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LYSYL tRNAs and LYSYL-tRNA synthetases of Glycine max , L.

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LYSYL tRNAs and LYSYL-tRNA SYNTHETASES OF *GLYCINE MAX*, L.

by

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ABSTRACT

Multiple isoacceptor tRNAs have been observed in the cells of many organisms (plant and animal) at different stages of differentiation, in tumor tissues, in transformed tissues or in cells grown under different culture conditions.

Fractionation of total cell $tRNA^{Lys}$ by RPC-5 column chromatography revealed the presence of five lysine-specific tRNA isoacceptors in soybean cotyledons. One isoacceptor appears to be localized in the chloroplasts ($tRNA^{Lys}_1$), three ($tRNA^{Lys}_2$, $tRNA^{Lys}_3$, and $tRNA^{Lys}_4$) are common to the mitochondria and the cytoplasm and $tRNA^{Lys}_5$ is cytoplasm-specific.

Total lysyl-tRNA synthetase from soybean cotyledons can be separated into three peaks of activity by hydroxylapatite column chromatography. Fractionation of the organelle lysyl-tRNA synthetases by HA column and cross-aminoacylation reaction with different tRNAs shows that isoenzyme 1 is present in the chloroplasts, isoenzyme 2 in the cytoplasm and isoenzyme 3 in the mitochondria. The chloroplast enzyme (isoenzyme 1) aminoacylates chloroplast $tRNA^{Lys}_1$ and E. coli tRNA. The cytoplasmic enzyme (isoenzyme 2) aminoacylates four lysyl-tRNAs, isoacceptors 2, 3, 4, and 5. The mitochondrial enzyme (isoenzyme 3) aminoacylated three $tRNA^{Lys}$ isoacceptors $tRNA^{Lys}_2$, $tRNA^{Lys}_3$, and $tRNA^{Lys}_4$ in the mitochondrial tRNA. These isoacceptors are common to the cytoplasm as well.

Experiments performed on aging soybean cotyledons have revealed age-related quantitative and qualitative changes in $tRNAs^{Lys}$ and lysyl-tRNA synthetases. These changes involve a loss in aminoacylation capacity in tRNAs from older cotyledons by synthetases from the same tissue and ultimately the inability of older enzymes to aminoacylate efficiently all isoaccepting tRNAs.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A_{260} (A_{280})	absorption at 260 (280) nm
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CTP	cytidine 5'-triphosphate
DEAE-cellulose	diethylaminoethyl cellulose
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
HA column	column made from a mixture of hydroxylapatite and cellulose powder
Lys	L-Lysine
mRNA	messenger ribonucleic acid
OD units	optical density units
rRNA	ribosomal ribonucleic acid
RPC	reversed phase chromatography
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
tRNA	transfer RNA

INTRODUCTION:

In replication and transcription, genetic information is transferred from one nucleic acid molecule to another. Information transfer in these steps is basically a copying process. At the stage of translation a more complex form of information transfer is taking place. The message is encoded in a triplet code which has to be translated into the sequence of single amino acids, a decoding mechanism requiring specific adaptor molecules which recognize and interact with both the trinucleotide codons and the corresponding amino acids.

Involvement of an "adaptor" in the transfer of amino acids to the site of protein synthesis was first hypothesized by Crick (Crick, 1957). The messenger RNA synthesis (transcription) and amino acid polymerization (translation) represent two sequential levels of control of protein synthesis, so possible qualitative and quantitative changes in tRNAs and aminoacyl-tRNA synthetases during differentiation may be of great importance. The studies performed in animal systems have suggested that the availability of one or several of isoaccepting tRNA species may be a limiting factor in the translation if the minor tRNA is the only tRNA whose anticodon messenger RNA is able to read in a given tissue or at a given time, (Osterman, 1979).

REVIEW OF LITERATURE:

Multiplicity of tRNA:

Ever since the initial work of Holley and his associates (Holley et al., 1959) showing the existence of multiple isoaccepting species of a particular tRNA in rat liver, followed by the work of Doctor (Doctor et al., 1961) on the multiple species of leucyl- and threonyl-tRNAs in yeast, ample evidence has accumulated regarding the multiplicity of tRNAs, raising a question of possible degeneracy of tRNA complement in a cell. The number of species of tRNA for each amino acid may vary considerably between different organisms (Caskey et al., 1968). The recognition of the importance of two macromolecules: tRNA and aminoacyl-tRNA synthetase in protein synthesis and regulation, initiated a tremendous amount of work in the past years. Many studies have been devoted to tRNAs in bacteria (Berg et al., 1961), fungi, and animal cells. As a result of technical difficulties in extraction and purification, plant tRNAs and aminoacyl-tRNA synthetases have been, in the past, studied less. However, in recent years much progress has occurred in the field of protein synthesis in green plants (Lea and Norris, 1977; Weil, 1979). During the last years multiplicity of isoaccepting tRNA species has been demonstrated in plants (Sueoka and Kano-Sueoka, 1970; Merrick and Dure, 1972; Hiat and Snyder, 1973; Cornelis et al, 1975). Isoacceptors have been characterized for the plant tRNAs specific for almost all twenty amino acids.

The multiplicity of tRNAs and aminoacyl-tRNA synthetases in green plants has been attributed to the fact that protein synthesis in a plant cell takes place in three cell compartments: in the cytoplasm, in the

chloroplast, and in the mitochondria. Each one of these cell compartments has a complete functional apparatus for protein synthesis. The smaller size of ribosomes (Lyttleton, 1962), the formylation of organelle initiator methionyl-tRNA (Bianchetti et al., 1971; Burkard et al., 1969) and the sensitivity of organelle protein synthesis to chloramphenicol (Boulter et al., 1972) have suggested that in many respects protein synthesis in organelles resemble protein synthesis in prokaryotes, especially bacteria (Fairfield and Barnett, 1971).

Aminoacyl-tRNA synthetases:

The aminoacyl-tRNA synthetases, first described by Hoagland and his co-workers (Hoagland, 1955; Hoagland et al., 1956) are a multisubstrate class of enzymes which catalyze the first step in protein biosynthesis. These enzymes attach amino acids to the 3' end of cognate tRNA, catalyzing the formation of aminoacyl-tRNA, thus they may be directly or indirectly involved in the regulation of the expression of certain genes. The great diversity of these enzymes in their subunit structure and size is puzzling since they all have a common function: the aminoacylation of tRNA (Schimmel and Soll, 1979). Growing evidence for the occurrence of multiple isoaccepting tRNAs prompted a search for multiple aminoacyl-tRNA synthetases. In prokaryotic cells there is usually only one synthetase for each amino acid (Bennet, 1969; Novelli, 1967). In higher plants, in addition to the enzymes in the cytoplasm, chloroplast and mitochondria contain their own synthetases different in their chromatographic mobility and tRNA specificity from their cytoplasmic counterparts. Multiplicity of these enzymes in eukaryotic cells has been

well established (Weil et al., 1977; Weil and Parthier, 1982).

In contrast to prokaryotic enzymes, where aminoacyl-tRNA synthetases occur as free diffusible enzymes, unbound to other material, eukaryotic enzymes are usually occurring in high molecular complexes (Soll and Schimmel, 1974). These complexes may contain proteins other than synthetases (Agris et al., 1976), lipids (Saxholm and Pitot, 1979), tRNA (Bandyopadhyay and Deutcher, 1971, 1973), rRNA and elongation factors (Smulson et al., 1975). The presence of these complexes has been well established in mammals and recently they have also been found in plants (Quintard et al., 1978). The physiological significance of these complexes is not completely understood. They may be important in stability or activity of the enzymes as shown, for example, in experiments performed on lupin seedlings (Jakubowski, 1979).

The studies on the origin of Euglena chloroplast-specific aminoacyl-tRNA synthetases suggests that they are transcriptional products of nuclear genes, synthesized on cytoplasmic ribosomes and then transported into chloroplasts (Reger et al., 1970; Parthier, 1973; Hecker et al., 1974). Distinct nuclear genes have been recently shown to exist for yeast cytoplasmic and mitochondrial Met-tRNA synthetase (Schneller et al., 1978), so it can be concluded with almost a certainty that all aminoacyl-tRNA synthetases are transcribed from the nuclear genome.

Organelles:

Chloroplasts:

Chloroplast DNA was first detected in the early 1950's (Chiba,

1951) but the discovery was not confirmed until 1962. In 1962 Ris and Plaut showed the existence of DNA in chloroplasts of Chlamydomonas (Ris and Plaut, 1962). Almost simultaneously Chun and his associates (Chun et al., 1963) found that DNA in chloroplasts of beet and spinach differs in its buoyant density from the one found in cytoplasm. The first knowledge of the possible existence of chloroplast-specific tRNAs different from their cytoplasmic counterparts was put forward by Aliev and Filipovich (Aliev and Filipovich, 1968). After a report showing the presence of formyl-methionyl-tRNA in a cell-free system on Euglena chloroplast ribosomes (Schwartz et al., 1967) N-formyl-methionine tRNA was characterized in the chloroplast of Phaseolus vulgaris (Burkard et al., 1969) and then in mitochondria and etioplasts of the same plant (Guillemaut et al., 1972). The absence of N-formyl-methionyl-tRNA in cytoplasm of the same plant suggested the possible prokaryotic nature of chloroplast protein-synthesizing machinery.

Several authors have pointed out striking differences in protein synthesis between chloroplast and cytoplasm. These differences are revealed in the differential influence several factors have on amino acid activation and on aminoacyl-tRNA formation (Burkard et al., 1970) in cytoplasm and chloroplast. Differences in the heat stability between cytoplasmic and chloroplastic aminoacyl-tRNA synthetases (Parthier and Krauspe, 1973, 1974); differences in their chromatographic mobilities and substrate specificities confirms this assumption. The presence of chloroplast-specific tRNAs was further substantiated by the discovery of two sets of tRNAs in Euglena: light-inducible and constitutive (Barnett et al., 1969).

The existence of different isoacceptor tRNAs raised a question of possible origin of chloroplast-specific tRNAs. The elucidation of this problem was made possible by DNA-tRNA hybridization experiments. Tewari and Wildman (Tewari and Wildman, 1970) first demonstrated that chloroplast tRNAs from tobacco hybridize with chloroplast DNA. Schwartzbach et al. (1976) concluded from DNA-tRNA hybridization experiments that Euglena chloroplast tRNAs are transcriptional products of chloroplast genome. In addition to work performed on Euglena (Schwartzbach et al., 1976), tea (Tewari and Wildman, 1970), maize (Haff and Bogorad, 1976), bean (Steinmetz and Weil, 1976) and other plants have suggested that a complete set of tRNAs can be transcribed from chloroplast DNA .

Mitochondria:

After the discovery of mitochondrial DNA in 1965 (Kislev et al., 1965) experimental evidence of the possible existence of tRNAs and aminoacyl-tRNA synthetases unique to mitochondria and different from those found in cytoplasm was provided by Barnett and Brown (Barnett and Brown, 1967) in Neurospora. Since this initial discovery mitochondrial tRNAs have been studied extensively in a large number of organisms, especially in yeast and animal cells, reviewed by Barnett (Barnett et al., 1978). Mitochondria contain a partially autonomous protein-synthesizing system, the mitochondrial genome coding for only a limited number of gene products (Borst and Grivell, 1978). Presently, little is known about mitochondrial tRNAs from higher plants except that plant mitochondria contain specific tRNAs and in some instances specific aminoacyl-tRNA synthetases (Guille-

maut et al., 1973; Shridhar and Pillay, 1976; Sinclair and Pillay, 1981).

Fewer studies have been devoted to the comparison of tRNAs and aminoacyl-tRNA synthetases in the three cell compartments. Such studies have been performed on Euglena (Kislev et al., 1972; Parthier et al., 1972), tobacco (Guderian et al., 1972), bean (Guillemaut et al., 1975; Guillemaut and Weil, 1976; Jeannin et al., 1976, 1978), lupin seeds (Augustyniak and Pawelkiewicz, 1978) and soybean (Shridhar and Pillay, 1976; Sinclair and Pillay, 1981; Swamy and Pillay, 1982). With regard to their tRNA specificity mitochondrial aminoacyl-tRNA synthetases show vast differences. Cross-aminoacylation experiments have suggested that in the case of bean $tRNA^{Met}$ (Guillemaut et al., 1975), $tRNA^{Lys}$, $tRNA^{Pro}$ (Jeannin et al., 1976) and $tRNA^{Phe}$ (Jeannin et al., 1978) mitochondrial tRNAs can be aminoacylated by mitochondrial, chloroplastic and E. coli enzymes but not by cytoplasmic, suggesting their prokaryotic nature. Experiments performed in this laboratory on soybean $tRNA^{Phe}$, $tRNA^{Trp}$, $tRNA^{Tyr}$ (Swamy and Pillay, 1982) produced similar results. In contrast to these observations mitochondrial $tRNA^{Leu}$ from the same tissue, soybean cotyledons, are readily aminoacylated by mitochondrial and cytoplasmic enzymes but not by chloroplastic or E. coli enzymes (Sinclair and Pillay, 1981). For the possible origin of mitochondrial tRNAs the same question can be raised: are mitochondrial tRNAs coded for by mitochondrial genes?

The existence of cistrons for some mitochondrial tRNAs on mitochondrial DNA has been established without doubt (Casey et al., 1974; Nass and Buck, 1970). The cistron number estimated by hybridization studies performed in different organisms is usually smaller than predicted by the "wobble" hypothesis (Crick, 1960). Several suggestions have been put

forward to account for the deficit. Chiu (Chiu et al., 1975) proposed the existence of two sets of tRNAs in Tetrahymena pyriformis, one transcribed on mitochondrial genome, the other transcribed on nuclear genome and transported into mitochondria. The import of one of lysyl-tRNA into mitochondria was reported in Saccharomyces cerevisiae (Martin et al., 1979). While several yeast mitochondrial tRNAs have been sequenced (Gauss and Sprinzl, 1981; Wesolonski and Fukuhara, 1979) no plant mitochondrial tRNA has been sequenced so far. Mapping of mitochondrial plant tRNA genes appears to be difficult as a result of heterogeneity of mitochondrial DNA (Bonen and Gray, 1980). Limitation of current techniques may be one of the possible reasons for the lower number of tRNA genes found on mitochondiral genome. Using cloned yeast mitochondrial $tRNA^{Ser}$ gene as a probe Martin and Underbrink-Lyon (1981) have shown that in Saccharomyces cerevisiae a mitochondrial locus is absolutely necessary for the synthesis of mitochondrial seryl-tRNA. The advancement of current techniques and new developments may soon help to solve this problem. It is possible that mitochondria contain most tRNA-specific genes.

All information gathered so far on translation and the coding properties of tRNAs have shown that in the plant kingdom the genetic code shows no deviation from the "universal". Two isoacceptors specific for $tRNA^{Lys}$ in black peas recognize AAA and AAG codons (Hague and Kofoid, 1971). In many instances chloroplast tRNAs recognize certain codon words, unrecognizable by cytoplasmic tRNAs (Augustyniak and Pawelkiewicz, 1978; Ramiasa et al., 1977). From these findings one may conclude that chloroplast tRNAs are coded for by the same or similar genes on chloroplast DNA.

Similar results with mitochondrial tRNAs in Tetrahymena pyriformis (Chiu et al., 1974; 1975) may indicate that organelle-specific tRNAs translate codons present in organellar mRNA which would not be recognized by cytoplasmic tRNA.

Aging:

The possibility that cell differentiation, cell specialization, or neoplasia are regulated at a translational level has been well substantiated. Cellular differentiation is thought to be associated with a loss in capacity for growth and cell division (Anderson and Cherry, 1969). Transfer RNAs and aminoacyl-tRNA aynthetases are key elements of regulation at a translational level. The levels of these two macromolecules in a cell determine which proteins, unique to a cell physiological state, can be made substantiating the proposition that translational control is a crucial part of control systems operating during development.

Quantitative variations in specific tRNA isoacceptors can markedly affect the rate of protein synthesis. Anderson (Anderson, 1969) concluded that the rate of protein synthesis could be regulated by the concentration of arginyl-tRNA in the reaction mixture. Extensive differences have been observed in tRNA and/or aminoacyl-tRNA synthetases between different plant tissues and in the senescence of plant organs. An organ-specific deficiency has been observed in $tRNA^{Leu}$ (Anderson and Cherry, 1969) and in leucyl-tRNA synthetase (Kanabus and Cherry, 1971) in soybean. A similar deficiency has been observed in germinating pea cotyledons (Patel and Pillay, 1976). Significant tRNA differences have been found between dividing and non-dividing cells in the root of pea seedlings (Vanderhoef

and Key, 1970). Marked differences occur in Tenebrio molitor (Ilan et al., 1970) and sea urchins (Taylor et al., 1971). Several examples exist which show similar changes in Amphibia (W. de Witt, 1971; Caston, 1971), in birds (Lee and Ingram, 1967; Portugal, 1972) and mammals (Wevers et al., 1966).

Senescence is the final phase of growth and development. Strehler (Strehler, 1967) suggests that senescence and cell death are the results of a loss of certain translational capacities, especially changes that occur in particular tRNA species and/or aminoacyl-tRNA synthetases. Moreover, it seems likely that different types and amounts of nucleic acids are produced in senescence. In the ultimate stage of development, in cell death, different cell types utilize only restricted code words, resulting in deleterious effects on a long-term cell function (Strehler et al., 1967).

Soybean cotyledons, being a highly differentiated tissue are a good model to study the mechanism of aging. During the germination, cotyledons support the growing embryonic axis with the hydrolytic products of stored material but do not themselves undergo further differentiation. Several laboratories have used soybean cotyledons to show pronounced changes in tRNAs and/or aminoacyl-tRNA synthetases with the seedling age. Striking changes in the relative amounts of $tRNA^{Leu}$ and $tRNA^{Tyr}$ were observed during the senescence of soybean cotyledons (Bick et al., 1970). Parallel changes have been observed in leucyl-tRNA synthetase of senescing soybean cotyledons (Bick and Strehler, 1971). As a consequence of senescence the relative amounts of chloroplast leucyl-tRNAs were decreased in yellowing pea leaves (Wright et al., 1972/73).

Previous work in our laboratory has shown that changes in tRNA population and in aminoacyl-tRNA synthetases seem to correlate with changes in growth, development and senescence (Pillay and Cherry, 1974). In aging soybean cotyledons there is a decrease in amino acid acceptor activity for a number of amino acids tested (Pillay and Gowda, 1981). At the same time few tRNAs have shown an increase in amino acid acceptor activity. The significance of such differences in amino acid acceptor activity is unknown. Fractionation of aminoacyl-tRNAs from young and old cotyledons revealed qualitative and quantitative changes in chromatographic profiles of these tRNAs (Pillay and Gowda, 1981). These changes (Pillay and Gowda, 1981) involve: displacement of chromatographic peaks, disappearance of some isoaccepting species, and formation of new isoaccepting species.

