Applequist, J., and Damle, V. (1965), *J. Amer. Chem. Soc. 87*, 1450.
Bangerter, B. W., and Chan, S. I. (1968), *Biopolymers 6*, 983.
Chan, S. I., Bangerter, B. W., and Heinrich, H. P. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 720.
Crothers, D. M. (1971), *Biopolymers 10*, 2147.
Felsenfeld, G., Sander, G., and Von Hippel, P. H. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 644.
Hershey, A. D., and Burgi, E. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 325.
Kartha, G., Bello, J., and Harker, D. (1967), *Nature (London) 213*, 862.
Kosaganov, Yu N., and Lazurkin, Yu S. (1967), *Mol. Biol. 1*, 75.
Kosaganov, Yu N., Lazurkin, Yu S., and Sidorenks, N. V. (1967), *Mol. Biol. 3*, 352.
Lazurkin, Yu N., Frank-Kamenetski, M. D., and Trifnov, E. N. (1970), *Biopolymers 10*, 2147.
Lee, C. H., and Wetmur, J. G. (1972), *Biopolymers 11*, 549.
Mandell, J. D., and Hershey, A. D. (1970), *Anal. Biochem. 1*, 66.
Raju, E. V., and Davidson, N. (1969), *Biopolymers 8*, 743.
Sander, C., and Ts'o, P. O. P. (1970), *Biopolymers 9*, 765.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 686.
Ts'o, P. O. P., and Lu, P. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 17.
Ts'o, P. O. P., Melvin, Ingelore, S., and Olson, Alfred C. (1963), *J. Amer. Chem. Soc. 85*, 1289.
Wang, J. C., and Davidson, N. (1966a), *J. Mol. Biol. 15*, 111.
Wang, J. C., and Davidson, N. (1966b), *J. Mol. Biol. 19*, 469.
Wu, R., and Kaiser, A. D. (1968), *J. Mol. Biol. 35*, 523.
Wu, R., and Taylor, E. (1971), *J. Mol. Biol. 57*, 491.
Yang, S. K., Soll, D. G., and Crothers, D. M. (1972), *Biochemistry 11*, 2311.

A Random Sequential Mechanism for Arginyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli*†

Takis S. Papas‡ and Alan Peterkofsky*

ABSTRACT: Using purified arginyl-tRNA synthetase and purified $tRNA^{Arg}$ (*Escherichia coli*) we determined kinetic patterns according to Cleland. Inverse plots of initial velocities at varying concentrations of arginine and different fixed levels of $tRNA^{Arg}$ gave a family of converging lines. Similar intersecting plots were also obtained when arginine was varied at several fixed levels of ATP. These studies as well as those involving the inhibitors homoarginine and 5′-adenylyl(methylenediphosphonate) indicate that all three substrates (ATP, arginine, and $tRNA^{Arg}$) interact in a random order with the enzyme but that all substrates must be bound before any product is released. Verification of the nature of substrate addition as well as the order of product release was investigated by studies of isotope exchange rates ($PP_i \leftrightarrow$ ATP; arginine $\leftrightarrow$ arginyl-$tRNA^{Arg}$; AMP $\leftrightarrow$ ATP; tRNA $\leftrightarrow$ arginyl-$tRNA^{Arg}$) at chemical equilibrium. Increasing concentrations of a variety of reactant pairs without perturbing the equilibrium led to an increase in exchange rate up to a plateau level. The lack of inhibition of the various exchanges at high concentrations of reactants indicate that not only do the substrates interact randomly with the enzyme but that the products are also released in a random order. The various exchange rates vary over a factor of 100-fold, therefore the rate-limiting step in the overall reaction involves release of some reactant or product, not interconversions of enzyme-bound quaternary complexes.

Most aminoacyl-tRNA synthetases catalyze an amino acid dependent ATP–pyrophosphate-exchange reaction in the absence of added tRNA. According to the nomenclature of Cleland (1963a–c), these enzymes operate by a Ping-Pong mechanism whereby tRNA adds to the enzyme subsequent to the release of pyrophosphate. The aminoacyl-tRNA synthetases for arginine (Mitra and Mehler, 1966, 1967; Mehler and Mitra, 1967), glutamine, and glutamic acid (Ravel *et al.*, 1965) have the unique property of requiring tRNA for the ATP–pyrophosphate-exchange reaction. This property of the arginine-activating enzyme to show an absolute requirement for a specific tRNA for the ATP–pyrophosphate-exchange reaction prompted us to look at the mechanism of the reaction by following the kinetic procedures proposed by Cleland (1963a–c) and Boyer (1959). Initial velocity studies were carried out at varying concentrations of the different reactants.[1] Similar experiments were also performed in the presence of the dead-end inhibitors AMPPCP[2] and homoarginine. A further study was carried out to verify the random sequential addition of reactants and to elucidate the mechanism of product release by using Boyer's method for measuring isotope exchange at chemical equilibrium. The data obtained sup-

† From the Laboratory of Biochemical Genetics, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received June 26, 1972.*

‡ Present address: National Cancer Institute, Bethesda, Md.

[1] In this paper, we have chosen to use the terminology of Wedler and Boyer (1972). *Substrates* refer to ATP, arginine, $tRNA^{Arg}$, AMP, PP_i, and arginyl-$tRNA^{Arg}$. *Reactants* refer to the first three and *products* to the latter three.

[2] Abbreviation used is: AMPPCP, 5′-adenylyl(methylenediphosphonate).

ports a random sequential mechanism for the enzymatic reaction. A preliminary report of some of this work has been presented (Papas and Peterkofsky, 1972).

