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The Hydroxamate Reaction of Aminoacyl-tRNA Synthetases

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THE HYDROXAMATE REACTION OF AMINOACYL-tRNA SYNTHETASES

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PREFACE

I am deeply indebted to Dr. Fritz Lipmann for his advice, criticisms, and encouragement throughout this study and throughout the duration of my stay in his laboratory. To study with him has been rewarding; to associate with him has been inspiring.

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ABSTRACT

Amino acids are activated as aminoacyladenylates which remain bound to the aminoacyl-tRNA synthetases that catalyze their formation. The activated amino acids are then esterified to specific transfer RNA molecules. By this reaction sequence, the specificity and energetics necessary for polypeptide synthesis are conferred upon the amino acids. The first method of determining acyl group activation was the hydroxamate assay. The activated amino acids can be trapped as the stable amino acid hydroxamates by carrying out the activation reactions in high concentrations of hydroxylamine.

This thesis comprises a study of the hydroxamate reaction of the aminoacyl-tRNA synthetases. Each of fourteen partially purified aminoacyl-tRNA synthetases from E. coli was assayed for the rates of formation of amino acid hydroxamate and aminoacyl-tRNA. These relative rates of amino acid hydroxamate formation were found to vary over a sixtyfold range. The methionyl- and aromatic aminoacyl-tRNA synthetases catalyze hydroxamate formation at rates comparable to the rates of acylation of tRNA. In descending order, Lys-, Ala-, Ile-, Gly-, Cys-, Leu-, Ser-, and Val-tRNA synthetases catalyze hydroxamate formation at rates below their respective rates of aminoacyl-tRNA formation. This variation is irrespective of the sources of the enzymes, and it is interpreted to reflect the differential protection that the aminoacyl-tRNA synthetases afford their respective aminoacyladenylates. The most extreme case in this regard is the threonyl-tRNA synthetase, which does not catalyze threonine hydroxamate formation. Therefore, this enzyme was studied in detail.

The E. coli threonyl-tRNA synthetase was purified 320 fold. The enzyme activates threonine, as determined by the ATP-PP$_i$ exchange reaction, and it readily esterifies threonine to tRNA. Only if tRNA supplements the threonyl-tRNA synthetase hydroxamate assay does threonine hydroxamate continuously form via the nonenzymatic reaction of hydroxylamine with the enzymatically produced threonyl-tRNA.

The threonyladenylate-enzyme complex was isolated. The complex transfers threonine to tRNA. This transfer requires Mg^{++}, although Ca^{++} or

Mn^{++} can substitute for Mg^{++}. The optimal Mg^{++} concentration for this transfer reaction is 2 mM whereas the optimum for the overall reaction is 10 mM. PCMBS inhibits the transfer reaction but NEM does not. The complex spontaneously hydrolyzes at 30°, pH 7, with a half life of 29 minutes. When the isolated complex is incubated with hydroxylamine, it immediately disintegrates. Threonine and not threonine hydroxamate is the reaction product. Hydrolysis instead of hydroxaminolysis occurs. Therefore, the enzyme is converted from a synthetase to a hydrolase in the presence of hydroxylamine.

For comparison, the E. coli phenylalanyl-tRNA synthetase was studied. This enzyme catalyzes phenylalanine hydroxamate formation at a rate within 20% of the rate of phenylalanyl-tRNA formation. This enzyme is also inhibited by PCMBS. The phenylalanyladenylate-enzyme complex was isolated. It requires Mg^{++} for transfer of phenylalanine to tRNA. It is fourfold less stable to spontaneous hydrolysis than is the threonyladenylate-enzyme complex under the same conditions. When incubated in hydroxylamine, phenylalanyladenylate-enzyme complex disintegrates. In contrast to the case of threonyladenylate-enzyme complex, phenylalanine hydroxamate is the reaction product.

As a result of these studies, it is proposed that the differential rates of amino acid hydroxamate formation by the various aminoacyl-tRNA synthetases are due to two factors: the sequestering of the aminoacyladenylates by the enzymes, and the conversion of the enzymes from synthetases to hydrolases. The latter feature is explicit in threonyl-tRNA synthetase. These may be protective mechanisms by which synthetases prevent nucleophilic attack by molecules other than the natural substrates. In this manner, false transfer reactions, which would prove lethal to the organism, are avoided.

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Abbreviations

The abbreviations accepted without definition by The Journal of Biological Chemistry have been used in this thesis (cf. J. Biol. Chem., 242, 1 (1967)). In addition, the following abbreviations have been used.

A_{260}; A_{280} Absorbancy at 260 mμ; Absorbancy at 280 mμ

DEAE-cellulose   Diethylaminoethyl-cellulose

DTT   Dithiothreitol

Hepes   N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

NEM   N-Ethylmaleimide

PCMBS   p-Chloromercuribenzenesulfonic acid

PCMB   p-Chloromercuribenzoic acid

TCA   Trichloroacetic acid

tRNA   transfer RNA

$tRNA^{cys}$ The specific tRNA which accepts cysteine

Thr-tRNA, Phe-tRNA, etc. threonyl-tRNA, phenylalanyl-tRNA, etc.

Thr-AMP-enz, Phe-AMP-enz, etc.   threonyladenylate bound to threonyl-tRNA synthetase, phenylalanyladenylate bound to phenylalanyl-tRNA synthetase, etc.

CHAPTER   ONE

The Biological Activation of Amino Acids

The studies on proteases done by Bergmann in the late 1930's clearly elucidated the degradative pathway from proteins to free amino acids (Bergmann, 1942). The pathway for the biosynthesis of polypeptides remained a mystery. At first sight, reversal of the peptidase reaction appeared to be a plausible synthetic route. However, the thermodynamic calculations done by Borsook and Dubnoff (1940) showed that the synthesis of a peptide bond by condensation of two free amino acids is an endergonic reaction. Therefore, it became apparent that energy must be supplied in the biosynthesis of the peptide bond.

The first insight into the biological activation of amino acids was provided by Lipmann (1941). Based on his studies of acetate activation and phosphoryl group transfer potentials, Lipmann proposed that amino acids might be activated at their α-carboxyl groups as phosphoryl derivatives, thereby harvesting some of the energy available in ATP. While studying the enzymatic synthesis of acetyl coenzyme A from acetate, coenzyme A, and ATP, Jones et al. (1953) observed the liberation of pyrophosphate during the reaction. At the same time, Maas and Novelli (1953) observed a similar pyrophosphate cleavage of ATP during the synthesis of the peptide bond linking β-alanine to pantoic acid in pantothenic acid.

These observations led Hoagland, Keller, and Zamecnik (1956) to seek a similar pyrophosphate release during the activation of amino acids in protein synthesis. Indeed, they found an amino acid dependent ATP-PP_i exchange reaction catalyzed by a rat liver supernatant fraction. Furthermore, the activated amino acid could be trapped as the stable amino acid hydroxamate when the reactions were carried out in high concentrations of hydroxylamine. This indicated that the α-carboxyl group of the amino acid was activated.

In 1956, DeMoss et al. chemically synthesized leucyladenylate and showed that a twentyfold purified leucine activating enzyme catalyzed the synthesis of ATP from leucyladenylate and pyrophosphate. This observation substantiated the concept of the activation of amino acids via formation of aminoacyladenylates and liberation of pyrophosphate.

Enzymes which activate each of the twenty common amino acids have

been found in the supernatant fractions of various organisms that synthesize proteins (Peterson, 1967). Initially, these enzymes were known as amino acid activating enzymes, but with the discovery that activation of the amino acid comprised only the first half of the reaction and transfer of the activated amino acid to a specific transfer RNA molecule was the second half, these enzymes became known as aminoacyl-tRNA synthetases. This is the recommended trivial name for the enzymes. The systematic name is amino acid:tRNA ligase (AMP). The enzymes belong to class 6.1.1 of the Enzyme Commission. Transfer RNA is a more recent and preferred nomenclature replacing the terms soluble RNA or sRNA.

Since there are numerous recent review articles on both aminoacyl-tRNA synthetases and tRNA (Stulberg and Novelli, 1962; Novelli, 1967; Peterson, 1967), only the major features of the reaction will be summarized here.

The first step in the aminoacyl-tRNA synthetase reaction is activation of the amino acid by condensation with ATP to form aminoacyladenylate. Pyrophosphate is a free reaction product.

$$\text{Enzyme + AA + ATP} \xrightleftharpoons{\text{Mg}^{++}} \text{AA} \sim \text{AMP...enzyme + PP}_i$$
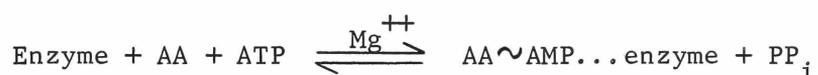
The evidence for this step is as follows. Most aminoacyl-tRNA synthetases catalyze an amino acid-dependent ATP-PP_i exchange reaction (Hoagland et al., 1956; De Moss and Novelli, 1956). The exceptions, in which tRNA is necessary to stimulate the ATP-PP_i exchange reaction, will be discussed in Chapter II. The enzymes catalyze ATP synthesis from chemically synthesized aminoacyl-adenylates and pyrophosphate (De Moss et al., 1956; Berg, 1957). ^{14}C-AMP does not exchange with unlabeled ATP in the absence of tRNA (Berg, 1956).

Reaction of amino acids, ATP, and some of the enzymes in the presence of high concentrations of hydroxylamine yields amino acid hydroxamates (Hoagland et al., 1956). Therefore, the amino acids are linked to AMP through the α-carboxyl group. Under these conditions, ^{18}O-labeled tryptophan forms tryptophan hydroxamate and AMP containing [18]O in the 5'-phosphate (Hoagland et al., 1957; Bernlohr and Webster, 1958). Consequently, the carboxyl oxygen of the amino acid must make a nucleophilic attack on the α-phosphorus atom of ATP,

and hydroxylamine must attack the carbonyl group of the aminoacyladenylate.

Aminoacyladenylates remain bound to the synthetases that catalyze
their formation. No free aminoacyladenylate can be detected using catalytic
quantities of enzymes. Only by using substrate amounts of enzymes followed
by acid precipitation of protein can free aminoacyladenylates be isolated
(Kingdon et al., 1958; Webster and Davie, 1959; Karasek et al., 1958).
Aminoacyl-AMP-enzyme complexes can be isolated (Allende et al., 1964; Norris
and Berg, 1964; Lagervist et al., 1966).

There exists a separate aminoacyl-tRNA synthetase for each amino
acid (Lipmann, 1958). Whether more than one exists for each amino acid is
presently a point of controversy. Neurospora contains two Phe-tRNA synthe-
tases. However, one is cytoplasmic and the other is mitochondrial (Barnett
et al., 1967). Recently, two separate Leu-tRNA synthetases have been isolated
from rat liver (Vescia, 1967). There is no evidence as to whether or not
these are from separate subcellular fractions. Yu and Rappaport (1966)
reported two Leu-tRNA synthetases from E. coli. Purification of enzymes from
various sources indicates that each enzyme catalyzes the activation of only
one amino acid. Two exceptions exist. The Ile-tRNA synthetase from E. coli
activates isoleucine and valine. The E. coli Val-tRNA synthetase activates
both threonine and valine (Bergmann et al., 1961). However, these enzymes
have much higher Michaelis constants for the wrong substrate than for the
correct one.

In the second step of the aminoacyl-tRNA synthetase reaction, the
activated amino acid is attached to a transfer RNA molecule according to the
following equation:

$$\text{Enzyme...AA} \sim \text{AMP} + \text{tRNA} \xrightleftharpoons{\text{Mg}^{++}} \text{AA} \sim \text{tRNA} + \text{AMP} + \text{Enzyme}$$
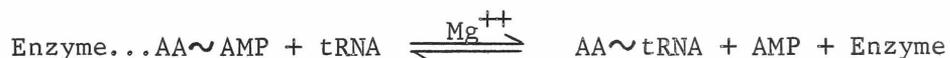
The existence of transfer RNA was predicted by Crick in 1956, who referred to
it as an adaptor molecule. Briefly, Crick's a priori reasoning was the follow-
ing: cellular RNA synthesis had been correlated with protein synthesis. It
was necessary to "translate" the genetic information encoded in DNA into a
linear array of amino acids to form a polypeptide. Messenger RNA containing

a base sequence complementary to that of DNA had been proposed, and some tentative evidence was available for it (for a review see Jacob and Monod, 1961). Crick proposed that at least twenty small nucleic acid molecules, each specific for one of the species of amino acids, accepted the activated amino acids and transferred them to the site of protein synthesis. By hydrogen bonding between the messenger RNA and the adaptor molecules, a necessary and sufficient translational mechanism existed. The following year, Hoagland et al. (1957) discovered transfer RNA when they observed that ^{14}C-leucine became bound to RNA which could be precipitated at pH 5 from the 105,000 x g supernatant fraction of rat liver extracts. The adaptor function of the transfer RNA in protein synthesis, as first proposed by Crick (1957), was subsequently proven to be true by the experiments of Chapeville et al. (1962).

The following experimental evidence reveals the role of tRNA as a substrate in the aminoacyl-tRNA synthetase reaction. Only with the addition of tRNA to the reaction mixture will ^{14}C-AMP exchange with unlabeled ATP (Holley, 1957; Holley and Goldstein, 1959). RNase eliminates the ATP-AMP exchange. Treatment of aminoacyl-tRNA with hydroxylamine yields amino acid hydroxamate, indicating that the carboxyl group of the amino acid participates in the linkage. The reaction rate of hydroxylamine with Leu-tRNA is faster than reaction with the 2' or 3' leucine ester of AMP. The aminoacyl-tRNA linkage is alkali labile. RNase digestion of aminoacyl-tRNA yields the 2' or 3' amino acid ester of adenosine. The amino acid therefore is transferred to the 2' or 3' hydroxyl of the terminal adenosine of tRNA (Zachau et al., 1958; Hecht et al., 1959). The ester linkage isomerizes too rapidly to permit unequivocal determination of which hydroxyl group is esterified (Wolfenden et al., 1964).

There are twenty species of tRNA, each capable of accepting a specific activated amino acid. By countercurrent distribution, several subclasses of tRNA are found in E. coli. For example, five different tRNAs exist capable of accepting leucine, and there are three tRNAs that accept serine (Goldstein et al., 1964; Kelmers et al., 1965). Periodate oxidation of tRNA destroys the cis diol configuration of the terminal adenosine residues (Preiss et al., 1959). Esterification of tRNA with an amino acid protects the terminal adenosine against periodate oxidation. Esterification of tRNA with one amino acid, followed by periodate oxidation and then removal of the

amino acid, yields the tRNA which accepts only that amino acid that served as a protecting agent.

The same enzyme that catalyzes the activation of the amino acid catalyzes the transfer to tRNA. Throughout extensive purification of several aminoacyl-tRNA synthetases, the ratio of the ATP-PP$_i$ exchange activity to the acylation activity remains constant (Lipmann et al., 1959; Berg et al., 1961). Despite the lack of specificity in the activation reactions of the Ile- and Val-tRNA synthetases from E. coli, there is no report of mistaken acylation. Whereas the incorrect amino acid is activated by these enzymes forming enzyme bound aminoacyladenylates, hydrolysis rather than esterification occurs in the presence of tRNA (Baldwin and Berg, 1966b). Evidently, the specificity of the second half of the synthetase reaction is greater than the first half.

Although the complete nucleotide sequence is now known for the alanine (Holley et al., 1965), tyrosine (Madison et al., 1966), phenylalanine (RajBhandary et al., 1967), and serine (Zachau et al., 1966) tRNA molecules, the site which the aminoacyl-tRNA synthetase recognizes, thereby conferring this remarkable specificity on the reaction, remains unknown. It is now apparent that the anticodon is not the site the enzyme recognizes since the same E. coli tyrosyl-tRNA synthetase esterifies tyrosine to both the normal and suppressor tyrosine tRNAs which differ only in their anticodon base compositions (Goodman et al., 1967). Once an amino acid is attached to a tRNA molecule, its insertion into the linear assembly of a polypeptide is specified by base pairing between the anticodon of the tRNA and the complementary triplet of nucleotides in the messenger RNA, the codon. The experiment of Chapeville et al. (1962) proved that this specificity during polymerization resides in the tRNA and not the amino acid. When cysteinyl-tRNA^{cys} was reduced to alanyl-tRNA^{cys}, the alanine was incorporated into polypeptide in the positions normally occupied by cysteine.

There is no evidence that the aminoacyl-tRNA synthetases play any role in the polymerization of the amino acids occurring on the ribosome (Schweet and Heintz, 1966).

To recapitulate, the reactions catalyzed by aminoacyl-tRNA synthe-

tases are pictured in Figure 1. Amino acids are activated by condensation with ATP to form enzyme bound aminoacyladenylates. In most cases, these reactions can be assayed by the ATP-PP$_i$ exchange reaction or by trapping the activated amino acid as the hydroxamate. Normally, the activated amino acid is esterified to a specific tRNA molecule. When this reaction has occurred, the entire aminoacyl-tRNA synthetase reaction has been carried out. The formation of aminoacyl-tRNA from amino acid, ATP, and tRNA is often referred to as the overall or complete reaction. Aminoacyl-tRNA is liberated for its subsequent role in polypeptide synthesis.

$$\text{enz}_1 \; + \; \text{AA}_1 \; + \; \text{ATP} \; \underset{\xrightarrow{\hspace{1cm}}}{\overset{\text{Mg}^{++}}{\xleftarrow{\hspace{1cm}}}} \; \text{AA}_1 \sim \text{AMP} \cdots \text{enz}_1 \; + \; \text{PP}_i$$

$+ \text{tRNA}_1$ $\qquad\qquad\qquad + \text{NH}_2\text{OH}$

$\text{AA}_1 \sim \text{tRNA}_1$ $\qquad\qquad\qquad\qquad \text{AA}_1 - \text{NHOH}$

$+ \text{enz}$ $\qquad\qquad\qquad\qquad\qquad\qquad + \text{enz}$

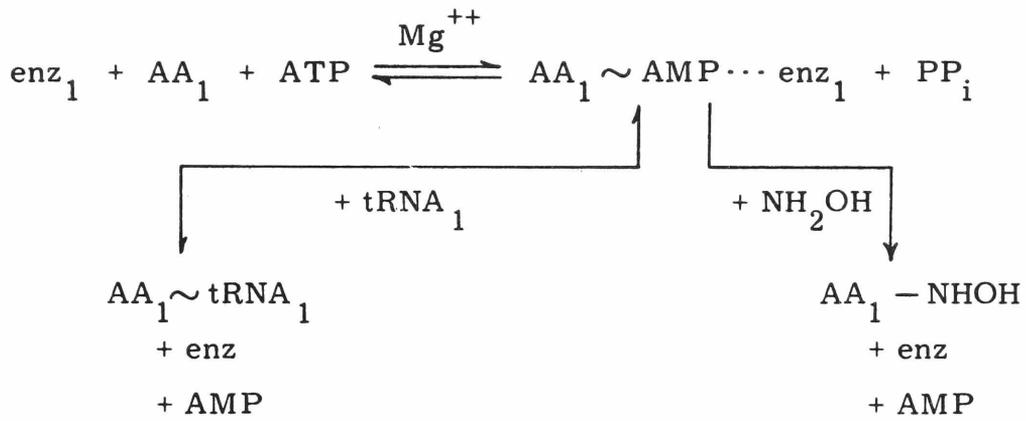$+ \text{AMP}$ $\qquad\qquad\qquad\qquad\qquad\qquad + \text{AMP}$

Figure 1. The equations for the aminoacyl-tRNA synthetase reaction and the amino acid hydroxamate reaction.

CHAPTER   TWO

The Hydroxamate Reaction

The hydroxamate assay was first devised by Lipmann and Tuttle (1945) to study the activation of the carboxyl group of acetate. However, when low concentrations of hydroxylamine were used, acetylhydroxamate only formed when the final acceptor, coenzyme A, was present. As was mentioned earlier, the hydroxamate assay was subsequently used by Hoagland et al. (1956) to prove that the carboxyl groups of amino acids were activated. In those cases, very high concentrations of hydroxylamine, from 1.5 to 3 M, were necessary to trap the aminoacyladenylates formed in the enzymatic reactions. By contrast, free aminoacyladenylates react instantaneously at room temperature with 0.1 M neutral hydroxylamine (Baldwin and Berg, 1966b). Even at 0° and pH 5, free aminoacyladenylates react rapidly with hydroxylamine (Zachau et al., 1958). Wolfenden (1964) calculated that the carboxyl transfer potential of aminoacyladenylates in the intracellular environment of protein synthesis is -16.5 kcal per mole. Therefore, these labile, activated intermediates appear to be sequestered within the active sites of the enzymes that catalyze their formation.

The original studies of Hoagland et al. (1956) showed that the rates of amino acid hydroxamate formation did not parallel the rates of the ATP-PP$_i$ exchange reactions catalyzed by five different amino acid activating enzymes from rat liver. It therefore appears that the different amino acid activating enzymes differentially protect their respective aminoacyladenylates from attack by hydroxylamine.

This proposition has been tested using fourteen partially purified aminoacyl-tRNA synthetases from Escherichia coli, and the results are presented in this chapter.

Materials and Methods

Materials   Escherichia coli B grown to the late logarithmic stage were purchased from General Biologicals Inc. and stored at -20°. E. coli B tRNA was purchased from Schwarz Bioresearch. DEAE-cellulose was a product of Biorad Laboratories. Hydroxylapatite was purchased from Clarkson Chemical Co. under the trade name of Hypatite C. Na$_2$ATP was purchased from Pabst Laboratories. Hepes and DTT were products of Calbiochem. Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co. ^{14}C-labeled amino

acids were obtained from New England Nuclear Corporation. ^{14}C-cysteine was prepared by mixing ^{14}C-cystine with a 1.5 fold molar excess of DTT in 0.1 M Tris·HCl at pH 8.0. ^{12}C-amino acids were purchased from Calbiochem. Amino acid hydroxamates were purchased from Sigma Chemical Co. Crystalline pyrophosphatase from yeast was prepared under the guidance of Dr. Moses Kunitz in his laboratories (Kunitz, 1961). Salt free hydroxylamine was prepared by Davie's modification of the method of Beinert (Davie, 1962; Beinert et al., 1953) and stored at -20° at a concentration of 10 to 20 M. Concentrations of hydroxylamine were determined according to Frear and Burrell (1955).

Enzymes Aminoacyl-tRNA synthetases from E. coli were partially purified using the methods described by Muench and Berg (1966). Whereas this procedure does not yield more than 10 to 20 fold purification of the synthetases, it separates the peaks of enzyme activities into individual fractions.

Enzyme assays The enzyme activities were located and assayed by measuring the rate of attachment of ^{14}C-amino acid to tRNA as described by Muench and Berg (1966), except that DTT replaced glutathione in the reaction mixture.

