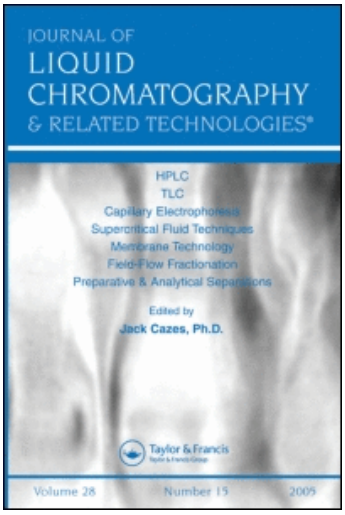

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Chromatography of *E. coli* tRNA on 5-20 µm Agarose Beads

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CHROMATOGRAPHY OF E. COLI tRNA ON 5-20 um AGAROSE BEADS

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ABSTRACT

Escherichia coli transfer RNAs have been separated by chromatography on 5-20 μm beads of divinylsulfone-crosslinked agarose with the use of isocratic elution combined with a negative salt gradient. For example, $tRNA^{Leu}$ was resolved into six isoacceptor species and $tRNA^{Ser}$ into four. Leucine-charged tRNA's were eluted after their corresponding uncharged isoacceptor species. By optimizing flow rate, column length and elution buffer the fractionation of tRNA could be performed within 3 hours.

INTRODUCTION

Many methods for fractionation and purification of individual tRNA species have been devised since the first successful purification of yeast $tRNA^{Ala}$ by means of a counter current distribution system (1). Among the most commonly used techniques so far are chromatography on benzoylated DEAE-cellulose (2), reversed-phase chromatography (3) and chromatography on Sepharose

4B with a negative salt gradient (4, 5). Most interestingly, the selectivity of these supports seems to depend to some extent on their hydrophobic properties. This is not surprising, since tRNA's have about the same sizes and negative charges but differ in hydrophobicity, often owing to the presence of modified bases.

Silica-based high-performance hydrophobic interaction chromatography for the purification of tRNA was introduced six years ago by Hjertén et al. (6). Since then there have been several reports on the fractionation of nucleic acids on HPLC columns of silica via electrostatic or hydrophobic interactions or both, as reviewed by McLaughlin (7). The present studies are focused upon the fractionation of tRNA's by isocratic elution in combination with a negative salt gradient (i.e, Holmes' method (4)) using a novel matrix: agarose beads crosslinked with divinyl sulfone (8). Furthermore, flow rate, column length and salt concentration in the eluent were studied in order to obtain good resolution in a relatively short time.

MATERIALS AND METHODS

Chemicals and Equipments

L-[^{14}C (U)] Leucine (344 mCi/mmol), phenylalanine (536 mCi/mmol), serine (151 mCi/mmol), valine (260 mCi/mmol) and glycine (91 mCi/mmol) and L-[4,5-^{3}H] leucine (5 Ci/mmol) were from New England Nuclear. Most chemicals used were of analytical grade. Agarose (Indubiose A 37) was bought from Réactifs IBF Villeneuve, la Garenne, France.

The chromatographic system, including a Model 2150 HPLC pump, a Model 2152 HPLC controller, a Model 2158 UV-detector and a Model 2210 recorder, was from LKB (Bromma, Sweden) and the loop injector from Rheodyne (Berkeley, CA, USA).

Preparation of Agarose Beads

15% agarose beads were prepared according to a method described previously (9). After preparation, the beads were sized by elutriation in water. The fraction containing beads with diameters between 5-20 μm was collected. The crosslinking with divinylsulfone was performed essentially as described by Hjertén et al. (8).

Bacterial Strain

Escherichia coli MRE 600 (RNase negative) was obtained from the Department of Molecular biology, University of Uppsala, Uppsala, Sweden. The cells were stored at -20°C.

Preparations of tRNA

Bulk tRNA was prepared from E. coli MRE 600 as described by Zubay (10). After deacylation at pH 9.0 in 1 M Tris-HCl for 1 h, high-molecular-weight RNA's were removed by chromatography on Sepharose® 6B (11).

Preparation of Aminoacyl-tRNA Ligases

A crude extract of aminoacyl-tRNA ligases was prepared from E. coli MRE 600 according to Muench and Berg (12). The preparation was stored at -20°C in aliquots.

