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PARTICIPATION OF ISOMERIC tRNA'S IN THE PARTIAL REACTIONS OF PROTEIN BIOSYNTHESIS†

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As first shown by Zamecnik *et al.*,[1] the initial step in protein biosynthesis involves the activation of individual transfer RNA's with their respective amino acids. This transformation is mediated for each tRNA by a cognate aminoacyl-tRNA synthetase (ligase) and results in the formation of an activated ester at the 3'-terminus of the tRNA (Fig. 1; eqn 1). The kinetics of the aminoacylation reactions have been studied in great detail and the mechanism has been a matter of considerable controversy and uncertainty.[2-9] Also ambiguous is the initial site of tRNA aminoacylation. Although the aminoacylation of a tRNA by its cognate aminoacyl-tRNA synthetase has been thought to involve the use of only a single (2'- or 3'-) OH group at the 3'-terminus of tRNA (eqn 1), the two positional isomers of aminoacyl-tRNA are believed to equilibrate rapidly in solution,[10] thus complicating attempts to determine the initial position of aminoacylation.

†This account is dedicated to Prof. NELSON J. LEONARD, on the occasion of an important birthday.

‡Research Career Development Awardee of the National Cancer Institute 1975–80. Alfred P. Sloan Research Fellow, 1975–77. John Simon Guggenheim Fellow, 1977–78.

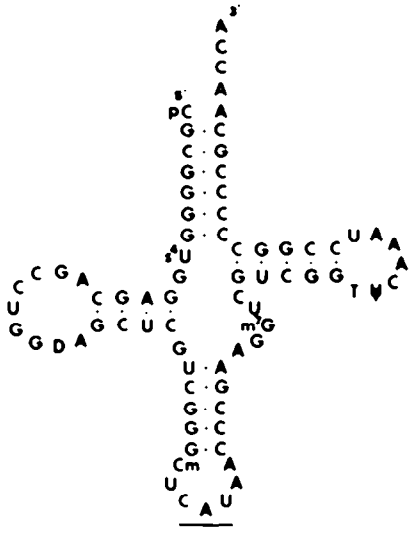
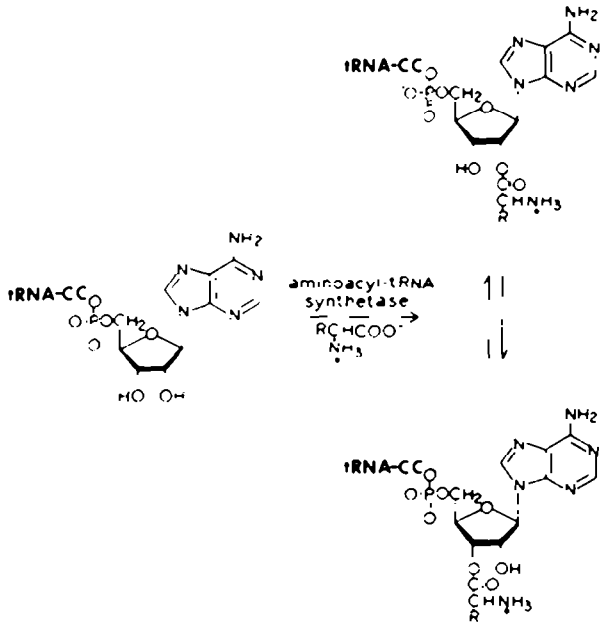


Fig. 1. Cloverleaf representation of *E. coli* $tRNA_f^{Met}$. The anticodon triplet is underlined.



(1)

The biosynthesis of polypeptides of defined sequence depends upon the availability of aminoacylated tRNA's as well as an appropriate messenger RNA (mRNA), ribosomes, GTP, Mg^{2+}, several protein factors and probably ATP. Protein biosynthesis begins with the assembly of an initiation complex; in a prokaryotic system such as *Escherichia coli* this involves the initiation factor f3-dependent binding of a 30S ribosomal subunit to the initiation site on the mRNA. The formation of this complex is stimulated by the presence of factor f1, a protein of molecular weight 9000, as is the subsequent binding to the subunit of N-formylated methionyl-$tRNA_f^{Met}$ from a factor f2 - GTP - N - formylmethionyl - $tRNA_f^{Met}$ complex. The complete initiation complex, containing the 30S ribosomal subunit, mRNA and N-formylmethionyl $tRNA_f^{Met}$, as well as f1, f2, f3 and GTP, then associates with the 50S ribosomal subunit with the concomitant release of initiation factors and hydrolysis of GTP. The P(peptidyl)-site of the resulting functional 70S ribosomal complex is occupied by N-formylmethionyl-$tRNA_f^{Met}$, the anticodon of which (Fig. 1) is hydrogen-bonded to the complementary initiator codon triplet (AUG) in the RNA via Watson-Crick base pairing. The second mRNA codon specifies the anticodon of the aminoacyl-tRNA which then fills the ribosomal A(aminoacyl)-site; actual introduction of that aminoacyl-tRNA into the ribosomal site is mediated by elongation factor Tu (EF-Tu), which forms a ternary complex with the aminoacyl-tRNA and GTP. In analogy with the introduction of N-formylmethionyl-$tRNA_f^{Met}$ into the P-site, as the EF-Tu·GTP-aminoacyl-tRNA complex is bound to the ribosome hydrolysis of GTP occurs and the factor is released from the ribosome as EF-Tu·GDP.[11-13]†

The introduction of an aminoacyl-tRNA into the A-site permits the formation of a peptide bond between the aminoacyl moieties of the two ribosome-bound tRNA's. This transformation is believed to involve nucleophilic addition of the amino group of the A-site tRNA to the activated ester of N-formylmethionyl-$tRNA_f^{Met}$ and is effected by peptidyl transferase, which is part of the 50S subunit. As shown in Scheme 1, completion of the reaction yields $tRNA_f^{Met}$ in the ribosomal P-site and N-formylmethionylaminoacyl-tRNA in the A-site.[11,12]

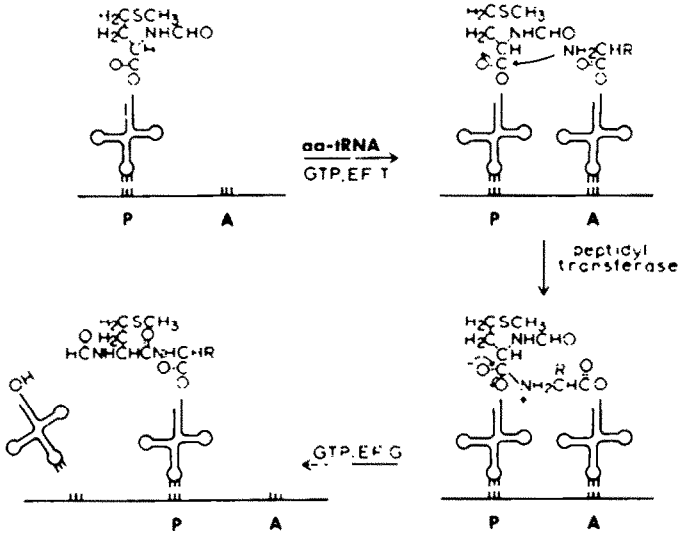
Although peptide bond formation itself does not involve the hydrolysis of ATP or GTP, the subsequent translocation step does. In addition to GTP, translocation requires elongation factor G and results in release of $tRNA_f^{Met}$ and movement of the mRNA relative to the ribosome. At the conclusion of this step, the peptidyl-tRNA has been moved from the A-site to the P-site and the next codon is positioned at the ribosomal A-site, where it will direct the binding of the appropriate aminoacyl-tRNA and lead to formation of an additional peptide bond. Since each of the 64 possible codons specifies no more than one tRNA isoacceptor, sequential translation of adjacent mRNA codons defines exactly the sequence of amino acids in the resulting protein.[11,12]

An increasing number of studies have been concerned with the mechanisms of the individual transformations which comprise protein biosynthesis; definition of the pathways utilized has been accomplished in part through the use of synthetic analogs of normal components of the protein-biosynthesizing system. One aspect of protein synthesis which has not been studied in detail is the (2'- or 3'-) position of the aminocyl (peptidyl) moiety of tRNA during each of the partial reactions. Although the amino acid is thought to equilibrate rapidly between the vicinal hydroxyl groups in solution, it is not unreasonable to acticipate that at least some of the individual reactions may require a single positional isomer of aminoacyl (peptidyl)-tRNA, if only to enhance the specificity and facility of such transformations.

Isomeric tRNA's can be used to determine positional specificity

On the basis of expected differences in chemical reactivity[14-16] of the 2'- and 3'-OH groups of adenosine, Zamecnik suggested that from a chemical point of view the 2'-OH group of the 3'-terminal nucleoside of tRNA would be the more likely point of attachment of the amino acid during aminocylation.[17] Also indicated was the likelihood of subsequent acyl migration between the vicinal hydroxyl groups and the probability that the 3'-O-aminoacyl isomer would be more stable thermodynamically. Early studies of the initial position of tRNA amino-

†A second elongation factor, EF-Ts, is used to re-generate EF-Tu·GTP from EF-Tu·GDP.



Scheme 1.

acylation, as well as the distribution of positional isomers at equilibrium, were carried out using toluene - *p* - sulfonyl chloride,[18] 2,3-dihydropyran[19] and 2 - cyanoethylphosphate[20] as trapping agents. All three studies gave products derived predominantly from the 3' - O - aminoacyl isomer of tRNA, although it is difficult to exclude the possibility that the observed isomer distributions may have been influenced by rapid migration of the aminoacyl moiety between the 2'- and 3'-positions or by differences in reactivity of the two hydroxyl groups.

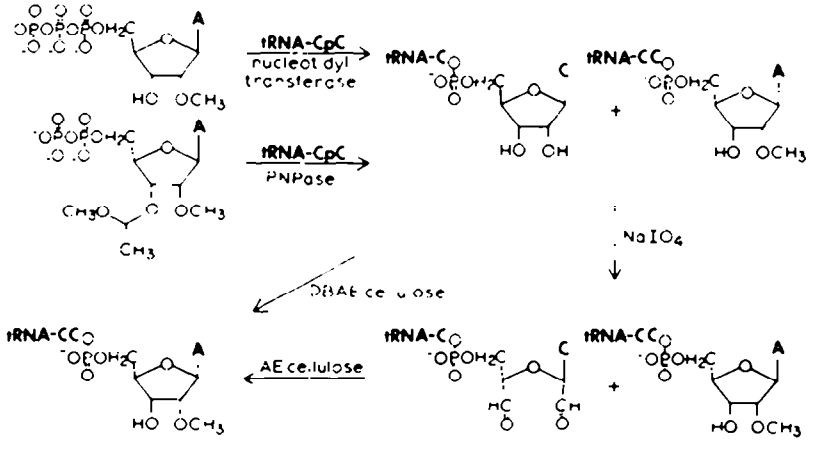
In 1973, three laboratories reported the preparation of tRNA's modified at the 3'-terminus such that the adenosine moiety had been replaced with a nucleoside analog containing only a single 2'- or 3'-hydroxyl group.[21-24] By the use of isomeric analogs, e.g. 2'- and 3'-deoxyadenosine, it was possible to prepare the corresponding isomeric tRNA's (**2** and **3**), the aminoacylated forms of which could not undergo isomerization between the 2'- and 3'-positions. Although the tRNA's terminating in 2'- and 3'-O-methyladenosine (**4** and **5**) were poor substrates for the cognate aminoacyl-tRNA synthetase activities,[23,25] the other modified tRNA's promised to be more useful. Modified yeast $tRNA^{Phe}$ species **3**, e.g., was found to be the sole substrate for the homologous phenylalanyl-tRNA synthetase. The K_m for aminoacylation of the modified tRNA was the same as that measured for the corresponding unmodified species (2.8 μM) and the associated V_{max} values were similar (0.28 μmoles min^{-1} for **1** *vs* 0.18 μmoles min^{-1} for **3**),[22] consistent with the interpretation that yeast $tRNA^{Phe}$ is normally aminoacylated on the 2'-OH group. The isomeric $tRNA^{Phe}$ species **2** was not a substrate for aminoacylation by the yeast phenylalanyl-tRNA synthetase, but was shown to be a competitive inhibitor of the aminoacylation of unmodified $tRNA^{Phe}$ with an apparent K_i of 2.16 μM.[22] Thus, these early results suggested strongly the probable utility of such modified tRNA's in studies of positional specificity during protein biosynthesis.

Preparation of modified tRNA's

As illustrated in Scheme 2, the modified tRNA's of interest may be obtained by enzyme-mediated reconstruction of abbreviated tRNA (tRNA–C–C$_{OH}$) with the appropriate nucleoside phosphates. Since the C–C–A sequence is common to the 3'-terminus of all tRNA's, the modification procedure is applicable to unfractionated mixtures of tRNA's, as well as single, purified species.

The abbreviated tRNA itself is accessible from intact tRNA by chemical or enzymatic removal of the 3'-terminal adenosine moiety. It has been shown, for example, that treatment of ribonucleoside 5'-phosphates successively with periodate and a primary amine under appropriate reaction conditions results in β-elimination of the phosphate moiety from the initially formed dialdehyde,[26-28] presumably via an imine[28] or enamine.[29] Application of this methodology to tRNA results in the elimination of tRNA–C–C$_p$,[29-31] which can be converted to tRNA–C–C$_{OH}$ by additional treatment with alkaline phosphatase. This procedure has the advantage that it results in the removal of a single nucleotide from tRNA; since the β-elimination has been of considerable mechanistic interest,[28,30] the optimal conditions for the transformation have also been studied in great detail. One may note, however, that the utility of this method is limited by the presence in tRNA's of modified nucleosides with additional *cis*-diol moieties[32,33] and more generally by the observed loss of amino acid acceptor activity upon treatment of certain tRNA's with periodate.[31,34]

Alternatively, the adenosine moiety may be removed from the terminus of tRNA by controlled digestion with venom exonuclease.[35] Although the removal of nucleotides does not occur to the same extent for all tRNA's, the progress of the hydrolysis for a given species can be determined by monitoring the loss of amino acid acceptor activity and degradation can thereby be limited largely to the single stranded C–C–A sequence. Since CTP(ATP):tRNA nucleotidyltransferase is responsible for the addition of this sequence to several tRNA's after transcription,[36,37] and is also capable of repairing partially degraded tRNA's,[38] the enzyme can be used to convert the mixture of tRNA's which results from venom treatment to tRNA–C–C$_{OH}$ by replacement of missing cytidine moieties. For example, by careful optimization of the venom exonuclease treatment of unfractionated *E. coli* tRNA, it was possible to prepare tRNA–C–C$_{OH}$ which had no phenylalanine acceptor activity and which could be reconstituted with [^{3}H]-ATP to the extent of 80–100% of the theoretical value (Fig. 2).[39] The reconstituted tRNA could be aminoacylated with phenylalanine at the same rate and to the same extent as unmodified tRNA. Similar procedures have been utilized for the conversion of purified yeast $tRNA^{Phe}$[22] and $tRNA^{Tyr}$[40] to the corresponding abbreviated tRNA's, and



Scheme 2.

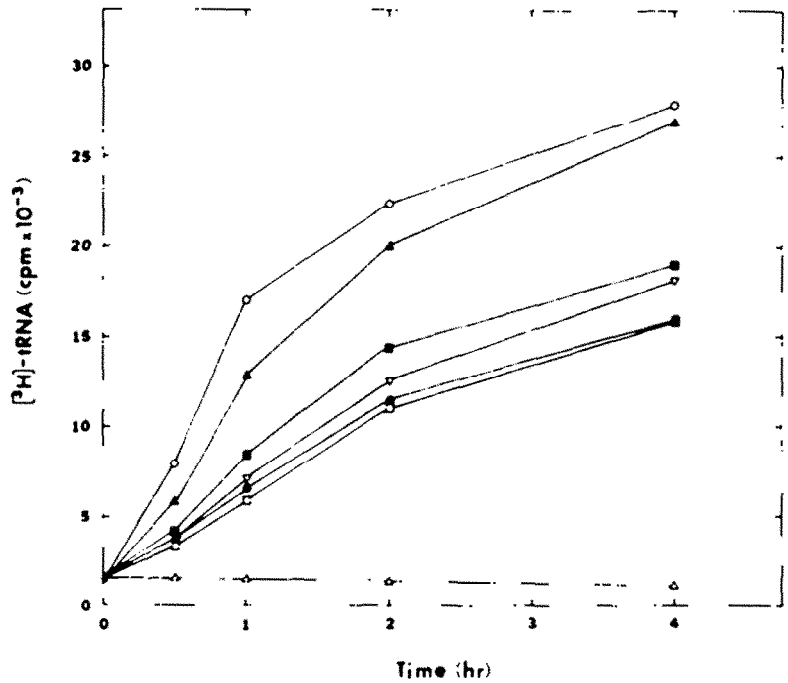


Fig. 2. Reconstitution with CTP and [³H]-ATP of samples of unfractionated *E. coli* tRNA treated with venom exonuclease for 30 (○), 40 (▲), 50 (■), 60 (▽), 70 (●) and 80 (□) min. A control was carried out in the absence of tRNA (△).

