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Investigation of the Transfer of Amino Acid from a Transfer Ribonucleic Acid Synthetase–Aminoacyl Adenylate Complex to Transfer Ribonucleic Acid*

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ABSTRACT: The kinetics of transfer of isoleucine (Ile) from Ile-tRNA synthetase (IRS)–aminoacyl adenylate complex to $tRNA^{Ile}$ was investigated. Complex of enzyme with isoleucyl adenylate (Ile∼AMP) was isolated on a Sephadex column and reacted with $tRNA^{Ile}$ under a variety of conditions. It is shown that the reaction proceeds *via* a rapid initial formation of IRS-bound Ile—$tRNA^{Ile}$ followed by a slow release of the charged $tRNA^{Ile}$ from IRS. Neither step is significantly altered by the absence or presence (up to 5 mM) of added Mg^{2+}. Experiments employing an excess of Ile∼AMP over IRS demonstrate that a new molecule of Ile∼AMP binds to IRS prior to the dissociation of the just synthesized Ile—$tRNA^{Ile}$. Furthermore, the Ile∼AMP stimulates the release of enzyme-bound Ile—$tRNA^{Ile}$. It is also shown that synthesis of Ile—$tRNA^{Ile}$ starting from ATP and isoleucine proceeds with a rapid initial production of IRS-bound Ile—$tRNA^{Ile}$, followed by a much slower production of additional molecules of Ile—$tRNA^{Ile}$ in subsequent catalytic cycles of the enyzme. The slow phase results from the necessity of completing release of bound Ile—$tRNA^{Ile}$ before new Ile—$tRNA^{Ile}$ can be made. The results obtained support and extend the evidence from nitrocellulose filtration studies (Yarus, M., and Berg, P. (1969), *J. Mol. Biol. 42*, 171) that release of aminoacyl-tRNA is the rate-determining step in the aminoacylation of tRNA and that this release is promoted by Ile∼AMP. In addition, an examination of various kinetic data provides strong evidence against the hypothesis advanced by others that aminoacylation of tRNA proceeds *via* a concerted mechanism in which the aminoacyl adenylate is not an intermediate. A slightly modified purification procedure for IRS is given which improves the yield of enzyme by about 250% over an earlier published procedure.

The AA-tRNA[1] synthetase reaction involves the specific attachment of an amino acid to its cognate tRNA, the reaction involving a distinct tRNA synthetase for each amino acid. It is customarily written as a two-step process in which an enzyme–aminoacyl adenylate complex is first formed, followed by reaction of the complex with tRNA (Berg, 1961; Novelli, 1967). The two steps are

$$E + AA + ATP \overset{Mg^{2+}}{\rightleftharpoons} E{\cdot}AA{\sim}AMP + PP_i \quad (1)$$

$$E{\cdot}AA{\sim}AMP + tRNA \rightleftharpoons AA\text{-}tRNA + AMP + E \quad (2)$$

Although the amino acid specificity of eq 1 can be somewhat irregular (Baldwin and Berg, 1966a; Mitra and Mehler, 1967; Calendar and Berg, 1967), the error in the transfer step (eq 2) is negligibly small (Berg *et al.*, 1961. Loftfield *et al.*, 1963).

The kinetic and mechanistic features of eq 1 have recently been investigated (Cole and Schimmel, 1970a,b) for IRS from

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[1] Abbreviations used are: AA, amino acid; AA∼AMP, aminoacyl adenylate; AA-tRNA, aminoacyl-tRNA; BSA, bovine serum albumin; E, enzyme; IRS, isoleucyl-tRNA synthetase; Ile∼AMP, isoleucyl adenylate; Ile—tRNA, isoleucine esterified to tRNA at the 2′- or 3′-terminal ribose hydroxyl; Val-ol-AMP, L-valinyl adenylate.

Escherichia coli. In this case, the rate of amino acid activation is eighty or so times more rapid than the overall rate of aminoacylation of tRNA (Yarus and Berg, 1969; E. W. Eldred and P. R. Schimmel, unpublished data). In fact, it is true for a number of synthetases that the amino acid activation step is not rate limiting (Loftfield, 1971). Interest naturally turns therefore to eq 2, the reaction between the enzyme aminoacyl adenylate complex and tRNA. This reaction must not only be rate limiting in the aminoacylation of tRNA, but it is more specific as well (See above).

We report here a kinetic investigation of the reaction between IRS·Ile~AMP and $tRNA^{Ile}$. A number of interesting questions and ideas can be attacked with this kind of study. For example, what is the *elementary* step in eq 2 that is rate limiting. Yarus and Berg (1969) suggest that release of newly synthesized Ile—$tRNA^{Ile}$ from IRS is the rate-determining step in the aminoacylation of tRNA. This proposal is easy to test by a kinetic study of eq 2, as is shown below. It is also possible to learn whether or not a new Ile~AMP molecule is synthesized on the enzyme prior to the release of the enzyme-bound nascent Ile—$tRNA^{Ile}$, and if so, whether or not the Ile~AMP can now promote dissociation of the aminoacylated tRNA from the enzyme, as has been suggested (Yarus and Berg, 1969). Finally, it is also possible to make an indirect test of the hypothesis that aminoacylation of tRNA proceeds *via* a concerted mechanism in which the adenylate is not an intermediate (Loftfield and Eigner, 1969). These and several other questions and ideas provide the motivation for the present work.