Aminoacylation of Fractionated tRNA

The aminoacylation mixture had a final volume of 0.1 ml. Conditions for aminoacylation of specific acceptor tRNA's are described elsewhere (13). After incubation for 20 min at +37°C, the samples were treated as described (14) and counted for radioactivity.

Preparation of Radioative Leucyl-tRNA

The reaction mixture contained, in a final volume of 1.68 ml: 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES buffer), pH 8.0; 2 mM ATP; 10 mM $MgSO_4$; 1.2 μM [^{14}C] leucine; 20 mM 2-mercaptoethanol (2-ME); appropriate amounts of ligases and 11 A_{260} units of total tRNA. After incubation at +37°C for 20 min, the reaction was terminated by adding 0.4 ml of 1 M sodium acetate buffer, pH 4.5. Following chromatography on DEAE-Sepharose (15) the aminoacylated tRNA was co-precipitated with carrier tRNA (about 70 A_{260}-units) by ethanol, dried and kept at -20°C.

EXPERIMENTS

Chromatography of tRNA's on 15% Crosslinked-Agarose Beads

Agarose beads with diameters of 5-20 μm were packed at constant pressure in distilled water in a Plexiglas column with an inner diameter of 5.5 mm to a height of 33 cm. The column was then equilibrated with a solution of ammonium sulfate in buffer A (10 mM sodium acetate buffer, pH 4.5 containing 6 mM 2-ME, 10 mM magnesium sulfate and 1 mM ethylenediaminetetraacetic acid). The

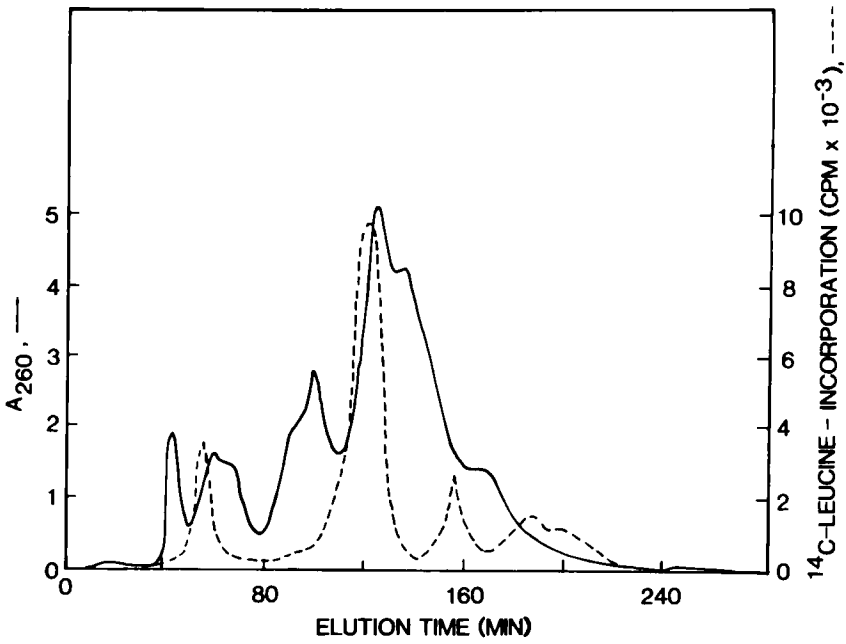

FIGURE 1: Fractionation of E. coli tRNA on DVS-crosslinked agarose beads using isocratic elution with 1.0 M $(NH_4)_2SO_4$ in buffer A. Aliquots of each fraction were assayed for leucine acceptance. The diameter of the 15% agarose beads: 5-20 µm. Bed dimensions: 0.55 x 33 cm. Flow rate: 0.2 ml/min.

flow rate was kept at 0.2 ml/min if not otherwise stated. The experiments were performed at room temperature.

For isocratic elution, 87 A_{260}-units of uncharged tRNA's in buffer A containing 1.0 M $(NH_4)_2SO_4$ was injected into the column equilibrated with buffer A containing 1.0 M $(NH_4)_2SO_4$ and eluted at room temperature with the same solution for 300 min. Fractions of 0.5 ml were collected for absorption and amino acid acceptor activity measurements (Fig. 1; for details see Materials and Methods).

