Solvent and Specificity. Binding and Isoleucylation of Phenylalanine Transfer Ribonucleic Acid (*Escherichia coli*) by Isoleucyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli*†

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ABSTRACT: A previous formulation of the mechanism of recognition of $tRNA^{Ile}$ (*Escherichia coli*) by isoleucyl-tRNA synthetase suggested that the interaction of noncognate tRNAs with isoleucyl tRNA synthetase might be facilitated by introduction of organic solvents. Here it is shown that, in 5–30% methanol, by volume, isoleucyl-tRNA synthetase will bind $tRNA^{Phe}$ with appreciable strength ($k \sim 0.13 \times 10^6$ to 1×10^6 l. per mole in 20% methanol), and also isoleucylate it, thus attaching isoleucine to the anticodon for phenylalanine. Binding competition experiments show that not only has $tRNA^{Phe}$ attained a higher absolute strength of interaction with isoleucyl-tRNA synthetase, but also that the ratio of the association constant to that of $tRNA^{Ile}$ has increased substantially. Thus the distinction made between $tRNA^{Ile}$ and $tRNA^{Phe}$ has lessened; this is a partial confirmation of the hypothesis that the compact structure of tRNAs, which may be loosened by methanol, is required for the specificity of this nucleic acid–protein interaction. This transcognate reaction can also be demonstrated and characterized in the complete absence of methanol.

A study of the effects of variation in the solvent on the affinity of $tRNA^{Ile}$ (*Escherichia coli*) for isoleucyl-tRNA synthetase led this author to the prediction (Yarus, 1972) that certain manipulations of the solvent, in particular, the addition of organic solvents, might alter the mechanism of specific binding (recognition) sufficiently that misrecognition and even misaminoacylation would occur. Because an experimental means of provoking mistakes would provide direct information about the nature of the normal process of discrimination between tRNAs, a study was undertaken to detect these atypical reactions. In this study, methanol has been used because it, among the organic solvents previously tried (Yarus, 1972), has the smallest inactivating effect on isoleucyl-tRNA synthetase.

Materials and Methods

Isoleucyl-tRNA synthetase is the homogeneous protein from *E. coli* B purified as by Baldwin and Berg (1966). Phenylalanyl-tRNA synthetase from *E. coli* B was supplied from an RNase-free preparation of mixed synthetases, derived by the method of Meunch and Berg (1966). The phrase $tRNA^{Ile}$ implies the majority species of isoleucine acceptor from *E. coli* B, purified to contain >80% isoleucine acceptor chains (Yarus and Berg, 1969), The phrase $tRNA^{Phe}$ refers to the species purified at Oak Ridge National Laboratory by extensive chromatography from mixed tRNAs in a phenol extract of *E. coli K12*. It is 78% pure as judged by a comparison of 3′-adenosine to Phe acceptance (Weeren *et al.*, 1970). Poly(U) is a product of Miles Laboratories. Poly(U,A) was made by supplying purified *Micrococcus lysodeikticus* polynucleotide phosphorylase with ADP and UDP in the molar ratio 1 to 4.16. Total nucleoside diphosphate concentration was 0.024 M at pH 9.3 in 0.20 M Tris-HCl, and 0.01 M $MgCl_2$. The polymer was purified by phenol extraction, ethanol precipitation, and exhaustive dialysis from 0.5 M NaCl into deionized, distilled H_2O. Its spectrum at pH 12 corresponds to the expected mixture of A and U (~1:4) but base composition has not been further checked.

Isotopic amino acids are from Schwarz-Mann. Usually, [^{14}C]Ile refers to lot no. WR-2127, of specific activity 312 Ci/mole: [^{3}H]Phe is lot no. 6901, of specific activity 1.65 Ci/mole.

Ribosomes were isolated from *E. coli* N464, a K12 derivative (Kuwano *et al.*, 1969) which lacks RNase I, has minimal polynucleotide phosphorylase, and has a temperature-sensitive RNase II. Extracts were heated to 50° for 5 min to inactivate RNase II. They have been isolated by pelleting at 105 kg, washed in 0.35 M NH_4Cl, and resolved into 30S and 50S subunits by the method of Takanami (1967). The term ribosomes, used below, refers to an equimolar mixture of these subunits, which were stored separately, frozen in a solution containing 0.01 M Tris-HCl, pH 7.8, 0.022 M NH_4Cl, and 0.010 M $MgAc_2$. S-100 is a tRNA-free preparation of supernatant enzymes from *E. coli* N464. It was obtained by DEAE-cellulose chromatography (Gold and Schweiger, 1969a) of a centrifugally cleared extract of a trimethoprim-treated culture (Gold and Schweiger, 1969b), and was stored frozen in 0.01 M Tris-HCl, pH 7.5, 0.05 M NH_4Cl, 0.010 M $Mg(OAc)_2$, and 0.001 M dithiothreitol.

Binding of aa-tRNA to ribosomes was measured by retention of the isotopic label after filtration of reaction mixtures onto Schleicher and Schuell 24 mm B-6 nitrocellulose filters (*e.g.*, Nirenberg and Leder, 1964). Binding reactions contained, in 125 μl, a final concentration of 0.05 M Tris-HCl, pH 7.8, 0.12 M NH_4Cl, 0.025 M $MgAc_2$, 0.001 M ATP, 0.0002 M GTP, 0.001 M dithiothreitol, 3.25 A_{260} of ribosomes, and either 0.25 A_{260} of poly(U), or 0.46 A_{260} of poly(U,A). Absorbance was measured in 0.01 N NaOH. Several picomoles of aminoacyl-tRNA was added, as required (see Results). [^{14}C]Ile-$tRNA^{Ile}$ carried 12 nmoles of amino acid/10 A_{260}, [^{14}C]Ile-

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tRNA^{Phe} 2.7 nmoles/10 A_{260}, and [^{3}H]Phe-tRNA^{Phe} 5.9 nmoles/10 A_{260}. The ribosomes were added to begin the reaction; incubation was carried out for 1 min at 37°, after which the reaction was filtered and the filter washed with 10 ml of iced buffer: 0.01 M Tris-HCl, pH 7.8, 0.020 M $Mg(OAc)_2$, and 0.060 M NH_4OAc. The nitrocellulose membrane was dried and counted by scintillation methods. A blank without message has been subtracted from binding data presented; this varies, but is always several tenths of a picomole. Values obtained this way are believed to consist of binding only, and not oligopeptide synthesis, as they were unaffected by 0.001 M chloramphenicol.

In order to observe synthesis of polypeptide, the ribosomal binding reaction mixture was used, but magnesium concentration was lowered to 0.0075 M, and a total of 10 μg of S-100 protein/3.25 A_{260} ribosomes was added. The resulting system is absolutely dependent on tRNA, S-100, and message. A mixture of S-100 and ribosomes was added to the other reactants at 37° to begin the reaction. Aliquots (100 μl) were diluted at intervals into 30 volumes of 10% trichloroacetic acid and incorporation was measured as insolubility in cold 10% trichloroacetic acid after 20 min at 95°. Scintillation spectrometry was used to resolve the ^{14}C and ^{3}H in these precipitates.

To identify amino acids bound to tRNA, a sample of aminoacyl-tRNA was precipitated with 2 N HCl in the presence of 100 μg of yeast carrier RNA, and then the acid- and ethanol-washed Whatman GF/C filter with the aminoacyl-tRNA embedded in it was eluted with 100 μl of 10% reagent NH_4OH containing 0.001 M amino acid as carrier. This liquid was placed at the origin of a Merck-Darmstadt precoated cellulose thin-layer chromatography plate, and, after drying and preequilibration over the eluent, chromatographed in a solvent consisting of absolute ethanol–*tert*-butyl alcohol–88% HCOOH–H_2O (60:20:5:15, v/v). The front was allowed to move 17.5 cm from the origin and then the plate was dried, the portion of the plate containing the standard amino acids was sprayed with a ninhydrin solution, and color was developed at 80° for 15 min. The lanes containing radioactivity were then marked off at measured intervals with a pencil and the cellulose was scraped into scintillation vials for radioactivity assay.

