Effects of Inorganic Pyrophosphate on *Neurospora crassa* Phenylalanine Transfer Ribonucleic Acid Ligase in Heterologous Aminoacylation Reactions*

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ABSTRACT: The cytoplasmic phenylalanine transfer ribonucleic acid ligase of *Neurospora crassa* is unique in that it can aminoacylate phenylalanine to the valine and alanine transfer ribonucleic acids of *Escherichia coli*. However, aminoacylation of these heterologous transfer ribonucleic acids is incomplete. Purified enzyme and purified transfer ribonucleic acids were used in the study of these heterologous reactions. The amount of phenylalanyl transfer ribonucleic acid$^{Val\ or\ Ala}$ (*E. coli*) formed was found to be a linear function of enzyme concentration; this was not due to irreversible enzyme inactivation. This relationship was not exhibited with homologous transfer ribonucleic acid (*N. crassa*) even at low levels of enzyme. Inorganic pyrophosphate at 10^{-8}–10^{-7} M was found to be a potent inhibitor of both the rate and extent of phenylalanyl transfer ribonucleic acid$^{Val\ or\ Ala}$ (*E. coli*) formation in the heterologous reactions. At low synthetase concentrations the presence of inorganic pyrophosphatase resulted in a doubling of the rate and extent of phenylalanyl transfer ribonucleic acid$^{Val\ or\ Ala}$ (*E. coli*) formation. Inorganic pyrophosphate at low concentrations did not have an effect on the extent of aminoacylation of transfer ribonucleic acid (*N. crassa*), and inorganic pyrophosphatase stimulated only slightly the rate of aminoacyl transfer ribonucleic acid production. The linear response to enzyme concentration and the incomplete aminoacylation observed in the heterologous reactions occurred in Tris-Cl buffer. In agreement with our previous findings, complete aminoacylation of transfer ribonucleic acid^{Val} (*E. coli*) was observed in potassium cacodylate buffered systems. In potassium cacodylate buffer the linearity with enzyme was not seen at comparable enzyme concentrations; inorganic pyrophosphate at 10^{-8}–10^{-7} M inhibited the rate but not the extent of phenylalanyl transfer ribonucleic acid^{Val} (*E. coli*) production, and inorganic pyrophosphatase stimulated the rate of phenylalanyl transfer ribonucleic acid^{Val} (*E. coli*) formation. The stimulation of the rate of the reaction by inorganic pyrophosphatase indicates inhibition by endogenously formed inorganic pyrophosphate.

In Tris-Cl buffer, inorganic pyrophosphate acts as a competitive inhibitor with respect to both of the heterologous transfer ribonucleic acids. The K_i values for the pyrophosphate are slightly smaller than the K_m's for the transfer ribonucleic acids. The presence of inorganic pyrophosphatase lowers both K_m's and K_i's and indicates that the inhibition by endogenous inorganic pyrophosphate is also competitive. In contrast to the results obtained in Tris-Cl buffer, inorganic pyrophosphate acts as a noncompetitive inhibitor with respect to transfer ribonucleic acid^{Val} (*E. coli*) in potassium cacodylate buffer. Apparent K_i and V_{max} values in the two buffers are comparable, but the K_m to transfer ribonucleic acid^{Val} (*E. coli*) in potassium cacodylate is 100-fold smaller than that in Tris-Cl.

These data show that some aspects of this heterologous aminoacylation reaction observed in Tris-Cl buffer are different in potassium cacodylate buffer. An important role for inorganic pyrophosphate was demonstrated in these heterologous reactions. This inhibitor should also be considered in studying other heterologous systems, especially those carried out in Tris-Cl buffer.

Several investigators have studied the formation of aminoacyl-tRNA catalyzed by heterologous aminoacyl-tRNA synthetases (for a review, see Novelli, 1967). Often the amount of aminoacyl-tRNA produced by the heterologous systems was found to be less than that formed in homologous reactions. In some cases this has been attributed to the preferential aminoacylation of one or more isoaccepting forms of tRNA; whereas, in others, all known isoaccepting forms are aminoacylated but not to the same extent as in homologous systems. The species of tRNA aminoacylated was generally assumed to be the same species of tRNA aminoacylated in the homologous systems, although this was not established in most instances.

We have studied a similar, though not strictly analogous, heterologous tRNA–tRNA synthetase system first recognized by Barnett and Jacobson (1964). The main difference between our system and others is that phenylalanine is aminoacylated to *Escherichia coli* tRNAs other than tRNA^{Phe} (*E. coli*) by the cytoplasmic phenylalanine tRNA ligase of *Neurospora crassa* (Syn^{Phe} (*N. crassa*)).[1] This enzyme has been shown to catlayze the aminoacylation of tRNA^{Val} (*E. coli*) and tRNA^{Ala} (*E. coli*), as well as its homologous cytoplasmic tRNA^{Phe} (*N. crassa*), with phenylalanine (Barnett and Jacobson, 1964; Barnett, 1965; Barnett and Epler, 1966a,b; Barnett and Brown, 1966; Barnett *et al.*, 1967).

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[1] Syn^{Phe} (*N. crassa*) = phenylalanyl-tRNA synthetase (L-phenylalanine tRNA ligase (AMP) (EC 6.1.1.4)) of *N. crassa*.