To assay for the rate of amino acid hydroxamate formation, each 2 ml reaction mixture contained 1.5 M NH_2OH, pH 7.5, 1 mM ATP, 10 mM $MgCl_2$, 2 mM DTT, 100 μg/ml bovine serum albumin, 10 μg/ml pyrophosphatase, 100 μM ^{14}C-amino acid (specific activity 4 μC/μmole), and an amount of enzyme which formed from 3 to 30 mμmoles of aminoacyl-tRNA in 10 minutes at 37° in the preceding tRNA assay.

After incubating at 37° for 0, 20, 40 and 60 min, 0.4 ml aliquots were removed to tubes containing equal volumes of 10% TCA at 0° and mixed. After 10 min, the precipitated protein was removed by centrifugation. The supernatant fluids were applied to 0.9 x 6.3 cm Dowex 50W x 2 columns (H^{+} form), to separate the ^{14}C-amino acids from any ^{14}C-amino acid hydroxamates that were formed. Tyrosine or tryptophan was eluted from the column with 31 ml of 0.5 N pyridinium formate, pH 6, followed by 1 N NH_4OH which eluted the corresponding hydroxamates. Lysine was separated from lysine hydroxamate using 31 ml of 2 N pyridinium formate, pH 6, followed by 1 N NH_4OH. All other amino acids shown in Table I were separated from their hydroxamates

using 12 ml of 2 N pyridinium formate, pH 6, followed by 1 N NH_4OH. One
ml fractions were collected when elution with NH_4OH was started. Ten ml
of Bray's scintillation fluid (1960) were added to each fraction and radio-
activity in the samples was determined in a Nuclear-Chicago scintillation
counter at about 75% efficiency. After use, each column was washed with 50 ml
of 1 N NH_4OH, regenerated with excess 2 N HC1, and washed with water until
the eluate was no longer acidic. These Dowex 50 columns were calibrated by
applying 0.8 ml of a 5% TCA solution containing 0.1 μC of ^{14}C-amino acid
and 20 μmoles of ^{12}C-amino acid hydroxamate to the column and collecting
1 ml fractions of the eluate. Half of each fraction was counted in the
scintillation system to locate the ^{14}C-amino acid. The other half was acidi-
fied with HC1 and then received 0.25 ml of a solution containing 0.37 M $FeCl_3$,
0.31 M TCA, 0.65 M HC1 in order to detect the amino acid hydroxamate as the
ferric salt. The absorbancy was determined at 540 mμ (Lipmann and Tuttle,
1945).

Results

Fourteen aminoacyl-tRNA synthetases from the bacterial extract were
separated into ten different fractions. The tyrosine and alanine enzymes
shared a common position in fraction 19 as did the tryptophan and lysine
enzymes in fraction 25, the isoleucine and threonine enzymes in fraction 28,
and the cysteine and serine enzymes in fraction 9.

In Table I are shown the initial reaction velocities of the four-
teen aminoacyl-tRNA synthetases measured in two ways, the attachment of
amino acid to tRNA and the formation of amino acid hydroxamate. The velocity
of amino acid hydroxamate formation was compared to the velocity of amino-
acyl-tRNA formation for each enzyme. These relative rates of amino acid
hydroxamate formation vary over a sixtyfold range among the fourteen enzymes.
They fall into three categories. The aromatic amino acid and methionine
activating enzymes catalyze the formation of hydroxamates at velocities with-
in 20% of the velocities at which they catalyze the formation of aminoacyl-
tRNA. Lysine and alanine enzymes form hydroxamates at less than half the
rates of the overall reactions. Also, the activating enzymes for isoleucine.

Table I

Assays of Aminoacyl-tRNA Synthetases from E. coli

Fraction		AA-NHOH	AA-tRNA	$\dfrac{\text{AA-NHOH}}{\text{AA-tRNA}}$ x 100
		mμmoles/10 min/mg protein		
19	Tyr	1205	975	124
14	Phe	308	375	82
25	Try	488	610	80
20	Met	583	732	80
25	Lys	136	433	32
19	Ala	84	373	22
28	Ile	122	1517	8
11	Gly	2	28	7
9	Cys	21	378	6
21	Leu	38	738	5
9	Ser	34	717	5
10	Val	12	607	2
13	Gln	*	214	*
28	Thr	*	230	*

The aminoacyl-tRNA synthetases were partially purified and separated into individual fractions by column chromatography. Each enzyme was assayed for the initial rates of amino acid hydroxamate and aminoacyl-tRNA formation. (*) No detectable amounts of hydroxamate formation were catalyzed by these enzymes unless tRNA supplemented the reactions. The details of the experiments are described in the text.

glycine, cysteine, leucine, serine, and valine form amino acid hydroxamates slowly. Finally, the threonine and glutamine enzymes do not catalyze amino acid hydroxamate formation.

The hydroxamate assays used in these experiments would have detected an initial velocity of approximately 0.5% of the velocity of aminoacyl-tRNA formation for these two enzymes. Therefore, valine hydroxamate formation is still twofold above the lower limit of the assay. This implies that under the assay conditions that were used, Thr- and Gln-tRNA synthetases form hydroxamates at less than 0.5% of the rate at which they catalyze aminoacyl-tRNA formation.

However, when tRNA (50 A_{260}/ml) supplemented the hydroxamate assays for these two synthetases, amino acid hydroxamates formed in the reaction. In the case of Gln-tRNA synthetase, glutamine hydroxamate formed at a rate of 112 mμmoles per 10 min per mg of protein or at about 50% of the rate at which the enzyme catalyzed Gln-tRNA formation in the absence of hydroxylamine. Formation of threonine hydroxamate in the presence of tRNA will be studied in greater detail in further chapters.

Discussion

Most of the published data on velocities of amino acid activation were obtained using supernatant fractions of crude extracts of microorganisms, plants, and animals (Hoagland, 1955; Novelli, 1958). These values must be viewed with extreme caution because tRNA can affect the ATP-PP$_i$ exchange and hydroxamate assays. Also, nonspecific activation, which is known to occur in the case of E. coli Ile- and Val-tRNA synthetases, can yield false activities (Bergmann et al., 1961). The E. coli aminoacyl-tRNA synthetases have been partially purified to remove nucleic acids and to separate the enzymes into individual fractions to avoid measuring nonspecific activation and competition between tRNA and hydroxylamine for the activated amino acid.

The sixtyfold range of hydroxamate activities implies that each of the various E. coli aminoacyl-tRNA synthetases protects its high energy intermediate to a different extent. Conversely, hydroxylamine has roughly a forty to sixtyfold greater accessibility to the aminoacyladenylates of

the aromatic aminoacyl- and methionyl-tRNA synthetases than it does to the valyl or seryl enzymes.

This differential protection of the activated amino acids by the synthetases is not limited to the E. coli enzymes. A predominance of hydroxamate forming activities by the aromatic amino acid and methionine activating enzymes was observed in rat liver supernatant fractions separated by acid precipitation (Hoagland et al., 1956). Try-tRNA synthetase from beef pancreas was the first aminoacyl-tRNA synthetase to be purified, and it rapidly catalyzes tryptophan hydroxamate formation (Davie et al., 1956). Purified Phe-tRNA synthetase from a special strain of E. coli catalyzes a rapid hydroxamate reaction (Conway et al., 1962). Tyr-tRNA synthetases purified from hog pancreas (Schweet and Allen, 1958) and yeast (van De Ven et al., 1958) readily catalyze tyrosine hydroxamate formation.

There are fewer comparative values available for enzymes which catalyze hydroxamate formation slowly, probably because it is more convenient to assay the enzymes by the faster ATP-PP$_i$ exchange or tRNA acylation reactions. Leu- and Val-tRNA synthetases in rat liver supernatant fractions form hydroxamates slowly (Hoagland et al., 1956). Gly-, Ile-, and Val-tRNA synthetases from Sarcina lutea form hydroxamates slowly relative to their rates of ATP-PP$_i$ exchange (Hahn and Brown, 1967).

In the survey of the E. coli aminoacyl-tRNA synthetases, Gln- and Thr-tRNA synthetases do not catalyze hydroxamate formation unless tRNA supplements the reaction. Ravel et al. (1965) studied the purified Gln-tRNA synthetase from E. coli W. Although no hydroxamate assays were performed, the enzyme failed to catalyze an ATP-PP$_i$ exchange reaction unless tRNA was added to the reaction mixture.

Hartmann purified Thr-tRNA synthetase from calf liver (Lipmann et al., 1959). The enzyme catalyzed an ATP-PP$_i$ exchange reaction and acylated tRNA with threonine. However, it failed to catalyze threonine hydroxamate formation (Lipmann, personal communication). Purified rat liver Thr-tRNA synthetase was studied by Allende (1964). This enzyme formed a threonyl-AMP-enzyme complex and rapidly acylated tRNA with threonine, but failed to catalyze threonine hydroxamate formation (Allende, personal communication).

The results of the survey of the E. coli aminoacyl-tRNA synthetases, taken together with these reports from the literature, indicate that the catalytic sites vary with respect to the individual enzymes but not with respect to the source of the enzymes. In general, those synthetases that catalyze hydroxamate formation rapidly in E. coli also catalyze rapid hydroxamate formation in animal tissues. Those enzymes that are slow or do not catalyze hydroxamate formation in the absence of tRNA in E. coli have these same properties in other organisms. This implies that there are fundamental differences in susceptibility to attack by hydroxylamine in the catalytic sites of the various aminoacyl-tRNA synthetases since these differences are independent of the source of the enzymes.

To a certain extent, the readiness with which certain enzymes catalyze hydroxamate formation also seems related to the structure of the amino acid involved. The enzymes which activate aromatic amino acids all rapidly form hydroxamates. Yet, valine is very slow and threonine does not catalyze hydroxamate formation. Valine is the structural analog of threonine, with a methyl group replacing the hydroxyl of threonine. The similarity in structure of these two substrates will be emphasized again in Chapter III. The general correlation of substrate structures with the susceptibility of the intermediate aminoacyladenylate to attack by hydroxylamine suggests that the catalytic sites for enzymes which activate structurally similar amino acids are themselves structurally similar.

It is also apparent that tRNA affects the ability of certain enzymes to catalyze amino acid hydroxamate formation. The most extreme example is Thr-tRNA synthetase. This property of Thr-tRNA synthetase raises several questions concerning the nature of the intermediates in the Thr-tRNA synthetase reaction. For example, does the enzyme activate threonine by the same mechanisms as other synthetases, particularly those whose intermediates can be trapped with hydroxylamine? Or, is the catalytic site of Thr-tRNA synthetase protected by some unique mechanism? With these questions in mind, a detailed study of Thr-tRNA synthetase from E. coli has been performed.

CHAPTER   THREE

Purification and Assays of Threonyl-tRNA Synthetase from

Escherichia coli

Over the past few years, several aminoacyl-tRNA synthetases have been purified (Peterson, 1967). Where they have been measured, molecular weights were near 100,000 with the exception of that of yeast Phe-tRNA synthetase. This was reported to have a molecular weight of 160,000 to 180,000, but this value may reflect protein aggregation (Makman and Cantoni, 1965). The occurrence of subunits has been mentioned only in the case of Met-tRNA synthetase (McElroy et al., 1967). The amino acids in the E. coli Ile-tRNA synthetase and Tyr-tRNA synthetases from E. coli and B. subtilis have been analyzed, and there were no uncommon amino acids present (Calendar and Berg, 1966).

To date the most thoroughly studied aminoacyl-tRNA synthetase is the E. coli Ile-tRNA synthetase. The interest in this enzyme stemmed from its ability to activate both valine and isoleucine but to transfer only iso-leucine to tRNA. Valyladenylate bound to Ile-tRNA synthetase hydrolyzes in the presence of tRNA (Baldwin and Berg, 1966a,b). The same is apparently true of the rat liver Ile-tRNA synthetase (Hele and Barth, 1966).

Several investigations have focused attention on how tRNA affects the first half of the aminoacyl-tRNA synthetase reaction (Ravel et al., 1965; Ogata, 1961; Hele, 1964; Loftfield and Eigner, 1965; Deutscher, 1967). Most often these effects have been examined in the ATP-PP$_i$ exchange reaction. However, Loftfield and Eigner (1965) studied the reactions of hydroxylamine and amino acids catalyzed by Ile- and Val-tRNA synthetases. Most of their efforts concentrated on the cross activation of valine by Ile-tRNA synthetase from E. coli.

In this chapter, the purification of E. coli Thr-tRNA synthetase is described. The enzyme catalyzes an ATP-PP$_i$ exchange reaction, esterifies threonine to tRNA, but does not catalyze threonine hydroxamate formation un-less tRNA supplements the reaction mixture. In this latter respect, the enzyme differs from other enzymes described above.

Materials and Methods

Materials   Amberlite SA 2 cation exchange paper was purchased from Reeve Angel. Diazomethane was kindly prepared and supplied by Dr. Paul Englund.

[32]P-sodium pyrophosphate was obtained from Nuclear-Chicago. Alumina Cγ gel which had been aged for 6 months was purchased from Biorad. Superbrite glass beads, 200 μ in diameter, were purchased from Minnesota Mining and Manufacturing Company. Crystalline ribonuclease A was obtained from Worthington. Crystalline beef liver catalase was obtained from Boehringer Mannheim Corp.

Standard enzyme assay   The standard assay used for Thr-tRNA synthetase measured the formation of Thr-tRNA. The 0.2 ml assay mixture contained 50 mM Hepes, pH 7.5, 4 mM ATP, 16 mM MgCl$_2$, 16 mM DTT, 100 μg/ml bovine serum albumin, 10 A$_{260}$ units of E. coli B tRNA, 100 μM uniformly labeled ^{14}C-threonine (specific activity 20 μC/μmole), and 1-2 units of enzyme. When dilution of the enzyme was necessary before assay, the diluting buffer contained 50 mM potassium phosphate, pH 7.5, 10% glycerol, 10 mM glutathione, 0.1 mM EDTA and 100 μg/ml bovine serum albumin.

After incubating for 3 min at 30°, the reactions were stopped by adding 3 ml of cold 5% TCA. After standing at 0° for 10 min, each sample was filtered through a Whatman GF/C glass fiber paper filter mounted on a Millipore filtration apparatus. The filter was washed five times with 5 ml of cold 5% TCA, dried at 100° for 10 min, and then counted in 5 ml of toluene containing 0.4% of 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene. Radioactivity was determined in a Nuclear-Chicago scintillation counter at 78% efficiency. Blank values were routinely obtained by omitting enzyme from one tube and tRNA from another tube. One unit of enzyme activity is defined as the formation of 1 mμmole of Thr-tRNA per hour under the conditions of the enzyme assay.

As shown in Figure 2, the reaction rate was strictly linear for 10 min. The rate then approached a plateau which was reached in 30 min. At that point, all of the available tRNA that would accept threonine had been esterified with the amino acid as determined by adding excess enzyme. The 3 min assay was also linear up to 2.7 units of added enzyme as shown in Figure 3.

Assays for hydroxamate formation   Unless otherwise noted, the standard hydroxamate assay mixture contained 4 mM ATP, 16 mM MgCl$_2$, 16 mM DTT, 100 μg/ml bovine serum albumin, 100 μM uniformly labeled ^{14}C-threonine (specific activity
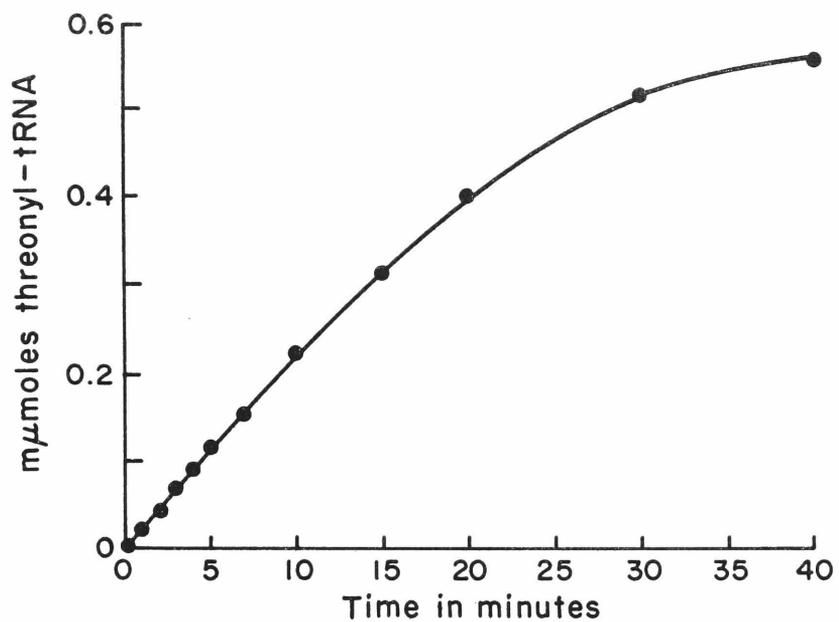
Figure 2. The Thr-tRNA synthetase assay with respect to time. 0.185 μg of enzyme protein (Fraction VI) were used. The conditions are given in the text.
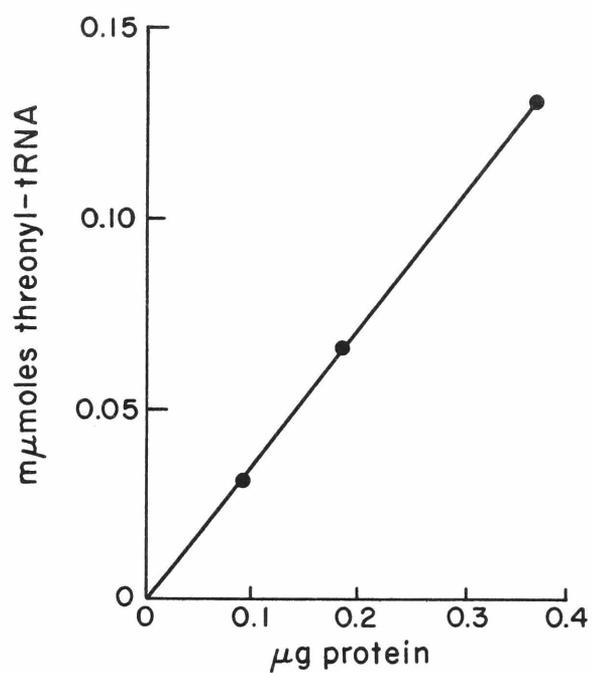
Figure 3.   Linearity of the standard enzyme assay for Thr-tRNA synthetase with respect to concentration of enzyme protein (Fraction VI). The conditions are given in the text.

20 $\mu C/\mu mole$), 1.5 M hydroxylamine, pH 7.5, 5 $\mu g/ml$ pyrophosphatase, and enzyme.

The reaction mixtures were incubated at 30° unless otherwise noted. At timed intervals, 100 μl aliquots were removed and placed in stoppered tubes in a boiling water bath. After 30 sec, they were placed in an ice bucket for 20 min. The resulting precipitate was removed by centrifugation.

The ^{14}C-amino acid was separated from its corresponding hydroxamate in the clarified supernatant fluid by paper chromatography, electrophoresis or chromatography on SA 2 cation exchange paper.

For the paper chromatographic separation of threonine from threonine hydroxamate, an aliquot of the clarified supernatant was applied to Whatman 3 MM paper, dried and developed for 5 hr in n-butanol, formic acid, water (75 : 15 : 10 $^V/v$).

Electrophoresis was performed at 2000 volts for 2 hr in 0.05 M sodium phosphate buffer, pH 7.0, on Whatman 3 MM paper. It was always necessary to lyophilize the supernatant samples to dryness and redissolve them in water before electrophoresis to remove the hydroxylamine, which interfered with the electrophoretic separation.

The method described by Loftfield and Eigner (1963) was used to separate the neutral amino acid from the basic hydroxamate by chromatography at pH 7 on Amberlite SA 2 cation exchange paper. ^{14}C-threonine hydroxamate remained at the origin of the SA 2 paper, whereas ^{14}C-threonine moved with the solvent front except for 1-2% which also remained at the origin. All efforts to eliminate the latter were unsuccessful. Even if the amino acid which traveled with the front was eluted and rechromatographed on SA 2 paper, 1-2% of the radioactivity remained at the origin. This also happened if the paper was washed with 1 mM threonine or if 1 mM threonine was present in the eluting phosphate solution. This was important because the evidence in Chapter II indicated that only very small amounts, if any, of threonine hydroxamate might be encountered. Loftfield and Eigner (1963) reported the same problem with isoleucine and valine but in those cases, the material remaining at the origin was only 0.1-0.2% of the total radioactivity.

Radioactivity was located by using a Packard Radiochromatogram Scanner. After scanning, or alternatively, the chromatograms were cut into 1 cm segments. The radioactivity on each segment was determined in 10 ml of toluene scintillation fluid at approximately 44% efficiency in the Nuclear-Chicago scintillation counter. Counting efficiency was determined by using a chemically synthesized ^{14}C-threonine hydroxamate standard. This standard was synthesized by reacting ^{14}C-threonine with diazomethane in methanol to form the methyl ester of ^{14}C-threonine, which, in turn, was reacted with hydroxylamine to form the hydroxamate. ^{14}C-threonine hydroxamate was purified by chromatography on SA 2 paper, followed by paper chromatography in butanol, formic acid, water as described above. The synthesized ^{14}C-threonine hydroxamate cochromatographed with threonine hydroxamate purchased from Sigma Chemical Co.

^{12}C-threonine was located by spraying chromatograms with ninhydrin. ^{12}C-threonine hydroxamate was identified as the ferric salt by spraying the paper with the acidic $FeCl_3$ solution described in Chapter II.

ATP-^{32}PP$_i$ exchange assay To determine if Thr-tRNA synthetase catalyzed an ATP-^{32}PP$_i$ exchange reaction, the assay method described by Calendar and Berg (1966) was used with the exceptions that the buffer was 100 mM Hepes, pH 7.5, and DTT replaced β-mercaptoethanol in the reaction mixture. The reactions were incubated at 30°.

Protein assays Protein was determined according to the procedure of Bennett (1967) using bovine serum albumin as a standard. The absorbancy of the column eluate was monitored at 280 mμ to estimate protein elution profiles (Warburg and Christian, 1941).

Hydrolysis of threonine hydroxamate Threonine hydroxamate was hydrolyzed in order to recover the free amino acid for further identification of the reaction product. Hydrolysis was carried out in vacuo in 6 N HCl for 1 hr at 110°. The solution was then dried in a rotary evaporator. The residue was dissolved in 1 ml of water. Hydroxamate was measured as the ferric salt after adding 2.3 ml of acidic $FeCl_3$ as previously described. After 10 min, the absorbancy at 540 mμ was measured. These conditions reduced the $FeCl_3$ positive material to less than 2% of its original value, indicating 98%

hydrolysis of the hydroxamate.

Periodate oxidation of tRNA General procedures have been published for oxidative cleavage of the cis diol bond of tRNA using sodium metaperiodate (Preiss et al., 1959; Hecht et al., 1959). However, the exact conditions for the reaction were determined in order to insure maximal destruction of acceptor activity of tRNA which had not been previously esterified with threonine and minimal destruction of acceptor activity of tRNA which had been esterified with threonine. The problem arose because threonine has vicinal amino and hydroxyl groups. Periodate oxidation can also cleave the bond between these groups. Capecchi and Gusson (1965) had determined the conditions for oxidation of tRNA with protection by serine, but their procedure was not effective for Thr-tRNA.

