p-nitrophenyl ester, it was possible to compare the room-temperature structure of the native enzyme in the sodium sulfate mother liquor with that in 70% aqueous methanol at -55 °C. The Fourier difference map indicated no changes in the polypeptide backbone and only changes on the surface, ascribed to immobilization at the low temperature of "floppy" surface residues. The formation of the acyl-enzyme intermediate could be monitored by changes in the intensity of selected reflections when the substrate was flowed in at -55 °C. A Fourier difference map of the acyl-enzyme minus the native enzyme at -55 °C showed the absence of the nitrophenyl group, the alanyl residue covalently attached to the active-site Ser side chain, the alanyl side chain in van der Waals contact with the Val-216, and the planar CBZ group in the anticipated location. No evidence of movement in the active-site His, Asp, and Ser residues was detectable at the resolution of the experiment.[10] No loss of acyl group was detected when cryosolvent was used to wash the crystalline acyl-enzyme at -55 °C for 4 days. When the temperature was raised to -10 °C the acyl group disappeared at the rate predicted for deacylation. For the crystalline enzyme both acylation and deacylation rates appeared decreased by a factor of 10–20 over the corresponding reactions with dissolved enzyme.[17]

In addition to the demonstration that it is possible to obtain high-resolution structural data of productive enzyme–substrate intermediates in this manner, this investigation established that, in the crystal at least, the structure (and function) of the enzyme is essentially the same at subzero temperatures in aqueous organic solvent as under normal conditions, and that the conformation of the enzyme in the free and acylated forms is very similar. The not unexpected implication of the latter observation is that those features responsible for the great efficiency of enzyme catalysis are to be found, in the case of the serine proteases, in the conversion of enzyme into acyl-enzyme, and acyl-enzyme into free enzyme. Experiments are now under way to extend these crystallographic studies to higher resolution (≤ 2.0 Å), to trapped intermediates prior to the acyl-enzyme, to polypeptide substrates, and to the enzymes papain, subtilisin, ribonuclease A, and γ-chymotrypsin. The significance of the anticipated results from these experiments should need no elaboration. Suffice it to say that we would appear to be on the threshold of an era when it will be possible to obtain a series of time-lapse "pictures" as the catalytic reaction proceeds.

Utilization of Isomeric Aminoacyl Transfer Ribonucleic Acids in Peptide Bond Formation

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The biosynthesis of proteins from their component amino acids is one of the most important and intensively studied biological processes. Peptide bond formation is mediated via the intemediacy of aminoacylated transfer RNAs (tRNAs), which contain specific amino acids covalently bound as activated esters.[1] At least one tRNA corresponds to each amino acid (Figure 1, for example, depicts *E. coli* tRNA$^{\text{Met}}_f$[2]), and a cognate aminoacyl-tRNA synthetase catalyzes the formation of the activated ester from the tRNA and its amino acid. Aminoacylation occurs on the 2′- or 3′-OH group of the adenosine at the 3′ end of tRNA (eq 1), and the ester initially formed equilibrates rapidly ($t_{1/2} \simeq 0.2$ ms)[3] between the vicinal hydroxyl groups. Although it has seemed likely that each aminoacyl-tRNA synthetase utilizes only a single tRNA OH group for aminoacylation, the rapid equilibration between the two isomers of aminoacyl-tRNA has made it difficult to

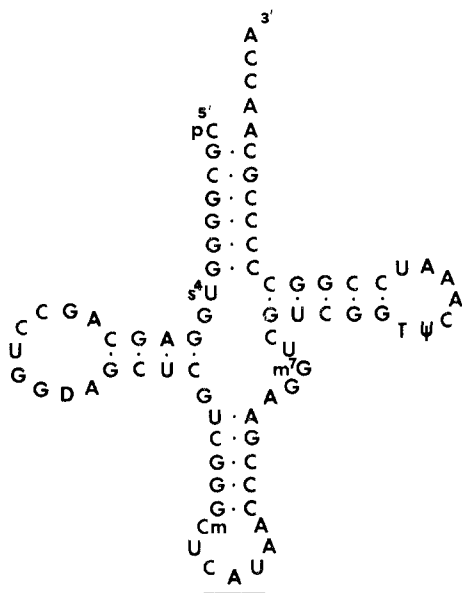

$$(1)$$
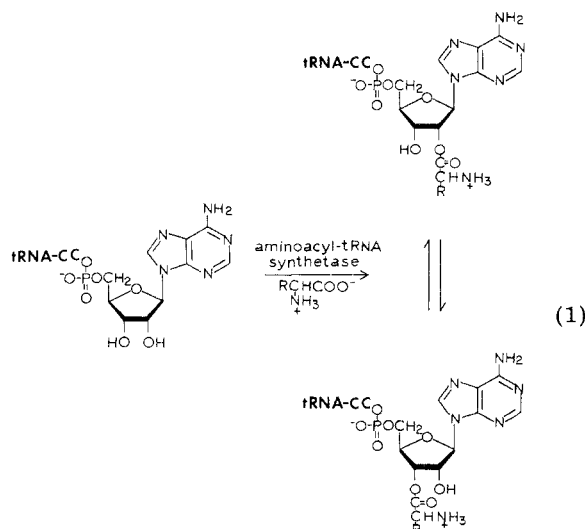
determine the initial site of aminoacylation.