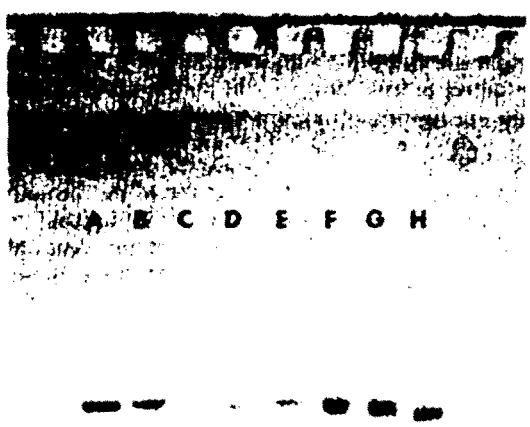


Fig. 3. Gel electrophoresis of *E. coli* $tRNA^{Arg}$ and $tRNA_f^{Met}$ after digestion with venom exonuclease. The digestion was carried out using 1.0 A_{260} unit of purified tRNA in 50 μl of Tris-HCl buffer at 37° for 20 min in the presence of varying amounts of venom exonuclease. Aliquots of each reaction mixture were applied to individual channels, which contained $tRNA^{Arg}$ (A); $tRNA^{Arg}$ digested with 5 (B), 10 (C) or 25 (D) μg/ml of nuclease; $tRNA_f^{Met}$ (E); $tRNA_f^{Met}$ digested with 5 (F), 10 (G), or 25 (H) μg/ml of nuclease.

also for $tRNA^{Ala}$ and $tRNA_f^{Met}$ from *E. coli*. For the latter two species, the extent of degradation by venom exonuclease was also monitored by polyacrylamide gel electrophoresis (Fig. 3); reconstitution of the abbreviated tRNA's with ATP in the presence of CTP(ATP):tRNA nucleotidyltransferase was shown to give functional tRNA's.[41]

TWO PROCEDURES CAN BE USED FOR THE RECONSTRUCTION OF tRNA-C-C_{OH}

Scheme 2 illustrates the methods which have been utilized for the preparation of modified tRNA's from tRNA-C-C_{OH}. One of these involves the incubation of tRNA-C-C_{OH} with CTP(ATP):tRNA nucleotidyltransferase and an appropriate ATP analog. Preparation of the analogs of interest (I-III) was accomplished starting from the respective nucleosides which were phosphorylated with pyrophosphoryl chloride,[42] labelled by exchange with ^3H_2O where necessary,[43] and converted to the respective nucleoside 5'-di and triphosphates via the 5'-phosphorimidazolidates.[44,45]

The incorporation of modified nucleotides onto tRNA-C-C_{OH} proceeds much less quickly than incorporation of the normal substrate, but a reasonable level of incorporation can generally be achieved after an extended period of incubation. CTP(ATP):tRNA nucleotidyltransferase preparations from different sources have different specific activities and substrate specificities and these must be considered when selecting an enzyme for tRNA reconstruction. For example, the incorporation of 2'- and 3'-deoxyadenosine 5'-triphosphates onto tRNA-C-C_{OH} could not be effected with the CTP(ATP):tRNA nucleotidyltransferase activities from *E. coli*,[41,46,47] rabbit liver[48] or rabit muscle,[49] but the yeast enzyme did catalyze the incorporation of both 2'- and 3'-deoxyadenosine 5'-triphosphates.[21,22] In the presence of the yeast enzyme, the deoxynucleotides were incorporated onto abbreviated tRNA derived from *E. coli*, yeast and calf liver in 45–65% yields;[21,34,50] virtually all of the tRNA isoacceptors from these three species could be modified in this fashion.[34,50] While the CTP(ATP):tRNA nucleotidyltransferase from *E. coli* did not utilize 2' or 3'-deoxyadenosine 5'-triphosphates as substrates, 2' - amino - 2' - deoxyadenosine and 3' - amino - 3' - deoxyadenosine 5' - triphosphates were utilized by the enzyme (and incorporated to the extent of 25–30%)[24,51] as were the isomeric 2' and 3'-O-methyladenosine 5'-triphosphates.[25] The latter two nucleotides were incorporated onto tRNA-C-C_{OH} in yields of 30 and 37%, respectively, and were also found to be competitive inhibitors of the incorporation of ATP (K_m measured as 70 μM) with apparent K_i's of 100 μM.[41]

Since extraneous nuclease activities in the CTP(ATP):tRNA nucleotidyltransferase preparations would degrade the abbreviated tRNA's during the relatively long period of incubation required for reconstruction with ATP analogs, the purity of the CTP(ATP):tRNA nucleotidyltransferase preparations utilized in such experiments has been a matter of special concern. Several procedures have been reported for purification of the *E. coli*[46,47,52-55] and yeast[56,57] enzymes, although none have dealt with the question of extraneous activities which could damage tRNA's during prolonged incubations. Recently, Chinault *et al.*[39] have described the purification of the *E. coli* enzyme by ammonium sulfate fractionation and successive chromatographies on Sephadex A-25 and DEAE-cellulose. This procedure gave an enzyme having a specific activity comparable to those of preparations reported previously and lacking nuclease activities sufficient to degrade tRNA-C-C_{OH}, as judged by the absence of radioactive breakdown products formed during the incubation of [^{32}P]-$tRNA^{Ser}$ with the preparation.

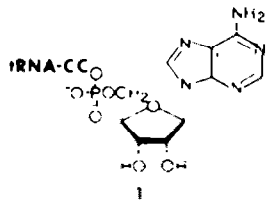
Preparation of yeast CTP(ATP):tRNA nucleotidyltransferase in a state of purity useful for the modification of unfractionated tRNA's has been accomplished by ammonium sulfate fractionation of the crude cell extract, followed by chromatography on DEAE-cellulose and then on phosphocellulose. Reconstruction of purified tRNA's can be effected after additional purification of the enzyme by chromatography on hydroxylapatite and Sephadex G-100,[41] or by the method of Sternbach *et al.*[57]

Another method for the preparation of modified tRNA's from tRNA-C-C_{OH} utilizes polynucleotide phosphorylase to catalyze the condensation with nucleoside 5'-diphosphates. Although nucleoside 5'-diphosphates having 3'-OH groups are polymerized by the enzyme, Gilham *et al.* have demonstrated that single additions of such ribonucleotides to a variety of substrates can be achieved by blocking the hydroxyl groups as the 2'(3') - O - (1 - methoxyethyl) derivatives (II);[58,59] a related procedure has been developed using 2'(3') - O - isovaleryl derivatives of nucleotides.[60,61] Application of the former methodology to the construction of tRNA species **2–5** was carried out using polynucleotide phosphorylase from *Micrococcus luteus*. Incorporation of 2' and 3'-deoxyadenosine 5'-diphosphates (II and III, R – H) proceeded to the extent of 4%, although the yield of the former was increased to 36% in the presence of 20% methanol. After incubation with polynucleotide phosphorylase and suitable deblocking, nucleoside 5'-diphosphate II (R – OCH_3) gave tRNA terminating in 2'-O-methyladenosine in 55% yield, while the incorporation of III (R – OCH_3) was found to be 17%.[25]

Improvements in the latter method of tRNA reconstruction may be possible by taking advantage of recent developments in the area. These include the isolation by Gillam *et al.* of an enzyme activity from *E. coli* B which, in the presence of Mn^{2+}, catalyzes the addition of a single deoxyribonucleotide onto primers consisting of oligodeoxyribonucleotides.[62-64] Also of interest in this context is the work of Sninsky *et al.*[65] which has resulted in a method for the incorporation of single ribonucleotides onto primers in high yield by suppressing unwanted phosphorolysis of the primers during the addition.

There was no special advantage associated with the use of polynucleotide phosphorylase, rather than CTP(ATP):tRNA nucleotidyltransferase, for the preparation of tRNA's terminating in 2' and 3' - deoxyadenosine or 2'- and 3' - O - methyladenosine. As discussed below, however, the technique utilizing polynucleotide phosphorylase was also applicable to the direct preparation of tRNA terminating in 2' - deoxy - 3' - O - phenylalanyladenosine,[25] and thus facilitated the preparation of isomeric phenylalanyl-tRNA's (**7** and **8**, R = $CH_2C_6H_5$).

Although the reconstruction of tRNA-C-C_{OH} with analogs of ATP does not proceed to completion, purification of the modified tRNA's can be accomplished by either of two methods, as shown in Scheme 2. One of these involved treatment of the tRNA's with periodate, which effected oxidation of the 3'-terminal nucleosides in the remaining tRNA-C-C_{OH}'s; conversion of the *cis*-diol moieties in these tRNA's to the corresponding dialde-



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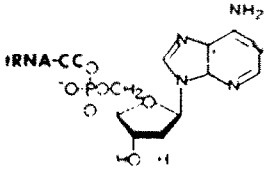

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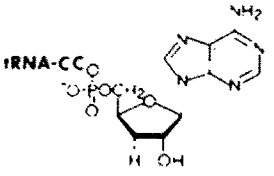

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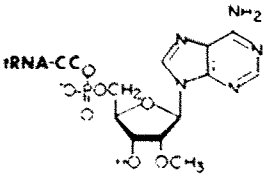

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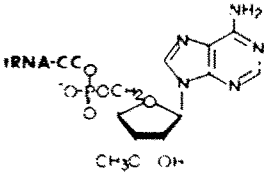

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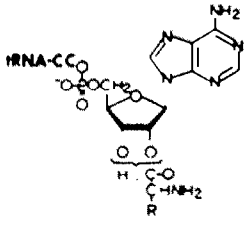

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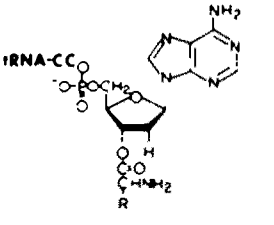

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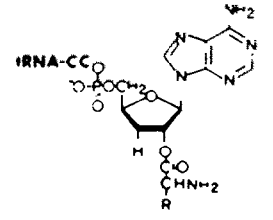

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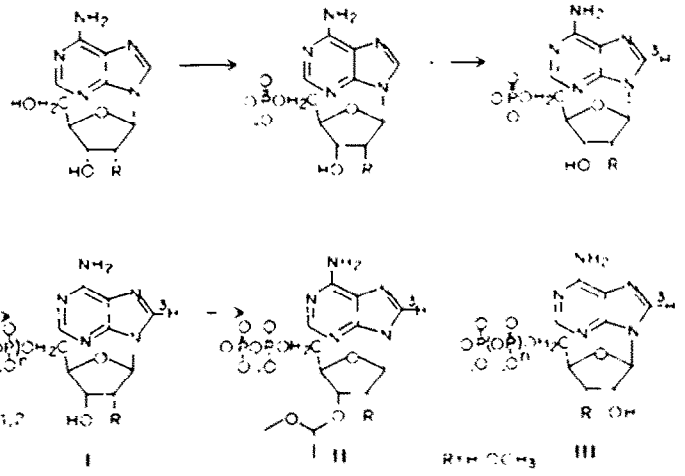
hydes caused these species to be retained during subsequent chromatography on aminoethyl-cellulose.[66] The deleterious effects of periodate noted earlier could be avoided by separation of the modified tRNA's and unreacted tRNA-C-C_{OH} on DBAE (N - [N' - (*m* - dihydroxyborylphenyl) - succinamyl]aminoethyl) - cellulose.[67,68] This support retains the latter species selectively by virtue of interaction with the vicinal glycol functionality of the 3'-terminal cytidine moiety. By elution with appropriate buffer solutions, it was also possible to separate the modified and abbreviated tRNA's derived from isoacceptors containing nucleoside Q (which have one and two *cis*-diol moieties, respectively; Fig. 4).[50]

Aminoacylation of modified tRNA's

Studies of the physical and biochemical properties of a number of aminoacyl-tRNA synthetases, of aminoacyl-tRNA synthetase recognition sites within the cognate tRNA molecules, and more generally of the aminoacylation process itself, suggest the probability of differences in the mechanisms of aminoacylation of individual tRNA's.[2-9,69] Although the individual positional isomers of aminoacyl-tRNA are thought to equilibrate at a rate well in excess of those of the partial reactions of protein biosynthesis,[10,70] so that the initial position of aminoacylation would probably have little effect on subsequent processes, utilization of a single (2' or 3')-OH group in tRNA for aminoacylation would seem not unlikely in that it could serve to enhance the specificity of tRNA-aminoacyl-tRNA synthetase interaction. Therefore, the choice of initial position of tRNA aminoacylation is of interest as a characteristic of individual enzymes and possibility as an aid in the identification of common tRNA-aminoacyl-tRNA synthetase recognition patterns among certain tRNA's. To the extent that the aminoacylation of tRNA species 2 and 3 can be considered analogous to the aminoacylation of the respective species 1, study of the former two should help to define the initial position of aminoacylation of individual tRNA isoacceptors and identify tRNA's whose aminoacylation occurs in related fashion.

THE INITIAL POSITION OF AMINOACYLATION IS NOT THE SAME FOR ALL tRNA ISOACCEPTORS

Subsequent to the finding that yeast $tRNA^{Phe}$ terminating in 3' - deoxyadenosine (3), but not 2' - deoxy-



I II R=H, OCH_3 III

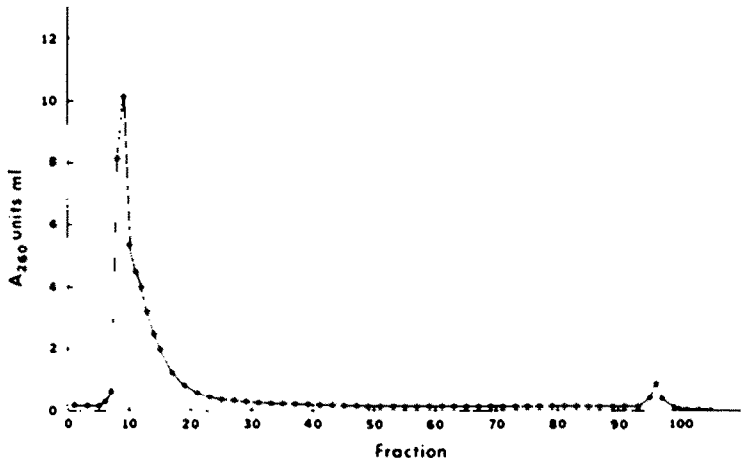
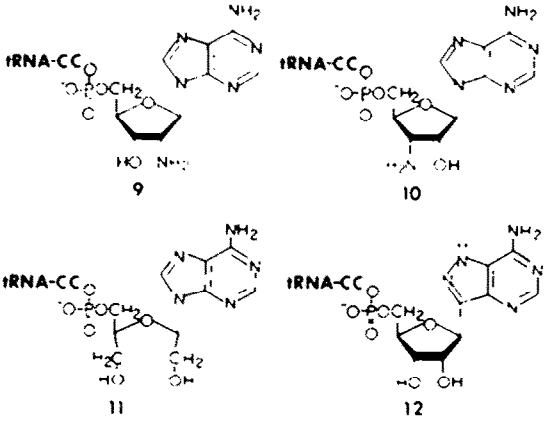


Fig. 4. Chromatography of unfractionated calf liver tRNA's on DBAE-cellulose to effect separation of the tRNA's containing multiple *cis*-diol groups. The tRNA was applied to the column in 50 mM morpholine-HCl buffer, pH 8.7, containing 1 M NaCl and 0.1 M $MgCl_2$ and washed with the same buffer until no more tRNA was eluted from the column. Transfer RNA's containing multiple *cis*-diol groups were then washed from the column with 50 mM sodium 2-(N-morpholino)ethanesulfonate buffer, pH 5.5, containing 1 M NaCl.

adenosine (**2**), was aminoacylated by yeast phenylalanyl-tRNA synthetase,[22] analogous results were obtained by Fraser and Rich using *E. coli* $tRNA^{Phe}$ terminating in 3' - deoxyadenosine (**10**)[23] and by Ofengand *et al.*[71] for *E. coli*, yeast and rat liver $tRNA^{Phe}$ and *E. coli* $tRNA^{Val}$ species (**11**) which had been treated successively with sodium periodate and then with sodium borohydride.

