# 2', 3' ISOMERIC SPECIFICITY AT THE C-C-A END OF tRNA DURING AMINOACYLATION AND PROTEIN SYNTHESIS

by

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## ABSTRACT

3'-amino-3'-deoxy adenosine was enzymatically converted into 3'-amino-3'-deoxy ATP. This ATP analog served as a substrate for E. coli tRNA-nucleotidyl transferase, which catalyzed incorporation of 3'-amino-3'-deoxy AMP into the 3' terminal AMP position of E. coli tRNA. The modified tRNA (tRNA-C-C-3'$A_N$) was enzymatically aminoacylated with phenylalanine. That the phenylalanine was attached to the 3'-amino group of the tRNA was shown by its stability to base-catalyzed hydrolysis, in contrast to the lability of normal aminoacyl-tRNA. Following ribonuclease digestion, a phenylalanyl-adenosine analog resistant to base-catalyzed hydrolysis was isolated from the phenylalanyl-3'-amino-3'-deoxy tRNA (Phe-3'N-tRNA).

When the Phe-3'N-tRNA was used in a poly(U) directed in vitro protein synthesizing system, it was not incorporated into polyphenylalanine. However, this aminoacyl-tRNA analog was capable of accepting an acetylphenylalanine from the ribosomal donor site. The product of this reaction, acetylphenylalanyl-phenylalanyl-3'-amino-3'-deoxy-tRNA (AcPhe-Phe-3'N-tRNA), was unable to continue chain elongation, possibly due to an inability of the ribosome to cleave an amide bond. It was also found that while elongation factor Tu stimulated the binding of Phe-3'N-tRNA to ribosomes, it was unable to form a stable ternary complex with the phenylalanyl-tRNA analog.

Phe-3'N-tRNA is structurally very similar to normal tRNA except that the amino acid is unable to freely migrate between the 2' and 3' adenosine sites as it does in normal tRNA. The results obtained using this molecule suggest that while 3'-O-aminoacyl-tRNA is used in some steps of protein synthesis, 2'-O-aminoacyl-tRNA may be necessary for other steps.

Additionally, E. coli tRNA has also been modified by replacement of the 3' terminal AMP with 2'-amino-2'-deoxy AMP (tRNA-C-C-2'$A_N$). This and the tRNA-C-C-3'$A_N$ analog have allowed a determination of the initial site of enzyme catalyzed aminoacylation for different tRNAs. The tRNA species specific for glutamic acid, glutamine, leucine, phenylalanine, tyrosine and valine all form stable amide bonds with tRNA-C-C-3'$A_N$, while the tRNA species specific for alanine, asparagine, aspartic acid, glycine, histidine, lysine and threonine all form stable amide bonds with tRNA-C-C-2'$A_N$. As all of the synthetases catalyzing these aminoacylations are apparently unable to catalyze the direct formation of an amide bond, it is concluded that the amino acids in the former group are initially attached to the 2'-hydroxyl, while those in the latter are initially attached to the 3'-hydroxyl. The stable amide bonds are then formed during a rapid, spontaneous acyl migration.

The amino acids arginine, isoleucine, methionine, proline, serine, and tryptophan form stable amide bonds with both amino tRNA analogs. This might suggest that the synthetases for these amino acids can acylate both the 2'- and 3'-hydroxyl groups, but it is more likely that these enzymes can acylate both hydroxyl and amino groups at either the 2' or 3' position of the tRNA. These results clearly illustrate a fundamental heterogeneity which is apparent in the mechanism of action of aminoacyl-tRNA synthetases.

Thesis supervisor: Alexander Rich

Title : Professor of Biophysics

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# BIOGRAPHICAL NOTE

The author was born March 19, 1948, in Dansville, N. Y. He was educated in the Rochester, N. Y. Public Schools, and graduated from the University of Rochester in 1970, majoring in Chemistry and Biology. In August, 1972, he was married to Janis Koehler. They are presently graduate tutors residing in Burton House on the M. I. T. campus.

Publications:

Fraser, T. H. and Rich, A. (1973). Synthesis and aminoacylation of 3'-amino-3'-deoxy transfer RNA and its activity in ribosomal protein synthesis. Proc. Nat. Acad. Sci USA, 70, 2671.

Fraser, T. H. and Rich, A. (1975). Amino acids are not all initially attached to the same position on transfer RNA molecules. Proc. Nat. Acad. Sci USA, in press.

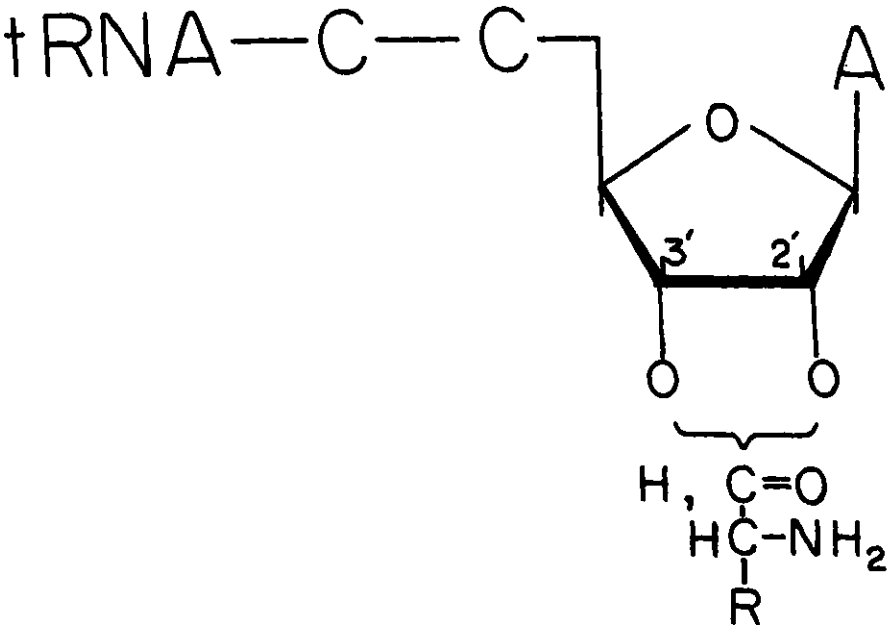
# INTRODUCTION

Transfer ribonucleic acid (tRNA) was first shown to be required in protein synthesis in the late 1950's. Work done in Zamecnik's laboratory demonstrated that ($^{14}$C) leucine was bound to a low molecular weight RNA (called soluble RNA or sRNA at the time) in a reaction dependent on ATP and a 105,000 x g supernatant from liver homogenate (Zamecnik et al., 1957). This aminoacyl-RNA was subsequently found to transfer its amino acid to protein in a cell-free protein synthesizing system (Hoagland et al., 1958).

This discovery bore out the prediction of F. H. C. Crick who had recognized the need for an "adapter" molecule in protein synthesis as early as 1955 (Hoagland et al., 1960). According to the adapter hypothesis, the 20 different amino acids would be linked to different adapter molecules capable of hydrogen bonding specifically with the nucleic acid template, enabling it to direct the synthesis of proteins.

The newly discovered tRNAs had a molecular weight of approximately 25,000 daltons. Where, then, was the amino acid bound to these tRNAs? It was found that the 3' terminal AMP, present in all tRNAs, was required in order to bind amino acids (Hecht et al., 1959). The amino acid was shown to be attached through an ester linkage at either the 2'-or 3'-hydroxyl group of the 3' terminal adenosine. A number of pieces of evidence supported this conclusion. The amino acids bound to tRNA had been shown to

have an activated carboxyl group by their ability to form aminoacyl hydroxamates when incubated with hydroxylamine at neutral pH (Hoagland et al., 1956). It was found that pancreatic ribonuclease treatment of ($^{14}$C) leucyl-tRNA released a fragment that migrated with synthetic 2'(3')-O-leucyl-adenosine when subjected to electrophoresis (Zachau et al., 1958). Furthermore, this fragment was found to contain both adenosine and amino acid in a 1:1 stoichiometric ratio. It was also shown that aminoacylation of the tRNA would protect the cis-hydroxyl groups of its 3' terminal adenosine from periodate oxidation (Hecht et al., 1959; Preiss et al., 1959). These results have been observed for all aminoacyl-tRNAs examined. Thus, the structure of the 3' (C-C-A) end of aminoacyl-tRNA was determined by 1959 to be the following:



A great deal of work has since been done to determine to which hydroxyl group on the terminal adenosine the amino acid is initially attached by the aminoacyl-tRNA synthetases. It was generally believed that the enzymes catalyzing aminoacylation would attach the amino acids specifically to either the 2'-or 3'-hydroxyl group. The approach initially taken to solve this problem was to examine the aminoacyl-adenosine fragment from the 3' end of aminoacyl-tRNA. This fragment was first split from the rest of the tRNA molecule by treatment with pancreatic ribonuclease and purified by electrophoresis or ion-exchange chromatography. The aminoacyl-adenosines were then reacted with chemical blocking reagents to prevent migration of the aminoacyl group during subsequent analysis and to facilitate separation of the pure 2' and 3' isomers. Thus, tosyl (Feldmann and Zachau, 1964), phosphoryl (McLaughlin and Ingram, 1965) and tetrahydropyranyl (Wolfenden et al., 1964) groups were all employed as blocking reagents in order to determine whether the amino acids were linked to the 2'-or 3'-hydroxyl group of tRNA. Regardless of the blocking groups or methods employed, a mixture of 2'-and 3'-aminoacyl-adenosine isomers was always found.

Most investigators found about 65% of the amino acid at the 3' position and 35% at the 2' position. It was generally believed that by improvement of the methods this chemical trapping approach would eventually determine the initial site of aminoacylation.

In 1966, however, a paper by Griffin et al. (1966) reported measurement of acyl migration in model systems designed to mimic the aminoacyl-tRNA. The rates of migration in 0.1M phosphate buffer at pH 7.0 of acetyl and formyl groups bound to adenosine and uridine were measured. They calculated that the half-time of equilibration between 2' and 3' isomers for an aminoacyl-tRNA would be approximately 0.2 milliseconds, although the value would vary somewhat depending on the amino acid side chain. The appearance of these results effectively stopped further attempts to determine the initial site of aminoacylation by chemical derivatization.

No progress has been made on this problem until recently when techniques employing modification of the tRNA molecules prior to aminoacylation were developed. These techniques and the results obtained using them will be discussed in detail in both the Results and Discussion sections of this dissertation.

Protein synthesis

Much of the interest surrounding tRNA involves its central role in protein synthesis. Based on known enzyme mechanisms, it was expected that the steps in ribosome-directed protein synthesis would involve a well-defined substrate. This substrate would have an aminoacyl or peptidyl group on either the 2'- or 3'-hydroxyl at the C-C-A end of tRNA.

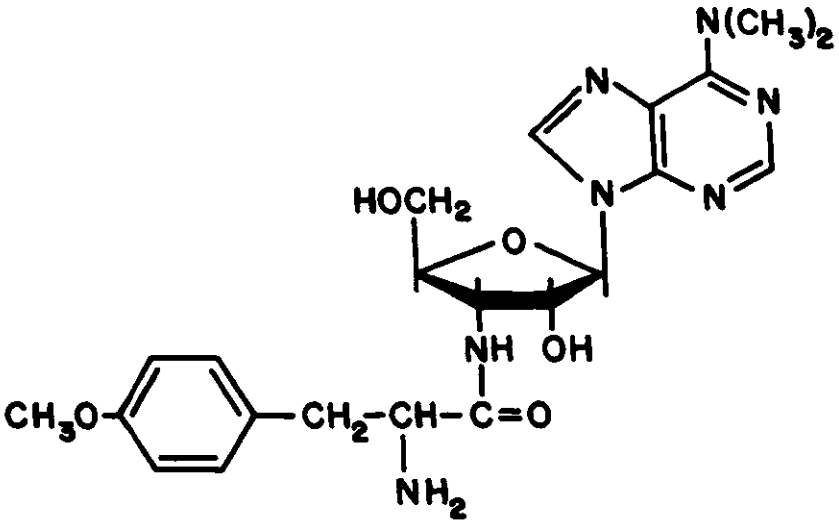
Until recently the only information bearing on the question of

isomeric specificity in protein synthesis involved the use of puromycin and the 2'-puromycin isomer. Figure 1 illustrates the structure of these compounds. Puromycin had been found to inhibit protein synthesis in all organisms tested. (Some of the early observations were made by Takeda et al., 1960; Hultin, 1961; Ferguson, 1962; Nemeth and de la Haba, 1962). The probable mode of this inhibition may be deduced by examining the structure of puromycin, as it appears to be a structural analog of phenylalanyl- (or tyrosinyl)-adenosine (Yarmolinski and de la Haba, 1959). Thus, one would expect that its inhibition of protein synthesis may be due to its ability to accept a growing polypeptide chain which, once attached to puromycin instead of tRNA, would dissociate from the ribosome. It has been experimentally verified that puromycin is incorporated at the carboxyl end of a polypeptide chain (Nathans, 1964; Smith et al., 1965). It has also been demonstrated that normal peptide bond formation and peptidyl-puromycin formation are similar in their substrate requirements (for example only the L-phenylalanyl analog of puromycin is active, the D-phenylalanyl analog being inactive) and reaction conditions (Maden et al., 1968). The conclusion, then, is that puromycin is a functional analog of phenylalanyl-adenosine and is a substrate for ribosomal peptidyl-transferase in the same way that the C-C-A end of aminoacyl-tRNA is a substrate.

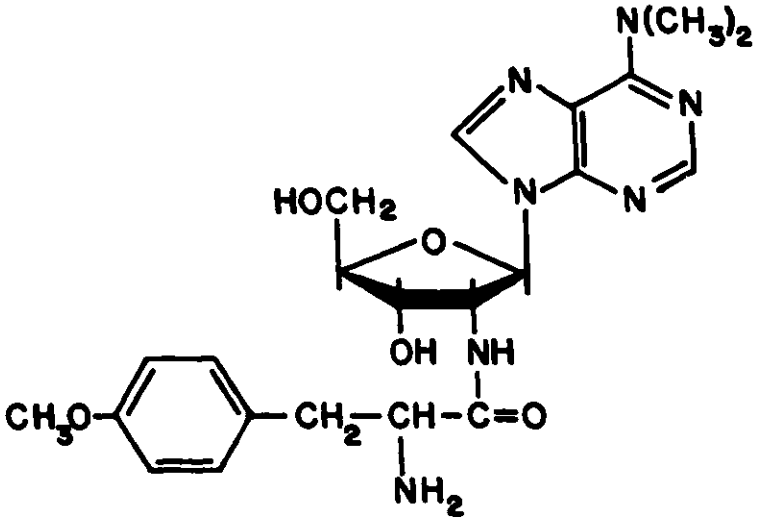
Nathans and Niedle (1963) determined that unlike puromycin, the

Figure 1: The structures of puromycin and the 2'-puromycin isomer

The base in puromycin is $N^6$, $N^6$- dimethyladenine, the sugar moiety is 3'-amino-3'-deoxyribose and the $\alpha$-amino acid is para-methoxy-phenylalanine. The 2'-puromycin isomer is identical except that the sugar is 2'-amino-2'-deoxyribose.




PUROMYCIN




2'-PUROMYCIN ISOMER

2'-isomer of puromycin was not an inhibitor of protein synthesis in E. coli. These results suggested that there was a specificity for the 3'-isomer of aminoacyl-tRNA in protein synthesis, or at least in the peptidyl transferase reaction.

Ribosomal protein synthesis in E. coli involves at least five different steps during which the 3' end of aminoacyl- or peptidyl-tRNA interacts specifically with ribosomal proteins or protein factors. These include initiation, aminoacyl-tRNA binding, peptide bond formation, translocation and termination. Each of these interactions may require the aminoacyl or peptidyl group to be attached to tRNA specifically on only one or the other hydroxyl groups. Although a great deal of work has been done to outline the details of ribosomal protein synthesis, virtually no descriptions of isomeric specificity have been reported until recently. The evidence for specific enzyme and factor interactions with the 3' end of aminoacyl- and peptidyl-tRNA will be reviewed here because these interactions may involve isomeric specificity. The most complete recent reviews on the details of protein synthesis are by Haselkorn and Rothman-Denes (1973) and by Lucas-Lenard and Lipmann (1971).

Initiation

The initiation of protein synthesis is encoded in the messenger RNA by an AUG or GUG triplet (Clark and Marcker, 1966; Ghosh et al., 1967) and in prokaryotes probably by a polypurine tract of from 3 to

6 residues on the 5' side of the initiation codon as well (Shine and Dalgarno, 1974, 1975). The initiator codons code for N-formyl-methionine in prokaryotic systems which is incorporated at the amino terminal end of the nascent polypeptide chains (Adams and Capecchi, 1966; Webster et al., 1966; Clark and Marcker, 1966b).

It has been found in in vitro systems that initiation factors are required in order to initiate protein synthesis at low $Mg^{2+}$ concentrations (about 4 mM). These factors have been designated IF-1, IF-2, and IF-3 in E. coli. There is still some controversy over the sequence of events in initiation, but the following represents some current views. IF-3 associates with the isolated 30 S ribosomal subunit and prevents its association with a free 50 S subunit prior to initiation (Sabol et al., 1970). It is also required in the binding of mRNA to the 30 S subunit. For this purpose, there are at least two different IF-3's, designated IF-3$\alpha$ and IF-3$\beta$. These different IF-3's recognize different mRNA's (Lee-Huang and Ochoa, 1973). The IF-3, mRNA and 30 S subunit together form a complex to which fmet-tRNA can be bound with the aid of IF-2 and GTP (Salas et al., 1967; Hershey and Thach, 1967). It is probable that fmet-tRNA, GTP and IF-2 form a complex prior to binding. This complex, although very unstable, has been isolated by gel filtration and is able to serve as an intermediate in the binding of fmet-tRNA to 30 S subunit initiation complexes (Lockwood et al., 1971). Whether or not this

complex is employed in vivo is not clear. Initially, fmet-tRNA, GTP and IF-2 are all bound to the 30 S subunit· mRNA· IF-3 complex in a 1:1:1 stoichiometric ratio (Benne et al., 1973). IF-1 is also bound in this complex (Thach et al., 1969). If a free 50 S ribosomal subunit is then allowed to bind to the entire complex, the previously bound GTP is hydrolyzed (Thach and Thach, 1971). IF-1 then allows the release of IF-2 and GDP from the initiation complex (Benne et al., 1973).

The result of this complicated initiation process is that the fmet-tRNA, bound in the donor or D ribosomal site, is able to begin peptide chain elongation by reaction with aminoacyl-tRNA, which will subsequently be bound in the acceptor or A ribosomal site. Functional binding of aminoacyl-or peptidyl-tRNA in the D site is assayed by its ability to react with added puromycin, resulting in aminoacyl- or peptidyl-puromycin formation. The D site has also been referred to as the P, or peptidyl, site because this is where peptidyl-tRNA is presumably bound prior to peptide bond formation. Because D site occupancy is determined in a functional assay (ability to react with puromycin), a functional definition (i. e. donor) is more appropriate than a structural definition.

IF-2 is very specific in its role of binding initiator tRNA into the D site. No aminoacyl-tRNA with an unblocked $\alpha$-amino group, including met-tRNA$_f^{met}$, is bound to the ribosome with IF-2

(Salas et al., 1967b), nor does blocking the amino group by formylation or acetylation allow most aminoacyl-tRNAs to function as initiators (Rudland et al., 1969). AcPhe-tRNA, however, is able to function as an initiator in a poly(U) system. This initiation takes place at low magnesium concentrations and is dependent upon initiation factors (Lucas-Lenard and Lipmann, 1967).

It is clear from examination of the substrate specificity of IF-2 that it must recognize a blocked $\alpha$-amino group on the amino acid. This recognition may require the amino acid to be attached exclusively to either the 2'-or 3'-hydroxyl group of the 3' terminal adenosine of $tRNA_f^{met}$ or $tRNA^{phe}$.

Aminoacyl-tRNA binding

The mRNA codon on the 3' side of the initiator codon specifies which aminoacyl-tRNA will be bound to the ribosome following initiation. The process requires two non-ribosomal protein factors (Ravel, 1967), designated EF-Ts and EF-Tu.

EF-Tu has been found to form a relatively stable complex with GTP and aminoacyl-tRNA (Shorey et al., 1969). Aminoacyl-tRNA which is complexed with EF-Tu and GTP will be preferentially bound to ribosomes over free aminoacyl-tRNA in an in vitro system (Shorey et al., 1969; Ravel et al., 1969). Thus it appears probable that the ternary complex is an in vivo intermediate in the binding of aminoacyl-tRNA to ribosomes. Hydrolysis of the GTP with release of EF-Tu·GDP,

follows immediately after a ternary complex is bound to a ribosome (Ono et al., 1969). The aminoacyl-tRNA is thus bound in the ribosomal A site. This is defined as a puromycin unreactive site because no aminoacyl-puromycin product is formed when puromycin is added. However, aminoacyl-tRNA which is bound in the A site will accept an aminoacyl or peptidyl group from aminoacyl- or peptidyl-tRNA bound in the D site.

The apparent function of EF-Ts is to recycle the EF-Tu that has been released from the ribosome as an EF-Tu·GDP complex (Shorey et al., 1969). The EF-Ts binds to this complex, displacing GDP and resulting in formation of an EF-Tu·EF-Ts complex. GTP in turn displaces EF-Ts, reforming an EF-Tu·GTP complex. It is this complex which is then able to bind an aminoacyl-tRNA molecule and catalyze its binding to the ribosome (Weissbach et al., 1970).

The initial selection of the molecules to be bound in the A site, then, is determined by their ability to form a complex with EF-Tu and GTP. The formation of this complex is easily assayed by Sephadex gel-filtration or Millipore filter binding. Deacylated tRNA (Gordon, 1967) will not form a complex, while all aminoacyl-tRNAs except met-$tRNA_f^{met}$ will form a complex (Ono et al., 1968). In addition, the amino acids which are bound to the tRNA must have an $\alpha$-amino group in order to interact with EF-Tu and GTP (Ravel et al., 1968). An exception to this is found if an $\alpha$-hydroxyl group is

substituted for the $\alpha$-amino group, in which case a ternary complex can be formed (Fahnestock et al., 1972).

It has already been pointed out that met-tRNA$_f^{met}$ cannot normally form a ternary complex with EF-Tu and GTP. However, this tRNA can be modified by reaction with sodium bisulfite so that it is able to form a ternary complex (Schulman et al., 1974). The bisulfite reaction converts cytidine to uracil. The important residue is apparently the 5' terminal cytosine of tRNA$_f^{met}$. This cytosine is not base-paired as are the 5' termini of all other tRNA's. Conversion to uridine allows it to form a U·A base pair. This result suggests that base pairing of the 5' terminal residue is required for ternary complex formation.

Other structural features of the tRNA have also been found to be important for EF-Tu binding. Removal of the 5' phosphate group significantly reduces ternary complex formation (Schulman et al., 1974). If the 3' end of yeast tRNA$^{phe}$ is lengthened to C-C-C-A, instead of C-C-A, ternary complex formation is greatly reduced (Thang et al., 1972). Cleavage of the C2'-C3' bond in the 3' terminal adenosine of yeast tRNA$^{phe}$ abolishes its ability to form a ternary complex (Chen and Ofengand, 1970). On the other hand, cleavage of E. coli valyl-tRNA$^{val}$ in the anticodon loop does not interfere with ternary complex formation (Krauskopf et al., 1972). Likewise, excision of the yeast tRNA$^{phe}$ Y base in the anticodon

loop does not affect its recognition by EF-Tu (Ghosh and Ghosh, 1970).

When aminoacyl-tRNA is associated with EF-Tu and GTP in a ternary complex, the stability to base-catalyzed hydrolysis of the ester bond linking amino acid and tRNA is increased more than ten-fold (Beres and Lucas-Lenard, 1973). This result, along with the previously described results on substrate specificity, suggest an intimate association between the aminoacyl end of aminoacyl-tRNA and EF-Tu. This association is very sensitive to particular structural features of the aminoacyl end of the molecule, one of which may be the attachment of the amino acid specifically to either the 2'- or 3'-hydroxyl of the terminal adenosine. This possible structural requirement could not be observed using normal aminoacyl-tRNA because of the ease of 2', 3' isomerization in this molecule.

Peptidyl transferase

Peptidyl transferase catalyzes peptide bond formation during protein synthesis. This enzymatic activity is associated with the 50 S ribosomal subunit (Monro, 1967) and cannot be localized to an individual ribosomal protein (Staehlin et al., 1969; Dietrich et al., 1974). It requires no added factors or GTP for activity.

In order for peptide bond formation to occur, an initiator or peptidyl-tRNA must be bound in the D site and an aminoacyl-tRNA bound in the A site. If these substrates are present in their proper sites,

the enzyme will immediately catalyze formation of a peptide bond between the carboxyl terminus of the D site reactant and the α-amino group of the A site reactant. This reaction probably involves direct nucleophilic attack by the α-amino group of aminoacyl-tRNA on the carbonyl carbon of the ester bond linking the aminoacyl or peptidyl moiety to tRNA. There is no evidence for any enzyme bound intermediate in this reaction. Peptidyl transferase has also been shown to catalyze the formation of ester bonds with α-hydroxyaminoacyl-tRNA in the A site (Fahnestock and Rich, 1971).

As would be expected, peptidyl transferase activity is very sensitive to modifications in the C-C-A end of its tRNA substrates. The key recognition features of the peptidyl transferase binding sites have been determined using the fragment reaction assay. It was initially found that fmet-tRNA was able to react with puromycin in the presence of isolated 50 S subunits, alcohol and salts (Monro et al., 1968). This reaction did not require 30 S subunits or mRNA. It was then determined that after T1 ribonuclease treatment of fmet-tRNA, the isolated 3' terminal fragment C-A-A-C-C-A-fmet was still able to react with puromycin (Monro et al., 1969). Variations of this basic reaction have been used as model systems to determine the minimum structural requirements for functional binding to both the D and A peptidyl transferase sites.

It was found that C-C-A-fmet was the smallest fragment which

had donor activity (Monro, 1968). At high concentrations (1mM), however, pA-fmet also has donor activity (Cerna et al., 1973). It is not necessary that the amino acid be methionine, but in order to bind to the D site the α-amino group of the amino acid attached to the fragment must be blocked, either by acetylation or formylation (Monro et al., 1968).

Binding and activity in the A site requires only an aminoacyl-adenosine or an aminoacyl-adenosine analog, such as puromycin. When aminoacyl-adenosine is used, the acceptor activity is a function of the nature of the amino acid side chain (Rychlik et al., 1970). A-Gly, which is inactive as an acceptor, can be made to react if C-A-Gly is used. If U, A, or G replaces C, the aminoacyl dinucleotide has no acceptor activity (Harris et al., 1971).

Binding in the A site apparently requires a free α-amino group. The aminoacyl-oligonucleotide C-A-C-C-A-(AcPhe) does not bind to the A site (Hishizawa and Pestka, 1971). This indicates that the ribosome itself is able to distinguish between a free and blocked α-amino group.

The fact that the amino acid side chains and the free α-amino group are apparently recognized at the A site suggests that the amino acid may be specifically bound to either the 2'-or 3'-hydroxyl group of adenosine while in the A site. Isomeric specificity of the peptidyl transferase A site was suggested long ago by the results of Nathans' and Nieldle's work with puromycin and the 2'-puromycin isomer (1963).

These results indicate that the A site is specific for the 3' isomer of aminoacyl-tRNA. It should be pointed out that although EF-Tu normally catalyzes the binding of aminoacyl-tRNA to the A site, it is not necessary that it require the same isomeric specificity (i.e. 3') as the A site. Transfer of aminoacyl-tRNA from EF-Tu to the A site could easily involve an acyl migration as the half-time of such a migration has been estimated at 0.2 milliseconds (Griffin et al., 1966).

The existence of isomeric specificity in the D site appears to be likely. The requirement for a blocked $\alpha$-amino group on the amino acid indicates that the D site recognizes this moiety, suggesting an isomerically defined linkage of the amino acid to tRNA. Furthermore, the carbonyl carbon in the ester bond must be properly positioned and possibly activated to allow attack by the acceptor $\alpha$-amino group. Although this is not a compelling argument, this process would appear to require the participation of a well-defined substrate molecule in the D site. Again, as was the case with EF-Tu, it is not necessary that IF-2 have the same isomeric specificity as the peptidyl transferase D site.

Translocation

After peptide bond formation, peptidyl-tRNA is bound in the A site and deacylated-tRNA is left in the D site on the ribosome. During translocation the deacylated tRNA is removed, the peptidyl-tRNA is

moved from the A to the D site and a new codon becomes available to direct binding of an incoming aminoacyl-tRNA in the A site (Erbe et al., 1969; Gupta et al., 1971).

This process requires the activity of a protein factor, EF-G, and the hydrolysis of GTP. Mechanistically, very little is known of this process. Results discussed previously regarding specificity of the A and D site binding with respect to blocked versus unblocked α-amino groups suggest that the C-C-A end of peptidyl-tRNA may spontaneously move from the A site to the D site once the deacylated tRNA in the D site is removed. The GTP molecule that is hydrolyzed during translocation is not the same GTP hydrolyzed in the EF-Tu dependent binding of aminoacyl-tRNA (Ravel et al., 1969). Two molecules of GTP, therefore, are hydrolyzed on the ribosome for each amino acid added to the nascent polypeptide chain.

There is no evidence that EF-G ever interacts directly with aminoacyl- or peptidyl-tRNA. The C-C-A end of the peptidyl-tRNA is apparently associated with first the A and then the D site during translocation. If the A site and the D site have different isomeric specificities, then translocation will be associated with a migration of the peptidyl group from one hydroxyl to the other on the 3' terminal adenosine. If the A and D sites have the same isomeric specificities, the migration will not be necessary during translocation.

Termination

Elongation of a nascent polypeptide chain continues until a termination codon on the mRNA is reached. At this point the ester bond linking the polypeptide chain to tRNA is hydrolyzed and the completed protein is released. In prokaryotes this process involves three protein factors RF-1, RF-2, and RF-3. RF-1 is bound to the ribosome in response to codons UAA or UAG, while RF-2 is bound in response to UAA or UGA (Scolnick et al., 1968. RF-3 alters the rate of hydrolysis in the presence of either RF-1 or RF-2 (Milman et al., 1969).

All evidence obtained is consistent with the conclusion that the 50 S subunit's peptidyl transferase center acts as the peptidyl-tRNA hydrolase. The reactions catalyzed by both enzymatic activities involve nucleophilic attack on the ester carbonyl of peptidyl-tRNA, in one case by an amino group and in the other by water. It has been shown that peptidyl transferase activity and peptidyl-tRNA hydrolase activity can be inactivated and reactivated in parallel by alteration of ionic conditions (Vogel et al., 1969). In addition, antibiotics that specifically inhibit peptidyl transferase activity also inhibit peptidyl-tRNA hydrolase activity (Vogel et al., 1969; Caskey et al., 1971). A suggestive finding is that the peptidyl transferase center is able to catalyze the hydrolysis of fmet-tRNA in the presence of acetone (Caskey et al., 1971). Thus, although the evidence is indirect, it appears likely that release factor

mediated peptidyl-tRNA hydrolysis requires the peptidyl transferase center. It has been speculated that the release factors may be directly involved in nucleophilic attack on the peptidyl-tRNA or that they may function to insure that the polypeptide chain is transferred only to water by the peptidyl transferase (Tate and Caskey, 1974).

Peptidyl-tRNA must be bound in the ribosomal D site in order for release factor mediated hydrolysis to occur (Capecchi and Klein, 1969; Tompkins et al., 1970). Thus, after the last peptide bond in a protein is formed, the peptidyl-tRNA must be translocated prior to release from the ribosome. One would therefore expect that any isomeric specificity in the release reaction would be the same as that of the peptidyl transferase D site.

Experimental approach

Until recently, many years of work on the mechanism of protein synthesis had yielded very little information regarding 2', 3' isomeric specificity. The difficulty has been the rapid equilibration of 2'-and 3'-aminoacyl-tRNA isomers in solution. If analogs of aminoacyl-tRNA could be synthesized which were functionally active and yet did not isomerize, the problem could be approached.

The E. coli enzyme tRNA-nucleotidyl transferase (EC 2. 7. 7. 25) will use, as a substrate, tRNA missing all or some of its 3' terminal C-C-A residues, completing this end of the molecule by accurately re-generating the C-C-A sequence. If this enzyme were able to incorporate

into the 3' terminus of tRNA-C-C an AMP analog that did not have cis-hydroxyl groups, it would represent the first step in synthesizing a non-isomerizable analog of aminoacyl-tRNA.

Some investigators had previously attempted to incorporate dAMP (2'deoxy AMP) into tRNA with the E. coli tRNA-nucleotidyl transferase and failed. If deoxyadenosine were substituted at the 3' end of a tRNA analog, any amino acid which was subsequently attached to the 3'-hydroxyl group would be unable to isomerize. If 3'-deoxyadenosine (cordycepin) could also be incorporated into tRNA, the resulting two analogs might prove valuable in deciphering isomeric specificity.

Since dATP was not a substrate for E. coli tRNA-nucleotidyl transferase, it was decided to attempt incorporation of 3'-amino-3'-deoxy adenosine into tRNA-C-C. If this were incorporated into the 3' terminus of tRNA, an amide bond might subsequently be made between the carboxyl group of an amino acid and the 3'-amino group on the tRNA. In the aminoacyl-tRNA synthetase catalyzed addition of an amino acid to analog tRNA there are two possible ways in which this amide bond could be formed:

1) Direct nucleophilic attack by the tRNA amino group on the activated carboxyl carbon of the amino acid;
2) Nucleophilic attack by the 2'-hydroxyl group on the activated carboxyl carbon of the amino acid (normal mechanism) followed by an O $\rightarrow$ N acyl migration forming an amide bond between the amino acid and tRNA.

Both possibilities appear chemically feasible, and it seemed that an aminoacyl-tRNA analog containing a stable amide bond could be synthesized.

Because N→ O shifts occur only under harsh acidic conditions (Welsh, 1949), it was unlikely that the amide bond once made would isomerize to make an ester bond. Thus, this analog of aminoacyl-tRNA would be non-isomerizable. The only difference between the analog and normal aminoacyl-tRNA would be the presence of an amide rather than an ester linking the amino acid and tRNA.

