columns of varying diameter, and at least 3.4 g of t-RNA may be fractionated with no decrease in resolution. Except for mechanical losses, recoveries of t-RNA and acceptor activities are complete.

Transfer RNA (t-RNA) is a mixture of many structurally similar polynucleotides each of which participates in the activation and transfer of a specific amino acid in protein synthesis (Berg, 1961; Brown, 1963). Complete resolution of the different t-RNA species and the determination of their primary and secondary structures are needed to establish how each aminoacyl RNA is formed and interacts specifically with an m-RNA–ribosome complex in peptide bond synthesis. Countercurrent distribution (CCD)[1] has proved to be the most successful method to date for resolving different amino acid specific t-RNA's on a preparative scale. First introduced for RNA fractionation by Warner and Vaimberg (1958), it was utilized for the isolation of yeast t-RNA_{tyr}, t-RNA_{val}, and t-RNA_{ala} (Holley *et al.*, 1963) with subsequent total primary sequence determination of the latter (Holley *et al.*, 1965). Others have obtained highly purified yeast t-RNA_{ser} (Tada *et al.*, 1962; Rushizky *et al.*, 1964) and yeast t-RNA_{phe} (Hoskinson and Khorana, 1965). t-RNA from *Escherichia coli* has also been highly resolved (Goldstein *et al.*, 1964) by partitioning through a large number of transfers. However, CCD requires elaborate and expensive equipment, and the isolation of t-RNA from each fraction is time consuming.

Chromatographic methods employing columns of methylated albumin-kieselguhr (Mandell and Hershey, 1960; Sueoka and Yamane, 1962), DEAE-cellulose and DEAE-Sephadex (Cherayil and Bock, 1965; Kawade

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[1] Abbreviations used in this work: t-RNA_{leu} or t-RNA_{ileu}, t-RNA which accepts leucine or isoleucine, respectively. Leu-RNA or ileu-RNA are used to designate the esterified forms of the t-RNA's. Where more than one species of t-RNA_{leu} occurs we have, in the absence of any systematic way of designating the multiple forms of RNA obtained in fractionation procedures, referred to them by the order of elution from the partition column, *e.g.*, t-$RNA_{leu,1}$ is the first peak of t-RNA_{leu}. Other abbreviations: PPO, 2,5-diphenyloxazole; dmPOPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; CCD, countercurrent distribution; AMP, adenosine monophosphate; CTP, cytidine triphosphate; ATP, adenosine triphosphate.

et al., 1963), and hydroxylapatite (Hartman and Coy, 1961; Muench and Berg, 1966) have offered supplementary approaches. The relative stability of aminoacyl t-RNA in the buffers used in such methods permits resolution of isotopically labeled aminoacyl t-RNA preparations, obviating the need for laborious enzymatic assays of all fractions to locate the desired t-RNA species. However, resolution has not equalled that of CCD, and low capacity usually limits most of these approaches to analytical procedures.

In an attempt to combine the resolution and capacity of CCD with the operational simplicity of column chromatography we have focused on column partition chromatography. Everett *et al.* (1960) had obtained partial resolution of t-RNA on silicic acid columns developed with the buffer system used in CCD, but there were excessive losses of t-RNA, presumably by adsorption to the column matrix. Because Sephadex is hydrophilic and does not adsorb t-RNA in the presence of dilute salt solutions its trial as a column matrix seemed worthwhile. Others (Tanaka *et al.*, 1962) have partially resolved t-RNA on partition columns supported by a Sephadex matrix.

Liquid–liquid anion exchange (Khym, 1963, 1965) offered a potentially useful modification of the partition process. In anion exchange, alkylammonium ions in a biphasic solvent system may form salts with a polyanionic species, thereby increasing its solubility in the organic phase.[2] The partitioning of the polyanion, in this case t-RNA, is therefore dependent on its ability to form alkylammonium salts as well as on solubility factors. Since the propensity to form such salts may differ for the various t-RNA chains, the process affords additional possibilities for resolution of t-RNA.

One distinction between column partition chromatography and CCD is the opportunity in the former for gradient elution by means of an increasing partition coefficient. The increase may be effected by raising the concentration of alkylammonium ion in the mobile phase. We have developed a fractionating method employing a biphasic solvent system in which t-RNA dissolved in an aqueous phase immobilized on a column of Sephadex G-25 beads is fractionally extracted by a mobile, organic phase containing triethylammonium as the alkylammonium cation (Muench and Berg, 1964). This system is capable of separating different amino acid acceptor t-RNA's and of resolving certain of the amino acid specific acceptors into multiple components. Those t-RNA's with the highest and the lowest partition coefficients are recovered with purities ranging from 25 to 40%.

Experimental Section

Materials. E. coli B cells, grown in complex medium and harvested in exponential phase, were purchased from Grain Processing Company, Muscatine, Iowa, and were used as a source for t-RNA and aminoacyl RNA synthetases. Certain aminoacyl RNA synthetases were purified from *E. coli* B grown on a glucose–mineral salts medium.

t-RNA was isolated essentially as described by Zubay (1962) except that the incubation at pH 10.3 was omitted. At this pH losses up to 30% in acceptor activity of the t-RNA were noted. Solution of t-RNA in the upper phase of the partition solvent hydrolyzes the aminoacyl linkages, leu-RNA having a half-life of 1 hr at 23°. Under the same conditions there is no detectable loss in acceptor activity for at least 2 weeks.

The t-RNA prepared through the 2-propanol step (Zubay, 1962) still contains variable amounts of protein, nonpentose sugar, and DNA, and more significantly, RNA, which does not accept amino acids. After solution and recovery of the RNA from the upper phase of the partition system the t-RNA preparation contains less than 2% protein (Lowry *et al.*, 1951), 1% DNA (Dische, 1955), and 0.5% nonpentose sugar (Seifter *et al.*, 1950). Most of the inactive RNA does not dissolve in the upper phase.

Usually, t-RNA from 2 kg of *E. coli* cell paste purified through the 2-propanol step (Zubay, 1962) was freeze dried. It was mixed at 23° with 200 ml of the upper phase of the partition system (made with 0.02 volume of triethylamine) until no solid material remained. Water (40 ml) was added to the two liquid phases and after equilibration at 23° the mixture was centrifuged to remove the resultant sediment. The sediment was mixed with 100 ml of upper phase, then with 20 ml of water, and after equilibration as above the mixture was centrifuged. The two supernatants were combined, and the t-RNA was recovered as described for the column fractions. The usual yield of t-RNA was at least 4 g from 2 kg of cell paste.[3]

The t-RNA preparation at this stage has a faint yellow color in concentrated solution. The yellow material moves as a single band on subsequent partition chromatography, emerging before t-RNA. Heating of the t-RNA at 100° for 5 min followed by rapid cooling produced no loss in $t\text{-RNA}_{tyr}$ or $t\text{-RNA}_{val}$ acceptor activity, a fact indicating the absence of hidden breaks in the polynucleotide chains. Over 95% of the t-RNA chains are probably terminated by the normal CpCpA end group since only 4% of the theoretical amount of AMP could be incorporated from [^{14}C]ATP as substrate with added CTP and mediated by t-RNA–pCpCpA pyrophosphorylase (Preiss *et al.*, 1961).

