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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TRANSFER RNAs

SEPARATION OF TRANSFER RNAs FROM MAMMALIAN SOURCES

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SUMMARY

A survey of recent advances in high-performance liquid chromatography (HPLC) of tRNA is presented here. The polystyrene and reversed-phase anion exchangers are discussed for their ability to resolve tRNAs without loss of the aminoacyl–tRNA bond. The HPLC of a tRNA of choice, based on the affinity principle, is studied. Both chemical (boronate) and biological (plant lectins) affinity groups for the tRNA interaction are described. A comprehensive scheme is presented for the separation of four mammalian tRNAs. Scope of future research in this area is also discussed.

INTRODUCTION

About two decades ago, reversed-phase columns were introduced for the separation of transfer ribonucleic acids (tRNAs)[1]. These columns, especially the so-called "RPC-5" were very useful for obtaining gram quantities of purified tRNA samples and also for analyzing minute differences in the tRNA structures. For example, separation of a tRNA mixture containing a common sequence with a difference in only one nucleotide position were resolved by these columns[2].

In recent years, the tRNA from mammalian sources has been isolated and purified by using new methods. Though the polystyrene anion exchanger of uniform and small beads produced superior resolution of the tRNAs[3], yield of the aminoacyl-tRNA from these columns was adversely influenced by residual amines found in certain brands of the polystyrene anion exchangers, causing base-catalyzed deesterification of the aminoacyl(AA)–tRNA bond[4]. More recently, affinity chromatography has been used to separate a given tRNA from nineteen other species in one step. This process is followed by resolution of the isoacceptors of the isolated tRNA by a combination of reversed-phase and anion-exchange high-performance liquid chromatography (HPLC)[5].

In this survey of the recent advances in the tRNA separation methods, poly-

styrene anion exchangers and the RPC-5 material are compared for HPLC of the tRNAs. A HPLC method is described here to separate a given AA-tRNA from other, uncharged tRNAs by using a combination of hydrophobic and affinity principles. This modification of the earlier affinity methods permits separation of the AA-tRNA under conditions stable for the AA-tRNA ester bond (pH 6.8). In addition, isolation of one specific tRNA or a group of tRNAs on the basis of the minor, modified nucleotide is described. A scheme for the separation of four mammalian tRNAs in mg amounts is presented.

BASICS OF THE tRNA STRUCTURE

A generalized structure of tRNA, as shown in Fig. 1, consists of four base-paired segments: the amino-acid-acceptor (AA) stem; the dihydrouridine (D) stem; the anticodon (AC) stem; and the TψCG (ψ) stem. The stems constitute two independent helices. The number of base pairs (BP) is specific for each stem (see Fig. 1). Non-hydrogen-bonded loop regions extend from the helical stems. The ψ and AC loops each contain seven nucleotides. The D loop contains two variable regions, α and β, each containing one to three nucleotides in addition to five constant purine nucleotides, found in all structures. The "variable" loop (also called the "extra" or the "small" loop, here called the V loop) may be short, containing as many as 21 nucleotides and creating a fifth double-helical stem of three to seven base pairs and a loop of three to five residues. The basic two-dimensional "cloverleaf" structure is thus characteristic of all tRNAs, yet they may vary in length from 75 to 93 nucleotides.

The tRNA sequences, as described in refs. 6 and 7, are numbered such that each tRNA can be compared directly with yeast $tRNA^{Phe}$, the three-dimensional structure of which is known in both orthorhombic[8] and monoclinic crystal forms[9–11]. To begin with residue no. 1 and end with residue no. 76 in each tRNA sequence, three gaps in the sequential numbering are introduced at residues 17, 20 and 47, respectively. Thus, the residue in the α and β regions of the D loop are numbered 17, 17:1 and 17:2, and 20, 20:1, 20:2 and 20:3, respectively. Similarly, "extra" residues of the V loop are numbered 47, 47:1, 47:2, 47:3, etc.

A generalized structure for the mammalian tRNA is derived in Fig. 1 from 48 known sequences of mammalian origin covering thirteen different amino acids and the initiator tRNA.

MINOR, MODIFIED RESIDUES IN MAMMALIAN tRNAs

Over 20% of the tRNA residues are modified in various ways (methylation, isomerization, thiolation or addition of the hydrophilic or hydrophobic "extra" structure to the heterocyclic ring). The nucleotide modification is confined to 29 specific positions of the tRNA structure (Fig. 2). The residues with high incidences of modification are located mostly in the short helix formed by D and AC stems.

The "wobble" base located in position 34 is modified in 30 sequences, while the so-called "hypermodified" base located in position 37 is modified in 37 sequences considering a total of 48 mammalian tRNA sequences[6,7]. Both positions, 34 and 37 are located in the "exposed region" of the tRNA structure[12], hence available for interaction with other molecules, for example, reactive groups of the HPLC matrices.

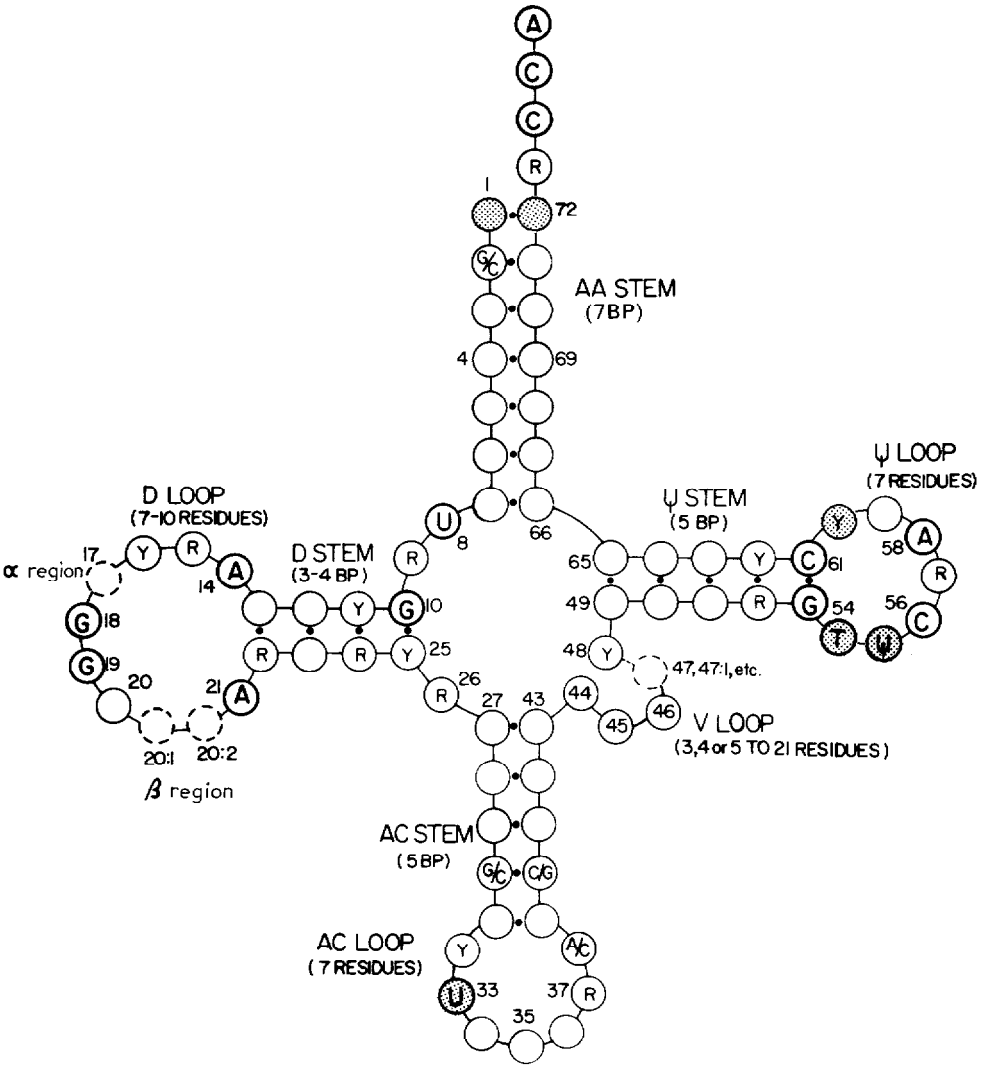


Fig. 1. A general structure of mammalian tRNAs based on 48 sequences with >90% probability, with the exception of the initiator tRNAs. Invariant (16) and semi-conserved (20) residues are shown in circles. (R = purine; Y = pyrimidine; G/C, G or C; A/C, A or C). Residues in boldface are the invariant nucleotides present in each mammalian tRNA sequence examined. Shaded circles of this general tRNA structure are the nucleotide positions in which the initiator tRNA structure varies. The circles with broken lines are the nucleotide positions not "occupied" in all the tRNAs examined.

In addition to a high incidence (70%) of the nucleotide modification, the greatest variation in the quality of modification also appears to exist in these two nucleotide positions of the tRNAs. This property of the tRNAs can be exploited for the separation of one specific tRNA (or a group of tRNAs) based on specific interactions between a tailor-made affinity group of the column matrix and the tRNA having a complementary reactive group. Indeed, a hydrophobic cellulose column (ben-