# MATERIALS AND METHODS

## Materials

3'-amino-3'-deoxy adenosine (9-β-3'-amino-3'-deoxy-D-ribofuranosyl adenine) and an α, β mixture of 2'-amino-2'-deoxy adenosine (9-α-2'-amino-2'-deoxy-D-ribofuranosyl adenine and 9-β-2'-amino-2'-deoxy-D-ribofuranosyl adenine) were supplied by Dr. Harry B. Wood and the NIH cancer chemotherapy program. Purified α and β isomers of 2'-amino-2'-deoxy adenosine were supplied by Dr. Derek Horton. Purified tRNA-nucleotidyl transferase was the gift of Dr. Georg R. Philipps. Purified EF-Tu was the gift of Mr. Eliot Jekowsky. E. coli B unfractionated tRNA was from Schwarz-Mann.

## Preparation of adenosine kinase

Adenosine kinase was prepared from rabbit liver by the method of Lindberg et al. (1967) and Lindberg (1969). A 6-8 pound New Zealand white rabbit was fasted for 48 hours prior to sacrifice by decapitation. The liver was immediately perfused in situ with cold 1 mM ethylenediaminetetracetic acid (EDTA).

The following steps were all done at 4°C. After perfusion the liver was cut into small pieces and homogenized in a precooled Waring blender with an approximately equal weight of 1 mM EDTA. The homogenate was centrifuged for 10 minutes at 7,000 x g and then for 60 minutes at 80,000 x g in order to remove cell debris and organelles. The pH of the supernatant was adjusted to 5.0 with 1.0 N acetic acid

and the precipitate removed by centrifugation at 40,000 x g for 5 minutes. The pH of the supernatant was made 6.8 with 1 N KOH, and the small amount of precipitate removed by centrifugation at 40,000 x g for 15 minutes. The supernatant was lyophilized and dissolved in 0.1 volume (pre-lyophilization) of distilled water.

Five-seven ml portions of this crude enzyme preparation (from approximately 40 gms of liver) were then passed over a 40 x 2.5 cm Sephadex G-100 column that had been previously equilibrated with elution buffer (5% (v/v) glycerol, 1 mM EDTA, and 4 mM potassium phosphate, pH 6.8). 10 ml fractions were collected and assayed for adenosine kinase activity.

Figure 2 shows the elution pattern from a typical preparation of adenosine kinase. The assays employed are described in the figure legend. The resulting adenosine kinase preparation is crude, but has been separated from major ATPase activities. The active fractions were made 20% in glycerol and stored at -90°C. Most fractions retained activity for one year under these storage conditions.

Preparation of 3'-amino-3'-deoxy ATP

3'-amino-3'-deoxy adenosine had been isolated from Helminthosporium species number 215 (Gerber and Lechevalier, 1962). This material is identical to chemically synthesized 3'-amino-3'-deoxy adenosine (Baker et al., 1955). The sample used in the experiments reported here was found to be homogeneous both by cellulose thin-layer

Figure 2: Elution of adenosine kinase from a Sephadex G-100 column.

Protein concentration ( —●— ) was estimated spectrophotometrically by the method of Warburg and Christian (1941).

Adenosine kinase activity ( —■— ) was assayed by the method of Lindberg et al. (1967). This assay employs the following reactions in a coupled system:

$$\text{ATP + Adenosine} \xrightarrow[\text{kinase}]{\text{adenosine}} \text{ADP + AMP}$$

$$\text{ATP + AMP} \xrightarrow[\text{myokinase}]{} \text{2 ADP}$$

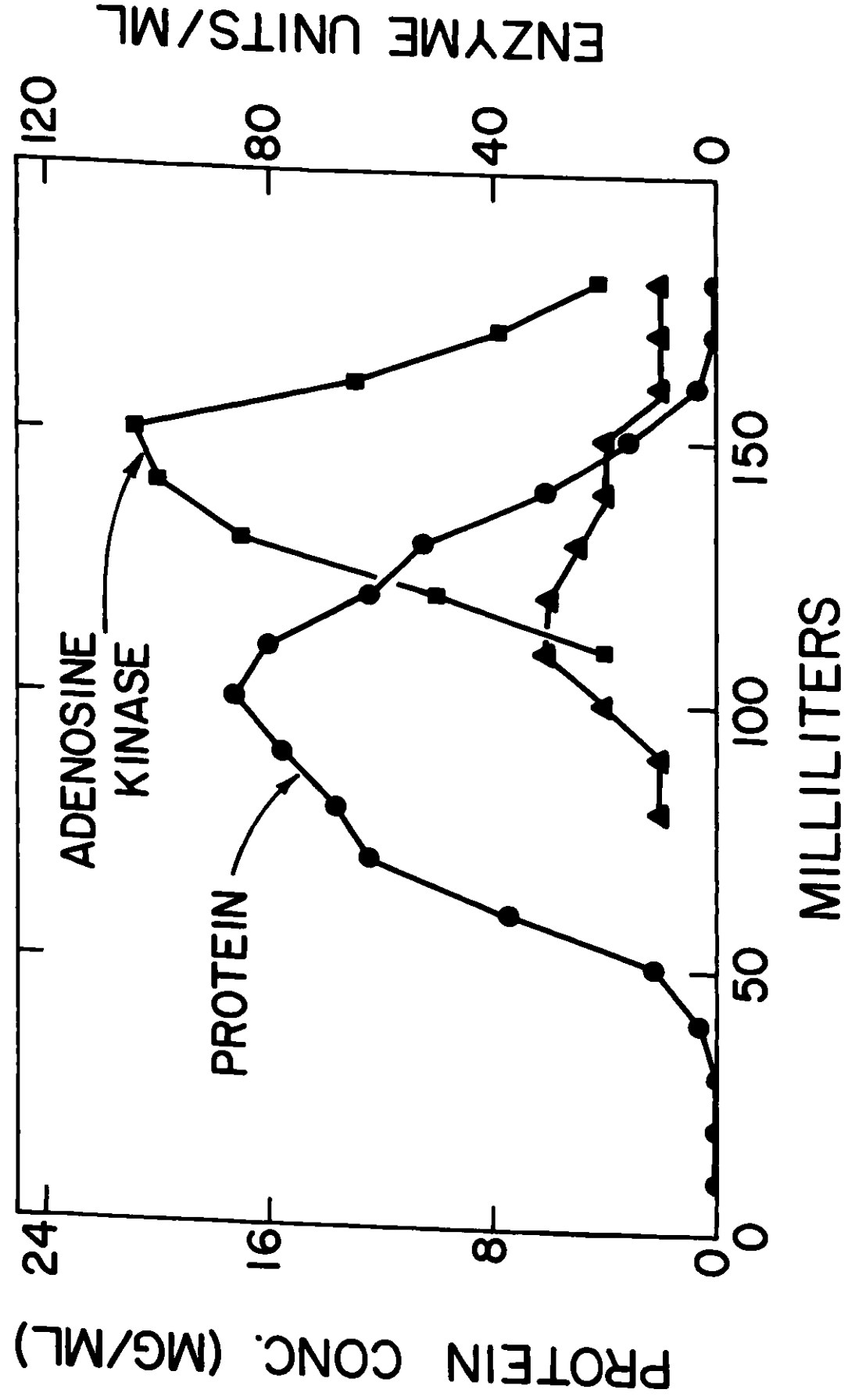
$$\text{3 ADP + 3 PEP} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{3 ATP + 3 Pyruvate}$$

$$\text{3 Pyruvate + 3 NADH} \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{3 Lactate + 3 } NAD^{+}$$

Thus three molecules of NADH are oxidized for each molecule of adenosine converted to AMP by adenosine kinase. This oxidation may be followed spectrophotometrically at 340 nm. The calculated

enzyme units were based on an extinction coefficient of $6.22 \times 10^3$ $cm^{-1}$ $M^{-1}$ for NADH at 340 nm with 1 unit able to phosphorylate 1 nmole of adenosine per minute. $NAD^+$ has no absorbance at 340 nm. The assay mixture contained in 1 ml: 0.7 μmole ATP, 0.07 μmole adenosine, 0.25 μmole PEP (tricyclohexylammonium salt), 4 μg pyruvate kinase (Sigma, 400 units/mg), 10 μg myokinase (Boehringer), 25 μg lactate dehydrogenase (Boehringer) and 0.35 μmole NADH. The reaction was buffered at pH 5.8 by 20 mM Tris-maleate and also contained 0.5 mM $Mg(C_2H_3O_2)_2$ and 50 mM KCl. The assay reaction was initiated by addition of 50 μl of the G-100 column fractions.

Blank activity ( —▲— ) is a measure of ATPase. It was desirable to separate ATPase activity from the adenosine kinase because the enzyme preparation was to be used in the synthesis of ATP analogs. The ATPase activity was assayed in a reaction mixture identical to that described for the adenosine kinase assay, except that no adenosine was added. If an ATPase were present, it would hydrolyze the added ATP to ADP, resulting in formation of pyruvate and ultimately $NAD^+$ by the coupled enzyme system.


ENZYME UNITS/ML
120
80
40
0
ADENOSINE KINASE
PROTEIN
150
100
50
MILLILITERS
24
16
8
0
PROTEIN CONC. (MG/ML)

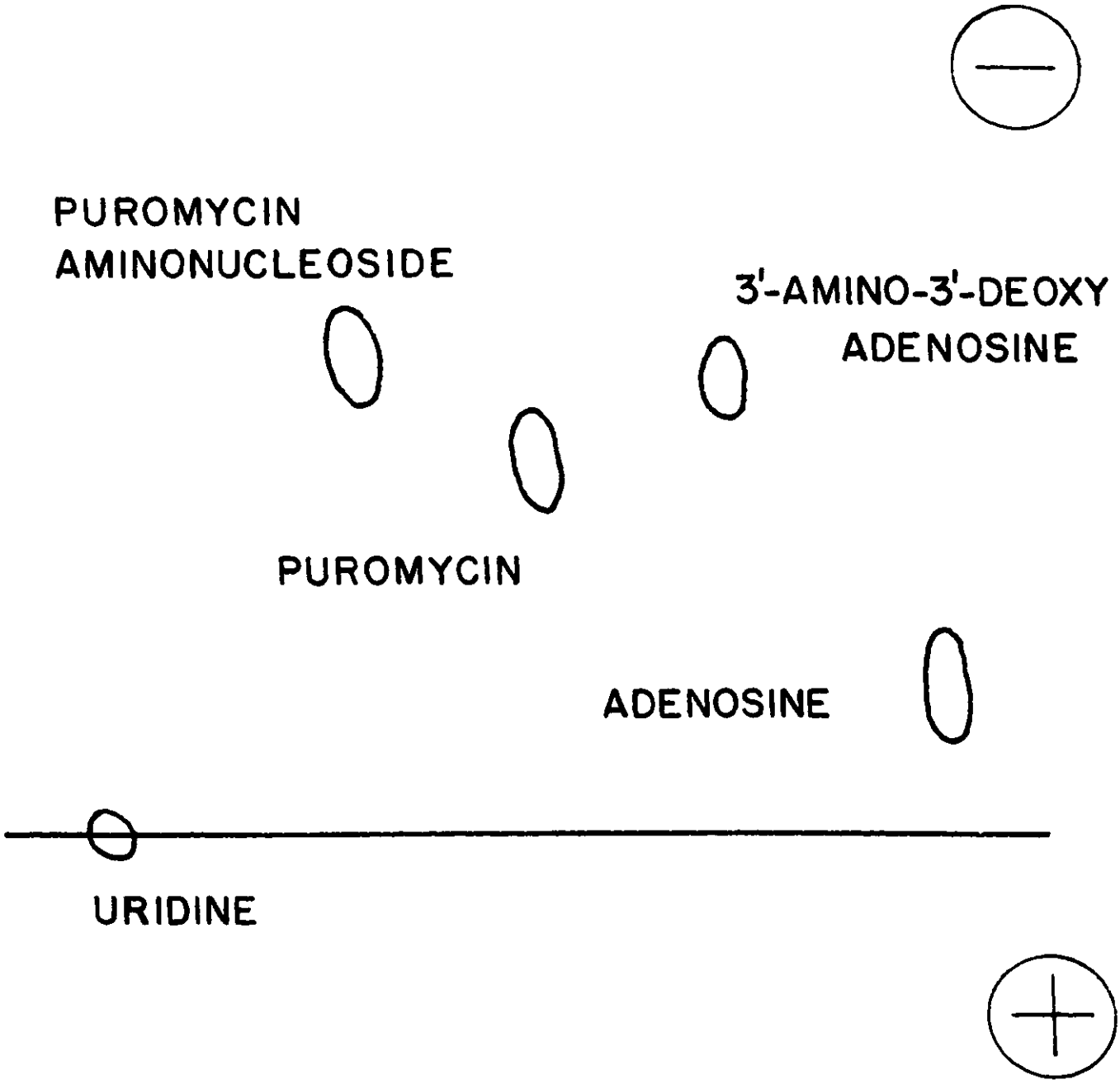
chromatography (isopropanol-concentrated $NH_4OH$-$H_2O$; 7:1:2) and thin-layer electrophoresis at pH 3.0. Figure 3 shows an electrophoreogram of this material. At pH 3.0, the amino group on 3'-amino-3'-deoxy adenosine is protonated and the compound is well separated from adenosine. Its migration rate is slightly slower than puromycin aminonucleoside, as would be expected from examination of the pK's of adenine and $N^6$, $N^6$-dimethyladenine.

In order to prepare 3'-amino-3'-deoxy ATP, 60 μmoles of 3'-amino-3'-deoxy adenosine were incubated with 8 μmoles ATP, 300 μmoles PEP, 0.1 mg pyruvate kinase (Sigma, 320 Units/mg), 10 μg rabbit muscle myokinase (Boehringer), 220 Units of adenosine kinase, 5 μmoles $MgCl_2$, 500 μmoles KCl and 1.25 ml glycerol in a total volume of 12.5 ml maintained at pH 5.8 by 7 mM Tris-maleate buffer.

After 20 hours at room temperature 3'-amino-3'-deoxy ATP was isolated from the reaction mixture by passage through either one of two different columns. In some cases the reaction mixture was applied to a 2 x 15 cm AG1-X2, (100-200 mesh) BioRad formate ion-exchange column and eluted with a 4 liter linear gradient of 0-2.2M ammonium formate, pH 4.5. The column fractions containing 3'-amino-3'-deoxy ATP were determined spectrophotometrically at 260 nm. The pH of the pooled fractions containing 3'-amino-3'-deoxy ATP was raised to 8.5 and the solution was made 50% in ethanol. The ATP analog

Figure 3: Electrophoreogram of 3'-amino-3'-deoxyadenosine and known standards.

Cellulose thin-layer electrophoresis was done in 20% acetic acid-ammonia buffer at pH 3. 0 for 75 minutes at 40 V/cm. Origin of the electrophoresis corrected for endosmosis is shown by the position of uncharged uridine. 20 nmole of each sample were applied to the cellulose thin-layer plate. After electrophoresis spots were visualized in ultraviolet light and traced.


PUROMYCIN
AMINONUCLEOSIDE
3'-AMINO-3'-DEOXY
ADENOSINE
PUROMYCIN
ADENOSINE
URIDINE

was precipitated by adding 0. 1 volume of saturated barium bromide and the precipitate recovered by low speed centrifugation. The barium salt of 3'-amino-3'-deoxy ATP was solubilized by batch treatment with the sodium form of AG 50W-X8 (Bio-Rad) at pH 6. 0. Alternatively, the reaction mixture was applied to a 1 x 5 cm AG1-X2 (100-200 mesh) chloride column and eluted with a 600 ml gradient of 0-0. 25 M LiCl containing 3 mM HCl. The pooled fractions containing 3'-amino-3'-deoxy ATP were neutralized with 1 M LiOH and reduced to 1 ml by flash evaporation. The preparation was desalted by repeated precipitation with acetone:methanol (20:1). The product was the lithium salt of 3'-amino-3'-deoxy ATP.

Preparation of 2'-amino-2'-deoxy ATP

2'-amino-2'-deoxy adenosine was chemically synthesized by Wolfrom and Winkley (Wolfrom and Winkley, 1967). The pure β isomer of 2'-amino-2'-deoxy adenosine was triphosphorylated in a reaction mixture identical to that described for the preparation of 3'-amino-3'-deoxy ATP. The product was then purified by elution from an anion-exchange column with a LiCl gradient as described in the preparation of 3'-amino-3'-deoxy ATP.

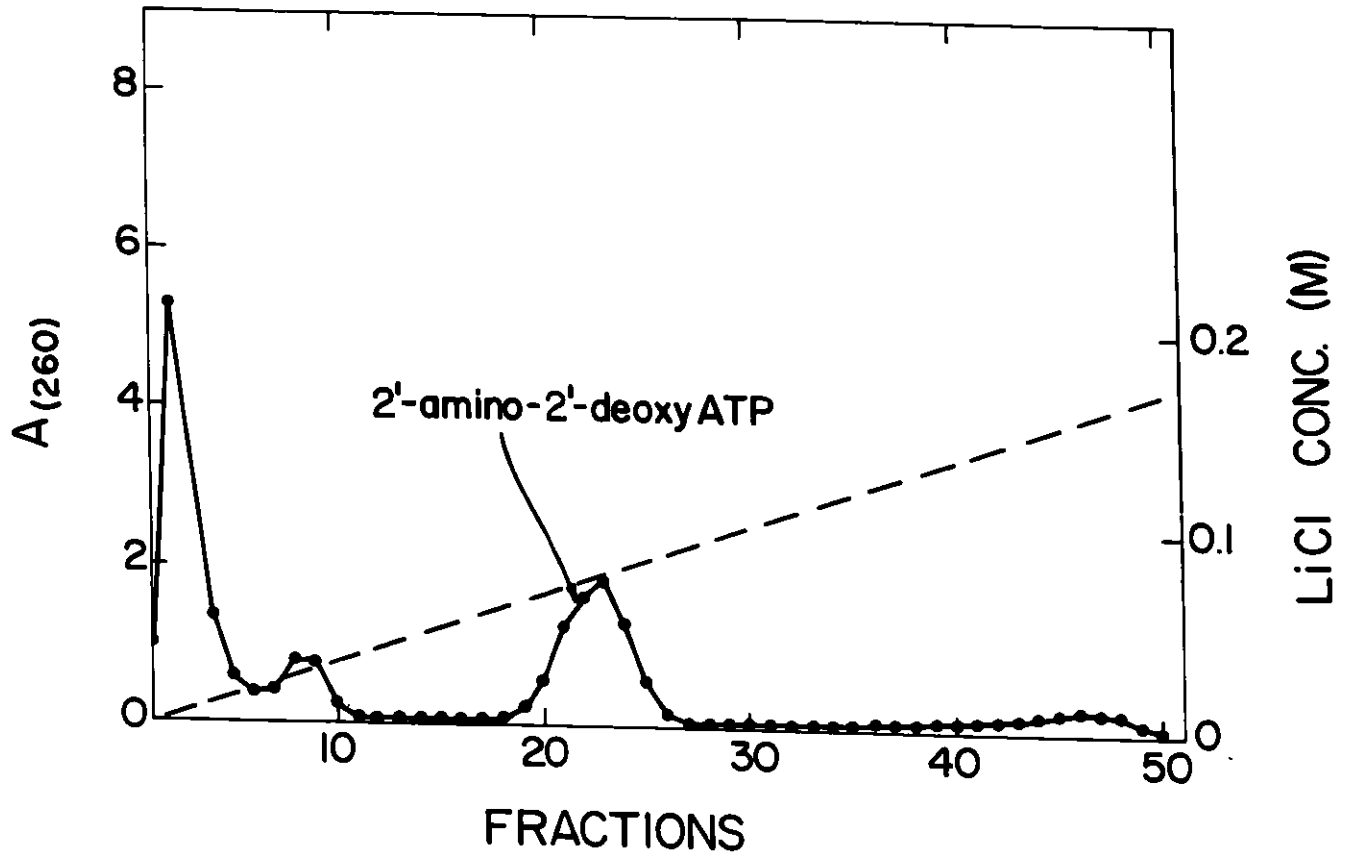
One of the more difficult problems a chemist faces in the synthesis of 2'-amino-2'-deoxy adenosine is the separation of the α-and β-isomers of the glycosidic linkage. Thus, although only a few milligrams of the pure β-isomer were available, a substantial amount of the unresolved

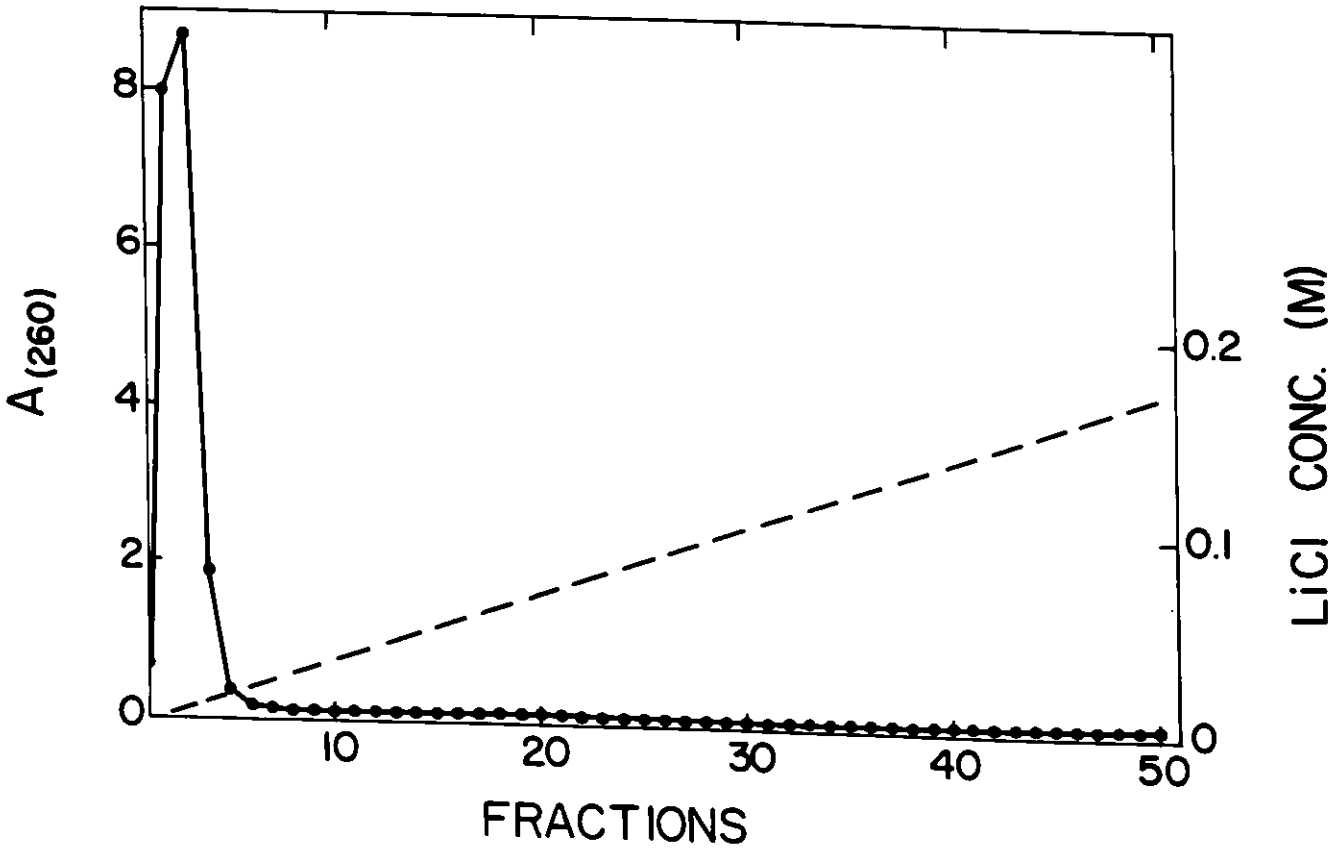
α, β isomer mixture of 2'-amino-2'-deoxy adenosine was available. It was found that the α-isomer is not a substrate for adenosine kinase, and furthermore it does not inhibit the phosphorylation of the β-isomer. This result is illustrated in Figure 4, which shows elution patterns from AG-1X2 chloride columns after incubation of either the pure α-isomer or the α, β isomeric mixture of 2'-amino-2'-deoxy adenosine with the coupled triphosphorylation system. The initial peak eluted at the beginning of the LiCl gradient is unreacted 2'-amino-2'-deoxy adenosine, which did not bind to the column at acid pH because it is positively charged. It can be seen in the top panel that when the α, β isomeric mixture of 2'-amino-2'-deoxy adenosine is used, approximately 50% of the material does not react, while the other 50% is triphosphorylated. When the pure α-isomer is used, as shown in the bottom panel, none of it is phosphorylated.

Figure 4: Elution of 2'-amino-2'-deoxy ATP from an AG1-X2 anion-exchange column.

Following incubation of the coupled triphosphorylation system, the pH of the reaction mixture was adjusted to 8.0 and it was adsorbed to a 1 x 5 cm AG1-X2 (100-200 mesh) chloride column which had been previously washed with distilled water. A 300 ml linear gradient of 0-0.25 M LiCl containing 3 mM HCl was then run through the column. The flow rate was 1 ml/min and fractions of 4 ml were collected. The dashed lines represent LiCl concentration and the solid lines are absorbance at 260 nm.

The top panel shows the elution pattern after triphosphorylation of an $\alpha,\beta$ isomeric mixture of 2'-amino-2'-deoxy adenosine. The bottom panel shows the elution pattern after attempted triphosphorylation of the pure $\alpha$-isomer of 2'-amino-2'-deoxy adenosine.


8
6
4
2
0
A(260)
2'-amino-2'-deoxy ATP
0.2
0.1
0
LiCl CONC. (M)
10
20
30
40
50
FRACTIONS


8
6
4
2
0
A(260)
0.2
0.1
0
LiCl CONC. (M)
10
20
30
40
50
FRACTIONS

Preparation of tRNA-C-C

Snake venom phosphodiesterase was used to remove the 3' terminal AMP from tRNA by a modification of the method of Miller and Philipps (1971) and Miller, Hirst-Burns, and Philipps (1970). 15 mg of E. coli B deacylated tRNA were incubated at 22 °C for 2 hours with 0.2 mg snake venom phosphodiesterase, 0.4 $\mu$moles glycine, pH 9.0, and 10 $\mu$moles $Mg(C_2H_3O_2)_2$ in a total volume of 1 ml. The snake venom phosphodiesterase was removed in either of two ways.

One deproteinization method is passage over an acid-washed silicic acid column. Two grams of silicic acid were used in the column for every milligram of protein in the reaction mixture. The experimentally determined adsorption capacity of silicic acid is 1 mg protein/gm silicic acid (Sueoka and Hardy, 1968). The tRNA passed unretarded through the column and fractions containing it were determined spectrophotometrically at 260 nm. The tRNA-containing fractions were pooled and precipitated by adding 0.1 times the volume of 20% potassium acetate and 2 times the volume of 100% ethanol. tRNA pellets were recovered after centrifugation for 15 minutes at 15,000 x g. The tRNA was then washed several times by dissolving in distilled water and reprecipitation as described above.

An alternative deproteinization method, which was used in the later stages of this work, is precipitation of the tRNA with

cetyltrimethylammonium bromide (CTAB) (Bellamy and Ralph, 1968). The insoluble cetyltrimethylammonium salt of tRNA was formed quantitatively when a two-to-fourfold excess (weight/weight) of CTAB was added. After centrifugation, the tRNA was solubilized in 1 M NaCl. The tRNA was then precipitated with ethanol and washed as described above.

It was not possible to adjust the venom phosphodiesterase reaction conditions so that all terminal AMP's and none of the penultimate CMP's are removed. The CMP's are added back in the following reaction, as described by Miller and Philipps (1971): 8 mg of venom phosphodiesterase-treated tRNA were incubated at 37°C in a volume of 2 ml with 7.7 μmoles CTP, 6 mg reduced glutathione, 25 μmoles $Mg(C_2H_3O_2)_2$, 60 μmoles KCl, 50 μgm bovine serum albumin, 100 μmoles glycine, pH 9.2, and 15 μgm purified tRNA-nucleotidyl transferase (EC 2.7.7.25) from E. coli. Following a 60 minute incubation, the mixture was deproteinized and washed as described above.

Preparation of tRNA-C-C-3'$A_N$ and tRNA-C-C-2'$A_N$

The reaction mixture for the preparation of tRNA-C-C-3'$A_N$ and tRNA-C-C-2'$A_N$ contained in 1 ml: 1.30 nm tRNA-C-C, 10 μgm purified E. coli tRNA-nucleotidyl transferase, 10 mg reduced glutathione, 0.3 mg bovine serum albumin, 15 μmoles $Mg(C_2H_3O_2)_2$,

60 μmoles potassium chloride, 50 μmoles glycine, pH 9.2 and 1.25 μmoles 3'-amino-3'-deoxy ATP or 2'-amino-2'-deoxy ATP. The reaction mixture was incubated for 75 min at 37°C. It was deproteinized either by passage through a silicic acid column or by CTAB precipitation as described in the preparation of tRNA-C-C. The tRNA was then washed by ethanol precipitation prior to storage at -20°C.

Growth of cells

Ribosomes, S-100 and initiation factors were isolated from either of two ribonuclease I deficient strains of E. coli. The first, D10, was originally isolated from its parent strain, AB301 (Hfr met$^-\lambda^+$), by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (Gesteland, 1966). The second, MRE600, is a naturally occurring strain isolated by Commack and Wade (1965).

The stock cells were kept on Dorset Egg Slopes (Difco). Large amounts of cells were grown in Luria Broth (10 gms/liter Tryptone, 5 gms/liter yeast extract, 5 gms/liter sodium chloride, 1 mM NaOH) which had been previously autoclaved. Thirteen liters of Luria Broth in a Microferm Laboratory fermentor (New Brunswick Scientific) were innoculated with 1 liter of an overnight growth of D10 cells in Luria Broth. The cells were grown to late logarithmic phase with constant mixing and aeration at 37°C. The cells were cooled to approximately 10°C when the $OD_{550}$ reached 3.5, harvested by

centrifuging the medium through a Sharples continuous flow centrifuge, frozen in liquid nitrogen and stored at -20°C. Yields ranged from 50-60 gms of cells.

Preparation of RNA-free S-100

All steps took place at 4°C. Frozen E. coli cells were broken open by grinding with 2.5 times their weight of alumina in a precooled mortar and pestle. The resulting paste was extracted with a minimal volume of buffer A (10 mM Tris-HCl, 10 mM $NH_4Cl$, 10 mM $Mg(C_2H_3O_2)_2$ and 6 mM β-mercaptoethanol, pH 7.8) to which 1 μgm/ml of electrophoretically purified DNAase (Worthington) was added. The alumina was removed by centrifugation at 40,000 x g for 20 min. The paste was extracted three times and the supernatants pooled. Ribosomes were pelleted by centrifugation for 2 hours at 260,000 x g. The upper two-thirds of the supernatant were removed and dialyzed against Buffer B (20 mM Tris-HCl, 1 mM $Mg(C_2H_3O_2)_2$, 2 mM β-mercaptoethanol, 10% (v/v) glycerol, pH 7.8). RNA was removed on a DE-52 (Whatman) column essentially according to Bretscher (1968). The dialyzed solution was made 200 mM in potassium chloride and 5 to 10 ml passed through a 1 x 15 DE-52 column which had previously been equilibrated with Buffer B containing 200 mM potassium chloride. The first two or three 2 ml fractions of material absorbing at 280 nm (void volume is approximately 10 ml) were pooled and dialyzed against Buffer B. The dialyzed RNA-free S-100 was divided in aliquots, frozen

in liquid nitrogen and stored at -90°C. A typical preparation had a protein concentration of 12.5 mg/ml and an $OD_{280}/OD_{260}$ ratio of 1.70 (approximately 0.1% nucleic acid). This RNA free S-100 was the source of aminoacyl-tRNA synthetase activity for all 20 amino acids, EF-Tu, EF-Ts and EF-G.

Preparation of phenylalanyl-tRNA-C-C-3'$A_N$ (Phe-3'N-tRNA) and phenylalanyl-tRNA

The charging reaction contained the following components in a volume of 1 ml: 0.107 μmoles ($^{14}$C)phenylalanine (NEN, spec. act. 472 mCi/mM), 1.5 mg 3'-amino-3'-deoxy tRNA, 0.42 mg RNA-free S-100, 4 μmoles dATP, 120 μmoles KCl, 20 μmoles $Mg(C_2H_3O_2)_2$, 20 μmoles β-mercaptoethanol and was buffered at pH 7.5 with 100 mM Tris-HCl. After incubation for 45 minutes at 37°C, the reaction mixture was deproteinized by passage over a silicic acid column, precipitated and washed by ethanol precipitation.

Normal phenylalanyl-tRNA was prepared essentially as described above, except that deacylated E. coli B tRNA was used instead of 3'-amino-3'-deoxy tRNA.

Preparation of acetyl phenylalanyl-tRNA-C-C-3'$A_N$ (AcPhe-3'N-tRNA) and acetylphenylalanyl-tRNA

AcPhe-3'N-tRNA was prepared essentially according to deGroot et al., (1966). The reaction mixture contained in 2 ml: 5.0 nanomoles ($^{14}$C) Phe-3'N-tRNA (in addition to all other uncharged tRNAs),

70 mg acetic acid N-hydroxysuccinimide ester, 0.4 ml 100 mM ammonium acetate buffer, pH 5.0, and 1.6 ml dimethylformamide (DMF). The incubation mixture was shaken on a wrist action shaker for 12-16 hours at room temperature (22°C). The acetylated Phe-3'N-tRNA was isolated by centrifugation at 40,000 x g for 20 minutes. The precipitate was washed three times with 3 ml of DMF and once with 3 ml of 100% ethanol.

Aminoacyl-tRNA which is acetylated by this method has been reported to bind an acetyl group only on the α-amino group of the amino acid. This is in contrast to acetylation by an alternative reaction with acetic anhydride (Haenni and Chapeville, 1966) in which it has been reported that only 50% of the acetyl groups attached to phenylalanyl-tRNA are bound to phenylalanine (deGroot et al., 1966).

Normal AcPhe-tRNA was acetylated by the same method described above. In order to determine whether acetylation was complete, Ac($^{14}$C) Phe-tRNA was incubated with pancreatic ribonuclease and subjected to electrophoresis at pH 2.7. Figure 5 shows the distribution of radioactivity on a cellulose thin layer plate after electrophoresis of the ribonuclease digest. Separately prepared Phe-adenosine was run as a marker.

