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THE CHARACTERIZATION OF LYSINE TRANSFER RNA
AND LYSYL-tRNA SYNTHETASE FROM
GERMINATING BARLEY ENDOSPERM

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ABSTRACT

Lysine tRNA and lysyl-tRNA synthetase were studied in barley (Hordeum vulgare L. cv. Atlas 57) endosperm. Six isoaccepting species of lysine tRNA were present in all tissues examined. RPC-5 chromatography revealed no differences in the lysine tRNA profiles from embryo-less half-seeds, starchy endosperm and isolated aleurone layers. Lysine tRNA profiles did not change during the first 48 hours of germination, in embryo-less half-seeds incubated in the presence and absence of gibberellic acid. The lysine analog S-2-aminoethyl-L-cysteine (AEC) was shown to be a competitive inhibitor of lysine in the lysyl-tRNA synthetase reaction. The K_m and K_i values for lysine and AEC were determined to be in the micromolar range. The usefulness of the parameters measured and the methods developed for the characterization of amino acid analog-resistant mutants is discussed.

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CHAPTER I

INTRODUCTION

Barley aleurone has been the subject of extensive physiological study over the past 20 years. Much of this effort has involved characterizing the effects of the plant growth regulator gibberellic acid (GA). Induction and synthesis of hydrolases such as α -amylase and ribonuclease (RNase), GA induction of poly(A)-messenger ribonucleic acid (mRNA) formation, and translation have been well established (Briggs, 1978; Chrispeels and Varner, 1967; Jacobsen and Zwar, 1974; and Jones and Stoddart, 1977). Although the effects of GA on many aspects of aleurone metabolism have been characterized, the effects of GA on transfer RNA (tRNA) have not been examined.

This thesis will characterize lysine tRNA in germinating barley (Hordeum vulgare L. cv. Atlas 57) endosperm tissue. Emphasis will be placed on the identification of lysine tRNA isoaccepting species present in dormant and germinating aleurone tissue and the ability of these species to aminoacylate the lysine analog S-2-aminoethyl-L-cysteine (AEC).

Figure I shows the structure of the barley seed. The endosperm tissue is composed of the starchy endosperm and the aleurone. The aleurone is usually a three cell thick layer surrounding the starchy endosperm. It is the only respiring part of the endosperm (Paleg, 1960). Upon imbibition in the quiescent seed, GA is secreted by the embryo and scutellum and moves to the aleurone where

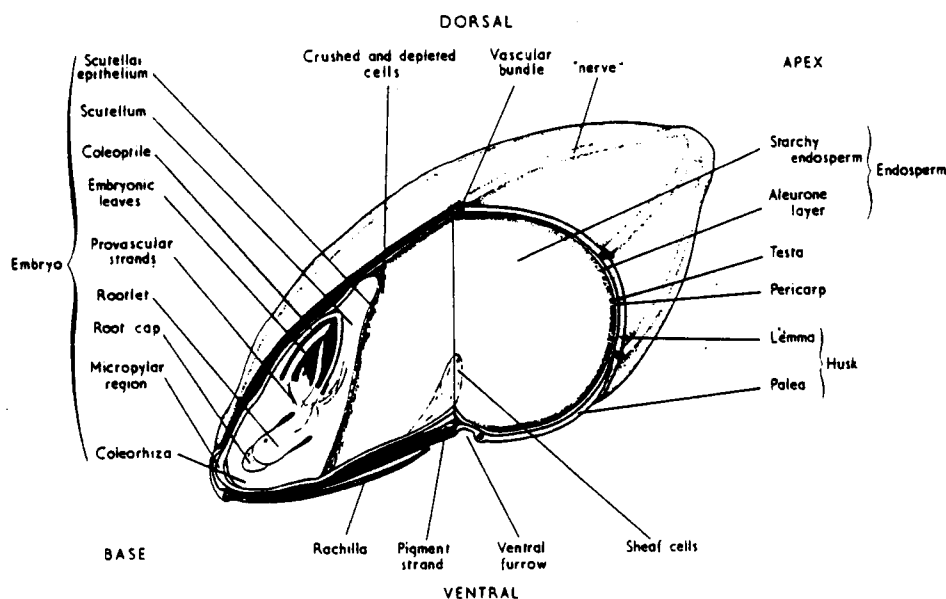
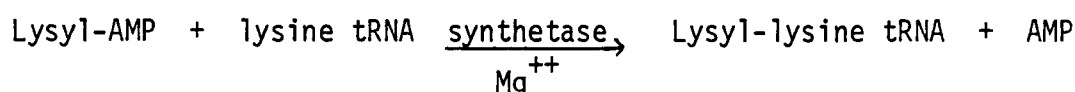
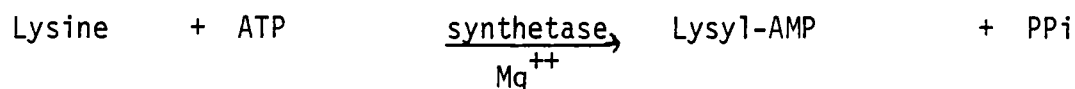


Figure I. Diagram of a barley grain. The aleurone layer, located between the testa and the starchy endosperm, is three cells thick and is the only respiring tissue in the endosperm. The aleurone layer originates from the fusion of the polar nuclei with one of the pollen nuclei. Source: Briggs, D. E., 1973, *Germinating cereal grains*, In *Biosynthesis and Its Control in Plants*, ed. B. V. Milborrow, Academic Press, London, pp. 219-277.

it initiates a number of metabolic events including the synthesis of mRNA and hydrolytic enzymes.

Alpha-amylase, one of the hydrolytic enzymes synthesized in response to GA, makes up 60% of the total protein synthesized by the aleurone tissue (Mozer, 1980). Twenty-two of its amino acid residues are lysine (Rodaway, 1978). In other systems where a tissue produces primarily a single protein, specific tRNAs have been shown to regulate protein synthesis (Sprague et al., 1977). Transfer RNAs have not been examined for their role in regulating protein synthesis in barley aleurone tissue.

Transfer RNAs are aminoacylated by the group of enzymes known as the aminoacyl-tRNA synthetases (EC 6.1.1). The aminoacylation reaction for lysine tRNAs is as follows:

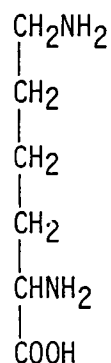


Both reactions are catalyzed by the enzyme lysyl-tRNA synthetase (EC 6.1.1.6). In the first reaction the pyrophosphate group of ATP is displaced by lysine to form lysyl-adenylic acid. This intermediate has the carboxyl group of lysine in a high energy anhydride bond with the 5'-phosphate of AMP. The second reaction is a transfer of the lysyl group from AMP to the 3' end of the lysine tRNA. The two reactions combined yield a small drop in free energy because much of the energy released by breaking the high energy bond of ATP, and subsequently the hydrolysis of the pyrophosphate, is conserved in the high energy ester linkage of lysine to the 3'-adenylic acid residue of the tRNA (Lehninger, 1975).

