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Changes in leucyl transfer ribonucleic acids and amino acyl transfer ribonucleic acid synthetases in developing and ageing soybean seedlings.

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CHANGES IN LEUCYL TRANSFER RIBONUCLEIC ACIDS AND AMINO ACYL TRANSFER RIBONUCLEIC ACID SYNTHETASES IN DEVELOPING AND AGEING SOYBEAN SEEDLINGS

BY

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ABSTRACT

Changes in leucyl tRNA isoaccepting species and multiple forms of amino acyl tRNA synthetases were determined in aging soybean cotyledons (variety Harrosoy 63) and in developing seed pods. Leucine specific tRNAs from these tissues can be fractionated into six species by Reverse Phase chromatography on a Plaskon column (RPC 5). The relative amounts of two of these tRNAs (species 5 and 6) are lower in seed pods than in cotyledons. Crude leucyl tRNA synthetase (leucyl: tRNA ligase (AMP); 6.1.1.4) from seed pods is less active than enzyme from cotyledons in the aminoacylating $tRNA_{5\text{and}6}^{leu}$.

Leucyl tRNA synthetases from cotyledons in a germinating seedling and in developing seed pods can be fractionated into three peaks of activity on hydroxylapalite (HA) columns. Enzyme peak 1 in seed pods amounts to about 36% less than in the five day old cotyledons. The amount of enzyme peaks 2 and 3 are more (about 10% and 18% respectively) in seed pods than in cotyledons.

Transfer RNA specificity of individual enzyme fractions from seed pods indicates, that peak 1 enzyme of seed pods acylates all six species of leucyl tRNA compared to the cotyledon enzyme fraction 1 which essentially acylates only leucyl $tRNA_{5\text{and}6}^{leu}$. The specificity of enzyme fractions 2 and 3 towards leucyl $tRNA_{1-4}^{leu}$ is identical in both the organs.

Preliminary experiments conducted to study the amino acid acceptor activity of synthetase in different developmental stages of cotyledon and developing seed pods suggested that the acceptor activity of different amino acids investigated decreases with senescence.

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ABBREVIATIONS

$A_{260\ (280)}$:	absorption at 260 (280) nm.
AMP:	adenosine 5' - phosphate.
ATP:	adenosine 5' - triphosphate.
aa:	amino acid.
DEAE - cellulose:	diethylaminoethyl cellulose.
DNA:	deoxyribonucleic acid.
Freon (1,1,1,3 Tetrachlorotetraflouro propane) column:	a reversed-phase chromatographic column (RPC-2).
HA column:	column made from a mixture of hydroxylapatite and cellulose powder at the ratio of 10:1.
MAK < column:	methylated albumin and kieselguhr column.
O.D. units:	optical density units.
Plaskon (Polychloro-triflouroethylene) column:	a reversed-phase chromatographic column (RPC-5).
PPi:	inorganic pyrophosphate.
RNA:	ribonucleic acid.
RPC:	reversed-phase chromatography
tRNA(s)	transfer ribonucleic acids.

INTRODUCTION

This investigation was undertaken to determine the multiplicity of leucyl-tRNA synthetases and leucyl-tRNA isoaccepting species in different stages of cotyledon senescence (5, 10, 15 and 20 day old cotyledons) and in developing stages of seed pods.

Alterations of specific tRNAs and appearance or disappearance of tRNA isoacceptors have been implicated as important steps in differenciation and regulation (41). Differences in the elution profiles of tRNAs and aminoacyl tRNA synthetases have been observed on phage infection of bacteria (26), in plant development (2), after administration of hormones (51) and during embryonic differenciation of echinoids (52), amphibians (19) and mammals. Previous studies of this enzyme in soybean revealed that the complement of $tRNA^{Leu}$ isoaccepting species changes during cotyledon senescence (11).

Protein synthesis in plants has been shown to involve the same reactions and general mechanisms as described for bacterial and animal systems (49). Usually in plant a quiescent or dormant state is associated with a low level of polyribosomes and certain enzymes in a repressed state (31, 45). A shift upwards in metabolic activity, such as with seed germination, is associated with increase in polyribosomes and certain enzymes involved in starch hydrolysis (16).

The activation of the protein synthesizing machinery with germination may be attributed to one or more of the following components: an increase in the availability of mRNA, changes in the amount of RNA species, aminoacyl tRNA species, aminoacyl-tRNA synthetase and/or various factors

(initiation, termination, etc.).

This report describes the changes in $tRNA^{Leu}$ species and aminoacyl $tRNA^{Leu}$ synthetases before (seed pod stage) and after germination until the cotyledon senescence stage. Differences in enzyme patterns shown here between seed pod stage and cotyledon stage, opens up a new area of investigation, to study differentiation process in seed germination through synthesis of specific enzymes and development of new organelles.

REVIEW OF LITERATURE

Multiplicity of Transfer RNA

The involvement of an "adaptor" in the transfer of aminoacids to the site of protein synthesis was first hypothesized by Crick in 1957 (17). The concurrent work on the genetic code, and its degeneracy in particular, raised the question of possible degeneracy of the tRNA populations of a cell. In 1961 Doctor et al (18) fractionated yeast tRNAs specific for leucine and threonine into two fractions each. At the same time Berg et al (6) showed the existence of two valine specific tRNAs in E. coli. Based essentially on these two types of experimental approach, evidence has accumulated since then, which leaves no doubt concerning the existence of multiplicity of isoaccepting tRNAs.

Aminoacyl tRNA Synthetases

The aminoacyl tRNA synthetases were discovered by Hoagland and his co-workers (22, 23) in 1955. Berg and Ofengand (7) established that this enzyme, or more precisely a large family of enzymes, with distinct substrate specificities is responsible for the first two reactions in protein biosynthesis.

(1) The activation of the aminoacid with ATP forming the aminoacyl adenylate anhydrides and

$$\text{aa (aminoacid)} + \text{ATP} \rightleftharpoons \text{aa-AMP} + \text{PPi}$$

(2) The incorporation of the activated aminoacid to the soluble portion of the nucleic acids

$$\text{aa} - \text{AMP} + \text{tRNA} \rightleftharpoons \text{aa} - \text{tRNA} + \text{AMP}.$$

It is evident, that in addition to the binding of ATP, which is common to all synthetases, each enzyme has to recognize both the correct amino acid and correct tRNA in order to produce a proper aminoacyl - tRNA combination.

Aminoacyl tRNA Synthetase Multiplicity in Bacteria

Growing evidence for the widespread occurrence of multiple isoaccepting tRNAs prompted a search for multiple aminoacyl - tRNA synthetases. In bacteria, only a few instances of synthetase multiplicity have been reported, Mahler and Jesensky (29) observed two peaks of activity eluting from DEAE cellulose column, while studying the proline specific enzyme in E. coli. The leucyl tRNA synthetases from the same bacterium were fractionated into three peaks of activity on a hydroxylapatite (53) by Chang-Tao Yu. The third fraction contained the major portion of the total activity (97%). The first two fractions contained only 3% of the total activity. Enzyme peak I differed from the other in the degree of stimulation by 5 mM NH_4Cl and in the degree of charging of the leucine accepting tRNAs.

Two synthetases specific for methionine (43), phenylalanine and aspartic acid and three leucine specific enzymes (42) have been found in Bacillus brevis by Surguchev and his co-workers. The methionine specific enzymes was isolated by the method of isoelectric focusing. Form I was found to be predominant in the lag phase while form II in the exponential phase of growth. The three leucine specific enzymes were separated from two strains of Bacillus brevis, by gel electrophoresis. The relative amounts of the three forms were different in the two bacterial strains. With the above exceptions, the majority of the results with bacteria favor the idea that only one synthetase exists for

each aminoacid (32).

Aminoacyl - tRNA Synthetase Multiplicity in Higher Organisms

Direct and indirect evidence exists for multiple synthetases in more or less homogenous cell populations of fungi, plants and animals, including man. Two lysyl tRNA synthetases in Baker's yeast were found by chromatography on a Bio-Rex 70 column (15). In rat liver, two synthetases were found specific for both threonine (1) and leucine (46). The leucine specific enzymes were fractionated on a hydroxylapatite column. They differed in their Km values and heat stabilities. Strehler et. al. (38) fractionated the leucyl tRNA synthetase from rabbit heart muscle into three fractions by DEAE cellulose chromatography. Two of these fractions displayed a complimentary range of specificity toward two $tRNA^{Leu}$ species as judged by MAK column profiles of the leucyl tRNAs. Some other differences have also been mentioned in this work between the enzymes specific for leucine, alanine, arginine and proline from five tissues of rabbit. In the only work of this type on plant tissues, Anderson and Cherry (2) showed that the product of tRNA aminoacylation by soybean cotyledon leucyl tRNA synthetase can be chromatographed into six leucyl tRNA species using a Freon column (RPC-2). The enzyme from soybean hypocotyls failed to aminoacylate two of the six tRNAs.

tRNAs and Synthetases in Aging and Developing Systems

Changes in the multiple aminoacyl tRNA synthetases or their specificities in the course of development and other related processes have been reported. Bick and Strehler (12) showed that the ability of the leucyl tRNA synthetase of soybean cotyledons to acylate four of the six $tRNA^{Leu}$ species of this tissue decreased disproportionately between

the 5th and 21st day of seed germination. Ceccarini et. al. (13) studied the specificity of synthetases of sea urchin during development from unfertilized egg until the stage of pluteus. MAK column chromatography of yeast tRNA acylated with ^{14}C labelled protein hydrolyzate using the enzyme from different stages of development showed drastic differences between pluteus and unfertilized egg or the early blastula.

Rennert (35) reported a leucyl-tRNA synthetase unique to the early embryonic tissue of mouse (gestational age of 5 to 8 days). The enzyme was more stable at 45°C than was the synonymous enzyme from liver of adult mouse. The "embryonic" enzyme was able to aminoacylate tRNA with trifluoroleucine much more efficiently than the "adult" enzyme. Gallo and Pestka (21), studying tRNAs of leukemic human lymphoblasts, observed an additional glutamyl-tRNA synthetase activity in normal cell that was absent from the leukemic cells.

Intracellular Distribution of tRNAs and Synthetases

The existence of organ specific tRNAs and aminoacyl-tRNA synthetases seems to be an important fact in connection with the problem of their multiplicity.

Nuclei and Mitochondria

As early as 1959 Hopkins (24) discovered the presence of aminoacyl-tRNA synthetases (the "pH 5 enzymes") in calf thymus nuclei. Webster reported (48) that the nuclear and cytoplasmic alanyl-tRNA synthetases from pig liver could aminoacylate tRNAs only from the same respective sources. The early work of Barnett and his co-workers (3, 4) on tRNAs and aminoacyl tRNA synthetases indicated the presence of a number of multiple tRNAs and of two synthetases specific for aspartic acid and phenylalanine. Barnett et al (3) also found a complete

set of aminoacyl tRNA synthetases in mitochondria for enzymes specific for aspartic acid, leucine and phenylalanine.

Chloroplasts

From the early work of Marcus (30) and Bove and Raacke (8) the presence of aminoacid activating enzymes specific for at least thirteen aminoacids in the chloroplasts from spinach leaves was established. Aminoacyl-tRNA synthetases and tRNAs were also found in chloroplasts from pea seedings (36) and tobacco leaves (20). The pea seedling system was later studied in more detail using five different aminoacids (36). Williams and Williams (50) reported that the $tRNA^{Leu}$ species of bean leaf exhibiting accelerated synthesis during chloroplast development were aminoacylated exclusively by one of a few leucyl-tRNA synthetase species from the same source. Clearly, the presence of specific tRNAs and aminoacyl tRNA synthetases in individual organelles contributes to the multiplicity of these molecules in higher organisms.

MATERIALS AND METHODS

Plant Material

Soybean seeds (Glycine max var-Harosoy 63) were surface sterilized in 10% chlorox and soaked in distilled water overnight and sown in moist vermiculite. Cotyledons were harvested after 5, 10, 15 and 20 days of germination in the dark at 27 - 29°C.

Samples of different developing stages of seed pods were collected after seven weeks from the day of planting. The harvested seed pods were frozen in dry ice and used for extraction later.

Transfer RNA

Transfer RNA was prepared from total RNA of 5, 10, 15, or 20 day old freshly harvested cotyledons or those stored in freezer for several days. The total RNA was extracted by the phenol technique of Cherry et al (11) with minor modifications. Homogenization buffer was prepared by shaking 10 mM Tris(hydroxymethyl aminomethane) - HCl buffer, pH 7.6, containing 60 mM KCl and 10 mM $MgCl_2$ (buffer A) with 90% solution of phenol at the ratio of 10:8 (v/v) for several hours and separating the phases. Batches of 100 gm of chilled tissue were ground in a Waring blender with a mixture of 100 ml of aqueous phase (buffer A), 100 ml of phenolic phase, 40 ml of 11% dupanol (sodium lauryl sulfate) and 20 ml of bentonite suspension (10) of 40 mg/ml. The homogenate was strained through four layers of cheesecloth and centrifuged for 10 minutes at 10,000 x g. The supernatant was shaken for two hours with an equal volume of the organic (phenolic) phase. The aqueous extract collected after centrifugation, was phenol extracted

four times using equal volumes of the organic phase containing 1/18 volume of the bentonite suspension. After each extraction, the aqueous phase was recovered by centrifugation after shaking in the cold for 30 minutes. Total RNA was precipitated with two volumes of cold 95% ethanol and collected by centrifugation. 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 absorption on a 2 ml column of DEAE-cellulose in buffer B (10 mM sodium acetate pH 4.5 containing 10 mM $Mgcl_2$) and elution with 1 M NaCl in the same buffer. The solution was dialyzed against water, and the concentration was determined based on the A_{260} absorbancy.