In 1975, Cramer and his coworkers reported on the aminoacylation of four additional purified yeast tRNA species terminating in 2' and 3'-deoxyadenosine.[40] While the modified $tRNA^{Ile}$ and $tRNA^{Val}$ species having a free 2'-OH group were the exclusive substrates for the cognate aminoacyl-tRNA synthetases, as had been observed for yeast $tRNA^{Phe}$, seryl-tRNA synthetase aminoacylated only $tRNA^{Ser}$ species 2 and the tyrosyl-tRNA synthetase utilized $tRNA^{Tyr}$'s 2 and 3. Further studies with modified tRNA's have been concerned with the position of aminoacylation of the remaining tRNA isoacceptors. Thus Sprinzl and Cramer described the aminoacylation of partially modified *E. coli* tRNA, which had been reconstructed with 2' or 3'-deoxyadenosine after removal of the 3'-terminal adenosine moiety by successive treatments with periodate, lysine and alkaline phosphatase.†[34] In a related study, Fraser and Rich prepared *E. coli* tRNA's terminating in 2' - amino - 2' - deoxyadenosine (**9**) and 3'-amino-3'-deoxyadenosine (**10**) and measured the amino acid acceptance of each with 19 amino acids.[41]

Perhaps the most comprehensive study of the position of aminoacylation of individual modified tRNA isoacceptors was reported by Hecht and his coworkers.[39,50] They prepared modified *E. coli*, yeast and calf liver tRNA's from the corresponding abbreviated tRNA's, the latter of which has been obtained by treatment of the unmodified species with venom exonuclease,



followed by incubation with CTP and CTP(ATP):tRNA nucleotidyltransferase. Prior to reconstruction of the abbreviated tRNA's, they were shown not to be damaged by virtue of their ability to incorporate an equivalent of ATP in the absence of CTP, and by the full restoration of amino acid acceptor activity to the reconstituted species. Reconstruction of the tRNA–C–C_{OH}'s was carried out with 2'- and 3'-dATP in the presence of yeast CTP(ATP):tRNA nucleotidyltransferase and each was purified chromatographically on DEAE-cellulose and DBAE-cellulose, as discussed above.

Aminoacylation of each of the modified tRNA's derived from *E. coli*, yeast and calf liver tRNA's was measured as a function of time for each of 20 amino acids, using homologous aminoacyl-tRNA synthetase preparations. Several of the aminoacylation curves obtained are shown in Figs. 5–12 and the extents of aminoacylation of individual modified tRNA's after 30 min are summarized in Table 1, relative to the corresponding unmodified tRNA's. As shown in Fig. 5, for example, after 30 min the *E. coli* glutamyl-tRNA synthetase had aminoacylated the modified $tRNA^{Glu}$ (species 3) having a 2'-OH group to the extent of 91% relative to unmodified $tRNA^{Glu}$, but the isomeric species (**2**) was not utilized as a substrate. The same was also true for the *E. coli*, yeast and calf liver (Fig. 6) valyl-tRNA synthetases and for the arginyl-, isoleucyl-, leucyl- and phenylalanyl-tRNA synthetases

†It is probably worth noting that after this treatment reconstruction of the abbreviated tRNA's with ATP resulted in incorporation of 78% of the theoretical amount of the nucleotide and restoration of only 62% of total amino acid acceptor activity, with a disproportionate loss of certain isoacceptors. This would seem to indicate at least some tRNA destruction during the preparation of tRNA–C–C_{OH} and possibly additional difficulties in reconstitution of some isoacceptor activities with ATP and CTP(ATP):tRNA nucleotidyltransferase.

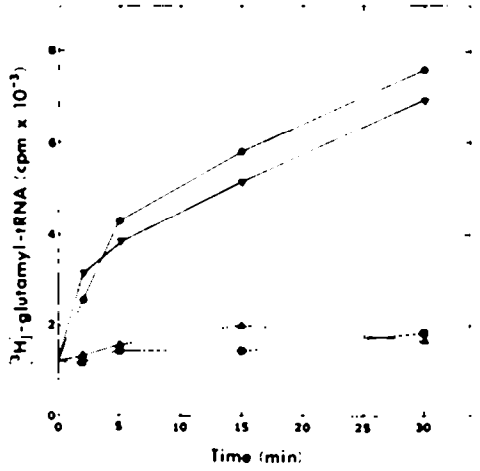


Fig. 5. Aminoacylation of *E. coli* $tRNA^{Glu}$ species 1 (●), 2 (▲) and 3 (▼), relative to a control lacking tRNA (■), in the presence of *E. coli* aminoacyl-tRNA synthetases.

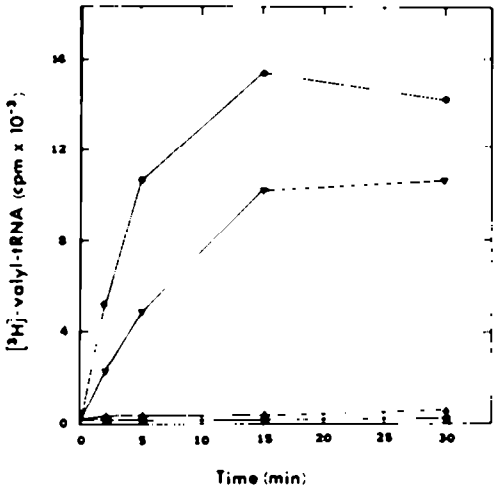


Fig. 6. Aminoacylation of calf liver $tRNA^{Val}$'s 1 (●), 2 (▲) and 3 (▼), relative to a control without tRNA (■), using a calf liver aminoacyl-tRNA synthetase preparation.

from all three species. Also specific for cognate tRNA's having a free 2'-OH group on the 3'-terminal nucleoside were *E. coli* and yeast methionyl-tRNA synthetases. On the assumption that aminoacylation of the unmodified tRNA's proceeds in the same fashion as that observed for the corresponding modified tRNA species, 2 and 3, these results indicate that all of the aminoacyl-tRNA synthetases discussed above normally aminoacylate their cognate tRNA's on the 2'-OH group.

Figure 7 depicts the aminoacylation of yeast $tRNA^{Pro}$ species 1–3. Although $tRNA^{Pro}$ species 3 was not a substrate for the prolyl-tRNA synthetase, after 30 min $tRNA^{Pro}$ species 2 was aminoacylated to essentially the same extent as the corresponding unmodified tRNA.† The same positional specificity was observed for *E. coli* $tRNA^{Pro}$ and for $tRNA^{Ala}$ (Fig. 8), $tRNA^{Gly}$, $tRNA^{His}$, $tRNA^{Leu}$, $tRNA^{Ser}$ and $tRNA^{Thr}$ from *E. coli*, yeast and calf liver (Tables 2–4).

†The slightly greater extent of aminoacylation of certain of the modified tRNA's, as compared with the respective unmodified species, can probably be attributed to removal of some tRNA species during the modification procedures, resulting in the enrichment of other tRNA isoacceptors.

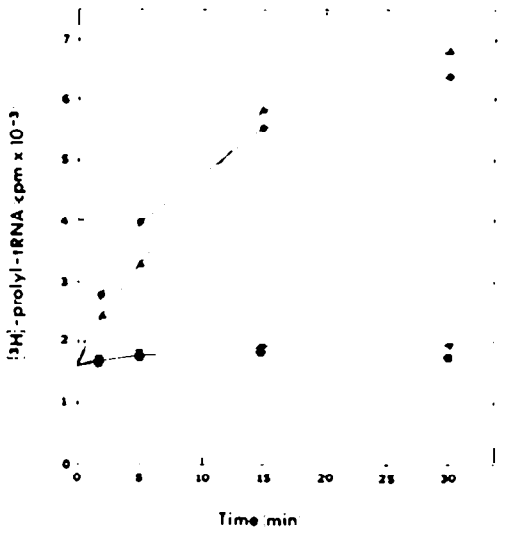


Fig. 7. Aminoacylation of yeast $tRNA^{Pro}$ species 1 (●), 2 (▲) and 3 (▼), relative to a control without tRNA (■), by a yeast aminoacyl-tRNA synthetase preparation.

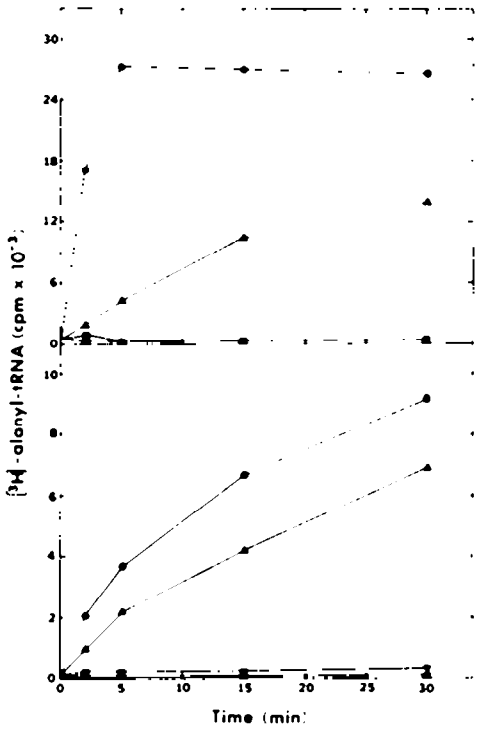


Fig. 8. Aminoacylation of *E. coli* (upper panel) and yeast (lower panel) $tRNA^{Ala}$'s 1 (●), 2 (▲) and 3 (▼), relative to controls lacking tRNA (■), in the presence of homologous aminoacyl-tRNA synthetases.

Certain aminoacyl-tRNA synthetases utilized both modified tRNA species 2 and 3, although generally at different rates. This is illustrated for yeast tyrosyl-tRNA synthetase in Fig. 9, and was observed for the corresponding *E. coli* and calf liver activating enzymes as well. The same was also true for the asparaginyl-tRNA synthetases from all three species and for the cysteinyl-

Table 1. Percent aminoacylation of modified tRNA species 2 and 3 relative to unmodified tRNA

	Transfer RNA terminating in:					
	2′-deoxyadenosine (2)			3′-deoxyadenosine (3)		
Amino Acid	*E. coli*	Yeast	Calf Liver	*E. coli*	Yeast	Calf Liver
Alanine	52	74	42	[illegible]	2	9
Arginine	[illegible]	5	22	[illegible]	25	100
Asparagine	5	85	53	15	81	56
Aspartic Acid	25	95	58	[a]	52	5
Cysteine	47	93	[illegible]	[illegible]	84	22
Glutamic Acid	0	[a]	[a]	91	[a]	[a]
Glutamine	[a]	45	[a]	[a]	0	[a]
Glycine	[illegible]	53[b] 28[c]	45	5	9[b] 6[c]	2
Histidine	57	24	22	4	2	[illegible]
Isoleucine	22	0	2	[illegible]	100	42
Leucine	2	0	2	100	100	79
Lysine	30	47	[illegible]	[illegible]	3	2
Methionine	32	0	[a]	76	22	[a]
Phenylalanine	2	2	2	107	72	36
Proline	27	130	[a]	2	5	[a]
Serine	77	58	79	2	4	4
Threonine	52	47[b] 27[c]	110	0	0[b] 44[c]	7
Tryptophan	5	50	28[b] 12[c]	130	7	6[b] 2
Tyrosine	27	2	10	24	10	13
Valine	5	1	3	109	88	84

[a] Uncertain.

[b] This value was obtained after 60 min. The 3′ min value is in parentheses.

[c] This value was obtained after 120 min. The 3′ min value is in parentheses.

Table 2. Initial site of aminoacylation of *E. coli* tRNA

Hydroxyl group at 3′-terminus of tRNA which is aminoacylated			
2′-OH	3′-OH	2′ and 3′-OH	Uncertain
Arg	Ala	Asn	Asp[a]
Glu	Cys	Gly	Gln
Ile	His	Ser	
Leu	Lys		
Met	Pro		
Phe	Ser		
Trp[a]	Thr		
Val			

[a] $tRNA^{Trp}$ species 2 was also 3′-aminoacylated to some extent.

[b] Aminoacylation does take place using $tRNA^{Asp}$ species 2 (which has a 3′-OH group); aminoacylation of the other isomer is uncertain.

tRNA synthetases from *E. coli* and yeast and the yeast aspartyl-tRNA synthetase.

That the observed aminoacylation of both isomeric tRNA's was not due to the presence of multiple tRNA species having the same accceptor activity but different positional specificities can be shown at least for yeast and calf liver $tRNA^{Tyr}$ and for yeast $tRNA^{Asn}$ and $tRNA^{Cys}$, since species 2 and 3 derived from each were aminoacylated to approximately the same extent as the respective unmodified tRNA's. While there is no conclusive evidence for the existence of more than one aminoacyl-tRNA synthetase activity corresponding to any amino acid in the organisms studied, it is difficult to exclude the possibility that the aminoacylation of both isomers (2 and 3) derived from a single tRNA isoacceptor is effected by different enzymes; in fact such isomeric tRNA's could be used in assays to determine the possible existence of multiple aminoacyl-tRNA synthetase activities after (partial) fractionation of the latter.

The effect of variable amounts of aminoacyl-tRNA synthetases on the relative rates of aminoacylation of modified and unmodified tRNA's is illustrated in Fig. 9 for yeast $tRNA^{Tyr}$ species 1–3. At the lowest concen-

Table 3. Initial site of aminoacylation of yeast tRNA

Hydroxyl Group at 3′ Terminus of tRNA which is Aminoacylated			
2′-OH	3′-OH	2′- and 3′-OH	Uncertain
Arg	Ala	Asn	Glu
Ile	Cys	Asp	
Leu	Gln	Lys	
Met	His	Tyr	
Phe	Leu		
Val	Pro		
	Ser		
	Thr		
	Trp		

Table 4. Initial site of aminoacylation of calf liver tRNA

Hydroxyl Group at 3′ Terminus of tRNA which is Aminoacylated			
2′-OH	3′-OH	2′- and 3′-OH	Uncertain
Arg	Ala	Asn	Glu
Ile	Asp	Ser	Gly
Leu	Cys		Met
Phe	Gln		Pro
Val	His		
	Leu		
	Ser		
	Thr		
	Trp		

tration of added tyrosyl-tRNA synthetase (0.4 μl; upper panel), $tRNA^{Tyr}$'s 1 and 3 were fully aminoacylated after 15 min, while species 2 was activated to the extent of only 14–15% after 30 min. However, as the amount of added tyrosyl-tRNA synthetase was increased (2 and 10 μl; lower panel), all three tRNA species were aminoacylated to approximately the same extent within 30 min. Thus the relative extent of aminoacylation of tRNA species 2 in this system, relative to 1 and 3, depended in a significant way on the amount of tyrosyl-tRNA synthetase present in the incubation mixture. Similar observations have been made for the aminoacylation of yeast $tRNA^{Cys}$ and it seems not unlikely that more concentrated aminoacyl-tRNA synthetase preparations (e.g. the *E. coli* and calf liver aspartyl-, calf liver cysteinyl- and yeast isoleucyl-tRNA synthetases) might effect the aminoacylation of both modified tRNA's (2 and 3) derived from certain additional tRNA isoacceptors. This is illustrated in Fig. 10 for the modified tRNA's structurally related to yeast $tRNA^{Ile}$. Species 3 was utilized to essentially the same extent as unmodified tRNA after a 30 min incubation period. Although aminoacylation of the isomeric tRNA species 2 was only 5% complete within 30 min, the extent of aminoacylation of 2 increased steadily during that period of time. Proper designation of the initial site of aminoacylation, then, would be easier to specify in

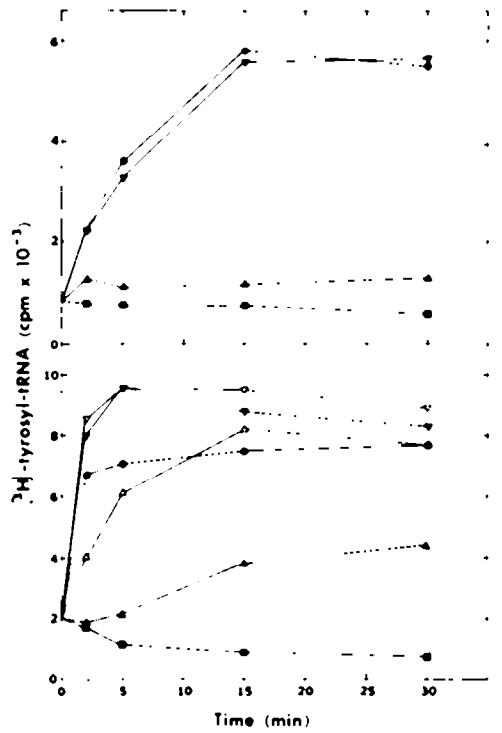


Fig. 9. Aminoacylation of yeast $tRNA^{Tyr}$ species 1 (●), 2 (▲) and 3 (▼), relative to a control without tRNA (■) in the presence of 0.4 μl (upper panel) and 2 μl (lower panel) of a yeast aminoacyl-tRNA synthetase preparation. Also shown in the lower panel are the results obtained for 2 (△) and 3 (▽) with 10 μl of enzyme.