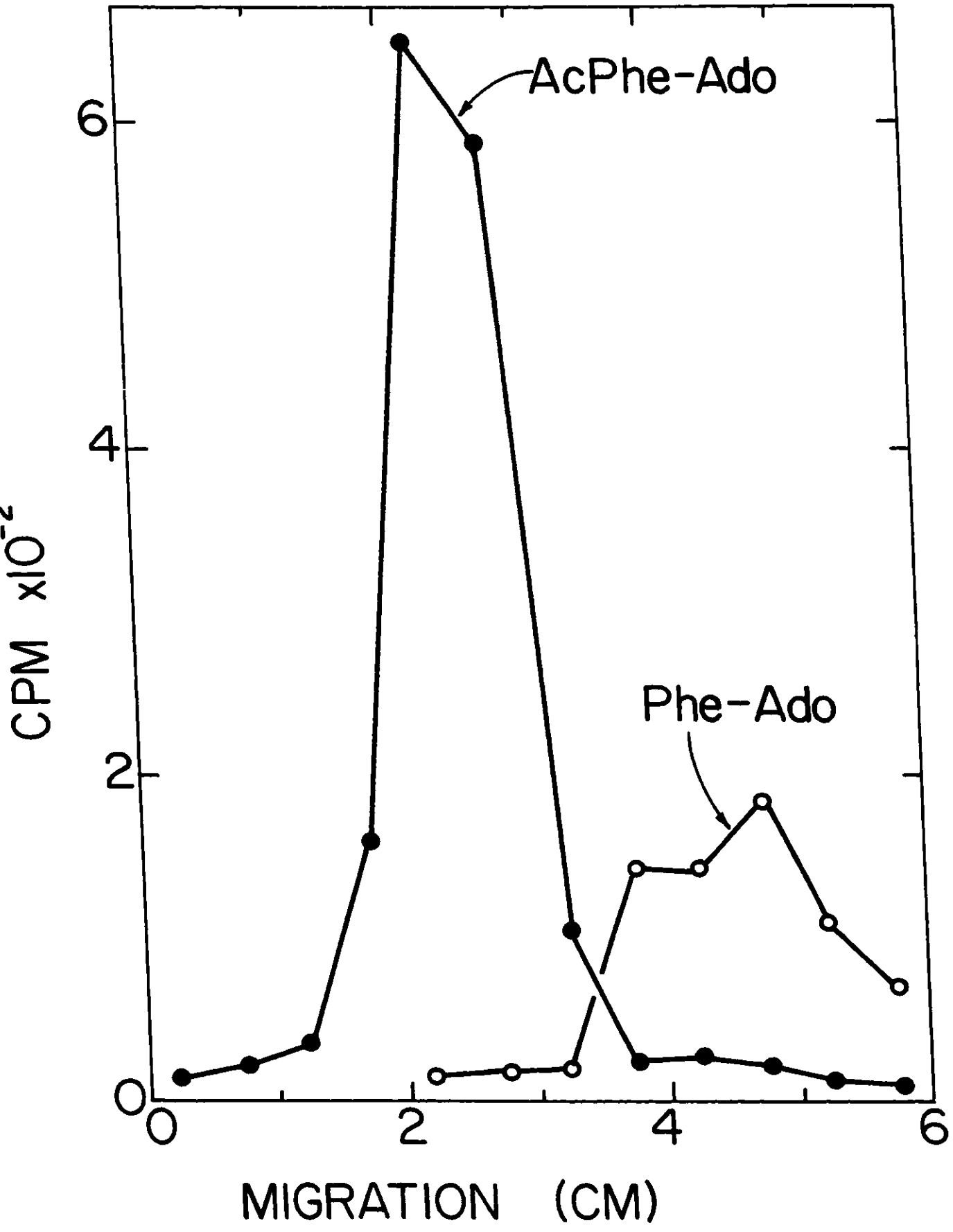
It can be seen that there is no contamination of the AcPhe-tRNA preparation with unreacted Phe-tRNA.

In some cases the Ac($^{14}$C) Phe-tRNA was purified on a BD-cellulose

column by the method of Lucas-Lenard and Haenni (1969). A 3 ml column was equilibrated with Buffer Z (10 mM $Mg(C_2H_3O_2)_2$ and 50 mM $K(C_2H_3O_2)$, pH 5.0) containing 40 mM NaCl. The Ac($^{14}$C) Phe-tRNA sample (1000 pmoles) in Buffer Z was made 0.4M in NaCl and adsorbed to the column. The column was eluted with 1M NaCl and 4.5% ethanol in Buffer Z until the absorbance at 260nm was zero. Elution was then continued with 1M NaCl and 12% ethanol in Buffer Z. Ac($^{14}$C) Phe-tRNA was eluted in this step and was concentrated by ethanol precipitation. The Ac($^{14}$C) Phe-tRNA was stored in distilled water at -20°C.

Figure 5: Electrophoretic separation of Acetylphenylalanyl-adenosine and Phenylalanyl-adenosine

($^{14}$C) Phe-tRNA and Ac($^{14}$C) Phe-tRNA were prepared as described in Methods and incubated separately at 37°C in 50 mM $NH_4(C_2H_3O_2)$, pH 5.5, with 500 $\mu$g/ml of pancreatic RNAase. After 15 minutes the reaction mixtures were precipitated in ethanol and centrifuged at 15,000 x g for 15 minutes. The supernatants were analyzed by thin-layer electrophoresis. The electrophoresis was done in 20% acetic acid-ammonia buffer at pH 2.7 for 1 hour at 34 V/cm. After drying, the cellulose thin layer plates were scraped and the radioactivity measured in a liquid scintillation counter. Phe-Ado is ($^{14}$C) Phenylalanyl-adenosine and AcPhe-Ado is ($^{14}$C) Acetylphenylalanyl-adenosine.


AcPhe-Ado
Phe-Ado
CPM x10⁻²
0
2
4
6
0
2
4
6
MIGRATION (CM)

Salt-washed ribosomes

The method used for the preparation of salt-washed ribosomes is essentially that of Anderson et al. (1967). All steps were done at 4°C. Frozen E. coli cells were broken open, extracted and subjected to high speed centrifugation exactly as described in the preparation of RNA-free S-100. The pellets from the high speed centrifugation were resuspended in Buffer C (10 mM Tris-HCl, pH 7.8, 10 mM $Mg(C_2H_3O_2)_2$, 1.0 M $NH_4Cl$, 6 mM β-mercaptoethanol) by gently shaking overnight on a wrist action shaker. After resuspension aggregated ribosomes and denatured proteins were removed by centrifugation at 40,000 x g for 20 minutes. The ribosomes were then pelleted at 230,000 x g for 3 hours. The supernatant was saved and used to prepare initiation factors. The ribosomal pellet was again resuspended in Buffer C and centrifuged at 40,000 x g. The ribosomes were repelleted at 230,000 x g for 1.5 hours and the supernatant was discarded. After one more high salt wash, the ribosomal pellet was resuspended in a small volume of Buffer A (see S-100 preparation). An equal volume of glycerol was added and the ribosomes stored at -20°C at a concentration greater than 1000 $A_{260}$/ml. Under these conditions ribosomes retained full activity in poly(U) directed polyphenylalanine synthesis, aminoacyl tRNA binding and initiation factor directed AcPhe-tRNA binding for up to 6 months.

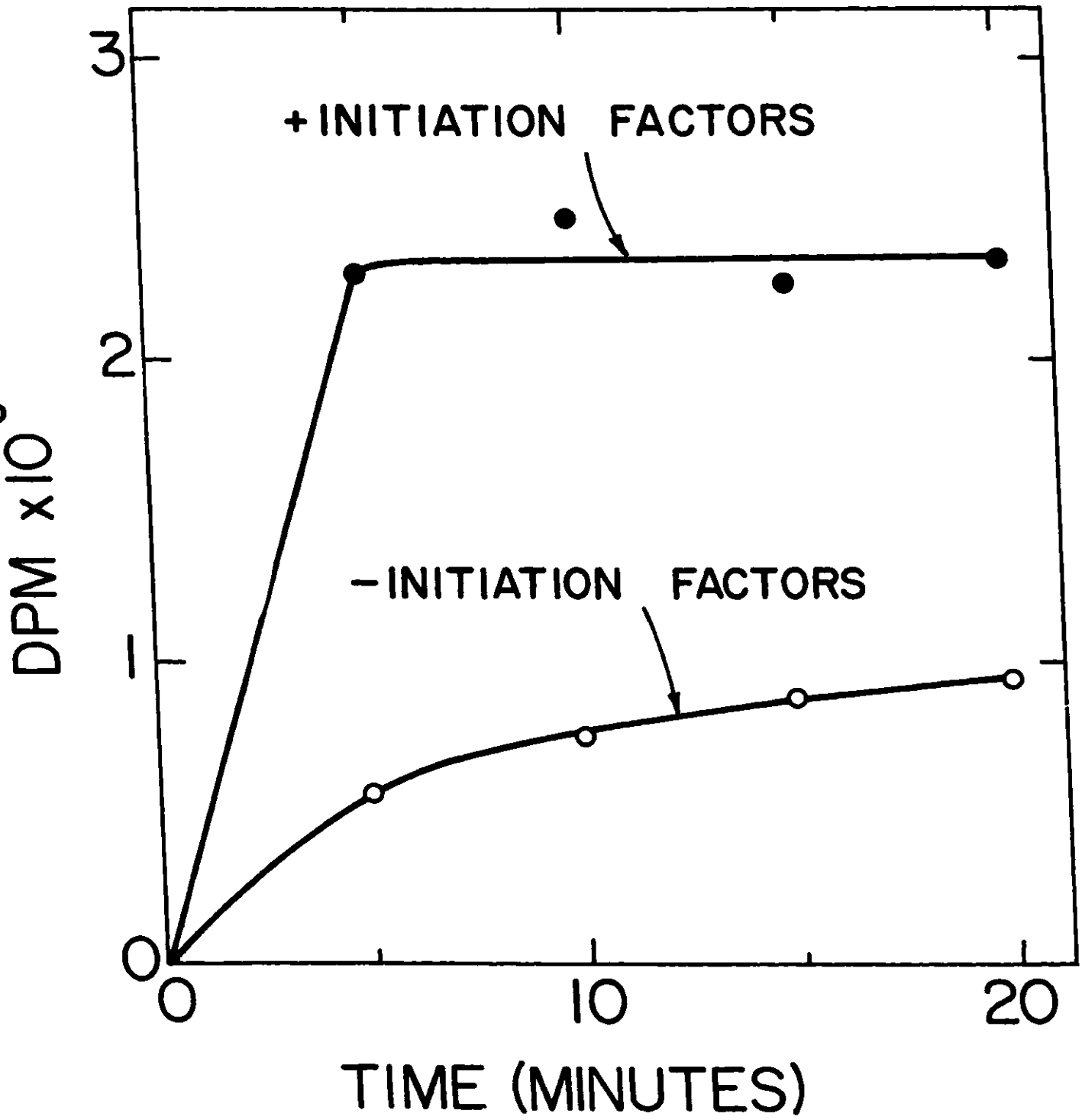
Initiation factors

All steps were performed at 4°C. The first high salt wash supernatant from the preparation of salt-washed ribosomes was further purified by ammonium sulfate fractionation to yield crude initiation factors. The wash was adjusted to 35% ammonium sulfate saturation (194 gm/ l ) by slow addition of solid ammonium sulfate. The resulting suspension was centrifuged, at 30,000 x g for 15 minutes, and the pellet discarded. An additional 291 gm/l of solid ammonium sulfate was slowly added to bring the final concentration to 80% saturation. The solution was centrifuged at 40,000 x g for 30 minutes, and the supernatant discarded. The pellet was dissolved in a small volume of Buffer D (10 mM Tris-HCl, 0.5 mM EDTA, 6 mM β-mercaptoethanol, and 10% glycerol, pH 7.8) and dialyzed against Buffer D. The crude initiation factors were then divided into aliquots, frozen in liquid nitrogen and stored at -90°C.

This preparation of crude initiation factors was shown to have activity in stimulating the poly(U) directed binding of AcPhe-tRNA to ribosomes. It has been known for some time that at low $Mg^{2+}$ concentrations this process requires initiation factor activity (Lucas-Lenard and Lipmann, 1967). Figure 6 compares the time-courses of poly(U) directed binding of AcPhe-tRNA to ribosomes with and without crude initiation factors added. It can be seen that the initiation factor preparation increases the rate as well as the extent of AcPhe-tRNA binding.

Figure 6: Dependence on added initiation factors of poly(U) directed binding of AcPhe-tRNA to ribosomes.

The assay incubation mixture contained in 1 ml:27.4 $A_{260}$ units of salt-washed ribosomes, 80 $\mu$g poly(U) (Sigma), 140 pmoles purified Ac($^{14}$C) Phe-tRNA (specific activity 466 $\mu$Ci/$\mu$M), 833 $\mu$g crude initiation factors and 0.2 $\mu$mole GTP. The mixture was buffered with 50 mM Tris-HCl, pH 7.2, and contained 80 mM $NH_4Cl$, 6 mM $\beta$-mercaptoethanol and 8 mM $Mg(C_2H_3O_2)_2$. After incubation at 37° for the indicated times 50 $\mu$l aliquots were removed and added to 3 ml of wash buffer (50 mM Tris-HCl, pH 7.2, 80 mM $NH_4Cl$, 8 mM $Mg(C_2H_3O_2)_2$ and 6 mM $\beta$-mercaptoethanol) which was immediately filtered through a nitrocellulose filter (Millipore Corp.). This was then washed with 10 ml of wash buffer and after drying counted in a liquid scintillation counter.


+INITIATION FACTORS
-INITIATION FACTORS
DPM x10^-3
TIME (MINUTES)
0
1
2
3
0
10
20

# RESULTS

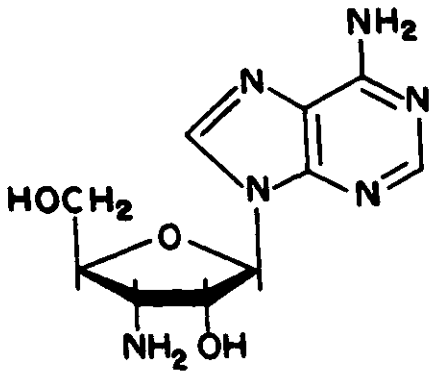
Preparation of 3'-amino-3'-deoxy ATP

3'-amino-3'-deoxy adenosine must be triphosphorylated to 3'-amino-3'-deoxy ATP in order to be a substrate for tRNA-nucleotidyl transferase and become incorporated at the 3' end of tRNA-C-C.

The preparation of 3'-amino-3'-deoxy ATP is outlined in Figure 7. 3'-amino-3'-deoxy adenosine was incubated with adenosine kinase, myokinase, pyruvate kinase, ATP and phosphoenolpyruvate as described in the "Methods" section. The reaction was carried out at pH 5.8 because this is the pH optimum of rabbit liver adenosine kinase when Tris-maleate is used as a buffer (Lindberg, Klenow and Hanson, 1967). Although this pH is optimal for adenosine kinase, pyruvate kinase has low activity under these conditions. A large excess of pyruvate kinase was found to be required in order to obtain a substantial yield of triphosphate product in this coupled system. The crude adenosine kinase isolated from rabbit liver has substantial myokinase activity, and it is not absolutely necessary to add myokinase to the reaction mixture, although this was done to insure the highest possible yields.

After incubation overnight at room temperature, the entire reaction mixture was applied to an anion-exchange column. For a typical reaction involving 60 micromoles of 3'-amino-3'-deoxyadenosine this column was a 2 x 15 cm AG1-X2 (100-200 mesh) formate column. After the reaction mixture was applied, the column was washed

Figure 7: Outline of the steps taken to convert 3'-amino-3'-deoxyadenosine to 3'-amino-3'-deoxy ATP.




3'-AMINO-3'-DEOXY ADENOSINE

↓ ADENOSINE KINASE

3'-AMINO-3'-DEOXY AMP

↓ MYOKINASE

3'-AMINO-3'-DEOXY ADP

↓ PYRUVATE KINASE

3'-AMINO-3'-DEOXY ATP

with 50 ml of water and then eluted with a linear gradient of 0-2.2M ammonium formate, pH 4.5.

The top panel in Figure 8 shows the elution pattern of products from the AG1-X2 formate column following conversion of adenosine to ATP in the coupled triphosphorylation system. Adenosine, AMP, ADP and ATP are all well resolved by this column. It can be seen that the bulk of the product is ATP, but significant amounts of ADP and AMP are also present in the product mixture. The driving force for formation of ATP is the splitting of phosphoenolpyruvate. Accordingly, a large excess of phosphoenolpyruvate is required in the reaction mixture in order to drive it as far as possible to completion and minimize the mono- and diphosphate products.

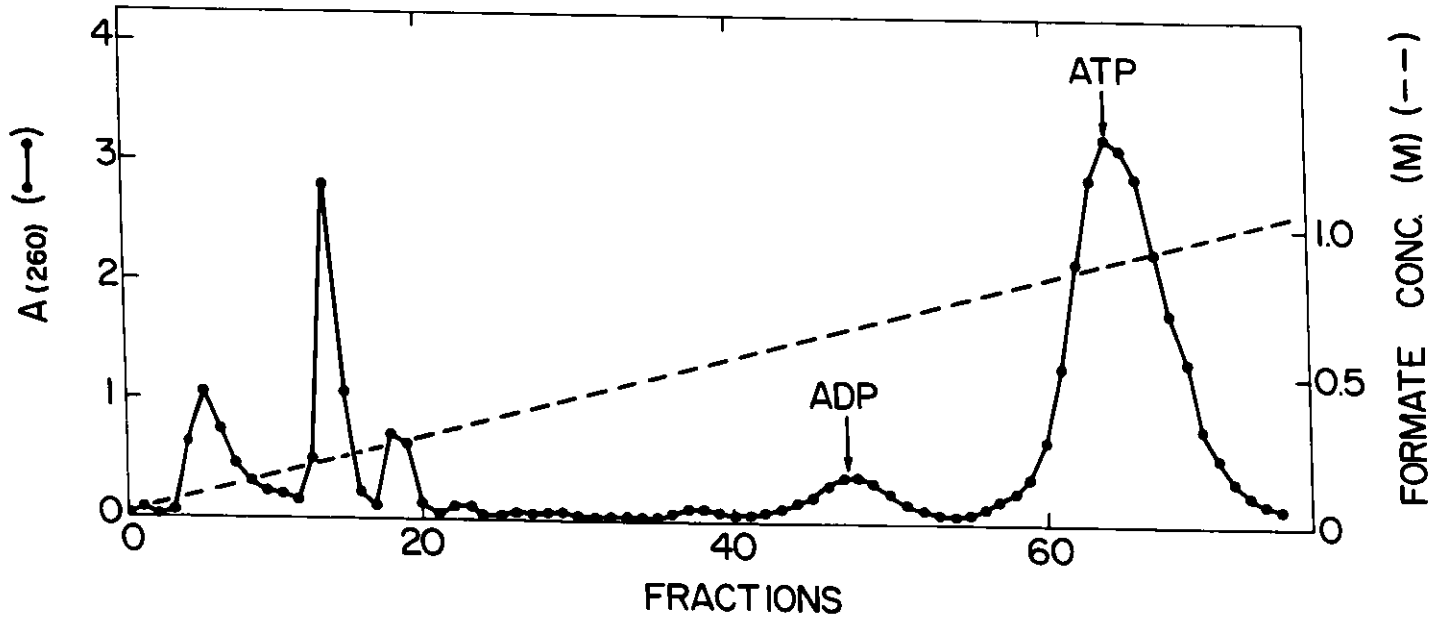
The bottom panel in Figure 8 shows the elution patterns of the product mixture from the AG1-X2 formate column after synthesis of 3'-amino-3'-deoxy ATP from 3'-amino-3'-deoxyadenosine. The large peak eluting at approximately 0.55M formate concentration has been identified as 3'-amino-3'-deoxy ATP. It can be seen by comparison with the top panel that normal ADP also elutes at approximately the same formate concentration. This occurs because the 3'-amino group on the analog ATP is protonated at acid pH and the molecules will have the same net charge as ADP.

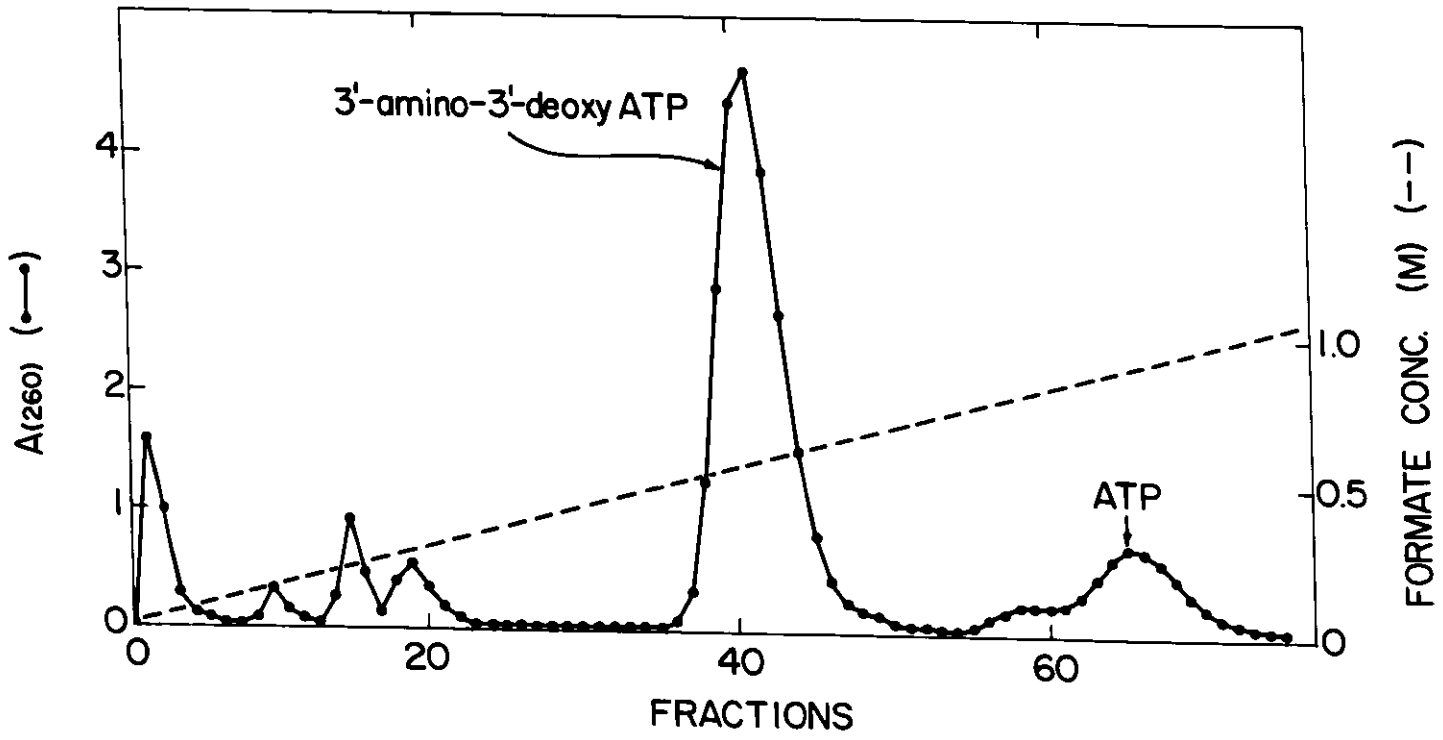
The product of the triphosphorylation reaction was characterized as 3'-amino-3'-deoxy ATP by a number of criteria. An important

Figure 8: Elution of phosphorylated derivatives of adenosine and 3'-amino-3'-deoxyadenosine from an AG1-X2 anion-exchange column.

Following incubation of the coupled triphosphorylation system, the reaction mixtures were applied to a 2 x 15 cm AG1-X2 (100-200 mesh) formate ion-exchange column. After washing the column with 50 ml of distilled water the products were eluted with a 4 liter linear gradient of 0-2.2M ammonium formate, pH 4.5. The flow rate was 5 ml/min and fractions of 25 ml were collected. The dashed lines represent ammonium formate concentration and the solid lines are absorbance at 260 nm.

The top panel shows the elution pattern after triphosphorylation of adenosine. The bottom panel shows the elution pattern after triphosphorylation of 3'-amino-3'-deoxyadenosine. The products were identified by polyethyleneimine thin-layer chromatography (see Figure 9).


A(260) (—)
FORMATE CONC. (M) (– –)
ATP
ADP
FRACTIONS
0
20
40
60
0
1
2
3
4
0
0.5
1.0


3'-amino-3'-deoxy ATP
A(260) (—)
FORMATE CONC. (M) (– –)
ATP
FRACTIONS
0
20
40
60
0
1
2
3
4
0
0.5
1.0

piece of evidence was that this material would act as a phosphate donor in the myokinase reaction:

$$ATP + AMP \xrightarrow[\text{myokinase}]{} 2ADP$$

This reaction can be assayed spectrophotometrically at 340nm if pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate and NADH are present. This sequence of reactions is described in the legend to Figure 2. It was found that addition of putative 3'-amino-3'-deoxy ATP and no AMP to this coupled assay system led to some oxidation of NADH. This indicated that the preparation was contaminated by a small amount of ADP, which would be expected from the similarity of the column elution positions of ADP and 3'-amino-3'-deoxy ATP. When AMP was subsequently added to the system, a substantial amount of NADH oxidation occurred. This result indicated that a phosphate donor such as ATP, or an ATP analog, was a major component of the preparation.

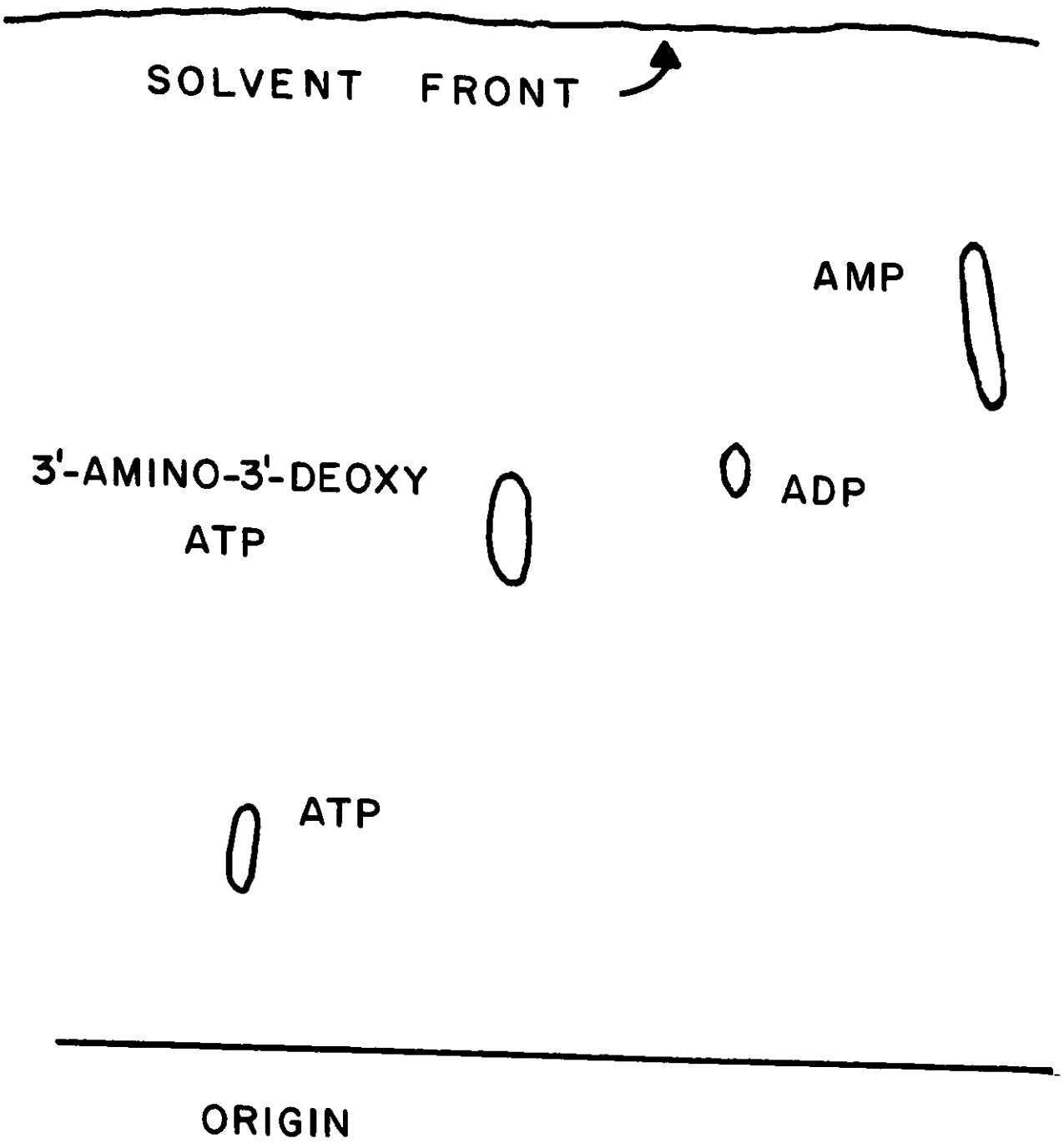
When analyzed by polyethyleneimine (PEI) thin-layer chromatography (Randerath and Randerath, 1967) at acid pH the product migrated with an $R_f$ slightly less than that of ADP. Figure 9 shows the migration of the product compared with ATP, ADP and AMP. The 3'-amino-3'-deoxy ATP preparation has no minor components that are resolvable on the PEI-cellulose plate.

These results suggest that 3'-amino-3'-deoxy ATP is the product

in the coupled triphosphorylation system. They further indicate that while there is probably some ADP contamination, there is no detectable ATP contamination of the analog ATP.

Figure 9: Polyethyleneimine thin-layer chromatography of ATP, ADP, AMP and 3'-amino-3'-deoxy ATP.

Polyethyleneimine thin-layer plates (EM laboratories, Inc.) were developed at room temperature (22°C) in 0.5M LiCl, 1M HCOOH. 20 nmoles AMP, 28 nmoles 3'-amino-3'-deoxy ATP and 10 nmoles of both ADP and ATP had been applied at the origin. After development the spots were visualized in ultraviolet light and traced.


SOLVENT FRONT
AMP
3'-AMINO-3'-DEOXY
ATP
ADP
ATP
ORIGIN

Incorporation of 3'-amino-3'-deoxy AMP into tRNA

Figure 10 outlines the synthesis of Phe-tRNA-C-C-3'$A_N$ (Phe-3'N-tRNA) from tRNA-C-C-A. In order to substitute the 3'-amino-3'-deoxy AMP at the 3' end of tRNA, the terminal AMP must first be removed. This is accomplished by incubation of tRNA with snake venom phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 2. 1. 4. 1). This is a 3'-exonuclease which consecutively hydrolyzes the tRNA to 5'-mononucleotides (Razzell and Khorana, 1959; Nihei and Cantoni, 1963). Using tRNA as substrate it has been found that the 3' terminal AMP is released four times faster than the first CMP and 65 times faster than the second CMP of the C-C-A end (Miller et al., 1970).

Removal of the terminal AMP was monitored, after deproteinization of the reaction mixture, by aminoacylation of the tRNA. The time and temperature of SVD treatment were adjusted until no amino acid accepting activity remained in the tRNA. This indicated that all of the terminal AMP's had been removed by the SVD. Under these conditions, however, a significant amount of the penultimate CMP was also removed. This CMP removal was assayed by incubation of a sample of the digested tRNA with E. coli tRNA-nucleotidyl transferase and ($^3$H) CTP. If CMP had been removed by SVD treatment, the tRNA-nucleotidyl transferase would catalyze incorporation of ($^3$H) CMP into the tRNA. It was found that conditions could not be adjusted

Figure 10: Outline of the steps taken to convert tRNA-C-C-A to Phe-tRNA-C-C-3'$A_N$.

tRNA-C-C-A

↓ SNAKE VENOM PHOSPHODIESTERASE

tRNA-C, tRNA-C-C

↓ CTP, tRNA-NUCLEOTIDYL TRANSFERASE

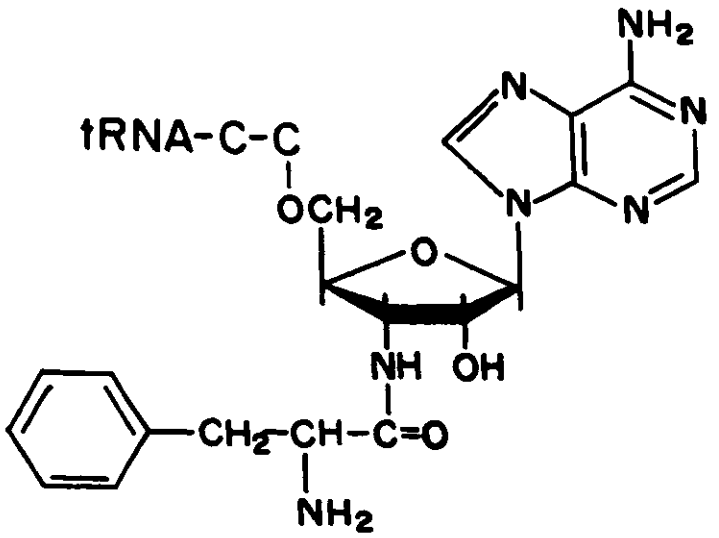
tRNA-C-C

↓ 3'-AMINO-3'-DEOXY-ATP, tRNA-NUCLEOTIDYL TRANSFERASE

tRNA-C-C-3'$A_N$

↓ PHENYLALANYL-tRNA SYNTHETASE

tRNA-C-C-3'$A_N$-Phe



so that all of the terminal AMP and none of the penultimate CMP were removed. SVD-treated tRNA preparations were therefore incubated with unlabeled CTP and tRNA-nucleotidyl transferase to synthesize homogeneous tRNA-C-C. If this material was subsequently incubated with ($^3$H) CTP and tRNA-nucleotidyl transferase, no radioactivity was incorporated into the tRNA. If ($^3$H) ATP was used instead of ($^3$H) CTP, radioactivity was incorporated into the tRNA. Calculations show that approximately 80% of the molecules (determined spectrophotometrically) in a homogeneous tRNA-C-C preparation accept an AMP.