After an amino acid is attached to its cognate tRNA, the position of insertion of the amino acid into a

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(1) J. Lucas-Lenard and F. Lipmann, *Annu. Rev. Biochem.*, **40**, 409 (1971).
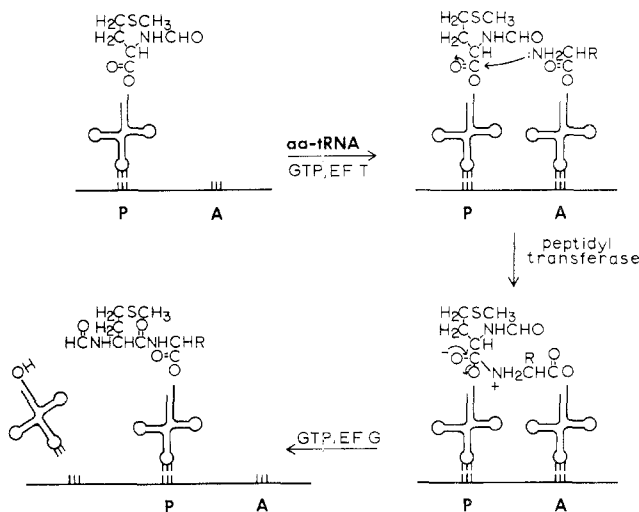
(2) tRNA$^{\text{Met}}_f$ denotes the (formylatable) tRNA specific for methionine; methionyl-tRNA$^{\text{Met}}_f$ denotes the corresponding aminoacylated tRNA.

(3) B. E. Griffin, M. Jarman, C. B. Reese, J. E. Sulston, and D. R. Trentham, *Biochemistry*, **5**, 3638 (1966).

Figure 1. Cloverleaf representation of *E. coli* tRNA$^{Met}_f$. The anticodon triplet is underlined.

Figure 2. Initiation of translation of mRNA in a prokaryotic system.

growing polypeptide is determined by the polynucleotide sequence of the messenger RNA (mRNA) which is being translated. As shown in Figure 2, translation begins with the formation of a hydrogen-bonded complex between the anticodon triplet (see Figure 1) of N-formylated methionyl-tRNA$^{Met}_f$ and the complementary mRNA initiator codon (AUG). The complex is formed initially on the 30S ribosomal subunit, which then combines with the larger 50S subunit to give the active ribosome–mRNA complex containing N-formylmethionyl-tRNA$^{Met}_f$ in the ribosomal P (peptidyl) site. The A (acceptor) site is then filled by the aminoacyl-tRNA whose anticodon is complementary to the three nucleotides on the mRNA immediately following the initiator codon. The aminoacyl-tRNA is introduced from a ternary complex with GTP and elongation factor Tu (EF-Tu), and GTP hydrolysis accompanies ribosomal binding.

Peptide bond formation itself is catalyzed by peptidyltransferase, a site on the 50S ribosomal subunit, and presumably involves nucleophilic addition of the amino group of the aminoacyl-tRNA in the A site to the

ester moiety of N-formylmethionyl-tRNA$^{Met}_f$, followed by loss of tRNA$^{Met}_f$ as a leaving group. Elongation factor G effects translocation of the N-formylated dipeptidyl-tRNA into the P site, after which a new aminoacyl-tRNA is introduced into the vacant A site and the cycle is repeated.

Many of the mechanistic features of the partial reactions of protein biosynthesis (i.e., tRNA aminoacylation, ternary complex formation with EF-Tu, A- and P-site binding, peptidyltransferase reaction) are known, but little information has been available regarding positional specificity (or lack thereof) for the amino acids on the aminoacyl- and peptidyl-tRNAs which are participants in these reactions. It would certainly not be necessary for a single isomer of aminoacyl- (peptidyl-) tRNA to be utilized in such partial reactions, but the specificity frequently associated with enzymatic transformations makes it reasonable to think that at least some of these reactions may involve only one positional isomer. If such positional specificity did exist, it might represent a relatively simple extension of known substrate specificities in such processes (e.g., for the proper amino acids and tRNAs in aminoacylation[4-9] and for structurally correct aminoacyl-tRNAs in ternary complex formation[10-15]) that are believed to assist in the maintenance of fidelity of protein biosynthesis.

Isomeric tRNAs Can Be Used as a Probe of Positional Specificity

Early efforts at determining the initial position of aminoacylation of tRNA, as well as the isomer (2′ or 3′) which predominates in solution, were carried out by treating the aminoacyl-tRNAs with reagents that reacted with the free hydroxyl group on the aminoacyladenosine moiety. These trapping experiments were carried out on the tacit assumptions that under the reaction conditions the reagents employed (p-toluenesulfonyl chloride,[16] 2,3-dihydropyran,[17] and 2-cyanoethyl phosphate[18]) reacted equally with both isomers of aminoacyl-tRNA and more rapidly than the two isomers interconverted. Since the $t_{1/2}$ for equilibration of a single isomer of aminoacyl-tRNA has subsequently been estimated as 2×10^{-4} s at 25 °C[3] and since the 2′- and 3′-hydroxyl groups have been shown to undergo certain chemical reactions at different

(4) T. W. Conway, E. M. Lansford, Jr., and W. Shive, *J. Biol. Chem.*, **237**, 2850 (1962).

(5) A. N. Baldwin and P. Berg, *J. Biol. Chem.*, **241**, 839 (1966).

(6) S. L. Owens and F. E. Bell, *J. Biol. Chem.*, **245**, 5515 (1970).

(7) T. S. Papas and A. H. Mehler, *J. Biol. Chem.*, **245**, 1588 (1970).

(8) D. V. Santi and P. V. Danenberg, *Biochemistry*, **10**, 4813 (1971).

(9) S. M. Hecht and S. D. Hawrelak, *Biochemistry*, **13**, 4967 (1974), and references therein.

(10) J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, **58**, 1574 (1967).

(11) J. M. Ravel, R. L. Shorey and W. Shive, *Biochem. Biophys. Res. Commun.*, **29**, 68 (1967).

(12) Y. Ono, A. Skoultchi, A. Klein, and P. Lengyel, *Nature (London)*, **220**, 1304 (1968).

(13) C. Jerez, A. Sandoval, J. E. Allende, C. Henes and J. Ofengand, *Biochemistry*, **8**, 3006 (1969).

(14) J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, **59**, 179 (1969).

(15) R. L. Shorey, J. M. Ravel, C. W. Garner, and W. Shive, *J. Biol. Chem.*, **244**, 4555 (1969).

