Interaction of Glycyl-L-phenylalanine with *Escherichia coli* Phenylalanyl-tRNA Synthetase†

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ABSTRACT: The interaction of glycyl-L-phenylalanine with the phenylalanyl-tRNA synthetase from *Escherichia coli* has been studied by the attempted enzymatic acylation of tRNA with the dipeptide and with the dipeptidyl-AMP anhydride. Also measured were inhibition of the formation of phenylalanyl-tRNA^{Phe} in the presence of glycyl-L-phenylalanine and the dipeptidyl-AMP analog, glycyl-L-phenylalaninol-AMP, as well as the inhibition of ATP-PP_i exchange in the presence of certain amino alcohol-AMP analogs of aminoacyl adenylates. The results, which indicated specific recognition of the dipeptide and dipeptidyl-AMP anhydride by the phenylalanyl-tRNA synthetase, are of interest in terms of the mechanism by which preexisting proteins are excluded from incorporation onto tRNA.

The transfer of an amino acid to its cognate tRNA by the appropriate aminoacyl-tRNA synthetase is a process which may be represented conveniently as proceeding in three steps (Owens and Bell, 1970): binding reaction

$$E \cdot ATP + aa \rightleftharpoons E \cdot ATP \cdot aa$$

carboxyl activation reaction

$$E \cdot ATP \cdot aa \rightleftharpoons E \cdot aa\text{-}AMP + PP_i$$

tRNA-aminoacylation reaction

$$E \cdot aa\text{-}AMP + tRNA \rightleftharpoons E + aa\text{-}tRNA + AMP$$

The transformation effected by a single synthetase is thought to involve specific recognition by that synthetase of its cognate amino acid. The study of several aminoacyl-tRNA synthetases, however, has shown that this is true only in an overall sense. For example, the initial binding reaction for *Escherichia coli* valyl-tRNA synthetase has been reported (Owens and Bell, 1970) to have a broader specificity than the subsequent carboxyl activation reaction in terms of the amino acid substrate. Calendar and Berg (1966) found that the tyrosyl-tRNA synthetase from *E. coli* and *Bacillus subtilus* supported the ATP-PP_i[1] exchange reaction with both D- and L-tyrosine, as well as 3-fluoro- and 3-hydroxy-L-tyrosine, but not with any other naturally occurring L-amino acids, nor with a large number of synthetic analogs.

The structural requirements for participation of the aminoacyl adenylate in the tRNA-aminoacylation reaction are thought to be even more stringent (Conway *et al.*, 1962). For example, isoleucyl-tRNA synthetase from *E. coli* activates L-valine, but does not utilize the activated adenylate in the aminoacylation of tRNA^{Ile} (Baldwin and Berg, 1966). Exceptions to this pattern do exist, however. Thus D-proline was not activated in the presence of *E. coli* prolyl-tRNA synthetase, but several other analogs were bound, activated, and transferred to tRNA^{Pro} (Papas and Mehler, 1970). Similarly, the tyrosyl-tRNA synthetases from *E. coli* and *B. subtilus* were found to effect the aminoacylation of tRNA^{Tyr} utilizing both D-tyrosine and 3-fluoro-DL-tyrosine (Calendar and Berg, 1966).

In the context of this variable specificity of the aminoacyl-tRNA synthetases toward amino acid substrates, it seemed of interest to consider the mechansim whereby proteins are excluded from direct incorporation onto tRNA. Such incorporations, if they occurred, might well yield functional tRNA species, in the sense that they would constitute N-blocked aminoacyl-tRNAs, at least one of which besides fMet-tRNA^{fMet} (*N*-acetylphenylalanyl-tRNA^{Phe}) is known to be capable of initiating protein biosynthesis after incorporation into the ribosomal P-site (Springer and Grunberg-Manago, 1972). The interaction of proteins with the aminoacyl-tRNA synthetase has been studied in terms of the dipeptide glycyl-L-phenylalanine, using aminoacyl-tRNA synthetase from *E. coli*. This report deals with the recognition and utilization of the dipeptide and the dipeptidyl-AMP anhydride by the synthetase.

Materials and Methods

N-Carbobenzyloxyglycine, inorganic pyrophosphatase, and Pipes buffer were obtained from Sigma Chemical Co. [^{3}H]Phenylalanine and [^{3}H]glycine were purchased from International Chemical and Nuclear Co. The materials for preparation of the RPC-5 column were obtained from Miles Laboratories and the BD-cellulose from Schwarz/Mann Biochemicals.

Radiolabeled samples were counted on Whatman 3 mm paper discs or GF/A glass fiber discs in a toluene-based scintillation fluid or as aqueous solutions in a xylene-based scintillation fluid (Anderson and McClure, 1973). The determinations were made on Packard 3375 and Beckman LS-100C liquid scintillation spectrometers.

N-Carbobenzyloxy-L-phenylalaninol Ester of Adenosine 5′-Monophosphoric Acid (**2a**). This compound was prepared by a modification of the method of Sandrin and Boissonnas (1966). To 54 mg (0.19 mmol) of *N*-carbobenzyloxy-L-phenylalaninol was added 54 mg (0.095 mmol) of pyridinium $N^6,O^{2'},O^{3'}$-triacetyladenosine 5′-monophosphate (Rammler and Khorana, 1962), 39.2 mg (0.19 mmol) of *N,N′*-dicyclohexylcarbodiimide, and 2 ml of dry pyridine. The anhydrous solution was maintained at room temperature for 2 days. The solution was filtered and concentrated under diminished pressure, and the solid residue was

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[1] Abbreviations used are: PP_i, inorganic pyrophosphate; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); P_i, orthophosphate.

TABLE I: Chromatographic Properties (R_F Values) of the Amino Alcohol Adenylates.[a]

Compd	Solvent[b] A	B	C
5′-AMP	0.28	0.28	0.14
L-Phenylalaninol-AMP (**3a**)[c]	0.59	0.64	
Glycinol-AMP (**3b**)[c]	0.61	0.48	0.14
Glycyl-L-phenylalaninol-AMP (**5**)[c,d]	0.54	0.66	0.33
Glycylglycl-L-phenylalaninol-AMP (**6**)[c,d]	0.54	0.61	

[a] Determined on Whatman No. 1 paper by descending chromatography. [b] Solvent A, 5:2:3 1-butanol–acetic acid–water; solvent B, 7:1:2 2-propanol–ammonium hydroxide–water; solvent C, 7:1:2 1-butanol–acetic acid–water. [c] Compound located by ultraviolet visualization as a single, ninhydrin-positive spot. [d] Bromocresol Green positive.

triturated with petroleum ether and ether. The residue was dissolved in 1 ml of pyridine and treated with 9 M ammonium hydroxide solution for 24 hr. The solution was concentrated under diminished pressure and the residue was purified by chromatography on a column of diethylaminoethyl (DEAE)-cellulose (HCO_3^- form; 2 × 20 cm), elution with an ammonium bicarbonate gradient (2 l., 0–0.25 M). The appropriate fractions were combined, desalted at 60°, and dried *in vacuo* to afford **2a** as a white solid: yield 38 mg (64%); λ_{max} 258 nm; negative ninhydrin, positive Bromocresol Green tests.

