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Divalent Cations in Transfer Ribonucleic Acid and Aminoacyl Transfer Ribonucleic Acid Synthetase Function and Structure†

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ABSTRACT: Mn^{2+} serves as well as Mg^{2+} to support the function of the isoleucyl-tRNA synthetase ($tRNA^{Ile}$) system of *Escherichia coli* B; therefore $^{54}Mn^{2+}$ has been used to determine the role of divalent ions in this process. These ions are easily removed from tRNA, isoleucyl-tRNA synthetase, and isoleucyl-tRNA synthetase (AMP-Ile), and the purified system containing deionized $tRNA^{Ile}$ and isoelucyl-tRNA synthetase (AMP-Ile) is still capable of aminoacylation, even in 0.2 M Na_2EDTA. We conclude that neither recognition of tRNA, nor stability of isoleucyl-tRNA synthetase (AMP-Ile), nor transfer of isoleucine from isoleucyl-tRNA synthetase (AMP-Ile) to tRNA requires stoichiometric or even catalytic participation of a divalent metal. Thus, in this system, divalent cations seem to be required only in the synthesis of isoleucyl-tRNA synthetase (AMP-Ile). Divalent metal ion(s) therefore cannot be a part of the static or dynamic structure of $tRNA^{Ile}$ which is required for recognition and aminoacylation.

The pronounced effects of divalent cations (M^{2+}) on tRNA structure (Millar and Steiner, 1966) have long been known (Penniston and Doty, 1963). The implications for tRNA function of the large increase in order and stability attained (Reeves *et al.*, 1970; Rosenfeld *et al.*, 1970) when divalent cations are bound to tRNA (Cohn *et al.*, 1969) are less clear, however. It appears that certain tRNAs may be trapped in an inactive conformation by depriving them of these ions (Gartland and Sueoka, 1966; Fresco *et al.*, 1966), but divalent cations, though they seem to catalyze a transformation from the "denatured" or inactive, to "native" and active disposition, may not be required for maintenance of the active state, once attained (Fresco *et al.*, 1966; Ishida and Sueoka, 1968). This behavior, however, is not general: the behavior of most tRNAs is unaffected by the treatments which affect the "denaturable" class. Further, divalent cations are persistent contaminants of many reagents used in studying

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tRNAs; and in particular, such ions are always required in reaction mixtures in which tRNA activity is measured by aminoacylation using ATP and amino acid as reactants. This introduces ambiguity into the interpretation of experiments designed to measure the influence of divalent metals. Simple physical chemical considerations (see Discussion) suggest that such ions must be reduced to extremely low concentrations if they are to be unavailable for the reactions of tRNAs as carried out under usual conditions. Thus it was difficult to know whether divalent metals were, in fact, required for tRNA activity or for a structural transformation which was a part of the process of aminoacylation, even in systems which had previously evinced no dependence on metals, *e.g.*, $tRNA^{Ile}$ (Yarus and Berg, 1970). We have tried to rigorously exclude and sequester these ions, and find that the recognition and aminoacylation of $tRNA^{Ile}$) (*Escherichia coli*) is completely independent of divalent cations after the enzyme-bound aminoacyl adenylate, isoleucyl-tRNA synthetase (AMP-Ile),[1] has been formed.

Materials and Methods

Purified Ile-tRNA synthetase is the homogeneous protein referred to by Baldwin and Berg (1966). Bovine serum albumin used as carrier for Ile-tRNA synthetase was obtained as a crystalline powder from Pentex, Inc., and freed of multivalent cations and RNase as described below. Preparations of tRNA consisting of unresolved acceptors were prepared from *E. coli* B by the method of Zubay (1962). Purified isoleucine acceptor, referred to as $tRNA^{Ile}$ (*E. coli* B) which is a mixture of the major species of $tRNA^{Ile}$ (*E. coli* B) which is at least 80% isoleucine acceptor, assuming 1.48 A_{260}/nmole of chains. A_{260} is measured by dilution of aliquots in 0.01 M NaOH. Purified tRNA was obtained by methods previously published (Yarus and Berg, 1969). Other purified tRNAs were a gift from the Oak Ridge National Laboratory. $tRNA^{Val}$ (*E. coli* K12) was 82% pure as judged by valine acceptance *vs.* total 3′-terminal adenosine; $tRNA_t^{Met}$ was 97% pure; $tRNA^{Phe}$ was 78% pure, $tRNA^{Arg}$ was 100% pure.

N-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid is the A grade of Calbiochem. Na_2EDTA was obtained from Sigma Chemical Co.; Chelex-100 analytical grade chelating resin from Bio-Rad Laboratories. Gel filtration of tRNA was performed on Sephadex G-25 coarse and isolation of isoleucyl-tRNA synthetase (AMP-Ile) on G-50 coarse from Pharmacia Fine Chemicals, Inc. Carrier-free $^{54}MnCl_2$ was obtained from the International Chemical and Nuclear Corp. and diluted with reagent grade $^{52}MnCl_2$ to give solutions of suitable specific radioactivity. [^{14}C]Isoleucine (Ile) was obtained from Schwarz BioResearch at a specific activity of 50 μCi/μmole. Other chemicals were of analytical or reagent grade.