It is, however, not necessary to prepare homogeneous tRNA-C-C in order to add a terminal AMP. If tRNA-C is incubated with CTP and ATP along with tRNA-nucleotidyl transferase, normal tRNA-C-C-A will be synthesized by the enzyme. The presence of CTP, however, inhibits the rate of incorporation of AMP into tRNA-C-C (Miller and Philipps, 1971; Carre et al., 1970). Because the 3'-amino ATP analog was not a good substrate for tRNA-nucleotidyl transferase under normal conditions, the rate of incorporation of the 3'-amino AMP had to be optimized. Accordingly, it was decided that no CTP should be added to the incubation mixture when 3'-amino-3'-deoxy AMP was incorporated into tRNA. This decision necessitated the use of homogeneous tRNA-C-C to obtain satisfactory yields.

Initial experiments measuring the incorporation of 3'-amino AMP

into tRNA were done with ($^{32}$P) 3'-amino-3'-deoxy ATP. This material was prepared using the same coupled reaction system described for the preparation of unlabeled 3'-amino-3'-deoxy ATP. If ( $\gamma$ - $^{32}$P) ATP is used in this system, the labeled phosphate is incorporated into both the $\alpha$ and $\beta$ phosphate positions of 3'-amino-3'-deoxy ATP. The $\gamma$ phosphate of 3'-amino-3'-deoxy ATP comes from phosphoenolpyruvate and does not become labeled. When the ($\alpha$,$\beta$-$^{32}$P) 3'-amino-3'-deoxy ATP is used as a substrate for tRNA-nucleotidyl transferase, the 3'-amino-3'-deoxy AMP incorporated into the tRNA is labeled with ($^{32}$P). Thus, incorporation into tRNA could be monitored directly by counting ($^{32}$P) label. If the ($^{32}$P) labeled tRNA was isolated and incubated with ($^{3}$H) ATP and tRNA-nucleotidyl transferase, some ($^{3}$H) AMP was incorporated into the tRNA. This ($^{3}$H) AMP will only be added to tRNA-C-C molecules which have not previously had a 3'-amino-3'-deoxy AMP added to them. Although tRNA-nucleotidyl transferase is able to catalyze the removal as well as the addition of AMP or CMP to tRNA (Hecht et al., 1958), it does not catalyze removal under the conditions used for these assays. As expected, ($^{32}$P) and ($^{3}$H) incorporations were inversely related; that is, if the tRNA had high incorporation of ($^{32}$P) (i. e. 3'-amino-3'-deoxy AMP), then subsequent incorporation of ($^{3}$H) AMP was low. The complementarity of the ($^{32}$P) and ($^{3}$H) incorporation data indicated that the ($^{32}$P) incorporation was an accurate measure of the

3'-amino-3'-deoxy AMP added to tRNA-C-C while the ($^{3}$H) incorporation reflected the amount of tRNA-C-C to which nothing had been added during the original incubation. In subsequent preparations, ($^{32}$P) label was not used, and incorporation of 3'-amino-3'-deoxy AMP into tRNA-C-C was determined by ($^{3}$H) AMP addition to the tRNA-C-C in the product tRNA mixture.

Preparation of Phe-3'-N-tRNA

When tRNA-C-C-3'$A_N$ and labeled phenylalanine were incubated with RNA-free S-100 containing phenylalanyl-tRNA synthetase, the phenylalanine became attached to the tRNA analog as indicated by TCA precipitability. The structure of Phe-3'N-tRNA shown in Figure 10 is the chemically feasible structure, regardless of the initial attachment site (2' or 3') of the amino acid to the 3' terminal adenosine. The equilibration half-time for isomerization of an amino acid between the 2' and 3' cis-hydroxyl groups in aminoacyl-tRNA has been estimated as 0.2 millisecond (Griffin et al., 1966). The increased nucleophilicity of an amino group relative to an hydroxyl group suggests that this acyl migration might occur more readily with the 3'-amino tRNA than with normal tRNA. Once an amide bond has been formed between the amino acid and 3'-amino tRNA, the amino acid will no longer be able to isomerize due to the stability of this bond. Thus, the amino acid should always be found attached to the 3'-amino group. Evidence that this is the case was obtained in the following experiments.

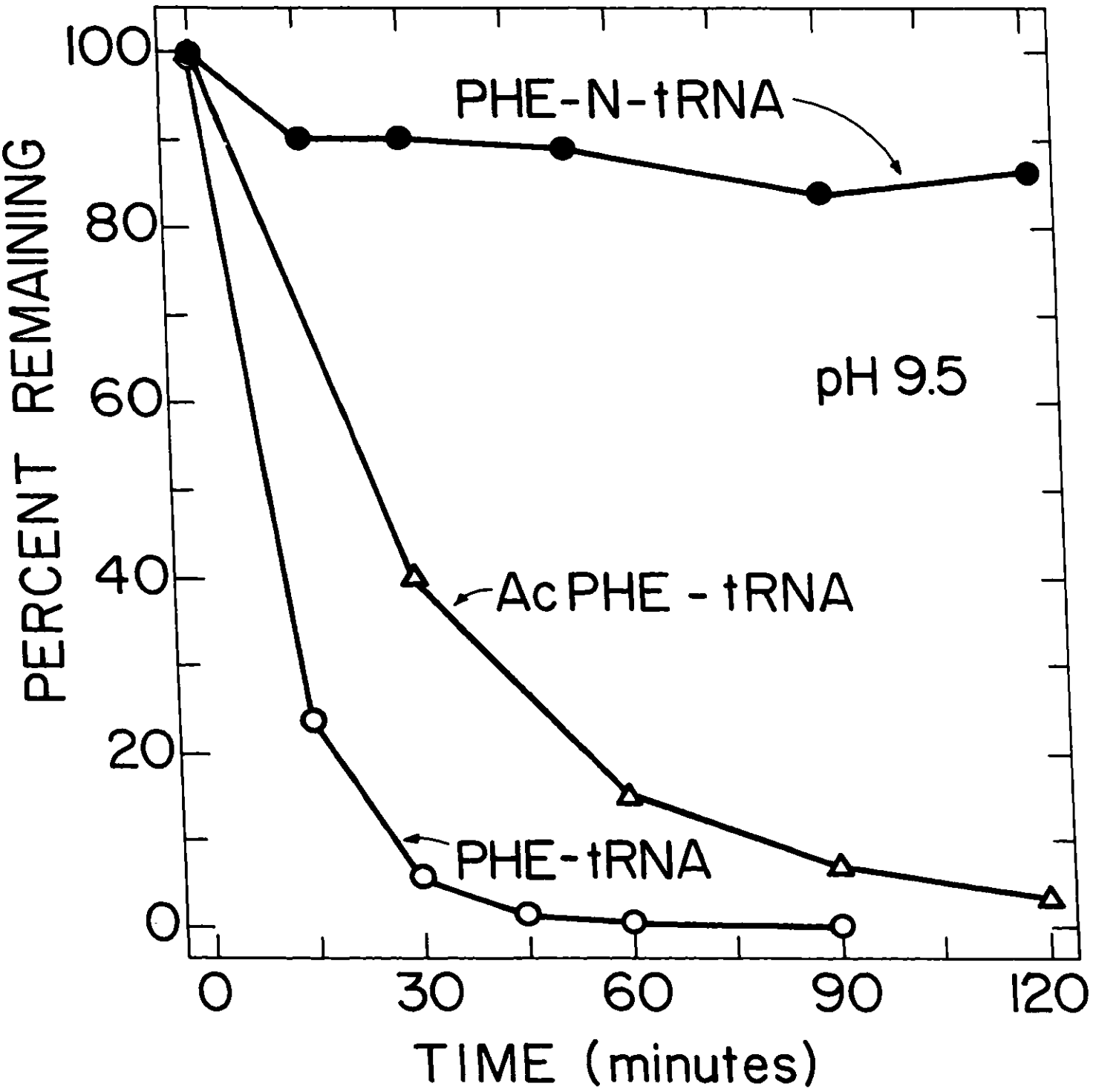
Figure 11 shows the base catalyzed hydrolysis of both Phe-3'N-tRNA and normal Phe-tRNA. On incubation at pH 9.5 and 37°C, the ester bond in Phe-tRNA is hydrolyzed with a half-life of 7 minutes. In contrast to this, the phenylalanine attached to tRNA-C-C-3'$A_N$ is largely resistant to hydrolysis. During the first 15 minutes, about 10% of the phenylalanine was no longer precipitable by TCA, but after that the amount of base-resistant Phe-tRNA analog did not change appreciably. This initial decrease varied with different preparations of tRNA-C-C-3'$A_N$ and has been observed to be as much as 50% of the total acid precipitable material. The decrease has been shown to be due to contaminating Phe-tRNA. The origins of this contamination will be discussed in the next section.

Acetylation of the $\alpha$-amino group of phenylalanyl-tRNA stabilizes it somewhat to base-catalyzed hydrolysis (Haenni and Chapeville, 1965) as Figure 11 shows. Although AcPhe-tRNA has a half-life of about 25 minutes under these conditions, it is distinctly more labile than the phenylalanine attached to tRNA-C-C-3'$A_N$. The half-life of Phe-tRNA which is lacking the 3'-hydroxyl group (Phe-tRNA-C-C-3'dA) has also been measured (Sprinzl and Cramer, 1973). It was found that this molecule is approximately three times as stable as normal Phe-tRNA to base-catalyzed hydrolysis. This would correspond to a half-life of 21 minutes under the conditions reported here.

The stability to base-catalyzed hydrolysis of phenylalanine bound to

Figure 11: Stability of various aminoacylated tRNAs as a function of time at 37°C and pH 9.5.

Normal tRNA and tRNA-C-C-3'$A_N$ were aminoacylated as described in Methods. Phe-tRNA was chemically N-acetylated as described. ($^{14}$C) Phenylalanine was used for AcPhe-tRNA (-△-) and Phe-tRNA (-○-), while ($^{3}$H) Phenylalanine was used for Phe-3'N-tRNA (-●-). The pH was maintained at 9.5 by 0.5M Tris-HCl. Samples were precipitated with 10% TCA at the indicated times, and the acid precipitable counts are expressed as a percentage of those precipitable at 0 time.


PERCENT REMAINING
100
80
60
40
20
0
0
30
60
90
120
TIME (minutes)
PHE-N-tRNA
pH 9.5
Ac PHE - tRNA
PHE-tRNA

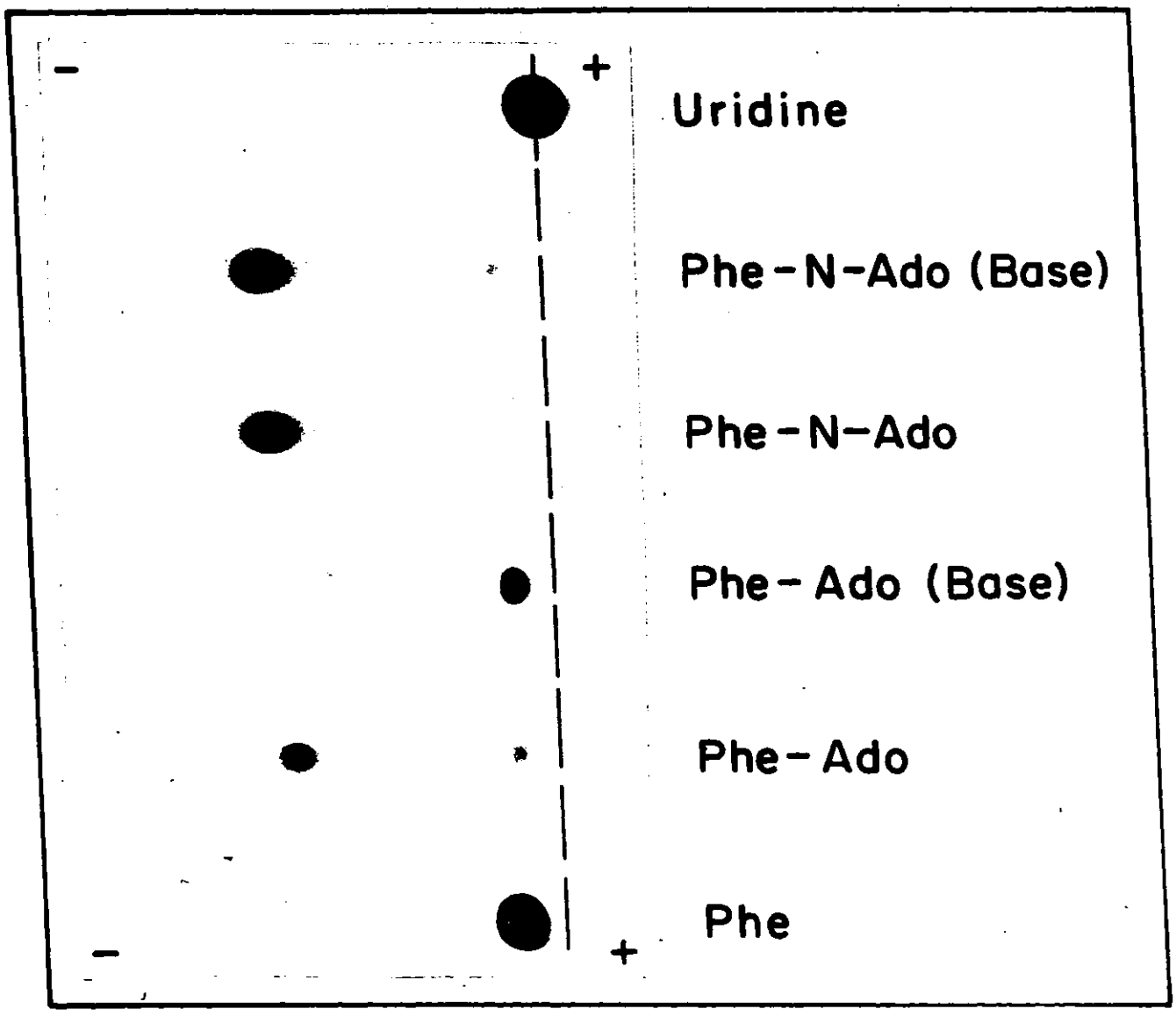
tRNA-C-C-3'$A_N$ indicates that the amino acid is attached to the terminal adenosine through a base-stable amide linkage rather than through the base-labile ester linkage found in normal phenylalanyl-tRNA.

The existence of an amide linkage in Phe-3'N-tRNA was verified after subjecting the analog phenylalanyl-tRNA to extensive digestion with pancreatic ribonuclease. With normal phenylalanyl-tRNA, this digestion liberates the terminal adenosine with its attached phenylalanine as an intact molecule. Both ($^{14}$C) Phe-tRNA and ($^{14}$C) Phe-3'N-tRNA were digested with ribonuclease and analyzed by thin-layer electrophoresis at pH 2.7. An autoradiogram of the thin-layer plate is shown in Figure 12. Phe-adenosine and Phe-3'N-adenosine have about the same mobility, although Phe-3'-N-adenosine migrates slightly more rapidly. This is probably due to somewhat more protonation of the amide carbonyl oxygen than the ester carbonyl oxygen at this pH. After incubation for 30 minutes in 1M triethylamine, the radioactivity in Phe-adenosine migrates with the phenylalanine marker, while Phe-3'N-adenosine is unaffected by this incubation. These results demonstrate in two different ways that the phenylalanine is attached to the amino group. The first is the absolute resistance to base-catalyzed hydrolysis at high concentrations of base (pH 12.5). The second is the demonstration that there is no free amino group in the Phe-3'N-adenosine molecule. If there were a free amino group, it would be protonated at pH 2.7 and substantially increase the rate of migration towards the cathode of

Phe-3'N-adenosine relative to Phe-adenosine. Alternatively, if the amino acid were linked to the 2'-hydroxyl group and the amino group were blocked by some additional unknown group, the ester linkage would be stabler to base-catalyzed hydrolysis than in normal aminoacyl-tRNA, but would still be labile.

Figure 12: Electrophoresis and base stability of phenylalanyl-adenosine and phenylalanyl-3'N-adenosine

($^{14}$C) Phe-tRNA and ($^{14}$C) Phe-3'N-tRNA were prepared and incubated separately at 37°C in 50 mM ammonium acetate pH 5.5 with 500 μg/ml of pancreatic RNAase. After 15 minutes the reaction mixtures were precipitated in ethanol and centrifuged at 15,000 x g for 15 minutes. The supernatants were analyzed by electrophoresis or cellulose thin-layer plates. The electrophoresis was done in 20% acetic acid-ammonia buffer at pH 2.7 for 1 hour at 34 V/cm. Where indicated (Base), the supernatants were incubated with 1M triethylamine for 30 minutes at 37°C. After electrophoresis the plates were dried and an autoradiographic record made using Kodak RP Royal X-omat medical X-ray film. The origin of electrophoresis corrected for endosmosis is shown by the position of uncharged ($^{14}$C) uridine. Phe is ($^{14}$C) Phenylalanine, Phe-Ado is ($^{14}$C) Phenylalanyl-adenosine, Phe-N-Ado is ($^{14}$C) Phenylalanyl-3'-amino-3'-deoxy adenosine.


-
+
Uridine
Phe-N-Ado (Base)
Phe-N-Ado
Phe-Ado (Base)
Phe-Ado
Phe
-
+

## Contamination of tRNA-C-C-3'$A_N$ with tRNA-C-C-A

As stated previously, different preparations of Phe-3'N-tRNA had varying percentages of TCA precipitable material to which phenylalanine was bound through a base-labile linkage. The most reasonable explanation for this material was that it represented contaminating normal tRNA-C-C-A which was enzymatically aminoacylated with phenylalanine during aminoacylation of the analog tRNA.

Preliminary investigation of the nature of this contaminating material indicated that phenylalanine became bound to it at approximately the same rate that it became bound to normal tRNA. In addition, the rate of base-catalyzed hydrolysis was identical to that of normal Phe-tRNA. It was also found that the material labeled with ($^{14}$C) phenylalanine was solubilized by pancreatic ribonuclease treatment. When this ribonuclease digested material was subjected to electrophoresis at pH 2.7, a single ($^{14}$C) labeled spot was found. This spot co-migrated with separately prepared phenylalanyl-adenosine. Thus, it was concluded that tRNA-C-C-A was contaminating the preparations of tRNA-C-C-3'$A_N$. This normal tRNA was responsible for the base-labile material observed in the base stability curve of Phe-3'N-tRNA in Figure 11.

The origin of the contaminating tRNA-C-C-A was investigated. The tRNA-C-C used as a substrate for the addition of 3'-amino-3'-deoxy AMP was entirely free of 3' terminal adenosine as indicated by its total

inability to be enzymatically aminoacylated by phenylalanine. Thus, it was thought that the preparation of 3'-amino-3'-deoxy ATP might have been contaminated by normal ATP which would serve as a substrate for the tRNA-nucleotidyl transferase and result in the addition of a normal AMP to tRNA-C-C. However, the large separation of the ATP and 3'-amino-3'-deoxy ATP peaks on the ion-exchange column, as well as the lack of apparent ATP contamination determined by PEI-cellulose chromatography, made this explanation unlikely.

The only nucleotide known to be contaminating the 3'-amino-3'-deoxy ATP preparation was ADP, as discussed previously. This ADP contamination was derived from ATP added to the triphosphorylation reaction mixture. The ATP acts catalytically as a phosphate donor, and is continually regenerated by phosphoenolpyruvate and pyruvate kinase. At the end of an overnight reaction, however, some ADP is present due to incomplete regeneration of the ATP. This ADP was though to pose no problem as it had been reported that ADP was not a substrate for E. coli tRNA-nucleotidyl transferase (Preiss et al., 1961). In some preparations of tRNA-nucleotidyl transferase low rates of ADP incorporation into tRNA-C-C had been attributed to myokinase contamination of the enzyme (Stan and Goldthwait, 1963; Best and Novelli, 1971).

Table 1 shows the results of a coupled assay system designed to

determine which nucleotides are added to tRNA-C-C and subsequently aminoacylated. The first step of the reaction consisted of incubation of the nucleotides with tRNA-nucleotidyl transferase under conditions favoring addition to tRNA-C-C. Phenylalanyl-tRNA synthetase, ($^{14}$C) phenylalanine, and dATP were then added to the system in order to aminoacylate the tRNA molecules to which an adenosine or adenosine analog had been added. It had previously been shown that tRNA with 2'-deoxyadenosine at the 3' end will not be aminoacylated by yeast phenylalanyl-tRNA synthetase (Sprinzl and Cramer, 1973). In addition, reports that dATP is not a substrate for E. coli tRNA-nucleotidyl transferase (Preiss et al., 1961) have been confirmed in this laboratory. The results reported in Table 1 indicate that ADP can serve as a substrate for tRNA-nucleotidyl transferase. These same results further indicate that the tRNA-nucleotidyl transferase is using the ADP as it would ATP, to correctly complete a C-C-A end which can subsequently be aminoacylated. It is possible that this is due to myokinase-like activity contaminating the enzyme preparation and the actual substrate is ATP. This enzyme preparation, however, yields apparently homogeneous enzyme when subjected to polyacrylamide gel electrophoresis at pH 9.3 and SDS-polyacrylamide gel electrophoresis (Miller and Philipps, 1971). When the gels are overloaded a minor component does appear, so that the presence of a small amount of contaminating enzyme activity cannot be eliminated on these grounds.

Table 1: Specificity of nucleotide addition to tRNA-C-C assayed by subsequent aminoacylation.

| Nucleotide tested | Nucleotide concentration (mM) | TCA precipitable ($^{14}$C) Phe after aminoacylation (DPM/assay) |
|---|---|---|
| ATP | 0.25 | 26,914 |
| ADP | 0.25 | 10,139 |
| 3'-amino-3'-deoxy ATP | 1.45 | 8,794 |
| dATP | 1.50 | 239 |
| dATP & ADP | 1.50<br>0.25 | 4,318 |

This assay proceeded in two steps. The first step allowed addition of the nucleotides to tRNA-C-C. This incubation mixture contained per ml: 4.2 $A_{260}$ units of purified yeast $tRNA^{phe}$-C-C, 6 mg reduced glutathione, 300 $\mu$g BSA, 5 $\mu$g purified E. coli tRNA-nucleotidyl transferase and added nucleotide in the concentration recorded in the table. The mixture was buffered with 50 mM glycine, pH 9.2, and contained 60 mM KCl and 15 mM $Mg(C_2H_3O_2)_2$. Incubation was carried out at 37°C for 75 minutes. The initial incubation was followed by the addition to each 40 $\mu$l assay of 1.77 $\mu$g purified yeast phenylalanyl-tRNA synthetase (a gift of R. Giege), 1.15 nmoles ($^{14}$C) phenylalanine (specific activity 200 $\mu$Ci/$\mu$M) and 0.384 $\mu$moles dATP, bringing the total assay volume to 50 $\mu$l. Incubation was

continued at 37°C for 30 minutes, and the reaction mixtures were precipitated by the addition of 1 ml 10% TCA containing 1% casamino acids. The precipitate was collected on glass fiber filter papers and counted in a liquid scintillation counter. The blank value (936 dpm) with no nucleotide added has been substracted from the values reported in the table.

If ADP were not a substrate for tRNA-nucleotidyl transferase, but simply a precursor of the actual substrate (i. e. ATP) then one might expect a lag in the kinetics of incorporation when labeled ADP is added to tRNA-C-C. Figure 13 clearly shows that the rate and final extent of incorporation of AMP into tRNA-C-C is nearly identical regardless of whether ADP or ATP is used as a substrate.

A straightforward way to remove ADP from the amino ATP preparations would be to convert it to ATP and separate it on an anion-exchange column. Consideration of the free energy liberated in the following reaction:

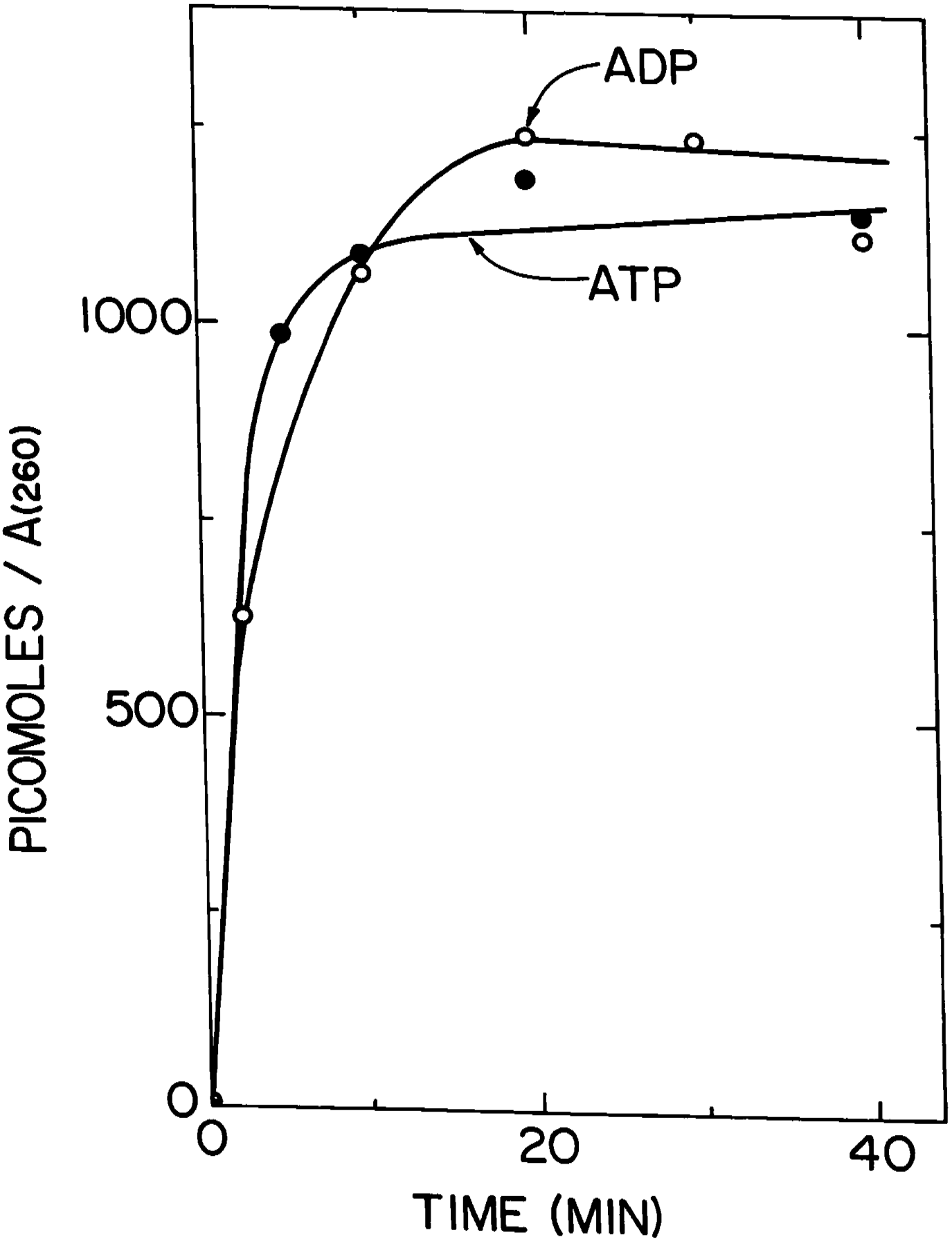
$$\text{PEP} + \text{ADP} \underset{\text{pyruvate kinase}}{\rightleftharpoons} \text{ATP} + \text{Pyruvate} \qquad \Delta G^{o\prime} = -7.5 \text{ Kcal.}$$

indicates that at equilibrium there should be no detectable ADP if an excess of PEP is added to the reaction mixture. Although this is the same reaction employed in the original triphosphorylation system, if it is carried out under conditions optimal for pyruvate kinase activity (as opposed to conditions optimal for adenosine kinase activity) equilibrium will be reached and essentially all contaminating ADP will be converted to ATP. The incubation for conversion of ADP to ATP was done in 5 mM HEPES-KOH pH 7. 4, 100 mM potassium chloride, and 25 mM magnesium acetate. This additional purification step has

been carried out with a recent preparation of 3'-amino-3'-deoxy ATP and after separation from ATP on an AG1-X2 column, the 3'-amino-3'-deoxy ATP was incorporated into the 3' end of tRNA-C-C. When this preparation of tRNA-C-C-3'$A_N$ was charged with phenylalanine, no contaminating base-labile material was found.

Figure 13: Kinetics of incorporation of ATP and ADP into tRNA-C-C.

The incubation mixture designed to assay incorporation of labeled ATP or ADP into tRNA-C-C contained per ml: 7.6 $A_{260}$ units of E. coli B tRNA-C-C, 6 mg reduced glutathione, 300 $\mu$g BSA, 5 $\mu$g purified E. coli tRNA-nucleotidyl transferase and either 0.8 $\mu$moles of ($^3$H) ATP (specific activity 12 $\mu$Ci/$\mu$M) or 0.68 $\mu$moles of ($^3$H) ADP (specific activity 16.7 $\mu$Ci/$\mu$M). The mixture was buffered with 50 mM glycine, pH 9.2, and contained 60 mM KCl and 15 mM $Mg(C_2H_3O_2)_2$. Incubation was carried out at 37°C for the indicated times. 75 $\mu$l aliquots were removed and added to 1 ml of 0.2 mM ATP, 20 mM EDTA. The samples were precipitated by the addition of 1 ml of 10% TCA, filtered, dried and counted in a liquid scintillation counter.


ADP
ATP
1000
500
0
0
20
40
PICOMOLES / A(260)
TIME (MIN)

Activity of Phe-3'N-tRNA in poly(U) directed polyphenylalanine synthesis

In order to determine whether the ribosome would accept the Phe-3'N-tRNA and use it in protein synthesis, a poly(U) directed polyphenylalanine synthetic system was utilized. Polymerization in this system was carried out at 15 mM $Mg^{2+}$ in the presence of RNA-free S-100 which was the source of elongation factors T and G (EF-Tu, EF-Ts and EF-G). Previously charged and purified aminoacyl-tRNA was added directly to the system. Both Phe-tRNA and Phe-3'N-tRNA were tested as substrates. Phe-3'N-tRNA had been preincubated for 1 hour in 0.5M Tris-HCl at pH 9.5 and 37°C to insure that there was no contamination by normal Phe-tRNA. Figure 14 shows the results obtained using the poly(U) system. After polymerization had proceeded for the indicated time periods, EDTA and pancreatic RNAase were added to the incubation mixtures and incubation was continued for 15 minutes at 37°C. The reaction mixtures were then precipitated with 10% TCA containing 1% casamino acids.

As shown in Figure 14, 60% of the ($^{14}C$) Phe-tRNA added to the system was converted into an RNAase stable, TCA precipitable product. Only about 4% of the ($^{14}C$) Phe-3'N-tRNA added to the system was converted into RNAase stable, TCA precipitable product. In the absence of poly(U), all of the Phe-tRNA was rendered TCA soluble by the EDTA/RNAase treatment. It can be seen that the small amount of incorporation of ($^{14}C$) phenylalanine into RNAase stable, acid precipitable

material from Phe-3'N-tRNA appears to be complete within two minutes.

As shown in Table 2, if ribosomes used in the poly(U) assay are preincubated with puromycin, the incorporation of ($^{14}$C) Phe-3'N-tRNA into RNAase stable, acid precipitable material is decreased by more than 50%, while incorporation with normal ($^{14}$C) Phe-tRNA increases slightly.

It is known that if ribosomes containing nascent polypeptide chains are incubated in vitro with puromycin, the chains will be released from the ribosome as peptidyl-puromycin (Pestka, 1972). These results suggest that the analog aminoacyl-tRNA may act as an acceptor of nascent polypeptide chains present on the ribosome. Accordingly, experiments were carried out to determine directly if the Phe-3'N-tRNA was able to act as an acceptor in the poly(U) system.

Figure 14: In vitro protein synthesis directed by poly(U) with either Phe-tRNA (—○—) or Phe-3'N-tRNA (—●—).

The incubation mixture contained per ml: 4.7 $A_{260}$ units of ribosomes washed three times with 1M $NH_4Cl$, 0.8 mg poly(U) (molecular weight >100,000), 0.2 μmoles GTP, 2 μmoles PEP, 35 μg pyruvate kinase (405 Units/mg), 0.34 mg RNA-free S-100 and either 7.4 mg ($^{14}C$) Phe-tRNA (2.2% charged with ($^{14}C$) phenylalanine, specific activity 466 μCi/μM) or 6.7 mg ($^{14}C$) Phe-3'N-tRNA (1.6% charged with ($^{14}C$) phenylalanine, specific activity 466 μCi/μM). The mixture was buffered with 50 mM Tris-HCl, pH 7.8, and contained 80 mM $NH_4Cl$, 6 mM β-mercaptoethanol and 15 mM $Mg(C_2H_3O_2)_2$. After the indicated times of incubation at 30°C, 50 μl aliquots were removed, and 50 μl of 0.1M EDTA and 5 μl of pancreatic RNAase (10 mg/ml) were added to each. Incubation was continued for 15 minutes at 30°C. The samples were then precipitated with 10% TCA and filtered on glass fiber filter paper. A control incubation was performed with the same incubation mixture minus poly(U) (—■—).

