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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF tRNAs ON NOVEL STATIONARY PHASES

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SUMMARY

Rapid separation of a group of tRNAs was carried out on novel siliceous bonded stationary phases with aqueous eluents by using gradient elution with increasing or decreasing salt gradient, as usual in electrostatic interaction chromatography or hydrophobic interaction chromatography, respectively. The stationary phases consist of microparticulate macroporous silica with surface-bound polar moieties, containing weak cationic and/or hydrophobic binding sites. Depending on the nature of the binding sites, the stationary phases exhibit different retention behavior and selectivity for tRNAs. Aqueous phosphate solutions were used as the eluent, and in many cases isocratic elution was sufficient to separate seven tRNAs. Addition of magnesium ions or *n*-decylbetaine to the eluent resulted in lower retention, the latter causing a greater increase in the eluent strength. The optimum pH range of the eluent was 5.5–6.5.

INTRODUCTION

The separation of transfer ribonucleic acids, tRNAs, was first carried out by anion-exchange chromatography on DEAE-cellulose[1–3] or benzoylated DEAE-cellulose[4,5] with increasing salt gradient. Later, partition chromatography in the anion-exchange mode on RPC-5 columns[6,7] has become widely used and recently, hydrophobic interaction chromatography on agarose-based stationary phases [8–11] was also introduced for the separation of tRNAs by conventional column chromatography.

In the first attempts to separate tRNAs by high-performance liquid chromatography (HPLC)[12] the stationary phase was microparticulate octadecyl-silica impregnated with trioctylmethylammonium chloride and elution was brought about with combined salt and pH gradients. The reproducibility and the operational stability of such anion-exchange columns, however, were rather poor in comparison to the bonded phases generally employed in HPLC. Therefore, the separation of tRNAs on siliceous bonded stationary phases by hydrophobic interaction chromatography with decreasing salt gradient was also explored recently[13].

The goal of the present study was to examine the separation of a group of tRNAs on certain new silica-bound stationary phases which have been described in a previous report from our laboratory[14]. These microparticulate siliceous stationary

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phases are "soft" with regard to their interaction with biopolymers as opposed to the "hard" stationary phases generally used for the separation of small molecules in HPLC. According to their electrostatic and hydrophobic binding properties they have been classified as follows: (i) weakly hydrophobic stationary phase, (ii) weak anion exchanger with weak hydrophobic sites, and (iii) relatively strong anion exchanger of weakly hydrophobic character. In the present work, we have investigated their chromatographic properties over a wide range of conditions and found that they are eminently suitable for the rapid separation of tRNAs.

EXPERIMENTAL

Materials

tRNAs specific for glutamic acid (Glu), lysine (Lys), phenylalanine (Phe) and valine (Val) from *E. coli* were purchased from Sigma (St. Louis, MO, U.S.A.). Oligoriboadenylic acid homologues, $(Ap)_nA$, with $n = 1, 2, 3, 4, 5$, and 7 were from Pharmacia (Piscataway, NJ, U.S.A.). *n*-Decylbetaine in 50% (w/w) aqueous solution was a gift from Lonza (Fair Lawn, NJ, U.S.A.). The solvent was removed under vacuum and the solid residue was used. The following chemicals were supplied by Fisher (Pittsburg, PA, U.S.A.): phosphoric acid, tetrahydrofuran (THF) and methanol (all three of HPLC grade), sodium citrate, disodium hydrogen phosphate, sodium sulfate, ammonium sulfate and ammonium acetate. Distilled water was prepared with a Barnstead unit. Vydac 300 Å and LiChrospher SI-1000 silica gels were from The Separations Group (Hesperia, CA, U.S.A.) and MCB-Merck (Cincinnati, OH, U.S.A.), respectively.

Instruments and columns

The liquid chromatograph consisted of a Model 750 pump with a Model 753 ternary solvent mixer and a Model 740 control module, supplied by Micromeritics (Norcross, GA, U.S.A.), a Model 7010 sampling valve with 100-μl sample loop (Rheodyne, Berkely, CA, U.S.A.) and a Model 770R variable-wavelength UV detector (Kratos, Ramsey, NJ, U.S.A.), set at 260 nm. A 100 × 4.6 mm I.D. precolumn packed with 40 μm naked silica was installed between the pump and the injector. In some experiments the column was thermostatted by using a recirculating water bath, Lauda Model K-2/RD (Brinkmann, Westbury, NY, U.S.A.). Chromatograms were obtained with a Model SR-204 strip-chart recorder (Schlumberger, Benton Harbor, MI, U.S.A.). All stationary phases[14] were packed into 100 × 4.6 mm I.D. No. 316 stainless-steel columns (Handy and Harman, Norristown, PA, U.S.A.) by using a methanolic slurry of the column material and methanol at 8000 p.s.i. and 5000 p.s.i. for the small-pore and large-pore materials, respectively.

