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LEUCYL TRANSFER RIBONUCLEIC ACIDS AND
AMINOACYL TRANSFER RIBONUCLEIC ACID
SYNTHETASES IN DEVELOPING PEA PODS AND
GERMINATING PEA SEEDLINGS

BY

HASMU KH V. PATEL

A THESIS

Submitted to the Faculty of Graduate
Studies through the Department of Biology
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
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WINDSOR, ONTARIO, CANADA.

1975

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ABSTRACT

Changes in leucyl-tRNA isoaccepting species were determined in germinating pea seedlings (Pisum sativum-var. Alaska) and in developing seed pods. Leucine specific transfer ribonucleic acids of pea cotyledons can be fractionated into four isoaccepting species by Reversed-phase chromatography on a Plaskon column (RPC-5). In contrast to this, only two species of leucyl-tRNAs were observed in the developing seed pods.

One of the objectives of this work was to investigate the possible multiplicity of leucyl-tRNA synthetase (leucyl:tRNA ligase (AMP); EC 6.1.1.4) in pea cotyledons. The procedure involved ammonium sulfate precipitation and DEAE cellulose column chromatography. The enzyme purified by this method retained the full range of specificity toward all four leucyl-tRNA species of pea cotyledons.

The partially purified cotyledon enzyme could be further separated on a hydroxylapatite (HA) column into two peaks of leucyl-tRNA synthetase activity. Pea cotyledon enzyme has two peaks designated peak 1 and peak 2. Enzyme peak 1 is dominant in seed pods while peak 2 is predominant in cotyledons. Enzyme peak 1 and 2 from

cotyledons were examined for the amino acid acceptor activity of twelve different amino acids. Both these fractions showed less than 3% acceptor activity for eleven other amino acids as compared to leucine-tRNA synthetase activity.

Enzyme peak 2 from cotyledons was further characterized by isoelectric focusing and polyacrylamide gel electrophoresis to detect the presence of any subspecies.

Preliminary results indicate that there are at least three species of enzyme peak 2.

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I am very grateful to Dr. H. B. Fackrell, Department of Biology for his constant encouragement and help. I would also like to thank Mr. C. Silinskas, Mrs. D. Veitch, Miss A. Vissochi and especially Mrs. V. Shridhar.

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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-23
DNA:	deoxyribonucleic acid.
Freon (1,1,1,3 Tetrachloro- tetrafluoropropane) 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 (Polychlorotri- fluoroethylene) column:	a reversed-phase chromatographic column (RPC-5).
PPi:	inorganic pyrophosphate.
RNA:	ribonucleic acid.
RPC:	reversed-phase chromatography.
tRNA(s)	transfer ribonucleic acids.
PI	Isoelectric point.

INTRODUCTION

This study was undertaken to determine the differences in leucyl-transfer RNA isoaccepting species and leucyl tRNA synthetases in two developmental stages, namely the germinating cotyledons and the seed pods of peas (Pisum sativum var. Alaska).

It is now well established that there are both qualitative and quantitative alterations in chromatographic elution profiles of certain isoaccepting tRNAs when a cell passes from one physiological state to another. Such changes occur in E. coli infected with bacterial phage T₂ (22) and in virus infected hamster cells (20). Similar changes have been observed at different developmental stages of various organisms. Previous studies on tRNAs in soybean have revealed that the complement of tRNA^{leu} isoaccepting species change during cotyledon senescence. (6)

There is some indication that aminoacyl-tRNA synthetases and tRNAs may be involved in developmental and regulatory functions of cellular systems. It has long been suggested that tRNA might play an important role in the regulation of protein synthesis at translational

level (34,36). Previous work in our laboratory (33) shows that the fractionation of leucyl-tRNA synthetases from soybean cotyledons and seed pods on hydroxylapatite (HA) column results in three peaks of activity. Bick and Strehler (7) demonstrated changes in these enzymes in respect to their ability to acylate certain transfer RNAs in aging soybean cotyledons. This implies that readout of certain messenger ribonucleic acids is not only controlled by the tRNA but also by the acylating enzyme. For this reason a study of the changes in leucyl-tRNA synthetase activity in the seed pods and germinating pea seedlings was undertaken.

The present work is concerned with partial purification and characterization of leu-tRNA synthetase from germinating pea cotyledons and seed pods as well as the changes in the relative amounts of leu-tRNA isoaccepting species, and aminoacyl tRNA^{leu} synthetases.

REVIEW OF LITERATURE

Transfer RNA

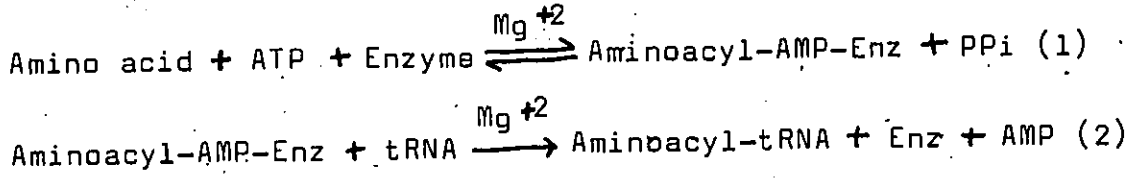
Transfer RNAs (tRNAs) are the "bilingual" adaptor molecules that, through the action of aminoacyl-tRNA synthetases, accept specific amino acids and transfer them to the nascent polypeptides, as directed by the messenger RNA on the ribosomes. These interesting polynucleotides which have a molecular weight of approximately 25,000 were first discovered in a mammalian system (19) and subsequently, in a bacterial extract (4). The formation of aminoacyl-tRNAs in plants was first reported by Webster (41). The extraction and purification of plant tRNAs has been achieved by methods adapted from those employed in bacterial and mammalian systems.

Multiple species of tRNA capable of accepting the same amino acid were initially reported by Doctor et al (13). At the same time, Berg et al (5) showed the existence of two valine specific tRNAs in E. coli. The existence of multiple isoaccepting tRNAs specific for a single amino acid appears not to show a uniform correlation with the degeneracy of the genetic code. The six species of leucyl tRNA from soybean cotyledons and hypocotyls

corresponds with the number of leucine codons (2) but pea cotyledons and leaves contain only four leu-tRNAs (44).

Aminoacyl-tRNA Synthetases

Aminoacyl-tRNA synthetases (E.C.6.1.1.-) were discovered by Hoagland and his coworkers (17,18) in 1955. Aminoacyl-tRNA synthetases catalyse the initial step in protein synthesis by attaching specific amino acids to their cognate tRNA molecules (Scheme 1).



Scheme 1: ATTACHMENT OF AMINO ACIDS TO TRANSFER RNAs
Aminoacyl-tRNA synthetases occupy a key role in protein biosynthesis, ensuring the accurate translation of the genetic message. These enzymes possess a high degree of specificity for amino acids and can discriminate between tRNA molecules of very similar structures.

Synthetase Multiplicity in Bacteria

Increasing evidence for the wide spread occurrence of multiple isoaccepting tRNAs led to a search for multiple aminoacyl-tRNA synthetases. In bacteria, a few instances of synthetase multiplicity have been reported. Mahler and Jesenski (27) observed two peaks of activity for proline tRNA synthetase in E. coli, using DEAE cellulose columns. The leucyl-tRNA synthetases from

E. coli were fractionated into three peaks of activity on a hydroxylapatite column by Chang Tao Yu (46). Two synthetases specific for methionine (39) and three leucine specific synthetases (38) have been found in Bacillus brevis by Surguchev and his coworkers. The methionine specific enzymes were separated by isoelectric focusing. The three leucine specific enzymes in Bacillus brevis were separated by gel electrophoresis (38). With the above exceptions, the majority of the results with bacteria favour the idea that only one synthetase exists for each amino acid.

Synthetase Multiplicity in Higher Organisms

Evolution of an increased specialization of cell function and the complex processes related to differentiation in higher organisms seem to involve an even more intricate organization of the protein synthesizing machinery including multiple tRNAs and synthetases, than that observed in bacteria. Direct and indirect evidence exists for multiple synthetases in more or less homogeneous cell populations of fungi, plants and animals.

Two lysyl-tRNA synthetases were found by chromatography on Bio-Rex 70 columns (10). In another study, two fractions of phenylalanine-tRNA synthetases were obtained from housefly larvae (14) by stepwise elution

from DEAE cellulose columns. In rat liver, two synthetases were found specific for both threonine (1) and leucine (40). The threonine specific enzymes were separated on a DEAE cellulose column, and the leucine specific enzymes (40) were fractionated on a hydroxylapatite column. Strehler et al (35) fractionated the leucyl-tRNA synthetase from rabbit heart muscle into three fractions by DEAE cellulose chromatography. Two of these fractions displayed a complementary range of specificity towards two tRNA^{leu} species as judged by MAK column profiles of the leucyl tRNAs. Working with plant tissues, Kanabus (23) showed the fractionation of leucyl-tRNA synthetase from soybean cotyledons on a hydroxylapatite column into three peaks of activity. However only two peaks of activity were found when soybean hypocotyl leucyl-tRNA synthetase was fractionated on hydroxylapatite column.

tRNAs and Synthetases in Aging and Developing Systems

It is now well documented that during the development of various organisms, both qualitative and quantitative changes occur in the chromatographic elution profiles of isoaccepting tRNAs (12, 30, 42) and aminoacyl-tRNA synthetases (7, 33, 35). Bick and Strehler (7) showed in soybean cotyledons that the ability of the leucyl-tRNA synthetase to acylate four of the six tRNA^{leu} species decreased disproportionately between the 5th and 21st day of seed germination. The elution profiles of

lysine and tyrosine transfer RNA during avian development (31) showed that these two tRNAs from whole chick embryos showed altered profiles when compared to the corresponding tRNAs from individual adult chicken organs. Studies of Ilan (21) on the development of an insect (Tenebrio molitor) indicated the appearance of both new tyrosine accepting tRNAs and the corresponding synthetases by the 7th day of pupation. Rehnert (32) reported a leucyl-tRNA synthetase unique to the early embryonic tissue of mice (gestational age of 5 to 8 days). The enzyme was more stable at 45°C than was the synonymous enzyme from liver of adult mice. The "embryonic" enzyme was able to amino acylate tRNA with trifluoroleucine much more efficiently than the "adult" enzyme. Gallo and Postka (15), studying tRNAs of leukemic human lymphoblasts, observed an additional glutaminyI tRNA synthetase activity in normal cells that was absent from leukemic cells.

MATERIALS AND METHODS

Plant Material

Green peas (Pisum sativum L var. Alaska) were surface sterilized in 10% chlorox, soaked in distilled water overnight and sown in moist vermiculite. Cotyledons were harvested after 5 days of germination in the dark at 27° - 29° C. The harvested cotyledons were either used immediately or stored at -20° C for tRNA and enzyme extractions.

Samples of mature seed pods were harvested from field grown Alaska peas. The harvested seed pods were stored at -20° C and used for extraction later.

Transfer RNA

Transfer RNA was prepared from total RNA of 5 day old freshly harvested cotyledons or those stored at -20° C. The total RNA was extracted by the phenol technique of Cherry et al (9) with minor modifications. Batches of 100 g of chilled tissue were ground for three minutes in a Waring blender with 100 ml of buffer A (10mM Tris HCl pH 7.6, 60 mM KCl and 10 mM MgCl₂), 100 ml of buffer washed phenol, 40 ml of 11% Dupanol (sodium lauryl sulfate) and 20 ml of bentonite suspension (40 mg/ml) (8). The homogenate was strained through four layers of cheese cloth and centrifuged for 10 minutes at 10,000 x g.

The supernatant was stirred in cold for two hours with an equal volume of organic (phenolic) phase. After centrifugation at 10,000 x g for 15 minutes, the aqueous phase was collected. Equal volume of buffer-washed phenol and 1/18 volume of bentonite was added to the aqueous phase. This mixture was vigorously stirred for 30 minutes in the cold followed by centrifugation at 10,000 x g for 15 minutes. The phenol extraction of the aqueous phase was repeated two more times and finally the aqueous phase was collected and the total nucleic acids precipitated by addition of two volumes of cold 95% ethanol and leaving in the freezer overnight. The precipitate was collected by centrifugation and the resulting pellet was extracted four times with 2.0 M potassium acetate, pH 6.5 by suspending with a glass mortar and pestle followed by centrifugation. The potassium acetate fraction was precipitated by addition of two volumes of cold 95% ethanol and leaving in the freezer overnight. The precipitate was collected by centrifugation at 10,000 x g for 15 minutes. This crude tRNA was further purified and concentrated by adsorption on a 2 ml column of DE-23 in buffer B (10 mM sodium acetate pH 4.5 containing 10 mM MgCl₂) and elution with 1 M NaCl in buffer B. The tRNA solution was dialysed against distilled water in the cold, and the concentration was

determined by U. V. absorbancy at 260 nm.