Transfer RNA isoaccepting species are families of tRNAs that may be aminoacylated to the same amino acid. These may arise from different gene products or may be from the same molecule that has undergone different post-transcriptional modifications (Littauer and Inouye, 1973). The number and relative amounts of tRNA isoacceptors has been demonstrated to change over development in some tissues (Shugart, 1972 and Chu-Der and Ortwerth, 1980).

Figure II shows that AEC is a structural analog of lysine. Henke et al. (1981) have shown that AEC inhibits the expression of α -amylase in aleurone. This inhibition may be reversed when higher concentrations of lysine are present. Furthermore, α -amylase synthesized in the presence of AEC has an altered electrophoretic mobility on nondenaturing gels suggesting that AEC may become incorporated into α -amylase (Mullin and Thompson, unpublished). For AEC to be incorporated into proteins, it is necessary that AEC be aminoacylated onto tRNA.

LYSINE



S-2-AMINOETHYL-L CYSTEINE

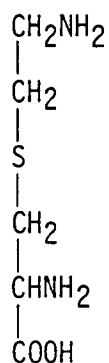


Figure II. Chemical structures of the amino acid lysine and one of its analogs S-2-aminoethyl-L-cysteine (AEC).

This thesis served the following objectives: To develop methods for the extraction of tRNA from barley endosperm tissue; To determine the number and relative amounts of lysine tRNA

isoaccepting species in dormant barley aleurone tissues; To examine lysine tRNA profiles during the first 48 hours of germination; To determine the effect of GA on the lysine tRNA profiles from germinating barley endosperm; To determine the effect of AEC on the lysyl-tRNA synthetase reaction; And to determine an apparent K_m for lysine and an apparent K_i for AEC in the lysyl-tRNA synthetase reaction.

CHAPTER II

LITERATURE REVIEW

Barley aleurone has proven amenable to physiological study. The aleurone layer is easily separated from the embryo, consists of only one cell type, and, in the mature grain, is fully developed and does not undergo further differentiation (Jones and Jacobsen, 1978). Upon imbibition, the aleurone of the quiescent seed begins a highly ordered sequence of metabolic events. Gibberellic acid is the only plant hormone known to participate in this sequence by inducing protein formation both in vivo and in vitro in aleurone cells. Finally, the response of this tissue to GA occurs independently and is not affected by other substrates (Jones and Jacobsen, 1978).

The effects of GA on barley aleurone metabolism have been extensively studied at several levels (e.g., enzyme activation, transcription, translation and secretion). Although GA has no effect on the total amount of tRNA (4S) in aleurone (Jacobsen and Zwar, 1974), studies on specific isoaccepting species have not been performed. Isoaccepting species of phenylalanine and leucine tRNA have been characterized from barley embryo (Hiatt and Snyder, 1973; Labuda et al., 1974; and Karwowska and Augustyniak, 1977).

Amino acid analogs have been widely used in biochemical investigations (Lea and Norris, 1976). Many plants naturally produce amino acid analogs; the metabolism of these plants has adjusted to function properly in the presence of these potential

inhibitors. In non-resistant plants, amino acid analogs have been known to decrease enzyme activity in two ways: by blocking enzyme synthesis or by becoming incorporated into proteins, causing changes in the final structure of the enzymes, and thus altering their catalytic activity (Lea and Norris, 1976).

The following is an overview of the literature in the areas of germination physiology, tRNA physiology and amino acid analogs.

I. GERMINATION PHYSIOLOGY--THE EFFECTS OF GA ON ALEURONE TISSUE

The role of GA in the germination of barley seeds has been studied extensively. The hydrolysis of the starchy endosperm was first observed in 1890 (Briggs, 1972). Paleg (1960) was the first to propose that this hydrolysis was due to enzymes synthesized in response to GA. As mentioned above, GA is a plant growth regulator secreted by the embryo and scutellar tissues after dormant seeds have undergone imbibition (Briggs, 1972). The hormone then moves to the aleurone tissue where it has been shown to affect the activation, synthesis and release of enzymes, the level of some mRNA species, the development of internal membranes and the breakdown of cell walls. Alpha-amylase, one of the first enzymes shown to be affected by GA, has been studied most intensively. Recently work has focused on the characterization of α -amylase mRNAs and the nucleotide sequences of the genes coding for α -amylase, on the identification and characterization of α -amylase isozymes, and on the elucidation of the mechanism of α -amylase secretion from aleurone cells. These topics will be discussed in more detail below.

The Effect of GA on Hydrolytic Enzymes

Chrispeels and Varner (1967) were the first to show that GA promoted the synthesis and release from barley aleurone of two hydrolytic enzymes, α -amylase and RNase. They showed that upon GA induction, α -amylase activity increased in a linear fashion after a 6 to 8 hour lag. Experiments that soon followed, using isopycnic centrifugation, showed GA induction of de novo synthesis of α -amylase, protease, RNase, and β -1,3-glucanase (Jacobsen and Varner, 1967 and Bennett and Chrispeels, 1972). Ashford and Jacobsen (1974) analyzed the effect of GA on phosphatase activity. Their results showed no increase in the enzyme activity or synthesis due to the presence of GA. However, GA did promote the breakdown of aleurone cell walls and thus the release of phosphatase that had collected outside of the aleurone cell protoplasm. Recently, Baisted and Stroud (1982) showed that lysophospholipase activity was enhanced by GA. The activity of this enzyme during germination was correlated with that of α -amylase. Lysophospholipase causes the release from amylose helices of compounds such as lysophosphatidylcholine, a known membrane fusing agent. Baisted and Stroud (1982) suggested that the increased activity of the lipase enzyme could play a role in the secretion of hydrolases from aleurone cells.

GA Induced Synthesis of α -Amylase mRNA

The transcription inhibitor actinomycin-D was shown to inhibit the GA stimulated activity of several hydrolytic enzymes (Chrispeels

and Varner, 1967 and Jacobsen and Varner, 1967). This led Jacobsen and Zwar (1974) to examine the possible role of GA in nucleic acid synthesis. They found that GA did not increase the overall amounts of DNA or RNA. Although the amounts of ribosomal RNA (rRNA), mRNA, 5S rRNA and tRNA did not increase in response to GA, they did find larger amounts of poly(A)-mRNA in GA treated aleurone cells.