Leucyl-tRNA Synthetase

Extraction, purification and fractionation of the enzyme was performed at 0-4°. Freshly harvested cotyledons, or frozen seed pods were chilled on ice and ground for a few minutes with insoluble polyvinylpyrollidone (Polyclar at: 200 mg/g tissue) using a mortar and a pestle. The grinding was continued the next 15 minutes with stepwise addition of the grinding medium. The grinding medium (1x concentration) consisted of 25 mM potassium phosphate, pH 7.8, in solution C (10^{-2}M 2-mercaptoethanol; 10^{-5}M phenylmethyl sulfonyl-flouride; ~10^{-6}M L-leucine) saturated to 30% with respect to ammonium sulfate. The homogenate was strained through cheesecloth and centrifuged for 15 minutes at 27,000 x g. The supernatant was collected through a miracloth filter and the ammonium sulfate concentration was increased to 60% saturation (195 mg added per ml) and stirred in the cold for 30 minutes. The enzyme pellet collected after

centrifugation at 10,000 X g for 10 minutes was dissolved in 25 mM potassium phosphate (pH 7.8) solution and dialyzed in buffer C for several hours. The solution was absorbed on a 10-ml column of DEAE-cellulose in 25 mM potassium phosphate, pH 7.8, in solution C. The column was washed with about 50 ml of the same solution and the enzyme eluted with 0.1 M phosphate, pH 7.8, in solution C. Material from the last fraction was diluted 1:1 with water and the pH was adjusted to 6.5 with 0.05 M KH_2PO_4 . The solution was applied to a column (HA column) made of a mixture of 10 g of hydroxylapatite and one gm of cellulose powder (Whatman CF 11) with small pads of cellulose at the bottom and top of the packing. The column had previously been equilibriated with 0.05 M potassium phosphate buffer, pH 6.5 in solution C. The column was washed with 50 ml of the above buffer followed by a gradient of potassium phosphate, pH 6.5, from 0.05 to 0.4 M in solution C. Fractions of 7 ml were assayed for leucyl-tRNA synthetase activity in a reaction mixture of 0.25 ml containing 0.05 ml of fractionated protein and other components as described in the aminoacylation assay. The reaction was allowed to proceed for 20 minutes at 30°C and then terminated by precipitation with trichloroacetic acid.

Transfer RNA Aminoacylation Assay

The reaction was carried out at 30°C. Unless otherwise stated, 1 ml of the reaction mixture contained: 10 μ moles Tris (hydroxymethyl aminoethane) - HCl, pH 7.8; 5 μ moles $MgCl_2$; 0.5 μ moles ATP; 0.2% soluble polyvinylpyrollidone - 0.2 mg/ml of tRNA, 0.2 mg/ml of enzyme and 20 μl unneutralized solution of L-(4,5-^{3}H) (leucine solution (60 Ci/m Mole).

Reversed-Phase Column Chromatography (RPC-5)

A mixture of 8 ml of Adogon 464 in 400 mls of chloroform was coated on to 200 g of polychlorotriflouroethylene (Plaskon) support, according to Pearson, Weiss and Kelmers (34). Finally the coated plaskon was suspended in 0.5 M NaCl in buffer B for packing of the columns.

For Plaskon column fractionation, tRNA was aminoacylated as described above in a 2 ml reaction mixture containing larger quantities of tRNA and protein. The concentration of other components were the same. Leucyl-tRNA was recovered using a small DEAE-cellulose column as described by Anderson and Cherry (2) and applied to a Plaskon column (2.5 x 30 cms) in 0.5 M NaCl in buffer B. Elution was performed at room temperature using a linear gradient of NaCl in buffer B from 0.5 - 0.9 M. Fractions of 10 ml were collected at a flow rate of 2 ml/min. The entire fraction was made 5% with respect to trichloracetic acid and filtered through glass fibre filters. Radioactivity was determined in Mark II Nuclear Chicago liquid scintillation counter.

Assay Procedure for Aminoacyl-tRNA Synthetase Activity

The reaction was carried out at 30°C. The reaction mixture contained in 300 µl, 4 n Moles tRNA, 200 µg protein, 200 p Moles aminoacid under study with concentration of the other components the same as used for tRNA-aminoacylation assay. Reactions were incubated at 30°C and 50 µl aliquots were withdrawn in duplicate after 2 and 4 minutes. The aliquots were spotted onto Whatman 3 mm paper discs and placed immediately into cold 5% TCA (w/v). Filters were washed a second time with cold 5% v/v, once with methanol ether (1:1 v/v), dried under a heat lamp, and put into vials containing a toluene scintillation fluid and radioactivity determined in liquid scintillation

counter. The aminoacids studied were arginine, aspartic acid, alanine, glycine, histidine, glutamic acid, isoleuceine, leucine, phenylalanine, tyrosine, and valine.

FIGURE 1a

Plaskon column chromatography of leucyl-tRNAs produced by leucyl-tRNA synthetase from soybean cotyledons (5 day old).

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 (5 day old) and 0.02 ml of ^{3}H-leucine (60 ci/mole) per ml. Other conditions were as given in Methods.

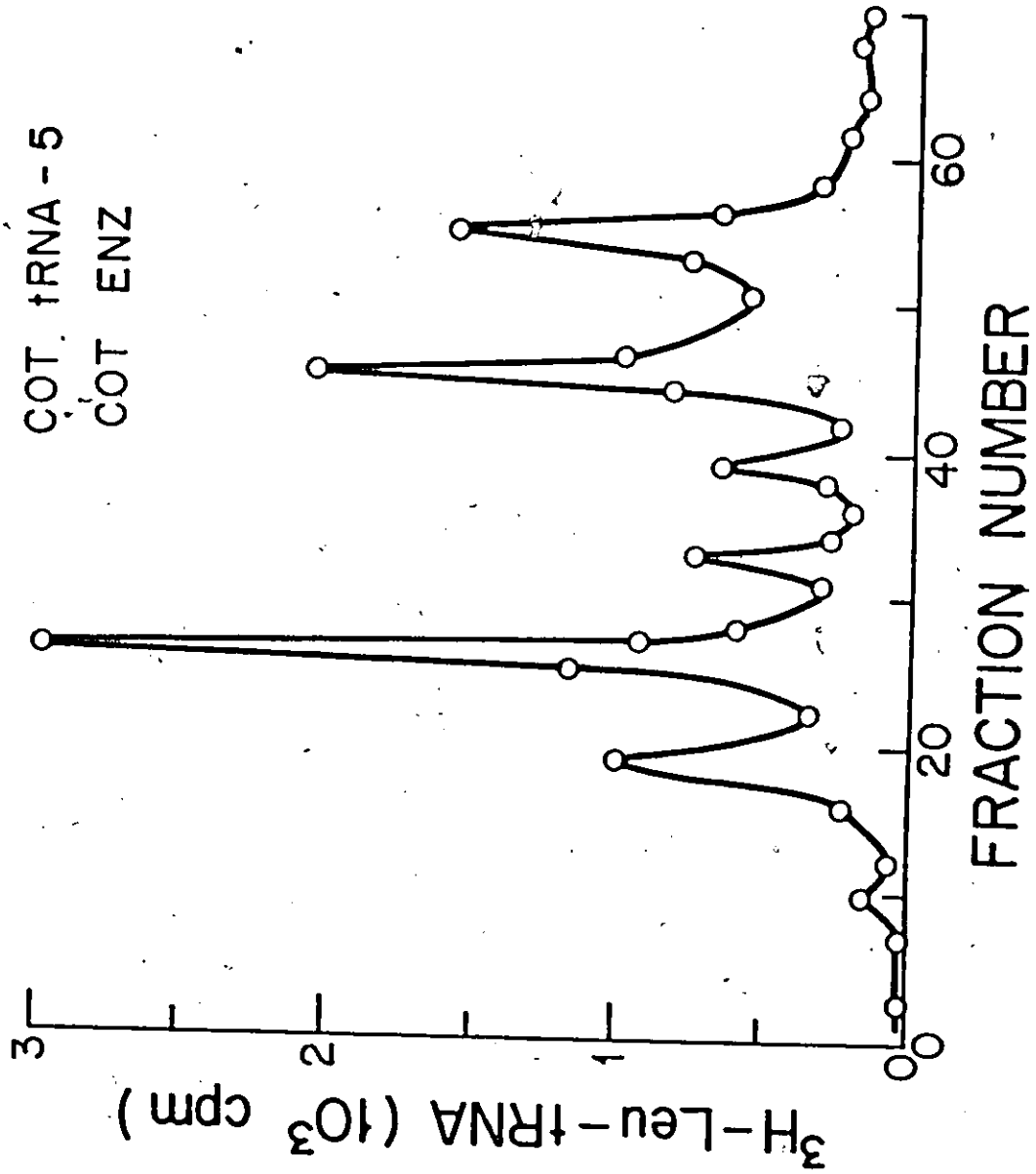
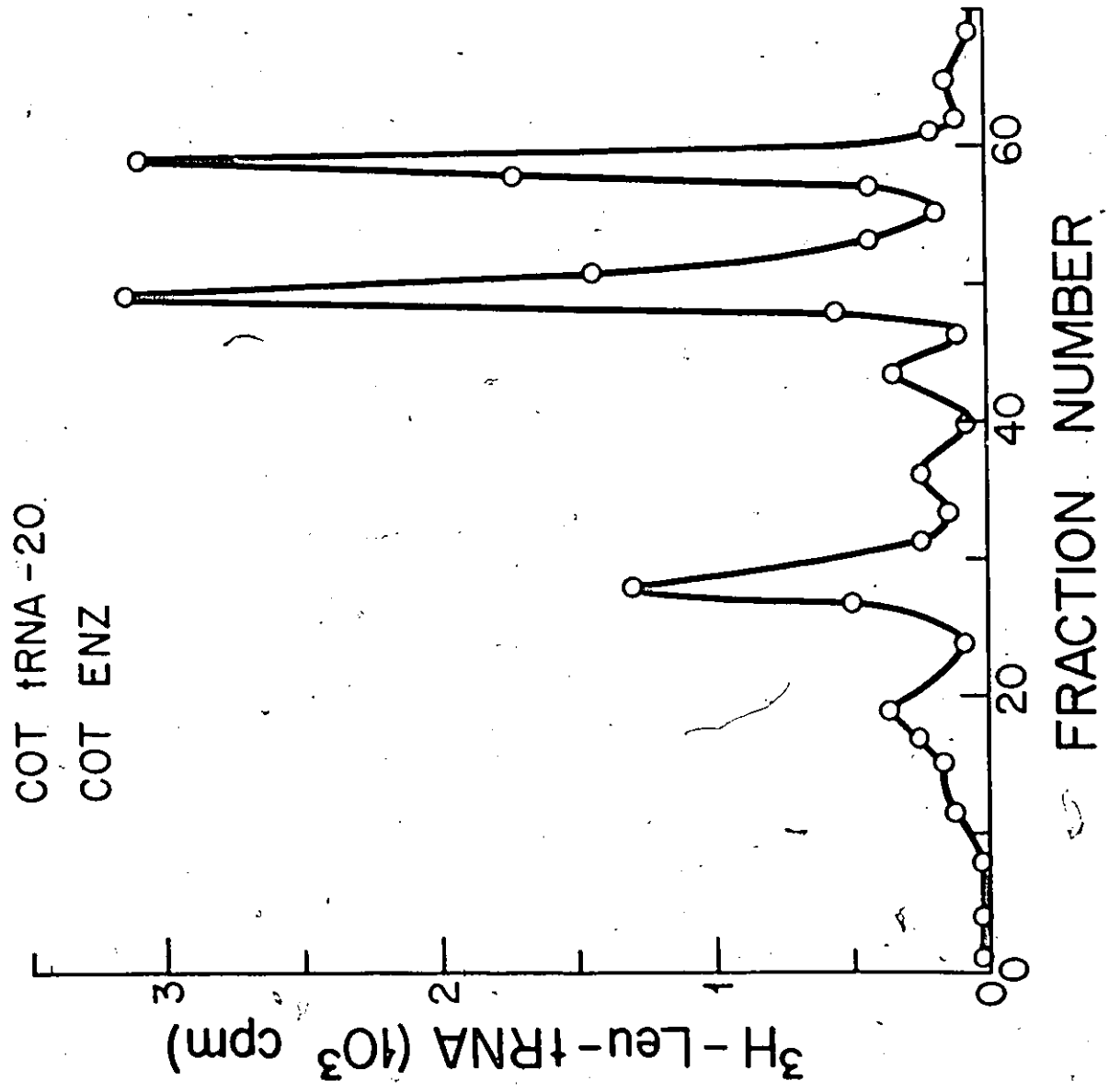

COT. tRNA-5
COT ENZ
^{3}H-Leu-tRNA (10^3 cpm)
FRACTION NUMBER
0
1
2
3
20
40
60

FIGURE 1b

Plaskon column chromatography of leucyl-tRNAs (20 day cotyledon) produced by leucyl-tRNA synthetase from soybean cotyledons. 0.2 mg protein from the peak of activity from a DEAE-cellulose column, 0.22 mg tRNA (20 day old) and 0.02 ml ^{3}H-leucine (60 Ci/mmole) per ml. Other conditions were as given in Methods.


COT tRNA-20
COT ENZ
^{3}H-Leu-tRNA (10^3 cpm)
0
1
2
3
0
20
40
60
FRACTION NUMBER

RESULTS

Changes in leucyl-tRNA species and leucyl-tRNA synthetase activities in aging soybean cotyledons following germination and in developing seed pods are described here. Primarily, leucyl tRNA synthetase activities were compared in aging cotyledons (5, 15 and 20 days old) and in developing seed pods, after the synthetase preparations were fractionated on HA columns as described under Materials and Methods.