Leucyl-tRNA Synthetase

Extraction, purification and fractionation of the enzyme was performed at 0-4° C. Freshly harvested or frozen cotyledons were chilled on ice and ground for a few minutes with insoluble polyvinyl pyrrolidone, (polyclar AT; 200 mg/g tissue) using a mortar and a pestle. The grinding was continued for the next 15 minutes with stepwise addition of the grinding medium. The grinding medium consisted of 25 mM potassium phosphate pH 7.8, in solution C (10^{-2} M 2-mercaptoethanol; 10^{-5} M phenyl methyl sulfonyl chloride; 10^{-6} M L-leucine) saturated to 30% with respect to ammonium sulfate. The homogenate was strained through cheese cloth and centrifuged for 15 minutes at 27,000 x g. The supernatant was filtered through miracloth 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 5 ml of 25 mM potassium phosphate pH 7.8 in solution C, and dialysed against the same buffer for several hours in cold.

DEAE Cellulose Chromatography

The DEAE cellulose chromatography was performed using a stepwise elution technique. The dialysed enzyme

was adsorbed on a 20 ml column of DE-23, which was previously equilibrated in 25 mM potassium phosphate, pH 7.8 in solution C. The column was washed with 50 ml of the same buffer. The enzyme was eluted with 100 mM potassium phosphate, pH 7.8 in solution C. Five-ml fractions were collected and assayed for leucyl-tRNA synthetase activity and absorption at 280 nm was recorded.

Hydroxylapatite Column Chromatography

Material from the DEAE cellulose column chromatography was diluted 1:1 with cold distilled 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 up of a mixture of 10 g hydroxylapatite and 1 g of cellulose powder (Whatman CF 11) with small pads of cellulose at the bottom and top of the packing. The column had previously been equilibrated with 50 mM potassium phosphate buffer pH 6.5 in solution C, containing 10% (v/v) glycerol. The column was washed with 50 ml of the above buffer and eluted by a linear gradient of potassium phosphate buffer, pH 6.5 from 50 to 400 mM in solution C, both containing 10% (v/v) glycerol.

Fractions of 6 ml were collected and assayed for leucyl-tRNA synthetase activity in a reaction mixture of 0.25 ml containing 0.05 ml of the fractionated protein, and all the other components as described in the amino acylation assay.

Transfer RNA Aminoacylation Assay

The reaction was carried out at 30°C. Unless otherwise stated, a 1 ml reaction mixture contained 10 μ moles Tris (hydroxymethyl aminoethane) -HCl, pH 7.5; 5 μ moles MgCl₂; 0.5 μ moles ATP; 0.2% soluble polyvinyl pyrrolidone; 0.2 mg tRNA; 0.2-0.5 mg of enzyme and 20 μ l unneutralized solution of L-(4, 5-³H) leucine solution (60 ci/mMole). Reaction was terminated after 20 minutes by the addition of 5 ml of ice cold, 5% trichloroacetic acid. The samples were filtered through glass fibre filters and counted in a liquid scintillation counter.

Assay Procedure for Aminoacyl-tRNA Synthetase Activity

The reaction was carried out at 30°C. The amino acids studied were arginine, aspartic acid, alanine, glycine, histidine, glutamic acid, isoleucine, leucine, methionine, phenylalanine, tyrosine, and valine. The reaction mixture consisted of 250 μ l, which included 4 nmoles tRNA, 100 μ g protein, 200 pmoles of the amino acid under study and the concentration of other components as used for tRNA-aminoacylation assay. Reactions were incubated at 30°C and 50 μ l 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. Filters were washed a second time with cold 5% TCA, once with Ethanol-ether (1:1 v/v), dried under a heat lamp, and put into vials containing a toluene scintillation fluid and the radioactivity determined in a liquid

scintillation counter.

Reversed Phase Chromatography (RPC-5) - Plaskon

A mixture of 4 ml of Adogen 464 in 400 ml of chloroform was coated on to 100 g of polychlorotrifluoroethylene (Plaskon) support, according to Pearson, Weiss and Kelmers (29). The coated plaskon was suspended in 0.5 M NaCl in buffer B for packing the columns. For plaskon column fractionation tRNA was aminoacylated as described above in a 2 ml reaction mixture containing larger quantities of tRNA and enzyme. The concentration of other components were the same. Reactions were run for 20 minutes at 30°C followed by chromatography on a DE-23 column (1 x 3 cm) as described by Anderson and Cherry (2). Approximately 50,000 cpm of the acylated tRNA sample were applied to RPC-5 column and eluted with a linear gradient of 0.5-0.9 M NaCl in buffer B. Fractions of 10 ml each were collected, made 5% with respect to trichloroacetic acid and filtered through glass fiber filters (Whatman GF/A). The amount of radioactivity on the filter was determined in Mark II Nuclear Chicago liquid scintillation counter.

Isoelectric Focusing

Isoelectric focusing was performed using the LKB 8100 Ampholine Electrofocusing equipment (LKB 8101, Column Capacity 110 mls). The electrofocusing was

carried out as described in LKB 8100 Ampholine Electrofocusing Equipment, Instruction Manual (25).

Briefly described, an electrofocusing experiment was carried out as follows. The column was mounted in a vertical position. Carrier ampholytes of pH 3-10 range was used. The enzyme was dialysed against 1% glycine, in cold for several hours. Two solutions were prepared, one heavy (Solution H) and one light (Solution L), of the following compositions:

Solution H: Ampholine 1.9 ml

Sucrose 28.0 gm

Water to 60.0 ml

Solution L: Ampholine 0.6 ml

Dialysed enzyme sample

(approximately 5 to 7 mg protein)

Water to 60.0 ml

The electrode solutions consisted of the following compositions:

For cathode at bottom of the column, the dense electrode contained:

Ethylene diamine 0.4 ml

Distilled Water 14.0 ml

Sucrose 12.0 gm

For anode at the top of the column, the light electrode solution contained:

Phosphoric Acid 0.1 ml

Distilled Water 10.0 ml

The dense electrode solution was poured into the central tube of the column, and then the solution H and solution L were introduced into the column using a density gradient mixer and a peristaltic pump at a flow rate of 4 ml/min. Finally the light electrode solution was poured into the electrofocusing compartment. The cooling jacket and the central cooling tube were connected to a cold water tap and the electrodes were connected to the power supply. The experiment was conducted for 72 hours at a voltage of 500 - 600 volts.

After completion of the experiment, the column was emptied by means of a pump, at a flow rate of 2 ml/min. Two ml fractions were collected and the pH measured immediately. The fractions were assayed for leucyl-tRNA synthetase activity in a reaction mixture of 0.25 ml containing 0.1 ml of the fractionated protein and all the other components as described in the aminoacylation assay.

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was performed as described by Davis (11) and Ornstein (28). A 7.5% gel, alkaline system with separation at pH 8.3 was used. After extrusion, the gels were stained with 1% Amido Schwartz in 7% acetic acid for several hours and then electrophoretically destained.

Analytical Ultracentrifugation

Sedimentation coefficient for the concentrated peak II synthetase from HA column chromatography (6 mg/ml) was measured from the schleiren patterns obtained using a Beckman Model E Analytical Ultracentrifuge equipped with schleiren optics and electronic speed control. The sample was analysed at 56,000 rpm and 4-8° C in an An-H rotor.



RESULTS

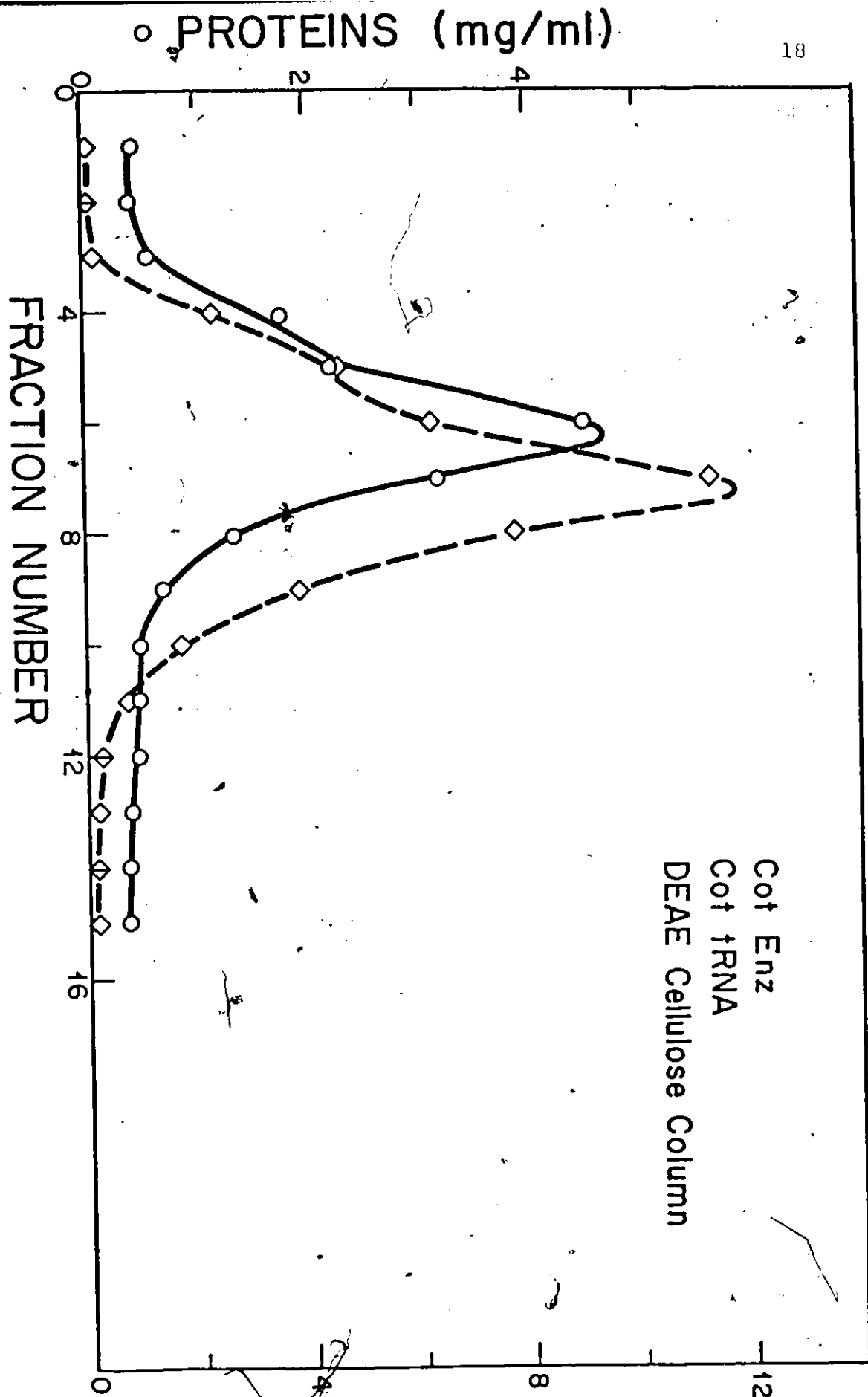
Differences in leucyl-tRNA species and leucyl-tRNA synthetase activities in two developmental stages of peas, cotyledons of germinating seedlings and in developing seed pods are discussed here. Partially purified leucyl-tRNA synthetase activity was investigated in these two systems, after the synthetase preparations were separated on HA columns. Transfer RNA preparations from these samples were acylated with enzyme fractions from HA column and finally the leucyl-tRNA species were separated on Plaskon (RPC-5) columns.

