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tRNA SEPARATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AN AGGREGATE OF ODS-HYPERSIL AND TRIOCTYLMETHYLAMMONIUM CHLORIDE*

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SUMMARY

High-performance liquid chromatography on a reversed-phase support treated with a tetraalkylammonium salt was used to separate tRNAs from baker's yeast. While resolution by this column appears to result from both anion-exchange and reversed-phase chromatography, it is the hydrophobic interactions which govern the separation of one tRNA from another. Chromatography of bulk tRNA resulted in a number of fractions with different amino acid acceptor activities and little cross-contamination. In some cases the column resolved several single nucleotide modifications of $tRNA^{Phe}$. Using a 250 × 6.2 mm column it has been possible to chromatograph a minimum of 100 A_{260} units of tRNA without serious loss in resolution. tRNAs isolated from this column as the last step of a purification procedure have very high amino acid acceptor activities.

INTRODUCTION

Bulk transfer ribonucleic acid (tRNA) isolated from a given organism, such as baker's yeast, consists of a family of some 60–90 unique species varying in length from about 75 to 90 nucleotides with molecular weights from 22,000 to 27,000 Daltons. Isolation of specific tRNAs from this mixture has previously been accomplished using anion-exchange columns usually containing a bound diethylaminoethyl (DEAE) moiety[1,2]. Following a number of separations by anion-exchange chromatography using various gradients, a final purification step by RPC-5 chromatography is often employed[3,4]. While RPC-5 chromatography is a powerful tool for purification of tRNAs, yields from the support tend to be low, it does not withstand pressure well and its commercial availability is poor. Recently separation of tRNAs based on hydrophobic interactions has been explored with some success[5,6].

Since nucleic acids in general are essentially polymeric anions containing hydrophobic bases, both anion-exchange and reversed-phase chromatography can be

* Dedicated to Professor Dr. Friedrich Cramer in honour of his 60th birthday

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potentially useful for separation and purification of a particular species. Chromatographic supports such as benzoylated DEAE-cellulose[7] and RPC-5 columns[4] which employ both anionic and hydrophobic interactions often result in better resolution of nucleic acids than those materials which employ solely ionic or non-ionic interactions.

In the present report we describe the use of an octadecasilyl (ODS) bonded phase silica support [C_{18} reversed-phase high-performance liquid chromatographic (HPLC) material] in the form of an aggregate with a tetraalkylammonium salt. This produces a material which appears to undergo both ion-exchange and reversed-phase chromatography. We have found this material of particular value for the separation and isolation of tRNAs.

EXPERIMENTAL

Materials

Trioctylmethylammonium chloride (Adogen 464) was purchased from Serva (Heidelberg, G.F.R.) and reagent grade ammonium acetate from E. Merck (Darmstadt, G.F.R.). ODS-Hypersil (5-μm particles) was a product of Shandon Southern (Runcorn, Great Britain) and bulk tRNA from baker's yeast was obtained from Boehringer (Mannheim, G.F.R.). The purified tRNAs specific for the amino acids serine and phenylalanine were prepared according to a previous procedure[8]. The purified tRNAs specific for the amino acids tyrosine, arginine and lysine were initially isolated using diethylaminoethyl anion exchangers according to a previous procedure[8] with a final purification step on trioctylmethylammonium chloride-treated ODS-Hypersil. tRNA specific for the amino acid aspartic acid was a gift from Dr. R. Giegé, Strasbourg, France. tRNAs specific for the amino acids valine and isoleucine were gift from Dr. H. Sternbach, Göttingen, G.F.R.

The modified derivatives of $tRNA^{Phe}$, $tRNA^{Phe}$ missing the AMP-76 and the wybutosine (Y) base[9], $tRNA^{Phe}_{-Y}$-C-C, $tRNA^{Phe}$ containing a 3′-deoxy-3′-aminoadenylic acid at position 76[10], $tRNA^{Phe}$-C-C-A(3′NH_2), and $tRNA^{Phe}$ missing the AMP-76 and 5′-phosphate[11], $tRNA^{Phe}_{-5'p}$-C-C, were prepared according to the procedures given in the corresponding references.