(16) H. Feldmann and H. G. Zachau, *Biochem. Biophys. Res. Commun.*, **15**, 13 (1964).

(17) R. Wolfenden, D. H. Rammler, and F. Lipmann, *Biochemistry*, **3**, 329 (1964).

(18) C. S. McLaughlin and V. M. Ingram, *Biochemistry*, **4**, 1442 (1965).

rates,[19,20] it is not clear that either of these assumptions was justified.

A more recent approach has involved the use of tRNAs with modified 3'-terminal nucleosides, such as tRNA species 2–5. In each of these, the adenosine
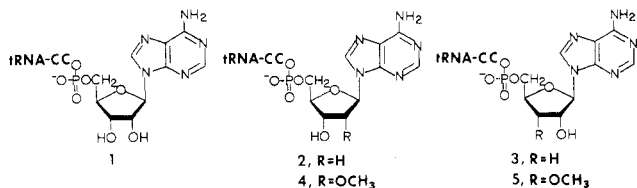
moiety has been replaced by an analogue containing only a single (2' or 3') OH group. The absence of a cis-diol moiety in these modified tRNAs would obviously preclude the isomerization of the corresponding modified aminoacyl-tRNAs (e.g., **7** and **8**). Thus,
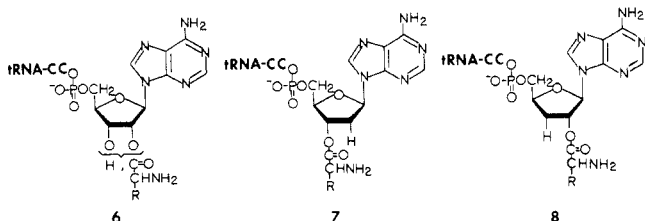
exclusive enzymatic aminoacylation of a single isomer of a modified tRNA isoacceptor (e.g., tRNA^{Ser} species **2**, but not **3**) with kinetics reasonably similar to those obtained for the corresponding unmodified tRNA might be considered indicative of the initial position of aminoacylation of the unmodified species.

Actual preparation of the modified tRNAs involves the initial removal of the 3'-terminal nucleoside, which can be accomplished by controlled treatment with venom exonuclease.[21] The loss of nucleotides does not occur uniformly over an entire population of tRNAs, so that a mixture of species of varying lengths is obtained, but hydrolysis can be confined largely to the single-stranded -C-C-A sequence which is the common terminus of all tRNAs. Since this sequence can be added posttranscriptionally to tRNAs by CTP-(ATP):tRNA nucleotidyltransferase, it is possible to obtain a uniform sample of tRNA-C-C$_{OH}$ ("abbreviated tRNA") by incubation of the venom-treated tRNA with CTP(ATP):tRNA nucleotidyltransferase and CTP, but not ATP. Alternatively, tRNA-C-C$_{OH}$ may be obtained by treatment of the intact tRNA with periodate to oxidize the vicinal diol moiety to the corresponding dialdehyde, and then with a primary amine to effect the elimination of tRNA-C-Cp.[22,23] Incubation with alkaline phosphatase then affords tRNA-C-C$_{OH}$. Unfortunately, periodate is destructive to some modified nucleosides in tRNA,[24] and possibly destructive in a more general sense.[25]

Two methods have been utilized for reconstruction of the abbreviated tRNAs with modified nucleotides
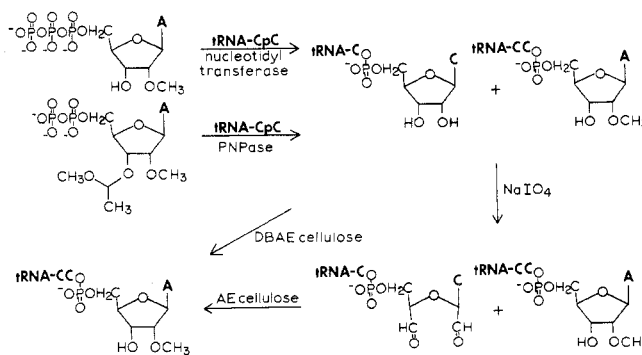

Figure 3. Reconstruction and purification of tRNAs modified at the 3' terminus.

(Figure 3). One of these simply involves additional incubation of tRNA-C-C$_{OH}$ with purified CTP-(ATP):tRNA nucleotidyltransferase in the presence of an ATP analogue (e.g., 2'- or 3'-deoxyadenosine 5'-triphosphate).[25–38] Although such analogues are incorporated onto tRNA-C-C$_{OH}$ with much less facility than ATP itself, an extended period of incubation with the yeast enzyme has been shown to effect 45–65% incorporation of the deoxynucleotides.[25,26,35,38] The *E. coli* enzyme did not utilize 2'- or 3'-deoxyadenosine 5'-triphosphates as substrates,[37] but did effect incorporation of 2'- and 3'-O-methyladenosine 5'-phosphates onto abbreviated *E. coli* tRNA in yields of 30 and 37%,[27,31,37] respectively, and was also used to prepare *E. coli* tRNAs terminating in 2'-amino-2'-deoxyadenosine and 3'-amino-3'-deoxyadenosine (**9**).[29,32] The
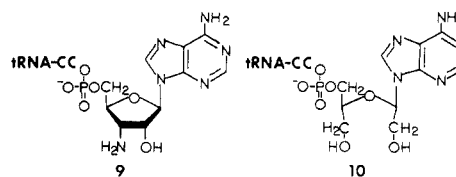
lack of incorporation of the deoxynucleotides onto tRNA-C-C$_{OH}$ by the *E. coli* enzyme may be indicative of a difference in substrate specificity of this enzyme as compared with the analogous yeast species or may simply reflect the greater specific activity of the yeast enzyme preparation.

The second method for tRNA-C-C$_{OH}$ reconstruction

(19) D. M. G. Martin, C. B. Reese, and G. F. Stephenson, *Biochemistry*, **7**, 1406 (1968).

(20) J. B. Gin and C. A. Dekker, *Biochemistry*, **7**, 1413 (1968).