Materials and Methods

E. coli B cells, $^3/_4$ log growth, were obtained from Grain Processing, Muscatine, Iowa, and stored at −20°. The [^{32}P]PP_i was purchased in 1-mCi lots from New England Nuclear; ATP was a product of Sigma Chemical Co. and L-isoleucine of Mann Chemical. [^{3}H]Isoleucine (34 Ci/mmole) was obtained from Schwarz BioResearch and [^{14}C]isoleucine (250 mCi/mmole) from New England Nuclear. The 2-mercaptoethanol was a product of J. T. Baker Chemical Co.

Superbrite glass beads (200 μ diameter) were purchased from Minnesota Mining and Manufacturing Co. The column materials employed in this work included DEAE-cellulose (Brown Co., Keene, N. H.), cellulose (W. and R. Balston, Ltd.), hydroxylapatite (Hypatite-C, Clarkson Chemical Co.), and Sephadex G-150, G-50, and G-25 (Pharmacia).

A modification of the Tener method (Gillam *et al.*, 1968) was used to isolate the $tRNA^{Ile}$ from crude *E. coli* tRNA (Schwarz BioResearch). (The isolation was performed by L. Dickson and A. Schreier of this laboratory.) The purified $tRNA^{Ile}$ had an amino acid acceptor ability of approximately 1025 pmoles/A_{260} in 0.1 N NaOH; concentrations were determined on this basis. Protein was determined by the method of Lowry (Lowry *et al.*, 1951), and also, during the later stages of enzyme isolation, by the approximate method of Warburg (Warburg and Christian, 1941). The published molecular weight of 112,000 was used to calculate enzyme concentration (Baldwin and Berg, 1966b).

The Whatman No. 3MM filter paper method was used for the detection of Ile—$tRNA^{Ile}$ (Hoskinson and Khorana, 1965). This technique was modified for the kinetic studies by presoaking the pads in 5% trichloroacetic acid so that the reaction is stopped immediately upon contacting the pad (a variation suggested by U. L. RajBhandary). Enzyme activity was ascertained by the amino acid dependent ATP–PP_i exchange assay. An adaptation of the Tyr-tRNA synthetase assay procedure was followed (Calendar and Berg, 1966), where 1 unit of activity is defined as the formation of 1 μmole of [^{32}P]ATP from [^{32}P]PP_i in 15 min at 37°. This exchange assay was also used in other buffer systems, at different temperatures, and at various pH values in order to determine rates of amino acid activation (*cf. seq.*).

The IRS was isolated by a modification of the method developed by Baldwin and Berg (1966b). Their published procedure consists of the following eight steps: (1) grinding with glass beads, (2) autolysis, (3) ammonium sulfate fractionation, (4) adsorption–elution from alumina C_γ gel, (5) DEAE-cellulose gradient chromatography, (6) concentrating against polyethylene glycol, (7) hydroxylapatite gradient chromatography, and (8) concentrating *via* vacuum dialysis. However, when this method was tried in our laboratory, we encountered difficulty with the alumina C_γ step. Therefore, a Sephadex G-150 column (97.5 × 5.4 cm) was substituted for the gel. This column resulted in a 2–2.5-fold purification with 95% recovery of active material as compared to a twofold increase in purity with 50% yield obtained with the alumina C_γ gel (Baldwin and Berg, 1966b). Another significant departure from the Baldwin and Berg scheme (1966b) was replacement of the polyethylene glycol concentration step with a mixed-bed column, consisting of 80% hydroxylapatite, 15% DEAE-cellulose, and 5% Sephadex G-25. (Concentrating of the enzyme is accomplished since the enzyme binds to hydroxylapatite and is eluted at a specific ionic strength. The ionic strength for elution of the enzyme can be varied by adjusting the proportion of DEAE-cellulose—higher ionic strengths being required with smaller amounts of DEAE.) The protein was adsorbed onto the column in the low ionic strength buffer (0.02 M potassium phosphate, pH 7.5), and eluted with a linear gradient of pH 7.5 potassium phosphate buffer—the mixing chamber containing 2 l. of 0.02 M buffer and the reservoir 2 l. of 0.1 M buffer. The column flow rate was adjusted to approximately 200 ml/hr and 20-ml fractions were collected. The enzyme eluted at approximately 0.15 M buffer. This step resulted in a significant concentration (about twofold) and purification (three- to fourfold) of the enzyme. The buffer was then changed to low ionic strength (0.02 M) by passage through a Sephadex G-25 column. Hydroxylapatite chromatography was conducted as described by Baldwin and Berg (1966b) except for the addition of 10% cellulose to the column material. The added cellulose gave much improved column flow rates and appeared to reduce enzyme denaturation for this step. These and other minor modifications led to a final isolation of 51 mg of pure IRS from 1 lb of *E. coli* B cells. This compares with a yield of 19 mg obtained with the earlier procedure (Baldwin and Berg, 1966b). A summary of the modified isolation procedure is presented in Table I.

