STUDIES ON TRANSFER RIBONUCLEIC ACIDS AND AMINOACYL-TRANSFER RIBONUCLEIC ACID SYNTHETASES OF AROMATIC AMINO ACIDS IN SOYBEAN (GLYCINE MAX L.).

G. SIVAKUMAR. SWAMY
University of Windsor

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STUDIES ON TRANSFER RIBONUCLEIC ACIDS AND AMINOACYL-TRANSFER RIBONUCLEIC ACID SYNTHETASES OF AROMATIC AMINO ACIDS IN SOYBEAN (GLYCINE MAX L.)

BY

C. SIVAKUMAR SWAMY

A DISSERTATION

Submitted to the Faculty of Graduate Studies and Research through the Department of Biology in Partial Fulfillment of the requirements for the Degree of Doctor of Philosophy at the University of Windsor

WINDSOR, ONTARIO, CANADA

1980
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>PART I - PURIFICATION OF PHENYLALANYL-tRNA SYNTHETASE</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS</td>
<td>5</td>
</tr>
<tr>
<td>Chemicals</td>
<td>5</td>
</tr>
<tr>
<td>Plant Material</td>
<td>6</td>
</tr>
<tr>
<td>METHODS</td>
<td>7</td>
</tr>
<tr>
<td>Preparation of whole cell tRNA</td>
<td>7</td>
</tr>
<tr>
<td>Preparation of chromatographic adsorbents</td>
<td>8</td>
</tr>
<tr>
<td>Polyacrylamide gel electrophoresis of native enzyme</td>
<td>10</td>
</tr>
<tr>
<td>SDS-polyacrylamide gel electrophoresis</td>
<td>11</td>
</tr>
<tr>
<td>Protein determination</td>
<td>11</td>
</tr>
<tr>
<td>Assay procedure</td>
<td>11</td>
</tr>
<tr>
<td>Preparation of affinity column</td>
<td>12</td>
</tr>
<tr>
<td>a) Aminoacylation of unfractionated tRNA</td>
<td>13</td>
</tr>
<tr>
<td>b) Oxidation of tRNA</td>
<td>15</td>
</tr>
<tr>
<td>c) Antiaffinity gel</td>
<td>15</td>
</tr>
<tr>
<td>d) Affinity gel</td>
<td>16</td>
</tr>
<tr>
<td>RESULTS</td>
<td>17</td>
</tr>
</tbody>
</table>
# Purification of Phe-tRNA synthetase by affinity chromatographic method

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Homogenization and ammonium sulfate fractionation</td>
<td>17</td>
</tr>
<tr>
<td>b) DEAE-cellulose chromatography</td>
<td>18</td>
</tr>
<tr>
<td>c) Sephadex G 150 chromatography</td>
<td>18</td>
</tr>
<tr>
<td>d) Hydroxylapatite chromatography</td>
<td>23</td>
</tr>
<tr>
<td>e) Anti-affinity chromatography</td>
<td>23</td>
</tr>
<tr>
<td>f) Affinity chromatography</td>
<td>28</td>
</tr>
<tr>
<td>g) Polyacrylamide gel electrophoresis of the purified enzyme</td>
<td>28</td>
</tr>
<tr>
<td>h) Gel filtration studies</td>
<td>34</td>
</tr>
</tbody>
</table>

# Regular Chromatographic purifications of Phe-tRNA synthetase

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Homogenization, ammonium sulfate fractionation and DEAE-cellulose chromatography</td>
<td>34</td>
</tr>
<tr>
<td>b) Sephacryl-S 200 chromatography</td>
<td>37</td>
</tr>
<tr>
<td>c) Hydroxylapatite chromatography</td>
<td>37</td>
</tr>
<tr>
<td>d) DEAE-Sephadex A 50 chromatography</td>
<td>42</td>
</tr>
<tr>
<td>e) Phosphocellulose chromatography</td>
<td>42</td>
</tr>
<tr>
<td>f) Polyacrylamide gel electrophoresis of the native enzyme</td>
<td>48</td>
</tr>
<tr>
<td>g) Molecular weight determination of the enzyme subunit</td>
<td>48</td>
</tr>
</tbody>
</table>

# Kinetic Characterization of Phe-tRNA Synthetase

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) pH optimum</td>
<td>48</td>
</tr>
<tr>
<td>b) ATP and Mg++ optima</td>
<td>53</td>
</tr>
</tbody>
</table>
c) Determination of Km for Phenylalanine, ATP and tRNA<sub>Phe</sub> ........................................... 53

d) Response to sulfhydryl reagents ........................................ 60

d) Thermal inactivation and nucleoside triphosphate interaction ............... 69

DISCUSSION ........................................................................ 75

PART II - LOCALIZATION OF CYTOPLASMIC, CHLOROPLASTIC AND
MITOCHONDRIAL tRNA<sub>Phe</sub>, tRNA<sub>Trp</sub> AND tRNA<sub>Tyr</sub> AND
CORRESPONDING SYNTHETASES

INTRODUCTION .................................................................. 81

MATERIALS ........................................................................ 88

Chemicals ........................................................................... 88

Plant material .................................................................... 88

E. coli .............................................................................. 88

METHODS .......................................................................... 89

Transfer RNA ..................................................................... 89

Isolation of chloroplasts .................................................. 90

Isolation of mitochondria ................................................. 91

Purity of organelles ........................................................ 91

E. coli ribosomes ............................................................. 92

Two-dimensional polyacrylamide gel electrophoresis of
chloroplast tRNA .............................................................. 93

Preparation of ribosomal RNA .......................................... 95

Isolation and partial purification of Phe, Tyr and
Trp-tRNA synthetases ..................................................... 96

Preparation of aminoacyl-tRNA synthetases from E. coli .......... 97
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoacylation with \textit{E. coli} aminoacyl-tRNA synthetases</td>
<td>98</td>
</tr>
<tr>
<td>Aminoacylation assays</td>
<td>98</td>
</tr>
<tr>
<td>Synthesis of UAC and UAU codons</td>
<td>99</td>
</tr>
<tr>
<td>Benzoylated-DEAE (B-D)-cellulose chromatography</td>
<td>100</td>
</tr>
<tr>
<td>Reversed-phase chromatography (RPC-5)</td>
<td>100</td>
</tr>
<tr>
<td>Protein determination</td>
<td>101</td>
</tr>
<tr>
<td>Scintillation fluid</td>
<td>102</td>
</tr>
</tbody>
</table>

### RESULTS

- Optimum ATP and Mg\textsuperscript{++} requirements for Phe, Tyr and Trp-tRNA synthetases from cytoplasm, mitochondria and chloroplasts... 103
- Aminoacylation by homologous and heterologous enzymes and tRNAs from the chloroplasts, the mitochondria and the cytoplasm... 105
- Fractionation of Phe, Trp and Tyr-tRNA synthetases from the three cell compartments... 107
- Characterization and aminoacylation properties of tRNA\textsubscript{Phe}, tRNA\textsubscript{Trp} and tRNA\textsubscript{Tyr} from the three cell compartments... 116
- Analysis of chloroplast tRNAs by two-dimensional polyacrylamide gel electrophoresis... 160
- B-D cellulose chromatography of tRNA... 161
- RPC-5 chromatography of ethanol fractions from B-D cellulose column... 165
- Codon recognition studies... 174
  - a) preparation of isoacceptors of tRNA\textsubscript{Tyr}... 174
  - b) ribosome binding assay... 175
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DISCUSSION</td>
<td>177</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>185</td>
</tr>
<tr>
<td>VITA AUCTORIS</td>
<td>199</td>
</tr>
</tbody>
</table>
ABSTRACT

Cytoplasmic phenylalanyl-tRNA synthetase from soybean (Glycine max L.) was purified using both affinity chromatographic and conventional chromatographic methods. The affinity chromatographic method involved the binding of the enzyme to tRNA^Phe covalently linked to Sepharose 4B. Before separation on the affinity column, the enzyme extract was fractionated on a similar column with non-cognate tRNA as the ligand in order to eliminate other aminoacyl-tRNA synthetases and various tRNA-binding proteins. Examination of the affinity purified enzyme by polyacrylamide gel electrophoresis indicated four additional proteins other than phenylalanyl-tRNA synthetase. These additional proteins were probably the enzymes and proteins involved in the metabolism specific to tRNA^Phe. Phenylalanyl-tRNA synthetase was purified to homogeneity by ammonium sulfate precipitation, DEAE-cellulose chromatography, Sephacryl-S 200 chromatography, hydroxylapatite chromatography and phosphocellulose chromatography. The enzymatically active subunit of the enzyme was found to have a molecular weight of 80,000 on SDS-polyacrylamide gel. The Km values for L-phenylalanine, ATP and tRNA^Phe were 4.5 x 10^{-7} M, 3.0 x 10^{-5} M, and 7.4 x 10^{-8} M respectively.

Transfer RNAs and aminoacyl-tRNA synthetases of phenylalanine, tyrosine and tryptophan were localized in the cytoplasm, the chloroplasts and in the mitochondria. The aminoacyl-tRNA synthetases of these three aromatic amino acids fractionated by hydroxylapatite chromatography from both chloroplasts and mitochondria were able to amino-
acylate Escherichia coli tRNA suggesting their prokaryotic nature. On the other hand, cytoplasmic enzymes were unable to aminoaclyl E. coli tRNA and they were found to be distinct from the organellar aminoaclyl-tRNA synthetases in chromatographic behaviour as well as in their kinetic properties. Three isoacceptors for each of tRNA^Phe and tRNA^Trp were resolved by reversed phase chromatography (RPC-5) and one isoacceptor was localized in each for cytoplasm, chloroplasts and mitochondria. tRNA^Tyr was resolved into five isoacceptors; two were localized in cytoplasm, two in chloroplasts and the remaining one in mitochondria. Organellar tRNAs in all the three cases could be aminoaclylated by E. coli aminoaclyl-tRNA synthetases while cytoplasmic tRNAs were not aminoaclylatable by E. coli enzymes. Codon recognition studies with tRNA^Tyr isoacceptors indicated that all the organellar isoacceptors recognize UAC codon whereas one of the cytoplasmic isoacceptors recognizes UAC codon and the other recognized UAU codon.
ACKNOWLEDGEMENTS

I wish to acknowledge my sincere thanks to Dr. D.T.N. Pillay, Department of Biology, University of Windsor, for his guidance and technical instruction during the course of my graduate education and the presentation of this dissertation.

I would like to thank Dr. M.J. Dufresne, Dr. D.A. Cotter of the Department of Biology, Dr. N.F. Taylor of the Department of Chemistry, University of Windsor, and Dr. R.J. Cedergren, Department of Biochemistry, University of Montreal, for critically reviewing this dissertation and for their participation as members of my examining committee.

I would like to thank Professor J.H. Weil, Institut de Biologie Moleculaire et Cellulaire, Universite Louis Pasteur, Strasbourg, France, for his generous help in giving me access to the facilities in his laboratory, and I am also indebted to his colleagues for their co-operation and help.

I also wish to acknowledge the financial assistance provided by the Department of Biology, University of Windsor, the University of Windsor, and the Government of Ontario.

Financial assistance from NSERC of Canada (Grant No. A-1984 to Dr. D.T.N. Pillay) is gratefully acknowledged.
LIST OF TABLES

PART I

1. Affinity purification of phenylalanyl-tRNA synthetase........... 33
2. Purification of phenylalanyl-tRNA synthetase................. 47
3. Effect of nucleoside triphosphates on the thermal inactivation of Phe-tRNA synthetase................................. 73
4. Effect of nucleoside triphosphates on the rate of enzymatic reaction of Phe-tRNA synthetase............................... 74
5. Km values determined for Phe-tRNA synthetase................... 74

PART II

1. Optimum ATP and Mg$^{2+}$ requirements of Phe, Tyr and Trp-tRNA synthetase from the cytoplasm, the chloroplasts and the mitochondria......................................................... 104
2. Aminoacylation by homologous and heterologous enzymes and tRNAs from the chloroplasts, the mitochondria and the cytoplasm................................................................. 106
3. Codon recognition by isoaccepting tRNA$^{Tyr}$ species........... 176
# LIST OF ILLUSTRATIONS

## PART I

<table>
<thead>
<tr>
<th>Illustration</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extraction of tRNA from the whole cell</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Mechanism of coupling of tRNA(^{\text{Phe}}) and non-cognate tRNA to the carrier matrix</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>DEAE-cellulose chromatography of Phe-tRNA synthetase</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Sephadex G 150 chromatography of Phe-tRNA synthetase</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>Hydroxylapatite chromatography of Phe-tRNA synthetase</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Anti-affinity chromatography of Phe-tRNA synthetase</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>Affinity chromatography of Phe-tRNA synthetase</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>Densitometric tracing of the polyacrylamide gel containing affinity purified enzyme preparation</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>Gel filtration studies with affinity purified Phe-tRNA synthetase</td>
<td>36</td>
</tr>
<tr>
<td>10</td>
<td>Sephacryl-S 200 chromatography of Phe-tRNA synthetase</td>
<td>39</td>
</tr>
<tr>
<td>11</td>
<td>Hydroxylapatite chromatography of Phe-tRNA synthetase</td>
<td>41</td>
</tr>
<tr>
<td>12</td>
<td>DEAE-Sephadex A 50 chromatography of Phe-tRNA synthetase</td>
<td>44</td>
</tr>
<tr>
<td>13</td>
<td>Phosphocellulose chromatography of Phe-tRNA synthetase</td>
<td>46</td>
</tr>
<tr>
<td>14</td>
<td>Densitometric tracing of the polyacrylamide gel containing purified Phe-tRNA synthetase under non-denaturing condition</td>
<td>50</td>
</tr>
<tr>
<td>15</td>
<td>SDS-polyacrylamide gel electrophoresis of the denatured enzyme</td>
<td>52</td>
</tr>
<tr>
<td>16</td>
<td>Aminoacylation kinetics of Phe-tRNA synthetase under the conditions of different pH</td>
<td>55</td>
</tr>
<tr>
<td>17</td>
<td>Aminoacylation kinetics of Phe-tRNA synthetase under the conditions of different levels of ATP</td>
<td>57</td>
</tr>
<tr>
<td>18</td>
<td>Aminoacylation kinetics of Phe-tRNA synthetase under the conditions of different levels of Mg(^{++})</td>
<td>59</td>
</tr>
</tbody>
</table>
19. Apparent Michaelis constant for L-phenylalanine .................. 62
20. Apparent Michaelis constant for ATP ............................ 64
21. Apparent Michaelis constant for tRNA\textsuperscript{Phe} .......... 66
22. Aminoacylation kinetics of Phe-tRNA synthetase in the presence of different sulphydryl reagents .................. 68
23. Kinetics of thermal inactivation of Phe-tRNA synthetase .... 71

PART II

1. Fractionation of the cytoplasmic, the chloroplastic and the mitochondrial Phe-tRNA synthetase ............................. 109
2. Fractionation of the cytoplasmic, the chloroplastic and the mitochondrial Trp-tRNA synthetase ............................ 111
3. Fractionation of the cytoplasmic, the chloroplastic and the mitochondrial Tyr-tRNA synthetase ............................. 113
4. RPC-5 fractionation of total cell tRNA\textsuperscript{Phe} ............................. 118
5. RPC-5 fractionation of total cell tRNA\textsuperscript{Trp} . ............................. 120
6. RPC-5 fractionation of total cell tRNA\textsuperscript{Tyr} .................................. 122
7. RPC-5 fractionation of chloroplast tRNA\textsuperscript{Phe} aminoacylated by homologous peak I enzyme ............................ 124
8. RPC-5 fractionation of chloroplast tRNA\textsuperscript{Trp} aminoacylated by homologous peak I enzyme ............................ 126
9. RPC-5 fractionation of chloroplast tRNA\textsuperscript{Tyr} aminoacylated by homologous peak I enzyme ............................ 128
10. RPC-5 fractionation of chloroplast tRNA\textsuperscript{Phe} aminoacylated by mitochondrial peak I enzyme ............................ 130
11. RPC-5 fractionation of chloroplast tRNA\textsuperscript{Trp} aminoacylated by mitochondrial peak I enzyme ............................ 132
12. RPC-5 fractionation of chloroplast tRNA_{Tyr} aminoacylated by mitochondrial peak I enzyme.......................... 134
13. RPC-5 fractionation of mitochondrial tRNA_{Phe} aminoacylated by homologous peak I enzyme.......................... 136
14. RPC-5 fractionation of mitochondrial tRNA_{Trp} aminoacylated by homologous peak I enzyme.......................... 138
15. RPC-5 fractionation of mitochondrial tRNA_{Tyr} aminoacylated by homologous peak I enzyme.......................... 140
16. RPC-5 fractionation of mitochondrial tRNA_{Phe} aminoacylated by chloroplast peak I enzyme.......................... 142
17. RPC-5 fractionation of mitochondrial tRNA_{Trp} aminoacylated by chloroplast peak I enzyme.......................... 144
18. RPC-5 fractionation of mitochondrial tRNA_{Tyr} aminoacylated by chloroplast peak I enzyme.......................... 146
19. RPC-5 fractionation of mixture of chloroplast and mitochondrial tRNA_{Phe} aminoacylated by chloroplast peak I enzyme.......................... 149
20. RPC-5 fractionation of mixture of chloroplast and mitochondrial tRNA_{Trp} aminoacylated by chloroplast peak I enzyme.......................... 151
21. RPC-5 fractionation of mixture of chloroplast and mitochondrial tRNA_{Tyr} aminoacylated by chloroplast peak I enzyme.......................... 153
22. RPC-5 fractionation of cytoplasmic tRNA_{Phe} aminoacylated by cytoplasmic (Peak II) enzyme.......................... 155
23. RPC-5 fractionation of cytoplasmic tRNA_{Trp} aminoacylated by cytoplasmic (Peak II) enzyme.......................... 157
24. RPC-5 fractionation of cytoplasmic tRNA_{Tyr} aminoacylated by cytoplasmic (Peak II) enzyme.......................... 159
25. Two-dimensional polyacrylamide gel electrophoresis of chloroplast tRNA.......................................... 162
26. Polyacrylamide gel electrophoresis map of chloroplast tRNA...... 164
27. B-D-cellulose chromatography of whole cell tRNA............... 167
28. B-D-cellulose chromatography of chloroplast tRNA.............. 169
29. RPC-5 fractionation of B-D-cellulose ethanol fractions of chloroplast tRNA............................................. 171
30. Sephadex G 25 chromatography of polyacrylamide gel extracts of tRNA$^{\text{TYR}}$ spots.............................. 173
**ABREVIATIONS**

- **A260**: absorbance at 260 nanometers
- **A280**: absorbance at 280 nanometers
- **OD280**: optical density at 280 nanometers (absorbance at 280 nanometers)
- **A**: adenosine
- **C**: cytidine
- **G**: guanosine
- **U**: uridine
- **AMP**: adenosine 5' - monophosphate
- **ATP**: adenosine 5' - triphosphate
- **CDP**: cytidine 5' - diphosphate
- **CTP**: cytidine 5' - triphosphate
- **GTP**: guanosine 5' - triphosphate
- **UA**: uridylyl (3'→5') adenosine
- **UDP**: uridine 5' - diphosphate
- **DNA**: deoxyribonucleic acid
- **RNA**: ribonucleic acid
- **tRNA**: transfer ribonucleic acid
- **AA-tRNA**: aminocyl-transfer ribonucleic acid
- **EME**: β-mercaptoethanol (2-mercaptoethanol)
- **GSH**: glutathione (reduced form)
- **DTT**: dithiothreitol
- **EDTA**: ethylenediaminetetraacetic acid
- **TCA**: trichloracetic acid
- **Tris**: tris-(hydroxymethyl) aminomethane
- **DEAE-**: diethylaminoethyl
CNBr-  cyanogen bromide
B-D-Cellulose  benzoylated diethylaminoethyl cellulose
MAK  methylated albumin on kieselguhr
PMSF  phenylmethylsulfonyl fluoride
RPC-5  reversed phase chromatography
PEI-  polyethyleneimine impregnated
TEMED  N,N,N',N'-tetramethylethylenediamine
CPM  counts per minute
M.W.  molecular weight
M  molar
pmol  pico moles
G  grams
xG  times gravity (relative centrifugal force)
mg  milligrams
µg  micrograms
ml  milliliters
µl  microliters
mm  millimeters
cm  centimeters
nm  nanometers
min  minutes
hr  hours
°C  degrees Celsius
PART I

PURIFICATION OF PHENYLALANYL-tRNA SYNTHETASE
INTRODUCTION

Ever since the suggestion of the need for an "adaptor" molecule (Crick, 1955) to mediate between the DNA code word and the amino acid, an incredible amount of information has accumulated on this intermediate reaction in protein biosynthesis. The first evidence for the amino acids being covalently linked to a small RNA molecule (of molecular weight 25,000 to 30,000) was obtained within two years of proposing the "adaptor" hypothesis (Hoagland et al., 1957).