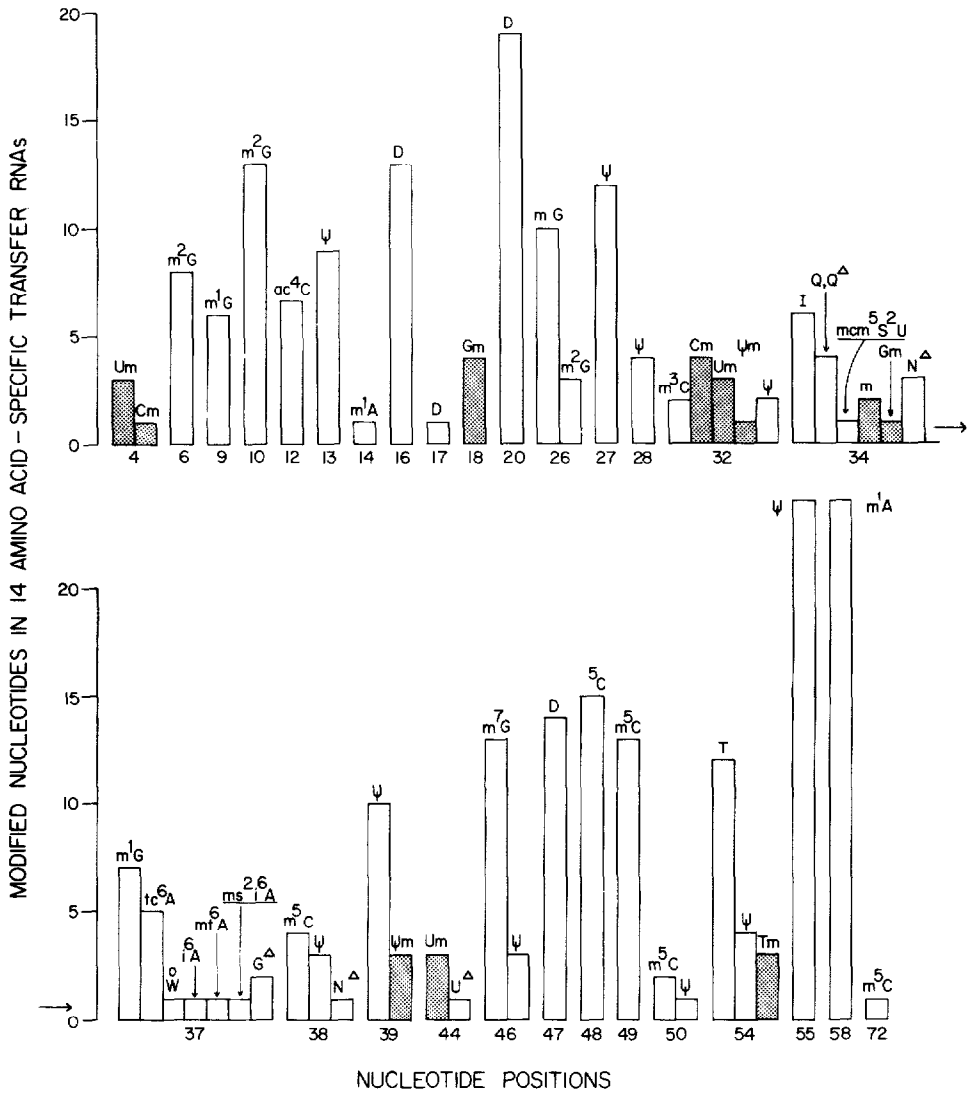


Fig. 2. Modification of the nucleotides in mammalian tRNAs. Numbers indicate nucleotide positions of the generalized structure (see Fig. 1). For a comprehensive list of the symbols for minor modified nucleotides, see ref. 6 or 7.

zoylated diethylaminoethylcellulose) matrix was used for the specific purification of the yeast $tRNA^{Phe}$ which contains a hydrophobic modified residue (so called "Wyosine") in position 37[13,14]. Atypical modifications in these two positions can be used for resolution of specific tRNAs. For example, queuosine (Q), found in position 34 of certain tRNAs is used for the group separation in order to separate Q-containing tRNAs from non-Q (or Q-deficient) tRNAs on a column matrix specific for interaction with the Q residue (see below for details).

POLYSTYRENE ANION EXCHANGER *VERSUS* RPC-5 MATRIX

Chemical and physical differences: both are anion exchangers

A recent study indicates that the smaller nucleic acid components separate on reversed-phase columns in a manner essentially identical with that observed in the usual anion-exchange chromatography on a polystyrene matrix[15]. The difference between reversed-phase chromatography and the conventional anion-exchange chro-

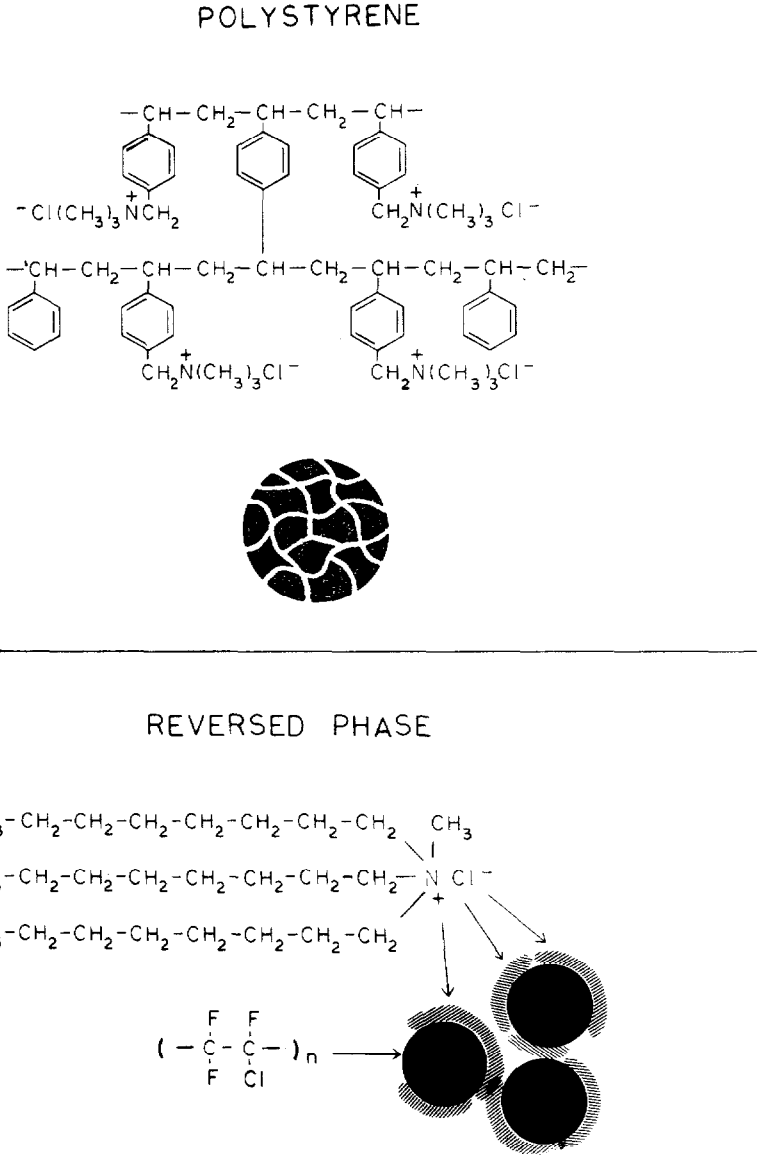


Fig. 3. Chemical and physical nature of two kinds of anion-exchange column matrices: top, polystyrene; bottom, reversed-phase RPC-5[4].

matography on polystyrene resins lies in the differences in the composition and physical character of the inert support holding the anion-exchange groups, which are quaternary ammonium derivatives in both cases[16]. For comparison, a schematic view of the polystyrene anion exchange (*e.g.*, Dowex 1) and the reversed-phase (RPC-5) beads is shown in Fig. 3. The polystyrene exchanger contains covalent functional groups of the type $[RN(CH_3)_3]^+$, where R represents a cross-linked porous vinylbenzene lattice. But in the reversed-phase matrix, the quaternary ammonium derivative [such as methyltrialkyl(C_8–C_{10})ammonium chloride] is immobilized on a solid inert support [for example, polytrifluorochloroethylene (Kel F) or polyfluoroethylene (Teflon, PTFE)]. Since the aryl residues of the polystyrene are more hydrophobic (organophilic) than the simple alkyl chains of the reversed-phase column, and because of the uniform, small bead size of the former material, improved resolutions were noted for oligonucleotides and monomers on the conventional anion exchanger[15].

Comparison of the separation efficiency: RPC-5 has lower efficiency

The separations of mixed tRNAs (*Escherichia coli*) by anion-exchange chromatography and reversed-phase chromatography were compared under identical conditions[3]. They were desorbed by a linear gradient of chloride ion, from 0.5 to 0.75 *M*, at 22°C. The elution profiles, monitored at two wavelengths, indicate that, while tRNAs from the reversed-phase column are eluted mostly as one large ultraviolet-absorbing band (mostly at the front of the gradient, with some peaks in the middle of the gradient, see Fig. 4b), this material on the anion-exchange column undergoes fractionation into numerous unresolved peaks throughout the salt gradient (Fig. 4a). They are eluted with higher chloride concentration. Some tRNAs are so strongly adsorbed on the polystyrene column that their elution requires organic solvents in addition to chloride ions (such as, 10% ethanol in 1 *M* sodium chloride). Thus, tRNAs are adsorbed more strongly by the polystyrene exchanger than by the reversed-phase matrix. The spread of tRNAs throughout the elution gradient indicates a higher resolving power of the polystyrene anion exchanger.

The anion exchanger (a) and the reversed-phase (b) matrices were examined for separation efficiency by using a purified tRNA preparation (Fig. 5). Glutamate tRNA was isolated in about 95% purity by extensive chromatography[12]. About five A_{260} units* of this pure $tRNA^{Glu}$ sample were chromatographed on columns a and b of identical dimensions (16.5 × 0.6 cm) and under identical conditions (0.53 ml/min, at 30°C); each column was eluted at constant ionic strength. Whereas the separation on the reversed-phase column indicates largely one tRNA peak, the same material, when chromatographed on the polystyrene anion-exchange column (Fig. 5a), showed heterogeneity. Multiple peaks perhaps did not represent specific glutamate isoacceptors (Fig. 5a); the RPC-5-purified isoacceptor appeared to be contaminated with denatured species or with identical sequences having different amounts of posttranscriptional modifications. Though the column efficiency could not be determined accurately from a heterogeneous peak, the height equivalent to a theoretical plate (HETP) of the major component (the middle peak) on Aminex A-28 (9-μm beads, 8% cross-

* One A_{260} unit is the amount of substance in 1 ml of 50 m*M* sodium acetate buffer with 10 m*M* $MgCl_2$ (pH 4.5) which gives an absorbance of 1.0 when measured at 260 nm with a light path of 10 mm.

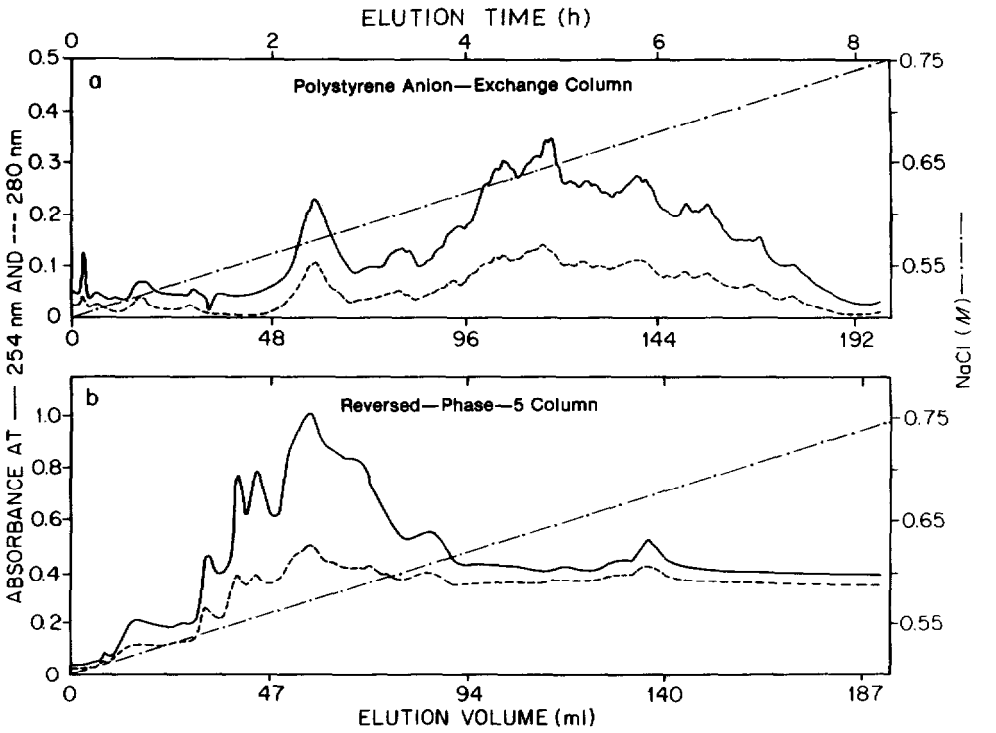


Fig. 4. Chromatography of unfractionated tRNAs on two kinds of anion-exchange column matrices: a, Bio-Rad polystyrene Aminex A-28, 27 × 0.6 cm column; b, reversed-phase RPC-5, 25 × 0.6 cm. In 200 μl, 2 mg of tRNAs were applied to each column. Both were eluted with a linear gradient of 0.5 to 0.75 *M* sodium chloride, 100 ml of each solution, at 22°C and 0.4 ml/min. The buffer used was 10 m*M* sodium acetate, 10 m*M* $MgCl_2$, 1 m*M* EDTA, and 1 m*M* $Na_2S_2O_7$, pH 4.5. The resultant pressures were 245 (a) and 105 (b) p.s.i.; see ref. 3 for details.

linked; Bio-Rad Labs., Richmond, CA, U.S.A.) was significantly lower than that on the RPC-5 column (Fig. 5b).

The separation of tRNAs on the anion-exchange column is superior to the reversed-phase chromatography in many ways. The chromatography of mixed tRNAs on the former indicates that different tRNAs tend to spread throughout the salt gradient. The plate heights, a quantitative measure of the separation efficiency[17] for the two matrices, indicate that the Aminex anion exchanger perhaps contains twice as many theoretical plates as does a RPC-5 column of the same dimensions.

Resolution of the isoacceptors: order of the elution is altered

Isoacceptors of the tRNA are characterized (numbered) in the order of their elution from the RPC-5 column (Fig. 6b). Is the order of elution of the isoacceptors from the polystyrene anion-exchange column the same as in the case of RPC-5? To answer this question, leucyl-tRNAs (six isoacceptors) separation was carried out on both the Aminex A-28 and the RPC-5 columns. Five [^{14}C]leucyl-tRNA peaks are generally obtained by reversed-phase chromatography under the best separation conditions (Fig. 6b). The anion-exchange column, not only resolved six species, but also

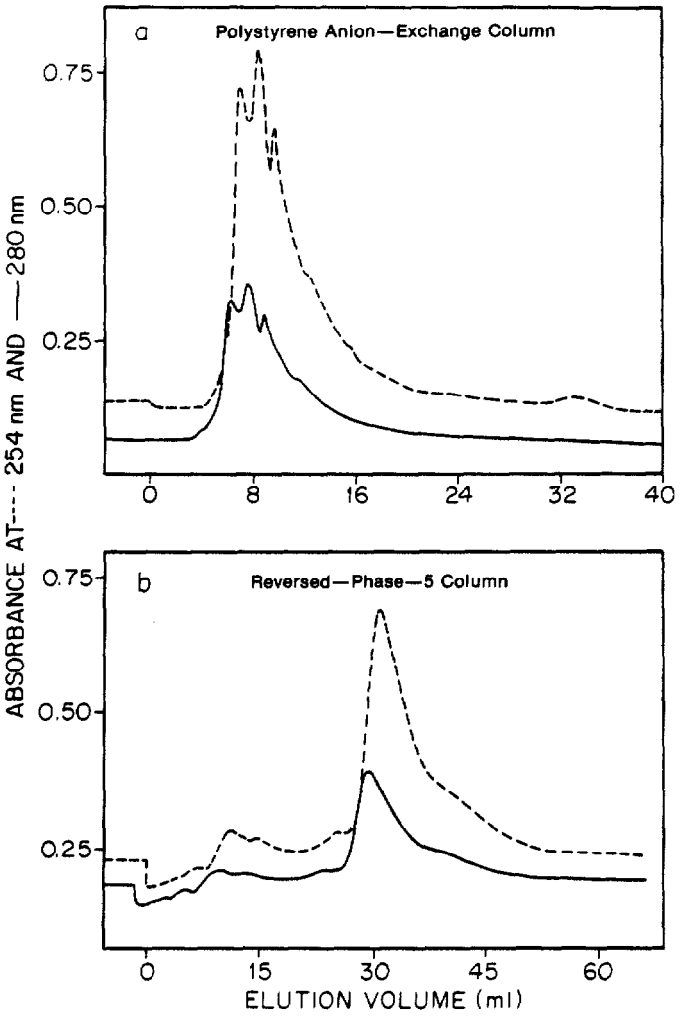


Fig. 5. A comparison of the separation efficiency (HETP values) of polystyrene anion-exchange, Aminex A-28 (a) and RPC-5 (b) columns. Column a was eluted with 0.57 *M* sodium chloride, while column b was eluted with 0.51 *M* sodium chloride, under the conditions as described in the next.

showed heterogeneity in two of the major ones (compare peaks 2 and 3 in Fig. 6). In addition, the elution pattern of these isoacceptors was different on the two columns[3]. For example, while the major isoacceptor (components 2 and 2′) was eluted by about 130 ml from the Aminex A-28 column, only 50% of the eluent was needed for its elution from the RPC-5 column.

Apparently, the isoacceptors containing both anionic and hydrophobic functions react differently with aryl anion exchangers (Aminex A-28) than with alkyl anion exchangers (RPC-5). Observed differences in the order of their elution from the two column matrices are important since characterization of each isoacceptor depends on the order of its elution.

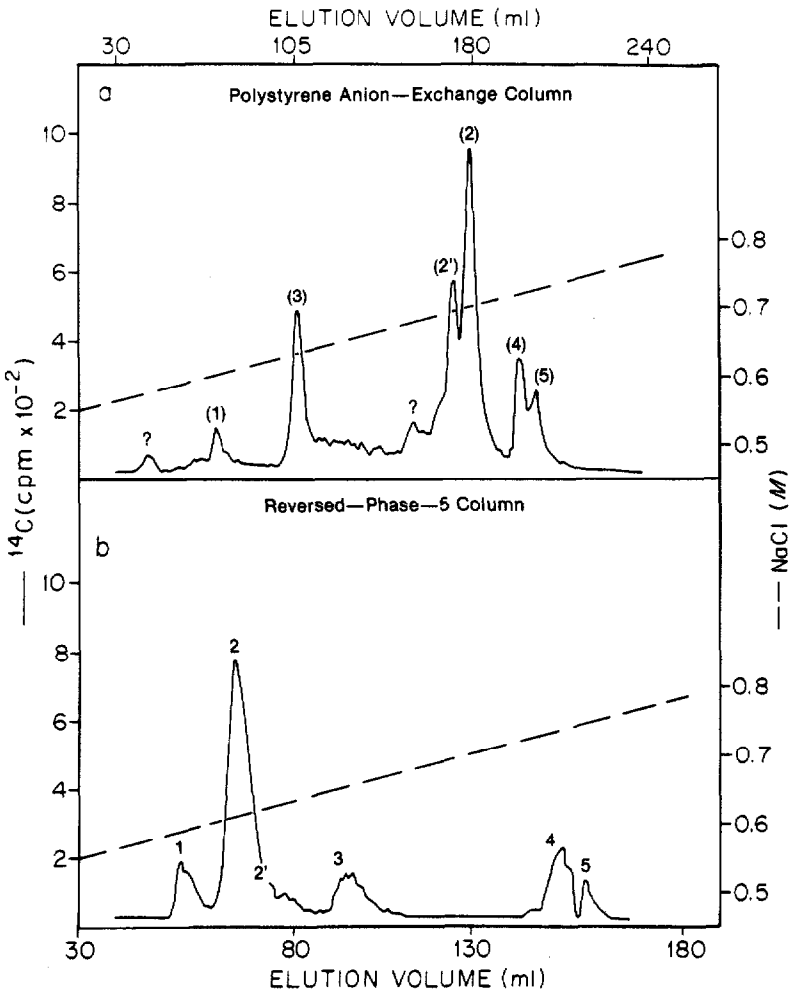


Fig. 6. Separation of [^{14}C]leucyl-tRNA isoacceptors (a) on a polystyrene anion-exchange column (Aminex A-28, 32 × 0.6 cm), eluted with a linear gradient of 0.55 to 0.80 *M* sodium chloride, 150 ml each at 0.5 ml/min, 200 p.s.i.; and (b) on a reversed-phase column (RPC-5, 30 × 0.6 cm), eluted with a linear gradient of 0.5 to 0.8 *M* sodium chloride, 100 ml each at 0.5 ml/min, 100 p.s.i., 37°C. Two A_{260} units of [^{14}C]leucyl-tRNAs were applied to each column (for buffer composition, see legend to Fig. 4).

STABILITY OF AMINOACYL-tRNA ON CHROMATOGRAPHY COLUMNS

The stability of the ester bond with regard to the presence of residual (primary, secondary or tertiary) amines, the structure of the resin bead and pH and temperature of the medium was examined[4].

Comparison of lysyl-tRNA isoacceptors on two column matrices

A comparison of chromatography of lysyl-tRNA isoacceptors on RPC-5 (Fig. 7b) and on a polystyrene anion exchanger (Aminex A-25, 18-μm beads, 8% cross-

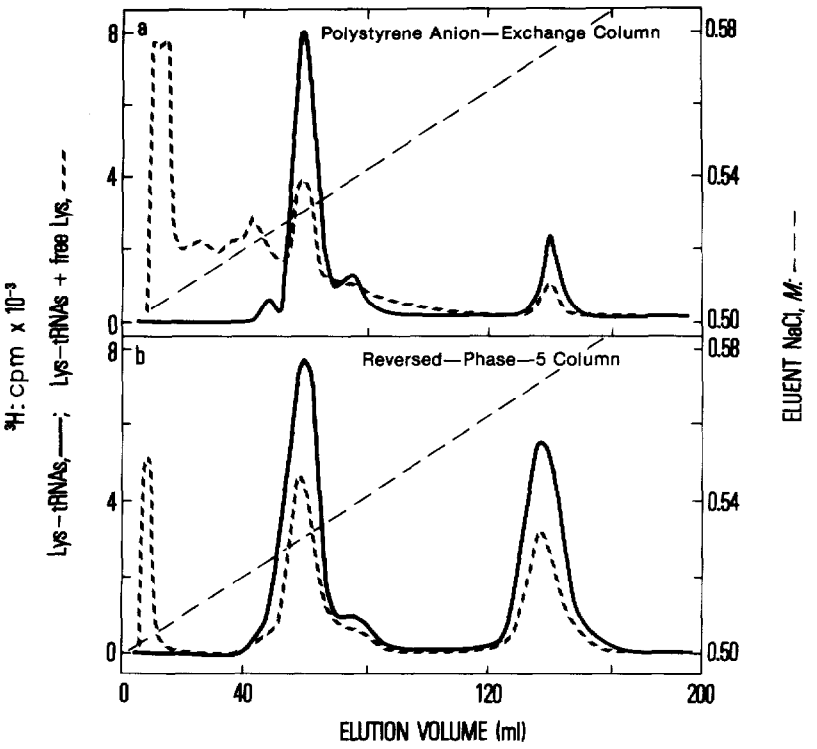


Fig. 7. Separation of rat liver lysyl-tRNAs on polystyrene anion-exchange, Bio-Rad Aminex A-25 (a) and RPC-5 (b) columns. About 0.5 A_{260} units of the [^{3}H]lysyl-tRNAs were applied and the column eluted with a linear gradient of 0.50 to 0.60 *M* sodium chloride, 100 ml each containing 10 m*M* magnesium acetate, 1 m*M* EDTA, 3 m*M* 2-mercaptoethanol and 10 m*M* sodium acetate, pH 4.5, at 20°C. Aliquots from each 2-ml fraction were assayed for lysyl-tRNA (———) and for total radioactivity (----)[4].

linked, Bio-Rad Labs.) (Fig. 7a) showed that the relative proportion of the two major peaks on the two matrices varied significantly. (Both total radioactivity due to AA-tRNA and free lysine and the acid-precipitable radioactivity due to AA-tRNA were studied in each case.) To ascertain the cause of this difference, both major peaks from the polystyrene column were chromatographed on the RPC-5 column. The results showed no deacylation on the RPC-5 polystyrene column. The two AA-tRNAs (peaks) on the latter column were deacylated at different rates, producing different ratios between the two peaks on the two columns.

Resolution and yields of the tRNA isoacceptors

Experiments distinguishing between the actual yield of isoacceptors (aminoacylated and free species) and the apparent yield of isoacceptors (aminoacylated species only) indicate that the apparent amount of an isoacceptor depends on the column matrix employed[4]. For example, two lysyl-tRNAs are resolved with different ratios (1.1 and 0.4) on two polystyrene exchangers of the same cross-linkage (8 %) and bead size (9 μm). The exchangers appear to influence the stability of the aminoacyl–tRNA bond differently (see below).

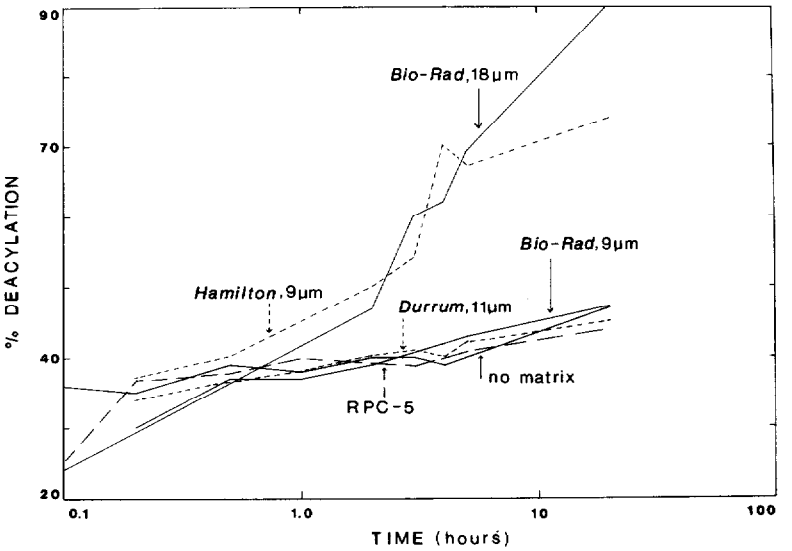


Fig. 8. Deesterification of labelled lysyl-tRNAs with time when incubated with various column matrices in the column buffer of pH 4.5 at 25°C: Bio-Rad, 18 µm; Hamilton, 9 µm; Bio-Rad, 9 µm; Durrum, 11 µm; RPC-5; no matrix.

How can certain matrices enhance deacylation?

To examine quantitatively the degree of deacylation occurring on various matrices during chromatography, [^{3}H]lysyl-tRNAs were incubated with each column matrix at 25°C, and aliquots of the same volume were withdrawn at various intervals and assayed for [^{3}H]lysyl-tRNA (acid-precipitable) and for the total radioactivity. The control experiment did not include any resin during the incubation.

Out of the five column matrices tested, chemical deacylation is observed for only two polystyrene anion-exchange matrices (both 8% cross-linked) [Bio-Rad Aminex A-25 and Hamilton HA-X8 (Hamilton, Reno, NV, U.S.A.)] (see Fig. 8). The exchangers causing deacylation are 18-µm beads of Bio-Rad (Aminex A-25) and 9-µm beads of Hamilton (HA-XA). However, no deacylation is caused by 9-µm beads of Bio-Rad (Aminex A-28) which have the same degree of cross-linking and bead size as the Hamilton packing. Therefore, deacylation is not caused by differences in the physical shape of the packing material. There must exist some other difference between the two exchangers. The AA-tRNA should react freely with the interior of the porous beads where the local concentration of the reactive and fixed residual (primary, secondary and tertiary) amines can vary from one preparation to another, especially when they are synthesized by different methods (which indeed was the case —Bio-Red Labs., personal communication). The residual amines of the beads appear to cause the base-catalysed deacylation of the AA-tRNA. The reader is advised to test the packing material for this before changing batches or using a new brand of packing material.

RESOLUTION OF A tRNA OF CHOICE BY AFFINITY METHOD

To separate one tRNA from up to nineteen other species, different types of affinity chromatography methods have been used, *e.g.*, immunoadsorbent columns of

anti-wye[18] and anti-i^{6}Ado[19–21], matrix-bound sugar-specific plant lectins[5,22,23], matrix-bound tRNA serving as the anticodon for a specific tRNA[24] and a matrix that reacts specifically with the amino acid of one aminoacyl-tRNA[25–27]. The boronate matrix interacts with all uncharged tRNAs[28] by complexing with *cis*-2′,3′-hydroxyl groups of the terminal adenosines[29,30]. Satisfactory binding is enhanced by a buffer of alkaline pH, but alkaline pH conditions cause hydrolysis of the aminoacyl–tRNA bond[4]. The free tRNA then binds like any other uncharged tRNA. To achieve satisfactory complex formation at a least alkaline pH, modification of the complexing solvent and postsynthesis acetylation of a boronate matrix, N-[N′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethyl-cellulose (AEB-cellulose), have been suggested[31,32].

Chromatography on CMB-cellulose columns

A separation of lysyl-tRNA from other tRNAs (uncharged) on N-(*m*-dihydroxyborylphenyl)carbamylmethyl-cellulose (CMB-cellulose) is shown in Fig. 9[33]. The specific activity of the material eluted by the basic buffer was 1.56 nmol of lysine per A_{260} unit of the tRNA. Each fraction was assayed for the sum of [^{3}H]lysine plus [^{3}H]lysyl-tRNAs and for acid-insoluble radioactivity, [^{3}H]lysyl-tRNAs. The difference between the two values indicated that about 15% of the radioactivity eluted with the lysyl-tRNAs was due to the free amino acid. (A very small amount of radioactivity was retained by the column —less than 3% of the total activity— and was eluted with the uncharged tRNA.) A long trail of apparent absorbance observed

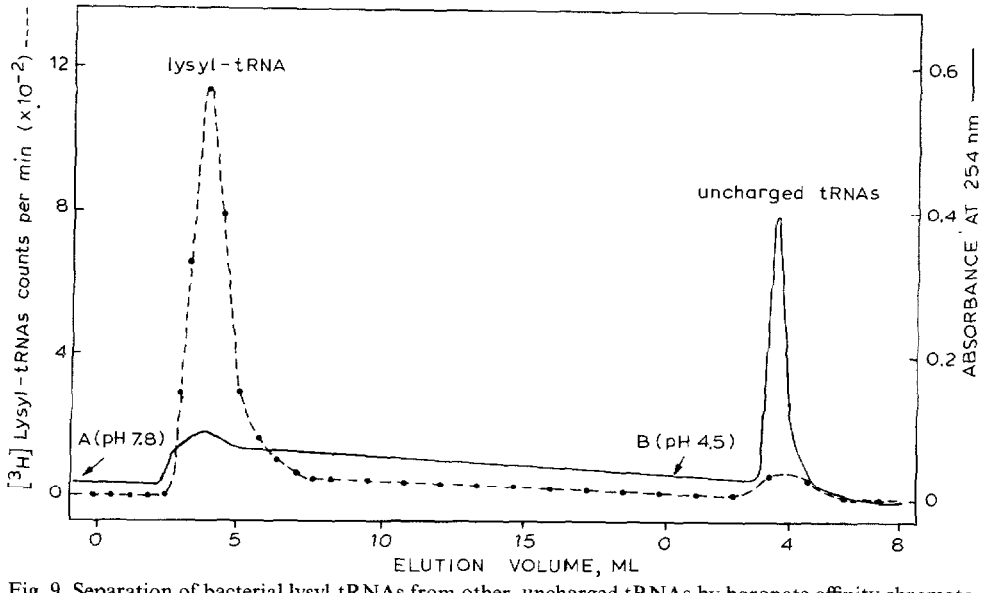


Fig. 9. Separation of bacterial lysyl-tRNAs from other, uncharged tRNAs by boronate affinity chromatography on a CMB-cellulose column. About 1 mg of *E. coli* tRNA containing [^{3}H]lysyl-tRNAs (40,000 cpm) in buffer A was applied to the column (10 × 0.5 cm). The column was preequilibrated and eluted first with buffer A [0.2 *M* sodium chloride, 50 m*M* magnesium chloride, 20% ethanol, 50 m*M* N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid (HEPES), pH 7.8] and then with buffer B (50 m*M* sodium acetate with 0.2 *M* sodium chloride, pH 4.5) at 4°C and 10 ml/h. 1-ml fractions were collected and assayed for radioactivity and absorbance monitored at 254 nm.

under peak 1 was caused by a baseline shift of the monitor; the corresponding fractions did not exhibit any absorbance at 260 nm when examined separately.

The results indicate appreciable (>15%) deacylation of the AA-tRNA under these elution conditions (Fig. 9). The deesterification is due to unstability of the ester bond even under these best stabilizing conditions of the eluent (ethanol, high salt and slight alkalinity).

The reversed-phase boronate matrix

A new kind of boronate chromatography matrix, RPB, was synthesized recently[33]. The active group is a phenylboronic acid adsorbed on the support by a 1-nm-long hydrophobic "tail". This material, N-(*m*-decanoyl)phenylboronic acid, is bound to (coated on) polychlorotrifluoroethylene beads by hydrophobic interactions (Fig. 10). Both uncharged and AA-tRNAs are retained by the RPB material by the hydrophobic nature of the matrix and the boronate groups. To weaken the interactions between AA-tRNAs and the matrix, relatively high concentrations of chloride and magnesium ions are necessary. However, these ionic conditions are not strong enough to weaken the boronate complex formed between the matrix and the uncharged tRNA. Strongly adsorbed uncharged tRNAs require significantly higher concentrations of chloride and fairly acidic conditions (low eluent pH) for desorption from the column.

Isolation of an AA-tRNA from other, uncharged tRNAs by RPB chromatography

In Fig. 11, purification of about 4 nmol of lysyl-tRNAs from other liver tRNAs (uncharged) in a single chromatogram is shown. Little radioactivity ($\approx 3\%$) due to unesterified lysine was detected; the specific activity was 1.56 nmol of lysine per 1.0 A_{260} unit under our aminoacylation conditions[15]. Lysyl-tRNA did not form a boronate anion; hence, there was no radioactivity in the uncharged tRNAs. Although no fractionation of lysine isoacceptors was observed, the uncharged tRNAs were eluted in two major peaks from this column. In each experiment, the level of purity of the AA-tRNA which is eluted in the front peak of the chromatogram is appreciably high. The non-Q-tRNAs esterified with amino acids of varying hydrophobicity can be

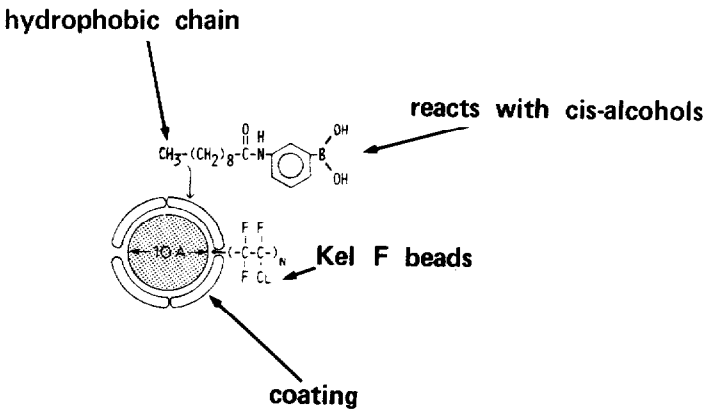


Fig. 10. Structure of reversed-phase boronate matrix and its interaction with the 3′-end of the uncharged tRNAs.

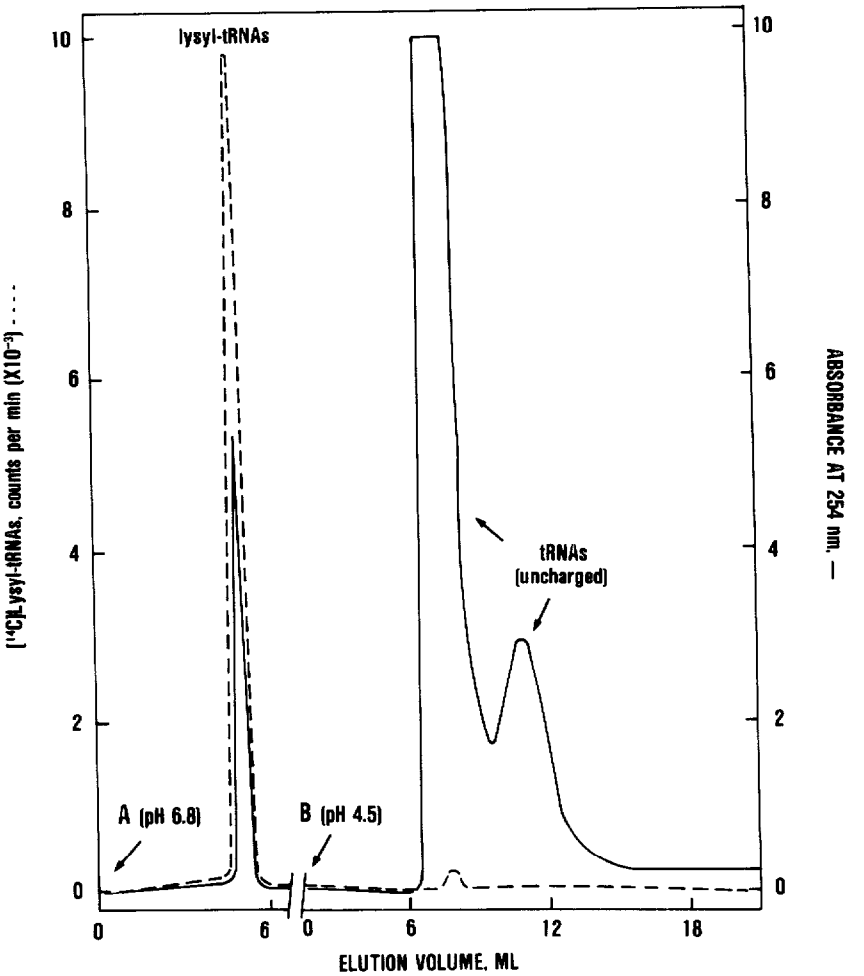


Fig. 11. Separation of an aminoacyl-tRNA from uncharged tRNAs by reversed-phased boronate chromatography at neutral pH (see legend to Fig. 9 for experimental details).

recovered in high yield (about 80%) in the column-excluded material. However, Q-tRNAs tend to interact with the boronate matrix more strongly than the non-Q-tRNAs and their yield in the front peak is extremely poor (6–17%).

Recovery of tRNAs from AEB-cellulose columns is incomplete (65–70%), but yields from CMB-cellulose and RPB column matrices are satisfactory (92–97%). Chromatography on other matrices (phenylboronate, linked to porous glass, agarose or Sepharose beads via >1.0-nm-long spacer arm) give little or no separation of AA-tRNA from uncharged tRNA, and recovery of the material is very low (about 30%). Low recovery of the tRNA is presumably caused by strong hydrophobic interactions between purine and pyrimidine bases (tRNA) and the long spacer arm of the matrix. However, such strong interactions are not exhibited by CMB-cellulose and RPB column matrices which contain only about 1-nm-long spacer arms.

Dialcohols of Q in the Q-tRNAs interact with the boronate matrix more

strongly than the dialcohols of adenosine at the 3′-end. Although only a small fraction (6-17%) of the Q-tRNA is purified by this method, purity of the tRNA is satisfactory. Elution conditions can be investigated in order to reduce the interaction between Q and the matrix while maintaining the complex formation between the ribose and the matrix.

Uncharged tRNAs form complexes with the boronate groups on RPB columns about two units away from the reported pK of the benzeneboronic acid. The presence

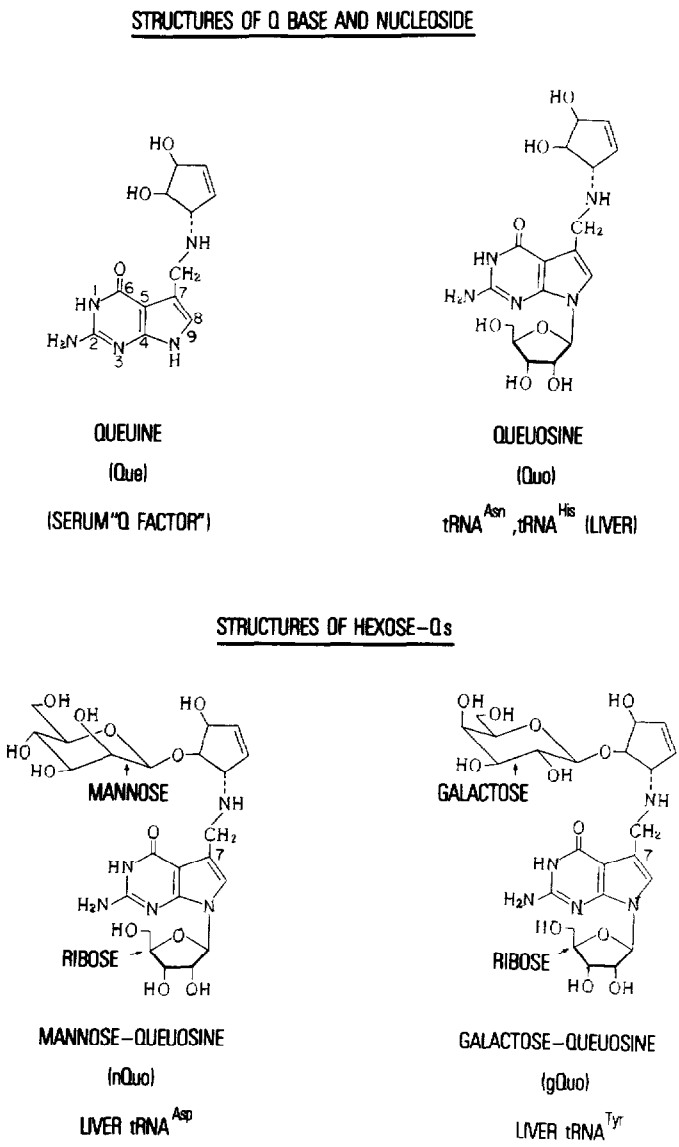


Fig. 12. Structure of queuosine (7-[(*cis*-4,5-dihydroxy-2-cyclopenten-1-yl)amino]methyl-7-deazaguanosine) and its derivatives.

of a 1-nm-long tail and the absence of negatively charged groups in the vicinity are apparently responsible for the lowering of the pK of the matrix.

RESOLUTION OF THE tRNA BASED ON THE PRESENCE OF A MINOR, MODIFIED COMPONENT — QUEUOSINE

The "wobble" base, located in position 34, is modified in 61 % of eukaryotic and 47 % prokaryotic tRNAs[6]. Similarly, out of 49 mammalian tRNAs, 31 contain a modified residue in this position (unpublished results). The replacement of guanine by queuine occurs specially at the wobble position in asparagine, histidine, aspartate and tyrosine tRNAs. Prokaryotes contain unmodified Q in each one of the four tRNAs, whereas mammals contain either an unmodified Q ($tRNA^{Asn}$ and $tRNA^{His}$) or a modified Q (manQ in $tRNA^{Asp}$ and galQ in $tRNA^{Tyr}$). Chemical structure of the base (queuine)*, nucleoside (queuosine) and the hypermodified nucleoside (mannosylqueuosine and galacetosylqueuosine) are shown in Fig. 12. The G to Q modification in bacteria occurs by substitution of a precursor or queuine, while in mammalian rRNAs it occurs by substitution of a preformed queuine (once called "Q factor") into the Q-deficient tRNAs[34–36].

Unsubstituted Q contains *cis* dialcohols which react with the boronate and form an anion at slightly alkaline pH — similar to the reaction of *cis* dialcohols of the ribose (3′-end of the tRNA) with the boronate. However, substituted Q (such as, hexosylQ present in $tRNA^{Asp}$ and $tRNA^{Tyr}$) does not contain dialcohols, and therefore fails to react with the boronate matrix. The bacterial tRNA contains only unsubstituted Q, and therefore, each one of the four Q-containing tRNAs reacts with the boronate matrix. However, only two mammalian Q-containing tRNAs contain unsubstituted Q ($tRNA^{Asn}$ and $tRNA^{His}$) and only these species can react with the boronate matrix. The other two mammalian Q-containing tRNAs, which in addition contain an hexose residue (manQ in $tRNA^{Asp}$ and galQ in $tRNA^{Tyr}$), can be made to react with specific column matrices especially designed with affinities for a specific hexose residue of the substituted-Q-tRNA.

HPLC of mammalian tyrosine tRNAs

Ricinus communis agglutinin, a lectin from castor beans has an affinity for β-D-galactose and tyrosine tRNAs of mammalian tissues have galactose in the galQ base of their anticodons. We have studied the interaction between tyrosine tRNAs and this lectin immobilized on solid supports with spacer arms of different lengths[23]. Tyrosine tRNAs are separated from nineteen other tRNAs of bovine liver by the affinity principle by using the lectin, immobilized on an agarose matrix. The results in Fig. 13 indicate that a spacer arm length of 10 Å between the agarose bead and the lectin gives the best separation. Two tyrosine tRNA isoacceptors are separated from each other and from other tRNAs in one step when this affinity principle is used (see legend to Fig. 13 for eluent composition).

HPLC of mammalian aspartate tRNAs

Fractionation of the mixed tRNAs on a concanavalin A–Sepharose column is

* 7-{[(*cis*-4,5-Dihydroxy-2-cyclopenten-1-yl)amino]methyl}-7-deazaguanine.

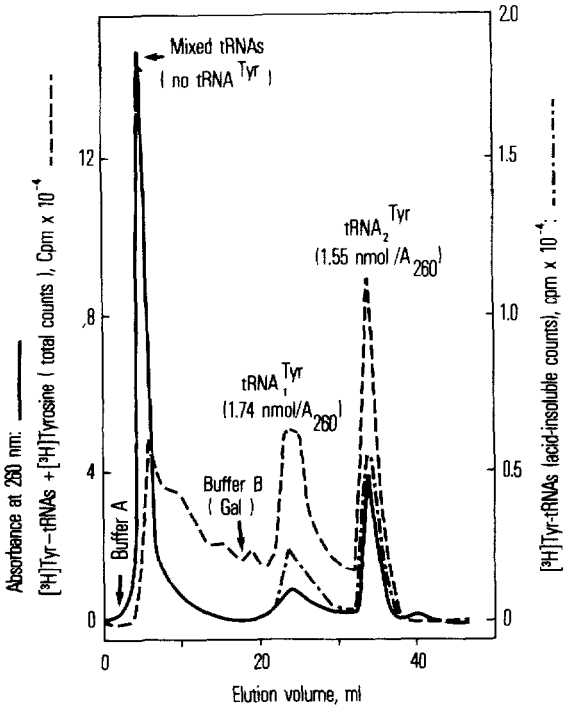


Fig. 13. Separation of liver tyrosyl-tRNAs from uncharged tRNAs by lectin affinity HPLC using a 10-nm spacer "arm" between the agarose matrix and the caster bean lectin. Eight A_{260} units of bovine liver tRNAs and [^{3}H]Tyr-tRNAs ($1.4 \cdot 10^4$ cpm) were applied to the column (7.5 × 1 cm). The column was eluted with buffer A (50 m*M* sodium acetate, 5 m*M* magnesium chloride, 0.15 *M* sodium chloride, pH 6.0), then with a buffer A containing 1 m*M* D-galactose and 0.7 *M* sodium chloride (indicated by an arrow) at a flow-rate of 3 ml/h. 1-ml fractions were collected at 4°C[3].

shown in Fig. 14. The column was preequilibrated, loaded and eluted with a buffer of pH 6.0, containing the metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}) necessary for binding between concanavalin A and the mannosyl residue of the manQuo in $tRNA^{Asp}$. Under these conditions, all other tRNAs and RNAs appeared in two peaks immediately after the breakthrough volume (peaks 1 and 2, in Fig. 14). The $tRNA^{Asp}$ was eluted with the same buffer containing α-methyl-D-glucopyranoside[5].

The interaction between manQuo of $tRNA^{Asp}$ and concanavalin A is very specific. Other tRNAs ($tRNA^{Tyr}$, $tRNA^{Asn}$ and $tRNA^{His}$) containing similar modifications (queuosine) in their structures do not complex with this particular lectin. The specific interaction is not influenced by the presence of five to six S RNAs and rRNA impurities in the tRNA preparation. Therefore, the time-consuming separation of tRNAs from other RNAs by gel-filtration chromatography can be avoided. Furthermore, the bound $tRNA^{Asp}$ can be recovered from the column without loss by incorporation of α-methyl-D-glucopyranoside into the eluent.

Using only 8 % of the capacity of a lectin column containing 17 μmol of bound concanavalin A, about 41 mg $tRNA^{Asp}$ (1.4 μmol) were purified from 2.2 g of the mixed RNAs in one step. For the separation of the two isoacceptors, further chromatographic separations on RPC-5 and Aminex columns were necessary. An RPC-5

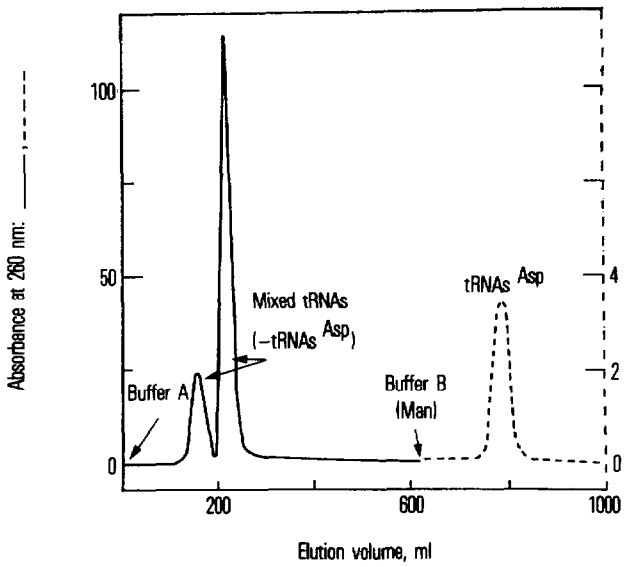


Fig. 14. Separation of aspartate tRNA from other tRNAs (and RNAs) by lectin affinity HPLC on a concanavalin A (Con A)–Sepharose column matrix. About 0.3 g of unfractionated RNA (70% of tRNAs), dissolved in 24 ml of buffer (50 m*M* sodium acetate, 0.25 *M* sodium chloride, 10 m*M* magnesium chloride, pH 4.5), were applied to a Con A–Sepharose column (32 × 2.5 cm; 0.1 μmol Con A per ml of gel). The column was first eluted with a buffer A (50 m*M* sodium acetate, 0.15 *M* sodium chloride, 5 m*M* magnesium chloride, 1 m*M* calcium chloride and 1 m*M* manganese chloride, pH 6.0). After removing unbound RNAs and tRNAs (peaks 1 and 2), the column was eluted with buffer B (buffer A, containing 50 m*M* mannose or α-methyl-D-glucopyranoside, indicated by an arrow). The column was eluted at 0.5 ml/min, 4°C and 10-ml fractions were collected, pooled and precipitated by ethanol[14].

column (bed volume 95 ml) gave preparations of $tRNA_1^{Asp}$ and $tRNA_2^{Asp}$ that were 88 and 97% pure, respectively. The two isoacceptors were purified to apparent homogeneity by further chromatography on an Aminex column[5].

Group separation of unsubstituted-Q-containing tRNAs

To achieve group separation of unsubstituted-Q-containing tRNAs in mammalian and bacterial tRNAs, unfractionated tRNAs were chromatographed on CMB-cellulose columns. About 0.5 g of bovine liver tRNA, after deacylation, were applied to a CMB column. The column was first eluted with a basic buffer (pH 8.7) and then with an acidic buffer (pH 4.5) (Fig. 15). Material from each peak was pooled and precipitated with ethanol. Levels of different Q-tRNAs in the two peaks were determined by aminoacylation. Analysis showed that about 92% of manQ-tRNA or galQ-tRNA was not retained by the column, but 87–98% of the Q-tRNAs ($tRNA^{Asn}$, $tRNA^{His}$) was specifically retained[33].