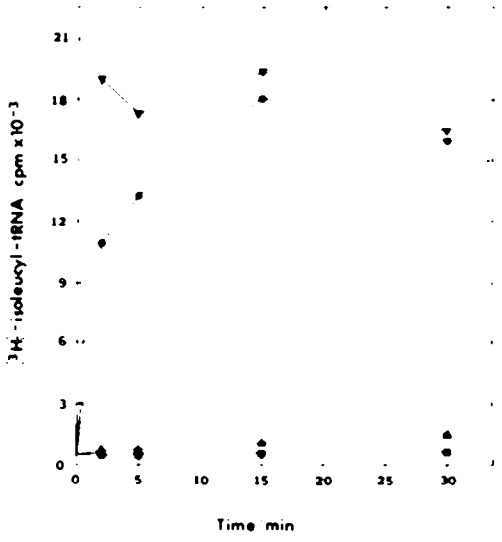


Fig. 10. Aminoacylation of yeast $tRNA^{Ile}$ species 1 (●), 2 (▲) and 3 (▼), relative to a control run without tRNA (■), by the homologous aminoacyl-tRNA synthetase.

terms of the K_m and V_{max} for aminoacylation of each of the modified tRNA's.

The observation that certain aminoacyl-tRNA synthetases utilized the cognate tRNA species 2 and 3 as substrates may be interpreted to mean either that the enzyme does not normally discriminate between the OH groups on the 3′-terminal adenosine moiety of the tRNA

or else that one OH is normally aminoacylated but in its absence the alternate position may be utilized (albeit, most likely at a diminished rate). If the latter were correct, and the relative rates of aminoacylation of 2 and 3 did accurately reflect the position at which species 1 was normally aminoacylated, then the initial position of aminoacylation of certain additional tRNA isoacceptors could be specified. One would conclude, for example, that *E. coli* and yeast tyrosyl-tRNA synthetases normally aminoacylated their cognate tRNA's at the 2'-position, since $tRNA^{Tyr}$'s 3 from both species were activated more quickly than 2, and that the alternate (3'-) position was utilized for *E. coli* and yeast $tRNA^{Asn}$ and $tRNA^{Lys}$, as well as for yeast $tRNA^{Asp}$. One should note, however, that these conclusions are based on the assumption that reconstruction of unfractionated tRNA–C–C_{OH} gave equal amounts of the isomeric tRNA isoacceptors of interest. That this assumption may be tenuous is illustrated by the finding that, unlike *E. coli* and yeast $tRNA^{Asn}$, calf liver $tRNA^{Asn}$ species 3 was activated more quickly than 2.

As shown in Figs. 11 and 12, one tRNA species for which the initial position of aminoacylation has changed during evolution is tryptophan. Thus, while *E. coli* $tRNA^{Trp}$ 3 was a substrate for *E. coli* tryptophanyl-tRNA synthetase,[14,39,50] incubation of modified yeast $tRNA^{Trp}$'s with a homologous aminoacyl-tRNA synthetase preparation resulted in the activation of species 2, but not 3,[39,50] and species 2 was also observed to be the sole substrate in the calf liver system. More recent experiments utilizing partially fractionated *E. coli* $tRNA^{Trp}$ species 2 and 3 have shown that both can be utilized by

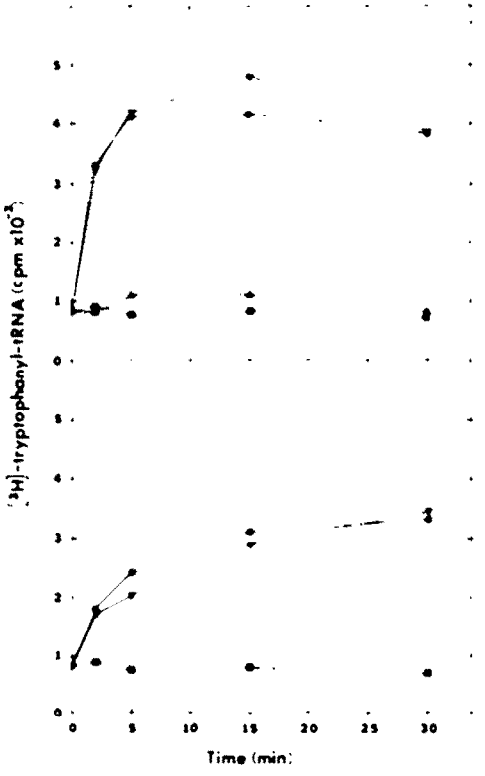


Fig. 11. Aminoacylation of *E. coli* $tRNA^{Trp}$ species 1 (■), 2 (▲) and 3 (▼), relative to a control lacking tRNA (■), by an *E. coli* aminoacyl-tRNA synthetase preparation in the presence of ATP (upper panel) and 3' - deoxyadenosine 5' - triphosphate (lower panel).

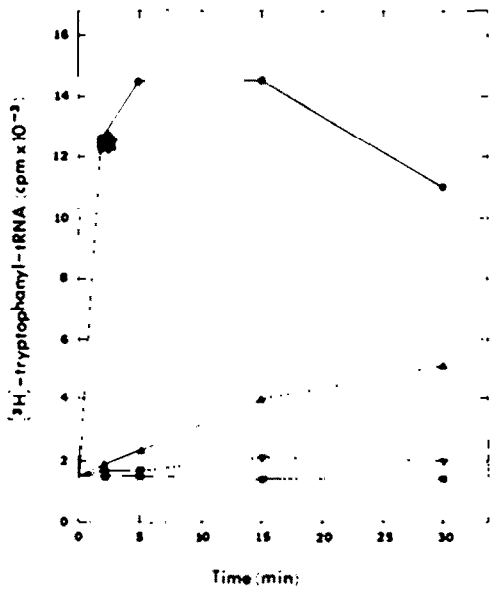


Fig. 12. Aminoacylation of yeast $tRNA^{Trp}$ species 1 (●), 2 (▲) and 3 (▼), relative to a control without tRNA (■), in the presence of yeast aminoacyl-tRNA synthetases.

E. coli tryptophanyl-tRNA synthetase, although aminoacylation of 3 was clearly more facile.[3] The successful heterologous aminoacylations of both *E. coli* and yeast $tRNA^{Trp}$'s have been reported[3] and it will be of interest to carry out the aminoacylations of *E. coli* and yeast $tRNA^{Trp}$ species 2 and 3 with the heterologous aminoacyl-tRNA synthetases since the results may help to indicate whether specificity for the initial position of aminoacylation is determined by the structures of the $tRNA^{Trp}$'s or the tryptophanyl-tRNA synthetases.

The results outlined in Tables 1–4 are those of Hecht and his coworkers;[39,50] comparison with those of other workers is instructive. Sprinzl and Cramer, e.g., reported that *E. coli* threonyl-tRNA synthetase utilized exclusively modified tRNA 3.[54] This was not in agreement with the assignment of positional specificity made by Hecht and Chinault,[50] who observed exclusive aminoacylation of *E. coli* $tRNA^{Thr}$ species 2, but reinvestigation at Göttingen has now verified species 2 as the sole substrate for *E. coli* threonyl-tRNA synthetase.[54] Sprinzl and Cramer also found that *E. coli* $tRNA^{Asn}$ species 3 was a substrate for the cognate aminoacyl-tRNA synthetase, but did not detect the additional aminoacylation of the isomeric $tRNA^{Asn}$ species 2, as reported by Hecht *et al.*[50] Presumably, the failure to observe aminoacylation of species 2 may have been due to an insufficient amount of asparaginyl-tRNA synthetase, as discussed above.

Fraser and Rich studied the aminoacylation of *E. coli* tRNA's terminating in 2' - amino - 2' - deoxyadenosine and 3' - amino - 3' - deoxyadenosine, to give the 2' and 3' - N - aminoacyl derivatives, respectively.[51] Interpretation of the results of these experiments is complicated by the fact that, in principle, aminoacylation of a single modified tRNA species (**9** or **10**) could occur either by direct aminoacylation on the 2'(3')-amino moiety of the terminal nucleoside or by aminoacylation of the adjacent hydroxyl group, followed by intramolecular aminoacyl transfer to the vicinal amino group. Therefore, the experimental observation that both isomers (**9** and **10**)

derived from several tRNA isoacceptors were substrates for the cognate aminoacyl-tRNA synthetases (Table 5) can be interpreted in at least two ways. One possibility would be that the aminoacyl-tRNA synthetases in question utilized exclusively the OH (or NH_2) moieties on the substrate tRNA's but had a relatively low degree of positional specificity, so that **9** and **10** were both aminoacylated. Alternatively, the observed aminoacylation could be attributed to an aminoacyl-tRNA synthetase which was specific for a given position on the 3'-terminal adenosine moiety, but which was capable of acylating either an OH or NH_2 group at that position. Clearly, any combination of these two possibilities could also have given the observed results.

In spite of the ambiguity inherent in the use of analogs **9** and **10**, a great deal of useful information has been accessible by comparison of the results obtained with these species and with tRNA's **2** and **3**. For example, $tRNA^{Thr}$ and $tRNA^{Gly}$ species **9**, but not **10**, were substrates for the respective aminoacyl-tRNA synthetases, enzymes which also activated $tRNA^{Thr}$ and $tRNA^{Gly}$ species **2** (but not **3**). This suggested strongly that the more facile process for the aminoacylation of **9** and **10** involved initial acylation on oxygen. Furthermore, investigation of $tRNA^{Met}$ species **9** and **10** revealed that while the ultimate extent of aminoacylation of the two did not differ markedly, the rate of aminoacylation of the latter (having a 2'-OH group) was much greater, and similar to that of unmodified $tRNA^{Met}$. This was consistent with the exclusive aminoacylation of $tRNA^{Met}$ species **3** (as compared with **2**) by the same enzyme and suggested that the more rapid aminoacylation of **10** was due to acylation on the 2'-OH group of that analog, while the slower activation of **9** involved direct 2' - N - acylation (a process not possible in the corresponding tRNA species **3**). This interpretation was reinforced by the finding that *E. coli* prolyl-tRNA synthetase, which activates $tRNA^{Pro}$ **2**, but not **3**, aminoacylated $tRNA^{Pro}$ species **9** at a much greater rate than **10**.

The only discrepancies noted between the results obtained with *E. coli* tRNA terminating in 2'(3') - deoxyadenosine and those terminating in 2'(3') - amino - 2'(3') - deoxyadenosine involved $tRNA^{Asn}$ and $tRNA^{Tyr}$. Isomeric tRNA's **2** and **3** derived from both isoacceptors were found to be substrates for the corresponding aminoacyl-tRNA synthetases. Although Fraser and Rich found that $tRNA^{Asn}$ species **9** and $tRNA^{Tyr}$ species **10** were the predominant substrates for their respective cognate synthetases some aminoacylation of the isomeric species were also obtained in each case (Table 5), so the results are not inconsistent with those determined for tRNA species **2** and **3**. In fact, the general agreement of the results obtained for the two types of analogs supports the belief that the initial position of aminoacylation of such species is probably the same as that of the corresponding unmodified tRNA's.

The aminoacyl-tRNA synthetases utilized for most of the experiments described above were generally unfractionated or partially fractionated preparations, which may have contained additional extraneous activities. Since the addition of ATP or ATP analogs to tRNA–C–C_{OH} by the CTP(ATP):tRNA nucleotidyltransferase is a reversible process, and since the aminoacylation of tRNA must be run in the presence of ATP, contamination of the aminoacyl-tRNA synthetase preparations by the CTP(ATP):tRNA nucleotidyltransferase could conceivably convert modified tRNA species (e.g. **2** or **3**) to tRNA species **1** (eqn 2). Control experiments, as well as the exclusive aminoacylation of single isomers of most tRNA's[39,50] suggested that this was not occurring to a detectable extent under the conditions utilized for aminoacylation of the modified tRNA's. To verify the results obtained with the modified tRNA's, additional aminoacylations were carried out using deoxyadenosine 5'-triphosphates as the energy source in the aminoacylation experiments.[39,50,51] Hecht and Chinault,[50] e.g., retested each modified *E. coli* tRNA which had behaved as a substrate in the initial aminoacylation experiment, using as an energy source the deoxynucleoside 5' - triphosphate corresponding to the deoxynucleotide at the 3'-terminus of that tRNA. As shown in Fig. 11 for *E. coli* $tRNA^{Trp}$, the aminoacylation of $tRNA^{Trp}$ species **3** proceeded equally as well, relative to species **1**, in the presence of ATP or 3' - deoxyadenosine 5' - triphosphate, thus verifying that the observed aminoacylation of $tRNA^{Trp}$ **3** was not due to the process outlined in eqn (2). All of the results presented in Table 1 for the modified *E. coli* tRNA's were confirmed qualitatively in the same fashion, as was much of the data obtained with modified yeast and calf liver tRNA's.

Table 5. Relative amounts of amino acid added to tRNA's **9** and **10**, expressed as the percentage of total base-stable amino acid[51]

	ATP energy source		ATP energy source		
Amino Acid	9	10	9	10	Class
Phe	5	100	4	100	
Gln	6	100	9	100	
Val	4	96			2*
Glu			5	95	
Leu	6	94	12	88	
Tyr	6	94	15	85	
Trp	23	77	33	67	
Arg	44	56	42	58	
Met	46	54			2*, 3*
Ser	58	42	58	42	
Pro	59	41	62	38	
Ile	72	28	62	38	
Ala	88	12	94	6	
Asn	91	9	100	0	
His	92	8	96	4	
Asp	97	3	100	0	3*
Cys	98	2	99	1	
Thr	100	0	100	0	
Gly	100	0	100	0	

$$\text{tRNA-CCdA} \xrightarrow[\text{nucleotidyl transferase}]{\text{CTP(ATP) tRNA}} \underset{\text{2 or 3}}{\text{tRNA-CC}_{OH}} \xrightarrow[\text{excess ATP}]{\text{aminoacyl-tRNA synthetase}} \underset{\text{1}}{\text{tRNA-CCA}_{OH}} \quad (2)$$

The aminoacylation results given in Table 1 indicate the extent of aminoacylation after 30 min. Although in most cases the data provide a reasonable approximation of the relative rates of aminoacylation of tRNA species 1–3, in several instances the experimental conditions required to activate the modified tRNA's to a reasonable extent within 30 min were such that aminoacylation of the corresponding unmodified species was complete within a few minutes. Incubation of some of these modified tRNA's for an additional period of time resulted in further aminoacylation, suggesting that their much lower rate of activation simply reflected an inherent property of these modified species. However, further incubation of other modified tRNA isoacceptors did not result in an increase in the extent of aminoacylation, an observation for which several explanations are possible. It has been noted, for example, that individual tRNA isoacceptor activities have different susceptibilities both to venom exonuclease and to nuclease activities associated with unfractionated aminoacyl-tRNA synthetase preparations, so that incomplete aminoacylation may have been due to disproportionate loss of certain amino acid acceptor activities during the modification or aminoacylation procedures.