The importance of amino acid lysine in plant nutrition and the lack of knowledge concerning localization and property of $tRNA^{Lys}$ and lysyl-tRNA synthetase prompted us to undertake this study. Localization and substrate specificity of lysyl-tRNAs and lysyl-tRNA synthetases have been investigated in only two other higher plants, in Phaseolus vulgaris (Jeannin et al., 1976) and in Lupinus luteus (Augustyniak and Pawelkiewicz, 1978).

MATERIALS AND METHODS:

Plant material:

Soybean seeds (Glycine max L. var. Harcor) were soaked in water for four hours and sown in moist vermiculite in shallow pans. Cotyledons were harvested 5, 10, 15, and 20 days following germination in the dark at 25-27°C. Hypocotyls were harvested after 5 days of germination in the dark. For chloroplast isolation soybean plants were grown in growth chambers at a temperature of 27°C with 12-14 hours of light periods and harvested 10-12 days following germination.

Chemicals:

All chemicals used were reagent grade. Acrylamide and N,N-methylenebisacrylamide were purchased from BDH. Xylene cyanole FF and N,N,N',N'-tetramethylendiamine (TEMED) were purchased from Eastman Co. Methylene blue, ATP, bovine serum albumin, β-mercaptoethanol and 2,5 Diphenyloxazole (PPO) were purchased from Sigma. Urea and hydroxylapatite (Biogel-HTP) were purchased from Bio-rad. E. coli tRNA, yeast tRNA and DNase were purchased from Boehringer-Manheim. Polyvinylpyrrolidone-insoluble form (trade name polyclar AT) was purchased from GAF corporation. Chromatographic adsorbent for RPC-5 column was purchased from Miles laboratories. DEAE-cellulose (DE-23 and DE-52), 3 MM filter paper and GF/A fiber filters were purchased from Whatman. 1,4 bis [2- (phenyloxazolyl) benzene] (POPOP), L (4,5- ^{3}H) lysine monochloride (1 Ci/mmol), L (U-^{14}C) lysine monohydrochloride (150 mCi/mmol) and α [^{32}P] ATP (1 mCi/ml) were purchased from Amersham.

Preparation of transfer RNA:

Transfer RNA was prepared from freshly harvested dark grown cotyledons or those stored for several days at -20°C according to the method described by Burkard et al. (1970) with minor modifications. The cotyledons were ground for 2-3 minutes in a Waring blendor in batches of 100 g with 150 mls of extraction buffer (10 mM Tris-HCl pH 7.6; 60 mM KCl; 10 mM $MgCl_2$ and 14 mM B-mercaptoethanol) and an equal volume of buffer saturated phenol, in the cold. The homogenate to which 4-5 g of solid duponol (SDS) was added, was stirred in the cold for two hours and centrifuged for 15 minutes at 10,000 x g at 4°C. The supernatant was collected and mixed with half volume of aqueous buffer saturated phenol (Buffer A) and stirred again for half an hour in the cold. Buffer A was prepared by shaking extraction buffer with phenol at the ratio of 10:8 (v/v) for several hours and the aqueous buffer phase separated from the phenol. The aqueous phase, recovered by centrifugation, was made 2.0 M with respect to potassium acetate and two volumes of cold 95% ethanol were added and stored overnight at -20°C. The precipitated material was collected the following day by centriguation (10,000 x g, 10 minutes, 4°C), extracted several times with 2.0 M potassium acetate, pH 6.5 (2 ml/100 g of tissue). All supernatants, collected by centrifugation were pooled, precipitated again with cold 95% ethanol and stored in the freezer overnight. The crude tRNA precipitate was dissolved in 1.0 M NaCl in buffer B (Buffer B contains: 10 mM sodium acetate buffer at pH 4.5 and 10 mM $MgCl_2$). After centrifugation the clear supernatant was made 0.3 M in respect to NaCl and applied to a small DEAE-cellulose column (2 ml bed volume per 100 g of plant material), previously equilibrated with several volumes of 0.3 M

NaCl in buffer B. The sample was washed with the same buffer until A_{260} dropped below 0.02. The soluble tRNA was eluted with 1.0 M NaCl in buffer B, deaminoacylated by incubation in 1.8 M Tris-HCl pH 7.8 at 37°C for 90 minutes, then precipitated with ethanol and stored in the freezer at -20°C for at least 12 hours. Pure tRNA, obtained after centrifugation was dialyzed against cold distilled water, the concentration of tRNA determined based on the A_{260} absorbancy and stored in small aliquots for the immediate use or lyophilized for the later use.

Preparation of lysyl-tRNA synthetase:

Freshly harvested cotyledons were ground with the addition of insoluble polyvinylpyrrolidone (Polyclar AT, 200 mg/g of tissue) using a chilled pestle and mortar. The grinding was continued for 10 minutes with stepwise addition of the grinding medium, which consisted of 25 mM potassium phosphate at pH 7.8 in buffer C (Buffer C: 10^{-2} M β-mercaptoethanol; 10^{-5} M phenylmethyl sulfonylfluoride (PMSF); 10^{-6} M L-lysine saturated to 30% with ammonium sulfate. The homogenate was strained through four layers of cheese cloth and centrifuged for fifteen minutes at 27,000 x g. The supernatant, after filtration through miracloth, was made 60% with the gradual addition of ammonium sulfate and stirred in the cold for 15 minutes. The enzyme pellet collected after centrifugation at 10,000 x g at 4°C for 15 minutes was either used immediately or stored in the freezer for the later use. The pellet was dissolved in 25 mM potassium phosphate in buffer C and dialyzed against the same buffer for four hours. The protein dialyzate was then adsorbed on a 15 ml DEAE-23 column. The column was equilibrated and the sample washed with 25 mM potassium phosphate in buffer C. Enzyme

elution was performed using 100 mM potassium phosphate pH 7.8 in buffer C, 5 ml fractions were collected and those fractions showing maximum protein content, measured as absorbance at 280 and 260 nm on a Beckman DBG spectrophotometer were pooled and used either as an enzyme source or with the addition of 50% glycerol for further enzyme purification. Using the A_{280} and A_{260} ratio in the table (Cherry, 1973), protein concentration was estimated.

Isolation of lysyl-tRNA synthetase from chloroplasts and mitochondria:

The method for isolation of lysyl-tRNA synthetase from chloroplasts and mitochondria is similar to that employed for extraction and purification of the enzyme from dark grown cotyledons with minor modifications. The organellar pellets were homogenized in extraction medium (25 mM phosphate buffer pH 7.8; 10^{-2} M β-mercaptoethanol; 10^{-5} M phenylmethyl sulfonylfluoride (PMSF); triton-x 100 and 20% glycerol) with slow addition of insoluble polyvinyl pyrrolidone (10% by weight of the plant material). After grinding, the extract was filtered through a cheese cloth and the filtrate was subjected to ammonium sulfate precipitation to a final concentration of 60% in respect to ammonium sulfate and stirred for 30 minutes in the cold. The enzyme pellet, collected after centrifugation was dissolved in extraction buffer, without triton-x 100 and then dialyzed against the same buffer in the cold. The dialyzed enzyme was subjected to DEAE-cellulose chromatography and fractions containing maximum enzyme activity were pooled and used either directly for charging tRNAs or loaded onto a hydroxylapatite column for further purification.

Preparation of chromatographic adsorbents:

DEAE-23 cellulose was swollen in deionized water and the fines removed by pouring off supernatant liquid. Precycling of chromatographic adsorbent was then completed according to the literature supplied by Whatman.

DEAE-52 (preswollen) was stirred in several volumes of appropriate buffer, the fines poured off and then thoroughly equilibrated before use.

Hydroxylapatite column was prepared by mixing hydroxylapatite and Whatman CF 11 cellulose powder in a 9:1 ratio in the starting buffer (80 mM potassium phosphate pH 7.5 in buffer C). The column was packed with the addition of 0.5 g of cellulose powder on the top and bottom of hydroxylapatite. Equilibration of the column was accomplished by washing the column with 20 volumes of the starting buffer. The protein sample, adjusted to pH 7.5 and with $KHPO_4^-$ concentration adjusted to 80 mM, was applied onto the column at 60 ml/h using a peristaltic pump, followed by 50 ml of the buffer. Elution was performed with 400 ml of a linear gradient 0.08M-0.4M of potassium phosphate at 4°C and 4 ml fractions were collected.

RPC-5 chromatography was conducted according to Pearson et al. (1971). A mixture of 4 ml of Adogen 464 in 200 ml of chloroform was coated onto 100 g of polychlorotrifluoroethylene (Plaskon) support, then suspended in 0.5 M NaCl in sodium acetate buffer pH 4.5 (Buffer B), dearated and packed under pressure in a 90 cm x 0.9 cm column. Before use the column was saturated with 200 ug of carrier yeast tRNA. Routinely 50,000 CPM of ^3H Lys-tRNA were applied to the column, $tRNA^{Lys}$ was

eluted with a 400 ml linear gradient of 0.5 M- 1.0 M of NaCl in buffer B at a flow rate of 35-40 ml/h and 4 ml fractions were collected. Each fraction was precipitated by the addition of 0.4 ml of 55% of TCA, the precipitates were collected on GF/A filters and radioactivity was determined in a Beckman liquid scintillation counter (LS 3150P) using a toluene based scintillation fluid.

Aminoacylation of transfer RNA:

The aminoacylation reaction was carried out at 30°C for 30 minutes. 1 ml reaction mixture contained 40 mM Tris-HCl pH 7.4; 6 mM ATP pH 7.0; 15 mM $MgCl_2$; 0.48 mg of glutathione pH 7.0; 0.08 mg of BSA; 24 mM of KCl; 4-6 units of tRNA; saturated levels of enzyme and 10 ul of L- (4,5 ^3H) lysine monohydrochloride. The reaction started with the addition of the enzyme. Aminoacylation of the tRNAs by E. coli enzyme was carried out at 37°C for 30 minutes. The radioactivity incorporated into tRNAs was counted either by the method of Mans and Novelli (1961) or by precipitating the tRNAs with 5% TCA and filtering on GF/A glass fiber filters.

Aminoacylation assays:

Aminoacylation assays were conducted in the same manner as for the aminoacylation of tRNA. The reaction was carried out at 30°C, aliquots of 100 ul of complete reaction mixture were taken at different time intervals, placed immediately onto Whatman paper disks and immersed in cold 5% TCA for 15 minutes. The paper disks were washed two more times in 5% TCA, followed by two washings in 95% ethanol, dried and trans-

ferred into scintillation vials containing toluene based scintillation fluid. All assays were usually done in duplicate and the average values determined.

Determination of lysyl-tRNA synthetase activity was carried out in 50 ul reaction mixture with 10 ul of the enzyme, purified on a hydroxylapatite column and with other necessary components. The reaction was allowed to proceed for 25 minutes at 30°C and terminated by precipitation with 5% TCA.

Isolation of organelles:

Chloroplasts:

Green leaves in batches of 200 g were quickly homogenized in a Waring blendor in 700 ml buffer containing 5 x 10^{-2} M Tris-HCl pH 8.0; 3 x 10^{-3} M EDTA: 10^{-3} M B-mercaptoethanol; 0.3 M mannitol and 1% BSA (Burkard et al., 1972). The slurry was then passed through nylon cloth of 50 um mesh size with gentle squeezing, followed by a passage through a nylon cloth of 25 um mesh size. The final filtrate was centrifuged at 10,000 x g for 90 seconds, the pellet was dissolved in extraction buffer (10 ml/100 g of tissue) and again centrifuged at 2,000 x g for 15 minutes. The resultant chloroplast pellet was either used immediately or stored in the freezer at -20°C. All the steps in extraction were carried out at 0-4°C. The purity of the chloroplast preparation was, at every step of extraction, checked under a light microscope.

Mitochondria:

The mitochondria were isolated according to the method of

Guillemaut et al. (1972). Freshly harvested dark grown hypocotyls were ground in batches of 200 g in a Waring blendor with 200 ml of extraction medium (0.7 M mannitol; 10^{-3} M EDTA; 4 x 10^{-4} M ATP and 1 mg/ml of BSA, pH adjusted to 7.2 with triethanolamine). The homogenate was passed through a series of nylon cloths of 50 um and 25 um mesh size and centrifuged at 1,000 x g for 5 minutes. The supernatant was transferred to fresh centrifuge tubes and a sucrose cushion, consisting of 27% sucrose; 10^{-4} M EDTA and 2 mg of BSA with pH 7.2 adjusted with triethanolamine, was gently introduced below the supernatant. The extract was then centrifuged at 8,000 x g for 10 minutes. The resulting mitochondrial pellet was either used immediately or stored in the freezer at -20°C. The entire process was done in the cold at 4°C and completed within 30 minutes to ensure intactness of the organelles.

Preparation of chloroplast and mitochondrial tRNA:

The tRNAs from the chloroplast and the mitochondria were isolated according to the method of Burkard et al. (1970). Chloroplast or mitochondrial pellets were quickly dissolved in a small quantity of extraction buffer (10^{-2} M Tris-HCl pH 7.4; 10^{-2} M $MgCl_2$ and 1% SDS) and poured into tubes containing the same volume of cold, water saturated phenol (8:2 v/v). The mixture was kept stirring for 30 minutes in the cold and then centrifuged at 4,000 x g for 15 minutes. The resulting aqueous phase was removed and mixed with 20% potassium acetate pH 5.0 to the final concentration of 2% in respect to potassium acetate. With the addition of two volumes of 95% ethanol, the solution was stored overnight at -20°C. The RNA precipitate, collected by centrifugation was dissolved in 1.0 M NaCl.

The solution was centrifuged at 1,000 x g for 15 minutes, the supernatant was then diluted with Tris-HCl buffer pH 7.5 and $MgCl_2$ to bring the final concentration to 10^{-2} M in respect to Tris-HCl, 10^{-3} M with respect to $MgCl_2$ and the NaCl concentration to 0.2 M. This solution was then incubated with DNase (10 ug/ml) at 4°C for 90 minutes followed by adsorbtion on a small DEAE-cellulose column previously equilibrated with 0.05 M Tris-HCl pH 7.4 and 0.2 M NaCl. The column was washed with the same buffer until A_{260} reached 0.03. The tRNA was eluted with 1.0 M NaCl and fractions showing A_{260} greater than 0.2 were pooled. The soluble tRNA was deaminoacylated by incubation with 1.8 M Tris-HCl pH 7.8 at 37°C for 90 minutes, precipitated with two volumes of cold 95% ethanol and stored overnight at -20°C. The pellet, collected the next day by centrifugation was dissolved in distilled water, then lyophilized in small aliquots and stored in the freezer for later use.

Two-dimensional polyacrylamide gel electrophoresis of chloroplast tRNA:

Chloroplast tRNA (150-300 ug), dissolved in distilled water and mixed with sample buffer (60% sucrose; 4 M urea; 0.1 M sodium acetate buffer pH 4.5 and 1% of xylene cyanole FF) was applied, with the aid of micropipette into one of the sample slots of the starting gel. Electrophoretic separation of chloroplast tRNA in the first dimension was conducted on 10% polyacrylamide vertical slab gel (40 cm x 20 cm) in the cold at 450 volts. The composition of the polyacrylamide separating gel in the first dimension was: 10% acrylamide, 0.4% N-N -methylenebisacrylamide; 4 M urea in 0.1 M Tris-borate buffer pH 8.3 with 4 x 10^{-4} M EDTA. The acrylamide gel was polymerized with 0.001% ammonium persulfate and 1

ul/ml N,N,N'-tetramethylethylenediamine (TEMED). The separation gel was poured into the space between the glass plates leaving the top 5 cm and allowed to polymerize. Over the separation gel 5% acrylamide was then poured leaving a space of 4 mm on the top. A wedge (2 cm x 1 cm x 0.2 cm) was placed between the glass plates on the top to create space in the gel for sample application. After the polymerization was completed the wedge was removed. The polymerization conditions of 5% starting gel were similar to that of the separation gel. The electrophoresis buffer was 0.1 M Tris-borate at pH 8.3 with 4×10^{-4} M EDTA. The electrophoresis was allowed to proceed for 40 hours or until the marker dye reached the bottom edge of the gel. First dimension polyacrylamide gel fractionates tRNAs into bands.

After completion of the first dimension polyacrylamide gel electrophoresis, a narrow strip of 3 cm width, containing bands was cut lengthwise enclosing the point of origin of the sample on the top and marker dye on the bottom. This strip was then placed horizontally between two glass plates (30 cm x 30 cm) approximately 5 cm from the top. A 20% polyacrylamide solution was poured into the set, leaving only 0.4 cm on the top. The thickness of this gel should be the same as in the first dimension. The composition of the 20% acrylamide gel was: 20% acrylamide; 0.8% N,N-methylenebisacrylamide; 4 M urea in 0.1 M Tris-borate buffer pH 8.3. Electrophoresis was conducted in the cold at 350 volts for 140 hours. The gel was then removed from the plates and stained with 0.2% methylene blue in 0.2 M sodium acetate buffer pH 4.5 at room temperature for 20-30 minutes. Destaining of the gel was carried out in running tap water for 6-8 hours. The tRNAs appear as blue spots.

Isolation of tRNAs from the gel:

The tRNA spots were excised from the gel and placed in numbered tubes. The tRNAs were then extracted from the gel with an extraction buffer containing 0.01 M sodium acetate; 0.3 M NaCl and 0.01 M $MgCl_2$, pH 4.6 using a glass rod and an equal volume of water saturated phenol. After centrifugation (600-700 x g) the aqueous phase was transferred into another set of tubes, tRNA was precipitated with the addition of two volumes of cold 95% ethanol and 200 ug of carrier rRNA. Transfer RNA was recovered by centrifugation, dissolved in distilled water and after the indentification of a particular chloroplast tRNA and the determination of the tRNA concentration, chloroplast $tRNA^{Lys}$ was purified on a small Sephadex G-50 column. Aminoacylation of chloroplast $tRNA^{Lys}$ was achieved using E. coli enzyme. This tRNA was co-chromatographed with total $tRNA^{Lys}$, aminoacylated independently, on RPC-5 column. 50,000 CPM of ^{3}H chloroplast $tRNA^{Lys}$ and 25,000 CPM of total ^{14}C $tRNA^{Lys}$ were loaded onto a RPC-5 column. Elution was performed with a gradient of 0.5 M - 1.0 M NaCl in buffer B.

Preparation of chloroplast $tRNA^{Lys}$ for hybridization with chloroplast DNA:

Removal of CCA terminus:

Removal of CCA terminus was achieved by the incubation of the tRNA with snake venom phosphodiesterase. Chloroplast $tRNA^{Lys}$, separated from total chloroplast tRNA by two-dimensional gel electrophoresis was subjected to the digestion by the enzyme in reaction mixture containing

50 mM Tris-Hcl buffer pH 8.0; 10 mM $MgCl_2$ and 0.1 ug of snake venom phosphodiesterase. Aliquots were taken 5, 10, and 15 minutes following the digestion. The tRNA was then re-extracted with chloroform: phenol (1:1) mixture and lyophylized overnight.