Periodate treatment was carried out on about 2 A_{260} of tRNAs in 0.9 ml of 0.10 M NaOAc, pH 5.0. These samples were the effluent at the void volume of small beds of Sephadex G-25 fine, to which had been applied the aqueous phase of a phenol extract of the aminoacylation or mock aminoacylation reaction mixtures described below. To the aminoacyl-tRNA^{Phe} or unacylated tRNA^{Phe} was added 200 nmoles of freshly prepared $NaIO_4$ in 4 μl. Following incubation at 25° for 45 min, 4 μmoles of ethylene glycol were added in 4 μl. After 15 min at 0°, tRNA was precipitated by adding NaCl to 0.5 M and then 2 volumes of absolute ethanol. The pelleted tRNA (10k*g* for 15 min) was taken up in 0.05 M Na_2CO_3 adjusted to pH 10.4 with CO_2, and incubated at 37° for 30 min to hydrolyze the aminoacyl ester. This is more stringent hydrolysis than is usually used, but it is necessary to remove the extremely stable ester of [^{14}C]Ile from both Ile-tRNA^{Ile} and Ile-tRNA^{Phe}

Aminoacylations (see also Yarus and Berg, 1967) were performed at 37° in 40–200 μl reactions containing: 0.10 M cacodylate–NaOH, pH 7.0, 0.080 M NH_4OAc, 0.005 M $MgCl_2$, 0.002 M ATP, 0.001 M dithiothreitol, 0.0001 M Na_2EDTA, and 50 μg/ml of carboxymethyl-bovine serum albumin (Yarus and Rashbaum, 1972). A reaction said to have been conducted in 5% methanol by volume was done by, *e.g.*, replacing 5 μl of the H_2O in an 100-μl acylation mixture with 5 μl of reagent grade methanol. [^{14}C]Ile was usually 2×10^{-5} M, [^{3}H]Phe 1.5×10^{-5} M. An aminoacylation unit of isoleucyl-tRNA synthetase transfers 1 nmole of Ile to tRNA in 15 min at 37° under these optimal conditions. Because isoleucyl-tRNA synthetase was stored in 50% glycerol, all aminoacylation reactions contain about 1% glycerol by volume.

The tRNA binding assay has been extensively described (Yarus and Berg, 1970); briefly, it is performed at pH 5.5 and 17°, in the presence of 0.01 M Mg^{2+}; it exploits the fact that filtered aminoacyl-tRNA is retained on a Schleicher and Schuell B-6 nitrocellulose filter only when it is bound to isoleucyl-tRNA synthetase, which sticks quantitatively. The binding competition assay is based on the generally valid equation for two tRNAs in equilibrium with Ile-tRNA synthetase

$$\left\{\frac{[\text{Ile-tRNA synthetase}_0] - [\text{Ile-tRNA synthetase}]}{[\text{Ile-tRNA synthetase (Ile-tRNA}^{Ile})]} - 1\right\} = \frac{k_c[\text{tRNA}_c]}{k[\text{Ile-tRNA}^{Ile}]}$$

in which brackets signify concentrations of the enclosed species; $tRNA_c$ is the competitor, and Ile-tRNA the standard tRNA, k_c is the association constant of the competitor and k that of Ile-tRNA^{Ile}, Ile-tRNA synthetase$_0$ is the total isoleucyl-tRNA synthetase, Ile-tRNA synthetase is free enzyme, not in complex, and Ile-tRNA synthetase (Ile-tRNA^{Ile}) is the enzyme in complex with the standard tRNA. As usually performed, a constant total concentration of the standard tRNA is used which is >[Ile-tRNA synthetase$_0$] and large with respect to k^{-1}. Thus two simplifications follow: [Ile-tRNA synthetase$_0$] $\simeq$ [Ile-tRNA synthetase (Ile-tRNA^{Ile})]$_{\text{no competitor}}$ and also [Ile-tRNA synthetase (Ile-tRNA^{Ile})$_{\text{no competitor}}$ $\gg$ [Ile-tRNA synthetase] since isoleucyl-tRNA synthetase has all been drawn into complex with tRNA. Thus we have

$$\left(\frac{[\text{Ile-tRNA synthetase (Ile-tRNA}^{Ile})]_{\text{no competitor}}}{[\text{Ile-tRNA synthetase (Ile-tRNA}^{Ile})]_{\text{at [tRNAc]}}} - 1\right) = \frac{k_c}{k}\frac{[\text{tRNAc}]}{[\text{Ile-tRNA}^{Ile}]}$$

and a plot of the competition function on the left *vs.* the ratio of competitor to standard tRNA concentrations on the right gives k_c/k directly.

Several embellishments and precautions are necessary to get acceptable data at small competitions and in methanol H_2O, as in the experiment of Figure 6 below: (1) a separate set of saturation measurements (constant isoleucyl-tRNA synthetase, increasing Ile-tRNA^{Ile}) was required to insure that the value used for [Ile-tRNA synthetase$_0$] was precise and represented a true saturation, since competitions observed may be of the order of the errors plus nonsaturation in ordinary duplicate measurements. The position of the origin (no competitor) in Figure 6 is based on the average of eight independent measurements, having a standard deviation of 2%. (2) Individual blanks (no isoleucyl-tRNA synthetase) must be done for each competition point, since the competitor tRNA depresses the background, and even though blanks are only ~5% of the total counts bound, differences between blanks can be significant when small amounts of competition are being measured, and methanol must be present in the wash buffer (Yarus and Berg, 1970) at the same concentration as in binding reactions, particularly in that aliquot of wash buffer used to presoak the filters (Yarus and Berg, 1970), or else the

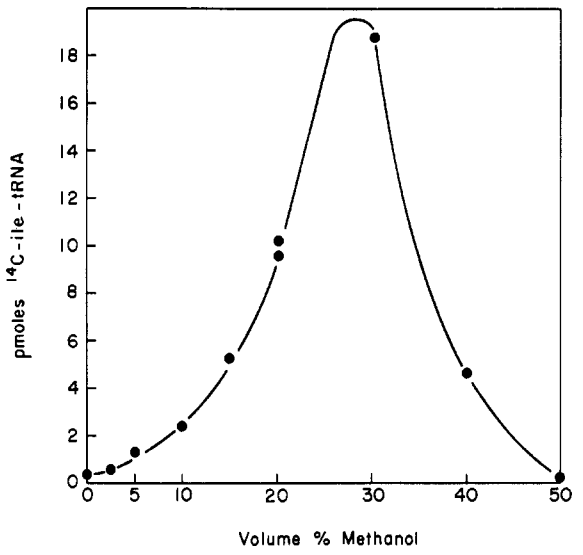

FIGURE 1: The effect of methanol concentration on transfer of [^{14}C]Ile to acid precipitability. Aminoacylation reactions contained methanol as shown, 85 aminoacylation units of pure isoleucyl-tRNA synthetase and 1.6×10^{-6} M tRNA^{Phe}. The data shown are the total transfer after 15 min at 37°.

Ile-tRNA synthetase (Ile-tRNA^{Ile}) seems to briefly sense an aqueous environment during filtration, and competition with tRNA^{Phe} becomes irreproducible.

Results

The phenomenon of heterologous acceptance is demonstrated in Figure 1, which shows the effect of methanol concentration on the amount of [^{14}C]isoleucine made acid precipitable in a reaction containing homogeneous isoleucyl-tRNA synthetase and highly purified tRNA^{Phe} (*E. coli*). At the left-hand margin (in 0% MeOH; that is, in the usual reaction conditions) there is a very small but measurable incorporation. We will return to consider this incorporation below. In any case, reactions in the presence of methanol greatly exceed this value (by up to 600-fold in other experiments), so there is no quantitative problem distinguishing the methanol effect from this background. The existence of this blank value does, however, raise the problem of cryptic or masked tRNA^{Ile}, which might be revealed by methanol. This would, of course, be a trivial explanation of the data in Figure 1. We will return below to experiments which make a qualitative distinction between Ile-tRNA^{Ile} and Ile-tRNA^{Phe}, and show that the latter is the species synthesized in these experiments. Methanol exhibits its effect at low concentrations; its stimulatory effect appears quite distinctly by 5% methanol by volume (mole fraction methanol = 0.023, 1.24 moles/l.). The transfer of isoleucine to tRNA^{Phe} increases more than linearly with increasing methanol concentration, up to a point around 30% methanol by volume (mole fraction methanol = 0.16, 7.6 moles of methanol/l.), then rapidly falls off. The suppression of the effect occurs at high enough methanol concentrations so that one may suspect inactivation of the enzyme, though this has not been specifically investigated. Most further work was done at 20% methanol. In previous studies on this system (Yarus, 1972), it has been shown that both mixed tRNA and tRNA^{Ile} are well behaved under conditions similar to these, existing as soluble monomers. Thus the isoleucine-accepting species is believed to be a single tRNA chain.

