Isoleucyl Transfer Ribonucleic Acid Synthetase. The Role of Magnesium in Amino Acid Activation*

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ABSTRACT: The effect of magnesium on the rate of the amino acid activation reaction of tRNA synthetases has been investigated. The reaction is eq 1a of the text. Measurements of the equilibrium velocity, V, of eq 1a were conducted with isoleucyl tRNA synthetase from *Escherichia coli* B, at pH 8.0, 0.4 M Na^+, 25°. V was investigated over a permutational variation of ATP, pyrophosphate, and magnesium concentrations, using the standard ATP-[^{32}P]pyrophosphate isotope-exchange reaction. It was found that magnesium can serve as both an activator and an inhibitor of eq 1a. There is no detectable reaction in the absence of magnesium. At a given level of ATP and pyrophosphate, increasing concentrations of magnesium give increased rates of amino acid activation until a maximal rate is achieved. At this point the rate is insensitive to further small increases in the level of magnesium, but high concentrations (10 mM) cause a drop in rate. Furthermore, the substrates ATP and pyrophosphate can activate or inhibit the reaction depending, in part, on the concentration of magnesium.

The measured rates are then correlated with the concentrations of the various ionic forms of ATP and pyrophosphate which exist under the conditions of the experiments. Equations are developed for calculating the concentrations of the various ATP and pyrophosphate species as a function of the total concentrations of ATP, pyrophosphate, and magnesium, and of pH and free salt cation concentration (*e.g.*, Na^+). Eleven different forms of ATP and pyrophosphate are simultaneously taken into account. Calculations are executed by using literature values for the various metal and acid dissociation constants. It is found that all of the experimental results can be quantitatively explained by postulating that $MgATP^{2-}$ and $MgP_2O_7^{2-}$ are the only principal substrate forms of ATP and pyrophosphate at pH 8.0. The analysis indicates that $NaATP^{3-}$, $HATP^{3-}$, ATP^{4-}, $NaP_2O_7^{3-}$, $HP_2O_7^{3-}$, and $(Mg)_2P_2O_7$ are all relatively or completely unreactive as substrates under the conditions of the experiments. Moreover, there is no suggestion of enzyme inhibition by any of these molecules. The decrease in V at high levels of Mg^{2+} is due to the decrease in $(MgP_2O_7^{2-})$ owing to the formation of the inert $(Mg)_2P_2O_7$ species. When magnesium is limiting, high concentrations of ATP inhibit the reaction by draining the metal away from pyrophosphate and thereby diminishing or preventing formation of the critical $MgP_2O_7^{2-}$ species. It thus appears that the enzyme is extremely specific for the monomagnesium complexes of ATP and pyrophosphate. In addition, no evidence was obtained for a critical enzyme–magnesium complex, although a tightly bound magnesium ion would have escaped detection.

The aminoacyl tRNA synthetase reaction is customarily written as[1]

$$E + aa + ATP \rightleftharpoons E\cdot AA\cdot AMP + PP \quad (1a)$$

$$E\cdot AA\cdot AMP + tRNA \rightleftharpoons E + AA\text{-}tRNA + AMP \quad (1b)$$

Although the rates of these reactions show a substantial magnesium dependence, the exact role of this metal has never been completely established. This dependence is presumably due at least in part to the fact that ATP, PP, and tRNA form stable complexes with magnesium.

Recently Cole and Schimmel (1970) have reported the results of an investigation of the ATP-[^{32}P]PP isotope-exchange kinetics of eq 1a with highly purified isoleucyl tRNA synthetase from *Escherichia coli* B. The exchange reaction was utilized to determine the *equilibrium* velocity V (moles/l. per sec) of eq 1a. It was found that at pH 8.0, 25°, V obeys the following rate law

$$V = k_{ap}(E)_0 \Big/ \left(\frac{\phi_1}{(Ile)} + \frac{\phi_2}{(ATP)} + \frac{\phi_3}{(Ile)(ATP)} + \frac{\phi_4(PP)}{(Ile)(ATP)} + \phi_5 + \frac{1}{(PP)} \right) \quad (2)$$

where the ϕ's are constants, k_{ap} is an apparent second-order (concentration independent) rate constant, and $(E)_0$ is the total enzyme concentration. The equilibrium constant of eq 1a and various enzyme–substrate dissociation constants have been calculated from the ϕ's (Cole and Schimmel, 1970). Equation 2 is applicable over a wide range of substrate concentrations, under conditions where the enzyme is present in catalytic quantities. In obtaining this rate expression, the magnesium concentration was always sufficient to have PP and ATP present largely as their respective monomagnesium complexes (Cole and Schimmel, 1970). The reactivities of the other species of ATP and PP were not ascertained.

Preliminary investigations established that magnesium can serve as both an activator and an inhibitor of eq 1a (Cole and

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[1] The abbreviations used are: E, an AA-tRNA synthetase; aa, amino acid; E·AA·AMP, enzyme–aminoacyl adenylate complex; AA-tRNA, aminoacyl-tRNA; PP, pyrophosphate.