Higgins et al. (1976) showed that mRNA for α -amylase was included in this GA induced poly(A)-mRNA. Alpha-amylase mRNA was first purified and characterized from barley by Mozer in 1980. Using a cDNA of α -amylase mRNA Bernal-Lugo et al. (1981) showed that exposure of barley aleurone to GA did cause an increase in the level of α -amylase mRNA. However, due to the fact that Bernal-Lugo used an enriched rather than a pure RNA preparation, their results were questionable. Using recombinant techniques, Muthukrishnan et al. (1983) obtained colonies of bacteria that have plasmids containing cDNAs of α -amylase mRNA. They characterized the largest clone by restriction mapping and used the cloned DNA to locate copies of the α -amylase gene in embryo DNA. They found multiple copies of the α -amylase gene. Analysis of the RNA extracted from seeds with the cDNA probe confirmed the results of Bernal-Lugo which had suggested the de novo synthesis of mRNA in response to GA. The RNA synthesis was blocked, however, by cycloheximide, a protein synthesis inhibitor. This led Muthukrishnan et al. to conclude that GA induced mRNA synthesis requires some protein factor for expression of the α -amylase gene.

α -Amylase Isozymes

Mozer (1980) stated that 60% of all of the protein synthesized in aleurone in response to GA was α -amylase. Jacobsen and Higgins (1982) showed that four α -amylase isozymes were found in Himalaya barley, the variety that has traditionally been used in GA-aleurone studies. They mentioned that the number of isozymes reported varies among barley varieties. Their work, in conjunction with that of Callis and Ho (in press), shows that the four isozymes fall into two groups based on differences in serological characteristics, peptide mapping, time course of production, response to levels of GA, separation using isoelectric focusing, calcium ion requirement, sensitivity to low pH and sensitivity to sulfhydryl reagents. Group A isozymes have a high pI (5.8), appear when a low concentration of GA is present and are more dominant during the first two days of germination. Group B isozymes have a low pI (4.5), require a higher concentration of GA, and are dominant after 4 days of germination. Similar differences in α -amylase isozymes exist in wheat (Triticum aestivum) (Sargeant, 1979) and rice (Oryza sativa) (Motojima and Sakaguchi, 1982). For wheat, the different groups of isozymes showed different substrate specificities. The isozymes synthesized early in germination hydrolyze insoluble polysaccharides, whereas those synthesized later hydrolyze only smaller oligosaccharides. Overall, these results suggest that the two groups of isozymes may originate from different genes with intra-group differences resulting from post-transcriptional processing of mRNA or post-translational processing of the protein.

α -Amylase Secretion

Early studies by Jones (1969) showed that rough endoplasmic reticulum (ER) was more prominent in aleurone tissue incubated in the presence of GA than in tissue incubated in water. Other experiments showed that GA did not induce the de novo synthesis of membranes and ribosomes. Jones and Chen (1976) explained that GA could promote the turnover of membranes rather than the synthesis of membranes from newly formed lipid molecules. An immunoprecipitation analysis of barley and wheat α -amylase synthesis in cell-free translation systems yielded an α -amylase molecule larger than the native active enzyme (Higgins et al., 1976 and Motojima and Sakaguchi, 1982). Jones and Jacobsen (1982) used ^{35}S -methionine to find the intracellular location of α -amylase in barley aleurone cells. They found α -amylase to be associated with ER membranes, and that once in the ER, the α -amylase was resistant to proteolytic degradation. These results, taken together with the knowledge that many secreted enzymes associated with rough ER, have been shown to contain an extra peptide sequence or signal sequence which aids in their secretion, demonstrate that the aleurone tissue is an ideal system to study enzyme secretion in plants. Jones and Jacobsen (1982) reported that out of five α -amylase isozymes detected, four were secreted. Further work on α -amylase secretion in barley is being done. The processing of α -amylase from rice scutellum is being analyzed by Miyata and Akazawa (1982). They analyzed the glycosylation reaction of α -amylase isozymes by inhibiting the process with the antibiotic

tunicamycin. Glycosylation is often done on the ER membrane during the secretion process. Barley and rice α -amylase are glycoproteins. Using an in vitro translation system, Miyata and Akazawa found that glycosylation had no effect on the number of α -amylase isozymes secreted. Boston et al. (1982) showed that α -amylase from wheat could be translated using the Xenopus laevis oocyte system, which has the capability of processing synthesized enzymes to their active forms. A comparison of the size of the oocyte synthesized α -amylase with that synthesized in a wheat germ cell-free translation system, showed that the cell-free system produced a larger (unprocessed) α -amylase molecule. However, when dog pancreas microsomal membranes (ER) were added to the wheat germ cell-free system, the α -amylase produced was of the same size as that synthesized by the oocyte. Wheat α -amylase is not a glycoprotein and was not affected by tunicamycin.

At the present time, it is known that GA affects the synthesis and release of several enzymes, that it affects the synthesis of mRNA for at least one of these enzymes, α -amylase, and that some protein factor must be synthesized before α -amylase mRNA synthesis can take place. It has been hypothesized that α -amylase is coded for by at least two different genes. Embryo DNA had been shown to contain multiple α -amylase genes. The number of genes and pseudogenes has not been determined. Also aleurone DNA has not been analyzed. More work needs to be done to elucidate the mechanism of action of GA in enzyme synthesis and secretion in aleurone tissue.

II. TRANSFER RNA PHYSIOLOGY

Jacobsen and Zwar (1974) noted that GA does not significantly increase the amount of any major group of RNA molecules in barley aleurone, including rRNA, mRNA and tRNA. By further separation of the mRNA preparation, they found that GA stimulated the increase of two poly (A)-mRNA species as discussed above. They did not further separate tRNA to identify specific isoacceptors. The effect of GA on specific tRNA isoaccepting species in barley aleurone is not known. The following section will present information on tRNAs in early germination, tRNAs and the regulation of protein synthesis, changes in tRNA profiles over time, hormones and tRNAs, the characterization of lysine tRNAs in plants, and tRNA profiles from barley tissue.

De Novo Synthesis of tRNAs

Transfer RNAs have been investigated in dormant and early developing grain tissue. Osborne (1977) isolated tRNAs able to be aminoacylated from viable and non-viable rye (Secale cereale) embryos. She concluded that the stability of tRNA molecules could be due to their small size and compact structure and that the availability of tRNAs does not limit protein synthesis at germination. Two studies have measured the initiation of tRNA synthesis in germinating tissue. Dobrzanska et al. (1973) found tRNA synthesis began after 18 hours of germination in wheat embryos. Rye embryos began de novo tRNA synthesis 20 minutes after the initiation of

imbibition (Sen et al., 1975). This large difference could be due to vast differences in methods used to label and extract nucleic acids.

The Regulation of Protein Synthesis by tRNAs

Transfer RNAs play a vital role in protein synthesis as the connecting element between the mRNA and the nascent protein. This interaction led investigators to examine the role of tRNA molecules as regulators of protein synthesis. Bray (1976) pointed out that this control could be on the level of the availability of amino acids, specific tRNA isoacceptors, or aminoacyl-tRNA synthetase isozymes, or on the level of amino acid activation or aminoacyl-tRNA formation both governed by the aminoacyl-tRNA synthetase.