(21) G. Zubay and M. Takanami, *Biochem. Biophys. Res. Commun.*, **15**, 2747 (1964).

(22) H. C. Neu and L. A. Heppel, *J. Biol. Chem.*, **239**, 2927 (1964).

(23) J. X. Khym and M. Uziel, *Biochemistry*, **7**, 422 (1968), and references therein.

(24) See, e.g., H. Kasai, Z. Ohashi, F. Harada, S. Nishimura, N. J. Oppenheimer, P. F. Crain, J. G. Liehr, D. L. von Minden, and J. A. McCloskey, *Biochemistry*, **14**, 4198 (1975).

(25) M. Sprinzl and F. Cramer, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3049 (1975).

(26) M. Sprinzl, K. H. Scheit, H. Sternbach, F. von der Haar, and F. Cramer, *Biochem. Biophys. Res. Commun.*, **51**, 881 (1973).

(27) S. M. Hecht, S. D. Hawrelak, J. W. Kozarich, F. J. Schmidt, and R. M. Bock, *Biochem. Biophys. Res. Commun.*, **52**, 1341 (1973).

(28) M. Sprinzl and F. Cramer, *Nature (London), New Biol.*, **245**, 3 (1973).

(29) T. H. Fraser and A. Rich, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2671 (1973).

(30) G. Chinali, M. Sprinzl, A. Parmeggiani, and F. Cramer, *Biochemistry*, **13**, 3001 (1974).

(31) S. M. Hecht, J. W. Kozarich, and F. J. Schmidt, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4317 (1974).

(32) T. H. Fraser and A. Rich, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3044 (1975).

(33) F. Cramer, H. Faulhammer, F. von der Haar, M. Sprinzl, and H. Sternbach, *FEBS Lett.*, **56**, 212 (1975).

(34) F. von der Haar and F. Cramer, *FEBS Lett.*, **56**, 215 (1975).

(35) S. M. Hecht and A. C. Chinault, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 405 (1976).

(36) S. M. Hecht, K. H. Tan, A. C. Chinault, and P. Arcari, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 437 (1977).

(37) A. C. Chinault, J. W. Kozarich, S. M. Hecht, F. J. Schmidt, and R. M. Bock, *Biochemistry*, **16**, 756 (1977).

(38) A. C. Chinault, K. H. Tan, S. M. Hassur, and S. M. Hecht, *Biochemistry*, **16**, 766 (1977).

utilizes nucleoside 5'-diphosphates and polynucleotide phosphorylase and has been reported to effect the incorporation onto *E. coli* tRNA-C-C$_{OH}$ of both 2'- and 3'-O-methyladenosine 5'-phosphates (in 4% yields) and 2' and 3'-deoxyadenosine 5'-phosphates (in yields of 55 and 17%, respectively).[27,31,37] The two substrates containing free 3'-OH groups were blocked with methyl vinyl ether prior to incubation with tRNA-C-C$_{OH}$ and polynucleotide phosphorylase to prevent polymerization.[39] These experiments were carried out with polynucleotide phosphorylase from *Micrococcus luteus*. More recently, an activity has been found in *E. coli* B cells which incorporates single deoxynucleotides onto oligodeoxyribonucleotide primers,[40,41] and Sninsky et al.[42] have described the single addition of ribonucleotides to oligoribonucleotide primers in very high yields by the removal of inorganic phosphate from the incubation medium as it is formed. Application of these findings to the reconstruction of modified tRNAs might well result in improved yields. Purification of the modified tRNAs has been effected both by chromatography on DBAE-cellulose (*N*-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl]aminoethyl-cellulose),[43,44] which retains selectively those tRNAs having vicinal diol moieties, and by treatment of the tRNAs with periodate, followed by chromatography on aminoethyl-cellulose (Figure 3).

Aminoacylation of the Modified tRNAs

The successful preparation of tRNAs modified at the 3' terminus permitted their testing as potential substrates for aminoacyl-tRNA synthetase. Sprinzl and Cramer demonstrated that yeast tRNA^{Phe} terminating in 3'-deoxyadenosine (3) was aminoacylated by phenylalanyl-tRNA synthetase with the same K_m as unmodified tRNA^{Phe} (2.8 μM), but with a slightly reduced V_{max} (0.18 vs. 0.28 μmol/min), suggesting strongly that tRNA^{Phe} is normally aminoacylated on the 2'-OH group.[28] This exciting result was extended to *E. coli* tRNA^{Phe} by Fraser and Rich,[29] who utilized tRNA terminating in 3'-amino-3'-deoxyadenosine. It was further verified by Ofengand et al.,[45] who studied the aminoacylation of *E. coli* tRNA^{Val} and *E. coli*, yeast, and rat liver tRNA^{Phe} which had been treated successively with periodate and borohydride (10). Although all of these tRNAs were aminoacylated exclusively on the 2'-OH group, additional experiments carried out with other tRNA isoacceptors revealed several which utilized tRNA species 2 exclusively and some for which both isomeric tRNAs were substrates. Studies of the position of aminoacylation of each of the tRNA isoacceptor activities from individual sources have been carried out with partially modified *E. coli* tRNAs,[25,32] with purified (but unfractionated) tRNA species 2 and 3 from *E. coli*,[35,38] yeast,[35,38] and calf liver,[38] and with modified tRNAs derived from four purified yeast tRNAs.[33] As

(39) J. K. Mackey and P. T. Gilham, *Nature (London)*, **233**, 551 (1971).
(40) S. Gillam and M. Smith, *Nucleic Acids Res.*, **1**, 1631 (1974).
(41) S. Gillam, K. Waterman, M. Doel, and M. Smith, *Nucleic Acids Res.*, **1**, 1649 (1974).
(42) J. J. Sninsky, G. N. Bennett, and P. T. Gilham, *Nucleic Acids Res.*, **1**, 1665 (1974).
(43) M. Rosenberg, J. L. Wiebers, and P. T. Gilham, *Biochemistry*, **11**, 3623 (1972).
(44) T. F. McCutchan, P. T. Gilham, and D. Söll, *Nucleic Acids Res.*, **2**, 853 (1975).
(45) J. Ofengand, S. Chládek, G. Robilard, and J. Bierbaum, *Biochemistry*, **13**, 5425 (1974).