Equipment

The chromatographic separations were performed on a DuPont 850 liquid chromatograph equipped with a variable-wavelength UV detector and a thermostatically controlled oven. Polyacrylamide gel electrophoresis was performed at 800 V for 8 h on 10% acrylamide–0.25% bisacrylamide 450 × 350 × 1 mm gels containing 7 *M* urea. The tRNAs were visualized using an ethidium bromide solution (four drops of a 1% aqueous ethidium bromide solution per litre of water).

Methods

The following procedure is adequate for a 250 × 4.6 mm column. To 3.3 g of ODS-Hypersil suspended in 20 ml chloroform was added 1.0 mmol (0.4 g) trioctylmethylammonium chloride in 10 ml chloroform and the suspension was shaken gently for a few minutes. The chloroform was removed using a gentle stream of nitrogen. The dry support was suspended in 20 ml of degassed 0.5 *M* ammonium

acetate pH 5.0 containing 0.02% sodium azide and further degassed with a water aspirator. The suspension was then transferred to a packing column followed by the attachment of the 250 × 4.6 mm chromatography column. The column was packed upwards at 200 bar for 10 min using the sodium azide-containing ammonium acetate buffer at pH 5.0. The column was then inverted and packing in the downward direction continued for 20 min.

Columns prepared in this manner were stored in the ammonium acetate buffer containing sodium azide. Prior to use the columns were washed with 0.5 *M* ammonium acetate at the appropriate pH until a stable baseline was obtained. With freshly packed columns a 2-h wash is often required. Columns which have already been in use produce stable baselines within two to three column volumes of the starting buffer. Columns prepared in this manner have been used for in excess of 50 injections without serious loss of resolution.

Generally two buffers were prepared. Buffer A was 0.5 *M* ammonium acetate at the desired pH and buffer B was 5.0 *M* ammonium acetate at the desired pH. A typical gradient at 35°C and 0.5 ml/min was from 100% buffer A (0.5 *M* ammonium acetate) to a mixture containing 60% Buffer A–40% buffer B (2.3 *M* ammonium acetate) in 1080 min (18 h).

Desalting of isolated tRNAs was accomplished by dialysis against distilled water, gel filtration using Sephadex G-10, ethanol precipitation followed by gel filtration, or lyophilization followed by gel filtration. Aminoacylation of purified tRNAs or tRNA mixtures was as previously described[12].

RESULTS

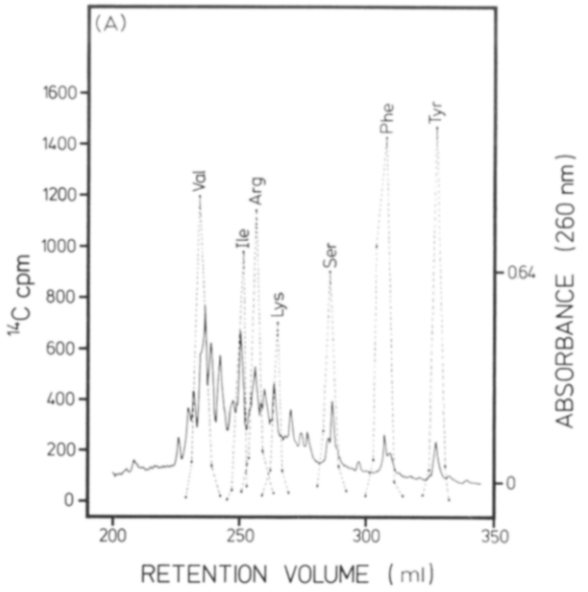
Bulk tRNA baker's yeast can be fractionated into a number of species using HPLC and a combined salt and pH gradient on trioctylmethylammonium chloride-treated ODS-Hypersil as shown in Fig. 1a. Testing of the eluted fractions for amino acid acceptor activity indicated that the individual tRNAs are eluted as well resolved species (Fig. 1a).

Polyacrylamide gel electrophoresis of the eluent (Fig. 1b) suggests that the resolution is not strictly a function of molecular weight as could be expected from purely anion-exchange chromatography.

