A New Chromatographic System for Increased Resolution of Transfer Ribonucleic Acids*

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ABSTRACT: A new reversed-phase chromatographic system yielding superior resolution of *Escherichia coli* transfer ribonucleic acids (tRNAs) has been developed. A quaternary ammonium extractant, tricaprylylmethylammonium chloride, in a Freon diluent, tetrachlorotetrafluoropropane, is supported as a thin film on hydrophobic diatomaceous earth. Chromatograms are developed by sodium chloride concave gradient elution at 37° in the presence of 0.01 M magnesium chloride. The position of 14 amino acid accepting tRNAs are shown in chromatograms at pH 4.5 and 7.0. Of these, 12 tRNAs are heterogeneous (show multiple peaks), with fivefold heterogeneity being shown for leucine tRNA. Large differences in the chromatographic position of several tRNAs were observed at the two different pH levels.

The method affords excellent resolution of tRNAs, experiments are easily conducted, and samples may be directly assayed for amino acid acceptor ability. These factors plus scale-up capability make this a valuable method for both preparatory and analytical experiments.

Many methods for the separation of tRNAs obtained from yeast and bacteria have been described in the literature. These methods include procedures based on differential solubility of specific tRNAs in two-phase systems, as in countercurrent distribution (Kirby, 1962; Apgar *et al.*, 1962; Wiesmeyer *et al.*, 1962; Tada *et al.*, 1962; Doctor and Connelly, 1963; Goldstein *et al.*, 1964) or partition chromatography (Kirby, 1960; Everett *et al.*, 1960; Tanaka *et al.*, 1962; Zachau, 1965; Nathenson *et al.*, 1965). Other chromatographic systems that have been employed for the fractionation of tRNAs utilized ion-exchanging celluloses or dextrans (Ofengand *et al.*, 1961; Bergquist *et al.*, 1965; Cherayil and Bock, 1965; Muench and Berg, 1966a; Smith, 1966), hydroxylapatite (Hartmann and Coy, 1961; Pearson and Kelmers, 1966; Muench and Berg, 1966b; Harding *et al.*, 1966), methylated albumin (Sueoka and Yamane, 1962; Okomoto and Kawade, 1963), and paper chromatography (Jacobson and Nishimura, 1964). Chemical methods of obtaining specific tRNAs have also been developed; these include techniques based on Schiff-base formation (Zamecnik *et al.*, 1960; Stephenson and Zamecnik, 1961; Zubay, 1962), sorption on polyacrylic acid hydrazide (von Portatius *et al.*, 1961; Grachev *et al.*, 1966), and coupling of polypeptide chains to aminoacylated tRNAs (Mehler and Bank, 1963; Simon *et al.*, 1964).

Our interest in preparing gram amounts of purified tRNAs has led to efforts concentrated on column chromatographic procedures, which are potentially more readily scaled up from an engineering viewpoint. A reversed-phase chromatographic system employing quaternary amines in an organic diluent was devised (Kelmers *et al.*, 1965) and applied to the preparation of highly purified *Escherichia coli* B phenylalanine tRNA (Kelmers, 1966a) and a leucine tRNA (Kelmers, 1966b). However, this system was not equally useful for other tRNAs, as many were nearly superimposed in the early portions of the chromatogram.

A new solvent system, employing quaternary ammonium extractants in a Freon diluent (Khym, 1966), was modified by selection of a high-boiling Freon[1] and has been tested as a reversed-phase column chromatographic system. This system is in principle no different from the reversed-phase system described earlier (Kelmers *et al.*, 1965). A new solvent, new ion exchanger, and exploration of gradient possibilities yielded superior resolution and a more even distribution of tRNAs throughout the chromatogram.

Materials and Methods

Materials. "Soluble ribonucleic acid"[2] from *E. coli* B was obtained from Schwarz BioResearch, Inc., and was used as received. Tricaprylylmethylammonium chloride (Aliquat 336) was obtained from General Mills, Inc., tetrachlorotetrafluoropropane (Freon 214) from E. I. duPont de Nemours and Co., and Chromosorb W, acid washed and dimethyldichlorosilane treated, of 100–120 mesh size, from Johns-Manville Products Corp. All other chemicals were of reagent grade.