Aggregates in t-RNA, which increase in relative amount as a consequence of CCD, have been detected during chromatography of t-RNA on Sephadex G-100 (Schleich and Goldstein, 1964). This aggregated material is inactive as amino acid acceptor but can be activated by heating in urea solutions (Schleich and Goldstein, 1964). t-RNA recovered from the partition solvent described here appears to be free of such ag-

[2] In the description of our biphasic solvent systems the words upper, organic, or mobile refer to one phase, and the terms lower, aqueous, and stationary refer to the other.

[3] We assume that 1 mg of sodium t-RNA dissolved in 1 ml of aqueous neutral buffer has an absorbance of 24 in a 1-cm light path.

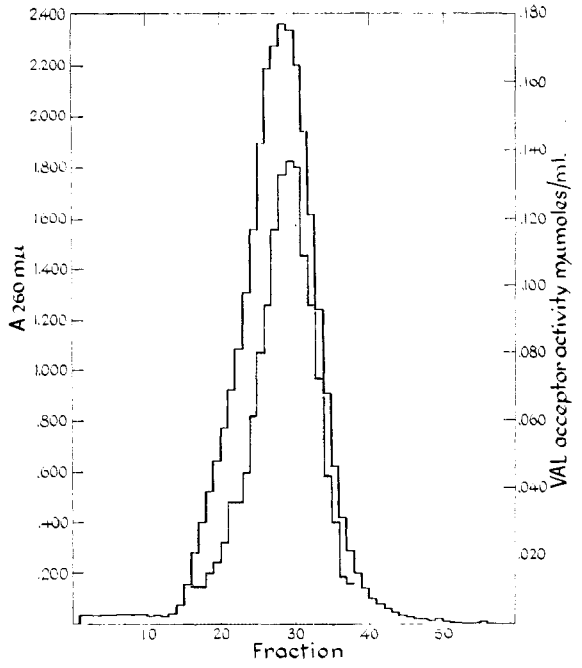

FIGURE 1: Gel filtration of t-RNA on Sephadex G-100. t-RNA (24 mg) in 1 M NaCl was passed over a column (1 cm × 103 cm) of Sephadex G-100 (bead form), previously equilibrated with 1 M NaCl, and eluted with the same salt solution at a flow rate of 11 ml/hr. RNA was recovered from each fraction by alcohol precipitation, and t-RNA_{val} in the recovered material was assayed as described in Methods.

gregates, since more than 97% of the t-RNA and 100% of t-RNA_{val} activity is recovered as a single peak from gel filtration on Sephadex G-100 (Figure 1). There does appear, however, to be slight separation of the t-RNA_{val} from the total RNA as judged by the change in valine-acceptor activity in fractions comprising the peak.

Valyl-, isoleucyl-, methionyl-, and leucyl-RNA synthetases were prepared as described by Bergmann *et al.* (1961) and tyrosyl-RNA synthetase was isolated according to Calendar and Berg (1965). Leucyl-RNA synthetase from yeast was prepared from commercial Fleischmann's yeast by a modification of the method of Lagerkvist and Waldenström (1964), with omission of the Sephadex and DEAE column steps. The final preparation, assayed with excess *E. coli* t-RNA, contained 78 units/mg of protein, was 17-fold purified over the initial extract, and contained no detectable t-RNA_{leu} (<0.0005 mμmole of t-RNA_{leu}/unit of enzyme). The preparation also contained 22 units of valyl-RNA synthetase/mg and 8.3 units of seryl-RNA synthetase/mg. One unit of aminoacyl RNA synthetase is equal to the formation of 1 mμmole of aminoacyl RNA in 10 min when assayed as described below. All aminoacyl RNA synthetases were stored in 50% glycerol at −15° with no detectable loss of activity for longer than a year.

Assay for arginine, aspartic acid, glutamic acid, glutamine, histidine, lysine, phenylalanine, and serine acceptor activities was done with a mixed aminoacyl RNA synthetase preparation made as follows: 30 g of *E. coli* B were suspended in 135 ml of 0.05 M glycylglycine, pH 7.0, and sonicated for 20 min (Raytheon Sonic Oscillator, 10 kc). The extract was centrifuged for 1 hr at 30,000*g*; the supernatant fluid was adjusted to 10 mg of protein/ml; and approximately 2 ml of freshly dissolved 5% streptomycin sulfate was added for each 10 ml of diluted extract over a 10-min period. After 10 min of stirring, the suspension was centrifuged as above for 20 min and the supernatant fluid was adjusted to pH 8 with 0.1 N KOH. The solution was passed over a DEAE-cellulose column (30 cm × 3 cm^2) previously equilibrated with 0.02 M potassium phosphate buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol. The column was washed with several column volumes of the phosphate–mercaptoethanol buffer and the mixed synthetases were eluted with 0.25 M potassium phosphate, pH 6.5. The fractions containing the bulk of the protein were pooled and concentrated to about 4 ml by dialysis against 0.01 M potassium phosphate, pH 6.8, containing 0.01 M reduced glutathione and 25% polyethylene glycol 6000. The dialyzed, concentrated solution was mixed with an equal volume of glycerol and stored at −15°. Aminoacyl RNA synthetase activities in units per mg for the amino acids cited were: arginine, 160; aspartic acid, 140; glutamic acid, 72; glutamine, 53; histidine, 11; lysine, 60; phenylalanine, 91; and serine, 16.

Alanyl-RNA synthetase of *E. coli* was isolated from the streptomycin supernatant described above by employing a phosphate gradient elution from the DEAE-cellulose column. The gradient was linear, using 1 l. each of 0.02 M potassium phosphate buffer, pH 7.5, and 0.25 M potassium phosphate buffer, pH 6.5, both containing 0.01 M 2-mercaptoethanol, at a flow rate of 40 ml/hr. The enzyme peak appeared after 800 ml, and upon concentration by dialysis against 0.05 M potassium phosphate containing 0.01 M 2-mercaptoethanol, 0.01 M EDTA (ethylenediaminetetraacetate), and 10% polyethylene glycol 6000, it was passed over a column of hydroxylapatite (15 cm × 0.8 cm^2) previously equilibrated with 0.1 M potassium phosphate, pH 6.8–0.01 M 2-mercaptoethanol. The enzyme was eluted with a linear gradient between 250 ml of the equilibrating buffer and the same volume of 0.2 M potassium phosphate buffer, pH 6.8–0.01 M 2-mercaptoethanol at a flow rate of 24 ml/hr. The peak fractions were pooled, concentrated, and stored as described above for the mixed enzyme preparation. The recovery and purification of alanyl-RNA synthetase activity relative to the sonic extract was approximately 25% and 25-fold, respectively (final specific activity, 105 units/mg of protein). t-RNA–pCpCpA pyrophosphorylase was isolated from *E. coli* and assayed as described by Preiss *et al.* (1961).

DL-[3-^{14}C]Phenylalanine and L-[3,5-^{3}H]tyrosine were purchased from New England Nuclear Corporation. DL-[1-^{14}C]Alanine, L-[G-^{14}C]arginine, L-[U-^{14}C]glutamine, DL-[1-^{14}C]valine, L-[1-^{14}C]isoleucine, DL-[3-^{14}C]-aspartic acid, and L-[2(ring)-^{14}C]histidine were pur-

chased from California Corporation for Biochemical Research. DL-[1-^{14}C]Lysine and DL-[3-^{14}C]serine were purchased from Isotopes Specialties Company, L-[^{14}C]-glutamic acid and DL-[1-^{14}C]leucine were from Volk Radiochemicals, and L-[methyl-^{14}C]methionine was purchased from Nuclear Chicago Corporation.