Materials

Frozen *Escherichia coli* cells were obtained from General Biochemicals. Unfractionated tRNA from *E. coli* was supplied by Schwarz–Mann. Purified $tRNA^{Arg}$ was prepared as described in Table I. The following radioactive compounds were obtained from Schwarz–Mann: L-[^{14}C]arginine, Stanstar (50 Ci/mole); [^{3}H]arginine (11 Ci/mmole); [^{3}H]AMP (24.9 Ci/mmole); [^{3}H]ATP (22.7 Ci/mmole). $^{32}PP_i$ was obtained from New England Nuclear Corp. Crystalline Na_2ATP was a product of Sigma. AMPPCP was obtained from Miles Laboratories. Homoarginine was the generous gift of Dr. Marco Rabinovitz. BD-cellulose and the phenoxyacetyl ester of *N*-hydroxysuccinimide were obtained from Schwarz–Mann. PEI-300 thin-layer plates were from Brinkmann.

Enzyme Preparations. Arginyl-tRNA synthetase was prepared according to Mitra and Mehler (1967) with omission of the hydroxylapatite step. The enzyme used was about 200-fold purified. A partially purified preparation of tRNA–CMP–AMP pyrophosphorylase (Hurwitz and Furth, 1966) was a generous gift of Dr. Tikvah Vogel. It was prepared as follows: S-100 from *Salmonella* LT-2 was prepared from 100 g wet weight of cells. It was applied to a column of DEAE-cellulose (500-ml bed volume) in 0.02 M potassium phosphate (pH 7.5), containing 0.02 M mercaptoethanol and 10^{-3} M $MgCl_2$ in 10% glycerol. A fraction containing tRNA–CMP–AMP pyrophosphorylase activity was eluted from the column with 0.25 M phosphate buffer (pH 6.5), containing 0.1 M mercaptoethanol, 5×10^{-4} M $MgCl_2$, and 10% glycerol.

Preparation of $tRNA^{Arg}$ Labeled in the 3'-Terminal Adenosine Residue. Purified $tRNA^{Arg}$ (see Table I) was treated with venom phosphodiesterase (Sigma) as described by Mehler and Mitra (1967). The tRNA was then separated from the phosphodiesterase by absorption to DEAE-cellulose. The protein and other reaction components were washed off the column with 0.3 M NaCl and the tRNA was then eluted with 1 M NaCl. The tRNA was dialyzed against 0.01 M $MgCl_2$ and 0.001 M Tris (pH 8), concentrated, and repaired by incubation with tRNA–CMP–AMP pyrophosphorylase in the presence of unlabeled CTP and [^{3}H]ATP (22.7 Ci/mmole, as described by Hurwitz and Furth, 1966). The repaired [^{3}H]tRNA was reisolated on DEAE-cellulose, dialyzed, and concentrated before use in exchange experiments. Measurements of specific activity of ^{3}H label and acceptance of arginine indicated that the tRNA contained 1 mole of [^{3}H]adenosine/mole of arginine acceptance activity. The yield of arginine acceptance activity was ~50%.

Methods

Kinetic Measurements. INITIAL VELOCITY STUDIES. Kinetic nomenclature and methodology were as described by Cleland (1963a–c). Initial velocity studies were performed by selecting enzyme concentrations that ensured linear reaction rates. Details of the methodology leading to the data of Figures 2, 3, and 4 are shown in the legend to Figure 1. Figure 1 represents the linear initial rate plots at various concentrations of $tRNA^{Arg}$ and arginine, representing the limits of concentration of these compounds used in the study shown in Figure 2 (right panel).

Deduction of reaction mechanisms can be made by inspection of reciprocal plots of velocity *vs.* substrate concentration at varying fixed concentrations of a second substrate. When the lines converge, the two substrates are sequentially connected in the reaction path. When the lines are parallel,

TABLE I: Purification of $tRNA^{Arg}$.[a]

Fraction	Total Acceptance, pmoles ($\times 10^{-5}$)	Total A_{260}	Sp Acceptance, pmoles of Arg/A_{260}
Crude tRNA	16.4	18,225	90
After sham acylation; 1.5 M NaCl fraction	6.6	2,750	240
After derivatization; ethanol fraction	5.3	338	1,568

[a] *E. coli* tRNA (Schwarz–Mann; 1 g) was sham acylated (Gillam *et al.*, 1968) as follows. It was dissolved in 80 ml of H_2O; 20 ml of 2.5 M triethanolamine buffer (pH 8.1) followed by 60 ml of a solution (50 mg/ml in dioxane) of the phenoxyacetyl ester of *N*-hydroxysuccinimide (Schwarz–Mann) was added. The solution was allowed to sit in ice for 15 min. Then, 50 ml of 1 M sodium acetate (pH 4.5), containing 1 M $MgCl_2$ was added and the tRNA was precipitated by the addition of 600 ml of ethanol. The precipitated tRNA was dissolved in 250 ml of buffer (0.01 M $MgCl_2$, 0.01 M sodium acetate (pH 4.5), 0.5 M NaCl, and applied to a column (120-ml bed volume) of benzoylated-DEAE-cellulose and the column was washed with the same buffer until the A_{260} of the eluting fluid was low and constant (requires about 300 ml of buffer). The buffer was then changed to 1.5 M NaCl containing the other components. A peak of tRNA was eluted in a volume of about 300 ml. This fraction was concentrated to a volume of 5 ml in a pressure filtration apparatus (Amicon) using a PM-10 membrane. The concentrated tRNA fraction was dialyzed for 1 hr against 0.01 M $MgCl_2$. The specific acceptance of this fraction was increased about threefold (sham acylation, 1.5 M NaCl fraction). The tRNA was acylated with [^{14}C]arginine of low specific activity. The [^{14}C]arginyl-tRNA was precipitated with ethanol, then dissolved in 10 ml of H_2O, and derivatized as follows. Triethanolamine buffer (2.4 ml) was added, followed by 7 ml of a dioxane solution of phenoxyacetylhydroxysuccinimide, as above. After 15-min incubation at 0°, 6 ml of 1 M sodium acetate–1 M $MgCl_2$ was added and the tRNA was precipitated with ethanol (three volumes). After dialysis, the derivatized tRNA was diluted to 50 ml with 0.5 M NaCl buffer and applied to BD-cellulose (22-ml bed volume) and the column was washed with 0.5 M NaCl buffer until the A_{260} was low and constant. A subsequent wash with 1.5 M NaCl buffer eluted the residual underivatized tRNA. Purified arginyl-$tRNA^{Arg}$ was then eluted with a linear gradient (600 ml) of 1.0 M NaCl to 2.0 M NaCl containing 30% ethanol. The symmetrical peak of radioactive tRNA was concentrated by ultrafiltration and precipitated with ethanol. The tRNA was then dissolved in 1 ml of H_2O and 0.6 ml of Tris (0.5 M, pH 8.2), 0.6 ml of 0.5 M $MgCl_2$, and 1.3 ml of H_2O were added. The tRNA was deacylated by incubation for 2 hr at room temperature. The deacylated tRNA was dialyzed for 1.5 hr at 4° against 0.001 M Tris (pH 8.0)–0.01 M $MgCl_2$. The specific acceptance of the purified tRNA was determined by aminoacylation with [^{14}C]arginine.