TABLE I: Effect of Added Amino Acids on the Apparent Velocity of Isoleucylation.[a]

Amino Acid Present	pmoles of ^{14}C Transferred/Min
1. 0.8 nmole of [^{14}C]Ile only	1.7
2. 0.8 nmole of [^{14}C]Ile + 2 nmoles of L-Phe	1.6
3. 0.8 nmole of [^{14}C]Ile + 10 nmoles of L-Phe	1.7
4. 0.8 nmole of [^{14}C]Ile + 1.4 nmoles of L-Ile	0.66
5. 0.8 nmole of [^{14}C]Ile + 8 nmoles of L-Ile	0.24

[a] Aminoacylation mixtures contained 20% methanol, 40 aminoacylation units of isoleucyl-tRNA synthetase, and 3.1×10^{6} M tRNA^{Phe}.

We turn now to the identification of the reacting species in experiments like those of Figure 1. First, isoleucine is the amino acid transferred to tRNA. Several independent preparations, including both [^{3}H]- and [^{14}C]isoleucine, have given similar results. More specifically, Table I shows the results of an isotope dilution experiment in which unlabeled L-Ile and L-Phe were added to methanol-containing reaction mixtures. While the addition of excess unlabeled Phe has no effect on the apparent velocity of aminoacyl transfer, the specific radioactivity of the amino acid transferred is diluted by unlabeled L-Ile in proportion to its abundance. This may be taken as evidence that the major species in the preparations of [^{14}C]Ile and unlabeled Ile are the reactive ones, and thus that isoleucine is being transferred. As a further proof of this point, tRNA^{Phe} was aminoacylated in 20% methanol to a specific level of 2.7 nmoles of [^{14}C]Ile/10 A_{260}, and the tRNA was isolated by phenol treatment and gel filtration to remove small molecules (see Methods). An aliquot of the resulting aminoacyl-tRNA preparation was precipitated with acid and the resulting precipitate deposited on a filter and washed, then eluted with 10% NH_4OH to release the amino acid from tRNA. The resulting radioactivity was chromatographed with amino acid markers and with a preparation of [^{3}H]Phe-tRNA^{Phe} treated in the same way. The result is shown in Figure 2; the radiocarbon bound to tRNA behaves as isoleucine.

What is the identity of the acceptor tRNA in Figure 1? The most usual experimental criterion for the identity of a tRNA is its aminoacylation under unperturbed, specific conditions. In Figure 3 is a flow chart and results of an experiment in which a preparation of tRNA^{Phe} was aminoacylated with [^{3}H]Phe under unperturbed conditions, using phenylalanyl-tRNA synthetase, and, alternatively, with [^{14}C]Ile in the presence of methanol and Ile-tRNA synthetase. Unacylated chains were oxidized with periodate (Baldwin and Berg, 1966), and a mock aminoacylated control is included to show that this periodate treatment does indeed destroy the acceptor activity of unacylated tRNA: phenylalanine acceptance is reduced >500-fold in this latter case (Figure 3). The crux of the experiment is the results in the lowest line of Figure 3; chains which were protected from periodate by aminoacylation with Phe will, after the Phe is removed in mild alkaline conditions, accept Ile in reactions containing methanol (Ile acceptor is

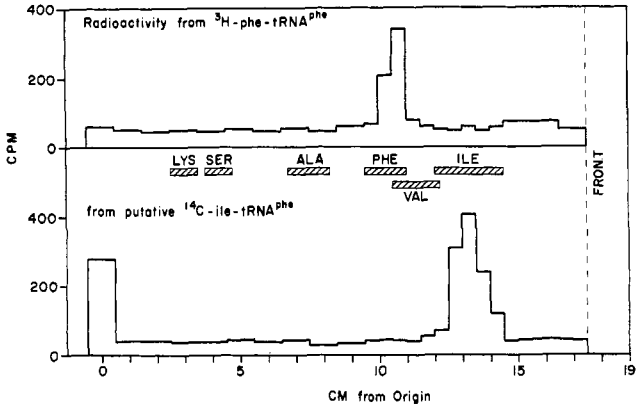

FIGURE 2: Chromatography of the radioactivity released from aminoacyl-tRNAs. Shaded bars mark the positions of amino acid markers run simultaneously. Counts per minute are observed values, without correction for background.

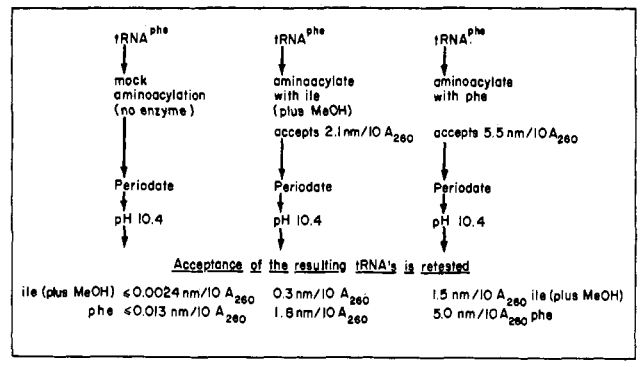

FIGURE 3: Flow chart for and results of an experiment to demonstrate the identity of the chains which accept Ile in 20% methanol with those that accept Phe under usual acylation conditions.

>500 times the mock aminoacylated blank (Figure 3)). This implies the synthesis of Ile-tRNA^{Phe}. Conversely, when chains are protected by aminoacylation with isoleucine in the presence of methanol, they later accept phenylalanine under unperturbed conditions. (Phe acceptor is >140 times the mock aminoacylated blank (Figure 3).) This result has the same implications as the first; the good recovery of acceptor activity[1] has the additional implication that most of the chains which accepted Ile in the presence of methanol were, in fact, tRNA^{Phe}.

A further test of the identity of the isoleucine acceptor in methanol–H_2O is provided by experiments which show that [^{14}C]Ile (in the form of the putative [^{14}C]Ile-tRNA^{Phe}) is bound to ribosomes in response to poly(U) (Table II) and (in Figure 4b) also incorporated into polypeptide linkage in response to poly(U). These assays are of course conducted under standard conditions (no methanol). Thus, isoleucine has become associated with the phenylalanine anticodon, and therefore, with tRNA^{Phe}.

Table II exhibits the net amount of aminoacyl-tRNA bound to nitrocellulose filters in the presence of ribosomes and message. [^{3}H]Phe-tRNA^{Phe} is bound, as expected, in response to poly(U) (entry 1), but no detectable [^{14}C]Ile-tRNA^{Ile} is bound (entry 3). A positive control (entry 4) shows that [^{14}C]Ile-tRNA^{Ile} will respond to poly(U,A), which contains the relevant (Yarus and Barrell, 1971) isoleucine codon, AUU. The critical entry is 2, which shows that the [^{14}C]Ile-tRNA synthesized when methanol is added to reactions containing tRNA^{Phe} and isoleucyl-tRNA synthetase binds to ribosomes in the presence of poly(U).

In Figure 4 are the kinetics of incorporation *in vitro* (into hot trichloroacetic acid resistant material) of [^{3}H]Phe from [^{3}H]Phe-tRNA^{Phe}, and [^{14}C]Ile from either [^{14}C]Ile-tRNA^{Ile} or [^{14}C]Ile-tRNA^{Phe}. Figure 4a shows the result when [^{3}H]Phe-

TABLE II: Binding of Aminoacyl-tRNAs to Ribosomes in Response to Message.[a]

Aminoacyl-tRNA	Message	pmoles Bound
1. Phe-tRNA^{Phe}	Poly(U)	1.48
2. Ile-tRNA^{Phe}	Poly(U)	1.34
3. Ile-tRNA^{Ile}	Poly(U)	<0.05
4. Ile-tRNA^{Ile}	Poly(U,A)	1.25

[a] Input was 8.7 pmoles of [^{14}C]Ile-tRNA^{Ile}, 6.6 pmoles of [^{3}H]Phe-tRNA^{Phe}, and 9.5 pmoles of putative [^{14}C]Ile-tRNA^{Phe}. A blank reaction mixture, lacking message, has been subtracted.