RESULTS AND DISCUSSIONS

Stationary phases

The chromatographic properties of the microparticulate bonded stationary phases employed in this study were characterized and their use in the separation of nucleic acid fragments was described previously[14]. The novel feature of these sorbents is that the organic material attached to the silica surface consists of two strata: a hydrocarbonaceous sub-layer of *n*-propyl chains and polar top layer. The hydrocar-

bonaceous sub-layer attenuates the hydrolytic degradation of the silica gel and is not accessible to eluites of high molecular weight as long as water-rich mobile phases are used in the chromatographic process. Yet, the retention behavior exhibited by the stationary phases toward small, non-polar eluites was the same as typically found with short alkyl silicas commonly employed in reversed-phase chromatography[14]. Whereas small, non-polar molecules can have access to the hydrocarbonaceous sub-layer at the surface, large biopolymer molecules interact only with the polar top layer, which typically contains relatively large, highly polar moieties bearing weak cationic and/or hydrophobic sites. Some of the key features of such "soft" stationary phases under investigation are listed in Table I.

TABLE I

STATIONARY PHASES EMPLOYED IN THIS STUDY FOR THE SEPARATION OF tRNAs

Column code	*Polar functions attached to C_3 hydrocarbon chains*	*Retention mechanism*
HI-300 I*	Polyol	Weak hydrophobic
HI-1000 I**	Polyol	
IE-300 I*	Amine and urea functions	Weak electrostatic and
IE-300 II*	Ethylene diamino tetra(2-propanol)	weak hydrophobic
IE-1000 I**	Ethylene diamino tetra(2-propanol)	
IE-300 III*	Polyethyleneimine mol.wt. 600	Relatively strong electro-
IE-300 IV*	Polyethyleneimine mol.wt. 1200	static and weak hydrophobic

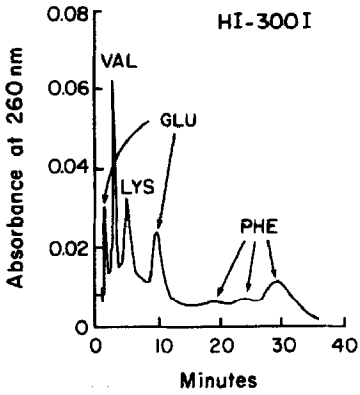
* Silica support: Vydac, spherical silica gel; particle size, 5 μm; specific surface area, 100 m^2/g; pore size, 330 Å.

** Silica support: LiChrospher, spherical silica gel; particle size, 10 μm; specific surface area, 30 m^2/g; pore size, 1000 Å.

Separation of tRNAs on different stationary phases

Column HI-300 I. The tRNAs were retained predominantly by hydrophobic interactions on this polyolic stationary phase. Fig. 1 illustrates the separation of four tRNAs by isocratic elution in less than 40 min with an aqueous mobile phase of relatively high salt concentration, 0.7 *M* disodium hydrogen phosphate. With eluents of low salt concentration the tRNAs were only weakly retained. As seen in Fig. 1, some of the tRNAs yielded several peaks believed to represent isoacceptors[4,6]. The resolution of the early peaks was improved by using decreasing salt gradients, as seen from the comparison of Figs. 1 and 2. Furthermore the isoacceptor tRNAs specific for Glu, Lys and Val are also better separated by gradient elution.

Column IE-300 I. Chromatograms depicted in Fig. 3a and b show the ability of this weak anion exchanger to separate the isoacceptor tRNAs specific for Val and Glu with a mobile phase of 0.15 *M* disodium hydrogen phosphate (pH 6.3) by isocratic elution at low ionic strength when the retention was mainly due to electrostatic interactions. The tRNA specific for Phe was very strongly retained under the isocratic condition used for the separation, shown in Fig. 3a and b, and was eluted from the column as a very broad peak. However, upon the addition of 10 m*M* Mg^{2+} to the



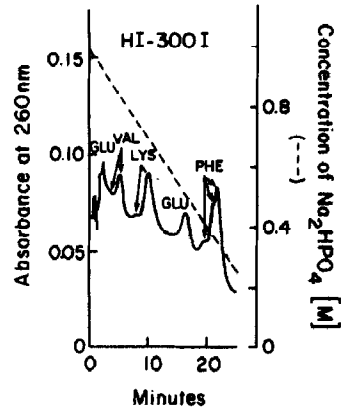
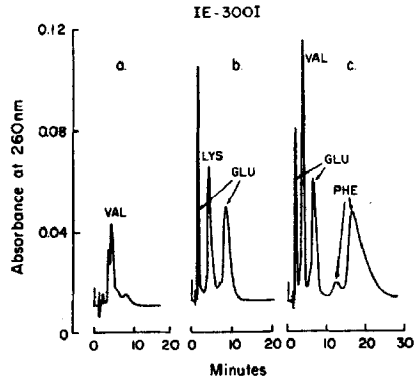


Fig. 1. Chromatogram of tRNAs. Column, 100 × 4.6 mm I.D., 5-μm HI-300 I; flow-rate, 1 ml/min; temperature, 25°C; eluent, 0.7 *M* disodium hydrogen phosphate, pH 6.3; sample volume, 100 μl.

