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An improved method for the purification of tRNA by chromatography on dihydroxyboryl substituted cellulose

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ABSTRACT

An improved method for the rapid separation of aminoacyl-tRNA from tRNA by chromatography on dihydroxyboryl-substituted cellulose has been developed. The method relies on the selective binding of unacylated tRNA to the cellulose support containing dihydroxyboryl groups. This binding is the result of complex formation between the *cis*-diol group of the 3'-terminal ribose in tRNA and the dihydroxyboryl groups immobilized on the resin. Aminoacyl-tRNA cannot undergo borate complex formation and is not retained on the resin. The separation is carried out at near neutral pH values ensuring stability of the aminoacyl ester linkage. The aminoacyl-tRNAs are obtained in very high purity. Aminoacyl-tRNA species containing the modified nucleoside Q are also retained on dihydroxyboryl cellulose. Conditions for isolating all Q base containing tRNA species from unfractionated tRNA are described.

INTRODUCTION

A number of rapid techniques for the isolation of aminoacyl-tRNA have been developed during the past few years. Most widely used is the derivatization method of Gillam, *et al.* (1). This procedure relies on the enzymatic aminoacylation of tRNA and the subsequent chemical attachment of an aromatic moiety to the amino group of the amino acid. The chemically modified tRNA can then be separated from unmodified tRNA by chromatography on benzoylated DEAE-cellulose. The variable yields of the chemical acylation reaction (1,2) as well as side reactions with some tRNAs (3,4) are drawbacks of the method. Another method uses the selective binding of aminoacyl-tRNA to elongation factor EF-Tu in order to separate aminoacylated tRNA from unacylated tRNA by gel filtration (5). This method depends on the availability of purified EF-Tu protein and is not applicable to $tRNA_f^{Met}$ (6). Recently a method has been described which involves the reaction of a bifunctional reagent with the amino acid of aminoacyl-tRNA and its subsequent attachment to an insoluble polymer (7). Unacylated tRNA does not attach to the resin because it can not react with the bifunctional reagent

and is therefore not retained on the resin. The purified tRNA is released from the polymer by deacylation at alkaline pH. As is the case with the method of Gillam, *et al.* (1), the yield of tRNA obtained by this method depends on the degree of chemical modification of aminoacyl-tRNA.

We have concentrated on improving a purification procedure that exploits the selective binding of uncharged tRNA to a column of DBAE-cellulose (8) in order to effect the isolation of particular aminoacyl-tRNA species (9,10). In the development of this method it had been shown that the predominant mechanism of binding the tRNA involves the formation of specific complexes between the *cis*-diol groups of the terminal adenosine in tRNA and the dihydroxyboryl groups attached to the cellulose. This mechanism is consistent with that invoked in the use of DBAE-cellulose for the separation of ribonucleotides and deoxyribonucleotides (11) and for the isolation of 3'-terminal polynucleotide fragments from large RNAs (12,13). However, the method described cannot be used as a general procedure for tRNA purification. In order to maximize the concentration of the active species in complex formation (the tetrahedral boronate anion) on the cellulose support, buffers of pH 8.7 were used; these conditions were shown to cause considerable deacylation of aminoacyl-tRNA (10,14). In addition, it was necessary to employ buffers containing relatively high concentrations of salt and Mg^{++} ion to inhibit the ionic binding of tRNA that arises through the presence of residual charged aminoethyl groups in the DBAE-cellulose. The presence of these components in the chromatographic buffers leads to a low solubility of nucleic acids in the buffer and hence to a low binding capacity of the cellulose.

In this paper we report the preparation of a modified DBAE-cellulose which allows the rapid purification of large amounts of aminoacyl-tRNA at near neutral pH.