tRNA^{Phe} and [^{14}C]Ile-tRNA^{Ile} are present in the same complete reaction mixture (see methods) with poly(U); only [^{3}H]-Phe is incorporated. In contrast, Figure 4b shows that when [^{3}H]Phe-tRNA^{Phe} and the supposed [^{14}C]Ile-tRNA^{Phe} are incubated together with a poly(U) message, both are incorporated. Thus, again, [^{14}C]Ile-tRNA responds to the message for Phe. Panel 4c is a positive control which demonstrates the competence of the system and of the [^{14}C]Ile-tRNA^{Ile} added to it: when the message added contains both Phe and Ile codons, isotope from both [^{3}H]Phe-tRNA^{Phe} and [^{14}C]Ile-tRNA^{Ile} is incorporated into polypeptide. In Figure 4b, the fraction of the label incorporated from [^{14}C]Ile-tRNA^{Phe} is smaller than from [^{3}H]Phe-tRNA^{Phe} (note the difference in scales), even though binding to ribosomes is comparable (Table II). This is not because of any intrinsic deficiency in the [^{14}C]Ile-tRNA^{Phe}, but because, in the complete reactions, there is an extremely rapid deacylation of [^{14}C]Ile-tRNA^{Phe} which does not similarly affect either [^{3}H]Phe-tRNA^{Phe} or [^{14}C]Ile-tRNA^{Ile}.[2]

Thus the aminoacyl-tRNA species of Figure 1 is clearly [^{14}C]Ile-tRNA^{Phe}. Not only is [^{14}C]Ile bound to tRNA^{Phe}, but it is clearly in the normal ester linkage to 3′-terminal ribose; acceptance is destroyed by pretreatment with periodate, isoleucine is released in mild alkali (Figure 1), and the product of the acylation reaction in methanol is a substrate for all the other reactions of polypeptide chain extension (Figure 4b), including peptidyl synthetase.

[1] Reactions in which Ile transfer is measured in the presence of methanol fall short of the true maximum *extent* of reaction because of the low maximal velocity of isoleucyl-tRNA synthetase in this reaction and because of the need for high tRNA concentrations to attain this velocity (see Figure 5 below), combined with a rapid inactivation of isoleucyl-tRNA synthetase in the presence of methanol at the high (37°) temperature used here. When true rates of Ile-tRNA^{Phe} synthesis are required they must therefore be measured in short incubations. On the other hand, reactions employing phenylalanyl-tRNA synthetase under usual acylation conditions (that is, measurements of tRNA^{Phe}) do go to a defined limit.

[2] M. Yarus, submitted to *Proc. Nat. Acad. Sci. U. S.*

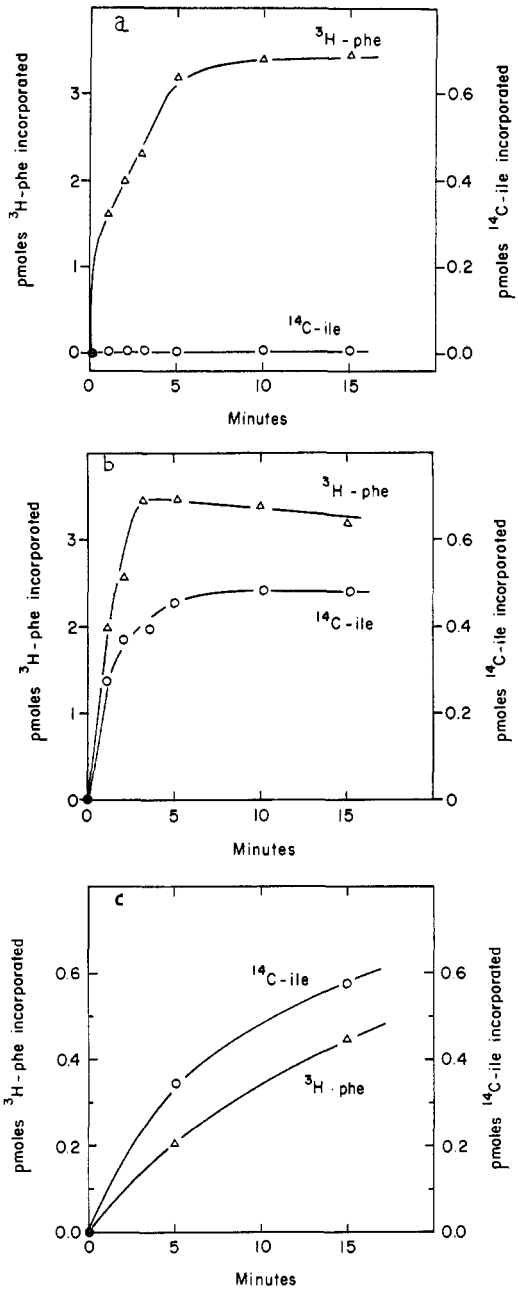

FIGURE 4: Kinetics of *in vitro* polypeptide synthesis in reactions containing aminoacyl-tRNAs. (a) Message: poly(U); aminoacyl-tRNA: [^{3}H]Phe-tRNA^{Phe} and [^{14}C]Ile-tRNA^{Ile}; (b) message: poly(U); aminoacyl-tRNA: [^{3}H]Phe-tRNA^{Phe} and putative [^{14}C]-Ile-tRNA^{Ile}; (c) message: poly(U,A); aminoacyl-tRNA: [^{3}H]Phe-tRNA^{Phe} and [^{14}C]Ile-tRNA^{Ile}. Input of aminoacyl-tRNAs for each kinetic point is the same as for the individual binding experiments of Table II.

Given that the product of the reaction is *bona fide* [^{14}C]Ile-tRNA^{Phe}, there remains the possible question of the enzyme responsible for its synthesis. In particular, one might ask if it is conceivable that phenylalanyl-tRNA synthetase contaminates the isoleucyl-tRNA synthetase preparations used here, homogeneous though they appear. Might it then be that the mistake is at the level of the amino acid, and that Ile is mistakenly activated and transferred to tRNA^{Phe} by the hypothetical phenylalanyl-tRNA synthetase?

This possibility is extremely unlikely. Some evidence has already been presented, in Table I, where it appears that addition of Phe has no effect on the incorporation of [^{14}C]Ile; if the enzyme concerned were phenylalanyl-tRNA synthetase, one would expect the formation of phenylalanyl-tRNA synthetase (Phe-AMP) at the expense of isoleucyl-tRNA synthetase ([^{14}C]Ile-AMP) and thus a decrease in the rate of [^{14}C]Ile transfer. Instead, the reaction is indifferent to the added Phe, and responsive to Ile, as would be expected of isoleucyl-tRNA synthetase.

TABLE III: A Comparison of Phenylalanyl-tRNA Synthetase Content and Velocity of [^{14}C]Ile-tRNA^{Phe} Synthesis.[a]

Aminoacylation Units of Phenylalanyl-tRNA Synthetase	Source	pmoles of [^{14}C]Ile-tRNA^{Phe}/Min
1. <0.00006	Basal isoleucyl-tRNA synthetase level	1.2
2. 0.0013	Basal + added phenylalanyl-tRNA synthetase	1.4
3. 0.012	Basal + added phenylalanyl-tRNA synthetase	1.2
4. 0.12	Basal + added phenylalanyl-tRNA synthetase	1.0

[a] All reaction mixtures contained 20% methanol, 17 aminoacylation units of isoleucyl-tRNA synthetase, and 4.7 $\times 10^{-6}$ M tRNA^{Phe}.

Secondly, the phenylalanyl-tRNA synthetase activity contaminating the preparations of pure isoleucyl-tRNA synthetase used in this work (measured by transfer of isotopic Phe to tRNA^{Phe}) is so low as to be difficult to measure, whether in the presence of methanol or not. Taking the low radioactivities observed at face value, the preparation of isoleucyl-tRNA synthetase used for most of this work (*e.g.*, for Figures 1–3, and Table I) has no detectable phenylalanyl-tRNA synthetase activity at all. The sensitivity of these measurements is such as to indicate that the rate of transfer of Phe to tRNA^{Phe} catalyzed by these isoleucyl-tRNA synthetase preparations is $<10^{-6}$ times that of the transfer of Ile to tRNA^{Ile}. Since (see below, Figure 5) the maximal rate of transfer of [^{14}C]Ile to tRNA^{Phe} is about 2.9×10^{-3} that of Ile to tRNA^{Ile}, I surmise that phenylalanyl-tRNA synthetase is unlikely to be involved because at least 2900 times as much activity as is detected would be required to carry out the reaction observed, even supposing that phenylalanyl-tRNA synthetase could mistakenly activate isoleucine, and then synthesize Ile-tRNA at the same rate it does Phe-tRNA. To make this point more clearly, Table III shows an experiment in which phenylalanyl-tRNA synthetase activity is purposely added to reactions containing isoleucyl-tRNA synthetase which has no detectable intrinsic phenylalanyl-tRNA synthetase activity. The table compares the total phenylalanyl-tRNA synthetase activity (measured in 20% methanol; phenylalanyl-tRNA synthetase activity is inhibited by the addition of methanol) with the observed velocity of transfer of Ile to tRNA^{Phe}. Increase of phenylalanyl-tRNA synthetase content at least 2000-fold has no significant stimulatory effect on the rate of synthesis of [^{14}C]Ile-tRNA^{Phe}.