However it was known earlier that energy is required for protein biosynthesis (Franz et al., 1948) and that ATP serves as the energy source for protein biosynthesis (Zamecnik and Keller, 1954) and also that living systems contain an enzymatic activity which activate amino acids to form aminoacyl-adenylates (Berg, 1956; Hoagland, 1955).

Subsequent experiments on this aspect of molecular biology established that each amino acid is attached to a specific soluble RNA molecule (transfer RNA) and the attachment is catalyzed by an individual enzyme with absolute specificity for the concerned amino acid and transfer RNA (tRNA). The overall reaction catalyzed by these enzymes (aminoacyl-tRNA synthetases) has been shown to occur in a two-step reaction. The first step is involved in amino acid "activation" and the second step results in the attachment of the amino acid to the cognate tRNA. This two-step mechanism of aminoacylation of tRNA is described by the following equation:
\[ \text{AA} + \text{ATP} + \text{E} \rightleftharpoons (\text{AA-AMP-E}) + \text{PPi} \] (1)

\[ (\text{AA-AMP-E}) + \text{tRNA} \rightleftharpoons \text{AA-tRNA} + \text{AMP} + \text{E} \] (2)

The reaction described by equation (1) has been called the "activation" step and it is assayed by ATP-PPi exchange or by hydroxamate formation, while the tRNA esterification step (equation 2) is followed by isolation of enzyme-bound aminoacyl-adenylates by gel filtration and reaction of this complex with tRNA. The overall reaction of AA-tRNA formation is assayed by the esterification of labelled amino acids to tRNA. (Soll and Schimmel, 1975; Lea and Norris, 1977).

Aminoacyl-tRNA synthetases, being multistep enzymes, have been the source of interest for enzymological studies because of the wide diversity and complexity in their nature. These enzymes have been known to exist in multiple forms in the cell including the ones existing in organelles of eukaryotic cells (Soll and Schimmel, 1974; Lea and Norris, 1977; Weil, 1979); the reason for the diversity among aminoacyl-tRNA synthetases which have the same major task (the aminoacylation of tRNA) is a puzzling problem.

Aminoacyl-tRNA synthetases have been purified from a wide variety of sources. In the majority of cases, conventional chromatographic methods have been employed for the purification of these enzymes and in a few cases (Bartkowiak and Pawelkiewicz, 1972; Befort et al., 1974; Faulhammer and Cramer, 1977; Gerö and Waller, 1972/ Hyashi, 1973; Remy et al., 1972; Van der Haar, 1976; Swamy and Pillay, 1979), affinity chromatographic techniques have been applied. A large number of
aminoacyl-tRNA synthetases have been purified to homogeneity (over
50 enzymes) from various organisms (especially from *E. coli* and
yeast) and only a few plant aminoacyl-tRNA synthetases have been
obtained pure (Weil, 1979). This has been considered (Weil, 1979)
mainly due to the highly labile nature of plant enzymes even at low
temperature and in the presence of their substrates, of sulfhydryl
reducing agents (such as glutathione, β-mercaptoethanol and Dithio-
threitol) and of polyols (such as glycerol or propylene glycol).

Purification of Phe-tRNA synthetase from soybean has been in-
cluded as the part of the present project on the studies on tRNAs
and aminoacyl-tRNA synthetases of aromatic amino acids. The purpose
of undertaking the project is discussed in part II. When this
project was initiated, none of these three aromatic aminoacyl-tRNA
synthetases (phenylalanine, tyrosine and tryptophan) was purified to
homogeneity from plant sources. Phe-tRNA synthetase was later pur-
ified by Carias and Julien (1976) from wheat germ and subsequently the
purification of this enzyme from lupin seeds was referred to (Barciszewski
et al., 1979) although these results have not yet been published.

Tyr-tRNA synthetase was purified from soybean (Locy and Cherry, 1978).
Phe-tRNA synthetase has been so far purified to homogeneity from
*E. coli* (Fayat et al., 1974; Hanke et al., 1974; Stulberg, 1967),
yeast (Fasolo et al., 1970; Schmidt et al., 1971), rat liver (Lanks
et al., 1971), rabbit liver (Dufresne, 1974), *Drosophila* (Christopher
et al., 1971), wheat germ (Carias and Julien, 1976), lupin (Barcisz-
zewski et al., 1979) and soybean cotyledons (Swamy and Pillay, 1980).
The following study demonstrates the purification of cytoplasmic Phe-tRNA synthetase from soybean cotyledons and the kinetic characterization of the enzyme. Conventional chromatographic methods of purification of aminoacyl-tRNA synthetases were considered to have "inherent undesirable features" (Soll and Schimmel, 1974) and therefore, the perfection of affinity chromatographic methods was warranted for the purification of these enzymes. In the present study, both the methods are employed for the purification of Phe-tRNA synthetases.
MATERIALS

Chemicals:

Acrylamide and N,N'-methylenebisacrylamide were purchased from BDH. Xylene cyanole FF and N,N,N', N'-tetramethylethylenediamine (TEMED) were purchased from Eastman Co. Bovine pancreas deoxyribonuclease, methylene blue, E. coli alkaline phosphatase, type 305 alumina, ATP, GTP, bovine serum albumin, β-mercaptoethanol, dithiothreitol, DEAE-Sephadex A 50, CNBr-Sepharose, Coomassie Brilliant Blue G 250 and Coomassie Brilliant Blue R 250 were purchased from Sigma. Micrococcus luteus polynucleotide phosphorylase, cytidine 5'-diphosphate, uridine 5'-diphosphate, uridylyl-(3'→5') adenosine, glutathione, GTP, E. coli tRNA and E. coli RNA polymerase were purchased from Boehringer-Mannheim. Urea and hydroxylapatite (Biogel-HTP) were purchased from Bio-Rad. Polyvinylpyrrolidone (insoluble form), trade name polyclar AT, was purchased from GAF corporation. RFC-5 chromatographic adsorbent was purchased from Miles Laboratories. Sephacryl S 200, phosphorylase b, carbonic anhydrase, Sephadex G 150, Sephadex G 25 and Sephadex G 100 were purchased from Pharmacia fine chemicals. Rabbit immunoglobulin G, hemoglobin and trypsin were kindly provided by Dr. H.B. Pachtel. PEI (Polyethyleneimine impregnated)-cellulose sheets for thin layer chromatography were purchased from Macherey-Nagel through Brinkmann Instruments, Inc. DEAE-cellulose (DE-23) and phosphocellulose P 11 were purchased from Whatman.
L - (G. - $^3$H) - phenylalanine, 1 Ci/m mol and
L - (5 - $^3$H) - Tryptophan, 21 Ci/m mol were purchased from Amersham.
L - (3,5-$^3$H) - Tyrosine, 40 Ci/m mol was purchased from New England
Nuclear. The remaining $^3$H-amino acids were purchased from Amersham
as labelled amino acid kits.

**Plant Material**

Soybean seeds (Glycine max L. var. Harcor) were imbibed in water
and sown in moist vermiculite. Cotyledons were harvested after 5 days
following germination in the dark at 25-27°C.
METHODS

Preparation of Whole Cell Transfer RNA

Transfer RNA was prepared from total RNA of 5 day old dark-grown cotyledons by the method described by Lester and Cherry (1978) with minor modifications. Extraction buffer was prepared by shaking Tris-HCl buffer $10^{-2}$ M at pH 7.6, containing $6 \times 10^{-2}$ M KCl and $10^{-2}$ M MgCl$_2$ with phenol at the ratio of 10:8 (v/v) for 2-3 hours. The aqueous buffer phase (Buffer A) was separated from the buffer saturated phenol (aqueous phenol). Chilled tissues were ground in 400 g batches with 800 ml buffer A, 50 ml aqueous phenol and 50 ml of 11% duponol (sodium lauryl sulfate). The homogenate was strained through four layers of cheese cloth and the filtrate was mixed with an equal volume of aqueous phenol and stirred in the cold for two hours. This phenol-aqueous extract was centrifuged to separate and recover the aqueous phase which was made 0.2 M with respect to potassium acetate, mixed with two volumes of cold 95% ethanol and stored at $-20^\circ$C overnight. The precipitated material was collected the next day by centrifugation and dissolved in buffer A using 30 ml of buffer for each 100 g of starting tissue. This solution was phenol-extracted four or five times using equal volumes of aqueous phenol. The final aqueous phase was recovered by centrifugation and made 2.0 M with respect to potassium acetate. This solution was allowed to stir for 3 hrs at 4$^\circ$C after which it was centrifuged at 10,000 xg for 15 min. The soluble RNA was precipitated from the supernatant liquid by adding two volumes of cold 95% ethanol
and storing overnight at \(-20^\circ C\). The precipitate was collected by centrifugation and dissolved in 100 ml of buffer B for every 100 g of starting tissue. The composition of buffer B was as follows: \(10^{-2}\) M sodium acetate buffer at pH 4.5 and \(10^{-2}\) M MgCl\(_2\). The resulting suspension was cleared by centrifugation at 10,000 g for 10 min and the supernatant was added to a DEAE-cellulose column containing a 2 ml bed volume for each 100 g of starting material. The DEAE-cellulose column was pre-equilibrated with buffer B. After sample application the column was washed with buffer B containing 0.3 M NaCl until the A\(_{260}\) dropped below 0.02. The soluble RNA was then eluted from the column with 1.0 M NaCl in buffer B. The eluate was dialyzed against cold deionized water and stored at \(-20^\circ C\). This procedure has been outlined in Fig. 1.

Preparation of Chromatographic Adsorbents

DEAE-cellulose was swelled in de-ionized water, and the fines removed by pouring off the supernatant liquid after a reasonable settling period. The remaining liquid was then suction-filtered from the DEAE-cellulose using a Buchner funnel fitted with a miracloth filter. The DEAE-cellulose was then stirred into 15 volumes of 0.5 M HCl, allowed to stand for 30 min and filtered from suspension as above. After washing in de-ionized water until the pH of the suspension was approximately 4, the DEAE-cellulose was stirred into 15 volumes of 0.5 M NaOH, allowed to stand for 30 min and filtered again. The DEAE-cellulose was washed with de-ionized water until the neutral pH of the filtrate was obtained and stored in a 0.02% sodium azide solution at 4\(^\circ C\).
COTTLEONS
Grind in buffer A = 5%
aqueous phenol = dioxane

SOLID (discard) FILTRATE
Add equal vol. of aqueous phenol; stir for 2 hrs at 4°C; centrifuge at 10,000 xg for 20 min.

AQUEOUS PHASE PHENOL PHASE
(discard)
Make 0.2 M with KOAc; add 2 vol.
95% EtOH; store at -20°C; centrifuge at 10,000 xg for 10 min.

SUPERNATANT PELLET
(discard)
Dissolve in buffer A;
extract with aqueous phenol

AQUEOUS PHASE PHENOL PHASE
(discard)
Extract with aqueous phenol 3-4 times

AQUEOUS PHASE
Make 2.0 M with KOAc; stir for 3 hrs; centrifuge at 10,000 xg for 15 min.

PELLET SUPERNATANT
(discard)
Add 2 vol. 95% EtOH;
store at -20°C; centrifuge at 10,000 xg for 15 min.

PELLET SUPERNATANT
(discard)
Dissolve in buffer B; pass through DEAE-cellulose; wash with 0.5 M NaCl
in buffer B; elute with 1.0 M NaCl in buffer B.

ELUATE
Dialyze against desalted water
STORE at -20°C

Figure 1. Extraction of tRNA from the whole cell.
Hydroxylapatite columns were prepared by mixing in the starting buffer, the hydroxylapatite and Whatman CF11 cellulose powder in a 9:1 ratio and packing an appropriate size column. A thin layer (1 cm thick) of cellulose powder alone was layered on the top as well as at the bottom of the hydroxylapatite. Equilibration of the column was accomplished by washing the column with 20 volumes of the appropriate starting buffer.

Sephadex G-25, Sephadex G-150 and Sephacryl S-200 were prepared according to the literature supplied by Pharmacia with the resin and equilibrated with the appropriate starting buffer.

Polyacrylamide Gel Electrophoresis of Native Enzyme

Polyacrylamide gel electrophoresis was performed at pH 8.3 on 5 and 7% acrylamide gel by a modification of the procedure of Jovin et al., (1964). The gels were made from a stock solution containing 20% acrylamide and 0.67% N,N-methylenebisacrylamide. The gel system was buffered in 0.375 M Tris-HCl buffer at pH 8.3 and the polymerization was catalyzed by 0.025% TEMED and 0.05% ammonium persulfate. Samples were concentrated and dissolved in 5 x 10^{-2} M Tris-HCl at pH 8.3 and 2 x 10^{-4} M dithiothreitol. Gels were run at 2 m amp per tube in the cold using bromophenol blue as a tracking dye. The electrophoresis was conducted in a buffer system containing 2 x 10^{-2} M Tris with 11.5 g glycine per 1000 ml at pH 8.3. Following electrophoresis, gels were stained in Coomassie Brilliant Blue. Destaining was carried out in several changes with a solution of 10% acetic acid and 5% methanol.
SDS-Polyacrylamide Gel Electrophoresis

Denaturing SDS gel electrophoresis was performed according to Laemmli (1970). Seven per cent polyacrylamide gels were made from a stock solution containing 30% acrylamide and 0.8% N,N-methylenebis-acrylamide. The gels (slabs of 15 cm x 15 cm and 1.5 mm thickness) were buffered with 0.375 M sodium dodecyl sulfate (SDS). The gel polymerization was catalyzed by 0.025% TEMED and 0.05% ammonium persulfate. Running buffer was 2 x 10^{-2} M Tris containing 11.5 g glycine per 1000 ml and 0.5% SDS. The samples were denatured prior to electrophoresis by incubating them for 2 min in a boiling water bath in 50 mM Tris-HCl at pH 8.3, plus 5% β-mercaptoethanol and 2% SDS. The gels were run at 15 m amp/slab at room temperature using bromophenol blue as the tracking dye. Staining and destaining of the gel was carried out by the same procedure described for native enzyme.

Protein Determination

The protein concentrations were determined according to the method of Lowry et al. (1951) for the estimations during the purification by affinity chromatographic method. For the regular chromatographic method of purification of the enzyme, the protein concentrations were determined using the method described by Bradford (1976).

Assay Procedure

Aminoacylation assays were conducted under the following conditions. Tris-HCl 10^{-5} M at pH 7.8, soluble polyvinylpyrrolidone 0.2% and ³H-
aminoacid 10 μCi/ml, ATP, 2 x 10^{-3} M and 100 μg/ml tRNA and varying concentrations of enzyme. The reaction mixtures (0.2 ml) were incubated at 30°C for 30 min and the tRNA was precipitated with 5% trichloroacetic acid (TCA). The TCA precipitate was then filtered on glass fiber filters (Whatman GF/A) and the radioactivity was counted in a liquid scintillation counter with a toluene based scintillation fluid.

**Preparation of Affinity Column**

Affinity column was prepared mainly based on the method described by Joyce and Knowles (1974) with minor modifications. The strategy adopted in this method involved the separation of cognate from non-cognate tRNAs without the laborious chromatographic procedures. When unfractionated tRNA is aminoacylated with a particular amino acid, the cis-glycol at the 3'-terminus is temporarily masked by aminoacylation in cognate-tRNA, while in the non-cognate tRNAs this feature remains intact. When this total unfractionated tRNA after aminoacylation is subjected to oxidative cleavage by sodium periodate, a dialdehyde is derived only in the non-cognate tRNAs. The resulting dialdehyde can be coupled to a hydrazide derivative of Sepharose 4B and this has been referred to as anti-affinity gel. Since the complete removal of oxidized non-cognate tRNAs cannot be achieved, further participation of these tRNAs can be curtailed by a borohydride reduction of the dialdehyde. Hydrolysis of the aminoacyl group leaves the cognate tRNA susceptible, in its turn, to oxidation and coupling to the carrier matrix. This provides the affinity gel. The mechanism of coupling
of the cognate and the non-cognate tRNAs to the carrier matrix has
been outlined in Fig. 2.

The anti-affinity and affinity columns were prepared as follows:

a) **Aminoacylation of Unfractionated tRNA:**

The tRNA (500 mg) extracted from the dark-grown cotyledons was
aminoacylated under the following conditions: Tris-HCl 10^-5 M at pH
7.8, 0.2% soluble polyvinylpyrrolidone, 2 x 10^{-3} M ATP, 2 x 10^{-2} M
MgCl₂, 25 μCi/ml ³H-phenylalanine (1 Ci/mM) in 10 ml of the reaction
mixture. The rest of the tRNA was aminoacylated with 10^{-4} M L-phenyl-
anine under similar conditions. Aminoacylation was started by
adding the enzyme (0.5 mg protein/ml) fraction obtained after purifying
on DEAE-cellulose. The reaction mixture was incubated at 30°C for
30 min. During incubation the reaction mixture was kept shaking at slow
speed. After the completion of the incubation period, 1.0 M potassium
acetate at pH 5.0 was added to bring the potassium acetate level to
0.2 M in the reaction mixture. Equal volume of water-saturated phenol
was added to the above and the mixture was stirred vigorously for 15
min at room temperature. Then the aqueous phenol mixture was centri-
fuged at 20,000 xg for 30 min. The resulting aqueous phase was saved
and the phenol phase was re-extracted with an equal volume of 1.0 M
potassium acetate at pH 5.0 containing 10^{-2} M MgCl₂ and centrifuged
to recover the aqueous phase. Both the aqueous phases were pooled
and the tRNA was precipitated by adding two volumes of 95% ethanol
and stored at -20°C.
FIGURE 2 - Mechanism of Coupling of tRNA\textsuperscript{Phe} and Non-Cognate tRNA to the Carrier Matrix
b) Oxidation of tRNA

The total tRNA after aminoacylation, deproteinization and precipitation was recovered by centrifugation and dissolved in 0.1 M acetate buffer at pH 5.0 containing $10^{-2}$ M sodium periodate and incubated at room temperature for one hour. Then the incubation mixture was made 0.2 M with respect to KCl by slowly adding solid KCl. The resulting precipitate of unreacted periodate was removed by centrifugation and the supernatant was added with three volumes of 95% ethanol and stored overnight at -20°C. The tRNA was recovered by centrifugation and dissolved in 0.1 M acetate buffer at pH 5.0 containing $10^{-2}$ M MgCl$_2$ (buffer C) and dialyzed extensively against the same buffer in the cold.

c) Anti-affinity Gel

Hydrazinyl Sepharose 4B was prepared by incubating CNBr-Sepharose 4B (from Sigma) with $2 \times 10^{-3}$ M hydrazine in 0.1 M sodium carbonate buffer at pH 10.0 for 12 hrs at 4°C under continuous stirring. Hydrazinyl Sepharose 4B thus obtained was then incubated with oxidized tRNA in buffer C for one hour at 37°C followed by further incubation at room temperature for 15 hours. The gel was then filtered and washed with 1 M NaCl till the $A_{260}$ of the washings fell below 0.02. The anti-affinity gel thus obtained was packed into a column (6 cm x 1 cm), equilibrated with buffer C containing 0.02% sodium azide and kept in the cold until required.
d) **Affinity Gel**

The filtrate and the washings of the anti-affinity gel were pooled, concentrated by vacuum dialysis and incubated with sodium borohydride (5 mg/mg tRNA) in 0.1 M potassium phosphate buffer at pH 7.0 for 2 hours at room temperature. The tRNA preparation was then dialyzed against 0.1 M glycine buffer at pH 10.0 for 5 hours at room temperature for deacylation. The deacylated tRNA was in turn oxidized and bound to hydrazinyl Sepharose 4B according to the method of preparation of the anti-affinity gel. The resulting affinity gel was packed into a pasteur pipette and washed extensively with 1 M NaCl in buffer C containing 0.02% sodium azide and kept in the cold until required.
RESULTS