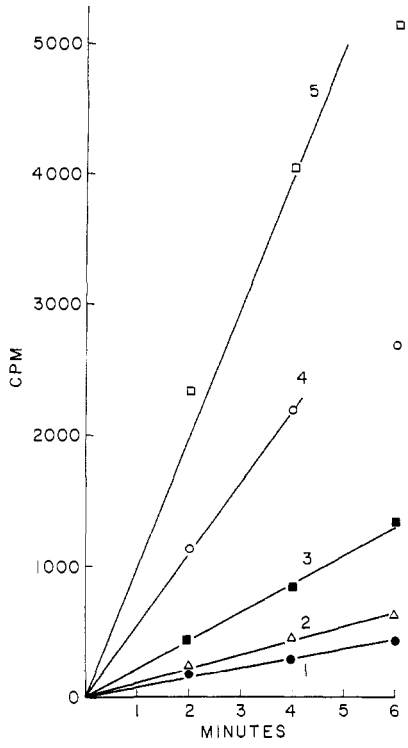


FIGURE 1: Initial rate measurements of acylation of [^{3}H]arginine to tRNA^{Arg}. Reactions mixtures contained in a total volume of 0.2 ml, the following: Tris buffer (pH 7.0, 20 μmoles), $MgCl_2$ (1 μmole), ATP (0.4 μmole), arginyl-tRNA synthetase (0.45 μg), and tRNA^{Arg} and [^{3}H]arginine (11 Ci/mmole) as specified for the different curves. Curve 1: 0.1 nmole of arginine and 2.48×10^{-4} mM tRNA^{Arg}; curve 2: 0.1 nmole of arginine and 5.07×10^{-4} mM tRNA^{Arg}; curve 3: 0.68 nmole of arginine and 2.48×10^{-4} mM tRNA^{Arg}; curve 4: 0.68 nmole of arginine and 5.07×10^{-4} mM tRNA^{Arg}; curve 5: 0.68 nmole of arginine and 10.1×10^{-4} mM tRNA^{Arg}. Incubation was at 30°. At 2-min time intervals, 20-μl aliquots were removed, diluted to 0.5 ml with 0.5 M KCl, and precipitated with 5% trichloroacetic acid. The precipitates were collected on glass fiber filters and washed with 5% trichloroacetic acid. Radioactivity on filters was determined by counting with 10 ml of counting fluid (Liquifluor–Triton–toluene, 1:6:12).

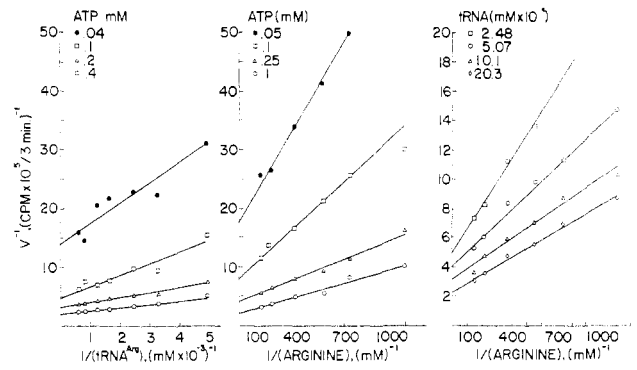


FIGURE 2: Effect of variations in substrate concentrations on the kinetics of aminoacylation of tRNA^{Arg}. Left panel: reciprocal plots of variations of rate *vs.* tRNA concentration at four different levels of ATP, as indicated. The arginine concentration was 0.08 mM. Experimental procedure is described in Methods. Each incubation mixture contained 0.33 μg of enzyme. Center panel: reciprocal plots of variations of rate with arginine concentration at four different levels of ATP, as indicated. The concentration of tRNA^{Arg} was 6×10^{-6} M. Each incubation mixture contained 0.27 μg of enzyme. Other experimental details are described in Methods. Right panel: reciprocal plots of variations of rate with arginine concentration at four different levels of tRNA^{Arg}, as shown. The concentration of ATP was 2 mM. Each incubation contained 0.45 μg of enzyme. Other experimental details are described in Methods.

the two substrates are interrupted by release of a product or addition of another substrate.

STEADY-STATE MEASUREMENTS. The methodology of Boyer (1959) was used. This method is based on the analysis of exchange rates that occur when a trace amount of highly labeled reactant or product is added to an enzyme-catalyzed reaction after it has reached chemical equilibrium. Measurements of isotope exchange rates at equilibrium can be used to distinguish ordered from random mechanisms in the following way. Measurements of isotope exchange rates are made at increasing concentrations of a reactant–product pair at a fixed ratio that does not disturb chemical equilibrium. In an ordered mechanism one expects the isotope exchange in the presence of some substrate pair to increase to a maximum and then decrease to zero. On the other hand, a random mechanism is characterized by an increase in exchange rate to a maximum value that does not decrease with an increase in concentration of the substrate pair.