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[1] The choice of Freon 214 was suggested by Mr. J. X. Khym

[2] Abbreviation used: "soluble RNA," crude material as received, containing tRNAs.

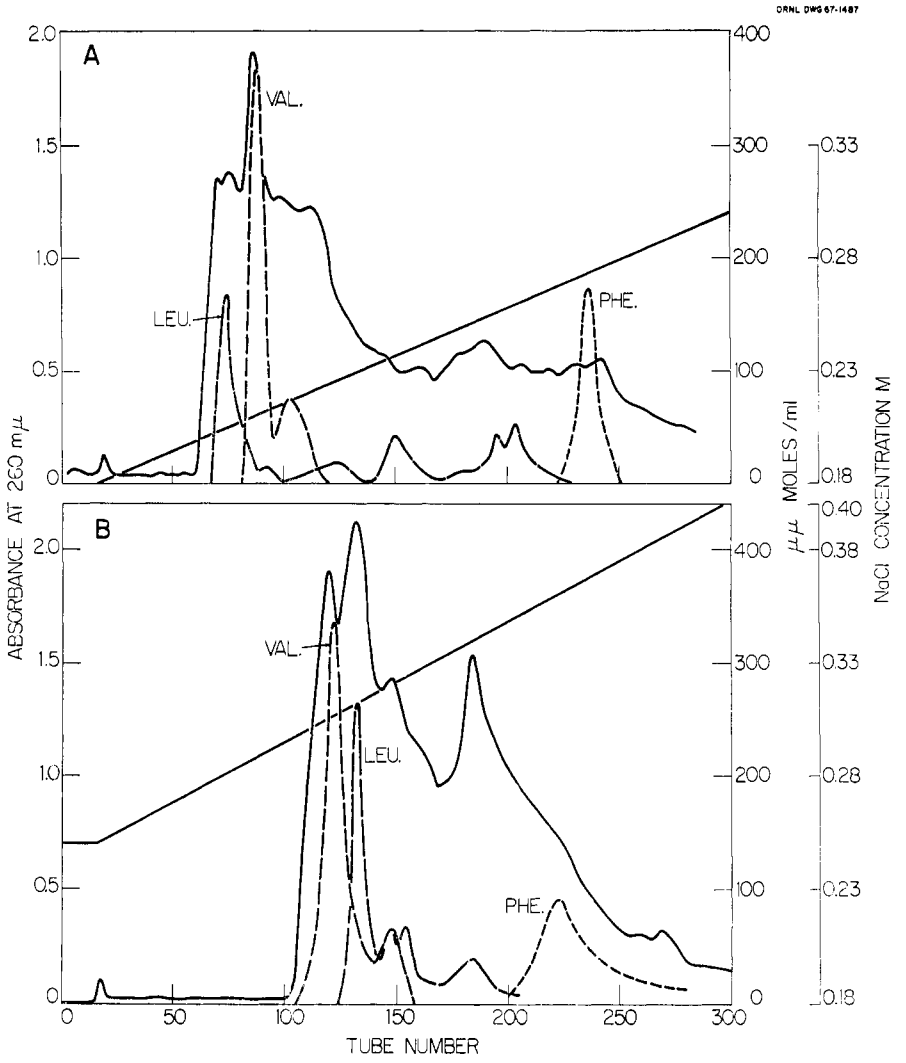

FIGURE 1: Effect of 0.01 M $MgCl_2$. Chromatograms at pH 7.0, 25°, 0.5-ml/min flow rate using linear gradient. RNA (100 mg) was applied to the column (1 × 240 cm). Fraction volume was 10 ml. (A) $MgCl_2$ present and (B) $MgCl_2$ absent.

Preparation of Organic Phase. Aliquot 336 was dissolved in Freon 214 on a 5% by volume basis and was washed successively with two volumes each of 1 M NaOH, 1 M HCl, and 0.5 M NaCl to remove any water-soluble contaminants. The organic phase was separated and dried over silica gel.

Preparation of Column Packing. The organic phase (336 ml) was slowly dropped into 600 g of Chromosorb W with constant manual mixing. The packing was then placed in a plastic bottle and mechanically tumbled for 2 hr and then was allowed to stand for 5 days to ensure even distribution of the organic phase on the solid support. It was then made into a slurry with aqueous elution solution and stored as a slurry.

