On the Rate Law and Mechanism of the Adenosine Triphosphate–Pyrophosphate Isotope Exchange Reaction of Amino Acyl Transfer Ribonucleic Acid Synthetases*

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ABSTRACT: The amino acid activation reaction of protein synthesis is conventionally investigated by studying the rate of adenosine triphosphate–pyrophosphate isotope exchange. The reaction is eq 1a of the text. A quantitative treatment of the kinetics of this reaction is given here, using methods and formalism developed previously. Rate laws for isotope exchange are derived for various possible mechanisms of amino acid activation.

These mechanisms may be distinguished by the concentration dependence of the isotope exchange kinetics. From a careful analysis of the data dissociation constants may also be obtained for various enzyme–substrate complexes as well as the equilibrium constant for the overall reaction. An experimental investigation with isoleucyl transfer ribonucleic acid synthetase from *Escherichia coli* is also described. The data strongly suggest that adenosine triphosphate, isoleucine, pyrophosphate, and isoleucyladenosine monophosphate each form 1:1 complexes with the enzyme. Dissociation constants for the enzyme–adenosine triphosphate, enzyme–isoleucine, and enzyme–pyrophosphate complexes have been obtained. The pyrophosphate complex is inhibitory, presumably because the bound pyrophosphate prevents or significantly lowers the binding of adenosine triphosphate and of isoleucine. Conversely, bound adenosine triphosphate or isoleucine reduces or eliminates pyrophosphate binding. The equilibrium constant of the overall amino acid activation reaction has also been extracted from the data. An analysis of various mechanisms shows that more than one kinetic scheme can satisfactorily account for the observed rate law. Preliminary investigations of the effect of magnesium on the reaction are described.

It is well known that in the first reactions of protein synthesis each amino acid is specifically attached to the 2′- or 3′-terminal ribose hydroxyl group of its cognate RNA molecule (Novelli, 1967). This reaction is catalyzed by AA-tRNA synthetases, one (or more) for each amino acid (Novelli, 1967). The reaction is generally believed to occur in two steps (Berg, 1961; Novelli, 1967)

$$E^{1} + AA + ATP \rightleftharpoons E\cdot AA\cdot AMP + PP \quad (1a)$$

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

The reactions generally require magnesium as a cofactor (Berg, 1961; Novelli, 1967). Reaction 1a has been conventionally studied by the ATP–PP* isotope exchange reaction: tracer amounts of PP* are simultaneously mixed with E, AA, PP, and ATP, and the initial rate of incorporation of PP* into ATP is then measured and subsequently used to compute the total rate, V, of production of ATP from pyrophosphate. Even though all of the reactants are frequently mixed simultaneously, equilibrium is rapidly established among all of the unlabeled species in eq 1a before significant isotope exchange takes place (this fact is demonstrated below with the Ile-tRNA synthetase). Hence, V is the equilibrium rate of production of ATP from pyrophosphate. The parameter V has been utilized in Lineweaver–Burk (1934) plots to obtain apparent Michaelis constants and maximal velocities for the various substrates in eq 1a (*cf.*, Novelli, 1967).

It is to be noted that the enzyme does not function as a catalyst in this reaction: pyrophosphate is stoichiometrically produced in an amount equal to that of the enzyme–adenylate complex, under the usual experimental conditions (Berg, 1961; Novelli, 1967). For this reason, and because the unlabeled species are at equilibrium while exchange occurs, the exchange reaction *prima facie* bears little resemblance to the usual circumstances of steady-state enzyme kinetics. It is, therefore, doubltess incorrect to interpret the exchange kinetics in the framework of Michaelis–Menten steady-state enzyme kinetics.

The purpose of this paper is to investigate quantitatively the aforementioned isotope exchange reaction, using the theoretical framework developed by previous investigators (Boyer, 1959; Alberty *et al.*, 1962; Morales *et al.*, 1962). Emphasis is placed on utilizing such data to distinguish between various possible mechanisms of amino acid activation, and to obtain the stoichiometry of and dissociation constants for the various enzyme–substrate complexes. An experimental study with Ile-tRNA synthetase is described in the final section of the paper. Mechanisms for amino acid activation are found which quantitatively account for all of the data; dissociation constants for various enzyme–substrate complexes and the equilibrium constant of the overall reaction have also been obtained.

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[1] The abbreviations used are: E, an AA-tRNA synthetase; E·AA·AMP, enzyme–aminoacyl adenylate complex; ATP*, ^{32}P-labeled ATP; PP*, ^{32}P-labeled pyrophosphate; P*, ^{32}P-labeled inorganic phosphate.

Treatment of Isotope Exchange

The theory of equilibrium isotope exchange is well established (see, for example, Boyer, 1959, Alberty *et al.*, 1962, Morales *et al.*, 1962, and Yagil and Hoberman, 1969); its salient features are briefly reviewed here in the context of the reaction of present interest. We make use of the usual assumptions that: (a) the total enzyme concentration is very much less than that of any of the substrates; (b) the isotope is present in tracer amounts; (c) all unlabeled species are at their equilibrium concentrations while exchange occurs; (d) the mechanism of the reaction is the same for labeled and unlabeled species; (e) isotope substitution has no effect on kinetic parameters; and (f) labeled enzyme intermediates are in a steady state while exchange occurs (the very short presteady state or transient period is not considered, because it is not amenable to experimental observation). We consider the isotope exchange reaction

$$\text{ATP} + \text{PP}^* \rightleftarrows \text{ATP}^* + \text{PP} \tag{2}$$

for which the rate of production of ATP* may be written

$$\frac{d(\text{ATP}^*)}{dt} = V\frac{(\text{PP}^*)}{(\text{PP})} - V\frac{(\text{ATP}^*)}{(\text{ATP})} \tag{3}$$

The first term on the right-hand side of eq 3 corresponds to the rate at which ATP* is produced from PP*, while the second term accounts for the rate at which ATP* is converted into PP*. The following relationships may be substituted into eq 3

$$(\text{ATP}^* - \overline{\text{ATP}}^*) = (\overline{\text{PP}}^* - \text{PP}^*) \tag{4a}$$

$$\frac{(\overline{\text{ATP}}^*)}{(\overline{\text{PP}}^*)} = \frac{(\text{ATP})}{(\text{PP})} \tag{4b}$$

and

$$F = \frac{(\text{ATP}^*)}{(\overline{\text{ATP}}^*)} \tag{4c}$$

where the bars denote concentrations at isotopic equilibrium. Using eq 4a–c, eq 3 may be integrated to give

$$F = 1 - \exp\left(-V\left(\frac{(\text{ATP}) + (\text{PP})}{(\text{ATP})(\text{PP})}\right)t\right) \tag{5}$$