Transfer RNAs in Aging Cotyledons

Leucyl-tRNA from cotyledons fractionate into six discrete peaks on plaskon columns (RPC-5). Figures 1a and 1b show the elution profiles of leucyl-tRNA species in 5 day and 20 day old cotyledons acylated with ^{3}H-Leucine, using 5 day cotyledon enzyme (crude preparation from DEAE column). The results presented here confirm previous data (11) that with aging, there is 12% increase in $tRNA_{5_{and}6}^{leu}$ and a 7% decrease in $tRNA_{2}^{leu}$ in 20 day old cotyledons.

However, when this 5 day old cotyledon enzyme is used to acylate tRNA preparations from immature seed pods and mature seed pods (Figures 2a and 2b), the percent acylation of $tRNA_{2}^{leu}$ increases by 17% to 18% and peaks 5 and 6 decreases by 12% and 13% respectively. The inadequate or poor charging of $tRNA_{5_{and}6}^{leu}$ and the maximum charging of $tRNA_{2}^{leu}$ indicates differences that could be based on one of the two rate limiting factors, i.e., synthetases or tRNA. No significant change in the acylation of $tRNA_{1\ 3_{and}4}^{leu}$ were noticed. This shows that $tRNA_{5_{and}6}^{leu}$ are not changing in developing system.

FIGURE 2a

Plaskon column chromatography of ^{3}H-leucyl tRNA isolated from immature seed pods acylated with 5 day old cotyledon enzyme (0.2 mg/ml), on a 2.5 x 30 cms Plaskon (RPC-5) column, using a linear gradient of 0.5 - 0.9m NaCl in buffer B. Fractions of 10 ml were collected and assayed for radioactivity as described in the Methods.

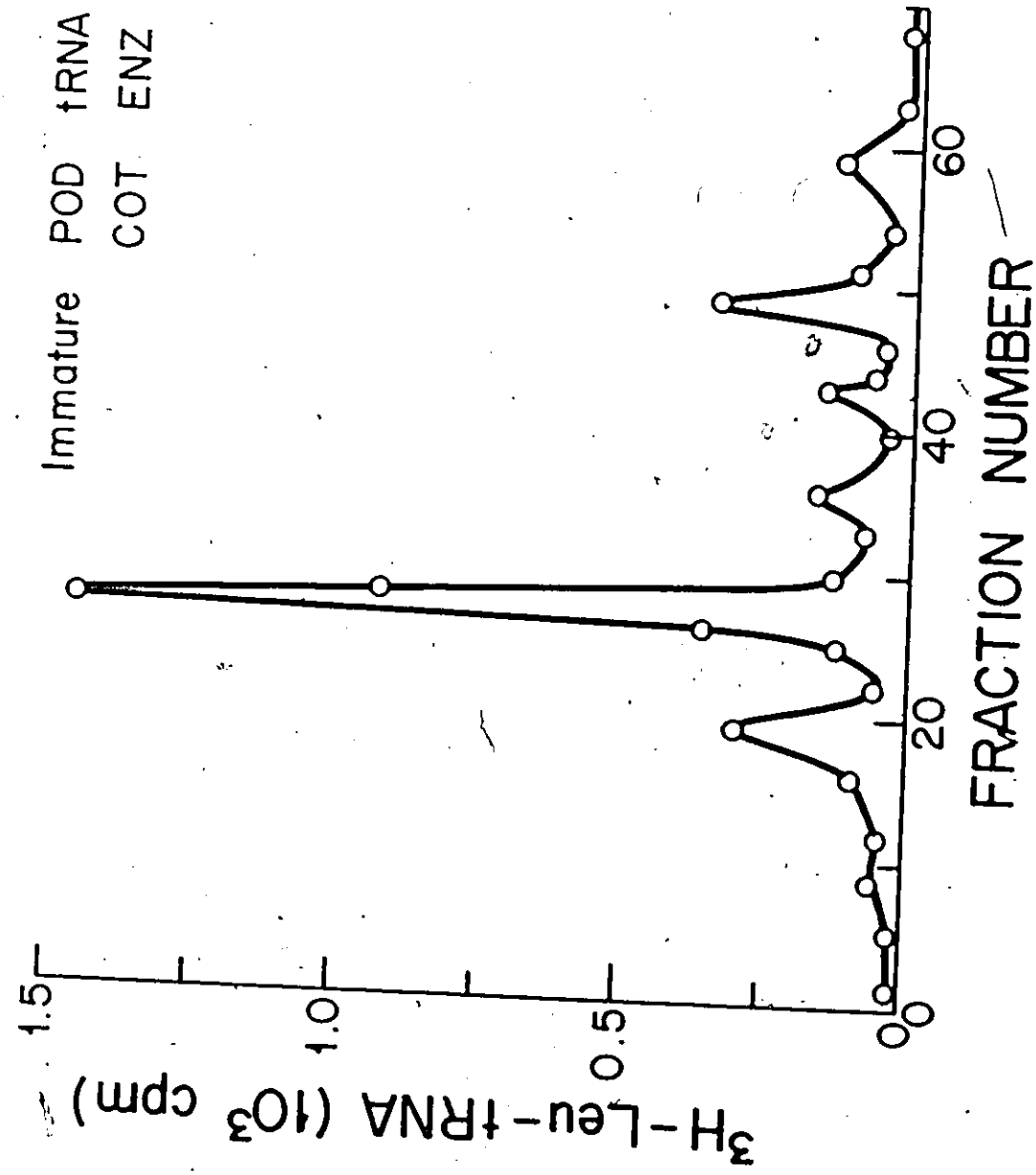
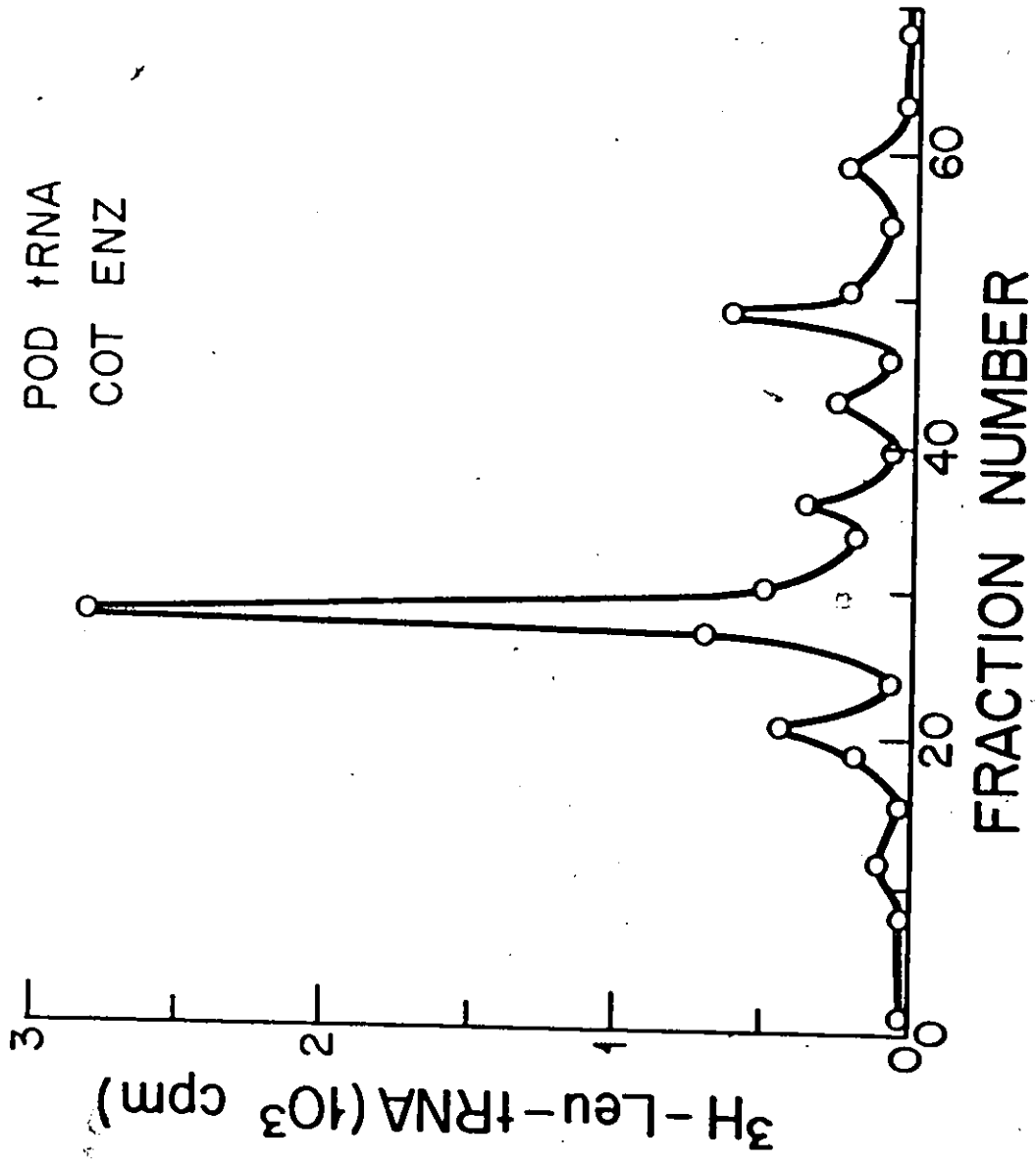

Immature POD tRNA
COT ENZ
^{3}H-Leu-tRNA (10^3 cpm)
1.5
1.0
0.5
0
20
40
60
FRACTION NUMBER

FIGURE 2b

Plaskon column chromatography of ^{3}H-leucyl tRNA isolated from mature seed pods, acylated with 5 day old cotyledon enzyme (0.2 mg/ml), on a 2.5 x 30 cms Plaskon (RPC-5) column, using a linear gradient of 0.5 - 0.9m NaCl in buffer B. Fractions of 10 ml were collected and assayed for radioactivity.


POD tRNA
COT ENZ
^{3}H-Leu-tRNA (10^3 cpm)
0
1
2
3
0
20
40
60
FRACTION NUMBER

Transfer RNAs in Developing Seed Pods

In another system, synthetase preparations from mature seed pods (Pod enzyme) were used to acylate tRNAs from 5 day and 20 day old cotyledons, as well as from immature and mature pod tRNAs (developing stages). Pod enzyme is capable of acylating all six species of $tRNA^{leu}$ obtained either from cotyledon system or from the developing seed pods (Figures 3a and 3b).

Leucyl $tRNA_2$ and $tRNA_{3\,and\,4}^{leu}$ increase by 26% and 5% respectively in 5 day old cotyledons (Figure 3a). But in 20 day old cotyledon $tRNA_2^{leu}$ and $tRNA_{3\,and\,4}^{leu}$ show greater increases by 39% and 10 and 15% respectively (Figure 3b). Acylation of $tRNA_{5\,and\,6}^{leu}$ decreases by 33% and 24% respectively.

It should be pointed out here that acylation of 20 day cotyledon tRNA with 5 day cotyledon enzyme (Figure 1a) shows an increase in $tRNA_{5\,and\,6}^{leu}$. Whereas, when the pod enzyme is used to charge the 20 day cotyledon tRNA, the acylation of $tRNA_{5\,and\,6}^{leu}$ decreases by 33% and 24% respectively. This clearly shows that it is the enzyme that is the limiting factor in seed pods and not transfer RNAs.

It could be argued here that acylation of 5 day and 20 day old cotyledons tRNAs with 5 day cotyledon enzyme shows a much higher acylation in $tRNA_{5\,and\,6}^{leu}$ compared to the acylation by the Pod enzyme. On the other hand, Pod enzyme is more specific in giving higher activities for $tRNA_{2,3\,and\,4}^{leu}$ compared to cotyledon enzyme.

In an homologous system, acylation of Pod tRNAs (immature and mature) with a Pod enzyme results in very low activity in $tRNA_{5\,and\,6}^{leu}$, about 16-20% less activity (Figures 4a and 4b) than one can expect with a similar homologous system of cotyledon tRNAs (5 and 20 day) acylated

FIGURE 3a

Plaskon column chromatography of leucyl-tRNAs produced by leucyl-tRNA synthetase from soybean seed pod (mature).

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 (5 day cotyledon) and 0.02 ml of ^{3}H-leucine (60 Ci/mole) per ml. Other conditions were as given in Methods.

