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Transfer Ribonucleic Acid Synthetase Catalyzed Deacylation of Aminoacyl Transfer Ribonucleic Acid in the Absence of Adenosine Monophosphate and Pyrophosphate†

Alan A. Schreier‡ and Paul R. Schimmel*,¶

ABSTRACT: Isoleucyl-tRNA synthetase has been found to catalyze *specific* deacylation of Ile-tRNA^{Ile} in the absence of AMP or pyrophosphate (PP_i). The K_m for the deacylation reaction is about 10^{-7} M and the turnover number is ~0.8 min^{-1} at pH 7, 37°. The rate of the enzyme-catalyzed deacylation decreases sharply with decreasing pH and exhibits a Mg^{2+} dependence which is substantially different from that of reverse aminoacylation. Unacylated tRNA^{Ile} is a potent (competitive) inhibitor of the reaction. In addition, natural substrates such as ATP, isoleucine, and the adenylate analog isoleucinyl-AMP are also inhibitors, but none are completely (100%) inhibitory. On the other hand, neither AMP nor PP_i, separately, affect the rate of deacylation. When aminoacylation of tRNA^{Ile} is carried out with approximately stoichiometric concentrations of Ile-tRNA synthetase, a considerable amount of tRNA^{Ile} is shown to be deacylated without going through reverse aminoacylation. This implies that the deacylase activity can function during aminoacylation with the result that considerably more ATP may be hydrolyzed to AMP than Ile-tRNA^{Ile} is produced. A cursory examination of a few different tRNA synthetases suggests that others may also possess the deacylation activity. Possible mechanistic and physiological implications of these findings are discussed.

Aminoacylation of tRNA is generally believed to proceed in a two-step reaction sequence in which an amino acid is condensed with ATP to form an enzyme-bound aminoacyl adenylate which in turn reacts with the 2′- or 3′-terminal hydroxyl group of tRNA to yield aminoacyl-tRNA. The reactions can be written[1] as

$$E + AA + ATP \rightleftarrows E \cdot AA\text{-}AMP + PP_i \quad (1)$$

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

In the course of investigations of isoleucyl-tRNA synthetase, it was discovered that under certain conditions the enzyme can catalyze deacylation of Ile-tRNA^{Ile} *in the absence of AMP and* PP_i. The reaction is

$$E + AA\text{-}tRNA \rightleftarrows E + AA + tRNA \quad (3)$$

Interest in this reaction stems from the fact that Lagerkvist *et al.* (1966) and Yaniv and Gros (1969) had suggested earlier that the valyl-tRNA synthetase from baker's yeast and from *Escherichia coli* B also possesses a similar activity. Moreover, a cursory examination of a few different tRNA synthetases (see below) gave evidence that other synthetases may also deacylate their cognate aminoacyl-tRNAs without going through reverse aminoacylation. Therefore, eq 3 may be a rather general reaction and its mechanism and possible significance bears investigation.

We report here an investigation of the Ile-tRNA synthetase catalyzed deacylation of Ile-tRNA^{Ile} according to eq 3. The general features of the reaction are first considered, followed by attempts to establish the existence of any relationship between the site(s) involved in aminoacylation and that for deacylation according to eq 3. Finally, some consideration of the possible significance of the deacylation reaction is given.

Materials and Methods

Materials. Unfractionated *E. coli* B tRNA (Schwarz BioResearch) was enriched threefold in isoleucine acceptance activity by BD-cellulose salt gradient chromatography. The

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*,¶ Alfred P. Sloan Fellow, 1970–1972.
‡ Predoctoral Trainee of the National Institutes of Health.

[1] Abbreviations used are: AA, amino acid; AA-AMP, aminoacyl adenylate; AA-tRNA, tRNA aminoacylated with AA; E, enzyme, an aminoacyl tRNA synthetase; EtOH, ethanol; Ile-ol-AMP, isoleucinyl-AMP; Ile-tRNA^{Ile}, tRNA^{Ile} aminoacylated with isoleucine; PCMB, *p*-chloromecuribenzoic acid; Phe-tRNA, phenylalanine presumably aminoacylated to phenylalanine-specific tRNA in a mixture of unfractionated tRNA; Tu, transfer factor; tRNA-CpCp, terminal adenosine-less tRNA; tRNA^{Ile}, tRNA specific for isoleucine; Val-ol-AMP, valinyl-AMP; Val-tRNA^{Ile}, tRNA^{Ile} aminoacylated with valine.

$tRNA^{Ile}$-enriched fractions were further purified by several modifications of the procedure of Yarus and Berg (1969; see also, Gillam *et al.* (1968); A. A. Schreier, 1972, in preparation). No attempt was made to separate isoacceptors.

[^{14}C]Ile-$tRNA^{Ile}$ was prepared by aminoacylation of approximately one micromole of purified $tRNA^{Ile}$ in a reaction mixture containing 30 mM Tris (pH 8), 10 mM $MgCl_2$, 2 mM ATP, and 40 μM [^{14}C]Ile (253 μCi/μmole, New England Nuclear), in 5-ml final volume. The reaction was started by adding about 40 μg of purified Ile-tRNA synthetase and incubating the components at 37° for 30 min. The reaction reached a plateau within 15 min. Enzyme was inactivated by adjusting the pH to 5.0 with glacial acetic acid. Sodium chloride was then added to a final concentration of 0.3 M and the reaction mixture was applied to a BD-cellulose column (0.8 × 5 cm) previously equilibrated with 0.3 M NaCl–0.1 mM cacodylate (pH 5.5). Elution of this column with 0.3 M NaCl–0.1 mM cacodylate (pH 5.5) removes all reaction components except Ile-$tRNA^{Ile}$ which is subsequently eluted with 1 M NaCl in 0.1 mM cacodylate (pH 5.5). The Ile-$tRNA^{Ile}$ was desalted by dialysis *vs.* 0.1 mM cacodylate (pH 5.5) and concentrated by lyophilization. The resulting powder was dissolved in a small volume of distilled water and the Ile-$tRNA^{Ile}$ concentration was determined from the acid-precipitable ^{14}C activity. The Ile-$tRNA^{Ile}$ contained about 1050 pmoles of [^{14}C]Ile/A_{260} unit (0.01 N NaOH). An aliquot of this aminoacylated $tRNA^{Ile}$ would not accept more [^{14}C]Ile when another aminoacylation was attempted. These stock Ile-$tRNA^{Ile}$ solutions (~50 μM) were subsequently stored at −20° and did not lose either acid-precipitable [^{14}C]Ile or biological activity on repeated freeze-thaw cycles.