Equation 5 is the familiar result that F is a simple exponential function of time, regardless of the mechanism by which exchange occurs (McKay, 1938; Duffield and Calvin, 1946; Norris, 1950; Harris, 1951). Information about the mechanism of exchange is contained in V, the concentration dependence of which is a function of the mechanism of amino acid activation. V may be obtained from the initial rate of exchange where $(\text{ATP}^*) \approx 0$, at $t \approx 0$, so that eq 3 becomes

$$\left.\frac{d(\text{ATP}^*)}{dt}\right|_{t\approx 0} = V\left.\frac{(\text{PP}^*)}{(\text{PP})}\right|_{t\approx 0} = Vs(0)$$

or

$$V = \frac{\left.\dfrac{d(\text{ATP}^*)}{dt}\right|_{t\approx 0}}{s(0)} \tag{6}$$

where $s(t)$ is the specific activity of pyrophosphate at time t. Thus, the equilibrium rate of the reaction V may be obtained from the known initial specific activity of pyrophosphate and the experimentally measured initial rate of exchange.

Before proceeding further, it is important to ascertain whether or not the above analysis is indeed applicable to the isotope exchange reaction of tRNA synthetases (eq 1a). This was accomplished in the following manner: the initial rate of exchange was experimentally measured (for a given concentration of the isoleucyl enzyme and of the various substrates) and used to compute V according to eq 6; V was then multiplied by the factor (ATP) + (PP)/(ATP)(PP) and the result was used in eq 5 to calculate the entire time course of exchange; finally, the time course of exchange was experimentally determined and compared with the calculated curves. The results of three representative experiments with the isoleucyl synthetase are shown in Figure 1. The curves represent calculated values of F and the points are the experimental values. The agreement between calculated and experimental values is excellent, thus confirming the validity of the above analysis for the case of interest here. Furthermore, in all cases it was found that the ratio $(\overline{\text{ATP}}^*)/(\overline{\text{PP}}^*)$ is correctly predicted by eq 4b, where the equilibrium concentrations of unlabeled ATP and pyrophosphate in eq 4b are the same as those added to the reaction mixture at $t = 0$. These results imply that no significant amounts of pyrophosphate are produced, which is consistent with the assertion of eq 1a that it is produced only in an amount equal to the production of enzyme–aminoacyl adenylate complex.

Mechanisms of Isotope Exchange

The concentration dependence of V may be used to distinguish between various possible mechanisms of amino acid activation. In the discussion given below, the effects of Mg^{2+} are not explicitly considered, although the analysis may easily be amended to include such effects (F. X. Cole and P. R. Schimmel, in preparation). In general, the equilibrium rate of the reaction may be written (see subsequent treatment) as

$$V = k_{app}(\text{E}\cdot\text{AA}\cdot\text{AMP})(\text{PP}) \tag{7}$$

where k_{app} is an apparent second-order rate constant which may be a function of concentration, depending on the mechanism of eq 1a. The equilibrium concentration of adenylate complex, (E·AA·AMP), may readily be expressed in terms of the total enzyme concentration and concentrations of the various substrates by applying the condition of mass conservation to the enzyme, in conjunction with dissociation constants for various enzyme–substrate complexes. Thus, the concentration dependence of V, which depends on the mechanism (see below), arises both in the kinetic parameter, k_{app}, and in the expression for the equilibrium concentration of (E·AA·AMP). Rate laws for some mechanisms of amino acid activation are now considered.

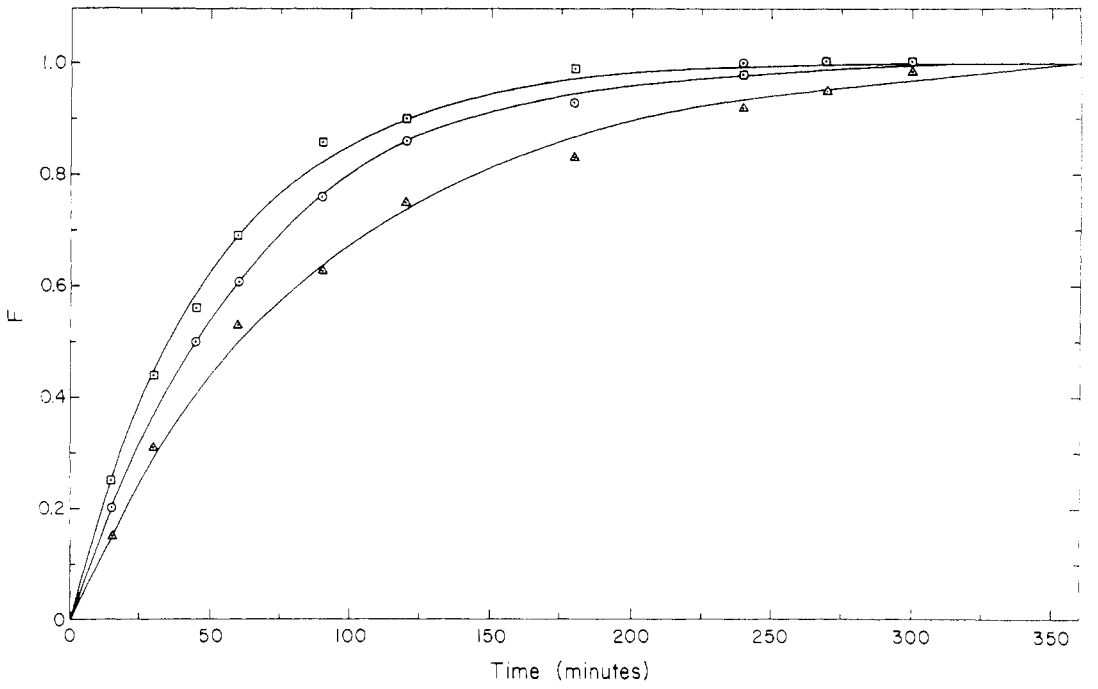

FIGURE 1: Fractional attainment of equilibrium F *vs.* time. The points are experimental values and the curves are calculated values (see text) Concentrations employed were: (□) — ATP (1 mM), pyrophosphate (3 mM); (○) ATP (2 mM), pyrophosphate (2 mM); (△) ATP (3 mM), pyrophosphate (1 mM); enzyme and isoleucine concentrations were, respectively, 5 mμM and 2 mM for all experiments.