Water used was double-distilled and then deionized by a Barnstead 24823-1 bed column. Water was stored in polyethylene carbuoys. It had very high specific resistance to 1000-cps current, about 1.8×10^6 ohms cm, characteristic of pure water in equilibrium with atmospheric CO_2, but containing no other ions. By a quantitative test for heavy metals, based on the A_{615}/A_{530} of carbon tetrachloride solutions of dithiazone (diphenylthiocarbazone), M^{2+} content was below detectability; that is, $<4 \times 10^{-9}$ M under our conditions (based on a Zn^{2+} standard). Spark mass spectra confirm that divalent ion contamination is absent.

Pyrex laboratory glassware was cleaned with sodium dichromate–H_2SO_4 cleaning solution and rinsed with double-distilled H_2O. Columns were constructed of Pyrex glass with fittings made of Tygon plastic from U. S. Stoneware, Inc., and nylon monofilament screen cloth from Tobler, Ernst, and Traver, Inc.

The albumin was freed of RNase by carboxymethylation. Albumin (200 mg) is treated with iodoacetic acid (100 mg) in 0.05 M Tris-HCl (66 ml) adjusted to pH 8.0. After incubation for 48 hr at room temperature the albumin is recovered by gel filtration on Sephadex G-50 coarse. The product, called CM-albumin, is absolutely inert with respect to RNase activity; this is untrue of any commercial albumin preparations in our hands. Multivalent cations in the albumin were then exchanged for Na^+ by passage of 10 mg over a 1×15 cm column of Chelex-100 (100–200 mesh) in the Na^+ form. The column, run at 12 ml/hr at 25°, was buffered with 0.01 M NaHepes (pH 7.0). ATP was also converted into NaATP by passage of an 0.03 M solution in water over a similar column.

Ile-tRNA synthetase treated to remove multivalent cations, referred to as "deionized isoleucyl-tRNA synthetase," was prepared by dialysis against three changes of several hundred volumes of 0.01 M Hepes, 0.001 M dithiothreitol, and 0.15 M EDTA (pH 7.0), allowing several hours between changes at 0°. EDTA was then removed by overnight dialysis *vs.* 1000 volumes of 0.01 M Hepes and 0.001 M dithiothreitol (pH 7.0). This treatment did not damage the enzyme as judged by measurement of the maximal rate of aminoacylation in reaction mixtures containing added divalent ions. Aminoacylation reactions were carried out in a solution containing 0.1 M sodium cacodylate buffer, 0.002 M NaATP, 0.005 M $MgCl_2$ or 0.006 M $MnCl_2$, 0.01 M dithiothreitol, 50 μg/ml of CM-albumin, 0.080 M NH_4OAc, and 0.1 mM EDTA, at pH 7.0 and 37°. Other details have been published previously (Yarus and Berg, 1967).

Quantitative conversion of isoleucyl-tRNA synthetase to its aminoacyl adenylate, isoleucyl-tRNA synthetase (AMP-Ile), was carried out in a total volume of 0.10 ml held at 37° for 2′ and containing about 0.20 nmole of deionized isoleucyl-tRNA synthetase, 0.002 M potassium phosphate buffer, 0.01 M dithiothreitol, 0.001 M EDTA, 4 nmoles of [^{14}C]Ile, 126 nmoles of NaATP, and 0.001 M $^{54}MnCl_2$ (specific radioactivity 90 cpm/pmole). The complex was isolated by gel filtration of the chilled reaction mixture on G-50 coarse at 4° in 0.05 M sodium citrate, 0.05 M KCl, and 0.001 M dithiothreitol (pH 6.1). The 0.8×50 cm column was run at 8 ml/hr. These conditions differ from those previously used for isolation of isoleucyl-tRNA synthetase (AMP-Ile) in the use of deionized reagents, substitution of 0.01 M $MnCl_2$ for 0.005 M $MgCl_2$, the use of 4 nmoles instead of 40 nmoles of [^{14}C]Ile, and the use of 0.05 M instead of 0.01 M sodium citrate in the column eluent. A comparison using the previous technique and the same preparation of isoleucyl-tRNA synthetase suggests that these changes cause no substantial alteration in the yield of isoleucyl-tRNA synthetase (AMP-Ile) or its ability to aminoacylate added tRNA.

tRNA was treated to remove multivalent cations by gel filtration over a jacketed 0.8×16 cm column of G-25 coarse held at 50° and eluted with 0.15 M EDTA (pH 7.0). To make $^{52}Mn^{2+}$-tRNA, this preparation was then passed over a similar column at 22° which was eluted with 10^{-4} M $^{52}MnCl_2$. $^{54}Mn^{2+}$-tRNA was made by a procedure more economical of $^{54}MnCl_2$. The product (10 A_{260}) of the first column above

[1] Abbreviations used are: isoleucyl-tRNA synthetase (AMP-Ile), the enzyme-bound aminoacyl adenylate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