MATERIALS AND METHODS

General. N-Cyclohexyl-N'-β-(4-methylmorpholinium)ethylcarbodiimide p-toluenesulfonate, m-aminophenylboric acid, and boric acid gel (particle size 0.1-0.4 nm) were purchased from Aldrich Chemical Co. Aminoethyl-cellulose (AE-11) was from Whatman. N-(m-dihydroxyborylphenyl)succinamic acid and DBAE-cellulose were synthesized by the method of Weith, *et al.* (11). Uniformly labelled [^{14}C]serine (128 mCi/mole) and [^{14}C]methionine (46 mCi/mmole) were obtained commercially. *E. coli* tRNA was obtained from Boehringer Mannheim Corp., New York, N.Y. Unfractionated aminoacyl-tRNA

synthetases were prepared from *E. coli* as described earlier (15). Pure *E. coli* seryl-tRNA synthetase (16) was a kind gift of Dr. J. Katze.

Aminoacylation of tRNA. The reaction mixture contained per ml: 100 µmoles of sodium cacodylate (pH 7.2), 10 µmoles of magnesium acetate, 5 µmoles of ATP, 10 µmoles of KCl, 20-40 nmoles of radioactive amino acid, 5 mg of tRNA and aminoacyl-tRNA synthetase. After incubation at 37° for 15 minutes, the mixture was extracted with phenol, dialyzed extensively against 0.4 M potassium chloride and precipitated with 2.5 volumes of cold ethanol.

Preparation of Acetylated DBAE-Cellulose. DBAE-cellulose was prepared and its capacity [0.65 mmoles N-(m-Dihydroxyborylphenyl)succinamide per gram of cellulose] determined as described by Weith, *et al.* (11).

DBAE-cellulose (5 g) was suspended in distilled water (100 ml), the pH of the suspension adjusted to 9.5 with 6N sodium hydroxide and the mixture was cooled to 0°C. Acetic anhydride (2 meq) was then added to the stirred mixture in a dropwise fashion over a period of 30 min while the pH was maintained at 9.5 by the addition of 1N sodium hydroxide. After an additional 60 min of stirring, the cellulose derivative was washed with a solution of 0.05 M sodium acetate (pH 5.0) - 1.0 M sodium chloride and then stored at 0° in the same solution.

Standard Analytical Chromatography. Routinely, 0.02 ml of a solution of unfractionated tRNA (300 A_{260}/ml) charged with one radioactive amino acid was cooled to 0° and mixed with 9 volumes of cold loading buffer (buffers B and C in Table I). This solution was applied to a column (0.4

TABLE I

Buffers Used in This Study

A	0.2 M NaCl - 0.01 M $MgCl_2$ - 0.05 M morpholine (pH 8.7) - 20% ethanol (v/v)
B	0.2 M NaCl - 0.01 M $MgCl_2$ - 0.05 M 4-methyl-morpholine (pH 7.7) - 20% ethanol (v/v)
C	0.2 M NaCl - 0.01 M $MgCl_2$ - 0.05 M 4-methyl-morpholine (pH 7.2) - 20% ethanol (v/v)
D	1 M NaCl - 0.1 M $MgCl_2$ - 0.05 M morpholine (pH 8.7) - 20% dimethylsulfoxide (v/v)
E	1 M NaCl - 0.1 M $MgCl_2$ - 0.05 M 4-methyl morpholine (pH 7.7) - 20% dimethylsulfoxide (v/v)
F	1 M NaCl - 0.1 M $MgCl_2$ - 0.05 M 4-methyl morpholine (pH 7.2) - 20% dimethylsulfoxide (v/v)
G	0.2 M NaCl - 0.05 M sodium acetate (pH 5.0)
H	1 M NaCl - 0.1 M $MgCl_2$ - 0.05 M 2-(*N*-Morpholine)ethanesulfonic acid (pH 5.4) - 20% dimethylsulfoxide (v/v)
I	1 M NaCl - 0.1 M $MgCl_2$ - 0.05 M morpholine (pH 8.7)

x 5 cm) of acetylated DBAE-cellulose which had been previously equilibrated at 4° in the same loading buffer. The aminoacylated tRNA was eluted and 0.5 ml fractions were collected every 15 min. If these fractions were kept for an extended period of time, they were collected into tubes containing 1 M sodium acetate (pH 5.0) in order to prevent chemical deacylation. After elution of the aminoacyl-tRNA, the bound uncharged tRNA was recovered by elution with low pH buffer (Buffer G). Examples are shown in Figures 1B and 3. The recovery of radioactive aminoacyl-tRNA is greater than 95% of the material applied to the column in Buffer B.

