N'-β-(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate (Aldrich Chemical Co., Milwaukee, Wis., 425 mg) was added and the suspension was stirred at 0° for 4 hr while the pH of the solution was maintained at 6.0 by the addition of 2 N hydrochloric acid. The reaction mixture was then allowed to come to room temperature and stirred for a further 4 hr after which time the pH was 6.2. The mixture was then placed in a small column containing a cotton plug and the cellulose was washed with 0.2 M ammonia (*ca.* 100 ml), then with 0.5 M sodium phosphate (pH 7), and finally with water. Spectrophotometric analysis at 260 nm of the combined ammonia washings (using ϵ_{260} 4800 for the substituted succinamic acid in dilute ammonia and ϵ_{260} 344 for the carbodiimide reagent) showed that 0.62 mmole of the boryl derivative had been incorporated onto the cellulose. This result was confirmed by boron analysis on a sample of the product dried *in vacuo* at room temperature. *Anal.* Found: B, 0.69%.

Retention Volumes. The conditions under which the column separations were carried out are indicated in Figure 2 and Tables I and II. The nucleosides were usually studied in 1-mg quantities and the sugars and other polyols in 10-mg quantities. The positions of elution of the nucleosides were detected spectrophotometrically and their identity confirmed by ultraviolet spectra, while the sugars were located by spotting the eluent on chromatography paper followed by spraying with a silver nitrate reagent (Trevelyan *et al.*, 1950).

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Effect of Divalent Metal Ions on the Reversed-Phase Chromatographic Separation of Transfer Ribonucleic Acids*

A. D. Kelmers

ABSTRACT: Divalent metal ions alter the elution sequence and position, multiplicity, and stability of *Escherichia coli* K-12 MO tRNAs during reversed-phase chromatography. The group IIA ions, Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}, were all equivalent in effect, compared to reversed-phase chromatography runs with no divalent metal or with EDTA. They shifted the elution position to lower sodium chloride concentrations sharpened some tRNA peaks while broadening others and stabilized certain tRNAs.

Practical application can be made of these divalent metal ion effects for the purification of individual transfer ribonucleic acids.

Magnesium ion has pronounced effects on the reversed-phase chromatographic[1] separation of tRNAs (Kelmers *et al.*, 1965; Weiss and Kelmers, 1967; Weiss *et al.*, 1968). Numerous publications describe the dramatic effect of magnesium ion on many tRNA properties, including conversion between active and inactive forms (Lindahl *et al.*, 1966; Gartland and Sueoka, 1966), protection from nuclease degradation (Nishi-

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[1] Abbreviations used are: RPC, reversed-phase chromatography; A_{260} unit, quantity of tRNA having an absorbance of 1 in 1 ml at 260 mμ in a 1-cm cell; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; BICINE, *N,N*-bis(2-hydroxyethyl)glycine.

TABLE I: Optimum Aminoacylation Conditions.

	Composition of 0.25-ml Assay Mixture						
	Arg	Leu	Met	Phe	Ser	Tyr	Val
Buffer, type	HEPES	HEPES	HEPES	Glycine	Cacodylate	HEPES	BICINE
μmoles	25	25	25	25	25	25	25
pH	6.6	8.0	8.0	8.4	7.2	8.0	7.5
Magnesium acetate (μmoles)	2.5	5	2.5	5.0	2.5	2.5	2.5
KCl (μmoles)	2.0	1.25	2.5	1.25	1.25	1.25	2.5
ATP (μmole)	1.0	1.0	1.0	0.25	1.0	1.0	0.25
L-[^{14}C]Amino acid (pmoles)	600	375	750	375	375	375	500
β-Mercaptoethanol (μmoles)	2.5	2.5	2.5	2.5	2.5	2.5	2.5
tRNA (A_{260} unit)	~0.04	~0.04	~0.04	~0.04	~0.04	~0.04	~0.03
Synthetase (mg)[a]	~0.1	~0.05	~0.1	~0.1	~0.1	~0.05	~0.1
Incubation (°C)	37	37	37	37	37	37	37
min	30	15	30	30	30	30	30

[a] Crude synthetase prepared as described (Kelmers *et al.*, 1965).

mura and Novelli, 1964), "annealing" of tRNA fragments to yield tRNA active in the aminoacylation reaction (Oda *et al.*, 1969; Bayev *et al.*, 1967; Thiebe and Zachau, 1969; Wintermeyer *et al.*, 1969), and stabilization of tRNA structure (Fuwa *et al.*, 1960; Monier and Gunberg-Manago, 1962; Mehler *et al.*, 1963; Nishimura and Novelli, 1964). The intracellular concentration of Mg^{2+} in *Escherichia coli* is the same as the extracellular concentration (Hurwitz and Rosano, 1967), and it seems probable that the intracellular concentration of other divalent metal ions is also significant. Less attention has been given to other divalent metals, although the presence of many metals in RNA was recognized early (Wacker and Vallee, 1959; Fuwa *et al.*, 1960). Of a number of divalent metal ions evaluated in the aminoacylation of *E. coli* tRNAs, only manganese could be substituted for magnesium (Rubin *et al.*, 1967), and the binding of Mn^{2+} to RNA has been shown to promote structural stability (Molin and Bekker, 1967).