TABLE I: Maximal Aminoacylation of Deionized tRNA with Isoleucine.[a]

Divalent Ion in Reaction	Final Level of Acylation
5 mM $MgCl_2$	0.71 nmole/10 A_{260}
6 mM $MnCl_2$	0.75 nmole/10 A_{260}

[a] tRNA used was a preparation of mixed acceptors, incubated 15 min at 37° with sufficient Ile-tRNA synthetase to aminoacylate a 1000-fold excess of tRNA.

was treated to remove EDTA by dialysis overnight against three changes of several hundred volumes of 0.1 M NaCl–0.01 M Hepes (pH 7.0, 4°). Then the tRNA was equilibrated with $^{54}MnCl_2$ by four repetitive 2-hr dialyses *vs.* 0.0001 M $^{54}MnCl_2$, 0.01 M Hepes, and 0.02–0.20 M NaCl (pH 7.0) with the NaCl concentration adjusted to the purpose of the experiment. It is necessary to take great care to exclude RNase from this procedure; we extensively boil dialysis tubing in dilute sodium bicarbonate and EDTA and rinse extensively in H_2O: it is stored in 50% EtOH. All materials in contact with tRNA are handled wearing disposable gloves. Reagents were autoclaved, glassware was baked at 180°, and pipets were cleaned with dichromate cleaning solution and baked. Under these conditions, these ion replacement procedures do not decrease the isoleucine acceptor activity of the tRNA.

To approximately measure bound Mn^{2+}, a small aliquot containing ~10 A_{260} of deionized tRNA in 0.2 ml, which had been reequilibrated with $^{54}MnCl_2$, was subjected to filtration on an 0.8 × 16 cm column of G-25 coarse at 25°. The eluent, depending on the experiment, was 0.01 M Hepes–0.02–0.20 M NaCl (pH 7.0), the tRNA peak at the void volume was collected, and A_{260} and Mn^{2+} were measured.

Radioactivity was measured by liquid scintillation spectrometry in a Packard 3310 instrument. Extensive experimentation was required to select a suitable scintillant and solvent for double-label counting of $^{54}MnCl_2$ and [^{14}C]Ile because many systems give time-dependent or irreproducible values for the channels ratio of $^{54}MnCl_2$. We finally settled on the addition of small volumes of aqueous solution to low ^{40}K glass vials containing 5 ml of a solution composed of 1 l. of reagent toluene, 5 g of 2,5-diphenyloxazole, and 100 ml of BBS scintillation detergent (Beckman Instruments 184983). Reconstruction experiments with solutions of dilute [^{14}C]Ile and $^{54}MnCl_2$ suggested that this method was well behaved, and gave efficiencies of 76% for ^{14}C and 36% for ^{54}Mn. The Mn γ emission gives light scintillations which resemble those from ^{3}H. Liquid scintillation was more efficient than NaI crystal scintillation, which was also surveyed.

Results

The data of Figure 1 show that Mn^{2+} serves as well as the more usual divalent ion, Mg^{2+}, to support the overall rate of aminoacylation catalyzed by isoleucyl-tRNA synthetase. Further, Table I shows that deionized tRNA is charged to the same level when Mg^{2+} or Mn^{2+} is used in the aminoacylation mixture. This suggests that the behavior of Mn^{2+} in the reaction will satisfactorily exemplify that of Mg^{2+}. This is convenient because $^{54}MnCl_2$, which emits gamma radiation, may be used to follow the fate of divalent ions in the tRNA recognition and aminoacyl-transfer reactions.

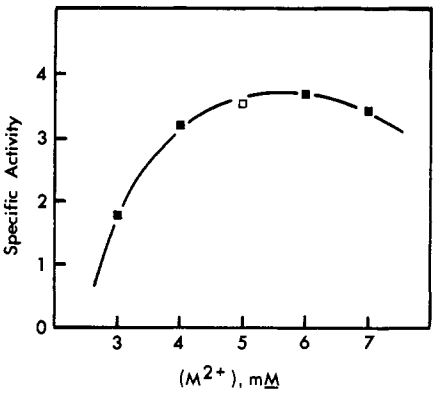

FIGURE 1: Specific velocity of aminoacylation *vs.* metal ion concentration. Closed squares, Mn^{2+}; open square, Mg^{2+}.

We begin by characterizing the affinity of tRNAs for $^{54}Mn^{2+}$ by measuring the amount of $^{54}Mn^{2+}$ bound to previously deionized tRNA.

tRNAs, deionized by gel filtration in 0.15 M EDTA at 50°, were allowed to equilibrate with 0.1 mM $^{54}MnCl_2$ by repetitive dialysis. This concentration was selected to be such as to equilibrate with the more strongly binding divalent ion sites on tRNAs, but to be too low to significantly load the weaker, more numerous sites, *e.g.*, the phosphates, which can acquire M^{2+} counterions (Cohn *et al.*, 1969). The resulting preparations are passed over gel columns and the ^{54}Mn migrating with the tRNA measured by scintillation spectrometry (Figure 2).