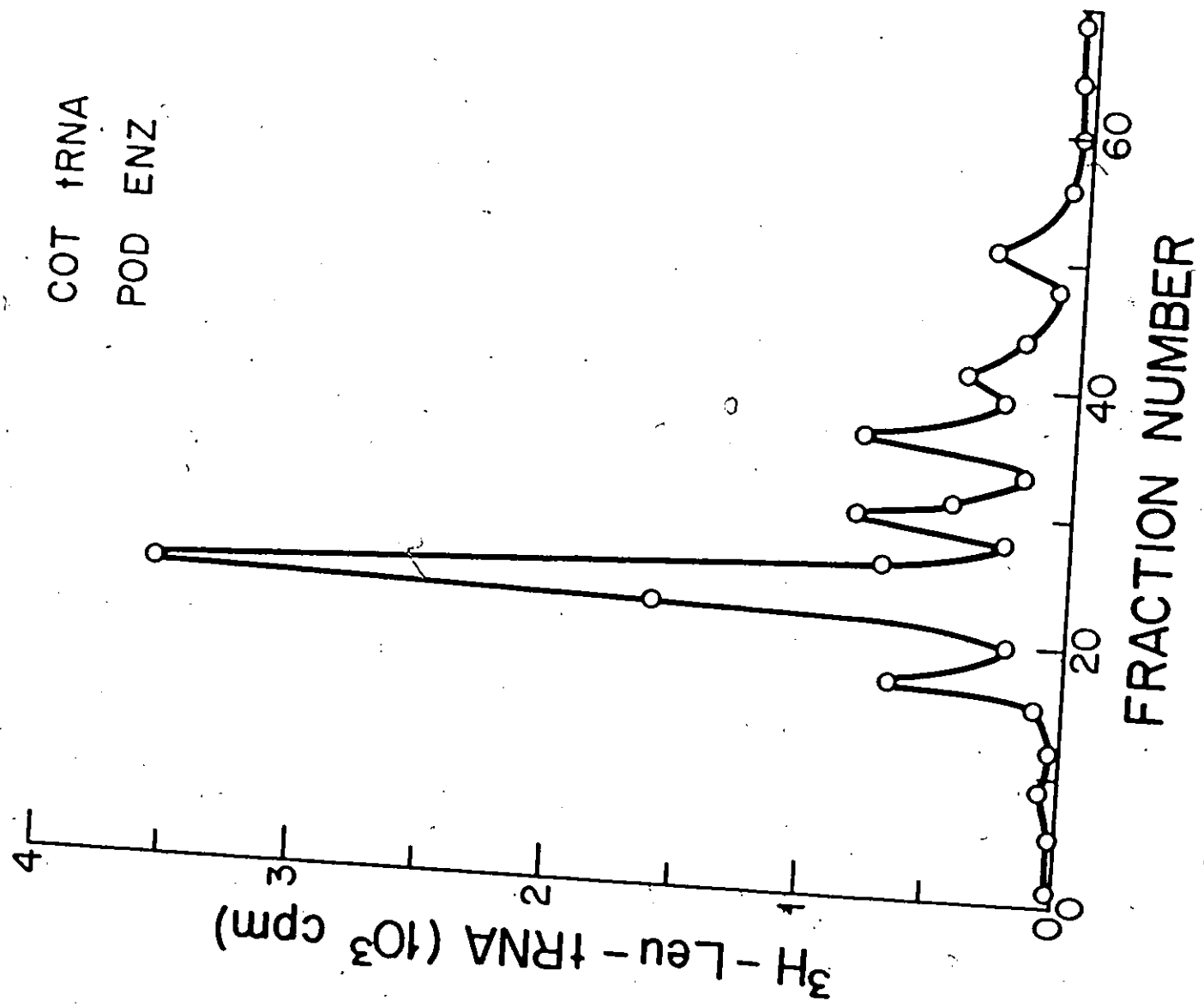

COT tRNA
POD ENZ
^3H – Leu – tRNA (10^3 cpm)
0
1
2
3
4
20
40
60
FRACTION NUMBER

FIGURE 3b

Plaskon column chromatography of ^{3}H-leucyl - tRNA isolated from 20 day cotyledons, acylated with mature pod enzyme (0.2 mg/ml), on a 2.5 x 30 cm Plaskon column (RPC-5), using a linear gradient of 0.5 - 0.9 M NaCl in buffer B. Fractions of 10 ml were collected and assayed for radioactivity.

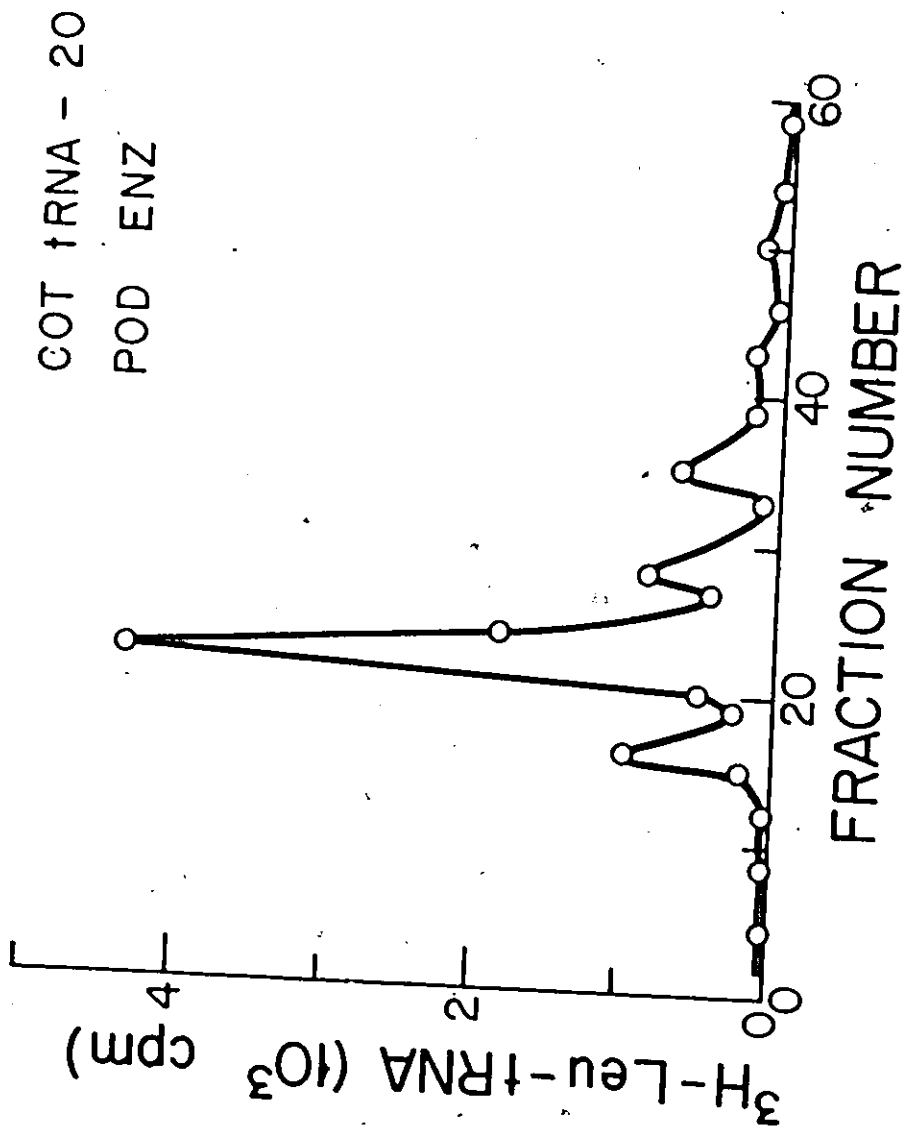

COT tRNA - 20
POD ENZ
^{3}H-Leu-tRNA (10^3 cpm)
0
2
4
0
20
40
60
FRACTION NUMBER

FIGURE 4a

Plaskon column chromatography of leucyl-tRNAs produced by leucyl tRNA synthetase from soybean seed pods.

0.2 mg protein from the peak of activity from a DEAE-cellulose column, 0.2 mg tRNA (immature seed pod) and 0.02 mg ^{3}H-leucine (60 Ci/mmole) per ml.

Other conditions were as given in Methods.

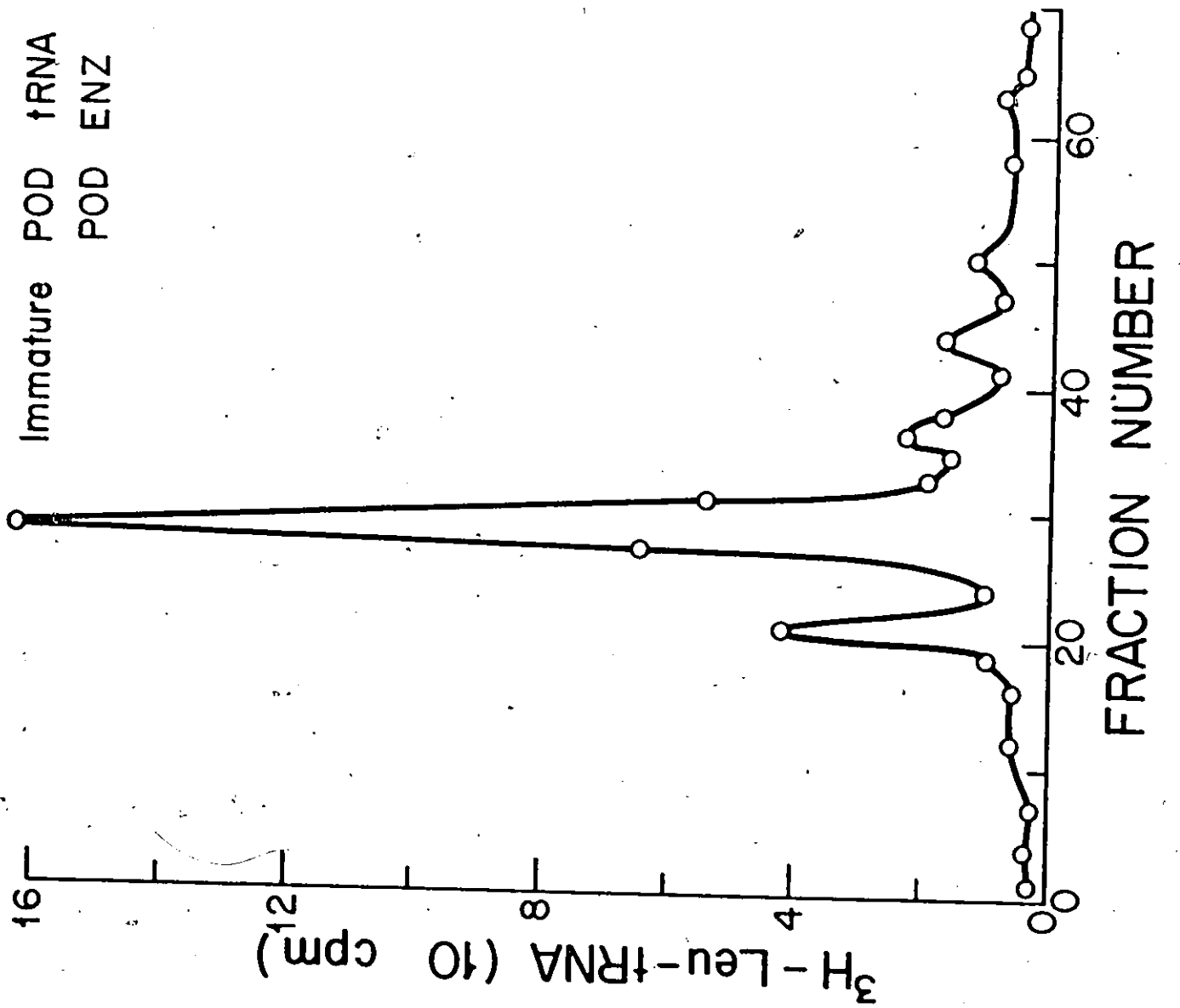
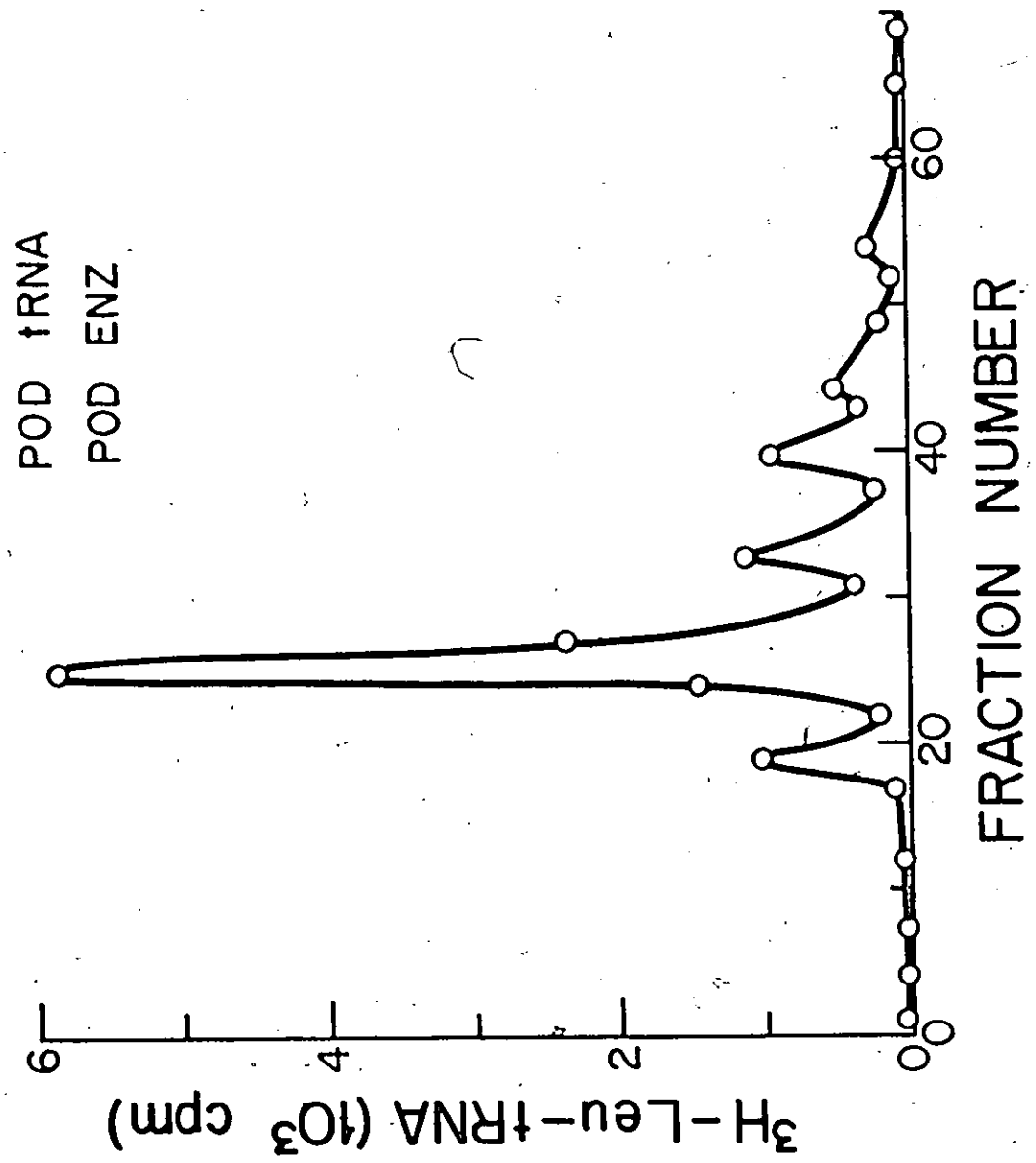

Immature POD tRNA
POD ENZ
^3H - Leu-tRNA (10 cpm)
16
12
8
4
0
0
20
40
60
FRACTION NUMBER

FIGURE 4b

Plaskon column chromatography of ^{3}H-leucyl-tRNA isolated from mature seed pods, acylated with mature pod enzyme (0.2 mg/ml) on a 2.5 x 30 cm Plaskon column, using a linear gradient of 0.5 - 0.9 M NaCl in buffer B. 10 ml fractions collected were assayed for radioactivity.