Labelling of $tRNA^{Lys}$ with $\alpha[^{32}P]$ ATP:

Labelling of chloroplast $tRNA^{Lys}$ was carried out at its 3' end in the reaction mixture containing 50 mM Tris-glycine buffer pH 8.9; 5 x 10^{-5} CTP; 8 mM DTT; 10 mM $MgCl_2$; 5.4 ug of yeast nucleotidyl transferase (Rether et al., 1974) and 40 uCi of $\alpha[^{32}P]$ ATP (1 mCi/ml) at 37°C for 60 minutes. Unattached label was removed by purification of tRNA on a small RPC-5 or DEAE-cellulose column, equilibrated with several volumes of 2 x SSC buffer (0.3 M NaCl and 0.2 M sodium citrate pH 7.8). Labelled tRNA was eluted with 300 ul of 8 x SSC buffer (1.2 M NaCl and 0.8 M sodium citrate pH 7.8), diluted to 2 x SSC and made 50% in respect to formamide. The $tRNA^{Lys}$ is now ready for the hybridization with the DNA.

DNA extraction:

DNA was extracted from chloroplast pellet according to the method of Kolodner and Tewari (1975) with minor modifications.

Digestion of DNA with restriction enzymes:

The digestion of DNA (1-2 ug) was carried out according to the instructions provided by the supplier (BRL) at 37°C for 60 minutes using the following restriction enzymes: Sac I, Kpn I, Xho I and Pvu II.

Fractionation of DNA fragments and transfer to nitrocellulose filters:

The DNA fragments obtained by digestion with restriction enzymes were separated by electrophoresis on 0.7% agarose gel (30 cm x 20 cm x 0.5 cm). After visualization of DNA bands under UV light the DNA fragments were denatured in alkali, neutralized and transferred to nitrocellulose strips, as described by Southern (1975).

Hybridization:

The nitrocellulose filters carrying DNA fragments were incubated with labelled $tRNA^{Lys}$ for 24 hours at 37°C in 2 x SSC and 50% formamide according to the procedure of Steinmetz and Weil (1976). Following the hybridization nitrocellulose filters were treated with RNase A to remove non-hybridized tRNA, washed and exposed to an X-ray film for autoradiography for 2-3 weeks.

RESULTS:

Optimal levels of ATP and Mg^{++}:

Binding of amino acid to tRNA, the type of reaction catalyzed by aminoacyl-tRNA synthetase shows an unquestionable requirement for ATP and Mg^{++} ions. In addition to tRNA and ATP there is a possibility that aminoacyl-tRNA synthetases may also be capable of binding Mg^{++} ions, therefore a number of experiments were carried out to optimize Mg^{++} and ATP concentrations in the assay system. A series of concentrations for ATP (1-10 mM) and Mg (2-20 mM) were tested. Results indicated that ATP and Mg^{++} requirement for all three systems (cytoplasm, chloroplast and mitochondria) tested, differed only slightly. However, mitochondria appears to require slightly higher ATP levels for aminoacylations (8 mM). Nevertheless, for all the three systems the aminoacylation mixture contained 6 mM ATP and 15 mM $MgCl_2$ at pH 7.4 (Tris-HCl buffer), which was satisfactory. The ATP/Mg^{++} ratio of 1/25 in our assays is similar to the requirements demonstrated in the case of Lupinus luteus seeds (Augustyniak and Pawelkiewicz, 1978).

Lysyl-tRNAs from cytoplasm, chloroplast and mitochondria:

The elution profile of ^3H $tRNA^{Lys}$ (from dark grown soybean cotyledons) charged by homologous enzyme and fractionated by reverse phase chromatography (RPC-5) is shown in Figure 1. Of the five peaks of activity, $tRNA^{Lys}_2$ and $tRNA^{Lys}_4$ are more pronounced than the rest ($tRNA^{Lys}_2$ 18.8% and $tRNA^{Lys}_4$ 11.8% of total).

In a plant cell protein synthesis occurs in three different com-

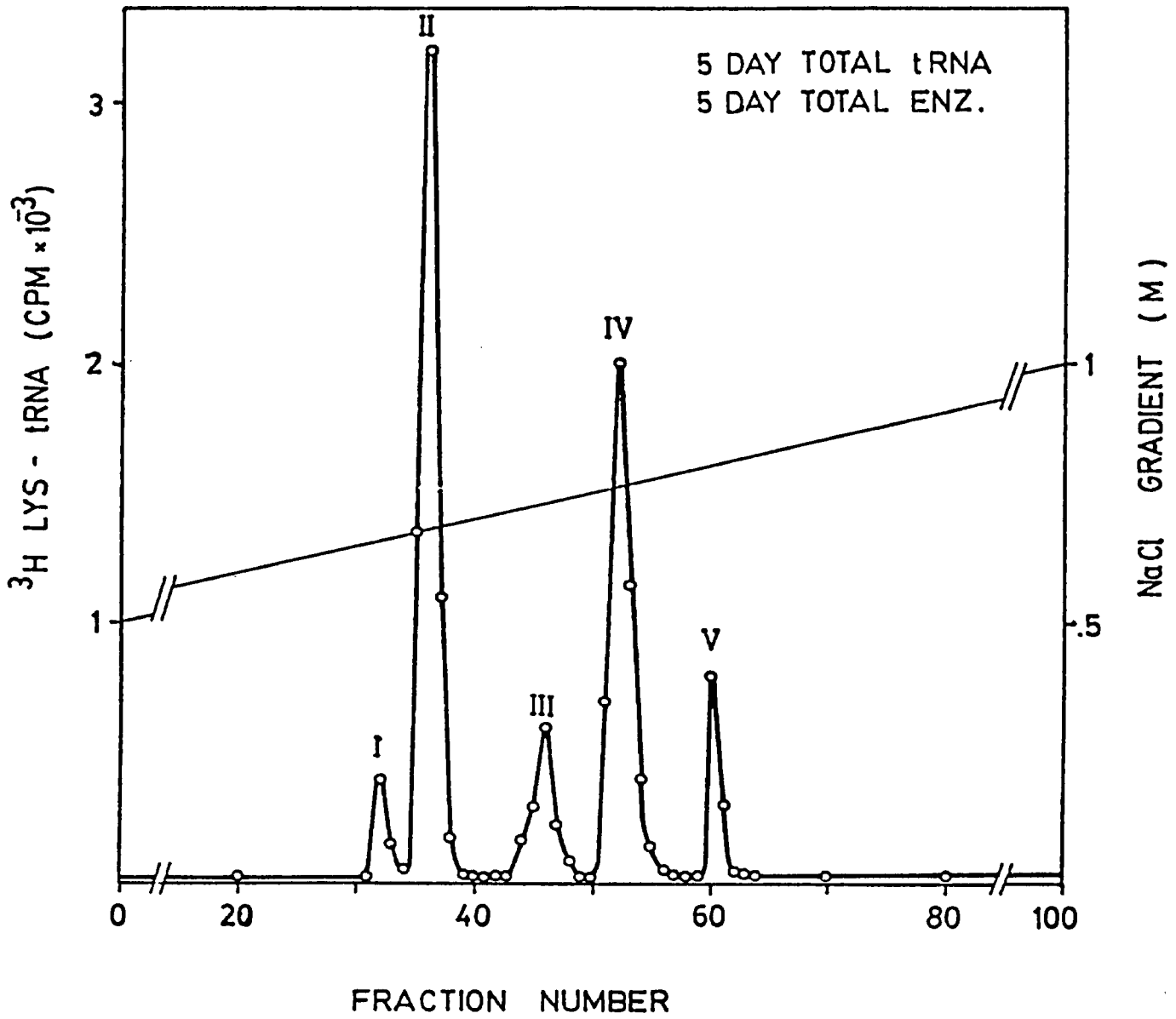

Figure 1 - RPC-5 Fractionation of total cell $tRNA^{Lys}$ aminoacylated with homologous enzyme

Unfractionated tRNA isolated from 5 day old dark grown cotyledons was aminoacylated with homologous enzyme in 1 ml reaction mixture containing 4 A_{260} units of tRNA, 0.3 mg of the enzyme, optimal levels of ATP, Mg^{++} and 0.01 mCi of ^{3}H L-Lysine at 30°C for 30 minutes as described in the methods. Elution was performed with a linear gradient of 2 x 200 ml of 0.5 M-1.0 M NaCl in buffer B, pH 4.5. Four ml fractions were collected with a flow rate of 35-40 ml/h.

partments: in the cytoplasm, in the chloroplasts and in the mitochondria. Occurrence of a number of isoaccepting tRNA species and multiple forms of aminoacyl-tRNA synthetases possibly indicate specificity within each compartment of the cell. With this in mind, we attempted to localize organellar and cytoplasmic tRNAs, after charging with homologous enzymes and fractionation on RPC-5 column. Mitochondrial tRNA charged with a crude mitochondrial enzyme and subsequently fractionated on RPC-5 column resulted in three peaks of activity as presented in Figure 2. These three mitochondrial tRNA peaks of activity coincide with the three total $tRNA^{Lys}$ ($tRNA^{Lys}_2$, $tRNA^{Lys}_3$ and $tRNA^{Lys}_4$) obtained after fractionation of total cell $tRNA^{Lys}$ charged by homologous enzyme. It is interesting to note that $tRNA^{Lys}_2$ and $tRNA^{Lys}_4$ are the most predominant isoacceptors showing 150% increase over the control (Figure 1) which indicates the exclusive efficient charging of mitochondrial tRNA by mitochondrial enzyme. Similarly, E. coli enzyme is used to charge the four isoacceptors in mitochondrial $tRNA^{Lys}$ (Figure 3), shows that $tRNA^{Lys}_2$ is the most predominant isoacceptor.

The elution pattern of chloroplast ^3H $tRNA^{Lys}$, charged with crude chloroplast enzyme indicated the presence of four peaks of activity (Figure 4). Peak 1 is the most predominant species. It should be cautioned here that the presence of four chloroplast $tRNA^{Lys}$ peaks indicates cross-contamination in the enzyme and the chloroplast tRNA. However, when chloroplast tRNA, charged with E. coli enzyme was fractionated on RPC-5 column (Figure 5) only one major peak of activity was observed, which coincides with $tRNA^{Lys}_1$ in the control (Figure 1), but with a 25 fold increase in the activity.

Cross-contamination in charging tRNAs from different cell com-

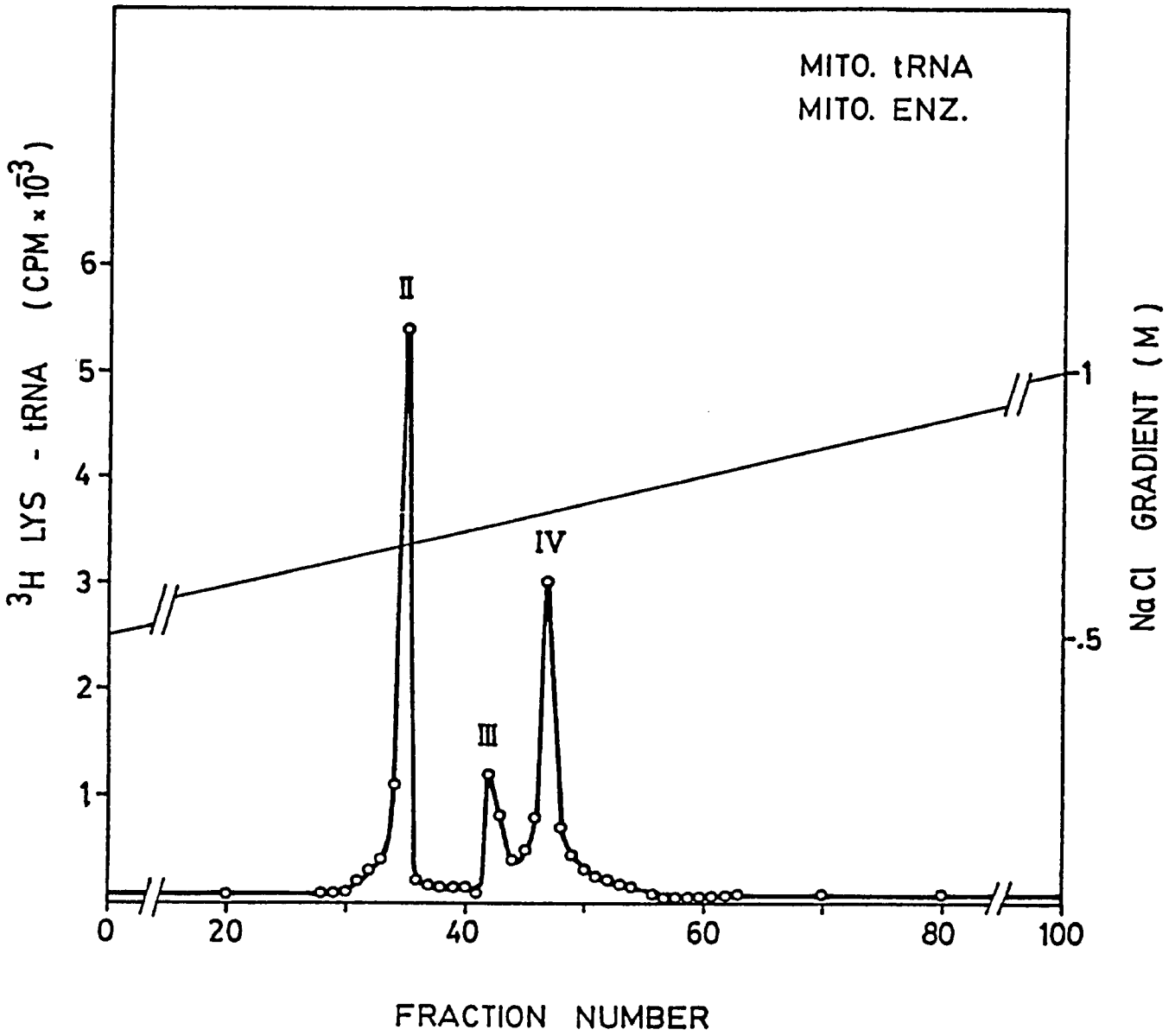

Figure 2 - RPC-5 Fractionation of mitochondrial $tRNA^{Lys}$ aminoacylated with crude mitochondrial enzyme

The aminoacylation reaction was carried out under the conditions described in the methods. Elution was performed with 2 x 200 ml gradient of 0.5 M - 1.0 M NaCl in buffer B with a flow rate of 35-40 ml/h as described in the methods.

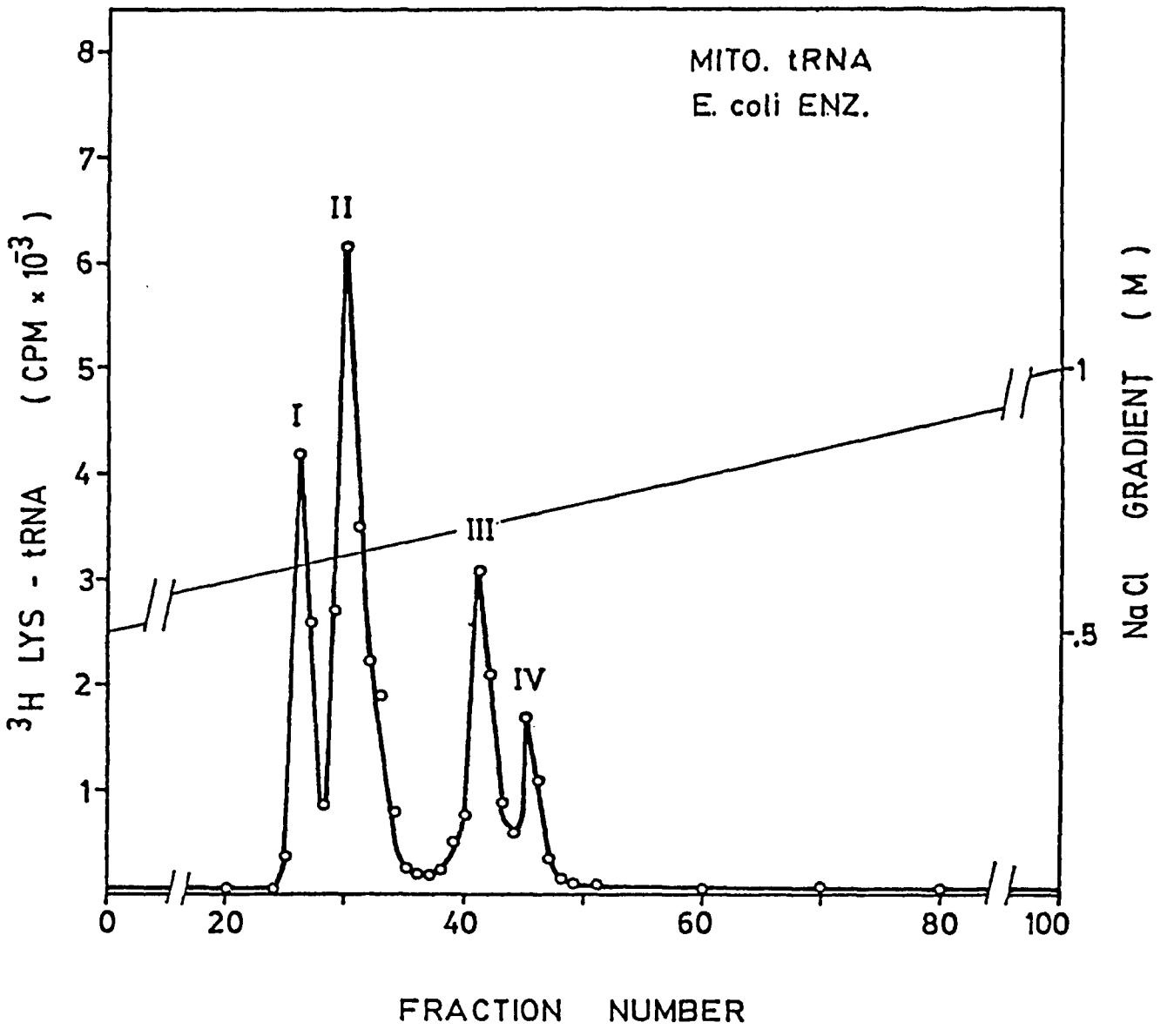

Figure 3 - RPC-5 Fractionation of mitochondrial $tRNA^{Lys}$ aminoacylated with lysyl-tRNA synthetase from Escherichia coli

The aminoacylation reaction was carried out at 37°C for 30 minutes in a standard reaction mixture containing 6 A_{260} units of tRNA and 0.3 mg of E. coli synthetase. Elution was performed as described in Figure 1.