Lastly, the observed reaction does not respond to variation of tRNA^{Phe} as does the *bona fide* phenylalanyl-tRNA synthe-

tase reaction. Since the kinetic properties of the reaction are of interest in themselves, these data are shown in Figure 5, which contains double reciprocal kinetic plots for the velocity of Phe-tRNA^{Phe} synthesis catalyzed by phenylalanyl-tRNA synthetase in 20% methanol, and of Ile-tRNA^{Phe} synthesis catalyzed by isoleucyl-tRNA synthetase in 20% methanol. The kinetic K_m's for tRNA are clearly quite different, suggesting that the element interacting with tRNA^{Phe} is different in the two cases. The maximal velocity of transfer of Ile to tRNA^{Phe} is 2.9×10^{-3} of the velocity at which the same amount of isoleucyl-tRNA synthetase could transfer Ile to tRNA^{Ile}. The K_m of isoleucyl-tRNA synthetase with respect to tRNA^{Phe} is about 7.7×10^{-6} M, rather higher than is usually observed in reactions between cognate synthetases and tRNAs under more usual conditions. The magnitude of this number will be discussed further below. I conclude that phenylalanyl-tRNA synthetase is not the active agent in reactions like those of Figure 1.

A further item of evidence is needed to characterize the interaction of isoleucyl-tRNA synthetase with tRNA^{Phe}. One might regard the synthesis of [^{14}C]Ile-tRNA^{Phe} as evidence that a certain absolute strength of binding had been achieved with isoleucyl-tRNA synthetase. However, the ideas previously expressed (Yarus, 1972) lead to a further prediction: that, under conditions in which the structure of tRNA is loosened by methanol, the distinction that is made between tRNA^{Phe} and tRNA^{Ile} should have declined; in other words, that the ratio of association constants (that of tRNA^{Phe} divided by that of tRNA^{Ile}) be a larger number in methanol–H_2O. To pose the alternative possibility, it could be that the interaction of all tRNAs with isoleucyl-tRNA synthetase has been enhanced (the association constant multiplied) by the same factor in 20% methanol. Thus the synthesis of [^{14}C]Ile-tRNA^{Phe} could occur, even though the isoleucyl-tRNA synthetase protein still distinguished the two tRNAs just as it did in the normal solvent. The effect would not pertain to the specificity of recognition, in this sense.

This is not the case for 20% methanol; instead, the specificity of the binding reaction has declined considerably. The relevant data are plotted in Figure 6, which compares the ability of tRNA^{Phe} to compete with [^{14}C]Ile-tRNA^{Ile} for binding to isoleucyl-tRNA synthetase in the presence and the absence of methanol. This method of analysis of competition data yields a straight line whose slope is directly the desired quantity (association constant for tRNA^{Phe}/association constant for [^{14}C]Ile-tRNA^{Ile}) (Yarus and Berg, 1969, 1967; see Methods also). In this figure it appears that no competition at all is detectable at the tested levels of tRNA^{Phe} without methanol: the level of [^{14}C]Ile-tRNA^{Ile} bound simply scatters (±2%) about the value obtained in the absence of added tRNA^{Phe}, even when there is several hundred times as much tRNA^{Phe} as Ile-tRNA^{Ile} present (Figure 6). This sets an upper limit on the affinity of tRNA^{Phe} for isoleucyl-tRNA synthetase in normal binding solvent: the association constant for tRNA^{Phe} and isoleucyl-tRNA synthetase is $\leq 4.1 \times 10^{-5}$ that of Ile-tRNA^{Ile}; the latter is 1.4×10^{8} l./mole (Yarus and Berg, 1969), so that the affinity of tRNA^{Phe} for isoleucyl-tRNA synthetase under these conditions corresponds to an association constant of $\leq 5.7 \times 10^{3}$ l./mole. Since the standard free energy of binding $\Delta G^\circ = -RT \ln k$ (R is the gas constant, T the temperature in degrees Kelvin, and k the association constant), this result implies that tRNA^{Phe} under unperturbed binding conditions can have, at most, only about half the free energy of interaction with isoleucyl-tRNA synthetase that tRNA^{Ile} does. As far as I am aware, this is the first rigorous

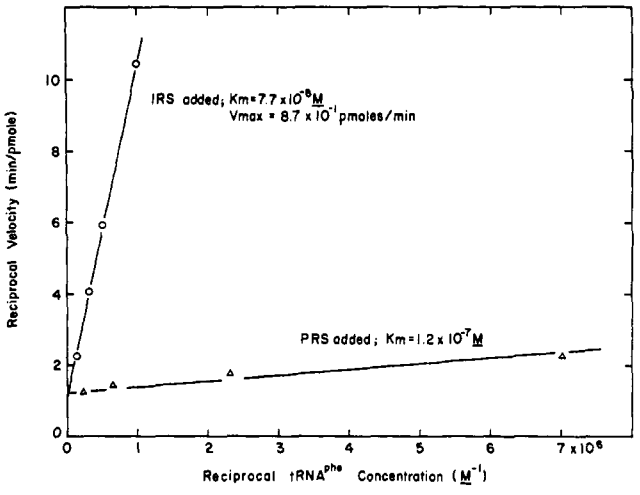

FIGURE 5: Double reciprocal plots of the variation of the rate o aminoacyl-tRNA synthesis (in 20% methanol) as tRNA^{Phe} concentration is varied. Triangles: transfer of [^{3}H]Phe to tRNA^{Phe} catalyzed by phenylalanyl-tRNA synthetase from *E. coli* B; circles: transfer of [^{14}C]Ile to tRNA^{Phe} catalyzed by isoleucyl-tRNA synthetase from *E. coli* B. Isoleucyl-tRNA synthetase catalyzed reactions contained 4.5 aminoacylation units of pure isoleucyl-tRNA synthetase, that is, isoleucyl-tRNA synthetase capable of a maximal velocity of 3×10^2 pmoles of Ile transferred to tRNA^{Ile} per minute under optimal conditions (see Methods). For conciseness, in the figure IRS and PRS are used as abbreviations for isoleucyl-tRNA synthetase and phenylalanyl-tRNA synthetase, respectively.

limit which has been set on transcognate affinity. A more complete discussion of this subject (M. Yarus, in preparation) will be presented shortly.

Turning now to the same competition (tRNA^{Phe} *vs.* Ile-tRNA^{Ile}) in the presence of methanol, a significant effect appears (Figure 6). The slope of the line is such as to suggest that tRNA^{Phe} has an association constant $\sim 10^{-3}$ that of Ile-tRNA^{Ile} itself. Since Ile-tRNA^{Ile} under these conditions is characterized by a k probably 8×10^8 to 10×10^8 l./mole (Yarus, 1972), this places the association constant for tRNA^{Phe} with isoleucyl-tRNA synthetase in 20% methanol around 0.8×10^6 to 1×10^6 l./mole. This is in reasonable agreement with another estimation[3] of this association constant, the kinetic K_m^{-1}, shown above (Figure 5) to be 0.13×10^6 l./mole under conditions which differ somewhat in temperature, [Mg^{2+}], and pH. Thus the addition of 20% methanol has increased the association constant of tRNA^{Phe} with isoleucyl-tRNA synthetase by at least 200-fold.

In addition, the specificity of the recognition process, as represented by the increase in slope in Figure 6 on addition of methanol, has declined. The association constant for tRNA^{Phe} has increased at least 25-fold more than has that for Ile-

[3] It appears that release of aminoacyl-tRNA is the rate-limiting step in the aminoacyl transfer reaction as usually measured between cognate enzymes and tRNA (Yarus and Berg, 1969; Helene *et al.*, 1971; also personal communication from E. W. Eldred and D. R. Schimmel). Under these conditions the kinetic K_m determined by variation of tRNA under conditions of saturation with other reagents should, in fact, be numerically similar to the true dissociation constant for tRNA. In the transfer of Ile to tRNA^{Phe}, the maximal rate of aminoacyl transfer is slower yet (Figure 5). Since the rate of dissociation of tRNA^{Phe} from isoleucyl-tRNA synthetase is probably at least as fast as tRNA^{Ile} from isoleucyl-tRNA synthetase, this suggests a new rate-limiting step, perhaps transaminoacylation (see footnote 4), which is even slower than tRNA dissociation. These latter conditions also would imply $k \simeq K_m^{-1}$, and so justify treating the kinetic quantity as a true dissociation constant.

