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Separation of Transfer Ribonucleic Acid by Sepharose Chromatography Using Reverse Salt Gradients

(reverse phase chromatography/hydrophobic bonding/tRNA isoaccepting species)

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ABSTRACT **The transfer ribonucleic acids of *Escherichia coli* bind to unsubstituted Sepharose in the presence of high concentrations of ammonium sulfate at pH 4.5. Transfer RNA species are eluted individually from the Sepharose by a gradient from high to low concentrations of ammonium sulfate; leucine tRNA is fractionated into five isoaccepting species. The order of elution of these isoaccepting species differs from that seen with reverse phase chromatography. By means of only these two procedures, one isoaccepting species of leucine tRNA can be purified to apparent homogeneity. Isoaccepting tRNA species for 9 amino acids have been resolved. This establishes the general utility of this chromatographic system for the separation and purification of specific isoaccepting transfer RNAs.**

As a result of the widespread interest in the biology and chemistry of tRNA, numerous techniques have been developed for the separation and purification of isoaccepting species of tRNA. Early methods involved partitioning of tRNA between two liquid phases by countercurrent distribution (1). Other techniques, including chromatography on hydroxylapatite (2), DEAE-Sephadex (3, 4), methylated albumin on kieselguhr (MAK) (5), benzoylated DEAE-cellulose (BD-cellulose) (6), and reverse phase chromatography (RPC) adsorbents (7) were later developed and have been used successfully for the isolation of over 40 tRNA species (8).

Recently Rimerman and Hatfield (9) reported that high concentrations of salts such as phosphate or sulfate caused many proteins in a cell-free extract of *Escherichia coli* to bind to Sepharose that had been substituted with L-valine, and that proteins in the mixture were subsequently eluted differentially by decreasing the concentration of phosphate. It was established that the order of elution of the various proteins was related to their solubility in ammonium sulfate solutions. It was suggested that hydrophobic interactions between the substituted gel and the proteins could account for the observed binding (9). Other studies have established the usefulness of this method in the chromatographic separation of polysaccharides and glycoproteins (10).

This communication demonstrates the salt-mediated chromatographic separation of nucleic acids on unsubstituted Sepharose and shows the general utility of this technique for the separation and purification of individual transfer RNA species.

Abbreviations: RPC, reverse phase chromatography.

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MATERIALS AND METHODS

Chemicals. Sepharose 4B was obtained from Sigma, and *E. coli* K-12 unfractionated tRNA was obtained from Schwarz/Mann (lot no. 6701). *E. coli* B tRNA (lot no. 73042) was purchased from Plenum Chemical Co. [^{3}H]- and [^{14}C]amino acids were purchased from ICN or Schwarz/Mann and were of the highest specific activities available. All other chemicals were reagent grade, and RPC-5 adsorbent was prepared by Miles Laboratories.

Preparation of Synthetases. Leucyl-, valyl-, and isoleucyl-tRNA synthetase enzymes were partially purified from *E. coli* B by ammonium sulfate fractionation, gel filtration, and DEAE-cellulose chromatography. Other aminoacyl-tRNA synthetases were partially purified by ammonium sulfate fractionation and/or DEAE-Sephadex chromatography.

Aminoacylation of Unfractionated tRNA. One hundred milligrams of crude tRNA was charged in a 22 ml reaction mixture containing the following: 5500 μmol of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.0), 2250 μmol of Mg acetate, 1.3 μmol of L-[^{3}H]leucine, 2 mg of bovine serum albumin, 100 units of leucyl-tRNA synthetase, (1 unit is that amount of enzyme which catalyzes the formation of 1 nmol of aminoacyl-tRNA in 10 min), 500 μmol of ATP, 300 μmol of KCl and 180 μmol of 2-mercaptoethanol. The reaction was initiated with enzyme and the mixture was incubated for 1 hr at 37°.

The reaction was terminated by the addition of 1 ml of 5 M NaCl and 2 volumes of cold absolute ethanol. Precipitated protein and charged tRNA were centrifuged at 4° and the pellet was resuspended in 5 ml of 0.01 M acetate buffer, pH 4.5, containing 6 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 1 mM EDTA (buffer A). The sample was applied to a 2.5 × 7 cm DEAE-cellulose column equilibrated with buffer A. Buffer A (200 ml) containing 0.3 M NaCl was next passed through the column. Then the charged tRNA was eluted from the column with 1.0 M NaCl in buffer A.