TABLE I: Acid and Metal Ion Dissociation Constants of Complexes of ATP and Pyrophosphate.

Reaction	Constant	$-\log K_{diss}$	Reference
$HATP^{3-} \rightleftarrows H^+ + ATP^{4-}$	K_{HATP}	6.95[a]	Smith and Alberty (1956a)
$MgATP^{2-} \rightleftarrows Mg^{2+} + ATP^{4-}$	K_{MATP}	4.00[b]	Martell and Schwarzenbach (1956)
$MgHATP^{-} \rightleftarrows Mg^{2+} + HATP^{3-}$	K_{MATPH}	1.49[a]	Smith and Alberty (1956a)
$NaATP^{3-} \rightleftarrows Na^+ + ATP^{4-}$	K_{NaATP}	1.06[a]	Smith and Alberty (1956b)
$HP_2O_7^{3-} \rightleftarrows H^+ + P_2O_7^{4-}$	K_{HPP}	8.93[c]	Lambert and Watters (1957b)
$MgP_2O_7^{2-} \rightleftarrows Mg^{2+} + P_2O_7^{4-}$	K_{MPP}	5.41[c]	Lambert and Watters (1957a)
$MgHP_2O_7^{-} \rightleftarrows Mg^{2+} + HP_2O_7^{3-}$	K_{MPPH}	3.06[c]	Lambert and Watters (1957a)
$Mg_2P_2O_7 \rightleftarrows Mg^{2+} + MgP_2O_7^{2-}$	K_{M_2PP}	2.34[c,d]	Lambert and Watters (1957a)
$NaP_2O_7^{3-} \rightleftarrows Na^+ + P_2O_7^{4-}$	K_{NaPP}	1.00[c]	Lambert and Watters (1957b)

[a] Tetra-*n*-propylammonium bromide (0.2 M), 25°. [b] KCl (0.1 M), 20°. [c] Tetramethylammonium chloride (1 M), 25°. [d] A value of 2.00 was used in the calculations (see text for discussion).

Schimmel, 1970). At given concentrations of ATP and pyrophosphate, increasing concentrations of magnesium give increased equilibrium rates of amino acid activation until a maximal rate is achieved. At this point the rate is insensitive to further small increases in the level of magnesium, but high concentrations (10 mM) cause a decrease in V. Furthermore, the substrates ATP and pyrophosphate can activate or inhibit the reaction depending, in part, on the concentration of magnesium. In order to quantitatively account for these and other observations, we have conducted a more detailed investigation of the effect of magnesium on eq 1a. We first develop the equations for calculating the concentrations of each of the various species of ATP and pyrophosphate which are present under the conditions of the experiments. Calculations are then carried out and compared with experimental data in order to make reasonable conjectures about the reactivities of the various ATP and PP species. Finally, the analysis is combined with eq 2 and certain hypotheses in order to quantitatively account for a variety of experimental data.

Theory

The experimental results in this and in the previous study of the isoleucyl tRNA synthetase isotope-exchange reaction were obtained at 25° in a solvent system containing 0.1 M Tris, 0.1 M sodium acetate, and 0.3 M sodium chloride titrated to pH 8.0 with acetic acid. Under these conditions there are at least nine equilibria involving ATP and pyrophosphate which must be considered. These reactions and their respective dissociation constants are given in Table I. In order to calculate the concentration of any given species, it is necessary to first consider the three mass conservation equations

$$(ATP)_0 = (ATP^{4-}) + (HATP^{3-}) + (NaATP^{3-}) + (MgATP^{2-}) + (MgHATP^{-}) \quad (3)$$

$$(PP)_0 = (P_2O_7^{4-}) + (HP_2O_7^{3-}) + (NaP_2O_7^{3-}) + (MgP_2O_7^{2-}) + (MgHP_2O_7^{-}) + Mg_2P_2O_7) \quad (4)$$

$$(Mg)_0 = (Mg^{2+}) + (MgATP^{2-}) + (MgHATP^{-}) + (MgP_2O_7^{2-}) + (MgHP_2O_7^{-}) + 2(Mg_2P_2O_7) \quad (5)$$

where the subscript zero signifies the total concentration. Inserting the relevant equilibrium constants (*cf.* Table I) in eq 3–5 and rearranging gives

$$(ATP)_0 = (MgATP^{2-}) \cdot f_{ATP} \quad (6)$$

$$(PP)_0 = (MgP_2O_7^{2-}) \cdot f_{PP} \quad (7)$$

and

$$(Mg)_0 = (Mg^{2+}) + \frac{(ATP)_0\left(1 + \frac{K_{MATP}}{K_{MATPH}}\frac{(H^+)}{K_{HATP}}\right)}{f_{ATP}} + \frac{(PP)_0\left(1 + \frac{K_{MPP}}{K_{MPPH}}\frac{(H^+)}{K_{HPP}} + 2\frac{(Mg^{2+})}{K_{M_2PP}}\right)}{f_{PP}} \quad (8)$$