Fig. 2. Chromatogram of tRNAs. Column, 100 × 4.6 mm I.D., 5-μm HI-300 I; flow-rate, 1 ml/min; temperature, 25°C. Linear gradient in 25 min from 0–100% gradient former. Starting eluent, 1.0 *M* disodium hydrogen phosphate, pH 6.3; gradient former, 0.25 *M* disodium hydrogen phosphate, pH 6.3; sample volume, 100 μl.

mobile phase the retention of all peaks was significantly reduced and three tRNA isoacceptor pairs were separated by isocratic elution in less than 30 min, as can be seen in Fig. 3c. Results obtained with gradient elution revealed the dual retention character of this stationary phase that was examined previously[14]. With an increasing salt gradient from 0.02 *M* to 0.25 *M* phosphate (pH 6.3), *i.e.* at low salt concentrations, where retention occurs mainly by electrostatic interactions, the resolution of



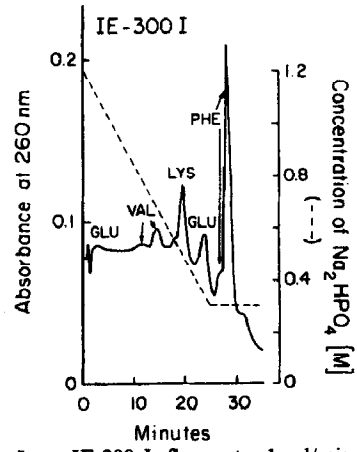
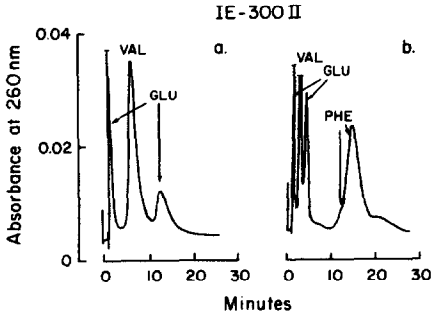


Fig. 3. Chromatogram of tRNAs. Column, 100 × 4.6 mm I.D., 5-μm IE-300 I; flow-rate, 1 ml/min; temperature, 25°C. Eluent, (a) and (b) 0.15 *M* disodium hydrogen phosphate, pH 6.3; (c) contains also 10 m*M* Mg^{2+}; sample volume, 100 μl.

Fig. 4. Chromatogram of tRNAs. Column, 100 × 4.6 mm I.D., 5-μm IE-300 I; flow-rate, 1 ml/min; temperature, 25°C. Linear gradient in 25 min from 0–100% (v/v) gradient former with 10 min isocratic elution. Starting eluent, 1.2 *M* disodium hydrogen phosphate, pH 6.3; gradient former, 0.3 *M* disodium hydrogen phosphate, pH 6.3; sample volume, 100 μl.

the tRNAs was very poor, and the peak of tRNA specific for Phe was very broad. In contradistinction, with decreasing salt gradient from 1.2 *M* to 0.3 *M* phosphate (pH 6.3), *i.e.* at high salt concentrations where retention was due to hydrophobic interactions, the four kinds of tRNA isoacceptors could be readily resolved as shown in Fig. 4.

Column IE-300 II. This stationary phase is a weak anion exchanger that retained the tRNAs by electrostatic and hydrophobic interactions at low and high salt concentrations in the eluent, respectively. By using isocratic elution with a mobile phase of low salt concentration (0.15 *M* disodium hydrogen phosphate, pH 6.3) the tRNAs were strongly retained mainly by electrostatic interactions. Addition of 10 m*M* Mg^{2+} to the mobile phase reduced the retention of all tRNAs and they were separated isocratically in less than 20 min as shown in Fig. 5a, although the tRNA specific for phenylalanine was still strongly retained. Addition of 3.75 m*M* *n*-decylbetaine to the mobile phase was more effective and allowed rapid isocratic elution of all tRNAs from the column with good resolution, as shown in Fig. 5b.