Preparation of other aminoacylated tRNAs in admixture followed a procedure similar to that outlined for Ile-$tRNA^{Ile}$, except that unfractionated tRNA, partially purified synthetases, and the appropriate [^{14}C]amino acid, were used. In some experiments a commercially prepared (New England Nuclear) [^{14}C]Phe-tRNA was employed.

Purified Ile-tRNA synthetase was prepared by E. Eldred (Eldred and Schimmel, 1972). It was stored at −20° at a protein concentration of ~1.9 mg/ml in 20 mM potassium phosphate (pH 7.2), 2 mM glutathione, and 50% glycerol. The ATP–pyrophosphate-exchange activity of Ile-tRNA synthetase was assayed by adopting the procedure of Baldwin and Berg (1966a). Although initially pure (see Eldred and Schimmel, 1972), variable amounts (up to 50%) of denaturation occurred over a period of time. Therefore, Ile-tRNA synthetase concentrations were calculated from the published molecular weight of 112,000 and the specific activity of 650 pyrophosphate exchange units/mg of pure protein (Baldwin and Berg, 1966a).

A partially purified mixture of synthetases was prepared by the method of RajBhandary and Ghosh (1969), except that the cells were disrupted in a French press and an $(NH_4)_2SO_4$ fractionation between 0.281 and 0.435 g per ml was performed in order to enrich the mixture with Ile-tRNA synthetase. The ammonium sulfate cut was dialyzed overnight *vs.* 4 l. of 2 mM potassium phosphate–20 mM mercaptoethanol–1 mM $MgCl_2$, in 10% v/v glycerol.

Bacterial alkaline phosphatase and inorganic pyrophosphatase were obtained from Worthington. One unit of pyrophosphatase activity liberates 1 μmole of orthophosphate/min under defined conditions at 25° (Heppel, 1955). Ile-AMP and Val-AMP were kindly donated by Dr. Daniel Santi. Purified transfer factor, Tu, was the kind gift of Dr. David Miller. Terminal adenosine less tRNA (tRNACpCp) was prepared by Mr. M. McNeil of this laboratory by using the technique of Roy and Tener (1967).

Methods. The extent of aminoacylation of $tRNA^{Ile}$ was measured in a reaction mixture identical with the one described above for the preparation of [^{14}C]Ile-$tRNA^{Ile}$. The reaction mixture was scaled-down to 120-μl final volume, and the partially purified synthetase preparation replaced the purified Ile-tRNA synthetase. After a 10-min incubation at 37°, 100 μl of this mixture was applied to Whatman No. 3MM filter paper disks which were then plunged into a beaker of ice-cold 5% trichloroacetic acid (modified from Hoskinson and Khorana, 1965). After an acid washing and drying of the disks, they were counted under 3 ml of toluene-based scintillation fluid in a Nuclear-Chicago scintillation counter. The efficiency of ^{14}C counting on filter paper disks was measured to be 60%.

The kinetics of aminoacylation was measured in a reaction mixture containing (in 60 μl) approximately 7 μM $tRNA^{Ile}$, 0.2–5 ng of purified Ile-tRNA synthetase, 2 mM ATP, 30 μM [^{14}C]Ile, 20 mM $MgCl_2$, and 0.05 M cacodylate (pH 7.0). After a 10-min incubation at 37°, a reaction mixture aliquot was applied to a filter paper disk which was previously soaked in 5% trichloroacetic acid and dried before use. (This procedure, suggested by U. L. RajBhandary, immediately quenches the reaction when it strikes the disk.) The disk was then washed in cold trichloroacetic acid and counted as above.

The deacylation activity of Ile-tRNA synthetase and of other synthetases was measured by two techniques. The usual conditions of the assays were 0.05 M cacodylate (pH 7) and 10–20 mM $MgCl_2$, with appropriate concentrations of synthetase and aminoacylated tRNA. The techniques involve either (1) measuring the disappearance of trichloroacetic acid precipitable [^{14}C]aminoacyl-tRNA or (2) the appearance of ethanol-soluble ^{14}C counts. The filter paper technique mentioned above was used to measure the disappearance of acid-precipitable [^{14}C]aminoacyl-tRNA. The appearance of ^{14}C-soluble counts was determined by quenching 50 μl of assay mixture in 1 ml of 95% EtOH at 4° and precipitating the tRNA by the addition of ~50 A_{260} units of carrier yeast RNA (in 0.15 ml of 10 mM $MgAc_2$, pH 5). After standing for 20 min at 4°, the mixture was centrifuged for 20 min at >1100*g*. The supernatant was then poured directly into 15 ml of 20% methyl Cellusolve–80% toluene scintillation fluid containing 4 g of 2,5-diphenyloxazole and 0.4 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. The samples were counted on a Nuclear-Chicago scintillation counter.

Both techniques work well for the determination of progress curves of the deacylation. However, the EtOH precipitation method simplified the measurement of initial velocities and it is considerably more reproducible. Before development of the EtOH precipitation technique, initial velocities were measured by the filter pad method; in these cases, many time points were taken on each sample in order to obtain accurate initial rates. Deacylation initial velocities were generally measured from the first 15% (or less) of the reaction.

Chromatography of ATP and AMP was performed essentially by the method of Gevers *et al.* (1968), using Whatman DEAE-cellulose strips (2.5 *vs.* 20 cm).

Experimental Results

Characteristics of the Reaction. When approximately stoichiometric concentrations of highly purified Ile-tRNA synthetase and Ile-$tRNA^{Ile}$ are mixed together at pH 7, in the absence of AMP or PP_i, there is a slow removal of the iso-