Column Preparation. Jacketed-glass columns of 1 × 240 cm dimensions, maintained at 25 or 37°, were filled with initial elution solution. The prepared packing was poured into the column as a thick slurry and allowed to settle, while maintaining maximum possible aqueous flow through the column. The bed was then subsequently compacted by pressurization under flow to about 60 psi. Equilibration was achieved prior to making a chromatographic run by pumping at least 1 l. of initial elution solution through the column.

Chromatographic Operation. The RNA was dissolved in 2 ml of initial elution solution and applied to the column. Sequential elution of the tRNAs was achieved by NaCl gradient elution at flow rates of 0.5 or 2 ml/min. In addition to NaCl, the eluent contained 0.01 M $MgCl_2$ and either 0.01 M Tris buffer at pH 7.0 or sodium acetate buffer at pH 4.5. A nine-chamber gradient generator (Phoenix Precision Instrument Co.) was employed to produce both linear and concave gradients. The solutions used in generating the concave gradients are shown in Table I.

The column eluate was collected in approximately

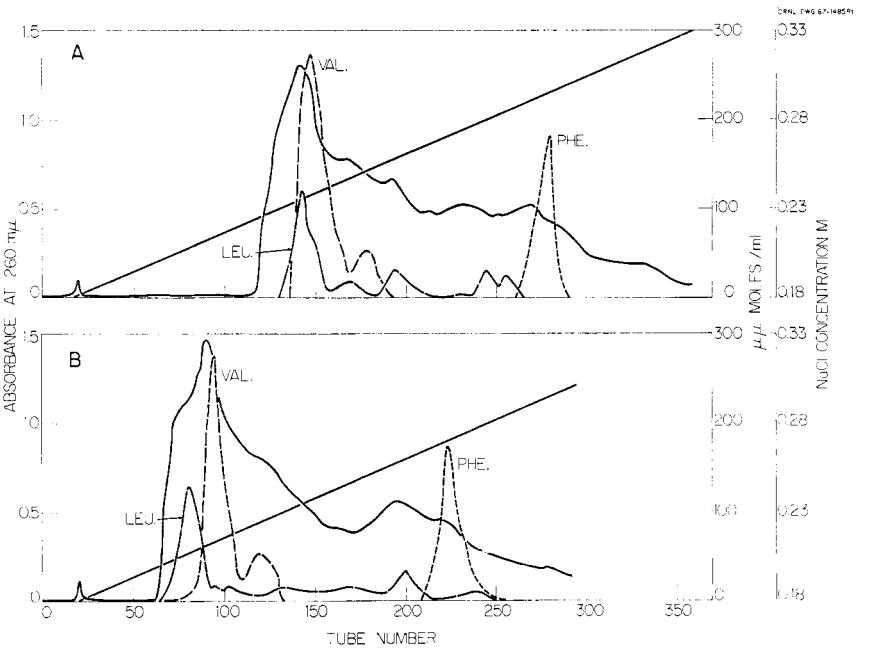

FIGURE 2: Effect of temperature. Chromatograms at pH 7.0, 2.0-ml/min flow rate, using linear gradient; 100 mg of RNA was applied to the column (1 × 240 cm). Fraction volume was 10 ml. (A) 37° and (B) 25°.

10-ml fractions and the absorbance at 260 mμ was measured with a Beckman DU spectrophotometer. The amino acid acceptance activity of selected fractions was directly determined as previously described (Kelmers *et al.*, 1965; Rubin *et al.*, 1967).

The columns were used repeatedly for up to eight runs. Any residual RNA (possibly RNA of higher molecular weight, known to be present in the soluble RNA) was discharged between runs by passing approximately 500 ml of a solution containing 0.5 M NaCl plus 0.01 M $MgCl_2$ and 0.01 M buffer through the column. As a precaution, columns were stored at 4° between experiments to minimize extraneous bacterial growth, although this never appeared to be a problem.

TABLE 1: Data for Generation of Concave Gradients.[a]

Chamber No.	Solution A (ml)	Solution B (ml)
1	325	0
2	298	27
3	288	37
4	271	54
5	246	79
6	217	108
7	183	142
8	120	205
9	0	325

[a] For experiment at pH 7.0 (Figure 3), solution A was 0.200 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.0); solution B was 0.350 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.0). For experiment at pH 4.5 (Figure 4), solution A was 0.225 M NaCl, 0.01 M $MgCl_2$, and 0.01 M sodium acetate (pH 4.5); solution B was 0.375 M NaCl, 0.01 M $MgCl_2$, and 0.01 M sodium acetate (pH 4.5).