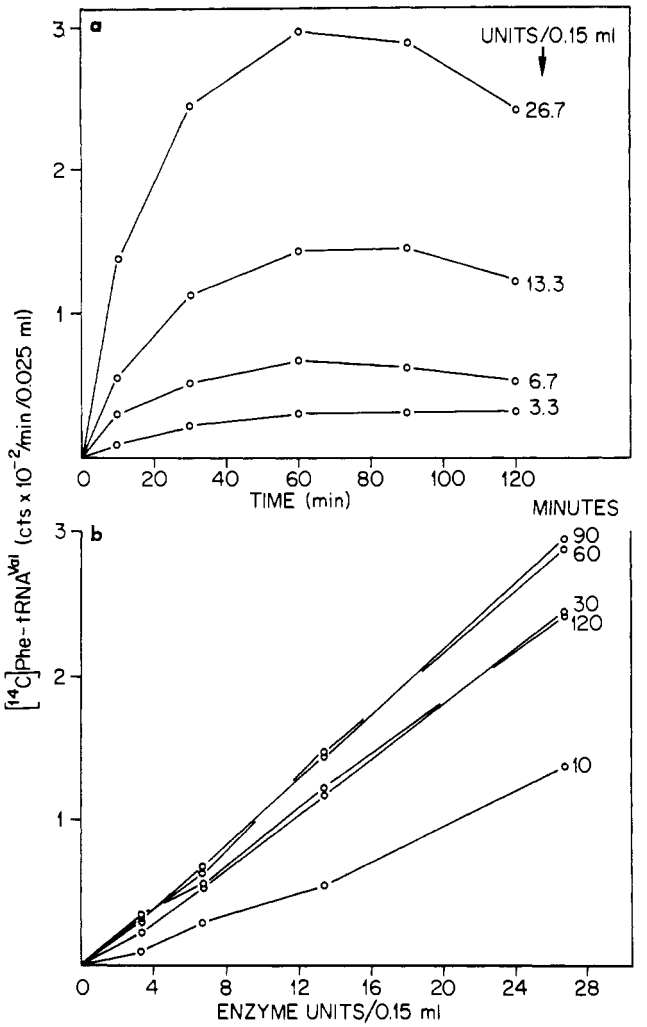

FIGURE 1: Syn^{Phe} concentration studies. (a) Effect of Syn^{Phe} (*N. crassa*) concentration on Phe-$tRNA^{Val}$ (*E. coli*) formation. Incubation mixtures in a final volume of 0.15 ml were 50 mM in Tris-Cl (pH 8.0), 15 mM in magnesium acetate, 10 mM in 2-mercaptoethanol, and 0.5 mM in ATP. They also contained 0.15 μCi of L-[^{14}C]phenylalanine (0.41 mμmole), 0.057 A_{260} unit of $tRNA^{Val}$ (*E. coli*), and the enzyme units shown. Enzyme dilutions were made in a buffer containing 50 μmoles of Tris-Cl (pH 8.0), 10 μmoles of 2-mercaptoethanol, and 0.5 mg of bovine serum albumin per ml. Enzyme was added to start the reaction, 0.025-ml portions were removed at the times indicated, and Phe-$tRNA^{Val}$ (*E. coli*) was determined. (b) The amount of Phe-$tRNA^{Val}$ (*E. coli*) formed as a function of Syn^{Phe} (*N. crassa*) concentration. Data were obtained from part a.

Holten and Jacobson (1969) demonstrated that with partially purified $tRNA^{Val}$ (*E. coli*) and $tRNA^{Ala}$ (*E. coli*) and crude Syn^{Phe} (*N. crassa*), the extent of aminoacylation was less than that obtained using Syn^{Val} (*E. coli*) and Syn^{Ala} (*E. coli*) and the appropriate amino acids. All separable isoaccepting forms of both tRNAs were aminoacylated, but again the amount of phenylalanine charged was less than half for each form. We have recently confirmed these observations using purified cytoplasmic Syn^{Phe} (*N. crassa*) and purified $tRNA^{Val}$ (*E. coli*) and $tRNA^{Ala}$ (*E. coli*) (Kull and Jacobson, 1969). Complete aminoacylation of a purified mixture of isoaccepting forms of $tRNA^{Val}$ (*E. coli*) by purified Syn^{Phe} (*N. crassa*) is achieved by adding dimethyl sulfoxide to the reaction or by replacing the Tris-Cl buffer with potassium cacodylate buffer (Ritter *et al.*, 1969). Although complete aminoacylation in this heterologous system is now possible, the factors responsible for incomplete aminoacylation in Tris-Cl buffer are not understood. An understanding of these factors may be pertinent to understanding the mechanism of other heterologous systems in which the extent of aminoacylation is not equivalent to that in homologous reactions. Such an understanding could also have a bearing on the mechanism by which a specific tRNA synthetase recognizes and interacts with its homologous tRNA.

We have observed that extremely low concentrations of PP_i have a pronounced inhibitory effect on the reaction of Syn^{Phe} (*N. crassa*) with $tRNA^{Val}$ (*E. coli*) or $tRNA^{Ala}$ (*E. coli*) in Tris-Cl buffer and with $tRNA^{Val}$ (*E. coli*) in potassium cacodylate buffer. Furthermore, the addition of inorganic pyrophosphatase significantly increases the rate of Phe-$tRNA^{Val}$ (*E. coli*) formation in both buffers and increases the extent of formation in Tris-Cl buffer. The type of inhibition, by PP_i with respect to *E. coli* tRNA, is different in the two buffers, but apparent inhibitor constants differ only by a factor of 6. However, Michaelis constants for $tRNA^{Val}$ (*E. coli*) differ by a factor of 100.

A preliminary report of some of this work has been made (Kull and Jacobson, 1968).

Materials and Methods

Enzymes. Crystalline inorganic pyrophosphatase was purchased from Worthington Biochemical Corp. An unfractionated *E. coli* tRNA synthetase preparation was obtained from *E. coli* strain B (Witkin) according to the procedure described by Kelmers *et al.* (1965). Cytoplasmic Syn^{Phe} C (*N. crassa*) purified at least 900-fold (Kull and Jacobson, 1969) was prepared from *N. crassa* wild-type strain OR23-1a, grown, and harvested as described by Barnett and Brown (1967). The preparation was free of mitochondrial Syn^{Phe} (*N. crassa*) as evidenced by its low activity toward mitochondrial $tRNA^{Phe}$ (*N. crassa*) or $tRNA^{Phe}$ (*E. coli*) and was free of ATP–tRNA adenylyltransferase activity. A unit of activity is defined as the amount of enzyme required to incorporate 1 $\mu\mu$mole of L-[^{14}C]phenylalanine/min at 20° into cold trichloroacetic acid insoluble form with *N. crassa* tRNA under assay conditions described in Figure 1. Units of Syn^{Phe} (*N. crassa*) activity were calculated from initial rates estimated by extrapolation of velocity *vs.* time increment curves to zero time. Plots of initial velocities, obtained in this manner, *vs.* enzyme concentration showed a strictly linear relationship under conditions of excess substrates when in the presence of 160–170 μg of bovine serum albumin/ml of incubation mixture.

tRNA. N. crassa tRNA was prepared according to Barnett (1965). Mitochondrial tRNA of *N. crassa* was a generous gift of Dr. W. E. Barnett. $tRNA^{Val}$ (*E. coli*) and $tRNA^{Ala}$ (*E. coli*) were free of $tRNA^{Phe}$ (*E. coli*). These and $tRNA^{Phe}$ (*E. coli*), free of $tRNA^{Val, Ala}$ (*E. coli*), were prepared by reversed-phase and hydroxylapatite chromatography as described by Pearson and Kelmers (1966), Weiss and Kelmers (1967), and Weiss *et al.* (1968), and were generous gifts of these workers. The $tRNA^{Val}$ (*E. coli*) was calculated to be 60% pure and the $tRNA^{Ala}$ (*E. coli*), 37% pure. Purity was based on acceptance of L-[^{14}C]valine and L-[^{14}C]alanine using the *E.*

TABLE I: Stability of Syn^{Phe} (*N. crassa*) at 20°.