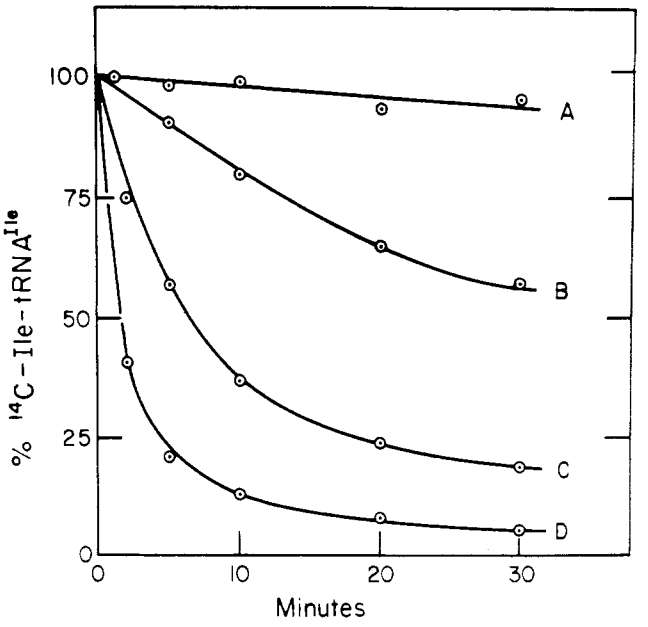

FIGURE 1: Per cent [^{14}C]Ile-tRNA^{Ile} remaining *vs.* time at 37° in the presence of several concentrations of Ile-tRNA synthetase. The reaction mixture contained in 0.1-ml total volume, 0.1 M cacodylate (pH 7.0), 10 mM $MgCl_2$, 1.4 μM [^{14}C]Ile-tRNA^{Ile}, and various quantities of Ile-tRNA synthetase: (A) no Ile-tRNA synthetase; (B) 40 nM Ile-tRNA synthetase; (C) 0.2 μM Ile-tRNA synthetase; and (D) 0.8 μM Ile-tRNA synthetase. The reaction was started by addition of [^{14}C]Ile-tRNA^{Ile}. Samples of 10 μl were periodically removed, spotted on filter paper disks and acid-precipitable counts were determined as in Materials and Methods.

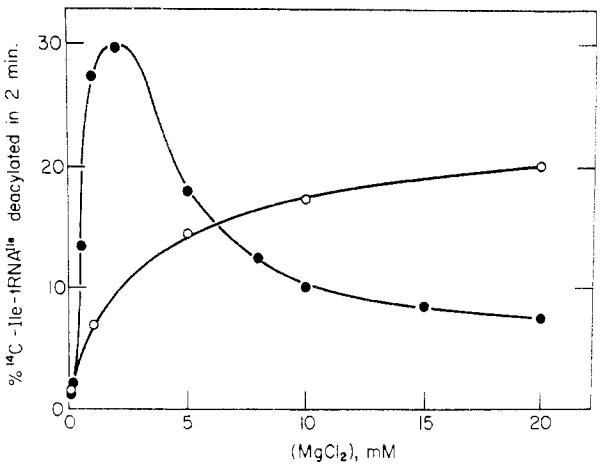

FIGURE 2: Magnesium dependence of the deacylation (eq 3) and reverse aminoacylation activities of Ile-tRNA synthetase. Each assay sample contained 50 mM cacodylate (pH 7), 1.4 μM [^{14}C]Ile-tRNA, and the indicated quantity of $MgCl_2$ in a final volume of 60 μl. Incubations were for 2 min at 37° and the EtOH precipitation method was used to determine the amount of [^{14}C]Ile released (see Materials and Methods). The zero $MgCl_2$ assays contained 1 mM EDTA. The assays contained (○) 70 nM Ile-tRNA synthetase, or (●) 6 nM Ile-tRNA synthetase with 1 mM AMP and 0.25 mM PP_i.

leucine moiety from the Ile-tRNA^{Ile}. The reaction is shown in Figure 1 which displays (Ile-tRNA^{Ile}) *vs.* time at pH 7 for various values of the ratio Ile-tRNA^{Ile}: Ile-tRNA synthetase. These data show that in the presence of Ile-tRNA synthetase[2] there is a slow, but complete deacylation of tRNA^{Ile} which is much more rapid than the spontaneous hydrolysis of the ester linkage. Furthermore, the rate of deacylation depends upon the Ile-tRNA synthetase concentration.

There are five obvious possibilities for the origin of this activity: (1) a contamination of the reaction components with AMP and PP_i so that the observed activity in fact represents reversal of aminoacylation; (2) a nuclease contamination of the Ile-tRNA synthetase which hydrolyzes the tRNA into small fragments so that the fragment containing the isoleucine is now soluble in trichloroacetic acid and ethanol; (3) the presence of trace amounts of a distinct "deacylase" enzyme; (4) a nonspecific hydrolysis of aminoacyl-tRNA linkages catalyzed by acidic and/or basic groups on Ile-tRNA synthetase; (5) a distinct, specific hydrolytic activity of Ile-tRNA synthetase.

The possibility that endogenous AMP and PP_i contamination causes hydrolysis *via* reverse aminoacylation was tested in several ways. It is important to appreciate that almost complete (>80%) deacylation of tRNA^{Ile} can be effected in less than 1 hr at pH 7 with an enzyme:tRNA ratio of 1:20. This means that the endogenous AMP and PP_i concentrations must be at least twenty times the enzyme concentration in order to support reverse aminoacylation. However, the hydrolysis of Ile-tRNA^{Ile} by Ile-tRNA synthetase was unaffected by addition of inorganic pyrophosphatase and bacterial alkaline phosphatase, enzymes which cleave PP_i and AMP, respectively. Furthermore, the addition of a heat-killed enzyme preparation to the normal assay mixture containing active Ile-tRNA synthetase had no effect on the hydrolysis. A stimulation of hydrolysis is anticipated if the enzyme preparation contained endogenous AMP and PP_i. In addition, the Mg^{2+} dependence of the deacylation reaction is quite different in the absence of added AMP and PP_i than in their presence, as shown in Figure 2. This result suggests that different reactions are being observed in the two experiments.

The second possibility is eliminated by the observation that the deacylated tRNA is readily recharged by the addition of ATP and isoleucine to the reaction mixture. This clearly shows that the amino acid acceptance ability has not been irreversibly altered.

The possibility that deacylation arises from the action of a distinct "deacylase" enzyme is controverted by several facts. First, it is shown below that the deacylation reaction is inhibited by the adenylate analog isoleucinol-AMP, as well as by other substrates of Ile-tRNA synthetase. There is certainly no reason to expect a "deacylase" enzyme to be inhibited by compounds such as isoleucinyl-AMP. Furthermore, when Ile-tRNA synthetase solutions are titrated with PCMB there is a loss of aminoacylation activity and of deacylation activity; although the aminoacylation activity is initially more sensitive to PCMB, both activities are completely abolished at the identical PCMB:Ile-tRNA synthetase ratio (see below). Finally, the Ile-tRNA synthetase used in these experiments was chromatographically pure (Eldred and Schimmel, 1972).