Extraction and purification of Leucyl-tRNA synthetase

Conditions for extraction, purification, storage and assay of the leucyl-tRNA synthetase of soybean cotyledons and seed pods, and pea cotyledons and leaves have been studied previously by several workers (23, 33, 44). Inactivation of enzyme by phenolic compounds in crude extracts can be partially prevented by addition of polyclar AT and a reducing agent to the extraction medium. (26). Therefore polyclar AT and 2-mercapatoethanol were routinely used in this work especially since their protective action has specifically been established in the

FIGURE 1

DEAE-cellulose column chromatography of leucyl-tRNA synthetase from pea cotyledons (stepwise elution technique). Enzyme preparation from 10 g tissue after ammonium sulfate precipitation and dialysis was applied to a column (20 ml) of DEAE-cellulose as described in methods. Protein  and leucyl-tRNA synthetase activity  were assayed in fractions of the eluate.



case of leucyl-tRNA synthetase from soybean tissue.(3). After extraction, the total leucyl-tRNA synthetase is purified by three consecutive steps: ammonium sulfate precipitation, DEAE cellulose chromatography and hydroxylapatite column chromatography. (Table I summarises the purification procedure of the leucyl-tRNA synthetase from 10 grams of five day old pea cotyledons. A 358 fold purification was achieved. At the end of hydroxylapatite column chromatography a 379% recovery was obtained.

DEAE Cellulose Chromatography

The protein precipitated with ammonium sulfate (30-60% fraction) was dissolved in 5 ml of buffer C, dialysed to remove the ammonium sulfate and chromatographed on a DEAE cellulose column. DEAE cellulose chromatography was routinely performed using a stepwise elution technique in the cold and the enzyme recovered in a single protein fraction by eluting with 0.1 M phosphate buffer (Figure 1). The enzyme recovery after DEAE cellulose chromatography was 269% and the specific activity increased about 2-3 fold as compared to the enzyme from the $(\text{NH}_4)_2\text{SO}_4$ fraction (Table I).

Hydroxylapatite Column Chromatography

Preliminary studies on the hydroxylapatite column chromatography was conducted using buffers without glycerol. This resulted in a poor separation and low

TABLE I: Purification of Leucyl-tRNA Synthetase

Enzyme Fraction	Volume ml	Protein mg/ml	Specific Activity (units/mg)	Total Activity	Purification Fold	Percent Recovery
Crude Extract	38.0	32.38	0.06	73.8	1.0	100
(NH ₄) ₂ SO ₄ Fraction	13.0	22.47	0.46	134.3	7.6	181
DEAE Cellulose Fraction	30.0	5.58	1.19	199.2	19.8	269
Hydroxylapatite Fraction	3.5	3.72	21.50	280.0	358.0	379

FIGURE 2

Hydroxylapatite column chromatography of leucyl-tRNA synthetase from 5 day old pea cotyledons. Approximately 50 mg protein was loaded onto a column (2.5 cm by 10 cm) in 50 ml of 0.05 M potassium phosphate (pH 6.5) and eluted with a linear gradient of potassium phosphate from 0.05-0.4M, without glycerol. Six ml fractions collected were assayed for leucyl-tRNA synthetase activity using tRNA from 5 day old cotyledons.

^3H -Leu-tRNA (10^3 cpm/ $50\ \mu\text{l}$ Enz/20 min)

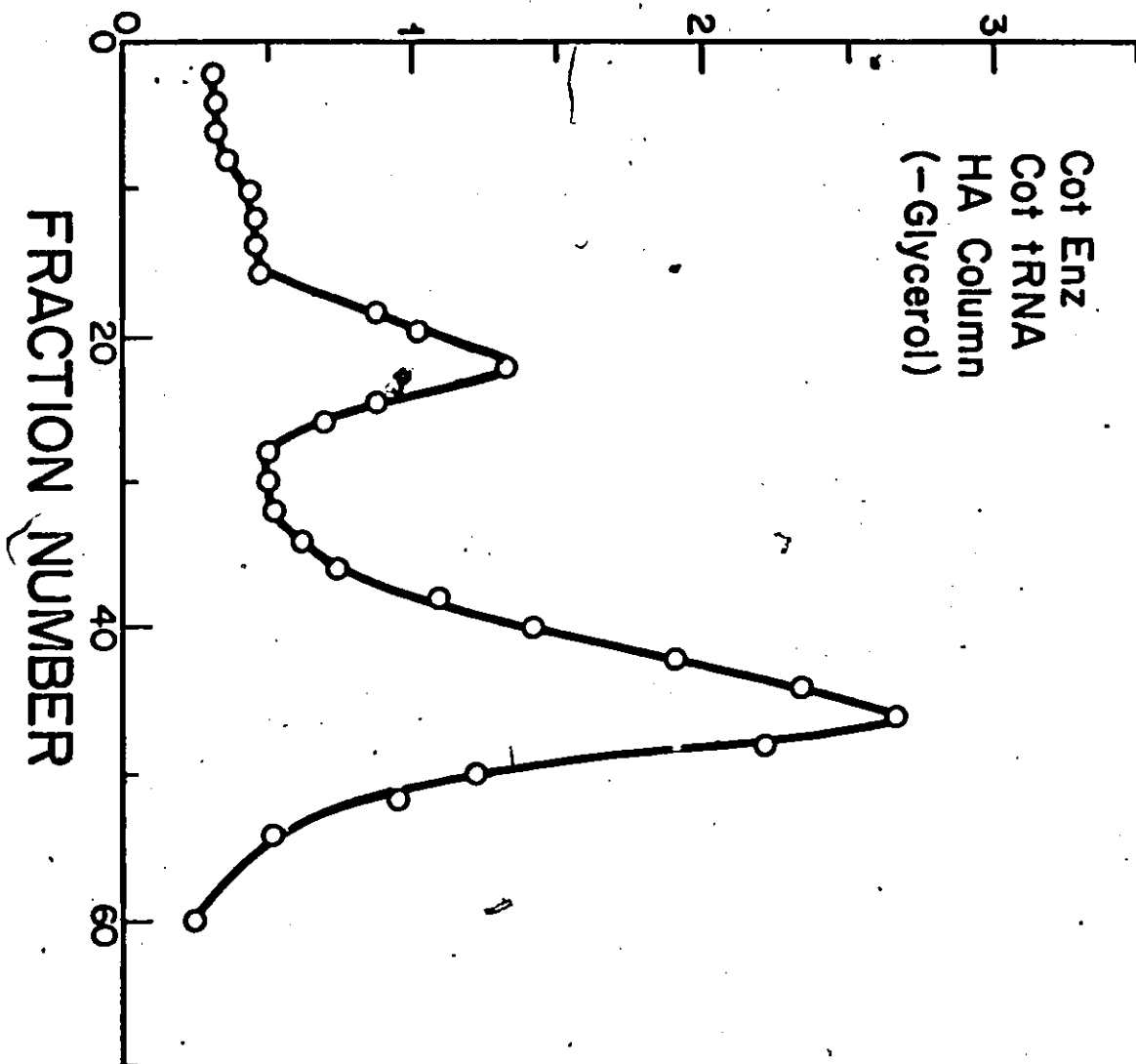


FIGURE 3

Hydroxylapatite column chromatography of leucyl-tRNA synthetase from 5 day old pea cotyledons. Approximately 50 mg protein was loaded onto a column (2.5 cm by 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, containing 10% (v/v) glycerol. Six ml fractions were collected and assayed for leucyl-tRNA synthetase activity using tRNA from 5 day old cotyledons.

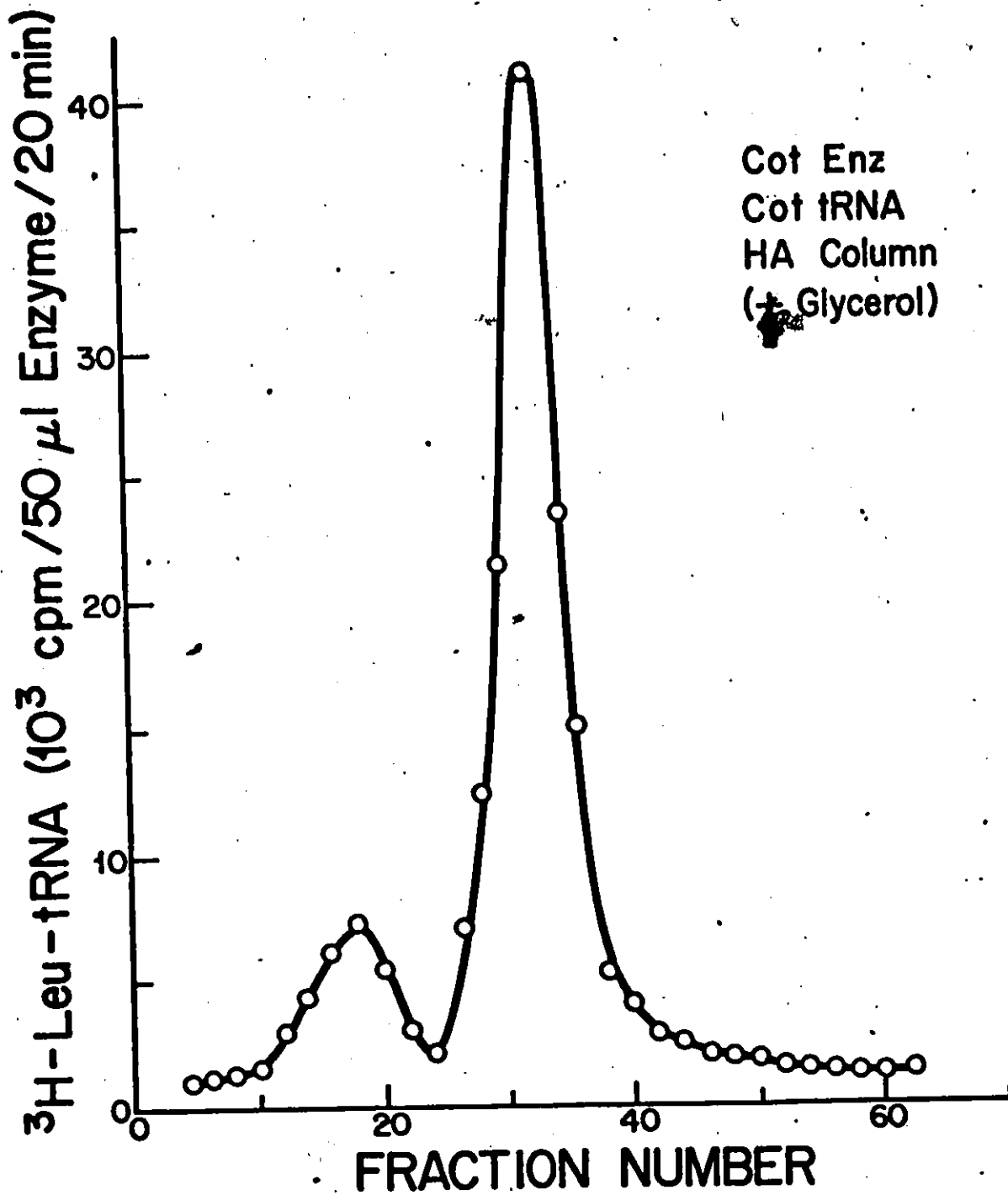
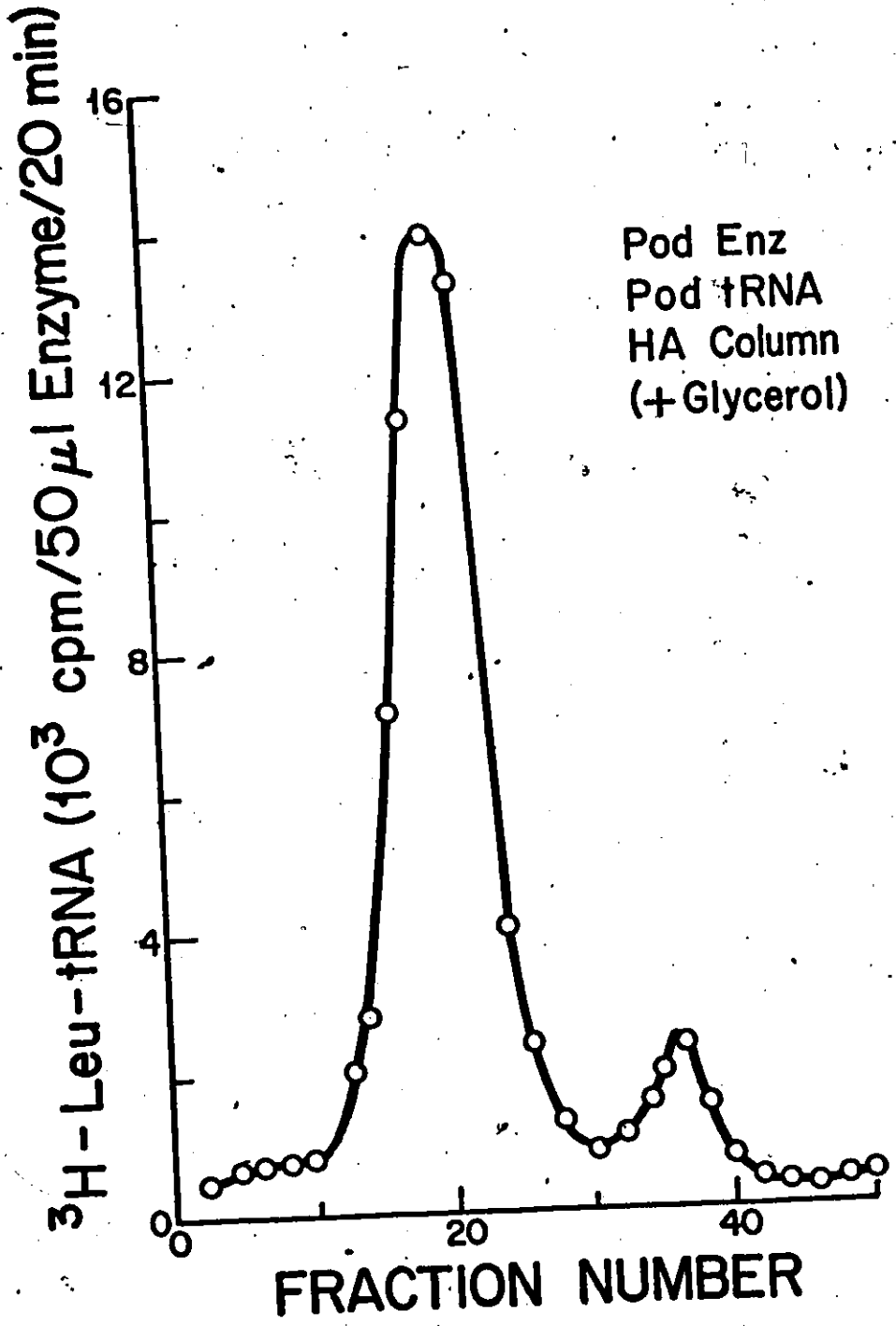