POD tRNA
POD ENZ
^{3}H-Leu-tRNA (10^3 cpm)
0
2
4
6
0
20
40
60
FRACTION NUMBER

with a 5 day cotyledon enzyme (Figures 1a and 1b). On a similar basis, if one compares $tRNA_2^{leu}$, its activity increases up to 37% in Pod system compared to the cotyledon system. Further, acylation of a cotyledon tRNA with a Pod enzyme (heterologous system) also brings about this substantial increase in $tRNA_2^{leu}$. Similarly the pod enzyme acylation of either cotyledon tRNAs or Pod tRNAs shows increases in $tRNA_{3\text{and}4}^{leu}$ and decreases in $tRNA_{5\text{and}6}^{leu}$.

It is very interesting to point out here that pod enzyme is capable of maximum or higher acylation activities in $tRNA_{2\ 3\text{and}4}^{leu}$ but not in $tRNA_{5\text{and}6}^{leu}$.

Data summarized in Table I clearly shows the differential activities of these two synthetase preparations and tRNAs. Now the question arises, are these differences in the enzyme activity primarily due to different stages of development of seed pods or the aging of cotyledons?

Fractionation of the Leucyl-tRNA Synthetase from Soybean Cotyledons and Seed Pods

It was of interest to determine, whether or not the variations in charging capacities of these two synthetase preparations could be due to specificities of the enzyme at the stage of development. To check this synthetase preparations from 5, 15 and 20 day cotyledon enzyme and pod enzyme were routinely prepared and their multiple forms separated on a HA column.

Aliquots were taken from every second fraction and assayed for leucine acceptor activity in a 0.2 ml reaction mixture as described in Materials and Methods. Figures 5a and 5b show the activity profiles obtained for 5 day cotyledon enzyme and pod enzyme respectively. It is also clear that the activity profiles corresponding to each of

TABLE 1: Aminoacyl-tRNA Synthetase Activity of Cotyledons and Seed Pods

Source of Enzyme	Source of tRNA	Relative Amount of Leucyl-tRNA Acylation of Each Peak (Percent of Total)					
		1	2	3	4	5	6
Cotyledon 5 days	Cotyledon (5 days)	10.96	23.37	6.11	7.22	25.49	19.07
	Cotyledon (20 days)	6.29	15.82	2.91	4.59	38.31	26.97
	Seed Pods (Immature)	13.46	41.58	6.66	4.50	13.68	5.58
	Seed Pods (Mature)	10.39	50.42	7.23	6.18	12.98	5.57
Seed Pods (Mature)	Cotyledon (5 days)	7.79	49.97	11.63	11.55	7.90	6.45
	Cotyledon (20 days)	9.80	55.42	13.01	10.24	4.75	2.26
	Seed Pods (Immature)	13.19	57.57	7.74	6.02	4.19	3.17
	Seed Pods (Mature)	8.34	59.62	10.30	9.12	4.96	2.14

tRNA was acylated in a 2 ml reaction mixture with ^{3}H-leucine and fractionated on a Plaskon column as described in Methods. The amount of radioactivity in each peak was summed and expressed as percent of the sum total in the six peaks.

FIGURE 5a

Hydroxylapatite fractionation of leucyl-tRNA synthetase from 5 day old soybean cotyledons. Approximately 50 mg protein was loaded onto a column (2.5 cm x 10 cm) in 50 ml of 0.05 M potassium phosphate (PH 6.5) and eluted with a linear gradient of potassium phosphate from 0.05 - 0.4 M. 7 ml fractions collected, were assayed for leucyl-tRNA synthetase activity using tRNA from 5 day old cotyledons.

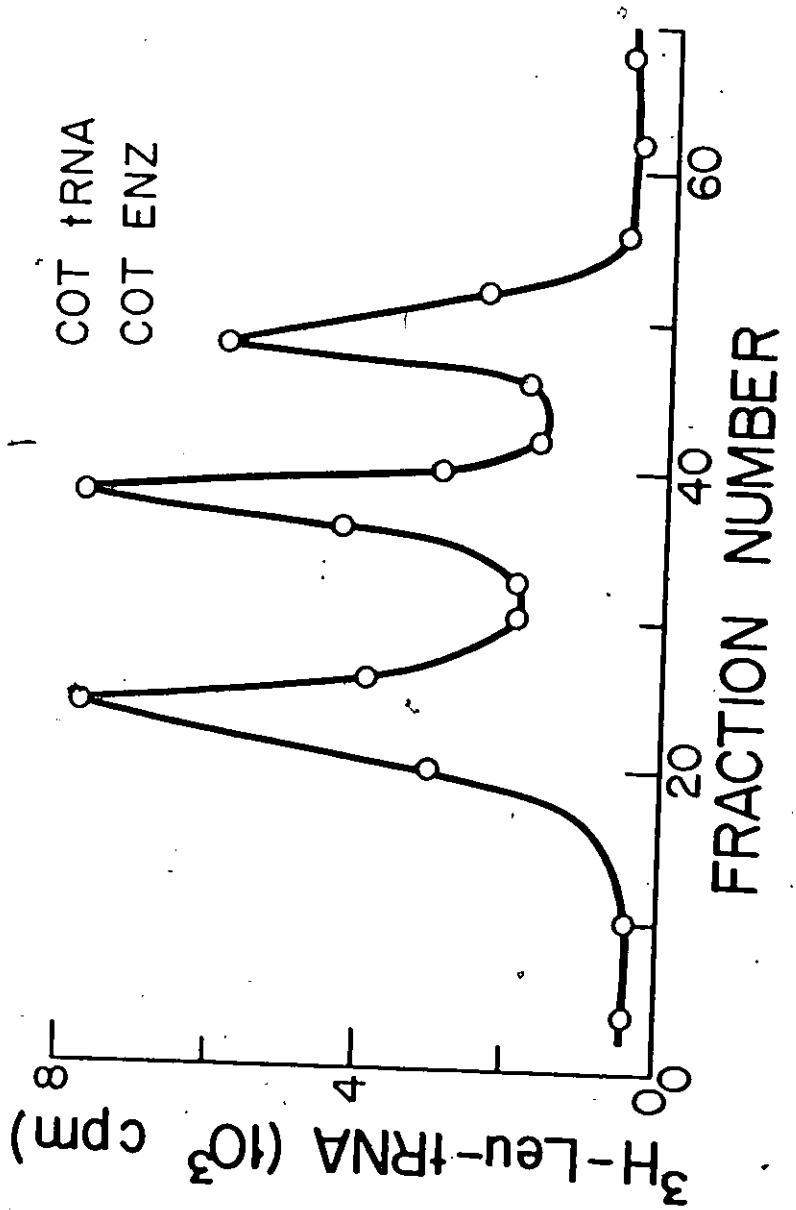

COT tRNA
COT ENZ
^{3}H-Leu-tRNA (10^3 cpm)
0
4
8
20
40
60
FRACTION NUMBER

FIGURE 5b

Hydroxylapatite fractionation of leucyl-tRNA synthetase from mature seed pods. Approximately 50 mg protein was loaded onto a column (2.5 cm x 10 cm) in 50 ml of 0.05 M potassium phosphate (PH 6.5) and eluted with a linear gradient of potassium phosphate from 0.5 - 0.4 M. 7-ml fractions collected, were assayed for leucyl-tRNA synthetase activity using tRNA from seed pods.

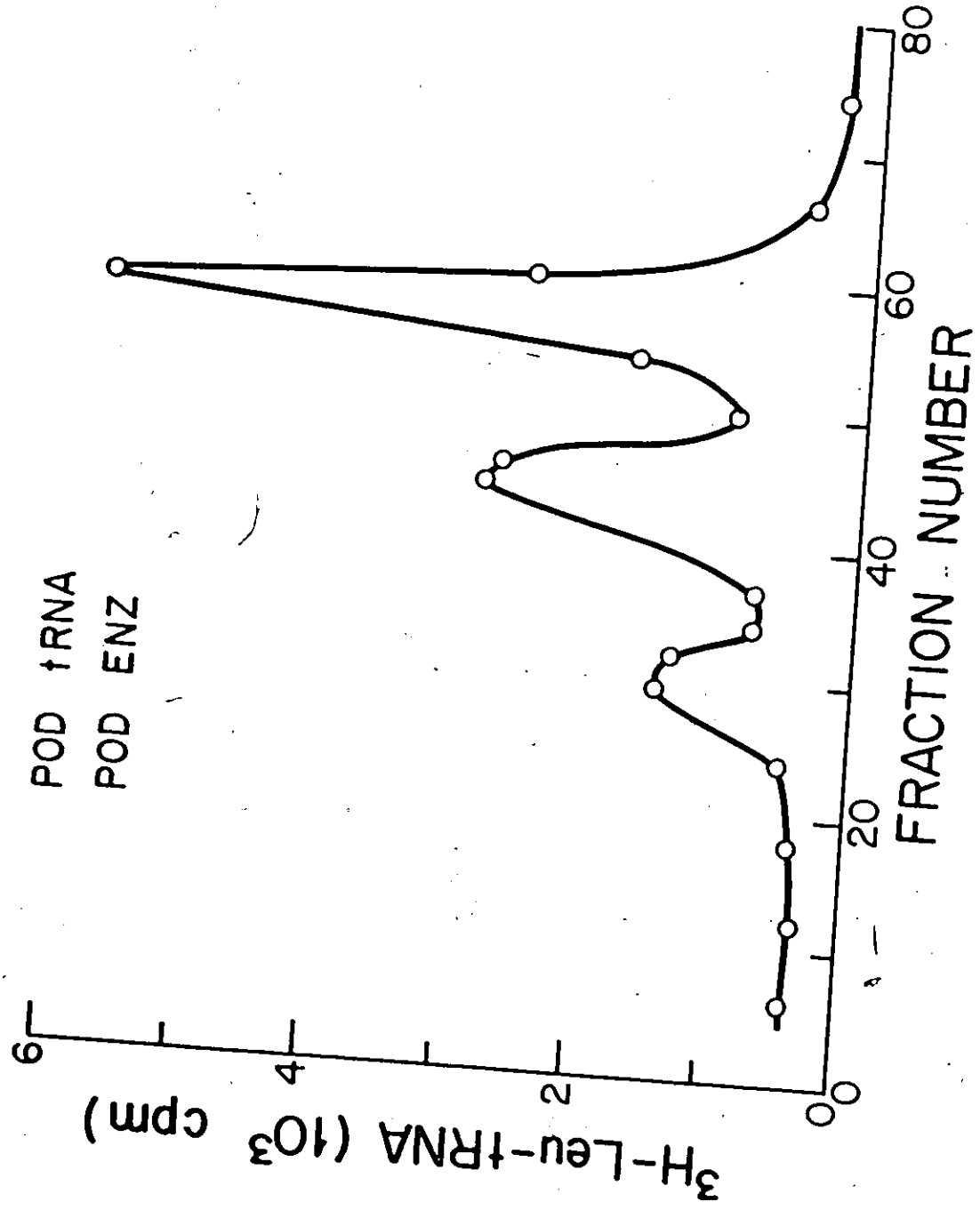

POD tRNA
POD ENZ
^{3}H-Leu-tRNA (10^3 cpm)
FRACTION NUMBER
0
2
4
6
20
40
60
80

TABLE 2: Aminoacyl-tRNA Synthetase Activity of Cotyledons and Seed Pods - Multiple Forms of Enzyme Separated on Hydroxylapatite (HA) Column

Source of Enzyme	Source of tRNA	Relative Amount of Leucyl-tRNA Acylation of Each Peak (Percent of Total)		
		1	2	3
Seed Pods (Mature)	Cotyledon (5 days)	17.54	45.81	36.63
	Seed Pods	15.45	41.83	43.12
Cotyledon (5 days)	Cotyledon (5 days)	41.77	32.52	25.70
	Seed Pods	39.32	32.91	27.74
Cotyledon (15 days)	Cotyledon (15 days)	48.44	28.63	24.07
	Seed Pods	44.96	24.21	23.51
Cotyledon (20 days)	Cotyledon (20 days)	48.67	26.43	24.88

Approximately 50 mg protein was loaded onto a HA column (2.5 cm x 10 cm) in 50 ml. of 0.05M potassium phosphate (pH 6.5) and eluted with a linear gradient of potassium phosphate from 0.05 - 0.4 M. Fractions were assayed for leucyl-tRNA synthetase activity as described in Methods.

the three elution peaks are present in both cotyledon and pod enzymes, although the relative amounts of the three peaks are somewhat different in the two systems. For clarity, the three peaks in these systems are designated as cotyledon enzyme 1, 2, and 3 and pod enzyme 1, 2, and 3 in order of elution from the column.

Successful isolation and separation of the three multiple forms of synthetases eliminates the possibility of any deficiencies in the pod enzyme compliment on a qualitative basis.