Preincubation Time (min)	Enzyme Activity[a] Phe-$tRNA^{Phe}$ (*N. crassa*) ($\mu\mu$moles)
0	3.10
30	3.04
60	2.98
120	3.24

[a] Phe-$tRNA^{Phe}$ (*N. crassa*) ($\mu\mu$moles) formed in 2 min/0.15 ml. Syn^{Phe} (*N. crassa*) was diluted, as described in the legend to Figure 1, to 500 units/ml. Portions (0.05 ml) were then assayed for enzyme activity under the conditions as also described in Figure 1 except that 0.25 A_{260} unit of *N. crassa* tRNA replaced the *E. coli* tRNA. Tubes were incubated at 20° for 2 min, 0.1 ml was removed and pipetted onto filter paper disks, and Phe-$tRNA^{Phe}$ (*N. crassa*) was determined as described in the Methods section.

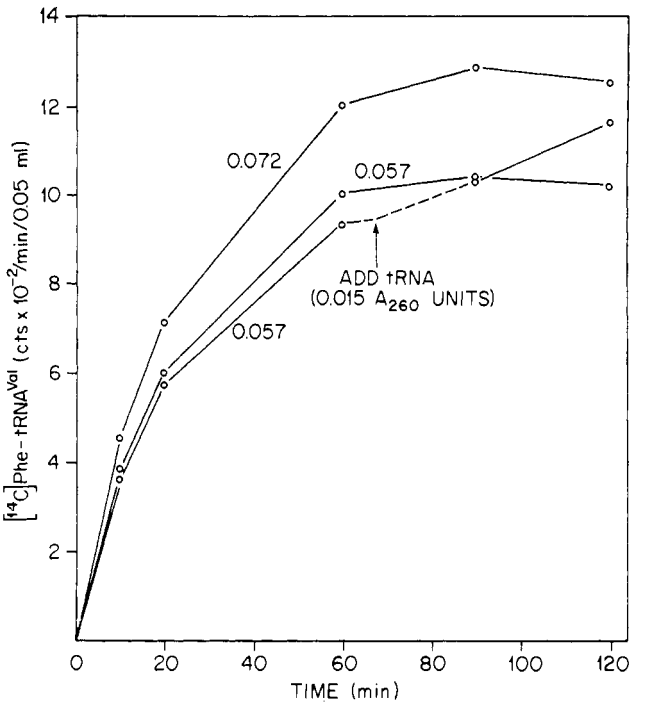

FIGURE 2: Effect of $tRNA^{Val}$ (*E. coli*) on Phe-$tRNA^{Val}$ (*E. coli*) formation by Syn^{Phe} (*N. crassa*). Assays were carried out under the conditions described in the legend to Figure 1 except that incubations in a volume of 0.3 ml contained the A_{260} units of $tRNA^{Val}$ (*E. coli*) indicated, 0.3 μCi of L-[^{14}C]phenylalanine (0.82 mμmole), and 30 units of enzyme activity. At the time indicated 0.015 A_{260} unit of $tRNA^{Val}$ (*E. coli*) was added in 10 μl. Phe-$tRNA^{Val}$ (*E. coli*) formation was determined on 0.05-ml portions.

coli aminoacyl-tRNA synthetase system. Under optimal conditions these tRNAs accepted 1200 $\mu\mu$moles of L-[^{14}C]valine and 740 $\mu\mu$moles L-[^{14}C]alanine, respectively, per A_{260} unit. An A_{260} unit is defined as the amount of tRNA which, if diluted to 1.0 ml in 0.01 M Tris-Cl (pH 7.5) and 0.01 M magnesium acetate, would have an absorbance of 1.0 optical density unit in a 1-cm light path at 260 mμ. By these criteria one A_{260} unit is equivalent to 50 μg of tRNA or 2000 $\mu\mu$moles. This assumes mol wt 25,000 and ϵ 5×10^5.

Assays. Incubation mixtures are described in the tables and figure legends. [^{14}C]Aminoacyl-tRNA was assayed by the filter paper disk method of Bollum (1959).

Radioactive Isotopes and Reagents. L-[^{14}C]Phenylalanine (specific activity 366 μCi/μmole), L-[^{14}C]alanine (specific activity 117 μCi/μmole), and L-[^{14}C]valine (specific activity 208.5 μCi/μmole) were purchased from New England Nuclear Corp., and L-[^{14}C]phenylalanine (specific activity 255 μCi/μmole) from Schwartz Biochemicals. The high specific activity L-[^{14}C]phenylalanine, supplied in 1 N HCl, was neutralized with NaOH prior to use. All isotopes were diluted to 50 μCi/ml. PP_i (reagent grade) ($Na_4P_2O_7 \cdot 10H_2O$) was used as purchased from Fisher Scientific Co. All other reagents were obtained commercially and used without further purification.

Results

Phe-tRNA Formation as a Function of Syn^{Phe} Concentration. A linear relationship between enzyme concentrations and initial reaction rates is usually observed, but it is unusual to observe such a relationship between enzyme concentration and the total amount of product formed. The proportionality between Syn^{Phe} (*N. crassa*) concentration and the extent of Phe-$tRNA^{Val}$ (*E. coli*) formation at various times in Tris-Cl buffer is shown in Figure 1a. The amount of Phe-$tRNA^{Val}$ (*E. coli*) formed is proportional to enzyme concentration at any time over a 2-hr time period (Figure 1b). The enzyme remained active after maximal Phe-$tRNA^{Val}$ (*E. coli*) had been formed, since addition of $tRNA^{Val}$ (*E. coli*) at 60 min results in additional Phe-$tRNA^{Val}$ (*E. coli*) formation (Figure 2).

Further evidence that the proportionality was not a result of enzyme inactivation was obtained by preincubating the enzyme in buffer at 20° and then assaying portions at various times during this 2-hr incubation (Table I). Other experiments showed that the enzyme is stable in the presence of $tRNA^{Val}$ (*E. coli*) in the absence of other substrates. The same experiments showed that the tRNA was not degraded by the enzyme. When the enzyme was preincubated in the presence of magnesium acetate, ATP, and L-[^{14}C]phenylalanine at 20° for 2 hr and $tRNA^{Val}$ (*E. coli*) added to initiate reaction, the amount of Phe-$tRNA^{Val}$ (*E. coli*) formed after 2 additional hr was at least 75% of that formed by the control (no preincubation).

When potassium cacodylate replaced Tris-Cl buffer, additional experiments showed that the amount of Phe-$tRNA^{Val}$ (*E. coli*) formed was not proportional to the concentration of Syn^{Phe} (*N. crassa*). The enzyme concentration did not affect the total amount of Phe-$tRNA^{Phe}$ (*N. crassa*) formed. Complete aminoacylation of either $tRNA^{Val}$ (*E. coli*) or $tRNA^{Phe}$ (*N. crassa*), with phenylalanine, occurred in less than 2 hr at Syn^{Phe} (*N. crassa*) concentrations at and above 6 units/0.15 ml.