Equilibrium conditions were established as detailed in the experiment shown in Table II. The concentrations at equilibrium are calculated on the basis of the knowledge that the amounts of PP_i, AMP, and arginyl-tRNA^{Arg} formed are equal to the amounts of arginine, tRNA^{Arg}, and ATP utilized. On the basis of these data, the apparent equilibrium constant (K_{eq})

$$K_{eq} = \frac{[\text{Arg-tRNA}^{Arg}][PP_i][AMP]}{[Arg][\text{tRNA}^{Arg}][ATP]}$$

was calculated to be 0.25.

AMP exchange into ATP (Figure 5, panel A) was determined as follows. Incubation mixtures (0.2 ml) similar to that described in Table II were allowed to reach equilibrium (2-hr incubation), then pulsed with 7.5 μCi of [^{3}H]AMP. The specific activity of the [^{3}H]AMP was so high that this addition did not perturb the chemical equilibrium. At time intervals of 0, 8, 16, 24, and 30 min, 30-μl aliquots were removed and mixed with 50 μl of 2 M HCOOH. Aliquots (20 μl) were spotted on sheets of PEI-cellulose. Ascending chromatography in 1 M potassium phosphate (pH 3.4) was carried out as described by Cashel *et al.* (1969). After development of the chromatogram (1 hr) the sheets were dried and the ultraviolet-absorbing spots corresponding to ATP were circled and cut out. The spots were counted with 10 ml of Liquifluor–toluene (1:24). A plot of radioactivity in ATP as a function of time was constructed to give the best straight line going through the origin. The specific activity of the AMP in the incubation mixture was calculated from the data of Table II and the input of [^{3}H]AMP (7.5 μCi). The exchange rate was expressed in the form nanomoles of [^{3}H]AMP exchanged into ATP per minute per milliliter of reaction mixture.

Pyrophosphate exchange into ATP (Figure 5, panel B) was determined as follows. Incubation mixtures (0.2 ml) similar to that described in Table II were allowed to reach equilibrium (2 hr), then pulsed with 1.8×10^6 cpm of $^{32}PP_i$. At time intervals of 5, 10, and 20 min, 20-μl aliquots were removed and mixed with 0.5 ml of 7% $HClO_4$. Acid-washed charcoal (20 mg) in 0.5 ml of water was added and the charcoal was collected on glass fiber filters and washed with 0.1 M PP_i. The filters were dried, glued onto planchets upside down, and

TABLE II: Determination of Equilibrium Constant for Arginyl-tRNA Synthetase.[a]

Substrate	Initial Concn (μM)	Concn at Equil (μM)
Arginine	200	198.7
$tRNA^{Arg}$	3.4	2.1
ATP	2000	1998.7
Arg-$tRNA^{Arg}$	0	1.3
PP_i	800	801.3
AMP	200	201.3

[a] Arginyl-tRNA synthetase (10.8 μg) and the substrates shown were incubated in a total volume of 0.2 ml at 30°. Samples were removed at different time intervals and the conversion of [^{14}C]arginine (10 Ci/mole) to [^{14}C]arginyl-$tRNA^{Arg}$ was determined as described in Methods. The reaction was followed until no further change in the amount of [^{14}C]arginyl-tRNA could be detected. The reaction was complete in 1.5 hr and no further change was measurable up to several hours. The equilibrium concentrations shown here are from a determination of the amount of [^{14}C]arginyl-tRNA found at 2-hr incubation. Under the conditions of these experiments (pH 7 and 30°), there was no appreciable deacylation of Arg-$tRNA^{Arg}$.

counted in a gas-flow counter. Calculation of exchange rates was done as above for AMP exchange into ATP.

Arginine ↔ arginyl-tRNA exchange (Figure 5, panel C) was determined as follows. Incubation mixtures (0.2 ml) were allowed to go to equilibrium as above, then pulsed with [^{3}H]-arginine (4.9×10^6 cpm). At time intervals of 0, 6, 12, and 30 min, 20-μl aliquots were removed, diluted to 0.5 ml with 1 M KCl, and then precipitated with 5% trichloroacetic acid. The precipitates were collected on filters, washed with 5% trichloroacetic acid and counted with 10 ml of Triton–toluene–Liquifluor. Exchange rates, expressed as nanomoles of [^{3}H]arginine exchanged into arginyl-tRNA per minute milliliter of incubation mixture were computed as above for AMP exchange into ATP.

All exchange reactions were measured at less than 10% approach to isotopic equilibrium.

Results

Figure 2 shows the results of studies on the kinetics of aminoacylation of arginine at varying concentrations of different reactants and different levels of a second reactant. The left panel shows the double-reciprocal plots when $tRNA^{Arg}$ was the variable reactant at different concentrations of ATP and arginine concentration was saturating. The double-reciprocal plots were linear, constituting a family of converging lines which would intersect at the left of the vertical axis. A linear converging pattern of reciprocal plots was again obtained when arginine was varied at different fixed concentrations of ATP (center panel). Similarly in the right panel a linear converging pattern was obtained when arginine was varied at different concentrations of tRNA. These converging kinetic patterns can be interpreted by Cleland's analysis as evidence for a mechanism in which all substrates, ATP, arginine, and tRNA must combine randomly with the enzyme before any