The fourth alternative mentioned above is ruled out by several lines of evidence which indicate that the hydrolytic activity of Ile-tRNA synthetase is specific toward Ile-tRNA^{Ile}. For example, it was found that Ile-tRNA synthetase causes no hydrolysis of unfractionated tRNA charged with valine, leucine, tyrosine, or phenylalanine, nor can it hydrolyze Ile-tRNA^{Ile} when the isoleucine amino group has been phenoxyacetylated according to Gillam *et al.* (1968). In addition, the

[2] The Ile-tRNA synthetase stock solutions contained 50% glycerol; therefore, in these and all following experiments (including blanks) a level of 1–3% glycerol was present; this amount of glycerol has no effect on the results.

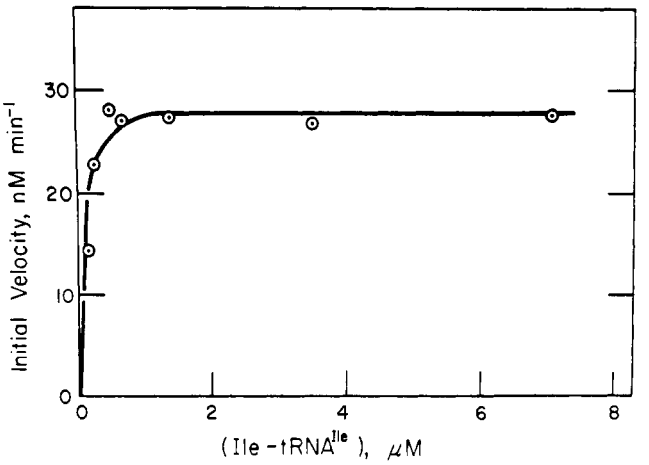

FIGURE 3: Dependence of deacylation on Ile-tRNA^{Ile}. Each assay sample contained in a final volume of 0.1 ml, 0.1 M cacodylate (pH 7), 10 mM $MgCl_2$, 30 nM Ile-tRNA synthetase, and the indicated concentrations of [^{14}C]Ile-tRNA^{Ile}. Incubation was at 37° and the reaction was started by adding [^{14}C]Ile-tRNA^{Ile}. Aliquots were removed at appropriate intervals and spotted on filter paper, and the acid-precipitable ^{14}C counts were then determined as in Materials and Methods. Each point on the plot is corrected for the spontaneous rate of hydrolysis of the corresponding concentration of [^{14}C]Ile-tRNA^{Ile}, as determined by an experiment in which bovine serum albumin replaced Ile-tRNA synthetase. See Materials and Methods for other details.

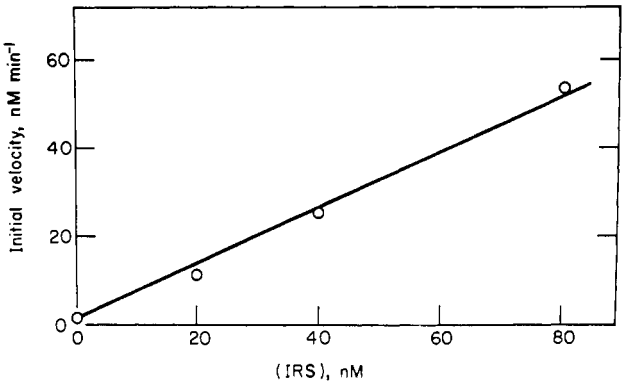

FIGURE 4: The relationship between the initial velocity of [^{14}C]Ile-tRNA^{Ile} deacylation and Ile-tRNA synthetase concentration. Conditions are the same as in the legend of Figure 1, except for enzyme concentrations given on the abscissa. IRS is Ile-tRNA synthetase.

deacylation activity is also inhibited by the addition of uncharged tRNA^{Ile} to the assay. This inhibition doubtless arises from competitive binding of tRNA^{Ile} since 50% inhibition occurs when the concentration of Ile-tRNA^{Ile} and the concentration of tRNA^{Ile} are about equal. (According to Yarus and Berg (1967), Ile-tRNA^{Ile} and tRNA^{Ile} have similar affinities for the enzyme.) A tenfold excess of tRNA^{Ile} over Ile-tRNA^{Ile} causes nearly complete inhibition. These results clearly demonstrate that the deacylation activity is specific. Finally, the hydrolytic activity is abolished when the enzyme is denatured by heat or exhaustive *N*-ethylmaleimide treatment. This indicates that the intact native structure of the enzyme is required, as expected for a specific catalytic process.

The above sets of experiments clearly indicate that the hydrolysis of Ile-tRNA^{Ile} in the absence of AMP and PP_i is a distinct activity of Ile-tRNA synthetase which is apart from its role in eq 1 and 2. This enzymatic activity may be further characterized in the usual fashion. The dependence of the initial velocity on Ile-tRNA^{Ile} concentration at pH 7 is shown in Figure 3. It is seen that the enzyme is half-saturated at about 10^{-7} M, a value which is comparable to the K_m for tRNA^{Ile} in the charging reaction (Baldwin and Berg, 1966b). Figure 4 shows that the initial rate of hydrolysis of Ile-tRNA^{Ile} is a linear function of (Ile-tRNA synthetase), as expected. From the data of Figures 3 and 4, a turnover number of 0.7–0.8 min^{-1} at pH 7, 37° is calculated. This is considerably less than the enzyme turnover number in the aminoacylation reaction (A. Schreier, unpublished).

The hydrolysis reaction (eq 3) is extremely sensitive to pH. Figure 5 shows that the rate of hydrolysis decreases sharply with pH to the point that no hydrolysis is observed below pH 5.5. In addition, the reaction is markedly temperature dependent and in fact exhibits a temperature sensitivity which is similar to that of the overall aminoacylation reaction. The ratio at pH 7 of the maximal initial rate of hydrolysis at 37° to that at 10° is 20, very close to that calculated by us (from data of Yarus and Berg, 1969) for the same temperature ratio of the aminoacylation maximum velocity. Finally, it should be mentioned that the hydrolysis reaction requires Mg^{2+} (see Figure 2); no hydrolysis above background levels was detected in the absence of added Mg^{2+}.

Effects of Substrates. Results cited above indicate that unacylated tRNA^{Ile} is a potent competitive inhibitor of the deacylation reaction. It is of obvious interest to investigate also the effects of ATP, PP_i, AMP, and Ile on the deacylation reaction described above (eq 3). From such investigations, it might be possible to gain additional information for the relationship between the active site(s) involved in the reactions of eq 1 and 2, and the catalytic site for the deacylation reaction.