Effect of ATP, AMP, and PP_i on Phe-tRNA Formation. Various explanations could account for the dependence of tRNA aminoacylation upon enzyme concentration seen with the heterologous system in Tris-Cl buffer. One possibility

TABLE II: Effect of ATP Concentration on Extent of Phe-tRNA^{Val} (*E. coli*) Formation by Syn^{Phe} (*N. crassa*).[a]

Concentration of ATP (mM)	0.17	0.33	0.50	0.67	0.83	1.00	1.67
Mg^{2+}/ATP	90	46.5	30	22.4	18	15	9
Phe-tRNA^{Val} (*E. coli*)[b] ($\mu\mu$moles)	2.84	3.33	3.29	3.55	3.36	3.20	2.80

[a] In addition to ATP, incubation mixtures were 50 mM in Tris-Cl (pH 8.0), 10 mM in 2-mercaptoethanol, and 15 mM magnesium acetate. They also contained 0.15 μCi of L-[^{14}C]phenylalanine (0.41 mμmole), 0.021 A_{260} unit of tRNA^{Val} (*E. coli*), and 15 units of Syn^{Phe}(*N. crassa*) in a final volume of 0.15 ml. Portions were removed at various times and assayed. [b] The amounts of Phe-tRNA^{Val} (*E. coli*) shown were calculated per 0.15 ml of incubation mixture after net formation had ceased (plateau values were reached in 60–120 min).

examined was that water was competing with tRNA^{Val} (*E. coli*) for the transfer of the activated amino acid of the assumed Syn–AMP–Phe complex, in effect converting the heterologous system into an ATPase and thereby making ATP limiting. However, varying the ATP concentration over a 10-fold range had no appreciable effect on the extent of Phe-tRNA^{Val} (*E. coli*) formation (Table II). Furthermore, addition of 75 mμmoles of ATP at 95 min had no effect during the following 90-min incubation.

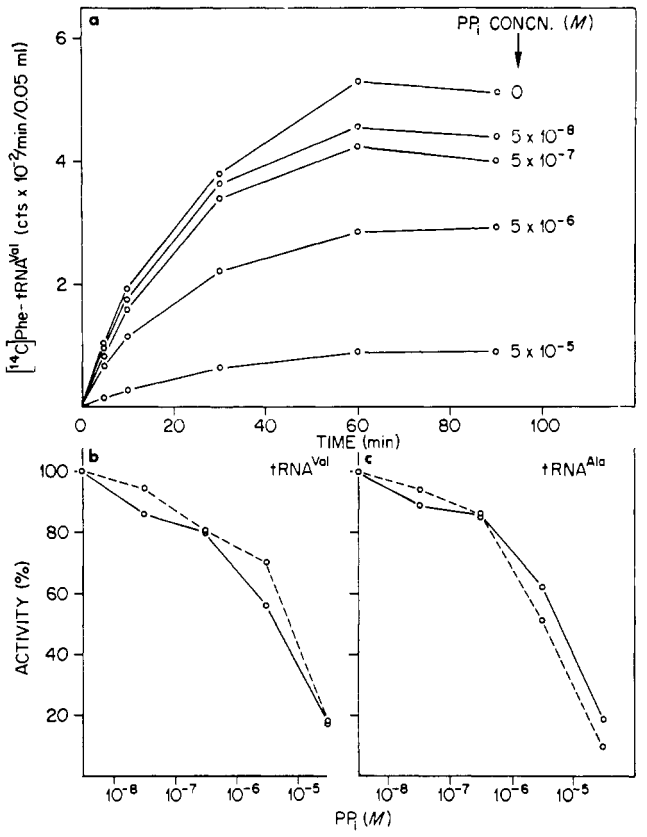

FIGURE 3: Effect of PP$_i$ on the formation of Phe-tRNA^{Val,Ala} (*E. coli*) in Tris-Cl. (a) Assays were as described in Figure 1 except that incubations in a volume of 0.3 ml contained 0.3 μCi of L-[^{14}C]phenylalanine (0.82 mμmole), the final concentrations of inorganic pyrophosphate shown, 30 units of enzyme, and 0.04 A_{260} unit of tRNA^{Val} (*E. coli*). (b and c) Rate (– – –) and extent (———) of Phe-tRNA^{Val,Ala} (*E. coli*) formation. (b) tRNA^{Val} (*E. coli*) assays were as described for part a. (c) tRNA^{Ala} (*E. coli*) assays were as described above except that they contained 0.06 A_{260} unit of tRNA^{Ala} (*E. coli*).

To test the possibility of product inhibition, PP$_i$ and 5′-AMP concentrations were varied over five orders of magnitude (5×10^{-9} to 5×10^{-4} M) and tested for their effect on the extent and rate of Phe-tRNA^{Val} (*E. coli*) formation in Tris-Cl buffer. This range of concentrations includes on the lower side the concentrations of 5′-AMP and PP$_i$ that would be present if produced in amounts equivalent to the Phe-tRNA^{Val} (*E. coli*) formed (about 2×10^{-8} M) and on the higher side the concentration of ATP originally present. The maximal inhibition seen with 5′-AMP (29%) occurred at 5×10^{-4} M; initial velocities at this concentration were inhibited 25%. At this concentration PP$_i$ inhibited both the rate and extent of Phe-tRNA^{Val} (*E. coli*) formation from 96 to 100%. Inhibition of the rate and extent at 5×10^{-7} M was 20 and 22%, respectively, and some inhibition of Phe-tRNA^{Val} (*E. coli*) formation appeared to exist at concentrations as low as 5×10^{-9} M.

The striking effect of PP$_i$ on the heterologous reaction was substantiated with both alanine and valine *E. coli* tRNAs (Figure 3). When the same concentrations of PP$_i$ and enzyme were used with *N. crassa* tRNA, no effect on either the rate or extent of Phe-tRNA formation was seen until the concentration of PP$_i$ was at 5×10^{-6} M. At this concentration the rate was inhibited only 4%, and the amount of the Phe-tRNA^{Phe} (*N. crassa*) formed was not affected. At 5×10^{-5} M the rate was inhibited 12% and the extent 6%. The effect of PP$_i$ on the heterologous reaction was also examined in potassium cacodylate buffer (Figure 4). Although the rate of aminoacylation in potassium cacodylate is appreciably affected at 5×10^{-8} M PP$_i$, the extent of aminoacylation is not. The amount of Phe-tRNA^{Val} (*E. coli*) formed in 2 hr at 5×10^{-7} M is virtually the same as the controls, and the maximum amount capable of being produced may not have been reached.

When PP$_i$ was added to a final concentration of 5×10^{-8} M 15 min after initiation of reaction, in Tris-Cl buffer, there was an immediate decrease in the rate of Phe-tRNA^{Val} (*E. coli*) production, and the maximum amount formed was much lower than that attained when the same concentration of the inhibitor was present from the time of initiation (Figure 5).