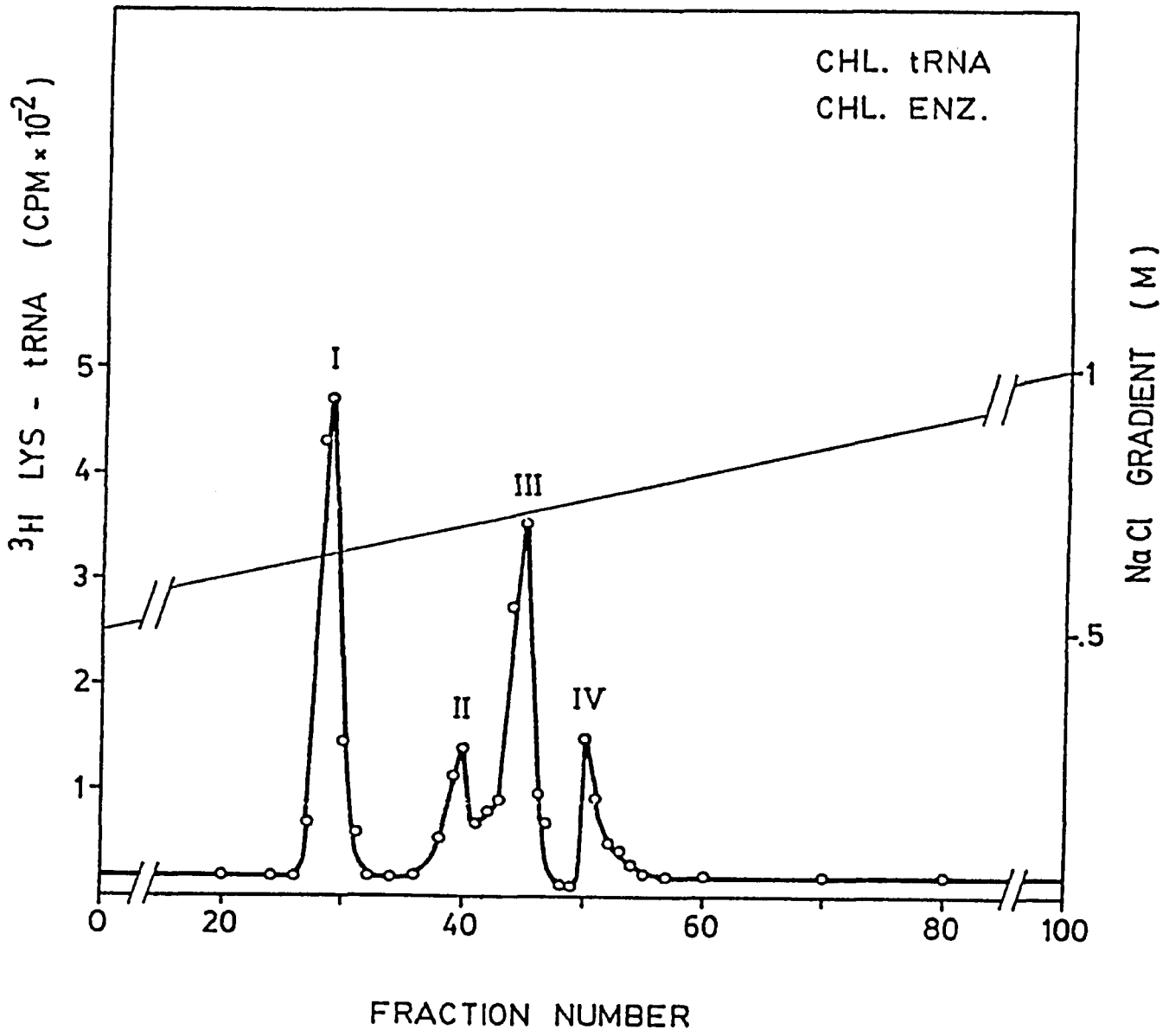

Figure 4 - RPC-5 Fractionation of chloroplast $tRNA^{Lys}$ aminoacylated with crude chloroplast enzyme

The aminoacylation reaction was carried out under conditions described in the methods and given in Figure 1. Elution was performed under same conditions given in Figure 1.

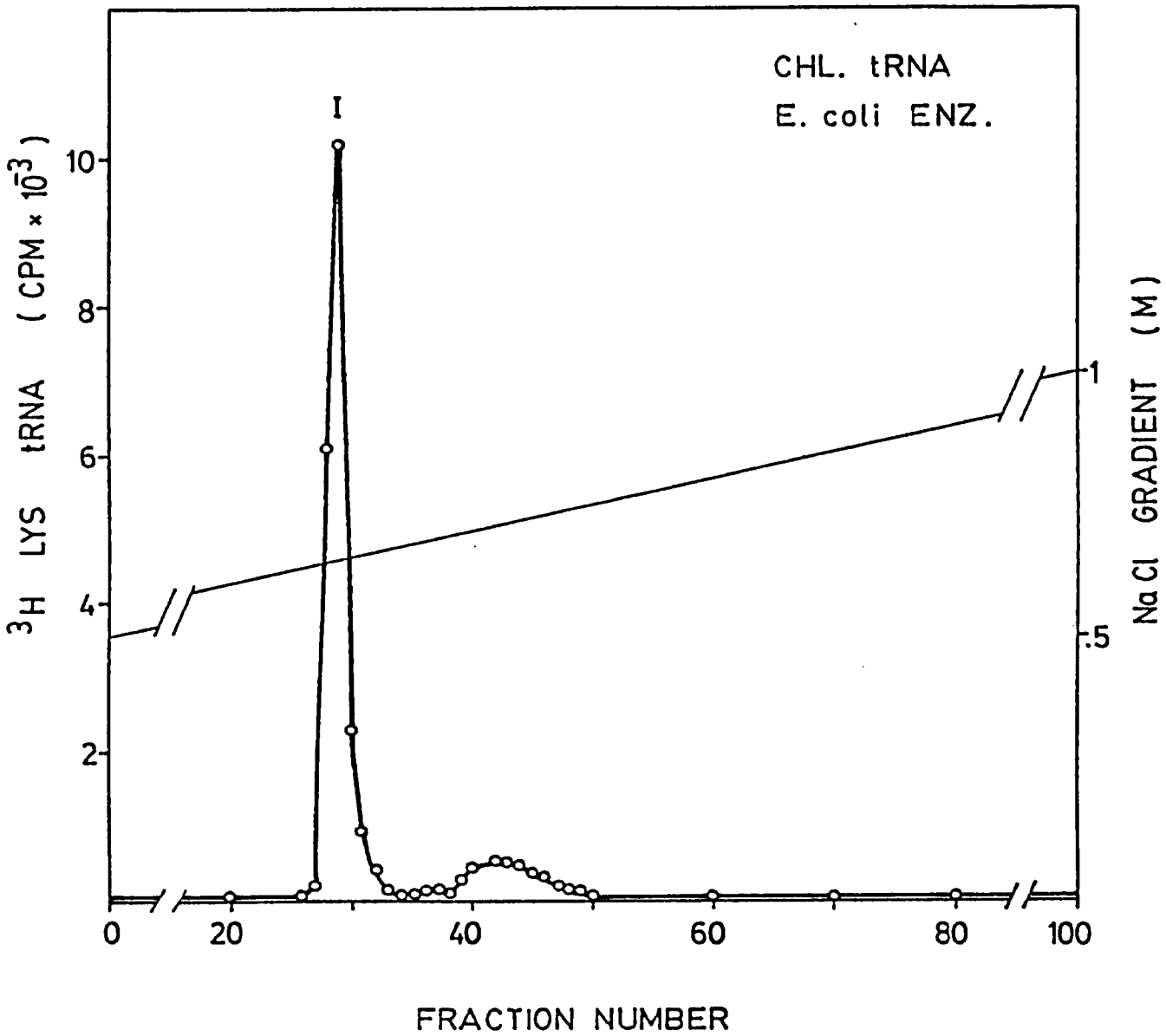

Figure 5 - RPC-5 Fractionation of chloroplast $tRNA^{Lys}$ aminoacylated with *E. coli* enzyme

The aminoacylation reaction was carried out at 37°C for 30 minutes in a standard reaction mixture containing 6 A_{260} units of tRNA and 0.3 mg of *E. coli* lysyl-tRNA synthetase. Elution was performed as described in Figure 1.

partments is usually unavoidable since it is virtually impossible to obtain pure organellar tRNAs and aminoacyl-tRNA synthetases. This is a widely acknowledged fact in the literature (Puttney et al., 1981, Guillemaut and Weil, 1975a, Guillemaut et al., 1975b, Jeannin et al., 1976). The possibility that lysyl-tRNA synthetases from the three cell compartments may possess different aminoacylation capacities had to be tested by eliminating cross-contamination from other cell compartments. To accomplish this, it was necessary to undertake the purification of lysyl-tRNA synthetases from three cell compartments by hydroxylapatite column chromatography.

Fractionation of lysyl-tRNA synthetase from the three cell compartments:

Fractionation of multiple aminoacyl-tRNA synthetases from various sources by hydroxylapatite column chromatography appears to be one of the most successful techniques. This fractionation is based on different chromatographic mobilities elicited by synthetases from different cell compartments. Using this technique the total cotyledon enzyme was separated into three peaks of activity as shown in Figure 6. Generally, 50 mg of crude enzyme, obtained after fractionation on a DEAE-cellulose column, was applied onto a hydroxylapatite column previously equilibrated with 0.08 M potassium phosphate buffer at pH 7.5 in buffer C as given in the methods. Following elution of the enzyme with a linear potassium phosphate gradient, alternate fractions were tested for enzyme activity using total tRNA in a standard reaction mixture as described in the methods.

Lysyl-tRNA synthetase appears to be a very labile enzyme, with its activity decreasing up to 40% within a few hours. The degradative

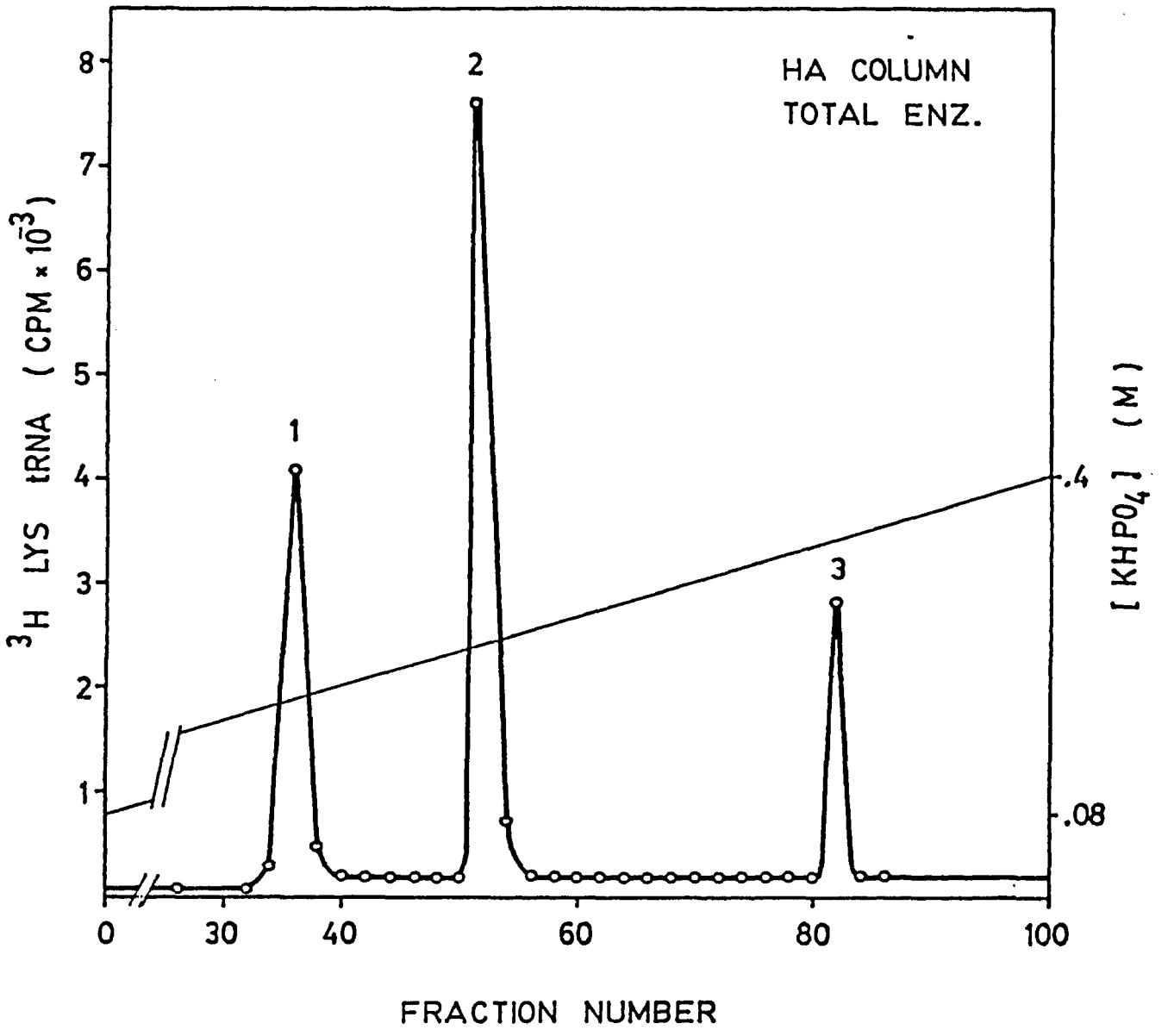

Figure 6 - Fractionation of total lysyl-tRNA synthetase by hydroxylapatite column

50 mg of the enzyme was applied onto a HA column in 0.08 M potassium phosphate pH 7.5. The enzyme was eluted with a linear gradient of 0.08 M - 0.4 M potassium phosphate in buffer C pH 7.5. Four ml fractions were collected at a flow rate of 50 ml/h at 4°C. Enzyme activity was tested using total cell $tRNA^{Lys}$ in 50 ul reaction mixture containing 0.5 A_{260} units of tRNA, 10 ul of the enzyme fractionated by HA column, optimal levels of ATP, Mg^{++} and ^{3}H L-Lysine.

action of proteolytic enzymes possibly present in the extract may be attributed to this effect. To minimize the action of proteolytic enzymes, the extraction of the enzyme and other subsequent steps in the purification as well as application on hydroxylapatite column and the assays were performed with increased speed at 0-4°C.

Upon hydroxylapatite column chromatography of chloroplast preparation one major and two minor peaks of enzyme activity were observed (Figure 7). When the fractions were assayed with chloroplast tRNA (o—o) and E. coli tRNA (Δ--Δ) the position of the major peak was identical to isoenzyme 1 of total cell synthetase fractionated on the HA column. Appearance of two minor peaks, similar to isoenzymes 2 and 3 may be the result of cross-contamination between cell compartments. Inclusion of 20% glycerol in the grinding medium, minimized contamination of organellar synthetases with cytoplasmic, but does not seem to have prevented it completely. Assay of enzyme fractions with E. coli tRNA (Δ--Δ) results in three peaks of activity, with isoenzyme 1 being the major peak with very low activities for peaks 2 and 3. These results lead us to conclude the prokaryotic nature of isoenzyme 1 and possibly isoenzyme 3. Isoenzyme 1 being the most prominent peak appears to be localized in the chloroplast.

Figure 8 shows the hydroxylapatite column fractionation of mitochondrial enzyme prepared from dark grown hypocotyls. The isolation and purification of lysyl-tRNA synthetase from mitochondria is described in the methods. Approximately 26 mg of mitochondrial lysyl-tRNA synthetase partially purified by DEAE-cellulose chromatography was applied onto hydroxylapatite column. Assay for enzyme activity with mitochondrial tRNA, carried out at 30°C for 25 minutes, resulted in three peaks of acti-

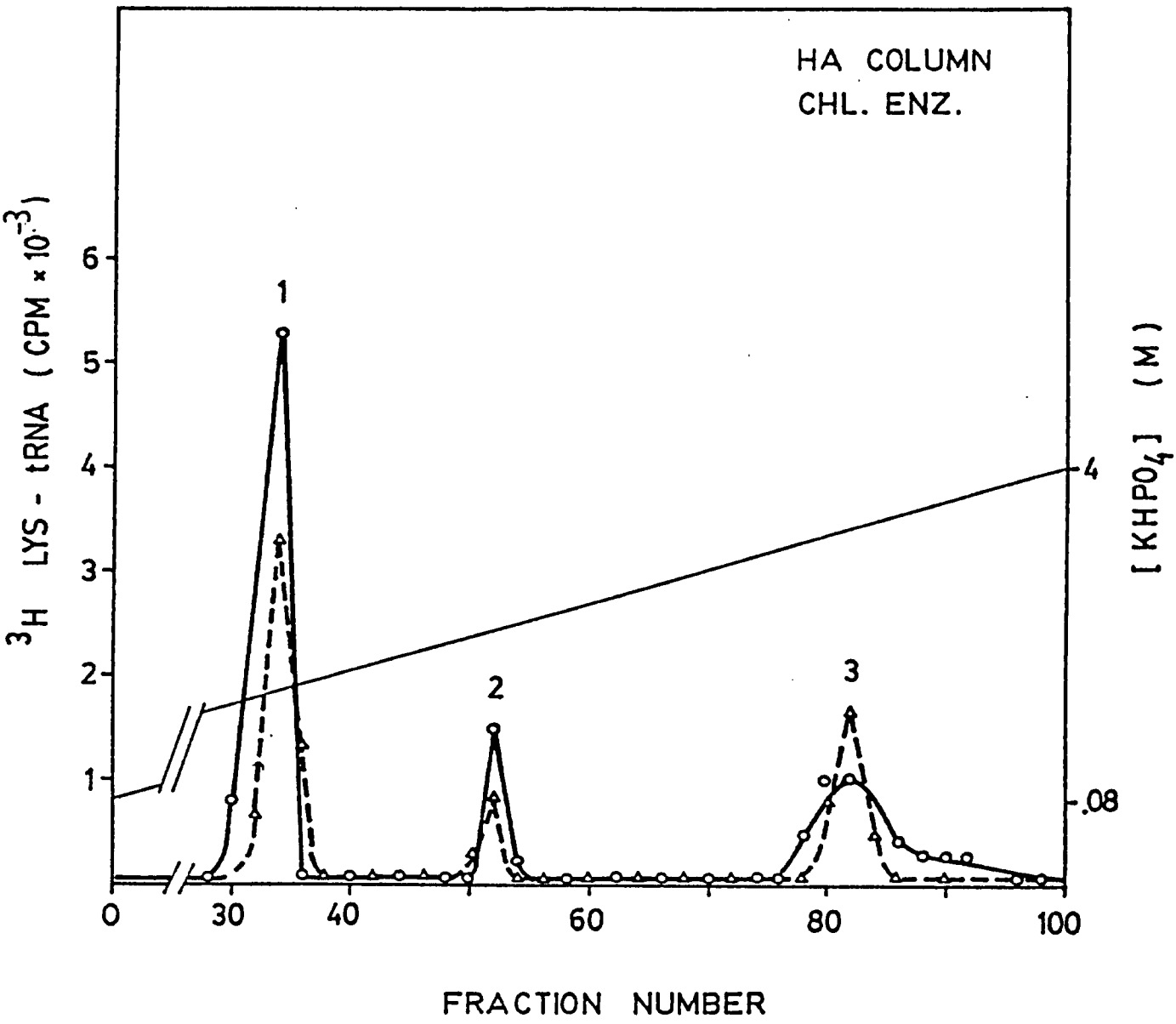

Figure 7 - Fractionation of chloroplast lysyl-tRNA synthetase by hydroxylapatite chromatography

36 mg of synthetase, purified on a DEAE-cellulose column was applied onto a HA column in 0.08 M potassium phosphate pH 7.5. The enzyme was eluted with a linear gradient of 0.08 M - 0.4 M potassium phosphate in buffer C, pH 7.5 as described in the methods. Four ml fractions were collected and assayed for enzyme activity using a) chloroplast tRNA (o——o) and b) *E. coli* tRNA (Δ--Δ).

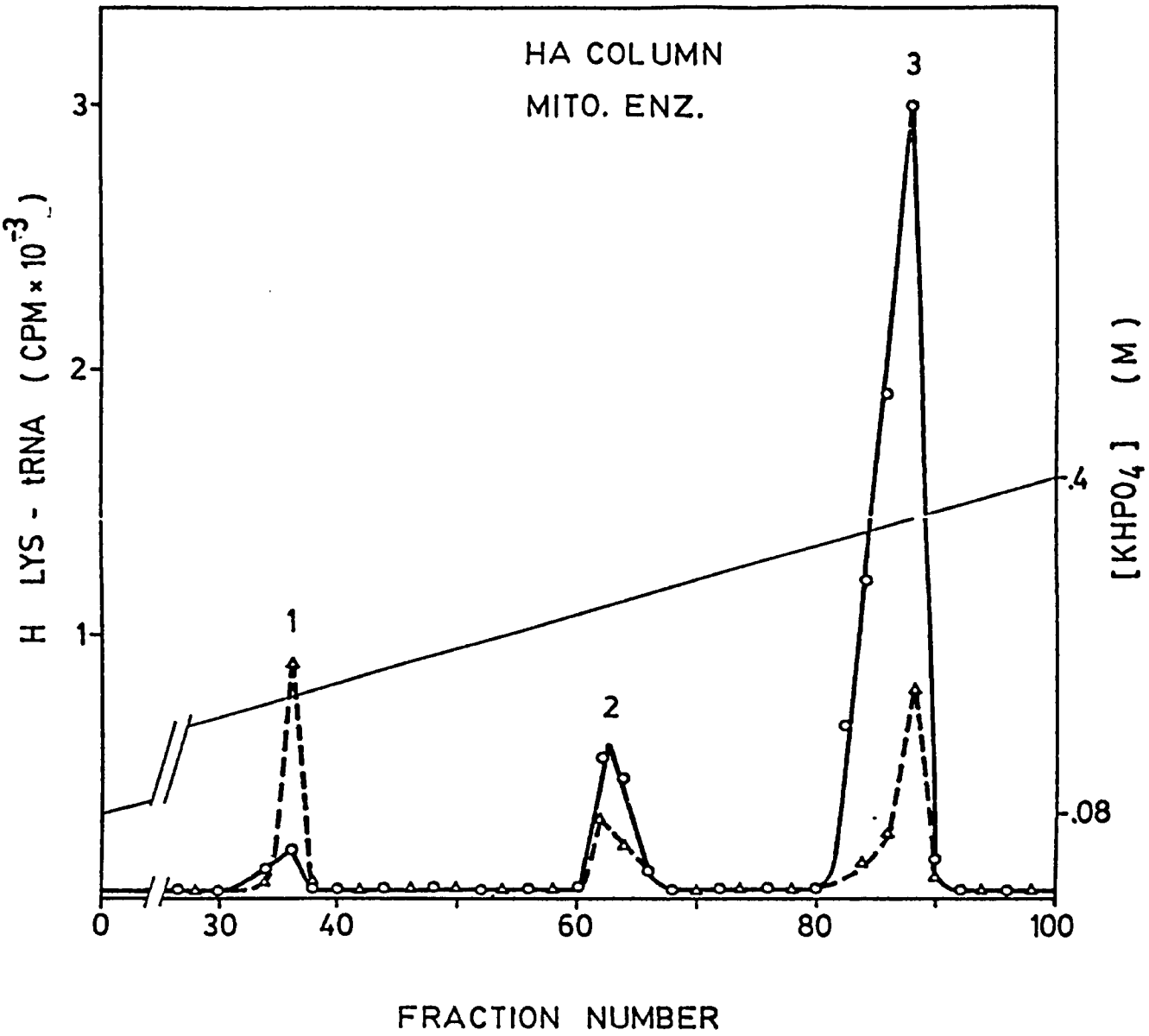

Figure 8 - Fractionation of mitochondrial lysyl-tRNA synthetase by hydroxylapatite chromatography

26 mg of synthetase was applied onto a HA column, after purification on a DEAE-cellulose column, in 0.08 M potassium phosphate buffer pH 7.5. Elution was performed with a linear gradient of 0.08 M - 0.4 M potassium phosphate in buffer C, pH 7.5 as described in the methods. Four ml fractions were collected and assayed for enzyme activity using a) Mitochondrial tRNA (o——o) and b) E. coli tRNA (Δ---Δ).

vity. Isoenzyme peak 3 appears to be localized in the mitochondria. When the fractions were assayed with E. coli tRNA (at 37°C for 25 minutes) three peaks of activity can be observed. Isoenzymes 1 and 3 are more pronounced than the isoenzyme 2. This fact may again indicate prokaryotic nature of isoenzymes 1 and 3. The presence of peaks 1 and 2 in the mitochondrial enzyme preparation may be due to cross-contamination from other cell compartments. Attempts to re-chromatograph each of the individual isoenzymes failed because the enzyme was labile during and after HA column chromatography.

Aminoacylation by homologous and heterologous enzymes and tRNAs from total cell, chloroplast and mitochondria:

Aminoacylation reactions were carried out with tRNAs and aminoacyl-tRNA synthetases from all three compartments. This set of experiments was undertaken to establish substrate specificity of lysyl-tRNA synthetases from the cytoplasm, the chloroplast, and the mitochondria. A preference to aminoacylate a tRNA from a particular cell compartment or E. coli tRNA may be an indicative of the prokaryotic or eukaryotic nature of a particular aminoacyl-tRNA synthetase. Various tRNAs were aminoacylated by individual isoenzymes fractionated on the HA column. The reaction mixture (50 ul) contained optimal levels of ATP and Mg^{++}, the concentration of tRNA was kept constant (0.5 A_{260} units) and 10 ul of enzyme (eluted from hydroxylapatite column). Table 1 shows the results of cross-aminoacylations with tRNAs and aminoacyl-tRNA synthetases from different cell compartments. We have previously shown that lysyl-tRNA synthetase from dark grown cotyledons (total cell) fractionates into three peaks of

Table 1: Aminoacylation by homologous and heterologous enzymes and tRNAs from chloroplast, mitochondria and total cell. The aminoacylation reaction was carried out with 10 ul of the enzyme fractionated by hydroxylapatite column as described in the methods.

Source of tRNA	Enzyme Peak No.	Percent of ^{3}H lysyl-tRNA formed/Total CPM Source of Enzyme Total Cell	Chloroplast	Mitochondria
Total Cell	1	7.6	-	1.8
	2	13.6	-	6.1
	3	4.4	-	4.1
Yeast	1	1.8	2.0	1.4
	2	10.8	3.1	2.0
	3	4.3	2.0	2.2
E. coli	1	5.9	5.9	1.6
	2	1.1	1.7	1.2
	3	4.3	3.0	1.4
Chloro.	1	2.2	9.5	0.7
	2	1.2	2.7	0.5
	3	1.1	2.3	1.6
Mitoch.	1	1.3	-	1.5
	2	0.7	-	0.7
	3	2.0	-	5.4

activity on the HA column (Figure 6). On a quantitative basis isoenzyme 2 shows a two and three fold increase in aminoacylation compared to isoenzymes 1 and 3 respectively when total cell tRNA was used as a substrate. Similar results were obtained when yeast tRNA was aminoacylated with isoenzyme 2 with a ten fold increase compared to the isoenzyme 1 and five fold increase compared to the isoenzyme 3. The preferential aminoacylation of E. coli tRNA and mitochondrial tRNA by isoenzymes 1 and 3 may indicate the prokaryotic nature of these isoenzymes, whereas isoenzyme 2 appears to be eukaryotic and localized in the cytoplasm.

Chloroplast enzyme fractionated on the HA column resolved into one major and two very minor peaks of activity (Figure 7). The first peak preferentially aminoacylated chloroplast tRNA and E. coli tRNA, as indicated in the Table 1. In the case of chloroplast tRNA there is a three-fold increase in enzyme activity compared to the activities of isoenzymes 2 and 3. Similar increase (three fold) in enzyme activity was observed when E. coli tRNA was used as a substrate, when isoenzymes 1 and 2 were compared. This affinity of chloroplast enzyme toward chloroplast $tRNA^{Lys}$ and E. coli $tRNA^{Lys}$ could indicate the prokaryotic nature of lysyl-tRNA synthetase localized in the chloroplast. The isoenzyme 3, which appears to be mitochondrial seems to aminoacylate E. coli tRNA quite well. The presence of some mitochondrial enzyme activity in chloroplast enzyme preparations may be attributed to the unavoidable cross-contamination between cell compartments. Similarly, in the aminoacylation of yeast tRNA by chloroplast isoenzyme 2 the same conclusion can be drawn suggesting enough cytoplasmic enzyme contamination in chloroplast enzyme extract. Earlier it was shown that mitochondrial enzyme preparation resolved into

three peaks of activity upon hydroxylapatite column chromatography, as presented in Figure 8. It should be pointed out that isoenzyme 3 showed maximal response in charging mitochondrial $tRNA^{Lys}$. However, the prokaryotic nature of mitochondrial isoenzyme 3 is not clearly established in this case, because the mitochondrial isoenzyme 3 aminoacylates chloroplast tRNA quite well hinting at its prokaryotic nature as well as the similarity between chloroplast and mitochondrial tRNA. Charging of E. coli tRNA by this isoenzyme 3 shows an increase in aminoacylation compared to mitochondrial isoenzyme 2. The capacity of mitochondrial isoenzyme 3 to efficiently aminoacylate total cell $tRNA^{Lys}$ and yeast $tRNA^{Lys}$ may reflect its eukaryotic nature or some contamination of cytoplasmic isoenzymes.

Comparison of $tRNA^{Lys}$ after aminoacylation by purified homologous enzyme (HA column):

Fractionation of chloroplast $tRNA^{Lys}$ after aminoacylation by chloroplast enzyme peak 1 (from HA column) is presented in Figure 9. The presence of one $tRNA^{Lys}$ peak which coelutes with total $tRNA^{Lys}_1$ (Figure 1) suggests the presence of one isoacceptor specific for lysine, in the pool of chloroplast tRNAs. When this chromatographic profile is compared to the profile in Figure 4, where chloroplast tRNA was charged with crude chloroplast enzyme, the presence of lysyl-tRNA isoacceptors 2, 3 and 4 in addition to isoacceptor 1 may indicate cross-contamination in the crude enzyme preparation. Comparison of chromatographic profile of chloroplast tRNA charged with E. coli enzyme (Figure 5) with the profile in Figure 9 suggests the similarity in chloroplast and E. coli $tRNA^{Lys}$, which elutes as the first peak on the RPC-5 column. Figure 10 shows the

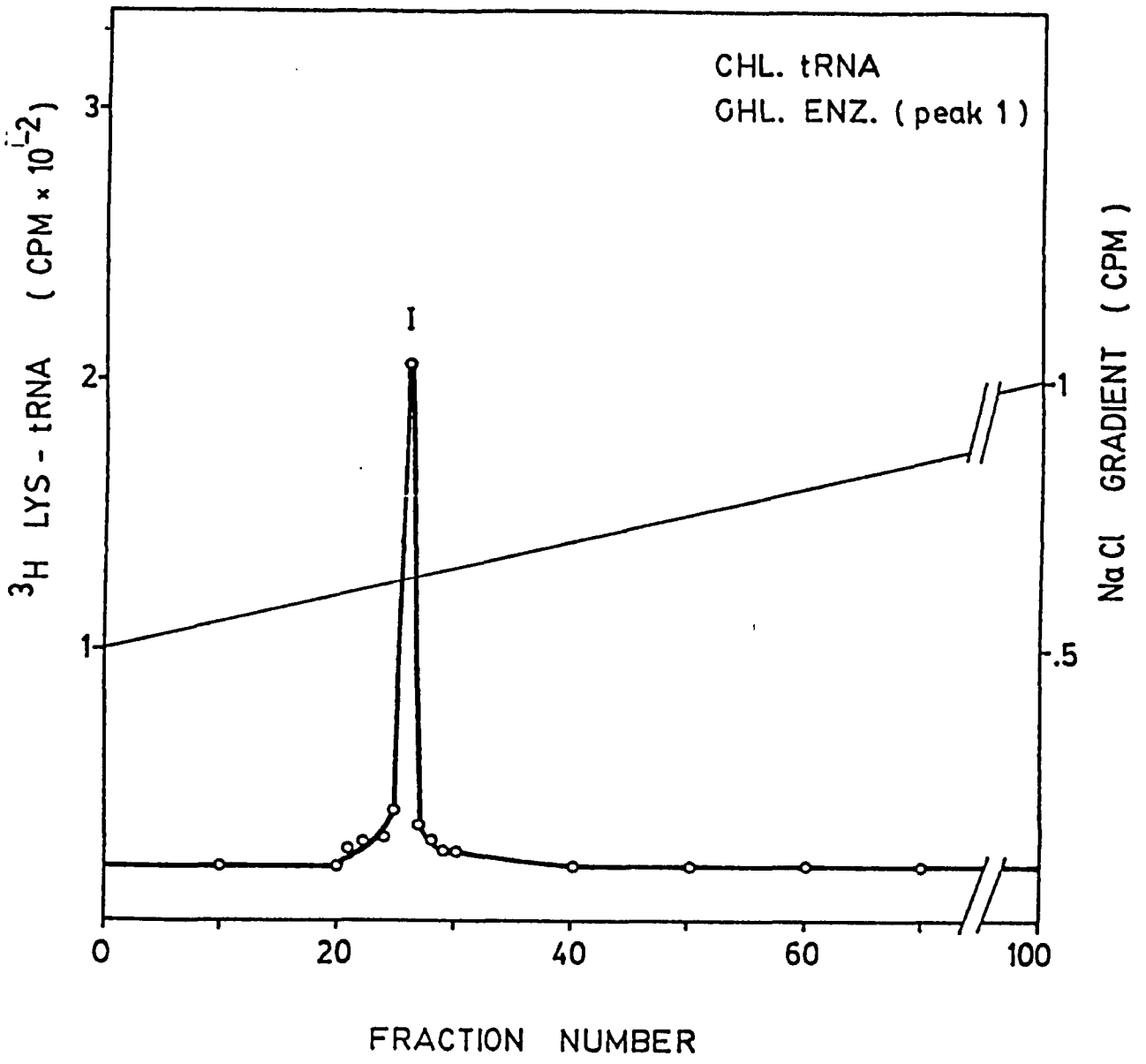

Figure 9 - RPC-5 Fractionation of chloroplast $tRNA^{Lys}$ aminoacylated with homologous enzyme peak 1

The aminoacylation reaction was carried out in a standard reaction mixture containing 4 A_{260} units of tRNA, 0.2 mg of synthetase, obtained after fractionation of mitochondrial enzyme on a HA column, optimal levels of ATP, Mg^{++} and 0.01 mCi of ^{3}H L-Lysine as given in Figure 1 and described in the methods. Elution conditions were given in Figure 1.

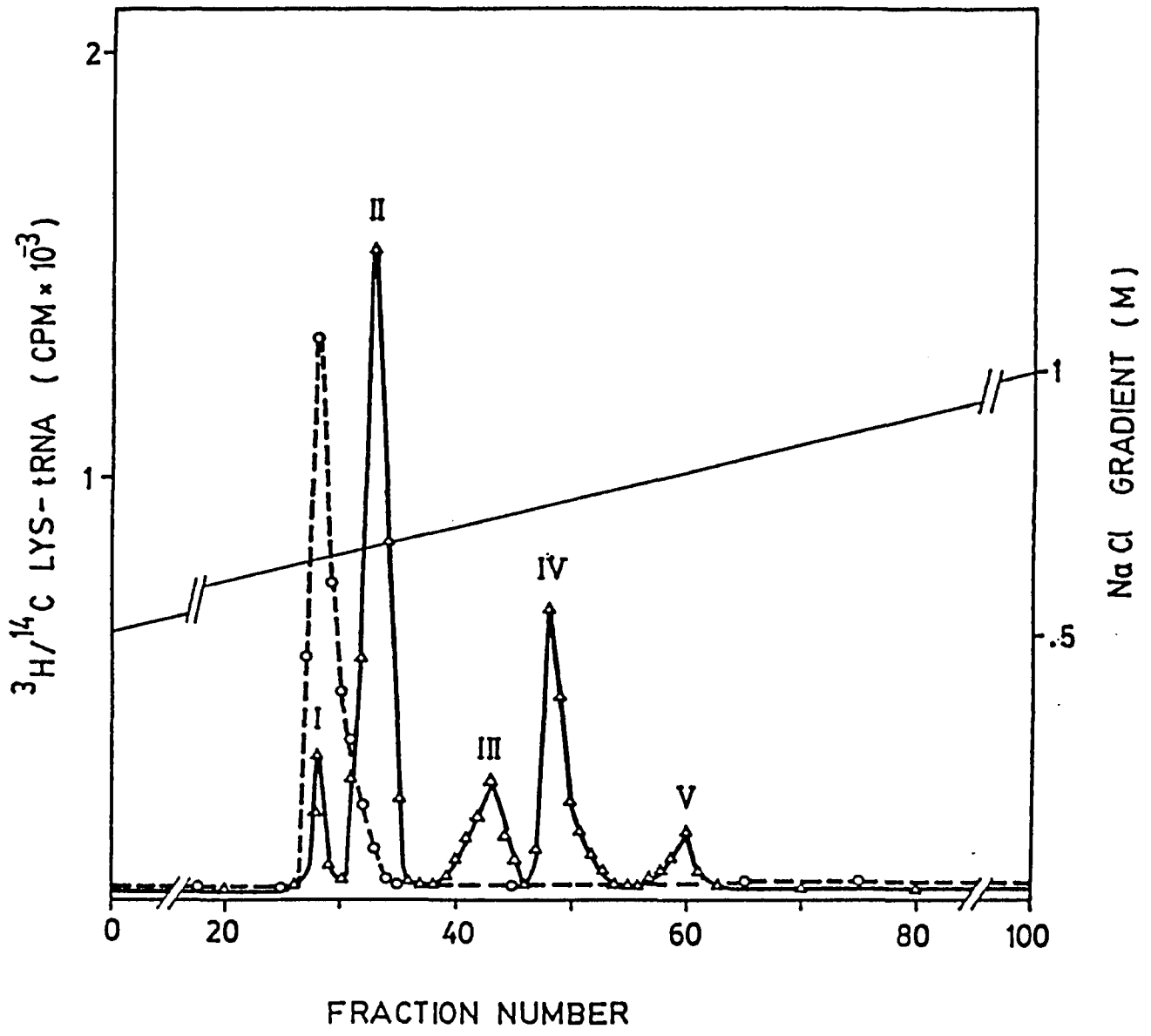

Figure 10 - RPC-5 Fractionation of double labelled ^3H chloroplast $tRNA^{Lys}$ and ^{14}C total cell $tRNA^{Lys}$

Co-chromatography of ^3H chloroplast $tRNA^{Lys}$, separated by two-dimensional polyacrylamide gel electrophoresis, and aminoacylated by E. coli enzyme (o---o) with ^{14}C total cell $tRNA^{Lys}$ aminoacylated by homologous enzyme (Δ——Δ). Aminoacylation reaction and elution were carried out in the same manner as described in Figure 1.

dual labelling and co-chromatography of total cell $tRNA^{Lys}$ (^{14}C) and chloroplast $tRNA^{Lys}$ (^{3}H). Chloroplast tRNA was first separated by two-dimensional polyacrylamide gel electrophoresis as described in the methods. Identification of individual tRNA spots showed the existence of only one lysine-specific isoaccepting tRNA (Figure 11). This finding is in agreement with past research (Swamy, Dr. Sc. Thesis, 1980, University of Windsor). ^{3}H chloroplast $tRNA^{Lys}$ coelutes with $tRNA^{Lys}_{1}$ of the total cell $tRNA^{Lys}$ preparation (^{14}C).

Fractionation of total cell tRNA on RPC-5 column, after charging with peak 2 enzyme, obtained after fractionation of total cell enzyme on the HA column yielded four peaks of activity (Figure 12). When this chromatographic profile is compared to the control (Figure 1), these peaks elute in the same regions as $tRNA^{Lys}$ peaks 2-5. These findings may indicate the presence of four isoaccepting $tRNA^{Lys}$ in the cytoplasm of soybean.

Upon fractionation of mitochondrial tRNA on RPC-5 column, charged with mitochondrial isoenzyme 3 (from HA column), three $tRNA^{Lys}$ isoacceptors were observed (Figure 13). These $tRNAs^{Lys}$ eluted in the same regions as isoacceptors 2, 3 and 4 in the control (Figure 1) and $tRNA^{Lys}_{2}$ seems to be the most abundant species. A similar chromatographic profile was observed when mitochondrial tRNA was charged with crude mitochondrial enzyme (Figure 2).

Age-related changes in lysyl-tRNAs in germinating cotyledons:

Transfer RNAs and lysyl-tRNA synthetases were isolated from 5, 10, 15, and 20 days old soybean cotyledons. Figure 14 shows a comparison

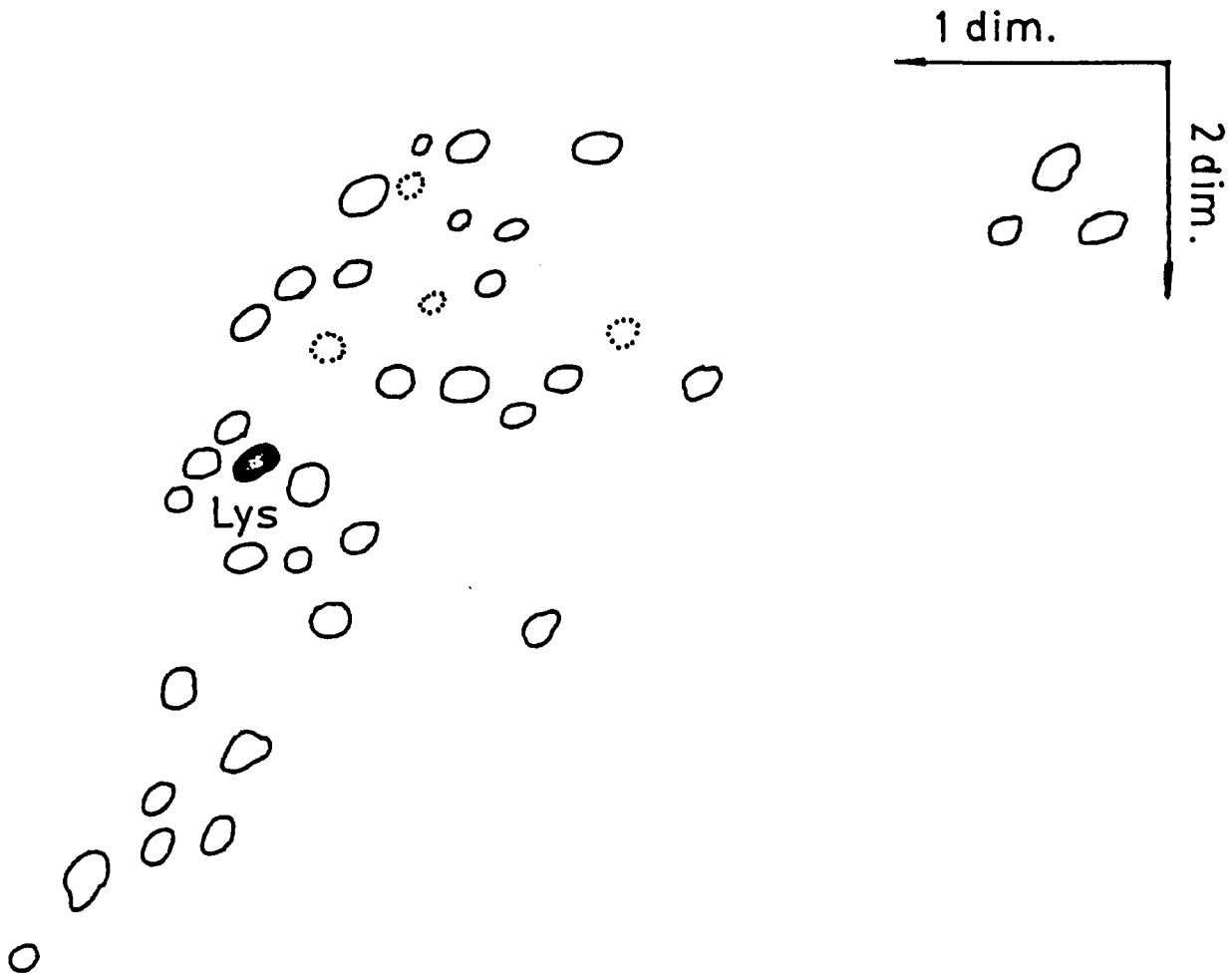

Figure 11 - Two-dimensional polyacrylamide gel electrophoresis of chloroplast tRNA.

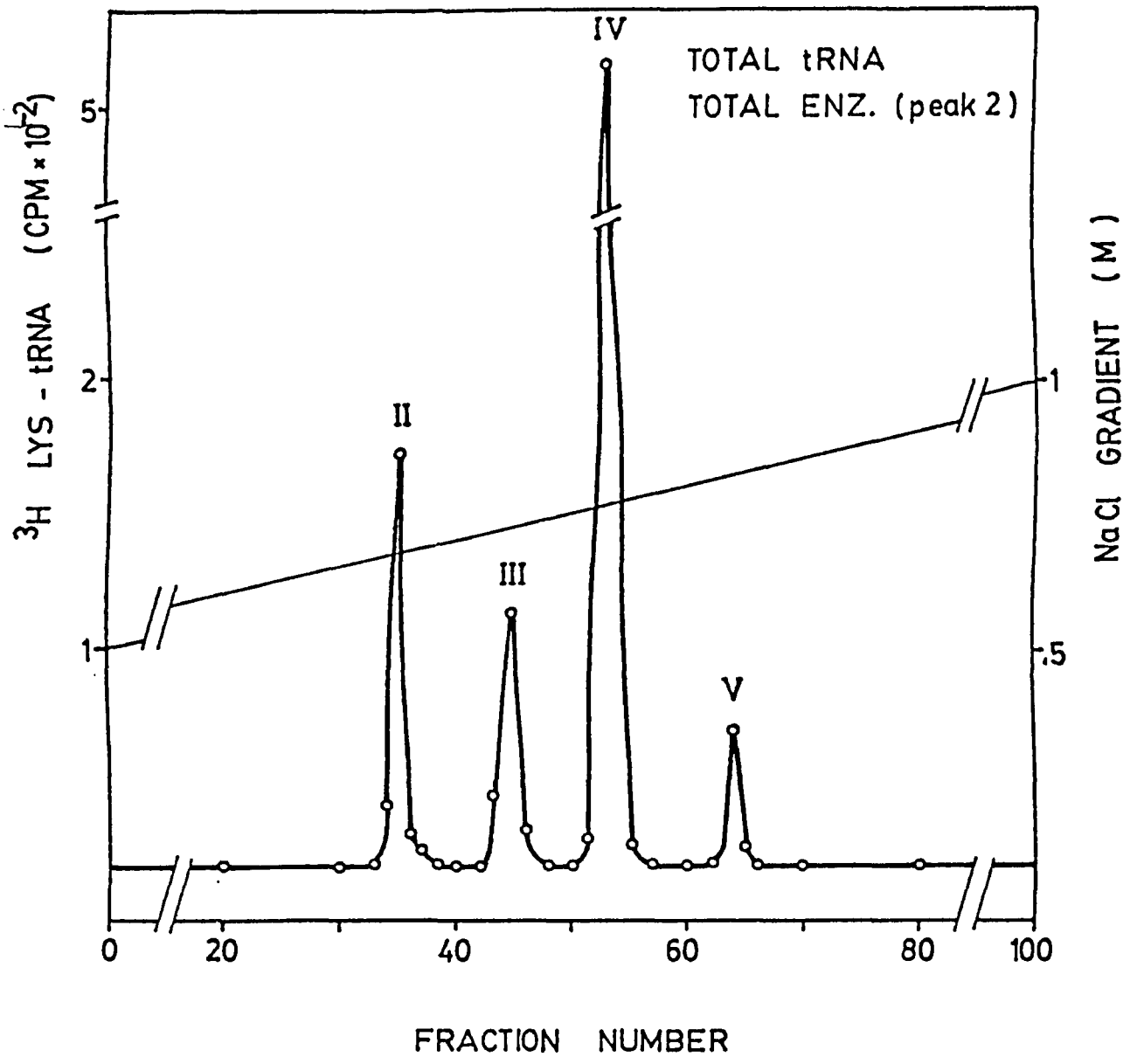

Figure 12 - RPC-5 Fractionation of total cell $tRNA^{Lys}$ aminoacylated by homologous enzyme peak 2

The aminoacylation reaction was carried out in a standard reaction mixture under conditions described in the methods and given in Figures 1 and 9. The enzyme used for aminoacylation was obtained after fractionation of total cell enzyme on a HA column. Elution conditions were given in Figure 1.

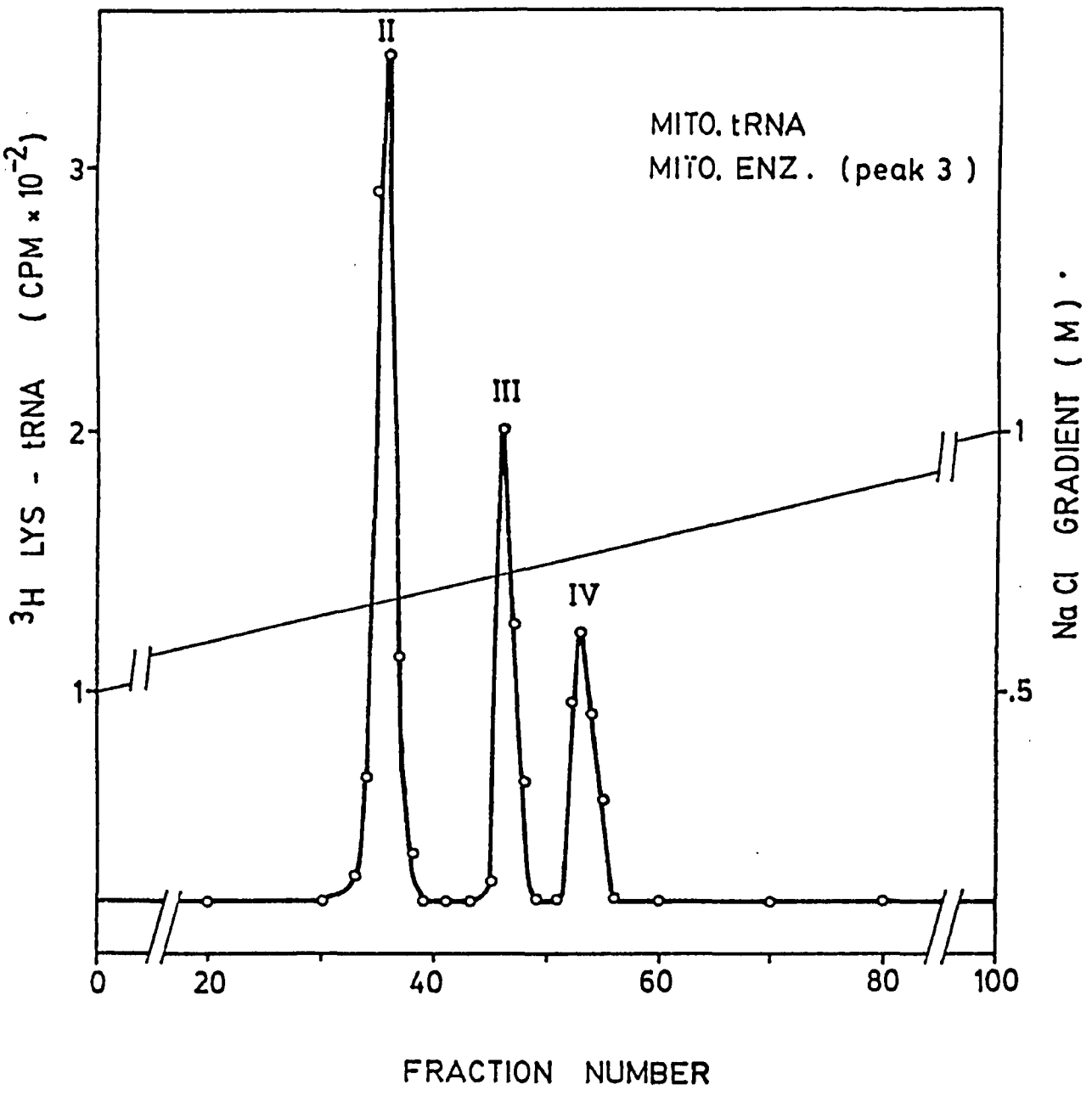

Figure 13 - RPC-5 Fractionation of mitochondrial $tRNA^{Lys}$ aminoacylated with homologous enzyme peak 3

The aminoacylation of mitochondrial $tRNA^{Lys}$ was carried out with the synthetase obtained after fractionation of mitochondrial enzyme on a HA column. The condition for aminoacylation and elution are given in Figure 1.

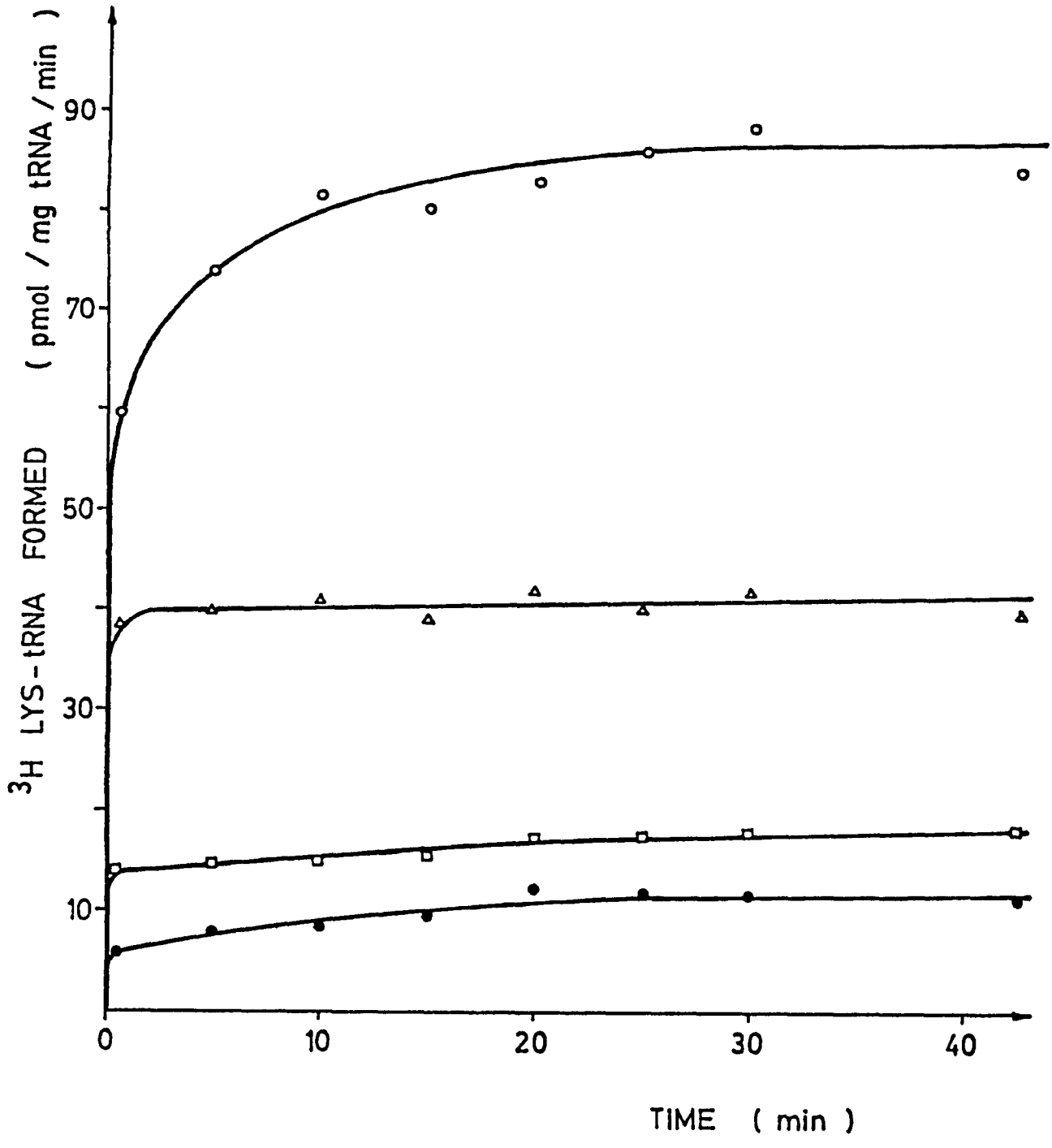

Figure 14 - Comparison of the incorporation of ^{3}H L-Lysine into ^{3}H tRNA^{Lys} of 5 day old tRNA^{Lys} (o——o), 10 day old tRNA^{Lys} (Δ——Δ), 15 day old tRNA^{Lys} (□——□) and 20 day old tRNA^{Lys} (●——●).

of the incorporation of ^{3}H lysine into ^{3}H lysyl-tRNAs from 5, 10, 15 and 20 day old cotyledons. A 53% loss of ^{3}H lysine incorporated into ^{3}H lysyl-tRNA can be noted between 5 day old and 10 day old tRNA. However, a more dramatic loss is observed when 5 and 15 day old tRNAs, charged by homologous enzymes, are compared (78.75%). This loss is even more pronounced between 5 and 20 day old tRNAs (85%). The cotyledons serve as nutrient suppliers for growing embryonic axis for 6-7 days following the onset of germination. With age this ability decreases. This loss of role as nutrient supplier may be reflected in decreased amino acid acceptor ability in older cotyledons. Figures 15 and 16 represent quantitative changes in 10 and 15 day old $tRNAs^{Lys}$ charged by homologous enzymes, with pronounced changes in 15 day old $tRNA^{Lys}$ (Figure 16). As cotyledons continue to age both quantitative and qualitative changes occur in $tRNAs^{Lys}$. RPC-5 chromatography of 20 day old $tRNA^{Lys}$, charged by homologous enzyme shows only four peaks of activity (Figure 17). The disappearance of one of isoacceptor tRNAs may be attributed to the inability of 20 day old lysyl-tRNA synthetase to aminoacylate efficiently all $tRNA^{Lys}$ isoacceptors in aging cotyledons. The relative amounts of each of five $tRNA^{Lys}$ isoacceptors are summarized in Table 2. It is clear from this table that the amount of lysyl-tRNAs in soybean cotyledons is reduced with age. Quantitative levels of lysyl-tRNA isoacceptors varied depending on the stage of germination. The changes are more pronounced in $tRNA^{Lys}_{2}$, $tRNA^{Lys}_{3}$, and $tRNA^{Lys}_{4}$. Decrease in the level of aminoacylation of these isoacceptors and the most dramatic change, the disappearance of $tRNA^{Lys}_{5}$, is most probably the result of decreased ability of lysyl-tRNA synthetase to aminoacylate certain isoacceptor tRNAs. In the case of $tRNA^{Lys}_{1}$ a slight

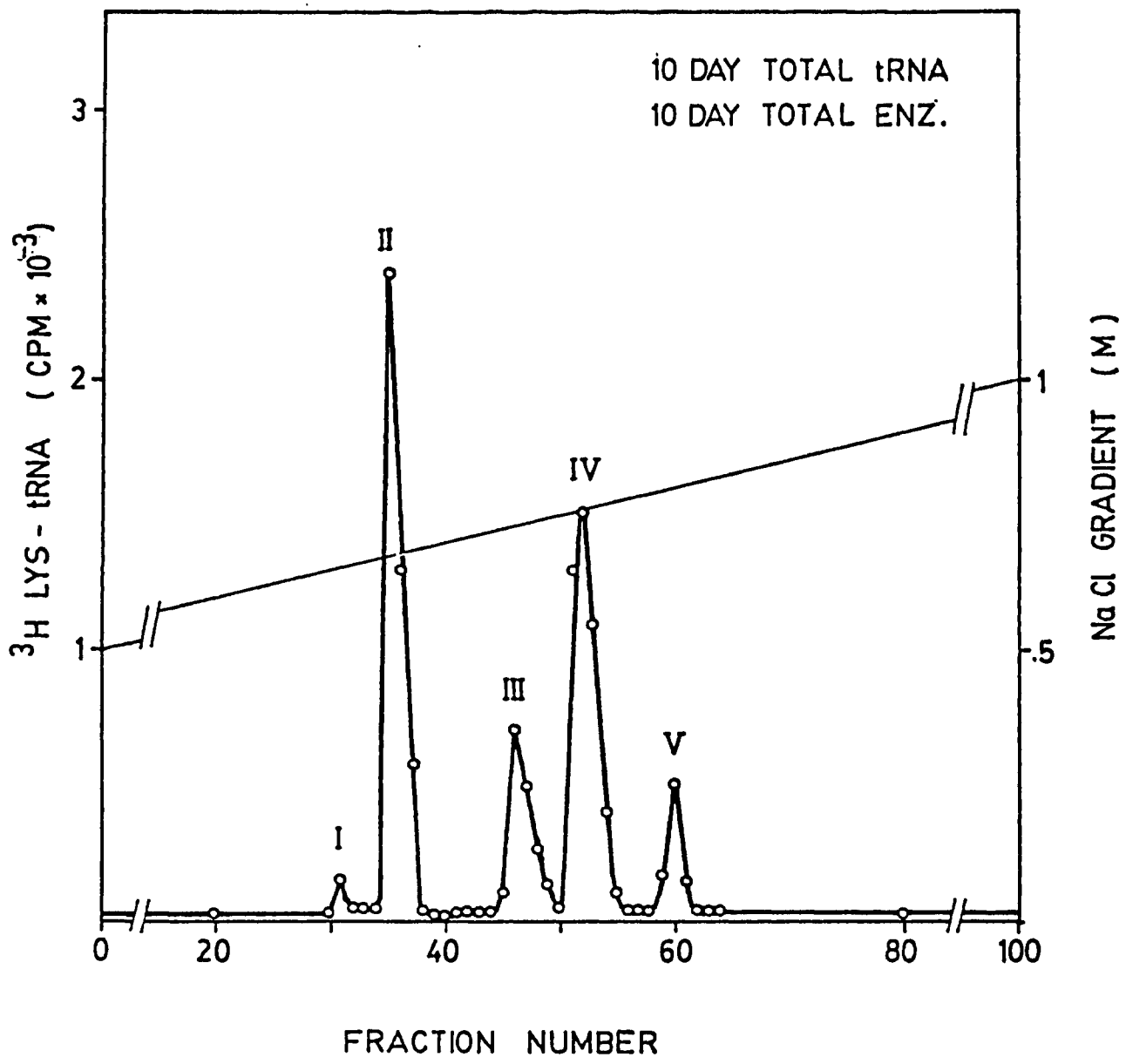

Figure 15 - RPC-5 Fractionation of total cell $tRNA^{Lys}$ aminoacylated by homologous enzyme

Unfractionated tRNA isolated from 10 day old dark grown cotyledons was aminoacylated with homologous enzyme as described in the methods and given in Figure 1. Elution was performed with 2 x 200 ml of a gradient of 0.5 M - 1.0 M NaCl in buffer B. Four ml fractions were collected with a flow rate of 35-40 ml/h as given in Figure 1.

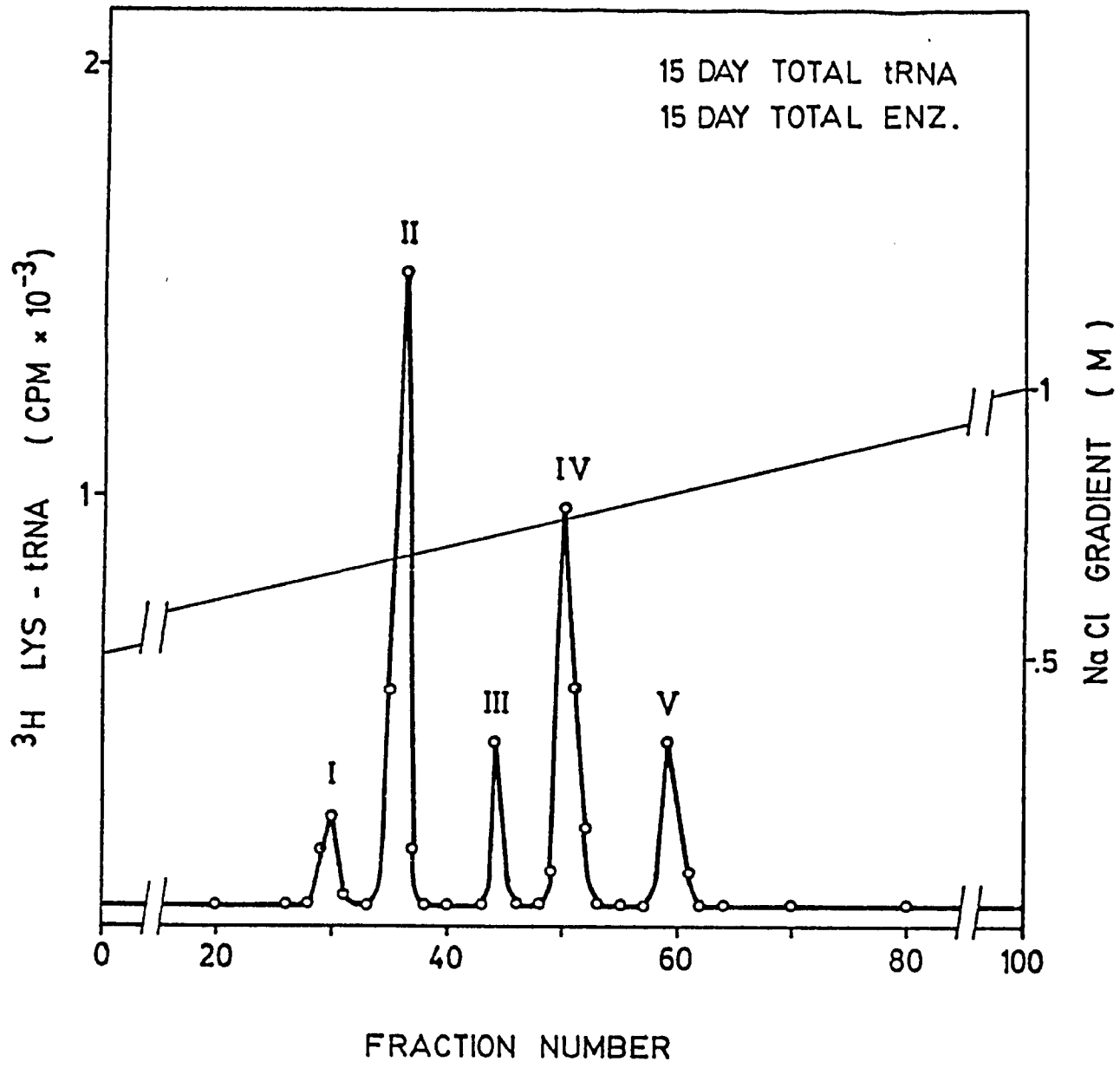

Figure 16 - RPC-5 Fractionation of total cell $tRNA^{Lys}$ aminoacylated with homologous enzyme

Total cell tRNA isolated from 15 day old dark grown cotyledons was aminoacylated with homologous enzyme under the conditions described in the methods and given in Figure 1. Elution was performed with a linear gradient of 0.5 M - 1.0 M NaCl in buffer B as given in Figure 1.

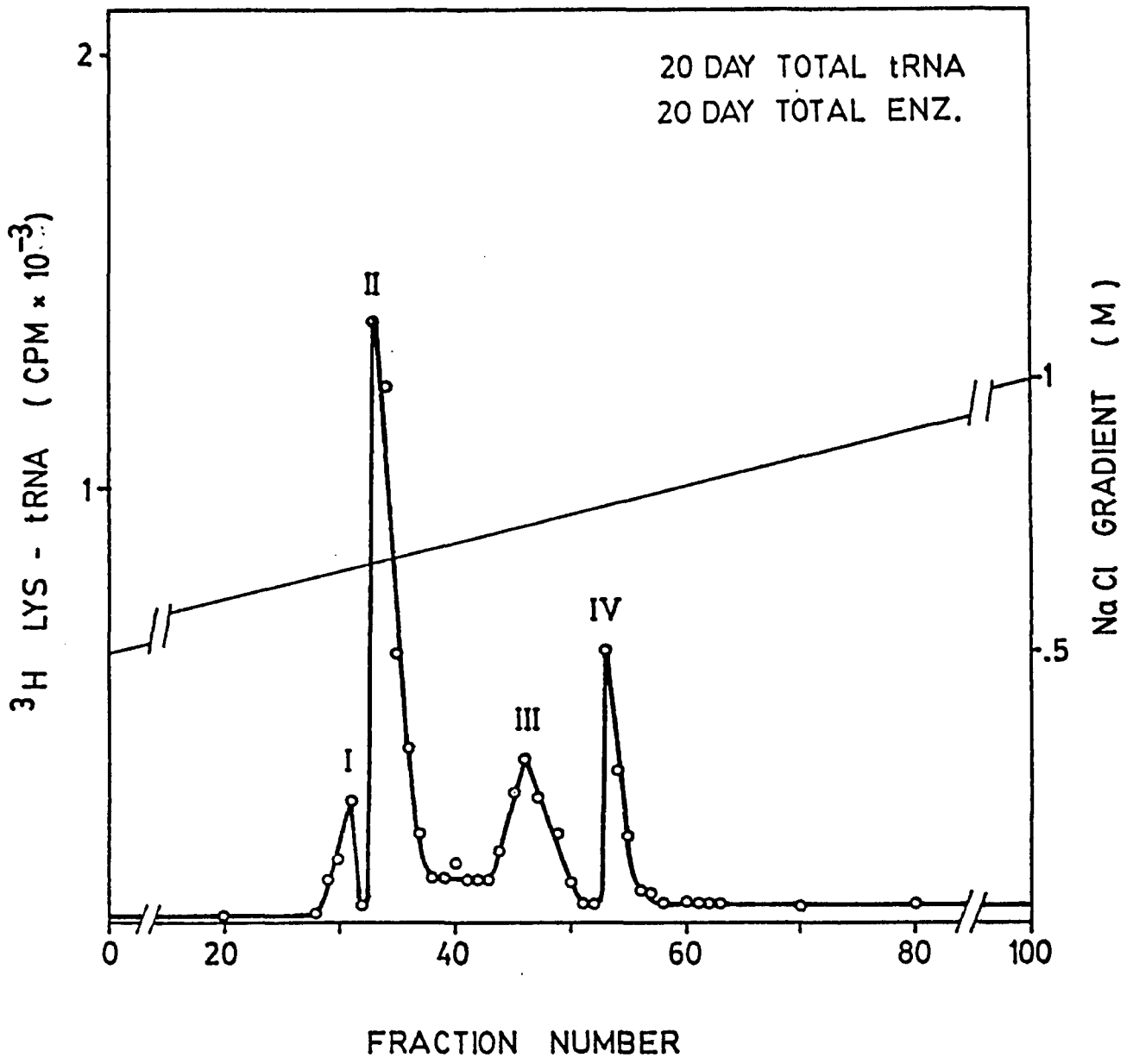

Figure 17 - RPC-5 Fractionation of total cell $tRNA^{Lys}$ aminoacylated with homologous enzyme

The aminoacylation of total cell tRNA isolated from 20 day old dark grown cotyledons was carried out in a standard reaction mixture with homologous enzyme under the same conditons as given in Figure 1. Elution was performed with a linear gradient of 0.5 M - 1.0 M NaCl in buffer B as described in Figure 1.

Table 2: Comparison of $tRNAs^{Lys}$ present in soybean cotyledons at different stages of germination

Relative amounts of each $tRNA^{Lys}$ species % of control

Source of tRNA	$tRNA^{Lys}_1$	$tRNA^{Lys}_2$	$tRNA^{Lys}_3$	$tRNA^{Lys}_4$	$tRNA^{Lys}_5$
5 day*	100	100	100	100	100
10 day	90.0	75.0	85.7	75.0	62.5
15 day	100	46.8	80.0	49.0	50.0
20 day	125	43.0	63.3	31.0	-

* 5 day old tRNA was used as a control

increase in aminoacylation was observed in 20 day old tRNA which could be attributed to an increase in the amount of chloroplast tRNA and chloroplast synthetase. Results presented in Figure 18 may confirm the possibility that age-related alterations in aminoacyl-tRNA synthetase are indeed the cause of inadequate aminoacylation of all $tRNA^{Lys}$ isoacceptors. Upon RPC-5 chromatography 20 day old $tRNA^{Lys}$, charged with 5 day old lysyl-tRNA synthetase yielded five peaks of activity. Elution pattern of these five isoacceptors is similar to elution patterns of 5, 10 and 15 day $tRNAs^{Lys}$ described before. This clearly shows that lysyl-tRNA synthetase from young 5 day old cotyledons has the capacity to aminoacylate all five isoacceptors efficiently whereas the synthetase from old 20 day cotyledons has lost this capacity. This enzyme had the capacity to aminoacylate only four out of five isoacceptors and these to a lesser degree. Moreover, a small increase in aminoacylation was observed in $tRNA^{Lys}_2$ and $tRNA^{Lys}_4$ when elution patterns in Figures 17 and 18 are compared.