A close examination of the Mn^{2+} and A_{260} distributions of Figure 2 shows that (a) counts trail from the tRNA peak, suggesting that, at this ionic strength, there is significant release of Mn^{2+} during gel filtration and (b) the Mn^{2+} peak is sharp, but displaced slightly to larger elution volume than the A_{260}; the leading edge of the tRNA peak is low in Mn^{2+}.

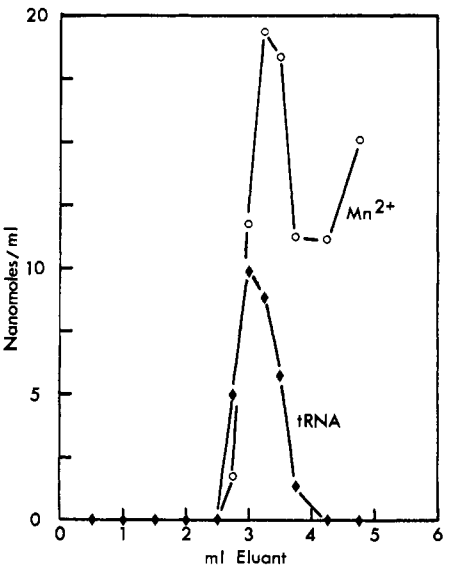

FIGURE 2: Mn^{2+} and tRNA in the eluate from a Sephadex G-25 column. Mixed deionized acceptor tRNAs equilibrated with 0.1 mM $^{54}MnCl_2$, 0.06 M NaCl, and 0.01 M Hepes (pH 7.0) were passed over a column of G-25 coarse at 25° and 8 ml/hr. The void volume was independently measured to be 2.7 ml. The eluent is 0.06 M NaCl–0.01 M Hepes (pH 7.0).

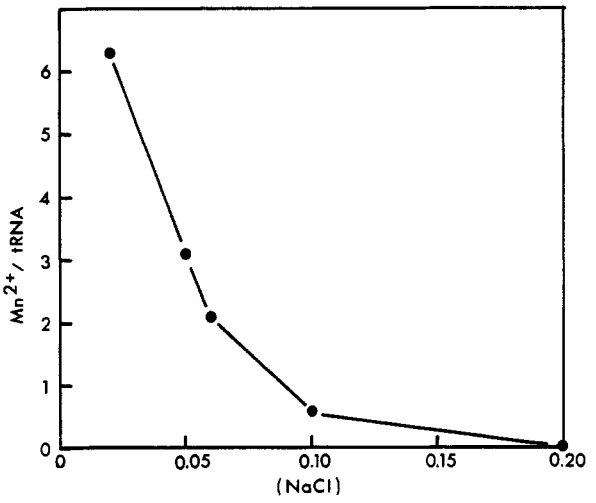

FIGURE 3: Mn^{2+} tRNA observed after gel filtration of tRNA equilibrated and filtered in varying concentrations of NaCl. Gel filtration performed as in Figure 2; mixed acceptors.

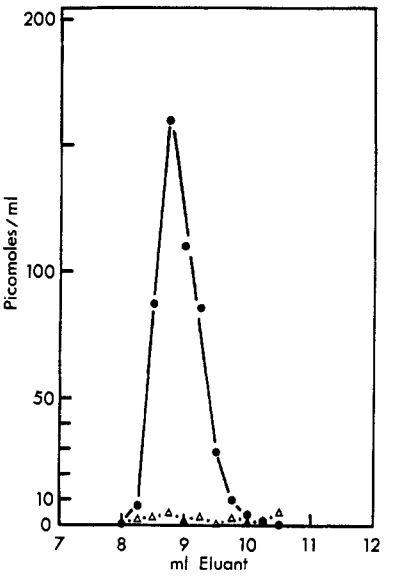

FIGURE 4: Gel filtration of isoleucyl-tRNA synthetase previously equilibrated with $^{54}MnCl_2$. Isoleucyl-tRNA synthetase equilibrated (24 hr at 4°) by dialysis aginst 0.5 mM $^{54}MnCl_2$, 1 mM dithiothreitol, and 0.01 M Hepes (pH 7.0) was passed over an 0.8 × 50 cm column of Sephadex G-50 coarse. Filled circles, concentration of enzyme as measured by aminoacylation velocity; open triangles, concentration of $^{54}Mn^{2+}$ as measured by scintillation spectrometry.