Effect of Inorganic Pyrophosphatase on the Formation of the Phe-tRNA. To determine if PP$_i$ was acting as an endogenous inhibitor of the heterologous reaction, experiments were carried out in the presence of inorganic pyrophosphatase. Figure 6 shows the stimulation of the rate and extent of Phe-tRNA^{Val} (*E. coli*) formation by inorganic pyrophos-

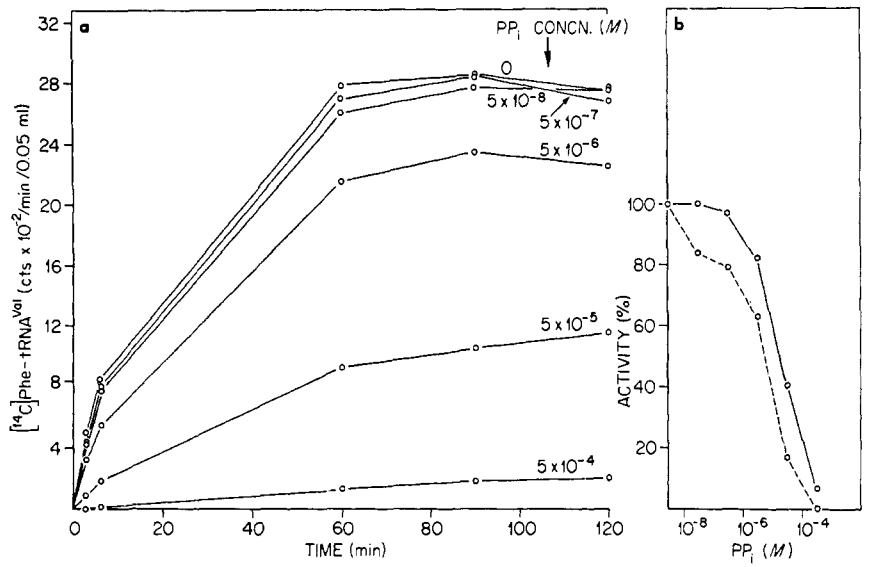

FIGURE 4: (a) Effect of PP_i on the formation of Phe-$tRNA^{Val}$ (*E. coli*) in potassium cacodylate buffer. Incubation mixtures in a final volume of 0.30 ml were 50 mM potassium cacodylate (pH 6.3), 5 mM magnesium acetate, 0.5 mM ATP, contained 0.3 μCi of L-[^{14}C]phenylalanine (1.18 mμmoles), 0.052 A_{260} unit of $tRNA^{Val}$ (*E. coli*), and the final concentrations of PP_i shown. The incubations also contained five units of enzyme previously diluted with a solution 10 mM in potassium cacodylate (pH 8.0) and containing 0.5 mg of bovine serum albumin/ml. Portions (0.05 ml) were removed at the various times for determination of Phe-$tRNA^{Val}$ (*E. coli*). (b) Per cent of control values of rate (— — —) and extent (——) of Phe-$tRNA^{Val}$ (*E. coli*) formation.

TABLE III: Kinetic Parameters for *E. coli* tRNAs using Syn^{Phe} (*N. crassa*).

	$tRNA^{Ala}$ (*E. coli*)	$tRNA^{Val}$ (*E. coli*)
Tris-Cl System		
V_{max}[a]	0.74	0.78
K_m (M)	2.9×10^{-6}	5.2×10^{-6}
K_m (in presence of inorganic pyrophosphatase) (M)	1.7×10^{-6}	3.5×10^{-6}
K_i (for PP_i) (M)	3.9×10^{-6}	3.8×10^{-6}
K_i (for PP_i calculated using K_m determined in presence of inorganic pyrophosphatase) (M)	1.7×10^{-6}	2.0×10^{-6}
Potassium Cacodylate System		
V_{max}		0.90
K_m (M)		3.5×10^{-8}
V_{max} (apparent, in presence of PP_i)		0.63
K_i (for PP_i) (M)		21.9×10^{-6}

[a] V_{max} = mμmoles/min per unit of enzyme. Details are given in Figures 7 and 8. Molar concentrations of $tRNA^{Val}$ (*E. coli*) and $tRNA^{Ala}$ (*E. coli*) were calculated assuming 60 and 37% purity, respectively, as described in Methods and Materials.

phatase in Tris-Cl buffer. Addition of inorganic pyrophosphatase after initiation of the reaction by Syn^{Phe} (*N. crassa*) showed an increase in aminoacylation but at a rate less than the control. In other experiments the maximum amount of Phe-$tRNA^{Val}$ (*E. coli*) formed was found to be less under these conditions than when inorganic pyrophosphatase was present from the time of initiation. A 10-fold increase in inorganic pyrophosphatase had no additional effect on either the rate or extent of aminoacylation. When 5×10^{-6} M PP_i and inorganic pyrophosphatase were added prior to initiation of reaction, no inhibition occurred, and the amount of Phe-$tRNA^{Val}$ (*E. coli*) formed was equal to that formed in the presence of inorganic pyrophosphatase alone.

In the presence of inorganic pyrophosphatase the highest level of Syn^{Phe} (*N. crassa*) used was able to acylate 50% of the $tRNA^{Val}$ (*E. coli*). This represents a doubling of the amount of Phe-$tRNA^{Val}$ (*E. coli*) formed in the absence of pyrophosphatase by the same amount of synthetase but is still short of complete aminoacylation. Inorganic pyrophosphatase had no effect on the extent of Phe-$tRNA^{Phe}$ (*N. crassa*) formed by Syn^{Phe} (*N. crassa*) but did stimulate its rate of formation (about 8%). In potassium cacodylate buffer the final amount of Phe-$tRNA^{Val}$ (*E. coli*) formed was not affected by the presence of inorganic pyrophosphatase since it was already maximal, but the rate of its formation was increased about 14%. This stimulation by inorganic pyrophosphatase suggests that

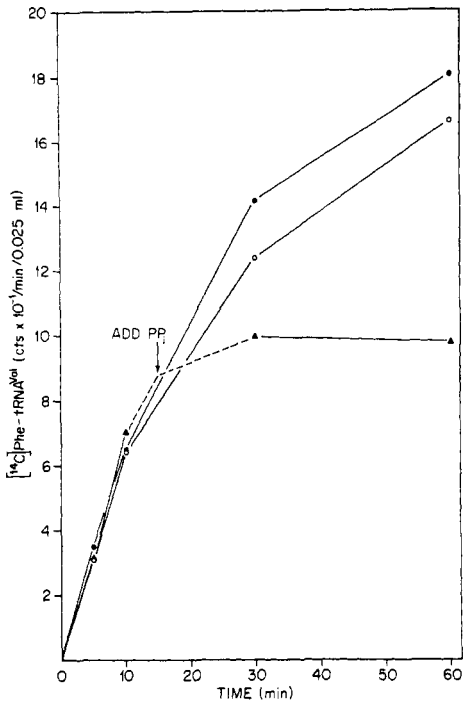

FIGURE 5: Effect of inorganic pyrophosphate when added before and after initiation of reaction. Assays were as described in Figure 1 except that incubation mixtures contained 15 units of enzyme and 0.02 A_{260} unit of tRNA^{Val} (*E. coli*). The control experiment had no additions (●). PP_i was present (5×10^{-8} M) either from the time of initiation (○) or was added at 15 min (▲) to 5×10^{-8} M. Portions (0.025 ml) were removed at various times and assayed.