The results obtained are summarized in Table I, which gives the per cent activity in the presence of various ligands, including tRNA^{Ile}. Most of these data were acquired in the presence of inorganic pyrophosphatase. (Pyrophosphatase was added because of apparent PP_i contamination of our

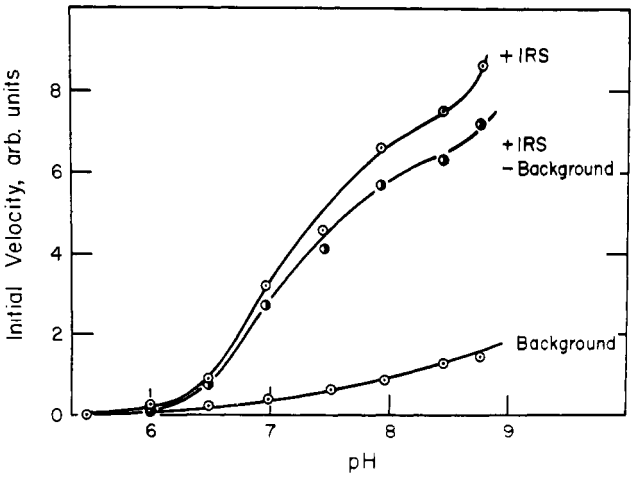

FIGURE 5: Effect of pH on the initial velocity of deacylation. The buffer contained 0.1 M NaCl, 10 mM $MgCl_2$, 10 mM cacodylate, and 10 mM Tris base. This buffer was titrated to the desired pH with NaOH for each experiment. Each assay also contained 1.5 μM [^{14}C]Ile-tRNA^{Ile} and 40 nM Ile-tRNA synthetase in a final volume of 80 μl. The reaction was started by the addition of [^{14}C]Ile-tRNA^{Ile} and the initial velocity determined by the filter paper technique as discussed in Materials and Methods. IRS is Ile-tRNA synthetase.

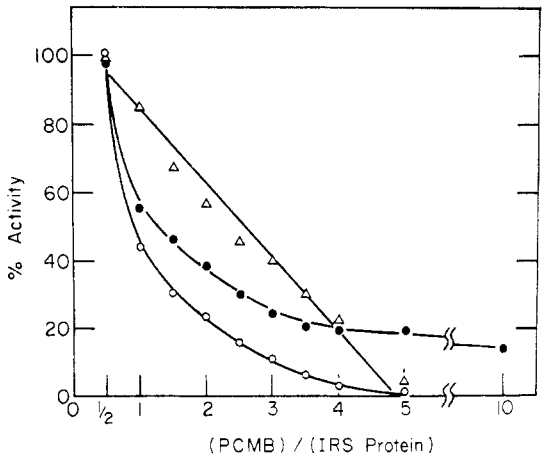

FIGURE 6: PCMB titration of Ile-tRNA synthetase activities. Samples of 5 μl of Ile-tRNA synthetase stock (1.9 mg/ml of enzyme protein) were diluted into 40 μl of a buffer consisting of 50 mM cacodylate (pH 7) in 50% glycerol. An aliquot of PCMB in H_2O was then added to these enzyme solutions to give the PCMB:Ile-tRNA synthetase protein ratios listed on the abscissa. The enzyme was incubated for 30, 60, or 90 min with PCMB with the same results being obtained in all cases. All operations were performed at 4°. Aliquots of the treated Ile-tRNA synthetase samples were assayed for various activities at pH 7, 37°: (○) aminoacylation of $tRNA^{Ile}$; (●) PP_i–ATP-exchange activity; (△) deacylation of [^{14}C]Ile-$tRNA^{Ile}$ according to eq 3. The deacylation activity was measured by the ethanol precipitation method; see Materials and Methods for the determination of aminoacylation and ATP–PP_i-exchange activities. A decrease in any of the activities does not occur until PCMB:Ile-tRNA synthetase approaches unity because of the presence of residual sulfhydryl reagent in the diluted enzyme solutions. IRS is Ile-tRNA synthetase.

TABLE 1: Effect of Various Ile-tRNA Synthetase Substrates on Ile-tRNA Synthetase Catalyzed Hydrolysis of [^{14}C]Ile-tRNA According to Equation 3.[a]

Substrate Additions			% Residual
1	2	3	Act.
$tRNA^{Ile}$ (1.1 μM)			52
$tRNA^{Ile}$ (11 μM)			9
Ile (0.1 mM)			93
Ile (1 mM)			76
Val (1 mM)			104
Val (10 mM)			86
ATP (0.1 mM)			72
ATP (1 mM)			37
ATP (2 mM)			23
dATP (1 mM)			69
ADP (1 mM)			56
AMP (1 mM)			96
PP_i (1 mM)			104
Ile-ol-AMP (0.01 mM)			56
ATP (0.01 mM)	Ile (0.01 mM)		67
ATP (0.05 mM)	Ile (0.01 mM)		49
ATP (0.1 mM)	Ile (0.1 mM)		42
ATP (2 mM)	PP_i (0.1 mM)		74
ATP (2 mM)	PP_i (0.5 mM)		114
ATP (2 mM)	PP_i (0.1 mM)	Ile (0.1 mM)	43
ATP (2 mM)	PP_i (0.5 mM)	Ile (1 mM)	42

[a] Except for experiments with $tRNA^{Ile}$ each assay contained in 60 μl, 50 mM cacodylate (pH 7.0), 20 mM $MgCl_2$, and 0.1 unit of inorganic pyrophosphatase when PP_i was not added, 80 nM Ile-tRNA synthetase, 1.4 μM [^{14}C]Ile-$tRNA^{Ile}$, and further additions as indicated. Initial velocities were measured by the EtOH precipitation technique (see Materials and Methods). In the experiments with $tRNA^{Ile}$, each assay contained 50 mM cacodylate (pH 7.0), 20 mM $MgCl_2$, 1.1 μM [^{14}C]Ile-$tRNA^{Ile}$, 80 nM Ile-tRNA synthetase, and the listed concentrations of $tRNA^{Ile}$ in a final volume of 60 μl. The acid precipitation technique was used to measure the reaction's progress.

preparations; when Ile-tRNA synthetase, $Ile\text{-}tRNA^{Ile}$, and AMP were mixed together there was a rapid deacylation of $Ile\text{-}tRNA^{Ile}$ with a concomitant production of ATP (identified by chromatography); in the presence of pyrophosphatase, the hydrolysis of the $Ile\text{-}tRNA^{Ile}$ ester linkage was not stimulated by AMP.) It is clearly seen in Table I that the effect of most of the ligands is inhibitory, but that except for $tRNA^{Ile}$ this inhibition is noncompetitive in nature. For example, presumably saturating quantities of Ile, ATP, or the alkyl adenylate Ile-ol-AMP reduce the activity to between 23 and 76% of its initial value. On the other hand, AMP and PP_i have no apparent effect by themselves. ATP is the most inhibitory ligand, but PP_i effectively reverses the ATP inhibition. The latter fact suggests that ATP and PP_i bind in a competitive fashion, which is consistent with the results of Penzer *et al.* (1971) and Holler *et al.* (1971). The small inhibition produced by isoleucine appears to be specific since comparable concentrations of DL-alanine, DL-threonine, or DL-leucine do not alter the rate of deacylation. In the case of valine, a barely significant inhibition is observed, even though valine is known to interact with the enzyme (Baldwin and Berg, 1966c). A mixture of ATP and isoleucine, which presumably yields Ile~AMP, gives a level of inhibition which is roughly commensurate with that produced by Ile-ol-AMP, but the exact amount of inhibition appears to vary with the relative amounts of isoleucine and ATP. It is interesting to note that although PP_i can effectively reverse ATP inhibition, it does so much less effectively when ATP is present with isoleucine.