The [^{14}C]phenylalanine was purified from material giving a high blank in the glass filter assay (see below) by adsorption from 2 N HCl to a Dowex-50 column (2 cm $\times$ 0.8 cm^2) and elution with 4 N HCl (Hirs *et al.*, 1954). After pooling of peak fractions and evaporation to dryness *in vacuo*, the phenylalanine was dissolved in 0.5 N acetic acid and washed through a Dowex-1 column (2 cm $\times$ 0.8 cm^2) with 0.5 N acetic acid. The peak fractions were pooled and evaporated *in vacuo* to yield the phenylalanine acetate. Glutamic and aspartic acids dissolved in 0.1 N acetic acid were passed over Dowex-1 columns as above and eluted with 0.5 N acetic acid after washing the column with 0.1 N acetic acid to assure removal of any contaminating glutamine or asparagine (Hirs *et al.*, 1954). Glutamine was freed of contaminating glutamic acid by the same procedure, except that the material eluted with 0.1 N acetic acid was collected. All other radioactive amino acids were used as supplied, diluted with their unlabeled counterparts (purchased from California Corporation for Biochemical Research) to specific activities of 3000 8000 cpm/mμmole. Other labeled substrates were [^{32}P]PP_i prepared according to Bergmann *et al.* (1961) and [8-^{14}C]ATP purchased from Schwarz BioResearch.

2-Ethoxyethanol (catalog no. 1697), triethylamine (catalog no. 616), and 2-mercaptoethanol (catalog no. 4196) were purchased from Eastman Organic Chemicals; 2-butoxyethanol (catalog no. E-179) was obtained from Fisher Scientific Company under the name ethylene glycol monobutyl ether. The triethylamine was dried over $CaCl_2$, filtered, and distilled. The fraction distilling between 88 and 90° was collected. All other solvents were used without purification. The variable amounts of peroxides detectable in the alkoxyethanols by KI (Moore and Stein, 1954) were neutralized by the addition of 0.0001–0.0005 volume of 2-mercaptoethanol. Phenol was purchased as 88% analytical reagent grade from Mallinckrodt Chemicals and was not redistilled. Polyethylene glycol 6000 was obtained from Union Carbide and Sephadex beads from Pharmacia. Hydroxylapatite was purchased from Clarkson Chemical Company and DEAE-cellulose from Brown and Company. Dowex-50-W-X4 from J. T. Baker Chemical Company was prepared as described by Moore and Stein (1951). Dowex-1-X8 from Bio-Rad was prepared according to Hirs *et al.* (1954). Dialysis tubing was purchased from Visking Corporation. Prior to use it was soaked in two changes of water and one of 0.01 M neutral EDTA. The knotted sacs were then refilled and suspended in water, autoclaved, and stored at 2° in 50% ethanol.

Methods. Aminoacyl RNA synthetases were assayed either by the amino acid dependent ATP–[^{32}P]PP_i exchange reaction or by measuring the rate of aminoacyl RNA synthesis with excess t-RNA as acceptor and limiting amounts of enzyme (Berg *et al.*, 1961). With excess enzyme and limiting levels of t-RNA the same assay served to measure the level of a particular species of t-RNA. The reaction mixtures described previously (Berg *et al.*, 1961) were incubated either 10 min (enzyme assay) or 30 min (t-RNA assay) at 37°, and then reactions were terminated by cooling to 0° and adding 1 mg of carrier RNA and 3 ml of cold 2 M HCl. The suspensions were filtered through Whatman GF/C glass filters, washed with cold 2 M HCl, dried, and counted in scintillation solution, 0.01% dmPOPOP and 0.4% PPO in toluene. Blanks without enzyme or t-RNA were run in each assay and were usually less than 10% of the experimental value. In every case direct proportionality was demonstrated between the amount of acid-precipitable radioactivity and the amount of enzyme or t-RNA.

Protein was assayed by the method of Lowry *et al.* (1951) and amino acids by the method of Moore and Stein (1954); pentose was determined with orcinol (Ashwell, 1957) and deoxypentose with diphenylamine (Dische, 1955). Nonpentose sugars were measured with anthrone (Seifter *et al.*, 1950) as modified by Roseman (S. Roseman, personal communication). The biphasic partition mixture for preparation of the column and for the chromatography was prepared by mixing at 23°: 6 volumes of 1.25 M potassium phosphate, pH 6.88 (equimolar in KH_2PO_4 and K_2HPO_4), 2 volumes of 2-ethoxyethanol, 1 volume of 2-butoxyethanol, 0.01–0.05 volume of triethylamine, and 0.0001–0.0005 volume of 2-mercaptoethanol (to neutralize peroxides in the alkoxyethanols). After equilibration at 23° the ratio of upper phase volume to lower phase volume ranged from 2.3 to 2.4, increasing with the amount of triethylamine added.

All column operations were performed at 23°. For constructing the column matrix, the mixture with 0.01 volume of triethylamine was utilized. Sephadex G-25 fine beads were suspended in the lower phase of this mixture and poured into the glass cylinder in the usual fashion with care to avoid entrapment of air and to achieve even packing under intrinsic hydrostatic pressure alone. Two column sizes were tested: 174 cm $\times$ 5 cm^2 and 193 cm $\times$ 52 cm^2. After construction in the lower phase, each column was washed with upper phase from the same mixture until no more lower phase appeared in the effluent. This washing procedure required approximately 2 column volumes of upper phase, which flowed under hydrostatic pressure at a rate of 14 ml/hr in the case of the smaller column. In the case of the larger column a Beckman Accu-Flow pump was employed to maintain the flow rate of 128 ml/hr. The resultant pressure in the system is without effect on relative phase volumes or resolution of the t-RNA. Polyethylene or glass tubing was used to carry the partition solvent.

For application to the column, t-RNA was dissolved in concentrations up to 30 mg/ml in a solution composed of 1 volume of water and 5 volumes of upper phase from the mixture containing 0.01 volume of triethylamine; 3.4 g was applied to the larger column

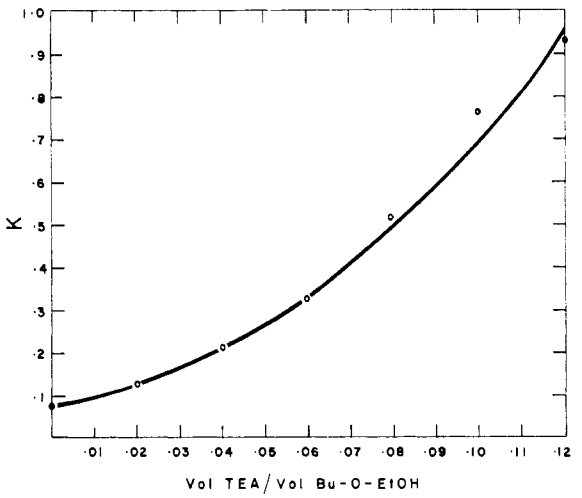

FIGURE 2: Partition coefficient (K) of t-RNA as a function of triethylamine concentration. Three-milliliter aliquots of 1.25 M potassium phosphate buffer, pH 6.88, containing 0.89 mg t-RNA, were equilibrated at 23° in graduated tubes with alkoxyethanols in the ratio described in Methods, in the presence of various amounts of triethylamine (TEA) relative to the butoxyethanol (BuO–EtOH). A_{260} and volume of each phase was measured. In every case at least 99% of the initial A_{260} units remained in solution.