The enzyme–aminoacyl adenylate complex used in these studies was isolated on a Sephadex G-50 (coarse) column (0.9 × 33 cm) at 4°. Reaction mixture (100 μg total volume) contained 0.4 nmole of [^{3}H]Ile (34 Ci/mmole), 0.2 nmole of IRS, 0.2 nmole of BSA, 0.5 μmole of $MgCl_2$, 0.2 μmole of ATP, and 1 μmole of 2-mercaptoethanol. The mixture was buffered at pH 8.0 with 0.02 M potassium phosphate, allowed to stand at 25° for 5 min, and directly applied to the column which was equilibrated at 4° with 0.01 M sodium cacodylate buffer (pH 6.0), 0.5 mM EDTA, and 0.05 M KCl. Figure 1 illustrates a typical Sephadex column separation, showing a complete resolution of complex from the small molecules. The isolated IRS·Ile~AMP complex was prepared fresh for each series of experiments, and in no case was kept for

TABLE I: Summary of the IRS-Modified Isolation Procedure from 1-lb *E. coli* B Cells.

Fraction	Total Units	Total Protein (mg)	Yield (%)	Sp Act. (Units/mg of Protein)
Crude extract	47,000	22,000		2.1
Autolysate	46,700	7,150	99	6.5
Ammonium sulfate	40,000	3,200	85	12.5
Sephadex G-150	38,300	1,540	82	25
DEAE-cellulose	34,400	284	74	121
DEAE-cellulose–hydroxylapatite	34,100	68	72	495
Hydroxylapatite-cellulose	33,200	51	70	650

more than 3 hr at 0°. Stability studies indicated little complex hydrolysis under these conditions (<5%).

Aminoacyl adenylate was synthesized enzymatically. Chemical synthesis, while easily accomplished (Berg, 1958), proved inadequate due to the impracticality of incorporating large amounts of radioactivity into the product. The enzymatic isolation procedure was as follows: (1) isolation of the enzyme aminoacyl adenylate on a Sephadex G-50 (coarse) column; (2) protein denaturation by lowering the solution to pH 1.0 with 1.0 N HCl; (3) centrifugation; (4) titration with 1.0 N NaOH to pH 6.0. This procedure destroyed all IRS activity. The Ile~AMP for each experiment was prepared fresh and showed little hydrolysis (<5% at pH 6.0 and 0°) when used within 1 hr of isolation.

The lower limit of radioactive accuracy in our experiments with commercially available [^{14}C]isoleucine (250 mCi/mmole) is about 10^{-8} M. This concentration of complex is not sufficiently low to allow the transfer reaction to be followed kinetically. Therefore, [^{3}H]isoleucine (34 Ci/mmole) was used to obtain meaningful data. The detection limit for [^{3}H]isoleucine in our studies is approximately 5×10^{-10} M.

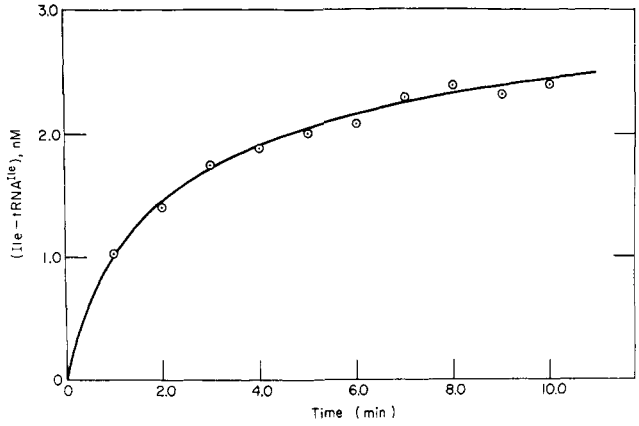

FIGURE 2: Time course for the transfer of isoleucine from the enzyme-adenylate complex to tRNA^{Ile} at pH 6.0 and 3°. Reaction mixture (1-ml total volume) contains 3.0 pmoles of IRS·[^{3}H]Ile~AMP, 1 nmole of tRNA^{Ile}, 5 μmoles of 2-mercaptoethanol, 0.5 μmole of EDTA, and 50 μmoles of KCl. Mixture buffered at pH 6.0 by 0.01 M sodium cacodylate buffer.

Experimental Results

Experiments were carried out in a temperature-controlled bath at 3°. The rapidity of the reaction under investigation, as well as the instability of the aminoacyl adenylate, precluded investigations at higher temperatures. The stability of the aminoacyl adenylate also decreases with increasing pH; a pH value of 6.0 was found to give sufficient stability for the kinetic studies discussed below.

Figure 2 gives the time course of the transfer of isoleucine from the enzyme-adenylate complex to tRNA^{Ile} (see eq 2) at pH 6.0, 3°. Under these conditions total (complex) = 3.0×10^{-9} M, total (tRNA^{Ile}) = 1.0×10^{-6} M), the reaction is almost complete in about 5 minutes. The final amount of Ile—tRNA^{Ile} produced is close to the amount of enzyme-adenylate complex added. Figure 3 displays the total concentration of Ile—tRNA^{Ile} synthesized *vs.* the concentration of enzyme-adenylate complex. A strictly linear relationship is obtained over a tenfold variation in enzyme complex concentration. The slope of this line indicates that about 85–90% of the isoleucine in the enzyme complex is transferred to

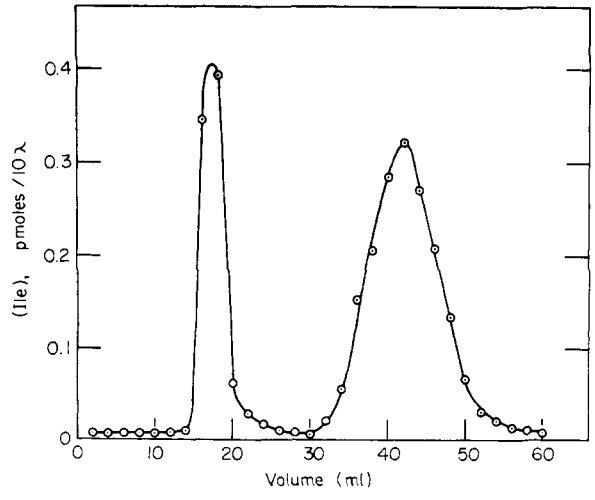

FIGURE 1: Isolation of IRS·Ile~AMP on Sephadex G-50 (coarse). The first peak on the left corresponds to the enzyme adenylate complex whereas the second peak is isoleucine. See text for details.