Tetraalkylammonium chloride loading, pH, gradient and temperature effects

In the initial phase of this study the reversed-phase support ODS-Hypersil was treated with various amounts of trioctylmethylammonium chloride. 300 μmol tetraalkylammonium salt per gram of support resulted in high resolution of tRNAs and homopolymer digests. Concentrations of either 150 μmol/g or 600 μmol/g did not produce satisfactory resolution.

Binding of the tRNA to the column is a pH-dependent phenomenon. At pH 4.0 no significant retention of the tRNA was observed. At pH 5.5 the binding of bulk tRNA to the support was so strong that it was difficult to elute all species from the column. Using ammonium acetate salt gradients at a constant pH (of 5.2) resulted in some resolution of the bulk tRNA. However, separation of the tRNA mixture was best effected with a combination salt and pH gradient. Using a starting buffer of 0.5 *M* ammonium acetate (A) a gradient was prepared by linear addition of 5.0 *M*



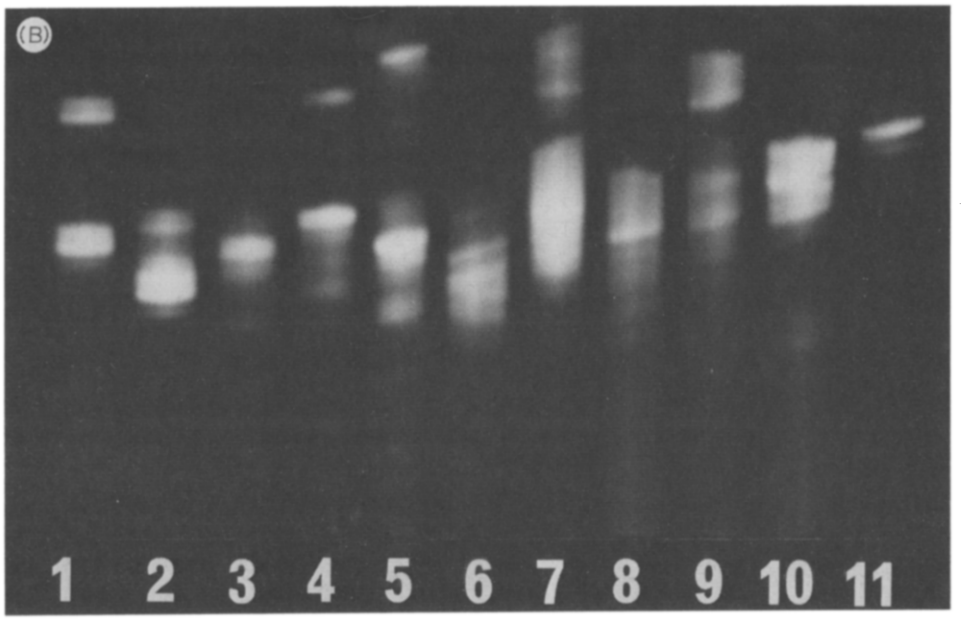


Fig. 1. (a) Resolution of 50 A_{260} units of bulk tRNA from baker's yeast, showing amino acid acceptor activity above background for valine (Val), isoleucine (Ile), arginine (Arg), lysine (Lys), serine (Ser), phenylalanine (Phe) and tyrosine (Tyr). Column: 4.6 × 250 mm trioctylmethylammonium chloride-treated ODS-Hypersil. Temperature: 35°C. Flow-rate: 0.5 ml/min. Gradient: buffer A, 0.5 *M* ammonium acetate pH 5.0; buffer B, 5.0 *M* ammonium acetate pH 5.5; 100% A to 60% A–40% B in 1080 min. (b) Polyacrylamide gel electrophoresis of the mixture obtained in the chromatogram (a). Numbers correspond to the eluent from (a): 1,229.5–232.2 ml; 2,234.9–237.6 ml; 3,240.3–243 ml; 4,248.4–251.1 ml; 5,253.8–256.6 ml; 6,261.9–264.6 ml; 7,bulk-tRNA; 8,275.4–278.1 ml; 9,286.2–288.9 ml; 10,307.8–310.5 ml; 11,326.7–329.4 ml.