Aminoacylation of Column Effluent Fractions. The amino acylation reaction mixture contained (in 0.05 ml) 5 μmol of Tris·HCl (pH 7.2), 2.5 μmol of $MgCl_2$, 0.5 μmol of ATP, 0.5 μmol of reduced glutathione, 10 μg of bovine serum albumin, 4 nmol of L-[^{14}C]amino acid (specific activity 9000 cpm/

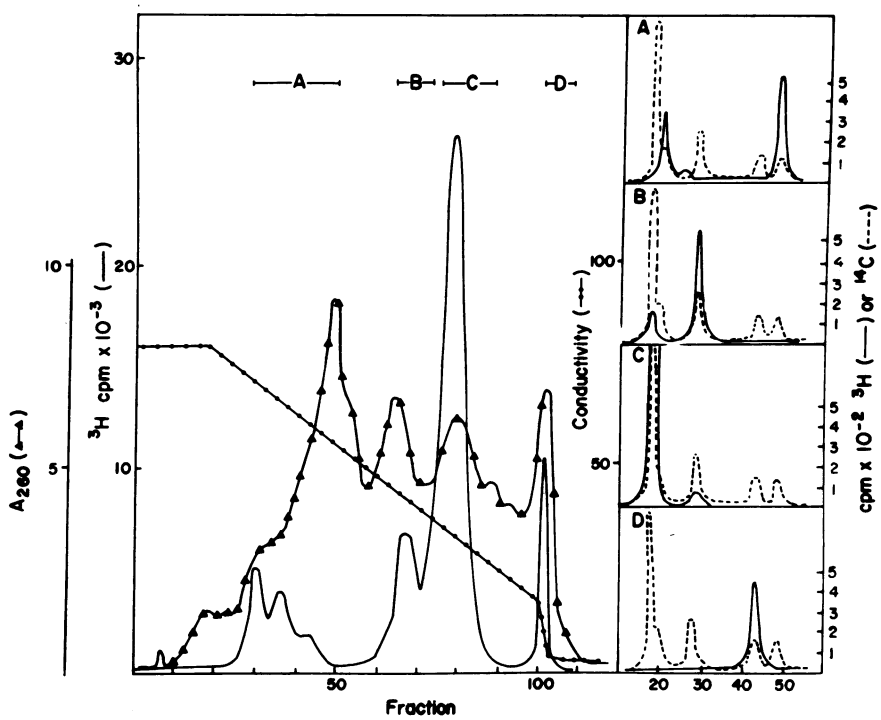

FIG. 1. Chromatography of aminoacylated $tRNA_{1-5}^{Leu}$ on Sepharose using a decreasing salt gradient. One hundred milligrams of unfractionated *E. coli* K-12 tRNA, aminoacylated with L-[^{3}H]leucine, was chromatographed as previously outlined. Lines designated with letters A to D indicate fractions pooled for each leucyl-tRNA peak. Graphs labeled A through D represent the chromatography of appropriate L-[^{3}H]leucine-labeled Sepharose pools on RPC-5 together with unfractionated K-12 tRNA aminoacylated with L-[^{14}C]leucine. It can be seen that pool A contains $tRNA_2^{Leu}$ and $tRNA_5^{Leu}$; pool B contains $tRNA_3^{Leu}$; pool C contains $tRNA_1^{Leu}$; and pool D contains $tRNA_4^{Leu}$. Conductivity is in units of mmho.

nmol), 2–5 units of partially purified aminoacyl tRNA synthetase, and 25 μl of the appropriate column fraction.

After 5 min incubation at 37°, a 40 μl aliquot was pipetted onto a 2.4 cm filter paper disk (Schleicher and Schuell no. 593A) which was then washed and the radioactivity was measured as described previously (11). In some instances tRNAs eluting in the early part of the profile failed to incorporate amino acid due to the high ammonium sulfate concentration in the assay; in these cases aliquots of the effluent fractions were diluted with two volumes of water prior to assay.

Sepharose Chromatography. Sepharose 4B was equilibrated with buffer A containing 1.3 M $(NH_4)_2SO_4$ and poured into a 1.5 × 40 cm column. One hundred milligrams of unfractionated tRNA (either *E. coli* B tRNA or *E. coli* K-12 tRNA) were dissolved in 5 ml of Buffer A and adjusted to 1.3 M $(NH_4)_2SO_4$ by adding 4 M $(NH_4)_2SO_4$ in Buffer A. Samples were applied to the Sepharose column and eluted at 4° with a gradient using 234 ml of 1.3 M $(NH_4)_2SO_4$ in Buffer A in the mixing chamber and 250 ml of Buffer A in the reservoir, at a flow rate of 40 ml/hr. Conductivity and absorbance (A_{260}) were determined in the effluent fractions. In addition, appropriate fractions were assayed for specific tRNA species using the aminoacylation assay outlined above.