The regulation of protein synthesis by tRNA has been shown in bacteriophage T4 by Wilson (1973). He demonstrated that T4-DNA codes for some of its own tRNA species. Bacterial mutants, that have low levels of tRNA, were infected with T4 strains that were deficient in tRNA genes; there was a decrease in burst size. This decrease was found to be correlated with a decrease in the amount of phage tail fiber protein. Wilson concluded that phage tRNA was needed to insure optimum rates of protein synthesis in host cells that might be deficient in tRNAs containing anticodons necessary for the synthesis of phage proteins. Sprague et al. (1977) isolated two alanine tRNA species from the posterior silk gland of Bombyx mori. Transfer RNA isolated from other tissues showed that one of the

alanine tRNA species occurred only in the posterior gland, where fibroin is the major protein produced. This result suggested that this alanine tRNA aids in translation of the fibroin protein. Chavancy et al. (1980) added support to Sprague's theory when they demonstrated that rates of in vitro synthesis of fibroin protein were highest when posterior silk gland tRNA was added to the cell-free protein synthesis system. Bovine lens fiber cells contain two major phenylalanine tRNA isoacceptors, whereas lens epithelial cells contain only one. Lin et al. (1980) sequenced these two tRNAs showing that the difference between the isoacceptors was in a guanosine substituted for an adenosine at position 57 of the T ψ CG loop. This change in sequence, rather than level of post-transcriptional modification, suggested that cell differentiation in the bovine lens is regulated by the expression of a new phenylalanine tRNA gene. Viotti et al. (1978) examined tRNAs from 30 day-post-pollinated corn (Zea mays) seeds. These seeds are actively synthesizing zein, a storage protein containing high levels of glutamine, leucine and alanine. Using homologous and heterologous enzymes, they found that for these three amino acids, endosperm tRNAs had higher charging efficiencies than embryo tRNAs. This increase in efficiencies was correlated with differences in the tRNA profiles for those amino acids extracted from the two tissues. Control amino acids, those not present at high levels in zein, extracted from the two tissues showed similar profiles and charging efficiencies.

Changes in tRNA Profiles Over Time

In several cases, changes in tRNA isoacceptors in a system have been shown during development. Chu-Der and Ortwerth (1980) have shown that the presence of lysine tRNA₄ is correlated with cell division in mouse LM cell cultures. Norris et al. (1975) examined lysine and proline tRNA from developing wheat grains. Endosperm tissue contained four lysine and three proline tRNA species, whereas embryo tissue contained three or four lysine and three proline tRNAs. Differences in amounts of tRNAs were not correlated with amino acid content of storage proteins. The profiles of tRNAs examined changed only slightly during pre-germination development. Shugart (1972) studied the change in tRNA profiles in developing wheat leaves. He isolated tRNA from the basal ends of leaves and compared it to that isolated from non-growing apical ends. The yield of tRNA from the older tissue was less than that obtained from the younger basal leaf tissue. As leaf tissue matured, the four phenylalanine tRNAs found appeared to lose their ability to be aminoacylated.

Hormones and tRNAs

A review by Littauer and Inouye (1973) points out several cases in animals where tRNA profiles and accepting capacities are affected by hormones. To date, most of the hormone related tRNA work in plants has dealt with the cytokinins. These molecules are substituted adenosines that promote cell division and are naturally

occurring modified bases in some tRNAs. Cherry and Anderson (1972) showed that the cytokinin 6-benzyladenine promoted the acylation of two leucine tRNA isoacceptors from soybean (Glycine max) hypocotyls. They further showed changes in serine and tyrosine tRNA profiles promoted by this cytokinin. Since a natural cytokinin is one of the bases in these tRNAs, it appeared that the exogenously supplied cytokinin blocked the breakdown of these tRNAs thus regulating the endogenous levels of cytokinins. Cytokinin active tRNAs have recently been isolated from wheat, mungbean (Phaseolus vulgaris) (Edwards and Armstrong, 1981) and cucumber (Cucumis sativus) seedlings (Jayabaskaran and Jacob, 1982).

Lysine tRNAs in Plants

Only a limited amount of work has been done on tRNA from plants. Much of this work has been reviewed by Weil (1979). In only a few cases, have specific tRNA isoacceptors been designated as being present in the chloroplast or mitochondria, and there has been little work reported on tRNA modifying enzymes from plants. Several studies have been done on lysine tRNA in plants. Five lysine isoacceptors have been isolated from lupin (Lupinus luteus) seeds and embryos (Augustynaik and Pamelkiewicz, 1978) and four from developing wheat grains, mentioned above (Norris et al., 1975). The lupin seeds have three major isoacceptors for lysine. One recognizes the codon AAG, the second recognizes AAA, and the third one both AAA and AAG. Hatfield and Rice (1978) studied codon

recognition of wheat germ tRNAs. Only one major lysine tRNA peak is evident and it recognizes only the AAG codon.

Barley tRNAs

The number of genes for barley tRNA and post-transcriptional modifications of barley tRNA have not been examined. However, profiles of tRNA have been generated from barley tissue. Hiatt and Snyder (1973) isolated nine different phenylalanine tRNA isoaccepting species from barley germ, root and leaves. Two of these species were isolated from chloroplasts. Changes in the profile were shown to occur over the first forty hours of germination. Labuda et al. (1974) found two phenylalanine tRNA isoaccepting species in barley embryos. The difference in results found by these two groups of investigators could be due to differences in the species of barley used. Janowicz et al. (1979) compared the structure of a major phenylalanine-tRNA isoacceptor from barley with that of wheat and found the two to be identical. Karwowska and Augustyniak (1977) isolated five species of leucine tRNA from barley seedlings grown in the light and four from those grown in the dark.

Aminoacyl-tRNA Synthetases

The enzyme responsible for the aminoacylation of tRNAs is the aminoacyl-tRNA synthetase (EC 6.1.1). Although the mechanism of this reaction has not been completely elucidated, the synthetase is thought to interact with the inner side of the tRNA molecule when the tRNA is in the L-conformation (Shimmel, 1980). Km values for cognate

amino acids have been determined for several of these enzymes in plants. Phaseolus cysteinyl-tRNA synthetase has a K_m of 62 μM for cysteine as determined using the phosphate exchange assay (Burnell and Shrift, 1977). For the same plant, the K_m for methioninyl-tRNA synthetase was 32 μM ; the aminoacyl-tRNA formation assay was used in this case (Burnell, 1981). Methioninyl-tRNA synthetase from wheat germ was characterized by Rosa and Sigler (1977). They measured the K_m for methionine at 18 μM . Carias et al. (1978) determined the K_m values for leucine and arginine with their cognate tRNA synthetases from wheat germ to be 10 and 2 μM , respectively.

In many eucaryotic systems aminoacyl-tRNA synthetases have been found to occur in complexes (Dang et al., 1982). One of these complexes, containing tRNA-synthetases for arginine, leucine, methionine and phenylalanine, was isolated from quiescent wheat germ (Quintard et al., 1978). This is the only report of a tRNA synthetase complex isolated from plant tissue.