ammonium acetate (B) until a final 60% A–40% B mixture was obtained (2.3 *M* ammonium acetate). A number of combination salt–pH gradients were investigated using an initial buffer of 0.5 *M* ammonium acetate pH 4.5 and a second buffer of 5.0 *M* ammonium acetate at pH 5.0, 5.5, 6.0 and 6.5. A mixture of the four tRNAs specific for the amino acids valine, isoleucine, serine and phenylalanine was analysed using these four pH gradients. Resolution was calculated according to retention volume and peak width as described[13]. Only the gradients produced using a second buffer at pH 5.5 or 6.0 provided high resolution of this tRNA mixture. The pH of the initial buffer could also be raised to 5.0. The combination gradient formed using an initial buffer of pH 5.0 and a second buffer at pH 5.5 provided high resolution not obtainable at constant pH. In most cases the pH gradient beginning at pH 4.5 provides slightly higher resolution. A typical combination salt–pH gradient as measured from the column eluent is plotted in Fig. 2.

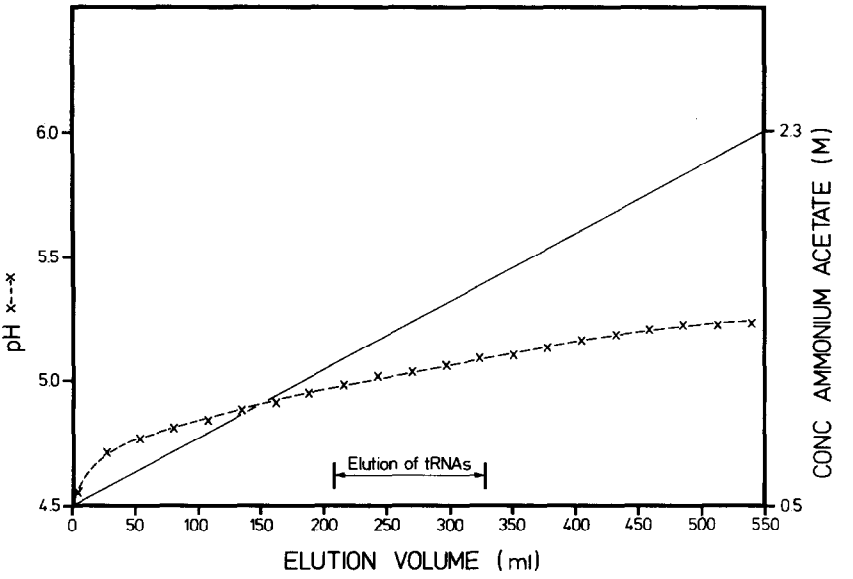
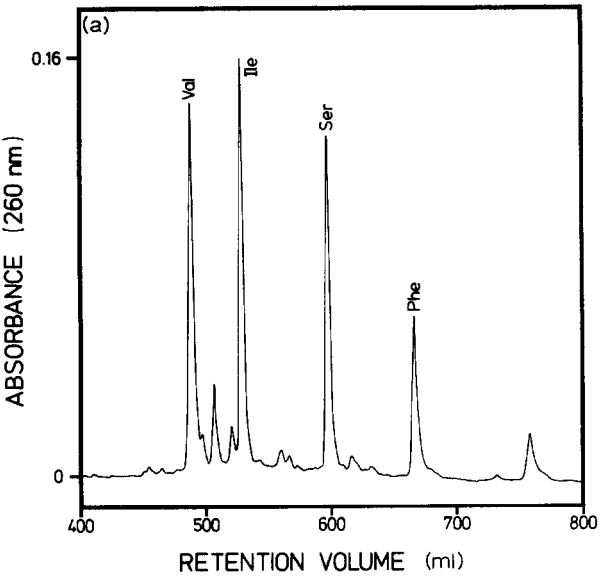


Fig. 2. pH of column eluent resulting from the combination salt–pH gradient described in the text for a 4.6 × 250 mm column with a flow-rate of 0.5 ml/min. x——x, using buffer A, 0.5 *M* ammonium acetate pH 4.5 and buffer B, 5.0 *M* ammonium acetate pH 6.0. Gradient: 100% A to 60% A–40% B in 1080 min; ——, corresponding salt gradient.