N-Carbobenzyloxyglycinol Ester of Adenosine 5′-Monophosphoric Acid (**2b**). Compound **2b** was synthesized from *N*-carbobenzyloxy-β-aminoethanol (Rose, 1947) and N^6,-$O^{2'}$,$O^{3'}$-triacetyladenosine 5′-monophosphate (Rammler and Khorana, 1962), as described above for **2a**, by slight modification of the published procedure (Sandrin and Boissonnas, 1966).

L-*Phenylalaninol Ester of Adenosine 5′-Monophosphoric Acid* (**3a**, L-*Phenylalaninol-AMP*). To 38 mg (0.06 mmol) of the *N*-carbobenzyloxy-L-phenylalaninol ester of adenosine 5′-monophosphoric acid (**2a**) was added 8 ml of water and 20 mg of 5% palladium on carbon. The suspension was shaken under 3 atm of hydrogen for 24 hr. The suspension was filtered through a Celite pad and the solution was concentrated under diminished pressure to afford **3a** as a white solid: yield 14 mg (48%); λ_{max} 258 nm, positive ninhydrin, negative Bromocresol Green tests.

β-Aminoethyladenosine 5′-Monophosphate (**3b**; *Glycinol-AMP*). Compound **3b** was prepared from the *N*-carbobenzyloxyglycinol ester of adenosine 5′-monophosphoric acid (**2b**), according to the literature procedure (Sandrin and Boissonnas, 1966).

*N-Carbobenzyloxyglycyl-*L*-phenylalaninol Ester of Adenosine 5′-Monophosphoric Acid* (**4**). To 58 mg (0.12 mmol) of L-phenylalaninol-*AMP* (**3a**) dissolved in 0.5 ml of dry dimethylformamide was added 102 mg (0.33 mmol) of the *N*-hyroxysuccinimidyl ester of *N*-carbobenzyloxyglycine (Anderson *et al.*, 1963) in 0.5 ml of dry pyridine. The anhydrous solution was stirred at room temperature for 12 hr. The reaction mixture was treated with water and then concentrated to dryness under diminished pressure. The residue was dissolved in 10 ml of water, adjusted to pH 3 with 1 M hydrochloric acid, and filtered. The filtrate was neutralized and purified by chromatography on a column of DEAE-cellulose (1.5 × 20 cm; HCO_3^- form), elution with 200 ml of water, and then with a linear gradient of ammonium bicarbonate solution (250 ml total volume; 0–0.3 M). The appropriate fractions were combined and desalted by repeated evaporation of water under diminished pressure to afford **4** as a white solid: yield 82 mg (100%); λ_{max} 258 nm; negative ninhydrin, positive Bromocresol Green tests; paper chromatography on Whatman No. 1 paper (5:2:3 1-butanol–acetic acid–water) afforded a single uv-absorbing spot, R_F 0.70 (R_F of **3a** was 0.59).

*Glycyl-*L*-phenylalaninol Ester of Adenosine 5′-Monophosphoric Acid* (**5**; *Glycyl-*L*-phenylalaninol-AMP*). To 165 mg (0.24 mmol) of the *N*-carbobenzyloxyglycyl-L-phenylalaninol ester of adenosine 5′-monophosphoric acid (**4**) in 50 ml of 10% aqueous ethanol was added 70 mg of 5% palladium on carbon. The suspension was shaken under 3 atm of hydrogen at room temperature for 24 hr and then filtered through a Celite pad. The solution was concentrated to afford **5** as a white solid: yield 85 mg (67%); λ_{max} 258 nm; positive ninhydrin and Bromocresol Green tests.

*N-Carbobenzyloxyglycylglycyl-*L*-phenylalaninol Ester of Adenosine 5′-Monophosphoric Acid.* This compound was prepared from **3a** and the *N*-hydroxysuccinimidyl ester of *N*-carbobenzyloxyglycylglycine (Pavar and Chimens, 1971) by the same procedure used for the synthesis of **4**: yield 87 mg (98%); λ_{max} 258 nm; paper chromatography on Whatman No. 1 paper (5:2:3 1-butanol–acetic acid–water) afforded a single uv-absorbing spot, R_F 0.71, which was Bromocresol Green positive (ninhydrin negative).

*Glycylglycyl-*L*-phenylalaninol Ester of Adenosine 5′-Monophosphoric Acid* (**6**; *Glycylglycyl-*L*-phenylalaninol-AMP*). To 121 mg (0.162 mmol) of the *N*-carbobenzyloxyglycylglycyl-L-phenylalaninol ester of adenosine 5′-monophosphoric acid in 45 ml of 10% aqueous ethanol was added 70 mg of 5% palladium on carbon. The suspension was shaken under 3 atm of hydrogen at room temperature for 24 hr and filtered through a Celite pad, and the filtrate was concentrated to give **6** as a white solid: yield 58 mg (60%); λ_{max} 258 nm; paper chromatography on Whatman No. 1 paper (5:2:3 1-butanol–acetic acid–water) afforded a single uv-absorbing spot, R_F 0.54, which was both ninhydrin and Bromocresol Green positive.

Characterization of Amino Alcohol Adenylates. To approximately 200 A_{258} units of each amino alcohol adenylate (**3a**, **3b**, **5**, and **6**), dissolved in 1.0 ml of 0.1 M sodium carbonate solution (pH 9.2) was added 50 mg of crude snake venom phosphodiesterase (*Crotalus adamanteus*). The resulting solution was incubated overnight at room temperature. A 50-μl aliquot was removed, diluted to 2 ml with water, and analyzed for P_i by a modification of the method of Allen (1940). Each of the adenylates had 1 mol of P_i/mol of adenosine.

A second aliquot (40 μl) of each of the reaction mixtures was diluted to 0.5 ml with water and utilized in a quantitative ninhydrin assay according to the method of Clark (1964). Compounds **3a**, **3b**, **5** and **6** gave measured absorbances of 0.75, 0.83, 0.84, and 0.78 per micromole, relative to a standard value of 0.77 obtained with phenylalaninol.

Paper chromatography of the four samples revealed that venom treatment had converted each from a single ninhydrin-positive compound (Table I) into two species. One of these was chromatographically identical with adenosine

(R_F 0.61 in solvent A, ninhydrin negative) while the other (R_F 0.79–0.82) could not be located by ultraviolet visualization but was ninhydrin positive.

[^3H]-L-Phenylalanyl Adenylate (**7**; *[^3H]Phenylalanyl-AMP*). To 14.2 A_{258} units (0.92 μmol) of anhydrous pyridinium adenosine 5′-monophosphate in 0.2 ml of dry pyridine was added 1.5 mg (2.58 μmol) of *N*-carbobenzyloxy-[^{3}H]-L-phenylalanine anhydride, 110 Ci/mol (Rammler and Khorana, 1963). The solution was stirred at room temperature for 10 hr and the reaction mixture was concentrated to dryness under diminished pressure and codistilled with portions of dry acetonitrile. The anhydrous residue was triturated with two 1.5-ml portions of dry acetonitrile to remove *N*-carbobenzyloxy-L-phenylalanine and anhydride and then dried briefly under diminished pressure. The solid residue was then dissolved in 0.3 ml of glacial acetic acid, which had been precooled to 20°, and treated with 0.2 mg of 5% palladium on carbon. The suspension was shaken under 3 atm of hydrogen for 20 min and filtered through a Celite pad, and the filtrate was concentrated under diminished pressure to afford **7** as a solid residue, yield 3.7 A_{258} units (26%). The product was utilized immediately for the aminoacylation of $tRNA^{Phe}$.