III. AMINO ACID ANALOGS; S-2-AMINOETHYL-L-CYSTEINE

By definition an amino acid analog is a compound that is structurally similar to its related amino acid. The two molecules are usually similar in most physical properties, such as size, shape and charge distribution. Therefore in biological systems, amino acid analogs normally interfere with the biochemical processes of the native amino acid and often inhibit the growth and development of the organism (Lea and Norris, 1976). This inhibition may be overcome

if a sufficient level of the related amino acid is available.

Aminoethylcysteine behaves as a lysine analog in barley aleurone tissue (Henke et al., 1981). The following discussion will elaborate on tRNA specificity in normal cell metabolism as well as in the presence of analogs and on the effects of analogs in analog-resistant tissues.

Specificity of the Aminoacyl-tRNA Synthetase Reaction

In nature aminoacyl-tRNA synthetases will not normally acylate any molecule other than their specific tRNA, and this aminoacylation can occur only with the cognate amino acid. Some mis-acylation can occur if the tRNA from one organism is acylated using the synthetase from another organism. An example of this lack of specificity has been found when phenylalaninyl-tRNA synthetase from Neurospora crassa is reacted with valine tRNA from Escherichia coli and phenylalanine. Phenylalanine was found in protein in place of valine (Holten and Jacobson, 1969).

A similar phenomenon occurs with amino acid analogs. Azetidine carboxylic acid, a proline analog, is normally found in Polygonatum multiflorum where the prolyl-tRNA synthetase and proline tRNA will not allow incorrect acylations (Bray, 1976). Bray stated that this analog is acylated onto tRNA and is incorporated into protein with Phaseolus aureus proline tRNA and prolyl-tRNA synthetase. He also points out that argininyl-tRNA synthetase from Canavalia ensiformis will not acylate canavanine onto C. ensiformis arginine tRNA; canavanine is an arginine analog naturally occurring in this plant.

Henke et al. (1981) have evidence that AEC is incorporated into protein when barley aleurone is incubated in the presence of this lysine analog. This suggests that AEC is acylated onto the lysine tRNA in aleurone cells.

Amino Acid Analogs in Resistant Tissues

Negrutiu et al. (1978) found AEC in protein synthesized by Arabidopsis thaliana callus cultures. Suspension cultures of A. thaliana were then used to screen for cells resistant to AEC. When resistant cell lines were analyzed for their free amino acid pools, it was found that the free lysine concentrations had doubled. It is not unusual for organisms resistant to analogs to have higher than normal levels of the related free amino acid. 5-methyl-tryptophan-resistant lines of Daucus carota, Nicotiana tabacum and Solanum tuberosum have been shown to accumulate over 20 times the amount of free tryptophan than is found in normal plants (Widholm, 1977).

Since cereal grains are generally low in lysine, an essential amino acid, much work has been done on cereals using AEC to select for high lysine seed. Using callus derived from rice anther cultures, Schaeffer and Sharpe (1981) selected for cells that were resistant to AEC. Plants were regenerated from resistant callus, and seeds were tested for amino acid and protein content. These seeds were found to have not only increased levels of proteins but also increased levels of lysine, alanine, arginine and asparagine.

For at least some of the mutant plants, no decrease in yield of grain was observed. Bright et al. (1979) screened barley embryos for resistance to AEC. Resistant plants exhibited normal morphology, growth and seed set. Progeny of resistant plants were found to tolerate higher concentrations of AEC than that used to screen the parent plants. Henke et al. (1981) have screened barley embryo-less half-seeds for resistance to AEC. Those half-seeds able to produce active α -amylase in the presence of GA and AEC were determined to be putative mutants. Their embryo halves were grown to maturity and progeny were tested. Of 98 plants that yielded seed, 12 had progeny that exhibited resistance to AEC. Three of these plants were α -amylase over-producers.

These mutant lines may have many possible alterations in their tRNA aminoacylation mechanisms. The synthetase may have been changed to allow the amino acid to out-compete the amino acid analog for its binding site or the synthetase may totally exclude the amino acid analog from the amino acid binding site. Transfer RNAs that formerly could bind the amino acid analog may change to lose this ability, or the amount of these tRNAs could decrease, leaving only the one(s) that cannot bind the amino acid analog available for protein synthesis. The last of these possible mutations could result in changes in tRNA profiles.

By characterizing germinating barley aleurone tRNA and tRNA synthetase, more information about the action of tRNAs in normal aleurone tissue will be learned. Methods demonstrated in this thesis can be used not only to study the effects of AEC on normal

aleurone lysine tRNA but also to study the effects of AEC on lysine tRNA in aleurone resistant to AEC. If AEC-resistant barley has free high lysine pools, then these resistant lines will be nutritionally valuable because lysine is an essential amino acid found in low levels in normal barley seeds. This study provides basic information about lysine tRNA in barley aleurone tissue and can serve as a foundation for future studies on tRNAs in normal and mutant cereal lines.

CHAPTER III

MATERIALS AND METHODS

I. PLANT MATERIALS: PREPARATION AND CULTURE CONDITIONS

Barley seed Hordeum vulgare L. cv. Atlas 57 was supplied by Dr. R. T. Ramage of the University of Arizona, Tucson. Seeds were stored in plastic bags at 12°C. Whole seeds were used for enzyme extractions, for preliminary tRNA extractions and for the source of tRNA used in enzyme kinetic studies. Embryo-less half-seeds were used for the remaining experiments. Embryo-less half-seeds were prepared according to the method of Chrispeels and Varner (1967). Seeds were cut transversely at approximately 4 mm from the extreme distal ends. Embryo halves were discarded.

Sterilization Procedure

Twenty grams of embryo-less half-seeds, approximately 800 half-seeds, were surface sterilized in a 100 x 80 mm pyrex storage dish containing a 1% sodium hypochlorate solution (commercial bleach diluted five times). Seeds were stirred for 20 minutes and then rinsed with sterile glass distilled water (ddH₂O) until no bleach odor could be detected. Seeds were dried overnight on sterile paper towels in a sterile transfer hood (Chrispeels and Varner, 1967).

Incubation Procedure

One hundred embryo-less half-seeds were placed in a 250 ml erlenmeyer flask containing 20 ml of incubation media consisting of 0.001 M sodium acetate pH 4.8 and 0.02 M CaCl_2 . When called for 10^{-6} M GA was added. Seeds were incubated at room temperature on a shaker (GCA/Precision Scientific Shaking Water Bath) set at 40 oscillations per minute (Chrispeels and Varner, 1967). At the end of the incubation period, the media was poured off and the seeds were frozen in liquid nitrogen and stored at -20°C .