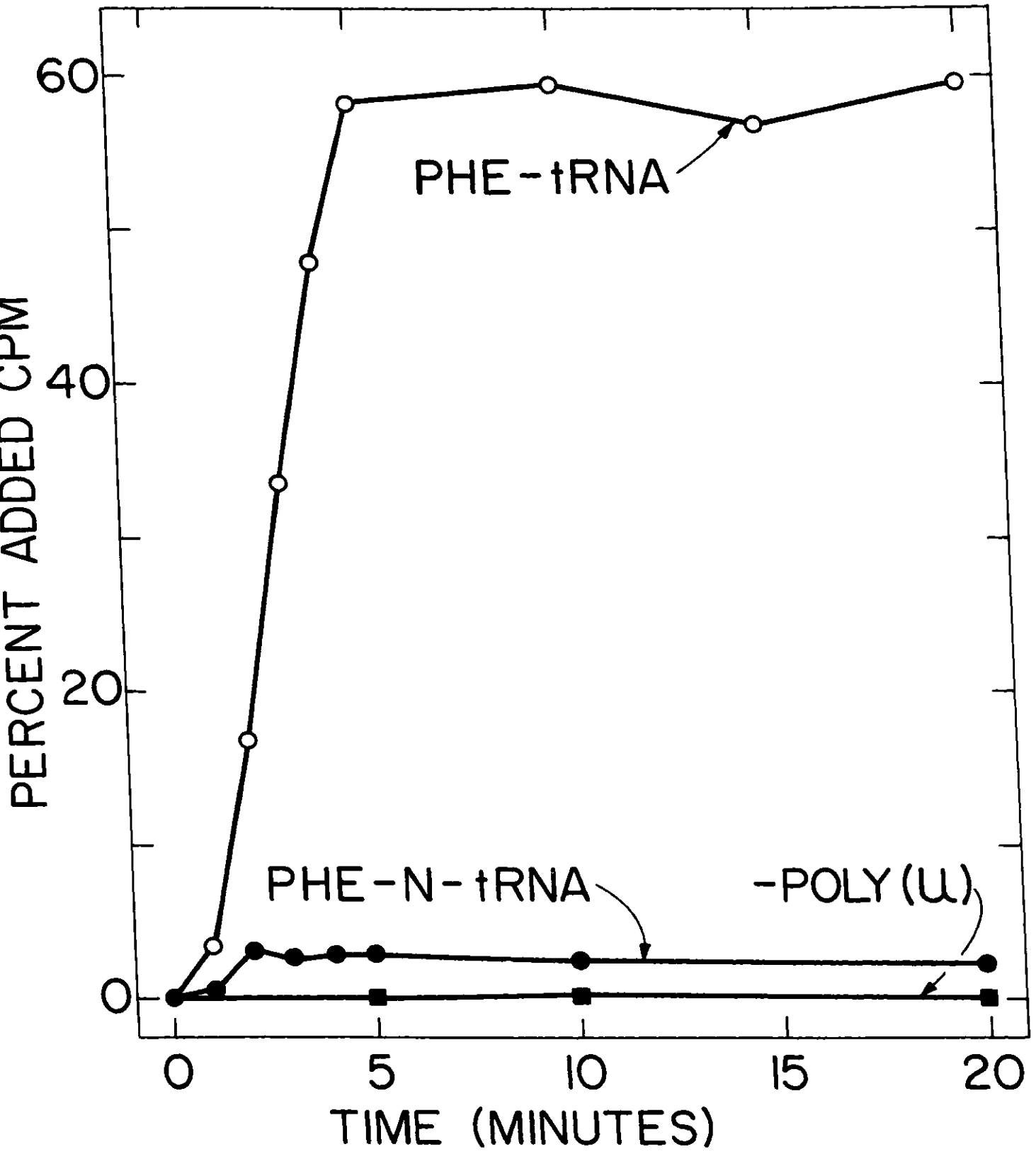

60
40
20
0
PERCENT ADDED CPM
PHE-tRNA
PHE-N-tRNA
-POLY(U)
0
5
10
15
20
TIME (MINUTES)

Table 2: Effect of puromycin pretreatment of ribosomes on poly(U) directed in vitro protein synthesis with either Phe-tRNA or Phe-3'N-tRNA.

| Aminoacyl-tRNA | Phenylalanine Polymerization(DPM) | | Percent polymerization after puromycin treatment |
|---|---|---|---|
| | untreated ribosomes | puromycin treated ribosomes | |
| Phe-tRNA | 5248 | 5986 | 114% |
| Phe-3'N-tRNA | 496 | 242 | 47% |

All ribosomes used had been washed three times with 1M $NH_4Cl$. The preparative incubation used in puromycin treatment of the ribosomes contained in 3 ml: 600 $A_{260}$ units of salt-washed ribosomes and 0.3 $\mu$moles puromycin. The mixture was buffered with 50 mM Tris-HCl, pH 7.2, and contained 20 mM $Mg(C_2H_3O_2)_2$, 80 mM $NH_4Cl$ and 6 mM $\beta$-mercaptoethanol. Incubation was for 60 minutes at 37°C. Puromycin was removed from the ribosomes by dialysis against buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM $Mg(C_2H_3O_2)_2$, 10 mM $NH_4Cl$ and 6 mM $\beta$-mercaptoethanol.

Poly(U) directed protein synthesis was then assayed in incubation mixtures identical to the ones described in the legend to Figure 14, except that 7.6 mg of ($^{14}C$) Phe-3'N-tRNA (2.0% charged with ($^{14}C$) phenylalanine) was added per ml when the analog aminoacyl-tRNA was tested. After incubation for 30 minutes at 37°C, 50 $\mu$l aliquots were assayed for RNAase stable, TCA precipitable ($^{14}C$) phenylalanine as described in the legend to Figure 14.

Assay of Phe-3'N-tRNA acceptor activity

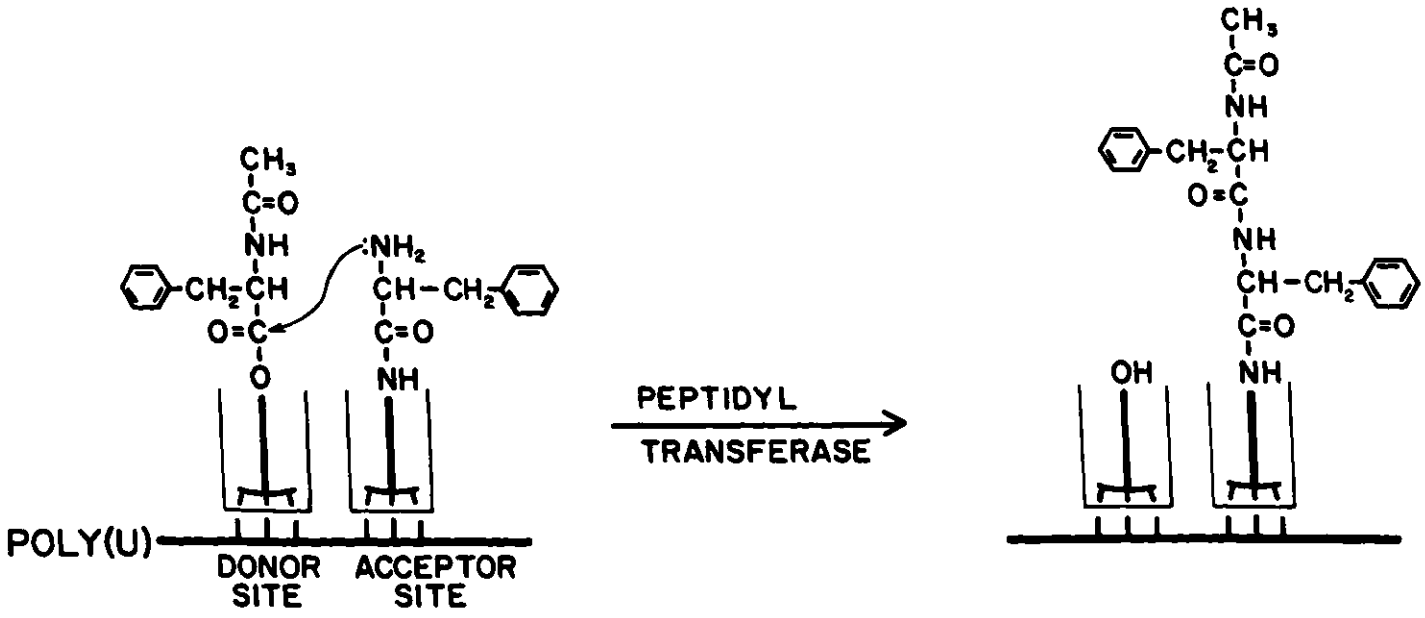
Figure 15 illustrates schematically the action of the peptidyl transferase center in the ribosome. In this case AcPhe-tRNA is bound in the donor site and Phe-3'N-tRNA is bound in the acceptor site. Although AcPhe-tRNA is not used by the ribosome in vivo, it is the only molecule other than fmet-tRNA which is known to initiate polypeptide synthesis with initiation factors at low $Mg^{2+}$ concentrations in vitro. The enzymatic activity of peptidyl transferase probably involves catalysis of a direct nucleophilic attack (no intermediates have been found) by the free $\alpha$-amino group of aminoacyl-tRNA on the esterified carbonyl carbon of AcPhe-tRNA. There is no reason to suspect that the mechanism with Phe-3'N-tRNA in the acceptor site would be different from the mechanism with normal Phe-tRNA in the acceptor site. The product of peptidyl transferase activity in the latter case would be AcPhe-Phe-tRNA, while the product in the former case would be AcPhe-Phe-3'N-tRNA (shown in Figure 15). In order to study this reaction, AcPhe-tRNA must first be bound to the D site of the ribosome.

Phe-tRNA was chemically acetylated with the acetic acid ester of N-hydroxysuccinimide to yield AcPhe-tRNA.

Poly(U) is able to direct the binding of AcPhe-tRNA to ribosomes in the absence of initiation factors, although this binding was stimulated about fourfold in the presence of initiation factors. In order to measure the acceptor activity of Phe-3'N-tRNA, the AcPhe-tRNA must be bound in the ribosomal donor or D site.

Figure 15: Schematic representation of ribosomal peptidyl transferase activity.

The ribosomal bound substrates in this peptidyl transferase catalyzed reaction are AcPhe-tRNA in the donor site and Phe-3'N-tRNA in the acceptor site. After peptide bond formation deacylated $tRNA^{phe}$ is bound in the donor site and AcPhe-Phe-3'N-tRNA in the acceptor site.


CH3
C=O
NH
CH2-CH
O=C
O
:NH2
CH-CH2
C=O
NH
POLY(U)
DONOR SITE
ACCEPTOR SITE
PEPTIDYL
TRANSFERASE
CH3
C=O
NH
CH2-CH
O=C
NH
CH-CH2
C=O
OH
NH

If initiation factors are not used, a high magnesium ion concentration of 15 mM is required to get satisfactory binding of AcPhe-tRNA to ribosomes. At high $Mg^{2+}$ concentrations, however, the AcPhe-tRNA is not bound exclusively in the D site. In studies done at both 18 and 20 mM $Mg^{2+}$, it was found that AcPhe-tRNA that had been nonenzymatically bound to ribosomes was almost totally unreactive with puromycin, indicating that it was not bound in the D site (Suarez and Nathans, 1965; Lucas-Lenard and Lipmann, 1967).

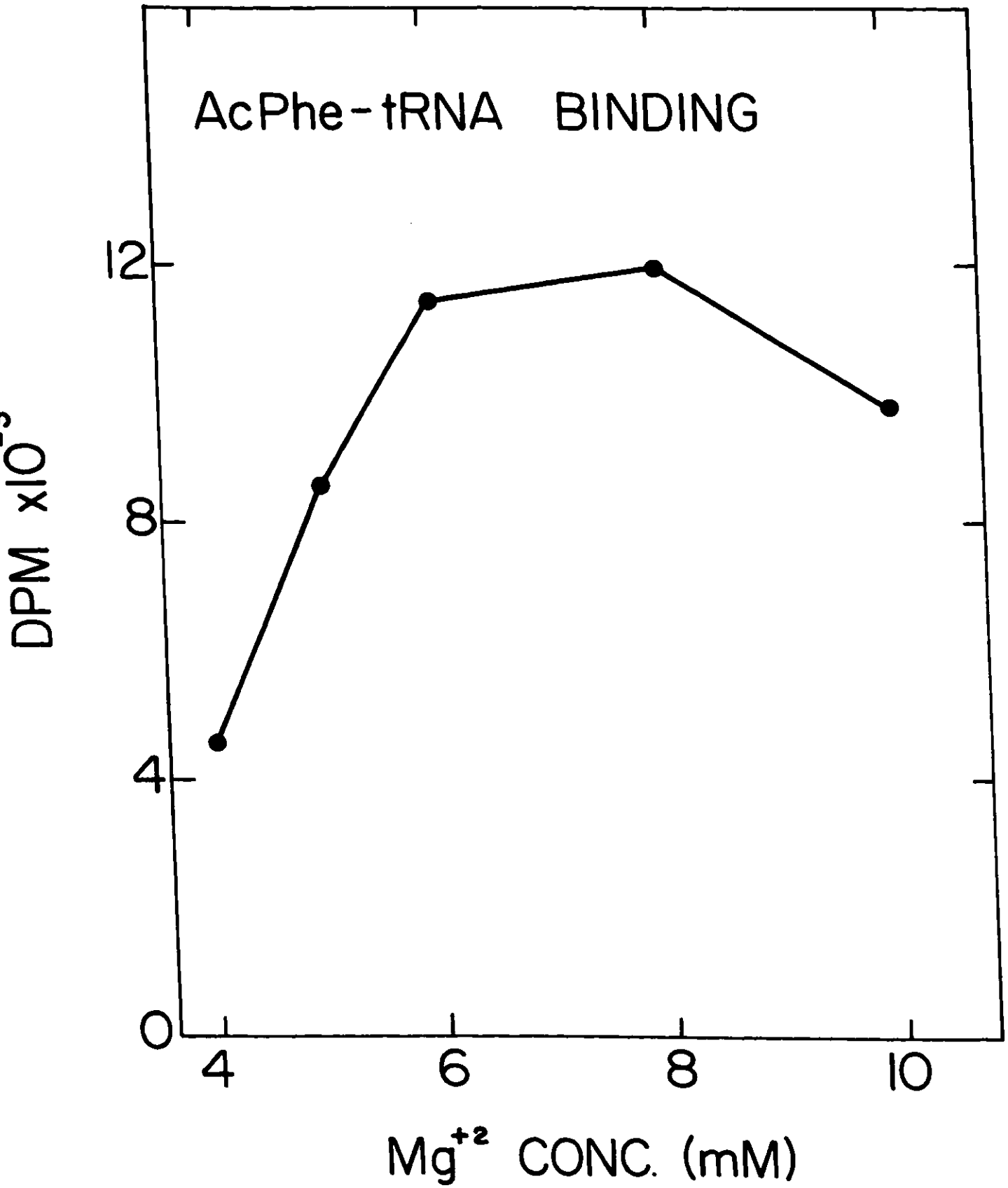
If AcPhe-tRNA is enzymatically bound to ribosomes with initiation factors, it will bind exclusively in the D site at low $Mg^{2+}$ concentrations. Figure 16 shows the binding of AcPhe-tRNA to ribosomes as a function of $Mg^{2+}$ concentration. The optimal $Mg^{2+}$ concentration in this system was between 6 and 8 mM. Very little nonenzymatic binding of AcPhe tRNA to ribosomes was observed at these $Mg^{2+}$ concentrations.

It may be parenthetically pointed out that not all ribosomes are active in binding AcPhe-tRNA, even under optimal binding conditions. If the assumption is made that only one AcPhe-tRNA molecule is bound per active ribosome (a reasonable assumption at low $Mg^{2+}$ concentration), then from 7% to 39% of the ribosomes used in these experiments were active. The percentage of active ribosomes varied both with different preparations and with time of storage prior to use.

The assay to measure the acceptor activity of Phe-3'N-tRNA was done in two steps. Ac($^{14}$C) Phe-tRNA was first bound to the ribosome

Figure 16: Poly(U) directed binding of AcPhe-tRNA to ribosomes as a function of magnesium ion concentration.

The assay incubation mixture contained per ml: 20.0 $A_{260}$ units of salt-washed ribosomes, 80 $\mu$g poly(U), 544 pmoles Ac($^{14}$C) Phe-tRNA (specific activity 466 $\mu$Ci/$\mu$M), 612 $\mu$g crude initiation factors and 0.2 $\mu$mole GTP. The mixture was buffered with 50 mM Tris-HCl,pH 7.8, and contained 80 mM $NH_4Cl$, 6 mM $\beta$-mercaptoethanol and the indicated concentrations of $Mg(C_2H_3O_2)_2$. After incubation for 15 minutes at 30°C, 3 ml of wash buffer containing 50 mM Tris-HCl, pH 7.8, 80 mM $NH_4Cl$, 6 mM $\beta$-mercaptoethanol and the tested concentration of $Mg(C_2H_3O_2)_2$ were added to each 50 $\mu$l assay. The mixture was immediately filtered through a nitrocellulose filter and washed with 10 ml of wash buffer. The radioactivity adhering to the filter was counted in a liquid scintillation counter.


AcPhe-tRNA BINDING
DPM x10^-3
12
8
4
0
4
6
8
10
Mg+2 CONC. (mM)

with poly(U) and initiation factors at 8 mM $Mg^{2+}$. Figure 6 has shown the time course of AcPhe-tRNA binding to ribosomes under these conditions. The binding is nearly complete after 5 minutes of incubation and does not change upon further incubation. In the two-step acceptor assay, the poly(U) directed binding of Ac($^{14}$C) Phe-tRNA was allowed to proceed for 10 minutes. Following this, Phe-3'N-tRNA was added directly to the Ac($^{14}$C) Phe-tRNA-poly(U)-ribosome complex. After incubation at 37°C the effect of the analog aminoacyl-tRNA addition was measured by treatment of the reaction mixture at pH 9.5 under conditions which have been shown to hydrolyze the ester linkage between AcPhe and tRNA, but are unable to break the amide linkage between phenylalanine and tRNA-C-C-3'$A_N$(demonstrated in Figure 11). If the Phe-3'N-tRNA were bound to the ribosome in the acceptor or A-site, then Ac($^{14}$C) Phe may be donated to the Phe-3'N-tRNA with the formation of AcPhe-Phe-3'N-tRNA. This material would be stable to the pH 9.5 incubation, whereas if normal Phe-tRNA were added to the ribosome (under conditions where polyphenylalanine will not be formed; i.e. no elongation factor G), the ester bond in AcPhe-Phe-tRNA would be hydrolyzed by the incubation and the radioactivity would no longer be precipitable by TCA. The crude initiation factors used to bind AcPhe-tRNA to the D site contain substantial EF-G activity, so this control was not done. As shown in Table 3, after 30 minutes of incubation, more than 95% of the Ac($^{14}$C) Phe is precipitable with

TCA after pH 9.5 incubation. This precipitability is dependent upon the presence of both poly(U) and Phe-3'N-tRNA. The time course of Ac($^{14}$C) Phe-Phe-3'N-tRNA formation is shown in Figure 17. It can be seen that the reaction is nearly complete after 5 minutes of incubation, continuing at a much slower rate thereafter. The results of these experiments show that Phe-3'N-tRNA is able to accept the Ac($^{14}$C) Phe from the D site of the ribosome even though the experiments described in Figure 14 and Table 2 suggested that it was unable to participate in the synthesis of polyphenylalanine.

Table 3: Acceptor activity of Phe-3'N-tRNA

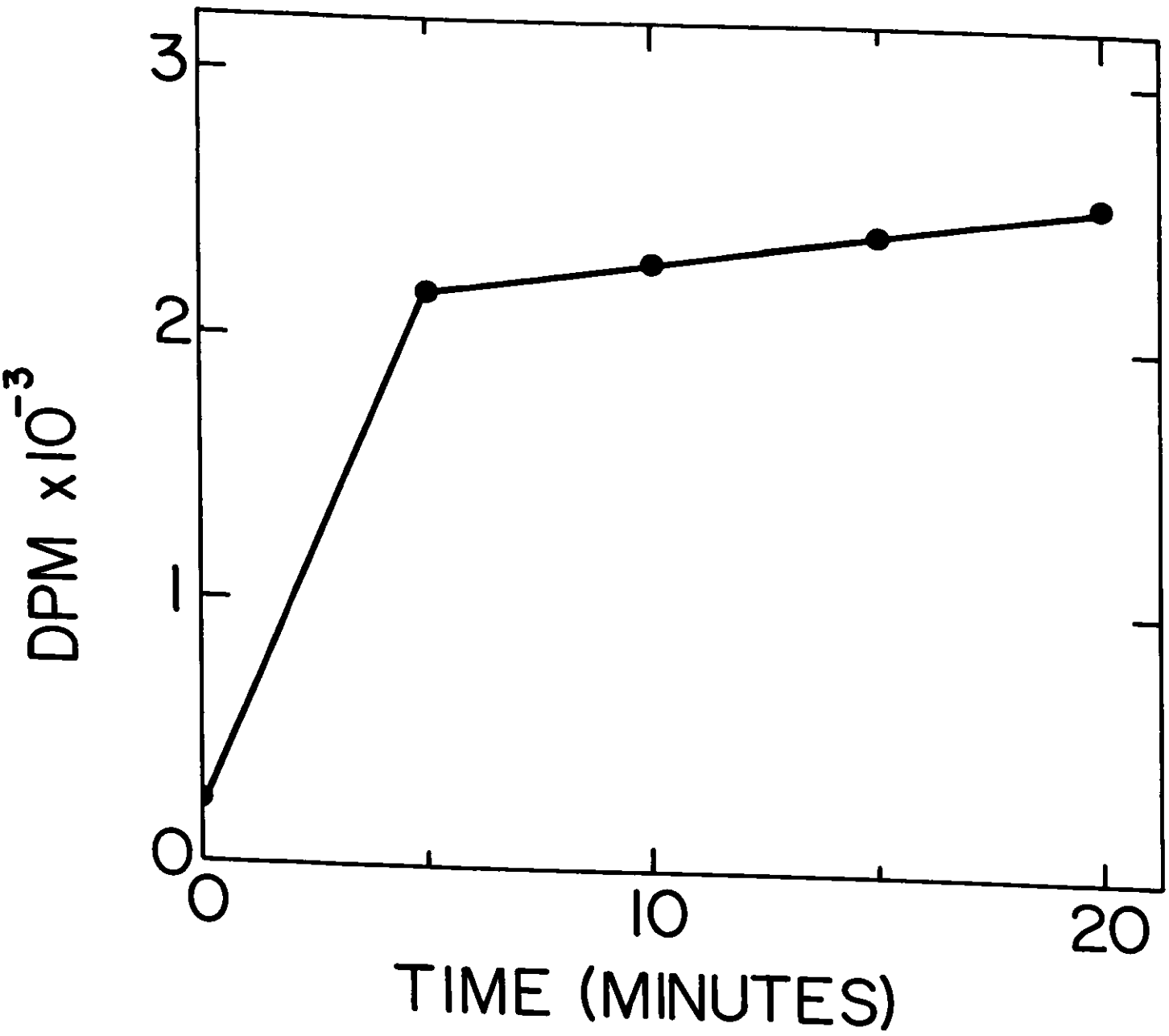
| Reaction Mixture | Incubation Time (minutes) | TCA precipitable Ac($^{14}$C)Phe | |
|---|---|---|---|
| | | dpm | % of bound Ac($^{14}$C)Phe-tRNA |
| complete | 0 | 230 | 8.2 |
| complete | 15 | 2405 | 85.9 |
| complete | 30 | 2671 | 95.4 |
| minus poly(U) | 15 | 251 | 5.4 |
| minus Phe-3'N-tRNA | 15 | 81 | 2.9 |

Acceptor activity of Phe-3'N-tRNA was determined in a two-step assay. In the first step, Ac($^{14}$C)Phe-tRNA was bound to the ribosome. The incubation mixture contained per ml: 27.6 $A_{260}$ units puromycin-treated, salt-washed ribosomes, 80 $\mu$g poly(U), 0.2 $\mu$moles GTP, 0.4 $\mu$moles phosphoenol pyruvate, 40 $\mu$g pyruvate kinase (320 units/mg), 750 $\mu$g crude initiation factors, 8 $\mu$moles $Mg(C_2H_3O_2)_2$, 80 $\mu$moles $NH_4Cl$, 6 $\mu$moles $\beta$-mercaptoethanol, 140 pmoles purified Ac($^{14}$C)Phe-tRNA. The mixture was buffered at pH 7.8 with 50 mM Tris-HCl. After incubation for 10 minutes at 30°C, binding of Ac($^{14}$C)Phe-tRNA was essentially complete. Approximately 2 pmoles of Ac($^{14}$C)Phe-tRNA were bound per $A_{260}$ unit of ribosomes. The binding was dependent on the presence of poly(U). The mixture was cooled to 0°C and 0.5 mg ($^{3}$H) Phe-3'N-tRNA (1% of this unfractionated tRNA was charged with ($^{3}$H) phenylalanine) and 450 pmoles of purified EF-Tu were added per ml.

The ($^{3}$H) Phe-3'N-tRNA had been preincubated for 1 hour at 37°C in pH 9.5 Tris-HCl to insure that no normal ($^{3}$H) Phe-tRNA was present. Incubation at 30°C was continued for the indicated times. 50 $\mu$l aliquots were removed, 150 $\mu$l of 0.5 M Tris-HCl, pH 9.5, added and the mixture incubated at 37°C for 2 hours, at which time 10% TCA was added and the precipitable counts were measured.

Figure 17: Time course of Ac($^{14}$C) Phe-Phe-3'N-tRNA formation.

The incubation mixture was identical to that described in Table 3. After allowing binding of Ac($^{14}$C) Phe-tRNA to proceed for 10 minutes, Phe-3'N-tRNA was added and incubation was continued at 30°C for the indicated times. The reaction was stopped by the addition of 0.5 M Tris-HCl, pH 9.5, and worked up as described in Table 3.


DPM x10$^{-3}$
3
2
1
0
0
10
20
TIME (MINUTES)

Donor activity of Phe-3'N-tRNA

The finding that Phe-3'N-tRNA is apparently inactive in a poly(U) directed polyphenylalanine synthesizing system and yet is able to bind to the ribosomal A site and accept an AcPhe from the D site suggests that this inactivity is due its inability to either initiate or donate in this system.

Initiation in this poly(U) directed protein synthesizing system is dependent upon a high $Mg^{2+}$ concentration. When the $Mg^{2+}$ concentration is 15 mM, unblocked Phe-tRNA will participate in polypeptide chain initiation as well as chain elongation. This initiation, however, is an artifact of a high $Mg^{2+}$ concentration and study of chain initiation in this poly(U) system at a high $Mg^{2+}$ concentration would probably not reveal much information about the normal initiation mechanism. It should be pointed out that a poly(U) system will initiate at a low $Mg^{2+}$ concentration if AcPhe-tRNA and initiation factors are used (Nakamoto and Kolakofsky, 1966). A study of this low $Mg^{2+}$ system with analog tRNA may help determine any isomeric specificity of initiation, but would not explain why Phe-3'N-tRNA is inactive in a poly(U) system at high $Mg^{2+}$ concentration. The results of an investigation into the donor activity of peptidyl-3'N-tRNA, however, might explain this inactivity.

Accordingly, an experiment was designed to measure donor activity with the aminoacyl-tRNA analog. A lack of donor activity would not necessarily suggest that the isomeric specificity of the donor site

in peptidyl transferase was different from that of the acceptor site. It is possible that the peptidyl transferase would be unable to catalyze the nucleophilic attack of the $\alpha$-amino group in aminoacyl-tRNA on the carbonyl carbon of the amide linkage. The amide bond is much more stable than an ester bond and there is no reason to expect that the peptidyl transferase will catalyze a transamidation at a measurable rate. It has been found that peptidyl transferase will catalyze the nucleophilic attack of an hydroxyl oxygen on the carbonyl carbon of the ester linkage (Fahnestock and Rich, 1971a; Fahnestock and Rich, 1971b). Although this discovery gives some clues to the catalytic mechanism of peptidyl transferase, we cannot predict whether an amide bond to tRNA will be cleaved by this enzyme.

In the donation experiment an incubation similar to that described in Table 3 was carried out, except that after binding of Ac($^{14}$C) Phe-tRNA to ribosomes and an additional 15 minute incubation with Phe-3'N-tRNA, a large amount of unlabeled normal Phe-tRNA was added to the system. If the Ac($^{14}$C) Phe-Phe-3'N-tRNA, which has previously been shown to form under these conditions, were able to donate to the unlabeled Phe-tRNA, then Ac ($^{14}$C) Phe-Phe-Phe-tRNA would be the product. This species, still bound to the ribosome, would continue to add phenylalanine residues at the normal rate until polymerization ceased. The products would then be Ac($^{14}$C) Phe-(Phe)$_n$-tRNA, where n represents the variable number of phenylalanine residues added to the polyphenylalanine

chain. If the products of this reaction were then subjected to a lengthy incubation at pH 9.5, the ester bond linking the Ac($^{14}$C) Phe-polyphenylalanine to tRNA would be cleaved. As in the acceptor experiment, however, the amide bond linking the Ac($^{14}$C) Phe-Phe to tRNA-C-C-3'$A_N$ would not be cleaved. If this experiment is done without adding Phe-3'N-tRNA (i.e. bind Ac($^{14}$C) Phe-tRNA and incubate with unlabeled Phe-tRNA), then, as shown in Table 4, approximately 50% of the ($^{14}$C) AcPhe bound to the ribosome is incorporated into polyphenylalanine which is TCA precipitable even after base incubation. It had been found in the acceptor experiment that at least 95% of the AcPhe-tRNA was functionally bound to ribosomes. The fact that only 50% of the Ac($^{14}$C) Phe is acid precipitable after polymerization probably indicates that the other 50% of the Ac($^{14}$C) Phe is incorporated into polyphenylalanine chains which are too short to be acid precipitable. If this experiment were done with added Phe-3'N-tRNA as described and the Ac($^{14}$C) Phe-Phe-3'N-tRNA were able to donate, one would expect that the amount of TCA precipitable ($^{14}$C) label stable to pH 9.5 incubation would decrease after addition of unlabeled Phe-tRNA. If all of the Ac($^{14}$C) Phe-Phe-3'N-tRNA had donor activity, the percentage of bound TCA precipitable Ac($^{14}$C) Phe stable to base would decrease from 95% to 50% after addition of Phe-tRNA. However, as seen in Table 4, this percentage did not decrease at all after addition of unlabeled Phe-tRNA.

Table 4: Donor activity of AcPhe-Phe-3'N-tRNA.

| Reaction Mixture | Incubation time after addition of unlabelled phe-tRNA (minutes) | TCA precipitable Ac($^{14}$C)Phe | |
|---|---|---|---|
| | | dpm | % of bound Ac($^{14}$C)Phe-tRNA |
| complete | 0 | 2580 | 92 |
| complete | 5 | 2626 | 94 |
| complete | 15 | 2662 | 95 |
| minus Phe-3'N-tRNA | 5 | 1304 | 47 |
| minus Phe-3'N-tRNA | 15 | 1400 | 50 |

($^{14}$C) AcPhe-tRNA was bound to ribosomes as described in the legend to Table 3. Phe-3'N-tRNA and EF-Tu were added and the mixture was incubated for 15 minutes at 30°C. The reaction mixture was then cooled to 0°C and 2.25 mg of tRNA charged with unlabeled phenylalanine was added per ml. Incubation at 30°C was then resumed for the indicated times. 50 $\mu$l aliquots were removed and 150 $\mu$l of 0.5 M Tris-HCl (pH 9.5) added. The mixture was incubated at 37°C for 2 hours, at which time 10% TCA was added and the precipitable counts measured.

This experiment shows that AcPhe-Phe-3'N-tRNA, once formed, is unable to continue polypeptide chain elongation. As previously discussed, this may be due to isomeric specificity of the D site or inability of peptidyl transferase to cleave an amide bond. Another possible explanation is that AcPhe-Phe-3'N-tRNA is unable to be translocated. Although this explanation is related to the isomeric specificity of the D site, it additionally implies an isomeric specificity associated with EF-G activity.

Initiator activity of AcPhe-3'N-tRNA

Initiation factors catalyze the binding of an initiator aminoacyl-tRNA into the ribosomal D site. The poly(U) coded binding of AcPhe-tRNA to ribosomes at low $Mg^{2+}$ requires initiation factors (Lucas-Lenard and Lipmann, 1967). Analysis of this process with AcPhe-3'N-tRNA would allow a direct approach to the isomeric specificity of D site binding and initiation.

Phe-3'N-tRNA was N-acetylated by the same method used for the N-acetylation of normal Phe-tRNA. Poly(U) directed binding of AcPhe-3'N-tRNA to ribosomes at low $Mg^{2+}$ concentration in the presence of initiation factors was measured. Table 5 shows the results of this binding with both AcPhe-tRNA and AcPhe-3'N-tRNA at 6 mM $Mg^{2+}$. It can be seen that approximately equal amounts of both species are bound in the absence of initiation factors. The bindingof AcPhe-tRNA is increased more than fivefold by the addition of initiation factors, while AcPhe-3'N-tRNA binding is increased only slightly. This suggests that the initiation factors do not interact strongly with the AcPhe-3'N-tRNA in order to catalyze its binding to the D site. An alternative explanation is that the AcPhe-3'N-tRNA does interact with IF-2, but is not subsequently bound in the ribosomal D site.

Table 5: Poly(U) directed binding of AcPhe-tRNA and AcPhe-3'N-tRNA to ribosomes at 6 mM $Mg^{2+}$

| | picomoles bound / $A_{260}$ ribosomes | |
|---|---|---|
| | AcPhe-tRNA | AcPhe-3'N-tRNA |
| complete system | 5.3 | 1.49 |
| minus initiation factors | 1.0 | 0.93 |
| minus ribosomes | 0.1 | 0.1 |

The assay incubation contained per ml: either 20.0 $A_{260}$ units of salt-washed ribosomes or no ribosomes as indicated, 80 $\mu$g poly(U), either 552 pmoles of Ac($^{14}$C)Phe-tRNA (specific activity 466 $\mu$Ci/$\mu$M) or 352 pmoles of Ac($^{3}$H) Phe-3'N-tRNA (specific activity 2.22 mCi/$\mu$M), 612 $\mu$g crude initiation factors or no initiation factors as indicated and 0.2 $\mu$mole GTP. The mixture was buffered with 50 mM Tris-HCl, pH 7.8, and contained 80 mM $NH_4Cl$, 6 mM $\beta$-mercaptoethanol and 6 mM $Mg(C_2H_3O_2)_2$. After incubation for 15 minutes at 30°C, binding of the aminoacylated tRNA to ribosomes was measured by filtration through nitrocellulose filters as described in the legend to Figure 16.

EF-Tu catalyzed binding of Phe-3'N-tRNA to ribosomes

As discussed in the Introduction, EF-Tu catalyzes the binding of aminoacyl-tRNA to ribosomes. This protein factor becomes closely associated with the 3' end of aminoacyl-tRNA and it is reasonable to suspect that it would exhibit isomeric specificity in its interaction with aminoacyl-tRNA.

The previously described experiments in which the acceptor activity of Phe-3'N-tRNA was measured had EF-Tu added to the reaction mixtures. It was not known prior to performing those experiments whether or not EF-Tu would catalyze the binding of Phe-3'N-tRNA to ribosomes. A control experiment without added EF-Tu was not done because the crude initiation factors also had EF-Tu activity associated with them. In any case, EF-Tu is not absolutely required to bind aminoacyl-tRNA to ribosomes at 8 mM $Mg^{2+}$ (Philipps, 1970; Shorey et al., 1971) so that the role, if any, of EF-Tu in the binding of Phe-3'N-tRNA to ribosomes was not elucidated in the acceptor experiment.