[^3H]Glycyl-L-phenylalanyl Adenylate (**8**). To 50 μg (140 nmol) of [^{3}H]carbobenzyloxyglycyl-L-phenylalanine (Anderson, 1958) (200 Ci/mol) was added 20.6 μg (100 nmol) of dicyclohexylcarbodiimide and 200 μl of dry ether. The anhydrous solution was stirred at room temperature for 2 hr and concentrated to dryness under diminished pressure. The residue was dissolved in pyridine and treated with 1.0 A_{258} unit (67 nmol) of pyridinium adenosine 5′-monophosphate. The anhydrous solution was maintained at room temperature overnight and then concentrated to dryness under diminished pressure. The solid residue was dissolved in 1 ml of cold glacial acetic acid and treated with 50 μg of 10% palladium on carbon. The suspension was shaken under 3 atm of hydrogen for 30 min and then filtered through Celite. The filtrate was concentrated under diminished pressure to afford 31.7 nmol of **9**, which was utilized immediately for the attempted acylation of $tRNA^{Phe}$.

Isolation of tRNA and Aminoacyl-tRNA Synthetase. The tRNA was isolated as previously described (Hecht *et al.*, 1971) from *E. coli* M72, from a tryptophan revertant of strain K12 with genotype F^- Su^- Lac^- Try^+ Sm^r $T_1{}^r$. The aminoacyl-tRNA synthetase was derived from the same strain of *E. coli*. It was prepared as described previously (Hecht *et al.*, 1971), except for additional purification by chromatography on Sephadex A-25 (1.8 × 20 cm), elution with a buffer (pH 7.0) containing 0.05 M ammonium citrate, 0.05 M imidazole, 0.25 M KCl, and 0.01 M $MgCl_2$, and then by chromatography on Sephadex G-75 (3.8 × 90 cm), elution with a solution containing 0.05 M KCl and 0.01 M $MgCl_2$. Before chromatography on Sephadex G-75 there was 8.2 μg of protein/μl of solution.

tRNA Fractionation. A sample of $tRNA^{Phe}$ was isolated by chromatography of *E. coli* tRNA on an RPC-5 column (1.5 × 23 cm, 37°) (Pearson *et al.*, 1971). The column was equilibrated in a buffer containing 0.01 M NaOAc (pH 4.5), 0.01 M $MgCl_2$, 0.002 M β-mercaptoethanol, and 0.5 M NaCl. Elution was effected with a linear gradient of sodium chloride (500 ml total volume; 0.5–0.9 M) in the same buffer solution and $tRNA^{Phe}$ was located by assay of individual fractions for phenylalanine acceptor activity. Alternatively, the entire tRNA sample could be aminoacylated with [^{3}H]phenylalanine prior to chromatography and the fractionated $tRNA^{Phe}$ located by scintillation counting of aliquots of alternate fractions. The tRNA isolated by this procedure was estimated to be 50–55% $tRNA^{Phe}$ on the basis of amino acid acceptance (Figure 1).

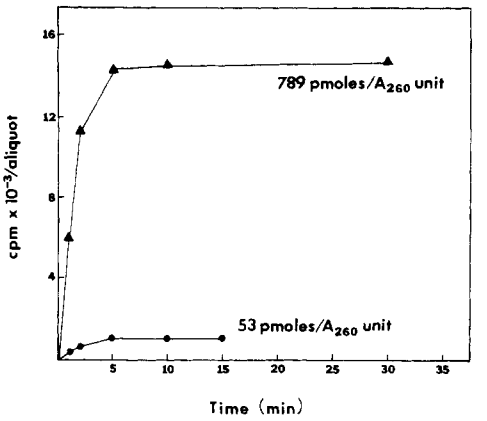


FIGURE 1: Aminoacylation of $tRNA^{Phe}$ before (53 pmol/A_{260} unit) and after (789 pmol/A_{260} unit) fractionation on an RPC-5 column. Experimental conditions for the fractionation and aminoacylation procedures are given in the Materials and Methods section.

Assay Procedures

tRNA Aminoacylation Assay. Amino acid incorporation was assayed by analogy with a method described previously (Hecht *et al.*, 1971). A solution (400 μl total volume) containing 0.1 M NH_4OH–piperazine-N,N′-bis(2-ethanesulfonic acid) monosodium monohydrate (Pipes; pH 7.0), 0.1 M KCl, 15 mM $MgCl_2$, 1 mM ATP, 0.4 mM CTP, 0.5 mM EDTA, 40 μM [^{3}H]phenylalanine (580 Ci/mol), and 38 A_{260} units of unfractionated *E. coli* tRNA (with or without added inhibitors) was equilibrated at 37°. The reaction was initiated by the addition of 10 μl of phenylalanyl-tRNA-enriched *E. coli* aminoacyl-tRNA synthetase solution. Aliquots (50 μl) were withdrawn at predetermined time intervals and quenched by addition to paper discs which had been presoaked with 100 μl of 0.05 M cetyltrimethylammonium bromide (CTAB) in 1% acetic acid solution. The discs were washed thoroughly with 1% acetic acid, dried, and counted.

Attempted Aminoacylation of $tRNA^{Phe}$ with Glycyl-L-phenylalanine. To 300 μl (total volume) of 0.10 M Pipes buffer (pH 7.0) containing 0.1 M KCl, 15 mM $MgCl_2$, 1 mM ATP, 0.4 mM CTP, and 0.5 mM EDTA was added 24.5 A_{260} units of *E. coli* $tRNA^{Phe}$ and 21 nmol of [^{3}H]glycyl-L-phenylalanine, 200 Ci/mol, obtained by hydrogenolysis of [^{3}H]carbobenzyloxyglycyl-L-phenylalanine (Anderson, 1958). The solution was equilibrated at 37° and the reaction was initiated by the addition of 4 μl of partially fractionated *E. coli* aminoacyl-tRNA synthetase solution (enriched in phenylalanyl-tRNA synthetase). The reaction mixture was incubated for 14 min at 37°, diluted to 1 ml with a 50 mM sodium acetate buffer (pH 5.0) containing 10 mM $MgCl_2$ and 0.5 M NaCl, and immediately applied to a column (6 ml) of BD-cellulose which had been preequilibrated with the same buffer. Elution was effected with a linear gradient of sodium chloride (150 ml total volume; 0.5–1.5 M) in 50 mM sodium acetate solution (pH 5.0) containing 10 mM $MgCl_2$, and then with a 20% ethanolic solution containing 50 mM sodium acetate (pH 5.0), 10 mM $MgCl_2$, and 1.5 M NaCl; 1-ml fractions were collected and these were assayed for optical density and radioactivity. An elution profile similar to that shown in Figure 4 was ob-

tained. The appropriate fractions from the ethanol purge, containing 6×10^5 cpm, were combined and dialyzed at 4° against 0.01 M sodium acetate (pH 4.5). Less than 2000 cpm was associated with the tRNA after dialysis. A control reaction, run with [^{3}H]phenylalanine and tRNA^{Phe}, was worked up in the same way and retained tRNA-associated radioactivity (12,000 cpm/A_{260} unit) after dialysis (8300 cpm/A_{260} unit) and RPC-5 chromatography (7700 cpm/A_{260} unit).