Preparation of Aleurone Layers and Endosperm Tissue

The following procedure to isolate aleurone layers is a modification of that used by Chrispeels and Varner (1967). All handling of seeds after sterilization was done in a sterile transfer hood. The extreme distal ends were cut off of embryo-less half-seeds. These half-seeds were placed in sterile 10 cm petri dishes containing 100 grams of sterile sand and 20 ml of sterile ddH_2O . The petri dishes containing half-seeds were wrapped in aluminum foil and incubated at room temperature for three days. Half-seeds were then removed and incubated in incubation media (above) with GA, for 24 hours. Then, using two small spatulas, the aleurone layers were removed from half of the seeds. Embryo-less half-seeds, aleurone layers and remaining starchy endosperm were frozen in liquid nitrogen and stored at -20°C until use.

II. tRNA EXTRACTION

The following procedure to extract tRNA is a modification of that used by Vanderhoef et al. (1970). All buffers were Millipore filtered and autoclaved. Reducing agents were added to buffers immediately prior to use. All glassware was autoclaved prior to use and all handling of phenol was done in a chemical hood.

Phenol Extraction

Phenol was freshly distilled (bp 181°C) and brought to a pH of 7.5 by equilibration with 0.2 M Tris hydroxymethyl aminomethane (Tris). To remove the Tris, the phenol was washed with ddH₂O. Freshly distilled cresol (bp 203°C) and 8-hydroxyquinoline were added to the water saturated phenol to make the final solution 10% (v/v) and 0.1% (wt/v), respectively. The organic phase was then equilibrated with tRNA extraction buffer (0.01 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.001 M EDTA, and 0.005 M β -mercaptoethanol (BME)).

Barley tissue, stored frozen, was ground to a fine powder in a mortar and pestle using liquid nitrogen. The original procedure of grinding seeds with buffer and phenol in an Omni Mixer was discarded due to low yields of broken cells.

Aleurone tissue was combined with five volumes of extraction buffer (g/ml) and five volumes of phenol. Half-seed, whole seed and starchy endosperm were combined with 10 volumes of each phase. In a preliminary extraction using embryo-containing half-seeds, tissues were extracted for 10 minutes. This was changed to extracting overnight by stirring tissue in both phases in a covered beaker.

Samples were centrifuged (Beckman Model J-21C) for 10 minutes at 1000-1500 x g, and the aqueous phase was removed and saved. The phenol phase and interface were re-extracted for 10 minutes with an additional half volume of extraction buffer after which the mixture was centrifuged for 10 minutes at 9500-13,000 x g. Phenol and interface were discarded. The combined aqueous phases were made to 0.1% dodecyl sodium sulfate (SDS) and one volume of fresh phenol was added. The mixture was stirred for 10 minutes and then centrifuged for 10 minutes at 9500-13,000 x g. Phenol and interface were discarded. The aqueous phase was re-extracted with fresh phenol in the same manner two more times. Then the aqueous phase was combined with 2.5 volumes of cold 95% ethanol and stored overnight at -20°C.

High Salt Separation of Low Molecular Weight RNA

The precipitated nucleic acids were pelleted by centrifugation for 30 minutes at 13,000 x g and the supernatant was discarded. The pellet was stirred overnight at 12°C in 2.5 M sodium acetate pH 6.0. Due to differential solubilities in high salt, the low molecular weight RNA dissolved in the buffer; the high molecular weight RNA did not. The sample was centrifuged for 10 minutes at 3000-4000 x g and the supernatant was saved. The pellet was washed for one hour in one-half volume of 2.5 M sodium acetate pH 6.0 and spun for 10 minutes at 9500-13,000 x g. The supernatants were combined and one volume of ddH₂O was added. Then 2.5 volumes of cold 95% ethanol

were added and the sample was allowed to precipitate overnight at -20°C . The sample was centrifuged for 30 minutes at $13,000 \times g$. The low molecular weight RNA pellet was taken up in a minimal amount of column buffer (0.05 M NaCl DEAE pH 7.5) containing 0.05 M NaCl, 0.01 M Tris-HCl pH 7.5, 0.01 M MgCl_2 , 0.001 M EDTA and 0.005 M BME. A DEAE column buffer with a pH of 7.5 gave tRNA with greater purity over buffers of pH 4.5, as originally proposed (data not shown). The absorbance of the sample was read at 260 and 280 nm using a Beckman DU spectrophotometer 2400 equipped with a Gilford #222 light source.

DEAE Cellulose Column Chromatography

Columns were prepared using sterile syringes with Miracloth disks as the column support. The washed DEAE-cellulose was pipetted into the syringe to the proper height, 5 ml high for small extractions or 1 ml/ A_{260} unit of RNA for large extractions (See Appendix for DEAE washing procedure). One A_{260} unit is equal to approximately 50 μg of pure RNA. The resin was then equilibrated with 10 column volumes of 0.05 M NaCl DEAE pH 7.5 buffer. The sample was layered onto the column. Five ml fractions were collected and monitored at 260 nm. The column was washed with five volumes of 0.05 M NaCl DEAE pH 7.5 buffer followed by 0.25 M NaCl DEAE pH 7.5 buffer until the absorbance was zero. The tRNA was eluted with 1 M NaCl DEAE pH 7.5 buffer. At this point 2 ml fractions were collected. The fractions containing the 260 nm peak were combined.

A 260/280 nm ratio was determined; a ratio of two indicated that the RNA was relatively free from protein contamination. Transfer RNA was precipitated overnight by adding 2.5 volumes of cold 95% ethanol and storing the sample at -20°C.

The sample was centrifuged for 60 minutes at 23,500 x g and the supernatant was filtered through a Swinney filtering apparatus using a Gelman metrical filter (0.20 μ m pore size). The tRNA on the filter and the pellet were then dried separately under nitrogen gas and the tRNA was taken up in a minimal amount of TnE buffer (0.01 M Tris-HCl pH 7.5, 0.01 M NaCl, 0.001 M EDTA and 0.005 M BME). An absorbance reading was taken at 260 and 280 nm. The tRNA solution was stored frozen at -20°C. Samples prepared in this manner were referred to as tRNA, even though they were undoubtedly contaminated with other low molecular weight RNAs.

Sucrose Density Gradient Fractionation

The purity of two samples was determined using sucrose density gradient centrifugation. Gradients of 10 to 30% RNase-free sucrose, in TnE plus 0.1% SDS, were prepared in 0.5 x 2.0 inch cellulose nitrate tubes. A small amount of tRNA or high molecular weight RNA (0.4 A₂₆₀ units) was layered on top of the gradients. Samples were centrifuged in a Beckman model L3-50 ultracentrifuge for 5 hours at 43,000 rpm using a SW 50.1 Beckman rotor. Gradients were then analyzed for their absorbance at 254 nm using an Isco density gradient fractionator model 640 and an Isco UA-5 absorbance/fluorescence monitor.

III. MIXED SYNTHETASE EXTRACTION

The following procedure for the extraction of aminoacyl-tRNA synthetases is based on that used by Labuda et al. (1974). All buffers were Millipore filtered (0.45 pore size) and reducing agents were added to buffers immediately prior to use. All solutions were kept at 4°C.