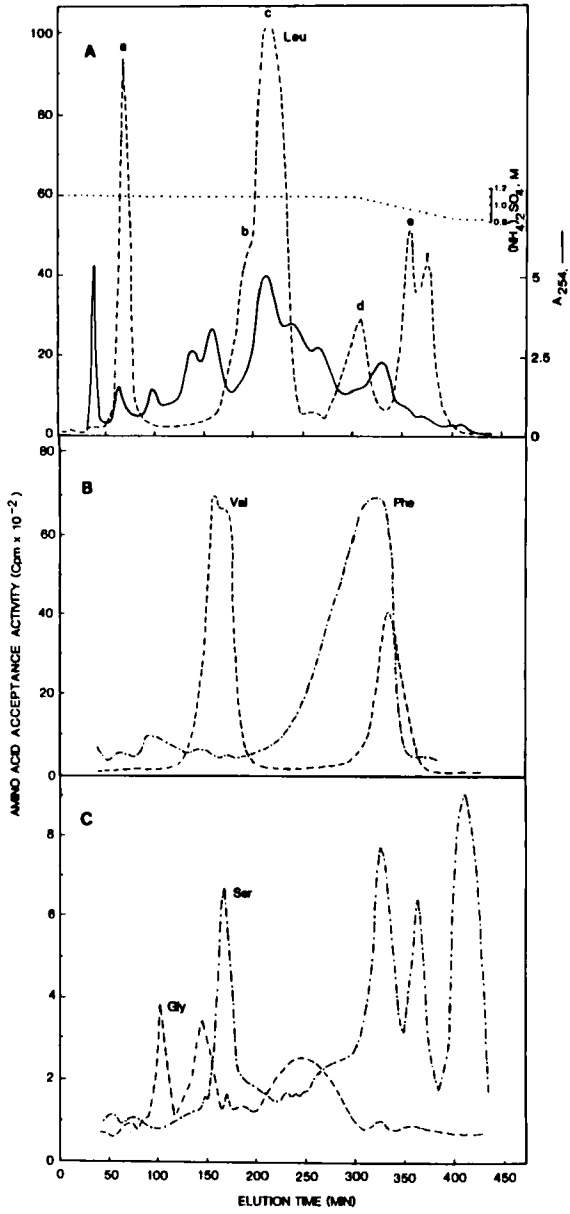

FIGURE 2: Fractionation of E. coli tRNA on DVS-crosslinked agarose beads using (1) isocratic elution with 1.1 M $(NH_4)_2SO_4$ in buffer A for 240 min, (2) a shallow negative salt gradient from 1.1 to 0.82 M $(NH_4)_2SO_4$ for 120 min and (3) isocratic elution with 0.82 M $(NH_4)_2SO_4$ in the same buffer for an additional 80 min. Aliquots of appropriate fractions were assayed for amino acid acceptance as indicated in panels A, B and C. For bead size, bed dimensions and flow rate, see the legend to Fig. 1.

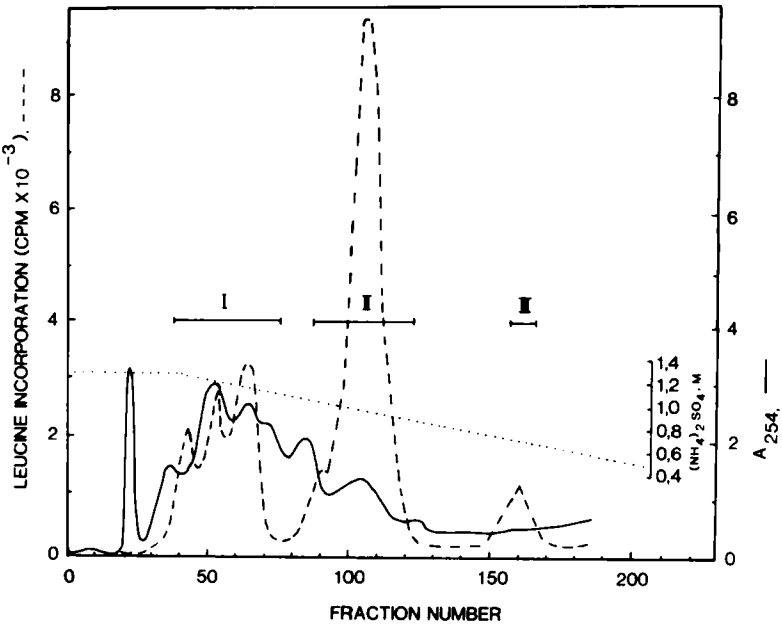

FIGURE 3: Fractionation of E. coli tRNA on Sepharose 4B with a negative linear gradient of $(NH_4)_2SO_4$ at 4°C. Bead size: 60-140 µm. Bed dimensions: 1.0 x 20.5 cm. Flow rate: 11.5 ml/min.