This paper describes the effect of group IIA ions (Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}), and a transition element (Mn^{2+}), on the chromatographic resolution of tRNAs on RPC-3 columns. The RPC-3 system was selected for these tests since it gives greatest resolution in the front of the chromatogram where the largest number of tRNAs are grouped (Weiss *et al.*, 1968) and has proved to be practical for large-scale (10 × 100 cm column) production of purified tRNAs (Kelmers *et al.*, 1970a).

Materials and Methods

Materials. Crude *E. coli* K-12 MO tRNA was prepared as previously described (Kelmers *et al.*, 1970b; Hancher *et al.*, 1969). Trioctylpropylammonium bromide was obtained from Eastman Organic Chemicals. Acid-washed, dimethyldichlorosilane-treated, 100–120 mesh Chromosorb G was obtained from Johns Manville. All chemicals were reagent grade, and double-distilled water was used in all tests.

Methods. RPC-3 packing containing 5 g of trioctylpropylammonium bromide/100 g of Chromosorb was prepared as previously described (Weiss *et al.*, 1968; Kelmers *et al.*, 1970a). A 1 × 120 cm jacketed glass column, maintained at 37 ± 0.1° (Lauda, Model MGW), was filled with equilibrating solution, and the prepared RPC-3 packing was slurried with additional equilibrating solution and poured into the column while the excess buffer was withdrawn from the bottom of the column. The final column bed was about 112–115 cm. One column volume of conditioning solution, 1.5 M NaCl, was pumped through the column at 3.3 ml/min (Milton Roy Mini-Pump) to remove a ultraviolet-absorbing contaminant present in some lots of Chromosorb. The column was equilibrated with one to two column volumes of equilibrating solution. Then 55–60 mg of crude tRNA was dissolved in 5 ml of equilibrating solution and applied to the column. A 3-l. concave gradient (Weiss and Kelmers, 1967) was generated with a nine-chamber box (Phoenix Precision Instrument Co.) and was pumped at 3.3 ml/min. The column effluent was monitored at 260 mμ and recorded. Fractions of 19 ml were collected and subsequently assayed for amino acid acceptance. After the gradient was completed, a column volume of conditioning solution was used to remove any residual RNAs. The column was then reequilibrated and used for up to four runs. When experiments were to be made with a different metal ion, the column was emptied, the entire system was flushed thoroughly with water, and a new column was poured from fresh packing.

The amino acid acceptance assay conditions (Table I) were optimized in a series of statistically designed and evaluated experiments[2] and were carried out as previously described (Rubin *et al.*, 1967). No inhibition of aminoacylation by the constituents of the chromatographic eluents was detected. Formyl acceptance assays for $tRNA^{fMet}$ identification were run as previously described (Weiss *et al.*, 1968).

Results

A series of RPC-3 runs were made to evaluate the effect of group IIA ions. The buffers contained either 0.01 M $MgCl_2$,

[2] I. B. Rubin, T. Mitchell, and G. Goldstein, manuscript in preparation.