Dry seeds were ground to a fine powder using a Waring blender. Tissue was then extracted for 3 hours in three volumes of extraction buffer (0.04 M sucrose, 0.05 M Tris-HCl pH 7.5, 0.1 M KCl, 0.005 M magnesium acetate, and 0.005 M BME). The sample was filtered through four layers of cheese cloth. The residue on the cheese cloth was re-extracted for 10 minutes in two volumes of fresh extraction buffer. Filtrates were combined and centrifuged for 30 minutes at 27,000 x g. The supernatant was made to 40% saturation of ammonium sulfate, stirred for 30 minutes and centrifuged for 20 minutes at 27,000 x g. The pellet was discarded. The supernatant was made to 70% saturation of ammonium sulfate, stirred for 30 minutes and centrifuged for 20 minutes at 17,300 x g. The resulting pellet was taken up into a minimal amount of Sephadex column buffer (15% glycerol, 0.01 M KPO₄ pH 7.4, 0.005 M KCl, 0.001 M MgCl₂ and 0.005 M BME). The sample was then layered onto a previously equilibrated Sephadex G-200 column (See Appendix for column preparation) and the column was washed with Sephadex column buffer. The eluate was monitored for absorbance at 280 nm, and

2 ml fractions were collected. Fractions were assayed for lysyl-tRNA synthetase activity using conditions for maximal aminoacylation of tRNA. Those fractions containing the first peak of enzyme activity were combined and stored at -80°C in 1 ml aliquots.

IV. ASSAY FOR LYSYL-LYSINE tRNA FORMATION

Assay Conditions for Maximal Aminoacylation

All reactions for maximal aminoacylation were done in duplicate. Unless otherwise indicated, the following conditions were used to obtain maximal aminoacylation of lysine tRNA. In a total volume of 50 μl there was 0.2 A_{260} units of tRNA, 20 μl of enzyme and the following: 100 mM Tris-HCl pH 7.5, 8 mM ATP, 20 mM MgCl_2 , 60 mM BME and 20 μM ^3H -lysine at 1 or 2 Ci/mmol. See the Appendix for preparation of the lysine stock. The reaction began with the addition of the enzyme. Samples were incubated for 30 minutes at 37°C . The reaction was terminated by pipetting 40 μl from each sample onto a Whatman number three 2.3 cm filter paper disk.

The filter disk washing procedure used was based on the method of Bollum (1966). Filters were washed for at least 15 minutes in an acid bath. This bath consisted of 5.0% acetic acid, 0.7% hydrochloric acid and 0.022 M sodium pyrophosphate stirring in a beaker on ice. This bath allowed the tRNA to precipitate onto the fibers of the filter disks. Disks were then washed two more times for 15 minutes each in fresh acid solution. Washing continued for two times, 10 minutes each, in a 1:1 solution of ethanol and diethylether

and once, for 5 minutes, in ether alone. Disks were then dried and radioactivity was measured by liquid scintillation spectroscopy. Disks were placed in 5 ml of a scintillation cocktail containing 0.4 g/l PPO and 0.05 g/l POPOP in Scintanalyzed (Fisher) toluene, and counted in a Beckman LS-200 B liquid scintillation system.

Assay Conditions for Kinetic Experiments

All reactions for the kinetic experiments were done in triplicate. The following conditions were used, 4 μ l of enzyme stock were used per 50 μ l assay. The lysine concentration ranged from 2 to 20 μ M using a stock of 5 Ci/mmol (See Appendix for lysine stock preparation). Some experiments also used a concentration of AEC of 6 or 12 μ M. The rest of the components were the same as above. Most assays were scaled up to a final volume of 100 μ l to allow three time points to be taken. At 3, 6, and 9 minutes, 30 μ l of the reaction mixture was pipetted onto Whatman 2.4 cm glass microfiber filters. The glass fiber filters were processed and counted as described in the preceding section.

V. RPC-5 SEPARATION OF LYSINE-tRNA ISOACCEPTING SPECIES

Preparation of the Sample

The sample was prepared for RPC-5 chromatography by scaling up the assay for maximal aminoacylation of tRNA to yield 10,000 cpm of labeled tRNA. To terminate aminoacylation, the sample was diluted in 5 ml of 0.05 M NaCl DEAE buffer pH 4.5 (0.01 M sodium

acetate pH 4.5, 0.01 M MgCl_2 , 0.001 M EDTA and 0.0025 M dithioerythritol) and was immediately layered onto a previously equilibrated 1 ml DEAE-cellulose column (prepared as described above). Five ml fraction were collected; these were monitored for radioactivity using the aqueous fluor system, consisting of 100 μl of the sample plus 300 μl of ddH_2O in 5 ml of Scintaverse (Fisher) (See Appendix for data on the quench curve for this system). The column was washed with 0.25 M NaCl DEAE pH 4.5 buffer until no radioactivity was detected. The tRNA was eluted by collecting 2 ml fractions using 1 M NaCl DEAE pH 4.5 buffer. Fractions were monitored as above and the peak fractions were combined. If not used immediately, the sample was stored at -20°C .

RPC-5 Column Chromatography

The following procedure for RPC-5 column chromatography is based on that used by Waters et al. (1975). See Appendix for preparation of the RPC-5 column. All glassware was autoclaved prior to use. Buffers were Millipore filtered and autoclaved prior to use. Buffers were Millipore filtered again and reducing agents were added immediately prior to use.

Immediately prior to use, the column was equilibrated at 37°C and 150 psi using 80 ml of 0.4 M NaCl RPC-5 buffer consisting of 0.01 M sodium acetate pH 4.5, 0.01 M MgCl_2 , 0.001 M EDTA and 0.005 M BME. The temperature was maintained using a Haake FJ water pump; the pressure was maintained using a Milton Roy mini-pump.

The sample was diluted to 0.4 M NaCl using RPC-5 buffer without NaCl. The total number of counts being loaded onto the column was determined by measuring the radioactivity of a small aliquot of the sample. The sample was loaded onto the column and 40 drop fractions were collected. The column was washed with 10 ml of 0.4 M NaCl RPC-5 buffer. Transfer RNA isoaccepting species were differentially eluted using a 200 ml gradient of 0.4 to 0.6 M NaCl in RPC-5 buffer. The column was then washed with 30 ml of 2 M NaCl RPC-5 buffer.

Fifty μ l samples of eluate were pipetted from every fifth fraction and were diluted into 25 ml of ddH₂O. Conductivity of these samples was determined using a YSI conductivity bridge. A standard curve allowed measurements to be converted into salt concentrations. These concentrations were plotted against fraction numbers and a line was fitted using the method of least squares.

Transfer RNA was precipitated from the fractions by adding 2 A₂₆₀ units of carrier DNA and one volume of an acid solution, containing 5.0% acetic acid and 0.2% hydrochloric acid, to each fraction. Fractions were shaken vigorously and allowed to sit for 30 minutes on ice. The samples were then filtered through glass microfiber filters to collect the precipitated tRNA. Each test tube was rinsed twice with 70% ethanol and the ethanol was filtered as above. Filters were dried and samples were counted for their radioactivity as described above, using the Beckman or a SEARLE isocap/300 6868 liquid scintillation spectrometer. After subtracting machine background, cps were plotted against fraction number.