FIGURE 4

Hydroxylapatite column chromatography of leucyl-tRNA synthetase from mature seed pods. Approximately 50 mg protein was loaded onto a column (2.5 cm by 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 containing 10% (v/v) glycerol. Six ml fractions were collected and assayed for leucyl-tRNA synthetase activity using tRNA from seed pods.



enzyme activity, less than 3,000 cpm in peak 2 (Figure 2). Addition of 10% glycerol to the buffers increased activity upto 40,000 cpm (Figure 3). Hydroxylapatite column chromatography gives an 18 fold enrichment of the DEAE cellulose fraction. The enzyme recovery after this stage of purification was also found to be high. Leucyl-tRNA synthetase from pea cotyledons fractionates into two discrete peaks of activity on HA column (Figure 2 and 3). Similarly, leucyl-tRNA synthetase from pea seed pods also fractionates into two peaks of activity (Figure 4). Addition of 10% glycerol increases the activity of these two enzyme peaks. It is of interest to note that pea cotyledons contain predominantly enzyme peak 2 whereas in pea seed pods enzyme peak 1 is predominant.

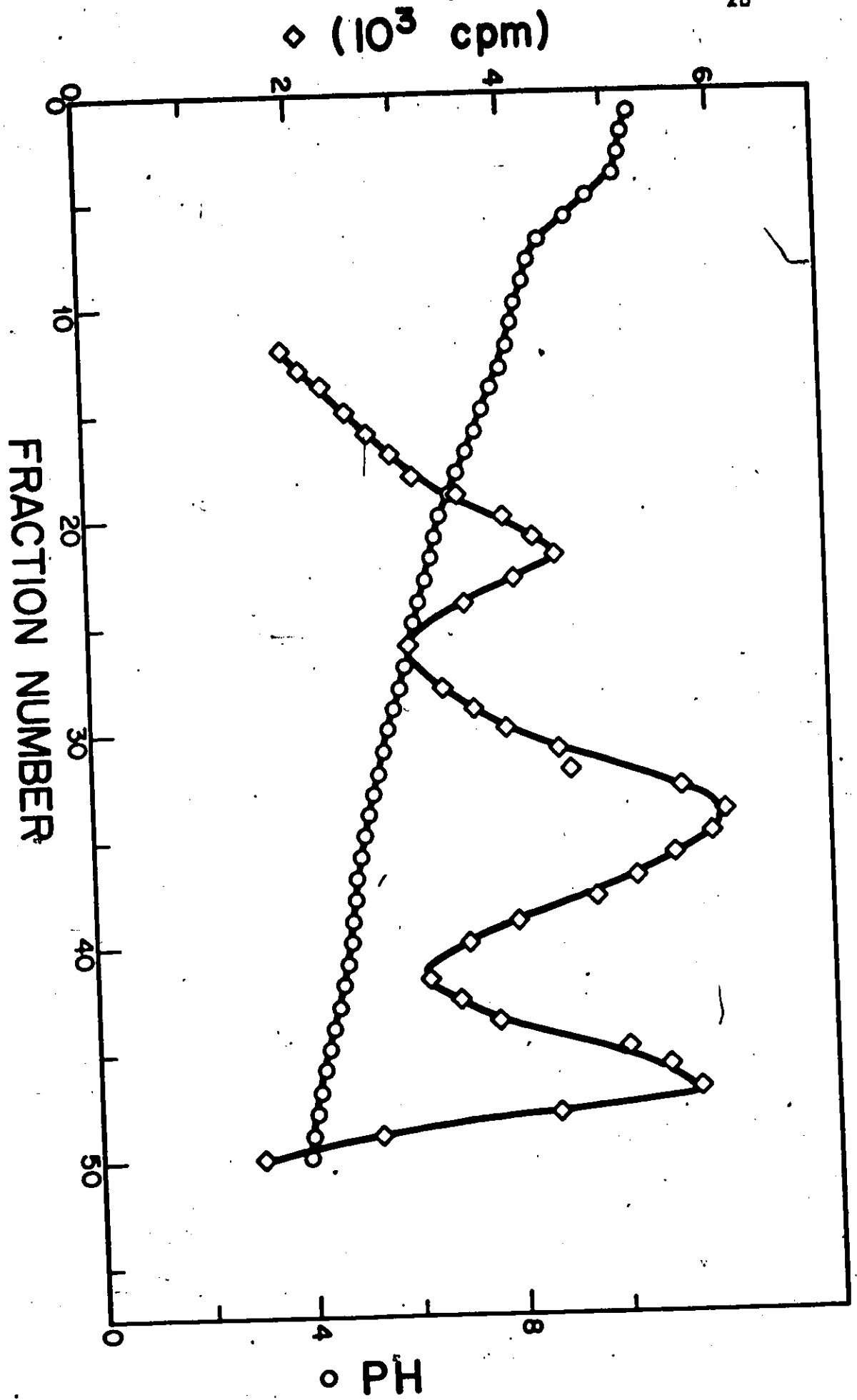
For further purification and characterization, it was necessary to have an enzyme preparation with little contamination. Therefore, the purity of peaks 1 and 2 from cotyledons following HA column chromatography was checked by testing their amino acid acceptor activity for 12 different amino acids (Table 2). It was of interest to note that both these peaks are essentially pure for leucyl-tRNA synthetase with less than 3% contamination from other synthetases. Enzyme peak 2 of cotyledons was pooled after hydroxylapatite column chromatography and concentrated by Diaflo ultrafiltration. This concentrated

TABLE II: Aminoacyl-tRNA Synthetase Activities of Peak I and Peak II Enzymes of Pea Cotyledons After HA Column Chromatography.

Amino acid	pmoles aminoacyl-tRNA formed per mg enz/min		% Activity of aminoacyl-tRNA synthetases relative to leu-tRNA synthetase	
	Enzyme I	Enzyme II	Enzyme I	Enzyme II
Alanine	10	5	0.20	0.04
Arginine	50	70	1.10	0.60
Aspartic Acid	30	160	0.70	1.50
Glutamic Acid	50	40	1.10	0.40
Glycine	7	7	0.15	0.06
Histidine	90	170	2.00	1.60
Isoleucine	60	60	1.30	0.60
Leucine	4410	10160	100.00	100.00
Methionine	20	32	0.40	0.30
Phenylalanine	10	20	0.20	0.20
Tyrosine	15	50	0.34	0.50
Valine	100	100	2.10	1.00

FIGURE 5

Isoelectric focusing of leucyl-tRNA synthetase fraction 2 from pea cotyledons. The pH gradient is represented by $\bullet-\bullet-\bullet$ and the leucyl-tRNA synthetase activity is represented by $\diamond-\diamond-\diamond$.



enzyme was used for isoelectric focusing, polyacrylamide gel electrophoresis and analytical ultracentrifugation studies.

Isoelectric Focusing

Isoelectric focusing of the enzyme peak 2 from cotyledons was performed over the pH range 3 to 10. Acceptor activity for leucyl-tRNA synthetase was measured for each fraction as described under materials and methods. Three peaks of activity were observed on isoelectric focusing of enzyme peak 2 from pea cotyledons (Figure 5). This illustrates that enzyme peak 2 possibly consists of three subspecies.

Disc Gel Electrophoresis

Samples of 50-100 μ g of concentrated peak 2 synthetase from pea cotyledons applied to polyacrylamide disc gel resulted in three protein bands (Figure 6). This suggests that enzyme peak 2 is a heterogeneous mixture of proteins and also confirms the findings with isoelectric focusing technique.

Analytical Ultracentrifugation

Results shown in Figure 3 indicate a single peak when enzyme peak 2 was examined by sedimentation velocity analysis. The S_{abs} obtained by a single experiment was 6.63. It was not possible to perform additional experiments with analytical ultracentrifuge, due to technical difficulties.

FIGURE 6

Polyacrylamide disc gel electrophoresis of leucyl-tRNA synthetase fraction 2 from pea cotyledons. 50-100 μ g of fraction 2 was applied on a polyacrylamide disc gel.



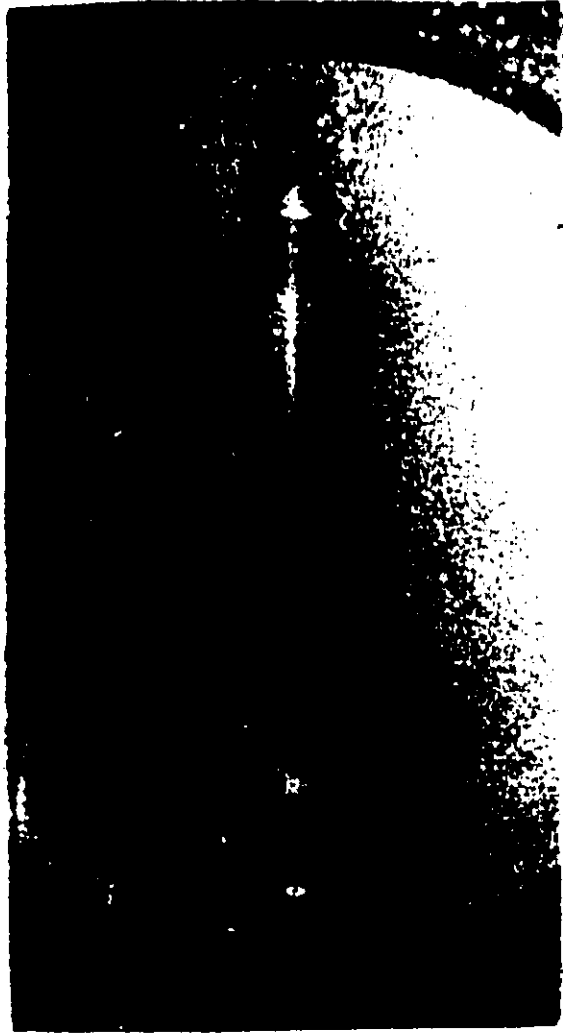


FIGURE 7

Schleiren pattern of concentrated leucyl-tRNA synthetase fraction 2 from pea cotyledons obtained 20 minutes after reaching a speed of 56,000 rev/min.