Sulfhydryl Titration. Ile-tRNA synthetase is known to possess a highly reactive sulfhydryl, the titration of which results in a severe reduction of the catalytic ability of the enzyme (Iaccarino and Berg, 1969; Kuo and DeLuca, 1969). Figure 6 displays the effect of PCMB titration on the Ile-tRNA synthetase activity in three reaction—aminoacylation, ATP-PP_i exchange (eq 1), and deacylation according to eq 3. The initial reduction in the activity of the aminoacylation reaction is much greater than that of ATP–PP_i exchange, as PCMB is added. In addition, the exchange activity plateaus at 14% of initial activity. These results are in accord with the results of Iaccarino and Berg (1969). The decrease in activity of the deacylation reaction lags behind that of the other two reactions, and in fact appears to be almost linear with the amount of PCMB added. This behavior is similar to that found for the effect of PCMB on the binding of $tRNA^{Ile}$ to Ile-tRNA synthetase (Iaccarino and Berg, 1969).

Deacylation of Charged tRNA in a Charging Mixture. The question arises as to whether or not eq 3 is an important reaction when aminoacylation of tRNA is being carried out in the presence of ATP and amino acid. The results of the experiments summarized in Table I show that ATP and Ile are

TABLE II: Hydrolysis of ATP to AMP during Aminoacylation.[a]

System	% ATP Hydrolyzed (−Background)	No. of ATP Hydrolyzed/ $tRNA^{Ile}$
1. Complete	45	12.3
2. −Ile, $-tRNA^{Ile}$	3.4 (= background)	
3. $-tRNA^{Ile}$	6.3	
4. $-tRNA^{Ile}$, +tRNACpCp	3.8	

[a] For the complete system, the conditions are identical with curve II of Figure 7, except that [^{14}C]ATP replaced ATP. Other additions and omissions are indicated by a + or −, respectively. The percentage of ATP required to acylate each $tRNA^{Ile}$ once is 3.6%, as measured from the final plateau level of aminoacylation. Amounts of [^{14}C]ATP and [^{14}C]AMP were determined as in Materials and Methods.

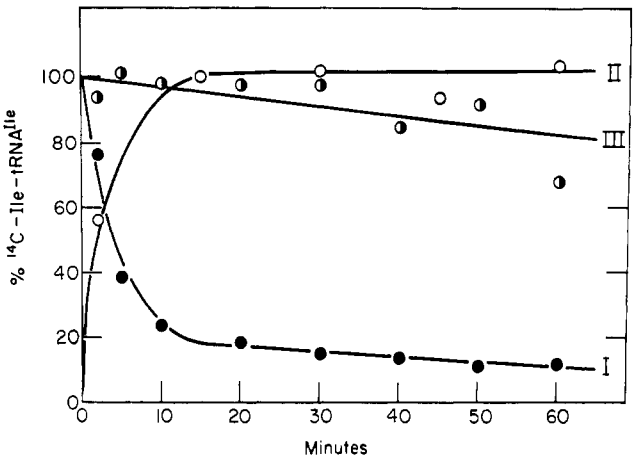

FIGURE 7: Turnover of Ile-$tRNA^{Ile}$ in an aminoacylation mixture. Each point represents a single assay which contained in 60 μl, 50 mM cacodylate (pH 7), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 unit of inorganic pyrophosphatase, 0.1 mM ATP, and 0.1 mM Ile. Other additions were as follows: curve I (●), 5 μM [^{14}C]Ile-$tRNA^{Ile}$ and 0.7 μM Ile-tRNA synthetase; curve II (○), about 4 μM $tRNA^{Ile}$, 0.7 μM Ile-tRNA synthetase, and tracer quantities of [^{14}C]Ile; curve III (◑), 5 μM [^{14}C]Ile-$tRNA^{Ile}$. Acid-precipitable counts were measured as described in Materials and Methods.

not capable of completely inhibiting the deacylation activity of Ile-tRNA synthetase. This suggests that eq 3 may indeed be taking place during the aminoacylation of tRNA when the Ile-$tRNA^{Ile}$:Ile-tRNA synthetase ratio is roughly stoichiometric. It is important to realize that activating enzymes and their cognate tRNAs are present in roughly equimolar amounts in the cell (Yarus and Berg, 1969) so that the question of the role of eq 3 during the charging of tRNA is of clear interest.

To test the deacylation activity (eq 3) during the aminoacylation of tRNA, precharged [^{14}C]Ile-$tRNA^{Ile}$ (5 μM) was added to 0.7 μM Ile-tRNA synthetase, 0.1 mM ATP, and 0.1 mM isoleucine, in the presence of inorganic pyrophosphatase (to prevent reversal of aminoacylation). The results of this experiment are given by curve I of Figure 7, which shows that a rapid removal of the [^{14}C]Ile from [^{14}C]Ile-$tRNA^{Ile}$ occurs until a new level of [^{14}C]Ile-$tRNA^{Ile}$ is approached. This new level approaches the ratio [^{14}C]Ile:total Ile, thus suggesting that the [^{14}C]Ile removed from $tRNA^{Ile}$ has been replaced by unlabeled Ile *via* the charging reaction (eq 1 and 2). From curve I of Figure 7, we calculate that 12% of the tRNA is deacylated per minute, under the conditions of this experiment.