Mechanism I

Simultaneous Binding of ATP and AA. The simplest, though perhaps most unlikely mechanism for eq 1a is

$$E + AA + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E\cdot AA\cdot AMP + PP \qquad (I)$$

where X is an enzyme intermediate and the k_i's are rate constants. This mechanism postulates that the enzyme, amino acid, and ATP come together in a ter-molecular reaction. This is a very unlikely possibility, but it provides a simple illustration of the derivation of the rate law. With the usual steady-state approximation on the radioactive intermediate (Boyer, 1959; Alberty *et al.*, 1962; Yagil and Hoberman, 1969), the rate of production of (ATP*) is given by

$$\frac{d(ATP^*)}{dt} = \frac{-d(PP^*)}{dt} = k_{-2}(E\cdot AA\cdot AMP)(PP^*) - k_2(X^*) \qquad (8)$$

and the rate equation for (X*) is

$$\frac{-d(X^*)}{dt} = 0 = (k_{-1} + k_2)(X^*) - k_1(E)(AA)(ATP^*) - k_{-2}(E\cdot AA\cdot AMP)(PP^*) \qquad (9)$$

At early times (ATP*) $\approx$ 0 and the term in eq 9 involving (ATP*) may be neglected, thus giving

$$(X^*) = \frac{k_{-2}}{k_{-1} + k_2}(E\cdot AA\cdot AMP)(PP^*) \qquad (10)$$

Substituting eq 10 into eq 8 and dividing both sides of the resulting equation by $s(0)$ (*cf.* eq 6) gives eq 7 with

$$k_{app} = \frac{k_{-1}k_{-2}}{k_{-1} + k_2} \qquad (11)$$

The equilibrium concentration of adenylate complex is given by

$$(E\cdot AA\cdot AMP) = \frac{(E_0)}{1 + K_{eq}\dfrac{(PP)}{(AA)(ATP)} + \dfrac{(PP)}{K_x}} \qquad (12)$$

where $(E_0)(= (E) + (X) + (E\cdot AA\cdot AMP))$ is the total enzyme concentration and the equilibrium constants K_{eq} and K_x are

$$K_{eq} = \frac{(E)(AA)(ATP)}{(E\cdot AA\cdot AMP)(PP)} \qquad (13a)$$

and

$$K_x = \frac{(E\cdot AA\cdot AMP)(PP)}{(X)} \qquad (13b)$$

Substituting eq 12 into eq 7 gives

$$V = \frac{k_{app}(E_0)(PP)}{1 + K_{eq}\dfrac{(PP)}{(AA)(ATP)} + \dfrac{(PP)}{K_x}} \qquad (14a)$$

or

$$\frac{(E_0)}{V} = \frac{1}{k_{app}}\left(\frac{1}{K_x} + \frac{1}{(PP)} + \frac{K_{eq}}{(AA)(ATP)}\right) \quad (14b)$$

with k_{app} given by eq 11.

Mechanism II

Sequential Addition of Substrates. ATP FIRST. An obvious possible mechanism for amino acid activation is the sequential addition of substrates to the enzyme. For the case that ATP is first bound the mechanism is

$$\begin{aligned} E + ATP &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot ATP \\ E\cdot ATP + AA &\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X \\ X &\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\cdot AA\cdot AMP + PP \end{aligned} \quad (II)$$

The rate law for this mechanism may be obtained in exactly the same fashion as that for I. In this case, however, the steady-state approximation is applied to both (X*) and (E·ATP*). The resulting rate law is

$$\frac{(E_0)}{V} = \frac{1}{k_{app}}\left(\frac{1}{K_x} + \frac{1}{(PP)} + \left(\frac{K_{eq}}{K_{ATP}}\right)\frac{1}{(AA)} + \frac{K_{eq}}{(AA)(ATP)}\right) \quad (15a)$$

where

$$k_{app} = \frac{k_{-1}k_{-2}k_{-3}}{k_2k_3(AA) + k_{-1}k_3 + k_{-1}k_{-2}} \quad (15b)$$

and K_{ATP} is the dissociation constant of the enzyme–ATP complex.

Mechanism III

Sequential Addition of Substrates. AA FIRST. For the case that amino acid is first bound to the enzyme the mechanism is

$$\begin{aligned} E + AA &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot AA \\ E\cdot AA + ATP &\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X \\ X &\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\cdot AA\cdot AMP + PP \end{aligned} \quad (III)$$

and the rate law is given by

$$\frac{(E_0)}{V} = \frac{1}{k_{app}}\left(\frac{1}{K_x} + \frac{1}{(PP)} + \left(\frac{K_{eq}}{K_{AA}}\right)\frac{1}{(ATP)} + \frac{K_{eq}}{(AA)(ATP)}\right) \quad (16a)$$

where

$$k_{app} = \frac{k_{-2}k_{-3}}{k_{-2} + k_3} \quad (16b)$$

and K_{AA} is the dissociation constant of the enzyme–amino acid complex.

Mechanism IV

Random Addition of Substrates. The random addition mechanism is

$$\begin{aligned} E + AA &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot AA \\ E + ATP &\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot ATP \\ E\cdot AA + ATP &\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} X \\ E\cdot ATP + AA &\underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} X \\ X &\underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E\cdot AA\cdot AMP + PP \end{aligned} \quad (IV)$$

The rate law for IV is

$$\frac{(E_0)}{V} = \frac{1}{k_{app}}\left(\frac{1}{K_x} + \frac{1}{(PP)} + \left(\frac{K_{eq}}{K_{AA}}\right)\frac{1}{(ATP)} + \left(\frac{K_{eq}}{K_{ATP}}\right)\frac{1}{(AA)} + \frac{K_{eq}}{(AA)(ATP)}\right) \quad (17a)$$

where

$$k_{app} = \frac{k_{-5}(k_{-3}k_4(AA) + k_{-2}k_{-3} + k_{-2}k_{-4})}{k_4(k_{-3} + k_5)(AA) + k_{-2}(k_{-3} + k_{-4} + k_5)} \quad (17b)$$

Discussion of Rate Laws and Mechanisms

The preceding analysis establishes a quantitative framework for the interpretation and analysis of experimental investigations of the isotope exchange reaction of eq 1a. Careful inspection of the rate laws for mechanisms I–IV reveals that their concentration dependencies are each distinct and unique. Therefore, in principle these mechanisms may be distinguished experimentally. The terms multiplying k_{app}^{-1} in eq 14a, 15a, 16a, and 17a are equal to $(E_0)/(E\cdot AA\cdot AMP)(PP)$, according to eq 7. Thus, the concentration dependence of these terms is determined solely by thermodynamic considerations; *viz.*, by the equilibrium concentrations of E·AA·AMP and PP. These terms can be examined, therefore, to obtain equilibrium constants. The concentration dependence of k_{app} is a function of the kinetic mechanism of exchange, however.