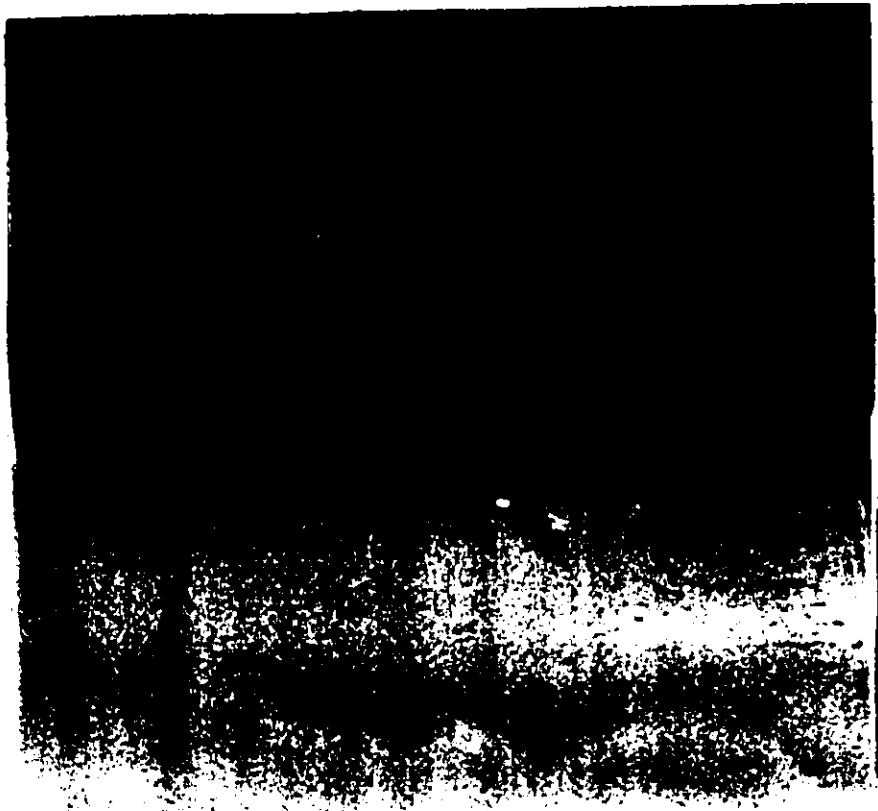


FIGURE 8

Plaskon column chromatography of ^3H -leucyl tRNAs isolated from 5 day old cotyledons, acylated with 5 day old cotyledon enzyme. 0.2 mg tRNA was acylated with 0.25 mg enzyme from the DE-23 column, keeping other conditions as given in Methods. RPC-5 chromatography was performed on a 2.5 x 30 cm column, using a linear gradient of 0.5-0.9 M NaCl in buffer B. Fractions of 10 ml were collected and assayed for radioactivity.

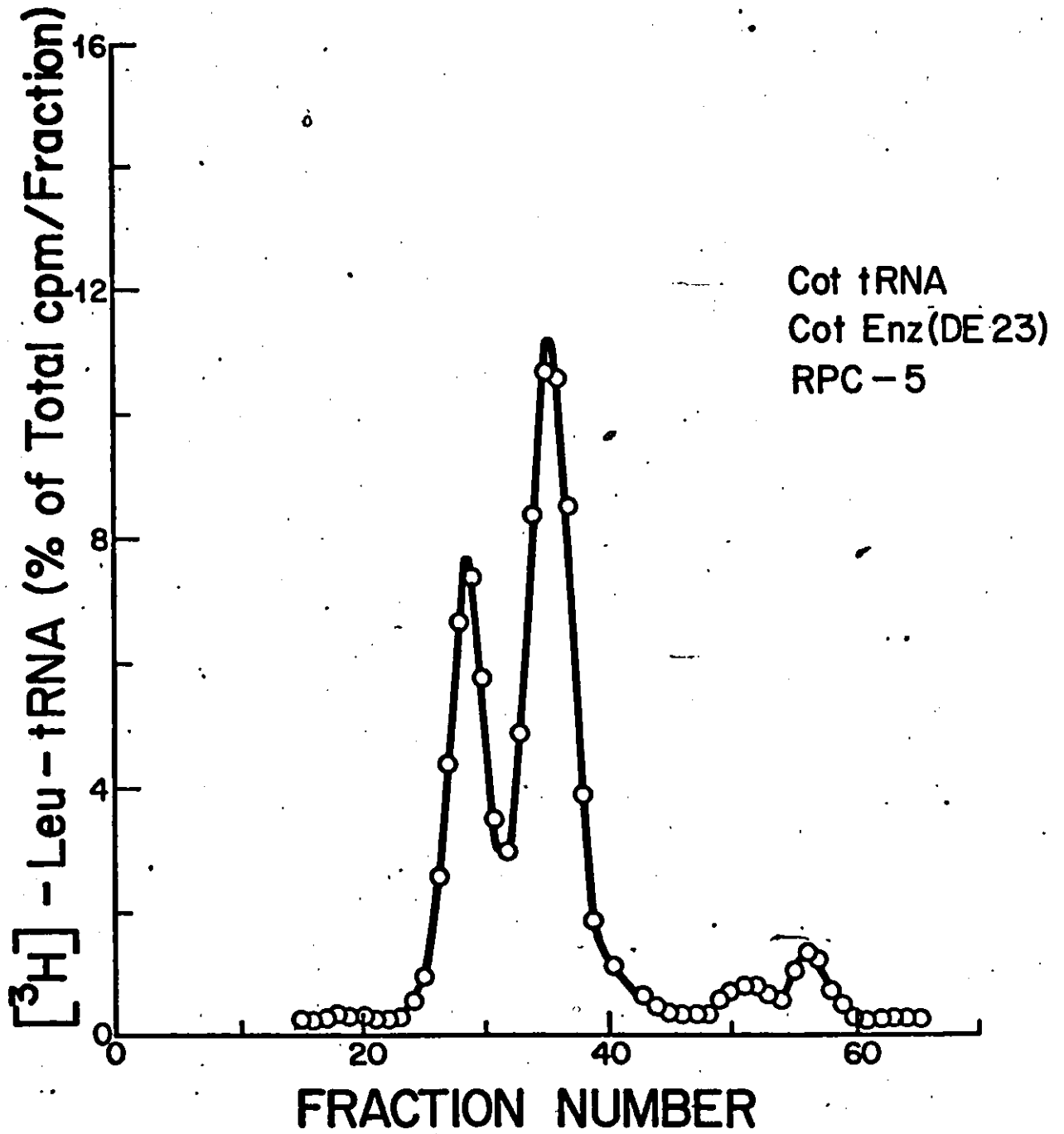
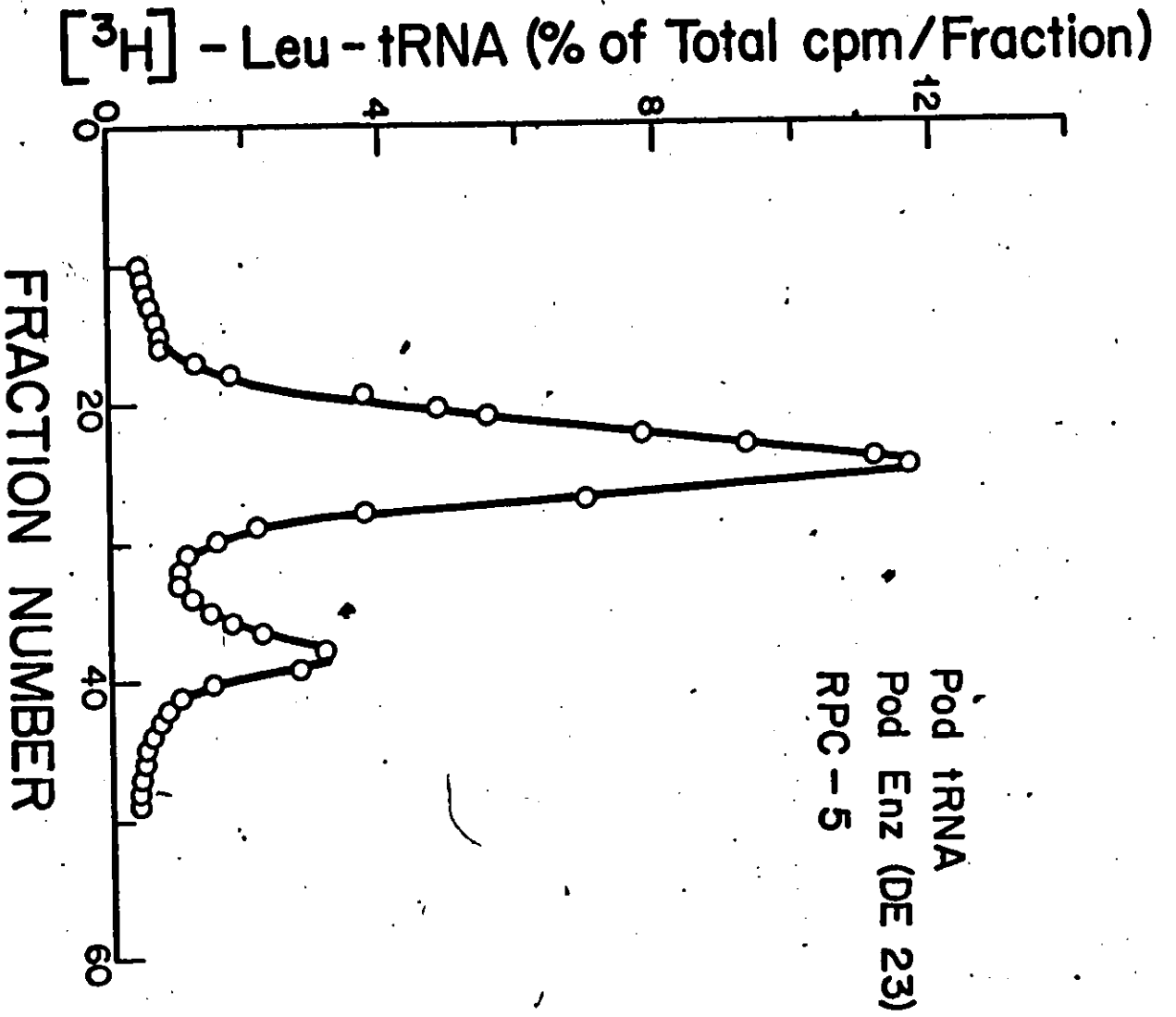


FIGURE 9

Plaskon column chromatography of ^3H -leucyl tRNAs isolated from seed pods, acylated with seed pods enzyme. 0.2 mg tRNA was acylated with 0.25 mg enzyme from DE-23 column, keeping other conditions as given in Methods. RPC-5 chromatography was performed on a 2.5 x 30 cm column, using a linear gradient of 0.5-0.9 M NaCl in buffer B. Fractions of 10 ml were collected and assayed for radioactivity.