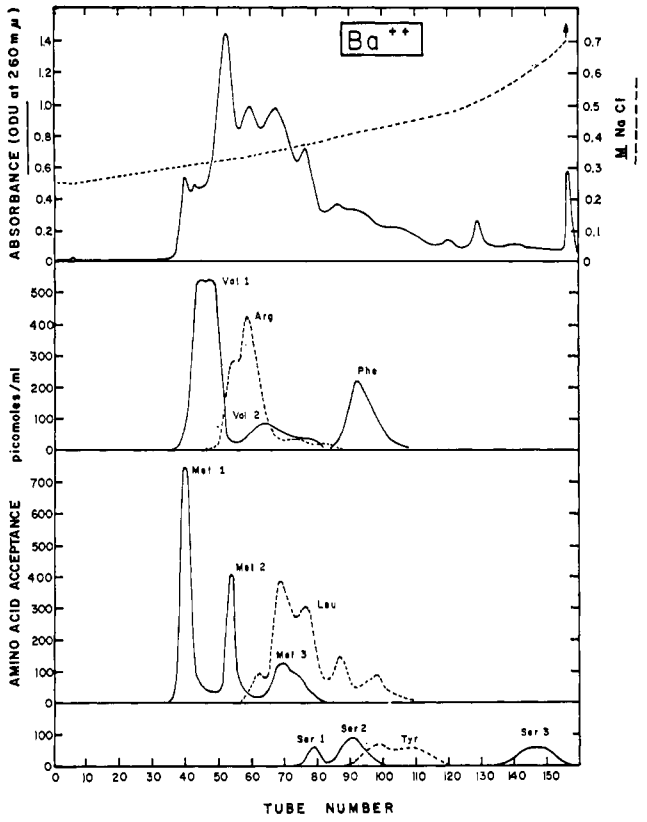

FIGURE 1: RPC-3 Chromatogram in the presence of Ba^{2+}. The column was equilibrated with an 0.25 M NaCl solution containing 0.01 M $BaCl_2$, 0.01 M Tris-HCl (pH 7.0), and 0.001 M $Na_2S_2O_3$. Crude tRNA (906 A_{260} units) was chromatographed by gradient elution from 0.25 to 0.70 M NaCl. The eluent solutions contained the same additional constituents.

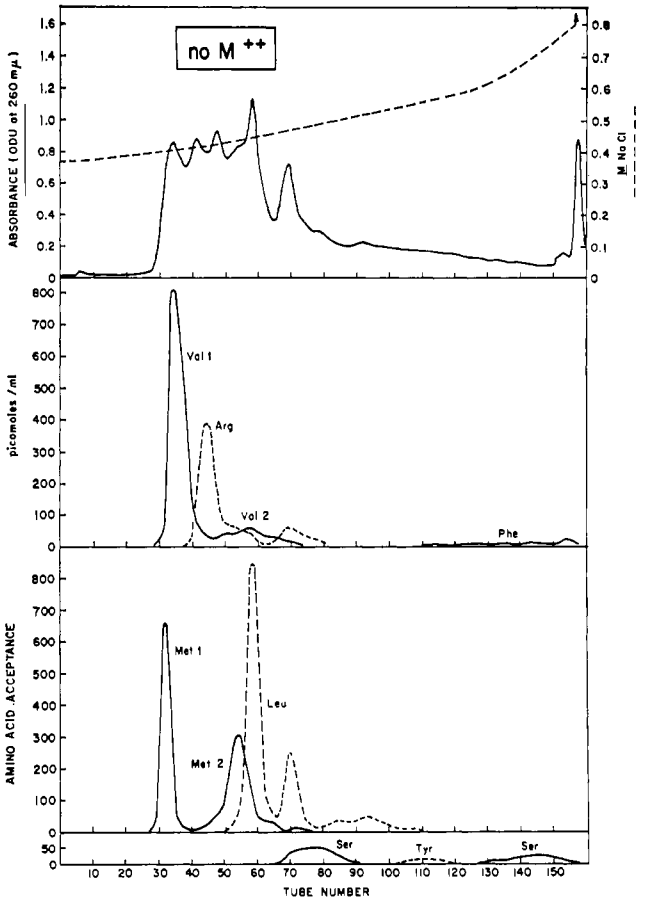

FIGURE 2: RPC-3 chromatogram with no divalent metal ion present. The column was equilibrated with an 0.37 M NaCl solution containing 0.01 M Tris-HCl (pH 7.0) and 0.001 M $Na_2S_2O_3$. Crude tRNA (881 A_{260} units) was chromatographed by gradient elution from 0.37 to 0.80 M NaCl. The eluant solutions contained the same additional constituents.