The reaction was repeated in a similar fashion, in the presence of 20% dimethyl sulfoxide. Work-up by chromatography on BD-cellulose as above afforded about 2×10^5 cpm in the ethanol purge, but very little radioactivity was associated with the tRNA after dialysis.

Aminoacylation of tRNA^{Phe} with Phenylalanyl Adenylate. To 0.53 nmol of [^{3}H]-L-phenylalanyl adenylate, 110 Ci/mol, was added 600 μl of a preequilibrated 0.1 M Pipes (pH 7.0) buffer solution containing 0.1 M KCl, 15 mM $MgCl_2$, 0.5 mM EDTA, 400 A_{260} units of unfractionated *E. coli* tRNA, and 30 μl of *E. coli* aminoacyl-tRNA synthetase solution. The solution was incubated at 37° and aliquots (100 μl) were withdrawn at predetermined time intervals and quenched by addition to Whatman GF/A glass fiber discs which had been soaked with 0.05 M CTAB in 1% acetic acid solution. The discs were washed thoroughly with 1% acetic acid solution, dried, and counted (Figure 3).

Also run at the same time was a reaction containing all of the components of the above mixture, but with 0.58 nmol of 5′-AMP and 0.58 nmol of [^{3}H]-L-phenylalanine, 110 Ci/mol, in place of the phenylalanyl adenylate. This run was used as a background for the above assay. Finally, a third reaction was run in the presence of the phenylalanyl adenylate and 6.0 units of inorganic pyrophosphatase. Aliquots were removed and analyzed as above. Immediately after these assays were completed, a normal aminoacylation assay was carried out (with ATP and [^{3}H]-L-phenylalanine) to verify the integrity of the assay system.

Attempted Acylation of tRNA^{Phe} with Glycyl-L-phenylalanyl Adenylate. To 23.4 nmol of [^{3}H]glycyl-L-phenylalanyl adenylate, 200 Ci/mol, was added 300 μl of a preequilibrated 0.1 M Pipes (pH 7.0) buffer solution containing 0.1 M KCl, 15 mM $MgCl_2$, 0.5 mM EDTA, 30 A_{260} units of *E. coli* tRNA^{Phe}, and 4 μl of phenylalanyl-tRNA enriched synthetase solution. The reaction mixture was incubated at 37° for 15 min, then diluted to 1 ml with 50 mM sodium acetate buffer (pH 5.0) containing 10 mM $MgCl_2$ and 0.5 M NaCl and immediately applied to a column (6 ml) of BD-cellulose which had been preequilibrated with the same buffer. Elution was effected with a linear gradient of sodium chloride (150 ml total volume; 0.5–1.5 M) in 50 mM sodium acetate solution (pH 5.0) containing 10 mM $MgCl_2$, and then with a 20% ethanolic solution containing 50 mM sodium acetate (pH 5.0), 10 mM $MgCl_2$, and 1.5 M NaCl; 1-ml fractions were collected and assayed for uv absorbance and radioactivity (Figure 4). That portion of the ethanol purge containing absorbance and radioactivity (total 7.3×10^5 cpm) was pooled and dialyzed at 4° against 0.01 M sodium acetate (pH 4.5). At this point 1.1×10^4 cpm were still associated with the tRNA. The solution was concentrated to 4 ml on a diaflo apparatus and applied to an RPC-5 column (2×20 cm) which had been preequilibrated at 37° with a 0.01 M sodium acetate solution (pH 4.5) containing 0.01 M $MgCl_2$, 0.5 M NaCl, and 2 mM β-mercaptoethanol. Elution was carried out with a linear gradient of sodium chloride (800 ml total volume; 0.5–0.9 M) in 0.01 M sodium acetate solution (pH 4.5) containing 0.01 M $MgCl_2$ and 2 mM β-mercaptoethanol; 4-ml fractions were collected and these were assayed for radioactivity. No peak of radioactivity was observed in the region in which (phenylalanyl-)tRNA^{Phe} is known to elute.

The reaction was repeated in a similar fashion, in the presence of 20% dimethyl sulfoxide. Analysis by successive BD-cellulose and RPC-5 chromatographies as above revealed no detectable [^{3}H]glycyl-L-phenylalanyl-tRNA^{Phe}. To confirm that the hydrogenolysis of [^{3}H]carbobenzyloxyglycyl-L-phenylalanyl adenylate had been successful, a portion of the sodium acetate buffer used for dialysis of the product from the BD-cellulose column was concentrated to a small volume, acidified to pH 2 with hydrochloric acid, and extracted with chloroform. Approximately 20% of the radioactivity was extracted into the chloroform (presumably as *N*-carbobenzyloxyglycyl-L-phenylalanine) indicating that the hydrogenolysis had proceeded about 80% to completion.

ATP-[^{32}P]PP$_i$ Exchange Kinetics. These determinations were carried out by the method of Cassio *et al.* (1967).

(a) DETERMINATION OF THE K_m OF PP$_i$. To a 0.1 M Pipes buffer solution (pH 7.0; 1 ml total reaction volume), containing 0.1 M KCl, 0.01 M $MgCl_2$, 10 mM KF, 0.5 mM EDTA, 5 mM ATP, and 1 mM phenylalanine, was added 0.1, 0.2, 0.5, or 1.0 mM inorganic pyrophosphate (containing 0.1 μCi of [^{32}P]PP$_i$ in each case). The solution was equilibrated at 37°, then 1.0 μl of aminoacyl-tRNA synthetase solution was added to initiate the reaction. A control (no phenylalanine) was also run. Aliquots (300 μl) were taken at 2, 4, and 8 min and quenched by addition to 0.50 ml of 0.4 M pyrophosphate in 15% $HClO_4$. Each aliquot was treated with 50 mg of charcoal suspended in 0.5 ml of water and then centrifuged. The supernatant was discarded and the charcoal was washed with portions of water and then suspended in 2 ml of 50% aqueous ethanol solution containing 0.15 M NH_4OH. The suspension was centrifuged and 1.5 ml of supernatant was counted. The apparent K_m value was calculated from the X intercept of a $1/v$ *vs.* $1/S$ plot of the data and found to be 260 μM.

(b) DETERMINATION OF THE K_m OF ATP. This K_m was determined as above except that each reaction mixture contained 2.0 mM sodium [^{32}P]pyrophosphate, 27.5 μCi/mol, and 0.16, 0.32, 0.64, or 1.56 mM ATP. A control experiment was also run without ATP. The apparent K_m was determined to be 570 μM.