I. Purification of Phe-\textit{tRNA} Synthetase by Affinity Chromatographic Method

Extraction and purification of the enzyme was performed at 0°C to 4°C. Initial experiments in small scale showed that most of the Phe-\textit{tRNA} synthetase could be extracted from tissue between 30–60% saturation with ammonium sulfate.

a) Homogenization and Ammonium Sulfate Fractionation

Freshly harvested dark-grown cotyledons were chilled on ice and ground in a Waring blender for 15 seconds. The enzyme extraction buffer consisted of $2.5 \times 10^{-2}$ M potassium phosphate at pH 7.8, $10^{-2}$ M β-mercaptoethanol, $10^{-5}$ M PMSF and $10^{-6}$ M L-phenylalanine (buffer D). Typically, three 200 g lots of cotyledons were ground with 1200 ml of buffer D plus 20 g of insoluble polyvinylpyrrolidone (Polyclar AT) each. The homogenate was strained through four layers of cheese cloth and to the resulting homogenate enough solid ammonium sulfate was slowly added to bring it to 30% saturation. After one hour of constant stirring the solution was centrifuged at 13,000 xg for 30 minutes. The clear supernatant was collected through a miracloth filter to remove the floating fat layer. To this filtered supernatant was slowly added enough solid ammonium sulfate to bring it to 60% saturation. The 30–60% fraction was collected by centrifugation at 13,000 xg for 30 minutes. The resulting pellet was dissolved in buffer D, dialyzed against the same buffer overnight and applied onto
a) DEAE-cellulose column.

b) DEAE-Cellulose Chromatography

A DEAE-cellulose column (2.5 x 60 cm) was equilibrated with buffer D and after application of the enzyme the column was washed with buffer D until the A_{280} of the fractions of the washings reached 0.05. These fractions showed negligible levels of enzyme activity. The enzyme from the DEAE-cellulose was then step eluted with 0.1 M potassium phosphate at pH 7.8 in buffer D. The enzyme activity of each fraction was assayed and the results are shown in Fig. 3. The enzyme fractions from the DEAE-cellulose were pooled and filled into dialysis bags. Solid polyethylene glycol (M.W. 20,000) was placed on the dialysis bags to concentrate the enzyme. The dialysis bags were emptied and the contents were centrifuged at 10,000 xg for 10 minutes. The clear supernatant was applied onto Sephadex G 150 column.

c) Sephadex G 150 Chromatography

The Sephadex G 150 column (2.5 x 90 cm) was equilibrated with 2 x 10^{-2} M potassium phosphate buffer at pH 6.5 containing 10^{-2} M β-mercaptoethanol, 10^{-5} M PMSF and 10% glycerol (buffer E). The enzyme was eluted with the same buffer. Each fraction was assayed for enzyme activity and the results are depicted in Fig. 4. The enzyme activity elutes slightly overlapping the first A_{280} peak and therefore the purification was not achieved to a significant level (Table 1), but still this gel filtration step was considered to be essential in order to reduce the protein concentration as far as possible for further fractionation on hydroxylapatite column. Furthermore, the Sephadex G-150 was
FIGURE 3 - DEAE-Cellulose Chromatography of Phe-tRNA Synthetase

Step elution at pH 7.8. Enzyme was prepared from 600 g soybean cotyledons. The protein was adsorbed onto the column after ammonium sulfate fractionation. After washing the column with extraction Buffer D, elution was carried out with 0.1 M potassium phosphate buffer (Buffer D). The flow rate was maintained at 60 ml/hr and 20 ml fractions were collected. A portion of each fraction was diluted 50 times with extraction buffer and assayed for Phe-tRNA synthetase activity. The absorbance at 280 nm (O.D. 280) of the diluted fractions was measured. The arrow pointing downwards shows the point of step elution with 0.1 M Buffer D. The solid line represents the absorbance at 280 nm and the broken line represents Phe-tRNA synthetase activity. The elution profile of the unbound protein from the column is not shown in the figure.
$3^H$-Phe-tRNA ($10^3$ CPM)

FRACTION

$-OD_{280}$
FIGURE 4 - Sephadex G 150 Chromatography of Phe-tRNA Synthetase

The enzyme fractions from DEAE-cellulose column were pooled, concentrated and applied onto the Sephadex G 150 column. Elution was carried at a flow rate of 20 ml/hr and 5 ml fractions were collected. Absorbance at 280 nm (---) and Phe-tRNA synthetase activity (○○○) were recorded for each fraction.
$^{3}\text{H-Phe-tRNA (10}^{3}\text{CPM)}$

![Graph showing fraction vs. $-\text{OD}_{280}$](image-url)
bedded in enzyme buffer at pH 6.5 so that the enzyme was in proper environment for the subsequent step.

d) **Hydroxylapatite Chromatography**

The hydroxylapatite column (20 g hydroxylapatite mixed with 2 g cellulose) was previously equilibrated with 0.2 M potassium acetate at pH 6.5 in Buffer E. The enzyme was adsorbed to the resin and eluted with potassium phosphate gradient of 0.2 - 0.6 M at pH 6.5 in Buffer E. The results are shown in Fig. 5. The enzyme fractions elute from the column overlapping one of the protein peaks.

e) **Anti-affinity Chromatography**

The enzyme fractions from the hydroxylapatite column were pooled and dialyzed against $5 \times 10^{-2}$ M acetate buffer at pH 5.5 containing $10^{-2}$ M β-mercaptoethanol, $10^{-4}$ M EDTA, $10^{-2}$ M MgCl$_2$ and 10% glycerol (Buffer F) in order to bring the enzyme to this environment, and applied onto anti-affinity column previously equilibrated with Buffer F. The elution was performed with Buffer F until the $A_{280}$ of the fractions reached near zero. Further elution of the column was conducted with 0.1 M Tris-HCl at pH 8.0 containing $10^{-2}$ M MgCl$_2$, $10^{-4}$ M EDTA, $5 \times 10^{-3}$ M β-mercaptoethanol, 0.5 M KCl and 10% glycerol (Buffer G). The results shown in Fig. 6 show the profile of elution on this column. Almost all the enzyme activity elutes in Buffer F fractions, and no activity of the enzyme was observed coincident with the protein peak obtained by the strongly bound proteins eluted by Buffer G from the column, thus indicating the purification of Phe-tRNA synthetase from other aminoacyl-tRNA synthetases. When one of the high $A_{280}$ fractions of Buffer G (from the affinity column) was tested
FIGURE 5 - Hydroxylapatite Chromatography of Phe-\(\text{tRNA}\) Synthetase.

The enzyme fractions from Sephadex G 150 column were applied onto the column and elution was carried out with 0.2 - 0.6 M potassium phosphate in Buffer E. The flow rate was maintained at 40 ml/hr and 10 ml fractions were collected. The solid line represents absorbance at 280 nm (O.D. 280) and the broken line represents Phe-\(\text{tRNA}\) synthetase activity.
FIGURE 6 - Anti-affinity Chromatography of Phe-tRNA Synthetase

The enzyme recovered from hydroxylapatite column was dialyzed against Buffer F and applied onto the column. Elution was carried out at a flow rate of 15 ml/hr and 2 ml fractions were collected. The solid line represents absorbance at 280 nm (O.D. 280) and the broken line represents phe-tRNA synthetase activity. The arrow indicates the beginning of the elution with Buffer G.
for the activity of leucyl, isoleucyl, seryl and tyrosyl-tRNA synthetase activities (not shown in Fig. 6) these enzymes were found to be present in substantial quantities.

f) Affinity Chromatography

The enzyme fractions from anti-affinity column were pooled and transferred to affinity column pre-equilibrated with buffer F. Elution was carried out similar to that of anti-affinity column. The results shown in Fig. 7 clearly show the highest activity for Phe-tRNA synthetase in buffer G fractions and no significant activity for the other enzymes tested. No significant activity for Phe-tRNA synthetase was observed in buffer F fractions (not shown in Fig. 7). Therefore the enzyme eluted from the affinity column appeared to be substantially pure. Table 1 summarizes all the purification steps; the unit of enzyme activity being defined as the amount of protein catalyzing the incorporation of 1000 cpm equivalent of 3H-phenylalanine into tRNA per minute.

g) Polyacrylamide Gel Electrophoresis of the Purified Enzyme

Polyacrylamide gel electrophoresis was conducted under non-denaturing conditions as described earlier in the methods section. The protein was stained with Coomassie Brilliant Blue and the gel was scanned at 550 nm in a Gilford spectrophotometer attached to a gel scanner and a Gilford Chart Recorder. The results shown in Fig. 8 indicate that the enzyme preparation obtained from the affinity column is not homogeneous and it contains altogether five proteins strongly bound to the tRNA Phe on the affinity column.
Affinity Chromatography of Phe-tRNA Synthetase

The enzyme fractions from anti-affinity column were pooled and applied onto the column. Elution was carried out at a flow rate of 10 ml/hr and 2 ml fractions were collected. The fractions were assayed for activity with $^3$H-phenylalanine, $^3$H-leucine, $^3$H-isoleucine, $^3$H-serine and $^3$H-tyrosine. The assay reaction mixture contained $10^{-5}$ M Tris-HCl at pH 7.8, $5 \times 10^{-4}$ M Mg$^{++}$, $5 \times 10^{-4}$ M ATP, 0.2% soluble polyvinylpyrrolidone, 0.2 mg/ml tRNA and 20 µl of enzyme from each fraction in 200 µl reaction mixture. The first and the second arrows (from left to right, represent the beginning of elution with Buffer F and Buffer G respectively.)
FIGURE 8 - Densitometric Tracing of the Polyacrylamide Gel Containing Affinity Purified Enzyme Preparation

Electrophoresis of the affinity purified preparation was conducted on 5% polyacrylamide gel at pH 8.3 and at 4°C. The gel was stained with Coomassie Brilliant Blue R 250 and scanned at 550 nm in a Gilford spectrophotometer attached to a gel scanner and a chart recorder.
TABLE 1: AFFINITY PURIFICATION OF PHENYLALANYL-cRNA SYNTHETASE

<table>
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<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Specific Activity</th>
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<th></th>
<th></th>
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<tr>
<td></td>
<td>mg</td>
<td>Units</td>
<td>Units/mg</td>
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<td>Yield %</td>
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<td>Crude extract</td>
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<td>60% (NH₄)₂SO₄</td>
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<td>0.6</td>
<td>1.2</td>
<td>70</td>
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<td>103.5</td>
<td>90.0</td>
<td>180.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>
h) **Gel Filtration Studies**

The purified enzyme from affinity column was subjected to filtration on Sephadex G-150 (Fig. 9) for estimation of the apparent molecular weight of the native enzyme. The gel column (35 x 1.5 cm) was equilibrated with potassium phosphate $10^{-2}$ M at pH 7.5 and the elution was carried out according to Easterday (1973). One ml fractions were collected at a flow rate of one ml per 30 min. The protein standards used were rabbit immunoglobulin G (M.W. 150,000), hemoglobin (M.W. 64,000) and trypsin (M.W. 10,000). The enzyme activity was eluted quite apart from rabbit immunoglobulin G and near to hemoglobin and an apparent molecular weight of 88,000 daltons is derived based on this elution. SDS-polyacrylamide gel electrophoresis could not be conducted with the preparation as this was found to contain more than one protein (Fig. 8). However, SDS gels were run with purified enzyme from regular chromatographic methods (described in the subsequent chapter) and those results in combination with results described in this chapter clearly indicate the subunit structure of the native enzyme.

II **Regular Chromatographic purification of Phe-tRNA Synthetase**

Since the purification of Phe-tRNA synthetase could not be achieved to complete homogeneity by the affinity chromatographic method, conventional methods were employed successfully to achieve complete purification of the enzyme.

a) **Homogenization, Ammonium Sulfate Fractionation and DEAE-Cellulose Chromatography**

The initial steps of purification are very similar to the previous
FIGURE 9 - Gel Filtration Studies With Affinity Purified Phe-tRNA Synthetase

The affinity purified preparation was applied onto a Sephadex G 150 column (35 x 1.5 cm). One ml fractions were collected at a flow rate of 1 ml/30 min. The protein standards used were 1. rabbit immunoglobulin G, 2. hemoglobin and 3. trypsin. The peak of enzyme activity is denoted as E.
procedure with only little modification. In this procedure, 1000 g of starting tissue was used and the ammonium sulfate fractionation and DEAE-cellulose fractionation of the enzyme remained exactly similar. After homogenization in buffer D and ammonium sulfate fractionation, the 30–60% ammonium sulfate fraction was dissolved in buffer D and desalted on a Sephadex G-25 column (5 x 80 cm) pre-equilibrated with buffer D and then adsorbed onto DEAE-cellulose column (5 ml DEAE-cellulose to 100 mg protein) and eluted as described earlier.

b) Sephacryl-S 200 Chromatography

The enzyme fractions from DEAE-cellulose were pooled and concentrated as described earlier and applied onto Sephacryl S 200 column pre-equilibrated with buffer E. Elution was carried out with the same buffer. Fig. 10 clearly shows that Sephacryl-S 200 separated the enzyme much better than Sephadex G-150 (Fig. 4; Table 1). The enzyme eluted between the two major protein peaks and this step has nearly doubled the purification of the enzyme (Table 2).

c) Hydroxylapatite Chromatography

This step of purification was conducted as described in the previous section. Fig. 11 depicts the results of hydroxylapatite fractionation of Phe-tRNA synthetase in this series of purification steps. These results show that the enzyme activity peak is not coinciding with any protein peak (unlike results shown in Fig. 5) and the purification is more than doubled by this step (Table 2). Therefore, the introduction of Sephacryl-S 200 instead of Sephadex G-150 has changed the entire profile of the subsequent step of purification and also resulted
FIGURE 10 - Sephacryl S 200 Chromatography of Phe-tRNA Synthetase

The enzyme fractions pooled from the DEAE-cellulose column were concentrated and applied onto the Sephacryl S 200 column. Elution was carried out at a flow rate of 20 ml/hr and 3 ml fractions were collected. A portion of each fraction was diluted 25 times with Buffer E and assayed with standard reaction mixture for Phe-tRNA synthetase activity. The $A_{260}$ of the diluted fractions is represented by open circles connected by solid line. The broken line represents Phe-tRNA synthetase activity.
FIGURE 11 - Hydroxylapatite Chromatography of Phe-tRNA Synthetase

The enzyme fraction obtained from Sephacryl-5 200 were applied onto the column. Elution was carried out with 0.2 - 0.6 M potassium phosphate in Buffer E. The flow rate was maintained at 40 ml/hr and 3 ml fractions were collected and alternate fractions were assayed for enzyme activity. The solid circles represent $A_{280}$ of the fractions and the open circles represent Phe-tRNA synthetase activity.
in higher purification.

d) **DEAE-Sephadex A 50 Chromatography**

The enzyme fractions from hydroxylapatite were pooled and applied onto a DEAE-Sephadex column (1.5 x 25 cm) equilibrated with 0.1 M potassium phosphate at pH 6.5 in Buffer E. The column was eluted with a gradient of 0.1 - 0.5 M potassium phosphate at pH 6.5 in Buffer E. A single peak of enzyme activity coincident with the second of the three major protein peaks eluted from the column (Fig. 12). Although the enzyme peak and protein peak coincide each other and are distinct from the remaining two protein peaks, the base line of the $A_{280}$ of the fractions is very high and they merge with each other resulting in the incomplete separation of the enzyme from the rest of the proteins.

e) **Phosphocellulose P 11 Chromatography**

Fractions of enzyme activity of DEAE-Sephadex column were pooled and dialyzed against $10^{-2}$ M Tris-Cl buffer at pH 7.5 containing $10^{-2}$ M β-mercaptoethanol and 10% glycerol (Buffer H). The enzyme was then applied onto phosphocellulose column equilibrated with Buffer E. Results in Fig. 13 show that the enzyme eluted coincident with the second of the two protein peaks. The $A_{280}$ of the fractions distinctly form two peaks and the enzyme peak coincident with the protein peak appears to contain Phe-tRNA synthetase purified to homogeneity. The entire purification process is summarized in Table 2. The unit of enzyme activity is defined as the amount of protein catalyzing the incorporation of one p mole L-3H-phenylalanine into tRNA
FIGURE 12 - DEAE-Sephadex - A50 Chromatography of Phe-tRNA Synthetase

The enzyme from hydroxylapatite column was adsorbed onto the column and the elution was carried out at 10 ml/hr. The fractions (3 ml fractions) were assayed for enzyme activity. Solid line connecting the closed circles represent $A_{280}$ values and open circles connected by broken line represent Phe-tRNA synthetase activity.
FIGURE 13 - Phosphocellulose Chromatography of Phe-tRNA Synthetase

Enzyme fractions from DEAE-Sephadex column were applied onto the column after equilibrating the enzyme with Buffer H. Elution was carried out with 0 - 0.3 M KCl in Buffer H and 3 ml fractions were collected. The flow rate was maintained at 10 ml/hr. The solid line joining the closed circles represents $A_{280}$ values and open circles connected by broken line represent Phe-tRNA synthetase activity.
Table 2: Purification of Phenylalanyl-tRNA Synthetase.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (enzyme Units/mg)</th>
<th>Total Activity (enzyme Units)</th>
<th>Purification Fold</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>74,500.00</td>
<td>3.0</td>
<td>223,500</td>
<td>1</td>
<td>100.0</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>1,165.00</td>
<td>133.0</td>
<td>154,945</td>
<td>44</td>
<td>69.0</td>
</tr>
<tr>
<td>Sephacryl-S200</td>
<td>435.00</td>
<td>250.0</td>
<td>108,750</td>
<td>83</td>
<td>48.0</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>129.00</td>
<td>585.0</td>
<td>75,465</td>
<td>195</td>
<td>33.0</td>
</tr>
<tr>
<td>DEAE-Sephadex A50</td>
<td>4.90</td>
<td>1766.0</td>
<td>8,653</td>
<td>588</td>
<td>3.8</td>
</tr>
<tr>
<td>Phosphocellulose PLL</td>
<td>0.54</td>
<td>6685.0</td>
<td>3,609</td>
<td>2228</td>
<td>1.6</td>
</tr>
</tbody>
</table>
per minute. A closer examination of the data presented in Table 2 indicates that very high levels of enzyme purification were obtained only in the last two steps with a substantial decrease in yield. This may be partly due to the loss of enzyme activity because of prolonged storage of the enzyme in the cold and this property of the enzyme is discussed in the chapter on discussion.

f) Polyacrylamide Gel Electrophoresis of the Native Enzyme

The enzyme fraction obtained after phosphocellulose fractionation was concentrated by vacuum dialysis and subjected to polyacrylamide gel electrophoresis in the cold. Staining and the rest of the operations were done as described earlier. Fig. 14 clearly shows that the enzyme is purified to homogeneity. Samples up to 50 µg of protein were loaded onto the gel and electrophoresis was conducted. No additional band(s) of protein was observed.

g) Molecular Weight Determination of the Enzyme Subunit

The molecular weight of the enzyme subunit was determined under denaturing conditions on SDS-polyacrylamide gels with appropriate standards as shown in Fig. 15. The enzyme was found to contain a single subunit of molecular weight 80,000 daltons. In comparison with the results obtained by gel filtration studies (Fig. 9) the enzyme appears to contain a single subunit.