On a quantitative basis, pod enzyme 1 amounts to about 30% less than the cotyledon enzyme 1. Conversely, the amount of pod enzyme 2 and 3 found is much higher in quantity (19% and 17% more than the cotyledon enzyme 2 and 3). Because of the variations in $tRNA^{leu}_{5 \text{ and } 6}$ in Figures 1a and 1b, it was necessary to show if there existed any variations in enzyme systems. Leucyl-tRNA synthetase from three stages of cotyledon growth (5, 15 and 20 day old) were extracted, purified and separated on HA columns. These were then charged with tRNAs either from cotyledons or seed pod.

From the results summarized in Table II it is clear that regardless the age of cotyledons or developmental stages, leucyl-tRNA synthetases have all the three basic peaks. However, there is a quantitative change from one stage to the next. In aging cotyledons there is an increase in cotyledon enzyme peak 1 while peaks 2 and 3 do not change to a greater extent. Thus the observed differences in the relative amounts of three enzyme species in seed pods and different stages of cotyledon senescence, could possibly explain the differences in the relative acylation patterns of their isoaccepting $tRNA^{leu}_{2,5 \text{ and } 6}$.

Transfer RNA Specificity of Individual Enzyme Fractions

Previous reports (28) have shown that cotyledon enzyme peak 1 was specific in acylating $tRNA_{5\text{and}6}^{leu}$ and peaks 2 and 3 were equally effective in acylating $tRNA_{1-4}^{leu}$. To determine whether or not synthetase peak 1, 2 and 3 in the pod enzyme and cotyledon enzyme differ in their specificities, tRNA samples from 5 day old cotyledons and mature seed pods were charged and cross charged and fractionated on RPC-5 columns. While the results in Figure 6b confirm the above observation (28) for the cotyledon system, it is further shown that the same specificity does not reside in pod enzyme, especially peak 1. Pod enzyme peak 1 acylates all six leucyl tRNA regardless the source of tRNA (Figure 6a-1 and 6a-2). This is contrary to reported and expected results that cotyledon enzyme peak 1 exclusively acylates $tRNA_{5\text{and}6}^{leu}$ with traces of activity in regions of $tRNA_{1-4}^{leu}$ possibly due to contamination from cotyledon enzyme peak 2 (Figure 6b). The range of specificity for pod enzyme peaks 2 and 3 in acylating $tRNA_{1-4}^{leu}$ is identical to cotyledon enzyme 2 and 3. (Figures 7a, 7b and 7c).

Differences in Acylation Activi-ies of Leucyl-tRNA Synthetase from DEAE Cellulose Column and HA column

For routine enzyme assays or acylation of tRNAs for fractionation on RPC-5 (Plaskon columns), a synthetase preparation, partially purified on DEAE cellulose columns was used. In view of maximum alterations in $tRNA_2^{leu}$ and $tRNA_{5\text{and}6}^{leu}$ in aging cotyledons a comparison of crude synthetase and synthetase fraction 1 from HA column was made by acylating tRNAs from different sources. Data presented in Table III clearly shows that crude enzyme from DEAE-cellulose column is less active in acylating $tRNA_{5\text{and}6}^{leu}$ compared to enzyme peak 1 from a HA column,

TABLE 3: Aminoacyl-tRNA Synthetase Activity in Cotyledons and Seed Pods. (Comparison of the enzyme from DEAE-Cellulose Column and Enzyme Peak 1 From Hydroxylapatite (HA) Column)

Source of Enzyme	Source of tRNA	Relative Amount of Acylation of Leucyl-tRNA. (Percent of Total)	
		$tRNA_2^{Leu}$	$tRNA_{5 \text{ and } 6}^{Leu}$
Cotyledon (5 days)	Cotyledon (5 days)	25.34	48.31
DEAE-Cellulose	Seed Pods	54.34	19.99
Cotyledon (5 days)	Cotyledon (5 days)	10.05	66.58
Peak 1 - HA Column	Seed Pods	11.09	65.46
Seed Pods DEAE-Cellulose	Cotyledon (5 days)	52.43	15.05
	Seed Pods	63.10	7.51
Seed Pods Peak 1 - HA Column	Cotyledon (5 days)	23.35	45.92
	Seed Pods	27.34	45.67

tRNA was acylated in a 2 ml reaction mixture with ^3H-leucine and enzyme from DEAE-cellulose column or enzyme peak 1 from HA column and fractionated on a Plaskon column. The amount of radioactivity in peaks 2, 5 and 6 was summed and expressed as percent of total counts.

FIGURE 6a-1

Plaskon column chromatography of leucyl-tRNA produced by leucyl-tRNA synthetase fraction 1 from soybean seed pods.

Pod tRNA ($\sim$ 4 A_{260} units/ml) was aminoacylated using enzyme fraction 1 (0.2 mg/ml) obtained from hydroxylapatite column.

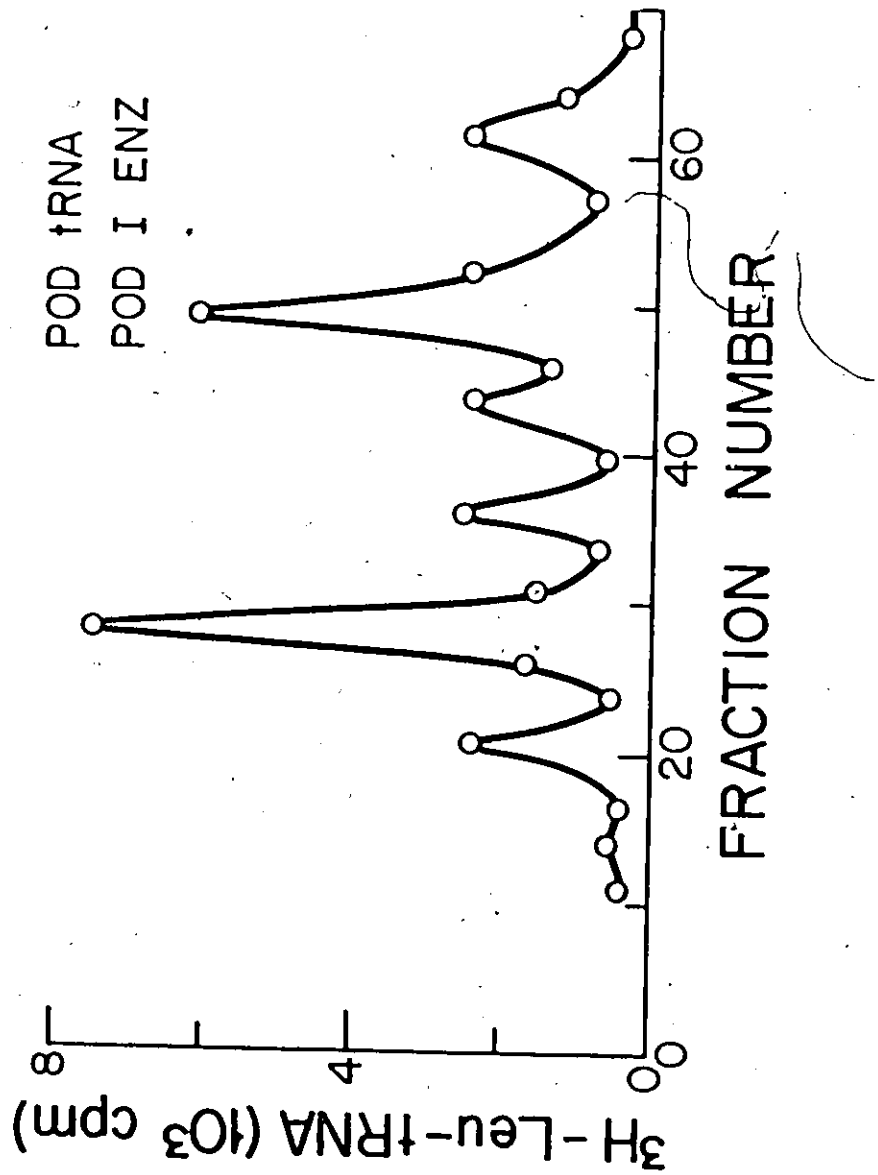

POD tRNA
POD I ENZ
^{3}H-Leu-tRNA (10^3 cpm)
0
4
8
20
40
60
FRACTION NUMBER

FIGURE 6a-2

Plaskon column chromatography of leucyl-tRNAs acylated by leucyl-tRNA synthetase fraction 1 from seed pods.

5 day cotyledon tRNA was aminoacylated using enzyme fraction (0.2 mg/ml) obtained from hydroxylapatite column.

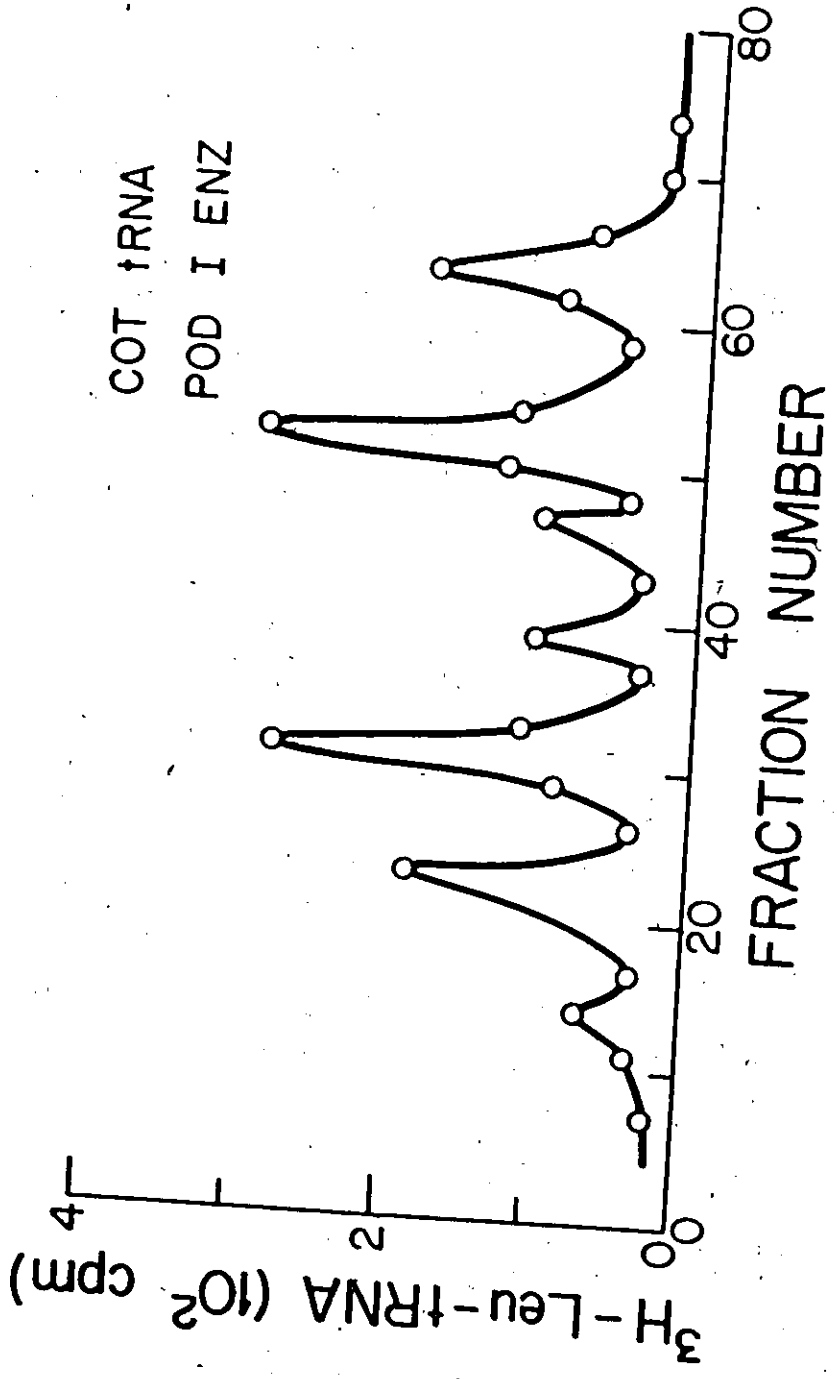

COT tRNA
POD I ENZ
^{3}H-Leu-tRNA (10^2 cpm)
0
2
4
FRACTION NUMBER
20
40
60
80

FIGURE 6b

Plaskon column chromatography of leucyl-tRNAs produced by leucyl-tRNA synthetase fraction 1 from soybean cotyledons.

Cotyledon tRNA (4.05 A_{260} units/ml) was amino-acylated using enzyme fraction 1 (0.2 mg/ml) obtained from hydroxylapatite column.