(c) DETERMINATION OF THE K_m OF PHENYLALANINE. This K_m was determined as above except that each reaction mixture contained 2.0 mM sodium [^{32}P]pyrophosphate, 27.5 μCi/mmol, and 10, 12.5, 15, 20, 25, 50, 75, or 100 μM phenylalanine. A control experiment, containing no phenylalanine, was also run. The apparent K_m for phenylalanine was found to be 50 μM.

(d) K_i DETERMINATIONS. To 1.0 ml (total volume) of a 0.1 M Pipes buffer solution (pH 7.0) containing 0.1 M KCl, 10 mM $MgCl_2$, 10 mM KF, 0.5 mM EDTA, 2.0 mM sodium pyrophosphate (containing 0.033–0.05 μCi of [^{32}P]PP$_i$), 4.0 mM ATP, and 10–100 μM phenylalanine was added varying concentrations of inhibitors such that three concentration levels of each inhibitor was tested against each phenylalanine concentration. The samples were equilibrated at 37° and reaction was initiated by addition of 1 μl of *E. coli* aminoacyl-tRNA synthetase solution. The reactions were quenched at the appropriate times by addition of 0.5 ml of

TABLE II: ATP–PP_i Exchange Kinetics.[a]

Compd	Apparent K_m (μM)	Apparent K_i (μM)
ATP	570	
PP_i	260	
L-Phenylalanine	50	
L-Phenylalaninol-AMP (**3a**)		0.51
Glycinol-AMP (**3b**)		250
Glycyl-L-phenylalaninol-AMP (**5**)		10
Glycylglycyl-L-phenylalaninol-AMP (**6**)		29

[a] Experimental procedure given in the Materials and Methods section.

TABLE III: tRNA Aminoacylation Kinetics.[a]

Compd	Apparent K_m (μM)	Apparent K_i (μM)
ATP	130	
L-Phenylalanine	3.2	
L-Phenylalaninol-AMP (**3a**)		1.5
Glycinol-AMP (**3b**)		400
Glycyl-L-phenylalaninol-AMP (**5**)		95
L-Phenylalaninol		7.2
Glycyl-L-phenylalanine		250
Glycine[b]		

[a] Experimental procedure given in the Materials and Methods section. [b] No inhibition noted at any tested concentration (up to 6.6 mM).

0.4 M sodium pyrophosphate in 15% aqueous $HClO_4$ and analyzed as indicated above. There was less than 1.5% exchange in every case and the data were corrected for background counts, which was determined by identical treatment of samples in the absence of phenylalanine. Controls which contained inhibitor at the highest tested concentration, but no phenylalanine, indicated that the inhibitors did not promote the exchange reaction to a detectable extent. Individual K_i values were determined from $1/v$ *vs.* $1/S$ plots, which indicated all of the inhibitors to be competitive in nature. The values are given in Table II.

Kinetics of tRNA Aminoacylation. (a) DETERMINATION OF THE K_m OF PHENYLALANINE. To a 0.1 M Pipes buffer solution (pH 7.0; 300 μl total reaction volume), containing 0.1 M KCl, 15 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, and 4.63, 1.85, 0.925, or 0.463 μM [^{3}H]phenylalanine, 3.6 Ci/mmol, was added 150 A_{260} units of unfractionated *E. coli* tRNA.[2] The reactions were initiated by the addition of 2 μl of aminoacyl-tRNA synthetase solution. At the appropriate times (20–100 sec) 50-μl aliquots were applied to Whatman GF/A discs which had been presoaked in 100 μl of 0.05 M CTAB in 1% aqueous acetic acid. The dried discs were washed with 1% aqueous acetic acid, dried, and counted. The data for each substrate concentration were plotted as [^{3}H]Phe-tRNA^{Phe} *vs.* time and used to calculate an initial velocity. The apparent K_m value was determined from the X intercept of a Lineweaver–Burk plot and found to be 3.2 μM.

(b) DETERMINATION OF THE K_m OF ATP. This K_m was determined as above except that [^{3}H]phenylalanine was utilized at a concentration level of 4.63 μM and ATP at 59.1, 39.4, 19.7, or 9.85 μM. The reactions were run for 0.5–1.5 min and assayed as described above. The apparent K_m of ATP was found to be 130 μM.

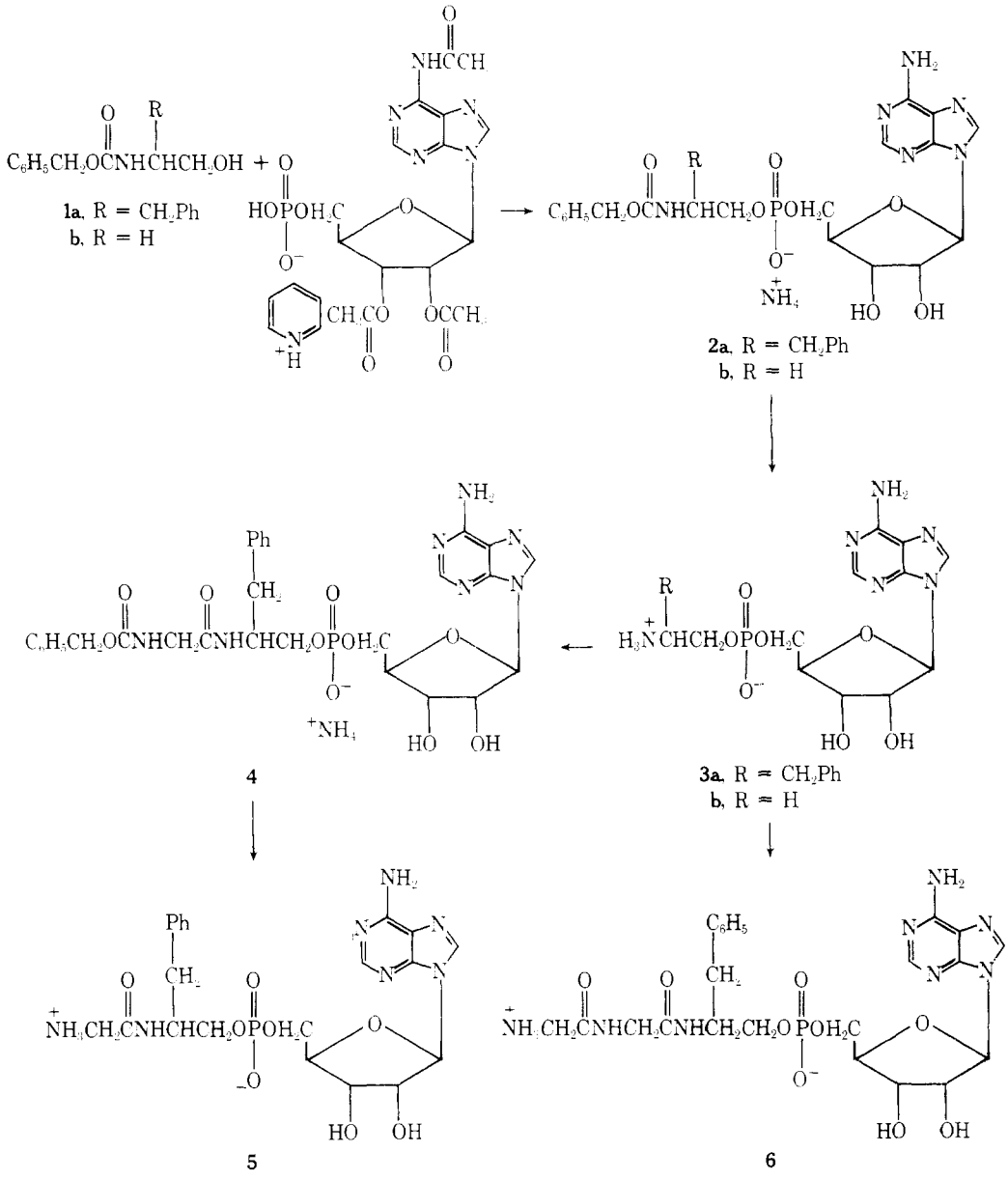
(c) K_i DETERMINATIONS. To a 0.1 M Pipes buffer solution (pH 7.0; 300 μl total reaction volume), containing 0.1 M KCl, 15 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, and 4.63, 1.85, 0.925, or 0.463 μM [^{3}H]phenylalanine, 3.6 Ci/mmol, was added 150 A_{260} units of unfractionated *E. coli* tRNA and the appropriate concentration of inhibitor (with three inhibitor concentrations utilized for each phenylalanine concentration). The reaction was initiated by the addition of 2 μl of synthetase solution and aliquots (50 μl) were removed at appropriate time intervals (0.5–17 min) and assayed as described above. The data were plotted as $1/v$ *vs.* I for each inhibitor, where four different concentrations of each inhibitor were utilized. The individual points on these plots were determined from the cpm *vs.* time plots obtained for each inhibitor and substrate concentration. Individual K_i values are given in Table III.