III Kinetic Characterization of Phe-tRNA Synthetase

a) pH Optimum: Fig. 16 shows the activity of Phe-tRNA synthetase when assayed at various pH values ranging between 6 and 8.5. The curve
FIGURE 14 - Densitometric Tracing of the Polyacrylamide Gel Containing Purified Phe-tRNA Synthetase Under Non-Denaturing Conditions

Electrophoresis was conducted on 7% polyacrylamide gel at pH 8.3 and at 4°C. The gel was stained with Coomassie Brilliant Blue R 250 and scanned at 550 nm in a Gilford spectrophotometer attached to a gel scanner and a chart recorder.
FIGURE 15 - SDS-Polyacrylamide Gel Electrophoresis of the Denatured Enzyme

SDS-polyacrylamide gel electrophoresis of the denatured enzyme was conducted on a slab gel (15 x 15 cm and 1.5 mm thickness) containing 7% polyacrylamide and 0.1% SDS. The standards used were the following: *E. coli* polymerase (subunits of M.W. $\alpha$ = 39,000, $\beta$-155,000 and $\beta'$-165,000), $\beta$-galactosidase (M.W. 130,000), phosphorylase b (M.W. 94,000), serum albumin (M.W. 67,000) and carbonic anhydrase (M.W. 30,000). The open square represents the position of Phe-tRNA synthetase subunit on the gel.
LOG MOLECULAR WEIGHT

10^5

10^4

0 0.2 0.4 0.6 0.8 1.0

RELATIVE MOBILITY

RNA POLYMERASE (β' & β)
β-GALACTOSIDASE
PHOSPHORYLASE b
SERUM ALBUMIN
RNA POLYMERASE α
CARBONIC ANHYDRASE
shows a wide pH optimum at about 7.8. Accordingly pH 7.8 was used for all subsequent assays.

b) **ATP and Mg**<sup>++</sup> **Optima**

Fig. 17 and 18 show the activity of the enzyme under different ATP and Mg<sup>++</sup> concentrations. Initial experiments were conducted with ranges of ATP and Mg<sup>++</sup> concentrations varying from 2 x 10<sup>-4</sup>M to 5 x 10<sup>-3</sup>M and 2 x 10<sup>-3</sup>M to 3 x 10<sup>-2</sup>M respectively. Each concentration of ATP tested included all the concentrations of Mg<sup>++</sup> and optimal levels of both ATP and Mg<sup>++</sup> were determined. Fig. 17 shows the aminoacylation reaction of the enzyme under different ATP concentrations with 2 x 10<sup>-2</sup>M<sup>++</sup> (previously determined to be optimal) concentration. There has been a sharp increase in enzyme activity with increase in ATP from 2 x 10<sup>-4</sup>M to 10<sup>-3</sup>M; further increase in enzyme activity at 2 x 10<sup>-3</sup>M ATP was minimal. Similar results were obtained with studies on the effect of Mg<sup>++</sup> concentrations (Fig. 18). These results were obtained with varying Mg<sup>++</sup> concentrations at optimal ATP level as determined earlier. There was only a slight increase in enzyme activity at 2 x 10<sup>-2</sup>M Mg<sup>++</sup> over the immediate lower concentration tested. Therefore, Phe-tRNA synthetase appears to have a considerably broad optimal range for both ATP and Mg<sup>++</sup>.

c) **Determination of Km for Phenylalanine, ATP and tRNA<sub>Phe</sub>**

The initial velocities of the enzyme were determined under conditions of optimal levels of different reaction components as described earlier. The tRNA used for these experiments was obtained from the ethanol fractions after fractionation through B-D - cellulose column.
FIGURE 16 - Aminoacylation Kinetics of Phe-tRNA Synthetase Under the Conditions of Different pH

The enzyme activity was measured in a reaction mixture (0.4 ml) containing optimal levels of various reaction components and varying pH. The enzyme (100 µg/ml) was obtained after affinity purification and the tRNA (0.4 mg/ml) was obtained from dark-grown cell. The reaction was terminated after 10 min of incubation.
$^3$H-Phe-tRNA (10 CPM)
FIGURE 17 - Aminoacylation Kinetics of Phe-tRNA Synthetase Under the Conditions of Different Levels of ATP

The reaction components were similar to those described in Figure 16 except that ATP concentrations varied and the pH was maintained at 7.8. Enzyme (100 µg/ml) was obtained after affinity purification.
FIGURE 18 - Aminoacylation Kinetics of Phe-tRNA Synthetase Under the Conditions of Different Levels of Mg$^{++}$

The reaction conditions were similar to those described in Figure 16 except that Mg$^{++}$ levels varied and pH was maintained at 7.8. Enzyme (100 μg/ml) was obtained after affinity purification. The open and closed circles represent the experiments conducted on two different occasions with enzyme preparations purified by the same procedure.
B-D-cellulose chromatography was performed as described in part II and 1 A₂₆₀ unit of tRNA per ml and 10⁻⁴ M ³H-phenylalanine were used for the assay. Enzyme fractions used for these studies were obtained after fractionation through hydroxylapatite column. Enough enzyme was added to insure that: (1) enzyme concentration was much less than substrate concentration, (2) that only 20% of the reaction was completed in the 3 minute assay period, (3) finally, that the enzyme concentration was in the linear portion of the initial velocity versus enzyme concentration plot. The reaction mixture was placed (0.2 ml) into 18 tubes representing three replications each for 0.5, 1.0, 1.5, 2.0, 2.5 and 3 minutes. A blank tube was also included. At the prescribed time, to each tube was added 1 ml of 5 x 10⁻² M L-phenylalanine in 10% trichloro acetic acid. Subsequently the samples were treated as described earlier to determine radioactivity.

A Lineweaver-Burk plot with phenylalanine as variable substrate gave a Km value for phenylalanine of 4.5 x 10⁻⁷ M (Fig. 19) and with ATP as the variable substrate as shown in Fig. 20, the Km value for ATP was found to be 3.0 x 10⁻⁵ M. While determining Km value for tRNAₚₗₑ the amount of tRNAₚₗₑ in the sample was estimated by aminoacylating the sample to a maximum degree under optimal conditions and at saturating levels of enzyme. The Km value for tRNAₚₗₑ was estimated as shown in Fig. 21 and it was 7.4 x 10⁻⁸ M.

d) Response to Sulphydryl Reagents

Aminoacylation kinetics of Phe-tRNA synthetase under optimal aminoacylation conditions in the presence of different sulphydryl reagents
Initial velocities were determined at various levels of L-phenylalanine (as described in methods). Each point in the figure represents the average of three measurements of initial velocity. Initial velocities are expressed as CPM/min x 10^-3, and substrate concentrations were varied as shown. Enzyme concentration was adjusted to yield 20% of the maximum reaction within 3 min. tRNA (1.0 A260) was obtained from ethanol fractions of B-D-cellulose chromatography.
FIGURE 20 - Apparent Michaelis constant for ATP

The reaction conditions were similar to those described in Figure 19, except that ATP was the variable substrate. Each point in the figure represents the average of three measurements of initial velocity. Initial velocities are expressed as CPM/min x 10^{-3}. 
FIGURE 21 - Apparent Michaelis constant for tRNA$^{\text{Phe}}$.

The reaction conditions were similar to those described in Figure 19. The tRNA$^{\text{Phe}}$ concentration was corrected by determining the maximum amount of tRNA acylatable with $^3$H - phenylalanylamine by the enzyme under optimal conditions. Each point in the figure represents the average of three measurements of initial velocity. Initial velocities are expressed as CPM/min x 10^{-3}. 
FIGURE 22 - *Aminoacylation Kinetics of Phe-tRNA Synthetase in the Presence of Different Sulfhydryl Reagents*

The rate of enzymatic reaction was measured in the presence of glutathione, \( \circ \rightarrow \circ \); dithiothreitol \( \Delta \rightarrow \Delta \) and \( \beta \)-mercaptoethanol \( \square \rightarrow \square \). The enzyme (12 µg) from DEAE-Sephadex fraction was used for these reactions.
is depicted in Fig. 22. β-mercaptoethanol (BME) was found to be the most effective sulfhydryl reagent among the commonly used ones. DTT was found to be comparatively less effective and GSH was the least effective of all the three sulfhydryl reagents tested. Both GSH and BME were found to be inhibitory at higher concentrations while DTT was not found to have inhibitory action even at \( 5 \times 10^{-2} \) M concentration.

e) Thermal Inactivation and Nucleoside Triphosphate Interaction

Thermal inactivation was carried out under the following conditions. The enzyme (12 µg from a DEAE-Sephadex A 50 column) was incubated at 55°C in 10⁻⁵ M Tris-HCl buffer (pH 7.8) containing 2 x 10⁻² M MgCl₂. Before inactivation, the reaction mixture was always allowed to equilibrate in ice for 10 min. After the tubes had been incubated at 55°C for different time intervals they were cooled rapidly in ice and the residual enzyme activity was determined. The thermal inactivation studies with nucleoside triphosphates were conducted at the level of approximately 50% residual activity of the enzyme (incubated for 15 min). The enzyme (5 µg) used in this case was from a phosphocellulose fraction.

Results presented in Fig. 23 illustrate the time course of thermal inactivation of the enzyme. The initial velocity of the reaction was reduced by 50% when incubated for 15 min at 55°C and this loss of activity followed first-order kinetics.

Among the nucleoside triphosphates tested representing both purine and pyrimidine groups, ATP was found to have a stabilizing effect against thermal inactivation of Phe-tRNA synthetase (Table 3) and the presence of this nucleotide resulted in retaining the enzyme activity by about
FIGURE 23. Kinetics of Thermal Inactivation of Phe-tRNA Synthetase

The rate of enzymatic reaction was measured after incubating the enzyme at 55°C for different time intervals. The enzyme used for these reactions (12 µg) was obtained from a DEAE-Sephadex column.
10% over the control. Under similar conditions GTP and CTP were found to have no such stabilizing effect. On the other hand, they were inhibitory to the enzyme action. The inhibitory action of GTP and CTP was further substantiated by studying the kinetics of aminoacylation with unheated enzyme (Table 4). These assays were conducted exactly under similar conditions as it was done with thermal inactivation studies except that the enzyme was from a DEAE-Sephadex fraction and it was not pre-heated before studying its kinetics. For all the studies on kinetics of thermal inactivation and nucleotide inhibition of Phe-tRNA synthetase, the aminoacylation reaction mixture included 10 A<sub>260</sub> units of unfractionated tRNA per ml and other components were as described for the determination of Km values. The inhibition of enzyme activity by GTP and CTP occurred at optimal levels of ATP. Therefore, these nucleoside triphosphates may compete with ATP for the same binding site on the enzyme and thus reduce the rate of the reaction. The stabilizing effect of ATP against thermal inactivation and the inhibitory effect of GTP and CTP was also observed in Leu-tRNA synthetase from Euglena (Krauspe and Parthier, 1975).
**TABLE 3: EFFECT OF NUCLEOSIDE TRIPHOSPHATES ON THE THERMAL INACTIVATION OF PHE-tRNA SYNTHETASE**

<table>
<thead>
<tr>
<th>Nucleoside Triphosphates</th>
<th>$^3$H-Phe-tRNA formed (p mol. min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-incubated with nucleoside triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>12.21</td>
</tr>
<tr>
<td>GTP</td>
<td>10.30</td>
</tr>
<tr>
<td>CTP</td>
<td>9.16</td>
</tr>
</tbody>
</table>

5 µg enzyme (from Phosphocellulose fraction) was incubated at 55°C for 15 min with or without the effectors. When the nucleoside triphosphate was included in the heating mixture, its concentration was maintained at 10 mM during heating and later diluted to 2 mM in the assay mixture. ATP was not added to the assay mixture when ATP was used in the heating mixture since it made up the optimal level (2 mM) for the reaction in the assay mixture. When the enzyme was pre-heated without nucleoside triphosphate, the respective nucleotide was later added (2 mM) to the assay mixture.
### Table 4: Effect of Nucleoside Triphosphates on the Rate of Enzymatic Reaction of Phe-tRNA Synthetase

<table>
<thead>
<tr>
<th>Nucleoside Triphosphates</th>
<th>$^3$H-Phe-tRNA formed (p mol.min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>18.30</td>
</tr>
<tr>
<td>ATP + CTP</td>
<td>13.70</td>
</tr>
<tr>
<td>ATP + CTP</td>
<td>12.66</td>
</tr>
</tbody>
</table>

Assay conditions were similar to Table 2 except that the enzyme was not pre-heated either with or without the nucleoside triphosphates. The nucleoside triphosphates were added (2 mM each) directly to the assay mixture. The enzyme from DEAE-Sephadex fraction (10 μg) was used for these reactions.

### Table 5: Km Values Determined for Phe-tRNA Synthetase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-phenylalanine</td>
<td>$4.5 \times 10^{-7}$ M</td>
</tr>
<tr>
<td>ATP</td>
<td>$3.0 \times 10^{-5}$ M</td>
</tr>
<tr>
<td>tRNA$^{Phe}$</td>
<td>$7.4 \times 10^{-8}$ M</td>
</tr>
</tbody>
</table>
DISCUSSION

When initial experiments were conducted, the enzyme preparation obtained after ammonium sulfate precipitation and DEAE-cellulose fractionation was directly applied onto the anti-affinity column. The anti-affinity gel was rendered useless after a single separation. This was thought to be mainly due to ribonuclease action still persisting in the enzyme preparation. Therefore additional steps of purification were introduced before taking the enzyme to affinity purification. The main drawback of affinity chromatography, particularly with Phe-tRNA synthetase, has been the very property of this enzyme. It has been well known that the substrate specificity of Phe-tRNA synthetase is less exacting than the other aminoacyl-tRNA synthetases (Anderson and Fowden, 1970; Smith and Fowden, 1968; Lea and Norris, 1972). This has been shown with Phe-tRNA synthetases obtained from different sources such as animal, microbial and also from some of the plant sources. The enzyme was shown to mischarge tRNAs of other amino acids such as tyrosine, isoleucine, leucine and threonine (Anderson and Fowden, 1970). The enzyme also reacted with various analogs of phenylalanine. Since separation of cognate tRNA from unfractionated tRNA for the preparation of affinity gel mainly depends upon the fidelity of the enzyme in its substrate recognition properties, Phe-tRNA synthetase, from this point of view, appears to be a less favorable enzyme. However, when the enzyme fractions obtained after separation on the affinity column were assayed for cross-charging abilities with the amino acids known (except threonine) for their cross-
recognition by the enzyme, no activity for these amino acids was observed.

Befero et al., (1974) employed affinity chromatographic procedure for the purification of rat liver Seryl-tRNA synthetase. This enzyme was found to bind very strongly to the affinity gel covalently charged with yeast tRNA deprived of tRNA$_{Ser}$. Such misacylation properties occur more readily in heterologous systems than in homologous ones (Ebel et al., 1973). Since the affinity column is prepared with homologous tRNA$^{Phe}$, in this case such a possibility seems to be remote and the enzyme appears to be free of other aminoacyl-tRNA synthetases. Various tRNA-binding proteins and enzymes involved in general metabolism of tRNA are eliminated by anti-affinity chromatography. Therefore, the additional proteins observed on polyacrylamide gels of the affinity purified enzyme preparation from soybean may be some of the other enzymes involved in tRNA$^{Phe}$ metabolism such as post-transcriptional modifications and also the possibility that these proteins are being involved in other functions of this tRNA cannot be excluded. Dyson et al., (1978) observed an additional protein of unknown function other than Tyrosyl-tRNA synthetase strongly bound to the affinity column prepared with homologous cognate tRNA for the purification of this enzyme from E. coli. Therefore, affinity purification of aminoacyl-tRNA synthetases does not seem to be a very successful method which can replace the conventional chromatographic methods.

In order to achieve purification of Phe-tRNA synthetase to homogeneity, conventional chromatographic methods were employed. The
enzyme purified by this method is composed of a single subunit of molecular weight 80,000. Sephadex G-150 gel filtration studies indicate that the enzymatically active molecule consists of a single subunit. However, the possibility of the enzyme occurring in the cell as aggregates of these subunits can not be ruled out and this has been considered as the general tendency among aminoacyl-tRNA synthetases (Soll and Schimmel, 1974). Phe-tRNA synthetase reported from yeast (Schmidt et al., 1971; Fasolo et al., 1970), rabbit liver (Dufresne, 1974) wheat germ (Carias and Julien, 1976) from lupin (Barciszewski et al., 1979) contain two non-identical subunits. The possibility that the other subunit in soybean enzyme is being lost during purification seems to be remote because the enzyme is in active condition even after final purification. When the specific activity of the purified enzyme from soybean is compared with that of wheat germ phe-tRNA synthetase, soybean enzyme is only seven times less active than its wheat germ counterpart. It has been a general observation with the soybean system that when the enzyme is allowed to stay in the cold for a week or 10 days, there is a gradual loss in the activity of the enzyme. This property of soybean Phe-tRNA synthetase has been found to be a common phenomenon in many of the plant aminoacyl-tRNA synthetases (Weil, 1979). Therefore, the observed low specific activity of the purified enzyme may be due to this phenomenon rather than due to the loss of one of the subunits. In addition, the loss of a subunit of the enzyme is generally expected to reduce the activity of the enzyme (if it retains any activity) to a far greater degree than just seven
times (Lapointe and Soll, 1972). It has also been a general observation that in multi-chain enzymes with dissimilar subunit sizes, no activity of the enzyme is retained when the subunits are separated (Soll and Schimmel, 1974).

Because of low activity of the enzyme after final purification due to the prolonged stay of the enzyme at 4°C, the Km values were determined with enzyme fractions obtained after fractionating through hydroxylapatite chromatography. It is not possible to compare the specific activity of this enzyme with that of the enzymes in other systems at the earlier steps of purification. This is mainly because cotyledons are storage organs and their level of physiological activity is generally much lower than in other systems, and also soybean is known to contain very high levels of storage proteins. Because of these reasons the amount of enzyme per mg protein is generally very low. Furthermore, the elimination of proteins other than the enzyme is always not uniform in all systems at different steps of purification since fractionation through these columns depends on the physical characteristics of each protein.

Reports on molecular weight range for aminoacyl-tRNA synthetases vary from 46,000 (Beikirch et al., 1972) to 270,000 (Fasiolo et al., 1970). The occurrence of single chain enzymes is not uncommon among this group of enzymes as it has been reported in the case of Ile-tRNA synthetase (Arndt and Berg, 1970) and Val-tRNA synthetase from E. coli (Berthelot and Yaniv, 1970) and yeast (Rymo et al., 1972) and E. coli Leu-tRNA synthetase (Hayashi et al., 1970). Single peptide aminoacyl-
tRNA synthetases are also reported for Arg-tRNA synthetase (Mitra and Mehler, 1968) and Asp-tRNA synthetase (Diller and Tener, 1971; Gangloff and Dirheimer, 1973) from yeast. Yeast Tyr-tRNA synthetase (Beikirch et al., 1972) has the lowest molecular weight reported (46,000) for any aminoacyl-tRNA synthetase.

Aminoacyl-tRNA synthetases have been known to require the protection of their sulfhydryl groups for their catalytic activity (Cassio, 1968; Iaccarino and Berg, 1969; Kuo and De Luca, 1969). Carías and Julien (1976) tested GSH, PME and DTT for their effect on purified Phe-tRNA synthetase from wheat germ and their data indicate that DTT completely inhibits the enzyme activity at 20 mM whereas GSH enhances the activity at that concentration and PME imparts slight inhibition at that level. Phe-tRNA synthetase from soybean exhibits an entirely different response to these sulfhydryl reagents. Km values for wheat germ Phe-tRNA synthetase are $6.6 \times 10^{-6}$ M and $2.7 \times 10^{-6}$ M for L-phenylalanine and tRNA$^{\text{Phe}}$ respectively. For lupin Phe-tRNA synthetase, the Km values are $0.9 \times 10^{-6}$ M, $4.7 \times 10^{-7}$ M and $0.3 \times 10^{-4}$ M for L-phenylalanine, tRNA$^{\text{Phe}}$ and ATP respectively (Barciszewski et al., 1979). Therefore, there appears to be a substantial difference in the properties of Phe-tRNA synthetase depending on the source of the enzyme even within the group of higher plants.

There has been a large variation in the nature and properties of aminoacyl-tRNA synthetases among various organisms studied (Soll and Schimmel, 1974) whereas there appears to be a conservation of structures among tRNAs (Barciszewski et al., 1979; Singhal and Fallis, 1979). The higher conservation of tRNA structures as compared to synthetases
was speculated by Barciszewski et al., (1979) as due to the result of the multiple biological functions of tRNAs that may place greater constraints on their evolution.
PART II

LOCALIZATION OF CYTOPLASMIC, CHLOROPLASTIC AND MITOCHONDRIAL
tRNA^Phè, tRNA^Trp and tRNA^Tyr AND CORRESPONDING SYNTHETASES
INTRODUCTION

The most sensational event in the history of biological sciences after the establishment of the evolutionary theory has been the elucidation of the "flow" of genetic message in the cell at the molecular level. The information flow from DNA to messenger RNA by transcription and the translation of messenger RNA into proteins, the mechanism by which genetic information is transformed into enzymatic activity, determining the destiny of the cell has been the focus of attention for the past twenty-five years. Although the overall control of the cell by nuclear material through "information transfer" was established beyond doubt, the hereditary continuity of plastids and mitochondria raised the possibility that these organelles possess their own genetic systems and to the supposition that these organelles contain their own genes.

The first report on organellar DNA was made by Ris and Plaut (1962) from the chloroplast of Chlamydomonas. At the same time Lyttleton (1962) reported the isolation of ribosomes from spinach chloroplasts, and he also showed that chloroplastic ribosomes have a lower sedimentation constant than cytoplasmic ribosomes. The possibility that the chloroplast soluble RNAs differed from plant cytoplasmic soluble RNAs was verified by comparison of the MAK elution profiles of total leaf tRNA, of chloroplast tRNA and of root RNA from Vicia faba (Dyer and Leech, 1968). Subsequently, the existence of chloroplastic tRNAs was demonstrated in Euglena (Barnett et al., 1969), and bean chloroplasts (Burkard et al., 1969). In addition, the
capacity of light to induce the formation of chloroplastic tRNA\(^{\text{Phe}}\), tRNA\(^{\text{Glu}}\) and tRNA\(^{\text{Ile}}\) was demonstrated (Barnett et al., 1969) in Euglena, further substantiating the genetic autonomy of chloroplasts. The existence of formylatable tRNA\(^{\text{Met}}\) in bean chloroplasts (Burkard et al., 1969) and the studies on the initiation of polypeptide synthesis in spinach chloroplasts (Bianchetti et al., 1971) have conclusively proved the prokaryotic nature of this organelle. The occurrence of chloroplast specific aminoacyl-tRNA synthetases and their properties were later reported both in Euglena and higher plant chloroplasts (Parthier et al., 1972; Burkard et al., 1974; Kislev et al., 1972). Although the occurrence of chloroplast specific aminoacyl-tRNA synthetases has been shown in many cases, at least some of these enzymes were found to be the transcriptional products of nuclear genes and they are probably "imported" into the chloroplasts from the cytoplasm (Hecker et al., 1974).

DNA was first detected in plant mitochondria by cytological methods (Kislev et al., 1965). The first experimental evidence for the occurrence of tRNAs in mitochondria was provided by Barnett and Brown (1967) from Neurospora. Subsequently, mitochondrial tRNAs were studied in yeast (Casey et al., 1974; Halbreich and Rabinowitz, 1971; Martin et al., 1976), Tetrahymena (Chiu et al., 1974, Chiu et al, 1975), insects (Fradin et al., 1975; Feldman and Kleinow, 1976), Xenopus (Dawid, 1972; Dawid and Chase, 1972), rodent liver (Leitman, 1968; Wallace and Freeman, 1974; Chia et al., 1976), cultured mammalian cells (Attardi and Attardi, 1969; Dubin and Friend, 1974), lupin
(Augustyniak and Pawelkiewicz, 1978), Phaseolus (Guillemaut et al., 1972) and soybean (Meng and Vanderhoef, 1972). Formylmethionyl-tRNA has been identified in mitochondria from yeast, rat liver, human tissue culture, Neurospora and Phaseolus (Smith and Macker, 1969; Gralper and Darnell, 1969; Epler et al., 1970; Guillemaut et al., 1972).

Cytoplasmic aminoacyl-tRNA synthetases from rat liver were found to be highly specific for cytoplasmic tRNAs, with 17-75 times more activity toward the homologous molecules than the mitochondrial tRNAs. On the other hand, mitochondrial enzymes were equally active with tRNAs from either of the cell compartments (Buck and Nass, 1969). HeLa cell cytoplasmic and mitochondrial aminoacyl-tRNA synthetases studied by Lynch and Attardi (1976) did not show any such distinct pattern in their substrate recognition properties and thus differed from rat liver enzymes (Buck and Nass, 1969). Mitochondrial and cytoplasmic Valyl-tRNA synthetases in Tetrahymena were found to be similar in their substrate recognition properties as well as in their chromatographic behaviour and other kinetic properties (Suyama and Hamada, 1968).

Much of the information available on mitochondrial tRNAs and aminoacyl-tRNA synthetases was derived from studies on organisms other than plants. Very little is known about the molecular biology of plant mitochondria and this may be partly due to the problems involved in obtaining plant mitochondria in high yield.

Various aspects of organelar tRNAs and aminoacyl-tRNA synthetases have been
discussed in recent reviews (Barnett et al., 1978; Lea and Norris, 1977; Weil, 1979; Bryant, 1976). Of all the different plant nucleic acids, tRNAs have attracted the attention of a vast number of investigators and an extensive survey on tRNAs and aminoacyl-tRNA synthetases have been made from plants belonging to different groups. With the advent of improved chromatographic techniques, tRNAs were found to occur in multiple species specific for most amino acids and it was also gradually established that some of these species are located in the organelles. This aspect has been reviewed very recently (Weil, 1979). Ever since the first elucidation of the nucleotide sequence of tRNA$^{\text{Ala}}$ by Holley (1965) and coworkers, an astonishingly large number of tRNAs (178 tRNAs until 1979) have been sequenced from various sources (Singhal and Fallis, 1979) including some from the organelles. However, very few tRNAs from plant sources have been sequenced. The following are the plant tRNAs that have been sequenced; tRNA$^{\text{Gly}}$ from wheat germ (Marcu et al., 1977) tRNA$^{\text{Met}}$ from Scenedesmus obliquus (Olin and Jones, 1978) tRNA$^{\text{Phe}}$ from wheat germ (Dudock et al., 1969) from Euglena gracilis chloroplasts (Chang et al., 1976) from peas (Everett and Madison, 1976), from barley embryos (Janowicz et al., 1979), from lupin seeds (Rafalski et al., 1977) and from bean chloroplasts (Guillemont and Keith, 1977).

Investigations in our laboratory (Shridhar and Pillay, 1976; Patel and Pillay, 1976; Pillay and Gowda, 1977, 1980; Pillay and Cherry, 1974) and other laboratories (Wright et al., 1973; Bick et al., 1970; Bick and Strehler, 1971; Cherry and Anderson, 1972) have estab-
lished that changes in the levels of tRNAs and aminoacyl-tRNA synthetases occur during development and senescence in plants. During the course of our investigations on senescence in soybean, such changes were found to be drastic in tRNA$^{\text{Leu}}_{5,6}$ isoacceptors resulting in the complete disappearance of these two species in the senescent cotyledons and the tRNA$^{\text{Leu}}_{5,6}$ were later found to be located in the chloroplasts (Shridhar and Pillay, 1976; Sinclair and Pillay, 1980). Similar observations are also made in tRNA$^{\text{Tyk}}$. (Pillay and Gowda, 1977, 1980). Quite interestingly, the levels of few tRNAs and some aminoacyl-tRNA synthetases such as Seryl-, Glycyl-, Methionyl- and all the three aromatic aminoacyl-tRNA synthetases were found to be increasing during senescence while the others were in the decline (Pillay and Gowda, 1977, 1980). Since the last three enzymes aminoacylate the tRNAs with a group of amino acids which are strongly hydrophobic in nature, they are bound to bring about a drastic change in the nature of proteins synthesized during senescence. It is also of interest to point out that during plant senescence, a gradual loss in some of the membrane lipids was observed (Ferguson and Simon, 1973) resulting in the partial dissolution of membranes causing leakage of intra-cellular substances.

The fact that chloroplastic tRNAs are degraded during early stages of senescence is tempting to speculate that these organellar tRNAs are exposed to cytoplasmic nucleases due to membrane dissolution. Since cytoplasmic tRNAs are always exposed to these nucleases they might be more resistant to cytoplasmic nucleases probably by post-
transcriptional modifications. Chloroplastic tRNAs which are always physically protected against these nucleases by the chloroplast envelope may not have modified bases in suitable positions so as to render the cytoplasmic nucleases less effective and this may result in the early degradation of chloroplastic tRNAs during senescence. Therefore, the following sequence of events can be speculated. The increase in the levels of aromatic aminoacyl-tRNA synthetases may bring about the synthesis of hydrophobic proteins which may act on membrane lipids. The concomitant dissolution of intracellular membranes may cause the early degradation of chloroplastic tRNAs during senescence. These speculations tempted us to undertake more detailed investigations on the tRNAs and aminoacyl-tRNA synthetases of aromatic amino acids from the three cell compartments as a basis for further investigations on the molecular mechanism of senescence at the translational level.

When the literature on organellar tRNAs and aminoacyl-tRNA synthetases for this group of three amino acids was surveyed, the total absence of information on organellar Trp-tRNA synthetases and mitochondrial tRNA_{Trp} was glaring. Information on higher plant mitochondrial tRNA_{Phe} was also not available until 1978 (Jeannin et al., 1978). Information on plant mitochondrial Tyr-tRNA synthetase was also lacking. After the initial report on mitochondrial tRNA_{Tyr} (Meng and Vanderhoef, 1972), a second report on chloroplastic tRNA_{Tyr} and Tyr-tRNA synthetase was made in 1978 (Locy and Cherry, 1978). Although both these reports on tRNA_{Tyr} are from soybean, there were some
inconsistencies. Whole cell tRNA\textsuperscript{Tyr} was resolved into four iso-
acceptors in both the cases. Meng and Vanderhoef (1972) located
one species in the mitochondria and the remaining three species
in the cytoplasm. These authors do not make any comment on chlor-
plastic species. Locy and Cherry (1978), on the other hand, located
two species in the cytoplasm and the remaining two in the chloroplasts
without making any reference to the mitochondrial species. Therefore,
detailed and comprehensive studies on tRNAs and aminoacyl-tRNA
synthetases of aromatic amino acids from the three cell compartments
were felt most essential to fill the gap in the knowledge on this
important group of tRNAs.

It has been established that coding properties of plant tRNAs are
similar to E. coli tRNAs and this confirms that the genetic code is
universal (Weil, 1979). According to the established genetic code,
tRNA\textsuperscript{Phe} recognizes two codons (UUU and UUC); tRNA\textsuperscript{Tyr} has a single
codon (UGG) and tRNA\textsuperscript{Tyr} recognizes two codons (UAC and UAU). Codon
recognition studies were conducted with cytoplasmic and chloroplastic
tRNA\textsuperscript{Phe} (Ramiassa et al., 1977) and the results indicated that these
tRNAs\textsuperscript{Phe} recognize different codons. Similar studies have not been
conducted so far with tRNA\textsuperscript{Tyr} from any plant source and in addition,
no such studies were made with plant mitochondrial tRNAs. Since
tRNA\textsuperscript{Tyr} recognizes two codons and this tRNA appears to have a regulatory
role during senescence (Pillay and Gowda, 1977, 1980) the need to
study the coding properties of tRNA\textsuperscript{Tyr} was felt compelling. Studies
were undertaken on this line in the present report.
MATERIALS

Chemicals: described in Part I

Plant Material

Soybean seeds (*Glycine max* L., var. Harcor) were imbibed in water for 4-5 hours and planted in moist vermiculite in shallow plastic pans. The pans were kept in the dark at 24-25°C for 6 days and the hypocotyls were harvested under dim red light, directly into ice and used immediately. For the light-grown seedlings, the pans containing the seeds were kept in growth chambers under the conditions of 12 hour daily light period for two weeks. The vermiculite was always kept moist by frequent watering. The green leaves were harvested along with the axes above the level of the cotyledons and used immediately.

E. coli

Two strains of *E. coli* were used. The BL 15 F-RNase rel (λ) strain was a gift from Dr. F.W. Studier of Brookhaven National Laboratory. This strain was used for the extraction of aminoacyl-tRNA synthetases and the strain FDA (ATCC 25922) was used for the isolation of ribosomes. Both the strains were grown in Trypticase soy broth (purchased from Baltimore Biological Laboratory) containing 1.7% trypticase; 0.3% soy peptone; 0.5% sodium chloride; 0.25% dipotassium phosphate and 0.25% dextrose. The cells were grown at 37°C and harvested in mid-log phase and washed with either synthetase extraction buffer or ribosome extraction buffer. The cells were then either used
immediately or stored at -20°C till required.

METHODS

Transfer RNA

The transfer RNAs from the dark-grown cotyledons and light-grown shoots were isolated as described in Chapter I. The tRNAs from the chloroplasts and mitochondria were isolated essentially according to the method of Burkard, et al., (1970) with minor modifications. The extraction buffer consisted of 10⁻² M Tris-HCl, (pH 7.4); 10⁻² M MgCl₂ and 1% SDS. For a preparation of organelles from 1000 g of plant material, 60-80 ml of extraction buffer was used. The chloroplast or the mitochondrial pellets were quickly dissolved in a small quantity of extraction buffer (about 5 ml) and poured into cold phenol. The phenol was saturated with water and contained 0.2% 8-hydroxyquinoline. The chloroplast or mitochondrial suspension was added while the phenol was kept stirring. The final ratio of extraction buffer to the phenol was maintained at 1:1 by volume. The mixture was kept stirring for 30 min in the cold and then centrifuged at 5,000 xg. The aqueous phase was removed with a syringe and the phenol phase was again extracted with an equal volume of extraction buffer. The aqueous phase from both the extractions were pooled and re-extracted with an equal volume of phenol and the aqueous phase was recovered. This aqueous phase was mixed with a 20% solution of potassium acetate at pH 5.0 and the final concentration of potassium acetate was brought to 2%. To this solution two volumes of cold 95% ethanol were added and stored overnight at -20°C. The
precipitate was collected by centrifugation and dissolved in 1 M cold NaCl. The resultant turbid solution was centrifuged at 5,000 xg for 5 min and the supernatant was collected and diluted with Tris-HCl at pH 7.5 to bring the Tris concentration to $10^{-2}$ M and the NaCl concentration to 0.2 M. This solution was also made $10^{-3}$ M with respect to MgCl$_2$ and DNase was added to bring the level of this enzyme to 10 µg/ml. The reaction mixture was incubated at 4°C for 90 min. The extraction mixture was then passed through a DEAE-cellulose column (3 ml bed volume for the extraction from 1000 g plant material) previously equilibrated with 0.1 M Tris-HCl at pH 7.5. The column was washed with the same buffer till the $A_{260}$ reached 0.05 and then eluted with 1 M NaCl. The fractions containing high $A_{260}$ were pooled and mixed with 2 volumes of cold 95% ethanol and stored overnight at -20°C. The precipitate was collected by centrifugation, dialysed against cold deionized water and stored in the freezer at -20°C.

Isolation of Chloroplasts

Chloroplasts were isolated from freshly harvested green leaves according to the method described by Hermann et al., (1975) with minor modifications. The extraction medium consisted of Tris-HCl at pH 8.0, $5 \times 10^{-2}$ M, EDTA $3 \times 10^{-3}$ M, β-mercaptoethanol $10^{-3}$ M, mannitol 0.3 M and bovine serum albumin 0.5%. Each 100 g of leaves were ground with 400 ml cold extraction medium in a Waring blender for 10 sec and the slurry was passed through nylon cloth of 50 µm mesh size by gently squeezing. The filtrate was passed through a series of nylon cloths of 25 µm and 10 µm mesh size respectively. The
final filtrate was centrifuged at 1000 xg for 90 sec and the supernatant was poured out. The pellet was dissolved in the extraction medium (10 ml per 100 g tissue) and again centrifuged at 1000 xg for 90 sec. The resultant chloroplast pellet was either stored at -20°C or used immediately if it was meant for the isolation of enzymes.

**Isolation of Mitochondria**

The mitochondria were isolated according to the method of Guillemaut et al., (1972). The extraction medium consisted of 0.7 M mannitol, 10^{-3} M EDTA, 4 x 10^{-4} M ATP and 1 mg/ml bovine serum albumin. The pH of the extraction medium was adjusted to 7.2 with triethanolamine. Freshly harvested hypocotyls were ground in batches of 200 g in a Waring blender with 200 ml of the extraction medium. The homogenate was passed through nylon cloths of 50 μm and 25 μm mesh size respectively and centrifuged at 100 xg for 5 min. The supernatant was transferred to fresh centrifuge tubes. A sucrose cushion consisting of 27% sucrose, 10^{-4} M EDTA and 2 mg/ml bovine serum albumin, pH 7.2 (adjusted with triethanolamine) was gently introduced below the extract. The tubes were then centrifuged at 8000 xg for 10 min. The resulting mitochondrial pellet was either used immediately or stored in the freezer at -20°C. The entire process was done in the cold and completed within 30 min.

**Purity of Organelles**

The purity of the chloroplast preparation was checked under a light microscope. Care was taken to ensure that the preparation was not contaminated by intact cells.

The intactness of mitochondria was determined by biochemical
methods. The rate of oxygen consumption at 30°C by mitochondrial suspensions (in Na-phosphate buffer 10^{-2} M at pH 7.2, KCl 10^{-2} M, MgCl_2 5 \times 10^{-3} M, mannitol 0.3 M and 0.1% bovine serum albumin) was measured polarographically using a Clark type oxygen electrode connected through an oxygen monitor (Yellow Springs Instruments, model 53) to a Heath chart recorder (Model SR-201A). The electrode was calibrated with air-saturated distilled water at 30°C and the dissolved oxygen content of the incubation medium was determined according to Beechy and Ribbons (1972). Rates of oxygen consumption was measured according to Douce et al., (1977).

The acceptor control ratio (ACR) was measured (Estabrook, 1967) in order to determine the intactness of mitochondria. With pyruvate as the substrate, the ACR recorded was 1.97 and with succinate the ratio was found to be 2.40. (Since the reaction medium did not contain thiamine pyrophosphate, ACR with pyruvate was lower than expected).

**E. Coli Ribosomes**

Extraction of ribosomes was carried out at 4°C. The *E. coli* cells (strain FDA) growing in mid-log phase were collected by centrifugation and washed through a buffer consisting of 10^{-2} M Tris-HCl at pH 7.5, 10^{-2} M MgCl_2, 6 \times 10^{-2} M KCl and 6 \times 10^{-3} M β-mercaptoethanol (this buffer will be referred to subsequently as ribosome extraction buffer). The cells were disrupted by grinding with twice their weight of type 305 Alumina (from Sigma) at 4°C for 15 min. The resulting paste was suspended in ribosome extraction buffer (three times the cell wet
weight) and centrifuged at 30,000 xg for 20 min. The supernatant was decanted and the pellet was again ground and re-extracted. The supernatants were pooled and centrifuged at 30,000 xg for 60 min. The final supernatant (S-30 fraction) was centrifuged at 105,000 xg for two hours in a Beckman 60 Ti rotor to pellet the ribosomes. The ribosomes were washed by being suspended in ribosome extraction buffer by gentle homogenization followed by centrifugation again. The final ribosome pellet was suspended in ribosome extraction buffer and used immediately.

Two-Dimensional Polyacrylamide Gel Electrophoresis of Chloroplast tRNA

The following was the composition of the polyacrylamide gel in the first dimension: 10% acrylamide, 0.4% N,N'-methylenebisacrylamide, 4 M urea in 0.1 M Tris-Borate buffer at pH 8.3, consisting of 4 x 10^{-6} M EDTA. The acrylamide gel was polymerized with 0.001% ammonium persulfate and 1 μl/ml N,N,N',N'-tetramethylethlenediamine (TEMED). Two glass plates of 40 cm x 20 cm were taken and two narrow strips of glass plates of the dimension 40 cm x 3 cm and 4 mm thickness were placed in between the larger glass plates lengthwise along their edges by applying silicone grease. Two pairs of binder clips were placed along the edges to keep the glass spacers in position. The apparatus was transferred to the electrophoresis stand made up of plexiglass and placed vertically by introducing one of the open ends into a small plexiglass boat. The inner dimensions of the boat were 25 cm x 2 cm x 2 cm:

A small portion of the 10% acrylamide gel was poured into the boat
and allowed to polymerize. Then the separation gel was poured into the space between the glass plates leaving the top 5 cm, and allowed to polymerize. A starting gel of the following composition was prepared: acrylamide 5%, N,N'-methylenebisacrylamide, 0.4%, 4 M urea, 2.5 x 10^{-3} M Tris-HCl buffer at pH 6.4. The polymerization conditions were similar to that of the separation gel. The starting gel was poured over the separation gel leaving a space of 4 mm on the top. A wedge (2 cm x 1 cm x 0.2 cm) was placed between the glass plates on the top to create space in the gel for sample application. The set up was removed from the polymerization boat and transferred to the buffer holding tank and electrophoresis buffer was poured into the tank. The electrophoresis buffer consisted of 0.1 M Tris-borate at pH 8.3 containing 4 x 10^{-4} M EDTA. The tRNA sample (200 μg) was mixed with the sample buffer containing the marker dye xylene cyanole FF. The composition of the sample buffer was as follows: 60% sucrose, 4 M urea, 0.1 M sodium acetate buffer at pH 4.5 and 1% xylene cyanole FF. One volume of the sample buffer was mixed with 2 volumes of the tRNA solution. The sample (80-100 μl) was introduced into the sample space in the starting gel. The top of the gel was connected to the upper electrophoresis tank by means of a wick created by a double fold of Whatman No. 3 chromatographic paper. The upper and lower electrophoresis tanks were connected to the power supply unit and the electrophoresis was conducted in the cold at 450 volts.