When isocratic elution and negative linear gradient elution were combined, 156 A_{260}-units of uncharged tRNA in buffer A containing 1.1 M $(NH_4)_2SO_4$ was applied to the column equilibrated in buffer A containing 1.1 M $(NH_4)_2SO_4$; tRNA was eluted isocratically with buffer A containing 1.1 M $(NH_4)_2SO_4$ for 300 min, then with a negative shallow linear gradient from 1.1 to 0.82 M $(NH_4)_2SO_4$ in the same buffer for 120 min and finally, isocratically with 0.82 M $(NH_4)_2SO_4$ in the same buffer for an additional 80 min. Fractions of 0.4 ml were collected. The absorbance at 254 nm was recorded (Fig. 2). From a standard curve the conduc-

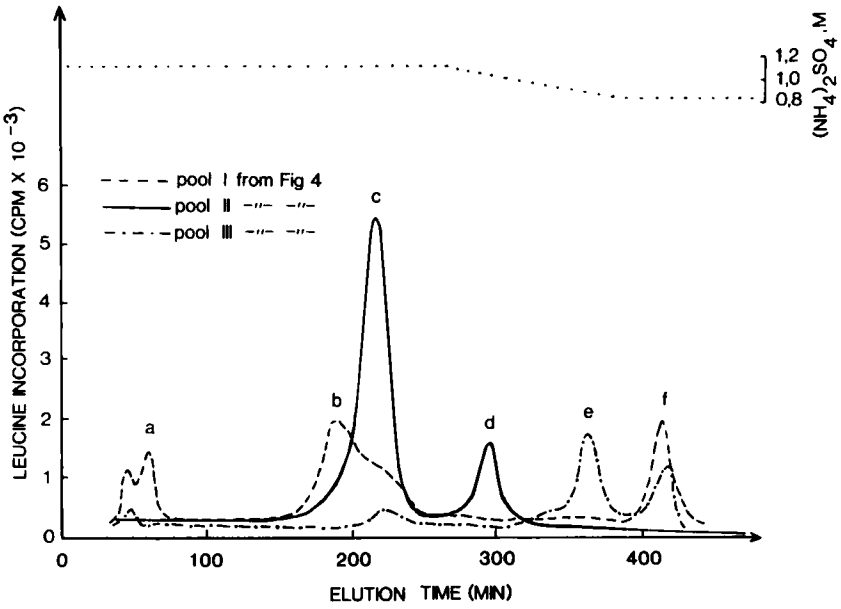

FIGURE 4: Fractionation on DVS-crosslinked agarose beads of leucine tRNA in pool I, II and III obtained from the Sepharose 4B experiment shown in Fig. 3. The experimental conditions were the same as those given in the legend to Fig. 2.

tivity values were transferred to molarities of the ammonium sulfate and plotted in the chromatogram.

After completion of the run, the column was washed with buffer A and then regenerated with buffer A containing 1.0 (or 1.1) M $(NH_4)_2SO_4$. The same gel bed was used for all of the experiments presented in the figures.

The influence of prelabelling (Fig. 5), and flow rate in combination with a change in the concentration of the eluent (Fig. 6) on the appearance of the chromatogram was also investigated.