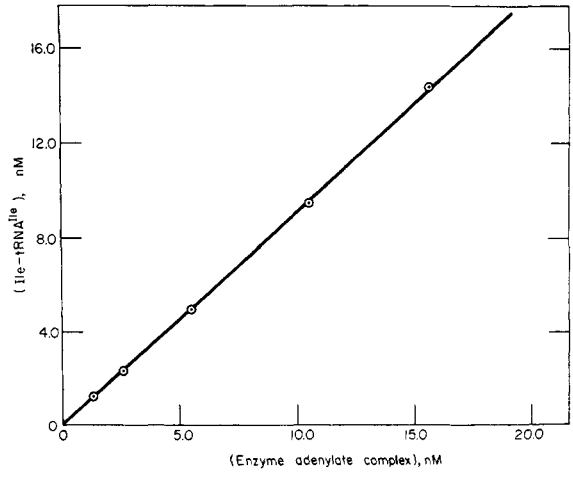

FIGURE 3: Total Ile—tRNA^{Ile} synthesized *vs.* enzyme-adenylate complex. Reaction conditions are the same as described in Figure 2, except for varying (IRS·[^{3}H]Ile~AMP).

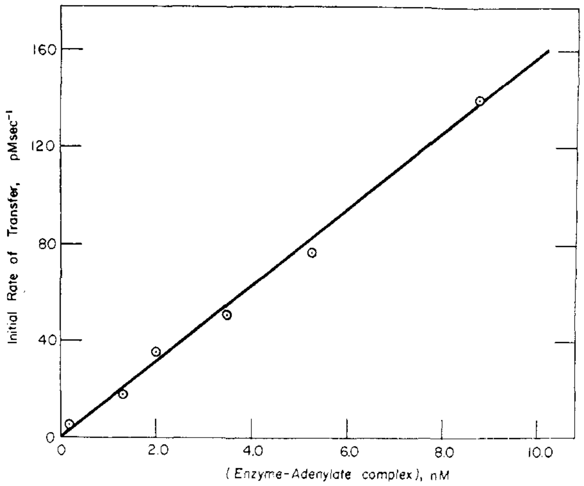

FIGURE 4: Initial velocity *vs.* (IRS·Ile∼AMP). Reaction mixture (1-ml total volume) contains IRS·[^{3}H]Ile∼AMP; 1 nmole of tRNA^{Ile}, 5 μmoles of 2-mercaptoethanol, 0.5 μmole of EDTA, and 50 μmoles of KCl. Mixture buffered at pH 6.0 by 0.01 M sodium cacodylate buffer.

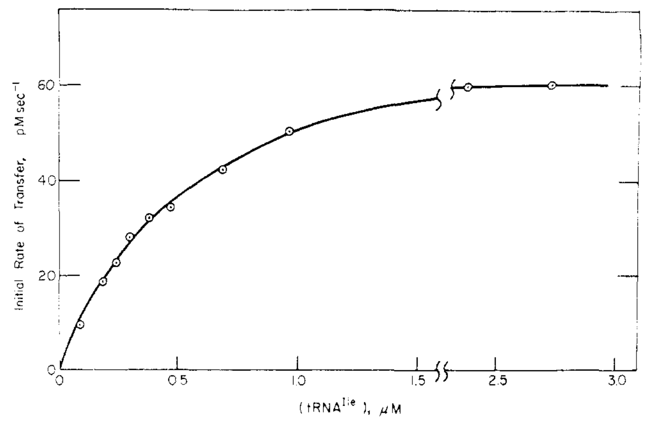

FIGURE 5: Initial velocity *vs.* tRNA^{Ile}. Reaction mixture (1-ml total volume) contains tRNA^{Ile}: 2.0 pmoles of IRS·[^{3}H]Ile∼AMP, 5 μmoles of 2-mercaptoethanol, 0.5 μmole of EDTA, and 50 μmoles of KCl. Mixture buffered at pH 6.0 by 0.01 M sodium cacodylate buffer.

tRNA^{Ile}. The fate of the remaining 10–15% is not known, but it is likely that this amount has been hydrolyzed by an unknown mechanism (see also Yarus and Berg, 1970).