These qualities suggest that Mn^{2+} reequilibrates during gel filtration, dissociating and reassociating with tRNA until it reaches the trailing edge of the peak where it can be released. Thus, the amount of Mn^{2+} found with the tRNA will be a function of tRNA concentration, rising to approximate the true equilibrium value as tRNA concentration rises. These ideas are supported by other experiments[2] and, in fact, it appears that the absolute values obtained in Figures 2 and 3 are numerically close to the true level of Mn^{2+} bound. They are also in reasonable agreement with values obtained by completely independent (spectroscopic) means in reference 5. In any case, values of Mn^{2+}/tRNA obtained in this way are experimentally correct indications of the amount of divalent metal which travels with tRNA on gel filtration. This quantity is quite sensitive to the monovalent cation concentration during preequilibration with $^{54}MnCl_2$ and gel filtration, and, in fact, Figure 3 shows that at moderate Na^+ concentration (0.20 M), the divalent metal contamination is reduced to undetectable levels (<0.004 Mn^{2+}/tRNA). Other experiments[3] have shown that individual, highly purified tRNAs ($tRNA^{Ile}$ (*E. coli*), $tRNA^{Val}$ (*E. coli*), $tRNA^{Met}$ (*E. coli*), $tRNA^{Arg}$ (*E. coli*), and $tRNA^{Phe}$ (*E. coli*)) behave in a fashion similar to the preparation of mixed acceptors used in the experiments of Figures 2 and 3. The effect of increasing Na^+ is unlikely to be due to a structural effect on tRNA. Since increasing monovalent ion concentrations at constant divalent ion concentration would be expected to progressively stabilize a compact conformation of tRNA, these data may be interpreted to indicate that increasing monovalent salt concentration speeds the release and/or weakens the binding of Mn^{2+} and that there is no site which is not subject to this exchange. This suggests that the very rigorous conditions of deionization. used for the preparation of deionized tRNA (elevated temperature, high Na^+, strong chelation: 0.15 M Na_2EDTA, 50°) should be effective in removing the divalent ions from tRNAs.

We now consider the ion content of the usual aminoacylating agent, isoleucyl-tRNA synthetase (AMP-Ile), since this might also carry a required metal ion and transfer it to tRNA. It is universally observed that divalent metals are required to carry out the complete aminoacylation reaction; that is, to produce aminoacyl-tRNA from a mixture of ATP, amino acid, tRNA, and aminoacyl-tRNA synthetase. This is also true of the isoleucyl-tRNA synthetase system, but we shall now show that isoleucyl-tRNA synthetase (AMP-Ile) may be obtained free of divalent metals.

Figure 4 displays the $^{54}Mn^{2+}$ content of pure isoleucyl-tRNA synthetase, measured by gel filtration after 24 hr in the presence of 0.5 mM $^{54}MnCl_2$. As can be seen, isoleucyl-tRNA synthetase does not carry a stably bound ion (<0.03 mole of Mn^{2+}/mole of isoleucyl-tRNA synthetase) after gel filtration under conditions which are used to measure the formation of isoleucyl-tRNA synthetase (AMP-Ile). We conclude that isoleucyl-tRNA synthetase has no site which can be stably occupied by Mn^{2+} under these conditions. This experiment does not exclude quickly exchanging ions or the possibility that isoleucyl-tRNA synthetase may be a metalloenzyme, having a very tightly bound, nonexchangeable metal ion.

Figure 5a displays the result of a typical experiment in which isoleucyl-tRNA synthetase (AMP-Ile) was synthesized in the presence of $^{54}MnCl_2$ and isolated by gel filtration. The profile of [^{14}C]Ile agrees reasonably well with that of enzyme activity, measured by aminoacylation velocity; there is ~0.067 Mn^{2+}/mole of isoleucyl-tRNA synthetase (AMP-[^{14}C]Ile) appearing as a resolved peak which does not quite superpose on the enzymatic activity of isoleucyl-tRNA synthetase (AMP-[^{14}C]Ile) profile. We suspect, therefore, that an appreciable part of the Mn^{2+} is not carried on the active enzyme which is recovered as isoleucyl-tRNA synthetase (AMP-Ile). In any case, Figure 5b exhibits the result when 10 A_{260} of deionized tRNA is added to the pooled peak fractions from this column at 0°; 34% is transferred to tRNA. We do not know the meaning of the partial yield, but partial yields are often observed in this reaction (*e.g.*, Yarus and Berg, 1970). Further, the partial yield is not a symptom of deionization or Mn^{2+} usage; neither the yield nor kinetics of transfer

[2] M. Yarus and P. Edwards, unpublished data.
[3] M. Yarus and S. Rashbaum, unpublished data.