Relatively long gradients are necessary for effective separations. We presently employ a gradient which is 1080 min (18 h) in length as it is convenient for overnight runs. Slow flow-rates are partly a necessity as the columns described here have high backpressures (40 bar for a 250 × 4.6 mm column running at 0.5 ml/min). Shorter columns with a larger diameter (100 × 6.2 mm) can be employed, generally with sufficient resolution and higher flow-rates.

To study the effects of temperature on this chromatographic support a mixture of four purified tRNAs was again used. A sample chromatogram showing the elution at 35°C of tRNAs specific for valine, isoleucine, serine and phenylalanine is shown in



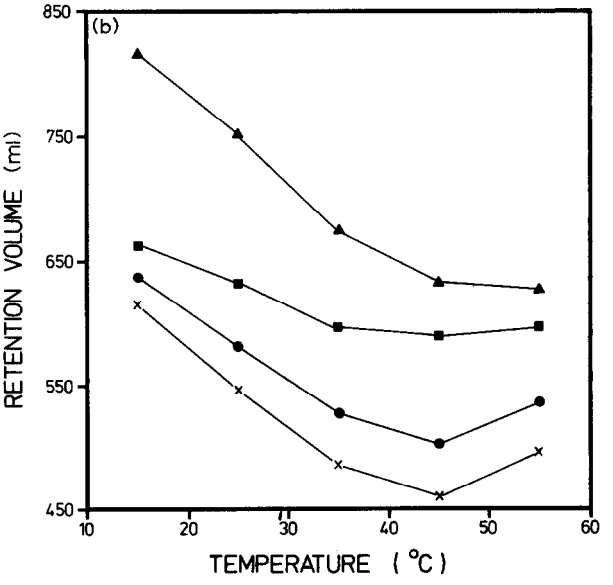


Fig. 3. (a) Separation of four specific tRNAs at 35°C. (b) Temperature dependence of the retention volumes of the tRNAs specific for valine (×), isoleucine (●), serine (■) and phenylalanine (▲). Column: 6.2 × 250 mm trioctylmethylammonium chloride-treated ODS-Hypersil. Flow-rate: 1 ml/min. Gradient: buffer A, 0.5 *M* ammonium acetate pH 4.5; buffer B. 5.0 *M* ammonium acetate pH 6.0; 100% A to 60% A–40% B in 1080 min.

Fig. 3a. Increasing the column temperature has a significant effect on the elution volume of a given tRNA as shown in Fig. 3b.

Resolution of modified tRNA

The retention volumes of a number of modified derivatives of tRNA specific for the amino acid phenylalanine are illustrated in Fig. 4. Removal of the wybutosine