Preparation of Seryl-tRNA (preparative scale). This was done in two steps: (i) isolation of pure uncharged tRNA by absorption onto acetylated DBAE-cellulose; (ii) aminoacylation of this tRNA and subsequent separation of Ser-tRNA from uncharged tRNA.

(i) 3 mg of unfractionated *E. coli* tRNA were dissolved in 2 ml of Buffer A and applied to a column (1 x 22 cm) of acetylated DBAE-cellulose. Elution with two column volumes of Buffer A at a flow rate of 12 ml per hr yielded a small amount of tRNA (approx. 1% of input). The remainder of the tRNA was eluted with Buffer G at the same flow rate. The tRNA was precipitated with 2.5 volumes of cold ethanol and collected by centrifugation. (ii) This tRNA was then acylated with [^{14}C]serine as described above using pure *E. coli* seryl-tRNA synthetase. The aminoacyl-tRNA preparation (30 A_{260} units) in Buffer B was applied to a column (1 x 22 cm) of acetylated DBAE-cellulose which had been previously equilibrated at 4° with the same buffer. Washing of the column with Buffer B (flow rate 12 ml/hr) yielded [^{14}C]Ser-tRNA (0.6 A_{260} units, eluting between 18 and 26 ml). Recovery of the bound, unacylated tRNA was effected by elution with Buffer G (Figure 2). After deacylation of [^{14}C]Ser-tRNA in Buffer B adjusted to pH 8.5 for 90 min at 37°, the material was dialyzed and then precipitated with 2.5 volumes of ethanol. The tRNA could be charged with [^{14}C]serine to a level of 1.51 nmoles/A_{260} unit.

RESULTS

The chromatographic behavior of tRNA on columns of DBAE-cellulose is the result of at least two different types of interactions; complex formation of borate groups with the 2'- and 3'-ribose hydroxyls of the terminal adenosine of tRNA, and ionic interactions of residual aminoethyl groups on the cellulose with negative charges of tRNA. All previous work (10)

involving separation of aminoacyl-tRNA and tRNA on DBAE-cellulose relied on the use of high sodium chloride concentrations in chromatographic buffers to reduce ionic interactions leaving the boric acid:ribose complex formation as the only retarding force (17). Furthermore, higher concentrations of Mg^{++} and also of sodium chloride stabilize the complex between RNA and DBAE-cellulose (9). However, in such chromatographic buffers tRNA had a very limited solubility.

We first attempted to alter the chemical properties of the resin to allow the use of low ionic strength buffers. Therefore we prepared acetylated DBAE-cellulose by treating DBAE-cellulose with acetic anhydride in alkaline medium. This treatment led to the acetylation of the residual aminoethyl groups; thus the strong ionic interactions at neutral pH were expected to be reduced. The acetylated DBAE-cellulose did not retain any tRNA in 0.05 M sodium acetate (pH 5.0), showing the lack of any significant ion-exchange properties.