The periodate oxidation reaction mixture contained 98 A_{260} units of E. coli B tRNA, 100 mM sodium acetate, pH 4.7, and 3.3 mM sodium metaperiodate. After incubating 10 min at 30° in the dark, 1 ml of 0.2 M glucose was added and the reaction chilled in ice for 5 min. The tRNA was recovered by ethanol precipitation. The precipitated tRNA was collected by centrifugation, dissolved in water, and dialyzed in water.

This method yielded tRNA which had less than 0.4% of its amino acid acceptor capacity. If the tRNA was first esterified with threonine, it then had less than 0.4% of its acceptor capacity for all amino acids other than threonine and maintained 50% of its threonine acceptor capacity. The latter was measured by esterification after removing the protecting threonine. This deacylation was accomplished by incubating the Thr-tRNA in 0.25 M Tris buffer, pH 9, for 60 min at 40°. The free amino acid was removed by dialysis in water.

Purification of the enzyme All operations were carried out at 0° - 4° unless otherwise stated. Frozen E. coli B cells (150 gm) were broken into small pieces and thawed. Buffer A (150 ml containing 25 mM K_2HPO_4, 10% glycerol and 7 mM β-mercaptoethanol) was added to the bacterial paste and the mixture blended for 30 sec at top speed in a stainless steel Waring Blendor. Then, 300 gm of acid washed glass beads were added. This mixture was blended 5 times at top speed for 2 min each time, with 2 min cooling intervals when

the Blendor cannister was placed in an ice water bath. Next, 150 ml of
Buffer A were added, and the suspension blended two more times. The beads
were allowed to settle and the supernatant fluid poured off. The residue and
beads were rinsed once with 150 ml of Buffer A. The combined wash and orig-
inal supernatant fluids were centrifuged at 16,000 x g for 60 min. The clar-
ified supernatant fluid was carefully siphoned off, diluted 2.8 fold with
Buffer A, and brought to 100 mM K_2HPO_4 by the addition of solid K_2HPO_4.

The extract was incubated at 37° with continuous stirring for 80
min. During this time, 0.2 ml aliquots were removed, precipitated with 4.8
ml of 5% TCA, centrifuged 10 min at 600 x g, and the supernatant fluid appro-
priately diluted with 5% TCA to measure the absorbancy at 260 mμ against a
blank of 5% TCA. Figure 4 shows the increase in the solubilized material
absorbing at 260 mμ throughout the autolysis. When the 260 mμ absorbancy
reached a plateau, the extract was quickly chilled to 0° in a dry ice-ace-
tone bath and centrifuged at 16,000 x g for 40 min to remove the flocculent
precipitate.

The supernatant fluid was decanted and stirred for 15 min with 1.4
volumes of a 25 mg/ml Alumina Cγ gel suspension. The mixture was then centri-
fuged for 15 min at 4000 x g and the supernatant fluid decanted. The gel was
successively eluted with 600 ml of each of the following concentrations of
pH 7.5 potassium phosphate buffers containing 10% glycerol and 7 mM β-mercap-
toethanol: two elutions with 0.08 M potassium phosphate, two elutions with
0.18 M potassium phosphate, one elution with 0.25 M potassium phosphate. For
each elution, the gel was resuspended and stirred for 15 min, and then collected
by centrifugation at 4000 x g for 10 min.

The last three eluates were combined, made 10^{-4} M in EDTA, and 22.6
gm of solid ammonium sulfate per 100 ml of extract were slowly added with
constant stirring. The precipitate that formed after 40 min was removed by
centrifugation at 16,000 x g for 30 min. For each 100 ml of clarified super-
natant fluid, 18.7 gm of solid ammonium sulfate were added and the precipitate
collected again by centrifugation. This precipitate was resuspended in 40 ml
of Buffer B containing: 0.05 M potassium phosphate, pH 7.5, 10% glycerol,
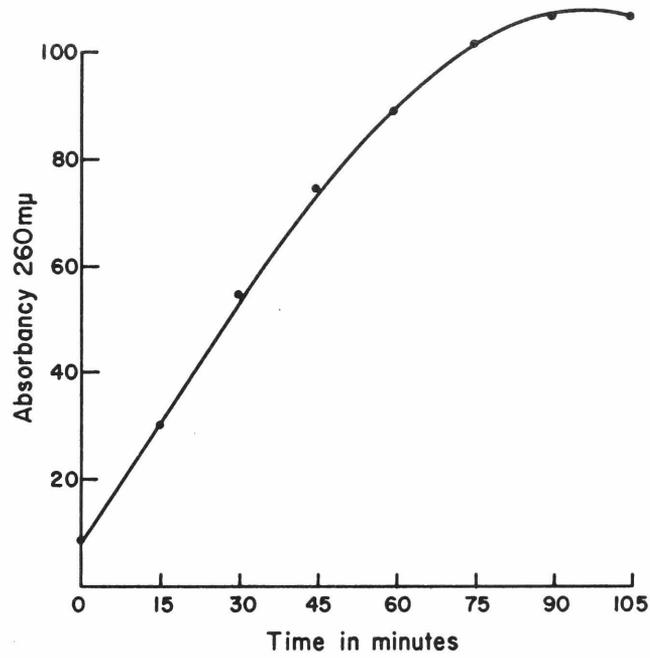10^{-4} M EDTA, and 7 mM β-mercaptoethanol, and dialyzed overnight against 4 liters

<u>Figure 4</u>. Autolysis of the crude extract from <u>E</u>. <u>coli</u> showing the increase in TCA soluble material absorbing at $260 \text{ m}\mu$ with respect to time of incubation at 37°. The experimental details are given in the text.

of the same buffered solution. The solution in the dialysis bag was collected and centrifuged at 30,000 x g for 10 min to remove any precipitated material.

The dialyzed extract was applied to a 1.7 x 79 cm DEAE-cellulose column, which had been equilibrated with Buffer B. After eluting the column with 210 ml of Buffer B at a flow rate of 50 ml/hr, a linear gradient was started with 500 ml of Buffer B in the mixing chamber and 500 ml of 0.35 M potassium phosphate, pH 7.5, 10% glycerol, 10^{-4} M EDTA, and 7 mM β-mercapto-ethanol in the reservoir. Fractions containing 7.5 ml were collected. The elution profiles of the absorbancy at 280 mμ and Thr-tRNA synthetase activity are shown in Figure 5. The tubes containing the peak of the enzyme activity were pooled and concentrated 6 fold by vacuum dialysis against Buffer C: 0.03 M potassium phosphate, pH 7.5, 10% glycerol, and 7 mM β-mercaptoethanol.

The concentrated solution was then applied to a 0.9 x 30 cm hydroxylapatite column which had been equilibrated with Buffer C. After eluting with 100 ml of Buffer C, a linear gradient was applied with 250 ml of Buffer C in the mixing chamber and 250 ml of 0.30 M potassium phosphate, pH 7.5, 10% glycerol, 7 mM β-mercaptoethanol in the reservoir. The column was run under pressure from a Buchler Polystaltic pump at a flow rate of 15 ml/hr. Five ml fractions were collected. The profiles of elution of protein and enzyme activity from the hydroxylapatite column are shown in Figure 6. Those tubes containing the peak of enzyme activity were pooled and concentrated by vacuum dialysis against 0.05 M potassium phosphate, pH 7.5, 10% glycerol, 10^{-4} M EDTA, and 0.01 M glutathione. The enzyme was stored frozen in small aliquots at -78°.

Molecular weight determination The molecular weight of Thr-tRNA synthetase was determined by the method of Martin and Ames (1961). A 5-20% linear sucrose gradient containing 20 mM triethylamine, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, and 0.25 mM EDTA was prepared in a total volume of 5.3 ml. A solution of 0.15 ml of 20 mM Tris, pH 7.2, 2 mM DTT containing 6.88 μg Thr-tRNA synthetase (Fraction VI), 2 μg yeast pyrophosphatase, 1.1 μg E. coli Ile-tRNA synthetase prepared according to Muench and Berg (1966), and 20 μg beef liver catalase was layered on top of the sucrose gradient. Centrifugation was performed for 10 hr at 50,000 rpm in a Model L2-65 Beckman Ultracen-
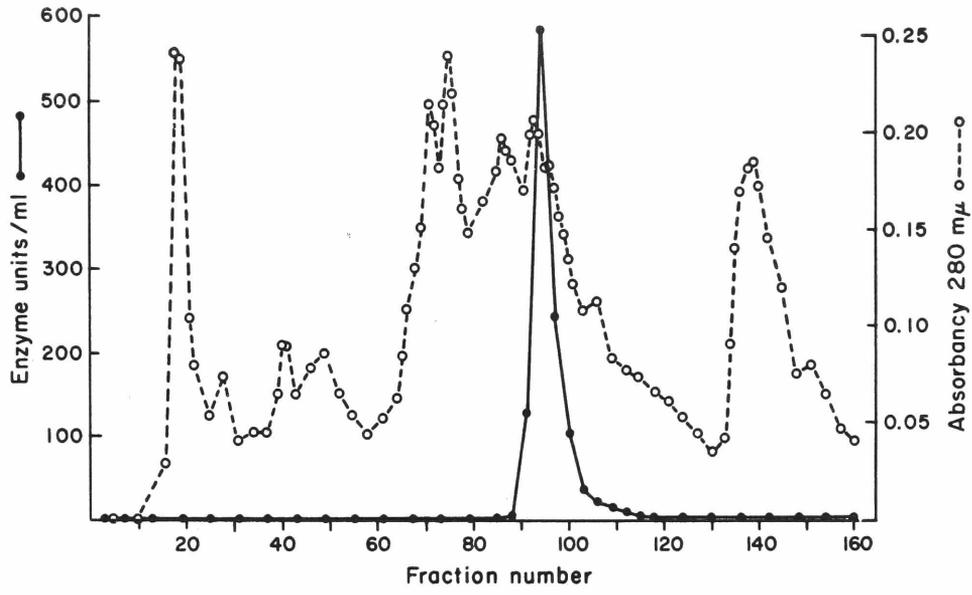
<u>Figure 5.</u> Chromatography of Thr-tRNA synthetase on a DEAE-cellulose column during the purification procedure (Fraction V). Experimental details are given in the text.
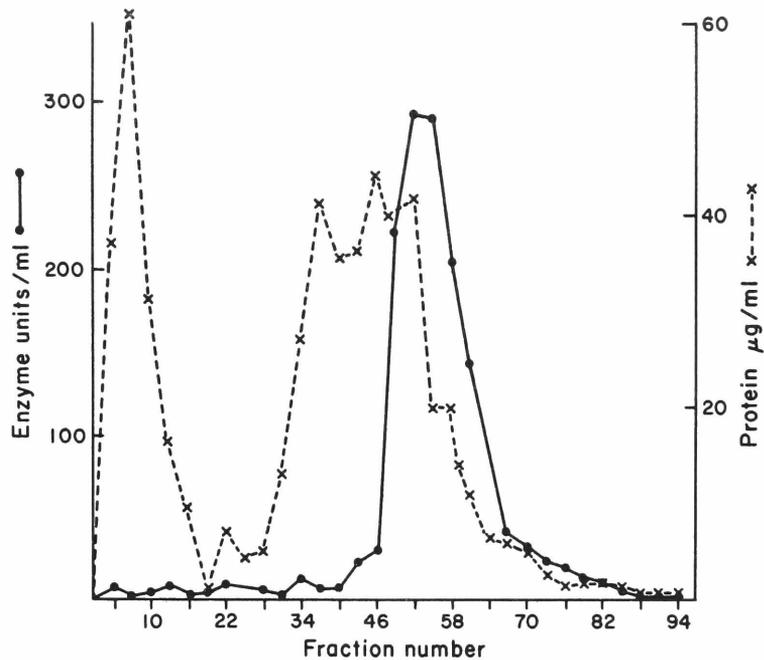
Figure 6. Chromatography of Thr-tRNA synthetase on a hydroxylapatite column during the final step of the purification of the enzyme (Fraction VI). Experimental details are given in the text.

trifuge at 2^O using an SW 50L rotor. Afterwards, the bottom of the tube was punctured and fractions containing 3 drops each were collected and assayed for each of the enzymes.

The standard enzyme assay described earlier measured Thr-tRNA synthetase activity. The same assay mixture was used for Ile-tRNA synthetase activity except 50 mM uniformly labeled ^{14}C-isoleucine (specific activity 10 μC/μmole) replaced ^{14}C-threonine. Catalase activity was measured according to Beers and Sizer (1952) and pyrophosphatase activity was assayed as described by Heppel (1955).

<div align="center">Results</div>

Purification procedure The entire purification procedure is outlined in Table II. Thr-tRNA synthetase was purified 320 fold with 10% yield. More recently, the total yield throughout the procedure has been increased twofold by substituting DTT for β-mercaptoethanol. If the enzyme was purified in the presence of β-mercaptoethanol and then preincubated with 10 mM DTT before assaying, the enzyme activity did not increase.

Glycerol has been included in every buffer throughout the purification. This helped stabilize Thr-tRNA synthetase. Initial attempts to purify Thr-tRNA synthetase without adding glycerol resulted in 80% loss of the enzyme activity within two to four hours after preparing the bacterial extract. Adding glycerol to the standard enzyme assay mixture up to a final concentration of 8% did not modify the activity of purified Thr-tRNA synthetase.

The autolysis (Fraction II of Table II) was originally described by Baldwin & Berg (1966a) in the purification of the isoleucine enzyme. It also proved effective here in the purification of Thr-tRNA synthetase. Evidently the high phosphate concentrations dissociate nucleic acids, and the potassium activates one class of ribonuclease that would otherwise remain dormant. Eighty percent of the material absorbing at 260 mμ was solubilized with no loss in enzyme activity.

All of the enzyme activity originally in the autolysate adsorbed to Alumina Cγ gel. However, 50% of the protein from the autolysate did not

Table II

Purification of Threonyl-tRNA Synthetase from E. coli

Fraction	Volume	Total protein	Total units*	Specific activity	Yield
	ml	mg		units/mg protein	%
I Crude extract	328	3910	89,300	22.8	100
II Autolysate	903	3745	91,100	24.4	102
III Alumina Cγ gel	1818	561	66,450	118.5	74
IV Ammonium sulfate	38	194	27,900	144	31
V DEAE-cellulose	45	13.3	14,990	1128	17
VI Hydroxyl-apatite	1.33	1.23	9,044	7350	10

* 1 unit = 1 mμmole threonyl-tRNA per hour at 30°.

adsorb to the gel. The first two phosphate buffer elutions removed 38% of the protein and about 15% of the enzyme. The final three phosphate buffer elutions removed the bulk of the enzyme activity from the gel (Fraction III).

The purified enzyme (Fraction VI) retained 100% of its activity after one month, and more than 50% after ten months, if it was stored frozen at -78° or if it was stored in 50% glycerol at -20°. However, only 10% of the original activity remained after 3 weeks if it was stored unfrozen in the final buffer at 0°. Fraction VI was assayed for the presence of other aminoacyl-tRNA synthetase activities. All twenty common amino acids were tested. Only Thr-tRNA synthetase activity was found. For reasons that will be apparent later, it is important to note that Fraction VI contained absolutely no Val-tRNA synthetase activity when assayed either by the esterification of tRNA or by the ATP-PP_i exchange reaction.

Kinetic constants of Thr-tRNA synthetase Thr-tRNA synthetase has a K_m of 100 μM for ATP in the overall reaction (Figure 7). At an ATP concentration of 4 mM, the maximal velocity is reached at 10 mM $MgCl_2$ (Figure 8). No detectable esterification of threonine to tRNA occurs in the absence of divalent metal ion and higher concentrations of $MgCl_2$ are slightly inhibitory. The K_m for threonine is 12 μM in the overall reaction (Figure 9).

Molecular weight of Thr-tRNA synthetase The results of the sucrose density gradient centrifugation analysis are shown in Figure 10. Two of the standard enzymes used in the molecular weight determination of Thr-tRNA synthetase were crystalline enzymes. Beef liver catalase was studied by Samejima and Shibata (1961) who reported a sedimentation coefficient of 11.15 S and a molecular weight of 244,000 as determined by velocity sedimentation. Yeast pyrophosphatase has a sedimentation coefficient of 4.4 S and a molecular weight of 63,000 as determined by velocity sedimentation (Schachman, 1952). Although Ile-tRNA synthetase has not been crystallized, its sedimentation behavior was well characterized by Norris and Berg (1966a). This enzyme has a sedimentation coefficient of 5.9 S and a molecular weight of 112,000 as determined by velocity and equilibrium sedimentation.

When the positions of the peaks of the standard enzymes in the
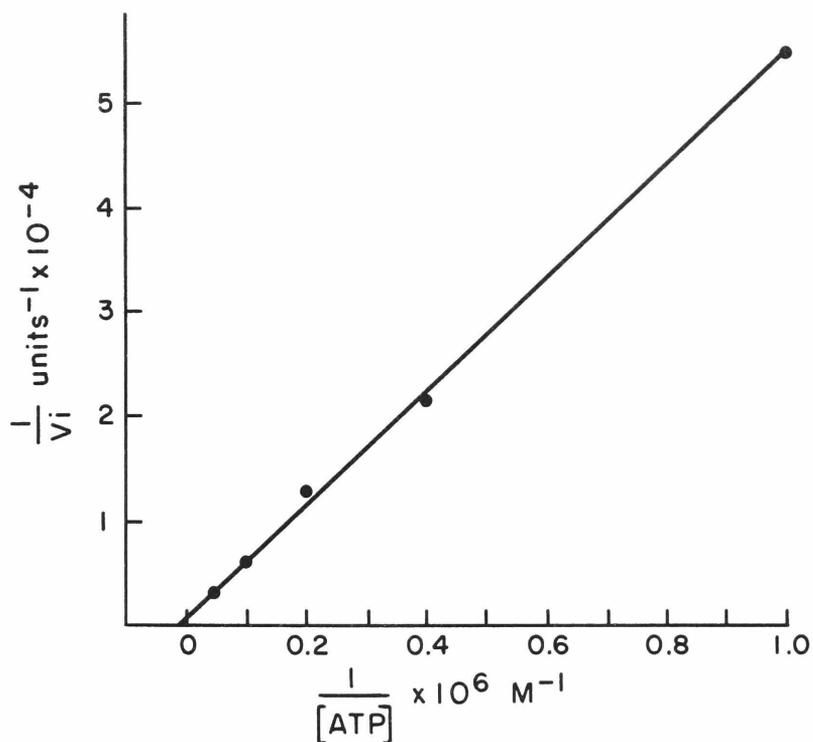
<u>Figure 7.</u> Double reciprocal plot of initial velocity of Thr-tRNA formation
versus ATP concentration. The standard enzyme assay mixture was
used to determine the initial velocity of Thr-tRNA synthetase at
varying ATP concentrations. For each ATP concentration, reactions
were incubated at 30° for 3, 6, 9, and 12 min and the velocity
calculated from the initial slopes. Each 0.2 ml reaction mixture
contained 0.688 μg enzyme protein (Fraction VI).
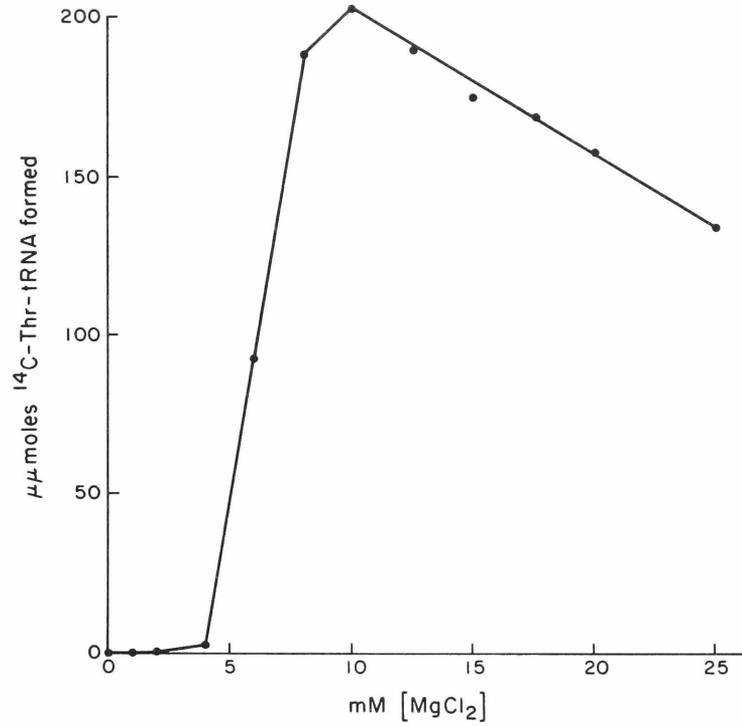
Figure 8. Mg^{++} optimum for the Thr-tRNA synthetase reaction. The standard enzyme assay was performed as described in the text except the concentration of MgCl$_2$ was varied as designated on the abscissa. Each point represents the product formation catalyzed by 0.688 μg enzyme protein (Fraction VI).
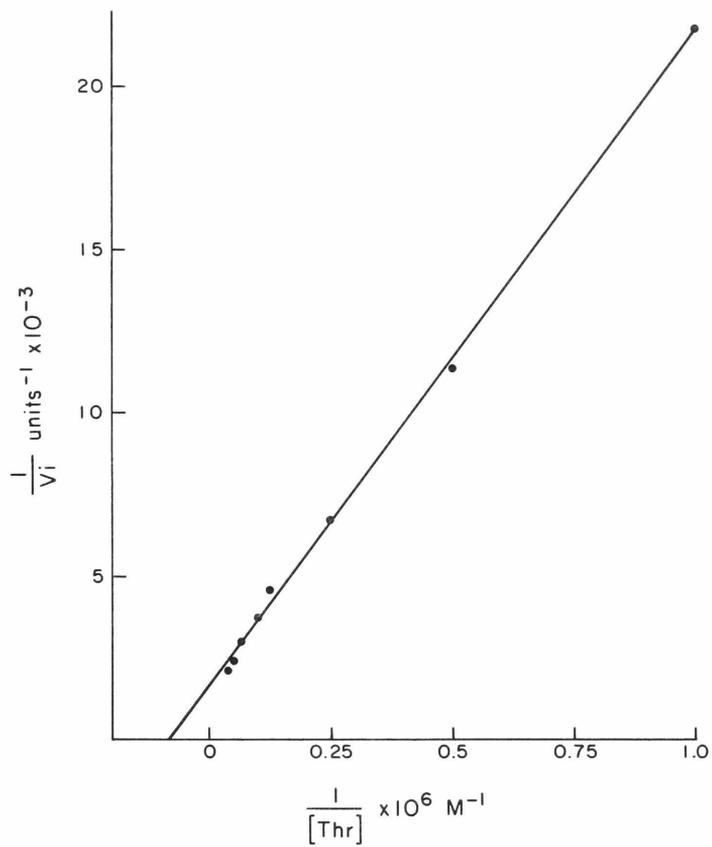
Figure 9. Double reciprocal plot of initial velocity of Thr-tRNA formation versus threonine concentration. The conditions were the same as described in Figure 7 except the ATP concentration was 4 mM and the $MgCl_2$ concentration was 10 mM. The ^{14}C-threonine concentration was varied as indicated on the abscissa.
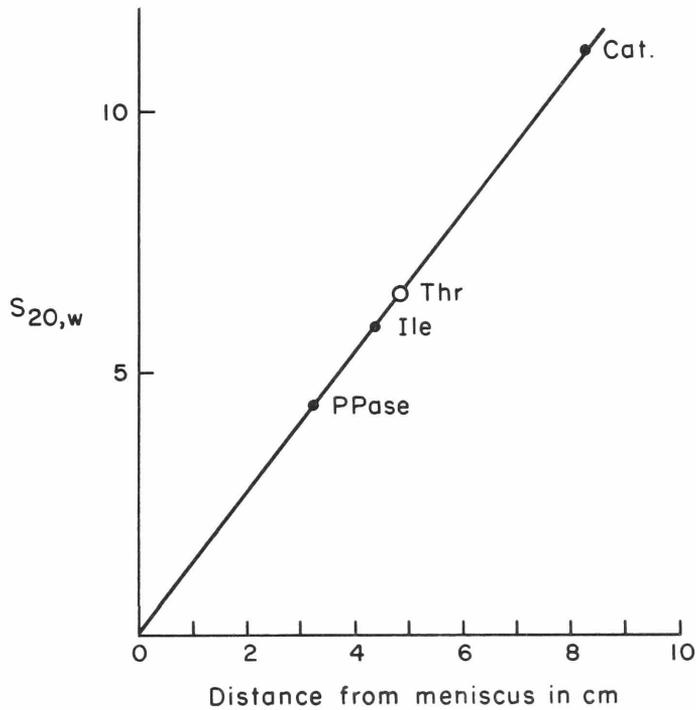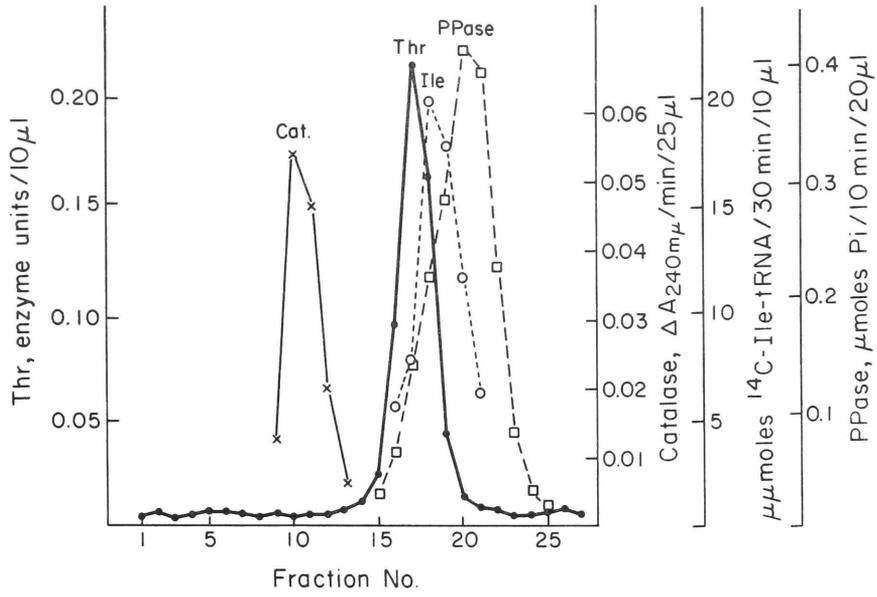
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Figure 10.   Sucrose density gradient analysis of Thr-tRNA synthetase.   The
standard enzymes used as reference markers for Thr-tRNA synthe-
tase (Thr) were catalase (Cat), Ile-tRNA synthetase (Ile), and
pyrophosphatase (PPase).   The details of the experiment are
given in the text.

sucrose density gradient are plotted against the known sedimentation coeffi-
cients, a straight line is obtained which extrapolates to zero at the origin
(Figure 10). From this graph, the sedimentation coefficient of Thr-tRNA syn-
thetase can be estimated to be 6.5 S and, using the relationships and assump-
tions described by Martin and Ames (1961), the molecular weight is found to
be 117,000 $\pm$ 9%.

Threonine hydroxamate formation with crude and purified enzyme
preparations In Chapter II, the distinguishing feature of the partially puri-
fied Thr-tRNA synthetase was the lack of threonine hydroxamate formation. There-
fore, two fractions from the purification procedure of Table II were assayed
for the formation of threonine hydroxamate. The relatively crude Fraction II
was assayed and then the purified enzyme (Fraction VI) was tested.

The autolysate (Fraction II) was assayed for threonine hydroxamate
formation using the SA 2 cation exchange paper chromatography procedure. As
shown in Figure 11, ^{14}C-threonine hydroxamate formed linearly with time. Pre-
incubation of the enzyme extract with ribonuclease did not affect the subse-
quent rate of formation of ^{14}C-threonine hydroxamate. This eliminated the
possibility that threonine hydroxamate formed via reaction of hydroxylamine with
Thr-tRNA. However, the addition of a mixture of nineteen ^{12}C-amino acids (threo-
nine missing) to the assay stopped the formation of ^{14}C-threonine hydroxamate.
When added singly, ^{12}C-valine eliminated the appearance of ^{14}C-threonine hy-
droxamate but ^{12}C-serine did not.

It therefore appeared that threonine hydroxamate formation was
catalyzed by the Val-tRNA synthetase. This mistaken activation had been re-
ported earlier (Bergmann et al., 1961). However, the possibilities also
existed that ^{14}C-valine contaminated the ^{14}C-threonine, or ^{14}C-threonine was
first converted to some other molecule which could be activated by the Val-tRNA
synthetase and react with hydroxylamine. Both of these possibilities were
eliminated by proving that the reaction product was threonine hydroxamate and
that it was not some other cationic molecule that remained at the origin of
the SA 2 paper chromatogram. As shown in Figure 12, if the radioactive mater-
ial remaining at the origin of the SA 2 paper chromatogram was eluted and
hydrolyzed, it was converted to ^{14}C-threonine as determined on the amino acid
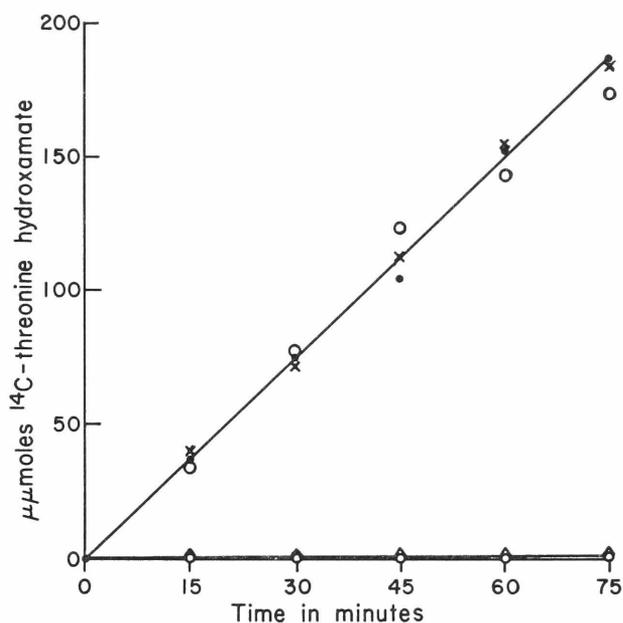analyzer. If it was not hydrolyzed, it could not be recovered as threonine.

Figure 11. Assay for formation of ^{14}C-threonine hydroxamate catalyzed by
the autolysate (Fraction II). Each point represents the product
formation catalyzed by 42 μg of autolysate protein (Fraction II).
Details of the procedure are given in the text. The autolysate
was assayed directly ($\bullet$) and after preincubation for 12 min at
30° with 5.4 μg of RNase A per mg of autolysate protein ($\times$).
It was also assayed in the presence of 20 mM ^{12}C-serine ($\bigcirc$) or
20 mM ^{12}C-valine ($\triangle$) or a mixture of 19 amino acids, 1 mM each,
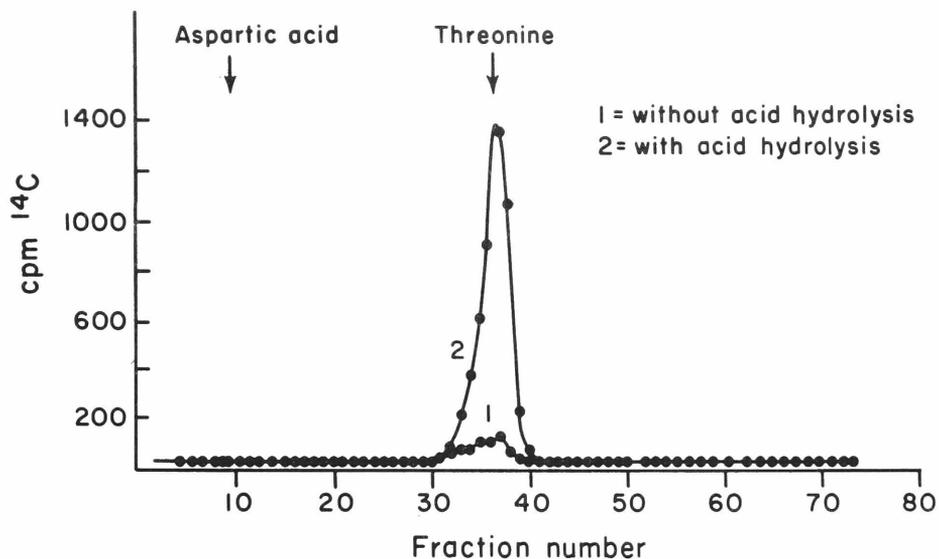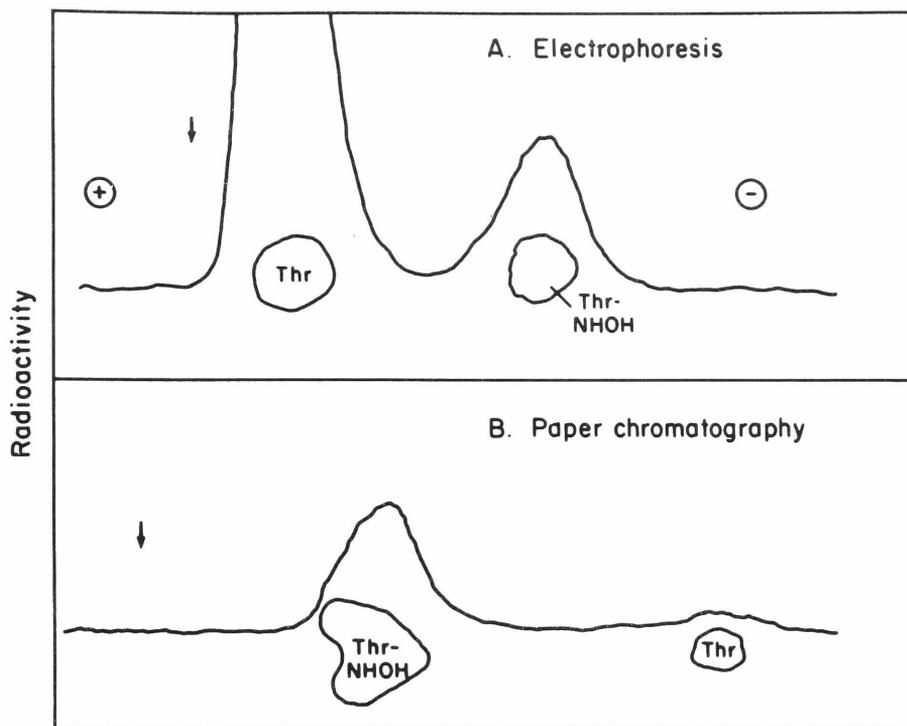threonine omitted ($\circ$).

Figure 12. Identification of the product formed in the assays of the autoly-
sate (Fraction II). Four 60 min hydroxamate assays, as described
in the text, were performed using 42 μg of autolysate protein
(Fraction II). The radioactive material at the origins of the
SA 2 paper chromatograms was eluted with 1 N NH_4OH, pooled, and
evaporated to dryness. Half the material was hydrolyzed and the
other half served as a control. ^{12}C-threonine and ^{12}C-aspartic
acid were added as internal markers. The samples were analyzed
on a Beckman amino acid analyzer with split flow of the column
eluate to measure radioactivity and ninhydrin color. The arrows
indicate the position of the ninhydrin peaks of aspartic acid
and threonine in the eluate.

The small amount of radioactivity which appeared coincident with threonine in the unhydrolyzed sample was probably due to elution of a small amount of threonine from the original SA 2 chromatogram. Furthermore, electrophoresis of a 60 min incubation mixture separated the radioactivity into two peaks. As shown in Figure 13A, the peak which moved rapidly toward the cathode cochromatographed with marker ^{12}C-threonine hydroxamate and the slower moving peak was the unreacted threonine. To assist further in identifying the faster moving peak, the electrophoresis paper was cut between the two peaks, and the portion containing the faster moving peak was subjected to paper chromatography. Once again, the major peak of radioactivity coincided with marker ^{12}C-threonine hydroxamate (Figure 13B). A trace amount of free threonine was present ahead of the threonine hydroxamate. Evidently, this was carried over from cutting the original electropherogram.

The foregoing evidence demonstrates that formation of ^{14}C-threonine hydroxamate was catalyzed by the relatively crude extract of E. coli (Fraction II). This formation can be totally accounted for by mistaken activation of threonine by the Val-tRNA synthetase.

By way of comparison with the crude extract, the purified Thr-tRNA synthetase in Fraction VI of the purification procedure was assayed for its ability to catalyze threonine hydroxamate formation. The results of these experiments are depicted in Figure 14. Thr-tRNA synthetase did not catalyze the formation of threonine hydroxamate in the absence of tRNA. The enzyme catalyzed the formation of threonine hydroxamate only in the presence of tRNA (Figure 14A). There was an initial lag period but then threonine hydroxamate formed linearly with time throughout the entire incubation period. In the absence of hydroxylamine, the enzyme catalyzed the formation of Thr-tRNA with a rapid initial velocity and then Thr-tRNA formation reached the plateau of maximal acylation of threonine to tRNA. However, if hydroxylamine was present, the initial velocity of Thr-tRNA formation was slower and a plateau was reached equal to 20% of the level of Thr-tRNA when hydroxylamine was omitted (Figure 14B). Threonine hydroxamate continuously formed in the reaction while this lower plateau was maintained. Periodate oxidized tRNA was ineffective in promoting threonine hydroxamate formation.

Figure 13. Identification of the product formed in the assays of the autoly-
sate (Fraction II). A 100 μl ^{14}C-threonine hydroxamate assay re-
action mixture containing 140 μg of autolysate protein (Fraction
II) was incubated for 60 min at 30°. The radioactive material in
the mixture was analyzed by (A) electrophoresis, and the peak of
radioactivity which cochromatographed with marker ^{12}C-threonine
hydroxamate was further analyzed by (B) paper chromatography as
described in the text. The arrows designate the origins. The non-
radioactive standard compounds were located by staining reactions
and their positions are designated.
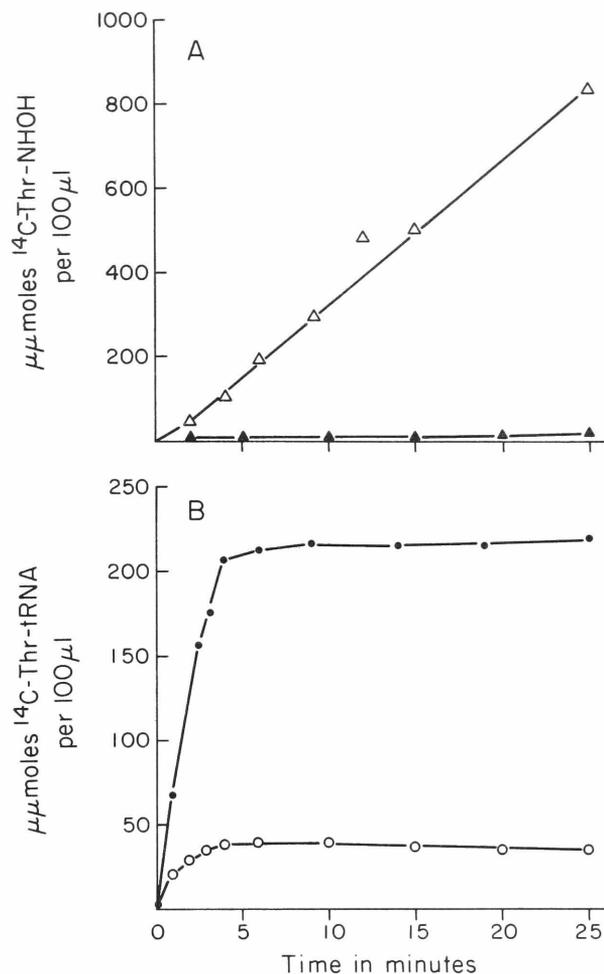
<u>Figure 14.</u> Threonine hydroxamate and Thr-tRNA formation catalyzed by Thr-tRNA
synthetase (Fraction VI). Four separate 2 ml reaction mixtures
were incubated at 30°. Each contained 50 mM Hepes, pH 7.2, 4 mM
ATP, 10 mM $MgCl_2$, 5 mM DTT, 50 μM ^{14}C-threonine (specific activi-
ty 20 $\mu C/\mu mole$), and 10.3 μg enzyme protein. (A) ^{14}C-threonine
hydroxamate formation in reactions containing 1.1 M NH_2OH, pH 7.2,
without tRNA (▲) and with 100 A_{260} units tRNA (△). (B) ^{14}C-Thr-
tRNA formation in reactions containing 100 A_{260} units tRNA with-
out NH_2OH (●) and with 1.1 M NH_2OH, pH 7.2 (○).

^{14}C-Thr-tRNA was determined as described in the text. To deter-
mine ^{14}C-threonine hydroxamate formation, each 100 μl aliquot was
removed at the designated time and added to a tube containing 0.12
ml of 4% potassium acetate, pH 5, and 10 A_{260} units tRNA. After
mixing, 0.5 ml of cold absolute ethanol were added. After standing
20 min at -20°, the precipitate was removed by centrifugation and
an aliquot of the supernatant fluid dried, redissolved in water,
and analyzed on SA 2 paper as described in the text.

Another variation of these experiments is shown in Figure 15. ^{14}C-threonine was enzymatically esterified to tRNA in the absence of hydroxylamine using two different concentrations of enzyme. As expected, the initial veloc-ities were proportional to the enzyme concentration and identical plateaus of maximal esterification were reached in both cases. Hydroxylamine was added at 26 min and the fate of Thr-tRNA followed. There was a rapid decrease of Thr-tRNA to new plateau levels. These new plateaus were proportional to the concentration of enzyme present in the reaction. After addition of hydroxyl-amine, there was a proportionally rapid appearance of threonine hydroxamate. The rate of formation of threonine hydroxamate then decreased slightly and threonine hydroxamate continuously formed at the new linear rate. This occurred concomitantly with the approach of the Thr-tRNA to the new plateau levels. The rate of threonine hydroxamate formation was also proportional to the enzyme concentration. Both the new plateau levels of Thr-tRNA after addi-tion of hydroxylamine and the rate of threonine hydroxamate formation were in-dependent of doubling or halving the tRNA concentration.

The kinetics of the nonenzymatic reaction of Thr-tRNA with hydroxyl-amine under the conditions of the above experiments are shown in Figure 16. The half-life of Thr-tRNA was 1 min. It is apparent that the initial rates of decrease of Thr-tRNA after the addition of hydroxylamine in the two enzy-matic reactions correspond to each other, to the initial rates of appearance of threonine hydroxamate, and to the rate of the nonenzymatic reaction.

The failure of Fraction VI enzyme to catalyze the formation of threo-nine hydroxamate in the absence of tRNA repeats the result observed with the partially purified enzyme fraction described in Chapter II. As long as Val-tRNA synthetase and tRNA are absent, threonine hydroxamate forms at a rate of less than 0.5% of the rate of Thr-tRNA formation, if it forms at all. The formation of Thr-tRNA and threonine hydroxamate in the presence of tRNA points out that the enzyme is active in 1.1 M hydroxylamine.

ATP-PP$_i$ exchange activity   The hydroxamate reaction is a measure of the first half of the overall aminoacyl-tRNA synthetase reaction. Since pur-ified Thr-tRNA synthetase failed to form hydroxamate in the absence of tRNA, it was assayed for ATP-PP$_i$ exchange activity which is another measure of this
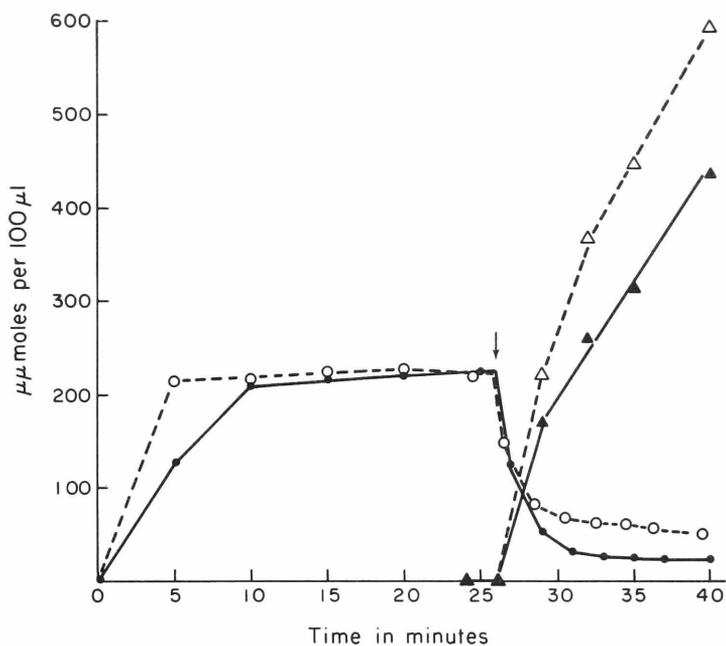
Figure 15. Kinetics of Thr-tRNA and threonine hydroxamate formation in the enzymatic reactions. Two 2 ml reaction mixtures were incubated at 30°. Each contained 50 mM Hepes, pH 7.2, 4 mM ATP, 10 mM $MgCl_2$, 5 mM DTT, 50 μM ^{14}C-threonine (specific activity 20 $\mu C/\mu mole$), and 100 A_{260} units tRNA. One reaction mixture contained 10.3 μg enzyme protein (Fraction VI) (——), the other contained 20.6 μg (-----). At 26 min (arrow), each reaction was made 1.1 M in NH_2OH, pH 7.2, by the addition of 22 M NH_2OH, pH 7.2. ^{14}C-Thr-tRNA ($\circ$, $\bullet$) and ^{14}C-threonine hydroxamate ($\triangle$,$\blacktriangle$) were determined as described in Figure 14.
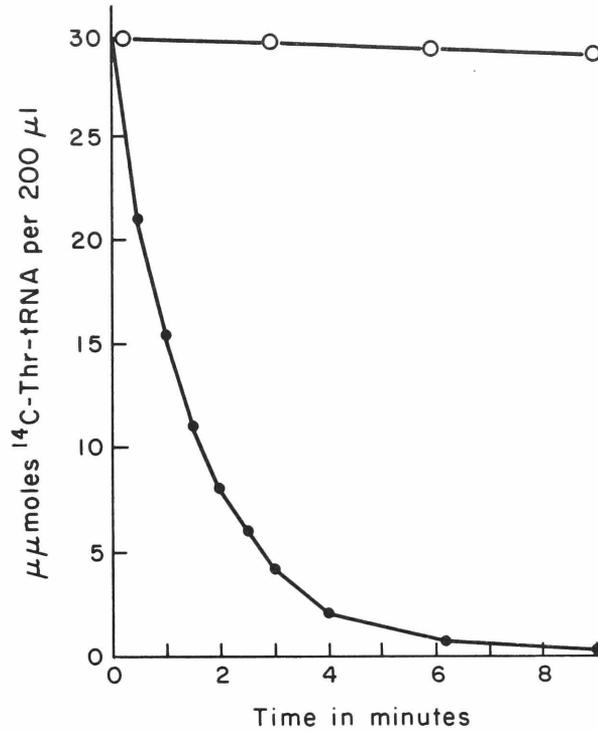
Figure 16. Reaction of ^{14}C-Thr-tRNA with hydroxylamine. Each 2 ml reaction mixture contained 50 mM Hepes, pH 7.2, 4 mM ATP, 10 mM MgCl$_2$, 5 mM DTT, 50 μM ^{14}C-threonine (specific activity 20 μC/μmole), and 100 units tRNA containing 297 $\mu\mu$moles ^{14}C-threonine (75,260 cpm) esterified to tRNA. The incubation was at 30° with 1.1 M NH$_2$OH, pH 7.2 (●) or without NH$_2$OH (○). 200 μl aliquots were removed at the designated times and ^{14}C-Thr-tRNA determined as described in the text.

phase of the overall reaction. As shown in Figures 17 and 18, Thr-tRNA synthetase catalyzes an ATP-PP$_i$ exchange reaction which is linear with respect to time and enzyme concentration. No tRNA is necessary for activation of threonine. The rate of the ATP-PP$_i$ exchange reaction is 12 times faster than the rate of the overall reaction.

Discussion

Purification of Thr-tRNA synthetase has been possible only by stabilizing the enzyme with glycerol and sulfhydryl compounds. The presence of glycerol was also necessary to stabilize Pro- and Gly-tRNA synthetases from E. coli (Muench and Berg, 1966). However, several E. coli aminoacyl-tRNA synthetases have been successfully purified without glycerol. The presence of glycerol in the assay of Ser-tRNA synthetase stimulated the enzyme activity. Although glycerol stabilized Thr-tRNA synthetase, it did not modify the velocity of the purified enzyme in the acylation reaction.

Baldwin and Berg (1966a) purified E. coli Ile-tRNA synthetase to homogeneity as determined by velocity and equilibrium sedimentation and analytical gel electrophoresis. This involved a 650 fold purification. E. coli Thr-tRNA synthetase was purified 320 fold. If Thr-tRNA synthetase represents an equivalent percentage of the E. coli proteins as Ile-tRNA synthetase does, then it is approximately 50% homogeneous. Preliminary studies of purified Thr-tRNA synthetase by analytical polyacrylamide gel electrophoresis in urea revealed a major protein band and three satellite bands, indicating that the purification has not reached the state of homogeneity. There existed only one peak of E. coli Thr-tRNA synthetase activity throughout the entire purification procedure, and it appears that there is only one Thr-tRNA synthetase in E. coli. Only one case of dual enzyme activities has been reported for an individual E. coli aminoacyl-tRNA synthetase (Yu and Rappaport, 1966). Two peaks of enzyme activity appeared during DEAE-cellulose chromatography in the purification of rat liver Thr-tRNA synthetase (Allende et al., 1966). It is unknown whether the two peaks represented two different enzymes, perhaps derived from subcellular fractions, or whether this was a protein aggregation phenomenon.

The molecular weight of E. coli Thr-tRNA synthetase is slightly
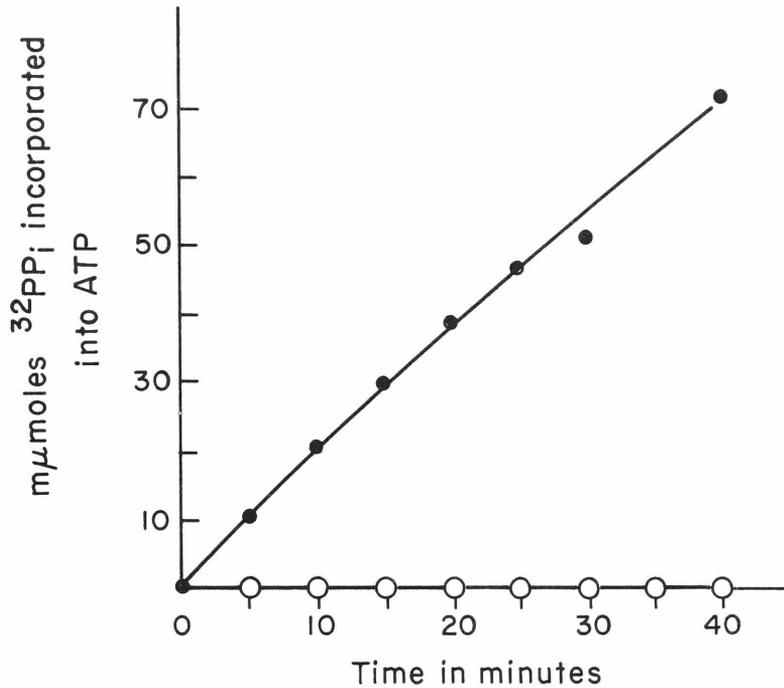
Figure 17. ATP-PP$_i$ exchange assay of purified Thr-tRNA synthetase with respect to time. Each point represents the product formation catalyzed by 1.38 μg enzyme protein (Fraction VI) either with threonine (●) or without threonine (○) added to the assay. This enzyme had a specific activity of 7670 units/mg protein in the standard enzyme assay which measured Thr-tRNA formation. ^{32}PP$_i$ specific activity, 105 cpm/mμmole. Experimental procedures are given in the text.
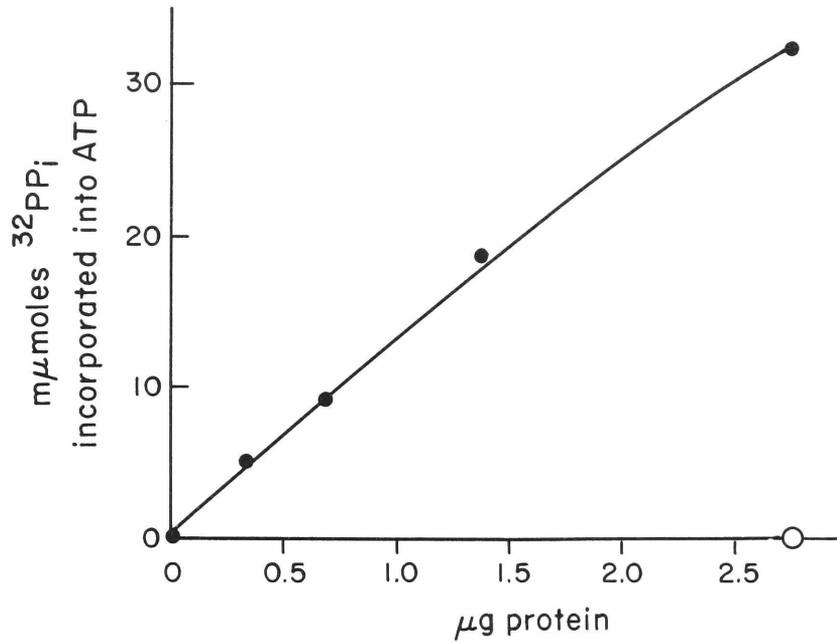
Figure 18. ATP-PP$_i$ exchange assay of purified Thr-tRNA synthetase with respect to the concentration of enzyme protein. Each point represents the product formation catalyzed by the enzyme either with threonine (●) or without threonine (○) added to the assay. The procedures are given in the text.

greater than that of E. coli Ile-tRNA synthetase but certainly falls into the general class of molecular weights reported for other aminoacyl-tRNA synthetases as being near 100,000 (Peterson, 1967). It is tempting to speculate that aminoacyl-tRNA synthetases may be composed of subunits in view of their large sizes. The stabilization of Thr-tRNA synthetase activity by glycerol may be related to stabilization of subunit association and not only maintenance of the proper conformation of a single polypeptide chain. This speculation can be answered only by future studies on the physical properties of aminoacyl-tRNA synthetases.

All E. coli aminoacyl-tRNA synthetases contain thiol groups (Stern et al., 1966). Therefore, the sensitivity to oxidation and stabilization by sulfhydryl compounds are not surprising. Thr-tRNA synthetase was irreversibly denatured in the absence of sulfhydryl compounds since incubation of the inactive enzyme with DTT failed to restore its activity.

Several arguments have been made for tRNA acting as an allosteric effector in the aminoacyl-tRNA synthetase reaction. Hele (Hele, 1964; Hele and Barth, 1966) reported that tRNA stimulated the ATP-PP$_i$ exchange reaction catalyzed by rat liver Ile-tRNA synthetase. Periodate oxidation of the tRNA did not alter its stimulatory activity. Prolonged storage of the enzyme or tRNA at -15° decreased the stimulatory effect of tRNA more rapidly than it decreased the basal activity of the ATP-PP$_i$ exchange activity measured in the absence of tRNA. Thirty percent of the tRNA could be digested with snake venom diesterase without affecting the stimulatory property. Hele suggested that the stimulatory effect was through an interaction of the tRNA with the enzyme at sites distinguishable from both the catalytic site of the enzyme and the acceptor terminus of the tRNA. This led to the suggestion that tRNA acted as an allosteric effector in addition to its amino acid accepting function.

In a similar vein, Loftfield and Eigner (1965) observed that either intact or periodate oxidized tRNA stimulated the hydroxamate activity of Ile-tRNA synthetase from E. coli. Transfer RNA increased the ability of the enzyme to discriminate between valine and isoleucine by changing the Michaelis constant and the maximal velocity. Ogata (1961) had reported earlier that

the ATP-PP$_i$ exchange reaction of Ile-tRNA synthetase from guinea pig liver was stimulated fourfold by tRNA.

The experiments on the Glu-, Gln-, and Arg-tRNA synthetases have focused attention on the participation of tRNA in the amino acid activation step. Ravel et al. (1965) reported that E. coli Gln-tRNA synthetase showed an absolute requirement for tRNA to catalyze an ATP-PP$_i$ exchange reaction. Gln-tRNA formed at one-tenth the rate that pyrophosphate was incorporated into ATP in the reverse reaction. This indirect result led them to propose that tRNA was acting as an allosteric effector. The Glu-tRNA synthetases from rat liver (Deutscher, 1967) and E. coli (Ravel et al., 1965) both showed thousandfold decreases in the Michaelis constants for glutamate when tRNA supplemented the ATP-PP$_i$ exchange reaction. Deutscher (1967) suggested that either tRNA acted as an allosteric effector or activation of glutamate occurred via a concerted reaction mechanism requiring the presence of tRNA.

Mitra and Mehler (1967) observed that Arg-tRNA synthetase from E. coli catalyzed an ATP-PP$_i$ exchange reaction only in the presence of tRNA. Furthermore, synthesis of ATP from the arginyl-AMP-enzyme complex and pyrophosphate, the reversal of the activation step, required tRNA.

Therefore, there is considerable evidence for tRNA having a second function in the aminoacyl-tRNA synthetase reaction as an effector molecule in addition to its role as an amino acid acceptor. McElroy et al. (1967) have recently reviewed the literature on several different activating enzymes, including aminoacyl-tRNA synthetases. They proposed a model of "homosterism" to explain these secondary functions of tRNA in the aminoacyl-tRNA synthetase reactions. Whereas allosteric effectors interact at some site distinct from the catalytic site, "homosteric" effectors are natural substrates, or very close resemblances, that combine at the catalytic site and modify the reaction of the bound activated intermediate. According to McElroy et al. (1967), tRNA could be such a "homosteric" molecule.

Thr-tRNA synthetase differs from all of these cases. Pyrophosphate can react at the catalytic site. Otherwise, no ATP-PP$_i$ exchange would be observed. This reaction is independent of tRNA. Transfer RNA can readily accept threonine from the catalytic site because the enzyme esterifies tRNA

in the overall reaction. However, it appears that hydroxylamine cannot react with enzyme bound threonyladenylate in the absence of tRNA.

In the presence of tRNA, threonine hydroxamate is continuously formed at a rate proportional to the enzyme concentration and independent of the tRNA concentration. Likewise, the formation of Thr-tRNA assumes a steady state level in the reaction which is proportional to enzyme concentration and independent of tRNA concentration. This can be expected because the enzyme is the limiting component in the reaction and the enzyme is saturated with respect to tRNA. In the presence of both tRNA and hydroxylamine, it therefore appears that a steady state reaction is achieved where threonine is activated, esterified to tRNA, and Thr-tRNA then reacts with hydroxylamine to form the stable threonine hydroxamate. The deacylated tRNA can again participate in the reaction sequence and, in this manner, a cyclic reaction of the tRNA occurs. However, it is not known whether tRNA somehow changes the conformation of the enzyme, thereby exposing threonyladenylate to direct attack by hydroxylamine, or whether Thr-tRNA accepts the activated amino acid and then hydroxylamine reacts with Thr-tRNA. The latter possibility is preferred since periodate oxidized tRNA cannot stimulate hydroxamate formation. Whereas periodate oxidized tRNA stimulates the Ile-tRNA synthetase to catalyze hydroxamate formation and the ATP-PP$_i$ exchange reaction (Loftfield and Eigner, 1965; Hele and Barth, 1966), it is ineffective in stimulating the arginine, glutamate or glutamine ATP-PP$_i$ exchange reactions (Ravel et al., 1965; Deutscher, 1967; Mitra and Mehler, 1967). It is also ineffective in stimulating the hydrolysis of valyladenylate bound to Ile-tRNA synthetase (Baldwin and Berg, 1966b). Therefore, periodate oxidized tRNA seems to have varying effects depending on which synthetase system is assayed. It is possible that cleavage of the cis diol bond of the terminal ribose by periodate oxidation may effect various species of tRNAs in different ways. Consequently, the failure of periodate oxidized tRNA to stimulate threonine hydroxamate formation can only be used as tentative evidence that hydroxylamine reacts with Thr-tRNA

Unfortunately, the equations for the steady state kinetics of the reaction cannot distinguish between hydroxylamine attack on the enzyme bound aminoacyladenylate and attack on the Thr-tRNA. However, the calcula-

tions do show that the rate of threonine hydroxamate formation and the steady state levels of Thr-tRNA are functions of only the enzyme concentration. Interestingly, the proportionating constant for the rate of hydroxamate formation is the rate constant for the first step of the reaction, the formation of the enzyme bound aminoacyladenylate.

Thus, only the natural substrates, pyrophosphate and tRNA, gain access to the activated threonine at the catalytic site of Thr-tRNA synthetase. Despite its marked nucleophilic character, hydroxylamine cannot react. This must be a property of the enzyme and not an unusual stability of threonyladenylate, because when threonine is activated by the Val-tRNA synthetase, threonine hydroxamate readily forms.

There still exists the possibility that the Thr-tRNA synthetase reaction involves intermediate steps which differ from the other synthetases, and that is why the intermediates are not susceptible to attack by hydroxylamine. In these experiments, inferences were made about the enzyme-substrate intermediate in the Thr-tRNA synthetase reaction by studying the catalytic properties of the enzyme. An important step in the elucidation of the properties of the enzyme-substrate intermediate of an enzymic reaction is to isolate the intermediate itself.

CHAPTER   FOUR

Isolation and Properties of Threonyladenylate-Enzyme Complex

The early experiments with the aminoacyl-tRNA synthetases clearly pointed to a tight binding of the aminoacyladenylate to the enzyme. It was necessary to use substrate levels of the enzymes to prove the existence of the aminoacyladenylates as intermediates in the synthetase reactions (Kingdon et al., 1958; Webster and Davie, 1959; Karasek et al., 1958).

More recently, three enzyme-bound aminoacyladenylates have been isolated. Baldwin and Berg (1966b) isolated the Ile-AMP-enzyme complex and studied it with special reference to the mistaken activation of valine by the Ile-tRNA synthetase, forming Val-AMP-enzyme complex. Lagervist et al., (1966) isolated the Val-AMP-enzyme complex using the yeast valine enzyme. Allende et al. (1966) used the purified Thr-tRNA synthetase from rat liver to study the Thr-AMP-enzyme complex. These results provide comparisons for the studies of the E. coli enzyme-substrate complex which are described in the following pages.

Isolation of the aminoacyladenylate-enzyme complex permits a closer study of the properties of the intermediate steps in the aminoacyl-tRNA synthetase reaction. In this regard, the mechanisms have been studied by which Thr-tRNA synthetase protects threonyladenylate against attack by hydroxylamine.

Materials and Methods

Materials   G-75 Sephadex (bead form) and G-50 Sephadex (coarse grade) were products of Pharmacia. Uniformly labeled ^{14}C-threonine (specific activity 163 μC/μmole or 167 μC/μmole) and generally labeled ^{3}H-threonine (specific activity 2.25 C/mmole) were purchased from New England Nuclear Corp. ^{3}H-ATP (specific activity 4.1 C/mM) was purchased from Schwarz Bioresearch. ATP-β, γ-^{32}P (specific activity 130 μC/μmole) was also a product of Schwarz. Before using it, it was necessary to repurify the compound since 10% of the total radioactivity existed as inorganic phosphate. The material was chromatographed on Whatman DE 81 paper with a solvent system of 0.25 M triethylammonium acetate, pH 3.56. In this system, inorganic phosphate moves with the solvent front and AMP, ADP, and ATP are well separated behind it. The ATP was eluted from the paper with 1 M triethylammonium acetate, pH 3.56, which was removed by lyophilization. The repurified ATP-β, γ-^{32}P had 0.2% ^{32}P$_i$ contamination as determined

by the method of Martin and Doty (1949). Radioactivity was determined in a
Nuclear-Chicago gas flow counter. NEM and PCMBS were products of Sigma Chem-
ical Co.

Formation of Thr-AMP-enz complex The standard reaction mixture
used for formation of the Thr-AMP-enz complex consisted of the following
ingredients in a final volume of 0.2 ml unless otherwise noted in individual
experiments: 25 mM Hepes, pH 7.5, 1 mM ATP, 4 mM $MgCl_2$, 10 mM DTT, 120 μM
^{14}C-threonine (specific activity 163 or 167 μC/μmole), 10 μg pyrophosphatase,
and from 340 to 1100 units of purified Thr-tRNA synthetase (Fraction VI).
After incubating for 12 min at 30°, the entire reaction mixture was applied
to a 0.9 x 20 cm G-75 Sephadex column equilibrated at 4° with 10 mM Hepes,
pH 7, 5 mM DTT, and 0.1 mM EDTA. In some experiments the reaction mixture
was applied to a 1 x 24 cm, G-50 Sephadex column equilibrated with 10 mM
Hepes, pH 7, 1 mM DTT, 0.1 mM EDTA. Fractions of 0.5 ml were collected. The
entire chromatographic procedure, from start until the enzyme emerged from the
column, required 25 min for the G-75 Sephadex column and 6 min for the G-50
Sephadex column.

Assays for Thr-AMP-enz complex Each fraction of the column eluate
was assayed for Thr-AMP-enz complex in two ways. First, 100 μl aliquots were
removed from each fraction and spotted on Whatman GF/C glass fiber paper discs
in scintillation vials. After the samples were dried for 10 min under a heat
lamp, 5 ml of toluene scintillation fluid were added. The radioactivity in
the samples was determined in a Nuclear-Chicago scintillation counter at about
78% efficiency for ^{14}C alone and at 40% for ^{14}C and 14% for ^{3}H when the two
isotopes were counted concurrently. This measures the total radioactive mater-
ial within each fraction.

The second assay that was used detected specifically the presence
of the Thr-AMP-enz complex by measuring the transfer of the amino acid from
the complex to tRNA. Aliquots of 100 μl were withdrawn from each fraction
and incubated for 10 min at 10° with 25 μl of a solution containing 0.25 μmoles
$MgCl_2$ and 5 A_{260} units of tRNA having a total threonine acceptor capacity of
310 $\mu\mu$moles. The reaction was stopped by adding 3 ml of 5% TCA and after
10 min at 0°, the precipitate was collected and washed on a Whatman GF/C

filter. The filter was dried and the radioactivity determined in the same scintillation system as above. This assay will be referred to as the standard transfer assay.

Thr-tRNA synthetase assay   Thr-tRNA synthetase was assayed by the standard enzyme assay which measured the overall reaction and was described in the preceding chapter.

Hydroxamate assays   The three assays for threonine hydroxamate formation described in the foregoing chapter were employed, namely, chromatography on SA 2 cation exchange paper, chromatography in the butanol, formic acid, water system, and electrophoresis at pH 7.

Results

Isolation of Thr-AMP-enz complex   When 680 units of purified Thr-tRNA synthetase were incubated with the substrates, ATP and ^{14}C-threonine, and the entire reaction mixture applied to a G-75 Sephadex column, some of the ^{14}C-threonine was excluded from the column in a well defined peak (Figure 19). The bulk of the ^{14}C-threonine was retained on the column. The excluded peak of ^{14}C-threonine coincided with the peak of enzyme activity as measured by the standard enzyme assay. When each fraction was incubated for 10 min at 10° with tRNA and magnesium, 85% of the excluded ^{14}C-threonine, but none of the retained ^{14}C-threonine, was TCA precipitable. However, if tRNA was omitted, ^{14}C-threonine was not TCA precipitable.

It therefore appeared that the excluded peak of ^{14}C-threonine represented the enzyme-substrate complex. Presenting the complex with the natural acceptor, tRNA, resulted in the attachment of the ^{14}C-threonine to tRNA. In Figure 20 are shown the kinetics of transfer of the amino acid from the complex to tRNA in the standard transfer assay. Within 2 min, the reaction reached a plateau in which 80% of the total ^{14}C-threonine was transferred to tRNA. Even by working rapidly and isolating the complex from a G-50 Sephadex column to minimize spontaneous hydrolysis, the transfer never exceeded 90% of the ^{14}C-threonine.

ATP, as well as ^{14}C-threonine, was part of the enzyme-substrate complex in addition to participating in the formation of the complex. As
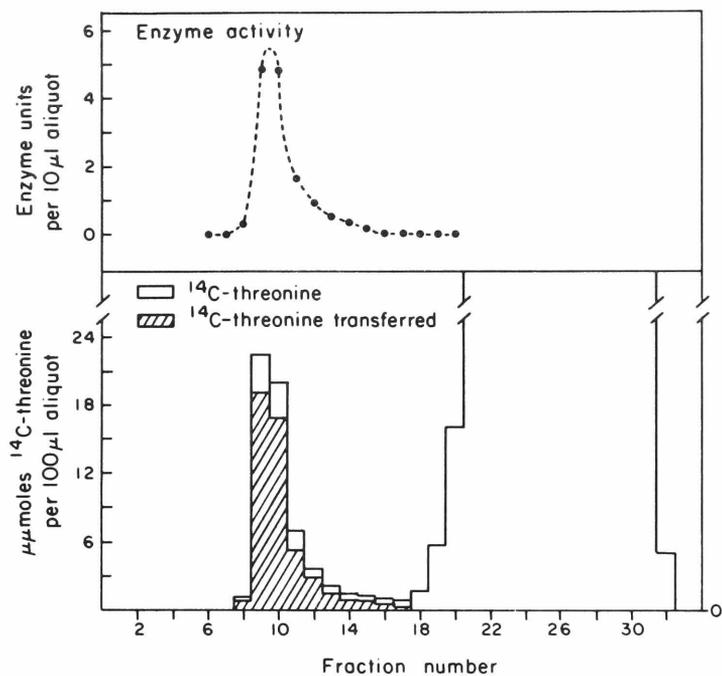
Figure 19. Isolation of ^{14}C-Thr-AMP-enz complex by G-75 Sephadex chroma-
tography. Each fraction of the eluate was assayed for the
presence of radioactivity (^{14}C-threonine) and for transfer of
radioactivity to tRNA (^{14}C-threonine transferred). The presence
of the enzyme was identified by the standard enzyme assay (Enzyme
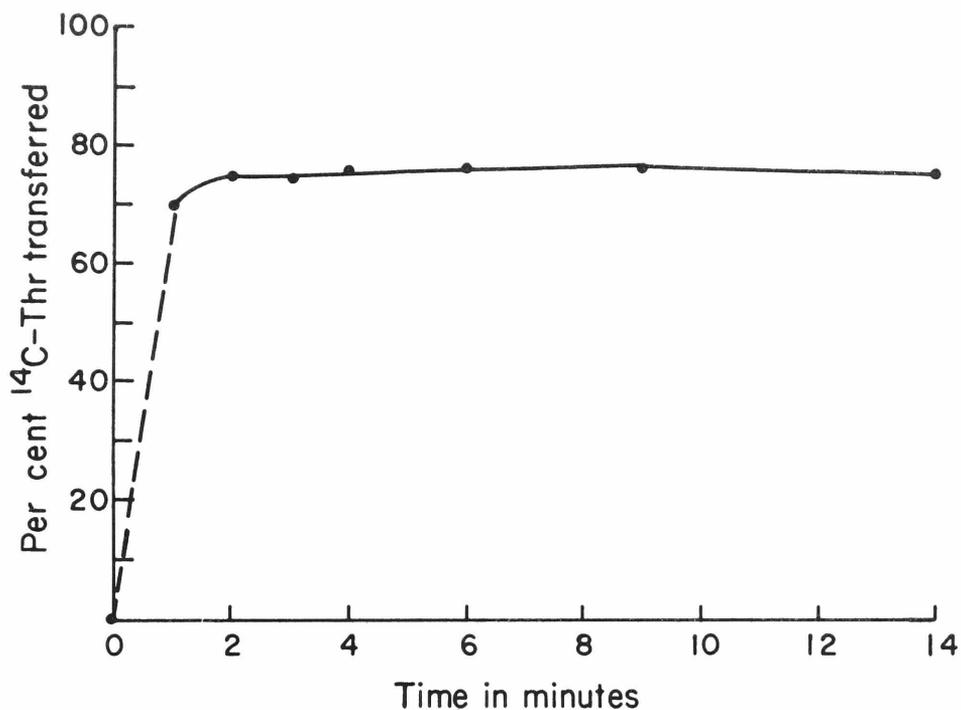activity). Experimental details are given in the text.

<u>Figure 20.</u> Kinetics of transfer of ^{14}C-threonine from ^{14}C-Thr-AMP-enz
complex to tRNA. The 0.375 ml reaction mixture contained 103
$\mu\mu$moles ^{14}C-Thr-AMP-enz complex (29,745 cpm ^{14}C-threonine),
1.5 μmoles MgCl$_2$, and 15 A$_{260}$ units of tRNA. After incubating
at 10° for the designated times, 50 μl aliquots were removed
and ^{14}C-Thr-tRNA determined as described in the text. The
zero time point was obtained by adding TCA to 1.5 $\mu\mu$mole of
the complex in 100 μl before adding the tRNA and MgCl$_2$.

shown in Table III, if both substrates were radioactive, ^{3}H-ATP was excluded from the column along with ^{14}C-threonine. One mole of ^{3}H-ATP was present for every mole of ^{14}C-threonine in the peak. However, when the enzyme-substrate complex was incubated with tRNA, 80% of the ^{14}C-threonine and none of the ^{3}H-ATP was converted to acid precipitable material. This is consistent with the view that the amino acid was transferred to the tRNA, but the ^{3}H-ATP, as expected, does not share this attachment. If ATP or enzyme was omitted, no complex formed.

A similar experiment was performed using a mixture of ^{3}H-ATP and ATP-β, γ-^{32}P to examine the phosphoryl participation in the reaction (Table IV). In the presence of threonine, AMP was the predominant nucleotide in the enzyme-substrate complex. However, 15% of the nucleotide which was excluded from the column existed as ATP which persisted even in the absence of the cosubstrate, threonine. This bound ATP cannot be attributed to contaminating threonine because (a) the enzyme fails to bind threonine in the absence of ATP, (b) the enzyme does not catalyze an ATP-PP$_i$ exchange reaction when threonine is omitted, and (c) ATP would be converted to AMP upon reaction with threonine. This bound ATP may be related to an ATPase activity in the enzyme preparation. Whether the ATPase activity is produced by Thr-tRNA synthetase itself or a separate contaminating protein cannot be resolved without further purification of the enzyme.

Stability of Thr-AMP-enz complex In Figure 21 is shown a semi-logarithmic plot of the percentage of the ^{14}C-threonine transferred to tRNA at various times after storage at 0° at pH 7 and at pH 6. The complex was considerably less stable at pH 6 than at pH 7. This is somewhat surprising in view of available information on the stability of free aminoacyladenylates (De Moss et al., 1956). However, the enzyme itself was less stable below than above neutrality, and this difference in stability of the complex probably reflects denaturation of the enzyme at the lower pH value.

The stability of the Thr-AMP-enz complex at three different temperatures is shown in Figure 22. The complex was destroyed within 30 sec when it was subjected to 100°. At 30°, the half life was 29 min. At 10°, half of the complex disappeared in 78 min. It is important to note that only 10% of

Table III

Stoichiometry of Substrates in Thr-AMP-enz Complex

Substrates	$\mu\mu$moles ^{14}C-Threonine		$\mu\mu$moles ^{3}H-ATP	
	Bound	Transferred	Bound	Transferred
Complete	44.2	35.3	42.5	< 0.05
- ATP	< 0.1	< 0.1	-	-
- Enzyme	< 0.1	< 0.1	< 0.1	< 0.1

The complex was formed as described in the text except 250 μM ^{3}H-ATP and 150 μM ^{14}C-threonine were used. The complex was isolated by G-75 Sephadex chromatography, and the radioactivity was determined in 100 μl aliquots from each fraction of the column eluate. Transfer of the radioactivity to tRNA was measured by the standard transfer assay. Where indicated, ATP was omitted or 100 μg of bovine serum albumin replaced Thr-tRNA synthetase.

Table IV

^{3}H-ATP-(β, γ)-^{32}P in Thr-AMP-enz Complex

	$\mu\mu$moles bound	
	- Threonine	+ Threonine
^{3}H	8.93	44.1
^{32}P	6.74	7.58

The reaction mixture for the formation of Thr-AMP-enz complex contained 75 μM ATP (8.88 x 10^6 cpm ^{3}H-ATP and 6.75 x 10^5 cpm ATP-β, γ-^{32}P) and 100 μM ^{12}C-threonine. Where indicated, threonine was omitted. Experimental details are contained in the text.
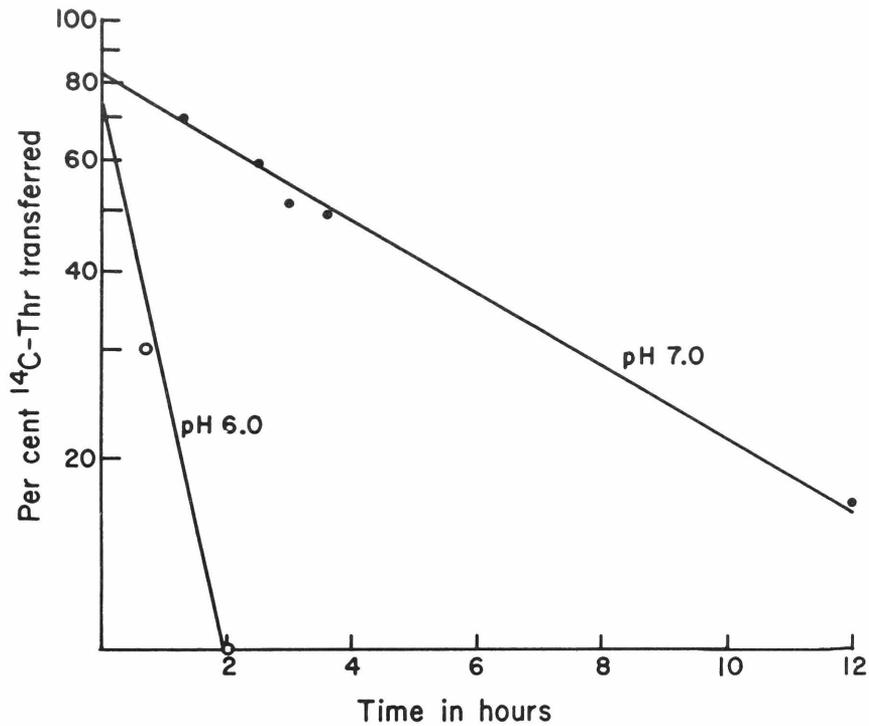
Figure 21. Stability of ^{14}C-Thr-AMP-enz complex at pH 6 and pH 7. Complex was formed and isolated as described in the text. (●) The complex was stored at 0°, and at the designated times the amount of ^{14}C-Thr-AMP-enz complex present was determined by the standard transfer assay. Each 100 μl aliquot contained a total of 7.8 $\mu\mu$moles ^{14}C-threonine (2250 cpm). (o) Same as above except the complex was isolated from a G-75 Sephadex column equilibrated with 50 mM potassium succinate, pH 6, 50 mM KCl, 5 mM DTT, and 0.1 mM EDTA. Each 100 μl aliquot contained 4.3 $\mu\mu$moles ^{14}C-threonine (1240 cpm).
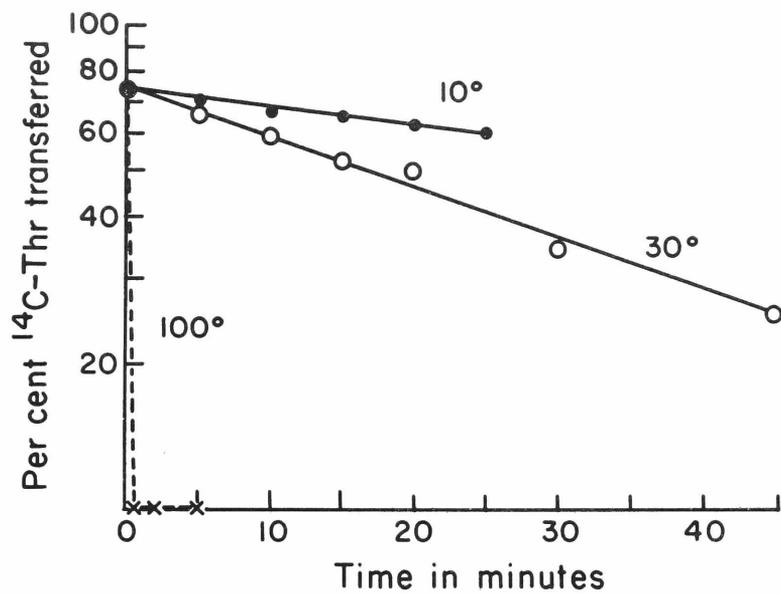
Figure 22. Stability of ^{14}C-Thr-AMP-enz complex with respect to temperature. The complex was isolated as described in the text and incubated at the designated temperature. The amount of complex present was determined by the standard transfer assay. Each 100 μl aliquot contained a total of 8.1 $\mu\mu$moles ^{14}C-threonine (2340 cpm).

the active complex hydrolyzed in 10 min at 10°, which were the incubation conditions used in the standard transfer assay and in some subsequent experiments. Incubating the complex with either Mg^{++} or tRNA alone did not alter the stability.

Metal requirements As shown in Table V, there was an absolute requirement for divalent metal ion in the formation of Thr-AMP-enz complex. When Ca^{++} replaced Mg^{++} in the standard reaction for complex formation, only 25% as much complex was recovered. It is unknown whether less complex was isolated in this case because the initial reaction was slower or because the complex was inherently less stable and decayed during its isolation. Reactions in which Ca^{++} can replace Mg^{++} usually show lower optimal concentrations for Ca^{++} than for Mg^{++}. The ^{14}C-threonine in the complex formed with either Ca^{++} or Mg^{++} could be transferred to tRNA in the standard transfer assay. The complexes did not transfer ^{14}C-threonine to tRNA if Mg^{++} was omitted from the transfer assay mixture. Therefore, a divalent metal ion was necessary both for formation of the complex and for esterification of the tRNA with threonine.

Figure 23 shows that the optimum Mg^{++} concentration for the transfer of threonine from Thr-AMP-enz complex to tRNA was 2 mM. Higher concentrations were only slightly inhibitory.

When other cations replaced Mg^{++} in the transfer assay, the results in column A of Table VI were obtained. Mn^{++} was as effective as Mg^{++}, and Ca^{++} was slightly less effective. Zn^{++} and Ni^{++} were very poor replacements for Mg^{++}. K^{+} and Na^{+} were completely ineffective. If instead of stopping the reactions after 10 min incubation, Mg^{++} was added to each assay and the incubation continued for another 10 min, then the results in column B of Table VI were observed. The complex survived the preincubation with tRNA in the absence of cation or in the presence of Na^{+} or K^{+} and efficiently transferred threonine as soon as Mg^{++} supplemented the reaction. By contrast, acylation could not be restored by adding Mg^{++} to the reactions in which Ni^{++} or Zn^{++} had been present with the complex and tRNA.

Effects of PCMBS and NEM Preincubation of the isolated Thr-AMP-enz complex with 4 mM PCMBS completely inhibited the transfer of the threonine from the complex to tRNA in the standard transfer assay. If 1.5 M hydroxyl-

Table-V

Thr-AMP-enz Complex Formation		Transfer Reaction	
Cation	Complexed ^{14}C-Thr	- Mg^{++}	+ Mg^{++}
	cpm	cpm	cpm
None	60	6	7
Mg^{++}	30,725	8	20,484
Ca^{++}	8,255	4	6,737

^{14}C-Thr-AMP-enz complex was formed as described in the text except 4 mM MgCl$_2$ was omitted or 4 mM CaCl$_2$ replaced MgCl$_2$ where designated. The complex was isolated by G-75 Sephadex chromatography. The standard transfer assay was used to determine the esterification of ^{14}C-threonine to tRNA except MgCl$_2$ was omitted from the transfer assay where indicated.
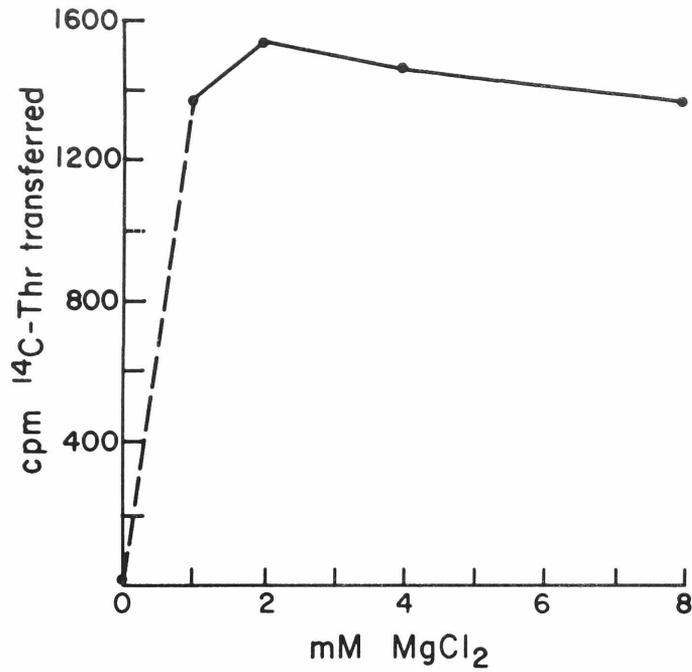
<u>Figure 23</u>. Transfer of ^{14}C-threonine from the complex to tRNA with respect to $MgCl_2$ concentration. The standard transfer assay was used except the $MgCl_2$ concentration was varied as indicated in the abscissa. Each assay contained ^{14}C-Thr-AMP-enz complex (6.6 $\mu\mu$moles ^{14}C-threonine, 1910 cpm).

Table VI

Effects of Cations on Transfer Reaction

Cation	^{14}C-Threonine transferred %	
	A	B
0	< 1	60
Mg^{++}	66	64
Mg^{++}(-tRNA)	< 1	-
Mn^{++}	66	63
Ca^{++}	62	62
Zn^{++}	7	7
Ni^{++}	2	8
K^{+}	< 1	63
Na^{+}	< 1	64

Each 0.25 ml reaction mixture contained ^{14}C-Thr-AMP-enz complex (14.2 $\mu\mu$moles ^{14}C-threonine, 4100 cpm), 2 mM metal chloride, and 10 A_{260} units of tRNA. Where indicated, metal or tRNA was omitted. After incubating 10 min at 10°, 100 μl were removed, mixed with 3 ml of 5% TCA, and the ^{14}C-Thr-tRNA determined (Column A). The remainder of the reaction mixture was made 2 mM in $MgCl_2$, and the incubation continued for 10 min at 10°. Three ml of 5% TCA were then added and the ^{14}C-Thr-tRNA determined (Column B).

amine, pH 7.5, instead of tRNA and $MgCl_2$, was added after the preincubation of the complex with PCMBS, threonine hydroxamate could be recovered as a reaction product in about 50% yield.

In contrast with the inhibitory effects of PCMBS, NEM, up to 40 mM concentrations, had no effect on either the formation of Thr-AMP-enz complex or the transfer of threonine from Thr-AMP-enz complex to tRNA.

Reaction of Thr-AMP-enz complex with hydroxylamine ^{14}C-Thr-AMP-enz complex was formed using the standard reaction and isolated from a G-75 Sephadex column. It was incubated for 10 min at 10° either in the presence or the absence of hydroxylamine and immediately thereafter, rechromatographed on a second G-75 Sephadex column. In the absence of hydroxylamine, 59% of the total ^{14}C-threonine that was applied to the second column could be recovered as active complex when measured by the standard transfer assay (Table VII). If, however, the isolated complex was incubated in 1.5 M hydroxylamine, pH 7.5, all of the ^{14}C-threonine was retained on the second Sephadex column, and none of it could be recovered as active Thr-AMP-enz complex. However, all of the enzyme activity, as measured by the standard enzyme assay, was recovered in the excluded volume of the column, indicating that the enzyme remained fully active. From the control experiment, it was expected that the disintegration of the complex in the presence of hydroxylamine would convert more than half of the threonine to threonine hydroxamate. However, when the radioactive material retained on the column was analyzed by paper chromatography and electrophoresis, all of it proved to be ^{14}C-threonine instead of ^{14}C-threonine hydroxamate (Figure 24).

These same results were obtained if Thr-AMP-enz complex was incubated for 20 min at 30° in 5 M neutral hydroxylamine, or if each of the metal ions shown in Table VI was present. Chemically synthesized ^{14}C-threonine hydroxamate was stable throughout incubation, lyophilization, and chromatography. Therefore, the failure to observe threonine hydroxamate as a product cannot be attributed to its formation and subsequent hydrolysis during the reaction or subsequent analyses.

Discussion

Despite the fact that Thr-tRNA synthetase does not catalyze the

<u>Table VII</u>

Reaction of Thr-AMP- enz Complex with Hydroxylamine

Preincubation	Total ^{14}C-Threonine $\mu\mu$moles	Recovered as complex %	^{14}C-Thr-NHOH %
- NH$_2$OH	76.1	59	-
+ NH$_2$OH	88.6	< 0.1	< 1

The details of the experiments are presented in the text. ^{14}C-threonine hydroxamate was determined as illustrated in Figure 24.
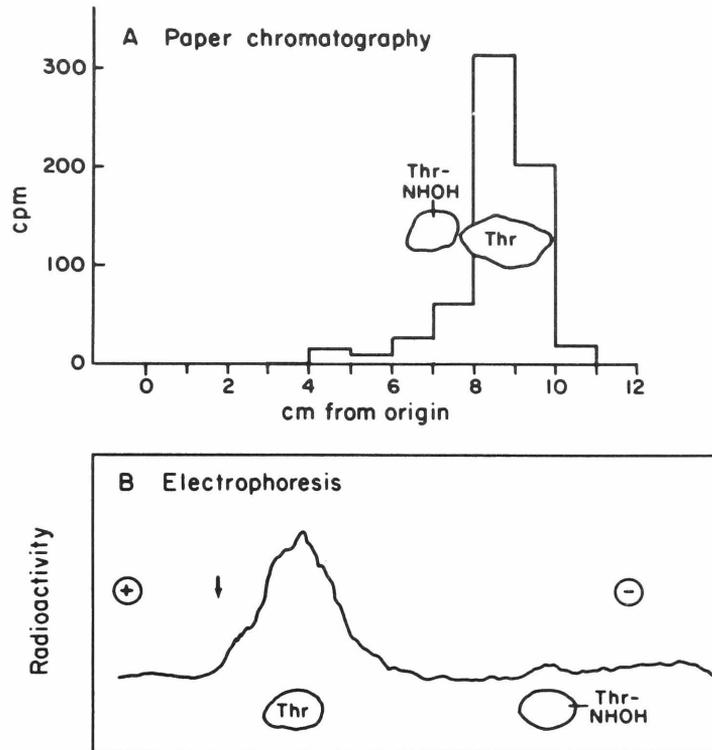
Figure 24. Identification of the product of the reaction of ^{14}C-Thr-AMP-enz complex with NH$_2$OH. The details of the experiment are described in the text. The arrows designate the origins. ^{12}C-threonine and ^{12}C-threonine hydroxamate markers were run concurrently, located by staining reactions, and their respective positions are indicated.

formation of threonine hydroxamate, the isolation of the Thr-AMP-enz complex indicates that Thr-tRNA synthetase activates threonine via the intermediate steps described in Chapter I for the aminoacyl-tRNA synthetase reaction. However, before commenting further on the studies of the hydroxamate reaction, the properties of the Thr-AMP-enz complex will be discussed, particularly with reference to the reports of other isolated aminoacyl-AMP-enz intermediates.

When Thr-AMP-enz complex is incubated with tRNA and $MgCl_2$, 90% of the threonine transfers to tRNA. The amount of threonine transferred to tRNA never reached 100%. This same incomplete transfer was observed in the studies with three other aminoacyl-AMP-enz complexes (Allende et al., 1966; Norris and Berg, 1964; Lagervist et al., 1966). The simplest explanation would be that 10% of the complex hydrolyzes before the transfer assay can be accomplished. However, Baldwin and Berg (1966) showed that in the case of Ile-AMP-enz complex, most of the enzyme bound isoleucine which does not transfer to tRNA remains in an activated form. Furthermore, increasing the amount of tRNA does not decrease this residual level of activated isoleucine.

As in all phosphate transfer reactions (Nordlie and Lardy, 1962), divalent metal ion is required for formation of the aminoacyl-AMP-enz complexes. It is an open question whether the Mg^{++} required for Thr-AMP-enz complex formation acts catalytically or whether it remains bound to the complex in a form which is inactive for amino acid transfer to tRNA. The probable role of Mg^{++} is to draw the electrons in the pyrophosphoryl group of ATP away from the α-phosphorus atom. In this way, it would facilitate the nucleophilic attack by the carboxyl oxygen of the amino acid onto the α-phosphorous atom. Subsequently, cleavage of the phospho-anhydride bond would occur and the new mixed anhydride bond with the amino acid would form. Mg^{++}-pyrophosphate would be eliminated from the catalytic site.

The various aminoacyl-AMP-enz complexes differ in their requirements for additional Mg^{++} in the transfer reactions. Ile-AMP-enz complex from E. coli (Norris and Berg, 1964) and Val-AMP-enz complex from yeast (Lagervist et al., 1966) do not require added Mg^{++} for the transfer reaction. Yet, Thr-AMP-enz complexes from rat liver (Allende et al., 1965) and E. coli, as shown here, both require supplementary Mg^{++} for the transfer reactions. If

those cases where no Mg^{++} is required are not due to contaminating Mg^{++} being inadvertantly present, then the Mg^{++} requirement in the transfer reactions of the two Thr-AMP-enz complexes implies a greater similarity between synthetase reactions of the same amino acids than between reactions of different amino acids in similar organisms.

Supplementary Mg^{++} is probably necessary to bind the tRNA to the complex or to maintain the proper tRNA conformation. It seems unlikely that it affects the Thr-AMP-enz complex directly, since the stability of the complex to spontaneous hydrolysis is unaffected by the presence of Mg^{++}. In this regard, the E. coli Thr-AMP-enz complex is similar to Thr-AMP-enz complex from rat liver (Allende et al., 1965). However, it differs from the yeast Val-AMP-enz complex which hydrolyzes rapidly in the presence of Mg^{++} (Lagervist et al., 1966). It is also interesting to note that Ile-AMP-enz complex does not require Mg^{++} for the transfer of isoleucine to tRNA, yet Mg^{++} is required to isolate the Ile-tRNA-enzyme complex (Norris and Berg, 1964; Yarus and Berg, 1967). The studies by Fresco et al. (1966) indicate that Mg^{++} stabilizes the biologically functional conformation of tRNA. Also, the presence of divalent metal ions affects the hyperchromicity of tRNA observed during its thermal denaturation (Mahler et al., 1963). Although the transfer reactions which require Mg^{++} are performed at 10°, the optical measurements of tRNA are affected by metal ions only above 15°. This demonstrates that the effects of the metal are subtler than physical methods can detect, and that the acylation reaction is a more sensitive assay of the state of the tRNA than physical measurements.

It is noteworthy to recall that maximal enzyme activity occurs at 10 mM $MgCl_2$ in the overall Thr-tRNA synthetase reaction. However, the maximal activity for the second half of the reaction, when the activated threonine is transferred to tRNA, occurs at 2 mM $MgCl_2$. This reveals a difference in the physical requirements of the chemical reactions composing the two separate halves of the enzymatic reaction. This same difference in $MgCl_2$ optima was noted by Allende et al. (1965) using the rat liver Thr-tRNA synthetase. This again reflects the general similarities of the particular properties of aminoacyl-tRNA synthetases from different organisms.

The E. coli Thr-AMP-enz complex is identical to the rat liver complex in its spectrum of transfer activity with different cations (Allende et al., 1965). It is now apparent that these cations have different effects on the complex. The presence of Ni^{++} or Zn^{++} renders the complex inactive by either irreversibly confining the reaction components to an inactive state or promoting hydrolysis. It is possible that Ni^{++} and Zn^{++} exert these effects by reacting with thiol groups on the enzyme. However, other cations either promote transfer of the amino acid to tRNA or are inert.

All aminoacyl-tRNA synthetases except the lysine enzyme are inhibited by PCMBS when assayed by the ATP-PP$_i$ exchange reaction (Stern et al., 1966). Therefore, an intact sulfhydryl group is necessary for these enzymes to catalyze amino acid activation. Since formation of the Thr-AMP-enz complex is the activation step of the Thr-tRNA synthetase reaction, it is to be expected that PCMBS should inhibit formation of the Thr-AMP-enz complex. Davie et al. (1965) showed several years ago that activation of tryptophan by the Try-tRNA synthetase from beef pancreas is completely inhibited by PCMB. More recently, De Luca and McElroy (1965) demonstrated that there are eight thiol groups per mole of Try-tRNA synthetase. However, only four of the thiol groups are available to the inhibitor in the presence of tryptophanyladenylate. Enzyme which is exposed to the inhibitor in the presence of tryptophanyladenylate loses only 30% of the enzyme activity for activating tryptophan.

Incubating the E. coli Thr-AMP-enz complex with PCMBS completely inhibits the transfer of the amino acid to tRNA. Furthermore, threonine hydroxamate can be recovered after incubation of the PCMBS treated complex with hydroxylamine. Under no other circumstances can threonine hydroxamate be recovered from the complex. Therefore, it appears that PCMBS reacts with a thiol group (or groups) on the enzyme that is essential not only for acylation of tRNA but also for maintenance of the structure of the intact complex. It is plausible to argue that the PCMBS either opens the enzyme or dissociates it into subunits, thereby freeing the bound threonyladenylate. Given the large molecular weight of the enzyme and the behavior of the complex in PCMBS, speculation again points toward the possibility of protein subunits. Only further studies can reveal whether this speculation is correct. It would also appear that this thiol group (or groups) has a distinctive reactiv-

ity, perhaps due to its location in the enzyme, since NEM inhibits neither formation of Thr-AMP-enz complex nor transfer of the amino acid to tRNA (Cecil, 1963).

In Chapter III, the distinguishing feature of Thr-tRNA synthetase was that it failed to catalyze threonine hydroxamate formation in the absence of tRNA although it catalyzed a threonine dependent ATP-PP$_i$ exchange reaction. In those experiments, limiting amounts of enzyme were added to excess substrate to determine the rate. Any product formation was dependent upon the enzyme going through many reaction cycles. However, with the isolation of the Thr-AMP-enz complex, the effects of hydroxylamine on this performed reaction intermediate could be scrutinized.

The results of the studies on the behavior of the Thr-AMP-enz complex in hydroxylamine corroborate the results in the preceding chapter. In hydroxylamine, the enzyme-substrate complex disintegrates. The enzyme remains active as defined by the assay for the overall reaction. However, threonine hydroxamate is not a reaction product. Instead, threonine is the product, indicating that hydrolysis takes precedence over hydroxaminolysis. Therefore, in the presence of hydroxylamine, the enzyme is converted from a synthetase to a hydrolase. Thus, the catalytic site of the Thr-AMP-enz complex must be selectively permeable to water. Or, as shown in the preceding chapter, the activated amino acid can be attacked by the terminal hydroxyl group of the tRNA.

There is no feature of the Thr-AMP-enz complex which would indicate that it should behave exceptionally in the presence of hydroxylamine. Much of the foregoing evidence, in fact, indicates that the enzyme activates threonine by the mechanism established for other aminoacyl-tRNA synthetases. Its sensitivity to thiol inhibitors, its stability properties, and its intermediate mechanisms give no hint of the hydrolase activity which the enzyme displays in hydroxylamine.

CHAPTER FIVE

Phenylalanyl-tRNA Synthetase and Comparison with Threonyl-tRNA Synthetase

In Chapter II it was pointed out that the aromatic aminoacyl-tRNA synthetases catalyze amino acid hydroxamate formation at rates comparable to the rates of the overall reactions in which the amino acids are esterified to tRNA. This is in direct contrast to the Thr-tRNA synthetase which does not catalyze formation of threonine hydroxamate, but instead, as shown with the isolated reaction intermediate, it is converted from a synthetase to a hydrolase in the presence of hydroxylamine. Therefore, it is to be expected that Phe-tRNA synthetase would maintain its synthetase activity and display little or no hydrolase activity, and consequently, the Phe-AMP-enz complex should react readily with hydroxylamine to form phenylalanine hydroxamate. This proposition is tested in this chapter. In addition, some of the properties of Phe-tRNA synthetase are examined to provide a comparison with Thr-tRNA synthetase.

Materials and Methods

Materials Ecteola-cellulose was obtained from Biorad Laboratories. Uniformly labeled ^{14}C-phenylalanine was purchased from Schwarz Bioresearch and New England Nuclear Corp.

Enzymes Partially purified Phe-tRNA synthetase from E. coli was prepared according to the procedure of McCorquodale (1964). The enzyme was purified 15 fold and was free of any other aminoacyl-tRNA synthetase activities. It contained no RNA as determined by the ratio of absorbancy at 280 mμ and 260 mμ (Warburg and Christian, 1941). Highly purified Phe-tRNA synthetase was a gift from Dr. Julian Gordon. A description of the purification procedure is in preparation for publication.

Enzyme assays The assay for the overall Phe-tRNA synthetase reaction measured the formation of Phe-tRNA. An 0.2 ml reaction mixture contained 100 mM Tris, pH 7.5, 16 mM MgCl$_2$, 16 mM DTT, 4 mM ATP, 5 mM NH$_4$Cl, 2 μM ^{14}C-phenylalanine (specific activity 100 μC/μmole), 6 A$_{260}$ units of tRNA, and enzyme. Incubations were at 30° for 3 min. The reactions were stopped by adding 5 ml of 5% TCA and the Phe-tRNA measured as described in Chapter III for Thr-tRNA except radioactivity was determined in a Nuclear-Chicago gas flow counter at 20% efficiency.

Phenylalanine hydroxamate formation was assayed using the same re-
action mixture as used in the assay of the overall reaction except that 1.8
M NH$_2$OH, pH 7.5, replaced tRNA. Incubations were at 30°. At time intervals,
20 μl aliquots were removed and assayed according to the method of Loftfield
and Eigner (1963) except that the SA 2 cation exchange papers were chromato-
graphed twice instead of once in the pH 7 sodium phosphate buffer. This im-
proved the separation of free phenylalanine from phenylalanine hydroxamate.

A standard ^{14}C-phenylalanine hydroxamate was prepared by the same
synthetic methods as described in Chapter III for ^{14}C-threonine hydroxamate.
The efficiency of scintillation counting for ^{14}C-phenylalanine hydroxamate
on the SA 2 paper was 30%.

Phe-AMP-enz complex formation and assays The Phe-AMP-enz complex
was formed and isolated using the same conditions described in Chapter IV for
the Thr-AMP-enz complex. However, G-50 Sephadex was routinely used instead
of G-75. Uniformly labeled ^{14}C-phenylalanine (specific activity 355 μC/μmole)
at a concentration of 27 μM and 100 units of purified Phe-tRNA synthetase were
used. Transfer of phenylalanine from the complex to tRNA was assayed by the
standard transfer assay described in the preceding chapter. The efficiency
of the scintillation counting for ^{14}C-phenylalanine was 70%.

Results

Phenylalanine hydroxamate and Phe-tRNA formation As shown in Fig-
ure 25, Phe-tRNA synthetase catalyzed the formation of phenylalanine hydroxa-
mate at an initial velocity within 20% of the initial rate of Phe-tRNA forma-
tion. Preincubating the Phe-tRNA synthetase with ribonuclease A did not change
the rate of phenylalanine hydroxamate formation, thereby eliminating the possi-
bility of tRNA participating in the reaction.

Effects of PCMBS Both the overall reaction, forming Phe-tRNA from
phenylalanine and tRNA, and the first half of the overall reaction, as meas-
ured by phenylalanine hydroxamate formation, were completely inhibited by
preincubating the enzyme with 4 μM PCMBS. Therefore, Phe-tRNA synthetase
shares the PCMBS sensitivity with the Thr-tRNA synthetase.

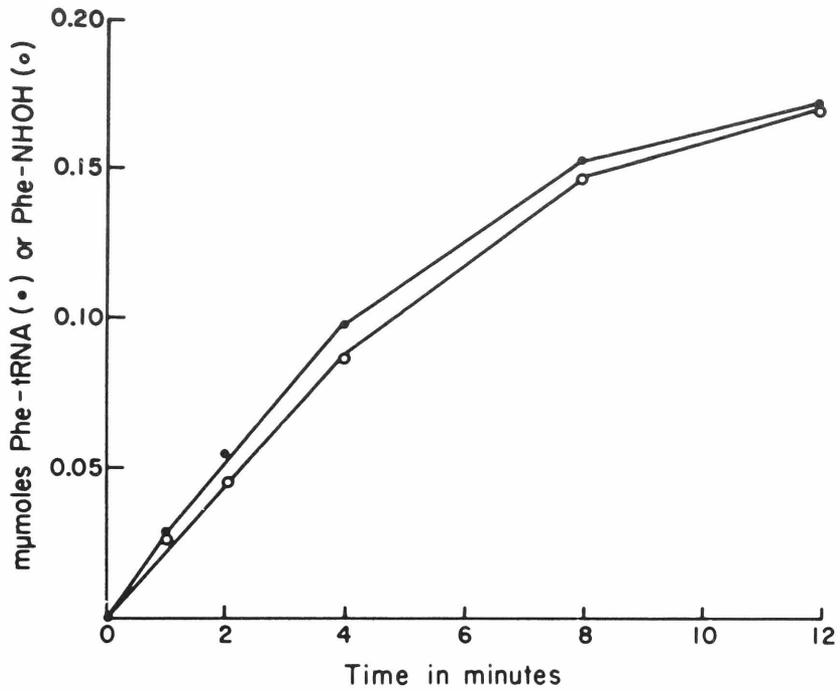Phe-AMP-enz complex The isolation of the Phe-AMP-enz complex by

<u>Figure 25.</u> Assays of <u>E</u>. <u>coli</u> Phe-tRNA synthetase for Phe-tRNA or phenylalanine
hydroxamate formation with respect to time. Each point represents
the product formation catalyzed by 12.3 μg enzyme protein. The
experimental procedures are given in the text.

gel filtration is illustrated in Figure 26. The Phe-AMP-enz complex trans-
ferred a maximum of 90% of the ^{14}C-phenylalanine to tRNA in the standard
transfer assay. This transfer reaction showed a requirement for Mg^{++}. There-
fore, in both the extent of amino acid transfer and the Mg^{++} requirement for
the transfer reaction, the Phe-AMP-enz and the Thr-AMP-enz complexes are sim-
ilar.

The stability of the Phe-AMP-enz complex was determined (Figure 27).
At 30°, pH 7, it had a half life of 7 minutes. Under the same conditions, it
therefore hydrolyzes about four times faster than the Thr-AMP-enz complex.

The behavior of the Phe-AMP-enz complex in hydroxylamine was examined
(Table VIII). As a control experiment, the isolated complex was incubated
for 10 min at 10° at pH 7.5 without hydroxylamine and rechromatographed on
Sephadex G-50. Fifty-five percent of the total ^{14}C-phenylalanine that was
applied to the column could be recovered in active complex that was transferable
to tRNA. However, when Phe-AMP-enz complex was incubated with 1.5 M hydroxyl-
amine, pH 7.5, none of the ^{14}C-phenylalanine was recovered in active complex.
Instead, when a sample was analyzed by chromatography (Figure 28), 51% of the
total ^{14}C-phenylalanine had been converted to ^{14}C-phenylalanine hydroxamate.
Therefore, most of the available phenylalanine in active Phe-AMP-enz complex
was converted to phenylalanine hydroxamate. This is in striking contrast to
the behavior of Thr-AMP-enz complex in hydroxylamine which produced threonine
instead of threonine hydroxamate.

Discussion

The Phe- and Thr-tRNA synthetases from E. coli appear to be similar
in their mechanisms of amino acid activation, sensitivity to thiol reagents,
and transfer of the activated amino acids to tRNA. However, the stabilities
of the two aminoacyladenylate-enzyme complexes are significantly different.
The Phe-AMP-enz complex is fourfold less stable to spontaneous hydrolysis
than the Thr-AMP-enz complex. Evidently, water gains entry to the Phe-AMP-
enz complex more readily than it does to the Thr-AMP-enz complex. Both the
Phe-AMP-enz complex and the Thr-AMP-enz complex rapidly disintegrate when
incubated with hydroxylamine. However, the reactions differ since hydroxyl-
amine reacts rapidly with Phe-AMP-enz complex to form phenylalanine hydroxa-
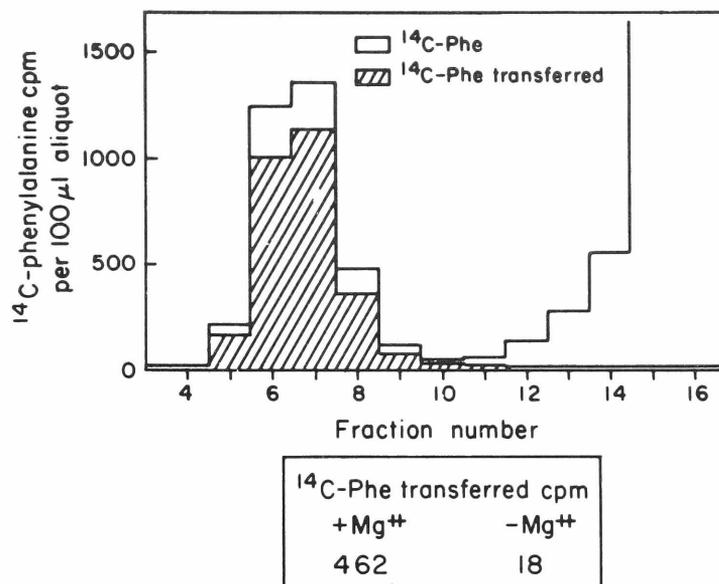
Figure 26. Isolation of ^{14}C-Phe-AMP-enz complex by G-50 Sephadex chromato-
graphy and dependency of the transfer reaction on the presence
of Mg^{++}. Experimental details are described in the text.
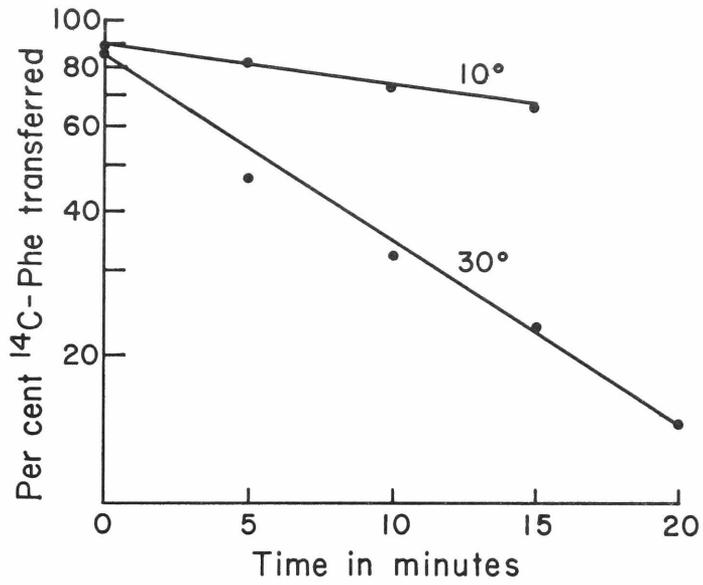
<u>Figure 27</u>. Stability of ^{14}C-Phe-AMP-enz complex with respect to temperature. For each curve, isolated complex, containing a total of 5.67 $\mu\mu$moles ^{14}C-phenylalanine (3150 cpm), was incubated in 0.6 ml from which 100 μl aliquots were removed at the designated times and transferred to the standard transfer assay.

<u>Table VIII</u>

Reaction of Phe-AMP- enz Complex with Hydroxylamine

Preincubation	Total ^{14}C-Phe $\mu\mu$moles	Recovered as complex %	^{14}C-Phe-NHOH %
- NH$_2$OH	5.5	55	-
+ NH$_2$OH	5.5	< 0.1	51

The details of the experiments are given in the text. ^{14}C-phenylalanine hydroxamate and ^{14}C-phenylalanine were separated and determined as illustrated in Figure 28.

<u>Figure 28.</u>   Analysis of the products of the reaction of ^{14}C-Phe-AMP-enz
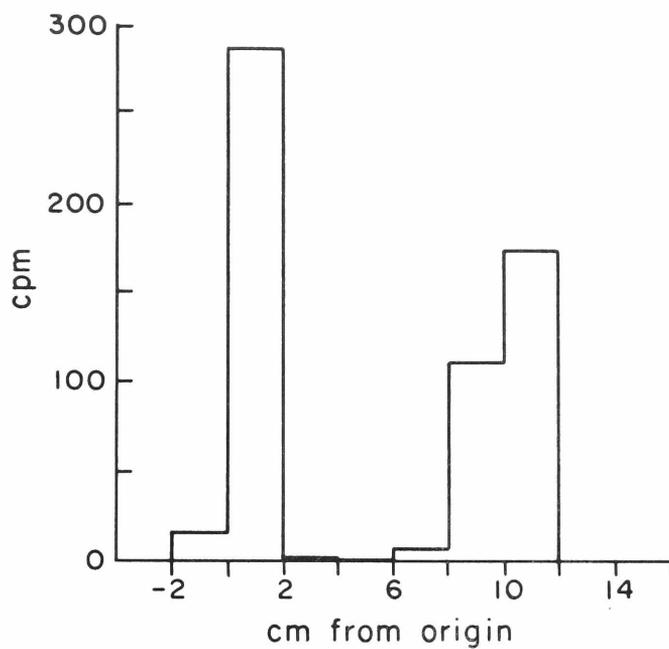complex with NH_2OH.   Chromatography was done on SA 2 cation
exchange paper (5 x 30 cm) as described in the text. ^{14}C-
phenylalanine hydroxamate remains at the origin, and ^{14}C-
phenylalanine moves with the solvent front.

mate as if there were easy access to the activated carbonyl group. By contrast, hydroxylamine cannot trap the activated threonine bound to Thr-tRNA synthetase because the hydroxylamine induces the enzyme to catalyze hydrolysis of threonyladenylate.

It is therefore apparent that the aminoacyladenylate within the Phe-AMP-enz complex is more exposed to the environment than is the aminoacyladenylate within the Thr-AMP-enz complex. Furthermore, Phe-tRNA synthetase lacks the protective devices that the Thr-tRNA synthetase employs to guard the activated amino acid. The Phe-AMP-enz complex displays minimal hydrolytic activity in hydroxylamine and presents a less formidable barrier to nucleophilic attack on the activated amino acid.

CHAPTER   SIX

Conclusion

Historically, the first method of assaying enzymatic activation of acyl groups was the hydroxamate assay. However, far higher concentrations of hydroxylamine were necessary to trap the acyladenylates formed in the enzymatic reactions than were required to form hydroxamates from free acyladenylates. It therefore appeared that acyladenylates formed in the enzymatic reactions were protected by the synthetases against attack by hydroxylamine.

The relative rates of amino acid hydroxamate formation catalyzed by the E. coli aminoacyl-tRNA synthetases vary over a sixtyfold range. From a practical point of view, this indicates that the hydroxamate assay alone may be an unreliable assay to search and quantitate for aminoacyl-tRNA synthetases but must be complemented by the aminoacyl-tRNA assay or the ATP-PP$_i$ exchange reaction. Even the latter method, like the hydroxamate assay, suffers from the problems of cross activation. The variation in the relative rates of amino acid hydroxamate formation catalyzed by aminoacyl-tRNA synthetases would imply that there is a great diversity in the degree of enzyme protection of the activated amino acids against attack by hydroxylamine. This diversity appears to be independent of the sources of the enzymes indicating that the aminoacyl-tRNA synthetases from higher organisms as well as bacteria have similar properties with respect to protecting their aminoacyladenylates.

The most extreme example in this regard is the threonyl-tRNA synthetase, which readily activates threonine and esterifies threonine to tRNA. But, this enzyme fails to catalyze any threonine hydroxamate formation. It has been demonstrated that this feature of the enzyme is not solely due to an exclusive protection of threonyladenylate against penetration by hydroxylamine but also due to the hydrolytic activity of the threonyladenylate-enzyme complex in the presence of hydroxylamine. Whereas the activated threonine within the valyl-tRNA synthetase can be trapped as threonine hydroxamate, the threonyladenylate bound to the threonyl-tRNA synthetase is hydrolyzed in the presence of hydroxylamine.

By contrast, the phenylalanyl-tRNA synthetase catalyzes formation of phenylalanine hydroxamate rapidly. In accordance with this observation, essentially all of the phenylalanine in the phenylalanyladenylate-enzyme complex can be trapped as phenylalanine hydroxamate. In addition, the phenylalanyl-

adenylate-enzyme complex is fourfold more susceptible to spontaneous hydrolysis in the absence of hydroxylamine than is the threonyladenylate-enzyme complex. This would imply that the phenylalanyladenylate is more exposed to the environment. Thus, it appears that either water or hydroxylamine gains access to the activated phenylalanine. Hydroxylamine, being a far better nucleophile than water, results in recovery of the activated phenylalanine as the hydroxamate.

Threonyladenylate-enzyme complex, on the basis of stability to spontaneous hydrolysis, is more resistant to attack by water, but in the presence of hydroxylamine, the threonyladenylate-enzyme complex is exclusively permeable to water. This is due to conversion of the enzyme from a synthetase to a hydrolase.

The conversion of synthetases to hydrolases is not unprecedented. Isoleucyl-tRNA synthetase activates valine to form valyladenylate bound to the isoleucine enzyme, but this complex hydrolyzes in the presence of tRNA rather than esterify the incorrect amino acid to tRNA (Baldwin and Berg, 1966b). The activation of luciferin with ATP is via luciferyladenylate bound to luciferase. In the presence of excess ATP, the luciferyladenylate-enzyme undergoes a reaction with water and thereby exhibits a hydrolase activity (McElroy et al., 1967).

Two speculations follow from these results. One is that the differential catalytic activities of the various aminoacyl-tRNA synthetases in forming amino acid hydroxamates is a function of both the accessibility of the aminoacyladenylates to attack from the environment and also is a function of the induced hydrolytic activities of these enzymes. It remains unknown what proportion of the protection can be attributed to the sequestering of the intermediate and what proportion is due to the hydrolase activity of each enzyme.

The other speculation concerns the biological significance of such a mechanism. One could imagine that in the intracellular milieu the very labile energetic aminoacyladenylates must be protected against attack by all nucleophilic molecules other than the natural acceptor, which in this case is tRNA. Therefore, the attempted entry of any other nucleophile would lead

to hydrolysis of the enzyme substrate intermediate rather than false transfer.

BIBLIOGRAPHY

Allende, J. E., Allende, C. C., Gatica, M., and Matamala, M. (1964). Isolation of threonyladenylate-enzyme complex. Biochem. Biophys. Res. Commun., 16, 342-6.

Allende, J. E., Mora, G., Gatica, M., and Allende, C. C. (1965). Metal ion requirement in the formation of threonyl-sRNA. J. Biol. Chem., 240, PC 3229-32.

Allende, C. C., Allende, J. E., Gatica, M., Celis, J., Mora, G., and Matamala, M. (1966). The aminoacyl ribonucleic acid synthetases. I. Properties of the threonyladenylate-enzyme complex. J. Biol. Chem., 241, 2245-51.

Baldwin, A. N., and Berg, P. (1966a). Purification and properties of isoleucyl ribonucleic acid synthetase from Escherichia coli. J. Biol. Chem., 241, 831-8.

Baldwin, A. N., and Berg, P. (1966b). Transfer ribonucleic acid-induced hydrolysis of valyladenylate bound to isoleucyl ribonucleic acid synthetase. J. Biol. Chem., 241, 839-45.

Barnett, W. E., Brown, D. H., and Epler, J. L. (1967). Mitochondrial-specific aminoacyl-RNA synthetases. Proc. Nat. Acad. Sci., 57, 1775-81.

Beers, R. F., Jr., and Sizer, I. W. (1952). Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem., 195, 133-40.

Beinert, H., Green, D. E., Hele, P., Hift, H., Von Korff, R. W., and Ramakrishnan, C. V. (1953). The acetate activating enzyme system of heart muscle. J. Biol. Chem., 203, 35-45.

Bennett, T. P. (1967). Membrane filtration for determining protein in the presence of interfering substances. Nature, 213, 1131-2.

Berg, P. (1956). Acyl Adenylates: The interaction of adenosine triphosphate and L-methionine. J. Biol. Chem., 222, 1025-34.

Berg, P. (1957). Chemical synthesis and enzymatic utilization of adenyl amino acids. Fed. Proc., 16, 152.

Berg, P., Bergmann, F. H., Ofengand, E. J., and Dieckmann, M. (1961). The enzymatic synthesis of amino acyl derivatives of ribonucleic acid. The mechanism of leucyl-, valyl-, isoleucyl-, and methionyl ribonucleic acid formation. J. Biol. Chem., 236, 1726-34.

Bergmann, F. H., Berg, P., and Dieckmann, M. (1961). The enzymatic synthesis of amino acyl derivatives of ribonucleic acid. The preparation of leucyl-, valyl-, isoleucyl-, and methionyl ribonucleic acid synthetases from Escherichia coli. J. Biol. Chem., 236, 1735-40.

Bergmann, M. (1942). A classification of proteolytic enzymes. Adv. Enzymology, 2, 49-68.

Bernlohr, R. W., and Webster, G. C. (1958). Transfer of oxygen-18 during amino acid activation. Archs. Biochem. Biophys., 73, 276-8.

Borsook, H., and Dubnoff, J. W. (1940). The biological synthesis of hippuric acid in vitro. J. Biol. Chem., 132, 307-24.

Bray, G. A. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem., 1, 279-85.

Calendar, R. and Berg, P. (1966). Purification and physical characterization of tyrosyl ribonucleic acid synthetases from Escherichia coli and Bacillus subtilis. Biochemistry, 5, 1681-90.

Capecchi, M. R., and Gusson, G. N. (1965). Suppression in vitro: identification of a serine -sRNA as a "nonsense" suppressor. Science, 149, 417-22.

Cecil, R. (1963). The intramolecular bonds in proteins. I. The role of sulfur in proteins. in The proteins, Neurath, H., ed., Academic Press, New York, V. 1, p. 379-476.

Chapeville, F., Lipmann, F., Ehrenstein, G. von., Weisblum, B., Ray, W. J., and Benzer, S. (1962). On the role of soluble ribonucleic acid in coding for amino acids. Proc. Nat. Acad. Sci., 48, 1086-92.

Conway, T. W., Lansford, E. M. Jr., and Shive, W. (1962). Purification and substrate specificity of a phenylalanine-activating enzyme from Escherichia coli 9723. J. Biol. Chem., 237, 2850-4.

Crick, F. H. C. (1957). Discussion of: M. H. F. Wilkins, Molecular structure of deoxyribose nucleic acid and nucleoprotein and possible implications in protein synthesis. in Biochemical Society, London, Symposium no. 14, The structure of nucleic acids and their role in protein synthesis, Cambridge, University Press, p. 25.

Davie, E. W., Koningsberger, V. V., and Lipmann, F. (1956). The isolation of a tryptophan activating enzyme from pancreas. Archs. Biochem. Biophys., 75, 299-308.

Davie, E. W. (1962). Tryptophan-activating enzyme. in Colowick, S. P., and Kaplan, N. O., eds., Methods in enzymology, New York, Academic Press, V. 5, p. 718.

De Luca, M., and McElroy, W. D. (1965). Sulfhydryl groups of tryptophan-sRNA synthetase. Fed. Proc., 24, 217.

De Moss, J. A., and Novelli, G. D. (1956). An amino acid dependent exchange between ^{32}P labelled inorganic pyrophosphate and ATP in microbial extracts. Biochim. Biophys. Acta., 22, 49-61.

De Moss, J. A., Genuth, S. M., and Novelli, G. D. (1956). The enzymatic activation of amino acids via their acyl-adenylate derivatives. Proc. Nat. Acad. Sci., 42, 325-32.

Deutscher, M. P. (1967). Rat liver glutamyl ribonucleic acid synthetase. II. Further properties and anomalous pyrophosphate exchange. J. Biol. Chem., 242, 1132-9.

Frear, D. S., and Burrell, R. C. (1955). Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal. Chem., 27, 1664-5.

Fresco, J. R., Adams, A., Ascione, R., Henley, D., Lindahl, T. (1966). Tertiary structure in transfer ribonucleic acids. Cold Spring Harbor Symp. Quant. Biol., 31, 527-37.

Goldstein, J., Bennett, T. P., and Craig, L. C. (1964). Countercurrent distribution studies of E. coli B sRNA. Proc. Nat. Acad. Sci., 51, 119-25.

Goodman, H. M., Smith, J. D., Abelson, J. N., Landy, A., Sanger, F., Barrell, B. G., and Brenner, S. (1967). The nucleotide sequence of an Escherichia coli amber suppressor transfer RNA. VIIth Intl. Congress of Biochemistry, Tokyo, p. 673.

Hahn, G. A., and Brown, J. W. (1967). Aminoacyl-tRNA synthetases from Sarcina lutea. Biochim. Biophys. Acta., 146, 259-63.

Hecht, L. I., Stephenson, M. L., and Zamecnik, P. C. (1959). Binding of amino acids to the end group of a soluble ribonucleic acid. Proc. Nat. Acad. Sci., 45, 505-18.

Hele, P. (1964). On the role of s-RNA on regulating the rates of amino acid-dependent pyrophosphate exchange reactions: a possible "allosteric control" phenomenon. Biochim. Biophys. Acta, 87, 449-59.

Hele, P., and Barth, P. T. (1966). Possible "allosteric" effects controlling the kinetic behavior of amino acid-dependent pyrophosphate exchange reactions. Biochim. Biophys. Acta, 114, 149-57.

Heppel, L. A. (1955). Inorganic pyrophosphatase from yeast. in Methods in enzymology, Colowick, S. P., and Kaplan, N. O., eds., Academic Press, New York, V. 2, p. 570-6.

Hoagland, M. B. (1955). An enzymatic mechanism for amino acid activation in animal tissues. Biochim. Biophys. Acta, 16, 288-9.

Hoagland, M. B., Keller, E. B., and Zamecnik, P. C. (1956). Enzymatic carboxyl activation of amino acids. J. Biol. Chem., 218, 345-58.

Hoagland, M. B., Zamecnik, P. C., Sharon, N., Lipmann, F., Stulberg, M. P., and Boyer, P. D. (1957). Oxygen transfer to AMP in the enzymatic synthesis of the hydroxamate of tryptophan. Biochim. Biophys. Acta, 26, 215-17.

Hoagland, M. B., Zamecnik, P. C., and Stephenson, M. L. (1957). Intermediate reactions in protein biosynthesis. Biochim. Biophys. Acta, 24, 215-16.

Holley, R. W. (1957). An alanine-dependent, ribonuclease-inhibited conversion of AMP to ATP, and its possible relationship to protein synthesis. J. Am. Chem. Soc., 79, 658-62.

Holley, R. W. and Goldstein, J. (1959). An alanine-dependent, ribonuclease-inhibited conversion of adenosine 5'-phosphate to adenosine triphosphate. J. Biol. Chem., 234, 1765-8.

Holley, R. W., Apgar, J., Everett, G. A., Madison, J. T., Marquisee, M., Merrill, S. H., Penswick, J. R., and Zamir, A. (1965). Structure of a ribonucleic acid. Science, 147, 1462-5.

Jacob, F. and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol., 3, 318-356.

Jones, M. E., Black, S., Flynn, R. M., and Lipmann, F. (1953). Acetyl coenzyme A synthesis through pyrophosphoryl split of adenosine triphosphate. Biochim. Biophys. Acta, 12, 141-9.

Karasek, M. P., Castelfranco, P., Krishnaswamy, P. R., and Meister, A. (1958). Enzymatic synthesis and reactions of tryptophan-adenylic acid anhydride. J. Am. Chem. Soc., 80, 2335-6.

Kelmers, A. D., Novelli, G. D., and Stulberg, M. P. (1965). Separation of transfer ribonucleic acids by reverse phase chromatography. J. Biol. Chem., 240, 3979-83.

Kingdon, H. S., Webster, L. T. Jr., and Davie, E. W. (1958). Enzymatic formation of adenyl tryptophan: isolation and identification. Proc. Nat. Acad. Sci., 44, 757-65.

Kunitz, M. (1961). An improved method for isolation of crystalline pyrophosphatase from baker's yeast. Archs. Biochem. Biophys., 92, 270-2.

Lagervist, U., Rymo, L., Waldenström, J. (1966). Structure and function of transfer ribonucleic acid. Enzyme-substrate complexes with valyl ribonucleic acid synthetase from yeast. J. Biol. Chem., 22, 5391-5400.

Lipmann, F. (1941). Metabolic generation and utilization of phosphate bond energy. Adv. Enzymology, 1, 99-162.

Lipmann, F., and Tuttle, L. C. (1945). The detection of activated carboxyl groups with hydroxylamine as interceptor. J. Biol. Chem., 161, 415-6.

Lipmann, F. (1958). Chairman's introduction: some facts and problems. Proc. Nat. Acad. Sci., 44, 67-73.

Lipmann, F., Hülsmann, W. C., Hartmann, G., Boman, H. G., and Acs, G. (1959). Amino acid activation and protein synthesis. J. Cell. and Comp. Physiol., 54, Supp. 1, 75-88.

Loftfield, R. B., and Eigner, E. A. (1963). A radioactive hydroxamate method for determining rates of amino acid activation. Biochim. Biophys. Acta, 72, 372-82.

Loftfield, R. B. and Eigner, E. A. (1965). A soluble ribonucleic acid-induced increase in the specificity of Escherichia coli isoleucine-activating enzyme. J. Biol. Chem., 240, PC 1482-4.

Maas, W. K., and Novelli, G. D. (1953). Synthesis of pantothenic acid by depyrophosphorylation of adenosine triphosphate. Arch. Biochem. Biophys., 43, 236-8.

Madison, J. T., Everett, G. A., and Klung, H. (1966). Nucleotide sequence of a yeast tyrosine transfer RNA, Science, 153, 531-4.

Mahler, H. R., Dutton, G., and Mehrotra, B. D. (1963). Effects of deuterium on the thermal transition of nucleic acids. Biochim. Biophys. Acta, 68, 199-210.

Makman, M. H., and Cantoni, G. L. (1965). Isolation of seryl and phenylalanyl ribonucleic acid synthetases from baker's yeast. Biochemistry, 4, 1434-42.

Martin, R. G., and Ames, B. N. (1961). A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem., 236, 1372-9.

Martin, J. B., and Doty, P. M. (1949). Determination of inorganic phosphate. Anal. Chem., 21, 965-7.

McCorquodale, D. J. (1964). The separation and partial purification of amino-acyl-RNA synthetases from Escherichia coli. Biochim. Biophys. Acta, 91, 541-8.

McElroy, W. D., De Luca, M., and Travis, J. (1967). Molecular uniformity in biological catalyses. Science, 157, 150-60.

Mitra, S. K., and Mehler, A. H. (1967). The activation of arginyl transfer ribonucleic acid synthetase by transfer ribonucleic acid. J. Biol. Chem., 242, 5495-99.

Muench, K. H., and Berg, P. (1966). Preparation of aminoacyl ribonucleic acid synthetases from Escherichia coli. in Procedures in nucleic acid research, Cantoni, G. L., and Davies, D. R., eds., Harper and Row, New York, V. 1, p. 375-83.

Nordlie, R., and Lardy, H. (1962). Phosphoryl group transfers. in The enzymes, Boyer, P. D., Lardy, H., and Myrbäck, K., eds., Academic Press, New York, V. 6, p. 3-46.

Norris, A. T., and Berg, P. (1964). Mechanism of aminoacyl RNA synthesis: studies with isolated aminoacyladenylate complexes of isoleucyl RNA synthetase. Proc. Nat. Acad. Sci., 52, 330-7.

Novelli, G. D. (1958). Some problems concerning the activation of amino acids. Proc. Nat. Acad. Sci., 44, 86-92.

Novelli, G. D. (1967). Amino acid activation for protein synthesis. in Annual review of biochemistry, Boyer, P. D., ed., Annual Reviews, Inc., Palo Alto, California, V. 36, p. 449-84.

Ogata, K., Nohara, H., Ishikawa, K., Morita, T., and Asaoka, H. (1961). The activation of isoleucine and leucine and their transfer to soluble ribonucleic acid (RNA) by a partially-purified enzyme fraction obtained from guinea pig liver. in Protein biosynthesis, Harris, R. J. C., ed., Academic Press, New York, p. 163-82.

Peterson, P. J. (1967). Amino acid selection in protein biosynthesis. Biol. Rev., 42, 552-613.

Preiss, J., Berg, P., Ofengand, E. J., Bergmann, F. H., and Dieckmann, M. (1959). The chemical nature of the RNA-amino acid compound formed by amino acid-activating enzymes. Proc. Nat. Acad. Sci., 45, 319-28.

RajBhandary, U. L., Chang, S. H., Stuart, A., Faulkner, R. D., Hoskinson, R. M., and Khorana, H. G. (1967). Studies on polynucleotides, LXVIII. The primary structure of yeast phenylalanine transfer RNA. Proc. Nat. Acad. Sci., 57, 751-8.

Ravel, J. M., Wang, S.-F., Heinemeyer, C., and Shive, W. (1965). Glutamyl and glutaminyl ribonucleic acid synthetases of Escherichia coli W. Separation, properties, and stimulation of adenosine triphosphate-pyrophosphate exchange by acceptor ribonucleic acid. J. Biol. Chem., 240, 432-8.

Samejima, T., and Shibata, K. (1961). Denaturation of catalase by formamide and urea related to the subunit make-up of the molecule. Archs. Biochem. Biophys., 93, 407-12.

Schachman, H. K. (1952). Ultracentrifugation, electrophoresis, and viscosity studies on crystalline pyrophosphatase. J. Gen. Physiol., 35, 451-4.

Schweet, R. S., and Allen, E. H. (1958). Purification and properties of tyrosine-activating enzyme of hog pancreas. J. Biol. Chem., 233, 1104-8.

Schweet, R., and Heintz, R. (1966). Protein synthesis. in Annual review of biochemistry, Boyer, P. D., ed., Annual Reviews, Inc., Palo Alto, California, V. 36, p. 723-58.

Stern, R., De Luca, M., Mehler, A. H., and McElroy, W. D. (1966). Role of sulfhydryl groups in activating enzymes. Properties of Escherichia coli lysine-transfer ribonucleic acid synthetase. Biochemistry, 5, 126-30.

Stulberg, M. P., and Novelli, G. D. (1962). Amino acid activation. in The enzymes, Boyer, P. D., Lardy, H., and Myrbäck, K., eds., Academic Press, New York, V. 6, p. 401-32.

van De Ven, A. M., Konigsberger, V. V., and Overbeek, J. T. G. (1958). Isolation of a tyrosine-activating enzyme from baker's yeast. Biochim. Biophys. Acta, 28, 134-43.

Vescia, A. (1967). Separation of two leucyl-ribonucleic acid synthetases from rat liver. Biochem. Biophys. Res. Commun., 4, 496-500.

Waldenström, J. (1968). Purification and some properties of lysyl ribonucleic acid synthetase from E. coli. Eur. J. Biochem., 3, 483-91.

Warburg, O. and Christian, W. (1941). Isolierung und Kristallisation des Garüngsferments Enolase. Biochem. Z., 310, 384.

Webster, L. T. Jr., and Davie, E. W. (1959). Enzymic formation of adenyl-serine and an unknown carboxyl-activated compound. Biochim. Biophys. Acta, 35, 559-60.

Wolfenden, R. (1964). Some characteristics of the linkage of amino acids to soluble RNA. Ph.D. Thesis, Rockefeller University.

Wolfenden, R., Rammler, D. H., and Lipmann, F., (1964). On the site of esterification of amino acids to soluble RNA. Biochemistry, 3, 329-38.

Yarus, M., and Berg, P. (1967). Recognition of tRNA by aminoacyl tRNA synthetases. J. Mol. Biol., 28, 479-90.

Yu, C.-T., and Rappaport, H. P. (1966). Multiple fractions of leucyl-transfer ribonucleic acid synthetase activity from Escherichia coli. Biochim. Biophys. Acta, 123, 134-41.

Zachau, H. G., Acs, G., and Lipmann, F. (1958). Isolation of adenosine amino acid esters from a ribonuclease digest of soluble liver ribonucleic acid. Proc. Nat. Acad. Sci., 44, 885-9.

Zachau, H. G., Dutting, D., and Feldmann, H. (1966). Nucleotide sequences of two serine-specific transfer ribonucleic acids. Angew. Chem. Internat. Edit., 5, 422.

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