Results

The effect of 0.01 M $MgCl_2$ in the eluent on the chromatographic resolution of tRNAs is shown in Figure 1. The solid curves show the over-all tRNA distribution, measured at 260 mμ, and the dashed lines show individual tRNAs, established by amino acid acceptance assay. Resolution was improved by the presence of 0.01 M $MgCl_2$, especially in the latter half of the chromatogram, where, for example, $tRNA^{Phe}$ is sharpened by a factor of five (ratio of peak height to width at half-height). Magnesium ion also shifts the entire elution range toward more dilute NaCl concentrations. At pH 7.0 and 37°, with 0.01 M $MgCl_2$ present, the operating range of the column for efficient tRNA elution was 0.20–0.35 M NaCl, while without $MgCl_2$, the range was 0.29–0.45 M NaCl. The NaCl concentrations at tRNA breakthrough were 0.215 and 0.315 M NaCl, respectively, with and without 0.01 M $MgCl_2$.

The effect of temperature is shown in Figure 2. Raising the temperature from 25 to 37° sharpened somewhat the tRNA peaks eluted toward the end of the chromatogram but may have broadened slightly those eluted near the beginning. At 37°, the tRNAs were eluted at higher NaCl concentrations. At 25° and pH 7.0 with

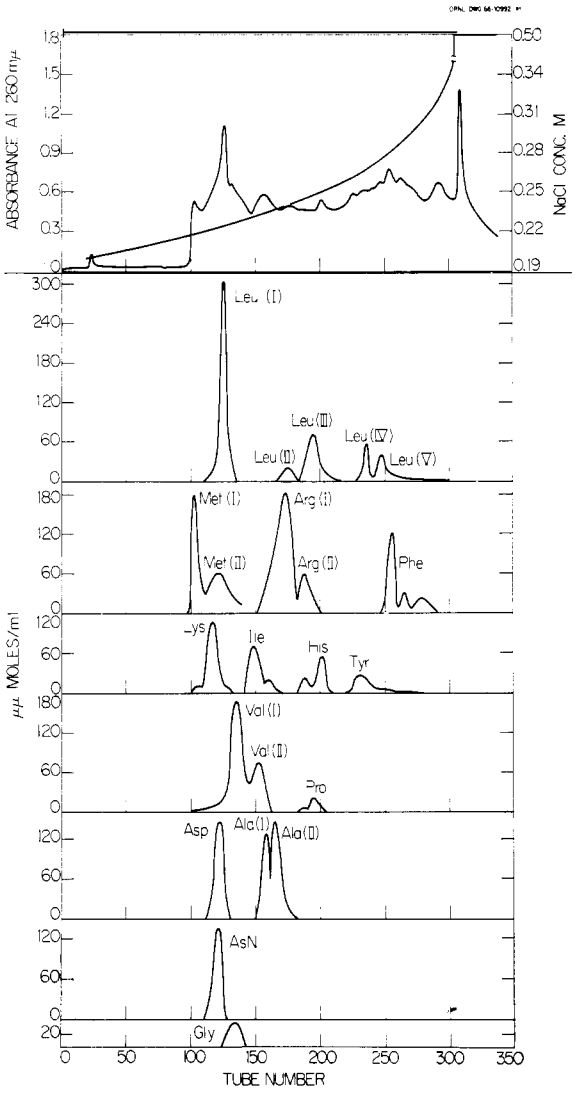

FIGURE 3: Chromatogram at pH 7.0, 37°, 0.5-ml/min flow rate using concave gradient (Table I). RNA (78 mg) was applied to the column (1 × 240 cm). Fraction volume was 9.5 ml. Peak at end of chromatogram was caused by regeneration solution.

0.01 M $MgCl_2$ present, the optimum operating range was 0.18–0.30 M NaCl, while at 37° the range was 0.20–0.35 M NaCl. The NaCl concentrations at tRNA breakthrough under these conditions were 0.199 and 0.215 M, respectively, at 25 and 37°.

Flow rates from 0.5 to 2.0 ml/min were investigated. The better chromatographic results, as indicated by sharpness of the individual acceptor peaks and detail of the absorbance curves, were obtained at 0.5 ml/min, where the ratio of peak height to width at half-height for a number of tRNAs was one-half to three-fourths of those observed at 2 ml/min. Figures 1 and 2 also illustrate this effect. The runs depicted in Figure 1

TABLE II: Effects of 0.01 M $MgCl_2$, Temperature, and pH on the NaCl Concentration Required for Elution of the tRNAs.

	NaCl Concentration (M)		
Temp (°C)	0.01 M Mg^{2+}	Mg^{2+} Absent	pH
25	0.215	0.316	4.5
	0.199	0.300	7.0
37	0.230	0.330	4.5
	0.215	0.315	7.0

were made at a flow rate of 0.5 ml/min, while those shown in Figure 2 were made at 2.0 ml/min.

Peak position is highly sensitive to small changes in NaCl concentration. In order to obtain maximum resolution, curved as well as linear gradients were investigated. Concave gradients yielded a more even distribution of the tRNAs by allowing those near the beginning of the chromatogram (most sensitive to small changes in NaCl concentration) to be eluted by a slowly increasing NaCl gradient, while maintaining sharpness near the end of the chromatogram with a more rapidly increasing concentration gradient.

Chromatograms were run at pH 4.5 and 7.0, under optimum conditions to investigate the distribution of 14 of the tRNAs. These experiments, shown in Figures 3 and 4, were carried out at 37°, with 0.01 M $MgCl_2$ present, at 0.5 ml/min and with a concave elution gradient. These conditions gave satisfactory resolution and distribution of the tRNAs.

The position of the 14 tRNAs was determined by amino acid acceptance assay and 12 were found to be heterogeneous.[3] For example, at pH 7.0, five leucine tRNAs were found, while at pH 4.5, three peaks each were detected for alanine, arginine, phenylalanine, and methionine and at least two peaks were present for aspartic acid, histidine, isoleucine, lysine, proline, tyrosine, and valine tRNAs. A greater degree of heterogeneity for many tRNAs was observed with this Freon solvent reversed-phase chromatographic system than was previously observed with a similar system using isopentyl acetate and dimethyldilaurylammonium chloride (Kelmers *et al.*, 1965).

Large changes in elution position, as a result of changing pH, were noted for certain tRNAs, in particular for alanine, arginine, leucine, methionine, and valine tRNAs. In addition to changing the elution pattern of the individual tRNAs, lowering the pH from 7 to 4.5 caused a general shift to slightly higher NaCl concentrations. The effects of Mg^{2+} ion, temperature, and pH on the NaCl concentration of tRNA

[3] The numbering of heterogeneous peaks refers only to the order in which they are eluted from the column in each particular chromatogram.

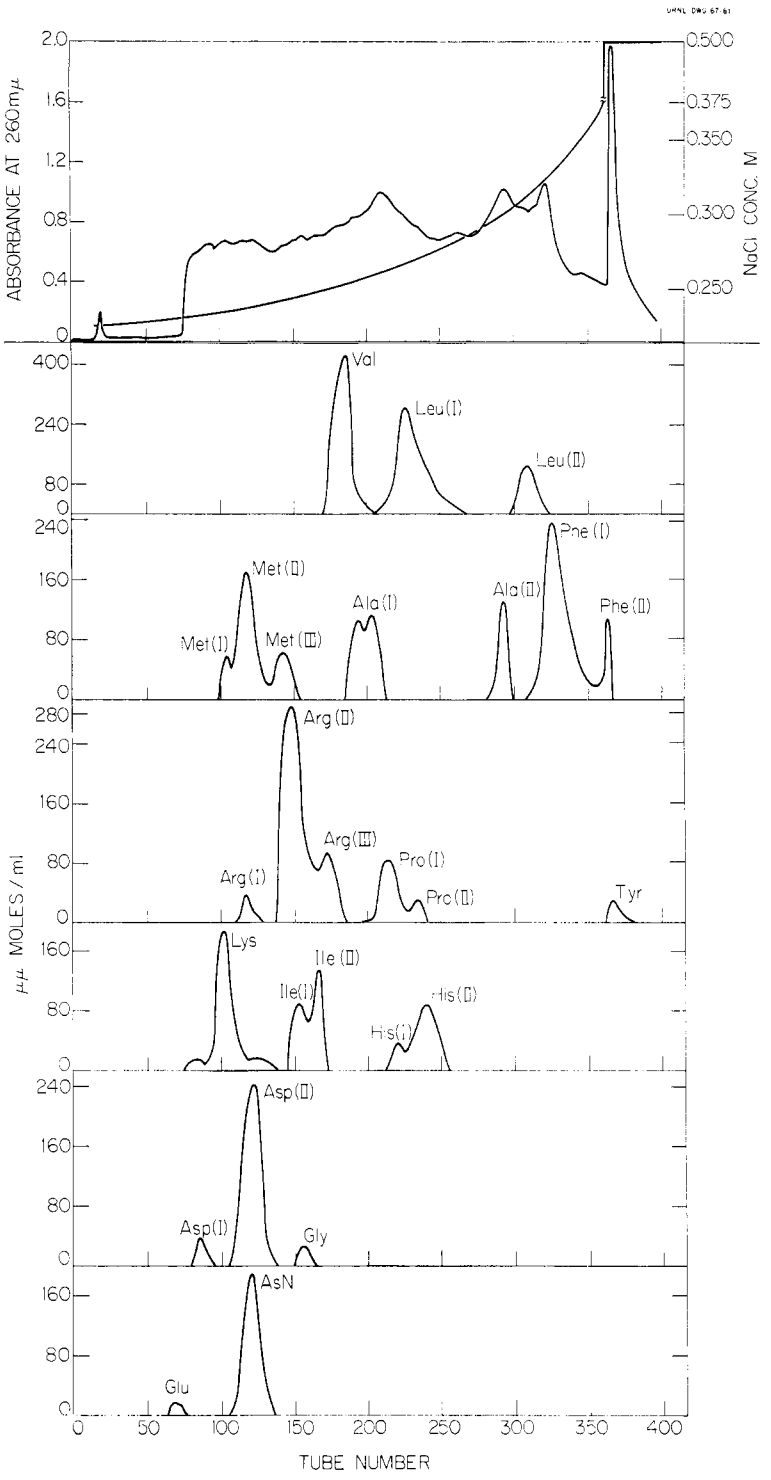

FIGURE 4: Chromatogram at pH 4.5, 37°, 0.5-ml/min flow rate using concave gradient (Table I). RNA (120 mg) was applied to the column (1 × 240 cm). Fraction volume was 8.2 ml. Peak at end of chromatogram was caused by regeneration solution.

breakthrough are summarized in Table II. Changes in the elution order with changes in pH have also been observed in DEAE chromatography (Cherayil and Bock, 1965).

About 82–92% of the RNA applied to the column was recovered in the eluate fractions, as determined by absorbance at 260 mμ. The specific activity of individual tRNAs (picomoles of amino acid accepted per absorb-

ance unit) was substantially increased. For example, using data from Figure 3, the following concentration factors were observed at peak fractions: $tRNA_1^{Ala}$, 15; $tRNA_2^{Ala}$, 19; $tRNA^{Asp}$, 8; $tRNA_1^{Leu}$, 13; $tRNA_1^{Met}$, 10; $tRNA_1^{Phe}$, 7; and $tRNA_1^{Val}$, 8.

Discussion

The heterogeneity of several *E. coli* tRNAs is confirmed. In fact, 12 of the 14 tRNAs assayed show such heterogeneity. Four (alanine, arginine, methionine, and phenylalanine tRNAs) gave triple peaks, and leucine gave five peaks. The presence of five leucine tRNAs in *E. coli* B has been noted previously (Apgar and Holley, 1964; Kelmers *et al.*, 1965; Pearson and Kelmers, 1966; Muench and Berg, 1966a). Multiple accepting species for a number of other *E. coli* tRNAs have been obtained by countercurrent distribution methods (Goldstein *et al.*, 1964) and DEAE or hydroxylapatite chromatography (Muench and Berg, 1966a,b). Such heterogeneity could result from differences in the nucleotide composition or changes in secondary or tertiary structure.

A magnesium ion concentration of 0.01 M shifts the entire tRNA elution range to lower NaCl concentrations and sharpens the resolution of the tRNAs in the latter portion of the chromatogram. Similar effects have been noted previously in reversed-phase chromatography (Kelmers *et al.*, 1965). It appears that the presence of magnesium ion causes the tRNAs, as a whole, to be less tightly bound to the column. Magnesium has recently been shown to affect tRNA structure or conformation (Lindahl *et al.*, 1966; Gartland and Sueoka, 1966) as well as apparent molecular weight (Millar and Steiner, 1966). Magnesium ion probably causes an increase in the tertiary structure and a consequent lowering of the effective charge of the polyanion. This behavior would produce the observed effect on elution range, since a lower binding capability of the tRNA molecules to the quaternary nitrogens would exist. Since those tRNAs eluting later in the chromatogram show the largest increase in sharpness, they are presumed to be most sensitive to conformational changes induced by Mg^{2+} ion.

The order of elution of the various tRNAs from the column at pH 7.0 roughly follows the order of increasing extraction coefficients determined by Khym (1966) in a biphasic distribution system. The differences between the two systems probably reflect basic variations in the partition coefficients of the individual tRNA species between the aqueous phase and the two different Freons employed.

Our work with linear gradients indicates some broadening of peaks toward the end of the chromatogram. This could indicate that the attainment of equilibrium between the phases, and hence removal of tRNA from the immobile organic phase, becomes increasingly slower as the NaCl concentration in the mobile eluent phase increases. The effect is corrected by the use of a concave curved gradient. The loss in resolving power of the system at higher flow rates indicates that interphase equilibrium, in this system, for the tRNAs as a whole is not reached very rapidly.

Elution positions in this system seem highly sensitive to small changes in NaCl concentration. This sensitivity explains in part the resolving power inherent in this method. In order to take full advantage of this sensitivity, a curved elution gradient significantly increasing the resolution of the system over that obtainable with linear gradient elution was developed. This gradient also yields a more even distribution of the individual acceptor tRNAs throughout the chromatogram. A nonlinear concave gradient brings about increased resolution in this system by having $d[NaCl]/dt$ increase slowly in the beginning and rapidly toward the end of the gradient, thereby allowing those tRNA species at the beginning of the chromatogram, which seem most sensitive to small changes in NaCl concentration, to be eluted under a very gradually increasing NaCl concentration. At the end of the chromatogram, the concave gradient accelerates the entry into the mobile phase of those species less sensitive to changes in NaCl concentration and more strongly bound to the column, thereby maintaining resolution.

The attraction of this Freon reversed-phase chromatographic system lies in the sharpness of the individual tRNA peaks obtained and in the simplicity of its operation. These factors along with direct assay capability and high recovery predict easy scale-up to an engineering level for production purposes.

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Titration Properties of Some Dinucleotides*

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ABSTRACT: The absorptivity changes produced by titrating all four dinucleotides containing adenine and uracil derivatives (ApA, ApU, UpA, and UpU) with acid and with alkali suggest that the ionized bases are unstacked. Using this result the titration curves are analyzed and the stacking association constants at pH 7 at 20° are calculated. The stacking association constants increase with the magnitude of the thermal hypochromicities in the order UpU, UpA, ApU, and ApA. The standard free energy of stacking of ApA at 20° is calculated to be −0.99 kcal/mole, a value which is similar to that obtained from the dependence of optical properties on temperature as reported in the literature.

Stacking interactions between adjacent bases in poly- and oligonucleotide chains have been demonstrated for poly- and oligoadenylic acids (Leng and Felsenfeld, 1966; Brahms *et al.*, 1966; Poland *et al.*, 1966), cytidylic acids (Brahms *et al.*, 1967a), and a variety of dinucleotides (Brahms *et al.*, 1967b). All the evidence supports the hypothesis that coooperative effects are minimal in that the interactions between adjacent bases are independent of one another; thus the thermodynamic parameters describing the stacking interactions in dinucleotides are nearly the same as those for a polymer.

A knowledge of the enthalpy and entropy of stacking for all 16 dinucleotides would thus be of considerable interest in the interpretation of the melting curves of RNA.

Several attempts have been made to determine these parameters for several of the dinucleotides (Leng and Felsenfeld, 1966; Brahms *et al.*, 1966, 1967a,b). These attempts have been based on measurements

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