As a result of the aminoacylation studies described above, it seems reasonable to assert that most, if not all, tRNA's are initially aminoacylated on a single, preferred (2′ or 3′) OH group by their cognate aminoacyl-tRNA synthetases. The utilization of a single OH group per se is not surprising, since it could increase the specificity of tRNA-aminoacyl-tRNA synthetase interaction and thereby help to suppress misacylations. However, this function could be accomodated within a system in which the initial position of aminoacylation for any given tRNA had been selected at random, since the isomerization of the aminoacyl moiety between the 2′ and 3′-positions of tRNA is probably faster than the subsequent partial reactions of protein biosynthesis. Therefore, the finding that there has been virtually complete maintenance of positional specificity during evolution, although other changes analogous to that observed for $tRNA^{Trp}$ could have occurred, suggests that some selective advantage is derived from the aminoacylation of one particular OH group, or from some other process which this maintenance of specificity reflects.

SECONDARY COGNITION MAY SUPPRESS THE FORMATION OF NON-FUNCTIONAL PROTEINS

Woese *et al.*[74] have discussed the appearance of certain regularities in the arrangement of the genetic code which minimize the consequences of DNA mutations. Codons assigned to the same amino acid, for example, tend to differ from each other in only one of the three codon positions (the third or "wobble" position[76]), so that mutations in that position can still result in insertion of the same amino acid into protein during translation. In addition, they noted that the second of the three deoxyribonucleotides in the codon, the corresponding ribonucleotide of which would probably hydrogen bond the most securely to its complementary ribonucleotide at the level of codon–anticodon interaction, seemed to be the most essential for determining the character of the amino acid inserted into a growing polypeptide. As is illustrated in Fig. 13, e.g., the presence of uridine in the second position of the codon virtually assures the appearance of a non-polar amino acid in the protein which results from translation of the corresponding mRNA, since all of the codon triplets having U in the second position specify hydrophobic amino acids. Even if U is misread as "C", or if there is a U→C mutation at this position, the resulting amino acid will be hydrophobic or neutral ("ambivalent"[77]).[74] Thus a mechanism is provided to minimize the possibility that DNA mutations, or errors caused by misreading during transcription or translation, would result in a protein having a hydrophilic amino acid in a position intended for a hydrophobic (or neutral) species.

In the sense that hydrophobic and neutral amino acids may be regarded as "inside" residues of proteins,[77] which contribute more directly to protein structure than to function, one might anticipate that a low level of translational errors (e.g., introduced via tRNA misacylations) would be acceptable for such amino acids, provided that the substitutions involved other amino acids of similar polarity. Since certain non-cognate tRNA's can compete with the cognate tRNA for its aminoacyl-tRNA synthetase,[78,79] this could be accomplished conveniently by providing tRNA's and aminoacyl-tRNA synthetases of a common type for activation of such non-polar amino acids. Thus the existing "mechanism" for ensuring the insertion of a non-polar amino acid into a protein by virtue of the relative positions of such species in the genetic code would be reinforced by the presence of a "class" or "family" of tRNA's and aminoacyl-tRNA synthetases which effected amino acid activation by a related mechanism. We denote this process "secondary cognition" and anticipate that it would reinforce the primary recognition mechanisms for suppressing tRNA misacylation and help to assure that any amino acid substitutions in proteins would occur with by far the greatest frequency among the same type of amino acid.

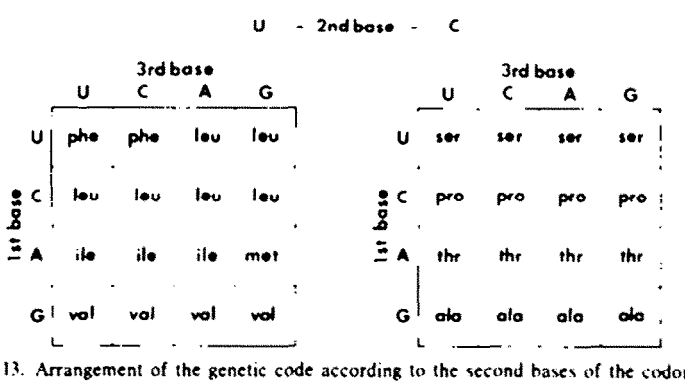


Fig. 13. Arrangement of the genetic code according to the second bases of the codons.

The misactivation of several tRNA's has been studied in some detail. It was shown, e.g., that the heterologous aminoacylation of unfractionated *E. coli* tRNA's with an unfractionated aminoacyl-tRNA synthetase preparation from *N. Crassa* resulted in three times more phenylalanyl-tRNA than was produced with the *E. coli* enzyme; identified among the products were phenylalanyl-$tRNA^{Val}$ and phenylalanyl-$tRNA^{Ala}$.[87,92] Ebel *et al.* established a set of experimental conditions which afford increased levels of misacylation and they have shown that the extent of misacylation increases at higher Mg^{2+}/ATP levels and in the presence of added dimethylsulfoxide.[82,83] In the absence of dimethylsulfoxide, the valyl-tRNA synthetase from *E. coli* utilizized as substrates yeast $tRNA^{Val}$, $tRNA^{Ile}$, $tRNA^{Met}$, $tRNA^{Thr}$ and $tRNA^{Phe}$, while the corresponding yeast enzyme aminoacylated the tRNA isoacceptors specific for valine, methionine, alanine, proline, isoleucine, threonine, leucine and phenylalanine. Similar results were obtained by Yarus *et al.*; both laboratories also appreciated the related nature of the amino acids involved and both postulated the existence of "families" of tRNA's and aminoacyl-tRNA synthetases. One may note that the products of misacylation in the experiments described above were derived exclusively from those tRNA's whose codons contained U or C in the second position. Even under experimental conditions which included dimethylsulfoxide and resulted in almost total loss of isoacceptor specificity, the most facile misacylations were those within the same two codons groups. Analogous misacylations were observed among some tRNA isoacceptors whose codons contained purines in the second position.

If secondary cognition were operative in the sense outline above, then tRNA's related to each other in their mechanism of aminoacylation would be expected to have a common nucleotide in the second position of the anticodon by virtue of the arrangement of amino acid codons in the genetic code. Under such circumstances, it would not be surprising if these nucleotides also participated in the aminoacylation process in some cases, thus further limiting the activation of a tRNA to those amino acids having similar polarity to that of the cognate species. In this regard, it is of interest that a C→U mutation in the second position of the anticodon of *E. coli* $tRNA^{Gly}$ greatly decreased its rate of activation by the cognate aminoacyl-tRNA synthetase.[97-100] Yaniv *et al.*[101] showed that the $tRNA^{Trp}$ of the *E. coli su*$^+_7$ mutation, which had a C→U mutation in the second position of the anticodon and therefore responded to the codon UAG, could not be activated by the tryptophanyl-tRNA synthetase but was aminoacylated with glutamine by the glutaminyl-tRNA synthetase. This result was later confirmed by Seno, who effected the same modification *via* bisulfite treatment of *E. coli* $tRNA^{Trp}$.[102] It should be noted, however, that active participation of the second base of the anticodon in the aminoacylation process is probably not general since, e.g., seryl-tRNA synthetase recognizes $tRNA^{Ser}$ species with quite different anticodons.[103]

Although aspects of the secondary cognition process have been noted previously, as indicated above, much stronger evidence for the functional nature of this process may be inferred from the observation (Table 6) that all tRNA isoacceptors specified by codons whose second base is U are initially aminoacylated exclusively on the 2'-OH group, while those having C as the second base are aminoacylated exclusively on the 3'-OH group. It seems reasonable to suggest that the use of a single OH group for tRNA aminoacylation enhances tRNA-aminoacyl-tRNA synthetase interaction, thereby facilitating aminoacylation and helping to suppress misacylations, while the particular pattern of tRNA acylation noted above for certain nonpolar amino acids may reflect

Table 6. Correlation between initial position of tRNA aminoacylation and second base of codon specifying each isoacceptor[a]

Hydroxyl group at 3' terminus of tRNA which is aminoacylated	Codon Assignments of Individual tRNA's			
	XUY	XCY	XAY	XGY
2'-OH	His		Gln	Arg
	Leu			
	Met			
	Phe			
	Val			
3'-OH		Ala	Glu	Gly
		Pro	Lys	Ser
		Ser	Lys	
		Thr		
2'- and 3'-OH			Asn	Cys
			Asp	Trp
			Tyr	

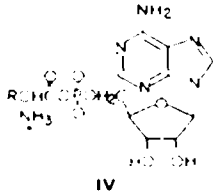
[a]The assignments of initial position of aminoacylation are based on the data from *E. coli*, yeast and calf liver tRNA's (Tables 2-4).

the existence of common mechanisms for the aminoacylation of such tRNA's (i.e., structurally related aminoacyl-tRNA synthetase active sites).

A CHEMICAL PROOFREADING MECHANISM IS POSTULATED TO CORRECT MISACYLATIONS

Von der Haar and Cramer have suggested a more explicit role for the individual (2' and 3') hydroxyl groups of tRNA in correcting misacylations.[104] They noted initially that in the presence of yeast isoleucyl-tRNA synthetase, valine was transferred to $tRNA^{Ile}$ terminating in 3'-deoxyadenosine.[105] Baldwin and Berg[106] had previously shown that the valyladenylate (IV, R = $(CH_3)_2CH$) underwent net hydrolysis in the presence of *E. coli* isoleucyl-tRNA synthetase and unmodified $tRNA^{Ile}$, although no valyl-$tRNA^{Ile}$ was detected during the course of the experiment so that hydrolysis of the valyladenylate could have occurred via a "kinetic proofreading"[107,108] process prior to formation of valyl-$tRNA^{Ile}$ or else by isoleucyl-tRNA synthetase-catalyzed deacylation of the misacylated species. The results obtained with the modified $tRNA^{Ile}$ species **3**, and the subsequent observation that $tRNA^{Ile}$ species **10** was also misacylated with valine by the isoleucyl-tRNA synthetase, suggested to von der Haar and Cramer that misacylation of unmodified tRNA with valine was corrected[106,109] rather than prevented, and that the presence of both hydroxyl groups might be necessary to permit correction of misacylations. Several experiments were carried out to test this hypothesis. For example, since a molecule of ATP is consumed in the aminoacylation of each molecule of tRNA, the extent of deacylation of several tRNA's could be followed conveniently by measuring the amount of ATP consumed in reacylation of the deacylated tRNA's. Also measured was the AMP-PPi-independent rate of hydrolysis of preaminoacylated tRNA's by the cognate aminoacyl-tRNA synthetases.

In support of the hypothesis that both OH groups on tRNA are requisite to enzyme-catalyzed deacylation, these experiments indicated significant deacylation by the cognate aminoacyl-tRNA synthetases of yeast $tRNA^{Phe}$, $tRNA^{Val}$ and $tRNA^{Ile}$, but not of the corresponding aminoacyl-tRNA's terminating in 2'(3')-deoxyadenosine. The same effect was noted for deacylation of preaminoacylated $tRNA^{Ser}$ species **6** and **7** (R = seryl) in the absence of AMP and PPi, although not for ATP consumption during the aminoacylation of $tRNA^{Ser}$ **1** and **2**. These observations prompted von der Haar and Cramer to postulate a general "chemical proofreading" mechanism whereby each of the two OH groups on the 3'-terminal nucleoside of tRNA corresponds to one of two sites: an aminoacylating site identical with the initial position of aminocylation and a hydrolyzing site. Release of initially aminoacylated tRNA from the aminoacyl-tRNA synthetase would permit rapid isomerization of the aminoacyl moiety between the aminoacylating and hydrolyzing sites (i.e., the vicinal OH groups) and subsequent recapture of the aminoacyl-tRNA with the aminoacyl group in the hydrolyzing site would result in rapid hydrolysis of the amino acid and eventual reaminoacylation, presumably with the cognate amino acid. The overall effect would thus be the same as that suggested by Yarus[110] and Schimmel[109,111] and their coworkers in their work on aminoacyl - tRNA - catalyzed deacylations of misacylated tRNA's and consistent with the kinetic scheme described by Fersht and Kaethner[112] for dissociation of the threonyladenylate by valyl-tRNA synthetase from *Bacillus stearothermophilus* in the presence of homologous $tRNA^{Val}$. Cited in support of this postulate was the observation that unmodified $tRNA^{Tyr}$, which was believed not to have a unique accepting site on the basis of studies with $tRNA^{Tyr}$ species **2** and **3**, was not deacylated by the tyrosyl-tRNA synthetase.



IV

Calendar and Berg[113] studied the substrate specificity of the tyrosyl-tRNA synthetases from *E. coli* and *B. subtilis* and demonstrated that a number of unusual substrates were converted to the corresponding aminoacyladenylates (IV) and that some were also transferred to the homologous $tRNA^{Tyr}$'s. While this behavior is not inconsistent with the interpretation that such misacylations occurred because of a deficient "chemical proofreading" system, it may simply reflect an inherent deficiency in substrate specificity of certain tyrosyl-tRNA synthetases in the initial aminoacylation process, which would parallel their demonstrated lack of positional specificity and hydrolytic capacity and suggest that these particular activating enzymes may be relatively atypical species and should not be used as a basis for generalization. It would help to establish a causal relationship between lack of a unique accepting site and consequent absence of hydrolytic capacity if it could be shown that yeast $tRNA^{Asp}$, $tRNA^{Asp}$ and $tRNA^{Cys}$, none of which has a unique accepting site, also fail to deacylate in the presence of their cognate aminoacyl-tRNA synthetases, since none of these species is known to be prone to misacylations. Another useful experiment would involve a "chemically aminoacylated" sample of $tRNA^{Phe}$ terminating in 2' - deoxy - 3' - O - L - phenylalanyladenosine (**7**, R = $(C_6H_5CH_2)$). According to the postulate of von der Haar and Cramer,[104] this species has the aminoacyl moiety in the "hydrolyzing site" and would be expected to undergo rapid deacylation in the presence of homologous phenylalanyl-tRNA synthetase.

In addition to modified tRNA's terminating in 2'- and 3'-deoxyadenosine, von der Haar and Cramer also studied tRNA's terminating in formycin (**12**).[104] Although these tRNA's have vicinal OH groups at their 3'-termini, in most cases the corresponding aminoacylated species were found not to be substrates for deacylation by their cognate aminoacyl-tRNA synthetases. Presumably, the lack of deacylation may be attributed to alterations of the sugar pucker of formycin as compared with adenosine[114] (and hence change in the positions of the 2'- and 3'-OH groups) or to conformational changes in the acceptor stems of the tRNA's resulting from differences in the preferred glycosyl torsion angles and base stacking properties of the two heterocycles. In fact, it is difficult to exclude the possibility that alterations in nucleoside conformation, and not the absence of the second hydroxyl group, account for the differences in the rate of deacylation of **2** and **3** as compared with **1**. In this context, the conclusions that can properly be drawn from the deacylation studies at present may be summarized as follows:

1. The presence of a vicinal hydroxyl moiety at the 3'-terminus of an aminoacyl-tRNA is a necessary, but not

sufficient, condition to permit its deacylation by the cognate aminoacyl-tRNA synthetase.

2. For whatever reason(s), the structural features required to facilitate activation of a tRNA by an aminoacyl-tRNA synthetase may not be the same as those required for deacylation by the same enzyme.

If "chemical proofreading" were to function as envisioned to correct tRNA misacylations, then the deacylation of a misacylated tRNA by its cognate aminoacyl-tRNA synthetase would have to proceed at a greater rate than deacylation of the same species which was properly aminoacylated. The aminoacyl-tRNA synthetase-catalyzed deacylation of aminoacyl-tRNA has been studied previously in three laboratories as a possible correction mechanism for tRNA misacylation;[82,109-111,115-117] the most compelling evidence was reported by Ebel *et al.*[115] As shown in Table 7,[82] no consistent pattern was noted between the presence of a cognate or non-cognate amino acid on a tRNA and the enzyme-catalyzed rate of hydrolysis of that amino acid from the tRNA. This observation led Ebel and his coworkers to conclude that the correction of misacylations is not a general function of aminoacyl-tRNA synthetases. One may note further that for "chemical proofreading" to function *in vivo* as described, the postulated reassociation of the misacylated tRNA and aminoacyl-tRNA synthetase[104] would have to occur in the presence of elongation factor Tu, which binds strongly to free aminoacyl-tRNA[118,119] and is present in *E. coli* at concentrations approximately equal to those of tRNA.[120] In fact, in their work on "kinetic proofreading" Hopfield *et al.*[108] were able to suppress aminoacyl-tRNA synthetase-catalyzed deacylation of aminoacyl-tRNA by adding EF-Tu·GTP to the incubation mixture used for tRNA aminoacylation.