We then decided to change the composition of the buffers to allow stable boric acid:*cis*-diol complexes at lower pH. For this reason the chromatographic properties of tRNA on columns of acetylated DBAE-cellulose were determined when temperature and buffer composition (pH, Mg^{++} ion concentration, and the nature of the organic solvent) were varied. Using Buffers A-F (Table I) it was shown that in every case the capacity of the cellulose derivative to bind tRNA decreased sharply with increasing temperature. For example, at room temperature in Buffer B one ml of acetylated DBAE-cellulose can quantitatively retain less than 1 A_{260} unit of *E. coli* tRNA while at 4°C the same amount of resin can retain over 20 A_{260} units (18). The buffers currently used for DBAE-cellulose chromatography (13) containing high concentrations of Mg^{++} ion and of sodium chloride, and 20% dimethylsulfoxide (e.g., Buffer D) will also bind tRNA effectively to acetylated DBAE-cellulose even at lower pH values (pH 8.7-7.0, Figure 1B). However, if ethanol replaces dimethylsulfoxide in the buffers, then much lower salt concentrations can be used (e.g., Buffer B) with no apparent loss in capacity. Under these conditions the binding appears to result solely from the interaction between the *cis*-diol group in tRNA and immobilized dihydroxyboryl groups, since bound tRNA can be displaced from the column by adding certain polyalcohols (e.g., ethylene glycol or sorbitol) to the high pH loading buffers (e.g., Buffer B). The presence of Mg^{++} ions is not necessary for binding; however higher NaCl concentrations (0.5 M) are needed to ensure good capacity in the absence of this divalent metal ion. Sodium

chloride can be omitted from the buffers, but it reduces the capacity of acetylated DBAE-cellulose for tRNA. For instance, one ml of resin binds at 4° only 9 A_{260} units of tRNA in Buffer B lacking sodium chloride. Ethanol is required for binding. If ethanol is omitted from Buffers A-C, tRNA is not retained on the resin.

In our studies with acetylated DBAE-cellulose we have chosen to work at pH 7.7 or pH 7.2 in 0.2 M NaCl - 0.01 Mg $MgCl_2$ (Buffers B and C) at 4°. Under these conditions there is no significant deacylation of aminoacyl-tRNA during the relatively short time required for chromatography. The recovery of tRNA or aminoacyl-tRNA from the column is at least 95% of the material applied. Usually we have worked with fast flowrates of 1-3 column volumes per hour. At faster flowrates much less tRNA will be retained on the resin. The resin is very stable under the conditions of chromatography. Our columns have been used at least 100 times over the period of one year.

We have compared the properties of acetylated DBAE-cellulose (using low salt buffers) with those of DBAE-cellulose using high salt buffers (conditions of reference 10) in separating aminoacyl-tRNA from tRNA. The relationship between binding of tRNA and the pH of loading buffers is exemplified

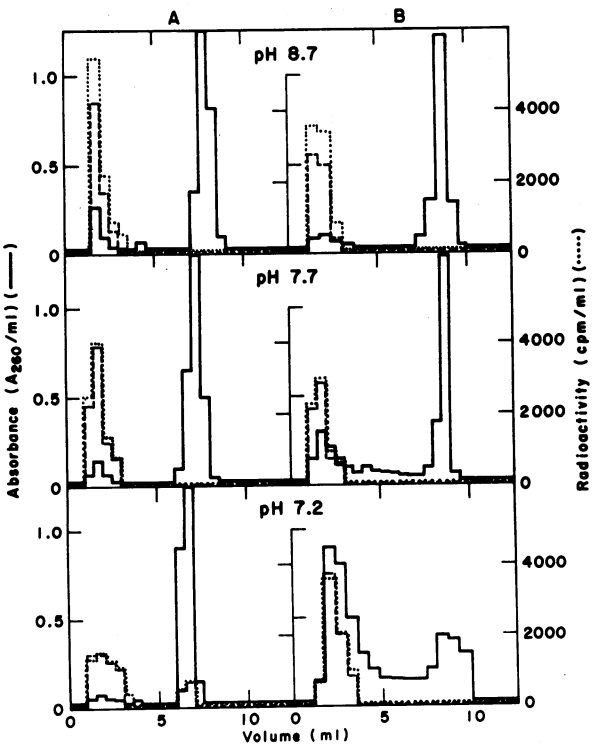

Figure 1. Chromatography of unfractionated *E. coli* [^{14}C]-Met-tRNA on DBAE-cellulose (B) and acetylated DBAE-cellulose (A). The column (0.4 x 5 cm) was eluted at 4° at a flow rate of 2 ml/hr. Fig. 1A shows the elution of [^{14}C]Met-tRNA with Buffers A, B, and C and the subsequent elution of unacylated tRNA with Buffer G. Fig. 1B shows the elution of [^{14}C]Met-tRNA with Buffers D, E, and F and the subsequent elution of unacylated tRNA with Buffer H.