The treatment of mechanisms given above considered only 1:1 complexes between enzyme and substrates. The presence of higher order complexes will give rise to different concentration dependencies for the various rate laws. Hence, the stoichiometry of the complexes can be inferred from a careful

TABLE I: Time for Establishment of Equilibrium among Unlabeled Species.[a]

Expt	Time of Incubn of Unlabeled Reactants before Addn of [^{32}PP]	0 min[b]	30 min	60 min	90 min
1	ATP, 2 mM; PP, 0.2 mM; Ile, 2 mM; Mg^{2+}, 3 mM	4.0	3.8	4.1	4.1
2	ATP, 2 mM; PP, 4 mM; Ile, 2 mM; Mg^{2+}, 8 mM	28.0	28.4	28.0	27.8
3	ATP, 2 mM; PP, 2 mM; Ile, 10 μM; Mg^{2+}, 5 mM	9.0	9.0	8.7	9.1

[a] Enzyme and all unlabeled reactants were mixed and incubated at 25°. At various times thereafter a pulse of [^{32}P]PP was added and the isotope exchange occurring in 15 min was measured in the usual fashion (*cf.* Materials and Methods). Numbers in the table refer to millimicromoles per milliliter of pyrophosphate synthesized from ATP in 15 min. In all experiments the enzyme concentration was 4.35×10^{-10} M. [b] In this case [^{32}P]PP was added prior to incubation.

study of the concentration dependence of the exchange kinetics.

Mention should be made of the fact that the rate laws for mechanisms I–IV are independent of the number of sequentially occurring ternary intermediates X_i, as is the case in steady-state enzyme kinetics (Peller and Alberty, 1959). In general, therefore, the parameter k_{app} is a complicated aggregate of rate constants, but it retains the same concentration dependence as given in the equations above. In addition, the total concentration ($\Sigma(X_i)$) of all species X_i replaces "(X)" in the expression for the equilibrium constant K_x in eq 13b.

It should be pointed out that some of the general predictions of the rate laws for mechanisms I–IV have been substantially confirmed. For example, all of the rate laws state that V is a linear function of (E_0); this prediction has been confirmed for several different synthetases (Bergmann *et al.*, 1961; Calendar and Berg, 1966a; Baldwin and Berg, 1966). Furthermore, in most instances the rate laws predict that a linear relationship exists between V^{-1} and $(AA)^{-1}$, $(ATP)^{-1}$, or $(PP)^{-1}$. This expectation has also been substantially verified (see, for example, Bergmann *et al.*, 1961, Calendar and Berg, 1966c, and Rouget and Chapeville, 1968; see also below).

The mechanisms discussed above may obviously be amended to include additional effects. For example, the activity of tRNA synthetases is known to be quite sensitive to the concentration of magnesium (Novelli, 1967). This phenomenon is considered elsewhere, within the framework of the preceding treatment (F. X. Cole and P. R. Schimmel, in preparation). Another interesting possibility is to account for the effect of amino acid specific tRNA on the exchange reaction. This is an important consideration since it is quite possible that the nucleic acid is nearly always associated with the enzyme *in vivo* (*cf.* Novelli, 1967). Although detailed quantitative studies have not been conducted, it has been shown that the effect of tRNA differs with different synthetases (see, for example, Hele, 1964, Ravel *et al.*, 1965, Hele and Barth, 1966, Lazzarini and Mehler, 1966, Calendar and Berg, 1966b, and Mitra and Mehler, 1966, 1967). In fact, a few tRNA synthetases do not even catalyze ATP–PP* isotope exchange in the absence of tRNA (Ravel *et al.*, 1965; Lazzarini and Mehler, 1966; Mitra and Mehler, 1966, 1967; Mehler and Mitra, 1967). Although the discussion given above does not explicitly consider such cases, it may easily be amended to treat tRNA-dependent exchange. It should also be mentioned that the considerations given above are also applicable to the analogous reactions of acetate thiokinase (Berg, 1956) and firefly luciferase (McElroy, 1962).

Results and Discussion of Experiments

All experiments were carried out with highly purified Ile-tRNA synthetase from *E. coli* B at pH 8.0, 25° (see Materials and Methods).

Time for Establishment of Equilibrium among Unlabeled Species. The preceding discussion applies to equilibrium isotope exchange. Experiments were conducted, therefore, to ascertain the time required for equilibrium to be established among the unlabeled species in eq 1a. Enzyme and unlabeled substrates were mixed and incubated at 25°; at varying times thereafter tracer quantities of PP* were added to the mixture, which was subsequently incubated for an additional 15 min. ATP was then isolated and the amount of PP* incorporated was determined. The results of such experiments, at several different extremes of substrate concentration, are summarized in Table I. In all cases it was found that the amount of PP* exchanged into ATP is independent of the length of the time of prior incubation of unlabeled species. This implies that equilibrium among the unlabeled species in eq 1a is essentially instantaneous (under the conditions and concentrations employed). In subsequent experiments, therefore, PP* was mixed almost simultaneously with the unlabeled substrates.

The Effect of Magnesium. The rate of isotope exchange is strongly dependent on the level of magnesium. In the absence of magnesium, no significant exchange occurs. At a given level of ATP and pyrophosphate (generally about 10^{-4}–10^{-3} M), increasing concentrations of magnesium give increased rates of exchange until a maximal rate is achieved. At this point the exchange rate is insensitive to further small increases in the level of magnesium, but very high concentrations (*ca.* 10 mM) cause a decrease in rate. In view of these findings, all experiments described below were done under conditions whereby the exchange rate is maximal, and insensitive to small variations in magnesium concentration. Under these conditions, ATP and pyrophosphate are present almost entirely as their monomagnesium complexes (F. X. Cole and P. R. Schimmel, in preparation). A detailed analysis of the effect of magnesium will be published in the future (F. X. Cole and P. R. Schimmel, in preparation).