SUMMARY OF THE AMINOACYLATION EXPERIMENTS

In contrast to the diversity in the initial positions of aminoacylation of different tRNA isoacceptors from a single source, for individual isoacceptor activities there has been a general conservation of the initial position of aminoacylation during the evolution from a prokaryotic to mammalian organism. Aside from the change in specificity for $tRNA^{Trp}$, and the failure to detect aminoacylation of the modified tRNA species **3** derived from *E. coli* $tRNA^{Asp}$ and calf liver $tRNA^{Asp}$ and $tRNA^{Cys}$, no differences were found in the initial position of tRNA aminoacylation for *E. coli*, yeast or calf liver. It is not unlikely that the use of a particular (2' or 3'-) position for the aminoacylation of a single isoacceptor in both prokaryotic and eukaryotic species reflects the development of certain discrete types of aminoacyl-tRNA synthetase active sites. Moreover, the data in Table 6 and misacylation data described above suggest that the tRNA's corresponding to certain structurally related amino acids may be aminoacylated by common mechanisms. The potential of this "secondary cognition" system for suppressing the synthesis of non-functional proteins may itself have provided sufficient pressure to effect the maintenance of positional specificity during evolution.

Regardless of the specific choice of initial position of aminoacylation, the utilization of a single OH group for aminoacylation would certainly be expected to increase the potential specificity of tRNA-aminoacyl-tRNA synthetase interaction, thereby minimizing misacylations. It is also possible that the two OH groups at the 3'-terminus of tRNA have more specialized editing functions and that the hydroxyl group not utilized for aminoacylation is the "hydrolyzing site" in a "chemical proofreading" scheme.

Isomeric aminoacyl-tRNA's

Since the partial reactions of protein biosynthesis subsequent to aminoacylation all involve the utilization of aminoacylated tRNA's, the availability of isomeric aminoacyl-tRNA's (**7** and **8**) is clearly a prerequisite for the determination of positional specificity of the aminoacyl moiety during such transformation. However, prior to the finding[34,39,50,51] that both modified tRNA's (e.g. **2** and **3**) derived from certain *E. coli*, yeast and calf liver tRNA isoacceptors were substrates for their cognate aminoacyl-tRNA synthetase activities, it was possible to obtain only a single isomer of any modified aminoacyl-tRNA (e.g. **7** or **8**) by enzymatic aminoacylation. Therefore, almost all of the experiments reported for modified aminoacyl-tRNA's have utilized only a single isomer; direct comparison between isomeric aminoacyl-tRNA's has been reported only once.[25]

The availability of tRNA species **7** and **8** derived from

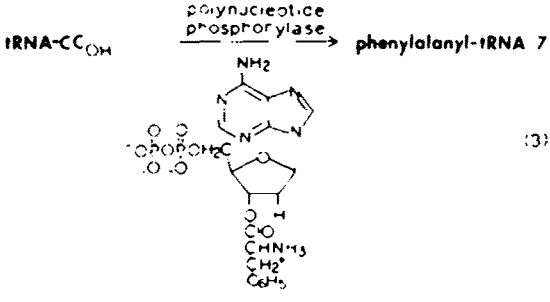
Table 7. Comparison of deacylation rates of various aminoacyl-tRNA species from yeast by different yeast aminoacyl-tRNA synthetases[a,82]

Aminoacyl-tRNA species	Rates of deacylation by methionyl-tRNA synthetase	phenylalanyl-tRNA synthetase	valyl-tRNA synthetase
Methionyl-$tRNA_f^{Met}$	26	55	
Phenylalanyl-$tRNA_f^{Met}$	24	98	
Valyl-$tRNA_f^{Met}$	5	2	
Phenylalanyl-$tRNA^{Phe}$	1	220	40
Valyl-$tRNA_1^{Phe}$		15,000	6
Valyl-$tRNA^{Val}$		2	70
Phenylalanyl-$tRNA_{1,2}^{Val}$		1	2

[a] The rates expressed in arbitrary units were corrected for spontaneous deacylation and normalized for enzyme concentration.

E. coli $tRNA^{Ala}$, $tRNA^{Cys}$, $tRNA^{Trp}$ and possibly $tRNA^{Tyr}$ should facilitate the determination of the positional specificity for the aminoacyl moiety during elongation factor Tu binding, ribosomal A- and P-site binding, etc. However, isomeric aminoacyl-tRNA's derived from the remaining 16 or 17 tRNA isoacceptors are not accessible by enzymatic aminoacylation; unfortunately, these include species for which synthetic messenger RNA's are readily available (e.g. $tRNA^{Phe}$ (polyuridylic acid) and $tRNA^{Lys}$ (polyadenylic acid)) and those isoacceptors which can be employed for both A-site and P-site studies ($tRNA^{Met}$ and $tRNA^{Phe}$). Hopefully, some of these tRNA's may become available in the future via "chemical aminoacylation",[21] which was employed for the preparation of phenylalanyl-tRNA species **7**.

"Chemical aminoacylation" refers to the process whereby a preaminoacylated nucleotide is incorporated enzymatically onto tRNA–C–C_{OH}. Although aminoacylated nucleoside 5'-triphosphates have not been found to be substrates for CTP(ATP):tRNA nucleotidyltransferase, at least one aminoacylated nucleotide (2' - deoxy - 3' - O - phenylalanyladenosine 5' - diphosphate) was incorporated onto abbreviated tRNA by the action of *Micrococcus luteus* polynucleotide phosphorylase (eqn 3).[25,121] Since the possible utility of this procedure was suggested by the work of Kaufmann and Littauer,[122] which showed that phosphorolysis of *E. coli* valyl-tRNA with *E. coli* polynucleotide phosphorylase resulted in the release of 2'(3') - O - valyladenosine 5'-diphosphate, it seems not unreasonable to anticipate that amino acids other than phenylalanine can also be introduced in the reverse reaction via "chemical aminoacylation".[25,121]



Of more serious concern at present than the substrate specificity of polynucleotide phosphorylase are certain technical limitations of the "chemical aminoacylation" procedure which must be overcome before it can be considered to be of general utility. These include the slow rate of incorporation of aminoacylated nucleotides onto tRNA–C–C_{OH} and the difficulty in separating large amounts of unreacted tRNA–C–C_{OH} from the limited amounts of many of the aminoacylated tRNA's which would be produced by the present procedure. Moreover, the length of the incubation period cannot be increased to compensate for the rate of incorporation, since the preaminoacylated nucleotides hydrolyze under the reaction conditions and the resulting deacylated nucleotides are better substrates for polynucleotide phosphorylase (and would give tRNA species **2** or **3**, which would be even more difficult to separate from **7** and **8** than tRNA–C–C_{OH}). Possible improvements in the "chemical aminoacylation" procedure might include recent modifications in the methodology for oligonucleotide synthesis[62-65] or the use of N-acylated aminoacyladenosine 5'-diphosphates, which would hydrolyze less quickly and permit a much longer period of incubation for incorporation of the nucleotide onto tRNA–C–C_{OH}.† That such N-acylated species can act as substrates for polynucleotide phosphorylase may be judged from the observation that the *E. coli* enzyme mediated the phosphorolysis of N-carbobenzyloxyvalyl-tRNA and N-acetylphenylalanyl-tRNA in the presence of phosphate or arsenate.[122]

Another approach to the production of isomeric aminoacyl-tRNA's might involve the use of tRNA's terminating in some nucleoside other than 2' or 3'-deoxyadenosine. For example, Fraser and Rich demonstrated that certain tRNA isoacceptors were substrates for their cognate aminoacyl-tRNA synthetase activities as both the 2' - amino - 2' - deoxyadenosine and 3' - amino - 3' - deoxyadenosine derivatives (**9** and **10**, respectively).[51] Although at least six isomeric *E. coli* aminoacyl-tRNA's were accessible by direct aminoacylation of **9** and **10**, the analogs tested exhibited unusual behavior in some of the partial reactions of protein biosynthesis and may therefore be of limited utility in defining the positional specificity for unmodified aminoacyl-tRNA's in these transformations.

ELONGATION FACTOR Tu BINDS BOTH ISOMERS OF AMINOACYL-tRNA's

As discussed above, aminoacyl-tRNA forms a ternary complex with EF-Tu and GTP. The aminoacyl-tRNA is subsequently transferred to the ribosomal A-site, with concomitant hydrolysis of GTP and release of EF-Tu·GDP. Measurement of the interaction of elongation factor and aminoacyl-tRNA can therefore be carried out either at the level of ternary complex formation or less directly in terms of EF-Tu-dependent binding to the ribosome. Ternary complex formation has been measured by the use of a nitrocellulose filter binding assay, which is based on the observation that the EF-Tu·GTP complex is retained by the filter, while the aminoacyl-tRNA·GTP complex is not.[123,124] Unfortunately, the physical basis of the binding which makes this assay possible is not well understood and it is not clear that the results obtained with aminoacyl-tRNA's **7** and **8** would necessarily reflect the actual interaction of those species with EF-Tu·GTP. The ternary complex may also be detected by gel filtration using Sephadex G-100, although complex formation is reversible and the Ka is such that relatively large amounts of material are required to permit chromatographic separation of EF-Tu·GTP and aminoacyl-tRNA–EF-Tu·GTP without dissociation of the latter.[124]

Although the extent of binding of individual isomeric aminoacyl-tRNA's to EF-Tu·GTP has been determined only recently,[125] the factor-dependent binding of certain positionally defined species to the ribosomal A-site was reported by Cramer and his coworkers. For example, Chinali *et al.*[126] studied the binding of yeast phenylalanyl-tRNA species **6** and **8** to *E. coli* ribosomes. They found that the binding of both species depended on the presence of added EF-T and that both were bound to a similar extent (16.8 pmoles of **8** was bound to 45 pmoles of ribosomes, vs 19.0 pmoles of **6**‡). Presumably due to

†The N-protecting group would be chosen such that it could be removed from aminoacyl-tRNA without effecting deacylation or other modification of that species.

‡This represents about 34 and 38%, respectively, of the phenylalanyl-tRNA's added to the incubation mixture.

the unavailability of yeast phenylalanyl-tRNA **7**, which would have the phenylalanyl moiety in the 3'-position, the same workers also measured the ribosomal A-site binding of yeast phenylalanyl-tRNA **10** and found little stimulation by EF-T. On the basis of these results it was suggested that tRNA's having the aminoacyl moiety at the 2'-position were the substrates for the elongation factor.[127,128] Consistent with this suggestion was the observation by Chládek and Ringer[129,130] that the "tRNA fragments" cytidylyl - (3'→5') - 2'(3') - O - phenylalanyladenosine and cytidylyl - (3'→5') - 2' - O - phenylalanyl - 3' - deoxyadenosine, but not cytidylyl - (3'→5') - 2' - deoxy - 3' - O - phenylalanyladenosine, effected dissociation of EF-Tu·GTP, ostensibly via the formation of unstable "ternary complexes".

Prior to these studies, however, Fraser and Rich had reported the results of their work with *E. coli* phenylalanyl-tRNA **10**.[24] These authors found that after preincubation of homologous ribosomes with polyuridylic acid, *E. coli* N-acetylphenylalanyl-tRNA^{Phe} and crude *E. coli* initiation factors to form a ribosomal complex having the unmodified tRNA in the ribosomal P-site, additional incubation with phenylalanyl-tRNA **10** and EF-Tu resulted in the formation of 95% of the theoretical amount of N-acetylphenylalanylphenylalanine. The efficient formation of dipeptide was a direct implication that phenylalanyl-tRNA species **10** had been bound to the ribosomal A-site. Also in apparent conflict with the findings of Cramer *et al.* was a study by Hecht *et al.*[25] which compared directly the binding of *E. coli* phenylalanyl-tRNA's **6–8** to *E. coli* ribosomes. One of the experiments reported involved the incubation of equimolar amounts of [^{3}H]-phenylalanyl-tRNA **6** and unlabeled phenylalanyl-tRNA's **6**, **7** or **8** in an EF-Tu-dependent system containing a limiting amount of ribosomes. Each of the three unlabeled species inhibited A-site binding of [^{3}H]-phenylalanyl-tRNA **6** to the extent of 50%, suggesting strongly that each was bound by the elongation factor.

Although the experiments described above gave results which seem inconsistent, two observations can be made regarding the implications of these studies for the interaction of EF-Tu·GTP and unmodified aminoacyl-tRNA. The first is that all but one of these studies have employed assays at the ribosomal level, so that the results may also reflect processes subsequent to ternary complex formation. Therefore, the finding that an aminoacyl-tRNA is bound to the ribosomal A-site in an EF-Tu-dependent system (or inhibits the binding of another aminoacyl-tRNA) would seem to be a direct implication that it interacts with EF-Tu·GTP to some extent, but may not provide a quantitative measure of the extent of interaction. On the other hand, failure to observe ribosomal binding of an aminoacyl-tRNA may imply lack of ternary complex formation or a deficiency in a subsequent step. The other observation is that it may not be reasonable to expect all tRNA analogs having an aminoacyl moiety in the same positionally defined site to behave similarly in EF-Tu-dependent ribosomal binding assays (i.e. some modified tRNA's may be much better analogs of unmodified tRNA's than others).

That some of the results described above may reflect processes at the ribosomal level (in addition to ternary complex formation) may be inferred from an extension of one of the experiments described above. In addition to measuring the inhibition of binding of [^{3}H]-phenylalanyl-tRNA **6** by unlabeled phenylalanyl-tRNA'S **6–8** in an EF-Tu-dependent assay system, Hecht *et al.* also repeated the experiment at 10 mM Mg^{2+} concentration in the absence of EF-Tu.[25] Each of the unlabeled species also effected 50% inhibition of binding in this assay, consistent with the interpretation that tRNA species **6–8** are all bound by the elongation factor, but that the observed extent of inhibition was mediated at the level of A-site binding.

Baksht *et al.*[131] have recently reported on the A-site binding of yeast phenylalanyl-tRNA species **6**, **8** and **10** in a heterologous eukaryotic system containing rabbit reticulocyte ribosomes and the appropriate eukaryotic elongation factor (EF-1) from rat liver. At 6 mM Mg^{2+} concentration, the use of approximately equimolar amounts of phenylalanyl-tRNA's and ribosomes resulted in only a small stimulation of the binding of tRNA's **8** and **10**, relative to controls which lacked EF-1. For all of the tRNA's tested, the small binding increment obtained in the presence of the factor reflected the fact that relatively good binding was obtained in the absence of the factor. This was especially true for phenylalanyl-tRNA species **10**, which was bound to at least twice the extent of unmodified phenylalanyl-tRNA (**6**) in the absence of the factor. At 20 mM Mg^{2+} concentration in the absence of EF-1, phenylalanyl-tRNA's **6**, **8** and **10** were bound rapidly and to roughly the same extent. Thus the differences reported for the factor-dependent A-site binding of phenylalanyl-tRNA species **10** may derive from differences in experimental conditions, especially as regards Mg^{2+} concentrations. As mentioned earlier, an unfortunate consequence of this variable behavior is that it may be difficult to decide which of the results determined for tRNA's **9** and **10** are analogous to those which obtain for unmodified aminoacyl-tRNA's.