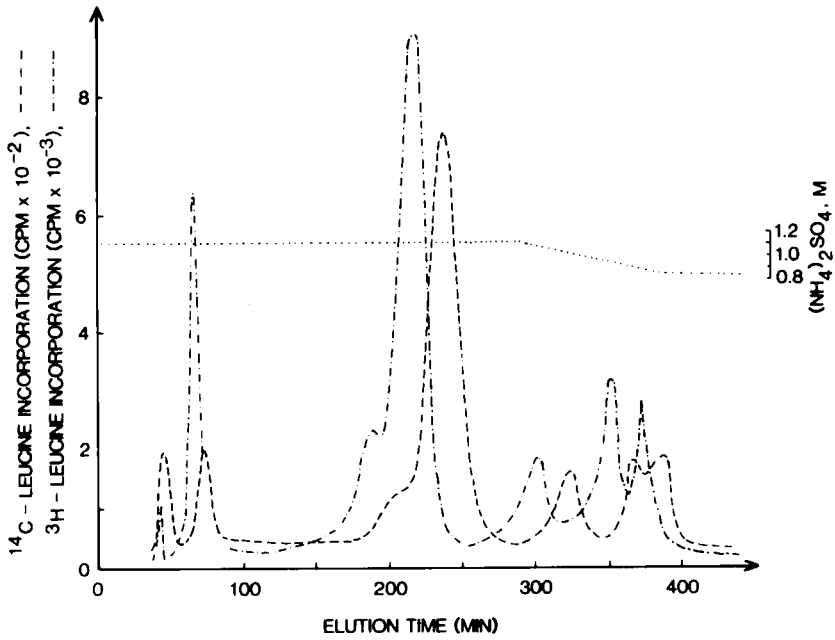

FIGURE 5: Fractionation of charged (----) and uncharged (-•-•-) leucine tRNA's on DVS-crosslinked agarose beads. For bead size, bed dimensions and flow rate, see the legend to Fig. 1.

Chromatography of tRNA on Sepharose 4B

Leucine-isoaccepting tRNA's were fractionated by chromatography on Sepharose 4B (batch no. 4763), according to Holmes' method (4) with minor modifications (15). The column (20.5 cm x 0.8 cm^2) was equilibrated with 1.3 M $(NH_4)_2SO_4$ in buffer A. Elution was performed at 4°C with a linear negative gradient (mixing chamber: 100 g of 1.3 M $(NH_4)_2SO_4$ in buffer A; reservoir: 100 g of buffer A). Fractions of 1.5 ml were collected at 8-min intervals and 25 µl aliquots were assayed for leucine incorporation

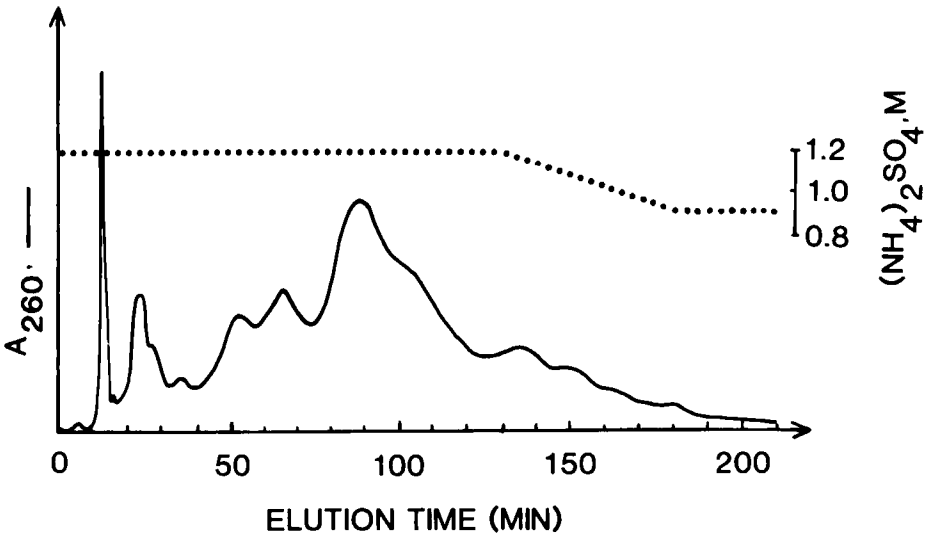

FIGURE 6: Fractionation of tRNA on DVS-crosslinked agarose beads at higher salt concentration and higher flow rate compared with the experiment shown in Fig. 2. The UV-patterns in Figs. 2 and 6 are similar, although the run time in the latter figure is about half that in the former. Isocratic elution with 1.2 M $(NH_4)_2SO_4$ in buffer A for 120 min followed by a negative salt gradient from 1.2-0.9 M $(NH_4)_2SO_4$ in the same buffer for 60 min. Flow rate: 0.6 ml/min. tRNA: 15 A_{260}-units. For bead size and bed dimensions, see the legend to Fig. 1.

(Fig. 3). Some pooled fractions were rechromatographed in the HPLC mode on the 15% agarose beads (Fig. 4).

RESULTS AND DISCUSSION

Fractionation of tRNA on agarose (Sepharose® 4B) at low pH and high ionic strength has been widely used since it was introduced in 1975 by Holmes (4). Although, the adsorption mechanism is not fully understood it probably involves, at least in part, hydrophobic interactions, since the elution is effected by a

decrease in the ionic strength of the buffer. In the present study we have exploited the usefulness of a recently described agarose matrix, based on crosslinking with divinyl sulfone (8), for fractionation of tRNA by Holmes' method.

Fractionation of tRNA by Isocratic Elution

Fig. 1 shows the separation pattern obtained upon isocratic elution of tRNA from the 15% agarose column (for detailed conditions see Experiments). The chromatogram illustrates that $tRNA^{Leu}$ was resolved into five peaks, most probably corresponding to five isoacceptor species. Since the concentration of salt in the eluent is lower than 1.3 M, tRNA does not precipitate on the column. Therefore, the fractionation obtained cannot be explained as a consequence of successive solubilization of the different tRNA's. The fact that the peaks in Fig. 1 do not show tailing is also evidence against such a separation mechanism.

Fractionation of tRNA by Isocratic Elution Followed by Gradient Elution

When isocratic elution was performed in 1.1 M (Fig. 2) instead of 1.0 M (Fig. 1) $(NH_4)_2SO_4$, the tRNA's were more retarded and the UV-pattern was more distinct and one extra peak (b) of $tRNA^{Leu}$ appeared in front of the large $tRNA^{Leu}$ peak (panel A). By applying a negative salt gradient after the isocratic run (for detailed conditions, see Experiments) the most strongly adsorbed tRNA species could be better resolved. Fig. 2 shows that about 80-85% of the tRNA was eluted isocratically in buffer A containing 1.1 M $(NH_4)_2SO_4$ and 15-20% by a shallow negative linear salt

gradient from 1.1 M to 0.82 M $(NH_4)_2SO_4$ followed by isocratic elution with 0.82 M $(NH_4)_2SO_4$.

Transfer RNA^{Leu} was separated into six peaks; a, b, c, d, e and f (Fig. 2A). In several different chromatographic systems (16, 17, 4), five peaks of $tRNA^{Leu}$ have been reported. However, six isoacceptors of $tRNA^{Leu}$ were found by Holmes et al. (4) using E. coli K tRNA, by Patel et al. (15) employing different batch preparations of E. coli and by Sindhuphak et al. (18) using a hydroxyapatite column. The orders of $tRNA^{Leu}$ were further analyzed and described below.

$tRNA^{Val}$ eluted in two completely separated peaks, the first of them showing heterogeneity in the form of a shoulder (Fig. 2B). Two papers (17, 4) report the fractionation of E. coli $tRNA^{Val}$ into one major and two minor peaks, whereas others (18) report separation of $tRNA^{Val}$ into four peaks.

$tRNA^{Phe}$ eluted as one composite peak in the later part of the chromatogram (Fig. 2B) and $tRNA^{Gly}$ gave three peaks (Fig. 2C).

$tRNA^{Ser}$ was split into four well separated peaks (Fig. 2C), the first peak appearing early and the last three peaks late in the chromatogram. Three serine isoacceptor peaks have been found upon chromatography on Octyl-Sepharose (19).

Identification of the Isoacceptor $tRNA^{Leu}$ Species

tRNA's from E. coli MRE 600 were resolved on Sepharose 4B by a slightly modified version (15) of Holmes' method (4) into six peaks of $tRNA^{Leu}$ (Fig. 3). According to Holmes (4), pool I from

Fig. 3 contains $tRNA_2^{Leu}$ and $tRNA_6^{Leu}$; pool II $tRNA_1^{Leu}$ and $tRNA_3^{Leu}$; and pool III $tRNA_4^{Leu}$.