The tRNAs containing non-substituted Q tend to interact with the boronate matrix more strongly than those containing no Q (or substituted Q). The yield of the former tRNAs in the front peak is very poor[33]. For example, Lys-tRNA in Fig. 9 is eluted as a front peak at pH 6.8 in only 6 ml; this is followed by elution of the uncharged tRNAs at pH 4.5. However, the latter tRNAs can be eluted from the column even at pH 7.8 by raising the salt concentration and the eluent volume, still

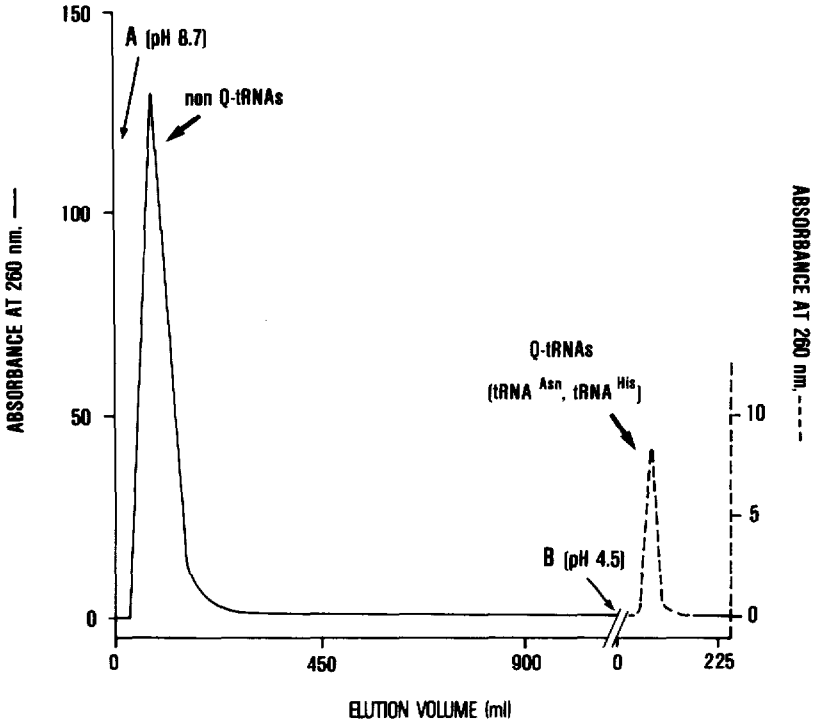


Fig. 15. Group separation of unsubstituted-Q-containing tRNAs ($tRNA^{Asn}$ and $tRNA^{His}$) from others by boronate HPLC. About 0.5 g of liver (or bacterial) tRNAs in 30 ml of buffer A were applied to a CMB-cellulose column (41 × 1.5 cm) and eluted first with buffer A (0.7 *M* sodium chloride, 0.1 *M* magnesium chloride, 50 m*M* morpholine, pH 8.7) and then with buffer B (0.2 *M* sodium chloride, 10 m*M* manganese chloride, 50 m*M* sodium acetate, pH 4.5). The column was eluted at 0.65 ml/min and 10-ml fractions were collected at 4°C[33].

leaving the two Q-tRNAs adsorbed on the column (Fig. 15; see legend for details).

Group separation of unsubstituted Q-containing tRNAs from non-Q-containing tRNAs by CMB-cellulose affinity chromatography is simple and can be scaled up to process large amounts of tRNA. Liver Q-containing tRNAs can be further resolved into $tRNA^{Asn}$ and $tRNA^{His}$ isoacceptors by column chromatography on reversed-phase (RPC-5) and anion-exchange columns (see below).

Resolution of unsubstituted-Q-containing liver tRNAs

The material obtained from CMB columns mostly contains asparagine and histidine tRNAs. To resolve the two tRNAs and remove any contaminant tRNA, the material from peak 2 of the CMB column (Fig. 15) was chromatographed on an RPC-5 column. In one experiment, a CMB column was overloaded and the material from peak 2 (Fig. 15) was applied to a RPC-5 column (200 × 1.0 cm) in a pH 4.5 buffer. The results in Fig. 16 indicate early elution of the Q-deficient liver tRNAs and in addition, partial resolution of the two Q-tRNAs. The material from each Q-tRNA peak was concentrated and further purified (see below).

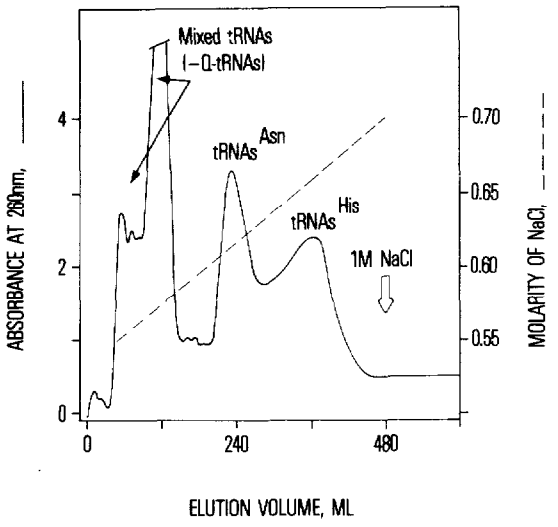


Fig. 16. Resolution of queuosine-containing tRNAs by reversed-phase HPLC. A RPC-5 column, 200 × 1.0 cm was loaded with a mixture of asparagine and histidine tRNAs, obtained by group separation on a CMB-cellulose column (see Fig. 15). The column was eluted with a linear gradient of 0.55 to 0.70 *M* sodium chloride, 250 ml, containing buffer, pH 4.5 (see legend to Fig. 9).

HPLC of asparagine tRNA

The material from the second peak in Fig. 16 was aminoacylated with labelled asparagine and the product was chromatographed on an Aminex A-28 column (64 × 0.9 cm) with a linear gradient of sodium chloride. The effluent was assayed for absorbance at 254 nm and radioactivity due to asparaginyl-tRNA (or free amino acid). The

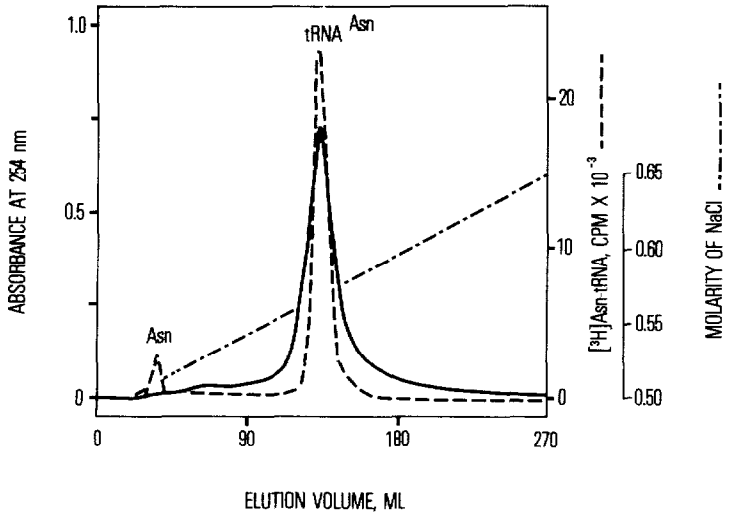


Fig. 17. Purification of asparagine tRNA by polystyrene anion-exchange HPLC. The material from the third peak of Fig. 16 was concentrated, aminoacylated with labelled asparagine, applied to a Bio-Rad Aminex A-28 (64 × 0.9 cm) column, and eluted with a linear gradient of 0.50 to 0.65 *M* sodium chloride, 150 ml each at 20°C. For buffer composition, see legend to Fig. 7.

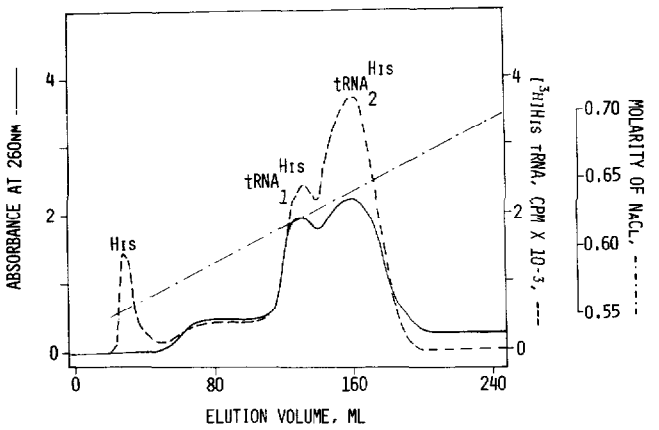


Fig. 18. Purification of histidine tRNAs by polystyrene anion-exchange HPLC. The material from the last peak of Fig. 16 was concentrated, aminoacylated with labelled histidine, applied to a Bio-Rad Aminex A-28 (64 × 0.9 cm) column and eluted with a linear gradient of 0.55 to 0.70 *M* sodium chloride, 150 ml each at 20°C. For buffer composition, see legend to Fig. 7.