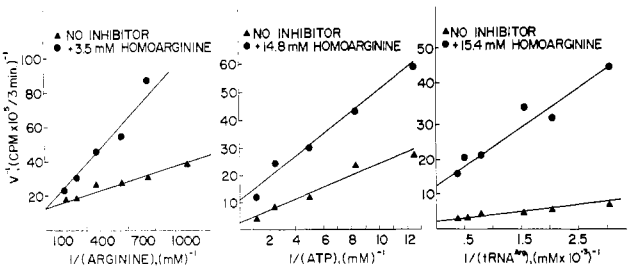


FIGURE 3: Effect of homoarginine on the kinetics of aminoacylation of $tRNA^{Arg}$. Left panel: reciprocal plots of variation of rate *vs.* arginine concentration in the presence or absence of 35 mM homoarginine. The concentration of ATP was 2 mM and of $tRNA^{Arg}$ was 6×10^{-6} M. Each incubation contained 0.54 μg of enzyme. Experimental procedure was as in Figure 1. Center panel: reciprocal plots of variation of rate with ATP concentration in the presence or absence of 14.8 mM homoarginine. The concentration of arginine was 0.08 mM and of $tRNA^{Arg}$ was 6×10^{-6} M. Each incubation contained 1.35 μg of enzyme. Experimental procedure was as in Figure 1. Right panel: reciprocal plots of variation of rate with $tRNA^{Arg}$ concentration in the presence or absence of 15.4 mM homoarginine. The concentration of arginine was 0.08 mM and of ATP was 2 mM. Each incubation contained 0.45 μg of enzyme. Experimental procedure was as in Figure 1.

product is released. Additional data which confirms the random addition of reactants was obtained by the use of two dead-end inhibitors, homoarginine, and AMPPCP. Figure 3 shows the data for homoarginine. Homoarginine is a competitive inhibitor with respect to arginine and is a noncompetitive inhibitor with respect to ATP and tRNA. These inhibition patterns establish that arginine must add to arginyl-tRNA synthetase either first or in a random fashion. Similarly, in Figure 4, AMPPCP is a competitive inhibitor with respect to ATP and demonstrates noncompetitive inhibition with arginine and tRNA. This again indicates that ATP binds either first or in a random fashion. The existence of an enzyme form which binds to either ATP or arginine is consistent only with a random order of addition for the reactants. The kinetic constants derived from these analyses are shown in

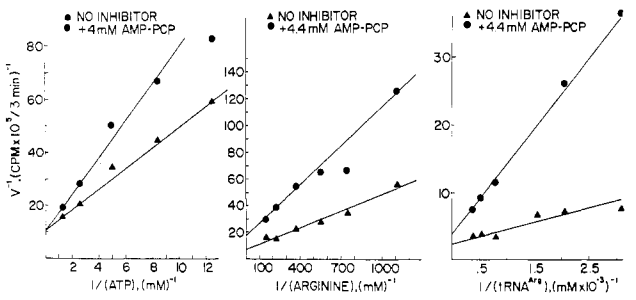


FIGURE 4: Effect of AMPPCP on the kinetics of aminoacylation of $tRNA^{Arg}$. Left panel: reciprocal plots of variation of rate *vs.* ATP concentration in the presence or absence of 4 mM AMPPCP. The concentration of arginine was 0.08 mM and of $tRNA^{Arg}$ was 6×10^{-6} M. Each incubation contained 1.35 μg of enzyme. Experimental procedure was as in Figure 1. Center panel: reciprocal plots of variation of rate with arginine concentration in the presence or absence of 4.4 mM AMPPCP. The concentration of arginine was 2 mM and of $tRNA^{Arg}$ was 6×10^{-6} M. Each incubation contained 0.54 μg of enzyme. Experimental procedure was as in Figure 1. Right panel: reciprocal plots of variations of rate with $tRNA^{Arg}$ concentration in the presence or absence of 4.4 mM AMPPCP. The concentration of ATP was 2 mM and of arginine was 0.08 mM. Each incubation contained 0.45 μg of enzyme. Experimental procedure was as in Figure 1.

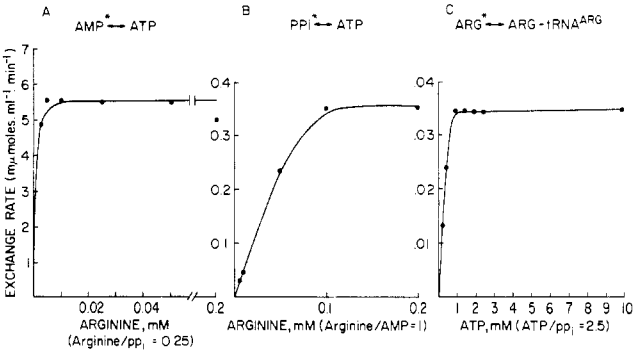


FIGURE 5: Isotope exchange reactions at chemical equilibrium. Panel A: the effect of increasing arginine and PP_i concentrations on the AMP ↔ ATP exchange rates. Panel B: the effect of increasing arginine and AMP concentrations on the PP_i ↔ ATP exchange rates. Panel C: the effect of increasing concentrations of ATP and PP_i on the arginine ↔ arginyl-$tRNA^{Arg}$ exchange rates. Details of the experiments are described in Methods. The asterisks denote those compounds that are initially radioactively labeled for the individual exchange experiments.

Table III. It is interesting to note that the K_m for arginine is about 100 times lower than that of ATP. Homoarginine, a competitive inhibitor of arginine, exhibits a K_i 500 times higher than the K_m for arginine. AMPPCP exhibits a K_i nine times higher than that of ATP. The K_m for tRNA of 10^{-7} M is in the range of that determined for the synthetases for other amino acids.