[2] The resulting tRNA concentration was shown to be well above the K_m for tRNA.

Results

The acylation of *E. coli* tRNA^{Phe} (in unfractionated tRNA) was attempted with *E. coli* aminoacyl-tRNA synthetase using glycyl-L-phenylalanine in place of L-phenylalanine. No significant dipeptide acceptance was observed under conditions which resulted in the aminoacylation of about 90% of the tRNA^{Phe} (Figure 1), when L-phenylalanine was employed in a control experiment. The experiment was repeated using fractionated tRNA^{Phe} and a partially fractionated aminoacyl-tRNA synthetase solution. This run was assayed by chromatography of the incubation mixture on a BD-cellulose column, pH 5.0, followed by dialysis against acetate buffer of the material eluted by the ethanol purge. The radioactivity associated with the dialyzed solution corresponded to less than 0.1% of that obtained in a control reaction, using [^{3}H]-L-phenylalanine. Additional experiments were carried out with the dipeptide under conditions similar to those which have been employed to promote misacylations (Giegé *et al.*, 1971; Kern *et al.*, 1972). Once again no significant dipeptide acceptance was observed.

The possible existence of a specificity for glycyl-L-phenylalanine by the phenylalanyl-tRNA synthetase, beyond that associated with a random amino acid, was assayed by the use of four amino alcohol analogs of the aminoacyl adenylate. The syntheses of the L-phenylalaninol and glycinol esters of adenosine 5′-monophosphoric acid (L-phenylalaninol-AMP (**3a**) and glycinol-AMP (**3b**)) were accomplished by modification of the method of Sandrin and Boissonnas (1966). The glycyl-L-phenylalaninol and glycylglycylphenylalaninol esters of 5′-AMP (**5** and **6**) were synthesized by treatment of **3a** with the *N*-hydroxysuccinimidyl esters of *N*-carbobenzyloxyglycine and *N*-carbobenzyloxyglycylglycine, respectively, followed by hydrogenolysis with 5% palladium on carbon.



Initial competition experiments were carried out for the phenylalanine and phenylalanyl-AMP binding sites on the aminoacyl-tRNA synthetase. tRNA was aminoacylated in the presence of 40 μM [^{3}H]phenylalanine with and without larger amounts of unlabeled glycine and glycyl-L-phenylalanine. The addition of larger amounts of random amino acids (*e.g.*, glycine) had no significant effect on the aminoacylation process, but the addition of glycyl-L-phenylalanine (1 mM) effected a substantial reduction in tRNA aminoacylation, indicating recognition of glycyl-L-phenylalanine. Figure 2 depicts the results of the aminoacylation of $tRNA^{Phe}$ in the presence of [^{3}H]-L-phenylalanine and the aminoacyl-AMP analogs L-phenylalaninol-AMP (**3a**), glycinol-AMP (**3b**), and glycyl-L-phenylalaninol-AMP (**5**). Glycinol-AMP was the least effective inhibitor of tRNA aminoacylation with L-phenylalanine,[3] while phenylalaninol-AMP was the most effective. Activation of $tRNA^{Phe}$ in the presence of glycyl-L-phenylalaninol-AMP was strongly inhibited, indicating specific recognition of the dipeptide terminating in the correct amino acid, relative to the binding for a random amino acid (glycine).

The apparent recognition of the dipeptide by the phenylalanyl-tRNA synthetase prompted the measurement of the initial velocity of ATP-PP_i exchange by the synthetase in the presence of phenylalanine and inhibitors **3a, 3b, 5,** and **6.** As shown in Table II, the apparent K_m's for PP_i, ATP, and phenylalanine were 260, 570, and 50 μM, respectively, in reasonable agreement with values reported by Cassio *et al.* (1967) and Santi *et al.* (1971). L-Phenylalaninol-AMP had an apparent K_i of 0.51 μM in the ATP-PP_i exchange assay, while the corresponding value for glycyl-L-phenylalaninol-AMP was 10 μM. In contrast, however, the apparent K_i for glycinol-AMP was 250 μM, indicating substantially less recognition for a random amino acid. The apparent K_i for the tripeptidyl-AMP analog, glycylglycyl-L-phenylalaninol-AMP, was 29 μM.

In light of the postulate (Loftfield, 1972) that tRNA aminoacylation is a concerted process and that ATP-PP_i exchange, which is carried out in the absence of tRNA, may

[3] Although the inhibition of tRNA aminoacylation by **3b** was not apparent under the conditions used to obtain the data for Figure 2, an additional experiment carried out at a lower concentration of phenylalanyl-tRNA synthetase resulted in 30% inhibition of phenylalanyl-$tRNA^{Phe}$ formation by **3b** after 5 min of incubation (*vs.* 85 and 95% inhibition by **5** and **3a**, respectively).