The effects of pH and magnesium ion on the chromatography of unfractionated *E. coli* K-12 tRNA were determined using a 1.5 × 15 cm Sepharose column. Ten milligrams of tRNA were dissolved in either 0.05 M Tris·HCl (pH 7.5) or in Buffer A (with and without 10 mM Mg^{2+}). Each sample was adjusted to 1.3 M with $(NH_4)_2SO_4$ and applied to the Sepharose column equilibrated with 1.3 M $(NH_4)_2SO_4$ in either 0.05 M Tris·HCl (pH 7.5) or Buffer A. The tRNA was eluted in each case with a 150 ml gradient of decreasing ammonium sulfate concentration in either 0.05 M Tris·HCl (pH 7.5) or Buffer A with or without 10 mM $MgCl_2$.

Reverse Phase Chromatography. Five milliliters of the appropriate pooled fraction from each peak of [^{3}H]leucyl-tRNA obtained by Sepharose chromatography of *E. coli* K-12 tRNA were mixed with 2 mg of unfractionated tRNA previously aminoacylated with L-[^{14}C]leucine. After dialysis the tRNA was precipitated by the addition of 2 volumes of cold ethanol, dried in a stream of nitrogen, and redissolved in 0.6 ml of Buffer A containing 0.5 M NaCl. The sample was applied to a 0.9 × 50 cm RPC-5 column equilibrated with Buffer A containing 0.5 M NaCl. Elution was carried out with a linear gradient of increasing NaCl concentration in Buffer A (125 ml of 0.5 M NaCl plus 125 ml of 0.8 M NaCl) at 300 pounds/inch^2 (2.1 MPa) and at a flow rate of 2 ml/min. Aliquots of each fraction were mixed with 1 ml of water, added directly to Aquasol scintillation fluid, and assayed in a Beckman LS-230 scintillation counter.

RESULTS

The data in Fig. 1 demonstrate that charged leucine tRNA is separated into at least five species by Sepharose chromatography. Other similar experiments, not shown here, demonstrate that charged isoaccepting species of valine and isoleucine tRNA are also rapidly and efficiently separated with this technique. In these analytical experiments, separations can be rapidly executed and each experiment usually takes

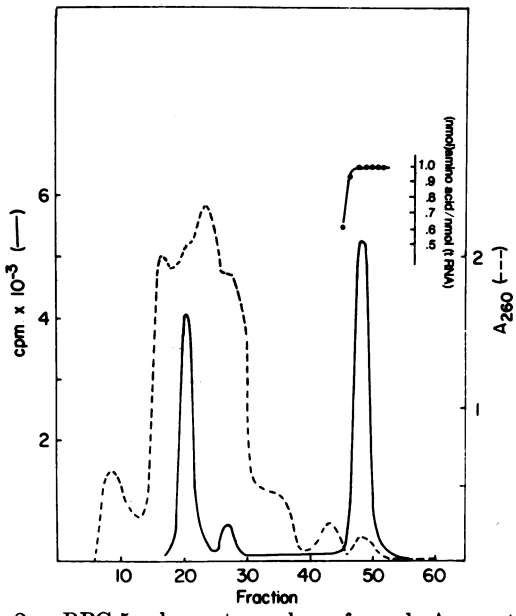

FIG. 2. RPC-5 chromatography of pool A, containing $tRNA_5^{Leu}$ and $tRNA_2^{Leu}$ (Fig. 1), obtained from Sepharose. Pool A was dialyzed against Buffer A, then pumped directly onto the RPC-5 column. Specific activities of fractions containing $tRNA_5^{Leu}$ were estimated by assuming the absorbance (A_{260}) of a 1 mg/ml of solution of $tRNA_5^{Leu}$ to be 18.0. Fractions 47 to 51 exhibit a leucine:tRNA stoichiometry of 1.0 and contain pure leucyl-$tRNA_5^{Leu}$.

about three hours to complete. It is apparent, therefore, that this technique is suitable for analytical purposes. Cochromatography of each leucyl-tRNA species with unfractionated leucyl-tRNA on RPC-5 demonstrated that the order of elution from Sepharose is markedly different from that seen on RPC-5 columns. As can be seen, the relative position of elution of $tRNA_5^{Leu}$ is quite different than that seen on RPC-5 columns. In view of this observation, we reasoned that this tRNA might be considerably purified using only these two procedures. Fig. 2 reveals that $tRNA_5^{Leu}$ is purified to apparent homogeneity in two steps, effecting a 390-fold