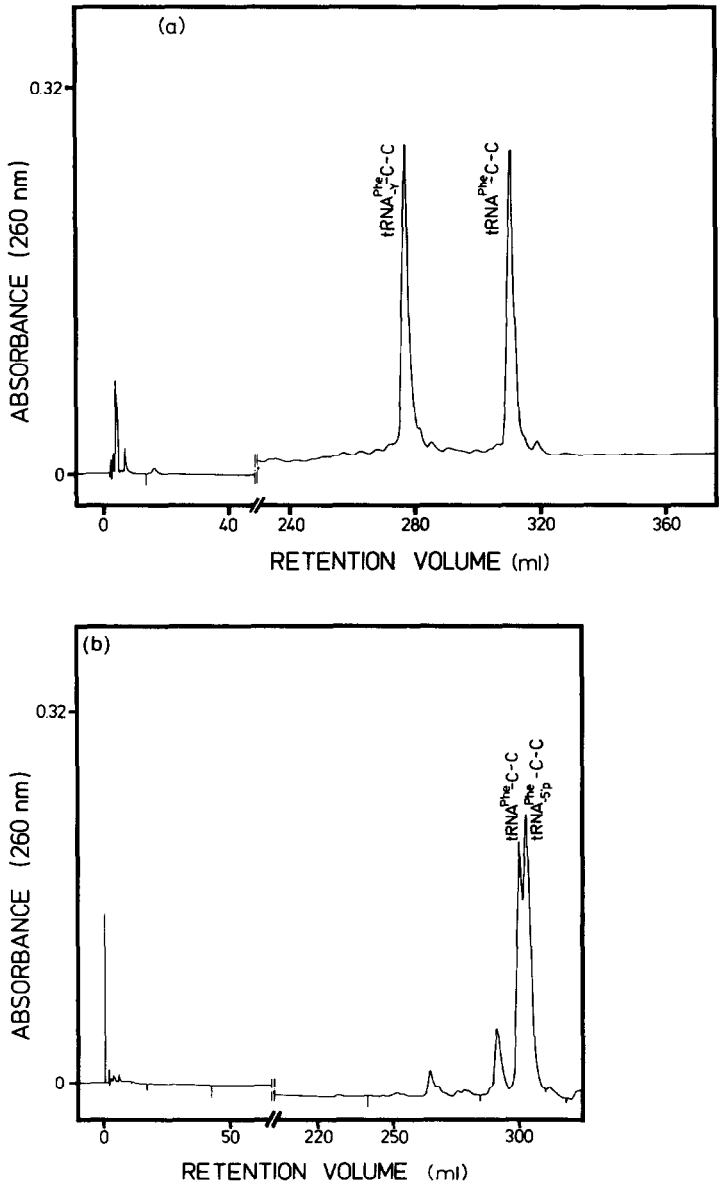


Fig. 4.

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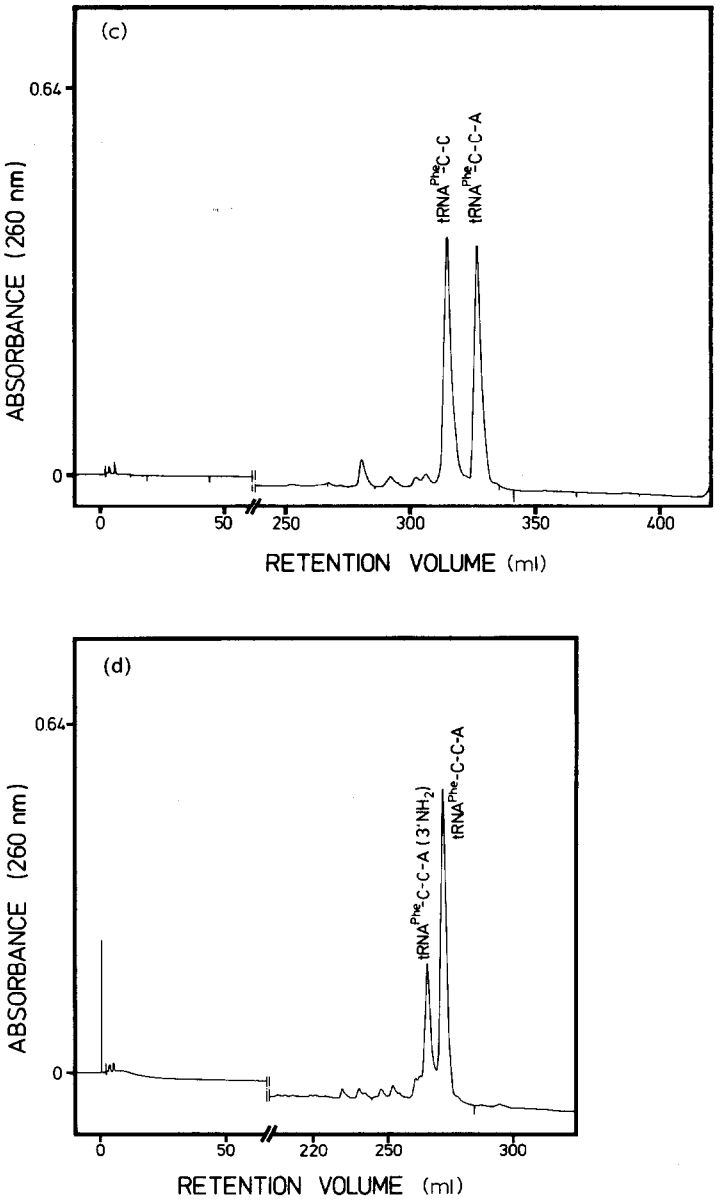