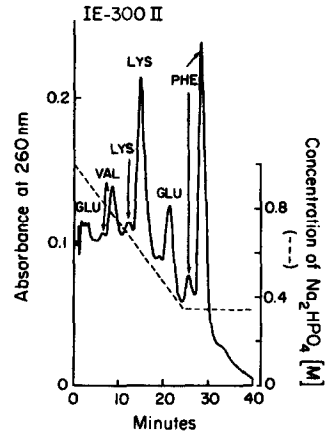


Fig. 5. Chromatogram of tRNAs. Column, 100 × 4.6 mm I.D., 5-μm IE-300 II; flow-rate, 1 ml/min; temperature, 25°C. Eluent, 0.15 *M* disodium hydrogen phosphate, pH 6.3, (a) containing 10 m*M* Mg^{2+}, (b) containing 3.75 m*M* *n*-decylbetaine; sample volume, 100 μl.

Fig. 6. Chromatogram of tRNAs. Column, 100 × 4.6 mm I.D., 5-μm IE-300 II; flow-rate, 1 ml/min; temperature, 25°C. Linear gradient in 25 min from 0–100% (v/v) gradient former, followed by 15 min of isocratic elution. Starting eluent, 1.0 *M* disodium hydrogen phosphate, pH 6.3; gradient former, 0.35 *M* disodium hydrogen phosphate, pH 6.3; sample volume, 100 μl.

The resolution of tRNAs was poor when an increasing salt gradient was used at relatively low salt concentrations where the retention was mainly due to electrostatic interactions. As seen in Fig. 6, however, under conditions of hydrophobic interaction chromatography with a decreasing salt gradient from 1.0 *M* to 0.35 *M* phosphate (pH 6.3) they were readily separated.

Columns IE-300 III and IV. Chromatograms depicted in Fig. 7 show the separation of tRNAs on the relatively strong anion-exchange columns, IE-300 III and

IE-300 IV, by using increasing linear salt gradients with phosphate. Both the starting eluent and the gradient former contained 5 m*M* *n*-decylbetaine, which improved resolution without any significant effect on the retention of tRNAs. As seen in Fig. 7, column IE-300 III has a lower retentive capacity for tRNAs than IE-300 IV under the conditions of the experiments and this may be the reason for the relatively fast separation on column IE-300 III as seen in Fig. 7c.

Effect of operating conditions

Ionic strength. Changes in the ionic strength of the mobile phase affected the retention of tRNAs on the stationary phases under investigation differently due to the difference in the prevailing retention mechanism. The effect was examined with stationary phases HI-300 I, HI-1000 I, IE-300 I. IE-300 II and IE-1000 I under isocratic elution conditions. The tRNA specific for Val was the test eluite and disodium hydrogen phosphate solutions (pH 6.3) at different concentrations were used as eluents.

The results are illustrated in Fig. 8 by plots of the logarithmic retention factor, κ, against the phosphate concentration in the eluent. With the two stationary phases, HI-300 I and HI-1000 I, that differ only in the mean pore size diameter of the silica gel, κ increases linearly with the ionic strength in the range from 0.1 *M* to 0.7 *M* disodium hydrogen phosphate. This is typical when hydrophobic interactions are mainly responsible for retention. On the other hand with the IE-300 I column κ first decreases with increasing phosphate concentration in the range from 0.1 *M* to 0.25 *M*, then the slope of the plot is reversed and κ increases in a linear fashion when the salt concentration increases from 0.25 *M* to 0.7 *M* as shown in Fig. 8. The observed decrease in retention with increasing salt concentration at low ionic strength suggests that retention is governed by electrostatic interactions. However, at high ionic strength the increase in retention with salt concentration indicates the predominance of hydrophobic interactions in the mechanism of retention.

The other two stationary phases, IE-300 II and IE-1000 I, have the same functionality but differ in the pore sizes of the silica matrix[14]. As seen in Fig. 8, the logarithmic retention factor, κ, of tRNA specific for Val first decreases with increasing salt concentration in the range from 0.1 *M* to 0.35 *M* phosphate. Then it passes through a minimum and finally increases when the concentration of disodium hy-

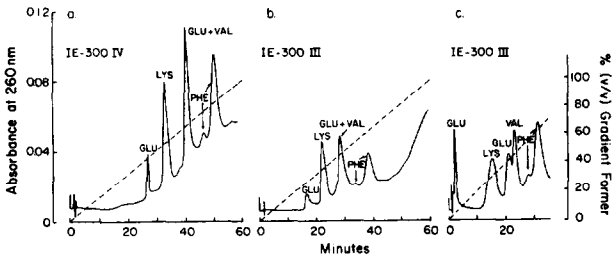


Fig. 7. Chromatograms of tRNAs. Columns, 100 × 4.6 mm I.D., 5-μm IE-300 III and IE-300 IV; flow-rate, 1 ml/min; temperature, 25°C. Linear gradient in 1 h from 0–100% (v/v) gradient former in (a) and (b) and in 35 min from 0–70% (v/v) gradient former in (c). In all cases, gradient former was 0.5 *M* disodium hydrogen phosphate, pH 6.3, containing 5 m*M* *n*-decylbetaine. Starting eluent was 0.2 *M* disodium hydrogen phosphate, pH 6.3, in (a) and (b) and 0.25 *M* disodium hydrogen phosphate, pH 6.3, in (c). Both eluents contained 5 m*M* *n*-decylbetaine.