Transfer RNAs in Pea Cotyledons and Seed Pods

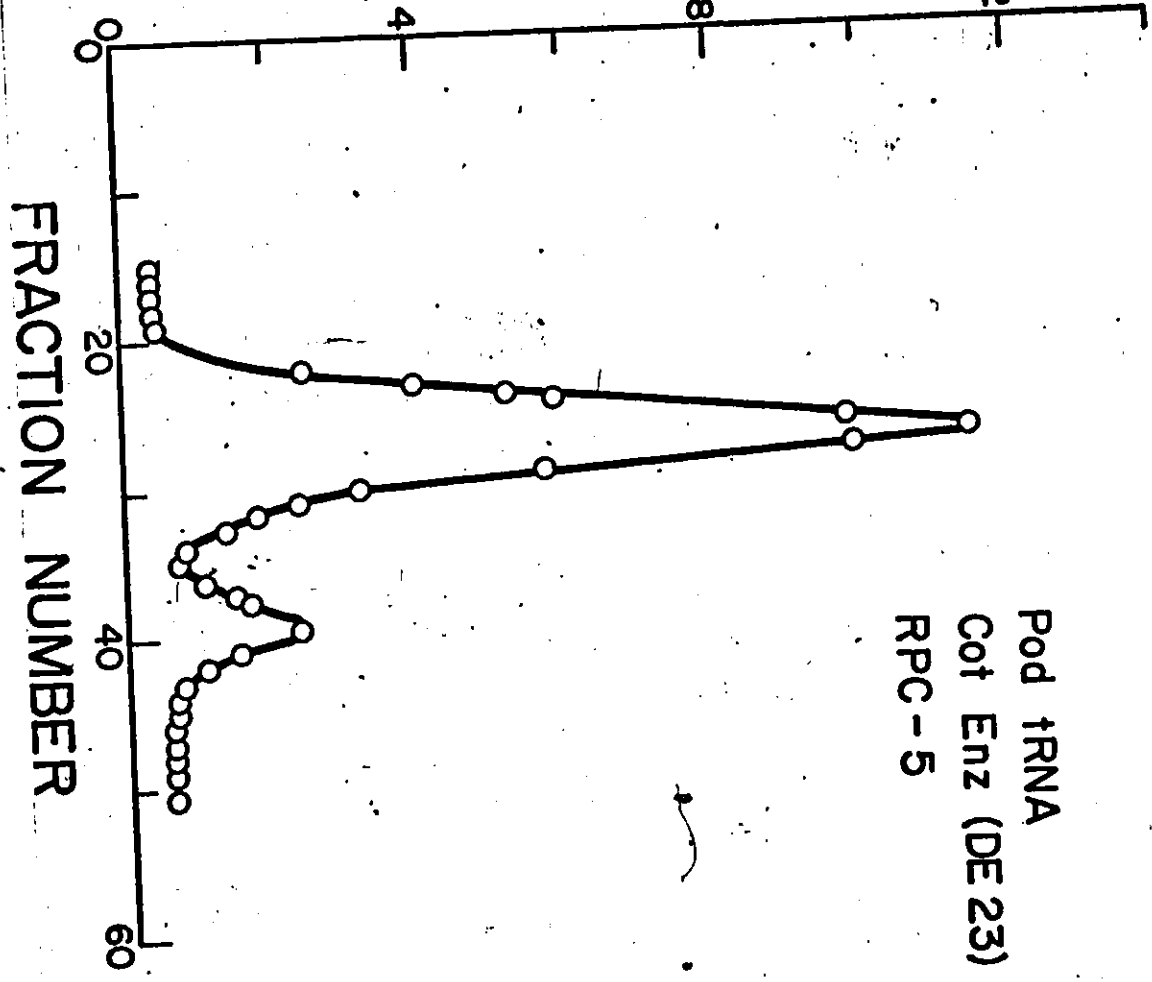
For routine enzyme assays or acylation of tRNAs for fractionation on RPC-5 (Plaskon) columns, a synthetase preparation, partially purified on DEAE cellulose columns was used. Leucyl-tRNA from cotyledons fractionate into four discrete peaks on RPC-5 (Plaskon) columns. Figure 8 shows the elution profile of leucyl-tRNA species in five day old cotyledons acylated with ^3H -leucine, using five day old cotyledon enzyme. The relative amounts of tRNA in peaks 1, 2, 3 and 4 are 32%, 57%, 5% and 6% respectively. Leucyl-tRNA from seed pods fractionates into two iso-accepting species on Plaskon columns (RPC-5). Figure 9 shows the elution profile of leucyl-tRNA species in seed pods acylated with ^3H -leucine, using seed pod enzyme. The relative amounts of leucyl-tRNA in peak 1 and peak 2 are 79.5% and 20.5% respectively. If one compares the relative amounts of tRNA₁^{leu} in cotyledons and pods, it is clear that in pods tRNA₁^{leu} is 47.5% more than in cotyledons.

The complete absence of tRNA_{3 and 4}^{leu} and the high amounts of tRNA₁^{leu} in seed pods indicate differences that could be based on one of the two rate-limiting factors; i. e. synthetases or tRNAs. Therefore, acylation of pod tRNA with cotyledon enzyme (heterologous system) and acylation of cotyledon tRNA with pod enzyme was performed. The charged tRNAs were then separated on RPC-5 columns. Figure 10 shows the elution profile of leucyl-tRNA species in seed pods charged with synthetase from five day old cotyledons. It is clear from this profile that only two

FIGURE 10

Plaskon column chromatography of ^3H -leucyl tRNAs isolated from seed pods, acylated with 5 day old cotyledon enzyme. 0.2 mg tRNA was acylated with 0.25 mg enzyme from the DE-23 column, keeping other conditions as given in Methods. RPC-5 chromatography was performed on a 2.5 x 30 cm column, using a linear gradient of 0.5-0.9 M NaCl in buffer B. Fractions of 10 ml were collected and assayed for radioactivity.

[³H] - Leu - tRNA (% of Total cpm/Fraction)



Pod tRNA
Cot Enz (DE 23)
RPC-5

FIGURE 11

Plaskon column chromatography of ^3H -leucyl tRNAs isolated from 5 day old cotyledons, acylated with seed pod enzyme. 0.2 mg tRNA was acylated with 0.25 mg enzyme from the DE-23 column. Other conditions were as given in Methods. RPC-5 chromatography was performed on a 2.5 x 30 cm column, using a linear gradient of 0.5-0.9 M NaCl in buffer B. Fractions of 10 ml were collected and assayed for radioactivity.

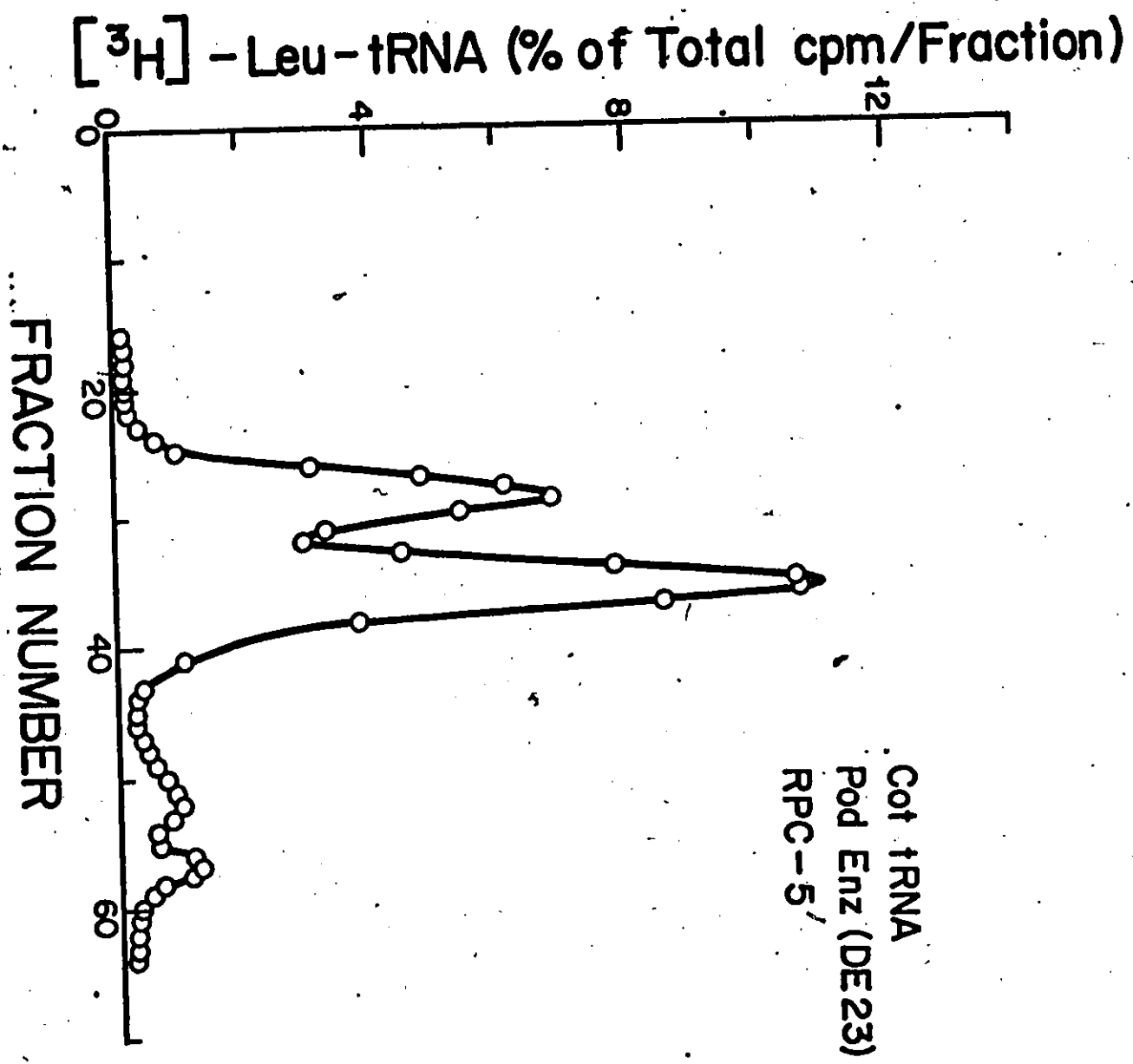


TABLE III: Variation of leucyl-tRNA isoaccepting species in germinating pea cotyledons and seed pods.

Source of Enzyme	Source of tRNA	Relative amount of leucyl-tRNA acylation of each peak (Percent total)			
		1	2	3	4
Cotyledons 5 days	Cotyledons 5 days	32.0	57.0	5.0	6.0
	Seed Pods	87.0	13.0	0.0	0.0
Seed Pods	Cotyledons 5 days	30.0	62.0	3.2	4.8
	Seed Pods	79.5	20.5	0.0	0.0

tRNA was acylated in a 2 ml reaction mixture with ^3H -leucine and enzyme from DEAE-cellulose column, and fractionated on a Plaskon column. The amount of radioactivity in peaks 1, 2, 3 and 4 was summed and expressed as percent of total counts.

species of tRNA^{leu} are obtained and the relative amounts of leu-tRNA₁ and 2 are 87% and 13% respectively. This shows that the pods are deficient in leu-tRNA₃ and leu-tRNA₄ indicating tRNAs as the limiting factor. Figure 11 shows the elution profile of leu-tRNA species in cotyledons charged with synthetase from seed pods. The seed pod enzyme charges all the four tRNA species of five day old cotyledons and the relative amounts of these four tRNA species are 30%, 62%, 3.2% and 4.8% respectively. This clearly shows that seed pod enzyme is capable of charging species 3 and 4 of tRNA from cotyledons.

Data summarized in Table III clearly shows the difference in proportions of the four tRNA isoacceptor species in cotyledons and seed pods. Seed pods contain 80% of tRNA₁ and 20% of tRNA₂ regardless of the source of enzyme, whereas cotyledons contain 32% of tRNA₁, 57% of tRNA₂, and 5% of tRNA₃, and 6% of tRNA₄. These results suggest that pea pods have only two species of tRNA and more important that there is a higher proportion of tRNA₁.

Transfer RNA Specificity of Individual Enzyme Fraction

Previous reports (44) have shown that RPC-2 (Freon) column chromatography of tRNAs amino acylated by individual leucyl-tRNA synthetase from HA column indicates that enzyme peak 1 was specific for tRNA^{leu}_{3and4} and peak 2

FIGURE 12

Plaskon column chromatography of cotyledon ^3H -leu-tRNAs produced by acylation with leucyl-tRNA synthetase peak 1 from pea cotyledons. Five day old cotyledon tRNA (0.2 mg) was aminoacylated using enzyme peak 1 obtained from hydroxylapatite column chromatography, and fractionated on a 2.5 by 30 cm plaskon column, using a linear gradient of 0.4-0.9 M NaCl in buffer B. Ten ml fractions were collected and assayed for radioactivity.

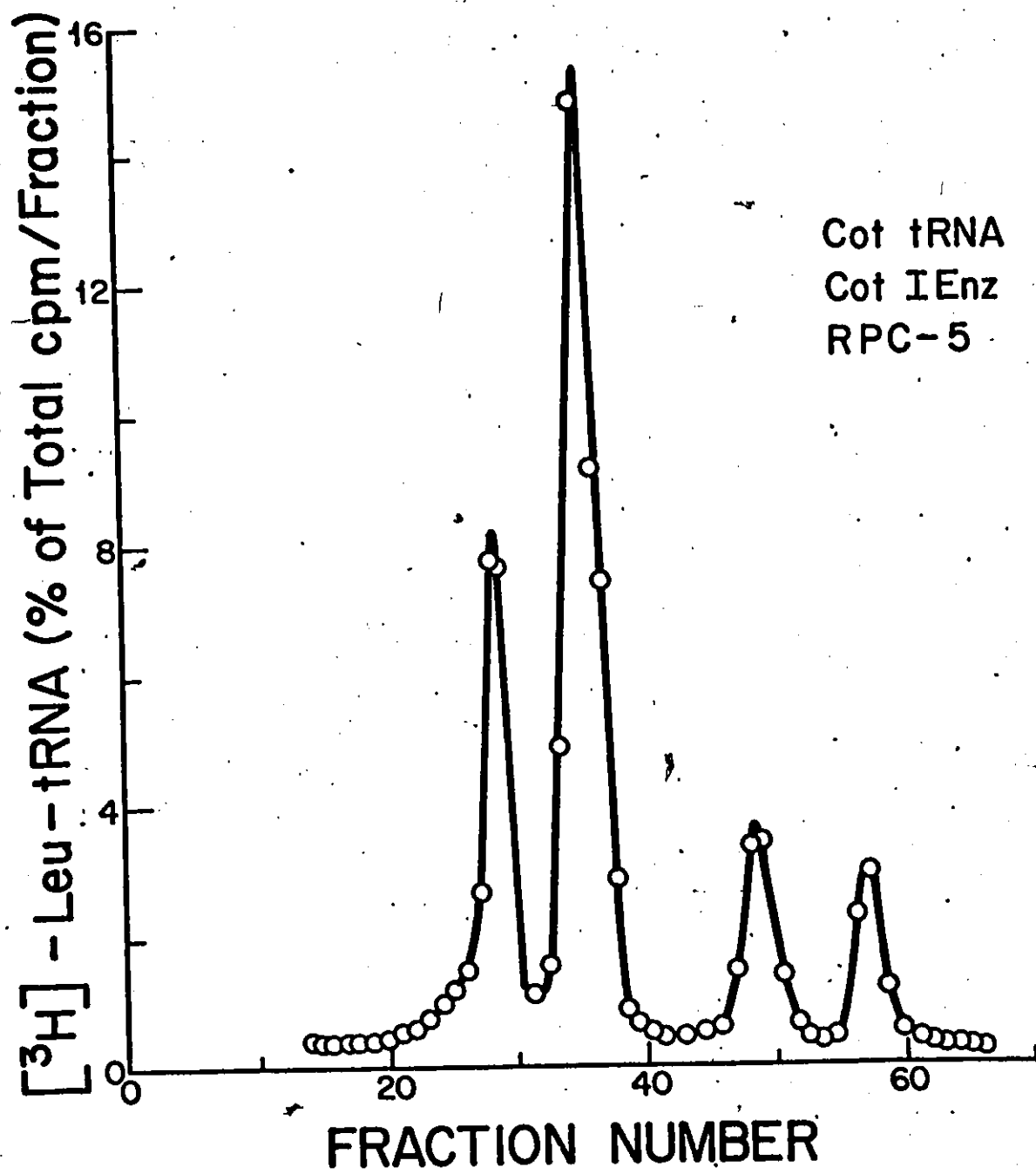


FIGURE 13

Plaskon column chromatography of cotyledon ^3H -leu-tRNAs produced by acylation with leucyl-tRNA synthetase peak 2 from pea cotyledons. Five day old cotyledon tRNA (0.2 mg) was aminoacylated using enzyme peak 2 obtained from hydroxylapatite column chromatography, and fractionated on a 2.5 x 30 cm plaskon column using a linear gradient of 0.4-0.9 M NaCl in buffer B. Ten ml fractions were collected and assayed for radioactivity.

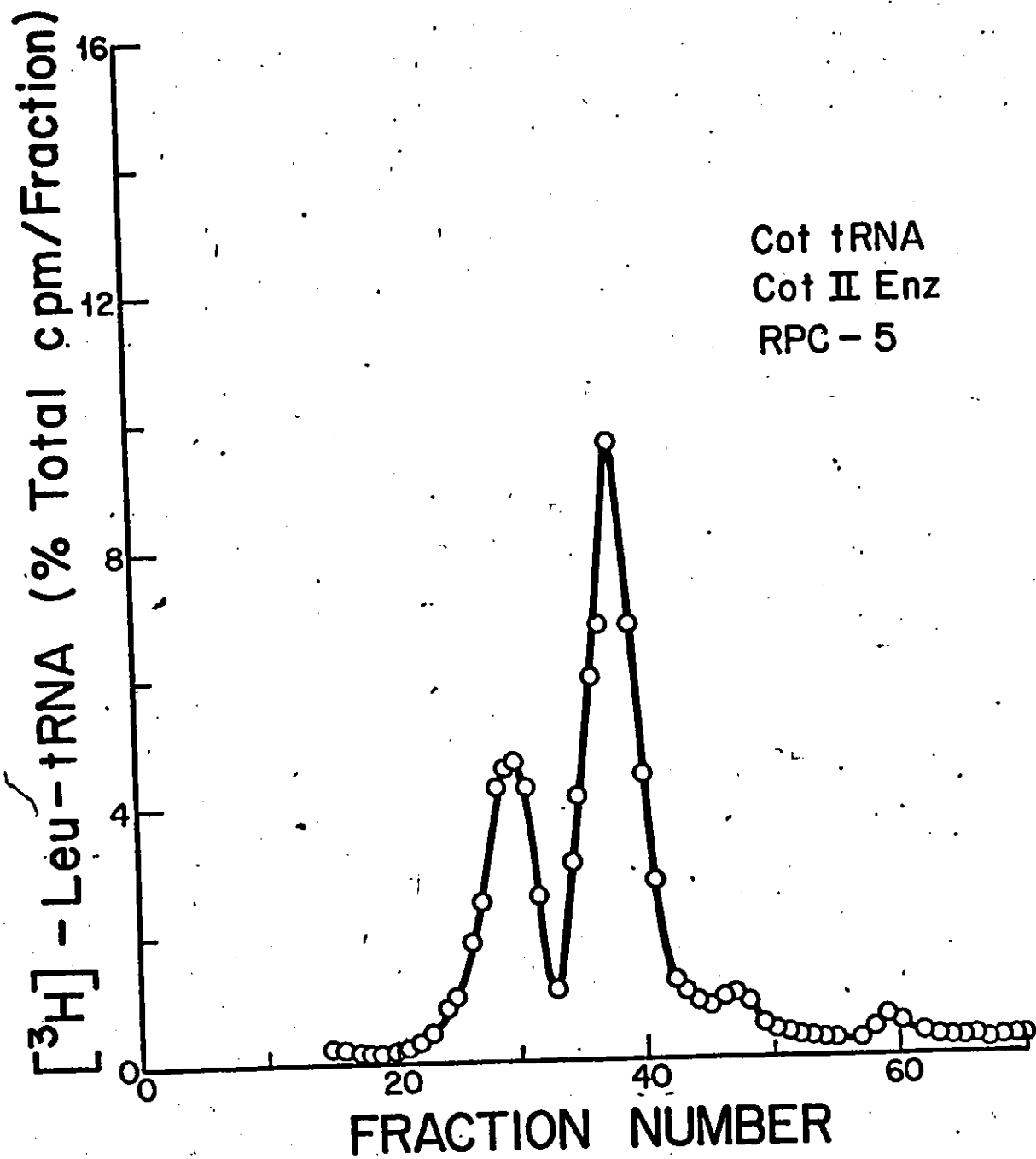


FIGURE 14

Plaskon column chromatography of seed pod ^3H -leu-tRNAs produced by acylation with leucyl-tRNA synthetase peak 1 from seed pods. Seed pod tRNA (0.2 mg) was aminoacylated using enzyme peak 1 obtained from hydroxylapatite column chromatography and fractionated on a 2.5 x 30 cm plaskon column, using a linear gradient of 0.4-0.9 M NaCl in buffer B. Ten ml fractions were collected and assayed for radioactivity.

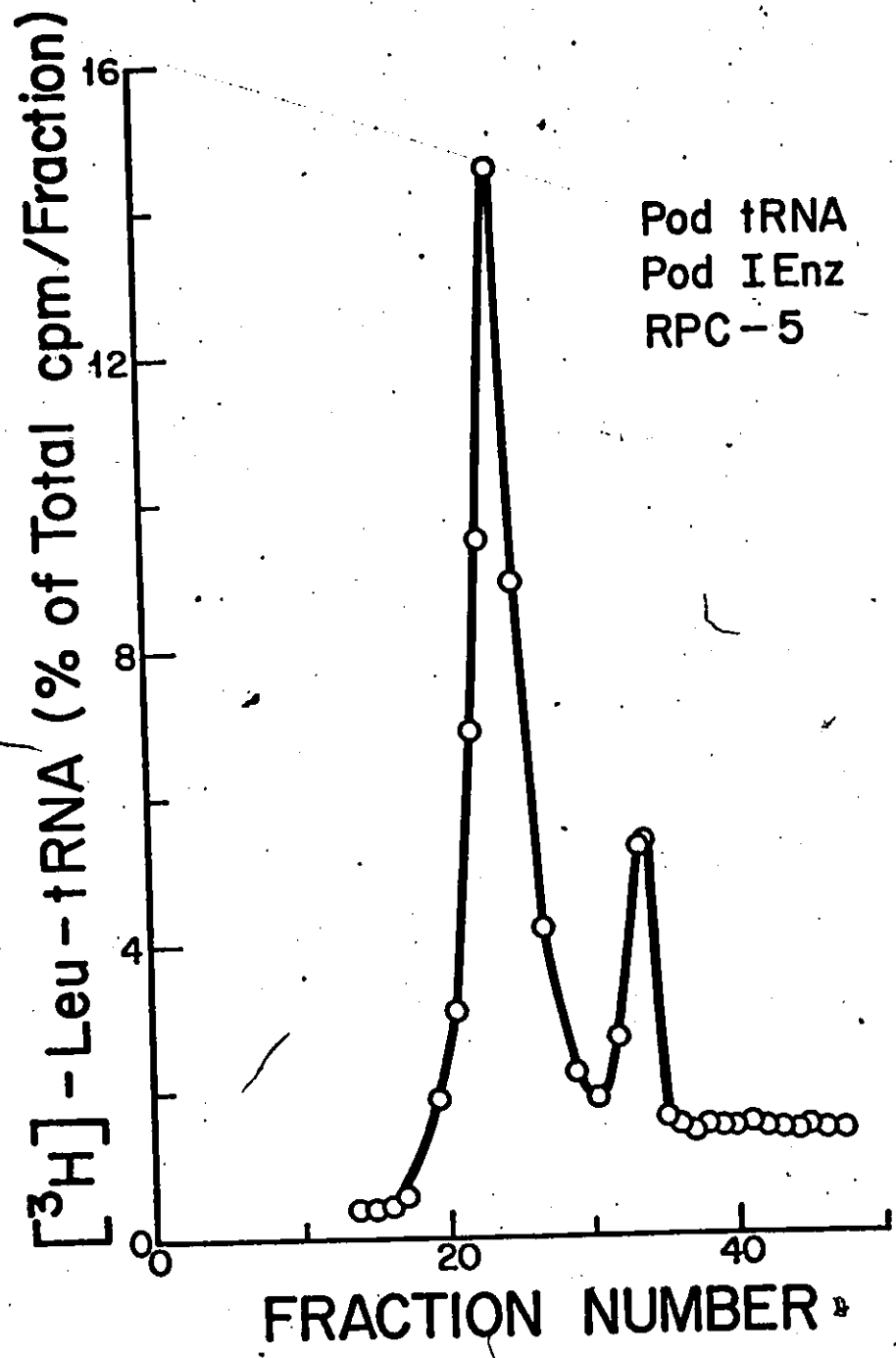
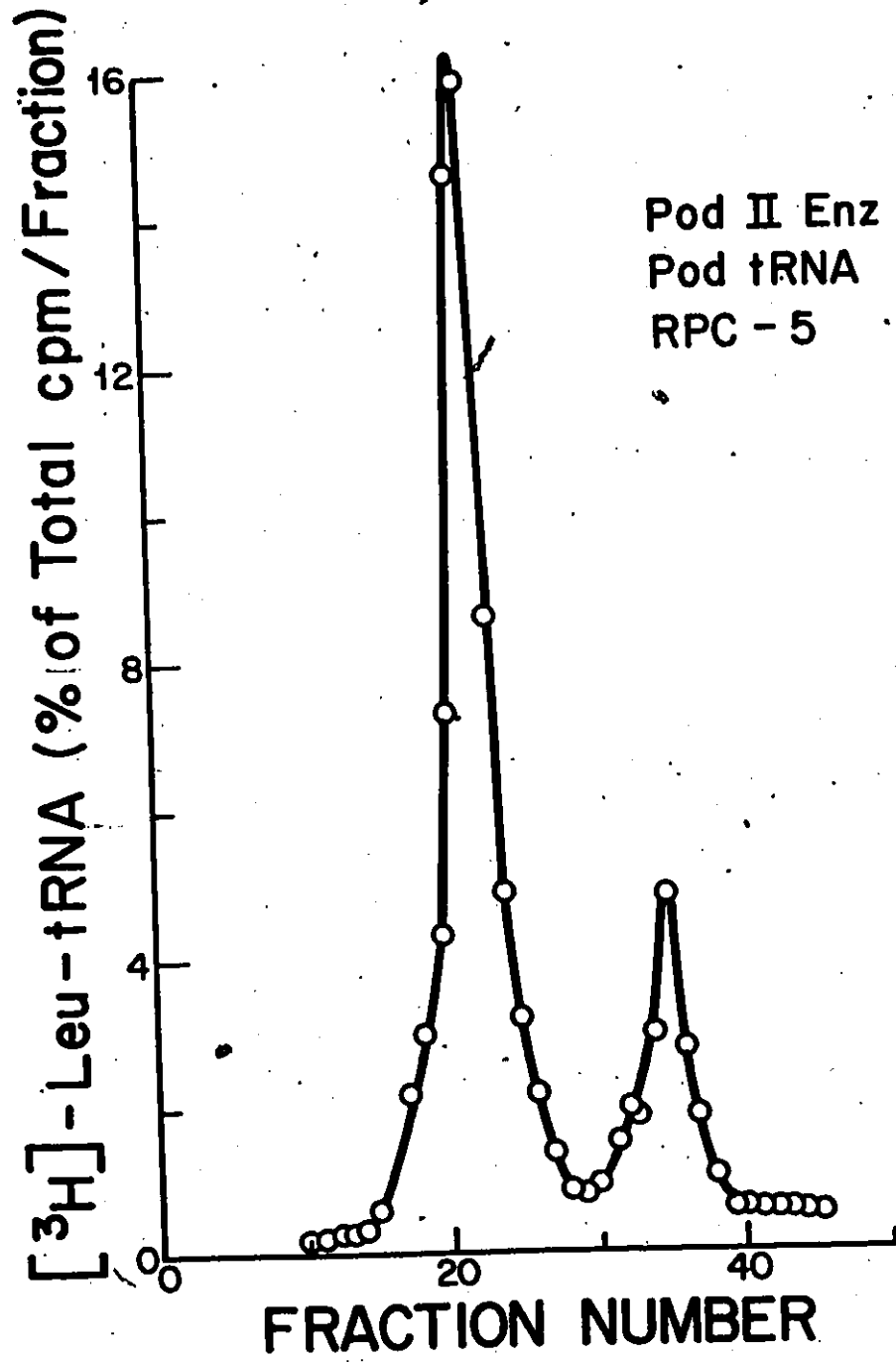