A series of experiments were designed to determine the manner in which the rate of exchange between reactants and

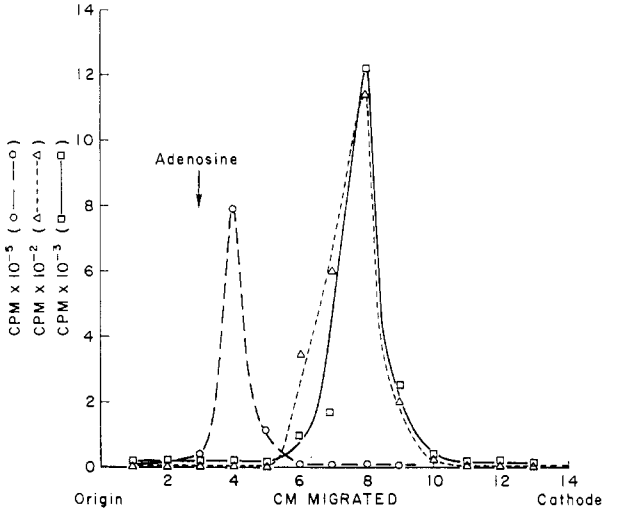


FIGURE 6: Electrophoretic separation of adenosine from arginyladenosine. $tRNA^{Arg}$ labeled with [^{3}H]adenosine at its 3′-terminal nucleotide was prepared as described by Mehler and Mitra (1967), using [^{3}H]ATP (specific activity 22.7 Ci/mmole). The [^{3}H]tRNA was aminoacylated with unlabeled arginine in a total volume of 0.2 ml, as described in Methods. Another preparation of unlabeled unfractionated tRNA was acylated with [^{3}H]arginine (specific activity 11 Ci/mmole). After aminoacylation was complete, 420 μg of pancreatic RNase (Worthington) was added and incubation was continued for 30 min. Aliquots (20 μl) were spotted on Whatman No. 3 MM paper and electrophoresis was run for 45 min at 12.5 V/cm at pH 2.0 (formic acid–glacial acetic acid–H_2O, 31.2:59.2:910). Standards of unlabeled adenosine and [^{3}H]arginine were also included. (○) [^{3}H]Arginine (cpm × 10^{-5}); (Δ) arginyl-[^{3}H]adenosine (cpm × 10^{-2}); (□) [^{3}H]arginyladenosine (cpm × 10^{-3}).

TABLE III: Kinetic Constants for Arginyl-tRNA Synthetase.[a]

Substrate or Inhibitor	K_m (M)	K_i (M)
Arginine	3×10^{-6}	1.6×10^{-3}
Homoarginine		1.6×10^{-3}
ATP	3×10^{-4}	
AMPPCP		2.8×10^{-3}
$tRNA^{Arg}$	4×10^{-7}	

[a] The K_m for arginine and K_i for homoarginine were derived from the data in Figure 3 (left panel). The K_m for ATP and K_i for AMPPCP were derived from the data in Figure 2 (left panel). The K_m for $tRNA^{Arg}$ was derived from Figure 2 (right panel).

products at equilibrium are affected by changes in concentration of the reactant–product pairs, arginine–pyrophosphate, arginine–AMP, and ATP–pyrophosphate. The data are shown in Figure 5. In all experiments, when equilibrium was reached, a trace amount of highly labeled [^{3}H]AMP, [^{32}P]pyrophosphate, or [^{3}H]arginine was added and the amount of exchange was determined at several time intervals, as described in Methods. Panel A shows the effect of increasing concentrations of the arginine–pyrophosphate pair on the rate of exchange of AMP into ATP at equilibrium. No decrease in the exchange rate was observed, even at concentrations of arginine in a 100-fold excess of its K_m value. Essentially the same exchange rates were obtained in a study (not shown) of the exchange of [^{3}H]ATP into AMP.[3] Panel B shows the results of a study of the rate of exchange of pyrophosphate into ATP. The concentration of the substrate pair arginine–AMP was varied. Again it can be seen that at concentrations of arginine well in excess of the K_m there is no inhibition of the exchange rate. Panel C details the study of the rate of exchange of [^{3}H]arginine into arginyl-tRNA at varying concentrations of a fixed ratio of ATP and pyrophosphate. Raising the ATP concentration to a value of approximately 25 times its K_m caused the arginine ↔ arginyl-tRNA exchange to increase to a maximum and then plateau.

A complete study of the various exchange reactions required the determination of the exchange of tRNA into arginyl-$tRNA^{Arg}$. This study required working out conditions for a separation of labeled adenosine from labeled arginyladenosine. We chose to do this by paper electrophoresis as shown in Figure 6. *E. coli* tRNA aminoacylated with [^{3}H]arginine was digested with ribonuclease and subjected to paper electrophoresis. A single peak of radioactivity was found at approximately 8 cm from the origin. This was presumed to be arginyladenosine. To confirm this presumption, we digested with ribonuclease a preparation of tRNA that had been labeled in its 3′-terminal adenosine residue and then aminoacylated with unlabeled arginine. The electrophoresis under the identical conditions also led to a peak of radioactivity at about 8 cm from the origin. Standard adenosine was well separated from the arginyladenosine and was found at a position of about 3 cm from the origin. Using this

[3] For measurements of exchange of ATP into AMP, it was necessary to purify the commercially obtained [^{3}H]ATP. [^{3}H]ATP was streaked in a band on a sheet of PEI-300 cellulose and chromatographed for 1 hr in 1 M KPO_4 (pH 3.4); the band of purified [^{3}H]ATP was eluted with 2 M NH_4OH, and then concentrated to remove the NH_4OH.

electrophoretic procedure as a means of determining the amount of arginyl-tRNA formed from adenosine-labeled tRNA, we set out to measure the kinetics of exchange of tRNA into arginyl-tRNA at chemical equilibrium. Table IV shows the results of such a study carried out at three concentrations of arginine and AMP, all at saturating levels. It can be seen that at all levels of arginine and AMP the exchange rate is essentially identical. There is no indication for an inhibition of the exchange rate at the highest levels of arginine and AMP used in this experiment. The data in Table IV together with the results of Figure 5 suggest a mechanism for the arginyl-tRNA synthetase reaction involving a random addition of reactants and a random release of products.