Concentration Dependence of Exchange Rate. The concentration dependence of the exchange rate was investigated by a permutational variation of the concentrations of ATP, pyrophosphate, and isoleucine. The range of concentrations inves-

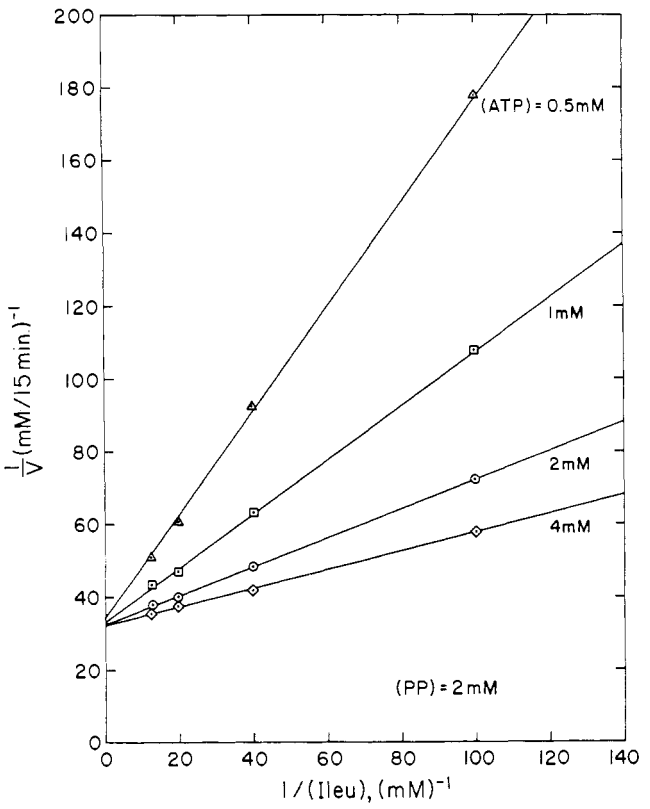

FIGURE 2: Double-reciprocal plots of V *vs.* isoleucine concentration at various indicated ATP concentrations; enzyme concentration, 0.65 mμM.

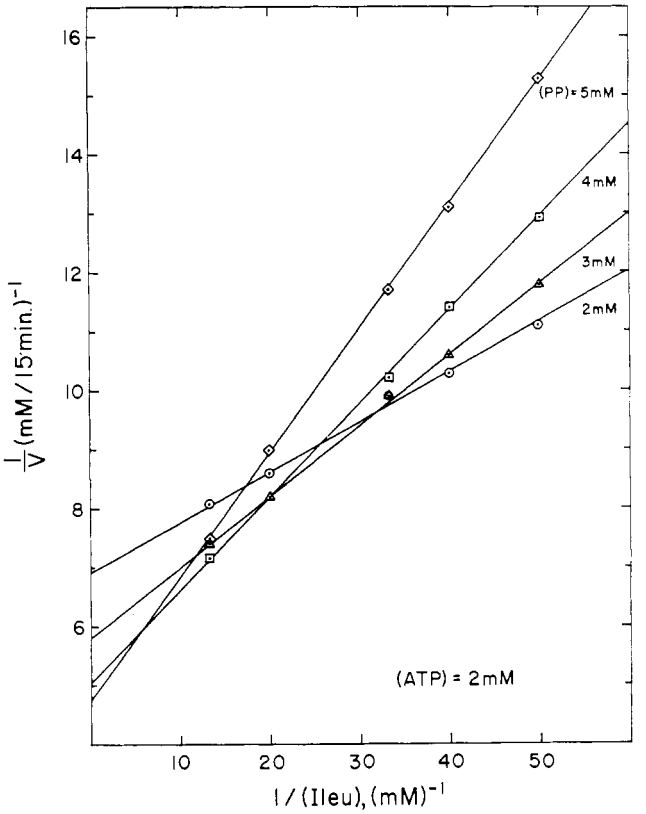

FIGURE 3: Double-reciprocal plots of V *vs.* isoleucine concentration at various indicated pyrophosphate concentrations; enzyme concentration, 3 mμM.

tigated were: ATP, 0.2–4 mM; pyrophosphate, 0.2–5 mM; and isoleucine, 5 μM–10 mM. It was not possible to investigate lower concentrations of ATP and pyrophosphate because of inherent difficulties in obtaining accurate and reproducible measurements at low concentrations of these substrates. Representative data obtained are summarized below.

Some plots of V^{-1} *vs.* $(AA)^{-1}$, at different ATP and pyrophosphate concentrations, are given in Figures 2 and 3. Analogous plots of V^{-1} *vs.* $(ATP)^{-1}$ at different concentrations of isoleucine and pyrophosphate are given in Figures 4 and 5. Many of the individual points in these figures represent the averages of several experimental determinations. It is apparent that the slopes and intercepts of these lines are themselves concentration dependent. Therefore, secondary plots of the data were constructed. In Figure 2, both the slopes and the intercepts are linear functions of $(ATP)^{-1}$; in Figure 3, the intercepts are linear functions of $(PP)^{-1}$, but the slopes are linear with (PP). A similar behavior is apparent in Figures 4 and 5, with $(ATP)^{-1}$ replacing $(AA)^{-1}$ as the abscissa variable. From these and additional data the rate V was found to obey the following relationship

$$\frac{(E_0)}{V} = \frac{1}{k_{app}}\left(\frac{1}{(PP)} + \frac{\phi_1}{(AA)} + \frac{\phi_2}{(ATP)} + \frac{\phi_3}{(AA)(ATP)} + \frac{\phi_4(PP)}{(AA)(ATP)} + \phi_5\right) \quad (18)$$

where k_{app} is an apparent (concentration independent) second-order rate constant and the ϕ_i's are constants. These parameters are readily obtained from plots such as those described above. This rate law quantitatively accounts for all of the data.