The initial rate of transfer is plotted *vs.* enzyme complex in Figure 4 and *vs.* tRNA^{Ile} in Figure 5. These points were obtained from measurements of Ile–tRNA^{Ile} production after 15 sec. The initial rate is linear in Figure 4 over a 100-fold variation in enzyme complex. Figure 5 shows that at high tRNA^{Ile} the initial rate becomes independent of tRNA^{Ile}, indicating that the enzyme adenylate complex has become saturated by the formation of a ternary complex of enzyme, aminoacyl adenylate, and tRNA^{Ile}. These data are consistent with eq 3. Assuming that the ternary complex is in a steady

$$\mathrm{IRS\cdot Ile{\sim}AMP} + \mathrm{tRNA^{Ile}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ternary complex} \quad (3)$$

$$k_2 \downarrow\uparrow k_{-2}$$

$$\mathrm{AMP + IRS + Ile{-}tRNA^{Ile}}$$

state or in rapid equilibrium with IRS·Ile∼AMP + tRNA^{Ile}, the initial rate V of disappearance of tRNA^{Ile} is given by

$$V = \frac{k_2(\mathrm{IRS\cdot Ile{\sim}AMP})_0}{1 + \dfrac{K}{(\mathrm{tRNA^{Ile}})_0}} \quad (4)$$

where $K = [(k_{-1} + k_2)/k_1]$ (or $K = k_{-1}/k_1$, if the bimolecular step is rapidly equilibrated compared to k_2). The subscript 0 denotes initial concentration, and we have also made use of the fact that $(\mathrm{tRNA^{Ile}})_0 \gg (\mathrm{IRS\cdot Ile{\sim}AMP})_0$. From the data of Figure 5 a value of $K = 3.5 \times 10^{-7}$ M and of $k_2 = 1.8 \times 10^{-2}$ sec^{-1} is obtained.

Interestingly enough, the value of k_2 is obtained from Figure 5 is considerably larger than the maximal velocity per enzyme molecule (turnover number) for aminoacylation under the identical conditions. In fact, we have found that at pH 6.0, 3° the maximal velocity per enzyme molecule of amino acid activation (eq 1) is $>10^{-1}$ sec^{-1}, the maximal rate of transfer (eq 2) per enzyme molecule is $k_2 = 1.8 \times 10^{-2}$ sec^{-1}, and the turnover number for aminoacylation (eq 1 and 2) is 2.0×10^{-3} sec^{-1}. (We also found that the rate of amino acid activation at pH 6.0, 3° in the presence of tRNA^{Ile}, as measured by ATP–PP$_i$ isotope exchange, is only about 25% slower than the rate measured in the absence of tRNA^{Ile}.) Thus, with the enzyme present in catalytic amounts the turnover number for aminoacylation is at least an order of magnitude slower than the rate of each of the two individual steps which comprise the overall reaction. This apparent paradox is not unique to the conditions employed, *i.e.*, pH 6.0, 3°. Although the rate of each of the reactions accelerates markedly with increasing temperature, we qualitatively determined that the paradox of the individual steps each being more rapid than the overall reaction is true over the range of 3–37° at pH 6.0 and also over the range of pH 6.0–8.0 at 3°.

A plausible explanation for this result is the evidence from nitrocellulose filter studies (Yarus and Berg, 1969) that release of the Ile—tRNA^{Ile} from IRS is the rate-determining step in aminoacylation. That is, eq 2 perhaps consists of two steps

$$\mathrm{E\cdot AA{\sim}AMP + tRNA \rightleftharpoons E\cdot AA\text{-}tRNA + AMP} \quad (2a)$$

$$\mathrm{E\cdot AA\text{-}tRNA \rightleftharpoons E + AA\text{-}tRNA} \quad (2b)$$

of which eq 2b is the rate-limiting process in the overall transfer reaction of eq 2. This process is not necessarily assayed in our investigation of transfer since the assay for charged tRNA does not discriminate between free and enzyme-bound Ile—tRNA^{Ile}. Furthermore, transfer was studied as a "one-shot" reaction in which the enzyme does not cycle catalytically. In studies of overall aminoacylation, catalytic amounts of enzyme go through many cycles each of which must pass through the release step before another cycle can be undertaken. Hence, it is conceivable that the data cited above can be reconciled by postulating that the release of Ile—tRNA^{Ile} is rate limiting.

This notion can be tested in several ways. For example, eq 2 can be studied under conditions whereby an excess of Ile∼AMP over enzyme is employed so that the enzyme is forced to go through more than one cycle in order to transfer completely all of the isoleucine to tRNA^{Ile}. If release of the nascent Ile—tRNA^{Ile} is rate determining, the apparent rate of esterification of isoleucine to tRNA^{Ile} should be much slower for all cycles after the first one. Thus the first "cycle" corresponds to eq 2a whereas subsequent cycles correspond to the entire reaction, eq 2, which includes both eq 2a and 2b. A

TABLE II: Maximum Rates per Enzyme Molecule of Various Reactions Involved in Acylation of tRNA at pH 6.0, 3°.

Rate of Amino Acid Activation (Eq 1)	Rate of Transfer (Eq 2a)	Rate of Overall Charging (Eq 1 and 2)	Rate of Transfer (Two Cycles, Eq 2)	
$>10^{-1}$ sec^{-1}	1.8×10^{-2} sec^{-1}	2.0×10^{-3} sec^{-1}	Cycle 1 (eq 2a)	2.0×10^{-2} sec^{-1}
			Cycle 2	2.2×10^{-3} sec^{-1}

direct way of measuring the apparent rate of reaction cycles subsequent to the first one is as follows. IRS·[^{14}C]Ile~AMP is isolated in the usual fashion (see Figure 1) and mixed with [^{3}H]Ile~AMP; this mixture is then reacted with tRNA^{Ile}. (Although the radioactive accuracy with [^{14}C]isoleucine is not really sufficient (see Materials and Methods), reasonable qualitative results can be obtained.) The first cycle of transfer is monitored by the incorporation of [^{14}C]isoleucine into tRNA^{Ile} whereas the subsequent cycles are monitored by incorporation of [^{3}H]isoleucine. (Interchange of [^{14}C]Ile~AMP bound to IRS with free [^{3}H]Ile~AMP before the reaction with tRNA^{Ile} does not occur in the time scale of the experiment.) The results of this kind of experiment are displayed in Figure 6 which shows a rapid production of [^{14}C]-Ile—tRNA^{Ile} (first cycle) followed by a roughly tenfold slower rate of production of [^{3}H]Ile—tRNA^{Ile} (subsequent cycles). These data clearly imply that between reaction cycles the rate-limiting release step gives rise to the slow apparent rate of the reaction after the first cycle.