Table I
Initial Site of tRNA Aminoacylation[35,38]

Hydroxyl group at 3' terminus of tRNA which is aminoacylated			
2'-OH	3'-OH	2'- and 3'-OH	Uncertain
E. coli tRNA			
Arg	Ala	Asn	Asp[b]
Glu	Gly	Cys	Gln
Ile	His	Tyr	
Leu	Lys		
Met	Pro		
Phe	Ser		
Trp[a]	Thr		
Val			
Yeast tRNA			
Arg	Ala	Asn	Glu
Ile	Gln	Asp	
Leu	Gly	Cys	
Met	His	Tyr	
Phe	Lys		
Val	Pro		
	Ser		
	Thr		
	Trp		
Calf Liver tRNA			
Arg	Ala	Asn	Gln
Ile	Asp	Tyr	Glu
Leu	Cys		Met
Phe	Gly		Pro
Val	His		
	Lys		
	Ser		
	Thr		
	Trp		

[a] tRNA^{Trp} species 2 may also be aminacylated to some extent.[46] [b] Aminoacylation does take place using tRNA^{Asp} species 2 (with a free 3'-OH group); aminoacylation of species 3 is uncertain.

shown in Table I, the *E. coli*, yeast, and calf liver aminoacyl-tRNA synthetase activities specific for arginine, isoleucine, leucine, phenylalanine, and valine, as well as the *E. coli* and yeast synthetases specific for methionine and the *E. coli* glutamyl-tRNA synthetase, aminoacylated only those cognate tRNA species having a free 2'-OH group (3). On the other hand, in all three systems the aminoacyl-tRNA synthetases specific for alanine, glycine, histidine, lysine, serine, and threonine aminoacylated only those species of type 2. This was also true for the *E. coli* and yeast prolyl-tRNA synthetases and for the glutamine-specific aminoacyl-tRNA synthetase from yeast. Although the relative rates of aminoacylation of these modified tRNAs varied significantly relative to the individual unmodified tRNAs, in most cases it was clear that one tRNA isomer was utilized by the enzyme and the other was not. This is illustrated in Figure 4 for *E. coli* tRNA^{Gly}. After a 30-min incubation, tRNA^{Gly} species 2 (having a free 3'-OH group) was aminoacylated to a slightly greater extent than the unmodified species, while tRNA^{Gly} species 3 was not a substrate for the glycyl-tRNA synthetase.

In a few cases, both isomeric tRNAs were aminoacylated, although at different rates. This was true for the modified tRNAs derived from *E. coli*, yeast, and calf liver tRNA^{Asn} and tRNA^{Tyr} as well as *E. coli* and yeast tRNA^{Cys} and yeast tRNA^{Asp}. That the observation was not due to the presence of individual species of a single tRNA isoacceptor activity with different positional
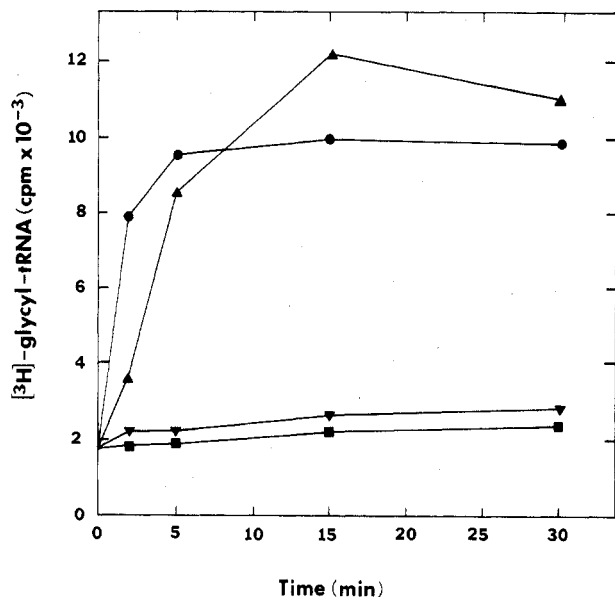
Figure 4. Aminoacylation of *E. coli* tRNA^{Gly} species 1 (●), 2 (▲), and 3 (▼), relative to a control lacking tRNA (■), in the presence of ATP and an *E. coli* aminoacyl-tRNA synthetase preparation.

specificity can be demonstrated for yeast tRNA^{Asn}, tRNA^{Cys}, and tRNA^{Tyr} and for calf liver tRNA^{Tyr}, since both isomers (2 and 3) of each were utilized to about the same extent as unmodified tRNA.

Although calf liver aspartyl- and cysteinyl-tRNA synthetases gave different results than were obtained with the *E. coli* and yeast enzymes, in that only a single modified tRNA species (2) was aminoacylated in the mammalian system, the apparent lack of amino-acylation of calf liver tRNA^{Asp} and tRNA^{Cys} species 3 may simply reflect differences in the relative rates of aminoacylation under the specific assay conditions utilized. Similar effects have been noted in some experiments with modified *E. coli* tRNA^{Tyr}. In fact, some of the tRNA isoacceptors listed in Table I as having substrate activity only in a single isomeric form may actually be slightly active as the other isomer as well. Yeast tRNA^{Ile} (3), for example, was aminoacylated at approximately the same rate and to the same extent as unmodified tRNA^{Ile}, and this presumably means that unmodified tRNA^{Ile} is aminoacylated initially on the 2′-OH group. While tRNA^{Ile} species 2 was amino-acylated to the extent of only 5% after 30 min, the extent of aminoacylation of this isomer increased steadily over that period of time, relative to a control lacking tRNA. Such effects have been observed repeatedly with some modified tRNA isoacceptors for incubation periods of up to 2 h. The use of purified aminoacyl-tRNA synthetases might well increase the extent of aminoacylation of such species, thus verifying their utilization as substrates.