It is clear that the rate law of eq 18 is somewhat different from any of those given for the three simple mechanisms II, III, and IV. However, careful inspection of eq 18 permits the identification of each term with a particular enzyme–substrate complex. This may be done by making use of the fact that according to eq 7 the terms multiplying k_{app}^{-1} in eq 18 are equal to $[(E\cdot AA\cdot AMP)(PP)]^{-1} \times (E_0)$ [*viz.*, eq 7 may be written as $(E_0)/V = (1/k_{app})((E_0)/(E\cdot AA\cdot AMP)(PP))$]. Using mass conservation of the enzyme we can obtain the six terms in eq 18 if we postulate the following six enzyme species

$$(E_0) = (E\cdot AA\cdot AMP) + (E\cdot ATP) + (E\cdot AA) + (E) + (E\cdot PP) + (X) = (E\cdot AA\cdot AMP)(PP)\left(\frac{1}{(PP)} + \frac{\phi_1}{(AA)} + \frac{\phi_2}{(ATP)} + \frac{\phi_3}{(AA)(ATP)} + \frac{\phi_4(PP)}{(AA)(ATP)} + \phi_5\right) \quad (19b)$$

where

$$\phi_1 = \frac{K_{eq}}{K_{ATP}} \quad (20a)$$

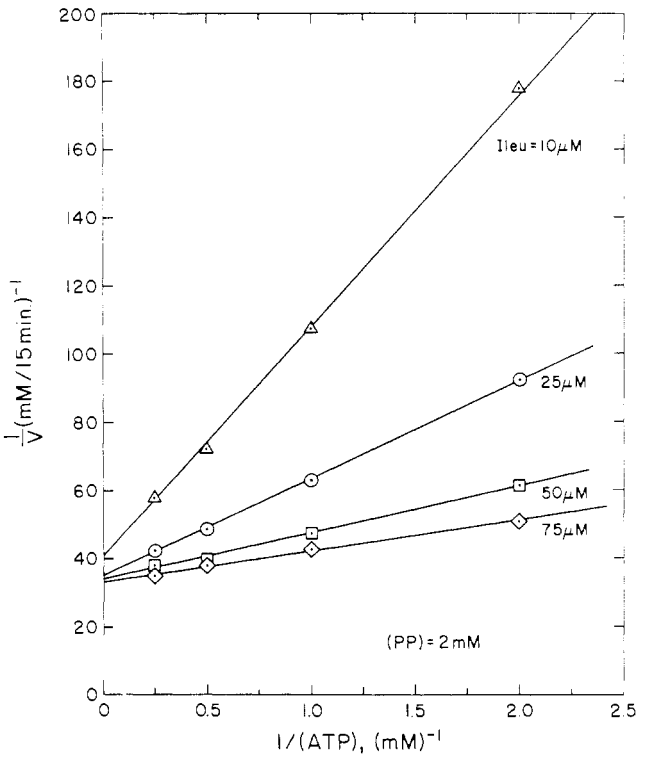

FIGURE 4: Double-reciprocal plots of V *vs.* ATP concentration at various indicated isoleucine concentrations; enzyme concentration, 0.65 mμM.

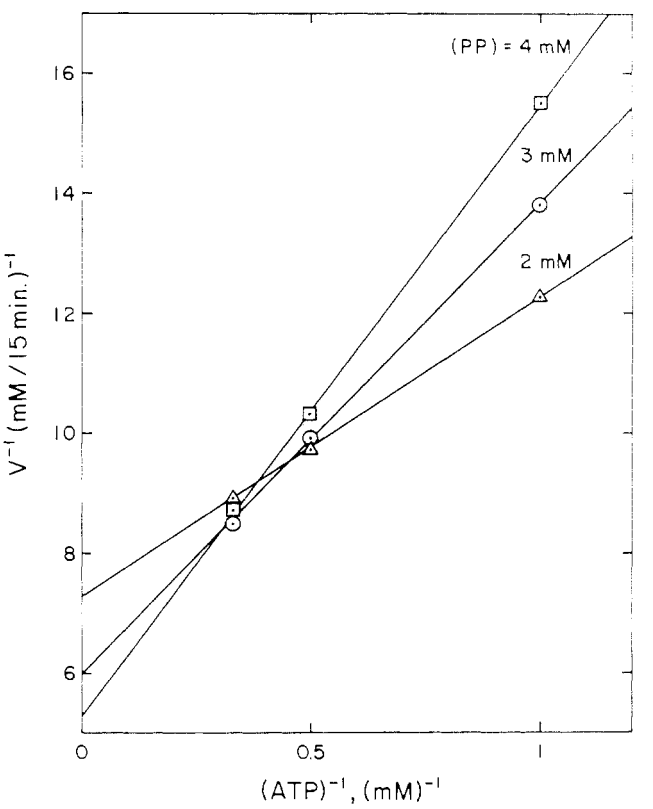

FIGURE 5: Double-reciprocal plots of V *vs.* ATP concentration at various indicated pyrophosphate concentrations; enzyme concentration, 3 mμM; isoleucine, 2 mM.

$$\phi_2 = \frac{K_{eq}}{K_{AA}} \tag{20b}$$

$$\phi_3 = K_{eq} \tag{20c}$$

$$\phi_4 = \frac{K_{eq}}{K_{PP}} \tag{20d}$$

$$\phi_5 = \frac{1}{K_x} \tag{20e}$$

and E·PP is an enzyme–pyrophosphate complex having a dissociation constant of K_{PP}. Note that the order of the terms in eq 19b is the same as that in eq 19a, *e.g.*, (E·AA) = $\phi_2 \times$ (E·AA·AMP)(PP)/(ATP).

The existence of these particular equilibrium enzyme complexes does not clearly delineate the *kinetic pathway* of amino acid activation, however. In fact each of the mechanisms (II, III, or IV) can be modified to give rise to the rate law of eq 18. For example, mechanism III gives rise to this rate law if two equilibria involving *abortive* complexes of ATP and PP are added to III. This mechanism (mechanism III′) thus involves the ordered addition of substrates, with isoleucine binding first; it also postulates the existence of abortive ATP– and pyrophosphate–enzyme complexes. However, a similar modification may be made of mechanisms II and IV to give II′ and IV′, respectively. II′ is obtained by adding equilibria for the formation of abortive E·AA and E·PP complexes to II; IV′ is obtained by adding to IV a step for the formation of an abortive E·PP complex. The three mechanisms (II′, III′, and IV′) each involve the same enzyme–substrate complexes. Consequently, the concentration dependence of the terms multiplying k_{app} are the same for the three mechanisms. However, each mechanism has a different kinetic pathway of amino acid activation and consequently each has a characteristic dependence of k_{app} on concentration. In the case of Mechanisms II′ and IV′, k_{app} is a function of amino acid concentration (*cf.* eq 15b and 17b, respectively); k_{app} is concentration independent in the case of III′ (*cf.* eq 16b). Experiments shown in Figure 6 demonstrate that the parameter k_{app} is constant over a very wide range (2000-fold) of amino acid concentration. The curves in this figure are calculated values of V using eq 18, and assuming that k_{app} is concentration independent; the points are experimental determinations. The agreement between calculated and observed quantities is excellent, thus demonstrating that k_{app} is essentially concentration independent. This fact by itself would tend to rule out mechanisms II′ and IV′. However, the relative values of the rate constants in the expressions for k_{app} (as given by eq 15b and 17b) could be such that this parameter is effectively constant over a wide concentration range. For this reason, no clear-cut choice can be made between the three mechanisms.