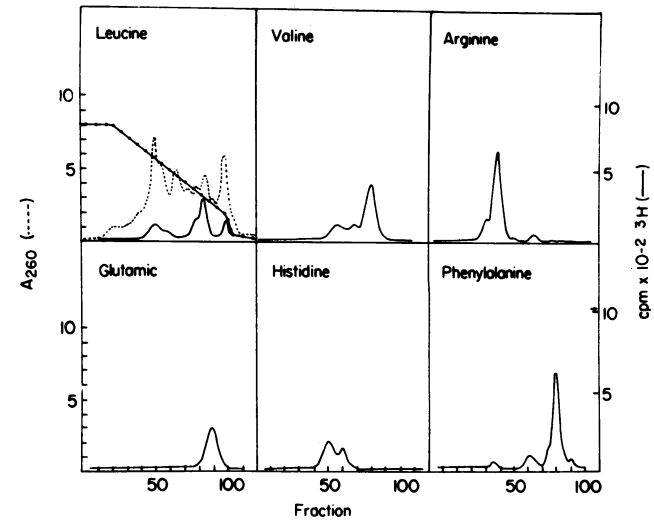

FIG. 4. Separation of tRNA isoaccepting species specific for several amino acids. All tRNAs were assayed in the effluent from a single Sepharose column used to fractionate 100 mg of *E. coli* K12 tRNA.

purification. Recovery was estimated at over 90% based on total radioactivity isolated.

Fig. 3 illustrates that the uncharged isoaccepting species of *E. coli* B tRNA specific for eight amino acids can be separated with this method. In many instances, the position and order of elution is different from that reported for RPC systems (7). Interestingly, a similar experiment carried out using tRNA isolated from *E. coli* strain K-12 reveals some differences in elution profiles of the leucine, valine, glutamate, and histidine tRNAs (Fig. 4). In addition, subtle differences in the overall elution profile of the total tRNA are evident.

Studies were conducted to determine the capacity of Sepharose 4B for tRNA. In the presence of 1.3 M $(NH_4)_2SO_4$ at 4°, 1 ml of Sepharose-4B adsorbed 3 mg of unfractionated tRNA. This capacity could be increased to 8 mg/ml in 1.6 M $(NH_4)_2SO_4$. At these concentrations of ammonium sulfate, tRNA remains soluble. It is apparent, therefore, that this technique is suitable for processing large quantities of tRNA.

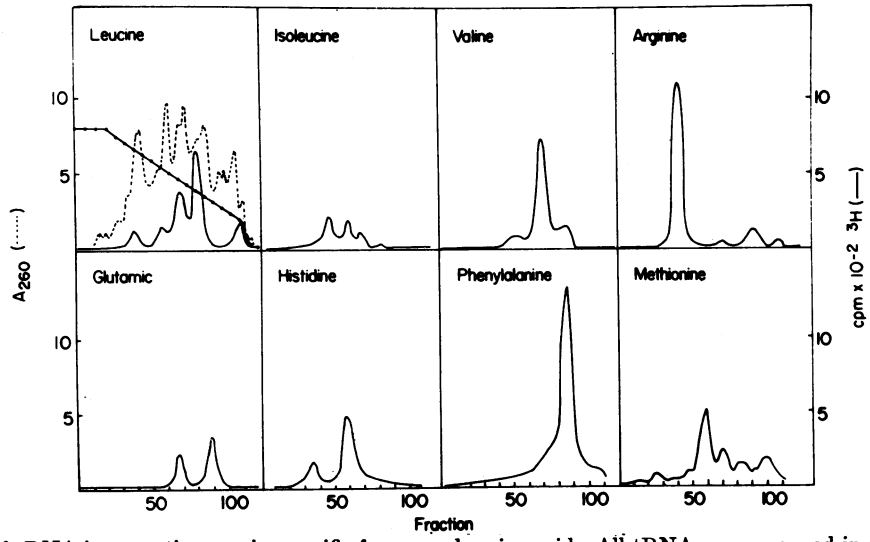

FIG. 3. Separation of tRNA isoaccepting species specific for several amino acids. All tRNAs were assayed in the effluent from a single Sepharose column used to separate 100 mg of *E. coli* B tRNA.