in the chromatography of unfractionated *E. coli* [^{14}C]Met-tRNA. Figure 1B shows a rapid decline in the capacity of DBAE-cellulose to bind tRNA as the pH of the loading buffer decreases. However, on aceylated DBAE-cellulose (Figure 1A) this relationship is not apparent, at least at these concentrations. Thus, acetylated DBAE-cellulose can be used successfully at lower pH values. The aminoacyl linkage of Met-tRNA is of moderate stability (14). It can be seen from Figure 1 that there is negligible deacylation after chromatography at pH 7.7 and pH 7.2, while a considerable amount of hydrolysis has occurred at pH 8.7. In a comparable chromatography of [^{14}C]Ala-tRNA no deacylation was observed, although the alanyl ester bond is very labile (14). It should be noted that the peaks are slightly more skewed in the chromatography on acetylated DBAE-cellulose at the lower pH values, necessitating the collection of tRNA in a larger volume. There is also retention of a small amount of aminoacyl-tRNA at pH values lower than 7.5 under conditions that should only bind *cis*-diol moieties (Figure 1A, pH 7.2). We have no explanation for this fact; the material can be released by buffers containing sorbitol. However, this affects only the yield, but not the purity, of the aminoacyl-tRNA preparation.

For the purification of tRNA on a preparative scale, two steps were performed. First, the unfractionted tRNA preparation was chromatographed on aceylated DBAE-cellulose in Buffer A. Any contaminating aminoacyl-tRNA or RNA fragments with a 3'-terminal phosphate group were eluted, while unacylated tRNA was retained on the column. The unacylated tRNA was eluted with Buffer G and enzymatically aminoacylated with the desired amino acid.

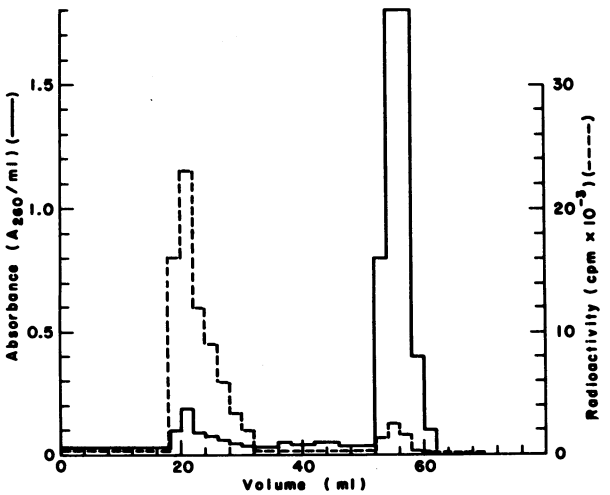

Figure 2. Chromatography of unfractionated *E. coli* [^{14}C]Ser-tRNA on acetylated DBAE-cellulose. For details see Materials and Methods. Buffer change was at 28 ml.