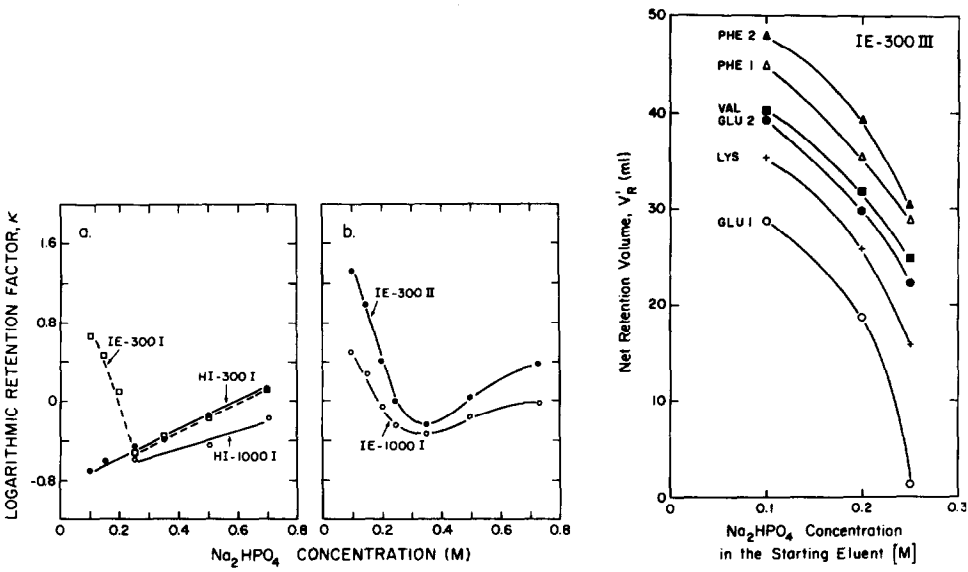


Fig. 8. Plots of logarithmic retention factor of $tRNA^{Val}$ on different stationary phases against disodium hydrogen phosphate concentrations in the eluent at pH 6.3.

Fig. 9. Plots of net retention volume of different tRNAs, obtained on the IE-300 III column against disodium hydrogen phosphate concentrations in the starting eluent. Linear gradient for 1 h at 1 ml/min from 0–100% gradient former, which was 0.5 *M* disodium hydrogen phosphate, pH 6.3. Both eluents contained 5 m*M* *n*-decylbetaine.

drogen phosphate increases from 0.35 *M* to 0.7 *M*. Again, these findings indicate that electrostatic and hydrophobic interactions are mainly responsible for the retention of tRNAs on the same stationary phase at low and high ionic strengths of the eluent, respectively.

Comparison of the retention behavior exhibited by the stationary phase pairs IE-300 II and IE-1000 I to that shown by IE-300 I suggests that the former two stationary phases are stronger anion exchangers than the latter. They yield higher retention factors than stationary phase IE-300 I at low salt concentrations, where retention is due to electrostatic interactions and exhibit the slope reversal of the κ *versus* salt concentration plots at a higher salt concentration.

The effect of the ionic strength of the mobile phase on the retention of tRNAs was also studied with stationary phase IE-300 III. In this particular case we separated the various tRNAs with linear salt gradients, starting at 0.1, 0.2 and 0.25 *M* disodium hydrogen phosphate, and increasing to a final salt concentration of 0.5 *M* disodium hydrogen phosphate. Both, the starting eluent and the gradient former, contained 5 m*M* *n*-decylbetaine. As seen in Fig. 9, the tRNAs are eluted faster upon increasing ionic strength of the starting eluent. This indicates that the retention of tRNAs on this stationary phase is governed mainly by electrostatic interactions. The retention of nucleotides and short oligonucleotides on these stationary phases was also attributed to electrostatic interactions[14] so that these phases behave as anion exchangers over a wide range of conditions.