The poly(U) directed binding of both Phe-tRNA and Phe-3'N-tRNA to ribosomes was measured directly, both in the presence and absence of EF-Tu and GTP. The results of these binding experiments are presented in Table 6. The binding of Phe-tRNA is stimulated nearly fourfold by the addition of EF-Tu. The binding of Phe-3'N-tRNA is stimulated only twofold. These results suggest that while EF-Tu

Table 6: Poly(U) directed binding of Phe-tRNA and Phe-3'N-tRNA to ribosomes at 6 mM $Mg^{2+}$.

| | picomoles bound / $A_{260}$ ribosomes | |
|---|---|---|
| | Phe-tRNA | Phe-3'N-tRNA |
| complete system | 1.62 | 1.20 |
| minus EF-Tu | 0.43 | 0.62 |
| minus ribosomes | 0.15 | 0.13 |

The assay incubations contained per ml: either 20.0 $A_{260}$ units of salt-washed ribosomes or no ribosomes as indicated, 80 μg poly(U), either 880 pmoles purified EF-Tu or no EF-Tu as indicated, 0.2 μmoles GTP, 60 μmoles PEP and 80 μg pyruvate kinase (400 Units/mg). The mixture was buffered with 50 mM Tris-HCl, pH 7.8, and contained 80 mM $NH_4Cl$, 6 mM β-mercaptoethanol and 6 mM $Mg(C_2H_3O_2)_2$. After an initial incubation for 5 minutes at 37°C, the reaction mixtures were cooled on ice and either 242 pmoles ($^{14}C$)Phe-tRNA (specific activity 466 μCi/μM) or 258 pmoles ($^{3}H$) Phe-3'N-tRNA (specific activity 1.1 mCi/μM) were added per ml. Incubation was continued on ice for 10 minutes, at which time the reaction mixture was filtered through a nitrocellulose filter. The filters were washed with 10 ml of wash buffer containing 50 mM Tris-HCl, pH 7.8, 80 mM $NH_4Cl$, 6 mM β-mercaptoethanol and 6 mM $Mg(C_2H_3O_2)_2$. After washing, the filters were dried and radioactivity determined in a liquid scintillation counter.

Table 6: Poly(U) directed binding of Phe-tRNA and Phe-3'N-tRNA to ribosomes at 6 mM $Mg^{2+}$.

| | picomoles bound / $A_{260}$ ribosomes | |
|---|---|---|
| | Phe-tRNA | Phe-3'N-tRNA |
| complete system | 1.62 | 1.20 |
| minus EF-Tu | 0.43 | 0.62 |
| minus ribosomes | 0.15 | 0.13 |

The assay incubations contained per ml: either 20.0 $A_{260}$ units of salt-washed ribosomes or no ribosomes as indicated, 80 $\mu$g poly(U), either 880 pmoles purified EF-Tu or no EF-Tu as indicated, 0.2 $\mu$moles GTP, 60 $\mu$moles PEP and 80 $\mu$g pyruvate kinase (400 Units/mg). The mixture was buffered with 50 mM Tris-HCl, pH 7.8, and contained 80 mM $NH_4Cl$, 6 mM $\beta$-mercaptoethanol and 6 mM $Mg(C_2H_3O_2)_2$. After an initial incubation for 5 minutes at 37°C, the reaction mixtures were cooled on ice and either 242 pmoles ($^{14}C$)Phe-tRNA (specific activity 466 $\mu$Ci/$\mu$M) or 258 pmoles ($^{3}H$) Phe-3'N-tRNA (specific activity 1.1 mCi/$\mu$M) were added per ml. Incubation was continued on ice for 10 minutes, at which time the reaction mixture was filtered through a nitrocellulose filter. The filters were washed with 10 ml of wash buffer containing 50 mM Tris-HCl, pH 7.8, 80 mM $NH_4Cl$, 6 mM $\beta$-mercaptoethanol and 6 mM $Mg(C_2H_3O_2)_2$. After washing, the filters were dried and radioactivity determined in a liquid scintillation counter.

does catalyze the binding of Phe-3'N-tRNA to the ribosome, it does not interact with it as strongly as normal Phe-tRNA.

Although one would expect EF-Tu to catalyze binding of aminoacyl-tRNA exclusively to the A site, this is not necessarily the case. During protein synthesis in vivo, EF-Tu will bind aminoacyl-tRNA to the A site on the ribosome. There is no need for a mechanism to insure that EF-Tu will not catalyze binding to the D site because the D site is always occupied, either with peptidyl-tRNA or deacylated tRNA. It is not surprising, then, that although EF-Tu directs binding of aminoacyl-tRNA mainly to the A site, some binding in the D site has been observed under conditions similar to those used in the experiments reported here (Weissbach et al., 1971; Ravel et al., 1969). This fact must be considered in the interpretation of the ribosome binding data. It is possible that the difference of 0.4 pmole of aminoacyl-tRNA per $A_{260}$ unit of ribosomes in the EF-Tu catalyzed binding of normal versus analog aminoacyl-tRNA represents binding of the normal tRNA in the D site. The results of attempted binding of AcPhe-3'N-tRNA to the D site suggest that the D site may not accept 3' isomeric species. If this were the case, it would be expected that the EF-Tu catalyzed binding of Phe-tRNA would exceed that of Phe-3'N-tRNA by the amount normally bound in the D site. Puromycin reactivity could not be used in this case to establish A or D site binding of Phe-3'N-tRNA unless it were already shown that this species is capable of donor activity.

Although the fact that EF-Tu stimulates the binding of Phe-3'N-tRNA to ribosomes emerges from these results, it is not clear whether the Phe-3'N-tRNA interacts with the EF-Tu as well as does normal Phe-tRNA. This question could best be answered by directly measuring the binding of Phe-tRNA and Phe-3'N-tRNA to EF-Tu.

EF-Tu binding with Phe 3'N-tRNA

The ternary complex, EF-Tu· GTP· aminoacyl-tRNA has been strongly implicated as an obligatory intermediate in the in vivo binding of aminoacyl-tRNA to ribosomes. This complex is stable enough to be isolated on a Sephadex column. Millipore nitrocellulose filters are the basis of another method for detecting ternary complex formation. The ternary complex will not stick to Millipore filters, but the binary complex EF-Tu· GTP does stick to the filters. The EF-Tu· GTP complex reacts directly with aminoacyl-tRNA to form the ternary complex. Labeled GTP is first used to form a binary complex and aminoacyl-tRNA is then added to the incubation mixture which is filtered through a Millipore filter. The decrease in radioactivity bound to the filter after aminoacyl-tRNA addition, relative to that bound when no aminoacyl-tRNA is added, is a direct measure of the amount of ternary complex formed. The molecular basis for this nitrocellulose filter assay is unknown. Because of this, the results must be interpreted with some caution. It is certainly conceivable that a molecule could interact with and bind to EF-Tu (detectable by Sephadex gel filtration) without inducing the

conformational change required so that the ternary complex will not bind to the nitrocellulose filter, although this uncoupling has not been observed for any aminoacyl-tRNA or aminoacyl-tRNA analog molecules examined.

Table 7 shows the results of Millipore filter assays for ternary complex formation. The experiments were done with both Phe-tRNA and Phe-3'N-tRNA. These results clearly indicate that no stable ternary complex is formed with Phe-3'N-tRNA, while a substantial percentage of the normal Phe-tRNA forms a ternary complex with EF-Tu and GTP.

These results appear paradoxical because ternary complex formation is believed to be a prerequisite for EF-Tu stimulated binding of aminoacyl-tRNA to ribosomes. It is possible, however, that a very weak ternary complex is formed with Phe-3'N-tRNA. This would result in some stimulation of Phe-3'N-tRNA binding to ribosomes in the presence of EF-Tu and would also account for the lower extent of ribosomal binding of the Phe-3'N-tRNA compared with Phe-tRNA.

Table 7: EF-Tu·GTP·aminoacyl-tRNA complex formation with Phe-tRNA and Phe-3'N-tRNA.

| Aminoacyl-tRNA | Picomoles aminoacyl-tRNA added | Picomoles complex forme |
|---|---|---|
| Phe-tRNA | 4.5 | 3.2 |
| | 12.0 | 9.5 |
| Phe-3'N-tRNA | 3.0 | 0.0 |
| | 6.0 | 0.0 |

The assay incubations contained per ml: 804 pmoles of purified EF-Tu, 0.01 $\mu$mole ($^3$H) GTP (specific activity 454 $\mu$Ci/$\mu$M), 60 $\mu$moles PEP and 80 $\mu$g pyruvate kinase (400 Units/mg). The mixture was buffered with 50 mM Tris-HCl, pH 7.8, and contained 80 mM $NH_4Cl$, 6 mM $\beta$-mercaptoethanol and 6 mM $Mg(C_2H_3O_2)_2$. After an initial incubation for 10 minutes at 37°C, the reaction mixtures were cooled on ice and the indicated amounts of either Phe-tRNA or Phe-3'N-tRNA were added. Incubation of the 50 $\mu$l assay mixtures was continued on ice for at least one minute at which time ternary complex formation was complete. Samples were then filtered through a nitrocellulose filter and washed as described in the legend to Table 6.

# 2', 3' Isomeric Specificity of Aminoacylation

## Introduction

The prospect of verifying the conclusions reached in studies with Phe-3'N-tRNA was enhanced by receipt of approximately three milligrams of 2'-amino-2'-deoxy adenosine. This was easily made into 2'-amino-2'-deoxy ATP and proved to be at least as good a substrate for tRNA-nucleotidyl transferase as 3'-amino-3'-deoxy ATP.

Since phenylalanine had been shown to aminoacylate almost exclusively at the 2'-position (Sprinzl and Cramer, 1973; Ofengand et al., 1974), it was expected that the aminoacyl-tRNA synthetase would have to catalyze the formation of an amide bond in order to aminoacylate the tRNA-C-C-2'$A_N$. This seemed very likely on chemical grounds, as the amino group is a much better nucleophile than the hydroxyl group. It was found, however, that this reaction was not catalyzed by phenylalanyl-tRNA synthetase.

In order to prepare Phe-2'N-tRNA, attempts were made to carry out the aminoacylation reaction under conditions known to induce mischarging of normal tRNA's. By "relaxing" the specific interactions between tRNA and synthetase, it was hoped that enzymatic aminoacylation might occur at the 3'-hydroxyl group. If this were to happen subsequent isomerization would result in an amide bond between phenylalanine and the 2'-amino group. Accordingly, aminoacylations were attempted in the presence of dimethylsulfoxide (DMSO), high enzyme concentrations, high $Mg^{2+}$/ATP ratios and

high pH (Giege et al., 1972). Even after two hour incubations, no base stable phenylalanyl-tRNA was ever detected under any of these conditions. It should be noted in this respect that one would not expect aminoacylation if the 2'-amino group were protonated. The pK of the amino group in 2'-amino-2'-deoxyadenosine has not been determined, but the pK of the closely related compound 2'-amino-2'-deoxyuridine is 6.2 (Verheyden et al., 1971). Although this pK seems low for a primary amino group, the measured pK range of different amino sugars is 6.1-7.7 (Barlow et al., 1969). Thus, the amino group in 2'-amino tRNA was almost certainly unprotonated under the conditions aminoacylation was attempted (pH 7.8-pH 9.0). The conclusion drawn from the failure of $tRNA^{phe}$-C-C-2'$A_N$ to charge is that the isomeric specificity of aminoacylation is invarient under a variety of conditions.

## Results

When unfractionated E. coli tRNA-C-C-2'$A_N$ was incubated with RNA-free S-100 and a mixture of labeled amino acids, a substantial amount of radioactivity was found attached to the tRNA through a base stable linkage. If unfractionated tRNA-C-C-3'$A_N$ was used instead of the 2'-amino tRNA analog, it was found that this, too, could form base stable linkages with the amino acids. These results suggested that the aminoacyl-tRNA synthetases may attach some amino acids to tRNA-C-C-2'$A_N$, and others to tRNA-C-C-3'$A_N$.

In order to determine if there were actually an amino acid dependent specificity, all 20 amino acids were tested for their ability to form a base stable linkage with both the 2'- and 3'-amino tRNA analogs. Figure 18 is a flow diagram of the assay used for all amino acids.

A 60 minute incubation period was chosen in order to approximate the extent of aminoacylation. It had been found that aminoacylation of either 3'- or 2'-amino tRNA analogs with an amino acid mixture plateaued after 45 minutes of incubation. A plateau is reached after only 20 minutes of incubation with normal tRNA. These results indicate that the amino tRNA's must be incubated for a relatively long time in order to become fully charged.

N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) was used as a buffer rather than Tris-HCl, which has been widely used

Figure 18: Flow diagram of aminoacylation assay for tRNA-C-C-3'$A_N$ and tRNA-C-C-2'$A_N$

All assays for acceptor activity with individual amino acids were done under the same conditions. The reaction mixtures contained the following components: 50 mM HEPES (N-2-Hydroxyethyl-piperazine-N'-2 ethanesulfonic acid), 50 mM deoxyadenosine triphosphate (or adenosine triphosphate as indicated), 20 mM $Mg(C_2H_3O_2)_2$, 50 mM KCl, 2.5 mM reduced glutathione, 100-125 $\mu$M labeled amino acid, 125 $\mu$M of 19 unlabeled amino acids. The pH was adjusted to 7.8 by the addition of KOH. A 40 $\mu$l reaction mixture contained 1.7 $\mu$g of RNA-free S-100 and 0.15-0.2$A_{260}$ units of tRNA. Incubation was carried out at 37°C for 60 minutes at which time 1 $\mu$l of 0.5M CTAB was added. After a short time at 0°C the reaction mixtures were centrifuged at 12,000 x g for 15 minutes and the supernatants discarded. The precipitates were dissolved in 1 M $K(C_2H_3O_2)$, pH 4.5 and precipitated with 100% ethanol. After at least two hours at -20°C the samples were again centrifuged and the supernatant discarded. The precipitates were dried in a vacuum dissicator and then dissolved in 50 $\mu$l of 0.5M Tris-HCl, pH 9.5, incubated at 37°C for 60 minutes, and precipitated with 10% trichloroacetic acid, 1% casamino acids.

The following radioactive L-amino acids were from New England Nuclear: (3-$^{3}$H) Ala (13.2 Ci/mM), (3-$^{3}$H) Arg (21.6 Ci/mM), ($^{14}$C) Asn (179 mCi/mM), (2,3-$^{3}$H) Asp (17.8 Ci/mM), (3-$^{3}$H) Glu (24.1 Ci/mM), ($^{14}$C) Gln (235 mCi/mM), (3-$^{3}$H) His (9.56 Ci/mM), (4,5-$^{3}$H) Ileu (63.4 Ci/mM), (4,5-$^{3}$H) Leu (5.0 Ci/mM), ($^{3}$H) Lys (2.2 Ci/mM), ($^{14}$C) Phe (466 mCi/mM), ($^{3}$H) Pro (3.3 Ci/mM), ($^{3}$H) Ser (3.38 Ci/mM), ($^{3}$H) Thr (2.09 Ci/mM), ($^{3}$H) Trp (7.9 Ci/mM), (2,6-$^{3}$H) Tyr (49.5 Ci/mM), ($^{14}$C) Val (242 mCi/mM).

From Amersham/Searle: (2-$^{3}$H) Gly (2.8 Ci/mM) and ($^{35}$S) Met (510 Ci/mM).

50 mM HEPES, pH 7.8
RNA-free S-100
1 labeled amino acid
19 unlabeled amino acids
ATP or dATP
Salts

Incubate 60 minutes at 37°C

Precipitate tRNA with CTAB
Centrifuge, discard supernatants

Dissolve pellets in 1.0 M $K(C_2H_3O_2)$, pH 4.5
Reprecipitate with 2 X volume of ethanol
Centrifuge, discard supernatants

Dissolve precipitates in 0.5 M Tris-HCl, pH 9.5
Incubate 60 minutes at 37°C

TCA precipitate

in aminoacylation reactions. When Tris-HCl was used to buffer an aminoacylation reaction in which a mixture of labeled amino acids were attached to tRNA, the TCA precipitable radioactivity reached a maximum after approximately 20 minutes at 37°C. After this time, the amount of aminoacyl-tRNA declined and usually decreased by at least 20% after 60 minutes. Under aminoacylation conditions that were identical except for the use of HEPES -KOH instead of Tris-HCl, no decline of TCA precipitable radioactivity after 20 minutes at 37°C was observed. This may be due to increased stability of the aminoacyl-tRNA synthetase activities in a solution buffered by HEPES-KOH. If a mixture of aminoacyl-tRNA's is incubated at pH 7.8 with 50 mM HEPES-KOH, the average half-life of these species at 37°C is approximately 22 minutes. Thus, a 60 minute incubation requires constant reaminoacylation of deacylated tRNA to maintain a plateau level of charged tRNA. If the activity of the aminoacylating enzymes deteriorates during the incubation, this plateau level may not be maintained. In the 60 minute incubation employed for these experiments, HEPES-KOH was therefore the more suitable buffer. At the end of the incubation period, cetyltrimethyl ammonium bromide (CTAB) was used to precipitate the tRNA. This reagent does not precipitate proteins, so that any radioactive amino acid which had become incorporated or entrapped would remain in the supernatant. After centrifugation the tRNA was washed with 67% ethanol to reduce the background radioactivity.

After drying in a vacuum dessicator, the tRNA was dissolved in 0.5M Tris-HCl, pH 9.5, and incubated for 60 minutes at 37°C. As discussed previously, the amino tRNA analog preparations were contaminated with normal tRNA. This pH 9.5 incubation insured that no normal aminoacyl-tRNA was measured in these experiments. When controls were done with normal aminoacyl-tRNA it was found that the pH 9.5 incubation reduced the TCA precipitable radioactivity to background level for all amino acids except isoleucine and valine. When bound to tRNA these amino acids form ester bonds which are more stable to base catalyzed hydrolysis than those of any other aminoacyl-tRNAs (Hentzen et al., 1972). The half-lives of isoleucyl-tRNA and valyl-tRNA were therefore measured at pH 9.5 and 37°C. These values were then employed to calculate the amounts of valine and isoleucine linked to an amino tRNA analog in the aminoacylation reaction. As has been clearly demonstrated in the case of Phe-3'N-tRNA, base stability of the aminoacyl derivative of a 2'-or 3'-amino tRNA analog indicates that an amide bond has been formed between the amino acid and the tRNA.

The results of the analysis for 19 amino acids are shown in Table 8. Normal $tRNA^{cys}$ was unable to be charged in this system. The first and fourth columns list the number of picomoles of the amino acid bound to normal tRNA per $A_{260}$ using two different energy sources. Differences in the extent of aminoacylation with normal tRNA-C-C-A

as substrate reflect differences in the amount of amino acid-specific tRNA in the unfractionated tRNA preparation, the stability of the various aminoacylating enzymes for this 60 minute long incubation, as well as the fact that the reaction conditions were not optimized for any particular amino acid. The extent of aminoacylation is not critically dependent on whether dATP or ATP is used as an energy source except in the case of glutamic acid, which is increased 30-fold when ATP replaces dATP as an energy source. dATP was originally used as an energy source in the aminoacylation assays because it is not a substrate for E. coli tRNA-nucleotidyl transferase. The RNA-free S-100 used as the source of aminoacyl-tRNA synthetases had tRNA-nucleotidyl transferase activity and would put ATP onto the 3' end of tRNA-C-C present in the amino tRNA preparations. The resulting tRNA-C-C-A would be immediately aminoacylated. Once it was recognized that normal tRNA was contaminating the amino tRNA analog preparations in any case, the assays were redone with ATP as an energy source. The final pH 9.5 incubation insures that none of the radioactivity in normal aminoacyl-tRNA is counted.

The other columns in Table 8 show the amount of aminoacyl-tRNA analog which is stable after incubation at pH 9.5 for 60 minutes at 37°. It can be seen that some amino acids form base stable 3'-analogs, some form base stable 2'-analogs, while others form base stable compounds with both amino tRNA analogs. In all cases, the amount of

Table 8: Extent of aminoacylation with normal and amino tRNA analogs expressed as picomoles amino acid/$A_{260}$.

| | dATP energy source | | | ATP energy source | | |
|---|---|---|---|---|---|---|
| Amino Acid | The 3' ends of the tRNA acceptor molecules | | | | | |
| | A | $3'A_N$ | $2'A_N$ | A | $3'A_N$ | $2'A_N$ |
| Ala | 5.10 | 0.29 | 2.05 | 6.21 | 0.11 | 1.53 |
| Arg | 65.2 | 15.8 | 12.5 | 78.7 | 19.3 | 14.1 |
| Asn | 13.0 | 0.25 | 2.49 | 22.0 | 0.00 | 3.33 |
| Asp | 22.9 | 0.16 | 5.46 | 38.7 | 0.02 | 8.40 |
| Cys | | | | | | |
| Glu | 2.20 | - | - | 64.3 | 3.45 | 0.18 |
| Gln | 21.1 | 1.60 | 0.00 | 31.0 | 3.52 | 0.00 |
| Gly | 37.7 | 0.00 | 7.00 | 35.7 | 0.00 | 4.36 |
| His | 21.9 | 1.22 | 14.4 | 35.1 | 0.62 | 13.5 |
| Ileu | 57.0 | 7.34 | 18.4 | 61.5 | 8.81 | 9.35 |
| Leu | 49.6 | 14.0 | 0.83 | 85.0 | 16.1 | 2.14 |
| Lys | 100.6 | 0.36 | 21.5 | 91.0 | 0.08 | 13.9 |
| Met | 35.0 | 3.94 | 3.39 | | | |
| Phe | 26.5 | 4.9 | 0.00 | | | |
| Pro | 81.2 | 17.2 | 25.1 | 72.1 | 13.1 | 21.4 |
| Ser | 67.9 | 1.11 | 1.50 | 44.8 | 1.86 | 2.62 |
| Thr | 60.4 | 0.00 | 1.11 | 45.7 | 0.00 | 1.20 |
| Trp | 9.40 | 1.56 | 0.46 | 13.0 | 0.92 | 0.46 |
| Tyr | 25.2 | 1.62 | 0.11 | 31.4 | 2.96 | 0.52 |
| Val | 45.7 | 16.4 | 0.29 | | | |

Aminoacylation was done as described in the legend to Figure 18.

base stable aminoacyl-tRNA analog is much lower than the corresponding normal aminoacyl-tRNA. This is largely due to the contamination of the analog tRNA's with normal tRNA-C-C-A. Only 25 to 30% of the tRNA in these amino tRNA analog preparations had an amino adenosine at the 3' end. Another 25% was unreacted tRNA-C-C. Approximately 50% of the preparation was found to be tRNA-C-C-A. This contamination of the amino tRNA analogs has been discussed in detail previously in this dissertation.

Although all aminoacyl-tRNA synthetases tested aminoacylated one or the other amino tRNA analog, the extent of the reactions relative to the normal tRNA controls varied widely. As shown in Table 8, the extent of reaction with serine and threonine was quite low. Even after applying an appropriate correction to the data in Table 8 due to the fact that only 25% of the modified tRNA had an amino analog on the 3' terminus, the extent of aminoacylation with these two amino acids was only 10 to 20% of that seen for normal tRNA. It is possible that a change of the reaction conditions would increase the amount of aminoacyl-tRNA analog formed with serine and threonine. However, the observations that the enzymes function well with normal tRNA under these conditions and that the amino tRNA analogs are good substrates for the other synthetases under these conditions suggests that a change in reaction conditions is unlikely to appreciably alter the extent of aminoacylation for serine and threonine.

Another possible explanation is that the tRNA-nucleotidyl transferase discriminates among the different tRNA species. Thus, $tRNA^{ser}$ and $tRNA^{thr}$ may be poor substrates for tRNA-nucleotidyl transferase and consequently have much less 2'- or 3'-amino adenosine at the 3' terminus than do the other tRNA's. The question of enzyme selectivity for different tRNA species in the addition of CMP and AMP to snake venom phosphodiesterase digested tRNA has been partially answered by Miller et al. (1970). They reported no significant differences in amino acid acceptor activity for the 15 amino acids which they tested, between normal tRNA and tRNA which had been digested with snake venom phosphodiesterase and reconstituted with tRNA-nucleotidyl transferase. Serine was among these amino acids, but threonine was not. Thus, at least when AMP is added to tRNA-C-C, the tRNA-nucleotidyl transferase does not appear to discriminate among different tRNA species. These results also rule out the possibility of inactivation of $tRNA^{ser}$ due to the high pH (9.2) conditions required for optimal tRNA-nucleotidyl transferase activity. Further studies will have to be carried out to determine why the extent of aminoacylation of the amino tRNA analogs is close to the level of normal tRNA for some synthetases, and very low relative to normal tRNA for others. However, although the amino tRNA analogs specific for serine and threonine are aminoacylated to a low extent relative to normal $tRNA^{ser}$ and $tRNA^{thr}$, the results obtained are both reproducible and clear.

In Table 9 the results of some of the data in Table 8 have been recalculated to show the base stability of the aminoacyl-tRNA analogs derived from either the 2'-amino or the 3'-amino tRNA analogs as a percent of the total amount of base stable 2'-plus 3'-aminoacyl-tRNA analogs. The amino acids fall into three different classes which are separated by dashed lines. These classes are not dependent on whether ATP or dATP is used as an energy source. Class 2' includes the amino acids phenylalanine, glutamine, valine, leucine, tyrosine, and glutamic acid. The percentage of base stable 3'-aminoacyl-tRNA analogs ranges from 85 to 100%. A second group, Class 3', includes the amino acids glycine, lysine, threonine, aspartic acid, histidine, asparagine, and alanine. These amino acids form base stable aminoacyl-tRNA's with the 2'-amino tRNA analogs, with percentages which vary from 88 to 100%. Finally, there is a group of amino acids, Class 2',3', in which both the 2'- and 3'-amino tRNA analogs form base stable compounds. In this group fairly substantial amounts of base stable aminoacyl-tRNA analogs are formed with both 2'- and 3'-amino tRNA analogs.

Although there appear to be clear demarcations among these three classes, the divisions are somewhat arbitrary. In both Class 2' and 3' synthetases, there is a clear and overwhelming preference for the 3'- and 2'- amino tRNA analogs respectively. Whether this preference amounts to exclusivity is not yet clear. The small percentages of

Table 9. Relative amounts of amino acid added to tRNA-C-C-3'$A_N$ and tRNA-C-C-2'$A_N$ expressed as the percentage of total base stable aminoacyl-tRNA.

| | dATP energy source | | ATP energy source | | |
|---|---|---|---|---|---|
| Amino Acid | tRNA-C-C-3'$A_N$ | tRNA-C-C-2'$A_N$ | tRNA-C-C-3'$A_N$ | tRNA-C-C-2'$A_N$ | Class |
| Phe | 100 | 0 | 100 | 0 | |
| Gln | 100 | 0 | 100 | 0 | |
| Val | 96 | 4 | | | |
| Glu | - | - | 95 | 5 | 2' |
| Leu | 94 | 6 | 88 | 12 | |
| Tyr | 94 | 6 | 85 | 15 | |
| Trp | 77 | 23 | 67 | 33 | |
| Arg | 56 | 44 | 58 | 42 | |
| Met | 54 | 46 | | | |
| Ser | 42 | 58 | 42 | 58 | 2, 3' |
| Pro | 41 | 59 | 38 | 62 | |
| Ileu | 28 | 72 | 48 | 52 | |
| Ala | 12 | 88 | 6 | 94 | |
| Asn | 9 | 91 | 0 | 100 | |
| His | 8 | 92 | 4 | 96 | |
| Asp | 3 | 97 | 0 | 100 | 3' |
| Lys | 2 | 98 | 1 | 99 | |
| Thr | 0 | 100 | 0 | 100 | |
| Gly | 0 | 100 | 0 | 100 | |

aminoacylation with the minor amino tRNA analog in some cases, as with histidine, certainly reflects a low level of charging. The high specific activity and low background of this amino acid permits the conclusion that the histidyl-tRNA synthetase, although placed in Class 3', is capable of using tRNA-C-C-3'$A_N$ as a substrate in addition to tRNA-C-C-2'$A_N$. In other cases, actual aminoacylation of the minor amino tRNA analog cannot be verified due to low specific activity or high background. Further experiments using highly purified amino acids with a high specific activity will have to be done before clear answers are found for all aminoacyl-tRNA synthetases.

Mechanisms of aminoacylation

The mechanism of an aminoacylation reaction is a function of the aminoacyl-tRNA synthetase. Thus, the classes defined in Table 9 refer to classes of synthetase enzymes. It is probable that the different classes of enzymes employ different aminoacylation mechanisms.

The interpretation of the mechanism of aminoacylation for synthetases in Classes 3' and 2' is shown in Figures 19 and 20. Figure 19(a) shows the mechanism of aminoacylation by a synthetase which is specific for the 3'-hydroxyl group. Normal tRNA is first aminoacylated on that position and the amino acid then isomerizes between the 2' and 3' sites as shown by the doubleheaded arrow. The nuclear magnetic resonance spectroscopic study of this isomerization has shown that the normal half-life for this process is approximately two-tenths of a millisecond (Griffin et al., 1966). These synthetases produce base stable compounds with the 2'-amino tRNA analog, involving a mechanism in which the 3'-hydroxyl group is initially aminoacylated as shown in Figure 19(b). Following aminoacylation, isomerization with an O $\longrightarrow$ N acyl migration results in attachment of the amino acid to the amino group of the terminal adenosine by an amide bond. The O $\longrightarrow$ N acyl migration is a well-characterized chemical reaction, and will occur under the slightly alkaline conditions of the aminoacylation incubation (Welsh, 1949; van Tamelen, 1951). N $\longrightarrow$ O shifts, on the other hand, will occur at a negligible rate under these conditions.

Measurable rates are usually obtained with dilute (1-5%) boiling, mineral acid (Welsh, 1949; van Tamelen, 1951). Thus, the amino acid will not migrate under the conditions employed in these experiments once it has formed an amide bond. Attempted aminoacylation of the 3'-amino tRNA analog with Class 3' synthetases produces no base stable compound, as illustrated in Figure 19(c). In this group of synthetases the aminoacylation site is specific. If this were not the case, and the enzymes were able to aminoacylate the hydroxyl groups in both the 3' and the 2' sites, then both the 2'- and 3'-amino tRNA analogs would produce base-stable derivatives. Both tRNA analogs would also form base stable compounds if the enzymes were able to aminoacylate the amino group directly, in addition to the hydroxyl group.

Figure 20 illustrates the mechanism of aminoacylation by a synthetase which is specific for the 2'-hydroxyl group. The reaction with normal tRNA is shown in 20(a), while the 2'-amino tRNA analog does not react (Fig. 20(b)). The 2'-hydroxyl specific aminoacylation results in a base stable product only with the 3'-amino tRNA analog, as shown in Figure 20(c) where the amino acid isomerizes from 0 to N as discussed above. By the same argument cited above, these enzymes are also site specific.

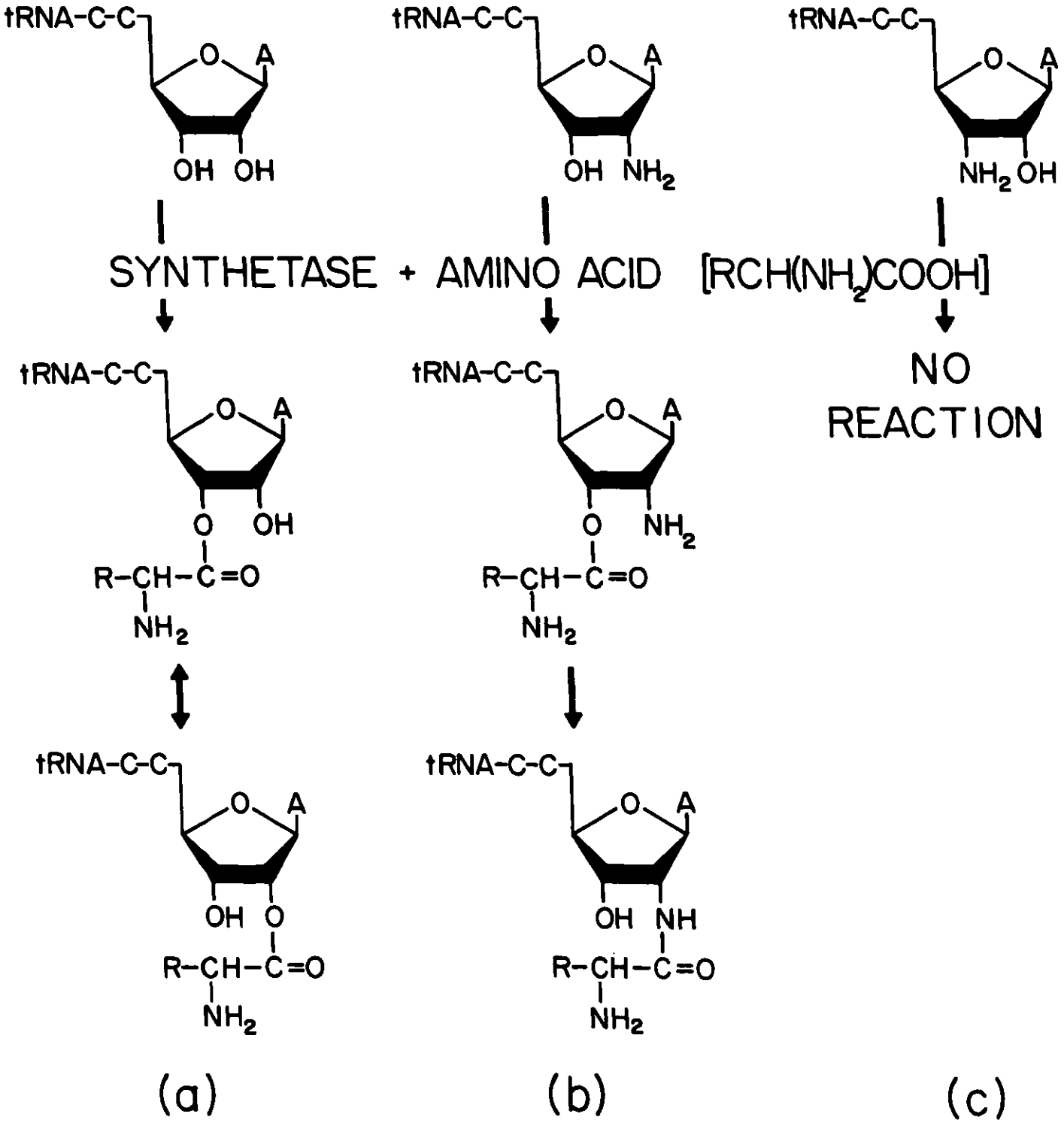
We are left with the question of the mechanism of Class 2', 3' enzymes, which produce base stable derivatives with both the 2'- and 3'-amino tRNA analogs. At least three possible explanations exist for

Figure 19: 3'-Hydroxyl specific aminoacylation of normal tRNA(a), tRNA-C-C-2'$A_N$(b) and tRNA-C-C-3'$A_N$(c).