Fig. 4. HPLC separation of modified tRNAs with conditions as described in Fig. 1a. (a) Resolution of tRNA^{Phe}-C-C and tRNA$^{Phe}_{-Y}$-C-C, (b) of tRNA^{Phe}-C-C and tRNA$^{Phe}_{-5'p}$-C-C, (c) of tRNA^{Phe}-C-C and tRNA^{Phe}-C-C-A and (d) of tRNA^{Phe}-C-C-A (3'NH$_2$) and tRNA^{Phe}-C-C-A.

(Y) base results in a tRNA species with the same ribose-phosphate backbone as the native tRNA but lacking a highly hydrophobic nucleobase. The difference in the elution volumes of these two species is shown in Fig. 4a. Removal of the 5′ terminal phosphate from tRNA^{Phe}-C-C produces a tRNA differing by one anionic phosphate

group. The ability of the column to discriminate between these two species is indicated in Fig. 4b. The column can also discriminate between native $tRNA^{Phe}$ with a normal CCA 3′ terminus and $tRNA^{Phe}$-C-C lacking the terminal adenosine nucleotide (Fig. 4c). Modification of the 3′ terminal adenosine to produce a 3′-amino-3′-deoxyadenosine moiety introduces an amino group in place of a hydroxyl group at the tRNA 3′ terminus. Separation of the modified and unmodified tRNA is illustrated in Fig. 4d.

tRNA purification

tRNAs of high specific activity can be obtained from this chromatographic support when it is used as the last step of a purification procedure. For example, tRNA specific for the amino acid arginine was isolated by chromatography on benzoylated DEAE-cellulose, Sephadex A-25 and Sepharose 4B columns. The fraction containing $tRNA^{Arg}$ could be aminoacylated with arginine to the extent of 1100 pmol amino acid per A_{260} unit of tRNA. This fraction also exhibited contamination by tRNA specific for the amino acid threonine. 50 A_{260} units of this fraction were chromatographed on a 6.2 × 250 mm column of trioctylmethylammonium-treated ODS-Hypersil as illustrated in Fig. 5. Collection of the major peak followed by dialysis to remove the ammonium acetate afforded 28 A_{260} units of $tRNA^{Arg}$ with a specific activity of 1800 pmol arginine per A_{260} unit tRNA.

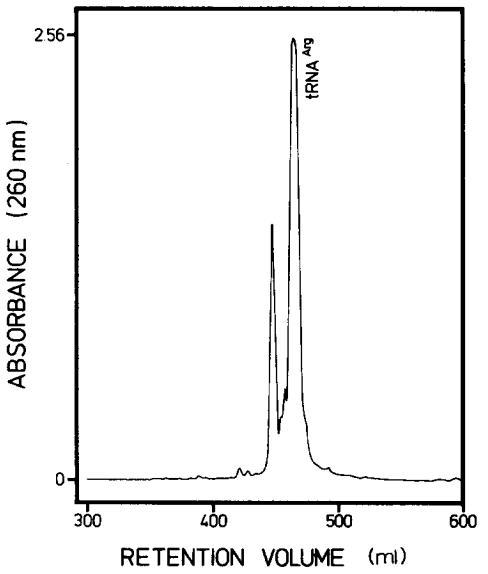

Fig. 5. Separation and isolation of tRNA specific for the amino acid arginine from a mixture containing in part tRNA specific for threonine. Chromatographic conditions at 35°C as described in Fig. 3 with a gradient of 960 min. Amino acid acceptor activity after chromatography: 1800 pmol amino acid per A_{260} unit.