TABLE IV: Exchange of $tRNA^{Arg}$ into Arginyl-$tRNA^{Arg}$ at Chemical Equilibrium.[a]

Concn of Arg and AMP (mM)	Exchange Rate (nmole/ml per min)
0.05	0.029
0.10	0.036
0.20	0.036

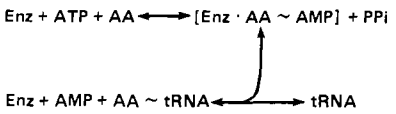
[a] Incubation mixtures (total volume 0.1 ml) contained: arginyl-tRNA synthetase (5.4 μg), $tRNA^{Arg}$ (343 pmoles), ATP (0.1 μmole), PP_i (0.04 μmole), Tris buffer (pH 7.0, 1.25 μmoles), and $MgCl_2$ (0.5 μmole). Arginine and AMP concentrations were varied as shown. After incubation at 30° for 2 hr to establish chemical equilibrium, $tRNA^{Arg}$ (6.15 pmoles) labeled with ^3H in the 3′-terminal adenosine residue (specific activity 22.7 Ci/mmole) was added in a volume of 0.05 ml. Incubation at 30° was continued. At time intervals of 10 and 20 min after pulsing with [^3H]$tRNA^{Arg}$, aliquots (50 μl) were removed and added to 20 μl of RNase (420 μg, Worthington) and incubation was continued for 30 min. The entire sample was spotted on Whatman No. 3MM paper and electrophoresed as described in Figure 5.

Discussion

Other studies in the literature involving three-reactant, three-product reactions provide analogies for the type of data we have obtained. The studies of Joyce and Himes (1966a,b) on the formyltetrahydrofolate synthetase reaction showed results similar to ours. Plots of their initial velocity studies by Cleland's (1963a–c) method gave intersecting lines, consistent with a random mechanism. Their study of exchange reactions at steady state also supported a random mechanism. In a recent study of the *E. coli* glutamine synthetase mechanism, Wedler and Boyer (1972) reasoned that the lack of detectable partial reactions or isolable intermediates points to a concerted mechanism for covalent interconversion and argues against a Ping-Pong mechanism.

The data we present here on the relative rates of exchange at equilibrium allows for an elimination of certain orders of binding. In a compulsory binding sequence, the most rapid exchange must occur between the last reactant to bind and the first product to dissociate. All other exchanges must be slower or equal in rate. The data show that the exchange reactions involving $tRNA^{Arg}$ and arginyl-tRNA are substantially slower than those involving ATP, AMP, and PP_i. Mehler and Mitra (1967) suggested a model in which binding of $tRNA^{Arg}$ to the enzyme is necessary for the initial activation step to form enzyme-bound arginyl–adenylate. However, the lack of inhibition of the exchange reactions by increasing the concentrations of arginine and AMP (Table IV), arginine and PP_i (Figure 5, panel A), arginine and AMP (Figure 5, panel B) or ATP and PP_i (Figure 5, panel C), exclude a reaction mechanism which involves a compulsory binding order with arginine, ATP, AMP, or PP_i as the last substrates to bind. Our studies of the exchange reactions at chemical equilibrium (Figure 5 and Table IV) also allow us to eliminate a "rapid-equilibrium" random mechanism and to make deductions about the relative rates of substrate association–dissociation. Thus, association–dissociations involving AMP and ATP are faster than enzyme interactions with PP_i, while association–dissociation involving arginine, $tRNA^{Arg}$, or arginyl-$tRNA^{Arg}$ are the slowest processes.

A generally accepted mechanism for the reactions carried out by aminoacyl-tRNA synthetases is expressed by eq 1.



(1)

There is substantial evidence for many aminoacyl-tRNA synthetases that supports this formulation. It has been shown that some enzymes will utilize chemically synthesized aminoacyl adenylates to form ATP (DeMoss *et al.*, 1956; Castelfranco *et al.*, 1958). Furthermore, the tryptophanyl-tRNA synthetase has been shown to form stoichiometric amounts of enzyme-bound tryptophanyl adenylate when incubated with ATP and tryptophan (Kingdon *et al.*, 1958; Karasek *et al.*, 1958). Another type of support for the adenylate intermediate mechanism comes from the wide observation that most aminoacyl-tRNA synthetases catalyze an amino acid dependent exchange of PP_i into ATP (see Papas and Mehler, 1971). In the cases of *E. coli* prolyl-tRNA synthetase (Papas and Mehler, 1971), rat liver threonyl-tRNA synthetase (Allende *et al.*, 1970), and *E. coli* tryptophanyl-tRNA synthetase (Penneys and Muench, 1972), initial velocity studies according to the analytical procedure developed by Cleland (1963a–c) have indicated ordered Ping-Pong mechanisms. In the case of the tyrosyl-tRNA synthetase of *E. coli* B, Santi and Pena (1971) present data supporting a mechanism in which tyrosine and ATP interact with the enzyme by a rapid-equilibrium random mechanism.

The aminoacyl-tRNA synthetases for arginine (Mehler and Mitra, 1967), glutamine and glutamic acid (Ravel *et al.*, 1965) present difficulties in accommodating to the general scheme outlined above. In the case of the arginyl-tRNA synthetase (Mehler and Mitra, 1967), ATP formation from arginyl adenylate does not occur in the absence of added $tRNA^{Arg}$. Further, in the case of all three enzymes (Mehler and Mitra, 1967; Ravel *et al.*, 1965), PP_i exchange into ATP does not take place unless tRNA is present. Lastly, the kinetic studies presented here give no support for a Ping-Pong mechanism.