Curve II of Figure 7 displays the time course of charging of about 4 μM $tRNA^{Ile}$ with 0.1 mM ATP and 0.1 mM Ile. A plateau value is quickly reached and maintained. According to curve I, however, this plateau is maintained *via* a constant recharging of $tRNA^{Ile}$ which has been deacylated according to eq 3. (Curve III gives the rate of spontaneous (no Ile-tRNA synthetase) deacylation of 5 μM [^{14}C]Ile-$tRNA^{Ile}$; this rate is negligible compared that shown by curve I.) This implies that a continuous hydrolysis of ATP to AMP must occur even when the $tRNA^{Ile}$ is completely charged. The data given in Table II confirm this expectation. The amount of hydrolyzed ATP was measured by using [^{14}C]ATP and chromatographing the reaction products after 1 hr to determine the amount of AMP produced. If the rate constant of 0.12 min^{-1}, cited above, arises from deacylation of Ile-$tRNA^{Ile}$ *via* eq 3, we calculate roughly that (0.12 min^{-1} × 60 min + 1) = 8.2 moles of ATP should be hydrolyzed to AMP per molecule of $tRNA^{Ile}$ per hr. The observed value is 12.3 ATP/tRNA, which is somewhat greater than the calculated value. However, control experiments cited in Table II rule out the possibility that a significant part of the hydrolysis of ATP arises from processes other than the continuous charging of $tRNA^{Ile}$. For example, it might be argued that the aminoacyl-adenylate complex formed in amino acid activation is sufficiently labile that in the period of one hour a substantial portion is hydrolyzed. However, experiments with Ile-tRNA synthetase, 0.1 mM ATP, and 0.1 mM isoleucine, in the presence and absence of tRNACpCp, suggest that little (*ca.* <15%) hydrolysis of the enzyme bound adenylate occurs in the presence or absence of tRNA (see Table II).

Other tRNA Synthetases. The results reported above clearly demonstrate the ability of the isoleucine enzyme to catalyze eq 3. Moreover, less exhaustive data of Lagerkvist *et al.* (1966) and of Yaniv and Gros (1969) suggest that the same activity is possessed by the valine enzyme from baker's yeast and *E. coli* B. To ascertain the generality of this phenomenon, it is of obvious interest to test other synthetases with respect to their ability to catalyze the deacylation reaction.

For this purpose, a relatively nuclease-free preparation (see Materials and Methods) containing various aminoacyl-tRNA synthetases in admixture was incubated with unfractionated tRNA which had been selectively charged with various amino acids, *i.e.*, leucine, tyrosine, valine, phenylalanine, and histidine. Except for histidine, these tRNAs were all deacylated at a rate substantially above background, when incubated with the synthetase mixture. The negative histidine result could have arisen from an insufficient histidyl-tRNA synthetase concentration. In all cases, the tRNAs were readily reaminoacylated, thus eliminating the possibility that the tRNA had been irreversibly altered. An experiment with tRNA aminoacylated with phenyalanine showed that inorganic pyrophosphatase and bacterial alkaline phosphatase did not reduce the rate of the deacylation reaction. This implies that contamination by AMP and PP_i is not responsible for the deacylation. These results suggest that the deacylation activity

(eq 3) may be common to most of the tRNA synthetases. However, other explanations for this deacylation, *e.g.*, nonspecific catalysis by substances in the crude enzyme preparation or the presence of specific deacylases (*e.g.*, D-tyrosyl-tRNA deacylase, Calendar and Berg, 1967), cannot be eliminated. Experiments with pure components are necessary for definite conclusions in this regard.

Discussion

The above experiments clearly demonstrate the existence of a deacylation activity of Ile-tRNA synthetase and point to the possibility that other tRNA synthetases possess this activity as well. In the case of Ile-tRNA synthetase several lines of evidence (see above) rule out other possible explanations for the origin of the observed deacylation of aminoacyl-tRNA.

The experiments cited in Table I indicate that the best inhibitor of the deacylation reaction is $tRNA^{Ile}$, which appears to be a competitive inhibitor. It seems likely, therefore, that the "deacylation site" is that area of the enzyme which is near or around the 3′-terminal adenosine region of the Ile-$tRNA^{Ile}$–Ile-tRNA synthetase complex. The fact that ATP and isoleucine, either separately or together (thus giving Ile-AMP), can partially inhibit the deacylation activity of Ile-tRNA synthetase (Table I) certainly suggests that some coupling exists between the sites for the small substrate molecules and the deacylation site. Because the inhibition is only partial, it is clear that this is not classical competitive inhibition, but probably arises from subtle changes in the geometry of the Ile-tRNA synthetase–Ile-$tRNA^{Ile}$ complex effected by the small ligands. The inhibition produced by ATP could actually be only apparent, since in the presence of ATP any Ile removed from Ile-$tRNA^{Ile}$ could be reattached to $tRNA^{Ile}$ *via* aminoacylation. However, dATP and ADP also act as inhibitors of deacylation (see Table I), even though they do not support aminoacylation. Therefore, it is probable that ATP is also acting at least partly as a genuine inhibitor. It is also worth noting that it has been shown with several tRNA synthetases that ATP can inhibit aminoacylation (Myers *et al.*, 1971). Interestingly enough, however, Yarus and Berg (1969) report that the presence of ATP does not alter the binding constant for the Ile-tRNA synthetase–Ile-$tRNA^{Ile}$ interaction at pH 5.5, 17°.

The magnesium dependencies of the reverse aminoacylation reaction and of the deacylation reaction (eq 3) are quite distinct (Figure 2). In the case of reverse aminoacylation, Mg^{2+} is required for the PP_i in order for it and the adenylate to form ATP and isoleucine (Cole and Schimmel, 1970a,b). Inhibition occurs because a dimagnesium pyrophosphate complex forms at high concentrations, and this complex does not react with the adenylate (Cole and Schimmel, 1970b). In addition, Mg^{2+} may have some effect on the Ile-$tRNA^{Ile}$ conformation which in turn alters the rate of reverse aminoacylation.

The pH dependence of the deacylation reaction, as shown in Figure 5, indicates that titration in the range of pH 6–8 of one or more groups on the enzyme, tRNA, or complex is necessary for deacylation activity. It is interesting to note that the binding of $tRNA^{Ile}$ to Ile-tRNA synthetase is also pH dependent in the same pH range (S. Lam and P. R. Schimmel, to be published). In this case, the binding decreases with increasing pH values. It is not yet possible to determine whether or not the same ionizable group(s) implicated in binding are also responsible for the deacylation reaction's pH dependence.