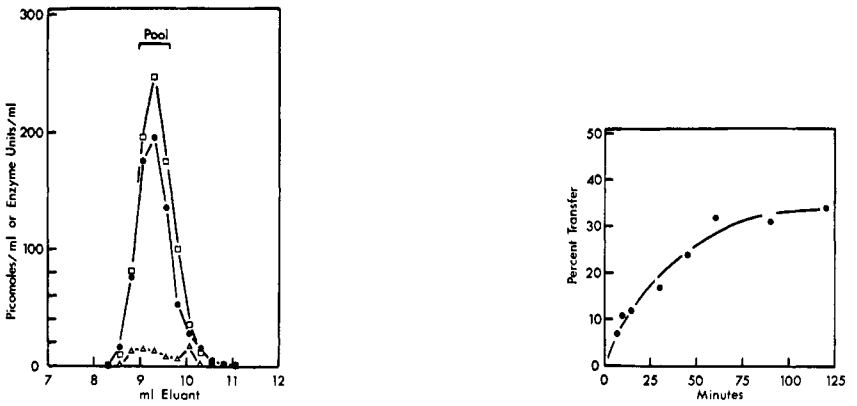

FIGURE 5: The measurement of Mn^{2+} which accompanies isoleucyl-tRNA synthetase (AMP-Ile). A reaction mixture containing deionized reagents and $^{54}MnCl_2$ as the only supplied divalent ion was passed through an 0.8 × 50 cm Sephadex G-50 column at 4°. The three peak fractions (bar marked pool) were combined and 10 A_{260} of deionized mixed acceptor tRNAs were added for measurement of isoleucyl transfer. (a, left) Isolation of isoleucyl-tRNA synthetase (AMP-Ile): open squares, enzyme units/ml, *e.g.*, nmoles of isoleucine transferred to tRNA/15 min at 37° under optimal conditions for this reaction; closed circles, pmoles of aminoacyl adenylate/ml measured by scintillation spectrometry of isoleucyl-tRNA synthetase (AMP-[^{14}C]Ile; open triangles, $^{54}Mn^{2+}$, measured by scintillation spectrometry. (b, right) Transfer of isoleucine from isoleucyl (AMP-Ile) to $tRNA^{Ile}$ at 0°—measured by acid precipitation of 50-μl aliquots of the 500-μl reaction mixture. Total [^{14}C]Ile was measured by drying an aliquot of the reaction on a filter and counting in the usual way with suitable standards.

under our conditions were changed by the substitution of Mg^{2+} for Mn^{2+} in the original reaction mixture, or by substituting highly purified $tRNA^{Ile}$ for mixed acceptors, or by substituting untreated for the deionized tRNA used here (but see below, Figure 8). In any case, the amount of transfer is fivefold greater than the amount of Mn^{2+} available in the reaction; we therefore conclude that, since neither tRNA nor isoleucyl-tRNA synthetase (AMP-Ile) carried a divalent metal into this reaction, that divalent metals cannot be required stoichiometrically for either recognition or aminoacyl transfer. It is still conceivable that Mn^{2+} might act catalytically in transfer; we will now argue on general physical chemical grounds from data obtained under more rigorous conditions, that divalent metals cannot serve catalytically either.

If Mn^{2+} is limiting, then the addition of more divalent ion should relieve the limitation, and conversely, the further limitation of divalent ions should make the limitation more severe. Several experiments of this kind are shown in Figures 6, 7, and 8. First, let us consider a reduction of Mn^{2+}. It is sometimes possible to pool and use isoleucyl-tRNA synthetase (AMP-[^{14}C]Ile) without including any detectible Mn^{2+}; Figure 6a,b show such an experiment; transfer to the purified and deionized $tRNA^{Ile}$ used as acceptor proceeds as before. The open circle of Figure 6b represents transfer to undeionized tRNA. As can be seen, it is not more efficient than the treated tRNA.

Secondly, one may increase the available divalent ion by adding Mn^{2+} to the reaction mixture. Figure 7 depicts an

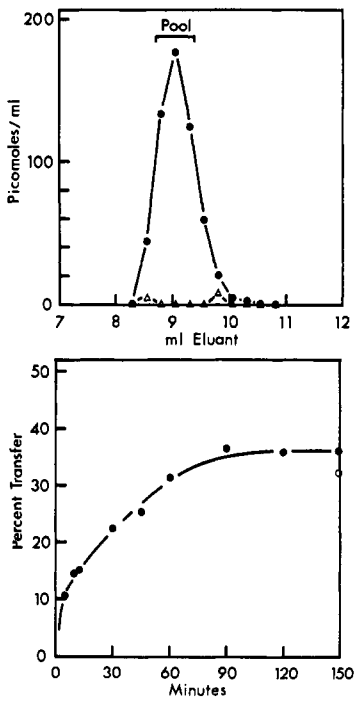

FIGURE 6: Isolation of and transfer of isoleucine from isoleucyl-tRNA synthetase (AMP-Ile) depleted of Mn^{2+}. (a, top) Gel filtration of the reaction in which isoleucyl-tRNA synthetase (AMP-Ile) was formed: symbols as in Figure 5. (b, bottom) Transfer of isoleucine to deionized $tRNA^{Ile}$ filled circles and technique as in Figure 5b. Open circle, transfer to undeionized $tRNA^{Ile}$ under the same conditions. Note that the tRNA used here is purified $tRNA^{Ile}$ (*E. coli*), not mixed acceptors.

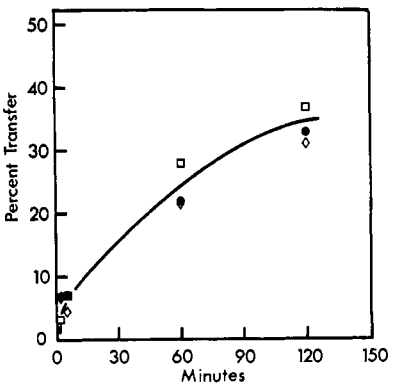

FIGURE 7: Transfer of isoleucine from isoleucyl-tRNA synthetase (AMP-Ile) to purified $tRNA^{Ile}$ under conditions of artificially increased Mn^{2+} concentrations. Filled circles, no Mn^{2+} added; that is, conditions as in Figures 5 and 6. Open squares, a final concentration of 3.8×10^{-6} M $^{52}MnCl_2$ added to transfer reaction mixture; open diamonds, 7.5×10^{-4} M $^{52}MnCl_2$ added.