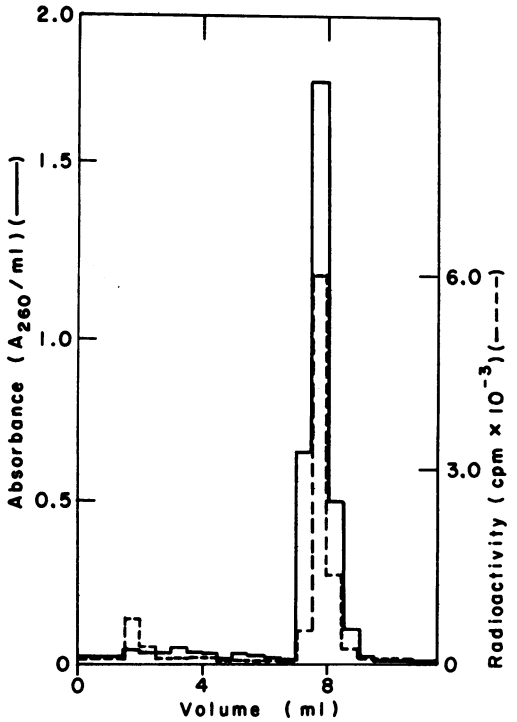

Figure 3.
Chromatography of *E. coli* [^{14}C]Tyr-tRNA on acetylated DBAE-cellulose. Conditions of standard analytical chromatography at 4° with Buffers B and G were used (see Materials and Methods). Essentially the same patterns were obtained with [^{14}C]Asn-tRNA, [^{14}C]Asp-tRNA and [^{14}C]His-tRNA.

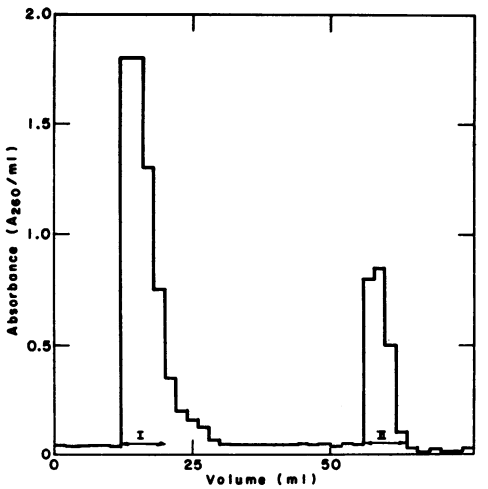

Figure 4.
Chromatography of unfractionated *E. coli* tRNA on acetylated DBAE-cellulose at 22°. tRNA (40 A_{260} units) was applied in Buffer I to a column (1 x 22 cm) and washed with the same buffer at a flowrate of 12 ml/hr. After 2 hr elution was effected with Buffer G. Peak fractions were pooled as indicated.

The resulting aminoacyl-tRNA was then separated from unacylated tRNA on acetylated DBAE-cellulose. The elution profile resulting from the preparative purification of *E. coli* Ser-tRNA is seen in Figure 2. The purified aminoacyl-tRNA eluted in the first peak. After deacylation this RNA could

accept 1.51 nmoles of serine/A_{260} unit. In view of the very long chain length of *E. coli* serine tRNA (19,20) we judge the preparation to be over 95% pure. *E. coli* leucine tRNA and methionine tRNA were obtained by the same procedure in over 90% purity.

Recently, the structure of the modified nucleoside Q was shown to contain a *cis*-diol group (21). In *E. coli* tRNA this nucleoside occurs in $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$, and $tRNA^{Tyr}$ (22). We found (Figure 3) that these *E. coli* tRNAs, even when aminoacylated, bind to acetylated DBAE-cellulose rendering borate substituted polymers ineffective for their separation by the procedure described above (at 0°C). It is plausible that the retention of these aminoacyl-tRNAs is the result of complex formation between borate groups and the *cis*-diol groups of the modified nucleoside Q. However, if the chromatography is done at room temperature in Buffer I, only the tRNAs containing the Q nucleoside are held on the resin. In this way unfractionated uncharged tRNA can be resolved into two peaks (Figure 4). The first peak (Pool I) contains most tRNAs while the late peak (Pool II) contains all the Q nucleoside containing tRNAs in high purity (Table II). Thus, while it is not possible to separate the individual tRNAs in this peak, a very easy purification of all Q base containing tRNAs can be done.