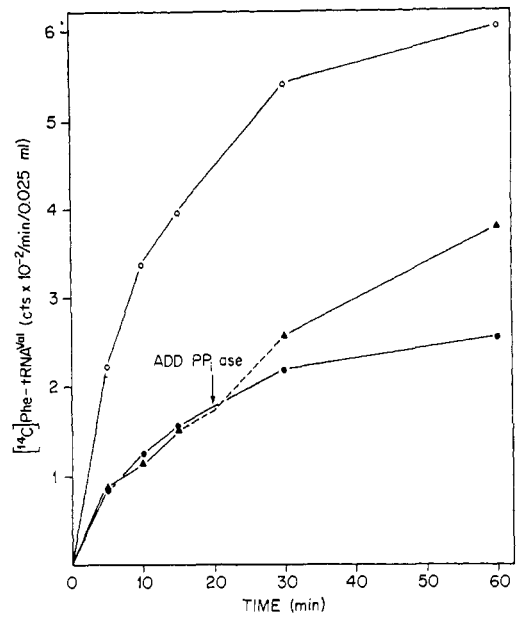

FIGURE 6: Effect of inorganic pyrophosphatase when added before and after initiation of reaction. Assays were as described in Figure 1 except that incubation mixtures contained 15 units of Syn^{Phe} (*N. crassa*) and 0.027 A_{260} unit of tRNA^{Val} (*E. coli*). Duplicate experiments contained no inorganic pyrophosphatase initially (●, ▲). In another experiment one unit or inorganic pyrophosphatase was present prior to initiation of reaction (○). Five units of pyrophosphatase were added to one control at the point indicated (▲). Portions (0.025 ml) were removed and assayed at various times.

PP_i acts as an endogenous inhibitor of Syn^{Phe} (*N. crassa*) when reacting with tRNA^{Val} (*E. coli*) and with tRNA^{Phe} (*N. crassa*) although there is less inhibition in the latter case. The effect is most pronounced when Tris-Cl is employed as buffer but is also demonstrable in potassium cacodylate buffer.

Kinetics of the Heterologous Reaction. The kinetics of the heterologous reaction were studied in greater detail to gain further insight into the effects of PP_i on the aminoacylation reaction.

PP_i, in the Tris-Cl system, showed kinetics consistent with its being competitive inhibitor with respect to either tRNA^{Val} (*E. coli*) or tRNA^{Ala} (*E. coli*) (Figure 7). Determination of Michaelis constants in the presence of inorganic pyrophosphatase substantiated our results indicating that PP_i is produced endogenously in high enough concentration to act as an inhibitor. Because V_{max}'s were found to be the same in the presence of inorganic pyrophosphatase, it is reasonable to conclude that the endogenous PP_i is also acting as a competitive inhibitor.

Table III shows apparent K_m values obtained in the presence and absence of inorganic pyrophosphatase, in the Tris-Cl system, for both species of *E. coli* tRNA. Also listed are the apparent K_i values calculated with each K_m. An estimation of the concentrations of PP_i necessary to give the apparent endogenous inhibition can be derived by use of the K_m obtained in the presence of inorganic pyrophosphatase, and the K_i calculated from this value. Then by the use of these values of K_m, K_i, and V_{max}, the concentration of endogenous inhibitor formed in the reaction to which neither PP_i nor inorganic pyrophosphatase was added can be readily calculated. The concentrations differ only slightly for the two species of *E. coli* tRNA and are approximately 10^{-6} M. Since the actual amount of PP_i produced in this reaction was not measured, the calculated K_i values are incorrect and probably lower than the actual values. Also, it is not known whether or not inorganic pyrophosphatase is able to completely remove all the PP_i produced during the reaction. Complete removal might not occur because inorganic pyrophosphatase is not acting under its optimal conditions, and although the K_m for this enzyme has not been determined, we estimate that it is in the order of 10^{-4} M (from Kunitz, 1952). This value suggests that the affinity of Syn^{Phe} (*N. crassa*) for PP_i is greater than the affinity of inorganic pyrophosphatase for inorganic pyrophosphate.

In contrast to its behavior in the Tris-Cl system as a competitive inhibitor, PP_i acts as a noncompetitive inhibitor in the potassium cacodylate system using tRNA^{Val} (*E. coli*) as the varied substrate (Figure 8). In this buffer the apparent K_m for this tRNA is approximately 100-fold smaller than that found in Tris-Cl buffer (Table III). However, the apparent K_i value for PP_i in potassium cacodylate is only sixfold greater than those obtained in Tris-Cl. The K_i value was calculated by assuming a linear noncompetitive inhibition. Apparent maximum velocities obtained in the two buffers are surprisingly close.

Discussion

A relationship between the amount of aminoacyl-tRNA formed and enzyme concentration seen in Tris-Cl buffer, as