These results are confirmed by experimental data given in Figure 7 which display the production of Ile—tRNA^{Ile} *vs.* time in a system in which the ratio of [^{3}H]Ile~AMP:IRS is about 2.6. The first cycle of the reaction corresponds to a rapid initial production of Ile—tRNA^{Ile} which is stoichiometrically equivalent to the amount of IRS present; the second cycle proceeds at a rate which is about tenfold slower, as is the case in Figure 6. It should be pointed out that essentially no reversal of transfer—back-reaction with AMP—occurs under these conditions. Addition of bacterial alkaline phosphatase in a few experiments had no discernable effect on the results.

The results obtained are summarized in Table II which gives the maximal rate of amino acid activation (eq 1), the maximal rate of a one-cycle transfer (eq 2a), the turnover number of aminoacylation, and the rates of the individual cycles obtained in the multicycle studies of eq 2 (see Figures 6 and 7). It is clear that the turnover number of aminoacylation agrees very well with that obtained for the apparent rate of the second cycle of the transfer reaction. These data thus add considerable support to the previous suggestion (Yarus and Berg, 1969) that release of Ile—tRNA^{Ile} from the enzyme is rate limiting in aminoacylation.

In the overall aminoacylation of tRNA from ATP and amino acid, we thus conclude that the initial burst of aminoacylation (which yields IRS-bound Ile—tRNA^{Ile}) proceeds at a rate which is a factor of ten or so more rapid than subsequent cycles. Ordinarily this fact is obscured because aminoacylation is carried out with catalytic amounts of enzyme and aminoacylation of tRNA is not monitored until the enzyme has completed 50 or more cycles. The contribution of the initial burst to the average rate is thus insignificant. These ideas may be tested by adjusting conditions so that it is possible to monitor the initial burst at early times and compare it directly with subsequent cycles. Figure 8 gives the results of such an experiment. The production of Ile—tRNA^{Ile} starting from isoleucine and ATP is plotted *vs.* time; a rapid initial production of Ile—tRNA^{Ile} occurs which is followed by a much slower and constant rate of synthesis of charged tRNA^{Ile}. The size of the initial burst corresponds to the amount of enzyme present and the rate of this burst coincides with that of the first cycle of transfer given in Table II. The rate of the second, slower phase agrees with that of the second cycle of transfer and the turnover number for charging (Table II).

According to the results described above, release of Ile—tRNA^{Ile} from IRS occurs at a rate of 2.0×10^{-3} sec^{-1} at

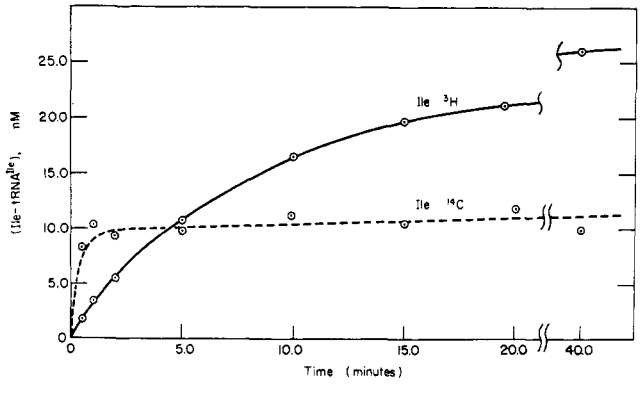

FIGURE 6: Time course of synthesis of [^{14}C]Ile—tRNA^{Ile} and of [^{3}H]Ile—tRNA^{Ile}. See text for details. Reaction mixture (1-ml total volume) contains 12 pmoles of IRS·[^{14}C]Ile~AMP, 31 pmoles of (^{3}H)Ile—AMP, 1 nmole of tRNA^{Ile}, 5 μmoles of 2-mercaptoethanol, 0.5 μmole of EDTA, and 50 μmoles of KCl. Mixture buffered at pH 6.0 by 0.01 M sodium cacodylate buffer.

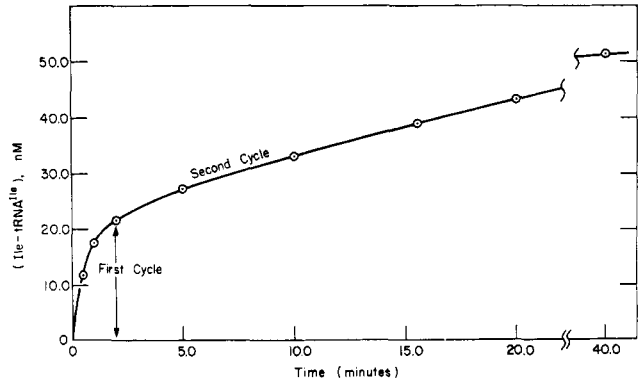

FIGURE 7: Time course of production of Ile—tRNA^{Ile} in a multicycle transfer reaction. See text for details. Reaction mixture (1-ml total volume) contains 22 pmoles of IRS, 58 pmoles of [^{3}H]Ile~AMP, 1 nmole of tRNA^{Ile}, 5 μmoles of 2-mercaptoethanol, 0.5 μmole of EDTA, and 50 μmoles of KCl. Mixture buffered at pH 6.0 by 0.01 M sodium cacodylate buffer.