and 250 mg to the smaller. After the applied sample had descended into the resin bed by gravity flow it was followed by a small volume of upper phase. Elution was begun with the upper phase flow set at the values mentioned for column washing. In each case the use of gradient elution was delayed, until 650 ml had coursed through the small column, or 7000 ml through the large column. Then a linear gradient was begun between the upper phases from the solvent mixtures employing 0.01 and 0.05 volume of triethylamine/volume of 2-butoxyethanol. In the case of the smaller column each gradient reservoir contained 1800 ml, whereas 18 l. was the comparable volume for the larger column. Fraction volumes were 26 ml for the smaller column and 128 ml for the larger; in the latter, every two fractions were pooled for assay.

To determine the A_{260} and isolate the t-RNA, $^1/_4$ volume of 1-butanol was added to each fraction, and after thorough mixing the phases were allowed to separate overnight at room temperature. The upper phase was removed and remaining traces were made miscible with the lower phase (which now contained the t-RNA in approximately 20% of the original volume) by addition of about $^1/_5$ the new volume of 50% ethanol. Each fraction was then brought to a convenient constant volume by addition of 10% ethanol and the A_{260} was read on an aliquot diluted 10-fold in water. The fraction was then dialyzed in sterile Visking sacs at 4° for 24 hr against 10 volumes of 10% ethanol, 10% polyethylene glycol 6000, and 0.01 M neutral EDTA, and for 24 hr against a similar volume of 2% potassium acetate, 10% polyethylene glycol 6000, and 0.01 M neutral EDTA. The collapsed sacs were then opened and rinsed with 2% potassium acetate, each rinse being added directly to 2 volumes of cold ethanol. The t-RNA was collected by centrifugation briefly at 10,000g and dissolved in an appropriate volume of water for storage at −15° and subsequent assay for amino acid acceptance. Unfractionated t-RNA isolated identically from upper phase served as control.

Results

Characteristics of the Solvent System. Our initial attempts to adapt several CCD solvent systems for partition chromatography were discouraging. In the case of Holley's system of 2-propanol, formamide, and phosphate buffer at pH 6 (Apgar *et al.*, 1962) the organic phase would not flow over a Sephadex column equilibrated with the aqueous phase. The mixture of 1-butanol, dibutyl ether, tributylamine, acetic acid, and water developed by Zachau (Zachau *et al.*, 1961) has been used in partition chromatography on Sephadex (Tanaka *et al.*, 1962); however, we found the organic phase to extract the aqueous phase from the Sephadex matrix during flow, altering the structure and composition of the column. Moreover, the partition coefficient of t-RNA in that system varies with t-RNA concentration.

The system of Kirby (1960), composed of 2-ethoxyethanol, 2-butoxyethanol, dibutylaminoethanol, and phosphate buffer, pH 7.5, overcame these operational difficulties but did not permit complete recovery of t-RNA from the column. Empiric modifications, namely, an increase in monobasic potassium phosphate to lower the pH, a substitution of triethylamine for dibutylaminoethanol, and an addition of 2-mercaptoethanol, have led to the system described in Methods. Increase of triethylamine in the biphasic mixture alters the partition coefficient as shown in Figure 2. Note that a linear increase in triethylamine level results in an exponential change in partition coefficient. The partition coefficient is independent of t-RNA concentration. Other desirable features are that the t-RNA is stable and completely soluble in the two phases, forming no precipitate at the interface.

In an attempt to adapt the system to partition chromatography on Sephadex columns, no single triethylamine concentration was found suitable for fractionating all t-RNA's. With a triethylamine level giving a partition coefficient of about 0.1, t-RNA was very slowly displaced from the column, the peaks of specific t-RNA's were very broad, and the recoveries of t-RNA's with low partition coefficients were poor. At triethylamine levels giving a partition coefficient of 0.25, the bulk of t-RNA was eluted early with poor resolution, although t-RNA chains with low partition coefficients were well separated, and recovery was complete.

To circumvent this difficulty we adopted a linearly increasing gradient of triethylamine in the mobile phase (effluent). This was accomplished with a mixing