The gel was removed after the marker dye reached the bottom edge of the gel which usually took 40 hours. A narrow strip of 3 cm width
was cut out lengthwise from the gel enclosing the marker dye at the bottom and the point of origin of the sample at the top. This strip of the gel was further trimmed by removing the top portion and retaining only the bottom 23 cm portion for the second dimension.

The gel strip from the first dimensional run was placed horizontally in between two glass plates of 30 cm x 30 cm dimension separated by spacers of 0.4 cm thickness as in the previous case. A 20% acrylamide solution was poured into this set-up till it covered the entire strip leaving only 0.4 cm at the top. The composition of the 20% acrylamide gel was as follows: 20% acrylamide, 0.8% NN'-methylenebisacrylamide, 4 M urea in 0.1 M Tris-borate buffer at pH 8.3. The polymerization conditions and electrophoresis buffer were similar to that of the first dimensional run.

Electrophoresis was conducted in the cold at 350 volts for 140 hours. The gel was then removed from the plates and immersed in a 0.2% methylene blue in 0.2 M sodium acetate buffer at pH 4.5. After one hour of staining at room temperature the gels were destained in running tap water for 15-20 hours. The tRNAs appeared as blue spots.

Preparation of Ribosomal RNA

The total RNA was extracted from the cotyledons using the phenol extraction method as described in Part I and the RNA was dissolved in 1 M NaCl. The turbid solution obtained was centrifuged and the pellet was re-extracted 2-3 times with 1 M NaCl. The final pellet was dissolved in 1 M NaCl and the RNA was precipitated with 2 volumes 95% ethanol. The
precipitate was dissolved in a small quantity of water and passed through a Sephadex G-100 column (60 cm x 2 cm) at room temperature. Elution was carried out with water. The fractions containing the highest A$_{260}$ values were pooled and the RNA was precipitated with 2 volumes of 95% ethanol and stored at -20°C.

**Isolation and Partial Purification of Phe-Tyr- and Trp-tRNA Synthetases**

The aminoacyl-tRNA synthetases of phenylalanine, tyrosine and tryptophan were partially purified from dark-grown cotyledons, light-grown shoots, chloroplasts and mitochondria. The method employed and the buffers used were similar to that of the purification of Phe-tRNA synthetase from the dark-grown cotyledons described in Part I, with only minor modifications. The extraction medium contained 25 mM potassium phosphate buffer at pH 7.8, $10^{-2}$ M 2-mercaptoethanol, $10^{-5}$ M phenylmethylsulfonyl fluoride (PMSF), 0.2% triton-X 100 and 20% glycerol.

Freshly harvested light-grown shoots were homogenized in the above mentioned buffer. Insoluble polyvinyl pyrrolidone was added to the proportion of 10% by weight of the plant material. After grinding, the extract was filtered through cheese cloth and the filtrate was subjected to ammonium sulfate precipitation. The 30%-60% ammonium sulfate precipitate was dialysed against the extraction buffer without triton-X 100 and subjected to fractionation through DEAE-cellulose as described in Part I. The enzyme from dark-grown cotyledons was extracted with no triton-X 100 in the extraction medium.

The enzyme fraction obtained from the DEAE-cellulose column was
either used directly for charging the tRNA or fractionated further through HA column.

The method employed for the isolation of aminoacyl-tRNA synthetases from chloroplasts and mitochondria was similar. The chloroplast or mitochondrial pellets were dissolved in the extraction medium and stirred for 30 min in cold and fractionated further on DEAE-cellulose column as described in Part I.

**Preparation of Aminoacyl-tRNA Synthetases from E. Coli**

Aminoacyl-tRNA synthetase fraction from _E. coli_ was prepared essentially according to Weil (1969).

_E. coli_ cells (BL15 F'-RNase-rel (λ) strain) were grown as described before. About 20 g of cells suspended in 20 ml extraction buffer were crushed in a French press at 14,000 psi in the cold. The extraction buffer contained the following ingredients: Tris-HCl 0.1 M at pH 7.8, MgCl₂ 10⁻² M, KCl 6 x 10⁻² M, glutathione (GSH) 10⁻³ M and 10% glycerol. The homogenate was centrifuged at 27,000 xg for 15 min and the supernatant was collected and again centrifuged for 2 hrs at 105,000 xg. This post-ribosomal supernatant was incubated with DNase (5 μg/ml) for 1 hr at 4°C.

The enzyme extract was then applied onto a DEAE-cellulose column (10 cm x 2 cm) previously equilibrated with the extraction buffer. Elution was carried out with 0.15 M NaCl in the extraction buffer. The _A₂₈₀_ of the fractions were monitored and each fraction was assayed for the enzyme activity with _E. coli_ tRNA. The fractions containing
the enzyme activity were pooled, made 50% with respect to glycerol and stored in the freezer at -20°C.

**Aminoacylation with E. Coli aminoacyl-tRNA Synthetase**

Aminoacylation of the tRNAs from *E. coli*, mitochondria and chloroplasts was carried out with the enzyme preparation from *E. coli*. The reaction medium consisted of the following components: Tris-HCl, $5 \times 10^{-2}$ M at pH 7.4, ATP $10^{-2}$ M, GSH 2.5 mg/ml, KCl $3 \times 10^{-2}$ M, bovine serum albumin 3 mg/ml, MgCl$_2$ $10^{-2}$ M, $^3$H-amino acids 5 µCi/ml, enzyme 0.5 mg protein/ml and varying concentrations of tRNAs. The reaction mixture was incubated at 37°C for 30 min in a water bath. The radioactivity incorporated into tRNAs were counted either by the method of Mans and Novelli (1961) or by precipitating the tRNAs with 5% trichloracetic acid and filtering on glass fiber filters.

**Aminoacylation Assays**

Aminoacylation assays were conducted under the following conditions. For all the three aromatic aminoacyl-tRNA synthetases, the buffer concentration (Tris-HCl, $10^{-5}$ M at pH 7.8), soluble polyvinylpyrrolidone (0.2%), and the $^3$H-amino acids (10 µCi/ml) remained the same. ATP and MgCl$_2$ concentrations were maintained for each enzyme as described in Table 1; tRNA and enzyme concentrations varied. The reaction mixture (0.2 ml) was incubated at 30°C for 30 min. The reaction was terminated by adding equal volume of 10% trichloracetic acid and filtered on glass fiber filters (Whatman GF/A) and radioactivity on the filters was counted.
Synthesis UAC and UAU Codons

The UAC and UAU triplets were synthesized according to the method of Thach and Doty (1965) with minor modifications. The reaction mixture (0.1 ml) was composed of the following: $10^{-5}$ M uridine diphosphate or cytidine diphosphate, $7.5 \times 10^{-3}$ M uridylyl - (3'→5') adenosine, $10^{-2}$ M magnesium acetate, 0.2 M glycine buffer at pH 9.3, $10^{-4}$ M CuSO$_4$, 0.4 M NaCl and 0.2 mg/ml polynucleotide phosphorylase. The reaction mixture was incubated at 35°C for 12 hours. The reaction mixture was heated at 100°C for 1 min to denature the enzyme and it was then treated with 20 ug of alkaline phosphatase for 2 hours at 37°C. The reaction mixture was then subjected to thin-layer chromatography on PEI-cellulose. The chromatograms were developed in a solvent system consisting of equal parts of 95% ethanol and 1 M ammonium acetate. The products were localized under ultra-violet light.

The reactants were also subjected to separation on the chromatogram for comparison. The traces of high polymers formed were found trailing from the point of origin. The nucleoside monophosphates created by the action of alkaline phosphatase were localized near the solvent front. The major new spots which were the synthesized trinucleotides due to the NaCl-inhibition of the enzyme were cut out from the chromatogram, washing it first with absolute ethanol to remove the salt and then with ether. The PEI-cellulose powder was then eluted with water and the UV absorbing eluate was lyophilized and stored at -20°C. A small portion of the synthesized product was subjected to alkaline hydrolysis in one of the cases (UAC). Three individual spots (U, A and C) were observed.
on the chromatogram developed with the product of alkaline hydrolysis.

**Benzoylated-DEAE (B-D) Cellulose Chromatography**

B-D cellulose chromatography was performed according to Gillam et al., (1967). B-D cellulose (Schwartz-Mann) obtained as a slurry in 2 M NaCl was packed into a column (25 cm x 2.5 cm) half filled with 2 M NaCl in sodium acetate buffer at pH 4.5 (buffer A) and allowed to settle slowly. After obtaining the desired bed volume (1 ml bed volume per mg tRNA) the column was washed extensively with 2 M NaCl in buffer A plus 25% ethanol until the A$_{260}$ was below 0.025. Then the column was equilibrated with 0.4 M NaCl in buffer A. Varying amounts of tRNA were pumped onto the column and washed with 0.4 M NaCl in buffer A. The column was then eluted with a gradient of 0.4 - 1.2 M NaCl in buffer A till the A$_{260}$ reached less than 0.05. Elution was then continued with 20% ethanol in buffer A containing 1.2 M NaCl. The flow rate was maintained at 60 ml/hr.

**Reversed-phase Chromatography (RPC-5)**

A mixture of 8 ml of Adogon 464 in 400 ml of chloroform was coated onto 200 g of polychlorotriflyoroethylene (plaskon) support according to Pearson et al., (1971). The coated plaskon was suspended in 0.4 M NaCl in sodium acetate buffer consisting of 10$^{-2}$ M sodium acetate at pH 4.5 and 10$^{-2}$ M MgCl$_2$ (buffer A) and packed under pressure into a 90 cm x 0.9 cm column. Prior to the initial chromatographic run, and periodically thereafter, the column was washed with several
volumes of buffer A containing $10^{-3}$ M EDTA, which improved chromatographic resolution.

Samples containing up to 10 mg of tRNA were acylated in a standard reaction mixture given in the legends to the figures. Following acylation, samples were deproteinized by elution on a small DEAE-cellulose column. The size of the column depended on the amount of tRNA used. Normally plastic disposable columns (Quik-sep columns from Isolab Inc.) with 3 ml bed volume were used. The DEAE-cellulose column was equilibrated with 0.3 M NaCl in buffer A. The reaction mixture incubated for aminoacylation was chilled on ice and applied onto the DEAE-cellulose column and washed with 0.3 M NaCl in buffer A until the $A_{280}$ had reached zero (normally 5-6 times the column volume was used). The acylated tRNA was then eluted from the column with 1 M NaCl in buffer A and diluted with buffer A to bring the NaCl concentration to 0.4 M for application to an RPC-5 column.

The sample was applied to the column and eluted with 400 ml linear gradient from 0.4 to 1.0 M NaCl in buffer A (described in the legend to figures) at a flow rate of 30 ml/hr. Four ml fractions were collected and chilled on ice and the RNA in each fraction was precipitated by the addition of 1/10 volume of 55% TCA. The precipitates were collected on Whatman GF/A type filters, and the radioactivity was determined in a Beckman Model LS 3150P liquid scintillation counter using a toluene based scintillation fluid.

**Protein determination**

All protein determinations were made using the ratio of $A_{280}/A_{260}$.
determined on a Beckman DB spectrophotometer. From this ratio a value for the amount of protein was estimated using the table given by Cherry (1973).

**Scintillation Fluid**

The scintillation fluid consisted of 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP) per 1000 ml of toluene.
RESULTS

Optimum ATP and Mg\(^{++}\) Requirements of Phe, Tyr and Trp-tRNA Synthetases

From Cytoplasm, Mitochondria and Chloroplasts

Since ATP and tRNA are both capable of binding Mg\(^{++}\) and with the possibility that the enzyme itself may bind Mg\(^{++}\), a series of assays were carried out to optimize the ATP and Mg\(^{++}\) concentrations in the standard assay system. The optimum levels of ATP and Mg\(^{++}\) requirements were determined for each enzyme from the cytoplasm, the mitochondria, and the chloroplasts. A range of concentrations was tested for Mg\(^{++}\) (2 \(\times\) \(10^{-3}\) M to 3 \(\times\) \(10^{-2}\) M) and ATP (5 \(\times\) \(10^{-4}\) M to 5 \(\times\) \(10^{-3}\) M). Each concentration of ATP tested included all the concentrations of Mg\(^{++}\) from 2 \(\times\) \(10^{-3}\) M to 3 \(\times\) \(10^{-2}\) M. The volume of the reaction mixture (0.3 ml), pH (7.8), tRNA (0.01 A\(_{260}\) units) and \(^3\)H-amino acids (0.1 mM) were kept constant. The enzyme preparations were in the range of 0.05 - 0.5 mg/ml protein. Each set was run with a control. The reaction was terminated by adding an equal volume of 10% trichloracetic acid after 3 min at 30°C.

Results summarized in Table 1, indicate that in general, organellar enzymes have a higher ATP requirement and with the exception of phenylalanyl-tRNA synthetase the organellar enzymes also require higher Mg\(^{++}\) concentrations compared to cytoplasmic enzymes. ATP concentrations higher than those indicated in Table 1 were found to be inhibitory to the enzyme action. Higher Mg\(^{++}\) concentrations were also found to be inhibitory but to a much lesser extent. Results in Table 1 show the conditions for maximum aminoaacylation by each enzyme under
TABLE 1: OPTIMUM ATP and Mg++ REQUIREMENTS OF PHE, TYR and TRP–tRNA SYNTHETASES FROM CYTOPLASM, CHLOROPLASTS AND MITOCHONDRIA

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cell Compartment</th>
<th>Optimum Concentrations M x 10^{-3}</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe-tRNA Synthetase</td>
<td>Chloroplast</td>
<td>20.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>20.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>20.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Tyr-tRNA Synthetase</td>
<td>Chloroplast</td>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Trp-tRNA Synthetase</td>
<td>Chloroplast</td>
<td>10.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>10.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>5.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
the levels of ATP and Mg$^{++}$ indicated; the increase over the immediate lower concentration tested being minimal. It is evident from these results that the properties of the organellar enzymes are different from those of the cytoplasmic enzymes and in addition, mitochondrial and chloroplastic enzymes are again indistinguishable based on these properties.

Mg$^{++}$/ATP ratios for aminoacyl-tRNA synthetases are as follows for organellar and cytoplasmic enzymes: 6.6 and 10 for Phe-tRNA synthetase, 3.3 and 5 for Tyr-tRNA synthetase and 5 for the both in case of Trp-tRNA synthetase. Mg$^{++}$/ATP requirements of soybean cytoplasmic Phe-tRNA synthetase and Tyr-tRNA synthetase are substantially higher than those required by same enzymes from Phaseolus vulgaris (Burkard et al., 1970).

Aminoacylation by homologous and heterologous enzymes and tRNAs from chloroplasts, mitochondria and cytoplasm

Aminoacylation reactions were carried out with enzymes and tRNAs obtained from all the three different cell compartments. Table 2 represents the results obtained by aminoacylating the tRNAs with homologous and heterologous enzymes. The reaction mixture (0.4 ml) contained optimal levels of ATP and Mg$^{++}$ for the enzyme from each source as determined earlier (Table 1). The concentration of tRNA was kept constant in all cases (0.05 $A_{260}$ units) but the enzyme concentration varied (0.1 mg-0.5 mg/ml protein). Care was taken to assure that the enzyme concentration was not rate limiting. The enzymes used for these reactions were obtained after DEAE-cellulose fractionation
**TABLE 2: AMINOACYLATION BY HOMOLOGOUS AND HETEROLOGOUS ENZYMES AND tRNAs FROM CHLOROPLASTS, MITOCHONDRIA AND CYTOPLASM**

<table>
<thead>
<tr>
<th>Source of Aminoacyl-tRNA Synthetase</th>
<th>Source of tRNA</th>
<th>C.P.M.</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>tRNA&lt;sub&gt;Ph&lt;/sub&gt;</td>
<td>tRNA&lt;sub&gt;Trp&lt;/sub&gt;</td>
<td>tRNA&lt;sub&gt;Tyr&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Chloroplast</td>
<td>Chloroplast</td>
<td>18,950</td>
<td>21,700</td>
<td>35,340</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>11,390</td>
<td>17,500</td>
<td>25,540</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>6,390</td>
<td>8,450</td>
<td>9,490</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Chloroplast</td>
<td>17,700</td>
<td>18,500</td>
<td>31,450</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>15,530</td>
<td>18,340</td>
<td>27,350</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>7,340</td>
<td>10,500</td>
<td>11,300</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Chloroplast</td>
<td>5,270</td>
<td>5,900</td>
<td>7,250</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>4,340</td>
<td>8,530</td>
<td>8,370</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>23,750</td>
<td>31,400</td>
<td>43,570</td>
<td></td>
</tr>
</tbody>
</table>

*Note:* The table lists the C.P.M. (counts per minute) for aminoacylation at different sources of tRNA and synthetases, indicating the efficiency of amino acid attachment to tRNA at various cellular compartments.
from each source. Results depicted in Table 2 illustrate that in each of the aromatic aminoacyl-tRNAs the organellar enzymes and tRNAs differ markedly from their cytoplasmic counterparts. There is a substantial similarity between chloroplastic and mitochondrial enzymes and tRNAs. This is evidenced by the extent of cross charging between the tRNAs and enzymes from these sources. On the other hand, the cytoplasmic tRNAs are not aminoacylated efficiently by the organellar enzymes and the organellar tRNAs are not aminoacylated by the cytoplasmic enzymes to a significant level. However, there has been some cross reaction between the cytoplasmic tRNAs and enzymes and their organellar counterparts to a limited extent. It should be pointed out here that this could be mostly due to cross contamination, since it was not possible to obtain the tRNAs and synthetases in a pure form from the cytoplasm without breaking the organelles during isolation. However, contamination of cytoplasmic aminoacyl-tRNA synthetases with organellar enzymes has been reduced to a minimum by grinding the cotyledons in a buffer system containing 20% glycerol. It should be noted here that the enzymes used for these reactions were obtained from DEAE-cellulose fractionation before applying it onto hydroxylapatite column.

Fractionation of Phe, Trp and Tyr-tRNA Synthetases from the three cell compartments

Amino-cyl-tRNA synthetases of aromatic amino acids (phenylalanine, tryptophan and tyrosine) were separated on hydroxylapatite columns (20 cm x 1.5 cm) after fractionating the crude enzyme on DEAE-cellulose
FIGURE 1 - Fractionation of Cytoplasmic, Chloroplastic and Mitochondrial Phe-tRNA Synthetases

About 50 mg total protein (eluted from DEAE-cellulose column) was loaded onto the hydroxylapatite column. Elution was carried out with a potassium phosphate (Buffer B) gradient (0.05 - 0.5 M) at pH 6.5; 2 ml fractions were collected and assayed for enzyme activity using cytoplasmic tRNA •—•, chloroplastic tRNA  —•, mitochondrial tRNA △—△ and E. coli tRNA X—X. Only about 10 mg total protein obtained after DEAE-cellulose fractionation of mitochondrial enzyme preparation was loaded onto the column. Reaction mixtures for assaying the mitochondrial enzymes included bovine serum albumin (1 mg/ml). I and II represent the elution peaks of organellar and cytoplasmic Phe-tRNA synthetases respectively.
Fractionation of Cytoplasmic, Chloroplastic and Mitochondrial Trp-tRNA Synthetases by Hydroxylapatite Chromatography.