In order to compare the retention of tRNAs to that of oligonucleotides on the weakly hydrophobic column HI-300 I under the same isocratic elution conditions, oligoadenylic acids $(Ap)_nA$ with n ranging from 1 to 7 and tRNAs were chromatographed. The results are presented as Ap retention indices in Table II. Whereas Glu 1 and Val are eluted before ApA, the first member of the homologous series, Lys is eluted together with ApA, Glu 2 is eluted between $(Ap)_2A$ and $(Ap)_3A$, and Phe 1 and Phe 2 are eluted between $(Ap)_3A$ and $(Ap)_4A$ and between $(Ap)_4A$ and $(Ap)_5A$, respectively. The data suggest that large tRNA molecules have limited access to binding sites available for the relatively small oligonucleotides and, thus, large biopolymers can be eluted faster than smaller residues. This may be of practical significance, for instance, in the study by HPLC of nucleic acid degradation.

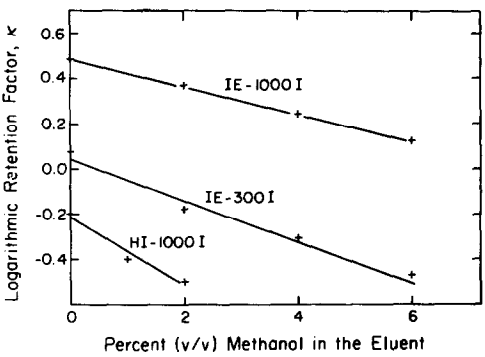
TABLE II

RETENTION INDICES, I_{Ap}, OF tRNAs

The retention index, I_{Ap}, was calculated from the relationship $I_{Ap} = 100\ (n-1) + 100\ [(\kappa_{tRNA} - \kappa_{(Ap)_{n-1}A})/(\kappa_{(Ap)_nA} - \kappa_{(Ap)_{n-1}A})]$ where $(Ap)_nA$ and $(Ap)_{n-1}A$ are oligoadenylic acid phosphate, containing n and $n-1$ residues. The logarithmic retention factors, κ, of various species are denoted by appropriate subscripts. Column, HI-300 I; temperature, 25°C; mobile phase, 0.7 *M* disodium hydrogen phosphate (pH 6.3).

tRNA	I_{Ap}
Glu	<100
Val	<100
Lys	100
Glu 2	286
Phe 1	397
Phe 2	493

Organic solvent. The effect of methanol in the mobile phase on the retention of tRNAs was studied with three different stationary phases: HI-1000 I, IE-300 I, and IE-1000 I. Isocratic elution was carried out with phosphate buffer (pH 6.3), and the ionic strength was adjusted in each case to obtain conveniently measurable retention values. tRNA specific for Val was chosen as the test eluite. As seen in Fig. 10, the logarithmic retention factor of Val decreases in a linear fashion at methanol concentrations where the retention factor can still be measured conveniently. This finding suggests that, although with IE-300 I and IE-1000 I and neat aqueous eluents of similar salt concentrations electrostatic interactions were mainly responsible for retention, as discussed above, hydrophobic interactions still play a role in the retention. The effect was also studied with the relatively strong anion exchanger, IE-300 III, by using a linear gradient with disodium hydrogen phosphate concentrations increasing from 0.02 *M* to 0.7 *M*. When 6% (v/v) THF were added to the starting eluent and the gradient former, the retention volume of Glu and Val decreased from 46 ml ot 28 ml and from 47 ml to 31 ml, respectively. Thus, the retention mechanism is mixed, and both electrostatic and hydrophobic interactions are involved. This is in agreement with reports[7] on the use of ethanol in conjunction with increasing salt gradient to elute tRNAs from a BD-cellulose anion-exchange column. Of course, in



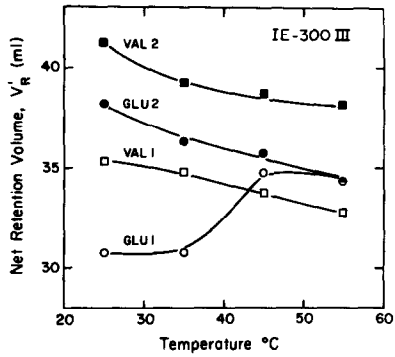


Fig. 10. Plots of logarithmic retention factor of $tRNA^{Val}$ against percent (v/v) methanol in the eluent. Eluents, 0.1, 0.2 and 0.7 *M* disodium hydrogen phosphate, pH 6.3 for columns IE-1000 I, IE-300 I and HI-1000 I, respectively.

Fig. 11. Plots of the net retention volume of tRNAs specific for Glu and Val against column temperature. Linear gradient in 1 h at 1 ml/min from 0–100% (v/v) gradient former. Starting eluent, 0.02 *M* disodium hydrogen phosphate, pH 6.3; gradient former, 0.7 *M* disodium hydrogen phosphate, pH 6.3. Both eluents contained 5 m*M* *n*-decylbetaine.

hydrophobic interaction chromatography, the addition of an organic solvent reduces retention, as was also reported recently by Pearson *et al.*[13] for tRNAs.