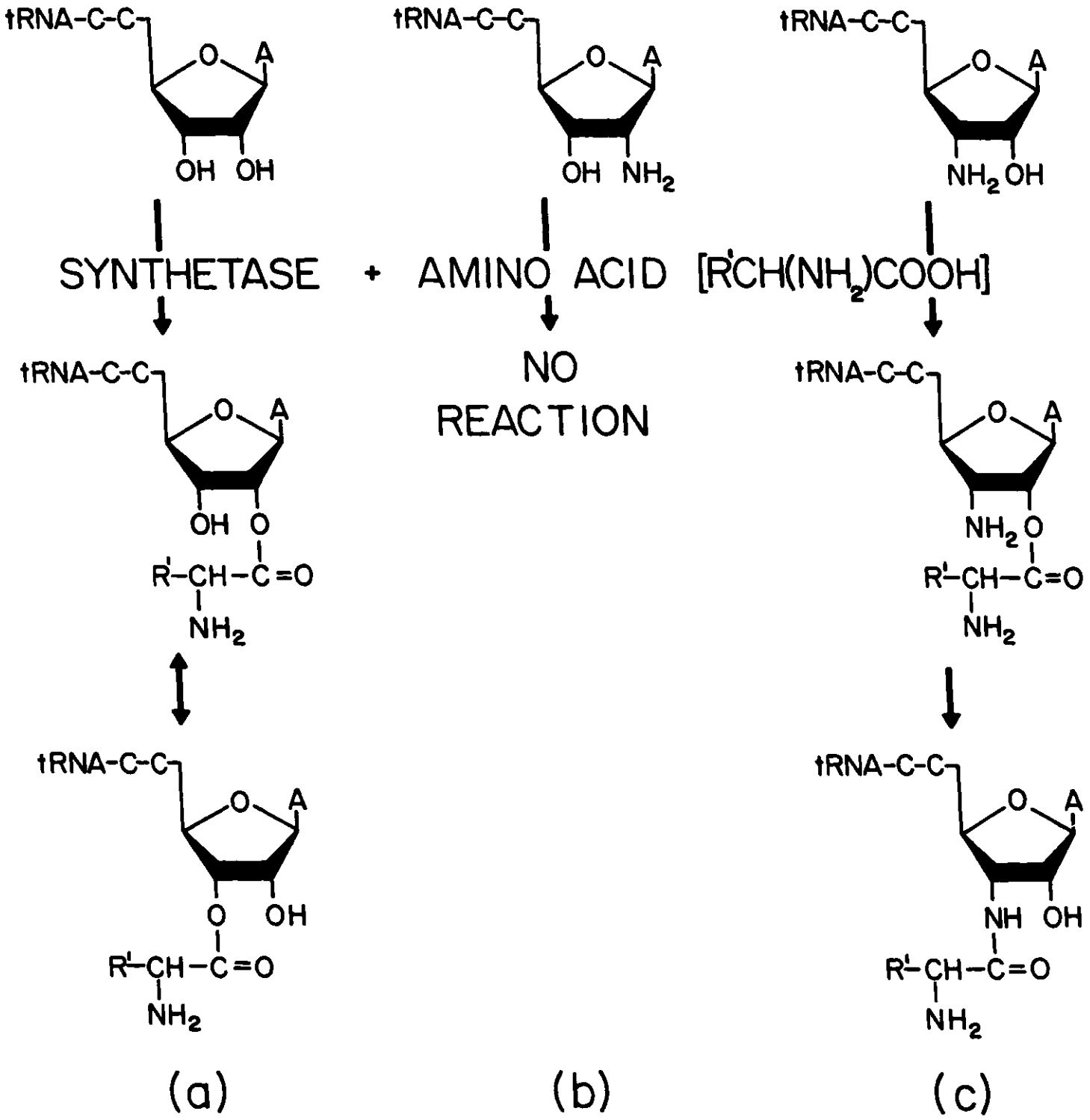
Figure 20: 2'-Hydroxyl specific aminoacylation of normal tRNA(a), tRNA-C-C-2'$A_N$(b) and tRNA-C-C-3'$A_N$(c).

These figures illustrate both the initial enzymatic attachment of an amino acid and subsequent non-enzymatic isomerization. These mechanisms are proposed to interpret the results of Class 3' and Class 2' aminoacyl-tRNA synthetases.

# 3'-HYDROXYL SPECIFIC AMINOACYLATION



# 2'-HYDROXYL SPECIFIC AMINOACYLATION



these enzymes. The enzymatic site may have some indeterminancy allowing aminoacylation to proceed at either the 2'-or the 3'-hydroxyl positions in the normal tRNA; alternatively, the enzymes may be restricted to aminoacylation exclusively at the 2' or the 3' site, but the enzyme may be able to aminoacylate the amino group directly as well as the hydroxyl group. These two interpretations are fundamentally different in that the first, the hydroxyl specific mechanism, assumes the possibility of substantial mobility of the terminal ribose on the enzymatic surface to allow access to both sites. In the second explanation, the site specific mechanism, the ribose is securely positioned in the activated complex, but the mechanism allows direct formation of an amide bond in addition to the ester bond which is normally formed. The third possibility is that Class 2', 3' contains tRNAs in which different isoacceptor species are specifically aminoacylated on either the 2' or the 3' site thereby giving rise to base stable compounds in both classes. This explanation seems unlikely, however, since tryptophan is in Class 2', 3' and E. coli has been shown to have only one isoaccepting species (Hirsh, 1971).

The mechanisms of aminoacylation for the hydroxyl specific and site specific hypotheses are shown in Figures 21 and 22.

Figure 21 illustrates the hydroxyl specific mechanism. A normal tRNA aminoacylated by a synthetase employing this mechanism would be expected to have its amino acid initially attached to the 2'-hydroxyl group in some cases and the 3'-hydroxyl group in others. Although there

might be a preference for either the 2'- or 3'-hydroxyl, the synthetase would not be able to unambiguously distinguish between them. When the 2'- and 3'-amino tRNA analogs are used as synthetase substrates, a base stable aminoacyl-tRNA analog will be formed with both of them as shown in Figure 21.

Figure 22 illustrates the 3' site specific mechanism. A synthetase employing this mechanism would catalyze aminoacylation only at the 3' position. Likewise a class of 2' site specific enzymes would aminoacylate only at the 2' position (not illustrated). The site specificity of these enzymes is unambiguous. They differ from the enzymes in Class 2' or Class 3' only in that they can catalyze the direct formation of an amide bond, whereas the Class 2' and 3' enzymes cannot.

The differences between the hydroxyl specific and site specific mechanisms are distinct. Experiments with the 2'- and 3'-amino tRNA analogs, however, cannot unambiguously distinguish between them. The use of a 2', 3'-diamino-2', 3'-dideoxy adenosine substituted at the 3' end of tRNA would allow a direct test of the ability of Class 2', 3' synthetases to catalyze formation of an amide bond. Other adenosine analogs at the 3' end of tRNA, such as 2'-deoxyadenosine and 3'-deoxyadenosine, would also allow one to distinguish between the hydroxyl specific and site specific mechanisms.

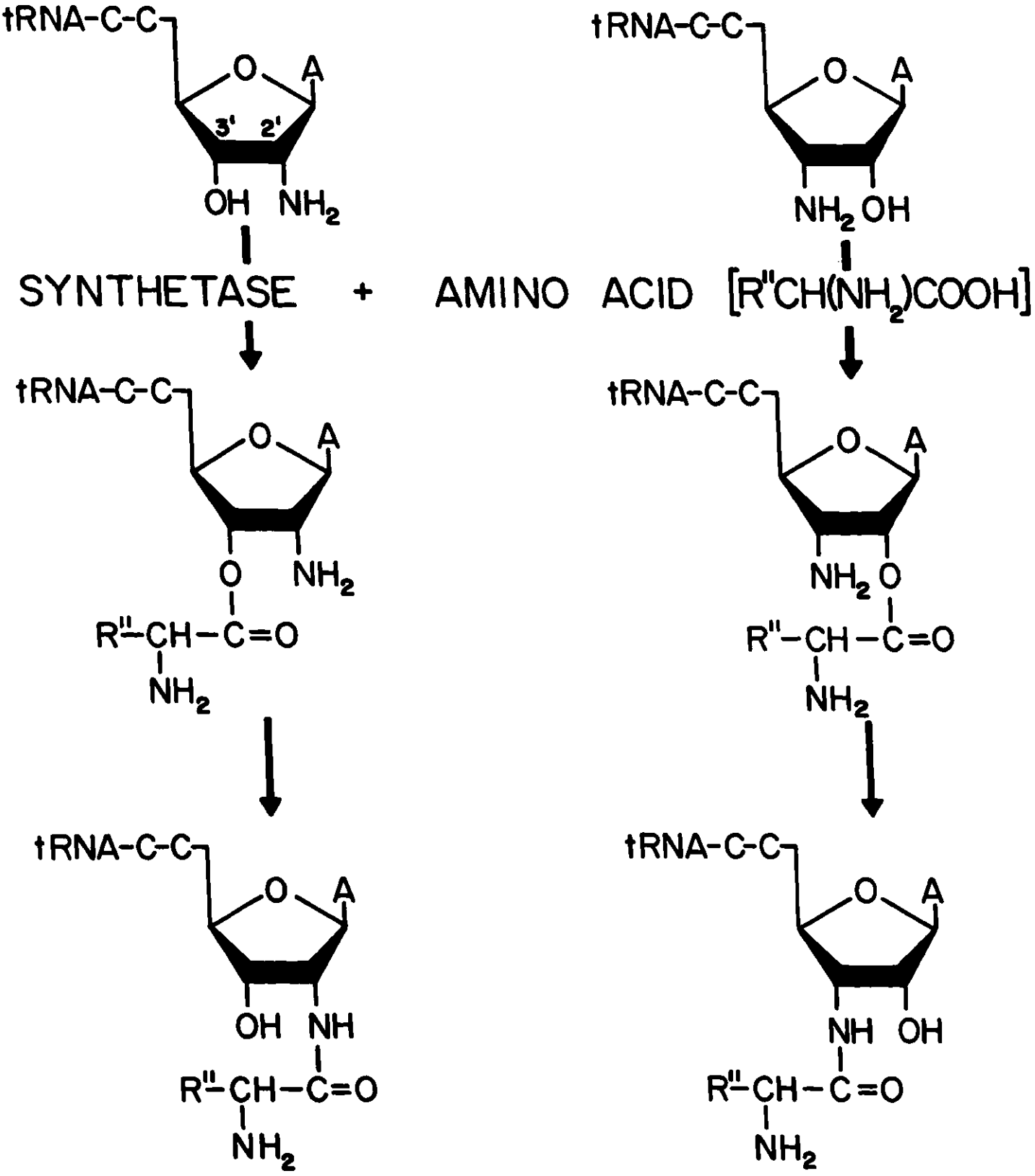
In order to determine which site is preferentially aminoacylated by the different synthetases in Class 2', 3', the kinetics of aminoacylation

Figure 21: Hydroxyl specific aminoacylation of tRNA-C-C-2'$A_N$ and tRNA-C-C-3'$A_N$.

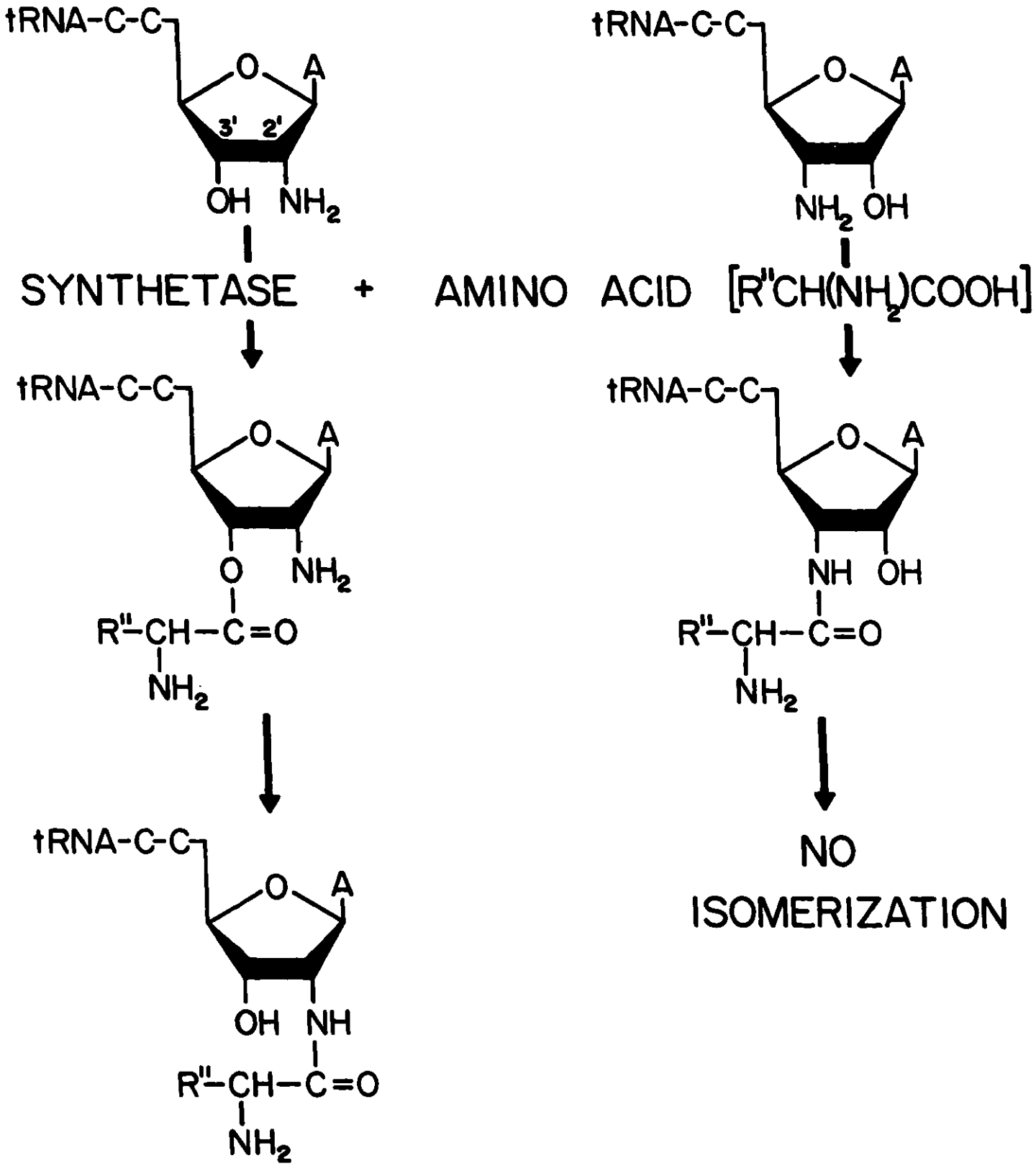
Figure 22: 3' site specific aminoacylation of tRNA-C-C-2'$A_N$ and tRNA-C-C-3'$A_N$.

These figures illustrate both the initial enzymatic attachment of an amino acid and subsequent non-enzymatic isomerization. These mechanisms are proposed to interpret the results of Class 2', 3' aminoacyl-tRNA synthetases.

# HYDROXYL SPECIFIC AMINOACYLATION



# 3' SITE SPECIFIC AMINOACYLATION



for both the 2'- and 3'-amino tRNAs with some Class 2', 3' synthetases have been examined. Figures 23 and 24 show these kinetics for methionyl-tRNA and arginyl-tRNA. As seen in Figure 23, the conversion of methionine to a base stable aminoacyl-tRNA with the 3'-amino tRNA analog is characterized by a very rapid aminoacylation which is largely completed in the first five minutes of incubation. In contrast to this, the rate of aminoacylation is about 15 times slower with the 2'-amino tRNA analog. Under these conditions, normal methionine-accepting tRNA (including $tRNA_m^{met}$ and $tRNA_f^{met}$) reaches a plateau level of aminoacylation between 2.5 and 5 minutes after incubation begins. These kinetics are similar to though somewhat faster than those observed with the 3'-amino tRNA analog. Figure 24 shows the kinetics of aminoacylation with arginine. Again, the 3'-amino tRNA is rapidly aminoacylated while the 2'-amino tRNA is aminoacylated approximately 20 times more slowly. The charging level of normal $tRNA^{arg}$ reaches a plateau after 2.5 minutes of incubation and the initial rate of aminoacylation is at least five times faster than with 3'-amino tRNA.

Figure 25 shows the kinetics of aminoacylation for proline. In this case the initial charging rate is much greater for the 2'-amino tRNA analog than for the 3'-amino-tRNA analog. This result suggests that there is no general feature of either the 2'- or 3'-amino tRNA's interactions with synthetases which would result in one of their always

being aminoacylated at a higher rate.

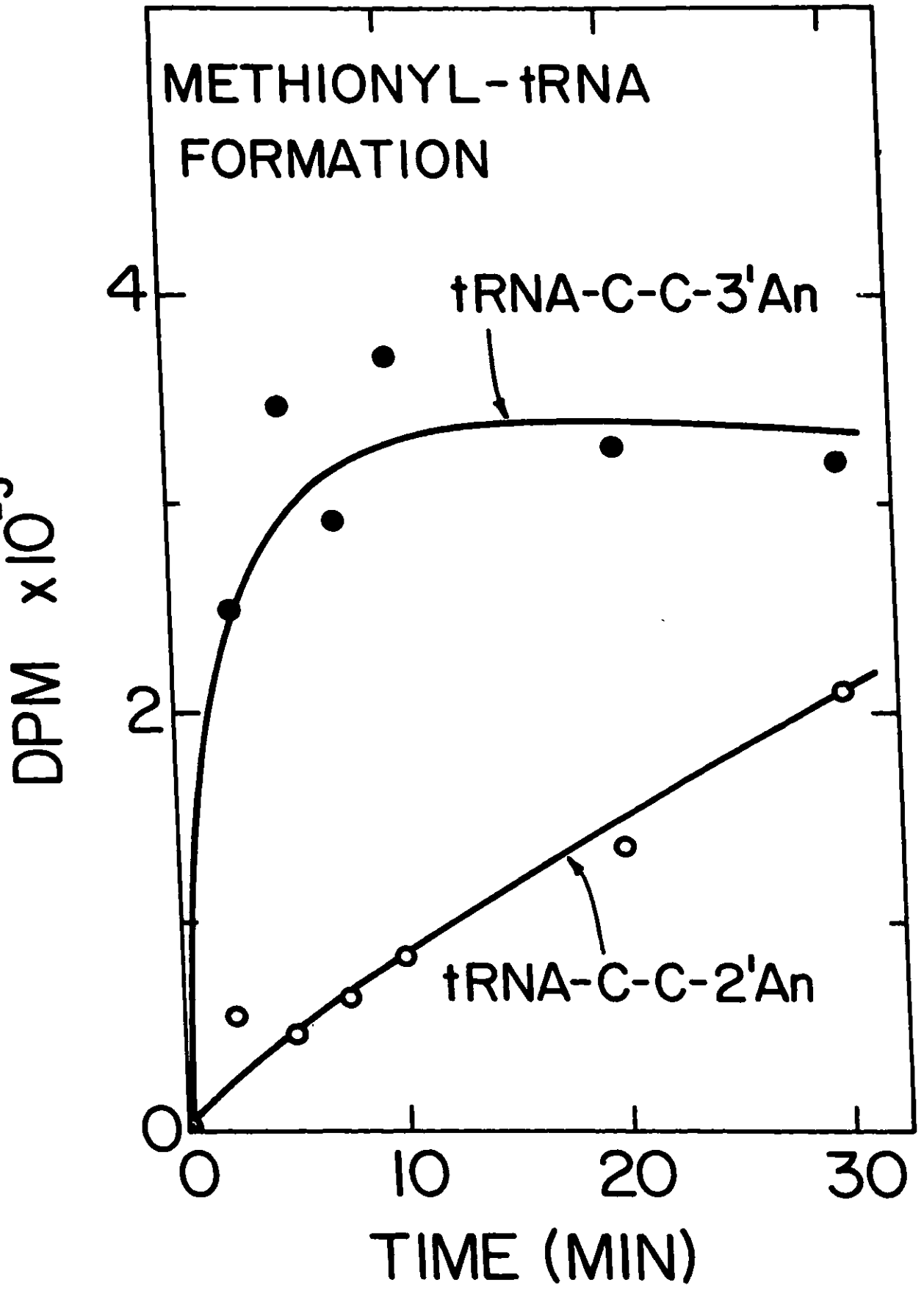
The rapidity of aminoacylation of the 3'-amino tRNA analogs for methionine and arginine and the 2' analog for proline, and the fact that this is similar to the kinetics using normal tRNA might suggest that the rapid kinetics represent a normal hydroxyl specific acylation mechanism, and the slower reaction may represent the direct aminoacylation of the amino group. This interpretation suggests that methionine and arginine belong to Class 2' and proline to Class 3'. However, these rate differences could also reflect differential binding of the 2'- and 3'-amino tRNA analogs to the enzyme, or a much lowered maximal velocity for the aminoacylation of one hydroxyl group compared with the other. As stated previously, then, the aminoacylation kinetics are unable to distinguish between the site specific and hydroxyl specific mechanisms, but it is clear that the Class 2', 3' synthetases which have been examined aminoacylate one amino tRNA analog at a significantly greater rate than the other.

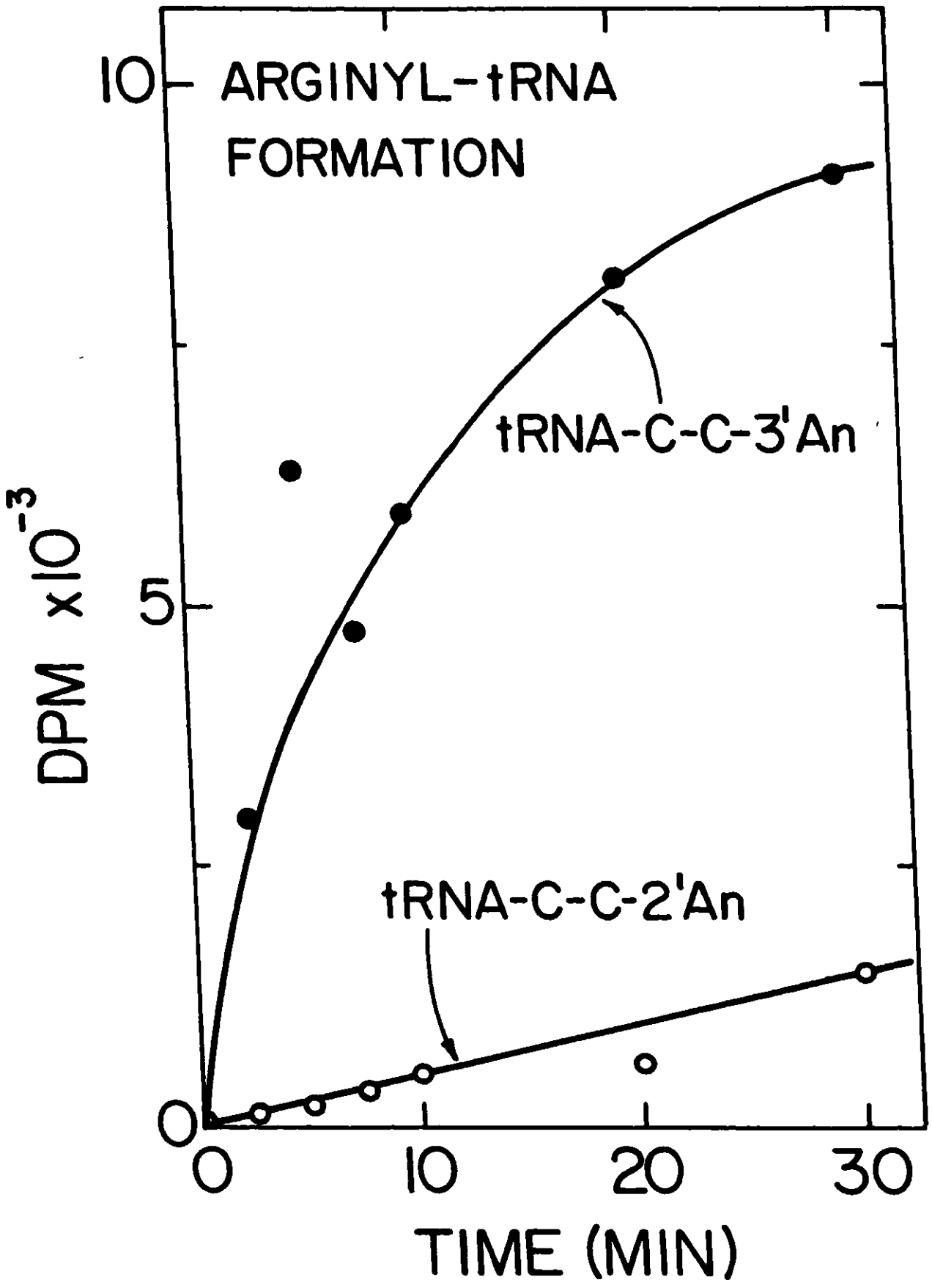
Figure 23: Kinetics of methionyl-2'N-tRNA and methionyl-3'N-tRNA formation.

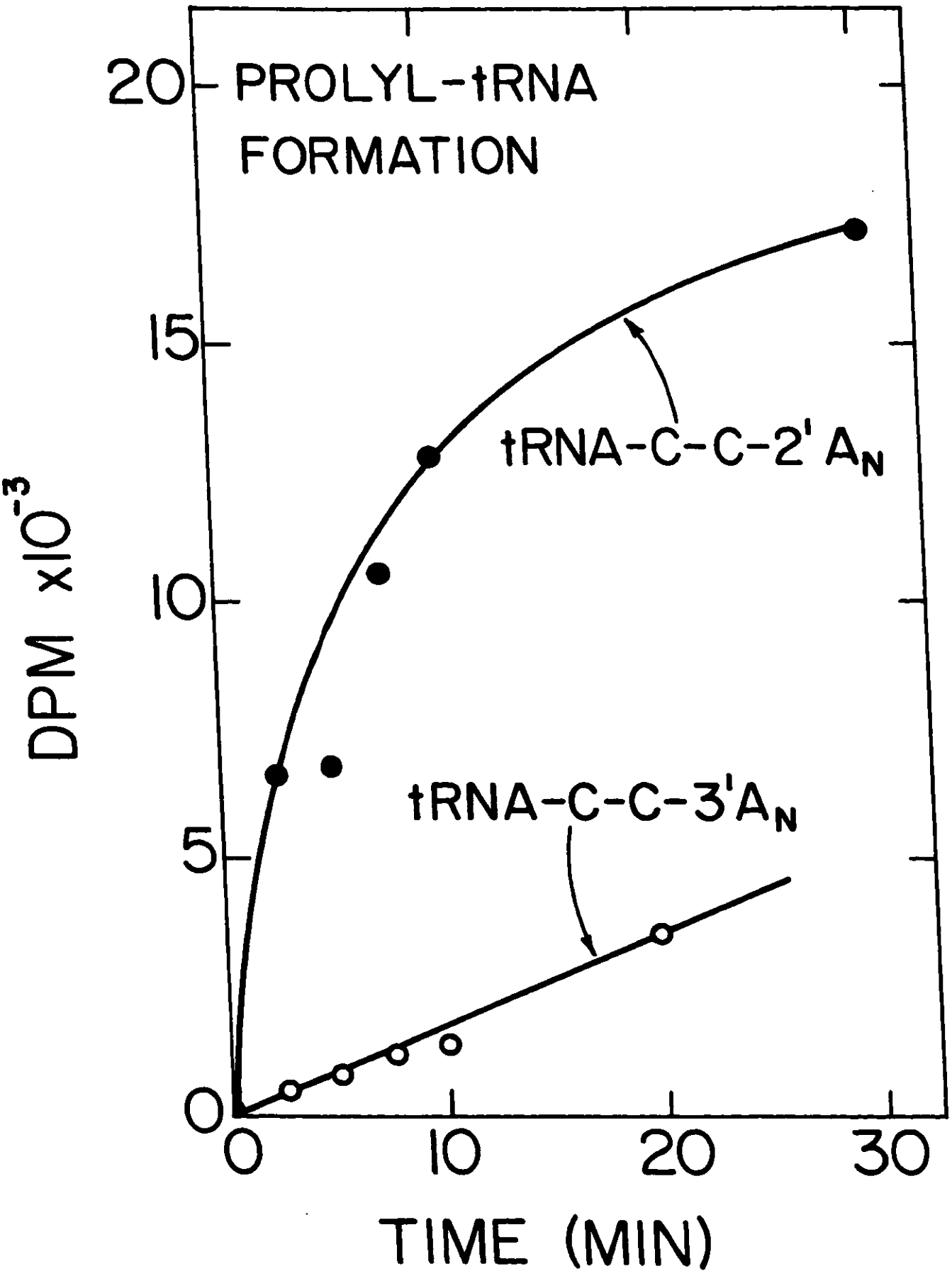
Figure 24: Kinetics of arginyl-2'N-tRNA and arginyl-3'N-tRNA formation.

Figure 25: Kinetics of prolyl-2'N-tRNA and prolyl-3'N-tRNA formation.

Aminoacylation conditions were as described in the legend to Figure 18, except that the incubations in Figures 23 and 24 were carried out at 30°C.


METHIONYL-tRNA
FORMATION
tRNA-C-C-3'An
tRNA-C-C-2'An
DPM x10^-3
4
2
0
0
10
20
30
TIME (MIN)


ARGINYL-tRNA
FORMATION
tRNA-C-C-3'An
tRNA-C-C-2'An
DPM x10^-3
10
5
0
0
10
20
30
TIME (MIN)


PROLYL-tRNA
FORMATION
tRNA-C-C-2' $A_N$
tRNA-C-C-3'$A_N$
DPM x$10^{-3}$
TIME (MIN)
0
5
10
15
20
0
10
20
30

# DISCUSSION

The possibility of isomeric specificity at the C-C-A end of tRNA during aminoacylation and protein synthesis has recently attracted considerable interest. A number of published reports, in addition to the work described in this dissertation, have addressed this problem. All laboratories involved in these studies have employed non-isomerizable analogs of aminoacyl-tRNA in their investigations.

Aminoacylation

Much work had previously been done on the question of the initial site of aminoacylation of tRNA; it was generally assumed that only one site would be involved (Wolfenden et al., 1964). There is considerable asymmetry between the two hydroxyl groups in that the 2'-hydroxyl is more nucleophilic than the 3' and has a lower pK for dissociation of the hydroxyl group proton (Martin et al., 1968; Gin and Dekker, 1968). It is well known, for example that chemical acylation takes place more readily on the 2' rather than the 3' position of ribonucleotides (Martin et al., 1968). However, it is not likely that this would provide a clue to the enzymatic site of aminoacylation as this would be determined largely by the mechanism of the aminoacylation enzyme.

Some recent results deal with the isomeric specificity of the aminoacyl-tRNA synthetases. In order to interpret these results, one must critically assess the ability of the analog tRNA's to substitute for normal tRNA's in the aminoacylation reactions and to yield accurate results concerning the isomeric specificity of these reactions. This

assessment may be guided by consideration of three general criteria, the most important of which is that the initial site of aminoacylation be unambiguously identified by examination of the analog aminoacyl-tRNA product. There should also be a functionally active interaction of the analog tRNA's with the different aminoacyl-tRNA synthetases under normal aminoacylation conditions. Finally, the analog tRNA's must resemble normal tRNA's closely enough so that observed results are actually due to isomeric specificity of the aminoacyl-tRNA synthetases rather than other modifications introduced into the analog tRNA's.

In their investigation of the initial site of aminoacylation, Hecht et al. (1973) have used tRNA in which 2'- and 3'-O-methyladenosine has been substituted for adenosine at the 3' end. This tRNA analog would clearly allow identification of the initial site of aminoacylation. If an amino acid were attached to the free hydroxyl group (either 2' or 3' depending on the analog) isomerization to the methoxy group would be impossible and the initial attachment site could be determined. Unfortunately, these tRNA analogs were found to have no acceptor activity when incubated with amino acids and aminoacyl-tRNA synthetases. The authors concluded that this result may indicate a requirement for both 2'- and 3'-hydroxyl groups in aminoacylation, possibly implying involvement of an orthoester structure in this process. In fact, no information regarding the isomeric specificity of aminoacylation may be gleaned from these negative results. As the authors pointed out,

the added methyl group may interfere with normal tRNA-synthetase interactions. Thus, there is no indication that these results are due to the isomeric specificity of the synthetases rather than to the additional methyl group introduced into the analog tRNA's.

Another tRNA analog that has been used in studying the initial site of aminoacylation is tRNA-C-C-$A_{oxi-red}$. To make this analog, normal tRNA is first subjected to periodate oxidation, which results in attack on the ribose ring of the 3' terminal adenosine, cleaving the C2'-C3' bond and forming a dialdehyde (Schmidt, 1968). If this periodate treated tRNA is then reduced with sodium borohydride, the corresponding dialcohol is formed (Leppla et al., 1968). Thus, the 2'- and 3'-hydroxyl groups are regenerated, but the bond between C-2' and C-3' is not reformed. Cramer et al. (1968) were the first to demonstrate that, at least in the case of yeast $tRNA^{phe}$, one could enzymatically aminoacylate tRNA-C-C-$A_{oxi-red}$. It was subsequently found that when E. coli tRNA was oxidized and then reduced, the tRNA species specific for phenylalanine, methionine and tyrosine could be aminoacylated, while the species specific for arginine, isoleucine, aspartic acid, histidine, serine, tryptophan and valine could not (Tal et al., 1972; Mehler, 1970). Ofengand and Chen (1972) were the first to propose that tRNA-C-C-$A_{oxi-red}$ might be useful in determining the initial site of aminoacylation. It was unlikely that a rapid acyl migration between the 2'- and 3'-hydroxyl groups would occur if the

C2'-C3' bond were cleaved, disrupting the structure of the ribose ring. It has subsequently been shown experimentally that acyl migration does not occur in phenylalanine esters of these open-ring analogs of adenosine (Chladek et al., 1973; Ofengand et al., 1974). Thus, if the tRNA-C-C-$A_{oxi-red}$ were used in aminoacylation, analysis of the products should reveal the initial site of aminoacylation. This analysis is easily done by pancreatic digestion of the aminoacylated tRNA and thin-layer chromatographic separation of the open-ring analogs of 2'- and 3'-aminoacyl-adenosine (Ofengand et al., 1974). It should be noted that when experiments of this type were carried out using yeast phenylalanyl-tRNA synthetase both under normal conditions with yeast $tRNA^{phe}$ and under mischarging conditions in 20% dimethylsulfoxide with E. coli $tRNA^{phe}$ and E. coli $tRNA^{val}$, phenylalanine was found attached to the 2'-hydroxyl group in all cases. In addition, when E. coli phenylalanyl-tRNA synthetase was used under normal conditions with E. coli $tRNA^{phe}$, as well as in the presence of 1.5 M ammonium sulfate with yeast $tRNA^{phe}$, phenylalanine was again attached to the 2'-hydroxyl group (Ofengand et al., 1974). These results suggest that isomeric specificity of aminoacylation is not sensitive to the reaction conditions employed.