It is also possible that the increase in rate as the pH is raised stems from a hydroxide ion attack on the aminoacyl linkage which has become more labile as a result of the tRNA being bound to the enzyme. If this is the entire cause of the pH dependence, the data of Figure 5 should give a linear plot when plotted as initial rate *vs.* (OH^-). However, although such a plot is linear from pH 8.6 (the highest pH examined) to pH 7.9, the points fall markedly below the pH 7.9–8.6 line at lower pH values. This suggests that a simple OH^- attack on a labilized aminoacyl linkage is certainly not the entire cause of the pH dependence. On the other hand, the rate of deacylation (eq 3) is stimulated by hydroxylamine at pH 7 and shows an approximately linear dependence on (NH_2OH) up to 0.1 M (A. A. Schreier, in preparation, 1972). Surprisingly, however, the main product is not isoleucyl hydroxamate but isoleucine. This suggests a more complicated role for the base than simple nucleophilic attack.

Another interesting aspect of the pH dependence shown in Figure 5 is that it closely resembles the pH profile reported by Yarus and Berg (1970) for the efficiency of the membrane filter binding assay. These authors observed that the efficiency of detecting the [^{14}C]Ile-$tRNA^{Ile}$–Ile-tRNA synthetase complex on nitrocellulose filter pads decreases markedly above pH 5.5. Conceivably the same group(s) which cause a variation in the pH dependence of the efficiency are also important for the deacylation reaction. In addition, it is possible that the efficiency of the membrane filter assay appears lower at pH values above 5.5 because the enzyme deacylates the [^{14}C]-Ile-$tRNA^{Ile}$ much more rapidly as the pH is raised (Figure 5) and, therefore, removes the radioactive marker used for detecting the complex.

The question naturally arises as to the significance of this reaction in relationship to the overall aminoacylation reaction itself. For example, is it possible that the deacylation reaction (eq 3) is somehow a part of or a side reaction of the catalytic mechanism for aminoacylation? One way in which this might be possible is if the deacylation reaction actually involved the breaking of the AA-tRNA linkage simultaneous with the formation of an "active" or labile linkage between the enzyme and the amino acid, *e.g.*, a thioester. At this point, the E–AA bond could be hydrolyzed to yield free amino acid (deacylation according to eq 3) or in the presence of AMP and PP_i reversal of aminoacylation could occur to yield ATP and AA. The formation of an E–AA bond thus has the advantage that both the aminoacylation and the deacylation reaction (eq 3) are tied together by a common intermediate. Moreover, such an enzyme intermediate has been discovered by Roskoski *et al.* (1970) in the synthesis of tyrocidin. This polypeptide antibiotic of *Bacillus brevis* is synthesized by a multienzyme complex; the enzyme complex first forms the aminoacyl adenylates which in turn give aminoacyl thioesters.

In spite of its attractiveness, we could obtain no evidence in support of the existence of a labile E–AA intermediate. Experiments carried out included attempts to directly precipitate such a complex in acid, as well as a variety of isotope-exchange experiments (A. A. Schreier, in preparation, 1972). In addition, the PCMB titration experiments of Figure 7 show that the deacylation activity is much less sensitive to PCMB additions than either aminoacylation or ATP–PP_i exchange activity. If the deacylation and aminoacylation reactions share a common intermediate (especially a thioester), one might expect a similar response for the two reactions to PCMB, although this is not necessarily required.

Apart from the unsupported possibility that the deacylation reaction is somehow part of or a side reaction of the aminoacylation reaction, the question also arises as to whether or not eq 3 has any physiological significance. One conjecture

is that the reaction is actually designed to prevent mistakes, *i.e.*, the attaching of the wrong amino acid to the tRNA. In the case of the "correct" amino acid, the deacylation reaction is very slow, but conceivably could be much more rapid if the wrong amino acid was attached. In this regard, it is interesting to consider the findings of a tRNA induced hydrolysis of adenylates formed and bound to the "wrong" synthetase. Baldwin and Berg (1966c) have shown that Ile-tRNA synthetase from *E. coli* B can form the Ile-tRNA synthetase–valyl-AMP complex from ATP and L-valine; when the complex is subsequently reacted with $tRNA^{Ile}$, hydrolysis of the valyl-AMP complex rapidly occurs and the wrong amino acid, valine, is not attached to $tRNA^{Ile}$. In the case of Ile-tRNA synthetase from *E. coli* K-12, both leucine and valine form adenylates with Ile-tRNA synthetase which are subsequently hydrolyzed when confronted with $tRNA^{Ile}$ (Kondo and Woese, 1969). It is not unreasonable to speculate that the deacylation activity of Ile-tRNA synthetase quickly hydrolyzes a nascent Val-$tRNA^{Ile}$ species, especially since small modifications of the 3′ end of $tRNA^{Ile}$ which destroy amino acid acceptor ability also destroy the ability of the $tRNA^{Ile}$ to induce hydrolysis of Ile-tRNA synthetase bound valyl-AMP (Baldwin and Berg, 1966c). A direct test of this hypothesis would be to attach valine to $tRNA^{Ile}$ and then present the Val-$tRNA^{Ile}$ with Ile-tRNA synthetase. This experiment has been sucessfully executed in this laboratory. The Val-$tRNA^{Ile}$ species is rapidly deacylated by Ile-tRNA synthetase, in the absence of AMP and PP_i. The rate of deacylation of Val-$tRNA^{Ile}$ is greater than ten times the rate of deacylation of Ile-$tRNA^{Ile}$. The results suggest the possibility of a Val-$tRNA^{Ile}$ intermediate in the hydrolysis of valyl-AMP. Further discussion is given elsewhere (E. W. Eldred and P. R. Schimmel, submitted for publication).

Of course, the amino acid activating enzymes occupy a central role in metabolism (Neidhardt, 1966), and acylated and/or unacylated tRNAs can participate in a number of control mechanisms (Ezekiel, 1964; Yegian and Stent, 1969; Iaccarino and Berg, 1970; Goldberg, 1970). With such a wide variety of interactions possible, one can always spectulate on various roles for the deacylation activity, but this is perhaps unwarranted until further experimental evidence is available.

Finally, the data in Figure 7 and Table II indicate that once tRNA is acylated by roughly stoichiometric amounts of enzyme, the fully charged state is only maintained by a constant hydrolysis of ATP. In the cell, however, where roughly stoichiometric quantities of enzyme and tRNA are present (Yarus and Berg, 1969), the acylated tRNA may be quickly drained off into the protein synthesis machinery. In particular charged tRNA next combines with the Tu-GTP factor (see Lipmann, 1969; Miller and Weissbach, 1970). Preliminary experiments in this laboratory have shown that the presence of sufficient Tu-GTP factor completely abolishes the deacylation (eq 3) of Ile-$tRNA^{Ile}$ by Ile-tRNA synthetase.

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