Column temperature. The effect of column temperature on the retention of tRNAs was examined with the relatively strong anion-exchange column IE-300 III. tRNAs specific for Glu and Val were chromatographed by using an increasing linear salt gradient of disodium hydrogen phosphate. Upon raising the column temperature from 25 to 55°C, the retention of tRNAs decreased slightly, except for the early-eluted tRNA specific for Glu, the retention of which increased with the temperature between 35 and 45°C, as seen in Fig. 11. The anomalous retention behavior is believed to arise from a conformational change of the tRNA in that temperature range. At column temperatures greater than 35°C, the peaks of all tRNAs studied were narrower than at room temperature.

Eluent pH. The effect of the eluent pH on the retention of tRNAs was examined with three different stationary phases: HI-300 I (weak hydrophobic), IE-300 II (weak anion exchanger and hydrophobic) and IE-300 III (relatively strong anion exchanger). With the first two columns isocratic elution was used, whereas with column IE-300 III a linear gradient elution with increasing salt concentration was used. In each case, the retention of tRNAs increased with decreasing pH of the eluent. The results, in Figs. 12 and 13, show that with IE-300 II and IE-300 III a pH range from 5.5 to 6.5 is the most suitable, whereas a wider pH range, between 4.0–6.0, could be used with HI-300 I without encountering impractically high retention. When the pH of the eluent is below 3.0 the tRNAs are very strongly retained on all stationary phases.

Nature of salt. The effect of the nature of the salt in the mobile phase was investigated by using the relatively strong anion-exchange column, IE-300 III. As seen in Table III, the concentrations of various anions required to bring about isochoric, *i.e.*, isochronal elution of tRNAs increase in the order of citrate < phosphate < sulfate < acetate. Best resolution was obtained by using phosphate, closely followed

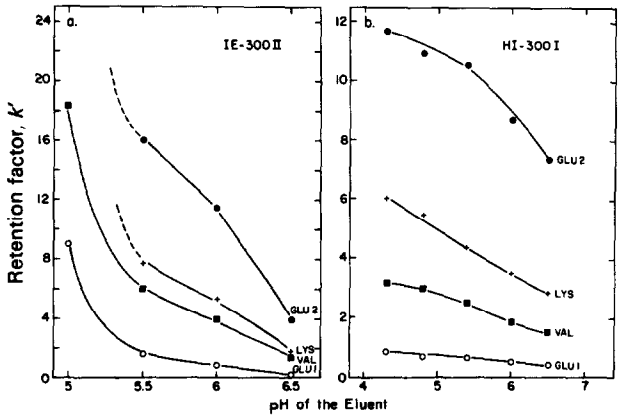


Fig. 12. Plots of retention factor of tRNAs obtained on HI-300 I and IE-300 II columns against the pH of the eluent. In case of HI-300 I, the eluent contained 0.02 *M* disodium hydrogen phosphate and 0.7 *M* sodium sulfate. In case of IE-300 II the eluent contained 0.02 *M* disodium hydrogen phosphate and 0.2 *M* sodium sulfate. In both cases the pH was adjusted with orthophosphoric acid.

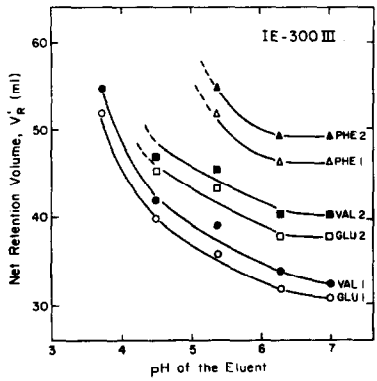


Fig. 13. Plots of net retention volume of different tRNAs obtained on column IE-300 III against the pH of the eluent. Linear gradient in 1 h at 1 ml/min from 0–100% (v/v) gradient former. Starting eluent, 0.02 *M* disodium hydrogen phosphate; gradient former, 0.7 *M* disodium hydrogen phosphate. Both eluents contained 5 m*M* *n*-decylbetaine and the pH was adjusted with orthophosphoric acid.

by citrate, whereas least satisfactory results were obtained with acetate, which gave rise to broad, strongly retained peaks.

Decylbetaine additive. The addition of the zwitterionic hetaeron *n*-decylbetaine significantly lowered the retention of tRNAs on the stationary phases examined. The effect was investigated on columns HI-300 I, IE-300 I and IE-300 II by using 0.15 *M* phosphate (pH 6.3) as the mobile phase with or without 3.75 m*M* *n*-decylbetaine under isocratic elution conditions. In the case of column IE-300 IV, linear gradient elution with increasing phosphate concentration was used, with or without 5 m*M* *n*-decylbetaine in the mobile phase. The results are given in Table IV in terms of the retention modulus, η_{DB}, which expresses the retention modulating effect of the hetaeron.