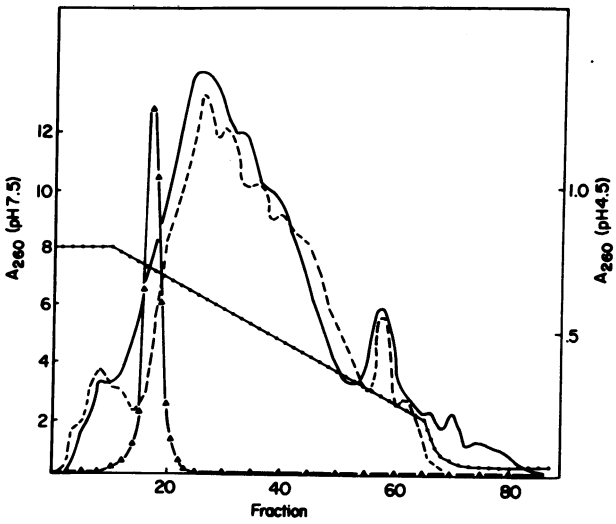

FIG. 5. The effects of pH and magnesium ion on the chromatography of tRNA. Conductivity, (●——●); A_{260} of fractions obtained at pH 7.5, (▲——▲); A_{260} of fractions obtained at pH 4.5 without magnesium, (——); A_{260} of fractions obtained at pH 4.5 in the presence of 10 mM Mg^{2+}, (- - -).

The data in Fig. 5 demonstrate that the salt-induced binding of tRNA to agarose is markedly influenced by pH. At pH 7.5 tRNA does not bind to agarose in high salt. In addition, magnesium does not substantially influence the chromatography of the tRNA under these conditions. It is possible, therefore, that tRNAs might be further resolved using an increasing pH gradient in the presence of high salt.

A final point worth mentioning regarding the convenience of this technique is that the columns can be used repeatedly without repouring; with simple washing with one or two column volumes of buffer followed by re-equilibration in the starting ammonium sulfate, the columns can be run at least 20 times without loss of resolution.

DISCUSSION

We have recently developed a general chromatographic method for the purification of proteins, glycoproteins, and neutral polysaccharides. The method involves the use of high concentrations of an antichaotropic ion (9–11), such as the anions phosphate or sulfate, to induce binding of these macromolecules to agarose gels substituted with aliphatic amino acids. The binding and fractionation of these macromolecules are thought to result from interaction of the nonpolar aliphatic chains of the gel with nonpolar sites on the macromolecules, and it has been suggested that these interactions may be hydrophobic in nature (9). In this report we show that this technique is also applicable to chromatography of tRNA; however, in this case it was not necessary to use substituted agarose. Presumably, the nonpolar character of the agarose itself is sufficient for tRNA binding at high $(NH_4)_2SO_4$ concentrations.

The two most widely used methods which were designed specifically for the separation of tRNA molecules are reverse phase chromatography (7) and benzoylated DEAE-cellulose chromatography (6). These methods utilize the principles of both ion exchange and hydrophobic interactions (6, 7); the method reported here is presumed to involve only the hydrophobic character of the tRNA.

An examination of the elution profiles obtained for a number of specific tRNA species derived from *E. coli* strains K-12 and B reveals several interesting differences. In the case of leucyl tRNAs, one species ($tRNA_2^{Leu}$) appears to differ in chromatographic behavior. In addition, one of the minor valine tRNA species in *E. coli* B tRNA is eluted much later than its K-12 counterpart. Marked differences in position of elution and relative composition of histidine tRNA species also can be seen. Interestingly, only one isoaccepting species of glutamate tRNA is seen in the case of K-12 tRNA. At present it is not clear what these differences indicate. However, several possibilities exist: (*i*) Actual strain differences in tRNA structures, (*ii*) differences in growth conditions of cells which in some instances can lead to alteration in tRNA profiles (7), or (*iii*) alteration of tRNA during the extraction procedures.

In view of the presumed nonionic nature of the gel–tRNA interaction we were surprised to find that binding did not occur at pH 7.5. At present, the significance of this observation is not clear. However, it could reflect pH-dependent changes in tRNA structure which could mask key sites on the molecule involved in binding.

In summary, the techniques described in this communication provide a method for separating tRNAs which can readily be adapted to both analytical and preparative purposes. Advantages of this method include: (*i*) low cost, (*ii*) simplicity of operation, and (*iii*) resolution which in some instances surpasses that seen with other methods such as RPC-5 and BD-cellulose. Furthermore, it has been shown that, when used in conjunction with other techniques such as RPC-5, salt-induced agarose chromatography provides a powerful tool for purifying individual species of tRNA.

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