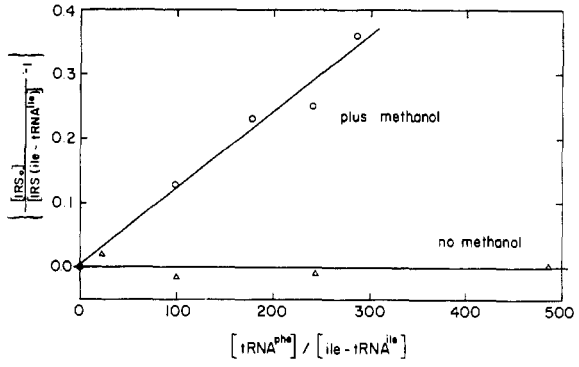

FIGURE 6: Competition between Ile-tRNA^{Ile} and tRNA^{Phe} for binding to limited isoleucyl-tRNA synthetase. Binding reaction mixtures contained 1.1×10^{-7} M isoleucyl-tRNA synthetase, 1.9×10^{-7} M Ile-tRNA^{Ile}, and 10^{-3} M L-Ile (Yarus and Berg, 1969).

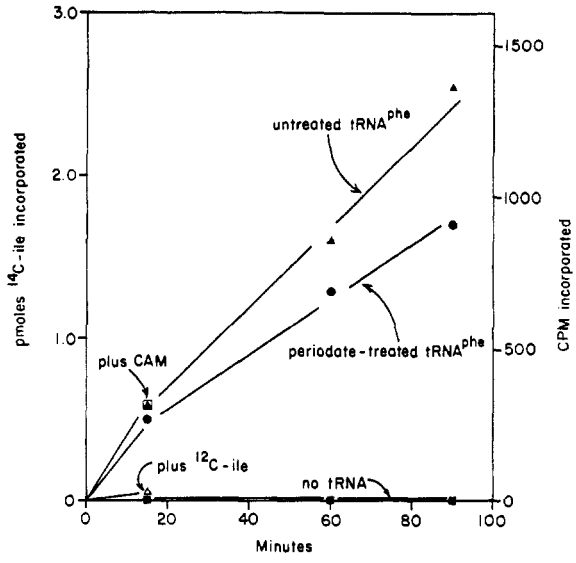

FIGURE 7: Kinetics of transfer of [^{14}C]Ile to tRNA^{Phe} in normal acylation conditions. Filled triangles represent an experiment employing untreated tRNA^{Phe} at 2.3×10^{-6} M; filled circles, a preparation of tRNA^{Phe} which has been aminoacylated with phenylalanine by phenylalanyl-tRNA synthetase, oxidized with periodate, then discharged; [tRNA^{Phe}] = 1.4×10^{-6} M; filled squares are measurements on a blank containing isoleucyl-tRNA synthetase, but no tRNA. These 40-μl reactions all contain 40 aminoacylation units of isoleucyl-tRNA synthetase. The open square represents an experiment to which 1×10^{-3} M chloramphenicol (CAM) has been added, the open triangle an experiment to which 10 nmoles of [^{12}C]Ile has been added. Reactions contain 0.8 nmole of [^{14}C]Ile. The point at the axis on the left (0 min) contains all reactants, but is unincubated.

tRNA^{Ile}. I conclude that addition of methanol has reduced the distinction which isoleucyl-tRNA synthetase usually makes between tRNA^{Ile} and tRNA^{Phe}. In addition, these data (Figure 6) are themselves an additional argument for the role of isoleucyl-tRNA synthetase in the synthesis of Ile-tRNA^{Phe}, rather than some hypothetical contaminant. The [^{14}C]Ile-tRNA^{Ile} is certainly bound to isoleucyl-tRNA synthetase; all the enzyme added occurs as complex in reactions which have no competitor added. Thus the effect of tRNA^{Phe} on the binding suggests that it must also interact with isoleucyl-tRNA synthetase.

Let us return now to the incorporation in the absence of methanol, that which was pointed out at the left margin of Figure 1. That point represents incorporation into 1 chain of every 1000 in the reaction mixture, and might plausibly have been attributed to occurrence of a small number of tRNA^{Ile} molecules as an impurity in the tRNA^{Phe} preparation. This, however, is not the case. The data of Figure 6, for competition in the absence of methanol, may be used to set a limit on the number of tRNA^{Ile} chains which can occur as contaminants in tRNA^{Phe}, as well as on the intrinsic association constant for tRNA^{Phe}. Unacylated tRNA^{Ile} is an excellent competitor with Ile-tRNA^{Ile} for isoleucyl-tRNA synthetase (Yarus and Berg, 1967), having an association constant 0.7–0.8 of the acylated molecule. Using the limit set on the slope in Figure 6 (slope $\leq 4.1 \times 10^{-5}$, see Methods), and the unbound Ile-tRNA^{Ile} concentration, 6.6×10^{-8} M, it is simple to calculate that less than one chain in 20,000 of the tRNA^{Phe} preparation can be tRNA^{Ile}. Thus I conclude that the incorporation in the absence of methanol is unlikely to be the synthesis of Ile-tRNA^{Ile}. This conclusion is confirmed by examination of the two major curves of Figure 7, which show that when experiments under normal acylation conditions are not restricted to very short times, one observes a slow but progressive incorporation of isoleucine. These kinetics are also inconsistent with the synthesis of Ile-tRNA^{Ile}, since the synthesis of the amount of Ile-tRNA present at 90 min would have required 60 msec under these conditions, instead of 5.4×10^6 msec, as shown. In addition, the two major curves provide a comparison of untreated tRNA^{Phe} and tRNA^{Phe} which has been acylated with Phe by phenylalanyl-tRNA synthetase under normal conditions, periodate treated, and deacylated at pH 10.4, just as in Figure 3; the suppression of acceptance by unacylated tRNAs by at least 500-fold (Figure 3) in this latter case has not notably affected Ile acceptance. At 90 min, the incorporations correspond to isoleucylation of one chain in 35 and one in 50. The slow decrease in rate is probably due to a slow inactivation of isoleucyl-tRNA synthetase, which has been previously observed in long incubations. This incorporation depends on addition of both tRNA^{Phe} and isoleucyl-tRNA synthetase, and ancillary measurements, shown in Figure 7, suggest that incorporation is unaffected by 1×10^{-3} M chloramphenicol, but is diluted when [^{12}C]Ile is added. When Ile-tRNA from a similar reaction was collected by ethanol precipitation, and dissolved in 0.05 M Na_2CO_3, pH 10.4, the distinctive slow hydrolysis of an isoleucyl ester in Ile-tRNA ($t_{1/2} \simeq 10$ min), which also characterizes Ile-tRNA^{Ile}, and Ile-tRNA^{Phe} (synthesized in 20% methanol), was observed. I conclude, by an argument similar to that used in the body of the text above, that the curves of Figure 7 represent the slow synthesis of Ile-tRNA^{Phe} catalyzed by isoleucyl-tRNA synthetase, even though the reaction conditions have not been modified by addition of methanol.

It seemed conceivable that the small amount of glycerol (final concentration 1.25% by volume) necessarily added with the isoleucyl-tRNA synthetase (see Methods) was serving as an "organic solvent" in these experiments. However, neither reduction of the glycerol concentration to 0.625% nor raising it to 3.75 or 6.25% perceptibly altered the velocity of Ile-tRNA^{Phe} synthesis in reactions like those of Figure 7. Thus it seems unlikely that glycerol is active in this respect.