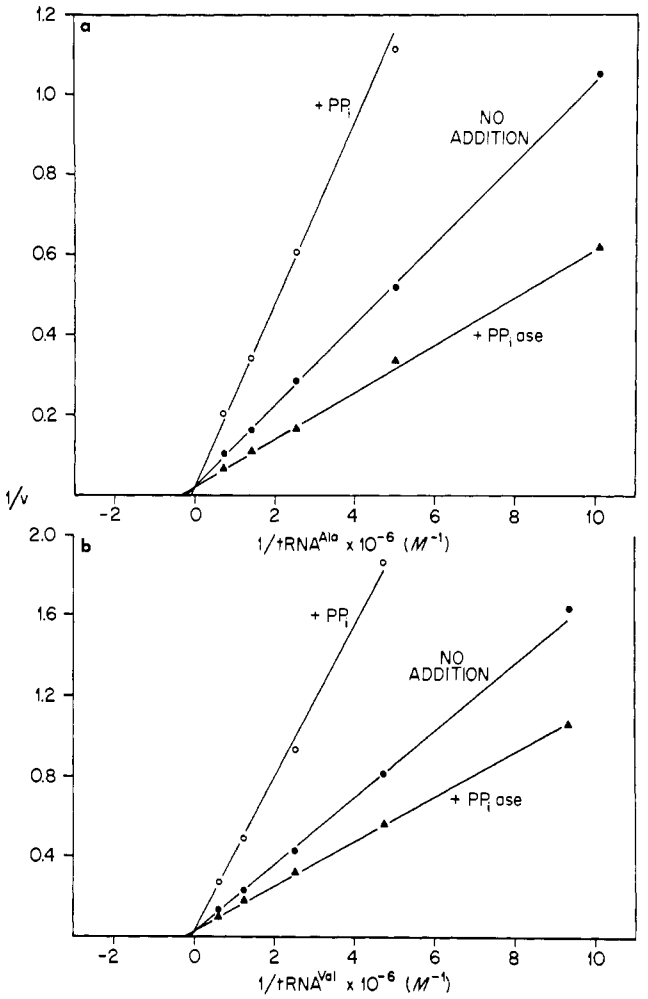

FIGURE 7: Plots of reciprocal velocity *vs.* reciprocal tRNA concentration in Tris-Cl. (a) $tRNA^{Ala}$ (*E. coli*); (b) $tRNA^{Val}$ (*E. coli*). Incubation mixtures were 50 mM in Tris-Cl (pH 8.0), 15 mM in magnesium acetate, 10 mM in 2-mercaptoethanol, 0.5 mM in ATP, and contained 0.3 μCi of L-[^{14}C]phenylalanine (0.82 mμmole). They also contained various concentrations of tRNA, 40 units of Syn^{Phe} (*N. crassa*), and additions as indicated, in a final volume of 0.3 ml. Portions (0.1 ml) were removed at 3 and 6 min, pipetted onto filter paper disks, and assayed. Velocities are expressed as $\mu\mu$moles of Phe-tRNA formed per min per 0.3 ml. (●) No addition; (○) 5×10^{-6} M PP_i; (▲) 2 units of inorganic pyrophosphatase.

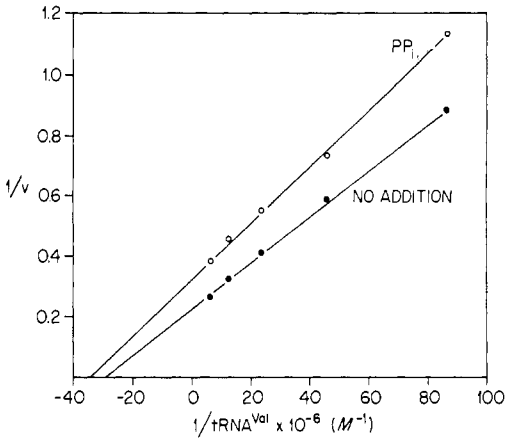

FIGURE 8: Lineweaver–Burk plots of velocity *vs.* $tRNA^{Val}$ (*E. coli*) concentration in potassium cacodylate. Incubation mixtures were as described in Figure 4, except that tRNA was varied as shown and when appropriate they contained PP_i. Portions (0.1 ml) were removed at 3 and 6 min for assay. (●) No addition; (○) 5×10^{-6} M, final concentration of PP_i.

reported here, was also observed by Svensson (1968) in the heterologous reaction between Syn^{Met} (yeast) and $tRNA^{Met}$ (*E. coli*). Previously Zillig *et al.* (1960) reported such a relationship which Berg *et al.* (1961) suggested could simply have been a result of enzyme inactivation; these workers had observed that inclusion of bovine serum albumin in incubation mixtures had eliminated a similar proportionality in their experiments. Svensson was aware of this possibility and showed that his enzyme was still active at the time net aminoacylation had ceased.

Various workers (Svensson, 1968; Rubin *et al.*, 1967; Peterkofsky *et al.*, 1966; Loftfield and Eigner, 1967) have investigated and discussed the effects of various salts, buffers, and ionic strengths on homologous and heterologous tRNA synthetase reactions. Peterkofsky *et al.* have reported that *E. coli* tRNA is incompletely aminoacylated by Syn^{Leu} (yeast) in the presence of high concentrations of Tris-Cl (above 0.05 M); yet Svensson, who discusses this observation, carried out his studies in 0.10 M Tris-Cl. Ritter *et al.* (1969) have shown that complete aminoacylation is achieved (in the same heterologous system examined in this report) when Tris-Cl buffer is replaced by potassium cacodylate buffer; they have also recognized the inhibitory effect of Tris-Cl and other salts on this heterologous system. It therefore appears likely that Tris-Cl has a pronounced effect on aminoacyl-tRNA formation in most heterologous reactions and may also have an effect on homologous reactions. Rubin *et al.* (1967) have seen decreased yields of aminoacyl-tRNA, in the presence of Tris-Cl buffer, in crude homologous systems. In addition to the inhibitory effects of salts and Tris-Cl, we have demonstrated in this report that inorganic pyrophosphate at low levels is a potent inhibitor of the heterologous reaction.

Most studies on the effects of PP_i on tRNA synthetases appear to have been carried out in the absence of tRNA (Davie *et al.*, 1956; Bublitz, 1966). As discussed by Stulberg and Novelli (1962), its effect, whenever measured in purified systems, has been inhibitory, as measured by hydroxamate formation. Bublitz found a greater inhibition of hydroxomate formation by AMP than by PP_i; Davie *et al.* found no inhibition by AMP. When PP_i inhibition was studied in the presence of tRNA, homologous enzymes were used. Allende *et al.* (1966) showed that 10^{-4} M PP_i inhibited the transfer of threonine from a threonyladenylate–Syn^{Thr} (rat liver) complex to tRNA (rat liver). The formation of Arg-$tRNA^{Arg}$ (*E. coli*) is also inhibited by PP_i, as reported by Mitra and Mehler (1967). These workers state that it acts as a noncompetitive inhibitor with respect to ATP, and report that at 4×10^{-4} M PP_i inhibition of Arg-$tRNA^{Arg}$ (*E. coli*) formation is 95%. They do not present a K_i. Loftfield and Eigner (1969, personal communication) have shown that PP_i inhibits the formation of Val-$tRNA^{Val}$ (*E. coli*) by Syn^{Val} (*E. coli*). The K_i is 10^{-4} to 10^{-3} M; and the inhibition is noncompetitive. M. P. Stulberg (1969, personal communication), using a highly purified system, has also observed that the formation of Phe-$tRNA^{Phe}$ (*E. coli*) by Syn^{Phe} (*E. coli*) is inhibited by inorganic pyrophos-

phate; however, in this system the inhibition is competitive with tRNA (K_i = 8 × 10^{-4}–1.5 × 10^{-3} M).

We observed a much more potent inhibition by PP_i in the heterologous system examined in this report. Furthermore, PP_i acts as a competitive inhibitor in Tris-Cl buffer at pH 8.0, but as a noncompetitive inhibitor in potassium cacodylate buffer at pH 6.3. A detailed explanation which could account for the change in the type of inhibition exhibited by PP_i is not possible using the information we now have available. A more detailed study of the reaction mechanism must first be carried out. The effects of dimethyl sulfoxide (Ritter *et al.*, 1969) and certain glycols (F. J. Kull and K. B. Jacobson, unpublished data) on this heterologous reaction in Tris-Cl suggest that conformational changes in the tRNA or synthetase, or both, are involved in determining the extent of aminoacylation. Also a suggestion of a referee of this paper is under consideration. It was pointed out that at pH 6.3 PP_i bears a net negative charge of 2.4 (1.6 of 4 possible sites are protonated) while at pH 8.0 it carries a negative charge of 3. Therefore the buffer-dependent change in type of inhibition could be a function of changes in charge balance between synthetase, PP_i, and possibly other reactants or products, or related to conformational changes of the synthetase and/or tRNA, or a combination of these effects.