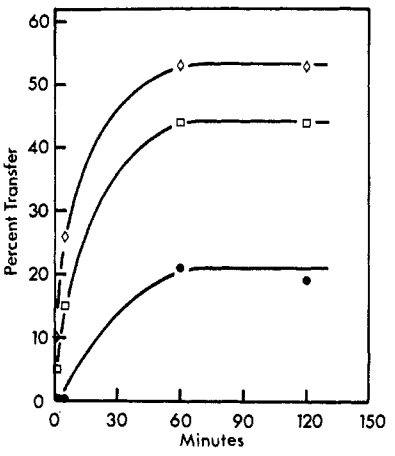

FIGURE 8: Transfer of isoleucine from isoleucyl-tRNA synthetase (AMP-Ile) to purified $tRNA^{Ile}$ in the presence of high concentrations of EDTA. Filled circles, conditions as in Figures 5 and 6, no added EDTA; open squares, EDTA adjusted to pH 6.1 was added to the control reaction mixture to a final concentration of 0.10 M; open diamonds, EDTA adjusted to pH 6.1 was added to the control reaction to a final concentration of 0.2 M. All reaction mixtures had a final volume of 720 μl and contained the same concentration of all reagents save EDTA.

aminoacyl-transfer experiment conducted with no, 3.8×10^{-6}, and 7.5×10^{-4} M $MnCl_2$ added; there is no substantial change in the kinetics or yield of transfer. The isoleucyl-tRNA (AMP-Ile) used in this experiment contained 0.061 Mn^{2+}/mole, or a final concentration of 4.1×10^{-9} M Mn^{2+} in the transfer reaction. Thus there is no change in kinetics or yield when total Mn^{2+} (before chelation by citrate) is elevated about 150,000-fold.

Thirdly, the free Mn^{2+} may be even more dramatically decreased by addition of EDTA. Figure 8 shows the kinetics of transfer in an experiment in which isoleucyl-tRNA synthetase (AMP-Ile) was synthesized from deionized isoleucyl-tRNA synthetase and contained 0.077 mole of Mn^{2+}/mole of complex. The addition of Na_2EDTA to 0.1 or 0.2 M did not inhibit the reaction; instead, it stimulated the rate and increased the extent of transfer somewhat; in the reaction which contains 0.20 M Na_2EDTA in addition to the usual reactants, 53% of the isoleucyl-tRNA synthetase has been converted to [^{14}C]Ile-tRNA at 120 min. The stimulation is not understood but it should be noted that the addition of 0.20 M EDTA considerably changes the composition and ionic strength of the transfer reaction solution; $EDTA^{2-}$ and $EDTA^{3-}$ become by far the most abundant anions so that a change in transfer kinetics might be expected.

Discussion

It appears that divalent ions are not required on a stoichiometric basis for (a) the stability of isoleucyl-tRNA synthetase (AMP-Ile), (b) for its actions during transfer of Ile to $tRNA^{Ile}$ or for (c) the maintenance of an active structure in $tRNA^{Ile}$; this tRNA is recognized and aminoacylated well when rigorous precautions have been taken to reduce the concentration of free divalent metals to very low levels. Thus, in particular, the information for the active structure of $tRNA^{Ile}$ seems to reside entirely in the nucleotide sequence.

