Separation of Transfer Ribonucleic Acids by Reverse Phase Chromatography*

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Numerous experimental techniques for the separation of transfer ribonucleic acids have been used successfully in preparing partially purified fractions of several specific tRNAs. Many of the existing methods have depended upon the differential solubility of specific tRNAs in complex two-phase systems (1-6), and the separations were achieved by means of countercurrent extraction techniques. Column chromatographic experiments with the use of cellulose exchangers (7, 8), methylated albumin (9, 10), or solvent phases supported on inert material (11-13) have also shown partial separation of specific tRNAs. Paper chromatographic procedures have produced partial resolutions of tRNA (14). Methods involving chemical treatment of specific tRNAs have been reported (15-19).

Because of our ultimate desire to prepare large quantities of highly purified specific tRNAs, we embarked on a procedure somewhat different from those employed by other investigators. We decided that a minimum number of components and ease of operation should be the primary criteria of our procedure. It seemed likely that column chromatography, combining both ion exchange and differential solubility phenomena, might produce significant resolutions. Furthermore, such a system could be readily adapted to large scale procedures.

Our choice of ion exchange agent was the quaternary ammonium compounds because of their demonstrated exchange properties at neutral pH and their solubility in organic solvents. It had been previously observed that quaternary ammonium compounds react with nucleic acids without modification of their activity (20, 21). The organic phase was selected after testing a series of water-insoluble solvents for their compatibility with the ion exchange agent. The organic phase was also selected for its ability to extract tRNA from aqueous solutions.

**EXPERIMENTAL PROCEDURE**

**Materials**—Soluble ribonucleic acid from *E. coli* was obtained from General Biochemicals. Alligal 204 (dimethyl-dialkylammonium chloride) was obtained from General Mills, Chromosorb W (acid-washed, Dimethyliodoiodinlana-treated, 120 to 140 mesh) was obtained from Johns Manville.

**Growth of E. coli**—E. coli strain B were grown in standard minimal medium, low in phosphate, with glucose as a carbon source. Vat fermenter growth was maintained at pH 6.5 to 7.0, and the cells were harvested at late log phase with a Sharples centrifuge.

**Preparation of Crude tRNA**—The crude tRNA was prepared by a modification of a method of Zubay (19). Packed wet cells, 400 g, were dispersed into 800 ml of 0.001 M Tris-HCl buffer, pH 7.4, containing 0.01 M magnesium acetate. This suspension was extracted with 688 ml of 88% phenol for 1 hour at room temperature and then centrifuged at 16,000 × g for 1 hour at 4° to recover the aqueous phase. All subsequent steps were at 4° unless otherwise indicated. The aqueous phase was adjusted to 2% potassium acetate, and 2 volumes of 95% ethanol were added to precipitate nucleic acids. After 1 hour the precipitate was recovered by centrifugation at 16,000 × g for 10 min and dissolved in 400 ml of 0.3 M sodium acetate at 20°. One-half volume of 2-propanol was added, the mixture was stirred at 20° for 1 hour, and the precipitate (the bulk of the DNA and large molecular weight RNA) was removed by centrifugation at 16,000 × g for 10 min at 20°. The supernatant solution was cooled to 4°, and an additional 0.5 volume of 2-propanol was added to precipitate tRNA. After 1 hour the precipitate was recovered by centrifugation at 16,000 × g for 10 min, dissolved in 500 ml of 0.05 M NaCl, and loaded on a DEAE-cellulose column (4 × 20 cm) in the chloride form. After the column had been washed with 2,000 ml of 0.2 M NaCl, the tRNA was eluted with 0.6 M NaCl, precipitated from the eluate by the addition of 2 volumes of 95% ethanol, and, after 4 hours, recovered by centrifugation at 16,000 × g for 10 min. The crude tRNA was dissolved in 50 ml of 0.2 M glycine buffer at pH 10.3 and incubated at 37° for 2 hours to discharge any bound amino acids. The solution was cooled to 4°, and 4 ml of 5 M NaCl and 2 volumes of 95% ethanol were added. After 2 hours the precipitate was recovered by centrifugation at 16,000 × g for 10 min, dissolved in 18 ml of H2O, and stored at -20°. A yield of ~500 mg of crude tRNA was obtained.