FIGURE 15

Plaskon column chromatography of seed pod ^3H -leu-tRNAs produced by acylation with leucyl-tRNA synthetase peak 2 from seed pods. Seed pod tRNA (0.2 mg) was aminoacylated using enzyme peak 2 obtained from hydroxylapatite column chromatography and fractionated on a 2.5 x 30 cm plaskon column, using a linear gradient of 0.4-0.9 M NaCl in buffer B. Ten ml fractions were collected and assayed for radioactivity.



was specific for tRNA^{leu}_{1and2}. Contrary to the above results enzyme peak 1 from cotyledons acylates all four isoaccepting species of tRNAs from cotyledons, but there is an increase in acylation of peak 3 and peak 4 tRNAs and a decrease in acylation of peaks 1 and 2. The % acylation of tRNAs 1, 2, 3 and 4 is 28%, 45%, 15% and 12% (Figure 12).

Figure 13 shows the elution profile of ³H-leu-tRNA species of cotyledons charged with synthetase peak 2 of cotyledons. Synthetase peak 2 acylates mainly tRNAs 1 and 2 with minor acylation of tRNAs 3 and 4. The % acylation of tRNAs 1, 2, 3 and 4 is 32.6%, 59%, 3.5% and 4.9% respectively.

The above results indicate that there is an increase in acylation of tRNA^{leu}_{3and4} when cotyledon tRNA is acylated with synthetase peak 1 from cotyledons as compared to the acylation of tRNA^{leu}_{3and4} using synthetase peak 2. When pod enzyme peaks 1 and 2 from HA column are used for acylation of pod tRNA and subsequent separation on RPC-5 columns, no preferential specificity for tRNAs is observed. (Figures 14 and 15). Both enzyme peaks are capable of acylating only tRNA^{leu}_{1and2}. On a quantitative basis 75-78% activity expressed as cpm is found in tRNA^{leu}₁ and 22-25% activity in tRNA^{leu}₂.

DISCUSSION

The purpose of this investigation was to study the differences, if any, in the multiple forms of synthetases and isoaccepting tRNAs in two stages of pea development; i. e. the pod stage and the germinating stage. A second objective was to partially purify and further characterize the synthetases for possible presence of subspecies. It is well documented that with the onset of seed germination, numerous biochemical changes occur. Some of these changes, like the increase in activity of specific enzymes, suggest that these changes are directly related to protein synthesis. It is also well established that transfer RNA serves as a functional link between the genetic information encoded in the messenger RNA and protein synthesis. Therefore, specific changes in tRNA population have obvious implication for the regulation of cellular events during differentiation and development. Many workers have suggested the possibility of regulation of protein synthesis at the translational level (34, 35, 37). Thus rate limiting quantities of tRNAs and/or synthetases can be the factors regulating the rate of messenger RNA out.

Results presented here show changes in amounts of isoaccepting leucyl-tRNAs and multiple forms of synthetases between seed pods, a stage in pea development before germination and the cotyledons, a stage after germination. Pea pods contain only two isoaccepting species of leucyl-tRNA, in contrast to pea cotyledons which contain four leucyl-tRNA species. In pods, the amount of $\text{tRNA}_1^{\text{leu}}$ is 79.5% and $\text{tRNA}_2^{\text{leu}}$ is 20.5%. Pea cotyledons, however, contain four isoaccepting species of leucyl-tRNAs. From this one can conclude that with the onset of germination two new tRNAs appear in the cotyledons. Further, if one considers the relative amounts of these four tRNAs, it is observed that $\text{tRNA}_1^{\text{leu}}$ decreases to 32%, while $\text{tRNA}_2^{\text{leu}}$ increases up to 57%; the two new species $\text{tRNA}_3^{\text{leu}}$ and $\text{tRNA}_4^{\text{leu}}$ amount to 5% and 6%.

RPC-5 chromatography of radioactivity labelled tyrosyl, aspartyl, asparaginyl, and histidyl tRNAs from developmental stages of wild type Drosophila melanogaster has revealed qualitative and quantitative changes in their major isoaccepting forms (42). These authors attribute these differences to post transcriptional modification of the same tRNA gene products (43). They propose a term "homogeneic" to describe tRNAs having the same sequence and presumably products of the same gene but which are chromatographically distinct because of different degrees

of post transcriptional modification. It is well established that there are tRNA modifying enzymes like tRNA methylases which modify tRNAs after the three dimensional structure of tRNA is already formed (16, 43). Therefore it is tempting to propose here, that with germination there is post transcriptional modification of tRNA₁^{leu} leading to observed changes (decrease or increase) in tRNA₁₋₄^{leu}, especially with the appearance of tRNA_{3and4}^{leu}. Further, all these isoaccepting tRNAs have the possibility of being "homogeneous". It should be pointed out here that in the absence of any conclusive evidence to the above suggestion, this hypothesis is speculative.

Partial purification procedures for leucyl-tRNA synthetase enzyme peak 2 from HA column resulted in 358 fold purification in pea cotyledons. It should be pointed out that at each step of purification the percent recovery is greater than 100 (Table I). Since crude plant extracts contain phenolic compounds which can inactivate various enzymes through binding and oxidation, the unusual high recovery at each purification step could be explained, if it could be accepted that inhibitor(s) are being removed from the extracts in these steps. Previous work in this laboratory shows that leucyl-tRNA synthetase from soybean cotyledons and pods can be fractionated into three peaks

of activity. Furthermore, differences in specificities of enzyme peak 1 from these two systems towards their cognate tRNAs were observed (33). Leucyl-tRNA synthetase from pea system on the other hand fractionates in two peaks of activity on HA column chromatography, which is in agreement with the results obtained by Wright et al (45). In germinating cotyledons, enzyme peak 2 of leucyl-tRNA synthetase is the dominant subspecies, whereas in the seed pods, enzyme peak 1 is the major subspecies. Similar results were obtained by Wright et al (44) for pea cotyledons. According to them enzyme peak 1 is chloroplastic in origin while enzyme peak 2 is cytoplasmic in origin. However their findings in the pea system and the work so far reported for the soybean system (23, 33) is contrary to the results obtained in this work, particularly in the area of the specificity of these two enzyme peaks and their acylating capacities. Enzyme peak 1 and 2 from pea cotyledons pods did not show any difference in their specificity toward leucyl-tRNA species. Both enzyme peak 1 and 2 from cotyledons acylate all four leucyl-tRNA species in a homologous system. Similarly pod tRNA which has only tRNA_{land2}^{leu} is equally acylated by enzyme peaks 1 and 2 in a homologous system. It is difficult at this stage to provide any additional proof to resolve this controversy,

except that it is possible to have had some cross contamination in enzyme peak 1 and 2 even after HA column purification. Only further work could provide additional clarification.

Aminoacylation of peak 1 and peak 2 enzyme fraction from cotyledons with twelve different amino acids reveals that there is less than 3% acylation of other amino acids with respect to leucine. Therefore enzyme peaks 1 and 2 are enzymatically pure fractions, free of the other eleven synthetases studied. However, this purification of leucyl-tRNA synthetase, from the foregoing observation does not preclude the possibility of cross contamination between enzyme peaks 1 and 2 from HA column.

In addition to the above, some preliminary attempts were made to characterize these enzymes by isoelectric focusing, polyacrylamide disc gel electrophoresis and sedimentation velocity analysis, by analytical ultracentrifuge. It should be emphasized that only enzyme peak 2, being the major enzyme in the cotyledons, was used in these studies. On the basis of isoelectric focusing, three subspecies with pI values 6.9, 5.3 and 4.4 were observed for enzyme peak 2. To date no other work in this area has been reported, in plant synthetases particularly in pea or soybean system. Therefore, no

definite conclusions could be drawn at this time.

Similarly, a preliminary observation on polyacrylamide disc gel electrophoresis of enzyme peak 2 indicates three protein bands. This observation in addition to the data obtained from isoelectric focusing suggests that the enzyme peak 2 from pea cotyledons is heterogenous, comprising of three isoenzymes. Again it is difficult to draw any comparisons or conclusions from the available literature.

Sedimentation velocity analysis of enzyme peak 2 resulted in a S_{abs} value of 6.63, which indicates that the subspecies obtained by isoelectric focusing or disc gel electrophoresis are approximately equal in their molecular weights. Caution is necessary here, because this result is from a single experiment only.

Further work involving additional purification and characterization should provide useful information.

SUMMARY

1. Leucine specific tRNAs of germinating pea cotyledons fractionate into four isoaccepting species on RPC-5 chromatography, whereas those of seed pods fractionate into two isoaccepting species.
2. The decrease of tRNA species 1, which is accompanied by the increase of species 2 and the appearance of species 3 and 4 during germination may be as a result of post-transcriptional modification of tRNA₁.
3. Leu-tRNA synthetase elutes in one peak of activity from DEAE cellulose chromatography.
4. Hydroxylapatite column chromatography of leucyl-tRNA synthetase from germinating cotyledons and pods results in two peaks of activity.
5. Pea cotyledons are found to contain predominantly enzyme peak 2 whereas pea pods are found to contain predominantly enzyme peak 1.
6. Enzyme peak 1 and peak 2 from cotyledons and pods do not show much difference in their specificity toward isoaccepting tRNAs.
7. Enzyme peak 1 and peak 2 from cotyledons are enzymatically pure with respect to eleven other aminoacyl synthetases.
8. Isoelectric focusing and polyacrylamide gel electrophoresis of enzyme peak 2 from cotyledons suggest that peak 2 synthetase is made up of three isoenzymes.

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