Now let us show that not only is less metal available than aminoacyl transfer performed, but also that the divalent cations available cannot support the observed reaction catalytically by circulating among otherwise active complexes. Aminoacylation is most rapid and conditions are most rigorous in the experiment of Figure 8, where, in the presence of 0.20 M EDTA and 0.02 M citrate at pH 6.1, aminoacyl transfer proceeds at a measured initial rate of $\sim 10^{-10}$ mole $l.^{-1}$ sec^{-1}. We begin by calculating the free Mn^{2+} concentrations in the aminoacyl-transfer reaction mixture. The necessary equilibrium constants for the affinity of EDTA and citrate for hydrogen ions and metals[4] were obtained from Sillén and Martel (Sillén and Martel, 1964), and the calculation performed by the methods of Schwarzenbach (1957). If we make the most liberal assumption, that all of the Mn^{2+} brought in with isoleucyl-tRNA synthetase (AMP-Ile) is free and able to serve in the recognition and aminoacylation reactions, then the total Mn^{2+} concentration is 2.5×10^{-9} M in these experiments. Because of chelation by EDTA and, to a smaller extent, citrate, at this pH the *free* concentration of Mn^{2+} will be about 1.7×10^{-17} M. We now estimate the rate at which this metal could react with a hypothetical aminoacylating reagent which is active but for addition of the metal. The maximum conceivable concentration of this latter reagent is that of the isoleucyl-tRNA synthetase (AMP-Ile), or 3.3×10^{-5} M in this experiment. Thus a bimolecular reaction would occur with a rate $= k$[isoleucyl-tRNA synthetase (AMP-Ile)]-[Mn^{2+}]. If the metal were required, the rate of aminoacyl transfer could be no faster than this. The second order rate constant, k, is limited because the reaction of metals with their ligands is limited by the rate at which the solvent shell around the metal can be rearranged to accomodate the ligand (McAulty and Hill, 1969). Observed rate constants vary from about 10^4 to 10^8 M^{-1} sec^{-1}, depending on the metal. We will maximize the calculated rate again by presuming that the rate of reaction is among the fastest, or $\sim 10^8$ M^{-1} sec^{-1}. Thus if the reaction of isoleucyl-tRNA synthetase (AMP-Ile) with a metal were limiting, the maximum rate at which aminoacyl transfer could go would be $10^8[3.3 \times 10^{-5}][1.7 \times 10^{-7}] = 5.6 \times 10^{-17}$ mole $l.^{-1}$ sec^{-1}. This is $\sim(2 \times 10^6)$-fold slower than the observed rate of aminoacyl transfer, and so Mn^{2+} cannot be required for this reaction. However, Mn^{2+} brought in with isoleucyl-tRNA synthetase (AMP-Ile) is probably not the main source of divalent ions. It is exceeded by the concentration of divalent cations contaminating the reagents used. The maximum level of contamination[5] which could be accumulated from this source is about 10^{-6} M, or about two to three orders of magnitude greater than the concentration of $^{5\text{-}4}Mn^{2+}$. The concentration of any free divalent metal should be proportional to the total metal present, at these concentrations. The result of this contamination could be, at a maximum, to support a rate of reaction greater by two to three orders of magnitude, keeping in mind that many of the conceivable contaminants which might be biologically active (*e.g.*, Co^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}), are also more strongly bound by EDTA than Mn^{2+}. Thus they would not increase the rate by a factor

[4] The alkaline form of EDTA was taken to bind Mn^{2+} with an affinity constant of $10^{13.8}$ and to have pK's of 10.26, 6.16, 2.67, and 1.99. The affinity constant of citrate for Mn^{2+} was taken to be 10^4 and pK's to be 6.1, 4.3, and 3.1.

[5] This has been calculated by assuming that all reagents have their minimal purities and also that all heavy metal or divalent metal contamination is in the form of biologically active ions. The first assumption has, in fact, been directly confirmed by spark source mass spectrometry on silver electrodes containing reactants in an Associated Electrical Industries MS-702 double-focusing mass spectrometer with electrical detection.

proportionate to their abundance. Magnesium is a special case. It is less strongly bound than Mn^{2+} (Sillén and Martell, 1964) but Mg^{2+} is among the slowest reacting ions of the biologically active group (McAulty and Hill, 1969). Thus the maximal conceivable rate of the ion reaction should still be at least three to four orders of magnitude slower than the observed rate of aminoacyl transfer. We conclude that a divalent cation cannot be an obligatory participant in the recognition or aminoacyl transfer from isoleucyl-tRNA synthetase (AMP-Ile) to $tRNA^{Ile}$ (*E. coli*). In particular, $tRNA^{Ile}$ does not seem to require a divalent ion to retain or regain its active structure after exposure to 0.15 M EDTA at high temperature (see Methods). This conclusion is consistent with unpublished binding (Yarus and Berg, 1970) measurements (by M. Y.) which suggest that the affinity of isoleucyl-tRNA synthetase for $tRNA^{Ile}$ is of the same order in the absence of added divalent metals as in the usual condition for these experiments (0.01 M $MgCl_2$). Similarly, experiments which demonstrated transfer of Ile from isoleucyl-tRNA synthetase (AMP-Ile) to tRNA in the absence of added divalent cations (Norris and Berg, 1964) are consistent with these observations, though in these latter two cases divalent metals were not excluded rigorously, and were surely present to an unknown extent.

It therefore appears that the requirement for a divalent cation observed when the overall isoleucylation reaction is studied reflects a requirement expressed during synthesis of isoleucyl-tRNA synthetase (AMP-Ile) from ATP and Ile, presumably because M^{2+}-ATP is the substrate for this reaction (Cole and Schimmel, 1970).

Previously published results suggest that some systems, under conditions in which ions are less rigorously controlled, behave like the Ile example (Lagerkvist *et al.*, 1966), and others do not: experiments performed by Allende (Allende *et al.*, 1966), Bluestein *et al.* (1968), and Hirsch (1968), suggest that the threonyl-tRNA synthetase from rat liver, seryl-tRNA synthetase from *E. coli*, respectively, all require Mg^{2+} for synthesis of aminoacyl-tRNA from tRNA and aminoacyl-tRNA synthetase (AMP-aa). This does not necessarily imply that the difficulty has to do with binding of the tRNA however; it would be of great interest to know which step of these reactions is blocked in the absence of the divalent cation.

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