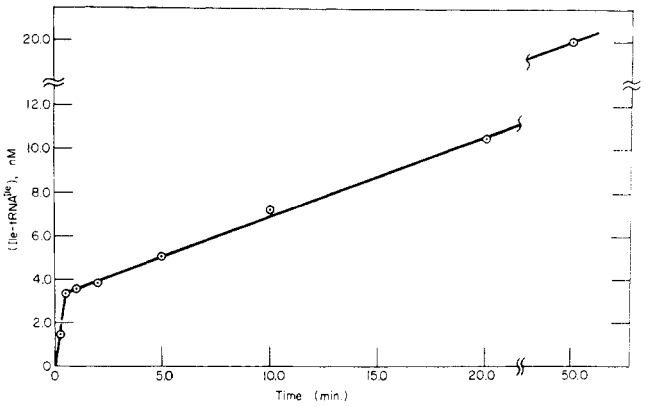

FIGURE 8: Time course of synthesis of Ile—tRNA^{Ile} from ATP, isoleucine, and tRNA^{Ile}. Reaction mixture (1-ml total volume) contains 3.5 pmoles of IRS, 1 nmole of tRNA^{Ile}, 30 nmoles of [^{3}H]Ile (34 Ci/mmole), 2 μmoles of ATP, 5.0 μmoles of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 0.5 μmole of EDTA, and 50 μmoles of KCl. Mixture buffered to pH 6.0 by 0.01 M sodium cacodylate buffer.

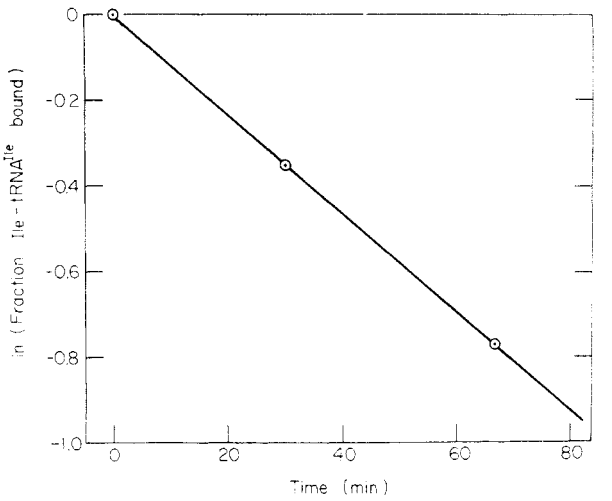

FIGURE 9: Plot of ln (fraction Ile-tRNA^{Ile} bound) *vs.* time. See text for details.

pH 6, 3°. Does this rate represent the release from IRS prior to the binding or synthesis of a new molecule of Ile~AMP, or conversely is a new molecule of Ile~AMP added to IRS prior to release of the Ile—tRNA^{Ile}? According to results of Yarus and Berg (1969), the release is stimulated about sixfold at 17°, pH 5.5 by isoleucine, somewhat greater by isoleucine plus ATP together (which may then give Ile~AMP), but not by ATP alone. This implies that at least isoleucine and possibly Ile~AMP is on the enzyme prior to the release of the nascent Ile—tRNA^{Ile}. Experiments were carried out to test further these ideas. The IRS·Ile~AMP complex was reacted with a large excess of tRNA^{Ile} to produce IRS·Ile—tRNA^{Ile} plus the excess uncharged tRNA^{Ile}. The Ile—tRNA^{Ile} gradually dissociates from the enzyme and is promptly replaced by tRNA^{Ile}. The rate of this replacement corresponds to the rate of release of Ile—tRNA^{Ile} from IRS, in the absence of any effector. To assay for the amount of Ile—tRNA^{Ile} replaced by tRNA^{Ile}, a pulse of additional Ile~AMP is added at various times and a quick burst of new Ile—tRNA^{Ile} is synthesized. The height of this burst corresponds to the amount of IRS which has been freed of its Ile—tRNA^{Ile} prior to the pulse of additional Ile~AMP. In this manner a quantitative assay for the rate of release of Ile—tRNA^{Ile} in the absence of Ile~AMP is obtained.

The results of this kind of experiment are shown in Figure 9 which gives a first-order plot of ln (fraction Ile—tRNA^{Ile} bound) *vs.* time. These data were obtained as described above and yield a rate constant for release in the absence of Ile~AMP of $k = 2.0 \times 10^{-4}$ sec^{-1}. This compares with the rate of release in the presence of Ile~AMP (see Table II, second cycle of transfer reaction) of 2.2×10^{-3} sec^{-1}. We thus conclude that release is stimulated about tenfold by Ile~AMP at pH 6.0, 3° and, therefore, that a new molecule of Ile~AMP binds to the IRS·Ile—tRNA^{Ile} complex prior to the release of Ile—tRNA^{Ile}. In the charging of tRNA from ATP and amino acid, the Ile~AMP is doubtless quickly synthesized on the enzyme prior to release of the charged tRNA^{Ile}.