Of particular interest in the aminoacylation studies was tryptophan, whose *E. coli* aminoacyl-tRNA synthetase was reported to utilize exclusively tRNA^{Trp} species 3, while the yeast and calf liver tryptopha-nyl-tRNA synthetases aminoacylated only tRNA^{Trp} species 2.[35,38] The same results for *E. coli* tRNA^{Trp} were obtained in two laboratories,[25,35] although recent results with partially purified *E. coli* tRNA^{Trp} indicate that species 2 may be utilized to a lesser extent as well.[46]

(46) K. H. Tan and S. M. Hecht, unpublished results.

Nevertheless, the aminoacylation of tRNA^{Trp} represents an example of change in specificity during the evolution from a prokaryotic to eukaryotic organism. It has been reported that both *E. coli* and yeast tRNA^{Trp} are aminoacylated with tryptophan by the heterologous aminoacyl-tRNA synthetase preparations.[47] Successful heterologous aminoacylation of a single isomer of *E. coli* or yeast tRNA^{Trp} would help to indicate whether positional specificity resides with the individual tRNA^{Trp}s or, perhaps more likely, with the trypto-phanyl-tRNA synthetases.

While there would seem to be no selective advantage to the aminoacylation of a single hydroxyl group on a particular tRNA, in the sense that equilibration of the 2′(3′)-*O*-aminoacyl isomers is believed to occur much faster than subsequent partial reactions of protein biosynthesis,[48] the utilization of one site for amino-acylation might well increase the specificity of tRNA–aminoacyl-tRNA synthetase interaction and thereby prevent misacylations.[9,34] Although the specific choice of OH group aminoacylated in any given tRNA isoacceptor could have been chosen at random and could simply reflect the nature of individual amino-acyl-tRNA synthetase active sites, the maintenance of positional specificity during evolution suggests that some advantage may be derived from the use of a particular OH group, or from some process which is reflected in the preservation of the initial position of tRNA aminoacylation.[49]

Isomeric Aminoacyl-tRNAs

Proper determination of positional specificity for transformations subsequent to aminoacylation would clearly require the use of isomeric aminoacyl- (peptidyl-) tRNAs (e.g., species 7 and 8). However, prior to the recent observation that both species (2 and 3) derived from three *E. coli* and four yeast tRNA isoacceptors could be aminoacylated by the cognate aminoacyl-tRNA synthetase activities (Table I), there was no facile method for obtaining isomeric aminoacyl-tRNAs, and most investigations have utilized only single isomers. Access to aminoacyl-tRNA analogues 7 and 8 derived from *E. coli* tRNA^{Asn}, tRNA^{Cys}, and tRNA^{Tyr} will certainly facilitate the study of positional specificity for these isoacceptors, but 17 other isomeric aminoacyl-tRNA analogues remain inaccessible by enzymatic aminoacylation, including those species for which there are readily available synthetic mRNAs (e.g., tRNA^{Phe} and tRNA^{Lys}) and those which can be utilized for both A- and P-site studies (tRNA^{Met} and tRNA^{Phe}).

Some of the modified aminoacyl-tRNAs (7 and 8) which cannot be obtained by direct aminoacylation of tRNA species 2 and 3 may be accessible via the "chemical aminoacylation" procedure that we developed for the preparation of tRNA terminating with 2′-deoxy-3′-*O*-L-phenylalanyladenosine (7). This proce-dure involved incubation of abbreviated tRNA with polynucleotide phosphorylase in the presence of chemically prepared 2′-deoxy-3′-*O*-L-[^{3}H]phenyl-alanyladenosine 5′-diphosphate (eq 2). Limitations of this procedure for the construction of modified aminoacyl-tRNAs include the poor activity of amino-acylated nucleotides as substrates for polynucleotide

(47) B. P. Doctor and J. A. Mudd, *J. Biol. Chem.*, **238**, 3677 (1963).
(48) A. Kepes and S. Beguin, *Biochim. Biophys. Acta*, **123**, 546 (1966).
(49) S. M. Hecht, *Tetrahedron*, in press.

tRNA-C-C$_{OH}$

$$\text{tRNA-C-C}_{OH} \xrightarrow[\substack{2'\text{-deoxy-}3'\text{-}O\text{-phenylalanyl-}\\ \text{adenosine } 5'\text{-diphosphate}}]{\substack{\text{polynucleotide}\\ \text{phosphorylase}}} \text{phenylalanyl-tRNA species } \mathbf{7} \quad (2)$$
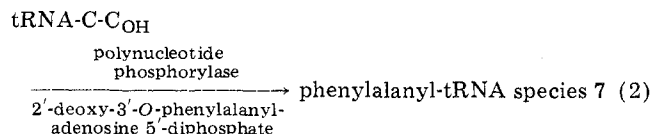
phosphorylase.[37] Also, the incubation period cannot be lengthened to compensate for the low yields since the nucleotides are unstable under the reaction conditions, undergoing hydrolysis to afford deoxynucleoside 5'-diphosphates, which are also better substrates for the enzyme. Nevertheless, a purified sample of phenyl-alanyl-tRNA species **7** was obtained by this method after chromatographic separation of reaction products, and other isoacceptors may also be accessible by this route.