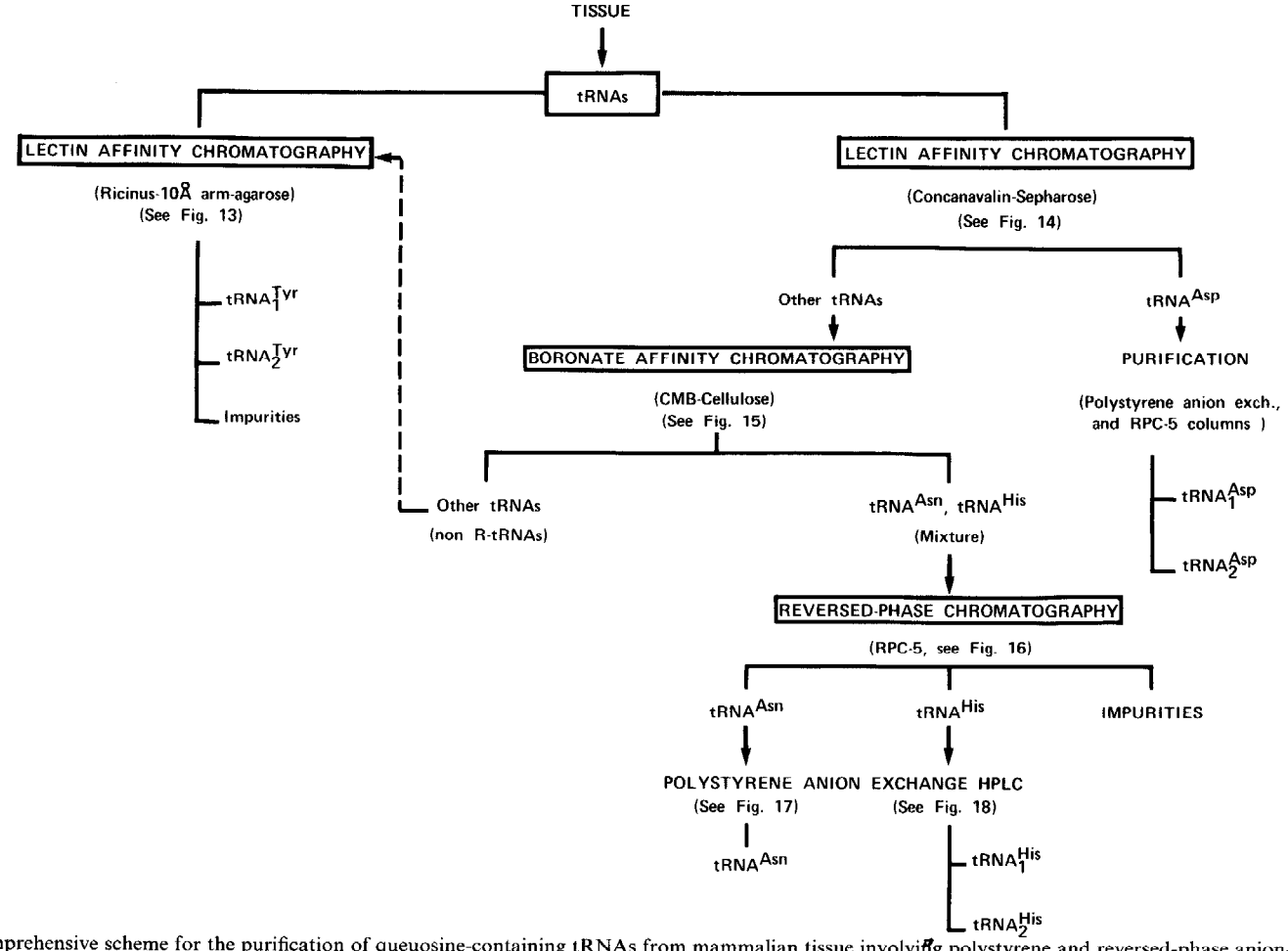
results in Fig. 17 indicate the high purity of the $tRNA^{Asn}$, free from other contaminants (no other 254 nm-absorbing material) and the presence of only one isoacceptor *in this tRNA preparation from bovine liver.*

HPLC of histidine tRNAs

The histidine tRNA peak was similarly aminoacylated with labelled histidine and also similarly chromatographed on a polystyrene anion-exchange column (see above). The results in Fig. 18 indicate high purity of the histidyl-tRNAs and absence of any contaminants, but poor resolution of the two isoacceptors. An addition of 5–10% ethanol to the reservoir of the gradient chambers did resolve the two isoacceptors (results not shown).

A GENERAL SCHEME FOR RESOLVING THE Q-CONTAINING tRNAs OF MAMMALIAN ORIGIN

In Scheme I, various steps are outlined for obtaining homogeneous isoacceptors of each Q-containing tRNA derived from the mammalian tissue. The tRNA is isolated and purified from mammalian livers according to the procedure described elsewhere[5]. First, tyrosine tRNAs are resolved by chromatography on the *Ricinus*–agarose matrix (Fig. 13) and aspartate tRNAs on the concanavalin A–Sepharose matrix (Fig. 14). In each case, hexose substituted-Q-containing tRNAs interact with the sugar-specific plant lectin and the tRNA is retained by the affinity column. The unadsorbed tRNA material from each lectin affinity column is concentrated by ethanol precipitation, dissolved in a buffer (pH about 9) and the unsubstituted-Q-containing tRNAs are separated from other tRNAs by the affinity principle by using a boronate column (CMB-cellulose or RPB). (Aminoacylation of the tRNAs is not required for this group separation.) The mixture of the asparagine and histidine tRNAs obtained in this manner (Fig. 15) is resolved by RPC-5 chromatography. In



Scheme I. A comprehensive scheme for the purification of queuosine-containing tRNAs from mammalian tissue involving polystyrene and reversed-phase anion-exchange HPLC and lectin affinity HPLC. Tyrosine and aspartate tRNAs were resolved by *Ricinus*– and concanavalin A–lectin affinity columns. Asparagine and histidine tRNAs were resolved by boronate affinity columns. isoacceptors of each tRNA were resolved by anion-exchange HPLC.

addition to the separation of these two tRNAs on this column, other tRNAs are eluted early and are separated from the two tRNAs of interest (see Fig. 16).

Isoacceptors of each Q-containing tRNA are resolved by HPLC on a polystyrene anion-exchange column (for example, Bio-Rad Aminex A-28; $tRNA^{Asn}$ in Fig. 17 and $tRNA^{His}$ in Fig. 18) or by using a combination of the ion-exchange column and the RPC-5 column (for $tRNA^{Asp}$, see ref. 5). No important deesterification of the applied aminoacyl-tRNA is observed on these columns. The purified isoacceptor in each case is fully active in the aminoacylation reaction and gives only one band in polyacrylamide gel electrophoresis (results not shown). The method can be scaled up to isolate large quantities of these tRNAs or scaled down for their detection and analysis at the picomole level.

NEW APPROACHES FOR THE tRNA SEPARATION

Polystyrene anion exchangers of uniform and small bead size have greater resolving power for the tRNAs. Unlike RPC-5 matrix, this exchanger can tolerate organic solvents and has greater capacity (functional groups: R_4N^+, mequiv.). The ion-exchange material is readily available from several manufacturers and their distributors, but caution is advised in using it. As discussed earlier, several brands of anion exchangers contain residual primary, secondary and tertiary amines which lower the tRNA yield from the column and catalyse deesterification of the AA–tRNA bond. In addition, in some instances the order of elution of the isoacceptor from the exchange columns may be different from the order of their elution from the RPC-5 columns (Fig. 6).

Affinity chromatography does indeed provide rapid isolation of tRNAs. For example, the reversed-phase boronate matrix described here allows chromatography at a neutral pH. However, the method is not entirely free from problems. The preparation of the matrix is tedious and time-consuming; inert supports of choice are not readily available; columns are unstable and light-sensitive, and finally, deesterification of several AA-tRNAs occurs even at the neutral pH used for chromatography. Presently, we are redesigning the affinity matrix in order that it can form a complex with the *cis* dialcohols (of the uncharged tRNAs allowing elution of AA-tRNA in the exclusion volume of the column) at a much lower pH value —about pH 4 to 5— where the AA–tRNA bond is most stable. The boronate matrix for group separation of the unsubstituted-Q-containing tRNAs does not require aminoacyl-tRNA, therefore, it is ideal for this purpose. Lectin affinity chromatography is indeed very specific for resolving aspartate and tyrosine tRNAs (of mammalian origin) from other tRNAs and RNAs. It is a powerful tool and can be used to prepare gram amounts of the two tRNAs.

Paired-ion reversed-phase chromatography, though not used for tRNA separation, can be employed successfully for tRNA work. Its application is limited to the separation of purine and pyrimidines[37], nucleosides[38,39], nucleotides[40] and short-chain oligonucleotides[41,42]. Recently, Nguyen *et al.*[43] reported the partial resolution of tRNA from globin mRNA by paired-ion reversed-phase HPLC by means of silica beads coated with a C_{18} hydrophobic material and an eluent containing tetrabutylammonium as the counter ion.

The principles of simple reversed-phase chromatography and of reversed-

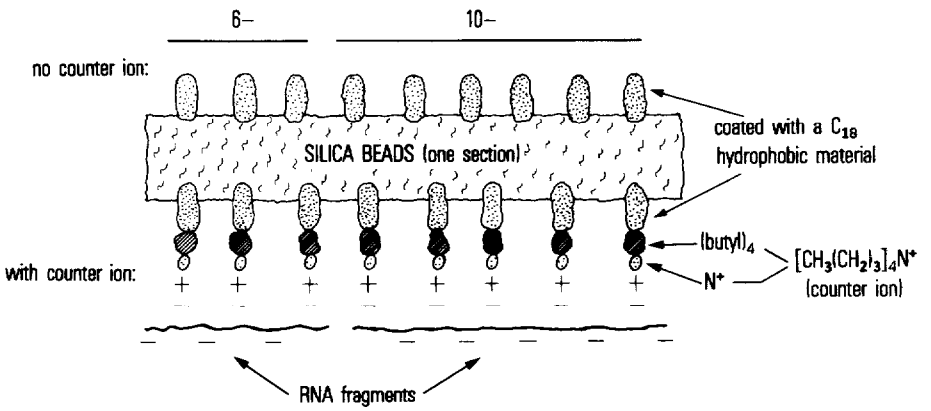


Fig. 19. Schematic representation of reversed-phase ion-paired HPLC. The top portion of the figure indicates simple absorption of a hexanucleotide and a decanucleotide by hydrophobic interactions on the non-polar, Si–$CH_2(CH_2)_{16}$–CH_3 residues of the matrix. The lower portion of the figure indicates that the two oligonucleotides react with the matrix via a counter ion which contains a non-polar group to react with the matrix and a polar "tail" to react with the phosphate anions of the nucleotides.

phase ion-pair HPLC are compared in Fig. 19 (see also Fig. 3 in ref. 43). In reversed-phase chromatography, purine and pyrimidine of the polynucleotides associate (often irreversibly) with the non-polar [*i.e.*, Si–CH_2–$(CH_2)_{16}$–CH_3] residues of the matrix. The oligonucleotides cannot be desorbed without recourse to organic solvents in the eluents. However, the solvents, abolish structural differences of the oligonucleotides and therefore adversely affect their resolution. When a counter ion, such as tetrabutylammonium ion is added to the eluent (*e.g.*, a linear gradient of methanol to water with 5 m*M* counterion), the hydrophobic portion of the counter ion associates with the non-polar groups of the matrix, and the polar cations (tails) become available for (ion) pairing with the phosphate anions of the polynucleotides. Polynucleotides with differences in the conformation allow different numbers of phosphate anions to pair with the bound counter anions. Thus, the polynucleotides are retained to a different degree in each case. The polynucleotides with the help of an appropriate gradient (salt or methanol, or both) can be separated from one another. This method needs to be explored for tRNA work.

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