The suggestion that not all modified tRNA's containing a common aminoacyl moiety in the same positionally defined site need behave similarly in a given assay system can be illustrated for phenylalanyl-tRNA species **11**. This species, which is believed to have the aminoacyl moiety in the same position as phenylalanyl-tRNA **8**, has been studied extensively and shown neither to form a ternary complex with EF-Tu·GTP[132] nor to undergo ribosomal binding under reasonable conditions.[126,131,132] Moreover, as a further indication that the comparison of positionally defined, but nonisomeric tRNA's (e.g. **8** and **10**) may give misleading results, Sprinzl and Cramer have recently shown that isomeric phenylanyl-tRNA's **9** and **10** are both inactive as substrates for elongation factor Tu and that neither undergoes factor-dependent ribosomal binding to *E. coli* ribosomes.[74]

The question of which positional isomer of tRNA is the preferred substrate for EF-Tu has recently been resolved by Hecht *et al.*[125] The extent of aminoacyl - tRNA - EF-Tu·GTP interaction was measured initially by means of a new assay, which was based on the observation that the factor binds specifically to aminoacylated tRNA's (as compared with N-acylated aminoacyl-tRNA or deacylated tRNA's[133,134]) and thereby diminishes the rate of chemical deacylation of those tRNA's.[118,119] This is illustrated in Fig. 14 for *E. coli* [^{3}H] - phenylalanyl - tRNA. In the absence of the elongation factor, or in the presence of EF-Tu·GDP, deacylation was essentially complete within 1 h at pH 7.5. However, the addition of 0.66 equivalent of EF-Tu·GTP substantially diminished the rate of chemical hydrolysis and the addition of more factor to the incubation mixture further reduced the rate of deacylation. Figure 15 shows

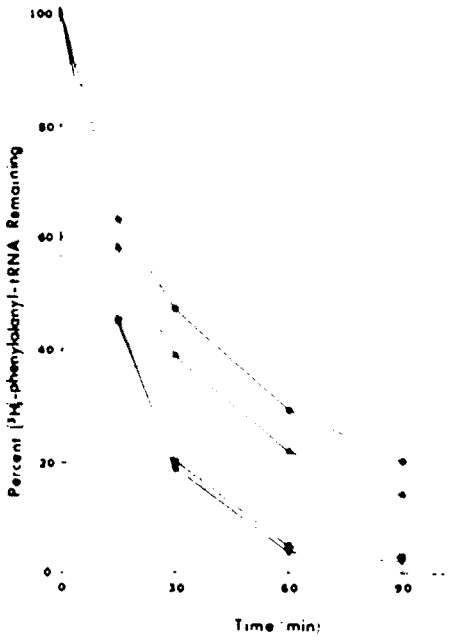


Fig. 14. Deacylation of [³H]-phenylalanyl-tRNA at pH 7.5 in the absence of EF-Tu·GTP (■), in the presence of 3.4 equivalents of EF-Tu·GDP (▼) and in the presence of 0.66 (▲) and 1.32 (●) equivalents of EF-Tu·GTP.

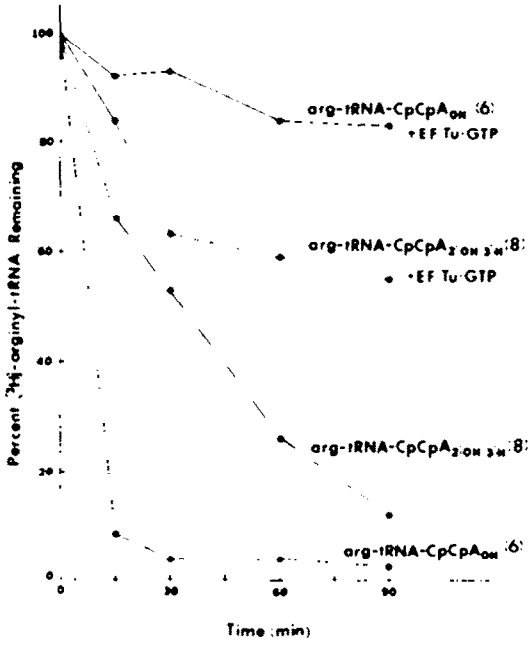


Fig. 15. Deacylation of [³H]-arginyl-tRNA **6** in the absence (---) and presence (——) of 1.3 molar equiv. of EF-Tu·GTP, and of [³H] arginyl-tRNA **8** in the absence (---) and presence (——) of 1.1 equiv of the factor.

the deacylation of unmodified *E. coli* arginyl-tRNA in the absence of EF-Tu·GTP and in the presence of five different concentrations of the elongation factor. As is evident from Fig. 15, arginyl-tRNA underwent more facile chemical deacylation than phenylalanyl-tRNA, but was also protected from deacylation more effectively. Because the rate of deacylation of a modified aminoacyl-tRNA in the presence and absence of EF-Tu·GTP is only meaningful in comparison with the results obtained for the corresponding unmodified aminoacyl-tRNA, and since the individual unmodified species exhibited variable behavior with respect to rate of deacylation and ternary complex formation, the study of each isoacceptor activity involved the simultaneous measurement of four deacylation curves.

The results for a typical deacylation experiment are shown in Fig. 15 for arginyl-tRNA's **6** and **8**. In the presence of an approximately equimolar amount of EF-Tu·GTP, the deacylation of arginyl-tRNA species **8** proceeded to the extent of 34% in 90 min, while that of unmodified arginyl-tRNA (**6**) was only 17% complete under the same conditions. In the absence of the factor, deacylation of arginyl-tRNA was 50% complete within 7–8 min. The somewhat greater protection from deacylation afforded the unmodified arginyl-tRNA (**6**) presumably reflected its greater affinity for EF-Tu·GTP, but interpretation of the data was complicated by the finding that, as expected,[22,121,134,136] chemical deacylation of aminoacyl-tRNA's terminating in 2'- or 3'-deoxyadenosine was inherently less facile than deacylation of unmodified aminoacyl-tRNA's. Therefore the deacylation of arginyl-tRNA **8** was also measured in the absence of EF-Tu·GTP to permit a more complete evaluation of the data. There was an additional difficulty in interpretation of the data in quantitative terms, which was the result of the use of unfractionated tRNA's and aminoacyl-tRNA synthetases for the preparation of the modified aminoacyl-tRNA species. Specifically, the reduced V_{max} values associated with the aminoacylation of many of the modified tRNA's made them more susceptible to misacylations than normal tRNA's[82,105] and each aminoacylation carried out in preparation for the deacylation studies was therefore done in the presence of the appropriate radiolabeled amino acid, plus unlabeled amino acids corresponding to the other tRNA isoacceptors present. Although this undoubtedly helped to suppress misacylations, and did not interfere with the observation of the aminoacyl-tRNA of interest, each of the unlabeled aminoacyl-tRNA's present in the incubation mixture would also be expected to complete with the labeled aminoacyl-tRNA for the limited amount of EF-Tu·GTP available for ternary complex formation, thus altering the apparent kinetics of deacylation.[125] Therefore, while the comparative data obtained for the various modified and unmodified tRNA isoacceptors probably do reflect their relative affinities for the elongation factor, a detailed kinetic analysis would be most difficult.

In addition to arginyl-tRNA's **6** and **8**, 14 other tRNA isoacceptors were studied in the same fashion, including three ($tRNA^{Asn}$, $tRNA^{Cys}$ and $tRNA^{Tyr}$) for which both isomeric aminoacyl-tRNA's were accessible by enzymatic aminoacylation.[125] In each case comparison of the deacylation of the corresponding modified and unmodified tRNA's was carried out using an amount of EF-Tu·GTP which gave reasonable, but not complete, protection of the unmodified species. The results are outlined in Table 8, in terms of the amount of time required for 50% deacylation of the modified aminoacyl-tRNA's in the presence and absence of EF-Tu·GTP. Although in some cases the protection from deacylation was not great, reflecting the chemical nature of the modified tRNA's and the way in which the assays were run, each of the modified tRNA's was protected from deacylation by EF-Tu·GTP and there was no obvious preference for a single positional isomer.

Table 8. Deacylation of modified tRNA's

	50% Deacylation Time (min)[a]			
	tRNA species 2		tRNA species 3	
	+EF-Tu	-EF-Tu	+EF-Tu	-EF-Tu
Alanine	38.5	15		
Arginine			5[b]	28[b]
Asparagine	45	21	50	28
Cysteine	120[b]	75[b]	64	52
Glutamic Acid			6[c]	25
Glycine	52.5	25		
Histidine	135	54.5		
Isoleucine			105[b]	75[b]
Leucine			120[b]	26
Lysine	88.5	49.5		
Phenylalanine			22[c]	8[c]
Serine	123.5[c]	1[c]		
Threonine	5[c]	4[c]		
Tyrosine	23[c]	59[c]	109	54.5
Valine			1[c]	0.4[c]

[a]Time required to effect 45% deacylation. >50% deacylation not reached in presence of EF-Tu.

[b]Time required to effect 20% deacylation.

[c]Time required to effect 40% deacylation.

[d]Less than 10% deacylation of the tRNA had occurred after 120 min.

[e]Time required to effect 30% deacylation.

[f]Time required to effect 35% deacylation.

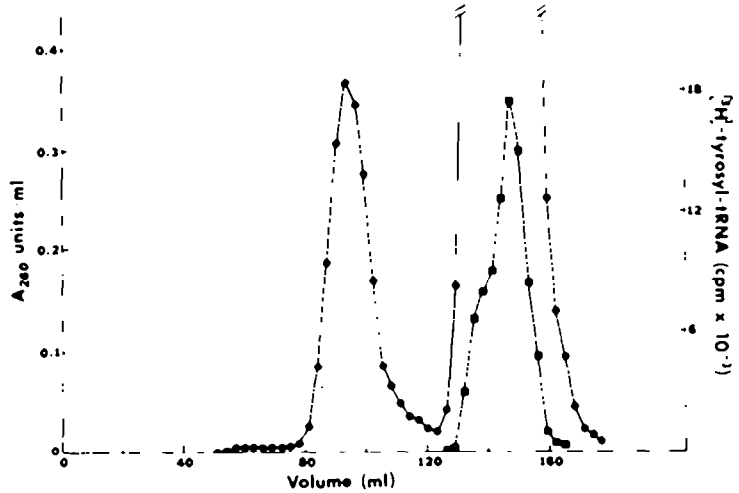


Fig. 16. Gel filtration on a Sephadex G-100 column (1.4 × 108 cm) of *E. coli* tyrosyl-tRNA species 7 (118 pmoles), which had been preincubated with 324 pmoles of EF-Tu-GTP. The ternary complex eluted at a volume of 93 ml.

To verify the results obtained in the deacylation studies and permit a more quantitative assessment of the extent of ternary complex formation with each of the positional isomers of the modified aminoacyl-tRNA's, several additional binding experiments were carried out and assayed by gel filtration.[125] For example, as shown in Fig. 16 for *E. coli* tyrosyl-tRNA 7, incubation of several modified aminoacyl-tRNA's (7 or 8) in the presence of EF-Tu·GTP, followed by chromatography on Sephadex G-100, revealed that each had formed a stable ternary complex with the factor. The experiments were repeated in the same fashion, except that equimolar amounts of the corresponding unmodified aminoacyl-tRNA's (6) having a different radiolabel in the aminoacyl moiety were also added. Since the incubation mixtures contained only about one-half as much EF-Tu·GTP as aminoacyl-tRNA on a molar basis, the relative affinities of the modified and unmodified aminoacyl-tRNA's for the elongation factor could be measured conveniently. As shown in Fig. 17, for example, the ternary complex formed in the presence of tryptophanyl-tRNA's 6 and 7 contained 62% of 6 and 38% of 7 while the complex formed in the presence of tryptophanyl-tRNA's 6 and 8 contained 71% of the unmodified species. For tyrosyl-tRNA's 6 and 7, the resulting complex contained 51% of unmodified tyrosyl-tRNA (6) while the complex formed in the presence of 6 and 8 consisted of 66% of 6 and 34% of 8. The competition for a limiting amount of EF-Tu·GTP was also carried out using equimolar quantities of unmodified *E. coli* alanyl-, arginyl-, lysyl- and phenylalanyl-tRNA's and the single isomers of the respective modified aminoacyl-tRNA's (7 or 8) available via enzymatic activation. All of the modified species were present in the individual ternary complexes in amounts ranging from 28% to 50%.

Sprinzl and Cramer have recently carried out experiments to measure the extent of ternary complex formation of certain modified aminoacyl-tRNA's. In qualitative agreement with the results reported by Hecht *et al.*,[125] they have also determined that both isomers (7 and 8) of tyrosyl-tRNA form ternary complexes with EF-Tu·GTP.[74]

RIBOSOMAL BINDING AND PEPTIDE BOND FORMATION

Although additional work remains to be done on the positional specificity of ribosomal A-site binding, on the basis of the experiments described above it seems not unlikely that both of the positional isomers of aminoacyl-tRNA can be bound enzymatically and nonenzymatically. One may note, however, that different isomeric analogs of aminoacyl-tRNA (e.g. 7 and 8 vs 9 and 10) may be expected to give somewhat different results when used to assess positional specificity and the conclusions reached on the basis of such studies will be subject to this limitation. It is probably reasonable to suggest that the use of the analogs whose behavior most closely paralleled that of the corresponding unmodified species would give the most reliable results.

The P-site binding of the N-acetylated analogs of phenylalanyl-tRNA's 6, 8 and 11 was studied by Chinali *et al.*[126] They showed that both modified species were bound to the P-site, although to a slightly lesser extent than unmodified N-acetylphenylalanyl-tRNA, and that subsequent incubation of the three N-acetylphenylalanyl-tRNA-poly U-ribosome complexes with unmodified phenylalanyl-tRNA resulted in the same extent of factor-dependent binding of phenylalanyl-tRNA to each of the three complexes. Similar results were obtained by Hecht *et al.*[27] for N-acetylated phenylalanyl-tRNA's 6–8, when these species were compared for their ability to inhibit the P-site binding of unmodified [^{3}H]-N-acetylphenylalanyl-tRNA. The use of equimolar concentrations of the three (unlabeled) potential inhibitors and of [^{3}H] - N - acetylphenylalanyl-tRNA resulted in 50, 36 and 35% inhibition of binding of the latter by the N-acetylated species derived from phenylalanyl-tRNA's 6, 7 and 8, respectively. Thus, while the positionally defined analogs were equally as effective as inhibitors of P-site binding, neither was so inhibitory as the respective unmodified species.

The finding that the naturally occurring 3'-N-aminoacylated nucleoside puromycin acted as a potent inhibitor of protein biosynthesis by virtue of its behavior as a tRNA analog, while its 2' - N - aminoacyl analog did not, led to the conclusion that the 3' - O - aminoacyl

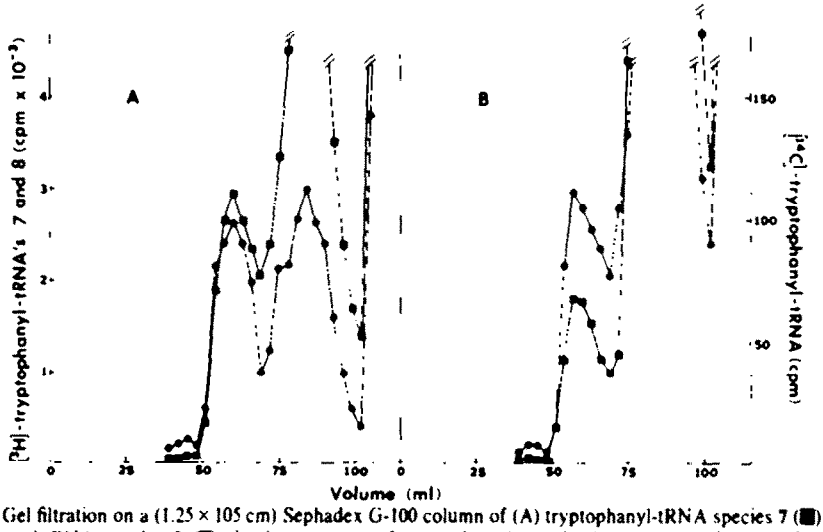


Fig. 17. Gel filtration on a (1.25 × 105 cm) Sephadex G-100 column of (A) tryptophanyl-tRNA species 7 (■) and (B) tryptophanyl-tRNA species 8 (■), in the presence of approximately equimolar amounts of unmodified tryptophanyl-tRNA (6) (●) and limiting amounts of EF-Tu·GTP. The ternary complex eluted at a volume of 58–60 ml, followed by unbound tryptophanyl-tRNA (V_t = 84 ml). The elution volume of aminoacyl-tRNA was also verified in the absence of the other components of this system, as were the values for aminoacyl-tRNA synthetase (V_t = 44 ml) and EF-Tu·GDP (V_t = 75 ml).

isomer of tRNA was acceptor in the peptidyl transferase reaction.[137,138] In 1973, Fraser and Rich demonstrated that an aminoacyl-tRNA analog having a 3'-terminus similar to puromycin (i.e., N-phenylalanyl-tRNA **10**) acted as an efficient acceptor in the peptidyl transferase reaction when added to an incubation mixture containing *E. coli* ribosomes with unmodified N-acetylphenylalanyl-tRNA prebound to the P-site, although the isomeric aminoacyl-tRNA analog was not tested in comparison.[24] Chinali *et al.*[126] later reported the results of experiments utilizing phenylalanyl-tRNA's **8** and **11** which have the aminoacyl moiety in the 2'-position. Surprisingly, these species also acted as acceptors in the peptidyl transferase reaction, but at lesser rates than phenylalanyl-tRNA **6**. Repetition of the experiment in similar fashion with reticulocyte ribosomes indicated that phenylalanyl-tRNA **8** was active as an acceptor, although not to the same extent as the corresponding unmodified species (**6**).[111] In this system, phenylalanyl-tRNA **11** was found to be without acceptor activity in the peptidyl transferase reaction. Direct comparison of the ability of *E. coli* phenylalanyl-tRNA's **6–8** to act as acceptors in the formation of a peptide bond was carried out by Hecht *et al.*[25] Each of the three phenylalanyl-tRNA's was added to a reaction mixture containing unmodified [^{3}H] - N - acetylphenylalanyl - tRNA prebound to *E. coli* ribosomes. After an additional 15 min of incubation, the three samples were analyzed chromatographically for dipeptide formation. As shown in Table 9, the modified phenylalanyl-tRNA (**7**) having a 3'-O-aminoacyl moiety accepted 17% of the theoretical amount of N-acetylphenylalanine, while the comparable figure for the unmodified phenylalanyl-tRNA (**6**) was 70%. The remaining phenylalanyl-tRNA species (**8**), having the aminoacyl group in the 2'-position, did not participate in dipeptide formation under the experimental conditions employed for the assay.