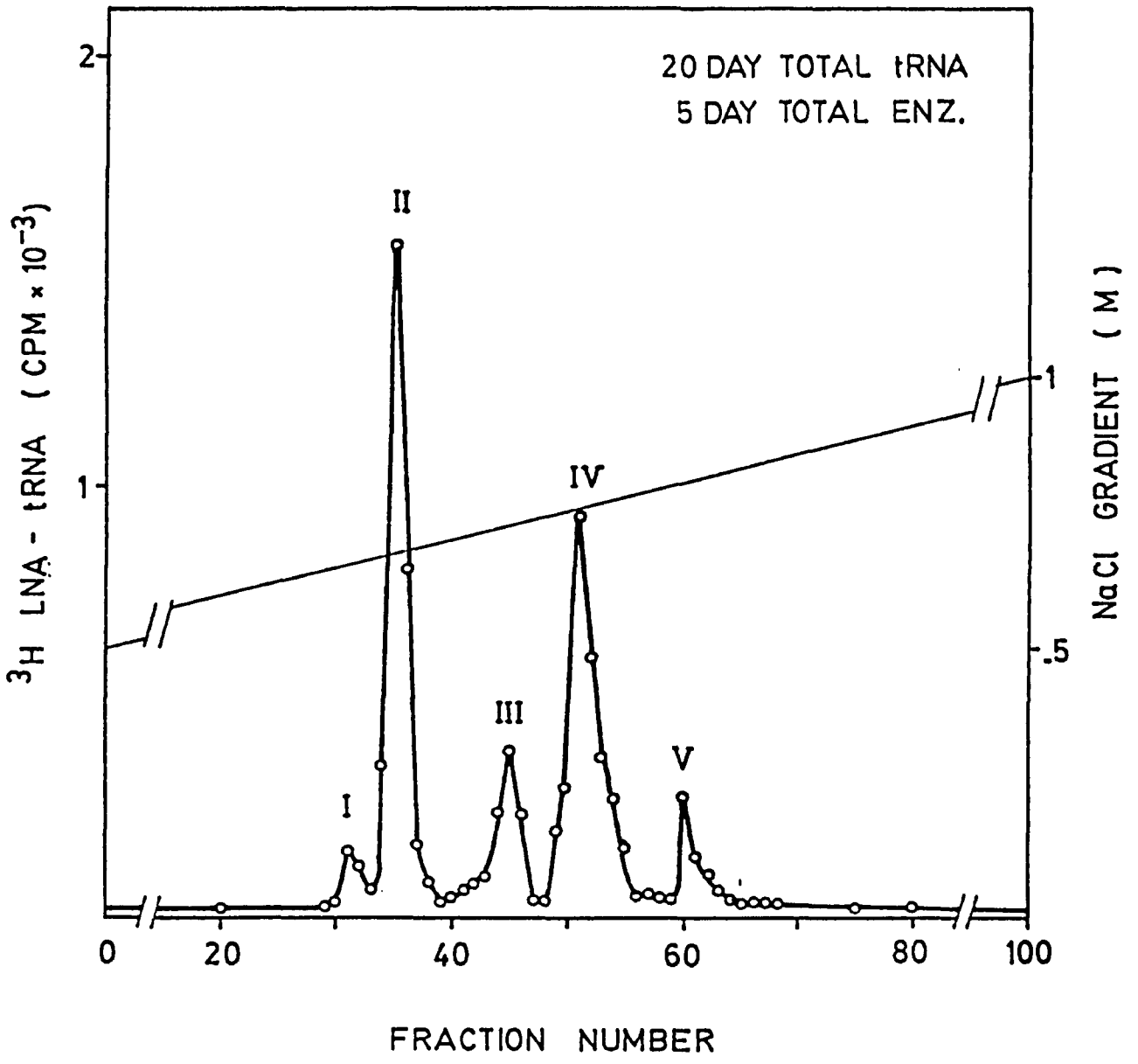

Figure 18 - RPC-5 Fractionation of total cell $tRNA^{Lys}$ aminoacylated with 5 day old enzyme

Total cell tRNA isolated from 20 day old dark grown cotyledons was aminoacylated with 5 day old synthetase. Conditions for aminoacylation are the same as given in Figure 1. Elution was performed in the same manner as given in Figure 1.

DISCUSSION:

Localization of $tRNAs^{Lys}$ and lysyl-tRNA synthetase in three cell compartments:

A great diversity exists in the number of isoaccepting tRNAs and aminoacyl-tRNA synthetases for the same amino acid, as well as for different amino acids among the related and unrelated species. This diversity is even greater when we consider the localization of tRNAs and aminoacyl-tRNA synthetases in each of the three cellular compartments in plant systems, which complicates the situation even further. Although, $tRNA^{Lys}$ respond to two codons (AAA and AAG) more than two isoaccepting $tRNA^{Lys}$ have been described in the eukaryotic systems.

Soybean cotyledons contain five $tRNA^{Lys}$ isoacceptors as revealed by RPC-5 chromatography. The same number of $tRNA^{Lys}$ has been found in the seeds and cotyledons of Lupinus luteus (Augustyniak and Pawelkiewicz, 1978), whereas in Phaseolus vulgaris (Jeannin et al., 1976), Triticum aestivum (Norris et al., 1975), cotton seeds (Merrick and Dure, 1972), apple and pear (Romani et al., 1975), and ethylene ripened tomato (Mettler and Romani, 1976) only four lysine isoaccepting tRNAs have been detected.

RPC-5 chromatography of soybean chloroplast ^{3}H $tRNA^{Lys}$ resulted in only one chloroplastic $tRNA^{Lys}$. Exactly similar findings were reported in Phaseolus vulgaris (Jeannin et al., 1976), Lupinus luteus (Augustyniak and Pawelkiewicz, 1978) and in cotton seedlings (Merrick and Dure, 1972). Investigations in our laboratory have shown that soybean cotains one chloroplastic $tRNA^{Phe}$ and $tRNA^{Trp}$ (Swamy and Pillay,

1982) and two chloroplastic $tRNA^{Tyr}$ (Swamy and Pillay, 1982), and $tRNA^{Leu}$ (Sinclair and Pillay, 1981). On the other hand in soybean mitochondria and the cytoplasm there are three and four $tRNAs^{Lys}$ respectively. Among them $tRNA^{Lys}$ species 2, 3 and 4 are common to both the mitochondria and the cytoplasm. Such observations have been made in other plants as in Lupinus luteus (Augustyniak and Pawelkiewicz, 1978) and Phaseolus vulgaris (Jeannin et al., 1976) and $tRNA^{Leu}$ in soybean (Sinclair and Pillay, 1981) and Phaseolus vulgaris (Guillemaut et al., 1975).

Aminoacyl-tRNA synthetases from the three cell compartments differ in their subcellular localization, substrate specificity and chromatographic mobility. This last property of aminoacyl-tRNA synthetases facilitates their separation on hydroxylapatite column. Total Lys-tRNA synthetase from soybean cotyledons resolves into three peaks of activity upon hydroxylapatite column chromatography. One isoenzyme appears to be located in each of the three cell compartments. Chloroplastic and mitochondrial enzymes are distinguishable from each other, based on their aminoacylation specificity. In contrast, Phaseolus vulgaris Lys-tRNA synthetases from chloroplast and mitochondria (Jeannin et al., 1976), Phe-tRNA synthetase, Tyr-tRNA synthetase and Trp-tRNA synthetase from soybean are undistinguishable from each other (Swamy and Pillay, 1982). In these systems only two enzyme peaks are revealed by hydroxylapatite column chromatography and isoenzyme 1 is localized in the chloroplast and in the mitochondria whereas isoenzyme 2 is localized in the cytoplasm.

In order to determine the specificity of each isoenzyme homologous and heterologous aminoacylations were performed. Total cell isoenzyme 1 and chloroplast enzyme preferentially aminoacylated $tRNA^{Lys}_1$,

which is localized in the chloroplast. Total cell isoenzyme 2 aminoacylated $tRNA^{Lys}$ isoacceptors 2, 3, 4, and 5, which are localized in the cytoplasm. Mitochondrial enzyme (peak 3 from HA column) aminoacylated $tRNA^{Lys}$ isoacceptors 2, 3 and 4. However, mitochondrial $tRNA^{Lys}_2$ is aminoacylated to a larger extent than the other two isoacceptors ($tRNA^{Lys}_3$ and $tRNA^{Lys}_4$). Similar results have been shown with $tRNA^{Leu}$ from soybean (Kanabus and Cherry, 1971; Sinclair and Pillay, 1981). Although, aminoacyl-tRNA synthetases show a strict specificity toward cognate tRNAs cross-aminoacylation reactions between tRNAs and aminoacyl-tRNA synthetases from different cell compartments are possible.

In any investigation concerning the localization of tRNAs and aminoacyl-tRNA synthetases, it is essential that pure intact organelles, free from cytoplasmic contamination are used. In our work this seemed to be a constant hindrance. It was virtually impossible to obtain pure organellar or cytoplasmic tRNAs and aminoacyl-tRNA synthetases without any cross-contamination. Fractionation on hydroxylapatite column did not achieve enzyme preparations entirely specific for cytoplasm or organelles. Similar problems have also been encountered by other workers (Puttney et al., 1981; Guillemaut and Weil, 1975a; Guillemaut et al., 1975b; Jeannin et al., 1976).

There appears to be more instances of recognition between chloroplast synthetase and prokaryotic tRNA than between these enzymes and eukaryotic tRNA (Weil, 1979). This is reflected in the charging of *E. coli* $tRNA^{Leu}$ by chloroplast Leu-tRNA synthetase from cotton (Brantner and Dure, 1975), bean (Guillemaut et al., 1975), soybean (Sinclair and Pillay, 1981) and *Euglena* (Parthier and Krauspe, 1973). We have found

that Lys-tRNA isoenzyme 1 from soybean chloroplast aminoacylated E. coli $tRNA^{Lys}$ and chloroplast $tRNA^{Lys}$ to a great extent. Similar results were reported in the case of Phaseolus vulgaris Lys-tRNA synthetase (Jeannin et al., 1976), and Phe-, Tyr-, and Trp-tRNA synthetase from soybean (Swamy and Pillay, 1982). The high degree of affinity between bacterial and chloroplast tRNAs and synthetases shows that the genome of higher plant chloroplasts are more closely related to genomes of prokaryotes than to higher plant nuclear genomes.

Most studies concerning organellar and cytoplasmic enzymes did not include mitochondrial tRNAs and synthetases, due to a low amount of these macromolecules in a plant cell and the inability to obtain pure preparations of mitochondrial tRNAs and aminoacyl-tRNA synthetases. As far as substrate specificity of mitochondrial synthetase is concerned, the enzyme shows in some instances affinity toward cytoplasmic tRNAs and in other cases toward E. coli and chloroplast tRNAs. Mitochondrial Leu-tRNA synthetase from Euglena gracilis (Muller et al., 1981) is similar to chloroplastic enzyme in its elution pattern, kinetic property and tRNA specificity. Similar results were obtained with isoenzymes of Pro-, Phe-, and Lys-tRNA synthetase of Phaseolus vulgaris (Jeannin et al., 1976, 1978) and Phe-, Trp-, and Tyr-tRNA synthetases of soybean (Swamy and Pillay, 1982). However, mitochondrial Leu-tRNA synthetase from soybean (Sinclair and Pillay, 1981) and from Phaseolus vulgaris (Guillemaut et al., 1975) appear to be similar to the cytoplasmic enzyme. It has been found that these isoenzymes readily aminoacylate mitochondrial as well as cytoplasmic tRNAs (Sinclair and Pillay, 1981; Guillemaut et al., 1975). Such similarity has also been reported in tobacco (Guderian et al., 1972) and

Euglena (Kislev et al., 1972). Thus it is clear that mitochondrial tRNA synthetase sometimes behaves like bacterial and/or chloroplastic synthetase and at other times like a cytoplasmic synthetase depending upon the amino acid in question.

Mitochondrial Lys-tRNA synthetase from soybean appears to resemble chloroplastic enzyme in its substrate specificity, as revealed by cross-aminoacylation reactions presented in Table 1. However, RPC-5 fractionation of mitochondrial tRNA, aminoacylated by isoenzyme 3, (obtained after fractionation of mitochondrial enzyme on HA column), revealed that this isoenzyme charged three $tRNA^{Lys}$ isoacceptors. The $tRNA^{Lys}$ isoacceptors 2, 3, and 4 are observed in the total cell as well as in the mitochondrial chromatograms. This raises the question whether these three species are common to both subcellular fractions. Similar problem has been encountered with $tRNA^{Lys}$ from Lupinus luteus (Augustyniak and Pawelkiewicz, 1978). These authors (Augustyniak and Pawkiewicz, 1978) speculated that certain $tRNA^{Lys}$ isoacceptors, which in Lupinus luteus were common to all three cell compartments, may belong to those organellar tRNAs that are coded for by the nuclear genome and appear in cytoplasm before they are imported into chloroplasts and mitochondria. Presently, nothing conclusive is known about the localization of all tRNA genes in mitochondria of higher plants. However, the nuclear origin of some mitochondrial tRNAs has been reported in Tetrahymena pyriformis (Chiu et al., 1975) and in Saccharomyces cerevisiae (Martin et al., 1979). Some or all of the mitochondrial $tRNA^{Lys}$ of Glycine max may have been transcribed on nuclear genome and then transported into mitochondria. In the mitochondria polynucleotide chains of these tRNAs may have been then post-transcrip-

tionally modified by methylation to such an extent that they are now recognized by the mitochondrial or E. coli enzyme (Dubois et al., 1974). Studies have shown that the mitochondria possess at least one methylase which is absent from the cytoplasm (Klagsbrun, 1973). This methylase may be capable of modifying the tRNA and rendering it recognizable by mitochondrial synthetase. However, it is not known whether these organellar tRNA methylases function in the tRNAs coded for by organellar or by nuclear DNA. Aminoacylation of $tRNA^{Lys}$ isoacceptors 2, 3, and 4 of mitochondrial tRNA by E. coli enzyme and by mitochondrial isoenzyme 3 may suggest that these three isoacceptors are common to cytoplasm as well as to mitochondria. The detection of modified (or unmodified) tRNAs by currently used chromatographic methods may be limited.

The solution to the problem of possible origin of mitochondrial lysyl-tRNAs in soybean may be obtained by evaluating the coding specificity of each of the individual $tRNA^{Lys}$ isoacceptors. If particular $tRNA^{Lys}$ isoacceptors are indeed transcribed on nuclear genome and then transported into the mitochondria, then they would exhibit the same or similar coding specificity as the corresponding cytoplasmic tRNAs. An additional tool in providing the evidence of the origin of mitochondrial $tRNAs^{Lys}$ is DNA-tRNA hybridization experiments. Selective hybridization of some or all of mitochondrial $tRNA^{Lys}$ isoacceptors to nuclear genome but not to mitochondrial may suggest the nuclear origin of these particular isoacceptors. In any one of these sets of experiments a great care should be taken to ensure pure DNA and tRNA preparations without any trace of cross-contaminations.

Aging:

Studies based on the chromatographic elution profiles of isoaccepting tRNAs from organisms at different stages of development and aging have shown alterations in tRNAs and aminoacyl-tRNA synthetases. It has been suggested (Strehler, 1966; Strehler et al., 1971) that changes in isoaccepting tRNAs and/or their synthetases may play a role in the aging process.

Quantitative and qualitative changes in $tRNA^{Lys}$ and in lysyl-tRNA synthetase have been observed in aging soybean cotyledons. Similar changes have been observed in $tRNA^{Leu}$ and $tRNA^{Tyr}$ during senescence of soybean cotyledons (Bick et al., 1970; Pillay and Cherry, 1974), in $tRNA^{Leu}$ in pea seeds (Patel and Pillay, 1976) and in $tRNA^{Lys}$ and $tRNA^{Pro}$ in wheat grain (Norris et al., 1975). Emergence and disappearance of certain tRNA isoacceptors specific for $tRNA^{Phe}$ has been observed during the seedling growth in barley (Hiatt and Snyder, 1973). Comparison of the elution profiles of $tRNA^{Lys}$ have revealed that the age-related changes are more pronounced in certain isoacceptors (Table 2). It is tempting to speculate here that $tRNA^{Lys}$ peak 2 and 4, in which these changes are more pronounced, could be of mitochondrial origin. The presence of these peaks in mitochondrial $tRNA^{Lys}$ (Figures 2, 12) is possibly a reflection of a number of hydrolytic enzymes synthesized for degradation of stored products in the cotyledons.

The increase in chloroplastic $tRNA^{Lys}$, observed in aging soybean cotyledons, also noted in the developing lupin seeds (Augustyniak and Pawelkiewicz, 1975) has been attributed to the increase in the amount of

chloroplast tRNA in these fractions especially as the levels of chloroplast enzymes increase in germinating cotyledons (Merrick and Dure, 1972).

Decrease in protein synthesis, a factor that often accompanies aging of tissues and organs may be attributed to one or more of the different components: decreased availability of mRNA, changes in the amount of tRNA species, aminoacyl-tRNA species and various factors (initiation, elongation, termination, etc.). Age-related changes observed in $tRNA^{Lys}$ of aging soybean cotyledons are probably a result of conformational changes (Rothstein, 1977) in aminoacyl-tRNA synthetases which may affect the extent of tRNA aminoacylation. There is also a possibility for the existence of repressors in old cotyledons, which may react with aminoacyl-tRNA synthetase. The enzyme conformation may thus be altered (Bick et al., 1972) which in turn may result in incomplete aminoacylation of all tRNA isoacceptors. The aminoacyl-tRNA synthetases of eukaryotic plant cells usually participate in large multimolecular complexes which contain various factors. Changes in these factors may affect the functioning of aminoacyl-tRNA synthetases. Enzymatic catalysis occurs by slightly different mechanism according to whether the enzyme is free or associated in complexes (Katchalki et al., 1971).

The presence of one $tRNA^{Lys}$ in the chloroplasts of soybean, revealed by RPC-5 chromatography raised a question of possible presence of $tRNA^{Lys}$ gene on the circular chloroplast DNA. Unfortunately, the attempts to localize this gene by DNA:tRNA hybridization experiments have failed. Several reasons may account for this failure, inefficient removal of CCA terminus by snake venom phosphodiesterase, the inability of yeast nucleo-

tidyl transferase to attach the radioactive label to the 3' end of tRNA and possible inability of radioactive probe to hydridize to the DNA. Our work was performed on the basis of previously reported results (Steinmetz and Weil, 1976). It may be necessary to carry out a full range of kinetic experiments for the removal of CCA terminus by snake venom phosphodiesterase and also for the attachment of [^{32}P] ATP by nucleotidyl transferase. The choice of hybridization temperature may be a crucial factor determining the success in these experiments. Our choice of hybridization temperature of 37°C, based on previous experience by other workers (Driesel et al., 1979; Steinmetz and Weil, 1976) may have not been the best choice.

SUMMARY

1. Total $tRNA^{Lys}$ fractionates into five peaks of activity on RPC-5 column.

2. Total lysyl-tRNA synthetase fractionates into three peaks of activity on HA column.

3. Organellar lysyl-tRNA synthetases (chloroplastic and mitochondrial) fractionate into one major peak each on HA.

4. Cross-aminoacylation reactions have revealed that:
   a) total enzyme peak 1 and chloroplastic enzyme preferentially aminoacylate chloroplast and E. coli $tRNAs^{Lys}$;
   b) total enzyme 2 aminoacylates total and yeast $tRNA^{Lys}$;
   c) mitochondrial and total enzyme 2 aminoacylate mitochondrial and to some extent E. coli $tRNA^{Lys}$.

5. Fractionation of chloroplast $tRNA^{Lys}$ on RPC-5 yielded one peak of activity after acylation with chloroplast (HA) or E. coli enzyme. This peak of activity co-incides with $tRNA^{Lys}_1$ of total.

6. Fractionation of total $tRNA^{Lys}$ on RPC after acylation with total enzyme peak 2 yielded four peaks of activity, isoacceptors 2, 3, 4, and 5.

7. Fractionation of mitochondrial $tRNA^{Lys}$ on RPC-5 after acylation with mitochondrial enzyme (HA) or E. coli enzyme revealed three isoacceptors in mitochondria, tRNAs 2, 3, and 4.

8. Age-related quantitative and qualitative in senescing soybean cotyledons:
   - a loss in amino acid acceptor activity;
   - disappearance of one of tRNA isoacceptors.

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