About 50 mg of total protein (eluted from DEAE-cellulose column) was applied onto the hydroxylapatite column. Elution was carried out with a potassium phosphate (Buffer B) gradient of 0.05 - 0.3 M at pH 6.5; 2 ml fractions were collected and assayed for enzyme activity using cytoplasmic tRNA ●● ●, chloroplastic tRNA □□□, mitochondrial tRNA △△△ and E. coli tRNA X X X. Only about 10 mg of total protein obtained after DEAE-cellulose fractionation of mitochondrial enzyme preparation was loaded onto the hydroxylapatite column. Reaction mixtures for assaying the mitochondrial enzymes included bovine serum albumin (1 mg/ml). I and II represent the elution peaks of organellar and cytoplasmic Trp-tRNA synthetases respectively.
FIGURE 3 - Fractionation of Cytoplasmic, Chloroplastic and Mitochondrial Tyr-tRNA Synthetases by Hydroxylapatite Chromatography

About 50 mg of total protein (eluted from DEAE-cellulose column) was applied onto the hydroxylapatite column. Elution was carried out with a potassium phosphate (Buffer B) gradient of 0.05 - 0.2 M at pH 6.5; 2 ml fractions were collected and assayed for enzyme activity using cytoplasmic tRNA •—•, chloroplastic tRNA •—•, mitochondrial tRNA Δ—Δ and E. coli tRNA X—X. Only about 10 mg of total protein obtained after DEAE-cellulose fractionation of mitochondrial enzyme preparation was loaded onto the hydroxylapatite column. Reaction mixtures for assaying the mitochondrial enzyme included bovine serum albumin (1 mg/ml). I and II represent the elution peaks of organellar and cytoplasmic Tyr-tRNA synthetases respectively.
$^{3}\text{H-Tyr-tRNA CPM} \times 10^{-3}$

[Fraction Number]

[0, 50, 100, 150]

[0.02, 0.04, 0.06, 0.08, 0.10]

$[\text{KHPO}_4] \text{ M}$
column as described in methods. Hydroxylapatite (HA) column was prepared as described in part I. Enzyme fractions from DEAE-cellulose column were dialysed against 0.05 M potassium phosphate buffer at pH 6.5 consisting of 10 mM β-mercaptoethanol, 10 μM phenylmethylsulfonylfluoride and 10% glycerol (buffer B). The dialyzed extract was applied onto a HA column and eluted with a linear potassium phosphate gradient. The linear gradient differed for different enzyme preparations as shown in the legend for Figs. 1, 2 and 3.

Enzyme preparations from dark-grown cotyledons, light-grown shoots, chloroplasts and mitochondria were fractionated on HA columns separately while maintaining the flow rate and other conditions constant. To allow further characterization, mixed enzyme preparations were also fractionated on HA columns obtained from different sources. Column fractions were assayed for enzyme activity with tRNAs from the dark-grown cotyledons, the chloroplasts, the mitochondria and E. coli.

In each one of these three aromatic aminocyl-tRNAs, two peaks of enzyme activity were resolved on HA column (Fig. 1, 2 and 3). When an enzyme preparation from dark-grown cotyledons was fractionated and assayed with the tRNA preparation from the same source, only peak II was resolved in all the cases and peak I was not resolved. When the fractions were assayed with the tRNAs from E. coli, chloroplasts and mitochondria, no enzymatic activity was observed in any of the fractions. When the enzyme preparation from either mitochondria or chloroplasts was fractionated and assayed for the activity with tRNAs from all the above-mentioned sources, peak I was resolved with tRNAs from E. coli,
mitochondria and chloroplasts and no significant enzyme activity
was observed in any of the fractions with the tRNA from the dark-
grown cotyledons. Similar results were obtained when a mixture of
chloroplastic and mitochondrial enzyme preparation was fractionated.
However, a slight activity of peak II enzyme was observed with the
chloroplast and the mitochondrial enzyme preparations on HA column,
probably due to some cross contamination and cross charging. On the
other hand, no activity for peak I enzyme was detected when the enzyme
preparation from the dark-grown cotyledons was fractionated.

Peak I activity was obtained at phosphate concentrations of
0.10 - 0.12 M, 0.07 - 0.08 M and 0.06 - 0.07 M for tRNA^{Phe}, tRNA^{Trp}
and tRNA^{Tyr} respectively. Peak II activity was obtained at phosphate
concentrations of 0.38 - 0.4 M, 0.22 - 0.24 M and 0.14 - 0.16 M for
those tRNAs in the same order. Therefore, these results indicate that
each of these three tRNAs of aromatic amino acids are acylated by two
enzymes, one being localized in the cytoplasm acylating only the
eukaryotic type tRNA from the cytoplasmic source, the other being
prokaryotic in nature which appears to be localized in the organelles.
However, no distinction could be made between the enzymes from mito-
chondria and chloroplasts as they were found to be superimposable in
their activity. When fractionation was carried out with a mixture of
chloroplastic and mitochondrial enzyme preparation expanding the phos-
phate gradient within the range of peak I activity (0.05 M - 0.1 M), a
single broad and diffuse peak was obtained. Therefore, the mito-
chondrial and the chloroplastic enzymes are under these conditions
chromatographically indistinguishable.
Characterization and Aminoacylation Properties
Of tRNA\textsuperscript{Phe}, tRNA\textsuperscript{Trp} and tRNA\textsuperscript{Tyr} From The Three Cell Compartments

Fractionation of tRNA\textsuperscript{Phe}, tRNA\textsuperscript{Trp} and tRNA\textsuperscript{Tyr} was carried out on RPC-5 columns with the aminoacylated tRNAs obtained from different sources. The NaCl gradient used is recorded in the legends of the respective figures. tRNA from the light-grown cells (whole) charged with a homologous enzyme and RPC-5 fractionation of tRNA\textsuperscript{Phe} results in three peaks as shown in Figure (4); peak III being the largest of all. Exactly similar results were obtained with tRNA\textsuperscript{Trp} when the tRNA, aminoacylated by a homologous enzyme, was fractionated on RPC-5 (Fig. 5). Although the elution profiles of these two tRNAs appear similar, the isoacceptors are eluted at different salt concentrations (Fig. 4 and 5). In the case of tRNA\textsuperscript{Tyr}, aminoacylated with homologous (isolated from light-grown cells) enzyme and fractionated on RPC-5, results in five distinct isoacceptors (Fig. 6).

RPC-5 fractionation of chloroplast tRNAs aminoacylated by chloroplast enzymes was carried out exactly under similar conditions. Figs. 7, 8 and 9 represent the results of these experiments with tRNA\textsuperscript{Phe}, tRNA\textsuperscript{Trp} and tRNA\textsuperscript{Tyr} respectively. Again the elution profiles of tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Trp} are similar closely indicating the existence of only one isoacceptor for these tRNAs in the chloroplasts (Figs. 7 and 8). It should be further stated here that the second tRNA peak (among the three isoacceptors) has been found to be localized in the chloroplasts in both the cases. On the other hand, the situation in tRNA\textsuperscript{Tyr}
FIGURE 4 - RPC-5 Fractionation of Total Cell tRNA\textsuperscript{Phe}

Unfractionated tRNA isolated from light-grown cell was aminoacylated with homologous enzyme (obtained after DEAE-cellulose fractionation) and fractionated using 0.4 - 0.9 M NaCl gradient in Buffer A. Peaks are numbered according to their sequence of elution.

Aminoacylation reaction mixture contained 10 A\textsubscript{260} units of tRNA, ATP and Mg\textsuperscript{++} levels were as shown in Table 1 for cytoplasmic tRNA\textsuperscript{Phe}. Saturating levels of enzyme was used. Other components and reaction conditions were as described in methods. Aminoacylation reaction mixture was incubated at 30\textdegree C for 30 min.
FIGURE 5 - RPC-5 Fractionation of Total Cell tRNA$^{Trp}$

Unfractionated tRNA isolated from the light-grown cell was aminocylated with the homologous enzyme (obtained after DEAE-cellulose fractionation) and fractionated using 0.4 - 0.9 M NaCl gradient in Buffer A. Peaks are numbered according to their sequence of elution. Aminoacylation conditions were as described in Figure 4 and with optimal levels of ATP and Mg$^{++}$ for cytoplasmic Trp-tRNA synthetase.
Unfractionated tRNA isolated from light-grown cell was aminoacylated with homologous enzyme (enzyme was obtained after DEAE-cellulose fractionation). The reaction mixture was deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.8 M NaCl in Buffer A. Peaks are numbered according to their sequence of elution. Aminoacylation conditions were as described in Figure 4 and with optimal conditions for Tyr-tRNA synthetase.
$^3$H-Tyr-tRNA CPM $\times 10^{-3}$

Fraction Number

Frac. No.

5 10 15

II

III

I

II

$100$

80

60

40

0
FIGURE 7  -  RPC-5 Fractionation of Chloroplast tRNA$^{Phe}$ Aminoacylated by Homologous Peak I Enzyme

Enzyme was obtained after fractionating as described in Figure 1. Aminoacylation reaction mixture included 0.20 A$^{260}$ units of chloroplast tRNA. Optimal levels of ATP and Mg$^{++}$ and saturating level of enzyme were used as described in Table 1. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution Peak (II) is numbered according to Figure 4.
Enzyme was obtained after fractionating as described in Figure 2. Aminoclaylation was carried out under optimal conditions as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoclaylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution peak (II) is numbered according to Figure 5.
FIGURE 9 - RPC-5 Fractionation of Chloroplast tRNA<sup>Tyr</sup> Aminoaecylated by Homologous Peak I Enzyme

Enzyme was obtained after fractionating as described in Figure 3. Aminoaecylation was carried out under optimal conditions as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoaecylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.8 M NaCl gradient in Buffer A. The elution Peaks (IV and V) are numbered according to Figure 6.
FIGURE 10 - RPC-5 Fractionation of Chloroplast tRNA\(^{\text{Phe}}\) Aminoacylated by Mitochondrial Peak I Enzyme

Enzyme was obtained as described in Figure 1. Aminoacylation was performed as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution peak (ii) is numbered according to Figure 4.
$^3$H-Phe-tRNA CPM $\times 10^{-3}$
FIGURE 11 - RPC-5 Fractionation of Chloroplast tRNA^{Trp} Aminoacylated by Mitochondrial Peak I Enzyme

Enzyme was obtained as described in Figure 2. Aminoacylation conditions were as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution Peak (II) is numbered according to Figure 5.
FIGURE 12 - **RPC-5 Fractionation of Chloroplast tRNA\textsuperscript{Tyr} Aminoacylated by Mitochondrial Peak I Enzyme**

Enzyme was obtained as described in Figure 3. Aminoacylation conditions were as described in Figure 7. The reaction mixture was deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.8 M NaCl gradient in Buffer A. The elution peaks (IV and V) are numbered according to Figure 6.
FIGURE 13 - RPC-5 Fractionation of Mitochondrial tRNA$_{\text{Phe}}$ Aminoacylated by Homologous Peak I Enzyme

Enzyme was obtained as described in Figure 1. Aminoacylation conditions were as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution Peak (I) is numbered according to Figure 4.
FIGURE 14 - RPC-5 Fractionation of Mitochondrial tRNA<sub>Trp</sub> Aminoacylated by Homologous Peak I Enzyme

Enzyme was obtained as described in Figure 2. Aminoacylation conditions were as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution peak (I) is numbered according to Figure 5.
FIGURE 15 - RPC-5 Fractionation of Mitochondrial tRNA$^{\text{Tyr}}$ Aminoacylated by Homologous Peak I Enzyme

Enzyme was obtained as described in Figure 3. Aminoacylation conditions were as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.8 M NaCl gradient in Buffer A. The elution peak (III) is numbered according to Figure 6.
$^{3}\text{H-Tyr-tRNA } \text{CPM} \times 10^{3}$

Fraction Number

0

40

60

80

100

I

II

III
FIGURE 16 - RPC-5 Fractionation of Mitochondrial tRNA\textsuperscript{Phe} Aminoacylated by Chloroplast Peak I Enzyme

Enzyme was obtained as described in Figure 1. Aminoacylation conditions were as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution peak (I) is numbered according to Figure 4.
FIGURE 17 - RPC-5 Fractionation of Mitochondrial tRNA$^{\text{Trp}}$ Aminoacylated by Chloroplast Peak I Enzyme

Enzyme was obtained as described in Figure 2. Aminoacylation conditions were as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution Peak (III) is numbered according to Figure 5.
FIGURE 18 - RPC-5 Fractionation of Mitochondrial tRNA$_{\text{Tyr}}$ Aminoacylated by Peak I Chloroplast Enzyme

Enzyme was obtained as described in Figure 3. Aminoacylation conditions were as described in Figure 7. The reaction mixture was incubated at 30°C for 30 min.
is different. Chloroplasts have been shown to contain two isoacceptors of tRNA\textsuperscript{TyT} (Fig. 9) which elute toward the end of the RPC-5 column run, out of the total five species.

Profiles presented here appear to be similar to the results obtained with chloroplast tRNAs aminoacylated by mitochondrial enzymes and fractionated on RPC-5 (Figs. 10,11,12). Irrespective of the source of aminoacylating enzyme, the same isoacceptor was acylated to a larger extent indicating that this species is localized in the chloroplasts. Further, this indicates a high degree of similarity between these organelles as far as these tRNAs and aminoacyl tRNA synthetases are concerned.

Aminoacylation of mitochondrial tRNAs by mitochondrial enzyme and fractionation on RPC-5 yields one peak for each amino acid. In the case of tRNA\textsuperscript{Phe} (Fig. 13) and tRNA\textsuperscript{Trp} (Fig. 14) it is the first peak in order of elution. In the case of tRNA\textsuperscript{TyT} (Fig. 15) it is the third peak in order of elution. Similar results were obtained when mitochondrial tRNAs were aminoacylated by chloroplast enzymes (Figs. 16,17,18). Therefore, these results clearly show that mitochondrial and chloroplast tRNAs and aminoacyl-tRNA synthetases have similar properties although these organelles contain different isoacceptors of tRNAs. The aromatic aminoacyl-tRNAs of chloroplast and mitochondria could be aminoacylated both by homologous and heterologous enzymes without any change in their chromatographic behaviour. In order to further test the cross charging abilities of chloroplastic and mitochondrial aminoacyl-tRNA synthetases, a mixture of chloro-
RPC-5 Fractionation of Mixture of Chloroplast and Mitochondrial tRNA<sub>Phe</sub> Aminoacylated by Chloroplast Peak I Enzyme

Enzyme was obtained as described in Figure 1. Aminoacylation conditions were as described in Figure 7. A mixture of chloroplastic and mitochondrial tRNAs (0.2 A<sub>260</sub> units) was incubated at 30°C for 30 min in an appropriate reaction mixture as described in the text. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution peaks (I and II) are numbered according to Figure 4.
Figure 20 - RPC-5 Fractionation of Mixture of Chloroplast and Mitochondrial tRNA\textsuperscript{Trp} Aminoacylated by Chloroplast Peak I Enzyme

Enzyme was obtained as described in Figure 2. Aminoacylation conditions were as described in Figure 7. A mixture of chloroplastic and mitochondrial tRNAs (0.2 A\textsubscript{260} units) was incubated at 30°C for 30 min in an approximate reaction mixture as described in the text. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution peaks (I and II) are numbered according to Figure 5.
Enzyme was obtained as described in Figure 3. Aminoacylation conditions were as described in Figure 7. A mixture of chloroplastic and mitochondrial tRNAs (0.2 A_{260} units) was incubated at 30°C for 30 min. Acylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4-0.8 M NaCl gradient in Buffer A. The elution peaks (III, IV and V) are numbered according to Figure 6.
RPC-5 Fractidnation of Cytoplasmic tRNA^Phe Aminoacylated by Cytoplasmic (Peak II) Enzyme

Enzyme was obtained as described in Figure 1 and the tRNA was obtained from dark-grown cell. Aminoacylation conditions were as described in Figure 4. The reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution Peak (III) is numbered according to Figure 4.
FIGURE 23 - RPC-5 Fractionation of Cytoplasmic tRNA<sub>Trp</sub> Aminoacylated by Cytoplasmic (Peak II) Enzyme

Enzyme was obtained as described in Figure 2 and the tRNA was obtained from dark-grown cell. Aminoacylation conditions were as described in Figure 4. The reaction mixture was incubated at 30°C for 30 min. The aminoacylation reaction mixture was then deproteinized on a DEAE-cellulose column and applied onto the RPC-5 column. Fractionation was carried out with 0.4 - 0.9 M NaCl gradient in Buffer A. The elution Peak (III) is numbered according to Figure 5.
FIGURE 24 - RPC-5 Fractionation of Cytoplasmic tRNA\textsubscript{Tyr} Aminoacylated
by Cytoplasmic (Peak II) Enzyme

Enzyme was obtained as described in Figure 3 and the tRNA
was obtained from dark-grown cell. Aminoacylation was as
described in Figure 4. The reaction mixture was incubated
at 30°C for 30 min. The aminoacylation reaction mixture was
then deproteinized on a DEAE-cellulose column and applied
onto the RPC-5 column. Fractionation was carried out with
0.4 - 0.8 M NaCl gradient in Buffer A. The elution
peaks (I and II) are numbered according to Figure 6.
plastic and mitochondrial tRNAs were aminoacylated by chloroplastic enzyme and fractionated on RPC-5 in each of the three aminoacyl-tRNAs. The elution profile of these tRNAs included both the chloroplastic and mitochondrial peaks, (Fig. 19, 20, 21).

RPC-5 fractionation of cytoplasmic tRNAs aminoacylated by cytoplasmic enzymes yielded one isoacceptor in each of tRNA\(^{\text{Phe}}\) and tRNA\(^{\text{Trp}}\) and two isoacceptors in the case of tRNA\(^{\text{Tyr}}\). Peak III in tRNA\(^{\text{Phe}}\) and tRNA\(^{\text{Trp}}\) and peak I and II in the case of tRNA\(^{\text{Tyr}}\) have been found to be localized in the cytoplasm (Figs. 22, 23 and 24). These results conclusively show that the three cell compartments possess different isoacceptors of tRNAs and that the chloroplastic and mitochondrial tRNAs are more similar to each other in their aminoacylation behaviour though they are distinct in their chromatographic behaviour. The organellar tRNAs in general are more dissimilar to cytoplasmic tRNAs in being different both in the chromatographic and aminoacylation behaviours.

**Analysis of Chloroplast tRNAs by two-dimensional polyacrylamide gel electrophoresis**

Two-dimensional polyacrylamide gel electrophoresis of chloroplast tRNAs was conducted as described in methods. The tRNAs were localized as blue spots after staining with methylene blue. The spots were cut out from the gel and each bit of the gel consisting of one spot was extracted with a sodium acetate buffer of the following composition: sodium acetate \(10^{-2}\) M at pH 4.6, sodium chloride 0.3 M and magnesium
chloride $10^{-2}$ M. This extract was agitated well with equal volume of aqueous phenol, centrifuged at 2,000 xg and the aqueous phase was recovered. The tRNAs from these gel extracts were precipitated by adding 200 μg of ribosomal RNA and three volumes of 95% ethanol. The precipitate was recovered by centrifugation and each sample was assayed with individual $^3$H-amino acids in a reaction mixture containing the standard reaction components needed for aminoacylation with E. coli enzyme as described in methods. By repeated analysis on polyacrylamide gels and assaying, the chloroplast tRNAs were mapped on the gel (Fig. 25, 26).

It has been found by two-dimensional polyacrylamide gel electrophoresis that tRNA\textsuperscript{Ty} is represented by a single spot (Fig. 26). When tRNA\textsuperscript{Ty} from the gel was extracted, aminoacylated and fractionated on RPC-5, it was resolved into two peaks (Fig. IV and V). Therefore the resolution on two-dimensional polyacrylamide gels does not seem to be as effective as with RPC-5. In addition, some of the tRNAs could not be aminoacylated after extracting from the gel, probably due to loss of activity of those tRNAs when subjected to this process of analysis.

**B-D-Cellulose Chromatography of tRNAs**

tRNAs extracted from light-grown cells as well as chloroplast tRNAs were subjected to fractionation on B-D-cellulose columns as described in methods. A sample from each fraction was diluted 10 times with water, as high salt concentration and ethanol were inhibitory to the enzyme, and assayed for the activity. B-D-cellulose fractionation of whole cell tRNAs yielded a typical profile and the ethanol fractions contained major part of tRNA\textsuperscript{Phe} and the salt fractions included very
FIGURE 25 - Two-Dimensional Polyacrylamide Gel Electrophoresis of Chloroplast tRNA
FIGURE 26 - Polyacrylamide Gel Electrophoresis Map of Chloroplast tRNA

tRNA extracted from each spot on the gel was tested for its activity with each of the 19 amino acids (activity for cysteine was not tested). E. coli enzyme preparation was used for all the assays. Activity for alanine (A1), asparagine (An), glycine (Gl), histidine (Hs), isoleucine (Il), leucine (Le), lysine (Ls), methionine (Mt), phenylalanine (Ph), serine (Sr), threonine (Th), tryptophan (Tp), tyrosine (Ty) and valine (Va) was observed. tRNA_Ile, tRNA_Leu and tRNA_Met are represented by two isoacceptors each. The remaining spots did not show activity for any of the amino acids tested.
little activity of this tRNA. When the same fractions were assayed for tRNA\textsuperscript{Tyr} the reverse condition was observed with the bulk of tRNA\textsuperscript{Tyr} being in salt fraction and only slight activity for this tRNA being detected in ethanol fractions (Fig. 27). Activity for tRNA\textsuperscript{Trp} was detected only in salt fractions (not shown in Fig. 27).

When chloroplast tRNAs were fractionated on B-D-cellulose column and assayed for activity with \textit{E. coli} enzymes, the entire tRNA\textsuperscript{Tyr} was eluted only in ethanol fractions with no detectable tRNA\textsuperscript{Tyr} in the salt fractions (Fig. 28).

**RPC-5 Chromatography of ethanol fractions from B-D-cellulose column**

Ethanol fractions obtained from fractionating chloroplast tRNAs on B-D-cellulose columns were pooled and the tRNA was precipitated with three volumes of ethanol. The tRNA precipitate was recovered, dissolved in sodium acetate buffer (buffer A) and fractionated on RPC-5 column. Fig. 29 shows the results of this experiment. tRNA\textsuperscript{Tyr} eluted as two peaks coinciding with the A\textsubscript{260} peaks. These two peaks were eluted at salt concentrations typical of peak IV and V of whole cell tRNA\textsuperscript{Tyr} fractionation.

Fractions containing these two peaks were pooled separately and the tRNAs were precipitated with three volumes of ethanol. The ethanol precipitates from these two peaks were subjected to two dimensional polyacrylamide gel electrophoresis.

Two-dimensional polyacrylamide gel electrophoresis of RPC-5 fractions of chloroplast tRNA\textsuperscript{Tyr} was conducted as described earlier.
FIGURE 27 - B-D-Cellulose Chromatography of Whole Cell tRNA

tRNA (500 mg) isolated from light-grown cell was fractionated (5 ml fractions were collected) and assayed for tRNA^Phe and tRNA^Tyr activity. The enzyme for the assays was obtained from light-grown cell after DEAE-cellulose fractionation. The reaction mixture for assays with 3H-phenylalanine included 2 \times 10^{-2} \text{M} \text{Mg}^{++} and 2 \times 10^{-3} \text{M} \text{ATP}. tRNA^Tyr activity was tested in a reaction mixture containing 5 \times 10^{-3} \text{M} \text{Mg}^{++} and 10^{-3} \text{M} \text{ATP}. All other components of the reaction mixture were as described earlier. The solid line represents A_{260} values of the fractions; open squares joined by solid line represent tRNA^Phe activity; open circles joined by broken line represent tRNA^Tyr activity.
FIGURE 28 - B-D-Cellulose Chromatography Chloroplast tRNA

Chloroplast tRNA (60 mg) was fractionated (5 ml fractions were collected) and assayed with \(^3\)H-tyrosine and E. coli enzyme. The solid line represents \(A_{260}\) values of the fractions and the broken line joining the open circles represent tRNA\(^{Tyr}\) activity.
FIGURE 29 - RPC-5 Fractionation of B-D-Cellulose Ethanol Fractions of Chloroplast tRNA

Elution was carried out with 0.4 - 0.8 M NaCl in buffer A and the fractions were assayed with E. coli enzyme. The solid line represents A260 values of the fractions and the broken line joining the closed circles represents tRNA Tyr activity.
FIGURE 30 - Sephadex G 25 Chromatography of Polyacrylamide Gel Extracts of tRNA\textsuperscript{Tyr} spota

The spots were phenol-extracted and applied onto the column (in 10 ml plastic pipette). One ml fractions were collected and assayed with \textit{E. coli} enzyme.

The solid line joining the the closed circles represents \(A_{260}\) values of the fractions and the broken line joining the open circles represents tRNA\textsuperscript{Tyr} activity.
When these two gel maps were compared, spots for tRNA\textsuperscript{TYR}\textsubscript{4} and tRNA\textsuperscript{TYR}\textsubscript{5} were superimposable. The tRNA\textsuperscript{TYR}\textsubscript{4} and 5 from the gel were extracted as described earlier and passed through a Sephadex-G 25 column and the column was eluted with water. Each fraction was assayed for tRNA\textsuperscript{TYR} activity with enzyme preparation from \textit{E. coli} (Fig. 30). Fractions containing tRNA\textsuperscript{TYR} activity were pooled and the tRNA was precipitated by adding the following materials: 20 µg ribosomal RNA, 0.3 M ammonium acetate (solid), 10^{-2} M magnesium acetate, 10^{-4} M EDTA and three volumes of ethanol.

**Codon Recognition Studies**

a) **Preparation of Isoacceptors of tRNA\textsuperscript{TYR}**

The isoaccepting species of tRNA\textsuperscript{TYR} were tested as to their ability to recognize the two codons (UAC and UAU) in an \textit{E. coli} ribosome directed binding assay. tRNA\textsuperscript{TYR}\textsubscript{4} and 5 obtained from two-dimensional polyacrylamide gel electrophoresis of the RPC-5 fractions of chloroplast tRNAs were aminoacylated with \textit{E. coli} enzyme and deproteinized on DEAE-cellulose column in a pasteur pipette. The tRNA\textsuperscript{TYR} was eluted from the column as described earlier and dialyzed extensively against 10^{-2} M sodium acetate buffer at pH 4.5 containing 10^{-2} M MgCl\textsubscript{2} to remove the salt and finally against cold de-ionized water. The samples were lyophilized and stored at -20°C. tRNA\textsuperscript{TYR}\textsubscript{1,2} and 3 were obtained by fractionating the aminoacylated samples from whole cell and mitochondrial tRNAs through RPC-5 column. 200 A\textsubscript{260} units of whole cell tRNAs and 10 A\textsubscript{260} units of mitochondrial tRNAs were aminoacylated with \textit{H}-Tyrosine and enzyme from cytoplasm and \textit{E. coli} respectively. The samples were
deproteinized on DEAE-cellulose columns and fractionated on RPC-5 as described earlier. $tRNA_{\text{Tyr}}^{\text{Tyr}}_{1,2}$ and 3 were pooled separately, concentrated on 1.0 ml DEAE-cellulose columns and desalted by dialysis. The samples were then lyophilized and stored at $-20^\circ$C.

b) Ribosome Binding Assay

The assay procedure was essentially that of Nirenberg and Leder (1964) with slight modifications. The reaction mixture consisted of 0.1 M Tris-HCl buffer at pH 7.5, $2 \times 10^{-2}$ M MgCl$_2$, $5 \times 10^{-2}$ M KCl, 4.0 A$_{260}$ units of E. coli ribosomes and 0.2 A$_{260}$ units of the triplets UAC and UAU. $tRNA_{\text{Tyr}}^{\text{Tyr}}$ isoacceptors (15,000 cpm) were added to reaction mixture. The reaction mixture was kept at 4°C before the tRNA was added to initiate the reaction. The reaction mixture was incubated at 30°C for 15 min after which it was chilled to 4°C and 3 ml of reaction buffer was added immediately. Cellulose nitrate membrane filters (Millipore HAWP, 0.45 mm pore size and 25 mm diameter) were washed with 5 ml reaction buffer. The reaction mixture was added and washed with three, 3 ml portions of buffer at 4°C. The filters were dried and the radioactivity on the filters was counted. The results are tabulated in Table 3. These results clearly show that both the chloroplastic and mitochondrial $tRNA_{\text{Tyr}}^{\text{Tyr}}$ species recognize a single codon (UAC). Among the two cytoplasmic species $tRNA_{\text{Tyr}}^{\text{Tyr}}$ recognizes UAC while $tRNA_{\text{Tyr}}^{\text{Tyr}}$ recognizes UAU.
<table>
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<th>Isoacceptor Species</th>
<th>UAU (%)</th>
<th>CPM Bound</th>
<th>% CPM Over Non-Specific Binding</th>
<th>UAC CPM Bound</th>
<th>% CPM Over Non-Specific Binding</th>
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<td>-</td>
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DISCUSSION

Studies on ATP and Mg"++ requirements of cytoplasmic, chloroplastic and mitochondrial aromatic aminoacyl-tRNA synthetases show that organellar enzymes require higher ATP levels compared to their cytoplasmic counterparts. Concentrations of ATP which are optimal for organellar enzymes are found to be inhibitory to cytoplasmic enzymes. This property of organellar aminoacyl-tRNA synthetases may be due to adaptation to the environment within their cell compartments, where higher levels of ATP are expected to occur due to photophosphorylation and oxidative phosphorylation, which otherwise would inhibit their action. This is also in conformation with the results obtained on higher tolerance of chloroplastic Leu-tRNA synthetase towards various free nucleotides when compared to cytoplasmic enzyme in Euglena (Krauspe and Parthier, 1975).

Aminoacylation studies with the cytoplasmic, the chloroplastic and the mitochondrial tRNAs by homologous and heterologous enzymes clearly show the close similarity between the tRNAs and enzymes of the mitochondria and the chloroplasts. It is also evident from these results that cytoplasmic enzymes are distinct from both the organellar enzymes. This is further substantiated by the fact that chloroplastic and mitochondrial enzymes are similar in their properties with regard to their ATP and Mg"++ requirements and they both differ from the cytoplasmic enzymes. In order to further investigate the distinct nature of organellar and cytoplasmic enzymes, these enzymes were fractionated on hydroxylapatite columns. Hydroxylapatite fractionation of these enzymes indicates a definite
elution pattern and aminoacylation characteristics common to all
the aromatic aminoacyl-tRNA synthetases. Peak I enzymes have been
found to be localized both in chloroplasts and mitochondria in all
three cases and it is prokaryotic in nature while peak II enzyme is
localized in the cytoplasm and it is eukaryotic, being unable to
aminoacylate bacterial tRNA. A similar situation was observed in
the case of bean tRNA\textsuperscript{Phe} (Jeannin et al., 1978); bean tRNA\textsuperscript{Met}
(Guillemaut et al., 1975), and tRNA\textsuperscript{Lys} and tRNA\textsuperscript{Pro} (Jeannin et al.,
1976). The similarity between chloroplastic and mitochondrial
enzymes regarding their chromatographic behaviour and both being
prokaryotic in nature as indicated in bean tRNA\textsuperscript{Phe} (Jeannin et al.,
1978) has been found to be the general rule for the entire family of
aromatic aminoacyl-tRNA synthetases in soybean.

Similarities between chloroplastic and prokaryotic tRNAs and
aminoacyl-tRNA synthetases were also suggested by cross-aminoacylation
reactions performed between cotton chloroplasts and \textit{E. coli} (Merrick
and Dure, 1973) and also between the blue green alga \textit{Anacystis nidulans},
\textit{E. coli} and \textit{Euglena} chloroplasts (Beauchemin et al., 1973; Parthier
and Krauspe, 1974). Among all these studies made with organelar and
cytoplasmic enzymes, none of them included mitochondrial tRNAs and
enzymes except in the case of bean tRNA\textsuperscript{Phe} (Jeannin et al., 1978).
However, there has been evidence to the contrary in some of the aminoacyl-
tRNA synthetases as far as mitochondrial enzymes are concerned. In
tobacco (Guderian et al., 1972) and soybean (Sinclair and Pillay, 1980)
and bean (Guillemaut et al., 1975), cytoplasmic and mitochondrial
tRNA\textsuperscript{Leu} species are aminoacylated by both cytoplasmic as well as mitochondrial Leu-tRNA synthetases. Such cross-aminoacylations between cytoplasmic and mitochondrial tRNA\textsuperscript{Leu} and Leu-tRNA synthetases are also recorded in case of Euglena (Kislev et al., 1972) and Tetrahymena (Chiu et al., 1974). Similarities between cytoplasmic and mitochondrial valyl-tRNA synthetases are established in Tetrahymena (Suyama and Hamada, 1978) based on chromatographic behaviours and kinetic properties of these two enzymes and the inability of both the enzymes to aminoacylate E. coli tRNA. The authors therefore conclude that tRNA\textsuperscript{Val} and valyl-tRNA synthetase are "imported" into the mitochondria from cytoplasm.

Cross-aminoacylation between cytoplasmic tRNA\textsuperscript{Phe} and chloroplast Phe-tRNA synthetase was reported in Euglena (Parthier and Krauspe, 1973). However, it is not clear whether this is due to possible contamination of chloroplast enzyme preparation with mitochondrial Phe-tRNA synthetase having similar chromatographic behaviour but different aminoacylation characteristics.

Therefore, there appears to be significant variation and diversity in the nature and distribution of aminoacyl-tRNA synthetases in these three cell compartments. Although mitochondrial and chloroplastic aromatic aminoacyl-tRNA synthetases in soybean are chromatographically indistinguishable, similar in their Mg\textsuperscript{++} and ATP requirements and share the prokaryotic nature in their aminoacylation behaviour, this itself is not conclusive proof that they are structurally similar. Further investigations are necessary in this direction.
There have been several reports on the occurrence of tRNA\textsuperscript{Phe} isoacceptors ranging from 1 to 9 in different plants (Vold and Sypherd, 1968; Dudock et al., 1969; Yoshikami and Keller, 1971; Barnett et al., 1969; Reger et al., 1970; Chang et al., 1976; Everett and Madison, 1976; Merrick and Dure, 1972; Hiatt and Snyder, 1974; Labuda et al., 1974; Janowicz et al., 1979; Augustyniak et al., 1974; Rafalski et al., 1977; Guillemaut et al., 1976; Jeannin et al., 1978; Guillemaut et al., 1977). Only in a few cases organellar tRNA\textsuperscript{Phe} species are reported. One isoacceptor of tRNA\textsuperscript{Phe} has been assigned to *Euglena* chloroplasts (Barnett et al., 1969; Reger et al., 1970; Chang et al., 1976). Two isoacceptors of tRNA\textsuperscript{Phe} have been assigned to the chloroplasts in case of cotton seedlings (Merrick and Dure, 1972) barley seedlings (Hiatt and Snyder, 1973) and bean leaves (Jeannin et al., 1978). Soybean tRNA\textsuperscript{Phe}, however, differs from cotton, barley and bean tRNA\textsuperscript{Phe} by having only one isoacceptor in the chloroplasts. There has been only one report on higher plant mitochondrial tRNA\textsuperscript{Phe} (Jeannin et al., 1978) other than the present report in soybean. In both the cases tRNA\textsuperscript{Phe} is represented by one species in the mitochondria.

Very little information is available on tRNA\textsuperscript{Trp} from plants. Three isoacceptors for tRNA\textsuperscript{Trp} are reported from cotton seedlings (Merrick and Dure, 1972) out of which two are located in the chloroplasts and the remaining one in the cytoplasm. The only other report on this tRNA is from *Chlamydononas reinhardtii* (Freddie et al., 1973) in which one species is located in the chloroplast and the other in cytoplasm. No information is available on mitochondrial tRNA\textsuperscript{Trp} except
the present report. tRNA$_{TYR}$ was isolated from peas (Vanderhoef and Key, 1970), tobacco cells (Cornelis et al., 1975) tomato fruits (Mettler and Romani, 1976), Mercurialis annua (Bazin et al., 1975) and soybean (Bick et al., 1970; Lacey and Cherry, 1978). Lacey and Cherry (1978) have reported four isoacceptors for tRNA$_{TYR}$ from soybean cotyledons and localized two of them in the chloroplasts and the remaining two in the cytoplasm. Meng and Vanderhoef (1972) resolved four tRNA$_{TYR}$ isoacceptors from soybean seedlings by RPC-2 fractionation and ascribed three of them to cytoplasm and the remaining one species to the mitochondria.

Although only four isoacceptors of tRNA$_{TYR}$ were detected from dark-grown cotyledons, tRNA$_{TYR}$ preparations from young light-grown shoots aminoacylated by enzyme preparation from the same source resolved into five isoacceptors, the additional peak resolved is localized in the mitochondria. It is interesting to note that tRNA preparations from soybean cotyledons when aminoacylated by homologous enzyme and fractionated on RPC-5, did not show the mitochondrial isoacceptor species in any of these aromatic aminoacyl-tRNAs. Mitochondrial species of tRNA$_{PHE}$, tRNA$_{TRP}$ and tRNA$_{TYR}$ are only resolved from whole cell tRNA preparations obtained from light-grown shoots. This is probably due to very low yield of mitochondrial tRNAs in the whole cell tRNA preparations from cotyledons. Cotyledons are physiologically less active compared to actively growing parts of the plant and thus may contain less mitochondria or the mitochondria may be less active. Therefore tRNA preparations from this source may contain very low percentage of
mitochondrial tRNAs, especially the tRNAs of aromatic amino acids and they may not be present in detectable quantities when fractionated with normal amounts of whole cell tRNAs. tRNA preparations from actively growing shoots may be enriched by mitochondrial tRNAs and thus made available in detectable quantities when whole cell tRNAs were fractionated. Therefore, identification of organellar tRNAs and aminoacyl-tRNA synthetases can best be achieved only by fractionating the tRNAs and the enzymes obtained from purified organelles.

These results are in contrast to the observations made with tRNA\textsubscript{Leu} from soybean cotyledons (Sinclair and Pillay, 1980), where cytoplasmic, mitochondrial and chloroplastic species aminoacylated by enzyme from cotyledons are easily resolved on RPC-5. There appears to be major differences between tRNA\textsubscript{Leu} and aromatic aminoacyl-tRNAs in soybean as far as mitochondrial species are concerned. Mitochondrial tRNA\textsubscript{Leu} could be aminoacylated by cytoplasmic Leu-tRNA synthetases and the mitochondrial Leu-tRNA synthetase closely resembles the cytoplasmic one. Similar results were obtained with bean Leu-tRNA synthetase (Guillemaut et al., 1975), tobacco Leu-tRNA synthetase (Guderian et al., 1972) and Euglena isoleucyl-tRNA synthetase (Kislev et al., 1972). Therefore, mitochondrial tRNA\textsubscript{Leu} and Leu-tRNA synthetase are probably "imported" from the cytoplasm and the cytoplasm may therefore share the mitochondrial species of tRNA\textsubscript{Leu} and Leu-tRNA synthetase which could be easily resolved from whole cell preparations from the cotyledons. The fact that mitochondrial tRNA\textsubscript{Leu} and Leu-tRNA synthetase closely resemble the cytoplasmic ones and differ markedly
from the chloroplastic enzyme and tRNA\textsubscript{Leu}, supports the hypothesis that they are "imported" from the cytoplasm.

Evidence for the cytoplasmic origin of some of the chloroplastic aminoacyl-tRNA synthetases has been provided by Becker et al., (1974). Therefore, cytoplasmic origin of some of the organellar aminoacyl-tRNA synthetases has been fairly an established fact. It should be pointed out here that to date, there is no experimental evidence available explaining the mechanism of transport of tRNAs and aminoacyl-tRNA synthetases across the membranes. However, a hypothesis has been presented by Suyama and Hamada (1978) in this direction, based on an analogous situation. It has been found (Blobel and Dobberstein, 1975) that extracellular proteins possess a highly hydrophobic N-terminal polypeptide sequence(s) that serves as a signal for their transport across the cell membrane and this terminal polypeptide is removed through the transport process. Therefore, it would be interesting to discover whether a signal polypeptide is also associated with those mitochondrial aminoacyl-tRNA synthetases which are presumably translated extramitochondrially and transported in through the mitochondrial membranes. It is also suggested (Suyama and Hamada, 1978) that aminoacyl-tRNA synthetases which are transported across the membranes may also play a role in the trans-membrane transport of tRNAs probably by associating themselves with their substrates in a condition similar to the encapsulated tRNAs as found in RNA virions (Elder and Smith, 1973).

The Codon recognition studies with the tyrosyl-tRNA codons UAC
and UAU indicate that the cytoplasmic tRNA_{TY} has two isoacceptors recognizing the two different codons while all the remaining organelar tRNAs recognize the UAC codon. Although the chloroplast possesses two isoacceptors, both the species recognize a single codon (UAC). Therefore, these two isoacceptors may differ only in their modified bases. The existence of a single gene for chloroplastic tRNA_{TY} in the chloroplastic genome (Steinmetz, 1979) is an added support to this view. In addition, the fact that the two-dimensional polyacrylamide gel electrophoresis fails to separate these two species indicate the close structural similarity between the two isoacceptors of chloroplastic tRNA_{TY}.
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