Regardless of which mechanism (II′, III′, or IV′) is operative, the parameters ϕ_i in eq 18 are readily interpreted in terms of the various equilibrium constants of the reaction (see eq 20). These equations provide five relationships in five unknowns, so that all the equilibrium constants may be deter-

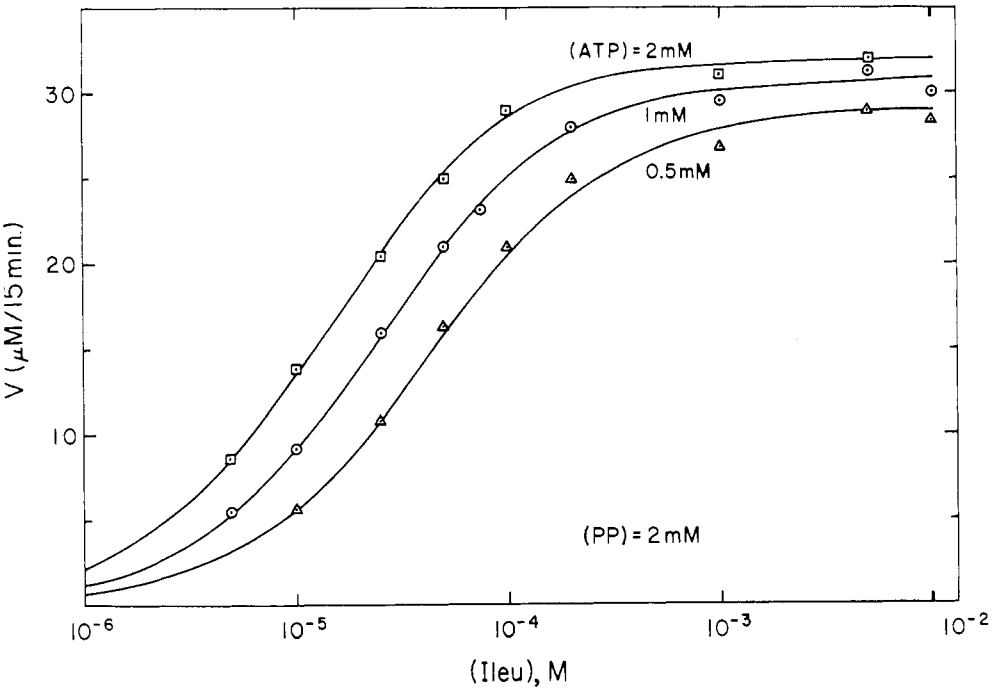

FIGURE 6: Plot of V *vs.* isoleucine concentration. The curves are calculated as described in the text and the points are experimental values; enzyme concentration, 0.65 mμM.

mined. These equilibrium constants and k_{app} are given in Table II.

The existence of the inhibitory pyrophosphate complex was implied from the behavior of the slopes and intercepts such as those given in Figures 2–5. In order to further substantiate the existence of the E·PP complex, we investigated the exchange rate over a range of pyrophosphate concentration sufficient to observe more directly both activation and inhibition, and to ascertain whether or not eq 18 correctly predicts the pyrophosphate concentration of maximal activity. The results are shown in Figure 7. The curve is calculated according to eq 18, using values of the parameters defined by eq 20a–e and given in Table II; the points are experimental values. The agreement between calculated and experimental values is good, thus providing additional support for the above analysis and interpretation.

Discussion of Experimental Results

Very little is known about the active site of tRNA synthetases. The data obtained here strongly suggest that only one molecule of each of the species in eq 1a binds per molecule of enzyme. It is to be noted that Norris and Berg (1964) found previously that only one molecule of AA-AMP is bound tightly per molecule of enzyme. It is interesting to note that the binding of amino acid to free enzyme is considerably stronger than that of ATP (*cf.* Table II). This is consistent with the results of Iaccarino and Berg (1969) who found that much higher concentrations of ATP than of amino acid are required

TABLE II: Equilibrium and Kinetic Parameters, pH 8.0, 25°.

K_{AA} (M)	4.0×10^{-6}
K_{ATP} (M)	1.5×10^{-4}
K_{PP} (M)	3.0×10^{-5}
K_{eq} (M)	3.3×10^{-7}
K_x (M)	2.0×10^{-3}
k_{app} (M^{-1} sec^{-1})	5.6×10^{4}

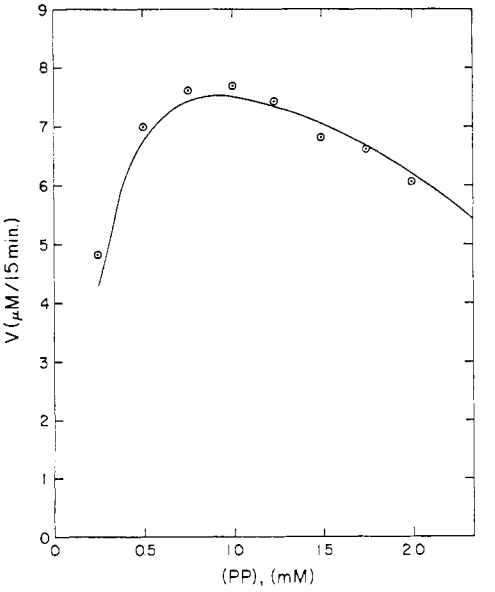

FIGURE 7: V *vs.* pyrophosphate concentration. The points are experimental values and the curve gives calculated values (see text). Concentrations of other reactants are: isoleucine (10 μM), ATP (1 mM), and enzyme (0.44 mμM).

to block a critical active-site sulfhydryl from reacting with *p*-hydroxymercuribenzoate. Binding of isoleucine, but not ATP, is believed to cause a conformational change in the enzyme which in turn causes an increase in the rate at which $tRNA_{Ile}$ enters and leaves its binding site (Yarus and Berg, 1969). Hence, the greater stability of the enzyme–isoleucine complex may be derived from a conformational change in the enzyme which accompanies binding.