where

$$f_{ATP} = 1 + \frac{K_{MATP}}{(Mg^{2+})}\left\{1 + \frac{(Na^+)}{K_{NaATP}} + \frac{(H^+)}{K_{HATP}}\left[1 + \frac{(Mg^{2+})}{K_{MATPH}}\right]\right\} \quad (9)$$

and

$$f_{PP} = 1 + \frac{K_{MPP}}{(Mg^{2+})}\left\{1 + \frac{(Na^+)}{K_{NaPP}} + \frac{(H^+)}{K_{HPP}}\left[1 + \frac{(Mg^{2+})}{K_{MPPH}}\right]\right\} + \frac{(Mg^{2+})}{K_{M_2PP}} \quad (10)$$

Rearrangement and manipulation of the above equations permits expression of the free magnesium concentration, (Mg^{2+}), as the appropriate root of the fourth-order polynomial

$$a_0 + a_1(Mg^{2+}) + a_2(Mg^{2+})^2 + a_3(Mg^{2+})^3 + a_4(Mg^{2+})^4 = 0 \quad (11)$$

where

$$a_0 = -K_{MATP}K_{MPP}\left[1 + \frac{(H^+)}{K_{HPP}} + \frac{(Na^+)}{K_{NaPP}}\right]\left[1 + \frac{(H^+)}{K_{HATP}} + \frac{(Na^+)}{K_{NaATP}}\right](Mg)_0 \quad (12a)$$

$$a_1 = K_{MATP}\left[1 + \frac{(H^+)}{K_{HATP}} + \frac{(Na^+)}{K_{NaATP}}\right] K_{MPP}\left[1 + \frac{(H^+)}{K_{HPP}} + \frac{(Na^+)}{K_{NaPP}}\right] + K_{MATP}\left[1 + \frac{(H^+)}{K_{HPP}}\frac{K_{MPP}}{K_{MPPH}}\right]\left[1 + \frac{(H^+)}{K_{HATP}} + \frac{(Na^+)}{K_{NaATP}}\right][(PP)_0 - (Mg)_0] + K_{MPP}\left[1 + \frac{(H^+)}{K_{HPP}} + \frac{(Na^+)}{K_{NaPP}}\right]\left[1 + \frac{K_{MATP}}{K_{MATPH}}\frac{(H^+)}{K_{HATP}}\right][(ATP)_0 - (Mg)_0] \quad (12b)$$

$$a_2 = \left[1 + \frac{(Na^+)}{K_{NaPP}} + \frac{(H^+)}{K_{HPP}}\right]\left[1 + \frac{K_{MATP}}{K_{MATPH}}\frac{(H^+)}{K_{HATP}}\right] K_{MPP} + K_{MATP}\left[1 + \frac{K_{MPP}}{K_{MPPH}}\frac{(H^+)}{K_{HPP}}\right] \times \left[1 + \frac{(Na^+)}{K_{NaATP}} + \frac{(H^+)}{K_{HATP}}\right] + \left[1 + \frac{K_{MPP}}{K_{MPPH}}\frac{(H^+)}{K_{HPP}}\right]\left[1 + \frac{K_{MATP}}{K_{MATPH}}\frac{(H^+)}{K_{HATP}}\right][(ATP)_0 + (PP)_0 - (Mg)_0] + \frac{K_{MATP}}{K_{M_2PP}}\left[1 + \frac{(Na^+)}{K_{NaATP}} + \frac{(H^+)}{K_{HATP}}\right] \times [2(PP)_0 - (Mg_0)] \quad (12c)$$

$$a_3 = \left[1 + \frac{K_{MATP}}{K_{MATPH}}\frac{(H^+)}{K_{HATP}}\right]\left[1 + \frac{K_{MPP}}{K_{MPPH}}\frac{(H^+)}{K_{HPP}}\right] + \frac{K_{MATP}}{K_{M_2PP}}\left[1 + \frac{(Na^+)}{K_{NaATP}} + \frac{(H^+)}{K_{HATP}}\right] + \frac{1}{K_{M_2PP}}\left[1 + \frac{K_{MATP}}{K_{MATPH}}\frac{(H^+)}{K_{HATP}}\right][(ATP)_0 + 2(PP)_0 - (Mg)_0] \quad (12d)$$

$$a_4 = \frac{1}{K_{M_2PP}}\left[1 + \frac{K_{MATP}}{K_{MATPH}}\frac{(H^+)}{K_{HATP}}\right] \quad (12e)$$

It is clear from eq 12a–e that the coefficients in eq 11 are functions of the hydrogen and sodium ion concentrations, as well as the total ATP, pyrophosphate, and magnesium concentrations. These concentrations are all known quantities (it may be assumed to an excellent approximation that the free sodium ion concentration equals the total sodium ion concentration (*cf. seq*)).

The roots of eq 11 may be extracted with a digital computer employing the standard Newton–Raphson procedure (Margenau and Murphy, 1956). Negative and imaginary roots are discarded, of course, and a real positive root is sought. The value of (Mg^{2+}) thus obtained can then be used to calculate the concentrations of all the various ionic forms of ATP and PP by means of expressions such as eq 6–10. With this information available it is then possible to correlate kinetic data with the concentration of each of the various ionic forms of ATP and PP.

Results of Experiments and Calculations

General Features of the Effects of Magnesium and the Reactivities of $MgATP^{2-}$ and $MgP_2O_7^{2-}$. All experiments and calculations reported here apply to pH 8.0 and a sodium ion concentration of 0.4 M. The usual investigations of eq 1a employ ATP and pyrophosphate concentrations of the order of millimolar. Using a typical set of substrate concentrations, we computed the concentration of each ATP and pyrophosphate species as a function of the total magnesium concentration, in order to ascertain the predominant species at each magnesium concentration. Figure 1 gives the concentration of each of the various species as a function of the total magnesium concentration, in a mixture containing $(ATP)_0$, 1 mM, and $(PP)_0$, 1 mM. These calculations show that $MgP_2O_7^{2-}$ and $MgATP^{2-}$ are the major monomagnesium complexes of ATP and pyrophosphate at pH 8.0. It is noteworthy that although the pK of the acid $HP_2O_7^{3-}$ is 8.95, addition of magnesium at pH 8.0 converts this species almost entirely into $MgP_2O_7^{2-}$, rather than $MgHP_2O_7^-$. Figure 1 also illustrates that high magnesium concentrations cause a decrease in the concentration of $MgP_2O_7^{2-}$ resulting from the formation of $Mg_2P_2O_7$.

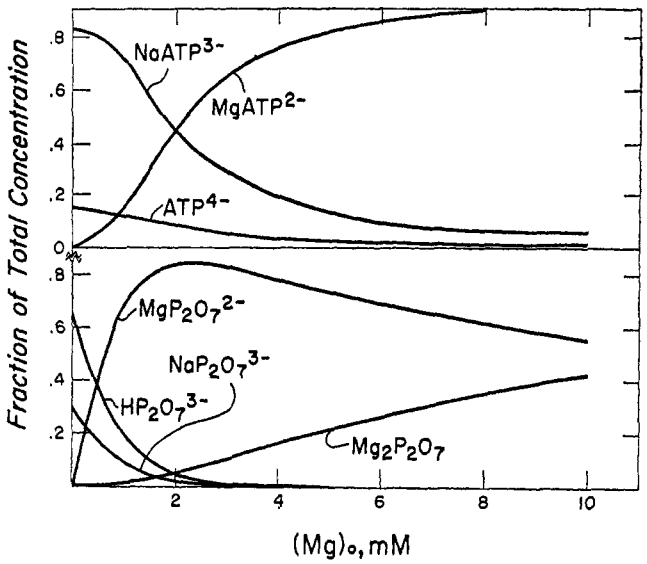


FIGURE 1: Calculated concentrations of various species of ATP and PP as a function of $(Mg)_0$, in a mixture containing 1 mM $(ATP)_0$ plus 1 mM $(PP)_0$. Concentrations are expressed as the fraction of $(ATP)_0$ or $(PP)_0$. Species present in very small amounts are omitted for clarity. See text for further details.

The equilibrium velocity of eq 1a was measured as a function of the total magnesium concentration at various fixed levels of enzyme, ATP, and pyrophosphate. Representative data are shown in Figure 2 where each curve of V *vs.* $(Mg)_0$ corresponds to a different permutation of ATP and pyrophosphate concentrations. Figure 2 shows that V increases rapidly with increasing $(Mg)_0$, reaches a maximum value, and then slowly decreases with increasing $(Mg)_0$. Comparison of these data with calculated concentrations such as are given in Figure 1 indicates that V increases as the concentration of $MgATP^{2-}$ and $MgP_2O_7^{2-}$ increases. The greatest rate occurs when $(MgATP^{2-})$ and $(MgP_2O_7^{2-})$ are both large; the rate decreases as $(Mg_2P_2O_7)$ becomes large, or equivalently, as $(MgP_2O_7^{2-})$ decreases. These observations suggest that $MgP_2O_7^{2-}$ and $MgATP^{2-}$ are major substrate species in the reaction at pH 8.0, and that inhibition by high concentrations of magnesium results from a decrease in $(MgP_2O_7^{2-})$. We attempted therefore to explain the data of Figure 2 on this basis, together with the assumption that all species other than $MgP_2O_7^{2-}$ and $MgATP^{2-}$ are inactive either as substrates or inhibitors. For each value of $(ATP)_0$, $(PP)_0$, and $(Mg)_0$ given in Figure 2, the concentrations of $MgATP^{2-}$ and $MgP_2O_7^{2-}$ were calculated from eq 6–12. The results were then substituted into eq 2 for the terms involving (ATP) and (PP), in order to

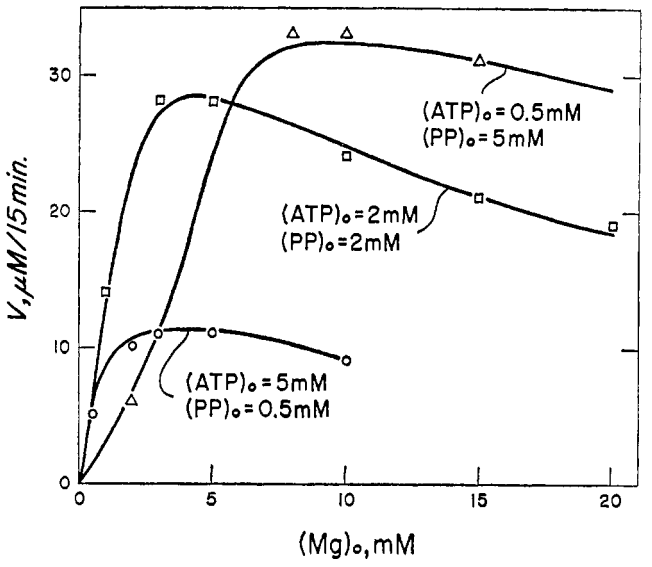


FIGURE 2: V *vs.* $(Mg)_0$, at various $(ATP)_0$ and $(PP)_0$. The points represent experimental observations and the solid curves were calculated as discussed in the text. In all cases the concentration of isoleucine and of enzyme was 2 mM and 0.65 mμM, respectively.

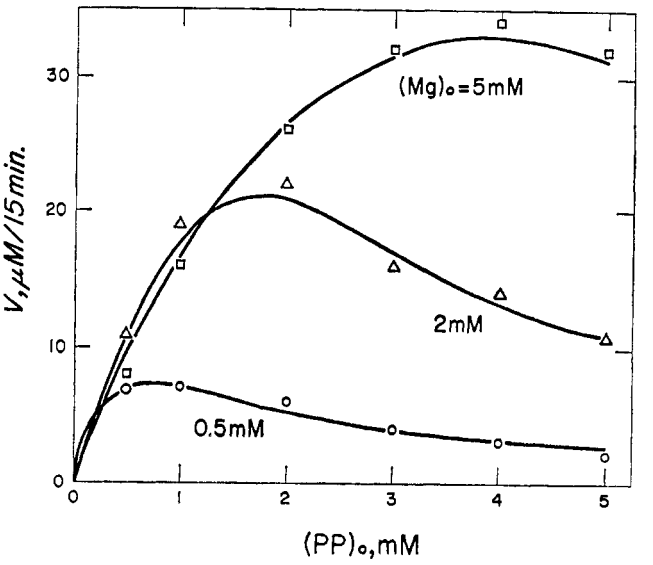


FIGURE 4: V *vs.* $(PP)_0$ at various $(Mg)_0$ with $(ATP)_0$, 1 mM. Other details are the same as are given for the legend to Figure 3.

compute the theoretical value of V corresponding to a given level of $(ATP)_0$, $(PP)_0$, and $(Mg)_0$. The equilibrium constants given in Table I were used for these calculations, with one exception: it was found necessary to adjust the constant K_{M_2PP} from $10^{-2.34}$ to $10^{-2.0}$ in order to satisfactorily account for all of the data. The solid curves in Figure 2 are values of V calculated in the described manner. The agreement between calculated and experimental values is quite good. In all cases, the positions of maximal activity occur when $(Mg)_0 \approx (ATP)_0 + (PP)_0$. Unfortunately, higher concentrations of magnesium cannot be investigated because of the limited solubility of the $Mg_2P_2O_7$ complex.

Employing exactly the same assumptions and methods, the dependence of V on $(ATP)_0$ and on $(PP)_0$ was calculated, for different values of $(Mg)_0$. Experimental determinations were also made and compared with the calculations. Figure 3 gives plots of V *vs.* $(ATP)_0$, at $(Mg)_0$, 2 mM and $(Mg)_0$, 0.5 mM.

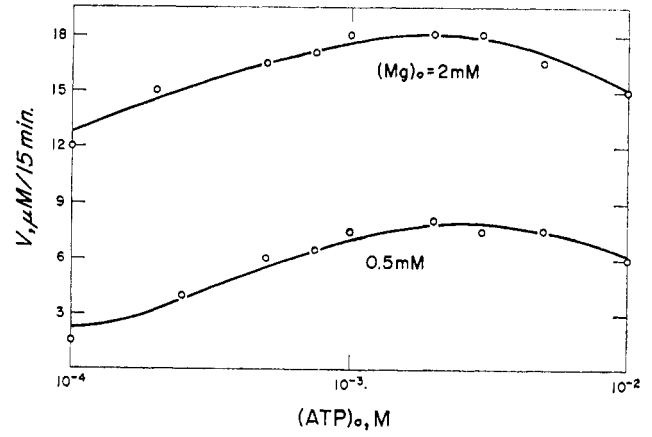


FIGURE 3: V *vs.* $(ATP)_0$ at various $(Mg)_0$. The points are experimental values and the curves were calculated as described in text. Concentrations of other reactants are: isoleucine 2, mM; $(PP)_0$, 1 mM; and enzyme, 0.65 mμM.

A 100-fold range of $(ATP)_0$ is covered. The curves are the theoretical values of V which agree well with the experimental points. The data demonstrate that high concentrations of ATP depress V, when Mg^{2+} is limiting. Figure 4 gives the analogous investigation of the dependence of V on $(PP)_0$, at $(Mg)_0$, 5 mM, 2 mM, and 0.5 mM. Theoretical curves and experimental points are again in good agreement.

The data in Figures 2–4 illustrate the complicated interdependence of V on $(Mg)_0$, $(ATP)_0$, and $(PP)_0$, over a widely permuted range of concentrations. The calculations demonstrate that all of these experimental results are explicable on the simple basis that $MgATP^{2-}$ and $MgP_2O_7^{2-}$ are the only active species of ATP and PP. It certainly seems clear that these two species are substrates. However, it is not clear if the calculations are sensitive enough to eliminate as substrates those other species which are present in significant concentrations. Therefore we further analyzed the data by considering in a one-by-one fashion the relative contribution to V that each of the other ionic forms of ATP and PP would make if they had activity comparable with that of $MgATP^{2-}$ and $MgP_2O_7^{2-}$, respectively.

Reactivities of Other Ionic Forms of ATP and PP. The reactivities of other ionic forms of ATP and PP can be tested by finding concentration ranges in which these other forms are present in substantial amounts and also in which the calculated rate (eq 2) changes significantly when changes are made in what is assumed to be the concentration of active ATP or PP. For this purpose, the data of Figures 2–4 together with the rate law of eq 2 were carefully examined. We found that reasonably critical tests of various hypotheses could be made with certain selected portions of the data given in Figures 2–4. Values of V were systematically computed by assuming that $MgATP^{2-}$ and $MgP_2O_7^{2-}$ are active substrate forms *together with* one of the other ionic forms of ATP or PP. These calculated values of V were then tested against the appropriate experimental data.

Figure 5 shows a plot of V *vs.* $(Mg)_0$ with two theoretical curves which are labeled so as to designate which species of

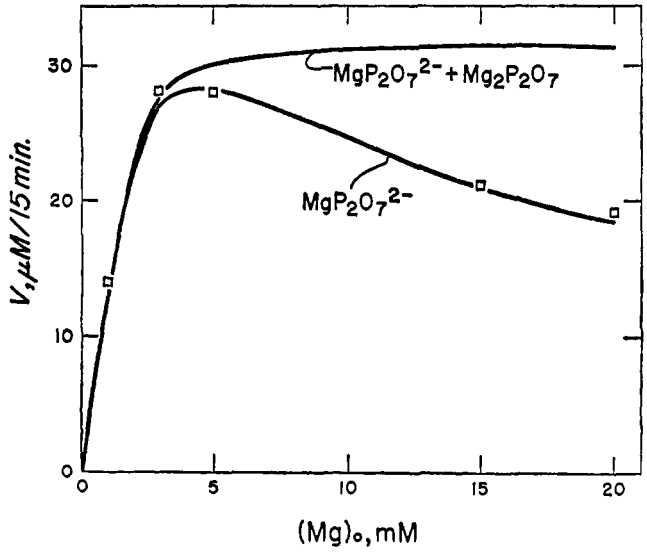
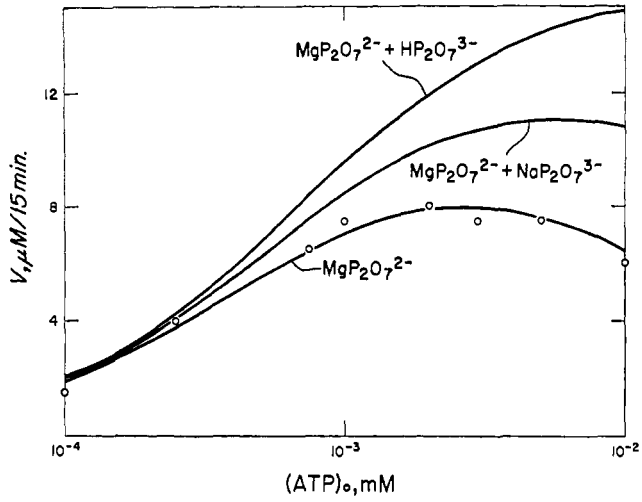


FIGURE 5: *V vs.* $(Mg)_0$ with $(ATP)_0 = (PP)_0 = 2$ mM (*cf.* Figure 2). Experimental points are compared with theoretical curves labeled so as to designate which species of pyrophosphate were assumed to be active.



FGURE 6: *V vs.* $(ATP)_0$ with $(PP)_0 = 1$ mM and $(Mg)_0 = 0.5$ mM (*cf.* Figure 3). Experimental points are compared with theoretical curves labeled so as to designate which species of pyrophosphate were assumed to be active.

PP were assumed to be active. This figure tests the hypothesis that $(Mg)_2P_2O_7$ reacts as well as $MgP_2O_7^{2-}$. Although the curves are indistinguishable at low $(Mg)_0$, they separate widely at high $(Mg)_0$ where the curve labeled $MgP_2O_7^{2-} + (Mg)_2P_2O_7$ falls well above the experimental points. Thus, $(Mg)_2P_2O_7$ is clearly established as being *relatively* unreactive as a substrate.

The relative activities of other species of PP, *i.e.*, $HP_2O_7^{3-}$ and $NaP_2O_7^{3-}$, are tested in the data displayed in Figure 6 where a plot of *V vs.* $(ATP)_0$ is given (*cf.* Figure 3). It is clear that the two curves labeled $MgP_2O_7^{2-} + NaP_2O_7^{3-}$, and $MgP_2O_7^{2-} + HP_2O_7^{3-}$, respectively, deviate widely from the experimental points at high $(ATP)_0$. These data, then, eliminate $HP_2O_7^{3-}$ and $NaP_2O_7^{3-}$ as substrates with reactivities anywhere near comparable with $MgP_2O_7^{2-}$.

Figure 7 displays *V vs.* $(PP)_0$ with four theoretical curves designed to test the relative activities of three nonmagnesium containing forms of ATP–$NaATP^{3-}$, ATP^{4-}, and $ATPH^{3-}$. Although the curves are coincident at low $(PP)_0$, they deviate greatly from each other at higher $(PP)_0$. Furthermore, as $(PP)_0$ increases there is simultaneously a large deviation from the experimental points of the three curves labeled $MgATP^{2-} + NaATP^{3-}$, $MgATP^{2-} + ATP^{4-}$, and $MgATP^{2-} + ATPH^{3-}$. Only the curve labeled $MgATP^{2-}$ passes through all of the points. Hence $NaATP^{3-}$, ATP^{4-}, and $ATPH^{3-}$ may each be categorized as relatively or completely unreactive as a substrate.

Eight of the eleven species of ATP and PP given in Table I have been tested for activity in the above analysis. Only $MgHATP^-$, $MgHP_2O_7^-$, and $P_2O_7^{4-}$ were not tested because none is present in sufficiently large amounts, under the conditions of the experiments.

Discussion

The method of calculating the distribution of magnesium in the presence of ATP and pyrophosphate which has been employed here is quite general, and may be used for any system containing these compounds. The procedure for treating a greater number of equilibria involving magnesium is altogether straightforward. In such cases, the order of the polynomial (eq 11) will increase, but the roots may readily be extracted with a computer. The algebraic complexity involved in computing (Mg^{2+}) could be circumvented by directly measuring the Mg^{2+} activity with one of the commercially available electrodes for divalent cations, although it is questionable if such electrodes are accurate at the high levels of Na^+ employed in this study.

The equilibrium constants used in the calculations (see Table I) are probably subject to some refinements, since these parameters were experimentally determined under somewhat different conditions of ionic strength than employed here. (A high ionic strength, ~0.45 M, was used in our studies in order to assure its approximate constancy over the substrate concentration range investigated.) Data applicable to the case of interest here are not available. In most cases we have used the value of the stability constant determined in the presence of a tetraalkylammonium salt, where effects due to salt cation binding to phosphates is minimal. We have then

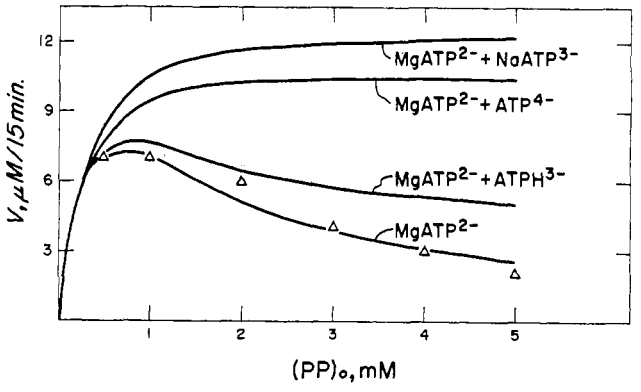


FIGURE 7: *V vs.* $(PP)_0$ with $(ATP)_0 = 1$ mM and $(Mg)_0 = 2$ mM (*cf.* Figure 4). Experimental points are compared with theoretical curves beled so as to designate which species of ATP were assumed to be active.