The utilization of positionally defined N-acetylphenylalanyl-tRNA's as donors in the peptidyl transferase reaction has been attempted without success in three laboratories.[24,25,126,131] Although the lack of activity observed for the N-acetyl derivatives of yeast and *E. coli* phenylalanyl-tRNA's **10**[24,131] is not surprising in view of the relative chemical stability of the N-acyl bond between the aminoacyl and tRNA moieties, the results obtained with the derivatives of tRNA species **7** and **8** are more difficult to interpret. The lack of donor activity could be due to insufficient activation of the aminoacyl moieties, e.g., since aminoacylated deoxyribonucleoside derivatives are known to be somewhat less susceptible to hydrolysis than the corresponding aminoacylated ribonucleosides.[121] Alternatively, the modified aminoacyl-tRNA's may bind to the ribosomal P-site in a fashion which is fundamentally different than the binding of the unmodified tRNA's and inappropriate for further participation in peptide bond formation, or there may simply be a requirement for the presence of the vicinal 2'(3')-OH group to facilitate the peptidyltransferase reaction. Whatever the reason for the lack of donor activity of such modified species, in light of their inability to function in this partial reaction it is not surprising that none of the modified aminoacyl-tRNA's studied to date supported protein biosynthesis.

Although none of the modified species acted as donors in the peptidyltransferase reaction, Baksht *et al.*[131] demonstrated that the presence of (glycyl)$_3$ - [^{14}C] - phenylalanyl - tRNA in the P-site of reticulocyte ribosomes resulted in a stimulation of subsequent elongation factor-dependent A-site binding of yeast phenylalanyl-tRNA to the extent of about 30% after an 8 min incubation period, relative to that obtained in the absence of the peptidyl-tRNA. No stimulation was observed when the corresponding peptidyl-tRNA derived from phenylalanyl-tRNA **8** was used in the same experiment, although it should be noted that no direct comparison was made using the isomeric (glycyl)$_3$ - [^{14}C] - phenylalanyl - tRNA structurally related to phenylalanyl-tRNA **7**. On the basis of this finding, and an additional experiment which indicated that yeast 2' - O - phenylalanyl - tRNA **8** was more easily displaced from reticulocyte ribosomes by nonaminoacylated tRNA^{Phe} than 3' - N - phenylalanyl - tRNA **10**, Baksht *et al.*[131] suggested that the 3'-isomer of peptidyl-tRNA is the form utilized in the ribosomal P-site. Given the confusion which has arisen in studies of EF-Tu and A-site binding due to the use of single isomers of modified tRNA's, and of tRNA's which were positionally defined, but not isomeric, it would seem judicious to suggest that no conclusions be drawn regarding the positional specificity for P-site binding and donor capacity in the peptidyl transferase reaction until such time as definitive experiments have been reported.

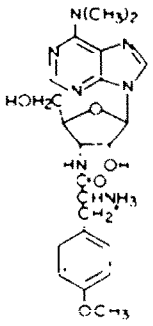
tRNA fragment reaction studies

The earliest experiment relevant to the question of positional specificity of the aminoacyl moiety of tRNA during a partial reaction of protein biosynthesis was reported by Nathans and Neidle.[117] Using the "aminoacyl-tRNA analog" puromycin (V) and its 2' - N - aminoacyl isomer VI, only the former was found to inhibit peptide bond formation when added to an *in vitro* protein biosynthesizing system. On the basis of this work and related experiments which followed, it has long been thought that the 3' - O - aminoacyl isomer of aminoacyl-tRNA is the acceptor in the peptidyl transferase reaction, a conclusion that has now been verified with some

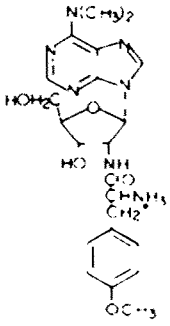
Table 9. Transfer of N-acetylphenylalanine from N-acetylphenylalanyl-tRNA to modified phenylalanyl-tRNA's **6–8**

Acceptor tRNA	cpm transferred[a]	Net % transfer of bound tRNA
tRNA-C-C-A$_{3'-O-Phe}$ (6)	[illegible]	70
tRNA-C-C-A$_{2'-H,3'-O-Phe}$ (7)	[illegible]	17
tRNA-C-C-A$_{2'-O-Phe,3'-H}$ (8)	[illegible]	[illegible]
[illegible]	[illegible]	

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



V



VI

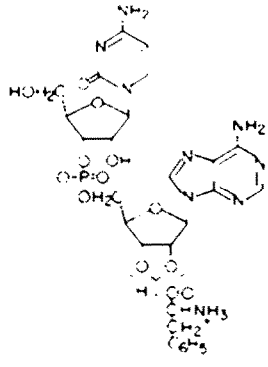
qualifications at the tRNA level. Fragment reaction studies were used extensively to probe the nature of the peptidyl transferase reaction;[139,140] it was shown, e.g., that N-acylated aminoacylhexanucleotides identical with the 3'-termini of certain tRNA's could suffice as "peptidyl" donors in a mRNA-independent system, providing only that high concentrations of the 50S ribosomal subunit were employed and that the incubation mixture contained 30–50% methanol or ethanol.[141–143] Similarly, a number of 2'(3') - O - aminoacylated ribonucleoside derivatives were found to act as puromycin analogs, although most accepted peptidyl moieties efficiently only when present at relatively high concentrations.[144–146]

Because these fragment reactions took place only under rather special conditions, it was also necessary to demonstrate that the results obtained did reflect processes normally operative during protein biosynthesis. This was done by studying the effects on the isolated system of known inhibitors of protein biosynthesis[146,147] and by showing that the structural requirements for the "tRNA fragments" were such that only those species structurally related to the 3'-termini of aminoacyl (peptidyl) tRNA's functioned in the isolated system.[137,148] While the successful uncoupling of the peptidyl transferase reaction from the overall process of protein biosynthesis,[140] with the concomitant simplification of the required components of the isolated system, was a remarkable achievement which facilitated the study of this partial reaction, it is less clear to what level of refinement the isolated system may be used as a model for the analogous transformation which occurs during protein biosynthesis.

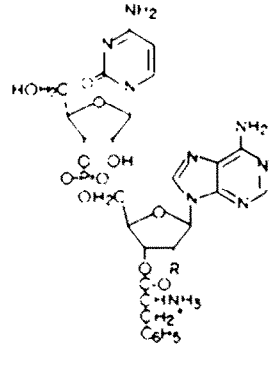
The difficulty in obtaining many isomeric aminoacyl-tRNA's for study makes the use of isomeric aminoacyl-nucleosides or dinucleoside monophosphates an ostensibly attractive alternative, since they can be prepared synthetically with relative facility. In previous sections it was noted that although tRNA's **2–5** were close structural analogs of unmodified tRNA (**1**), many of the modified isoacceptors were much less active as substrates for their cognate aminoacyl-tRNA synthetases than the respective unmodified species from which they were derived.[19,50] This property of the modified tRNA's was even more apparent in EF-Tu and A-site binding studies, in which the type of positionally defined analog employed largely determined the outcome of certain experiments.[24,126,131] As demonstrated amply by Monro and others, small fragments of tRNA suffice even less well than tRNA's **2–5** as analogs of unmodified tRNA and must be used at high concentrations or under special conditions to function as peptidyl donors or acceptors. Therefore, while the study of tRNA fragments which have been additionally modified in the sugar moiety (such that they are identical to the 3'-termini of modified tRNA's **2–5**) is certainly of interest, one should not be surprised if the results obtained do not parallel findings at the tRNA level. Under such circumstances, the modified aminoacylnucleosides could not be regarded as adequate analogs of aminoacyl-tRNA.

ELONGATION FACTOR Tu AND RIBOSOMAL A-SITE BINDING

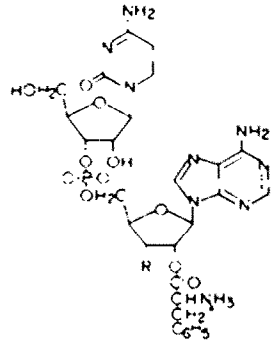
Ringer and Chládek studied the interaction of cytidylyl - (3'→5') - 2'(3') - O - phenylalanyladenosine (VII), cytidylyl - (3'→5') - 2' - deoxy - 3' - O - phenylalanyladenosine (VIII) and cytidylyl - (3→5') - 2' - O - phenylalanyl - 3' - deoxyadenosine (IX) with EF-Tu·GTP.[129,130] Although fragments of tRNA had not previously been found to interact with EF-Tu·GTP, dinucleoside monophosphate VII was shown to effect dissociation of the complex when employed at 10^{-6}–10^{-4} M concentration, as judged by assay of the incubation mixture on nitrocellulose filters. The same result was obtained at slightly higher concentrations using dinucleoside monophosphate IX, but not with the 3' - O - phenylalanyl analog VIII. The authors conjectured that dissociation of EF-Tu·GTP by VII and IX may have occurred via the intermediary of an unstable ternary complex, analogous to the one observed with aminoacyl-tRNA. In support of the thesis that interaction of the aminoacylated dinucleoside monophosphates with the EF-Tu·GTP complex was essentially analogous to the interaction of the complex with aminoacyl-tRNA, it was also shown that neither cytidylyl - (3'→5') - adenosine nor the N-acetylated derivative of VII interacted with EF-Tu·GTP at any tested concen-



VII



VIII, R = H
X, R = OCH_3



IX, R = H
XI R = OCH_3

tration.[151,154] One may note, however, that the amount of VII required to effect dissociation of 16 pmoles of [^{3}H]-EF-Tu·GTP was 500 times greater than the amount of phenylalanyl-tRNA needed to bind the same quantity of the factor and that compounds VII and IX also dissociated EF-Tu·GDP, an interaction which is without precedent at the aminoacyl-tRNA level.

Consistent with results obtained at the tRNA level, compounds VII–IX were all found to inhibit the EF-Tu-dependent binding of phenylalanyl-tRNA to the A-site of *E. coli* ribosomes and the associated hydrolysis of GTP: 50% inhibition of binding was observed at 27, 58 and 5.8 μM concentrations of VII, VIII and IX,[149] respectively, and it was stated that "the enzymatic binding of Phe-tRNA was inhibited 10× more by C-A(2'-Phe)H than by its 3'-isomer; the non-enzymatic binding of Phe-tRNA shows a similar pattern of inhibition".[159] In another recent report from the same laboratory, however, values of 3.5, 5.2 and 2.5 μM were given for what would seem to be the same factor-dependent interactions and it was concluded that "the effect of all three compounds is similar".[150] In the latter report, non-enzymatic ribosomal binding of phenylalanyl-tRNA was reported to be inhibited to a significant extent only by compounds VII and IX. Of the two, 2' - O - phenylalanyl dinucleoside monophosphate IX was the better inhibitor, giving 49% inhibition of binding at 10^{-4} M concentration vs 19% for VII. Inexplicably, the same group had previously reported that the non-enzymatic ribosomal binding of the phenylalanyl ester of the pentanucleotide C-A-C-C-A was inhibited equally as well by VII–IX, but more strongly by 3' - O - phenylalanyl derivative X than by its isomeric 2' - O - phenylalanyl dinucleoside monophosphate XI.[151]

THE PEPTIDYL TRANSFERASE REACTION

Subsequent to the initial experiments with tRNA fragments, which suggested that the 3' - O - aminoacyl isomer of tRNA was the acceptor molecule in the peptidyl transferase reaction, Hussain and Ofengand provided additional evidence. They found that the phenylalanyladenosine analog isolated after pancreatic ribonuclease digestion of phenylalanyl-tRNA **11**, which was believed to be the 2' - O - aminoacyl derivative, did not act as an acceptor in the peptidyl transferase reaction, but that a synthetic sample of the same analog (presumably a mixture of 2'- and 3' - O - phenylalanyl derivatives) did have acceptor activity.[152] These results have been extended by Chládek *et al.*, who have assayed several aminoacylated nucleosides and dinucleoside monophosphates as potential substrates.[153,154] Consistent with the results of earlier work, the 3' - O - phenylalanyl derivatives tested (e.g. VIII and X) were all bound to the ribosomal A-site in the presence of poly U and accepted N - acetyl - L - phenylalanine from N - acetyl - L - phenylalanyl - tRNA which has been prebound to the P-site. Under the same conditions, none of the corresponding 2' - O - phenylalanyl species had any activity.

Also studied were the effects of several aminoacylated dinucleoside monophosphates as potential inhibitors of the peptidyl transferase-catalyzed formation of N-acetylphenylalanylpuromycin.[151,155] Both 2'- and 3' - O - aminoacylated dinucleoside monophosphates were tested and all were found to inhibit the formation of N-acetylphenylalanylpuromycin, consistent with the authors' interpretation that the ribosomal A-site could bind aminoacylated nucleotides (and perhaps tRNA's) with a 2'- or 3' - O - aminoacyl moiety. However, a closer inspection of the data (Fig. 18)[151] illustrates the difficulties inherent in interpreting such data and the risk involved in extrapolating the results to the tRNA level. While both cytidylyl - (3'→5') - 2' - O - phenylalanyl - 3' - deoxyadenosine (IX) and the corresponding 2' - O - glycyl dinucleoside monophosphates were equally as inhibitory to the formation of N-acetylphenylalanylpuromycin at all tested concentrations, the shape of the inhibition curve obtained for cytidylyl - (3'→5') - 2' - deoxy - 3' - O - phenylalanyladenosine (VIII) was different than those obtained for the 2' - O - aminoacyl derivatives. Depending upon the concentration chosen for comparison, VIII could be considered to be more, less or equally as inhibitory as the two 2' - O - aminoacylated species. While the behavior of VIII in this assay might have been attributed to some special property of 3' - O - aminoacylated dinucleoside monophosphates, the corresponding 3' - O - glycyl derivative was also tested and found to be lowest in activity of the four analogs and to give an inhibition curve most similar in shape to those of the 2' - O - aminoacylated dinucleoside monophosphates. Moreover, in an earlier publication from the same laboratory,[155] compound IX was reported to be more inhibitory to N-acetylphenylalanylpuromycin formation than VIII at all tested concentrations.

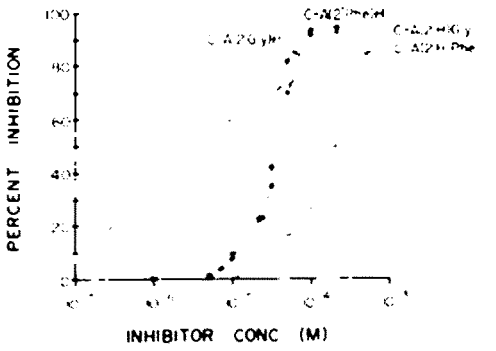


Fig. 18. Inhibition of the formation of N-acetylphenylalanylpuromycin in the presence of 10^{-5} M puromycin and varying concentrations of cytidylyl - (3'→5') - 2' - deoxy - 3' - O - phenylalanyladenosine (○) (VIII; C-A(2'H)Phe), cytidylyl-(3'→5') - 2' - O - phenylalanyl - 3' - deoxyadenosine (●) (IX; C-A(2'-Phe)H), cytidylyl - (3'→5') -2' - deoxy - 3' - O - glycyladenosine (△) and cytidylyl - (3'→5') - 2' - O - glycyl - 3' - deoxyadenosine (▲). Percent inhibition represents the difference in amount of N-acetylphenylalanylpuromycin formed in the presence and absence of inhibitor.[151]

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