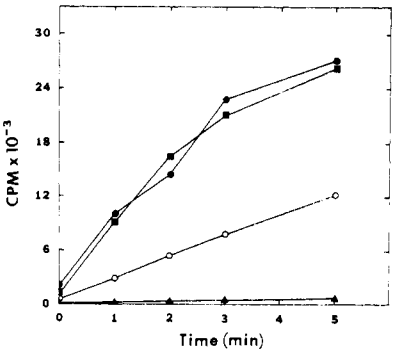


FIGURE 2: Aminoacylation of $tRNA^{Phe}$ by [^{3}H]phenylalanine (40 μM, ■) in the presence of 1 mM glycinol-AMP (**3b**; ●); 1 mM glycyl-L-phenylalaninol-AMP (**5**; ○); or 1 mM L-phenylalaninol-AMP (**3a**; ▲).

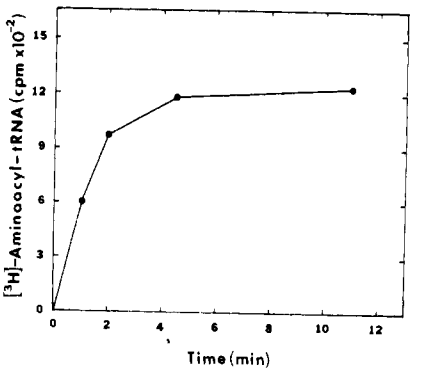
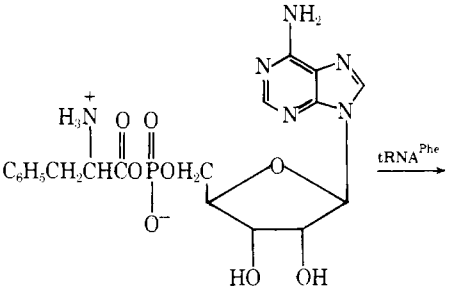


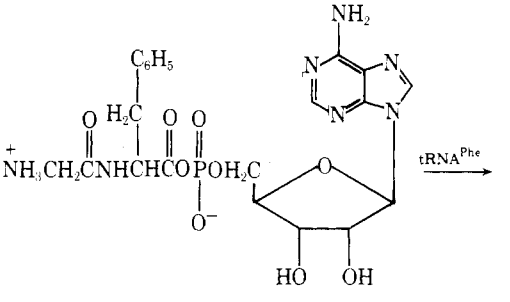
FIGURE 3: Aminoacylation of $tRNA^{Phe}$ with [^{3}H]-L-phenylalanyl adenylate (**8**), 110 Ci/mol. Experimental procedures are given in the Materials and Methods section.

be an artifact not normally associated with tRNA aminoacylation,[4] apparent K_i's were also determined for the inhibitors by assaying their effects on the overall formation of phenylalanyl-$tRNA^{Phe}$.[5] The apparent K_m's for ATP and phenylalanine were determined as 130 and 3.2 μM, respectively (Table III), while the apparent K_i's for L-phenylalaninol-AMP (**3a**), glycyl-L-phenylalaninol-AMP (**5**), and glycinol-AMP (**3b**) were 1.5, 95, and 400 μM, respectively. Also measured were the apparent K_i's for L-phenylalaninol (7.2 μM) and glycyl-L-phenylalanine (250 μM). Glycine was found not to be an inhibitor at any tested concentration.

In an effort to circumvent the lack of dipeptide activation by the phenylalanyl-tRNA synthetase, the acylation of $tRNA^{Phe}$ was attempted utilizing chemically synthesized glycyl-L-phenylalanyl adenylate (**8**). The acylation was run under conditions which had resulted in the transfer of ~45% of the phenylalanine from [^{3}H]phenylalanyl adenylate (**7**) to $tRNA^{Phe}$ (Figure 3), with or without added inorganic pyrophosphatase. The incubation mixture containing glycyl-L-phenylalanyl adenylate (**8**) was assayed for possible formation of glycyl-L-phenylalanyl-$tRNA^{Phe}$ by chromatography on BD-cellulose in acetate buffer (pH 5.0) (Figure 4). The material eluted by the ethanol purge was dialyzed at 4° against acetate buffer (pH 4.5) and the resulting desalted solution, containing 1.1×10^4 cpm, was further purified by chromatography on RPC-5 column in acetate buffer (pH 4.5). The column was assayed for radioactivity corresponding to glycyl-L-phenylalanyl-$tRNA^{Phe}$, but no dipeptidyl-tRNA was observed. Less than 0.1% of this species could easily have been detected. The experiment was repeated under conditions known to promote misacylations (Giegé *et al.*, 1971; Kern *et al*, 1972). Once again, no dipeptide acceptance was observed. To show that glycylphenylalanyl-tRNA would have been stable to the reaction conditions if it had formed, an authentic sample of [^{3}H]glycylphenylalanyl-tRNA was prepared by modification of published procedures (Lapidot *et al.*, 1967; Schmidt *et al.*, 1972) and incubated under the same conditions employed in the attempted formation of dipeptidyl-tRNA from tRNA and adenylate **8**. No breakdown of the authentic dipeptidyl-tRNA could be detected.



7 phenylalanyl-$tRNA^{Phe}$



8 glycylphenylalanyl-$tRNA^{Phe}$

Discussion

A substantial number of amino acid analogs have been assayed for binding and activation by individual aminoacyl-tRNA synthetases; 17 analogs of L-valine, for example, were found to bind to the valyl-tRNA synthetase and six of these, including L-threonine, L-α-aminobutyric acid, and L-norvaline, catalyzed the ATP–PP_i exchange reaction at a significant rate (Owens and Bell, 1970). The tyrosyl-tRNA synthetases from *E. coli* and *B. subtilus* have been reported to activate both D- and L-tyrosine as well as 3-fluoro- and 3-hydroxy-L-tyrosine (Calendar and Berg, 1966). Conway *et al.* (1962) prepared a number of analogs of phenylalanine and reported nine which were converted to the corresponding hydroxamate by the action of *E. coli* phenylalanyl-tRNA synthetase in the presence of hydroxylamine. The requirements of the *E. coli* phenylalanyl-tRNA synthetase for the binding and carboxyl activation reactions

[4] See, however, Eldred and Schimmel (1972a).

[5] Differences in the absolute values of the apparent K_m's and K_i's as determined by the two methods undoubtedly derive from the nature of the two systems. Aminoacylation of tRNA, *e.g.*, involves a number of steps, any one of which could be rate limiting under certain circumstances, although dissociation of aminoacylated tRNA from synthetase is ordinarily rate limiting (Eldred and Schimmel, 1972a). The ATP-PP_i exchange assay is likewise complicated by the absence of tRNA from the assay system (Loftfield, 1970) and by the presence of a saturating level of PP_i which itself inhibits aminoacyl adenylate formation (Loftfield and Eigner, 1969).