The velocity of the reaction is very responsive to increase in [tRNA^{Phe}] and, in fact, attempts (based on 15 min incubations) to determine the characteristic kinetic parameters of this reaction have been unsuccessful because the velocity of the reaction is still increasing linearly with [tRNA^{Phe}]

TABLE IV: A Summary of the Characteristic Constant for Some Reactions of Isoleucyl-tRNA Synthetase from *E. coli* B.[a]

Reaction	Rel Max Acylation Velocity[b]	Ass. Constant, k (l./mole)[c]	Diss. Constant, K (mole/l.)	Method of Measurement	Ref
1. Isoleucylation of tRNA^{Ile}, normal acylation conditions	1.0			Kinetics, acylation	Methods, this work
2. Binding of Ile-tRNA^{Ile}, normal binding conditions		1.4×10^8	7×10^{-9}	Binding	Yarus and Berg (1969)
3. Isoleucylation of tRNA^{Phe}, normal acylation conditions	$\geqslant 0.79 \times 10^{-3}$	$\leqslant 7 \times 10^3$	$\geqslant 1.4 \times 10^{-4}$	Kinetics, acylation	Figure 8
4. Binding of tRNA^{Phe}, normal binding conditions		$\leqslant 5.7 \times 10^3$	$\geqslant 1.8 \times 10^{-4}$	Binding competition	Figure 6
5. Isoleucylation of tRNA^{Phe}, acylation conditions plus 20% methanol	2.9×10^{-3}	1.3×10^5	7.7×10^{-6}	Kinetics, acylation	Figure 5
6. Binding of tRNA^{Phe}, binding conditions plus 20% methanol		$\sim 10 \times 10^5$	$\sim 1 \times 10^{-6}$	Binding competition	Figure 6

[a] Blank entries are not relevant to either the discussion or to the reaction. [b] Values normalized to that of isoleucyl-tRNA synthetase aminoacylating tRNA^{Ile} under optimal conditions. [c] See footnote 3.

at the highest practical concentrations (Figure 8). This presumably implies that the concentrations used are still far below K_m, in the region where $v = (V_{max}/K_m)[tRNA^{Phe}]$. These data can nevertheless be used to calculate limits for V_{max} and K_m as follows. The dependence of v on [tRNA^{Phe}] should depart from linearity by -5% at the point where [tRNA^{Phe}] $\simeq 0.05K_m$. If we may say that the velocity of the reaction in Figure 8 is still increasing linearly to this precision at the highest [tRNA^{Phe}] used, $\sim 8 \times 10^{-6}$ M, then: $K_m \geqslant$ [tRNA^{Phe}]/0.05 or $K_m \geqslant 8 \times 10^{-6}/0.05 = 1.6 \times 10^{-4}$ M. Insofar as this conclusion goes, it is in agreement with the conclusion derived from the competition experiment of Figure 6. Since (Figure 8) $V_{max} = 0.2 \times 10^6 K_m$, $V_{max} \geqslant 32$ pmoles/15 min. Corrected to the same amount of enzyme (40 aminoacylation units), and cast in the same velocity units, the maximal velocities of isoleucylation of tRNA^{Phe} are: $V_{max} \geqslant 2.1$ pmoles/min (no methanol); $V_{max} = 7.7$ pmoles/min (20% methanol, Figure 5). Thus we have the striking conclusion that the reaction of tRNA^{Phe} with isoleucyl-tRNA synthetase in the absence of methanol probably proceeds with a maximal velocity which is of the same order as that in the presence of methanol; the effect of the added organic solvent is, most importantly, to increase the affinity[3] (to decrease K_m, or increase k) of the protein for tRNA^{Phe}. The numerical conclusions of this work are summarized in Table IV.

Discussion

One can now give an account of the normal mechanism by which isoleucyl-tRNA synthetase distinguishes tRNA^{Ile} from tRNA^{Phe}, and of the effects of methanol on this distinction, based on the data in Table IV.

Under usual conditions *in vitro*, both tRNA^{Ile} and tRNA^{Phe} have detectable affinities for isoleucyl-tRNA synthetase, though the association constant for tRNA^{Ile} is more than 10^4 as large as for tRNA^{Phe}. This implies a standard free energy of binding greater by at least 5.5 kcal/mole. We therefore expect to find ultimately that tRNA^{Ile} makes at least 2 or 3 favorable contacts (resulting in secondary bonds) with isoleucyl-tRNA synthetase that are unavailable to tRNA^{Phe}. This statement assumes that the region of contact of tRNA^{Phe} with isoleucyl-tRNA synthetase can be considered homologous to the region of contact of tRNA^{Ile}. There is strong internal evidence that this is true, at least for a major part of tRNA^{Phe}, because isoleucyl transfer to its 3′ end can

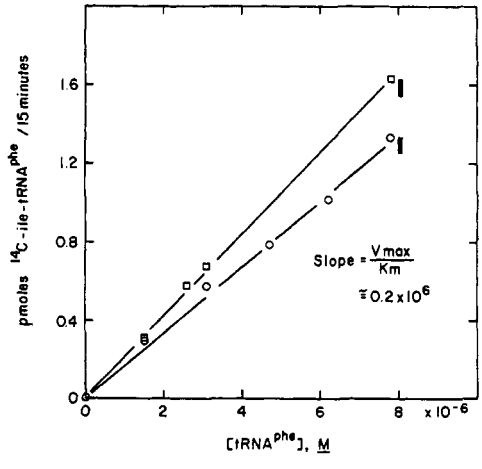

FIGURE 8: The effect of tRNA^{Phe} concentration on the velocity of Ile-tRNA^{Phe} synthesis. The vertical bar beside the lines represents 5% of the extent of reaction at that point. These 40-μl reactions contained 40 aminoacylation units of isoleucyl-tRNA synthetase, and represent incorporation after 15 min at 37°. The two lines represent two experiments performed at different times.

occur. This presumably implies that, even in the absence of methanol, the terminal ribose of $tRNA^{Phe}$ is placed precisely, within atomic dimensions[4] of the correct position for the terminal ribose of $tRNA^{Ile}$.

A similar impression is also derived, though less directly, from the substantial affinity of $tRNA^{Phe}$ for isoleucyl-tRNA synthetase, even though, under usual conditions, it has only been possible to calculate an upper limit: ≤ 0.47 the total free energy of binding of $tRNA^{Ile}$ is shared by $tRNA^{Phe}$. The existence of this affinity, and the argument for homologous contacts, suggest a radical change may be in order in the logic of sequence comparison among tRNAs which interact with the same enzyme: this has been a standard method in the search for areas of interaction with aminoacyl-RNA synthetases (*e.g.*, see Dudock *et al.*, 1971). We now expect a very substantial fraction of the areas of contact between the protein and the tRNA to be homologous, *even* when noncognates are compared. Thus, sequences common to many tRNAs, insofar as these enzymes show sequence specificity (M. Yarus, in preparation), not only cannot be excluded from consideration, but become particular candidates for participation in that part of the interaction which is shared among noncognates. The unshared portion of the interaction, which is probably considerably less than the total, can be plausibly sought elsewhere in the sequence. The situation in 20% methanol is particularly pointed: one presumes that the influence of shared regions has been expanded in this solvent, and it is observed that $tRNA^{Phe}$ has fully two-thirds the free energy of interaction of $tRNA^{Ile}$ itself.

What, in fact, are the effects of methanol? As indicated above, they are mainly to increase the association constant (up approximately 200-fold in 20% methanol); it is quite clear that the stimulation of the maximal reaction velocity, if any, must be much smaller. To put this another way, this work shows that the obstacles to reaction between isoleucyl-tRNA synthetase and $tRNA^{Phe}$ can be separated into two classes; it binds relatively poorly and is not aminoacylated quite as easily, even after being bound. Methanol relieves the first obstacle, but probably not the second.

This behavior provides confirmation of certain aspects of a proposed scheme for tRNA binding (Yarus, 1972). There it was pointed out that the effects of solvent are large and appear to be conformational; organic solvents are supposed to make accessible a novel conformational state of the tRNA which is better able to conform to the surface of the enzyme; this enhances the affinity of a tRNA–isoleucyl-tRNA synthetase pair, whether or not they be cognate. The conformation of $tRNA^{Phe}$ has not been directly studied in this work, but the author wishes to point to the following congruencies: (1) the addition of methanol enhances the affinity of the $tRNA^{Phe}$–isoleucyl-tRNA synthetase pair, indicating that the conclusions of the previous study extend to a transcognate pair. (2) There is no large enhancement of the maximal velocity of aminoacylation; that is, there is no large (favorable) rearrangement of the bound state. The explanation of the effect at the level of the free solution state of tRNA is therefore supported. (3) The addition of methanol suppresses the distinction which is usually made between $tRNA^{Ile}$ and $tRNA^{Phe}$. This is consistent with attribution of the increased binding free energy to a changed conformation, but not with some other conceivable explanations which would imply multiplication of both cognate and noncognate binding free energies by a similar factor.