It is interesting to observe that no terms in the rate law were found which correspond to the formation of an E·PP·ATP or an E·PP·AA complex, over the concentration range investigated. If the binding of ATP or of isoleucine to enzyme is independent of pyrophosphate binding, then the concentrations of the ternary complexes (E·PP·ATP and E·PP·AA) would be so large—at high pyrophosphate concentrations—that the discrepancy between the calculated curve and the experimental points in Figure 7 would be considerable. In fact, there is no suggestion of such complexes in any of our experiments. Therefore, bound pyrophosphate prevents or lowers considerably the binding of ATP or isoleucine, and conversely bound ATP or isoleucine must prevent or lower pyrophosphate binding. We estimate that the respective dissociation constants must be increased at least an order of magnitude when the antagonist is bound. The precise mechanism of this reciprocal binding phenomenon is not clear, although some intriguing possibilities exist.

We have obtained a value of 3.3×10^{-7} M for the equilibrium constant of eq 1a (*cf.* Table II). There are no other published equilibrium constants for the activation reaction of AA-tRNA synthetases. However, eq 1a is exactly analogous to the reaction of firefly luciferase in which a luciferase–acyladenylate complex is formed (with the concomitant release of pyrophosphate) from oxyluciferin, ATP, and luciferase (Rhodes and McElroy, 1958). The equilibrium constant for this reaction has been determined by Rhodes and McElroy (1958) to be 4×10^{-6} M at pH 7.1 (about room temperature). This equilibrium constant is reasonably close to that determined here, considering the differences in the reactants and the conditions employed.

Loftfield and Eigner (1969) have recently reported data which they believe cast doubt on the existence of the enzyme–aminoacyl adenylate complex as an intermediate in the synthesis of AA-tRNA. In the presence of tRNA, they postulate a concerted mechanism of formation of AA-tRNA, without an enzyme-bound aminoacyl adenylate intermediate. In the absence of tRNA, the adenylate complex is believed to form, however, since ATP–PP* exchange occurs. These conclusions are based largely on observations of inhibition of ATP–PP* exchange by certain nucleophiles and inhibition of hydroxamate formation by pyrophosphate. The patterns of inhibition observed are interpreted as being inconsistent with enzyme–aminoacyl adenylate complex formation. However, it appears that their observations can be more simply explained as being due to the formation of inhibitory complexes—for example, the case of the E·PP complex discussed above—without postulating a concerted reaction mechanism. Furthermore, the assumption is made that eq 1a is the rate-determining step in the formation of AA-tRNA (Loftfield and Eigner, 1969); this assumption is in direct opposition to the results of Berg *et al.* (1961). In addition, Iaccarino and Berg (1969) have recently reported data which imply that the rate of formation of isoleucyl hydroxamate is limited by the rate of reaction of hydroxylamine with the adenylate complex, and not by the rate of formation of the adenylate complex.

Materials and Methods

E. coli B cells were purchased from Grain Processing Co., Muscatine, Iowa. ATP (Sigma) was purified by chromatography on DEAE-cellulose utilizing linear gradients of triethylammonium bicarbonate buffers (Khorana, 1963). [^{32}P]PP was a product of New England Nuclear. L-Isoleucine (Mann) was determined to have a specific rotation of $[\alpha]_D^{25}$ +39° in 5 N HCl, which compares with the published value of 39.5° under the same conditions (Meister, 1965). All other chemicals used were of reagent quality.

Ile-tRNA synthetase was isolated from *E. coli* B by a modification of the method of Baldwin and Berg (1966). Protein was determined by the method of Lowry *et al.* (1951). The specific activity of the freshly purified enzyme was 650 units/mg, where one unit is defined as the formation of 1 μmole of [^{32}P]ATP from [^{32}P]PP in 15 min at 37° in the standard assay mixture (Baldwin and Berg, 1966). The published value of the molecular weight (112,000, Baldwin and Berg, 1966) was used to calculate the enzyme concentration.

Unless otherwise noted all isotope exchange data reported here were obtained by the procedure of Calendar and Berg (1966a) in a buffer system which contained 0.1 M sodium acetate, 0.3 M sodium chloride, and 0.1 M Tris, titrated to pH 8.0 with acetic acid. All experiments were done at 25 ± 0.2°.

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Cell-Free Transfer of Leucine by Transfer Ribonucleic Acid from Mouse Liver and Plasma Cell Tumors into Rabbit Hemoglobin*

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ABSTRACT: Chromatographic differences in leucyl transfer ribonucleic acids have been previously demonstrated among several mouse plasma cell tumors which synthesize κ-type immunoglobulin light chains. To investigate whether these chromatographically different transfer ribonucleic acids also differed functionally, transfer ribonucleic acid from two such tumors as well as from normal mouse liver was used to transfer leucine into hemoglobin in a cell-free system derived from rabbit reticulocytes. The two tumors chosen, MOPC 46B and MOPC 149, secrete light chains differing in leucine placement in at least one position and have different leucyl transfer ribonucleic acid patterns when chromatographed on a reversed-phase column. The leucyl transfer ribonucleic acid from MOPC 46B was like that from mouse liver except for differences in the relative amounts of each distinguishable isoaccepting transfer ribonucleic acid. MOPC 149, on the other hand, showed the apparent loss of a component peak of leucyl transfer ribonucleic acid compared with MOPC 46B. Despite these qualitative and quantitative differences in chromatographic pattern, all the mouse leucyl transfer ribonucleic acid preparations, normal and neoplastic, transferred labeled leucine into the proper hemoglobin α-chain peptides.

Transacylation under these conditions of protein synthesis was ruled out by demonstrating that two fractions of rabbit liver leucyl transfer ribonucleic acid separated on DEAE-Sephadex transferred leucine into different hemoglobin peptides. Thus, it is concluded that transfer ribonucleic acid from tumors as well as normal tissue can be utilized by protein-synthesizing components from another mammal. Further, these experiments show no evidence for variation in translation of natural hemoglobin α-chain message when transfer ribonucleic acid from either of two different mouse plasma cell tumors is used.

Previous work from this laboratory has demonstrated significant, stable, and reproducible differences in Leu-tRNA from κ-type-light-chain-producing mouse plasma cell tumors analyzed chromatographically on a reversed-phase column (Mushinski and Potter, 1969). For the purpose of investigating the functional significance of these findings, the Leu-tRNAs from two such tumors were studied for their ability to transfer leucine into protein as directed by a natural mammalian messenger.

The possibility of translational variations playing a role in shaping mammalian protein sequences has been postulated for immunoglobulins (Potter *et al.*, 1965; von Ehrenstein, 1966; Mach *et al.*, 1967; Campbell, 1967) and also for rabbit hemoglobin (von Ehrenstein, 1966). One of the possibilities for shaping protein structure during translation would be a regulation of the populations of tRNAs available during polypeptide synthesis. If certain exceptional messengers had regions or codons which could be translated by several tRNAs

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