included in our calculations the known binding of Na^+ to ATP^{4-} and $P_2O_7^{4-}$ (Smith and Alberty, 1956b; Lambert and Watters, 1957b). In preliminary studies sodium ion binding was not taken into account. This results in a predicted dependence of V on the total magnesium concentration which is substantially different from that found. For example, in a plot of V *vs.* $(Mg)_0$ (Figure 2) these calculations give a predicted maximum in V at considerably lower total magnesium concentrations than observed. In addition, we found it necessary to use a somewhat smaller value for K_{M_2PP} than given in Table I. The value of $10^{-2.0}$ M for K_{M_2PP} which we found to fit the data does not appear unreasonable, however, in view of the differences in conditions employed here from those used to determine K_{M_2PP} (Lambert and Watters, 1957a). The value K_{M_2PP} $10^{-2.34}$ M, determined in 1 M tetramethylammonium chloride (*cf.* Table I), does not satisfactorily reproduce the data at high magnesium concentrations; nor does a value significantly below $10^{-2.0}$ M, *e.g.*, $10^{-1.5}$ M. Finally it should be mentioned that the value selected for K_{MgATP} is somewhat arbitrary, since a range of experimental values are available (see Phillips *et al.*, 1966). However, this choice (from Martell and Schwarzenbach, 1956) gives a good fit to our data whereas the significantly higher or lower values which have been published are unsatisfactory.

A single set of parameters (see Table I), together with the hypothesis that $MgATP^{2-}$ and $MgP_2O_7^{2-}$ are the active substrate species, satisfactorily accounts for all of the data obtained at pH 8.0. It also appears clear that ATP^{4-}, $HATP^{3-}$, $NaATP^{3-}$, $HP_2O_7^{3-}$, and $NaP_2O_7^{3-}$ are relatively or completely unreactive as substrates of the reaction. This conclusion is established by the analysis given in Figures 5–7. Moreover, there is no suggestion of enzyme inhibition by any of these species. In addition, the $(Mg)_2P_2O_7$ species appears to be inert since significant concentrations of this complex do not alter the reaction rate beyond that caused by the concomitant decrease in $(MgP_2O_7^{2-})$. We conclude, therefore, that $MgATP^{2-}$ and $MgP_2O_7^{2-}$ are virtually exclusive substrates of the enzyme, under the conditions of the experiments employed here.

Figure 3 shows that high concentrations of ATP can inhibit the reaction under certain conditions, even though ATP itself is not an inhibitor of the enzyme. The inhibition results from competition between ATP and pyrophosphate for magnesium. When magnesium is limiting, high concentrations of ATP drain the metal away from $MgP_2O_7^{2-}$ and decrease the concentration of this species of pyrophosphate which is required for the reaction. As Figure 3 shows, the magnitude of the effect depends upon the concentration of magnesium.

The situation with respect to pyrophosphate is more complicated, however (see Figure 4). Previous work has established that $MgP_2O_7^{2-}$ acts as both a substrate and an inhibitor of eq 1a (Cole and Schimmel, 1970). It acts as a substrate by reacting with E·AA·AMP to form ATP, isoleucine, and free enzyme; it acts as an inhibitor by binding to the active site of the free enzyme and thereby inhibits binding of both ATP and isoleucine (Cole and Schimmel, 1970). The inhibition by high pyrophosphate concentrations shown in Figure 4 thus arises from two sources: (a) direct inhibition of the free enzyme by $MgP_2O_7^{2-}$ and (b) drainage of Mg^{2+} from $MgATP^{2-}$ by pyrophosphate.

Finally, it should be emphasized that it was not necessary to assume that a critical enzyme–magnesium complex is required for enzymatic activity. However, our data are not sufficiently sensitive to detect a firmly bound enzyme–magnesium complex with a dissociation constant less than *ca.* 10^{-4} to 10^{-3} M. Finally, no definitive statement can be made with regard to the dissociation of magnesium from enzyme-bound $MgATP^{2-}$ or $MgP_2O_7^{2-}$ but from a simple analysis it appears that the dissociation constants must also be less than *ca.* 10^{-4} to 10^{-3} M.

Materials and Methods

Highly purified isoleucyl tRNA synthetase was isolated from *E. coli* B (Grain Processing Co., Muscatine, Iowa) by a modification of the method of Baldwin and Berg (1966). The details concerning reagents and kinetic measurements are given in a previous paper (Cole and Schimmel, 1970). All experiments were done in a buffer system containing 0.1 M sodium acetate, 0.3 M sodium chloride, and 0.1 M Tris, titrated to pH 8.0 with acetic acid. The temperature of all solutions was regulated at 25 ± 0.2° by a thermostated water bath.

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