The results obtained with the tRNA-C-C-$A_{oxi-red}$ must be interpreted cautiously for several reasons. The amino acid accepting hydroxyl groups in tRNA-C-C-$A_{oxi-red}$ are in a very different structural

environment than the hydroxyl groups in normal tRNA. These primary alcohol groups are free to rotate about the C1'-C2' bond and the C3'-C4' bond. This free rotation results in disruption of the original planar structure of the ribose due to rotation about the C1'-O-C4' acetal linkage (von der Haar et al., 1971). The synthetase enzyme may, of course, have a binding site for the normal ribose ring which constrains the cleaved ribose to the normal planar conformation. If this is not the case, however, it is possible that one of the hydroxyl groups on the cleaved ribose may bind at the "wrong" enzyme site and yield spurious results on the isomeric specificity of aminoacylation. However, the fact that nearly 100% of the enzymatically attached phenylalanine was found on the 2'-hydroxyl group of tRNA-C-C-$A_{oxi-red}$ suggests that this is probably not the case.

The aminoacylation of some amino acid specific tRNA-C-C-$A_{oxi-red's}$ apparently requires an intact C2'-C3' bond because, as previously stated, they do not functionally interact with the aminoacyl-tRNA synthetases. Control experiments were done which demonstrated that periodate treatment or sodium borohydride reduction alone could not account for the loss of amino acid accepting activity (Tal et al., 1972). These results indicate that different synthetase enzymes may employ different mechanisms of aminoacylation.

By substitution of 2'-deoxy AMP or 3'-deoxy AMP into the 3' end of tRNA Sprinzl et al. (1973) were able to synthesize tRNA analogs

in which either the 2'- or the 3'-hydroxyl group on the terminal adenosine was missing (tRNA-C-C-2'dA and tRNA-C-C-3'dA). This substitution was accomplished by the use of 2'- or 3'-deoxyadenosine triphosphates as substrates for yeast tRNA-nucleotidyl transferase-catalyzed addition to yeast tRNA-C-C. It was found that phenylalanine could be enzymatically attached to tRNA-C-C-3'dA, but not to tRNA-C-C-2'dA (Sprinzl et al., 1973; Sprinzl and Cramer, 1973). Thus, isomeric specificity of aminoacylation was determined by attempted charging of both deoxy-tRNA analogs with phenylalanine. The fact that aminoacylation of phenylalanine occurred exclusively with the 3'-deoxy tRNA analog suggested that the 2'-hydroxyl group was specifically aminoacylated.

When kinetic studies were done with the yeast $tRNA^{phe}$-C-C-3'dA, it was found that aminoacylation proceeded with the same $K_m$ ($2.86 \times 10^{-6}$ M) and about two-thirds of the $V_{max}$ ($0.18\ \mu M\ min^{-1}$ versus $0.28\ \mu M\ min^{-1}$) found with normal yeast $tRNA^{phe}$ (Sprinzl and Cramer, 1973. This indicates that although the 3'-hydroxyl normally contributes to the rate of aminoacylation, its absence probably does not reduce the binding of $tRNA^{phe}$ to its synthetase. Also, it was found that $tRNA^{phe}$-C-C-2'dA is a competitive inhibitor of the yeast phenylalanyl-tRNA synthetase. The $K_i$ for this inhibition ($2.16 \times 10^{-6}$ M) showed that this 2'-deoxy tRNA, although not a substrate, bound to the enzyme as tightly as normal tRNA (Sprinzl and Cramer, 1973). It should be noted in this respect that periodate oxidized yeast $tRNA^{phe}$ does not bind to

the synthetase (Cramer et al., 1968). These binding results, along with the near-normal maximal velocity of $tRNA^{phe}$-C-C-3'dA charging, suggest that the $tRNA^{phe}$-C-C-3'dA is recognized and used by yeast phenylalanyl-tRNA synthetase in the same way that normal $tRNA^{phe}$ is recognized and used. This relationship, however, does not hold for all aminoacyl-tRNA synthetases. It has been observed using E. coli enzymes that neither the 2'- nor 3'-deoxy tRNA analogs specific for alanine, aspartic acid, glutamic acid, glutamine and proline are aminoacylated (Sprinzl and Cramer, 1975). As a control, normal tRNA was subjected to the same preparative reactions as the deoxy tRNA analogs, except that a normal AMP residue was added at the 3' terminus instead of a deoxy AMP residue: normal tRNA was incubated with periodate, and the terminal adenosine removed by subsequent β-elimination at pH 9.0. Alkaline phosphatase was then employed to remove the remaining 3'-phosphate group. The resulting tRNA-C-C served as a substrate for yeast tRNA-nucleotidyl transferase in the addition of AMP at the 3' terminus. tRNA treated in this way showed reduced accepting capacity for nearly all amino acids. In particular, the accepting capacity of $tRNA^{asp}$ was nearly completely abolished (Sprinzl and Cramer, 1975). This result, however, is not in agreement with the results of Tal et al. (1972), who found that aspartic acid accepting capacity was reduced only slightly by the same treatment described above. Thus, it is not clear whether the inability to aminoacylate either deoxy tRNA analog is due to

a requirement by the synthetases for cis-hydroxyl groups or to a secondary modification in the tRNA structure. Further experiments will be needed to resolve this point. The use of snake venom phosphodiesterase, rather than periodate treatment, to remove the 3' terminal AMP may prove useful in this investigation.

When Sprinzl and Cramer (1975) attempted to aminoacylate both the 2'- and 3'-deoxy tRNA analogs with each individual amino acid, they found that some were attached almost exclusively to the 2'-deoxy tRNA and some almost exclusively to the 3'-deoxy tRNA. Cysteine and tyrosine were attached to both 2'- and 3'-deoxy tRNA analogs. These results are summarized in Table 10. Some of Sprinzl and Cramer's data have been recalculated to express the chargeability of both the 2'- and 3'-deoxy tRNA analogs for each amino acid as a percentage of the total amount of charged 2'-deoxy tRNA plus 3'-deoxy tRNA for that amino acid. Although these results do not allow one to conclude that aminoacylation of a given amino acid occurs exclusively on either the 2'- or 3'-hydroxyl, it can be seen that in most cases over 90% of the total charging for each amino acid is done with only one of the deoxy tRNA analogs. The low percentage charging with the other deoxy tRNA analog may or may not be real. This system has a relatively high background, apparently due to contamination of the deoxy tRNA preparations with normal tRNA. For this reason, the tRNA preparations were subjected to periodate oxidation both before and after addition of

Table 10. Relative amounts of amino acid added to tRNA-C-C-3'dA and tRNA-C-C-2'dA expressed as the percentage of total base stable aminoacyl-tRNA.

| Amino Acid | tRNA C-C-3'dA | tRNA C-C-2'dA | Class |
|---|---|---|---|
| Phe | 86 | 14 | |
| Val | 95 | 5 | |
| Leu | 94 | 6 | |
| Trp | 98 | 2 | |
| Arg | 98 | 2 | 2' |
| Met | 90 | 10 | |
| Ileu | 95 | 5 | |
| Thr | 83 | 17 | |
| Asn | 95 | 5 | |
| Tyr | 56 | 44 | |
| Cys | 59 | 41 | 2', 3' |
| Ser | 14 | 86 | |
| His | 12 | 88 | 3' |
| Lys | 2 | 98 | |
| Gly | 23 | 77 | |
| Ala | | | |
| Asp | | | |
| Glu | | uncertain | |
| Gln | | | |
| Pro | | | |

Data from Sprinzl and Cramer (1975).

the deoxy AMP's. Whether all of the contaminating normal tRNA was rendered inactive by these treatments is unclear.

Results of the aminoacylation experiments using deoxy tRNA analogs clearly suggest that the aminoacyl-tRNA synthetases have different isomeric specificities. These results, however, must be interpreted with some caution. The fact that one of the adenosine hydroxyl groups is missing may alter the mechanism of aminoacylation. A major effect would be a change in the hydrogen bonding potential at the enzyme active site. An hydroxyl group is capable of forming three different hydrogen bonds, two as an acceptor and one as a donor. It is probable that this hydrogen bond-forming capacity is utilized to some extent in the interaction between tRNA and the active site of its cognate synthetase. The lack of aminoacylation activity of the tRNA's with 2'- or 3'-O-methyl adenosine at the 3' end may be explained on these grounds. If an hydroxyl group hydrogen were normally used as a hydrogen bond donor with the synthetase, then substitution of a methyl group for the hydrogen might result in steric hindrance to binding of the 3'-terminal adenosine in the synthetase active site. The negative results obtained in attempted aminoacylation of O-methyl tRNA's are consistent with this hydrogen bonding model. A hydrogen bond normally made between the synthetase and the non-aminoacylated adenosine hydroxyl group cannot be made if a deoxy tRNA analog is used. The consequences of missing a hydrogen bond at the

enzyme active site cannot be fully assessed. The fact that the $V_{max}$ of yeast phenylalanine aminoacylation is only slightly reduced suggests that with this synthetase the effect is small. The inability of several synthetases to use either deoxy tRNA analog indicates that this may not be true in all cases. Whether the absence of the second hydroxyl group can result in exclusive aminoacylation of the "wrong" hydroxyl group is an open question, although this possibility seems remote.

Another set of tRNA analogs that has been used to determine the initial site of aminoacylation is the 2'- and 3'-amino tRNAs, described in this dissertation. It was initially found that the 3'-amino tRNA was aminoacylated with E. coli phenylalanyl-tRNA synthetase (Fraser and Rich, 1973). This result alone did not allow any conclusions to be drawn about the site of aminoacylation. The amino acid could have been acylated onto the 3'-amino group or the 2'-hydroxyl group. It was subsequently found, however, that the 2'-amino tRNA analog was not aminoacylated with E. coli phenylalanyl-tRNA synthetase. This result was apparently due to the inability of the synthetase to catalyze direct formation of an amide bond. If the inability to form an amide bond were general among the synthetases, then the amino tRNA analogs could be employed to determine the isomeric specificity of the synthetases. If the amino acid were initially attached to the 2'-hydroxyl group of the 3'-amino-3'-deoxyadenosine, at the 3' terminus, it would migrate to the

amino group to form a base-stable amide bond. If isomeric specificity required it to be acylated onto the site occupied by the amino group, there would be no charging. Thus, the initial site of aminoacylation could be easily determined.

Aminoacylation has been attempted with both amino tRNA analogs for 19 individual amino acids (Fraser and Rich, 1975). The results of this work showed that all of the E. coli aminoacyl-tRNA synthetases tested interact functionally with at least one of the amino tRNA analogs. The activity of the amino tRNA analogs requires no special reaction conditions. The aminoacylation rates were measured for a mixture of amino acids and for several individual amino acids. In all cases, it was found that the charging rates are somewhat lower than for normal tRNA. It was not determined whether this was due to an increased $K_m$ or a decreased $V_{max}$. Although the E. coli synthetases aminoacylate normal and amino tRNA's at different rates and possibly to different extents, the distinguishable structural differences between these tRNA's are apparently small enough so that all synthetases can use at least one of the amino tRNA analogs. It should be pointed out that the amino group would be capable of forming hydrogen bonds, both as a donor and an acceptor, with the synthetase. Although it has one more hydrogen, it is sterically similar to the hydroxyl group. Experimentally determined hydrogen bonding distances from nitrogen to oxygen (N-H. . . . O) tend to be somewhat longer than, but overlap with,

the distances from oxygen to oxygen (O-H. . . . O). The hydrogen bonding angles ( ∢ NHO and ∢ OHO) are very similar in both cases (Hamilton and Ibers, 1968). Thus, if the non-aminoacylated hydroxyl group on the 3' terminal adenosine were to normally serve as a hydrogen bond donor during aminoacylation, the amino group may functionally substitute for it. In addition, the electron pair on the amino nitrogen may act as a hydrogen bond acceptor, as may the electron pairs on the hydroxyl oxygen. There is no evidence that any of these hydrogen bonds are actually operative in tRNA-synthetase interactions, but if they were, it may explain why the deoxy tRNA analogs are not used by all the synthetases while the amino tRNA analogs are.

The previously discussed results in Table 9 showed that some amino acids would acylate 2'-amino tRNA only and some 3'-amino tRNA only, while others were attached to both analogs. If these results are compared with those of Sprinzl and Cramer (1975), one notes that the two sets of aminoacylation classes disagree in the isomeric specificities of threonine, asparagine and tyrosine synthetases. In the case of tyrosine, Sprinzl and Cramer found that the amino acid was attached to both the 2'- and 3'-deoxy tRNA analogs. They have also found this to be the case with yeast $tRNA^{tyr}$ and yeast tyrosinyl-tRNA synthetase (Cramer et al., 1975). When aminoacylation kinetics were done in the yeast system, it was found that the 2'-hydroxyl group was aminoacylated 20 times faster than the 3'-hydroxyl group. Thus, although both deoxy

tRNA analogs were aminoacylated, there was a preference for the 3'-deoxy tRNA. A clear preference for the 2'-hydroxyl group was also observed in the results described in this dissertation. Whether the ambiguous aminoacylation observed by Sprinzl and Cramer was a function of the deoxy tRNA analogs or an accurate reflection of the synthetase mechanism has not been determined. The only other ambiguous synthetase which Sprinzl and Cramer reported was cysteinyl-tRNA synthetase. Unfortunately $tRNA^{cys}$ could not be aminoacylated in the system described here, so that results cannot be compared. In the cases of threonine and asparagine opposite results were obtained using the deoxy tRNA and amino tRNA analogs. Further work will have to be done to resolve the isomeric specificity of these synthetases.

The results of Sprinzl and Cramer (1975) suggest that most aminoacyl-tRNA synthetases utilize a site specific mechanism. Thus, the synthetases previously found to be in Class 2', 3' using the amino tRNA's are probably able to catalyze the direct formation of an amide bond. This suggests that the kinetic studies reported in "Results" may allow reclassification of these synthetases into either Class 2' or Class 3'. On this basis, methionine and arginine would be in Class 2' and proline in Class 3'. Sprinzl and Cramer (1975) have reported that methionine and arginine are in Class 2', but they were unable to draw a conclusion concerning proline.

The general agreement of the results on isomeric specificity of

aminoacylation using widely different tRNA analogs allows a rather firm conclusion regarding this specificity. A large number of enzymatic studies have been carried out on the mechanism of aminoacylation (Loftfield, 1972). These studies will now have to be reviewed with the recognition that there are at least two and possibly five different types of reaction mechanisms which this class of enzymes is capable to catalyzing.

To a certain extent it could be argued that there is very little biological significance to aminoacylation at either the 2' or 3' site in view of the very rapid isomerization between these two sites which seems to have a half life for migration estimated as two-tenths of a millisecond in model studies (Griffin et al., 1966). Attempts to determine the speed of protein synthesis in prokaryotes such as E. coli have yielded numbers ranging from 10 to 80 amino acids added per second at 37°C (Goldstein et al., 1964; Kepes and Beguin, 1966; Lacroute and Stent, 1968). Most estimates are around 15 amino acids per second, or one amino acid every 67 milliseconds. Nonenzymatic acyl migration may thus be expected to result in an equilibrium mixture of 2' and 3' isomers (1:2) on a time-scale much shorter than that required to add an amino acid to a growing polypeptide chain. It is possible, however, that aminoacyl-tRNA may never be free in solution after charging. Thus, EF-Tu may immediately associate with newly charged aminoacyl-tRNA, inhibiting free acyl migration. The evidence

for EF-Tu association with a particular isomer will be fully discussed later. In any case, aminoacyl-tRNA probably participates in peptide bond formation exclusively as one isomer.

Thus, there appears to be no important evolutionary selection pressure to maintain a mechanism resulting in aminoacylation on either the 2'- or 3'-hydroxyl group for a particular amino acid. It has been found, however, that tyrosine, serine, valine and isoleucine are initially aminoacylated on the same site by yeast synthetases and E. coli synthetases (Cramer et al., 1975). In addition, phenylalanine is aminoacylated on the 2'-hydroxyl group by E. coli, yeast and rat liver synthetases (Sprinzl and Cramer, 1973; Sprinzl and Cramer, 1975; Fraser and Rich, 1975; Ofengand et al., 1974). It would be worthwhile to extend these studies of aminoacylation mechanisms to other species in order to determine whether all synthetases have maintained the same specificity throughout evolution or have drifted from one mechanism to another.

The primary and secondary structural features of the different amino acid specific tRNA's have been examined in an attempt to correlate the isomeric specificity of aminoacylation with one or more of these features. No correlation has been found. It is interesting to note, however, that there is a partial correlation between the middle base in the tRNA anticodon and the isomeric specificity of charging in E. coli. This correlation is dependent upon the presence of a purine in this middle position. If the

base is adenine, charging is on the 2'-hydroxyl group and if the base is guanine, charging is on the 3'-hydroxyl group. The correlation does not hold if the middle base is a pyrimidine.

It is clear that further information will be required to obtain a proper biological perspective on the differences in isomeric specificity of the aminoacyl-tRNA synthetases.

Protein synthesis

Determination of the isomeric specificities of aminoacyl- and peptidyl-tRNA in the different steps of ribosomal protein synthesis is central to a full understanding of this process.

The conclusion of Nathans and Neidle (1963) regarding the isomeric specificity of the ribosomal A site has recently received close scrutiny. Some of these recent experiments have utilized a puromycin-type reaction to assay acceptor activity. A N-blocked aminoacyl-tRNA serves as a donor, while chemically synthesized non-isomerizable analogs of the 3' terminus of aminoacyl-tRNA are tested as acceptors. Two such compounds are the open-ring analogs of 2'- and 3'-O-phenylalanyl-adenosine (Chladek et al., 1973). The open-ring adenosine moiety in these compounds has no C2'-C3' bond and is identical to the periodate oxidized and borohydride reduced adenosine at the 3' end of tRNA-C-C-$A_{oxi-red}$. It was found that when phenylalanine was esterified to the 3'-hydroxyl group, the phenylalanyl-adenosine analog was able to accept an AcPhe residue from AcPhe-tRNA bound in the D site. When phenylalanine was bound to the 2'-hydroxyl group, there was no acceptor activity (Chladek et al., 1973). The acceptor activity of the 3'-O-phenylalanyl-adenosine analog was much lower than normal phenylalanyl-adenosine. This was interpreted by the authors to indicate that an intact ribose ring is required for maximal activity. The cleavage of the C2'-C3' bond subjects these

analogs to the same potential structural ambiguities previously discussed for tRNA-C-C-$A_{oxi-red}$. Thus, while the results obtained with these analogs cannot by themselves prove that the A site is specific for the 3' isomer of aminoacyl-tRNA, they do support this conclusion.

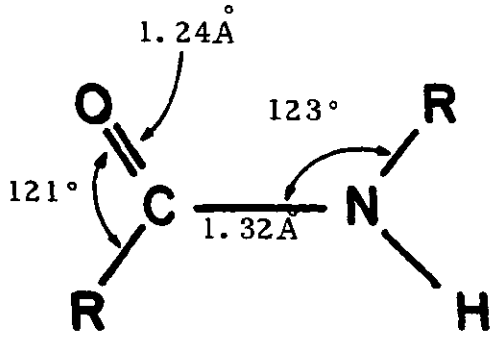
In order to reinforce these results, Chladek and co-workers measured acceptor activity with several non-isomerizable analogs of the 3' terminus of phenylalanyl-tRNA. These analogs included chemically synthesized C-A(2' Phe)Me, C-A(2'Me)Phe, C-A(2'Phe)H and C-A(2'H)Phe (Ringer and Chladek, 1974; Chladek et al., 1974). The first two analogs have the non-aminoacylated hydroxyl group blocked by methylation. The 2'- or 3'-O-methyladenosine moieties are identical to the 3' end of the previously discussed tRNA analogs which Hecht et al. (1973) found inactive in aminoacylation reactions. The latter two analogs lack a functional group at the non-aminoacylated sites, the adenosine analogs being either 2'- or 3'-deoxy. Using these analogs it was found that when phenylalanine was esterified to the 3'-hydroxyl group it would accept AcPhe in a peptidyl transferase catalyzed reaction. It was found that the 2'-O-aminoacyl analogs had no activity. This work was extended by measuring the ability of these analogs to inhibit the peptidyl transferase catalyzed transfer of AcPhe-tRNA to puromycin (Ringer and Chladek, 1974). It was found that all four previously described C-A (2'- or 3'-O-phenylalanyl) analogs inhibited

this reaction. These results suggest that 2'-O-aminoacyl-tRNA isomers may be bound at the A site, but will be unable to participate in peptide bond formation. While the results obtained with these abbreviated phenylalanyl-tRNA analogs are consistent with a 3' isomeric specificity of acceptor activity, the limitations of these fragment analogs as models of aminoacyl-tRNA in the peptidyl transferase reaction cannot be fully evaluated at the present time.

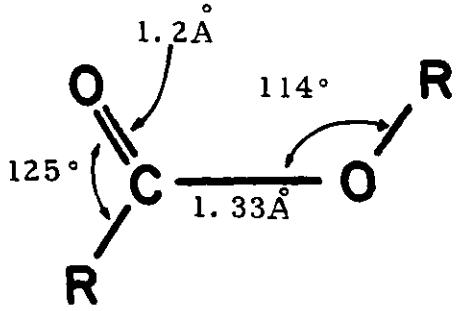
In order to more closely mimic the in vivo reaction catalyzed by peptidyl transferase, non-isomerizable analogs of aminoacyl-tRNA have been examined for acceptor activity. Chinali et al. (1974) synthesized yeast Phe-tRNA-C-C-3'dA and Phe-tRNA-C-C-$A_{oxi-red}$. In both of these analogs, aminoacylation resulted in phenylalanine being esterified to the 2'-hydroxyl group. The complementary analogs, with phenylalanine bound to the 3'-hydroxyl group, were not tested. In the acceptor experiments poly(U) directed the binding of AcPhe-tRNA to ribosomes and AcPhe-Phe formation was measured after incubation with a phenylalanyl-tRNA analog. The results of these experiments showed that both Phe-tRNA-C-C-3'dA and 2'-O-Phe-tRNA-C-C-$A_{oxi-red}$ were capable of accepting an AcPhe residue. Neither analog was as active as normal Phe-tRNA, and the 3'-deoxy tRNA analog was somewhat more active than the oxidized-reduced analog. This result does not agree with the isomeric specificity obtained using non-isomerizable aminoacyl-tRNA fragments. It is

somewhat puzzling that this apparent ambiguity was not detected in the experiments with C-A-aminoacyl analogs as one might expect less specificity from the fragments. Furthermore, the results of Chinali et al. (1974) are in direct disagreement with those subsequently obtained by Hecht et al. (1974) using both Phe-tRNA-C-C-3'dA and Phe-tRNA-C-C-2'dA. When acceptor activity of these aminoacylated deoxy tRNA analogs were measured, it was found that only the 3'-phenylalanyl-tRNA isomer was capable of accepting AcPhe although both isomers were able to bind in the A site. Although a firm conclusion is not yet possible, it appears that the A site may bind both 2'- and 3'-aminoacyl-tRNA isomers, but that only the 3'-isomer is able to participate in peptide bond formation.

The fact that Phe-3'N-tRNA has acceptor activity (Fraser and Rich, 1973) is consistent with the conclusion that 3'-O-aminoacyl-tRNA is the acceptor substrate in peptidyl transferase catalyzed peptide bond formation. This result also indicates that the differences in geometry and hydrogen bonding properties between the ester and amide linkages are not significantly distinguished at the A site. This is not surprising since the two bonds are stereochemically very similar. The structures of generalized amide and ester bonds are illustrated.




(Roberts and Caserio, 1965)




(Simonetta and Carra, 1969)

Both bonds are planar. One difference between these bonds is the hydrogen bonding potential of the amide nitrogen versus the ester oxygen. While both are capable of accepting a hydrogen bond, only the nitrogen may act as a hydrogen bond donor. Whether or not this difference is important in any step of protein synthesis cannot be predicted at present.

Non-isomerizable analogs of aminoacyl-tRNA have also been used to help determine the structural requirements for interaction with EF-Tu.

Ofengand and Chen (1972) found that Phe-tRNA-C-C-$A_{oxi-red}$ was unable to form a ternary complex with EF-Tu and GTP. Furthermore, EF-Tu and GTP did not stimulate the binding of this aminoacyl-tRNA analog to the A site. Although phenylalanine was esterified exclusively with the 2'-hydroxyl group in this analog, a conclusion regarding isomeric specificity could not be drawn from these results. Cleavage of the normal C2'-C3' bond in the 3' terminal adenosine may have been responsible for the inactivity with EF-Tu, and since 3'-O-phenylalanyl-tRNA-C-C-$A_{oxi-red}$ was not tested, this possibility could not be ruled out.

Chinali et al. (1974) have measured the EF-Tu catalyzed binding of Phe-tRNA-C-C-3'dA to ribosomes. They have found that over a wide range of magnesium concentrations (4-14 mM), EF-Tu stimulates the binding of this phenylalanyl-tRNA to ribosomes. In addition, this binding was found to be coupled with GTP hydrolysis. They did not, however, attempt to directly measure formation of a ternary complex. These results suggest that a vicinal hydroxyl group on the ribose ring is not necessary for interaction of aminoacyl-tRNA with EF-Tu. As an analog of 3'-aminoacyl-tRNA was not used, these authors were unable to reach a conclusion concerning the isomeric specificity of this interaction.

The interaction of EF-Tu with Phe-3'N-tRNA has been examined, and the results presented in this dissertation. EF-Tu and GTP form no detectable ternary complex with this analog, but EF-Tu does stimulate

its binding to the ribosome at low magnesium concentrations. Whether or not a weak ternary complex is an intermediate in this binding has not been determined.

Although further experiments will be necessary, these results along with those of Chinali et al. (1974) suggest that ternary complex formation may be specific for 2'-O-aminoacyl-tRNA.

It has recently been found (Ringer and Chladek, 1975) that interaction between EF-Tu·GTP complexes and aminoacyl-dinucleoside phosphates can be measured. At concentrations higher than one micromolar, C-A-Phe, C-A-Pro and C-A-Asp will prevent binding of EF-Tu·GTP to Millipore filters. Sephadex gel filtration studies suggest that the interaction of these compounds with EF-Tu·GTP results in dissociation of the GTP, rather than stable ternary complex formation. The filter release, however, does not occur with C-A, C-A-(Ac-Phe), or A-Phe. Since this interaction between model compounds and EF-Tu appeared to retain normal specificity for the 3' end of aminoacyl-tRNA, the isomeric requirements for the interaction were examined. When phenylalanine was bound to the 3'-hydroxyl group (C-A(2'H)Phe) there was no detectable interaction with EF-Tu. A 2'-aminoacyl derivative (C-A(2'Phe)H), however, was nearly as active as normal C-A-Phe. Although these results must be interpreted with some caution due to the nature of the substrates tested, they suggest that EF-Tu specifically recognizes 2'-O-aminoacyl-tRNA.

If EF-Tu is specific for the 2'-isomer of all aminoacyl-tRNA's, then two transacylations may be required for some amino acids prior to incorporation into a nascent polypeptide chain. Amino acids that are aminoacylated on the 3'-hydroxyl would have to isomerize to the 2'-hydroxyl in order to interact with EF-Tu. EF-Tu may then catalyze binding of the 2'-O-aminoacyl-tRNA directly into the A site, as this site will bind both isomers. Prior to peptide bond formation, however, a transacylation may be required resulting in a 3'-O-aminoacyl-tRNA substrate for peptidyl transferase. This hypothesis suggests a possible explanation for the observed EF-Tu stimulated binding of Phe-3'N-tRNA to ribosomes in the apparent absence of ternary complex formation. At high concentrations of EF-Tu (as used in the ribosome binding assay), the EF-Tu· GTP binary complex may interact with ribosomes directly to partially open the A site. Since the A site itself is able to bind both 2' and 3' isomers, nonenzymatic Phe-3'N-tRNA binding may then be enhanced.

Once a peptide bond has been formed and the aminoacyl-tRNA has been incorporated into a growing polypeptide chain, this peptidyl-tRNA is translocated from the A to the D site. If the D site is specific for 3'-O-peptidyl-tRNA, then a transacylation must occur prior to or during translocation. If the D site is non-specific, or specific for 2'-O-peptidyl-tRNA, then acyl migration will not be required. Thus, a study of D site isomeric specificity may also tell us something about the poorly understood process of translocation.

Chinali et al. (1974) have found that both 2'-O-AcPhe-tRNA-C-C-$A_{oxi-red}$ and AcPhe-tRNA-C-C-3'dA will bind to the D site in the absence of added initiation factors at 10 mM magnesium concentration. Both of these analogs bind nearly as well as normal AcPhe-tRNA and once bound they do not interfere with EF-Tu catalyzed binding of normal Phe-tRNA to the A site. Despite the fact that both of these analogs bind in the D site, neither serves as a donor. It is possible to explain this lack of activity on grounds other than isomeric specificity. If a vicinal hydroxyl group were required for donor activity, then neither analog would be expected to be a D site substrate for peptidyl transferase. Thus, although it is clear that the D site will bind 2'-isomers, no conclusions can be reached from these results regarding isomeric specificity of peptidyl transferase donor activity.

In their work with AcPhe-tRNA-C-C-3'dA and AcPhe-tRNA-C-C-2'dA, Hecht et al. (1974) found that both of these analogs competed with normal AcPhe-tRNA for binding in the D site at 10 mM magnesium concentration. Although neither analog binds as strongly as AcPhe-tRNA, both apparently bind to the same extent. An attempt was made to measure the donor activity of these analogs, but none was detectable. As pointed out by the authors, however, this lack of detectable activity may be associated with a low level of binding in the D site.

Initiation factor dependent binding to the D site has been studied only with AcPhe-3'N-tRNA, as reported in this dissertation. Using this analog, it was found that binding was only slightly stimulated by initiation factors. In the absence of initiation factors, however, nearly equal amounts of AcPhe-3'N-tRNA and AcPhe-tRNA are bound to the ribosomes (Table 5). Although it was not determined in these experiments whether this binding was to the D site, one would expect this to be the case (Lucas-Lenard and Lipmann, 1967).

Although no conclusive experiments have been done, preliminary indications are that the D site may normally bind both 2'- and 3'-O-peptidyl-tRNA. It is unknown whether one or the other of these isomers exclusively is then used in peptide bond formation. The results obtained so far are consistent with a model analogous to that of EF-Tu directed binding of aminoacyl-tRNA to the A site; that is, IF-2 may be specific for N-blocked 2'-O-aminoacyl-tRNA, but the D site itself may accommodate both N-blocked 2'- and 3'-O-aminoacyl-tRNA. Future experiments will easily allow resolution of this problem.

No experiments have been done to directly determine the isomeric specificity of peptidyl-tRNA hydrolysis in the release reaction of chain termination. As suggested in the introduction, this may have the same specificity as peptide bond formation from the D site.

## Concluding remarks

The results presented in this dissertation strongly suggest that the enzymatic aminoacylation of most tRNAs is a site specific reaction, 2' for some and 3' for others. The full significance of the different mechanisms of aminoacylation among different aminoacyl-tRNA synthetases is not obvious at the present time. However, the general utility of the amino tRNA analogs with these enzymes suggests that it may be worthwhile to explore the mechanism of aminoacylation in species other than E. coli to see whether they have been invariant throughout evolution.

On the basis of the results of experiments employing Phe-3'N-tRNA, firm conclusions cannot be drawn regarding isomeric specificity in protein synthesis. In order to make a strong statement, the experiments must also be done with Phe-2'N-tRNA. Unfortunately, this analog was not available, although both enzymatic and chemical phenylalanylation of tRNA-C-C-2'$A_N$ were attempted. The discovery that synthetases do not all aminoacylate at the 2' position, however, will allow construction of the proper tools for several new experiments dealing with isomeric specificity of protein synthesis. The amino acids which are enzymatically attached to both amino tRNA analogs will be particularly useful. The isomeric specificity of EF-Tu·GTP-aminoacyl-tRNA complex formation may be examined, as well as EF-Tu stimulated binding to ribosomes. Prolyl-tRNA synthetase will aminoacylate both tRNA-C-C-2'$A_N$ and

tRNA-C-C-3'$A_N$. The use of a poly(C) directed polyproline in vitro protein synthesizing system may be valuable in determining the isomeric specificity of the D site and to answer conclusively whether or not the ribosome is capable of breaking an amide bond. The fact that methionyl-tRNA synthetase will also aminoacylate both tRNA-C-C-2'$A_N$ and tRNA-C-C-3'$A_N$ suggests that this may prove useful in examining the isomeric specificity of initiation.

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# ABBREVIATIONS

tRNA-C-C-A: normal tRNA

tRNA-C-C: tRNA missing the normal 3' terminal AMP

tRNA-C-C-3'$A_N$: tRNA with 3'-amino-3'-deoxy AMP substituted for AMP at the 3' terminus

tRNA-C-C-2'$A_N$: tRNA with 2'-amino-2'-deoxy AMP substituted for AMP at the 3' terminus

Phe-tRNA-C-C-3'$A_N$: tRNA-C-C-3'$A_N$ aminoacylated with L-phenylalanine

Phe-3'N-tRNA: Phe-tRNA-C-C-3'$A_N$

AcPhe-tRNA: Phenylalanyl tRNA in which the phenylalanine $\alpha$-amino group has been acetylated

CTAB: cetyltrimethyl ammonium bromide

Tris: tris-(Hydroxymethyl)-aminomethane

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid