Localization of tRNAs and aminoacyl-tRNA synthetases in cytoplasm, chloroplast and mitochondria of Glycine max, L.

Douglas A. Sinclair
University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation
https://scholar.uwindsor.ca/etd/2634
NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

Ottawa, Canada
K1A 0N4
LOCALIZATION OF tRNAs AND AMINOACYL-tRNA SYNTHETASES IN CYTOPLASM, CHLOROPLAST AND MITOCHONDRIA OF GLYCINE MAX, L.

by

Douglas A. Sinclair

A Thesis
Submitted to the Faculty of Graduate Studies and Research through the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

1980
ABSTRACT

Soybean leucyl-tRNAs and synthetases were localized in the cytoplasm, in the mitochondria and in the chloroplasts. Total leucyl-tRNA synthetase (cytoplasmic) resolves into three enzyme peaks when chromatographed on a hydroxylapatite column (HA) while the mitochondrial and the chloroplastic enzymes result in one peak each. Cytoplasmic enzyme peak 1 preferentially charges cytoplasmic tRNA^Leu^ species 5 and 6 and chloroplastic tRNA^Leu^ species 5 and 6. Cytoplasmic enzyme peak 2 preferentially charges cytoplasmic or mitochondrial tRNA^Leu^ species 1-4. Similarly, cytoplasmic enzyme peak 3 preferentially charges cytoplasmic or mitochondrial tRNA^Leu^ species 1-4. Chloroplast enzyme acylates only tRNA^Leu^ species 5 and 6 of chloroplast tRNA and preferentially charges peaks 5 and 6 of total cytoplasmic tRNA. Mitochondrial enzyme acylates four mitochondrial tRNA^Leu^ species 1-4 and the same four cytoplasmic tRNA^Leu^ species 1-4. However, the acylation of tRNA^Leu^ species 3 and 4 using mitochondrial tRNA is considerably increased. E. coli tRNA, charged with homologous synthetase and separated on RPC-5 column yields five tRNA^Leu^ species. Cytoplasmic enzyme (crude) acylates all five tRNA^Leu^ species of E. coli. Cytoplasmic enzyme 1 from H.A. column or purified chloroplastic enzyme acylate all five tRNA^Leu^ species of E. coli whereas cytoplasmic enzyme 2 and 3 and/or purified mitochondrial enzyme preferentially acylate tRNA^Leu^ species 1 and 2 of E. coli.
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 thesis.

For critically reviewing this thesis, I extend my sincere appreciation to Dr. N. Taylor, Department of Chemistry and Dr. D.G. Wallen, Department of Biology, both at the University of Windsor.

I am very grateful for the help and encouragement freely given by Miss L. Barakett, Mr. S. Gowda and Mr. S. Swamy.

I also gratefully acknowledge the financial assistance provided by the Department of Biology, the University of Windsor and by Grant No. Grant No. A-1984 to Dr. Pillay from the National Research Council of Canada.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>11</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>Eukaryotic Organelles</td>
<td>4</td>
</tr>
<tr>
<td>Aminoacyl-tRNA synthetase</td>
<td>5</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>6</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>7</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>10</td>
</tr>
<tr>
<td>Plant materials</td>
<td>10</td>
</tr>
<tr>
<td>Transfer RNA</td>
<td>10</td>
</tr>
<tr>
<td>Preparation of Leucyl-tRNA synthetase</td>
<td>11</td>
</tr>
<tr>
<td>Hydroxylapatite column chromatography</td>
<td>12</td>
</tr>
<tr>
<td>Transfer RNA aminoacylation assay</td>
<td>13</td>
</tr>
<tr>
<td>Reverse Phase Chromatography</td>
<td>13</td>
</tr>
<tr>
<td>Isolation of Organelles i) Mitochondria</td>
<td>14</td>
</tr>
<tr>
<td>ii) Chloroplast</td>
<td>15</td>
</tr>
<tr>
<td>Measurement of oxygen consumption</td>
<td>15</td>
</tr>
<tr>
<td>Extraction of Chloroplast and Mitochondrial tRNA</td>
<td>16</td>
</tr>
<tr>
<td>Aminoacylation of tRNA</td>
<td>17</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Stoichiometric acylation of cytoplasmic, mitochondrial and chloroplastic tRNAs at optimum enzyme concentrations.</td>
<td>33</td>
</tr>
<tr>
<td>2. Reverse phase column chromatography of cytoplasm leucyl-tRNA produced by leucyl-tRNA synthetase from cytoplasm of soybean cotyledons.</td>
<td>35</td>
</tr>
<tr>
<td>3. Reverse phase column chromatography of chloroplast leucyl-tRNA produced by leucyl-tRNA synthetase from cytoplasm of soybean cotyledons.</td>
<td>37</td>
</tr>
<tr>
<td>4. Reverse phase column chromatography of mitochondrial leucyl-tRNAs produced by leucyl-tRNA synthetase from cytoplasm of soybean cotyledons.</td>
<td>39</td>
</tr>
<tr>
<td>5. Reverse phase column chromatography of cytoplasmic leucyl-tRNAs produced by leucyl-tRNA synthetase from mitochondria.</td>
<td>41</td>
</tr>
<tr>
<td>6. Reverse phase column chromatography of mitochondrial leucyl-tRNAs produced by leucyl-tRNA synthetase from mitochondria.</td>
<td>43</td>
</tr>
<tr>
<td>7. Reverse phase column chromatography of chloroplast leucyl-tRNAs produced by leucyl-tRNA synthetases from mitochondria.</td>
<td>45</td>
</tr>
<tr>
<td>8. Reverse phase column chromatography of cytoplasmic leucyl-tRNAs produced by leucyl-tRNA synthetase from chloroplast.</td>
<td>47</td>
</tr>
<tr>
<td>9. Reverse phase column chromatography of chloroplast-tRNAs produced by leucyl-tRNA synthetase isolated from chloroplast.</td>
<td>49</td>
</tr>
</tbody>
</table>
10. Reverse phase column chromatography of mitochondrial tRNAs produced by leucyl-tRNA synthetase from chloroplast .............................................. 51

11. Hydroxylapatite fractionation of leucyl-tRNA synthetase from cytoplasm of soybean cotyledons .......... 53

12. Reverse phase column chromatography of cytoplasmic leucyl-tRNA produced by leucyl-tRNA synthetase fraction 1 from cytoplasm ............................................. 55

13. Reverse phase column chromatography of leucyl-tRNA (chloroplast acylated by leucyl-tRNA synthetase fraction 1 from cytoplasm) ............................................. 57

14. Reverse phase column chromatography of mitochondrial leucyl-tRNA acylated by leucyl-tRNA synthetase fraction 1 from cytoplasm .................................................. 59

15. Reverse phase column profile of cytoplasmic leucyl-tRNA acylated with hydroxylapatite fraction 2 enzyme from cytoplasm ............................................................. 61

16. Reverse phase column chromatography of mitochondrial leucyl-tRNA acylated with leucyl-tRNA synthetase fraction 2 from cytoplasm .................................................. 63

17. Reverse phase column chromatography of chloroplast leucyl-tRNA produced by leucyl-tRNA synthetase fraction 2 from cytoplasm .......................................................... 65

18. Reverse phase column chromatography of $^3$H-leucyl-tRNA (cytoplasmic) acylated with synthetase fraction 3 ................................................................. 67

19. Reverse phase column chromatography of mitochondrial leucyl-tRNA acylated with leucyl-tRNA synthetase fraction 3 ................................................................. 69
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>20. Reverse phase column chromatography of chloroplast-leucyl-tRNA acylated with synthetase fraction 3</td>
<td>71</td>
</tr>
<tr>
<td>21. Hydroxylapatite column chromatography of leucyl-tRNA synthetase from mitochondria</td>
<td>73</td>
</tr>
<tr>
<td>22. Reverse phase column chromatography of cytoplasmic leucyl-tRNA acylated by mitochondrial leucyl-tRNA synthetase fractionated by hydroxylapatite column chromatography</td>
<td>75</td>
</tr>
<tr>
<td>23. Reverse phase column chromatography of mitochondrial leucyl-tRNA acylated with hydroxylapatite fractionated mitochondrial synthetase</td>
<td>77</td>
</tr>
<tr>
<td>24. Reverse phase column chromatography of chloroplast leucyl-tRNA acylated by mitochondrial leucyl-tRNA synthetase fractionated by hydroxylapatite column chromatography</td>
<td>79</td>
</tr>
<tr>
<td>25. Hydroxylapatite column chromatography of leucyl-tRNA synthetase from chloroplast</td>
<td>81</td>
</tr>
<tr>
<td>26. Reverse phase column chromatography of cytoplasmic leucyl-tRNA acylated by hydroxylapatite fractionated chloroplast synthetase</td>
<td>83</td>
</tr>
<tr>
<td>27. Reverse phase column chromatography of chloroplast leucyl-tRNA acylated by chloroplast synthetase fractionated by hydroxylapatite column chromatography</td>
<td>85</td>
</tr>
<tr>
<td>28. Reverse phase column chromatography of mitochondrial leucyl-tRNA acylated by hydroxylapatite fractionated chloroplast synthetase</td>
<td>87</td>
</tr>
<tr>
<td>29. Reverse phase column chromatography of leucyl-tRNAs (E. coli) produced by leucyl-tRNA synthetase from E. coli</td>
<td>89</td>
</tr>
</tbody>
</table>
30. Reverse phase column chromatography of $^3$H-leucyl-tRNA isolated from *E. coli*, acylated with cytoplasmic enzyme.

31. Reverse phase column chromatography of leucyl-tRNAs produced by leucyl-tRNA synthetase (mitochondrial).

32. Reverse phase column chromatography of *E. coli* leucyl-tRNAs produced by leucyl-tRNA synthetase from chloroplast.

33. Reverse phase column chromatography of leucyl-tRNAs acylated by leucyl-tRNA synthetase fraction 1.

34. Reverse phase column chromatography of *E. coli* leucyl-tRNA acylated with leucyl-tRNA synthetase fraction 2.

35. Reverse phase column chromatography of leucyl-tRNAs acylated by leucyl-tRNA synthetase 3.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A260(280)</td>
<td>absorbance at 260 (280) nm</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5'-phosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>aa</td>
<td>aminoacid</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>diethylaminoethyl cellulose</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>H.A.</td>
<td>hydroxylapatite</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>O.D. units</td>
<td>optical density units</td>
</tr>
<tr>
<td>Plaskon</td>
<td>polychlorotrifluoroethylene</td>
</tr>
<tr>
<td>PPI</td>
<td>organic pyrophosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPC</td>
<td>reversed-phase chromatography</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
</tbody>
</table>
INTRODUCTION

When a cell passes from one physiological state to another, both qualitative and quantitative changes in certain isoaccepting tRNAs and aminoacyl-tRNA synthetases have been observed. Previous studies on tRNAs and synthetases in soybean have revealed that the complement of tRNA\textsuperscript{leu} isoaccepting species change during ageing of the cotyledons (15). The relative amounts of six tRNA\textsuperscript{leu} species in young and older cotyledons change, with such change being more pronounced in tRNA\textsuperscript{leu} species 5 and 6. This raised the question as to what brings about the changes in species 5 and 6 and further, since most of these studies were conducted with the total tissue including the cytoplasmic and organelar tRNAs and synthetases it was difficult to assess these changes in terms of their localization.

In a plant cell, protein synthesis takes place in three compartments; in the cytoplasm, in the mitochondria and in the chloroplast. Localized within the organelles (mitochondria and chloroplast) are precisely the same categories of informational, transcriptional and translational macromolecules that have been characterized in the cytoplasm of eukaryotes and in prokaryotes. In many respects, protein synthesis in organelles resemble protein synthesis in prokaryotes as seen by the sensitivity of organelar protein synthesis to certain antibiotics such as chloramphenicol, or the formylation of organelle initiator methionyl tRNA (18) rather than that taking place in the plant cell cytoplasm.

In order to provide a definitive answer this investigation was
undertaken to determine the presence of unique tRNAs and synthetases in the chloroplasts, the mitochondria and in the cytoplasm of Glycine max through the use of homologous and heterologous aminoacylation experiments. In addition, E. coli tRNA^leu and leu-tRNA synthetases were included in these comparative studies to provide further proof that tRNAs and synthetases from plant organelles resemble those from bacteria and thus add credence to the endosymbiont theory.
REVIEW OF LITERATURE

The involvement of an "adaptor molecule" in the transfer of amino acids to the site of protein synthesis was first proposed by Crick (28). Subsequent discoveries of soluble RNA in E. coli (14) and rat liver (41, 53) offered credence to the theory that transfer RNA could play a major role in metabolic regulations (16, 57). At the same time it could recognize the nucleotide code in messenger RNA and the specific amino acid involved in protein synthesis.

The concurrent work on the genetic code and its degeneracy raised the question as to the possible degeneracy of the tRNA complement of a cell. In 1959 Holley et al., (42) showed the existence of multiple isoaccepting species of a particular tRNA in rat liver. Based on this information, evidence has accumulated which attests to the existence of multiple isoaccepting tRNAs.

Aminoacyl tRNA synthetases were first isolated by Hoagland et al., (39, 40). This led to the elucidation of the first two steps of protein biosynthesis; namely 1) the activation of the amino acid with ATP forming an aminoacyl adenylate anhydride and 2) the incorporation of the activated amino acid to the soluble portion of the nucleic acids, by Berg and Olfengand (14).

```
\[
\text{AA} + \text{ATP} \xrightarrow{E_1} \frac{\text{AMP}}{\text{PPi}} \xrightarrow{E} \text{tRNA} \xrightarrow{E} \text{aminoacyl-tRNA}
\]
```
These two steps are extremely important for the fidelity of translation since in later steps of protein synthesis, the aminoacyl – tRNA is recognized through its polynucleotide moiety. Experiments by Chapeville et al., (25) have shown that any mistake in the nature of the amino acid attached to a tRNA would result in an error in the protein synthesized.

For the correct aminoacylation of a tRNA, it must be catalyzed by an appropriate aminoacyl – tRNA synthetase. In prokaryotic cells, there are 20 different aminoacyl – tRNA synthetases; however, there are about 60 different tRNAs. The various isoaccepting tRNAs are recognized by the same aminoacyl – tRNA synthetase. These differences in isoacceptors may be due to either sequence differences, or structural dissimilarities (62).

The situation is more complicated when one considers a eukaryotic cell. Pioneering studies by Barnett et al., (6,7) showed that mitochondria contains tRNAs and aminoacyl-tRNA synthetases which are different from the cytoplasmic counterparts. With the addition of chloroplast organelles in plants the situation becomes further complicated.

Eukaryotic Organelles

Historically, mitochondria and chloroplasts have been the subject of extensive research. Recent research stems around the revelation that localized within these organelles are precisely the same areas of informational, transcriptional and translational macromolecules that have been characterized in prokaryotes and in the cytoplasm of eukaryotes. Thus in the organelles a functional apparatus for protein synthesis
is present complete with ribosomes, tRNAs and synthetases. It is accepted that organelles have a molecular biology of their own, although dependent upon interaction with the host cytoplasm. In many respects protein synthesis in organelles resemble protein synthesis in prokaryotic organisms; the small size of the ribosomes, the formulation of organelle initiator methionyl-tRNA and the sensitivity of organelle protein synthesis to antibiotics such as chloramphenicol (10).

Existence of organelle specific tRNAs and aminoacyl-tRNA synthetases plays an important role in connection with their multiplicities.

Aminoacyl tRNA synthetase

With the increasing evidence showing the widespread occurrence of multiple isoaccepting tRNA species, investigations to search for the existence of multiple aminoacyl-tRNA synthetases have been on the rise.

In prokaryotic organisms only a few instances of synthetase multiplicity have been reported. In *Bacillus brevis* Surgachev et al., (58,59) have reported the occurrence of multiple methionine, aspartic acid, leucine and phenylalanine specific enzymes. Mahler and Jenenski (47) have reported multiple proline specific enzymes in *E. coli*. In general, however, the majority of the results with prokaryotes point to the finding of only one synthetase for each amino acid.

In eukaryotic organisms the occurrence of multiple isoenzymes is well documented (2,4,65).
In general then, individual synthetases have been shown to recognize both specific amino acids and their cognate tRNAs, in addition to performing three basic catalytic functions:

1) the hydrolysis of ATP to AMP + inorganic pyrophosphate
2) tRNA acylation
3) tRNA deacylation

While each synthetase is specific for a particular amino acid, structurally related tRNA can be acylated by one enzyme (13,45). In higher organisms the situation is complex (2,44) due to the presence of multiple forms of amino acid-specific synthetases which acylate different isoacceptors. One reason for this multiplicity of synthetases in eukaryotic cells is that both cytoplasmic and organelle associated forms may exist (17,27) which exhibit differential activities with different isoacceptor tRNAs.

Mitochondria

The first evidence that mitochondria have molecular pieces of tRNA and synthetases which differ from those of the cytoplasm was presented by Barnett et al., (5-8). In Neurospora chromatographically separable synthetases for certain aminoaicyl-tRNAs were discovered. These results seemed to defy the central dogma which stated that all organisms contain only one synthetase for each amino acid. Further work using mitochondrial tRNA and chromatography of soluble protein preparations from mitochondria has shown that one of the synthetases was of mitochondrial origin and the other cytoplasmic. Since this
time a full complement of tRNA species of similar size (4S) to eukaryotic or prokaryotic cytoplasmic tRNAs has been identified in a number of types of mitochondria (8, 48, 55).

With regard to the acylation specificities of mitochondrial synthetases the recognition between organelle and cytoplasmic tRNAs and synthetases is analogous to that in other heterologous systems; some cross reactions are complete, others have low rates of acylation and still others show no cross-recognition.

Chloroplasts

Aliev and Filipovich (1) first demonstrated that pea chloroplasts contain a species of tRNA which is chromatographically distinct from cytoplasmic tRNA. In a study of tRNAs and aminoacyl-tRNA synthetases of Phaseolus vulgaris Burkard et al., (21, 35, 36) described a chloroplast tRNA that can be acylated and formulated by either chloroplast enzymes or E. coli synthetase but not by the corresponding cytoplasmic or mitochondrial enzymes. In addition there are four other leucyl-tRNA's in chloroplasts that can be charged by either the cytoplasmic or mitochondrial enzymes but not by the chloroplast or E. coli enzymes.

Barnett et al., (9, 56) studying the effects of light on the pattern of tRNAs present in Euglena observed that for all amino acids examined, two sets of tRNA species exist: one that is "constitutive" in that its cellular levels are independent of growth conditions and one that is light inducible. Further experimentation showed that light does not induce the synthesis of these new species of tRNA in the U.V. bleached mutant, w3 BUL, which lacks a chloroplast DNA and an identifiable
chloroplast structure. These findings infer that the new tRNA species which are light inducible may be of chloroplastic origin. This finding was borne out by reverse phase column chromatography of the tRNAs from isolated chloroplasts. Similar experiments were done with respect to the aminoacyl-tRNA synthetases in which case some, but not all, of the chloroplast synthetases were shown to be light inducible and could not be detected in either the dark grown or the W3BUL bleached mutants. Weil et al., (36, 43, 62) have shown in aminoacylation experiments that mitochondria-specific and chloroplast-specific prolyl-tRNAs can be charged by mitochondrial, chloroplastic or E. coli enzymes, but not by the cytoplasmic enzyme. The two cytoplasmic tRNAs Pro on the other hand can be charged by the cytoplasmic enzyme but not by either the E. coli or organelle enzymes. Similar cross-reaction experiments using chloroplastic prokaryotic and cytoplasmic tRNAs and aminoacyl-tRNA synthetases have been performed on algae (11, 52) bean (35, 36, 43) and cotton (51).

In mitochondria the situation is less resolved. In some cases the mitochondrial aminoacyl-tRNA synthetase resembles chloroplast and bacterial enzymes (36, 43). But in other cases, the mitochondrial enzymes behaves similar to the cytoplasmic enzyme as shown in Euglena isoleucyl-tRNA synthetase, (46) tobacco leucyl-tRNA synthetases (32) and bean leucyl tRNA synthetases (36).

Winterberger and Viehhauser (63) first demonstrated that yeast mitochondrial tRNA hybridizes to yeast mitochondrial DNA. Similar experiments by Tewari and Wildman (60) showed that tobacco chloroplast tRNA hybridizes to chloroplast DNA. Since then the existence of organelle
specific tRNAs coded for by organellar DNA has been well established (49).

Recently, Guillemaut and Keith (37,38) have determined a tentative primary sequence for chloroplast tRNA^Phe of Phaseolus. Surprisingly, it differs from Euglena chloroplast tRNA in only 5 parent nucleotides, 3 occurring in the acceptor stem. Thus the chloroplast tRNA^Phe from two phylogenetically widely divergent organisms are strikingly similar. All tRNA^Phe of known sequence are similar and contain 76 nucleotides. However, in studying sequence homology there is a greater degree of homology among prokaryotes and among eukaryotes than between the two groups; in this regard chloroplast tRNA^Phe is more similar to prokaryote tRNA^Phe. In the majority of cases when studying the terms of modified nucleotide content (49,50) chloroplast tRNA^Phe lacks m^2G, m^2C, Cm, w' and m^1A all considered characteristic of eukaryotic cytoplasmic tRNA^Phe (exceptions) (33,38).

In looking at mitochondrial tRNA^fMet sequence homology it does not appear to fit logically into either group (10), showing only slightly more homology to prokaryotic species. However, unlike prokaryotic tRNA^fMet it contains a Watson and Crick base pair at the end of the acceptor as do the eukaryotic tRNAs^fMet. If the mitochondrial tRNA^fMet has been subjected to the same selective pressures for conservation of sequence as those of extant prokaryotes and eukaryotes, it is possible that mitochondria evolved from a primitive organism not represented in current sampling. Barnett et al., (10) hypothesize that based upon the sequence of chloroplast tRNA^Phe and its similarity to other Phe-tRNA that chloroplasts evolved considerably later than mitochondria during the course of evolution.
MATERIALS AND METHODS

Plant Material

Soybean seeds (Glycine max var-Harcor) were soaked in distilled water for 3-4 hours and sown in moist vermiculite. Cotyledons and hypocotyls were harvested after 5 days of germination in the dark at 27-29°C. Leaves were harvested from plants grown in the light in a plant growth chamber after 10 days of germination.

Transfer RNA

Transfer RNA was prepared from total RNA of cotyledons from either freshly harvested or those stored for several days at -20°C. The total RNA was extracted according to procedures described by Pillay and Cherry (54). The homogenization buffer was prepared by shaking 10 mM Tris (hydroxymethyl aminoethane) – HCl buffer, pH 7.6, containing 60 mM KCl and 10 mM MgCl₂ with 90% aqueous solution of phenol at the ratio of 10:8 (v/v) for several hours and separating the phases. The cotyledons were ground in a Waring blender in batches of 100 grams with a mixture of 100 ml of aqueous phase buffer, 100 ml of phenolic phase, 40 ml of 11% dupanol (sodium lauryl sulfate) and 20 ml of bentonite suspension (20) of 40 mg/ml. The homogenate was strained through four layers of cheese cloth and then centrifuged for 10 min at 10,000 xg. The supernatant was stirred for one hour with an equal volume of the organic (phenolic) phase. The aqueous phase was collected by centrifugation at 10,000 xg for 15 min, made to 0.2 M with
respect to potassium acetate and mixed with two volumes of cold 95% ethanol and stored overnight in the freezer at -20°C. Next day the precipitate was collected by centrifugation at 10,000 xg for 15 min, dissolved in buffer A (30 ml/100 gm of tissue) and phenol–extracted four to five times using equal volumes of organic phase containing 1/18 volume of the bentonite suspension. After each extraction, the aqueous phase was stirred in the cold for 30 min and recovered by centrifugation. The total RNA was then precipitated with 2 volumes of cold 95% ethanol. The pellet was extracted several times with 2.0 M potassium acetate pH 6.5 (1 ml/100 g tissue) by suspending with a mortar and pestle followed by centrifugation. The crude tRNA was further purified and concentrated by adsorption on a small DEAE-cellulose column in buffer B (10 mM sodium acetate pH 4.5 containing 10 mM MgCl₂) and eluted with 1 M NaCl in the same buffer. The solution was dialyzed against distilled water, and the concentration determined based on the A\textsubscript{260} absorbancy.

**Preparation of Leucyl-tRNA Synthetase**

Extraction, purification and fractionation of the enzyme was performed at 0-4°C. Freshly harvested tissue was ground on ice for a few minutes with insoluble polyvinylpyrrolidone (Polyclar AT:200 mg/g tissue) using a cold mortar and pestle. The grinding was continued for 10 min, with stepwise addition of the grinding medium, which consisted of 25 mM potassium phosphate, pH 7.8, in solution C (10^{-2} M 2-mercaptoethanol; 10^{-5} M phenylmethyl sulfonylfluoride (PMSF): 10^{-6} M L-leucine)
saturated to 30% with ammonium sulfate. The homogenate was strained through four layers of cheesecloth and centrifuged at 27,000 xg for 15 min. The supernatant was then filtered through miracloth and the ammonium sulfate concentration adjusted to 60% and stirred in the cold for 30 min. After centrifugation at 10,000 xg for 15 min the enzyme pellet was either stored in the freezer or used immediately. The pellet was dissolved in 10 ml of 25 mM potassium phosphate, pH 7.8 and dialyzed in buffer C for 3-4 hours. The proteins were adsorbed onto a 15 ml column of DEAE cellulose in 25 mM potassium phosphate, pH 7.8, in buffer C. The column was then washed with the same solution and the enzyme eluted using 0.1 M potassium phosphate pH 7.8 in buffer C. The absorbance of each fraction at $A_{280}$ and $A_{260}$ was measured and those fractions showing maximum protein content were pooled and used either as an enzyme source or for further enzyme purification.

**Hydroxylapatite Column Chromatography**

In order to separate the isoenzymes, the enzyme solution from the DEAE column was applied to a hydroxylapatite column (HA column) made of a mixture of 10 grams of hydroxylapatite and 1 gram of cellulose powder (Whatman DF 11) with 0.5 gram portions of cellulose at the top and bottom of the packing. The column was equilibrated by washing with 200-300 ml of 0.05 M potassium phosphate buffer, pH 6.5 in solution C. The enzyme adjusted to pH 6.5, and the $\text{PO}_4^{3-}$ concentration adjusted to 0.05 M was applied to the column followed by 50 ml of
buffer. Elution was performed using a potassium phosphate gradient from 0.05 M to 0.4 M (linear) containing β-mercaptoethanol, leucine and phenylmethylsulfonylfluoride at the constant concentration equal to that of the starting buffer and 5 ml fractions were collected. For leucyl-tRNA synthetase activity a reaction mixture of 0.4 ml containing 200 µl of fractionated enzyme plus other components were used as described in the aminoacylation assay. The reaction was allowed to proceed for 20 minutes at 30°C and then terminated by the addition of 10% TCA.

Transfer RNA Aminoacylation Assay

The reaction was carried out at 30°C. Unless otherwise stated 1 ml of the reaction mixture contained 100 mM Tris (hydroxymethyl aminoethane)–HCl pH 7.8; 10 mM MgCl₂; 1 mM ATP; 0.2% soluble polyvinylpyrrolidone, 200 µg/ml tRNA, 200 µg/ml of enzyme and 20 µl unneutralized solution of l-(4,5-3H) leucine solution 60 Ci/m mole.

Reverse Phase Column Chromatography (RPC-5).

A mixture of 8 ml of Adogen 464 in 400 ml of chloroform was coated on to 200 g of polychlorotrifluoroethylene (plaskon) support, according to Pearson et al. (60). Finally the coated plaskon was suspended in 0.5 M NaCl in buffer B for packing of the column.

Aminoacyl-tRNA for RPC-5 chromatography was prepared essentially as stated above. Normally a 1-2 ml reaction mixture was incubated at 30°C for 30 min and the reaction stopped by immersing in an ice
bath for 20 minutes. The mixture was passed through a small DEAE-
cellulose column (18), washed with 0.3 M NaCl in buffer B, (10 mM
sodium acetate pH 4.5 containing 10 mM MgCl₂) and eluted with 10 ml
buffer containing 1.0 M NaCl. The eluate was diluted three times
and applied to an RPC-5 column (1.5 x 100 cm). Elution was performed
under pressure, at room temperature with a linear gradient from 0.5 M
to 1.0 M of NaCl in buffer B and 5 ml fractions were collected at a
flow rate of 1 ml/min, made 5% with respect to cold trichloracetic
acid (TCA). The TCA insoluble material was filtered through glass
fibre filters, washed with 3 volumes of 5% TCA, and the filters dried.
Radioactivity was determined in a Beckman liquid scintillation counter
using a toluene based scintillation fluid (0.4% PPO and 0.005% POPOP).

Isolation of Organelles

Mitochondria:

Seeds of Glycine max were grown in the dark and the hypocotyls
harvested after 5 days. Mitochondria were extracted as described by
Guillemant et al., (35). Lots of 200 grams of hypocotyls were quickly
homogenized in a Waring blender at 0⁰-4⁰C in 200 ml buffer containing
0.7 M mannitol, 4 x 10⁻⁴ M ATP, 10⁻³ M EDTA and 1 mg/ml of bovine serum
albumin, with the pH adjusted to 7.2 with triethanolamine. The homo-
genate was then filtered through several layers of fine nylon cloth
and centrifuged at 1,000 x g for 5 min. The supernatant was layered
onto a sucrose cushion containing 27% sucrose, 10⁻⁴ M EDTA and 2 mg/ml
of bovine serum albumin with the pH adjusted to 7.2 with triethanol-
mine. The ratio of 5:2 supernatant to cushion was centrifuged at
8,000 xg for 10 min. The mitochondrial pellet was then resuspended in the appropriate buffer for either enzyme preparation or tRNA extraction.

**Chloroplasts:**

Seeds of *Glycine max* were grown in the light and the leaves harvested after 10 days. Batches of 100 grams of leaves were quickly homogenized in a Waring blender in 350 ml buffer containing 0.05 M Tris-HCl pH 8.0, 3 x 10^{-3} M EDTA; 10^{-3} M β-mercaptoethanol, 0.3 M mannitol and 1% BSA (22). The resultant slurry was then passed through several layers of 50 μ nylon cloth squeezing gently. The filtrate was then passed through several layers of 25 μ nylon cloth followed by 10 μ mesh nylon cloth without squeezing. The final filtrate was centrifuged at 1,000 xg for 90 sec. The supernatant was quickly poured off and the pellet resuspended in the extraction buffer (1 ml for 10 g of tissue). This was followed by centrifugation at 1,000 xg for 90 sec. The supernatant was discarded and the pellet stored in the freezer. All operations were done at 0°-4°C.

**Measurement of Oxygen Consumption**

The rate of oxygen consumption at 30°C by mitochondrial suspensions were 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 deter-
mined according to Beechey and Ribbons (12). Rates of oxygen consumption by mitochondrial suspensions were measured according to Estabrook (30) in a 3 ml reaction mixture (29).

**Extraction of Chloroplast and Mitochondrial tRNA**

Generally 60-80 ml extraction medium containing $10^{-2}$ M Tris-HCl pH 7.4, $10^{-2}$ M MgCl$_2$ and 1% SDS was used for organelle preparation from a kilogram of tissue. To each tube containing the organelle pellet, 5 ml extraction buffer was added; the pellet quickly dissolved and immediately poured into water saturated phenol. The phenol buffer was in a 1:1 volume with the extraction medium. These operations were done on ice, keeping the phenol buffer cold and the extraction buffer at room temperature, to prevent the SDS precipitation. The mixture was stirred for 30 min and then centrifuged at 3,500 xg for 15 min. The aqueous phase was extracted once again. A 1/10 volume of 20% potassium acetate pH 5.0 was added to the aqueous phase, followed by 2 volumes of 95% ethanol. The next day the pellet was collected by centrifugation at 1000 xg and dissolved in 4 ml of 1 M cold NaCl. The solution was again centrifuged at 1,000 xg for 15 min. The supernatant was then diluted with Tris pH 7.5 to bring the Tris concentration to $10^{-2}$ M, keeping the MgCl$_2$ concentration at $10^{-2}$ M and the final NaCl concentration to 0.2 M. This is the optimum condition for DNase activity. 10 ug of DNase/ml was added and the reaction allowed to proceed for 90 minutes at 4°C. The solution was then adsorbed on a DEAE cellulose column in 0.1 M Tris. Washing continued till the absorbance at 260 was less than 0.03 O.D. The tRNA was eluted with 1 M Tris and fractions showing an O.D. greater than 0.2
were pooled. Two volumes of 95% ethanol were added to the pooled fractions. The pellet was collected the next day by centrifugation, the tRNA suspended in distilled water and the solution stored in the freezer.

**Aminoacylation of Organellar tRNA**

Aminoacylation of organellar tRNA was carried out at 30°C for 30 min in a 0.5 ml reaction mixture containing 10 mM Tris-HCl pH 7.8 and optimal concentrations of Mg++, ATP and the aminoacid. ATP was tested at 0.05, 0.25, 0.5, 1, 10 and 20 mM while the Mg++ concentrations were 10, 50, 100, 150 and 200 mM. Concentrations which elicited maximum response were selected for aminoacylation. The reaction was started by the addition of synthetase enzyme. After incubation 50 ul of the reaction mixture was applied to Whatman No. 3 filter paper discs, dried and immersed in cold 5% TCA. The discs were washed three times with cold TCA, once each with ethanol; ether (3:1), ethanol: ether (2:1), and ethanol: ether (1:1) and finally, ether alone. The discs were then dried, transferred into scintillation vials, and radioactivity determined in a scintillation counter.
RESULTS

The localization of tRNAs and aminoacyl-tRNA synthetases in the cytoplasm, the chloroplast and the mitochondria of *Glycine max* together with the aminoacylation of leucyl-tRNA species by homologous and heterologous enzymes are described here.

Optimum ATP and Mg$^{++}$ levels

A series of assays were carried out to optimize Mg$^{++}$ and ATP concentration in our assay system. Using a pH optimum of 7.8 for chloroplast assay system a range of concentrations for ATP (1-2.5 mM) and for magnesium (0.05 to 5 mM) were tested. For the mitochondrial assay system concentrations of ATP (0.5-2 mM) and magnesium (0.05-5 mM) were tested. Activity in both organelle systems was inhibited only slightly (10%) when ATP and Mg$^{++}$ levels were varied from 0.5-10 mM ATP and 0.5-15 mM Mg$^{++}$. Based on the maximum activity at optimal ATP and Mg$^{++}$ concentrations, the standard assay reaction mixture used contained 1 mM ATP and 10 mM Mg$^{++}$ for all the three systems tested.

Time course experiments were conducted using cytoplasmic, chloroplastic and mitochondrial preparations in order to determine the time required for maximum acylation. Figure 1 shows the results of these experiments. Maximum acylation for the cytoplasmic tRNA occurs at 20 minutes, while the optimal acylation for the mitochondrial and the chloroplast tRNAs occur after 15 and 10 minutes respectively. In all the experiments reported here the aminoacylation reactions were allowed
to proceed for 20 minutes.

Purity of Organelles

In order to determine the purity of the organelles, samples of chloroplast preparations were taken during different stages of purification and examined under the light microscope. Filtration through a fine mesh nylon cloth was continued until no further decrease in cellular contamination was achieved.

Mitochondria were biochemically tested to determine the purity of the organelle preparation. The acceptor control ratio (A.C.R.) was measured in order to determine the intactness of the organelles. The greater the ratio of broken to intact organelles the lower the respiratory rate and this results in a subsequently lower acceptor control. When pyruvate was used as the substrate the acceptor control ratio averaged 1.97, with succinate as the substrate the A.C.R. was 2.40. An acceptor control ratio of about 2 or greater indicates the organelles are intact to a large degree.

Comparison of tRNA\textsuperscript{Leu} from the Different Cell Compartment

A comparison of the elution profile of leucyl-tRNAs from the cytoplasm, the chloroplast and the mitochondria after reverse phase column chromatography suggests the localization of leucyl-tRNA species in the organelles. Charged leucyl-tRNA preparations from the cytoplasm of soybean cotyledons fractionate into six discrete peaks on plaskon chromatography (RPC-5). Figure 2 shows this elution profile which will be used as the control to make the necessary comparisons in the dis-
cussion that follows. Aminoacylation of chloroplast tRNA with a total crude cytoplasmic enzyme and chromatography on RPC-5 (Figure 3) indicates a three fold increase in tRNA^{Leu} species 5 and 6 when compared to the control. Although tRNA^{Leu} species 3 and 4 (believed to be mitochondrial) essentially disappear, small peaks of tRNA^{Leu} species 1 and 2 are still present, which may be indicative of some cytoplasmic contamination. Irrespective of the source of tRNA (cytoplasmic or chloroplastic) the enzyme is able to charge leucyl-tRNA species 5 and 6.

Aminoacylation of mitochondrial tRNA with a crude cytoplasmic enzyme and chromatography on RPC-5 shows only tRNA^{Leu} species 1 to 4 (Figure 4). A sharp increase in peaks 3 and 4 is observed when compared to the cytoplasmic tRNA^{Leu} elution profiles. These results suggest that among the isoaccepting leucine-specific tRNAs some may be localized in the chloroplast or in the mitochondria.

Cross Aminoacylation Reactions

In order to determine whether differences exist in both isoaccepting tRNAs and synthetases in the organelles, homologous and heterologous aminoacylation reactions were studied. Figure 5 indicates that a crude mitochondrial enzyme preparation acylates all 6 peaks of cytoplasmic tRNA. When a mitochondrial tRNA preparation is acylated using a crude mitochondrial enzyme, Leu-tRNA species 1-4 are the most pronounced peaks (Figure 6). The fact that tRNA^{Leu} 5 and 6 are present to a minor degree may be due to contamination in the crude preparation, which requires further study. When a chloroplast tRNA was acylated by
a crude mitochondrial enzyme, tRNA\textsuperscript{Leu} 1 and 2 were observed (Figure 7).

Next a chloroplast enzyme preparation was used to acylate tRNA\textsuperscript{Leu} from the three sources. Figure 8 indicates the presence of tRNA\textsuperscript{Leu} 1, 2, 5 and 6 when a cytoplasmic tRNA is acylated using a crude chloroplast synthetase and chromatographed on RPC-5. If a chloroplast tRNA is acylated by a chloroplast enzyme and chromatographed on RPC-5 tRNA\textsuperscript{Leu} 5 and 6 (Figure 9) are the predominant isoacceptors present (46% and 39% respectively). The elution profile shown in Figure 10 indicates that a crude chloroplast enzyme preparation does acylate mitochondrial tRNA but then only tRNA\textsuperscript{Leu} 1 and 2 are the dominant species. This may be indicative of some contamination of the enzyme source. These results suggest that the tRNA species may be localized in the organelles and that the three plant cell compartments may also house different isoenzymes.

In view of the fact that organelle synthetases appear to possess differences in their acylation capabilities, synthetase preparations from the three cell compartments were further purified by fractionation on a hydroxylapatite column.

**Fractionation of the Aminoacyl-tRNA Synthetase from the Three Cell Compartments**

The aminoacyl-tRNA synthetases specific for the same amino acid but located in three cell compartments can be separated on the basis of their chromatographic mobilities (62).

When a crude cytoplasmic leucyl-tRNA synthetase preparation is
chromatographed on a hydroxylapatite column and assayed for enzyme activity using cytoplasmic tRNA as substrate, three peaks of leucyl-tRNA synthetase activity are observed (Figure 11) and the individual peaks are designated as enzyme 1, 2 and 3. Individual isoenzymes were then used to acylate the compartmentalized tRNAs. Results presented in Figure 12 indicates that cytoplasmic enzyme 1 charges only tRNA$^{\text{Leu}}$ 5 and 6 of the cytoplasmic tRNA, compared to the total crude cytoplasmic enzyme preparation which charges all six tRNAs. Similarly, when cytoplasmic enzyme 1 is used to acylate chloroplast tRNA, again only tRNA$^{\text{Leu}}$ 5 and 6 elute on RPC-5 (Figure 13). If a mitochondrial tRNA preparation is charged with a cytoplasmic enzyme 1 again tRNA$^{\text{Leu}}$ 5 and 6 are present (Figure 14). However, the amount of charging is extremely low, which may be due to impurity of tRNA source. Cytoplasmic enzyme 1 then appears to be chloroplast specific, since it acylates tRNA$^{\text{Leu}}$ species 5 and 6 if the substrate used is either cytoplasmic or chloroplast tRNA.

Figure 15 shows the elution profile of cytoplasmic tRNA acylated by cytoplasmic enzyme 2 after passage through an RPC-5 column. Four isoacceptors corresponding to tRNA$^{\text{Leu}}$ 1-4 of control cytoplasmic preparation are present. When a mitochondrial tRNA preparation is acylated with cytoplasmic enzyme 2 the four isoacceptors are again prevalent (Figure 16). On a quantitative basis peaks 3 and 4 show a marked percentage (110%) increase in the mitochondrial preparation compared to the preceding figure. Figure 17 shows the elution profile
of chloroplast tRNA acylated by cytoplasmic enzyme 2. The ability of cytoplasmic enzyme 2 to charge chloroplast tRNA is very minor and this could be due to some cross contamination.

Figure 18 shows that cytoplasmic enzyme 3 acylates four isoaccepting cytoplasmic leucyl-tRNAs. On a quantitative basis peaks 1 and 2 are the prevalent isoacceptors, accounting for 25% and 50% respectively of the total charging. When a mitochondrial tRNA is charged using a cytoplasmic enzyme 3 the elution profile again shows that four isoacceptors are present (Figure 19). However, on a quantitative basis peaks 3 and 4 have increased markedly (125% and 100% respectively). Chloroplastic-tRNA acylated by cytoplasmic enzyme 3 and chromatography on RPC-5 results in only tRNA$^{\text{Leu}}$ 1 and 2 (Figure 20).

Hydroxylapatite chromatography of a mitochondrial enzyme preparation and assay of the column using cytoplasmic tRNA as substrate, only one peak of activity is observed which appears similar to cytoplasmic enzyme 2 (Figure 21). When a cytoplasmic tRNA is acylated by this mitochondrial enzyme, tRNA$^{\text{Leu}}$ 1-4 are observed, with peaks 1 and 2 being the most prevalent (Figure 22). Mitochondrial tRNA acylated using the purified mitochondrial enzyme exhibits a similar elution profile; however, quantitatively there is an increase (110%) in peaks 3 and 4 (Figure 23). When chloroplast tRNAs are aminoacylated using the mitochondrial enzyme two small peaks corresponding to tRNA$^{\text{Leu}}$ 1 and 2 in the control are present (Figure 24). Again this may be due to contamination in tRNA source.
When a chloroplastic enzyme preparation is chromatographed on hydroxylapatite and a cytoplasmic tRNA is used as a substrate to determine the leucyl-tRNA synthetase activity of the column fractions only one peak of activity is observed (Figure 25). This chloroplast enzyme is similar to cytoplasmic enzyme 1 in its ability to charge leucyl-tRNA species 5 and 6 when either cytoplasmic or chloroplastic tRNA is used as substrate (Figures 26, 27). When mitochondrial tRNA is aminoacylated by this chloroplast enzyme, two small peaks of tRNA$^{\text{Leu}}$ 5 and 6 are present (Figure 28); however, these could be due to contamination in the tRNA source. Chloroplast tRNA charged with this chloroplast enzyme, leucyl-tRNA species 5 and 6 are observed upon RPC-5 chromatography (Figure 27), accounting for 51% and 49% respectively of the charging.

**Studies Using a Prokaryotic tRNA**

A prokaryotic tRNA from *E. coli* was used to study the similarities, if any, among the different enzyme sources in the plant cell compartments, to those of a prokaryotic enzyme source. Charged *E. coli* leucyl-tRNA fractionates into five discrete peaks on plaskon columns (RPC-5). The elution profile of *E. coli* leucyl-tRNA acylated by *E. coli* enzyme is shown in Figure 29 which will be used for comparison in the following studies.

When *E. coli* tRNA is charged by a soybean cytoplasmic synthetase five tRNA$^{\text{Leu}}$ peaks upon RPC-5 chromatography are obtained (Figure 30). Isoaccepting species 1 and 2 are the prevalent peaks. When *E. coli*
tRNAs are aminoacylated using soybean mitochondrial enzyme, two mitochondrial specific leucyl-tRNA peaks are observed (Figure 31). Compared to the control profiles these two peaks elute similar to peaks 1 and 2. Using *E. coli* tRNAs aminoacylated by the soybean chloroplastic enzyme, the elution profile shows 5 isoacceptors present (Figure 32). It is readily noticeable that peaks 3-5 are quantitatively greater (400% increase) than in the cytoplasmic acylated profile.

Figure 33 shows the elution profile upon RPC-5 chromatography of *E. coli* tRNA aminoacylated by the cytoplasmic enzyme 1. Five *E. coli* specific leucyl-tRNA peaks are observed. When charged by cytoplasmic enzyme 2, *E. coli* tRNA yields two peaks upon chromatography using RPC-5 (Figure 34). When compared to the control elution profile these isoacceptors correspond to peaks 1 and 2. The elution profile of *E. coli*-tRNA charged by cytoplasmic enzyme 3 results in 2 peaks eluting similar to peaks 1 and 2 in the control (Figure 35).
Summary of Figures 11, 25, 21

A comparison of hydroxylaaptite column chromatography of leucyl-tRNA synthetases from the cytoplasm, the chloroplast and the mitochondria. Approximately 20 mg of protein was loaded onto a column (2 cm x 30 cm) in 50 ml of 0.05 M potassium phosphate (pH 6.5) and eluted using a linear gradient (0.05-0.4 M) of potassium phosphate.
Summary of Figures 12, 13, 26, 27

A comparison of the RPC-5 elution profiles of cytoplasmic and chloroplastic leucyl-tRNA acylated by leucyl-tRNA synthetase (hydroxylapatite purified) from chloroplast or enzyme 1 from the cytoplasm. All other conditions are as given in Methods.
Summary of Figures 2, 15, 18

Reversed phase column chromatography of cytoplasmic leucyl-tRNA acylated by leucyl-tRNA synthetases from 1) crude cytoplasmic enzyme, 2) enzyme 2 of cytoplasm obtained from hydroxylapatite chromatography and 3) enzyme 3 of cytoplasm obtained from hydroxylapatite chromatography. All other conditions are as given in Methods.
Summary of Figures 4, 16, 19

Reversed phase column chromatography of mitochondrial leucyl-tRNA acylated by leucyl-tRNA synthetase from 1) crude cytoplasmic preparation 2) enzyme 2 obtained from hydroxylapatite chromatography and 3) enzyme 3 obtained from hydroxylapatite chromatography. All other conditions were as given in Methods.
Summary of Figures 4, 6, 22, 23

A comparison of the elution profile following reverse phase column chromatography of mitochondrial and cytoplasmic leucyl-tRNA acylated by leucyl-tRNA synthetase from cytoplasm, mitochondria and mitochondrial enzyme obtained from hydroxylapatite chromatography. All other conditions were as given in Methods.
Summary of Figures 29, 30, 32, 33

Reversed phase column chromatography of E. coli leucyl-tRNA acylated by leucyl-tRNA synthetase from 1) E. coli, 2) enzyme I obtained from hydroxylapatite chromatography; 3) chloroplast and 4) cytoplasm of soybean. All other conditions were as given in Methods.
Summary of Figures 31, 34, 35

Reversed phase column chromatography of *E. coli* leucyl-tRNA acylated by leucyl-tRNA synthetase from 1) enzyme 2 obtained from hydroxylapatite chromatography 2) enzyme 3 obtained from hydroxylapatite chromatography and 3) mitochondria. All other conditions were as given in Methods.
Figure 1

Stoichiometric acylation of cytoplasmic (●——●), mitochondrial (X——X) and chloroplast (◆——◆) tRNAs at optimum enzyme concentrations. The conditions for aminoacylation were as follows: 0.2 mg protein from the peak of activity from a DEAE-cellulose-column, 0.2 mg tRNA and 0.02 mls of $^3$H-leucine (60 Ci/mmol) per ml.

Other conditions were as given in Methods.
Figure 2

Reverse phase column chromatography of cytoplasm leucyl-
tRNA acylated by leucyl-trNA synthetase from cytoplasm of
soybean cotyledons.

The conditions for aminoaacylation were as follows:
0.2 mg protein from the peak of activity of a DEAE-cellulose
column, 0.2 mg tRNA (cytoplasm) and 0.02 ml of $^3$H-Leucine
(60 Ci/m mole) per ml.

Other conditions were as given in Methods.
Reverse phase column chromatography of chloroplast leucyl-tRNA produced by leucyl-tRNA synthetase from cytoplasm of soybean cotyledons.

The conditions for aminoacylation were as follows:
0.2 mg protein from the peak of activity of a DEAE-Cellulose column, 0.2 mg tRNA (chloroplast) and 0.02 mls of $^3$H-Leucine (60 Ci/m mole) per ml.

Other conditions were as given in Methods.
Reverse phase column chromatography of mitochondrial leucyl-tRNAs charged by leucyl-tRNA synthetase from cytoplasm of soybean cotyledons.

The conditions for acylation were as follows:
0.2 mg protein from the peak of activity from a DEAE column;
0.2 mg tRNA (mitochondrial) and 0.2 mls of $^3$H leucine (60 Ci/mmol) per ml.

Other conditions were as given in Methods.
$^{3}\text{H} \cdot \text{LEU} \cdot \text{tRNA} \ (10^5 \text{cpm})$

Fraction Number vs. NaCl Gradient (M)

Mito tRNA

CyT Enz
Figure 5

Reverse phase column chromatography of cytoplasmic leucyl-tRNAs acylated by leucyl-tRNA synthetase from mitochondria.

The conditions for aminoacylation were as follows:
0.2 mg protein from the peak of activity from a DEAE-cellulose column, 0.2 mg tRNA (cytoplasm) and 0.02 mls of $^3$H-Leucine (60 Ci/mmole) per ml.

Other conditions were as given in Methods.
Reverse phase column chromatography of mitochondrial leucyl-tRNAs acylated by leucyl-tRNA synthetase from mitochondria.

The conditions for aminoacylation were as follows:
0.2 mg protein from the peak of activity from a DEAE-cellulose column, 0.2 mg tRNA (mitochondria) and 0.02 ml $^3$H-leucine (60 Ci/mmole) per ml.

Other conditions were as given in Methods.
Reverse phase column chromatography of chloroplast leucyl-tRNAs charged by leucyl tRNA synthetase from mitochondria.

The conditions for aminoacylation were as follows: 0.2 mg proteins from the peak of activity from a DEAE-cellulose column, 0.2 mg tRNA (cytoplasm) and 0.2 mls of $^3$H-leucine (60 Ci/mmol.) per ml.

Other conditions were as given in Methods.
Figure 8

Reverse phase column chromatography of cytoplasmic leucyl-tRNAs charged by leucyl tRNA synthetase from chloroplast.

The conditions for aminoacylation were as follows:
0.2 mg protein from the peak of activity from a DEAE-cellulose column, 0.2 mg of tRNA (chloroplast) and 0.02 mls of $^3$H-leucine (60 Ci/mmol) per ml.

Other conditions were as given in Methods.
Reverse phase column chromatography of chloroplast tRNAs acylated by leucyl tRNA synthetase isolated from chloroplast.

The conditions for aminoacylation were as follows:

0.2 mg of protein from the peak of activity from a DEAE-cellulose column, 0.2 mg tRNA (chloroplast) and 0.2 mls of $^3$H-Leucine (60 Ci/mmoles) per ml.

Other conditions were as given in Methods.
Figure 10

Reverse phase column chromatography of mitochondrial tRNAs acylated by leucyl tRNA synthetase from chloroplast.

The conditions for aminoacylation were as follows:

0.2 mg protein from the peak of activity from a DEAE-cellulose column, 0.2 mg tRNA (mitochondrial) and 0.02 mls of $^3$H-leucine (60 Ci/mmol) per ml.

Other conditions were as given in Methods.
Figure 11

Hydroxylapatite fractionation of leucyl-tRNA synthetase from cytoplasm of soybean cotyledons. Approximately 20 mg of protein was loaded onto a column (2 cm x 30 cm) in 50 mls of 0.05 M potassium phosphate (pH 6.8) and eluted using a linear gradient of potassium phosphate from 0.05 — 0.4 M. 5 ml fractions collected were assayed for leucyl-tRNA synthetase activity using cytoplasmic tRNA.
Reverse phase column chromatography of cytoplasmic leucyl-tRNA acylated by leucyl-tRNA synthetase fraction 1 from cytoplasm. Cytoplasmic tRNA (4 A_{260} units/ml) was aminoacylated using enzyme fraction 1 (0.2 mg/ml) obtained from hydroxylapatite column.
Figure 13

Reverse phase column chromatography of leucyl-tRNA (chloroplast) acylated by leucyl-tRNA synthetase fraction 1 from cytoplasm. Chloroplast tRNA (4 A_{260} units/ml) was aminoacylated using enzyme fraction 1 (0.2 mg/ml) obtained from hydroxylapatite column chromatography.
Figure 14

Reverse phase column chromatography of mitochondrial-leucyl tRNA acylated by leucyl-tRNA synthetase fraction 1 from cytoplasm.

Mitochondrial tRNA (0.2 mg/ml) was aminoacylated using enzyme fraction 1 (0.2 mg/ml) obtained from hydroxylapatite column.
$^{3}\text{H} \cdot \text{LEU} \cdot \text{tRNA} \ (10^3 \text{cpm})$

![Diagram of NaCl gradient (M) with fraction number and tRNA distribution](image)

- Fraction Number:
  - 20
  - 40
  - 60

- NaCl Gradient (M):
  - 0.5
  - 1.0

- tRNA Distribution:
  - Mitochondrial tRNA
  - Cytoplasmic tRNA

- cpm: Counts per minute
Reverse phase column profile of cytoplasmic leucyl-tRNA acylated with hydroxylapatite fraction 2 enzyme from cytoplasm.

The conditions for acylation were as follows:

0.2 mg protein from peak 2 of hydroxylapatite column,
0.2 mg tRNA (cytoplasm) and 0.02 mg mls of (60 Ci/n mole) $^3$H-leucine per ml.

Other conditions were as given in Methods.
$^{3}H$ - LEU - tRNA (10^5 cpm)

NaCl GRADIENT (M)

FRACTION NUMBER
Figure 16

Reverse phase column chromatography of mitochondrial leucyl-tRNA acylated with leucyl-tRNA synthetase fraction 2 from cytoplasm. Mitochondrial tRNA (4 A₂₆₀ units/ml) was aminoacylated using enzyme fraction 2 (0.2 mg/ml) obtained from hydroxylapatite column chromatography.
Reverse phase column chromatography of chloroplast leucyl-tRNA charged by cytoplasmic leucyl-tRNA synthetase (enzyme 2). Chloroplast tRNA (4 A₂₆₀ units/ml) was aminoacylated using cytoplasmic enzyme fraction 2 (0.2 mg/ml) obtained from hydroxylapatite column. Charged samples were chromatographed on RPC-5 columns.
$3^\text{H} - \text{LEU - tRNA (10}^3\text{cpm}$

![Diagram with axes labeled and data points](image-url)
Reverse phase column chromatography of $^{3}$H-leucyl tRNA (cytoplasmic) acylated with synthetase fraction 3 (0.2 mg/ml), on a 0.9 x 100 cms Plaskon (RPC-5) column using a linear gradient of 0.5 - 0.9 NaCl in buffer B. Fractions of 5 mls were collected and assayed for radioactivity as described in Methods.
Reverse phase column chromatography of mitochondrial leucyl-tRNA acylated with leucyl-tRNA synthetase fraction 3 from cytoplasm.

Mitochondrial tRNA (4 A_{260} units/ml) was aminoacylated using enzyme fraction 3 (0.2 mg/ml) obtained from hydroxylapatite column chromatography.
$^{3}$H - LEU - tRNA (10$^3$ cpm)

FRACTION NUMBER

NaCl GRADIENT (M)

MITO tRNA

CYT 3 ENZ
Reverse phase column chromatography of chloroplast-leucyl tRNA acylated with synthetase fraction 3 (0.2 mg/ml), on a 0.9 x 100 cm Plaskon (RPC-5) column, using a linear gradient of 0.5 — 0.9 m NaCl in buffer B. Fraction of 5 mls. were collected and assayed for radioactivity as described in Methods.
Figure 21

Hydroxylapatite column chromatography of leucyl-tRNA synthetase from mitochondria. Approximately 20 mg of protein was loaded onto a column (2 cm x 30 cm) in 50 mls of 0.05M potassium phosphate (pH 6.5) and eluted using a linear gradient of potassium phosphate from 0.05 - 0.4 M. 5 ml fractions collected were assayed for leucyl-tRNA synthetase activity using cytoplasmic tRNA.
Reverse phase column chromatography of cytoplasmic leucyl-tRNA acylated by mitochondrial leucyl-tRNA synthetase fractionated by hydroxylapatite column chromatography.

The conditions for aminoacylation were as follows:

0.2 mg protein from hydroxylapatite column, 0.2 mg tRNA (cytoplasm) and 0.02 mls of $^3$H-leucine (60 Ci/m mole) per ml. Other conditions were as given in Methods.
Reverse phase column chromatography of mitochondrial leucyl-tRNA acylated with hydroxylapatite fractionated mitochondrial synthetase (0.2 mg/ml), on a 0.9 x 100 cm Plaskon (RPC-5) column, using a linear gradient of 0.5 - 0.9 M NaCl in buffer radioactivity as described in Methods.
Reverse phase column chromatography of chloroplast leucyl-tRNA acylated by mitochondrial leucyl-tRNA synthetase fractionated by hydroxylapatite column chromatography.

The conditions for aminoacylation were as follows: 0.2 mg of protein from hydroxylapatite column, 0.2 mg tRNA (chloroplast) and 0.02 mls of $^3$H-Leucine (60 Ci/mmol) per ml. Other conditions were as given in Methods.
Hydroxylapatite column chromatography of leucyl-tRNA synthetase from chloroplast. Approximately 20 mg of protein was loaded onto a column (2 cm x 30 cm) in 50 ml of 0.05 M potassium phosphate (pH 6.5) and eluted using a linear gradient of potassium phosphate from 0.05 - 0.4 M. 5 ml fractions collected were assayed for leucyl-tRNA synthetase activity using cytoplasmic tRNA.
Figure 26

Reverse phase column chromatography of cytoplasmic leucyl-tRNA acylated by hydroxylapatite fractionated chloroplast synthetase.

Cytoplasmic tRNA was aminoacylated using enzyme fraction (0.2 mg/ml) obtained from hydroxylapatite column chromatography.
Figure 27

Reverse phase column chromatography of chloroplast leucyl-tRNA acylated by chloroplast synthetase fractionated by hydroxyapatite column chromatography.

Chloroplast tRNA was aminoacylated using enzyme fraction (0.2 mg/ml) obtained from hydroxyapatite column chromatography.
Figure 28

Reverse phase column chromatography of mitochondrial leucyl tRNA acylated by hydroxylapatite fractionated chloroplast synthetase. Mitochondrial tRNA was aminoacylated using enzyme fraction (0.2 mg/ml) obtained from hydroxylapatite column chromatography.
Figure 29

Reverse phase column chromatography of leucyl-tRNAs (*E. Coli*) acylated by leucyl tRNA synthetase from *E. Coli*.

0.2 mg. protein from the peak of activity from a DEAE-cellulose column, 0.2 mg tRNA (*E. Coli*) and 0.02 mg $^3$H-leucine (60 Ci/mmol) per ml.

Other conditions as given in Methods.
Reverse phase column chromatography of $^3$H-leucyl-tRNA isolated from E. Coli, acylated with cytoplasmic enzyme (0.2 mg/ml) on a 0.9 x 100 cm Plaskon column, using a linear gradient of 0.5 - 0.9 M NaCl in buffer B. 5 ml fractions collected were assayed for radioactivity.
Figure 31

Reverse phase column chromatography of leucyl-tRNAs acylated by leucyl-tRNA synthetase (mitochondrial)

_E. Coli_ tRNA (4 A₂₆₀ units/ml) was aminoacylated using mitochondrial enzyme (0.2 mg/ml).

Other conditions were as given in Methods.
Reverse phase column chromatography of E. Coli leucyl-tRNAs produced by leucyl-tRNA synthetase from chloroplast. The conditions for aminoacylation were as follows:
0.2 mg proteins from the peak of activity from a DEAE-cellulose column, 0.02 mg tRNA (E. Coli) and 0.02 mls of $^3$H-leucine (60 Ci/m mole) per ml.

Other conditions were as given in Methods.
Reverse phase column chromatography of leucyl-tRNAs acylated by leucyl-tRNA synthetase fraction I from cytoplasm of E. Coli tRNA was aminoacylated using enzyme fraction I (0.2 mg/ml) obtained from hydroxylapatite column chromatography.
Figure 34

*E. coli* leucyl-tRNA acylated with leucyl-tRNA synthetase fraction 2 from cytoplasm (0.2 mg/ml) and chromatography on RPC-5 column (0.9 x 100 cm), using a linear gradient of 0.5 - 0.9 M NaCl in buffer B. Fractions of 5 mls were collected and assayed for radioactivity as described in Methods.
Figure 35

Reverse phase column chromatography of leucyl-tRNAs acylated by leucyl-tRNA synthetase 3 from cytoplasm.

E. Coli tRNA was aminoacylated using enzyme fraction 3 (0.2 mg/ml) obtained from hydroxylapatite column chromatography.
DISCUSSION

The present investigation was undertaken to study the differences in the soybean leucyl-tRNAs and leucyl-tRNA synthetases in the various compartments of the plant cell, i.e. the cytoplasm, the chloroplast and the mitochondria, and to compare these with a prokaryotic system (E. coli). Aminoacylation of cytoplasmic, chloroplastic and mitochondrial leucyl-tRNAs by homologous and heterologous enzyme clearly show that both tRNAs and synthetases are organelle-specific.

Results presented here for the soybean are in agreement with the findings in Phaseolus (35), tobacco (32), Euglena (10), peas (1), cotton (50) and lupin (3). All of these studies confirm the existence of chloroplast-specific and mitochondria-specific tRNAs and aminoacyl-tRNA synthetases. It is only in the case of Phaseolus (62) that organelle-specific tRNAs have been studied for a number of amino acids, while other reports have looked at only individual amino acids.

The first experimental evidence that mitochondria contain molecular species of tRNA and synthetases which differ from those found in the cytoplasm was found in Neurospora (8). It was shown that the mitochondrial tRNA\textsuperscript{Leu} species could be charged by either cytoplasmic or mitochondrial synthetase while the cytoplasmic tRNA\textsuperscript{Leu} species could only be acylated by a cytoplasmic enzyme. In the rat liver (19), mitochondrial leucyl-tRNA synthetase recognizes both mitochondrial and cytoplasmic tRNA\textsuperscript{Leu} species whereas the cytoplasmic enzyme acylates
only the cytoplasmic tRNA\textsuperscript{Leu} species; a reverse situation of the results obtained in \textit{Neurospora}. In tobacco (32) cytoplasmic and mitochondrial tRNA\textsuperscript{Leu} are charged by the homologous and heterologous leucyl-tRNA synthetases. Cytoplasmic tRNA\textsuperscript{Leu} species are charged by cytoplasmic and mitochondrial synthetase in \textit{Tetrahymena} (26) whereas mitochondrial tRNA\textsuperscript{Leu} species are preferentially charged by the mitochondrial enzyme. In \textit{Euglena} (46) a partial aminoacylation was found where cytoplasmic tRNA\textsuperscript{Leu} species were charged only partially (75%) by the mitochondrial enzyme and the cytoplasmic enzyme partially (40%) acylates mitochondrial tRNA\textsuperscript{Leu}. Our results in the soybean system demonstrates that cytoplasmic and mitochondrial tRNA\textsuperscript{Leu} species are aminoacylated by the homologous and the heterologous enzymes.

In photosynthetic organisms the situation is further complicated by the existence of chloroplasts. In \textit{Euglena} (56) there are chloroplast-specific light inducible species of tRNA and aminoacyl-tRNA synthetase. Tobacco (32) contains two tRNA\textsuperscript{Leu} species found in chloroplast which are exclusively acylated by chloroplast leucyl-tRNA synthetase. Merrick (50) et al., have described the occurrence of cotton chloroplast tRNA\textsuperscript{Leu} species which increase during early germination and capable of being acylated by \textit{E. coli} synthetase. Work by Kanabus (44) has shown that a leucyl-tRNA synthetase was present in the soybean cotyledons, but absent in the dark grown hypocotyls. In the present study it is shown that chloroplast enzyme preferentially acylates tRNA\textsuperscript{Leu}5 and 6 of a cytoplasmic tRNA preparation. In the homologous reaction chloroplast tRNA acylated by a chloroplast leucyl-
tRNA synthetase also results in recognizing tRNA Leu 5 and 6.

It is clear from these observations that the number of tRNA species for a particular amino acid localized in the chloroplast is different for different amino acids. In the case of leucyl-tRNA in Phaseolus there are three species in the chloroplast, whereas in the soybean, peas and tobacco, only two species exist. The difference in an extra chloroplastic tRNA species in the same family of leguminosae is not clear at this time. Further work is necessary to study other amino acids in the soybean.

Further it appears that the cytoplasmic and mitochondrial leucyl-tRNA synthetases are similar in their tRNA recognition patterns. This is not to imply that what is true for one set of aminoacyl-tRNA synthetases for a particular amino acid applies to other amino acids even in the same organism. Therefore, the complement of tRNAs and aminoacyl-tRNA synthetases found in the cytoplasm and in the mitochondria of a given organism may be similar or different, depending upon the amino acid considered.

The specificity of E. coli tRNAs and enzyme used for charging organelle tRNAs and synthetases has been used as an evolutionary indicator. In soybean, the results show a similarity between cytoplasmic and mitochondrial enzymes on the one hand and between chloroplast and E. coli enzymes on the other. These results are in agreement with Euglena isoleucyl-tRNA synthetases (7), tobacco leucyl-tRNA synthetases (32) and P. vulgaris leucyl-tRNA synthetase (62).
The situation in mitochondria is markedly different. The present study shows that mitochondrial enzyme is able to acylate only two of the five E. coli tRNA isoacceptors. The enzyme in this case resembles cytoplasmic enzyme. Studies show mitochondrial aminoacyl-tRNA synthetases in some cases resembles the cytoplasmic one, as illustrated for bean (35) and tobacco (32) enzymes. In other cases the mitochondrial enzyme resembles the chloroplastic and bacterial enzymes, as shown for bean lysyl-tRNA synthetase (38).
SUMMARY

1) Cytoplasmic enzyme resolves into three enzyme peaks (enzyme 1, 2 and 3) when chromatographed on H.A. column, while mitochondria and chloroplast yield one peak each.

2) Cytoplasmic enzyme 1 was found to preferentially charge leucyl-tRNA species 5 and 6 of the cytoplasmic tRNA and the same 2 isoaccepting species for chloroplast tRNA (tRNA-Leu 5 and 6).

3) Cytoplasmic enzyme 2 was found to preferentially charge leucyl-tRNA species 1-4 of cytoplasmic tRNA and resolve 4 isoaccepting species for mitochondrial tRNA (tRNA-Leu 1-4).

4) Cytoplasmic enzyme 3 was found to preferentially charge leucyl-tRNA species 1-4 of cytoplasmic tRNA and resolve 4 isoaccepting species for mitochondrial tRNA.

5) Chloroplast enzyme was found to acylate 2 isoaccepting species (tRNA-Leu 5 and 6) for chloroplast tRNA and preferentially charge species 5 and 6 of cytoplasmic tRNA.

6) Mitochondrial enzyme was found to acylate 4 isoaccepting species (tRNA-Leu 1-4) for mitochondrial tRNA and preferentially charge species 1-4 of cytoplasmic tRNA.

7) E. coli tRNA when separated on RPC-5 chromatography after charging with homologous enzyme yields 5 isoaccepting leucyl-tRNA species.

8) Cytoplasmic enzyme (unfractionated) was found to acylate 5 leucyl-tRNA species for E. coli tRNA.

9) Cytoplasmic enzyme 1 was found to charge 5 leucyl-tRNA species for E. coli tRNA.

10) Cytoplasmic enzyme 2 and 3 were found to preferentially charge 2 isoaccepting species for E. coli tRNA (tRNA-Leu 1 and 2).

11) Chloroplast enzyme was found to acylate the 5 leucyl-tRNA species for E. coli tRNA.

12) Mitochondrial enzyme was found to preferentially charge 2 leucyl-tRNA species for E. coli tRNA (tRNA-Leu 1 and 2).


VITA AUCTORIS

Born:
October 30, 1954, Chatham, Ontario
Son of Mr. & Mrs. James D. Sinclair

Elementary Education:
Oriole Parkway Public School
Queen Elizabeth II Public School
Chatham, Ontario
1959-1968

Secondary Education:
Chatham Collegiate Institute
Chatham, Ontario
1968-1973

University Education:
University of Windsor
Windsor, Ontario
B.Sc Biology, 1973-1977