$CaCl_2$, $SrCl_2$, or $BaCl_2$. The results were similar and only the chromatogram for Ba^{2+} (Figure 1) is shown. The upper panel shows the absorbance at 260 mμ and the calculated sodium chloride concentration of the eluate. The lower panels show the position of the arginine, leucine, methionine, phenylalanine, serine, tyrosine, and valine tRNAs. Relatively sharp chromatographic peaks were obtained for all the tRNAs except tyrosine, which gave broad, ill-defined peaks in all tests. In the presence of 0.01 M Ba^{2+}, compared to Mg^{2+}, the $tRNA^{Met}$ peaks became sharper; valine tRNA peaks also became sharper and tended to split into two major peaks with Ba^{2+}. Three well-separated $tRNA^{Met}$ peaks were present; the first two accepted formyl groups equal to methionine aminoacylation and thus were $tRNAs^{fMet}$. The second peak occupied a different chromatographic position than the abnormal $tRNA_2^{fMet}$ (Shugart *et al.*, 1969), and may represent a different modified form. In the chromatograms with Ca^{2+} and Sr^{2+}, not shown, the position of the first methionine tRNA was shifted forward, relative to the other tRNAs, and use of either of these ions would favor the preparation of purified $tRNA_1^{fMet}$. Multiple peaks can be seen for arginine and leucine tRNAs, although they are not completely resolved. Three well-separated peaks were detected for serine tRNA, and a single, rather broad peak for phenylalanine tRNA. Two phenylalanine tRNAs are present in this crude tRNA (Kelmers *et al.*, 1970a), but are not resolved on RPC-3 columns. Good recoveries of 85–115% (total tRNA accounted for by aminoacylation after each run compared to that in the feed) were recorded for all tRNAs except tyrosine for the runs with Mg^{2+}, Ca^{2+}, Sr^{2+}, or Ba^{2+}. Tyrosine tRNA recoveries were lower, ranging from 41 to 74%.

A reference RPC-3 run was made with no divalent metal ion and the results are shown in Figure 2. The entire chromatogram was shifted to higher NaCl concentrations. Substantial changes occurred in the shape of some of the chromatographic peaks. The methionine, valine, and leucine tRNA peaks were much sharper than in the presence of any divalent cation. The serine tRNAs were very broad and ill defined, and the phenylalanine and tyrosine tRNAs almost vanished. Recoveries of tRNAs after the run ranged from excellent (91–102%) for methionine, valine, and leucine tRNAs, to fair (52–80%, respectively) for serine and arginine tRNAs, to almost nil (11–13%) for phenylalanine and tyrosine tRNAs. The number of isoaccepting peaks for leucine changed; two very sharp peaks plus a small indefinite minor peak or peaks were seen. Also, only two methionine-accepting peaks were seen.

A similar RPC-3 run was made in the presence of 0.01 M EDTA, adjusted to pH 7.0. The number of isoacceptor peaks for the various tRNAs and the sharpness of the individual

chromatographic peaks were essentially identical with the reference run with no divalent ion.

An RPC-3 run was made in the presence of 0.01 M $MnCl_2$. The tRNA elution positions were shifted to lower NaCl concentrations with Mn^{2+} than with group IIA ions. Chromatographic peaks were broader and less clearly defined, with the exception of valine tRNA, and the number of isoacceptors was similar to that for group IIA ions.

Discussion

Although the atomic weights and crystal radii increase substantially in the group IIA elements from magnesium through barium, the size of the hydrated divalent ions do not vary greatly. One set of calculated hydrated radii shows a systematic decrease from Mg^{2+}, 4.28 Å, through Ba^{2+}, 4.04 Å, and a value of 4.38 Å for Mn^{2+} (Nightingale, 1959). The addition of any divalent metal ion decreased the concentration of sodium chloride required to elute a given tRNA, compared to the reference run with no metal ion added. All of the group IIA ions were equivalent in effect in this respect. These results are consistent with the hypothesis that size or charge density of the hydrated divalent ion plays an important role in metal ion binding with the tRNA and in modulating the tRNA interaction with the quaternary ammonium chloride extraction. The hydrated divalent ions tested have similar radii, and large differences in chromatographic behavior were not observed. Major changes occurred only in the absence of any divalent ion.

The stability of tRNAs under chromatographic conditions, measured by the recovery of amino acid accepting activity after the runs, shows systematic differences. The late-running tRNAs, phenylalanine, tyrosine, and serine, were stable only when a divalent metal ion was present. Since these tRNAs were not denatured in the presence of group IIA ions or Mn^{2+}, these ions may be stabilizing a specific region of these tRNAs, perhaps similar to site-bound Mg^{2+} (Lindahl *et al.*, 1966). It is interesting to note that these tRNAs, phenylalanine, tyrosine, and serine, contain a modified nucleoside, 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine, next to the anticodon (Harada *et al.*, 1968; Nishimura *et al.*, 1969). The tRNAs appearing near the front of the RPC chromatograms, methionine, valine, leucine, and arginine, were equally stable with or without a divalent metal ion present. However, they gave sharper chromatographic peaks in the absence of metal ions. This was particularly marked with the leucine tRNAs.

The RPC chromatograms suggest a number of practical applications for the preparation of samples of purified tRNAs. Previously, purification has been achieved on rechromatography by changing pH or shifting from RPC-3 to RPC-4 (Kelmers *et al.*, 1970a). These experiments show that changing the divalent metal ion can also be used to favor the purification of certain tRNAs.

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