Ternary Complex Formation and A-site Binding

EF-Tu interacts with aminoacyl-tRNA and GTP to form a ternary complex from which the aminoacyl-tRNA is subsequently transferred to the ribosomal A site. The interaction of EF-Tu and aminoacyl-tRNA can therefore be measured by studying the ternary complex itself or the factor-dependent binding of the tRNA to the ribosome. Chládek and Ringer utilized the "tRNA analogues" cytidylyl-(3'→5')-2',3'-O-phenyl-alanyladenosine, cytidylyl-(3'→5')-2'-O-phenylalanyl-3'-deoxyadenosine, and cytidylyl-(3'→5')-3'-O-phenylalanyl-2'-deoxyadenosine as probes of possible EF-Tu·GTP specificity for a single isomer of amino-acyl-tRNA.[50-52] The former dissociated the EF-Tu·GTP complex, ostensibly via the intemediacy of an unstable ternary complex, and this was interpreted as evidence that formation of the EF-Tu·GTP–amino-acyl-tRNA complex normally involves utilization of 2'-O-aminoacyl-tRNA. More recently, we have assayed the EF-Tu·GTP binding of modified *E. coli* amino-acyl-tRNAs (**7** and **8**) derived from 16 different iso-acceptors. Binding was measured both in terms of the ability of EF-Tu·GTP to diminish the rate of hydrolysis of the modified aminoacylated tRNAs and by gel fil-tration of several of the ternary complexes. All of the modified aminoacyl-tRNAs tested were found to bind to the elongation factor, although generally to a lesser extent than the corresponding unmodified amino-acyl-tRNAs.[36] Our results are in agreement with those of recent studies at Göttingen that employed modified *E. coli* tRNA^{Tyr}s **7** and **8**.[53]

Chinali et al.[30] studied the A-site binding of yeast phenylalanyl-tRNA^{Phe} species **6** and **8** in the presence of *E. coli* EF-T and ribosomes. At 10 mM Mg^{2+} con-centration, the binding of species **8** was stimulated to almost the same extent as species **6** (34 vs. 38%). Also studied were the 3'-N-phenylalanyl derivative of tRNA species **9**[54] and the 2'-O-phenylalanyl derivative of species **10**,[30] neither of which was bound to a significant extent. Although no direct comparison was made

between isomeric aminoacyl-tRNAs, the implication of the experimental observations was that EF-T-directed A-site binding was specific for 2'-O-aminoacylated tRNAs having an intact ribofuranosyl moiety.[54] In fact, the finding for tRNA species **10** was consistent with the results of earlier studies[55,56] which utilized the same modified tRNA species, but the lack of A-site binding obtained with the 3'-N-phenylalanyl derivative of **9** was surprising in view of the published work of Fraser and Rich.[29] These authors had reported that, after preincubation of *E. coli* ribosomes in the presence of poly(U), crude initiation factors, and unmodified N-acetylphenylalanyl-tRNA from *E. coli* (8 mM Mg^{2+}), further incubation with purified EF-Tu and the 3'-N-phenylalanyl derivative of **9** resulted in the formation of 95% of the theoretical amount of N-acetylated di-peptide. It would be difficult to envision efficient dipeptide formation without prior binding of phenyl-alanyl-tRNA **9** to the A site. A possible resolution of the differences in experimental results has now been provided by Baksht et al.,[57] who have shown that, at 6 mM Mg^{2+} in the absence of elongation factor, the extent of A-site binding of yeast phenylalanyl-tRNA **10** to reticulocyte ribosomes was at least twice that of unmodified phenylalanyl-tRNA **6** (and almost 70% of theoretical), so that added elongation factor effected little additional stimulation of binding of the former. An unfortunate consequence of this observation is that the behavior of aminoacyl-tRNAs **9** (and also of the isomeric 2'-N-aminoacyl species) may differ funda-mentally from those of unmodified aminoacyl-tRNAs under certain circumstances and give results not analogous to those which are obtained for the unmo-dified species.

We have carried out a direct comparison of the extent of participation of isomeric aminoacyl-tRNAs in EF-Tu mediated A-site binding by studying the effects of unlabeled phenylalanyl-tRNAs **6–8** on the binding of equimolar amounts of [^{3}H]phenylalanyl-tRNA (**6**, R = CH$_2$C$_6$H$_5$). All three unlabeled species were found to inhibit A-site binding to the extent of 50%, consistent with the interpretation that both positional isomers of phenylalanyl-tRNA (**7** and **8**) are bound to the A site as well as normal phenylalanyl-tRNA.[31] Repetition of this experiment at 10 mM MgCl$_2$ concentration in the absence of EF-Tu gave the same results, suggesting that the observed inhibition was effected at the level of A-site binding rather than at the level of ternary complex formation.

P-Site Binding and Peptide Bond Formation

The acceptor tRNA in the peptidyltransferase re-action has long been thought to be the 3'-O-aminoacyl species on the basis of the observation that the 3'-N-aminoacyl-tRNA "analogue" puromycin was a potent inhibitor of protein biosynthesis, while the isomeric 2'-aminoacyl analogue was not.[58-60] Additional evi-dence in support of the role of the 3'-O-aminoacyl

(50) S. Chládek and D. Ringer, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **34**, 559 (1975).

(51) D. Ringer and S. Chládek, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2950 (1975).

(52) D. Ringer, S. Chládek, and J. Ofengand, *Biochemistry*, **15**, 2759 (1976).

(53) Professor F. Cramer, personal communication.

(54) F. Cramer in "Recent Developments in Oligonucleotide Synthesis and Chemistry of Minor Bases of tRNA," International Conference, Sept 13–14, 1974, Poznan-Kiekrz, Poland, pp 175–191.

(55) C.-M. Chen and J. Ofengand, *Biochem. Biophys. Res. Commun.*, **41**, 190 (1970).

(56) J. Ofengand and C.-M. Chen, *J. Biol. Chem.*, **247**, 2049 (1972).

(57) E. Baksht, N. deGroot, M. Sprinzl, and F. Cramer, *Biochemistry*, **15**, 3639 (1976).

(58) D. Nathans and A. Neidle, *Nature (London)*, **197**, 1076 (1963).

(59) R. J. Suhadolnik, "Nucleoside Antibiotics", Wiley-Interscience, New York, N.Y., 1970, p 3 ff.

(60) R. J. Harris and R. H. Symons, *Bioorg. Chem.*, **2**, 286 (1973).