The fact that the type of inhibition by PP_i, with respect to tRNA, is different in the two buffers does emphasize that all individual homologous (as well as heterologous) reactions should not be expected to exhibit the same form of inhibition by PP_i. The homologous reaction (Syn^{Phe} (*N. crassa*) and $tRNA^{Phe}$ (*N. crassa*)) in Tris-Cl buffer is not inhibited by these very low levels of PP_i; instead it requires concentrations similar to the other homologous reactions already discussed.

Inorganic pyrophosphatase has been shown to affect the rate of hydroxamate formation (Bublitz, 1966; Davie *et al.*, 1956; Attwood and Cocking, 1965) and the formation of threonyladenylate–enzyme complexes (Allende *et al.*, 1966), but its effect on the formation of aminoacyl-tRNA has not been previously reported. Inorganic pyrophosphatase had the effect of increasing the rate of aminoacyl hydroxamate formation and, as reported here, of increasing the rate of both Phe-$tRNA^{Phe}$ (*N. crassa*) formation in Tris-Cl and of Phe-$tRNA^{Val(or\ Ala)}$ (*E. coli*) formation in either Tris-Cl or potassium cacodylate buffers. In the heterologous reaction we are studying, inorganic pyrophosphatase has the additional effect of increasing the amount of Phe-$tRNA^{Val\ (or\ Ala)}$ (*E. coli*) formed in the Tris-Cl-buffered system. We have interpreted these effects as being the result of overcoming an inhibition by the low levels of PP_i formed during the reactions.

The effects seen with inorganic pyrophosphatase appear to be similar in many respects to those seen by Makman and Cantoni (1966) and Pearlman and Bloch (1967), who have described factors, presumably protein, which when added to incubations cause an increase in aminoacyl-tRNA formed by heterologous systems. It would be of interest to know if either of the factors reported by these workers contains inorganic pyrophosphatase activity. In the purification procedure used by Makman and Cantoni the "enhancing" factor is obtained from the same $(NH_4)_2SO_4$ fraction that contains yeast inorganic pyrophosphatase (Heppel, 1955). In their sucrose gradient the "enhancing" factor has a Svedberg constant of about 4.7 S, while inorganic pyrophosphatase from yeast has been shown to have a Svedberg constant of 4.2 S (Schachman, 1952). Thus, there is a distinct possibility that the "enhancing" factor represents inorganic pyrophosphatase activity that is relieving inhibition by PP_i in their heterologous system. For the mammalian enzyme described by Pearlman and Bloch (1967), the factor elutes from DEAE-cellulose under similar conditions of ionic strength and pH as the yeast factor and therefore could also represent inorganic pyrophosphatase.

Loftfield and Eigner (1967) have demonstrated that the aminoacylation of $tRNA^{Val}$ (*E. coli*) by Syn^{Val} (*E. coli*) undergoes general base catalysis by various nucleophiles (Tris, NH^+_4, and imidazole) and that the nature of catalysis is similar to the action of hydroxamate formation. Ritter *et al.* (1969) showed that the aminoacylation of phenylalanine to $tRNA^{Val}$ (*E. coli*) by Syn^{Phe} (*E. coli*) is inhibited by Tris-Cl buffer and concluded that its effect is not due to deacylation of Phe-$tRNA^{Val}$ (*E. coli*). It seems reasonable that in this heterologous system Tris-Cl could be acting as a general base catalyst in the breakdown of some aminoacyl intermediate (Syn^{Phe} (*N. crassa*)–AMP–Phe?) in the presence of heterologous tRNA. Such a base-catalyzed hydrolysis of an acyl intermediate, as suggested here, would, in effect, convert the heterologous system into a partial ATPase. Baldwin and Berg (1966) observed such an ATPase action (recognized by Novelli, 1967) when they demonstrated than an isolated Syn^{Ile} (*E. coli*)–AMP–Val complex is hydrolyzed in the presence of $tRNA^{Ile}$ (*E. coli*) and that no transfer to $tRNA^{Ile}$ (*E. coli*) takes place. It is therefore reasonable that in the heterologous reaction we studied, in Tris-Cl buffer, PP_i could be produced in amounts sufficient to account for the effects seen.

Several heterologous reactions previously studied, that led to incomplete aminoacylation relative to homologous reactions, were carried out using Tris-Cl buffer (see Novelli, 1967; Niyomporn *et al.*, 1968; Makman and Cantoni, 1966). This buffer was also used where the amount of aminoacylation was found to be related to enzyme concentration (Svensson, 1968). In view of our experiments, it seems possible that these observations could be explained by a combination of a Tris buffer effect and a potent inhibition by the PP_i produced during the course of the reaction. The Tris-Cl buffer effect appears to exaggerate the effect of PP_i. This was demonstrated by comparing the kinetic constants for the heterologous reaction in Tris-Cl and potassium cacodylate buffers. In Tris-Cl PP_i seems to be bound to Syn^{Phe} (*N. crassa*) as strongly as $tRNA^{Val}$ (*E. coli*), while in potassium cacodylate buffer the tRNA is bound three orders of magnitude more tightly than PP_i. In contrast, in the homologous reaction, the affinity of the synthetase for tRNA is far greater than for PP_i even in Tris-Cl buffer.

The competition between $tRNA^{Val}$ (*E. coli*) and PP_i for Syn^{Phe} (*N. crassa*) shown by kinetic constants obtained using initial rates also exists, in the Tris-Cl-buffered system, after net aminoacylation has ceased. Increasing concentrations of PP_i resulted in decreases in the amount of Phe-$tRNA^{Val}$ (*E. coli*) formed. That is, the extent of aminoacylation varied inversely with PP_i concentration. Changes in apparent steady-state levels of Phe-$tRNA^{Val}$ (*E. coli*) were also observed when enzyme concentrations were varied but here the extent of aminoacylation was directly related to enzyme concentration. In both cases the extent of aminoacylation was clearly not a result of irreversible enzyme inactivation. The similarity of the two situations suggests an intimate relationship between synthetase, PP_i, and the extent of aminoacylation that is buffer

dependent. Possibly bearing on this very complex situation are the results concerning the order of addition of inorganic pyrophosphate and inorganic pyrophosphatase. Inorganic pyrophosphatase is less effective when added after the heterologous reaction is already underway than when present before the reaction is initiated. On the other hand, the addition of PP_i during the reaction results in greater inhibition than when the inhibitor is present initially. These observations which appear to be related to each other and also involved with the steady-state phenomena are, like the steady-state situations, not at present readily understood.

Not recognizing the effects of buffer and PP_i when studying heterologous reactions could lead to confusing results. In our experiments PP_i exhibits two classically different types of inhibition with respect to tRNA. This observation could be interpreted as an indication that in the two different environments the reaction could proceed by entirely different mechanisms. Whether such a conjecture is justified is currently under investigation.

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