DISCUSSION

The fractionation of bulk tRNA from baker's yeast on trioctylmethylammonium chloride-treated ODS-Hypersil appears to be a result of both anion-exchange and reversed-phase chromatography. The elution pattern is not purely a function of molecular weight as indicated by the polyacrylamide gel analysis in Fig. 1b. Two relatively small tRNAs, $tRNA^{Tyr}$ (78 nucleotides) and $tRNA^{Phe}$ (76 nucleotides), are observed relatively late in the gradient. $tRNA^{Tyr}$ contains a hydrophobic 6-(Δ^2-isopentenyl)adenosine nucleotide and $tRNA^{Phe}$ contains the hydrophobic Y base both of which have been observed to be very tightly bound to chromatographic supports which employ hydrophobic interactions such as benzoylated DEAE-cellulose[7]. Non-ionic interactions clearly then play an important rôle in the observed fractionation.

A ratio of 300 μmol tetraalkylammonium salt per gram of ODS-Hypersil appears to be an optimum for tRNA resolution. However, since new columns require a 2-h wash before a stable baseline is obtained it is likely that some of the tetraalkylammonium salt is lost and the amount remaining bound to the ODS support is somewhat less than 300 μmol/g. The pH gradient described by Fig. 2 provides adequate resolution of the tRNAs. Although the pH gradient is very flat it may result in selective protonation–deprotonation during chromatography of functional groups having high solvent accessibility. While we employ an 18-h gradient conveniently for overnight runs, the gradient length can be reduced to 9 h without significantly affecting resolution.

The temperature dependence of the elution volumes (Fig. 3b) from 15 to 45°C indicates that the binding of the tRNAs to the support is weaker at higher temperatures. The observation that the elution volumes increase at 55°C may reflect the breakdown of tertiary and secondary structure such that more anionic phosphate groups or hydrophobic bases have access to the support.

The high resolving power of trioctylmethylammonium chloride-treated ODS-Hypersil is of particular value in the analysis and/or isolation of modified tRNAs. The modified tRNAs specific for the amino acid phenylalanine shown in Fig. 4 also give some insight into the mechanisms of separation. Removal of the Y base from $tRNA^{Phe}$-C-C produces a species which elutes from the column much earlier than the native $tRNA^{Phe}$-C-C and indicates that non-ionic or hydrophobic interactions play a pivotal rôle in the separation of one tRNA from another. The chromatogram of Fig. 4b indicates that ionic interactions are secondary in importance to the separation mechanism. If anion-exchange interactions were of primary importance one would expect that the native $tRNA^{Phe}$-C-C possessing the anionic phosphate on the 5′-terminal nucleotide would have the larger elution volume. In fact the opposite is observed. Removal of the 5′-terminal phosphate results in a less polar species which by non-ionic interactions is more tightly bound to the column.

It is useful that $tRNA^{Phe}$-C-C and $tRNA^{Phe}$-C-C-A can be separated from one another as indicated by the chromatogram of Fig. 4c. The later elution of $tRNA^{Phe}$-C-C-A clearly reflects the greater hydrophobicity of adenosine compared to cytidine rather than the difference of the additional internal phosphate group. This separation is of particular value when preparing tRNAs with a modified 3′-terminal adenosine. The starting material for such a modification is often $tRNA^{Xxx}$-C-C. It is naturally

desirable to ensure that no contaminating $tRNA^{Xxx}$-C-C-A is present prior to the addition of the modified adenosine.

In some cases the support will discriminate single nucleotide modifications as shown in Fig. 4d. The replacement of the 3′-hydroxyl group of the terminal adenosine from $tRNA^{Phe}$ with an amino group results in a species with a smaller retention volume. Since the aliphatic amino group will be extensively protonated under the chromatographic conditions, it is more polar and elutes earlier from the column than the native $tRNA^{Phe}$-C-C-A.

We have begun using the trioctylmethylammonium chloride-treated ODS-Hypersil as a last step for purification of small amounts of tRNA. A 6.2 × 250 mm column can easily bind 100 A_{260} units of tRNA without serious loss of resolution. Column yields after desalting for pure tRNAs generally vary from 60 to 90%.

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