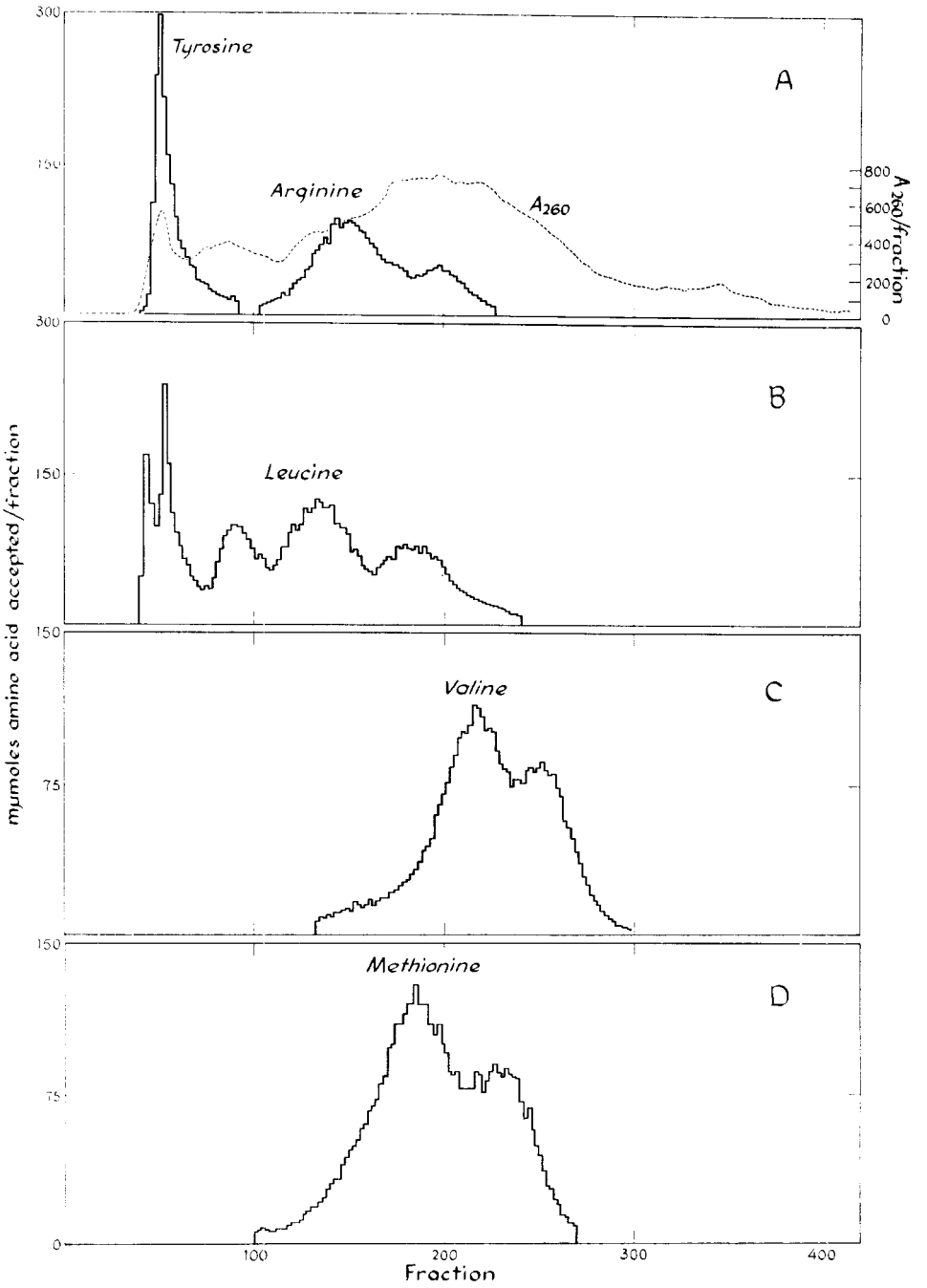

FIGURE 3: Partition column profiles of t-RNA$_{tyr}$ and t-RNA$_{arg}$ (panel A), t-RNA$_{leu}$ (panel B), t-RNA$_{val}$ (panel C)' and t-RNA$_{met}$ (panel D). The recoveries of the different t-RNA values were as follows: 87% of t-RNA$_{tyr}$ (1.8 μmoles), 100% of t-RNA$_{arg}$ (3.1 μmoles), 92% of t-RNA$_{leu}$ (7.4 μmoles), 89% of t-RNA$_{val}$ (3.9 μmoles), and 86% of t-RNA$_{met}$ (4.9 μmoles).

chamber containing the upper phase of the mixture prepared with 0.01 volume of triethylamine and a reservoir containing upper phase of the mixture made with 0.05 volume of triethylamine. Such a gradient more effectively fractionates t-RNA's having a wide range of partition coefficients, and the elution profiles of the individual t-RNA's are sharpened as they are displaced from the column.

The triethylamine gradient is possible because minute increments in triethylamine raise partition coefficients enough to quantitatively elute t-RNA but do not significantly alter the solvent system. Thus, in the 0.01–0.05 volume range of triethylamine, pH increases less than 0.05 unit, and phase-volume ratio increases only from 2.3 to 2.4. The absence of extraction of stationary phase by mobile phase during flow permits reuse of

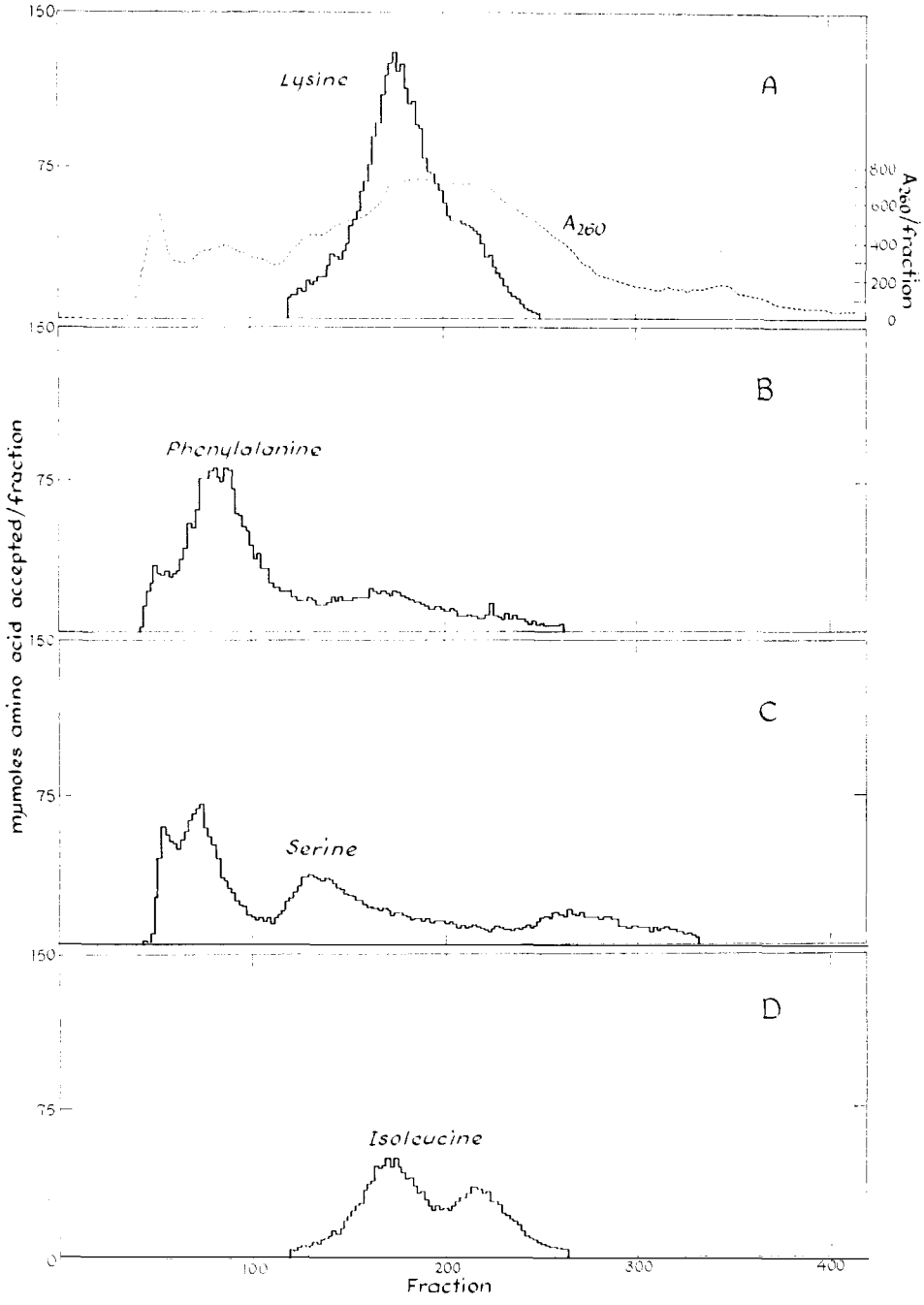

FIGURE 4: Partition column profiles of t-RNA_{lys} (panel A), t-RNA_{phe} (panel B), t-RNA_{ser} (panel C), and t-RNA_{ileu} (panel D). The recoveries of the different t-RNA's were as follows: 76% of t-RNA_{lys} (3.2 μmoles), 68% of t-RNA_{phe} (2.5 μmoles), 91% of t-RNA_{ser} (2.7 μmoles), and 79% of t-RNA_{ileu} (1.6 μmoles).

columns without regeneration by stationary phase.