It is not possible to ascertain by these methods whether or not isoleucine itself is capable of stimulating the release. In studies of the transfer reaction (eq 2), it was found that high concentrations of isoleucine act as an inhibitor (probably competitive) of the binding of Ile~AMP to IRS. Other studies are currently underway to investigate the effects of isoleucine.

Finally, it should be mentioned that the transfer reaction proceeds well without added Mg^{2+}. Moreover, addition of up to 5 mM Mg^{2+} had little effect (<10%) on the rate of transfer.

Discussion

When measured separately, the rates of amino acid activation (eq 1) and of transfer (eq 2a) are found to exceed by tenfold or more the observed rate of aminoacylation of tRNA. The experiments shown in Figures 6–8, and the data in Table II, clearly indicate that the slow rate of aminoacylation is due to the rate-limiting release of nascent Ile—tRNA^{Ile} from the IRS·Ile—tRNA^{Ile} complex (eq 2b). This release is promoted about tenfold at pH 6.0, 3° by the binding of Ile~AMP to the IRS·Ile—tRNA^{Ile} complex. This conclusion follows from analysis of the data obtained in Figures 6–8 in which the rate of synthesis of Ile—tRNA^{Ile} after the first cycle is limited by the release in the presence of Ile~AMP, and of the data in Figure 9 in which release is assayed in the absence of Ile~AMP. These results further confirm the evidence obtained by the nitrocellulose filter technique that release of enzyme-bound Ile—tRNA^{Ile} is rate-limiting in aminoacylation and that Ile~AMP can promote release (Yarus and Berg, 1969).

Since synthetase–tRNA complexes are generally quite stable with association constants in the range of 10^7–10^9 M (Yarus and Berg, 1969; Hélène *et al.*, 1971), it might be expected that release of aminoacylated tRNA is in general the rate-limiting step in the charging of most if not all tRNAs. In support of this expectation, Hélène *et al.* (1971) have found that *E. coli* Val-tRNA synthetase·Val-tRNA^{Val} complex dissociates ten times more quickly in the presence of saturating amounts of valine (or of Val-ol-AMP) than in the absence of these effectors at pH 5.5, 20°. The rate of dissociation observed is commensurate with the overall rate of aminoacylation of tRNA^{Val} at the same pH (Hélène *et al.*, 1971).

The fact that Ile~AMP can promote release of Ile—tRNA^{Ile} from the enzyme obviously implies that an Ile~AMP·IRS·Ile—tRNA^{Ile} complex is formed in which two isoleucine moieties are associated with the enzyme. Probably the isoleucine moiety of enzyme-bound Ile—tRNA^{Ile} actually hangs

off the enzyme or at least swings free of any specific binding site. This follows from the fact that phenoxylation of the free amino group of the valine of Val-tRNA^{Val} (Yaniv and Gros, 1969), or naphthoxylation of the free amino group of the isoleucine of Ile—tRNA^{Ile} (D. C. Lynch and P. R. Schimmel, unpublished data), does not prevent binding of the tRNA to its cognate enzyme.

Loftfield and Eigner (1969) have proposed that aminoacylation of tRNA proceeds through a concerted mechanism in which the adenylate is not an intermediate of the reaction. When viewed in detail, the results reported here argue strongly against this proposal. For example, when IRS·Ile~AMP is reacted with tRNA^{Ile} to produce enzyme bound Ile—tRNA^{Ile} in a "one-shot" reaction (eq 2a), the rate observed (see Table II) coincides with the initial burst of synthesis of enzyme-bound Ile—tRNA^{Ile} when starting from ATP, isoleucine, and tRNA^{Ile} (see Figure 8). Since the rates of both reactions are the same, it appears that the same rate-limiting step is being observed in both cases. This step is eq 2a, the transfer of isoleucine from Ile~AMP to tRNA^{Ile} to yield IRS-bound Ile—tRNA^{Ile}. Hence, we conclude that the adenylate intermediate is formed as a necessary intermediate in the synthesis of Ile—tRNA^{Ile}.

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Acidic Hydrolysis of Deoxycytidine and Deoxyuridine Derivatives. The General Mechanism of Deoxyribonucleoside Hydrolysis*

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ABSTRACT: The kinetics of the acidic hydrolyses of deoxycytidine and deoxyuridine, and their 5-bromo and 5-methyl derivatives have been studied, in the range pH 6 to $H_0 = -6$. The pH–rate profiles, substituent effects, and other parameters of the reactions are consistent with a mechanism involving the formation of mono- and dications of the nucleoside, followed by rupture of the *N*-glycosyl bond. An alternative, widely accepted, mechanism involving sugar-ring opening (initial C–O cleavage) is excluded. As the *N*-glycosyl cleavage pathway has already been established for the uncatalyzed hydrolysis of thymidine, and for the acidic hydrolysis of deoxyadenosine and deoxyguanosine, this process is general for the major naturally occurring deoxyribonucleosides. The pK_A value of 0.5 reported for uracil is incorrect. Another value reported at −3.38 has been confirmed.

Acidic hydrolysis of the *N*-glycosyl bonds of DNA has been of importance as a method for base composition analysis (Tittensor and Walker, 1967) and base sequence determination (Shapiro, 1967; Mushynski and Spencer, 1970) of DNA. The cleavage of one of these bonds *in vivo* may lead to mutations (Freese, 1963) or to inactivation of the DNA *via* chain breakage (Strauss *et al.*, 1969).

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