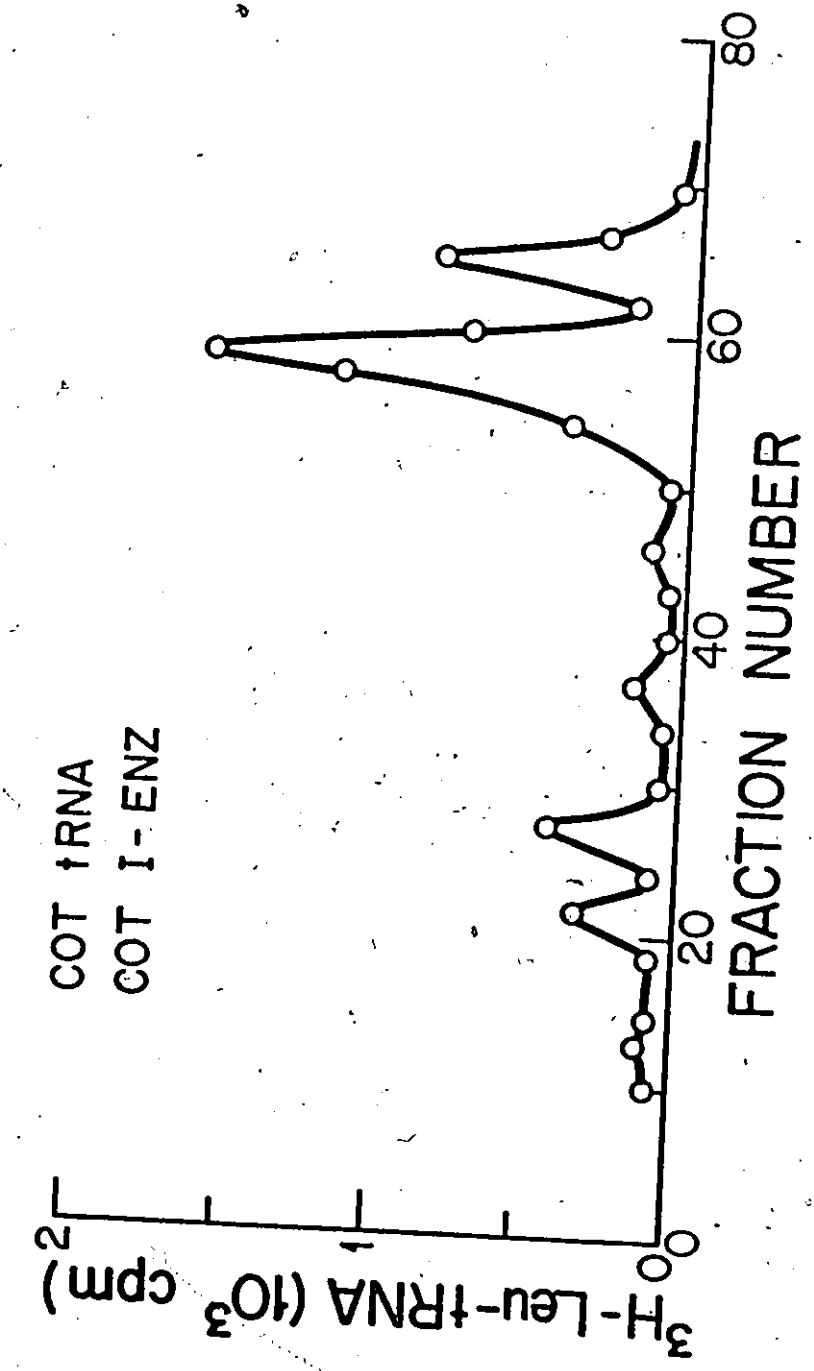

COT tRNA
COT I-ENZ
^{3}H-Leu-tRNA (10^3 cpm)
0
1
2
20
40
60
80
FRACTION NUMBER

TABLE 4: Comparison of Aminoacyl-tRNA Synthetase Activity in Cotyledon Enzyme Peak I and Seed Pod Enzyme Peak 1 from Hydroxylapatite Column

Source of Enzyme	Source of tRNA	Relative Amounts of Acylation of Leucyl-tRNA (cpm in peaks)		
		$tRNA_2^{Leu}$	$tRNA_5^{Leu}$	$tRNA_6^{Leu}$
Cotyledon (5 days) Peak I	Cotyledon (5 days)	400	1500	800
	Seed Pods	200	500	210
Seed Pods Peak I	Cotyledon (5 days)	2775	2990	1850
	Seed Pods	750	610	250

tRNA was acylated with peak I enzyme from HA column and fractionated on a Plaskon column. Cpm in peaks 2, 5 and 6 are expressed.

which acylates roughly 3-4 times more. This observation is true for both 5 day cotyledons enzyme and pod enzyme fractions 1. Conversely, the enzymes (cotyledon or pod) from DEAE column is more active in acylating $tRNA_2^{leu}$ as compared to enzyme peak I from HA column.

Regardless the source of enzyme I from HA column, or the tRNA used for aminoacylation reaction, the decrease in peak 2 and increase in peaks 5 and 6 is obvious as is summarized in Table IV.

Preliminary attempts (27) to locate the organelle containing synthetase fraction 1 in 5 day old cotyledons indicates that mitochondrial fraction did not contain any measurable synthetase activity. Synthetase preparations from chloroplast fraction of both etiolated and green cotyledons contained enzyme fraction 1 that acylated $tRNA_{5\text{ and }6}^{leu}$. No attempt was made to identify the organelle containing synthetase fraction 1 in seed pods or cotyledons.

Aminoacid Acceptor Activity of Aminoacyl-tRNA Synthetase During Development

The differences in enzyme patterns observed from HA column in aging cotyledons and developing seed pods prompted us to look into the acceptor activity of the different synthetase preparation. Aminoacids were acylated with enzymes from aging cotyledons (5, 10 and 20 days) and pod enzyme with tRNA from 5 day cotyledons, as described in Materials and Methods. Data presented in Table V clearly shows that the acylation of aminoacyl-tRNA synthetase decreases with aging. Though these results are of a preliminary nature, they do indicate that synthetase activity in accepting different aminoacids decreases with senesence.

TABLE 5: Variations in Aminoacyl-tRNA Synthetase Activity of Different Amino Acids in Soybean Seedlings

AMINO ACID	pMoles Aminoacyl-tRNA Formed/mg Protein/min.			
	E N Z Y M E			
	Seed Pods	Cotyledons (5 days)	Cotyledons (10 days)	Cotyledons (20 days)
Alanine	210	98	64	60
Arginine	1143	726	377.7	321
Aspartic Acid	1973	959	981	577
Glutamic Acid	563	146	336	205
Glycine	299	113	124	77
Leucine	157	61	93	86
Isoleucine	1215	574	337	250
Phenylalanine	649	239	252	317
Tyrosine	1055	269	444	232
Histidine	2503	1125	1043	1175
Valine	1421	684	650	371

FIGURE 7

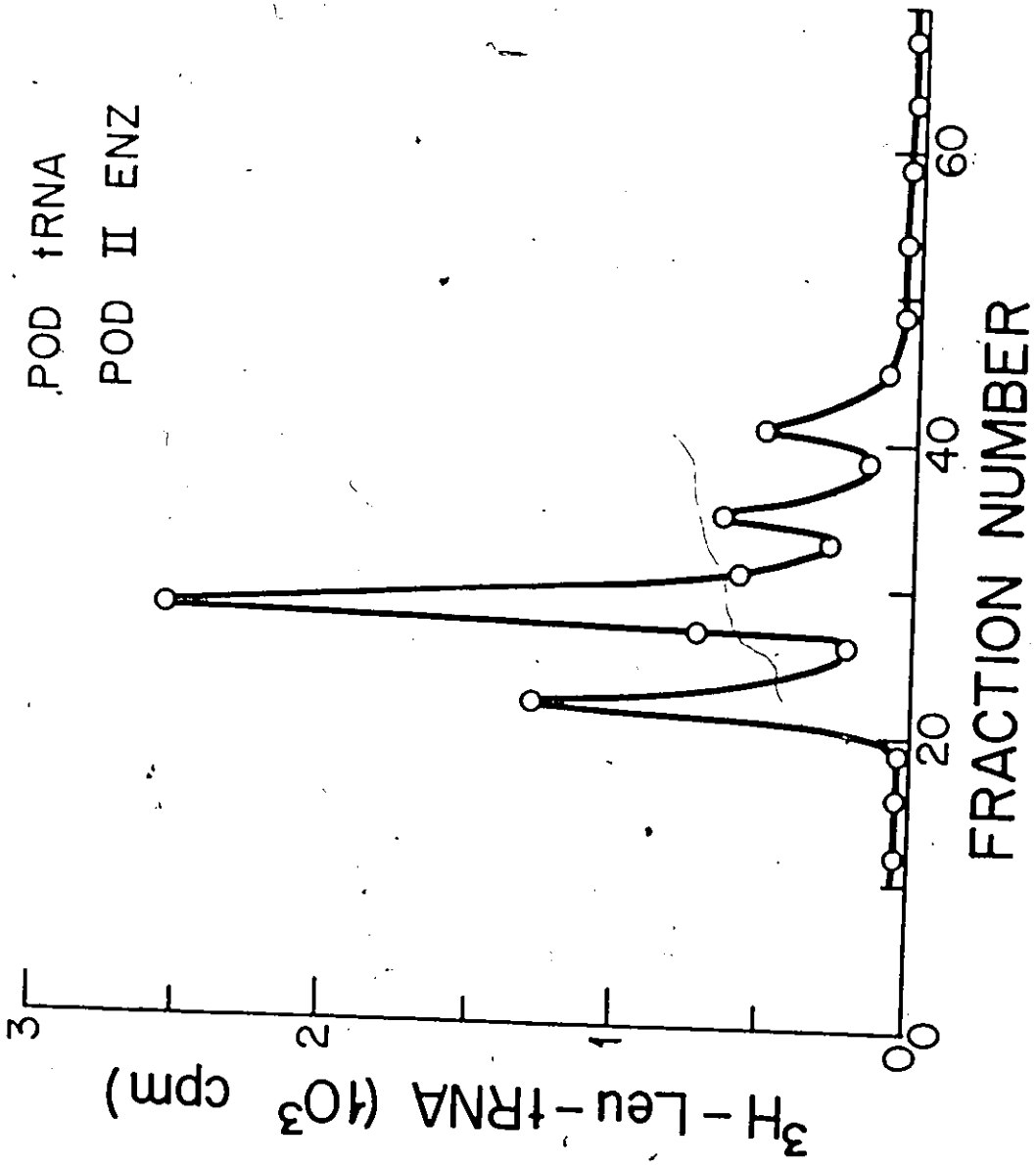
Plaskon column profile of leucyl-tRNA acylated with hydroxylapatite-fractionated pod synthetase.

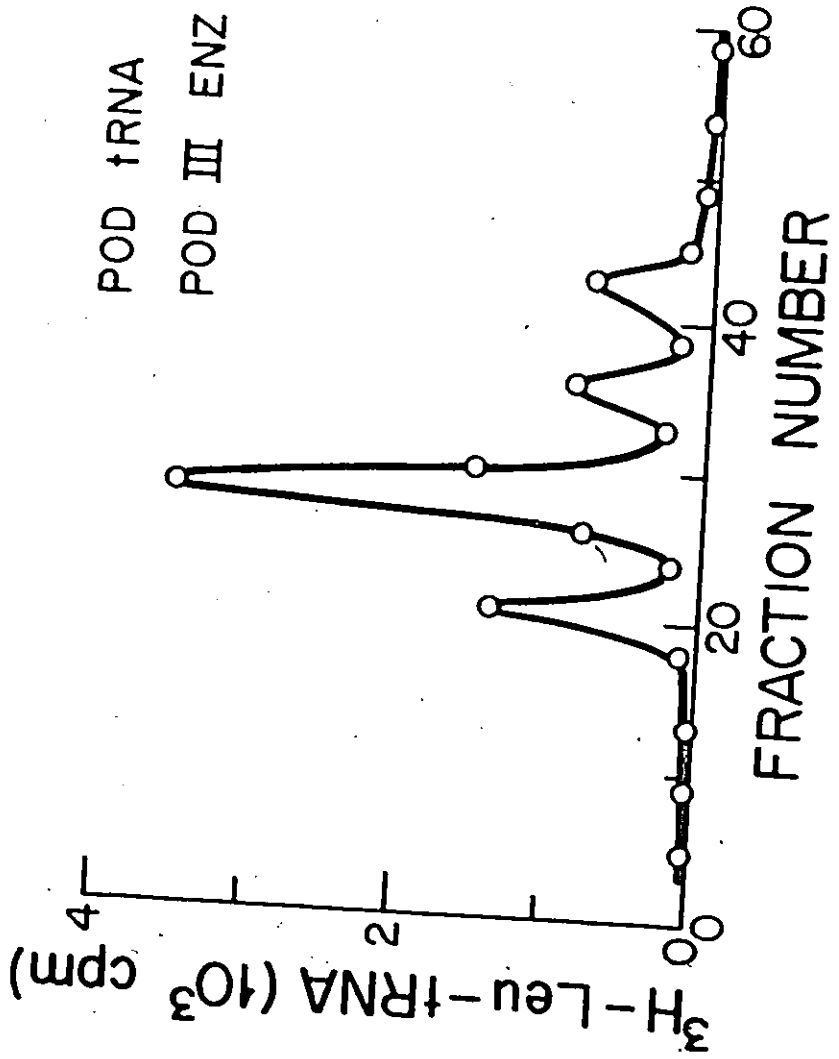
7a: Pod tRNA acylated with peak II synthetase.

7b: Pod tRNA acylated with peak III synthetase.

7c: Cotyledon tRNA acylated with peak III synthetase.

Elution was with a linear gradient of 0.5 - 0.9 M NaCl in buffer B.