To summarize: the novel suggestion which these observations make is that the barrier to reaction between these noncognates is slighter than might have been supposed. Their reluctance is largely overcome by manipulations which only provide moderate increases in the strength of binding (Yarus, 1972, and Results, this work). This makes implausible certain, particularly the more extreme, possibilities for the molecular origins of the specificity of this system. As a hypothetical example, the selectivity under normal conditions cannot have depended on steric incompatibility between the bases in a certain critical region of the tRNA and the sites which must *necessarily* acccept them on isoleucyl-tRNA synthetase. It is highly unlikely that the addition of 5% methanol to the solvent, as in Figure 1, could, *e.g.*, make an A acceptable in a site whose occupancy was required but which had been evolved as a stringent fit for a U.

The implications of this work differ somewhat from those of previous observations of misaminoacylation (*e.g.*, Jacobson, 1971; Taglang *et al.*, 1970; Dudock *et al.*, 1971) because in these latter cases, heterospecific systems were used: *Neurospora* phenylalanyl-tRNA synthetase and $tRNA^{Val}$ (*E. coli*) and $tRNA^{Ala}$ (*E. coli*) (Jacobson, 1971), *Saccharomyces* and $tRNA^{Val}$ (*E. coli*) and $tRNA^{Phe}$ (*E. coli*) (Taglang *et al.*, 1970; Dudock *et al.*, 1971). The isoleucylation in this work of $tRNA^{Phe}$, in contrast, surmounts the barrier to reaction between tRNAs within a species. However, it is conceivable that this discussion can aid interpretation of those experiments in which a heterologous synthetase reacts with several tRNAs (*E. coli*) (*e.g.*, Dudock *et al.*, 1971). I suggest that such cases of widespread reactivity arise when an aminoacyl-tRNA synthetase has a particular affinity for the common elements in tRNA structure, and thus that sequence elements shared by all the cross-reacting tRNAs may represent elements in contact with the aminoacyl-RNA synthetase in several, if not many, systems. This present work has made it appear likely that there are common structural elements in tRNAs which do interact with an aminoacyl tRNA synthetase. This may be the case with the dihydro-U stem (Dudock *et al.*, 1971), whose structure is indeed common to several tRNAs capable of transpecific misaminoacylations (Dudock *et al.*, 1971). Again, it should be emphasized that comparison of a one-dimensional string of letters (A, G, U, C) may not

[4] What meaning is to be attached to the fact that the maximum velocity of aminoacyl transfer to $tRNA^{Phe}$ could be several hundred times slower than to the cognate tRNA? It is known from previous work that both activation of isoleucine and aa-tRNA release are much faster than the rates of aa-tRNA synthesis observed here (Yarus, 1972; Yarus and Berg, 1969), and that the latter process, release of Ile-tRNA, is probably the rate-limiting step under ordinary acylation conditions (*cf.* footnote 2). Thus this new, much slower, rate most likely implies a novel rate-limiting step in aminoacyl transfer to $tRNA^{Phe}$. The obvious possibility is that transaminoacylation itself, from the aminoacyladenylate to the terminal ribose of $tRNA^{Phe}$, is slow enough to become the new rate-limiting step. This is most likely to mean, since the enzymatic apparatus is identical for $tRNA^{Phe}$ and $tRNA^{Ile}$, that the $tRNA^{Phe}$ terminus is, in fact, displaced slightly or available only part of the time. But a 1000-fold difference in rate corresponds to a difference in standard free energy of activation of 4 kcal/mole. This amount of free energy corresponds to that required to stretch a single bond only about 0.1 Å (*e.g.*, see Ramachandran and Sasisekharan, 1968). Thus even though the rates are more different than indicated (transaminoacylation is faster than the rate-limiting step for Ile-$tRNA^{Ile}$ synthesis), and even if all the difference is attributed to a misplaced tRNA which must be deformed in order to react, it is still likely that the occurrence of the reaction with the velocities observed implies that the 3′ end of $tRNA^{Phe}$ is placed correctly within atomic dimensions (<1 Å) and this justifies the argument above, that at least a substantial part of the contact area with isoleucyl-tRNA synthetase which is accessible to both $tRNA^{Ile}$ and $tRNA^{Phe}$ must be homologous in structure.

stimulate very well the chemical comparison made by the enzyme (*e.g.*, see Yarus, 1969).

As a final point of relation to the heterologous systems, I point to the emergent coincidence that all known cases of misaminoacylation involve tRNAs for the chemically similar hydrophobic amino acids Phe, Val, Ala, Ile: these tRNAs may have had a common evolutionary precursor. Perhaps this means that an event in the evolution of the genetic code is suffering a recrudescence as an event in the evolution of our understanding of it.

The sequences of tRNA^{Ile} (*E. coli*) (Yarus and Barrell, 1971) and tRNA^{Phe} (*E. coli*) (Barrell and Sanger, 1969) are reproduced in Figure 9. There is only a moderate resemblance, totaling 39 homologies in all, and many of the homologies are in sequences also shared with other *E. coli* tRNAs; this is the case (see above) with much of the dihydro-U loop, for example. They are not, in any case, promising *a priori* candidates for equivalence from the point of view of isoleucyl-tRNA synthetase. Yet the one part of tRNA^{Phe} which is located by these experiments, the 3′ end, must spend some time placed very precisely indeed in the tRNA site of isoleucyl-tRNA synthetase so that transaminoacylation from isoleucyl adenylate can occur (see above).

It is notable, therefore, that the homologies between the tRNAs are not, in fact, concentrated or even very marked in the CCA stem, save for the common terminal ACCA-OH sequence (Figure 9).

In 20% methanol, the standard free energy of binding of tRNA^{Phe} to isoleucyl-tRNA synthetase is fully two-thirds that of the cognate Ile-tRNA^{Ile}, and thus roughly speaking, tRNA^{Phe} in 20% methanol possesses two-thirds of the favorable interactions of tRNA^{Ile} itself, and it is unlikely that two-thirds of the total can be attributed entirely to the ApCpAOH region. For example, the removal of the terminal pCpAOH has only a small effect on the association constant of tRNA^{Ile} (Yarus and Berg, 1969), addition of the bulky phenoxyacetyl group to the amino group of isoleucine in Ile-tRNA^{Ile} yields a derivative which is still tightly bound (M. Yarus, unpublished data). Also, binding by another purified tRNA having the same ApCpCpAOH end group which is not stimulated by organic solvents has been observed (M. Yarus, unpublished data). Thus there is reason to believe that the reaction in 20% methanol must include another part of the tRNA^{Phe}.

Since the sequences of tRNA^{Ile} and tRNA^{Phe} are not themselves two-thirds homologous, this may be taken as direct evidence that (a) the region of interaction is a selected group of nucleotides smaller than the whole or (b) the sequence specificity (in 5–30% methanol, at least) is not high, and alternative nucleotides are accepted by the protein. This latter type of situation has been previously suggested as a likely one on general grounds (Yarus, 1969). Both these possibilities, of course, could exist at once.

Finally, previous work (So and Davie, 1964) has shown that methanol and other alcohols derange the translation of the genetic code *in vitro* and in particular, that isoleucine incorporation into polypeptide in response to poly(U) is stimulated. These experiments were conducted under conditions which are not particularly favorable to the synthesis of Ile-tRNA^{Phe} (M. Yarus, in preparation), but perhaps they should nevertheless be reconsidered in the light of Figure 4 of this work, which shows that incorporation of isoleucine in response to poly(U) can occur because of misaminoacylation, rather than miscoding, as originally supposed.

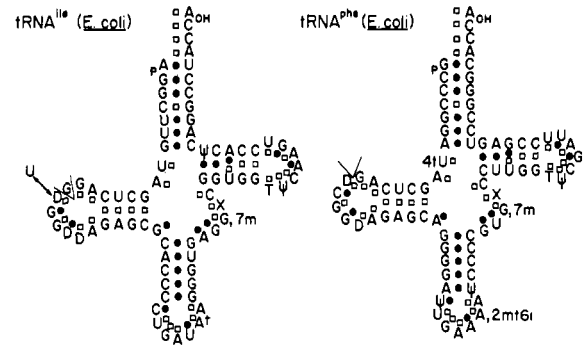

FIGURE 9: The sequence of nucleotides in tRNA^{Ile} (*E. coli*) and tRNA^{Phe} (*E. coli*). An open square next to a letter indicates that the same nucleotide is found at that position in the other tRNA; a closed circle indicates a nucleotide which is different in the two. The two lines joined at an angle in the left-hand loop of each figure indicate the site of a deletion in the sequence of tRNA^{Ile} which is made to maximize the homology between the two sequences.

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