In addition to the crude tRNA prepared as described above, commercially available "soluble ribonucleic acid" prepared from *E. coli* strain B was used for many of the preliminary chromatographic experiments. In most cases the commercial material was approximately 65% as active as our crude tRNA.

**Preparation of Crude Aminoacyl-RNA Synthetases**—The following steps were carried out at 4°. *E. coli* strain B cells, 30 g, were dispersed in 80 ml of buffer (0.01 M Tris-HCl, pH 7.4, containing 0.01 M magnesium acetate and 0.001 M glutathione) and broken in a French pressure cell at 10,000 to 12,000 psi. The broken cell preparation was diluted with 4 volumes of buffer and centrifuged at 35,000 × g for 40 min and then at 75,500 × g for 3 hours to remove particulate matter. The supernatant solution was dialyzed overnight against 2 liters of buffer. Nucleic acids were then precipitated from the dialyzed solution by the addition
Fig. 1. Chromatography of a 100-mg sample of commercial tRNA on a column (9 mm X 8 feet) in the absence of MgCl₂. All methods are described in "Experimental Procedure." Column capacity is approximately 350 mg of tRNA.

Fig. 2. Chromatography of a 100 mg sample of commercial tRNA on a column (9 mm X 8 feet) in the presence of 0.91 M MgCl₂. All methods are described in "Experimental Procedure."
Fig. 3. Chromatography of a 100-mg sample of commercial tRNA on a column (9 mm X 8 feet) with a MgCl₂ gradient from 0 to 0.01 M. All methods are described in ‘Experimental Procedure.”

of buffer. Finally, 0.25 volume of glycerol was added to the enzyme solution, and it was stored at -20°. Crude aminoacylRNA synthetases prepared in this manner contained about 4 mg of protein per ml and were stable for 3 to 6 months.

Amino Acid Acceptor Activity—The crude tRNA mixture or chromatographed tRNA fractions were assayed for amino acid-accepting activity by a slight modification of the methods of Bollum (22), Mans and Novelli (23), and Nishimura and Novelli (24). A 0.5-ml reaction mixture contained 50 μmoles of Tris-HCl buffer at pH 7.4; 5 μmoles of magnesium acetate; 1 μmole of ATP; 2.5 μmoles of KCl; 0.75 pmole of a 14C-labeled L-amino acid, tRNA, 0.02 to 2.0 absorbance units at 260 μm; and sufficient crude aminoacyl-RNA synthetase (approximately 0.04 mg) to obtain maximal formation of aminoacyl-RNA. The reaction was initiated by the addition of the aminoacyl-RNA synthetase and incubated for 10 to 30 min at 37°. The optimum incubation time for each of the amino acids was previously determined. A 50-μl sample was then pipetted onto a 2.3-cm filter paper disk (the disks were exposed to a constant stream of warm air during sampling) and dropped into cold 10% trichloroacetic acid. The disks were then successively washed for 10 min, twice in cold 5% trichloroacetic acid and once each in ether-alcohol and ether.

The disks were then dried by exposure to infrared light for 15 min and counted in a Packard Tri-Carb scintillation spectrometer.

Preparation of Reverse Phase Chromatographic Columns—After preliminary screening experiments the organic phase selected consisted of 4% w/v dimethyldilaurylammonium chloride (Aliquat 204) in isoamyl acetate. This phase was washed successively with 3 volumes of 1 M NaOH, 1 M HCl, 1 M NaCl, and 0.3 M NaCl to remove soluble contaminants. The chromatographic column packing consisted of one part organic phase thoroughly mixed with two parts (w/w) of hydrophobic diatomaceous earth (Chromosorb W). To prepare a chromatographic column, the glass column was first filled with an aqueous solution containing 0.3 M NaCl and 0.05 M Tris buffer, pH 7.4, which had been saturated with isoamyl acetate. All aqueous solutions used in the chromatographic experiments were saturated with isoamyl acetate to prevent depletion of the isoamyl acetate on the column packing. The packing was slowly poured into the column and allowed to settle. These columns permitted a free flow of the mobile aqueous phase and could be used repeatedly for many experiments by regeneration with 1 M NaCl after each run.

The chromatographic experiments were performed by adding the tRNA (dissolved in an aqueous solution containing 0.25 or
Fig. 4. Rechromatography of a pooled phenylalanyl-tRNA peak. Original sample was 70 mg of crude tRNA, first chromatographed on a column (9 mm X 8 feet), with a 0.6 to 0.9 M NaCl gradient containing 0.01 M MgCl₂ and 0.05 M Tris-HCl, pH 7.4. The pooled phenylalanyl-tRNA fractions were then rechromatographed on a column (9 mm X 2 feet), with a 0.5 to 0.9 M NaCl gradient. All methods are described in "Experimental Procedure."

Table I

<table>
<thead>
<tr>
<th>Recovery of phenylalanine acceptor activity</th>
<th>Concentration factor</th>
</tr>
</thead>
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<tr>
<td>Pooled fractions</td>
<td>Peak fraction</td>
</tr>
<tr>
<td>Initial</td>
<td>%</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>After first column</td>
<td>91</td>
</tr>
<tr>
<td>Pooled for second column</td>
<td>74</td>
</tr>
<tr>
<td>After second column</td>
<td>54</td>
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</tbody>
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0.3 M NaCl and 0.05 M Tris buffer at pH 7.0 to 7.4) to the column. At this NaCl concentration the tRNA was tightly bound to the column. A NaCl gradient was then utilized to elute the tRNAs from the column. The eluate was collected in 10-ml fractions and measured for absorbance at 260 mp. Appropriate fractions were then tested for amino acid acceptor activity. It was found that up to 0.1-ml samples could be assayed directly for this activity without prior treatment to remove the NaCl or isoamyl acetate. This lack of interference by the components of the system increases the value of this procedure for the fast and simple assay of amino acid acceptor activity when one is dealing with many chromatographic fractions.

RESULTS AND DISCUSSION

Fractionation of tRNA—Magnesium ion was found to have a pronounced effect upon the reverse phase column chromatography, as shown in two experiments in which 100-mg samples of commercial tRNA were chromatographed on a column, 9 mm X 8 feet. In the first experiment (Fig. 1), no MgCl₂ was present in the NaCl elution gradient. Five distinct leucyl-tRNAs were found, and alanyl- and phenylalanyl-tRNAs were also well separated. In this experiment, the tRNAs eluting first from the column formed sharp peaks both in absorbance and in amino acid acceptor ability, whereas those peaks eluting later were rather broad and poorly defined. In the second experiment (Fig. 2) 0.01 M MgCl₂ was added to the NaCl elution gradient. Again five leucyl-tRNAs were observed, and alanyl- and phenylalanyl-tRNAs were well separated. However, the initial fractions did not contain well defined absorbance and amino acid acceptor peaks, whereas the later eluting tRNAs produced sharp peaks. Apparently the magnesium ion causes specific changes in specific tRNAs, as observed in the chromatographic patterns. Magnesium ion is known to have a pronounced effect on tRNA, presumably due to changes in conformation, since effects have been noted on RNase resistance (24), sedimentation rates (25), and melting behavior (26).

A concentration gradient from 0 to 0.01 M MgCl₂ was used in addition to the 0.35 to 0.75 M NaCl gradient to obtain satisfactory chromatographic resolution of all of the specific tRNAs. This eluting solution also contained 0.05 M Tris buffer at pH 7.4. The results are shown in Fig. 3. The locations of 16 specific tRNAs were determined. A single peak was obtained for alanyl-, histidyl-, isoleucyl-, lysyl-, methionyl-, phenylalanyl-, prolyl-, threonyl-, tyrosyl-, and valyl-tRNAs. Asparagine and aspartic acid activities coincided in a single peak, suggesting that both may be accepted by a common tRNA. Glutamine charging was obtained only with the first column volume breakthrough fractions (other experiments have confirmed the shape and position of this peak). If this is glutamyl-tRNA, it must be quite different from the other tRNAs since it was not retarded by the
column. Two distinct arginyl-tRNA peaks were obtained, and again five peaks for leucine acceptance were observed. Seryl-tRNA appeared spread over many fractions, and insufficient charging was obtained with cytosine, glutamic acid, glycine, and tryptophan to indicate the presence of tRNAs for these amino acids.

The separation of five leucyl-tRNA peaks is in agreement with published results (15, 27). The distribution of 10 specific tRNAs from E. coli B in a countercurrent solvent extraction system has been previously reported (6), and multiple peaks were found for alanyl-, glycyl-, lysyl-, methionyl-, prolyl-, seryl-, threonyl-, and tyrosyl-tRNA, all of which gave single peaks in our experiments. Since it is not known whether multiple amino acid acceptor peaks are a reflection of degeneracy or heterogeneity of the amino acid code, or are artifacts caused by the fractionation system or by partial degradation of the tRNA sample, it is difficult to make a meaningful comparison of the two results.

Recovery of Purified Phenylalanyl-tRNA—Crude tRNA, 70 mg, prepared as described under “Experimental Procedure,” was chromatographed on a column, 9 mm × 8 feet. The resulting fractions were assayed for phenylalanine acceptance, and the main portion of the phenylalanyl-tRNA chromatographic peak was pooled and rechromatographed on a column (9 mm × 2 feet) with an elution gradient from 0.5 to 0.9 M NaCl. The results are shown in Fig. 4. After the second chromatographic treatment, 54% of the initial phenylalanine-accepting ability was recovered as a symmetrical ultraviolet-absorbing peak, followed by a small second peak which was not active. The concentration of the phenylalanyl-tRNA was increased by a factor of 24 for the pooled fractions and by a factor of 29 for the peak fractions (Table I). These values are considerably higher than the 3- to 5-fold concentration of E. coli phenylalanyl-tRNA and 10-fold concentration of yeast phenylalanyl-tRNA previously reported (28).

Coincidence of amino acid acceptor activity and the 260 μ absorption peak shows that the phenylalanyl-tRNA was substantially purified. However, the absolute activity of the phenylalanyl-tRNA (moles of phenylalanine accepted per mole of tRNA) cannot be definitely established because the molar extinction coefficient for phenylalanyl-tRNA is not known. Calculations were made on the assumption that tRNA contains 80 nucleotides. Thus, 1.25 mmoles of tRNA per ml produces an absorbance of 1 at 260 μm (29). The peak tube, tube 136, was extensively assayed to obtain maximum phenylalanine charging and to detect the presence of other tRNAs. With these data and the above assumptions, the 260 μm absorbing material in the peak tube was 74.2% phenylalanyl, 21.8% leucyl, and 2.9% tyrosyl-tRNA, totaling 98.9% active tRNA. No other aminoacyl-tRNAs were detected. When Fractions 130 through 145 were pooled and analyzed in the same manner, the total peak consisted of 61.2% phenylalanyl, 25.2% leucyl, and 2.1% tyrosyl-tRNA. Again no other tRNAs were detected. These percentages total 98.5% tRNA in the pooled peak. The contamination must be due to inactive tRNA or other RNA that is typhified by the shoulder seen to the right of the main peak in Fig. 4.

Experiments to isolate completely the phenylalanyl-tRNA are now in progress. Since we have noticed separations of these three species in early experiments under different conditions, we expect to obtain 100% pure phenylalanyl-tRNA in the near future.

We consider this chromatographic system as a general preliminary method for the separation of tRNAs. Experiments will be initiated to test variations of metal ion, ion exchange agent, organic solvent, and column support material (30) in order to improve the resolution of the method as well as to isolate completely other species of tRNA.

SUMMARY

A simple method has been developed for the separation of transfer ribonucleic acid by reverse phase chromatography. Sixteen tRNAs have been detected and partially separated. Multiple forms of leucyl-, arginyl-, and seryl-tRNA have been observed. The presence of magnesium ion in eluting solutions differentially affects the chromatographic peaks of the tRNAs. Phenylalanyl-tRNA has been concentrated 29-fold and is 74% pure in peak chromatographic fractions.

REFERENCES

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