POD tRNA
POD II ENZ
^3H - Leu - tRNA (10^3 cpm)
0
1
2
3
0
20
40
60
FRACTION NUMBER


POD tRNA
POD III ENZ
^{3}H-Leu-tRNA (10^3 cpm)
0
2
4
20
40
60
FRACTION NUMBER

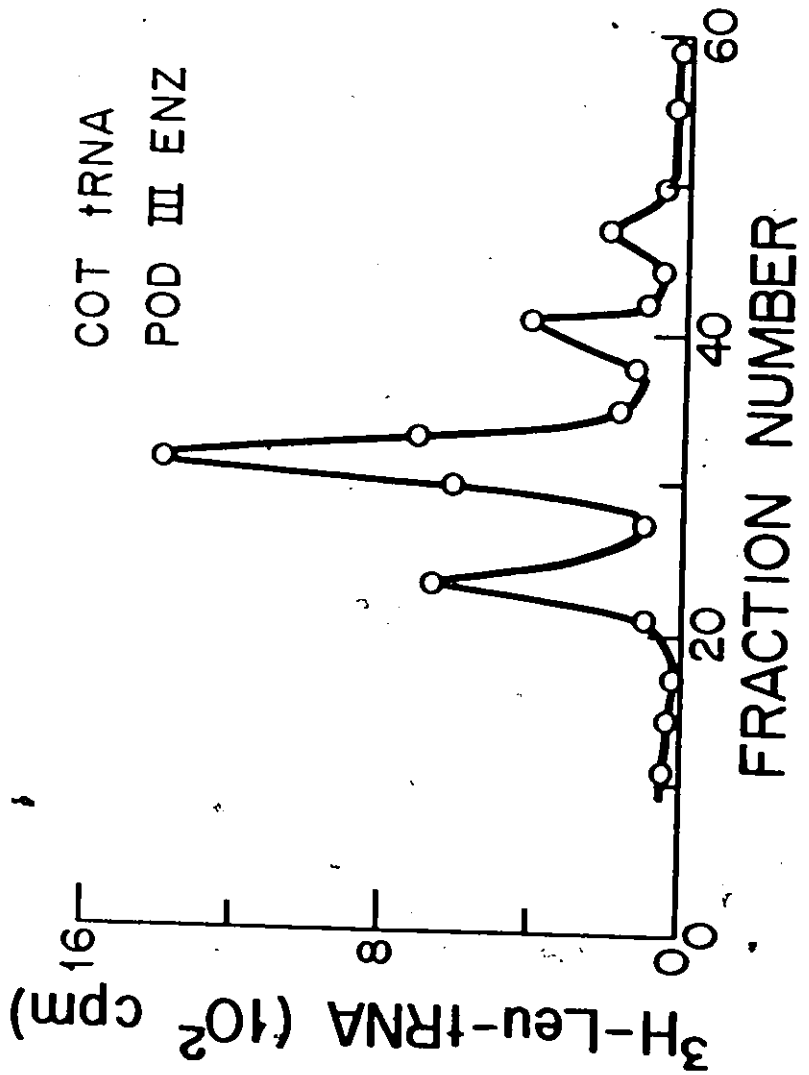
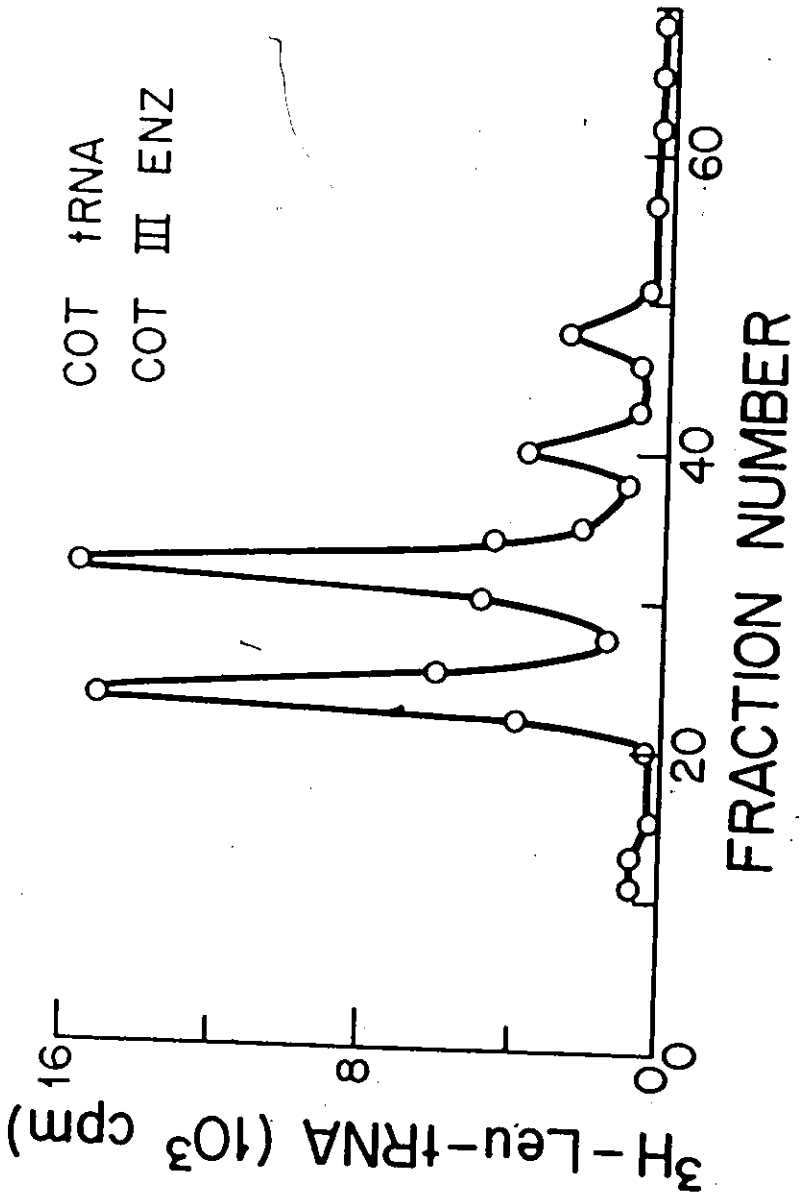

COT tRNA
POD III ENZ
^{3}H-Leu-tRNA (10^2 cpm)
16
8
0
0
20
40
60
FRACTION NUMBER

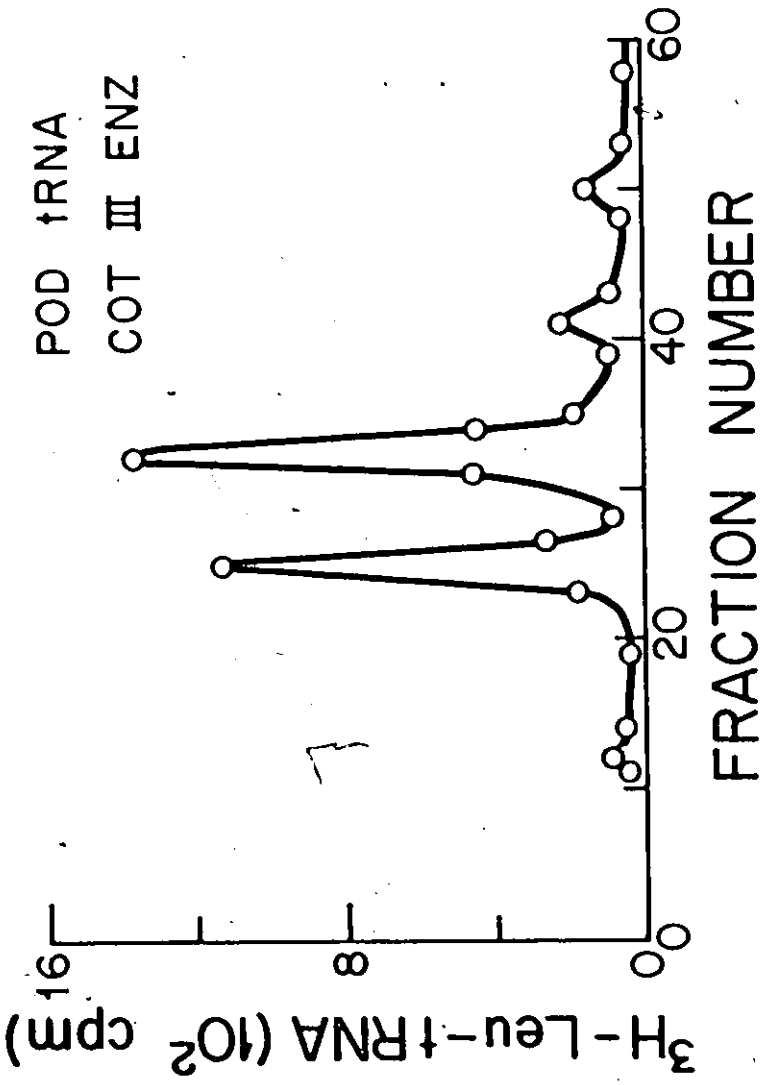
FIGURE 8

Plaskon column profile of leucyl-tRNA acylated with hydroxylapatite-fractionated 5 day old cotyledon synthetase.

8a: 5 day cotyledon tRNA acylated with peak III synthetase.

8b: Pod tRNA acylated with peak III synthetase.


COT tRNA
COT III ENZ
^{3}H-Leu-tRNA (10^3 cpm)
16
8
0
0
20
40
60
FRACTION NUMBER


POD tRNA
COT III ENZ
FRACTION NUMBER
^{3}H-Leu-tRNA (10^2 cpm)
0
8
16
20
40
60

DISCUSSIONS

The present investigation was undertaken to study the differences, if any, in the multiple forms of synthetases and isoaccepting tRNAs in two different stages of soybean development and germination.

Buds and seeds of many species exhibit dormancy. Newly formed apical bud will not sprout, and freshly shed seeds will not germinate, even when placed under optimal environmental conditions for growth. Distinctive biochemical changes which occur during the onset of dormancy are reversed when the organ emerges from dormancy. The onset of germination is accompanied by an increase in respiration rate (37), the activity of certain enzymes (45), the formation of new organelles (44), changes in nucleic acid metabolism (25), formation of polyribosomes (31) in vivo and their associated capacity to support aminoacid incorporation.

A study of the biochemical events associated with protein synthesis should provide some insight into the phenomena of growth and differentiation at the molecular level. Because tRNA serves as a functional link between the genetic information encoded in the messenger RNA, a specific change in the tRNA population has obvious implications for the regulation of cellular events (40). The possibility of regulation of protein synthesis at the level of translation has been suggested by many workers (38). It is apparent that the rate of mRNA readout, and hence the rate of synthesis of protein can be regulated by rate limiting quantities of tRNAs and/or synthetases.

Results presented here clearly show differences in the amount of isoaccepting leucyl tRNAs and multiple forms of synthetases between seed pods (a stage in soybean development before germination) and the

cotyledons (a stage after germination).

The results and observations reflect a gradual increase in peak 1 enzyme capable of acylating leucyl $tRNA_{5 and 6}$ with cotyledon senescence. Contrary to this, peak 1 enzyme in seed pods is limiting; yet it is capable of acylating all six leucyl tRNA species. Some reports exist to show that aminoacyl tRNA synthetases are especially located in the nuclei, mitochondria and chloroplasts.

There are two tempting explanations for the differences in the enzyme patterns between the seed pods and cotyledons.

(1) Regardless the identity of the organelle containing enzyme fraction 1 preliminary experiments (27) on etiolated and green cotyledons point towards the possibility of chloroplasts or proplastids being involved. Since the cotyledons serve as first leaves, until true leaves emerge, an increase in enzyme peak 1 could indicate an increase in the amount of chloroplasts.

(2) However, the difference in enzyme patterns between the cotyledons and hypocotyls and their specificity towards their cognate tRNAs (2), draw attention to a group of organelles collectively called microbodies, which include glyoxysomes and peroxisomes. These particles enclose specific enzymes involved in the β-oxidation of fatty acids and appear in abundance at the onset of germination of fat containing seeds (9). These organelles are absent in hypocotyls. The origin of the marker enzymes like isocitrate lyase and malate synthetase in the glyoxysomes and in fact the mechanism of formation of these organelles themselves is not known (47).

The assessment of a specific leucyl tRNA synthetase fraction 1 in these organelles would then provide the most tempting explanation

for differences in enzyme patterns between seed pods and cotyledons. With the onset of germination, along with other biochemical events, it can be proposed here that these glyoxysomes keep increasing in quantity in cotyledons, providing a source of energy by their β-oxidation system until the cotyledons senesce. Whereas the seed pods ready to go into a period of dormancy do not require these organelles in large quantity.

This difference in the amount of these organelles, with the assessment of enzyme fraction 1 then would open up a whole new area of investigation with the development of new organelles and enzymes with differentiation and germination. As to the question of difference in specificity of enzyme fraction 1 from seed pods and cotyledons towards their cognate tRNAs, the results can be interpreted on the basis of an inhibitor or a repressor like molecule covering part of the peak 1 enzyme in cotyledons so that it probably loses the capacity to acylate all six peaks of leucyl tRNA, but acylates only peaks 5 and 6 more. The absence of any such inhibitor forming a complex with peak 1 enzyme of seed pods would explain the capacity of this enzyme fraction to acylate all six peaks of leucyl tRNA. Moreover the higher acylation of $tRNA^{leu}_{5 \text{ and } 6}$ seen in a homologous cotyledon system could result in increased translation of certain hydrolytic enzymes, needed to hydrolyze the storage componds in the cotyledons.

In the cotyledons, there is hydrolysis and release from the compartments of a number of intracellular components (14). These compounds may act as inhibitors, covering part of enzyme peak 1. Such a complex effectively would prevent enzyme peak 1 from entering into further rounds of charging, yet is not formed until at least some charging has taken place to produce sufficient amounts of

aminoacyl-tRNA ($tRNA^{leu}_{5 \text{ and } 6}$). Some implications of such a model have already been discussed by Bick, Strehler and Hirsch (39).

SUMMARY

Leucine specific tRNAs of soybean cotyledons and seed pods fractionate into six discrete peaks on a plaskon column (RPC-5). The relative amounts of two of these tRNAs (species 5 and 6) are lower in seed pods than in cotyledons. Crude leucyl tRNA synthetase from seed pods is less active than the enzyme from the cotyledons in aminoacylating $tRNA_{5 \text{ and } 6}$.

Leucyl tRNA synthetases from cotyledons in a germinating seedling and in developing seed pods can be fractionated into three peaks of activity on hydroxylapatite columns. Enzyme peak 1 in seed pods amounts to about 36% less than in the five day old cotyledons. The amount of enzyme peaks 2 and 3 are more (about 10% and 18% respectively) in seed pods than in cotyledons.

Transfer RNA specificity of individual enzyme fractions from seed pods indicates that peak 1 enzyme of seed pods acylates all six species of leucyl tRNA compared to the cotyledon enzyme fraction 1 which essentially acylates only $tRNA^{leu}_{5 \text{ and } 6}$. The specificity of enzyme fractions 2 and 3 towards leucyl $tRNA_{1-4}$ is identical in both the organs.

Amino acid acceptor activity of the various synthetase preparations studied decreases with cotyledon senescence.

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