Fractionation of t-RNA. Figures 3–5 show the elution profiles for 13 of the amino acid specific t-RNA's relative to the distribution of the bulk RNA (A_{260}). These results were obtained with 3.4 g of t-RNA on a column of Sephadex G-25 (193 cm × 52 cm^2) and 43 l. of eluent. The distribution of the RNA and the resolution of different t-RNA's reflects their differential solubilities in the mobile phase. A virtually identical partition profile of A_{260} and specific t-RNA's was obtained when 250 mg of t-RNA was partitioned on a smaller column (174 cm × 5 cm^2) with approximately one-tenth the volume of eluent (Muench and Berg, 1964).

t-RNA, 80 to 85%, was recovered from the column prior to dialysis. Recovery of individual t-RNA activities was in most cases greater than 75% of that in the starting material (see legends to Figures 3–5) when

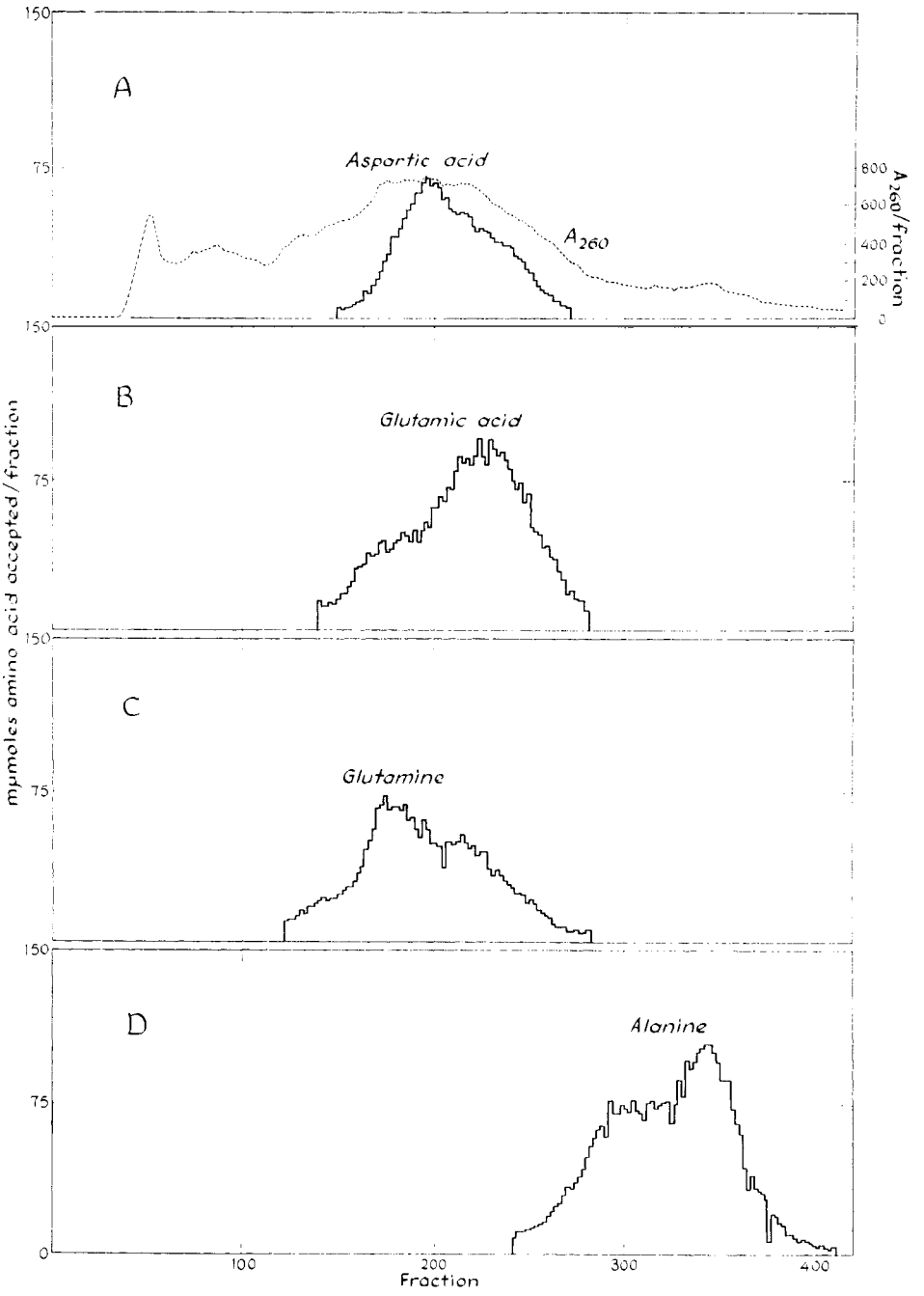

FIGURE 5: Partition column profiles of t-RNA_{asp} (panel A), t-RNA_{glu} (panel B), t-RNA_{gluNH_2} (panel C), and t-RNA_{ala} (panel D). The recoveries of the different t-RNA's were as follows: 84% of t-RNA_{asp} (2.1 μmoles), 70% of t-RNA_{glu} (3.6 μmoles), 70% of t-RNA_{gluNH_2} (2.7 μmoles), and 79% of t-RNA_{ala} (4.1 μmoles).

the precautions described in Methods were followed. In spite of these precautions, progressive loss of acceptor activity has been observed over a period of a year in recovered samples kept frozen in aqueous solution most of the time. Since this loss has not been consistent or proportional to the initial acceptor activity we assume that bacterial contamination or inadvertent introduction of nucleases during handling (Holley *et al.*, 1961) may be responsible for the inactivation.

Heterogeneity of Specific t-RNA. With the possible exception of t-RNA_{tyr} (Figure 3A) each of the specific t-RNA's is heterogeneous. t-RNA_{leu} reproducibly appears as five discrete peaks in the first half of the profile (Figure 3B) and t-RNA_{phe} (Figure 4B) and t-RNA_{ser} (Figure 4C) show three or four peaks of acceptor activity for the particular amino acid. t-RNA_{arg} (Figure 3A), t-RNA_{val} (Figure 3C), t-RNA_{met} (Figure 3D), t-RNA_{ileu} (Figure 4D), and t-RNA_{ala}

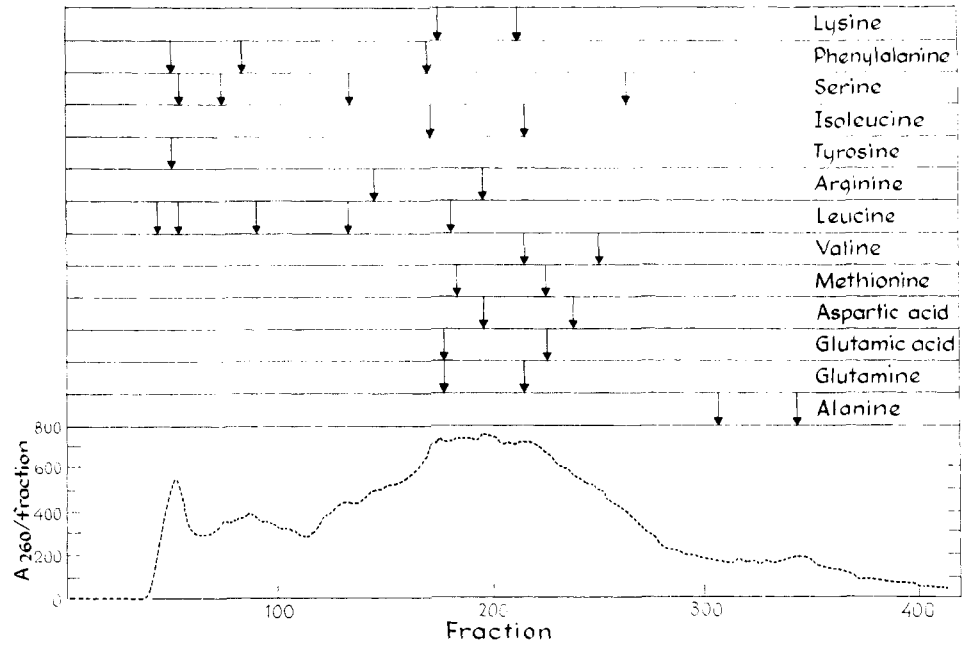

FIGURE 6: Summary of positions and number of peaks for the different t-RNA acceptors. The arrows indicate the position of peaks for each of the amino acid specific t-RNA's.