Pools I, II and III recovered from the Sepharose 4B experiment shown in Fig. 3 were prelabelled with leucine and run on a column of DVS-crosslinked agarose using almost the same conditions as in Fig. 2. The results are shown in Fig. 4: pool I is split into three peaks: a, b (with a shoulder) and f (the peak appearing before peak a corresponds to the void volume); pool II into the peaks c and d; and pool III into two main peaks, e and f. It can be concluded that peaks a and b contain $tRNA_2^{Leu}$ and $tRNA_6^{Leu}$, peak c and d $tRNA_1^{Leu}$ and $tRNA_3^{Leu}$. Since peak c is the major peak of $tRNA^{Leu}$ which is known to be $tRNA_1^{Leu}$, peak d must therefore correspond to $tRNA_3^{Leu}$. Peak e is probably $tRNA_4^{Leu}$. Peak f, found in pool I and III (Fig. 4) might contain $tRNA_6^{Leu}$ or denatured tRNA or tRNA dimers. The order of $tRNA^{Leu}$ observed on columns of DVS-crosslinked agarose is the same as that on columns of Sepharose 4B, with the exception that $tRNA_1^{Leu}$ elutes ahead of $tRNA_3^{Leu}$.

Positional Differences in the Chromatograms Between Uncharged and Charged Isoacceptor $tRNA^{Leu}$ Species

A mixed prelabelling and postlabelling technique in the experiments illustrated in Fig. 2 was adopted. 130 A_{260}-units of uncharged tRNA was mixed with about 8 A_{260}-units of tRNA charged with ^{14}C-leucine. This partially charged ^{14}C-labelled material was chromatographed as described in Experiments. For measurement of prelabelled leucyl-tRNA, 0.2 ml aliquots of the chromato-

graphic fractions was mixed with 1 ml of liquid scintillation cocktail (Quickszint 212, Scintvaruhuset, Uppsala, Sweden) and counted for radioactivity. For postlabelling measurement, 25-µl aliquots of the same fractions were then assayed for ^{3}H-labelled leucyl-tRNA. The results are shown in Fig. 5. It is clearly demonstrated that charged isoacceptor $tRNA^{Leu}$ species elute after their corresponding uncharged species, which may be explained by the greater hydrophobicity of the leucine residue of prelabelled leucyl-tRNA. These positional changes of leucine-charged tRNA were not observed upon chromatography on Sepharose 4B (15). The agarose column used in this study is therefore very effective for fractionation of tRNA's with small differences in hydrophobicity. Separation of valyl-tRNA from its cognate non-aminoacylated tRNA's was reported (20) when using mixed-mode chromatography (ion-exchange and hydrophobic-interaction chromatography), whereas charged-tRNA with hydrophilic amino acids did not allow separation of the aminoacylated from the non-aminoacylated tRNA.

Shortening the Run Time

Fractionation of tRNA as illustrated in Fig. 2 takes about 7-8 hours. By increasing the flow rate three-fold and changing the elution conditions (using isocratic elution in 1.2 M $(NH_4)_2SO_4$ in buffer A for 120 min followed by a negative salt gradient from 1.2 to 0.9 M $(NH_4)_2SO_4$ in the same buffer for 60 min) the run time could be shortened to about 3 hours (Fig. 6). A comparison of UV-chromatograms in Fig. 2 and Fig. 6 shows that the reduction in the experimental time only slightly decreased

the resolution. When the bed height used in Fig. 2 was reduced two-fold (and thus the elution time) the UV-pattern and the fractionation time of tRNA's were similar to those shown in Fig. 6.

Conclusion

Fractionation of E. coli tRNA isoacceptors on 5-20 μm 15% agarose beads, crosslinked with DVS, was achieved by using either isocratic elution or isocratic elution combined with a negative salt gradient. $tRNA^{Ser}$ was split into four well separated peaks on this adorbent whereas only three peaks have been found upon chromatography on Octyl-Sepharose (19). $tRNA^{Leu}$ was resolved into six isoacceptor species. The elution order for the leucine-isoacceptor tRNA's is not exactly the same as that obtained on Sepharose 4B. $tRNA_1^{Leu}$ thus elutes earlier than $tRNA_3^{Leu}$ on the 15% agarose beads. Isoacceptor tRNA's^{Leu} charged with leucine bound stronger to the matrix and hence were eluted after their uncharged counterparts. This observation can be used to distinguish between aminoacylated tRNA's and non-aminoacylated tRNA's. Under appropriate experimental conditions the fractionation of tRNA can be performed within 3 hours (Fig. 6) instead of 7-8 hours (Fig. 2).

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