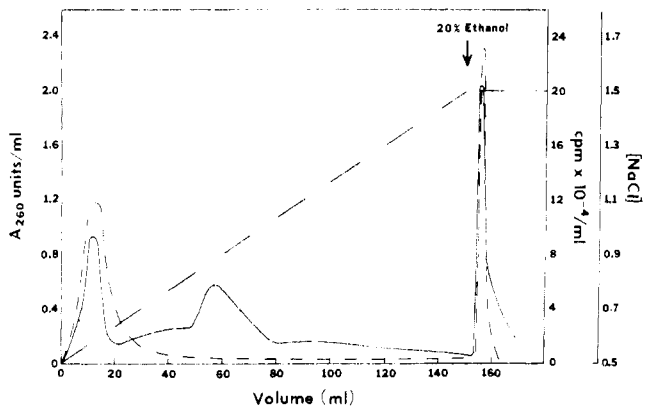


FIGURE 4: Chromatography on a 6-ml BD-cellulose column of the reaction mixture resulting from the attempted acylation of fractionated $tRNA^{Phe}$ with glycyl-L-phenylalanyl adenylate (**8**). That region of the ethanol purge containing both cpm (- - -) and ultraviolet-absorbing material (A_{260}; —) was further purified by dialysis and chromatography on an RPC-5 column.

have been investigated by Santi and Danenberg (1971) using a large number of structural analogs of phenylalanine. They identified those structural features in the analogs requisite for synthetase binding, as well as amino acid activation and inhibition of such activation.

The synthesis of peptidyl-tRNAs may be effected both enzymatically and chemically (Lapidot *et al.*, 1967, 1969a,b, 1970; Yankofsky *et al.*, 1970; Igarashi and Paranchych, 1970; Rubenstein *et al.*, 1970) from aminoacyl-tRNAs. However, the enzymatic synthesis of peptidyl-tRNA by direct attachment of a peptide to nonaminoacylated tRNA has never been reported. In the sense that peptides, especially those of limited size, may be regarded as analogs of their C-terminal amino acids, it seemed of interest to consider the mechanism whereby such peptides are normally excluded from incorporation onto tRNA, as well as methods for circumventing that mechanism.

Attempts at direct activation of $tRNA^{Phe}$ with glycyl-L-phenylalanine were unsuccessful, as expected. Utilization of either unfractionated or purified $tRNA^{Phe}$ afforded no activation relative to a control sample, even when the conditions were varied to approximate those known to have previously afforded misacylations (Giegé *et al.*, 1971; Kern *et al.*, 1972). this was consistent with the findings of previous workers that N-blocked amino acids were generally poor substrates for the synthetase (Santi and Danenberg, 1971) and that glycyl-L-phenylalanine in particular (Conway *et al.*, 1962) could not be converted to the corresponding hydroxamate in the presence of hydroxylamine. Experiments were therefore carried out to determine whether any synthetase specificity existed for the dipeptide, beyond that which might be associated with a random amino acid. This was done initially by assaying glycyl-L-phenylalanine for its ability to inhibit L-phenylalanine activation, both at the amino acid and aminoacyl adenylate levels, relative to a random amino acid (glycine). It was shown that while glycine (1 mM) had no effect on the aminoacylation of tRNA with phenylalanine, glycyl-L-phenylalanine caused substantial inhibition at the same concentration level.

The results of competition for the synthetase at the aminoacyl adenylate level is shown in Figure 2, utilizing amino alcohol adenylates **3a, 3b,** and **5.** Analogs of this type are known to bind tightly to their cognate aminoacyl-tRNA synthetases, thus preventing normal amino acid activation and subsequent tRNA aminoacylation (Sandrin and Boissonnas, 1966; Cassio *et al.*, 1967). As is shown in Figure 2, glycyl-L-phenylalaninol-AMP was bound to the synthetase more efficiently than a random aminoacyl-AMP analog (glycinol-AMP), although not as well as the normal substrate analog L-phenylalaninol-AMP.

A more direct measure of the relative specificities for the adenylate analogs by the phenylalanyl-tRNA synthetase was obtained from the initial velocities of ATP-PP_i exchange in the presence of phenylalanine and inhibitors **3a, 3b, 5,** and **6.** As indicated in Table II, the apparent K_i's for L-phenylalaninol-AMP (**3a**) and glycyl-L-phenylalaninol-AMP (**5**) were 0.51 and 10 μM, respectively, indicating more efficient binding of the normal substrate analog than the dipeptidyl analog. The apparent K_i for glycinol-AMP (**3b**), however, was 250 μM, indicating that the K_i of 10 μM for the dipeptidyl analog did reflect a specificity for **5** beyond that associated with a random amino acid. It seemed reasonable to expect that lengthening of the "amino acid" analog **5** beyond the dipeptide level would result in less efficient binding to the phenylalanyl-tRNA synthetase. Indeed, the K_i value of 29 μM associated with glycylglycyl-L-phenylalaninol-AMP (**6**) was consistent with this expectation. The same order of K_i values was obtained from measurement of the initial velocities of tRNA aminoacylation in the presence of **3a, 3b,** and **5** (Table III), supporting the foregoing arguments.

One question which has not been examined in detail is whether aminoacyl species which are specifically recognized by a given aminoacyl-tRNA synthetase at the aminoacyl adenylate level, but which do not participate in the formation of that aminoacyl adenylate from "amino acid" and ATP, might be transferred to tRNA under appropriate conditions if they were introduced into the tRNA-aminoacylating system at the aminoacyl adenylate level. Although a few experiments have described the lack of aminoacylation of tRNA achieved with preformed aminoacyl adenylates (Krishnaswamy and Meister, 1960; Kondo and Woese, 1969; Berg *et al.*, 1961), only one example involved an analog which was specifically recognized by the synthetase (Berg *et al.*, 1961; Kondo and Woese, 1969) and numerous examples of misacylation do exist (Allende and Allende, 1964; Calendar and Berg, 1966; Papas and Mehler, 1970; Giegé *et al.*, 1971; Kern *et al.*, 1972). The acylation of $tRNA^{Phe}$ with glycyl-L-phenylalanine was therefore attempted utilizing chemically synthesized [^{3}H]glycyl-L-phenylalanyl adenylate under conditions which had resulted in the transfer of ~45% of the phenylalanine from [^{3}H]phenylalanyl adenylate to $tRNA^{Phe}$ (Figure 3). The extent of acylation was assayed by successive chromatographies of the incubation mixture on BD-cellulose (Figure 4) and RPC-5 columns. Although less than 0.1% of theoretical dipeptidyl-tRNA formation could have been detected, none of the acylated species was observed. Repetition of the experiment under conditions similar to those known to afford misacylation (Giegé *et al.*, 1971; Kern *et al.*, 1972) gave the same results.

Since it has been shown that *E. coli* contains at least one enzyme activity capable of deacylating acylated aminoacyl- and peptidyl-tRNAs (Cuzin *et al.*, 1967; Vogel *et al.*, 1968; Kössel and RajBhandary, 1968) and that certain aminoacyl-tRNA synthetases can specifically and rapidly deacylate their cognate tRNAs when the latter are misacylated (Yarus, 1972; Eldred and Schimmel, 1972b), an authentic sample of [^{3}H]glycyl-L-phenylalanyl-$tRNA^{Phe}$ was pre-

pared (Lapidot *et al.*, 1967; Schmidt *et al.*, 1972) to demonstrate that this species would have been stable if it had been formed from the glycyl-L-phenylalanyl adenylate (**8**) and tRNA. Incubation of the authentic dipeptidyl-tRNA under conditions used in the attempted formation of glycylphenylalanyl-tRNA from **8** and tRNA resulted in no detectable breakdown of the authentic sample.

Acknowledgments

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