(Figure 5D) show at least two resolvable species, while the profiles for t-RNA_{lys} (Figure 4A), t-RNA_{asp} (Figure 5A), t-RNA_{glu} (Figure 5B), and t-RNA_{gluNH_2} (Figure 5C) suggest multiple acceptors for the same amino acids.

Multiple forms of t-RNA specific for a single amino acid have been detected previously by enzymic criteria (Berg *et al.*, 1961), base sequence differences (Berg *et al.*, 1962), and by differences in fractionation behavior in CCD (Goldstein *et al.*, 1964; Apgar and Holley, 1964). Partition chromatography reveals heterogeneity of virtually every amino acid specific t-RNA tested. With t-RNA_{leu} this is most striking in that there are five discrete and separable components (see Figure 6 for a summary of the positions and number of discrete t-RNA's for a single amino acid).

The multiple peaks of t-RNA_{leu} do not result from an artifact of the partitioning procedure or from existence of an equilibrium mixture of t-RNA_{leu} isomers with different partition coefficients. When material taken from peak 2 of the t-RNA_{leu} cluster (t-$RNA_{leu,2}$) was repartitioned on the same column with an identical gradient almost all of the A_{260} and recovered t-RNA_{leu} activity was eluted in the position it occupied originally (Figure 7). Thus, there appears to be no significant interconversion of one component into another by the partition column procedure. A similar result was obtained when t-$RNA_{leu,4}$ was repartitioned.

We have also verified that in the assay of each of the t-RNA_{leu} peaks it is leucine and not some other radioactive amino acid contaminating the [^{14}C]leucine substrate that esterifies the t-RNA. Following the usual loading reaction with aliquots from each of the t-RNA_{leu} peaks, the esterified amino acid was recovered and chromatographed on a thin layer of silica gel G in 1-butanol–acetic acid–water (3:1:1) (Randerath, 1963). At least 60% of the [^{14}C] from each t-RNA_{leu} peak chromatographed as leucine.

Specific Acceptor Activity of t-RNA's Recovered from the Partition Column. Each of the peaks of t-RNA_{leu} activity serves as leucine acceptor with about equal effectiveness when the activity of *E. coli* leucyl-RNA synthetase is measured (Table I). Thus, the rate of leu-RNA formation with an excess of either peaks 1, 2, or 3 of t-RNA_{leu} is about 70% that found with bulk t-RNA; the rate with peaks 4 and 5 is equal to that found with the bulk t-RNA. By contrast, peaks 1

TABLE I: Comparison of Rate of leu-RNA Formation Using Resolved Fractions of t-RNA_{leu} and Leucyl-RNA Synthetases of *E. coli* and of Yeast.[a]

t-RNA_{leu} Peak	Rate of leu-RNA Formation[b]	
	E. coli Enzyme	Yeast Enzyme
1	0.7	<0.01
2	0.7	0.03
3	0.7	1.0
4	1.0	1.3
5	1.0	1.2

[a] Assays of the rate of leu-RNA formation were performed with the partially purified leucyl-RNA synthetases as described in Methods. [b] Activity is expressed as a fraction of the rate found with the initial unfractionated t-RNA.

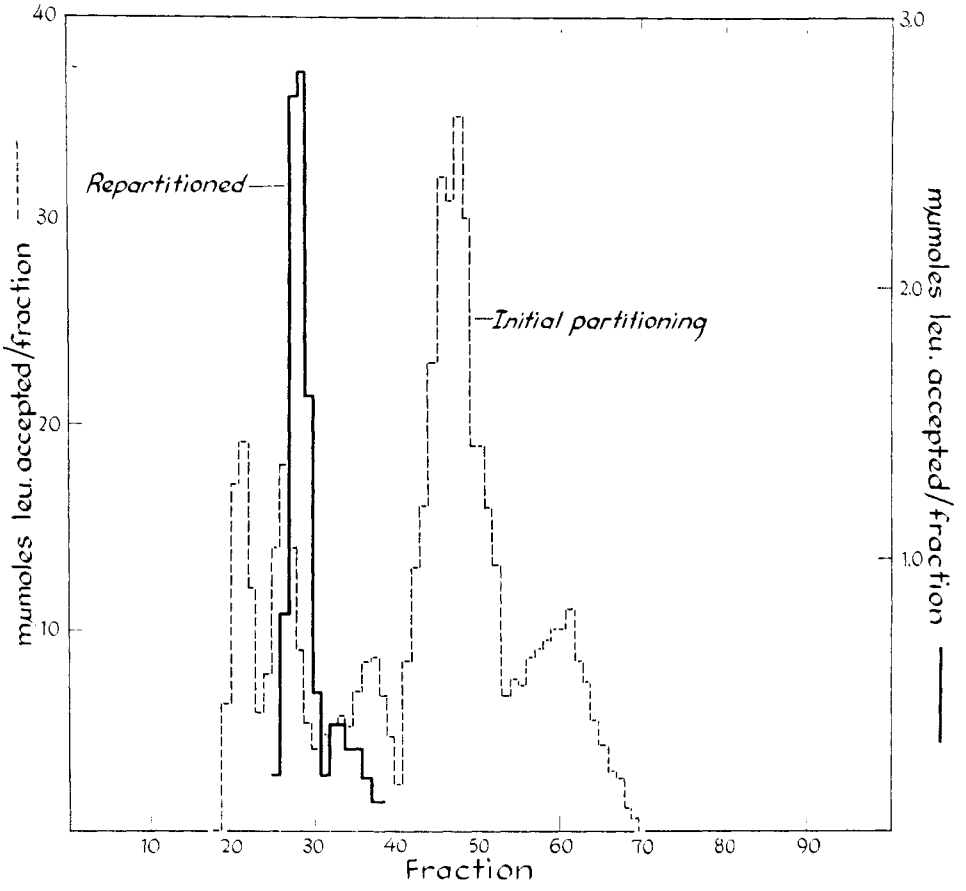

FIGURE 7: Repartitioning of t-RNA$_{leu,2}$. The dashed profile represents the elution pattern of t-RNA$_{leu}$ obtained with the smaller column (174 cm × 5 cm^2, see Methods). t-RNA$_{leu,2}$ (2.6 mg) was repartitioned on the same column using the same elution gradient. The solid profile represents the pattern of t-RNA$_{leu}$ from the repartitioning; 84% of the A_{260} units applied were recovered in fractions 23–38.

and 2 are virtually inactive for leu-RNA formation with yeast leucyl-RNA synthetase, whereas peaks 3, 4, and 5 function as well or slightly better than the bulk t-RNA. The failure to detect leu-RNA formation with peaks 1 and 2 is not because the yeast enzyme modifies or inactivates the t-RNA$_{leu}$ in these peaks. Thus, when leucyl-RNA synthetase from *E. coli* is added with the yeast enzyme the expected level of leu-RNA is formed (Table II). Moreover, since the amount of leu-RNA formed with peaks 3, 4, or 5 in the presence of both enzymes is equal to that formed in the presence of the *E. coli* enzyme alone, we conclude that the yeast enzyme loads leucine on only the t-RNA$_{leu}$ chains. Similar differences in the activity of different t-RNA$_{leu}$ fractions with the leu-RNA synthetases from *E. coli* and yeast have been reported earlier (Yamane and Sueoka, 1963; Bennett *et al.*, 1963).