Table II
Transfer of N-Acetylphenylalanine from
N-Acetylphenylalanyl-tRNA to Modified
Phenylalanyl-tRNAs 6–8[31]

Acceptor tRNA	cpm transferred[a]	Net % transfer of bound tRNA
tRNA-C-C-A$_{O}$-Phe (6)	2960	70
tRNA-C-C-A$_{2'}$-H,$_{3'}$-O-Phe (7)	760	17
tRNA-C-C-A$_{2'}$-O-Phe,$_{3'}$-H (8)	100	
None		90

[a] Approximately 4100 cpm of N-[^{3}H]-acetylphenyl-alanyl-tRNA was bound to the P site initially.

isomer derives from the work of Hussain and Ofengand,[61] who found that the aminoacylated adenosine moiety isolated from phenylalanyl-tRNA^{Phe} species 10 (presumably the 2'-O-phenylalanine derivative) could not participate in the peptidyltransferase reaction, whereas a chemically prepared mixture of the 2'- and 3'-O-aminoacylated nucleosides did function as an acceptor. These results were verified and extended by Chládek et al.,[62] who carried out experiments with single isomers of the same aminoacylated nucleosides and later with isomeric aminoacylated dinucleoside monophosphates structurally related to the 3' termini of tRNA species 2–5.[63–65] Only those analogues having a 3'-O-phenylalanyl moiety functioned as acceptors in the peptidyltransferase reaction, although all of the aminoacylated analogues were bound to the A site, as judged by their ability to inhibit both the binding of C-A-C-C-A$_{2'(3')-O-Phe}$[65] and the transfer of N-acetylphenylalanine to puromycin by peptidyltransferase.[63,65]

Fraser and Rich[29] demonstrated that the 3'-N-phenylalanine derivative of *E. coli* tRNA species 9 accepted N-acetylphenylalanine from unmodified N-acetylphenylalanyl-tRNA in the peptidyltransferase reaction. While the isomeric phenylalanyl-tRNA species was not tested in comparison, Chinali et al.[30] later reported that yeast 2'-O-phenylalanyl-tRNA^{Phe} species 8 and 10 also participated as acceptors in peptide bond formation, although at a diminished rate as compared with unmodified phenylalanyl-tRNA^{Phe}. Perhaps the most definitive study was carried out at MIT using isomeric *E. coli* phenylalanyl-tRNAs 7 and 8 in direct comparison to unmodified phenylalanyl-tRNA.[31] As shown in Table II, after a 15-min incubation period 3'-O-phenylalanyl-tRNA species 7 had accepted 24% as much N-acetylphenylalanine as the unmodified aminoacyl-tRNA (6, R = CH$_2$C$_6$H$_5$), while no dipeptidyl-tRNA formation was noted for the isomeric phenylalanyl-tRNA species 8.

The P-site binding of 2'-O-(N-acetylphenylalanyl)-tRNA species 8 and 10 was studied by Chinali et al.,[30] both species were bound, although neither was bound so well as unmodified N-acetylphenylalanyl-tRNA^{Phe}. A similar result was obtained in my laboratory for isomeric N-acetylated phenylalanyl-tRNAs 7 and 8.[31] These species were compared for their ability to inhibit the binding of unmodified N-[^{3}H]acetylphenylalanyl-tRNA. When used in equimolar concentration with the unmodified species, N-acetylated phenylalanyl-tRNAs 7 and 8 effected 36 and 35% inhibition of binding, respectively.[66] Thus the analogues were equally as effective as inhibitors of the P-site binding of N-acetylphenylalanyl-tRNA, although neither bound to the P site as strongly as the unmodified species. Utilization of the modified N-acetylphenylalanyl-tRNAs as donors in the peptidyltransferase has also been attempted, but without success.[29–31,57] It is unclear at present whether this is due to insufficient activation of the N-acetylphenylalanyl moieties in the modified species, to an enzymatic requirement for the adjacent 2'(3')-OH group in the peptidyltransferase reaction, or possibly even to an alteration in the nature of the binding of the N-acetylphenylalanyl-tRNA analogues to the P-site.

Summary

To the extent that the results obtained for the modified tRNAs are applicable to the corresponding unmodified species, several conclusions may be drawn. For example, while the initial position of tRNA aminoacylation shows considerable diversity from one isoacceptor to another, the aminoacylation of any single tRNA is probably specific for a single hydroxyl group at the 3' terminus of tRNA. Moreover, that specificity has generally been conserved through the evolution from prokaryotic to mammalian species. Both positional isomers of tRNA can bind to EF-Tu·GTP, although it is possible that one isomer may normally be bound preferentially. Similarly, both of the modified 2'- and 3'-aminoacyl-tRNA analogues were bound equally to both the A and the P sites, although neither analogue was bound as well to the P site as the corresponding unmodified tRNA. While the acceptor species in the peptidyltransferase reaction is almost certainly the 3'-O-aminoacyl derivative, it has not been possible to determine which positional isomer acts as the donor. It seems reasonable to conclude that there is positional specificity in those partial reactions of protein biosynthesis which involve formation or breaking of the O-acyl bond, but much less specificity when only the binding of aminoacyl(peptidyl)-tRNA is involved.

(61) Z. Hussain and J. Ofengand, *Biochem. Biophys. Res. Commun.*, 50, 1143 (1973).
(62) S. Chládek, D. Ringer, and J. Žemlička, *Biochemistry*, 12, 5135 (1973).
(63) D. Ringer and S. Chládek, *Biochem. Biophys. Res. Commun.*, 56, 760 (1974).
(64) S. Chládek, D. Ringer, and K. Quiggle, *Biochemistry*, 13, 2727 (1974).
(65) D. Ringer, K. Quiggle, and S. Chládek, *Biochemistry*, 14, 514 (1975).

(66) As anticipated, the use of an equimolar amount of unlabeled N-acetylphenylalanyl-tRNA (6) resulted in 50% inhibition of binding of unmodified N-[^{3}H]acetylphenylalanyl-tRNA.