TABLE II

Separation of Q Base Containing tRNAs

Unfractionated E. coli tRNA was chromatographed on acetylated DBAE-cellulose as described in Figure 4. The concentration (expressed in pmoles amino acid accepted/A_{260} unit of tRNA) of seven acceptor RNAs in unfractionated tRNA and in Pools I and II are given. Acceptance for the remaining thirteen amino acids combined was less than 20 pmoles/A_{260} unit in Pool II.

Amino Acid	Unfractionated tRNA	Pool I	Pool II
Asn	48	0	358
Asp	70	5	409
His	66	5	340
Tyr	54	0	310
Val	61	81	0
Phe	50	60	0
Glu	62	75	0

DISCUSSION

The primary aim of this investigation was to develop a rapid method for the separation of aminoacyl-tRNA from tRNA which does not depend on a selective chemical modification of the molecule. Insoluble polymers derivatized with boric acid, which bind unacylated tRNA, have already been shown to be useful for this purpose (10,23). However, their effectiveness has been compromised mainly by the fact that the alkaline conditions necessary for efficient binding of tRNA also result in deacylation of the aminoacyl-tRNA.

In this paper we have shown that efficient binding of unacylated tRNAs to acetylated DBAE-cellulose can be achieved under conditions that are compatible with the chromatography of aminoacyl-tRNA. Under our conditions, in the presence of ethanol, large amounts of tRNA can be bound to this cellulose derivative in buffers of relatively low concentrations of magnesium chloride and sodium chloride. There is some flexibility in adjusting the Na^{+} or Mg^{++} concentration, but each of them enhances the stability of complex formation with tRNA. Ethanol is required for tRNA binding which has been shown to result from *cis*-diol:boric acid interactions.

Based on the principle of separation this method provides an easy way of isolating families of isoacceptors from unfractionated tRNA. Resolution of the individual isoacceptor species can be achieved by subsequent chromatography using other techniques (e.g., reversed phase chromatography). A serious complication to the general usefulness of this technique arises from the fact that several tRNAs bind to acetylated DBAE-cellulose even when aminoacylated. Each of these tRNAs contains in its anticodon the modified nucleoside Q (22). The structure of this compound, which contains a *cis*-diol group, has recently been elucidated as 7-[4,5-dihydroxy-1-cyclopenten-2-yl-(3,4,5-*cis*)aminoethyl]-7-deazaguanosine (21). The majority of tRNA species does not contain this nucleoside and these can be purified by the method described in this paper. The choice of different conditions for chromatography on acetylated DBAE-cellulose allowed the purification of all *E. coli* tRNA species containing the Q base (Figure 4 and Table II). It might be interesting to characterize the Q base containing tRNAs in other organisms by this technique, since tRNA species possessing this modified nucleoside may be involved in regulatory processes (24,25).

Acetylated DBAE-cellulose and its high capacity in the procedure described here should also prove useful in the purification of the 3'-terminal fragment of RNA (9,12,13), or the 5'-terminal fragments of viral or

mammalian messenger RNAs (26), or in the separation of deoxyribo- from ribonucleotides (11,23). Although procedures of this type have previously been quite successful using boric acid gels and DBAE-cellulose, it is expected that most of the advantages outlined in this paper for tRNA separation, such as increased capacity to bind *cis*-diol containing compounds, elimination of ionic interactions, recovery of material in high yield, use of low salt buffers usually suitable for subsequent chromatographic procedures (e.g., direct application of material to RPC-5), and rapid rate of chromatography will also be applicable to use with other molecules.

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17. The Boric acid gel [Schott, H. (1972) *Angew. Chem. 84,* 819-820], which has no ion exchange capacity and thus does not require high ionic strength buffers, showed only a low capacity for tRNA in our hands. Under the conditions described by Schott less than 2 A_{260} units bound to one ml of resin. Therefore its use in preparative applications is limited.

18. Four separate preparations of DBAE-cellulose were synthesized and acetylated. The capacity to bind tRNA varied between 12 and more than 20 A_{260} units per ml of resin. Overacetylation reduced the capacity.

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