Whereas there is only partial cross reaction between yeast and *E. coli* leucyl-RNA synthetases with each of the t-RNA$_{leu}$ peaks, valyl-RNA synthetases from yeast and *E. coli* esterify both peaks of t-RNA $_{val}$ with equal rates and to the same extent. The amount of ser-RNA formation with the respective enzymes from yeast and *E. coli* was also equivalent. These latter findings conflict with others reported (Doctor and Mudd, 1963).

The elution profiles for t-RNA$_{glu}$ and t-RNA$_{gluNH_2}$ (Figures 5B and C), each of which was determined by loading with the appropriate [^{14}C]amino acid in the presence of a 5-fold excess of the other, are clearly distinguishable, although there is considerable overlap between them. This shows, in agreement with the conclusions of others (Ravel *et al.*, 1965; Lazzarini and Mehler, 1964), that there are separate t-RNA chains for glutamic acid and glutamine.

Purity of t-RNA's Obtained by Partition Column Chromatography. From an examination of the RNA (A_{260}) profile and the specific t-RNA profiles obtained from the partition column it is clear that the major part of the t-RNA appears in the mid-portion of the distribution (fractions 125–275). As a consequence, t-RNA's which are eluted early and late in the partitioning are more purified than those appearing in the middle of the distribution; *e.g.*, compared to the starting RNA, t-RNA$_{tyr}$ is more than 17-fold enriched in fractions 45–56 and 24-fold purified at the peak (fractions 47–50). If we assume that each t-RNA chain con-

TABLE II: Comparison of Extent of Leu-RNA Formation with Resolved Fractions of t-RNA$_{leu}$ and Leucyl-RNA Synthetases of *E. coli* and of Yeast.[a]

t-RNA$_{leu}$ Peak	Extent of leu-RNA Formation		
	Yeast Enzyme	*E. coli* Enzyme	Yeast Enzyme and *E. coli* Enzyme
	(mμmoles leucine/μmole RNA)		
1	0.3	6.9	6.0
2	0.3	2.8	2.5
3	2.0	2.2	2.1
4	2.7	2.9	2.7
5	1.3	1.4	1.1

[a] Assays for the extent of leu-RNA formation were performed with the partially purified leucyl-RNA synthetases as described in Methods.

sists of 80 nucleotides, mol wt 27,500, the maximum acceptor capacity for a pure t-RNA chain is 36 mμmoles amino acid/mg of t-RNA. On this basis we estimate that the t-RNA$_{tyr}$ is about 40% pure in fractions 47–50.

Similarly, the material in fractions 41–44 (Figure 3B) is about 40% t-RNA$_{leu,1}$, whereas material in fractions 51–54 of the second peak, which overlaps the first A_{260} peak, is about 25% t-RNA$_{leu,2}$. t-RNA$_{leu,3}$, t-RNA$_{leu,4}$ and t-RNA$_{leu,5}$ are progressively less pure as the bulk of the t-RNA is eluted. At the other end of the distribution, the second peak of t-RNA$_{ala}$ (fractions 331–356; Figure 5D) is approximately 10-fold enriched over the starting RNA. The estimated purity of this material is 38%. Maximum purities of the other 10 t-RNA's range from 5 to 15%, depending on their position in the elution profile.

Discussion

The partition chromatographic system described here provides a simple and reproducible method for resolving different amino acid acceptors of *E. coli* t-RNA. The separations achieved compare quite favorably and for certain t-RNA's exceed those found with approximately 1000 transfers in CCD (Goldstein *et al.*, 1964). An advantage of the partition column is the combination of high resolving ability and high capacity. With the larger column 3.4 g of unfractionated t-RNA was partitioned, but the maximum amount of t-RNA which can be fractionated by the column without sacrifice of resolving power may be even higher and remains to be determined. High capacity CCD fractionators can now partially resolve 4–8 g of t-RNA (Holley *et al.*, 1963; Hoskinson and Khorana, 1965).

Because the gradient eventually adopted was designed to obtain the best results for all t-RNA's in a single run, the purification of any single t-RNA may not be optimal. Improvements could probably be made to accentuate separations of certain t-RNA's by modifying the steepness of the triethylamine gradient. Other variables which still need exploration include the optimum matrix for supporting the immobile aqueous phase, pH, temperature, and other amines.

Another possibility, which needs further study and which has already proved successful in CCD procedures, is that redistribution of the t-RNA from peak fractions with the same or different gradients will further enrich for a selected t-RNA. An alternate approach for further enrichment of partially purified t-RNA's is the use of a procedure which depends upon a completely different property of the t-RNA. Such alternatives might be chromatography on DEAE-cellulose (or Sephadex) columns with variations in ionic strength, pH, or urea concentrations (Cherayil and Bock, 1965), or chromatography on hydroxylapatite (Hartman and Coy, 1961; Muench, 1965). Results of chromatography on hydroxylapatite are reported in detail in the next paper (Muench and Berg, 1966).

One distinct disadvantage of the partition column and CCD methods is the relatively time-consuming procedure for isolating and assaying each of the fractions for the different t-RNA's. Labeling a desired t-RNA chain with an isotopic amino acid, as was used by Sueoka and Yamane (1962) to locate particular t-RNA's in the effluent from methylated albumin-kieselguhr columns, is not possible because of the instability of the ester linkage under the conditions used. For this reason the partition column is less well suited to analytic procedures than to preparative purposes. Nevertheless, in certain types of studies such as examination of the fate or modifications of the different t-RNA$_{leu}$ species, the partition column offers promise because of its high resolving power.

A striking feature of the partition column profile is the similarity in the order of elution of different t-RNA's from the column compared to their order in the CCD train using a phosphate–2-propanol–formamide system (Apgar *et al.*, 1962). These similarities probably reflect characteristic solubility differences among t-RNA's in a wide range of relatively nonpolar solutions, but clearly further study is needed to provide a systematic rationale for differences in the partition coefficients of different t-RNA's.

The results obtained in the present work further emphasize the very widespread heterogeneity of t-RNA's for the same amino acid. Indeed there appear to be multiple forms of t-RNA for virtually each amino acid and quite possibly the degree of heterogeneity will be even more evident as methods of fractionation improve. According to current notions this heterogeneity is necessary to translate the degenerate codons for the same amino acid during protein assembly (Weisblum *et al.*, 1965). Whether this is the sole reason for t-RNA heterogeneity and what the structural bases are for the heterogeneity remain to be seen.

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