Several models have been suggested to resolve the complication introduced by the tRNA requirement for the "partial" reactions for some aminoacyl-tRNA synthetases. Loftfield (1972) has recently presented an extensive summary of data in conflict with the enzyme-bound adenylate mechanism. He suggests that all aminoacyl-tRNA synthetases normally operate by a concerted reaction mechanism in which all three reactants interact directly with the enzyme to form amino-

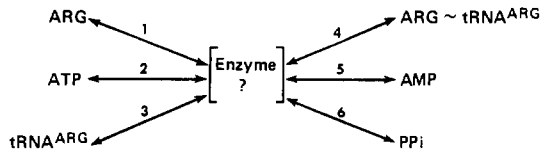


FIGURE 7: A random substrate binding mechanism for *E. coli* arginyl-tRNA synthetase. The numbers 1–6 refer to the rates for association–dissociation of the substrates arginine, ATP, $tRNA^{Arg}$, arginyl-$tRNA^{Arg}$, AMP, and PP_i, respectively.

acyl-tRNA. To various degrees, some of these enzymes may also carry out a related reaction, the formation of enzyme-bound aminoacyl adenylates, with possibilities for PP_i exchange into ATP in the absence of tRNA. Loftfield argues that the adenylate is not an obligatory intermediate in the reaction.

The hypothesis that aminoacyl adenylates are not involved in the aminoacylation reaction has recently received further support (Igarashi *et al.*, 1971; Pastuszyn and Loftfield, 1972). The Mg^{2+} requirement for the aminoacylation reaction can be replaced by spermine. Under these conditions, no $PP_i \leftrightarrow$ ATP exchange can be detected.

Mehler and Mitra (1967) suggested a model for the arginyl-tRNA synthetase in which $tRNA^{Arg}$ serves as an activator of the enzyme. They proposed that enzyme-bound arginyl adenylate was an intermediate, but that the initial activation step required $tRNA^{Arg}$. As discussed above, this mechanism appears unlikely.

The work of Rochovansky and Ratner (1967) suggests another possible explanation of the tRNA requirement for the PP_i-exchange reaction. Their study of argininosuccinate synthetase, a reaction formally analagous to aminoacyl-tRNA synthetases, showed that both citrulline and aspartate were required for the exchange of PP_i into ATP. Using stoichiometric levels of enzyme, it was possible to show the formation of tightly bound citrulline adenylate. The powerful inhibition by pyrophosphate led them to suggest a mechanism in which aspartate promotes the release of tightly bound PP_i from the enzyme. The stimulation of the PP_i–ATP exchange by α-methyl aspartate, which inhibits the overall reaction, provides support for this model. Thus, in the case of arginyl-tRNA synthetase, it might be postulated that tRNA is required for release of firmly bound PP_i. In this connection, Mitra and Mehler (1967) have found PP_i to be a potent inhibitor of the aminoacylation reaction.

On the basis of the available information, the mechanism of arginyl-tRNA synthetase is best represented as shown in Figure 7. The scheme shows a random sequential mechanism in which the three reactants and three products interact with enzyme at discrete rates. The differences in the various exchange rates at equilibrium indicate that the interconversion of enzyme-bound substrates is not the only rate-determining step. As shown in the figure, the nature of such enzyme-bound interconversions is not yet clear. A resolution of whether the mechanism involves an arginyl adenylate or is "concerted" must await further studies.

Acknowledgments

An immeasurable contribution to these studies was derived from discussions with a number of our colleagues. These include Drs. John Westley, Alan Mehler, William Jakoby, and John Folk. The assembly of the manuscript was patiently conducted by Mrs. Helmi Carpenter.

References

Allende, C. C., Chaimovich, M., Gatica, M. and Allende, J. E. (1970), *J. Biol. Chem. 245*, 93.
Boyer, P. D. (1959), *Arch. Biochem. Biophys. 82*, 387.
Cashel, M., Lazzarini, R. A., and Kalbacher, B. (1969), *J. Chromatogr. 40*, 103.
Castelfranco, P., Moldave, K., and Meister, A. (1958), *J. Amer. Chem. Soc. 80*, 2335.
Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67*, 104.
Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67*, 173.
Cleland, W. W. (1963c), *Biochim. Biophys. Acta 67*, 188.
DeMoss, J. A., Genuth, S. M., and Novelli, G. D. (1956), *Proc. Nat. Acad. Sci. U. S. 42*, 325.
Gillam, J., Blew, D., Warrington, R. C., Von Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7*, 3459.
Hurwitz, J., and Furth, J. J. (1966), *Procedures Nucl. Acid Res. 1*, 347.
Igarashi, K., Matsuzaki, K., and Takeda, Y. (1971), *Biochim. Biophys. Acta 254*, 91.
Joyce, B. K., and Himes, R. H. (1966a), *J. Biol. Chem. 241*, 5725.
Joyce, B. K., and Himes, R. H. (1966b), *J. Biol. Chem. 241*, 5716.
Karasek, M., Castelfranco, P., Krishnaswamy, P. R., and Meister, A. (1958), *J. Amer. Chem. Soc. 80*, 2335.
Kingdon, H. S., Webster, L. T., and Davie, E. W. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 757.
Loftfield, R. B. (1972), *Progr. Nucl. Acid Res. 12*, 87.
Mehler, A. H., and Mitra, S. K. (1967), *J. Biol. Chem. 242*, 5495.
Mitra, S. K., and Mehler, A. H. (1966), *J. Biol. Chem. 241*, 5161.
Mitra, S. K., and Mehler, A. H. (1967), *J. Biol. Chem. 242*, 5490.
Papas, T. S., and Mehler, A. M. (1971), *J. Biol. Chem. 246*, 5924.
Papas, T. S., and Peterkofsky, A. (1972), *Fed. Proc., Fed. Amer. Exp. Biol. 31*, 450.
Pastuszyn, A., and Loftfield, R. B. (1972), *Biochem. Biophys. Res. Commun. 47*, 775.
Penneys, N. S., and Muench, K. H. (1972), *Fed. Proc., Fed. Amer. Exp. Biol. 31*, 450.
Ravel, J. M., Wang, S. F., Heinemeyer, C., and Shive, W. (1965), *J. Biol. Chem. 240*, 432.
Rochovansky, O., and Ratner, S. (1967), *J. Biol. Chem. 242*, 3839.
Santi, D. V., and Pena, V. A. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 157.
Wedler, F. C., and Boyer, P. D. (1972), *J. Biol. Chem. 247*, 984.