TABLE III

SALT CONCENTRATION IN THE COLUMN EFFLUENT AT THE ELUTION OF tRNAs WITH THE NET RETENTION VOLUME, V_R, FROM COLUMN IE-300 III BY USING INCREASING LINEAR SALT GRADIENTS

Flow-rate, 1 ml/min; column temperature, 25°C. Gradient elution was carried out from the starting eluent to the gradient former in 60 min.

tRNA	*Phosphate**		*Sulfate***		*Acetate****	
	V_R	*Concn. (M)*	V_R	*Concn. (M)*	V_R	*Concn. (M)*
Glu 1	18.3	0.30	16.3	0.50	24.3	1.84
Lys	25.8	0.33	25.3	0.60	27.8	1.96
Glu 2	29.8	0.35	29.8	0.66	31.8	2.08
Val	31.8	0.36	30.8	0.67	31.8	2.08
Phe 1	35.8	0.38	35.8	0.72	34.8	2.20
Phe 2	39.8	0.40	37.8	0.74	34.8	2.20

* Starting eluent, 0.2 *M* disodium hydrogen phosphate, pH 6.3; gradient former, 0.5 *M* disodium hydrogen phosphate, pH 6.3. Both contained 5 m*M* *n*-decylbetaine.

** Starting eluent, 0.3 *M* ammonium sulfate; gradient former, 1.0 *M* ammonium sulfate. Both contained 20 m*M* disodium hydrogen phosphate, pH 6.3, and 5 m*M* *n*-decylbetaine.

*** Starting eluent, 1.0 *M* ammonium acetate; gradient former, 3.0 *M* ammonium acetate. Both contained 20 m*M* disodium hydrogen phosphate, pH 6.3, and 5 m*M* *n*-decylbetaine.

As seen in Table IV, the retention-attenuating effect of *n*-decylbetaine is much larger in isocratic than in gradient elution. The observed decrease in tRNAs retention upon the use of the dipolar hetaeron is in agreement with earlier findings[15] that dipolar hetaerons reduce the retention of zwitterionic eluites similar to the retention lowering of acidic and basic eluites by acidic and basic hetaerons.

Mg^{2+} in the eluent. The effect of magnesium ions on tRNAs retention was studied on two columns, IE-300 I and IE-300 II, by using isocratic elution and a mobile phase of 0.15 *M* disodium hydrogen phosphate with or without 10 m*M* Mg^{2+}.

TABLE IV

RETENTION MODULI, η_{DB}, OF tRNAs REPRESENTING THE CHANGE IN RETENTION UPON ADDITION OF *n*-DECYLBETAINE TO THE ELUENT

Column	*Retention modulus, η_{DB}*					
	Glu 1	*Lys*	*Val*	*Glu 2*	*Phe 1*	*Phe 2*
HI-300 I*	–	–	–	0.50	0.40	0.40
IE-300 I*	0.34	0.19	0.25	0.17	0.21	0.18
IE-300 II*	0.50	0.18	0.23	0.12	–	–
IE-300 IV**	1.0	0.90	0.98	0.91	0.87	0.90

* $\eta_{DB} = k'$ (with *n*-decylbetaine)/k' (without *n*-decylbetaine). Isocratic elution by the use of 0.15 *M* disodium hydrogen phosphate, with or without 3.75 m*M* *n*-decylbetaine.

** $\eta_{DB} = V_R$ (with *n*-decylbetaine)/V_R (without *n*-decylbetaine). Linear gradient in 1 h at 1 ml/min from 0.2 *M* to 0.5 *M* disodium hydrogen phosphate, pH 6.3, with or without 5 m*M* *n*-decylbetaine.

TABLE V

RETENTION MODULI, η_{Mg}, OF tRNAs REPRESENTING THE CHANGE IN RETENTION UPON ADDITION OF MAGNESIUM IONS TO THE ELUENT

The retention modulus is defined as $\eta_{Mg} = k'$ (with Mg^{2+})/k' (without Mg^{2+}). Mobile phase: 0.15 *M* disodium hydrogen phosphate (pH 6.3) with or without 10 m*M* Mg^{2+}.

Column	*Retention modulus,* η_{Mg}					
	Glu 1	*Val*	*Lys*	*Glu 2*	*Phe 1*	*Phe 2*
IE-300 I	0.27	0.60	0.55	0.49	0.34	0.41
IE-300 II	0.40	0.49	0.48	0.32	–	–

The results are reported in Table V in terms of retention the moduli, η_{Mg}. The retention of tRNAs decreased significantly by addition of 10 m*M* Mg^{2+} to the mobile phase which is in agreement with earlier reports[16–18].

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