CHAPTER IV

RESULTS AND DISCUSSION

I. TRANSFER RNA EXTRACTION

Optimization of the Extraction Procedure

The preliminary extraction, referred to in the materials and methods, was done using embryo-containing half-seeds. Due to a very low yield of tRNA (0.3 A_{260} units per 20 grams) the procedure was examined to determine which steps might be changed to increase the yield. One of the steps chosen for examination was the phenol extraction. Since all of the residual phenol and interfaces had been kept during the preliminary extraction, it was decided to try to extract more tRNA from the interface and also from the remaining phenol and interfaces combined. Extraction buffer was added to each of these two samples, and they were stirred overnight. The following day the samples were centrifuged, and RNA was recovered from the aqueous phase by ethanol precipitation. From that point on the samples were processed according to the original procedure. Table I shows the yield of tRNA from these two samples. The initial phenol phase and interface and the combined phenol and interfaces yielded 15.0 and 19.6 A_{260} units of tRNA, respectively. Combined, the two samples resulted in 115 times the amount of tRNA that was recovered during the original extraction. Thus the first step of the phenol extraction procedure that involved shaking the sample

Table I. Yields of tRNA from a Preliminary Extraction.

Sample	Yield ^a (A ₂₆₀)	Percent of Total	Increase Over the Initial Sample
Initial sample	0.3	0.9	--
Initial phenol phase and interface	15.0	43.0	49.8
Combined remaining phenol phases and interfaces	19.6	56.1	65.2
Total	34.9	100.0	--

^aFrom an extraction of 20 grams of dry embryo-containing half-seeds.

for 10 minutes was modified to stirring the sample overnight. In order to reduce the amount of tRNA left behind in the remaining phenol extractions, care needed to be taken to pipet off as much of the aqueous layer as possible without contaminating it with the interface.

DEAE Cellulose Column Chromatography

Figure III shows a typical elution pattern of a tRNA sample during purification by DEAE cellulose chromatography. Before proceeding to elution at a higher concentration of salt, the column was washed with buffer until the eluate had no absorbance at 260 nm. The tRNA was eluted with a 1 M salt wash. The absorbance peak, obtained in fractions 28 through 33 for the representative sample in Figure III, was collected in preparation for ethanol precipitation of tRNA.

Purity of tRNA Samples

Sucrose density gradients were used to determine the extent of high molecular weight RNA contamination of tRNA preparations. Figure IV shows an absorbance profile of a typical sample that was centrifuged for 5 hours at 43,000 rpm on a 10 to 30% sucrose gradient. As indicated by the position of the peak at the beginning of the profile, the low molecular weight RNA barely entered the gradient. Gradients were not spun long enough to separate the 4S tRNA from the 5S rRNA. Arrows indicate the position of the 18 and 25S rRNA in a control gradient run under identical conditions. The absorbance detected after 0.7 minutes was background, as determined

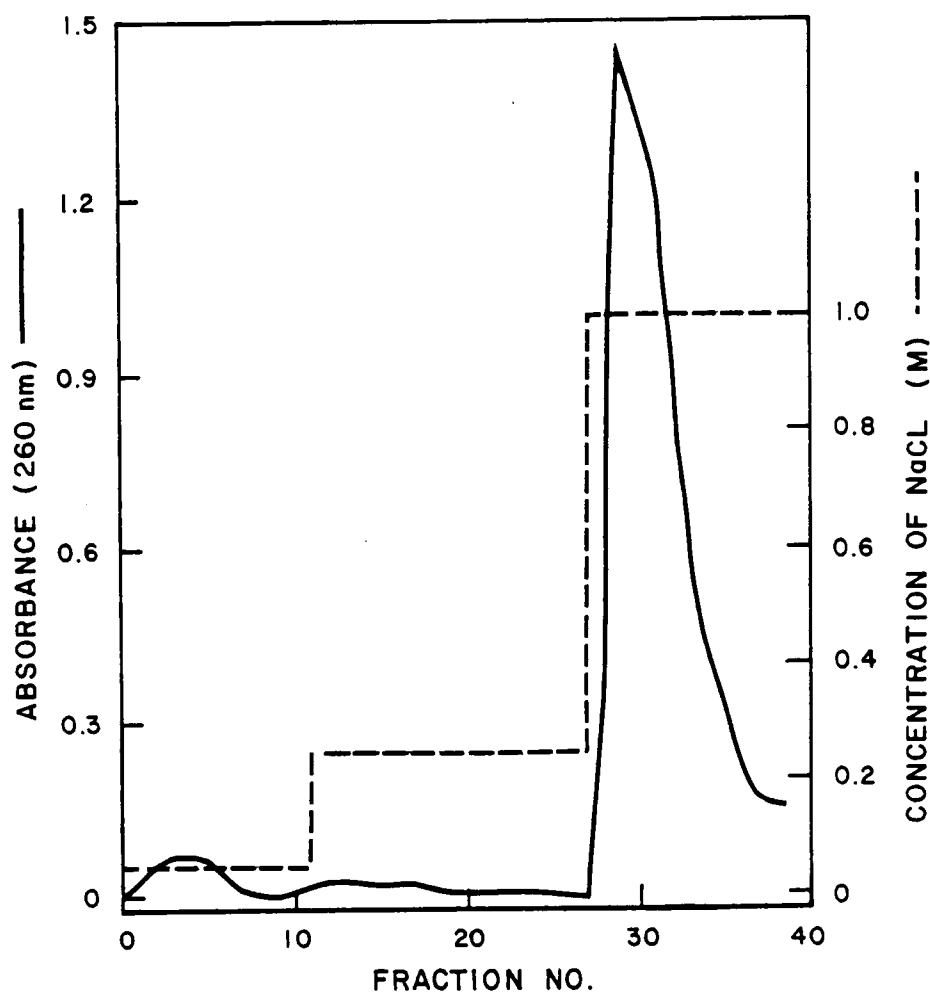


Figure III. Purification of a tRNA sample from starchy endosperm, by DEAE cellulose column chromatography. The fraction of nucleic acids that remained soluble in high salt was applied to a 1 x 5 ml column in DEAE 7.5 buffer (0.01 M Tris-HCl pH 7.5, 0.01 M MgCl₂, 0.001 M EDTA, and 0.005 M BME) containing 0.05 M NaCl. The column was washed with 25 ml of the same buffer, and 5 ml fractions were collected. The column was then washed with DEAE 7.5 buffer containing 0.25 M NaCl until no absorbance at 260 nm was detected in the eluate. Collecting 2 ml fractions, the tRNA was eluted in DEAE 7.5 buffer containing 1 M NaCl. Fractions 28 through 33 which contained the maximal absorbance were pooled.

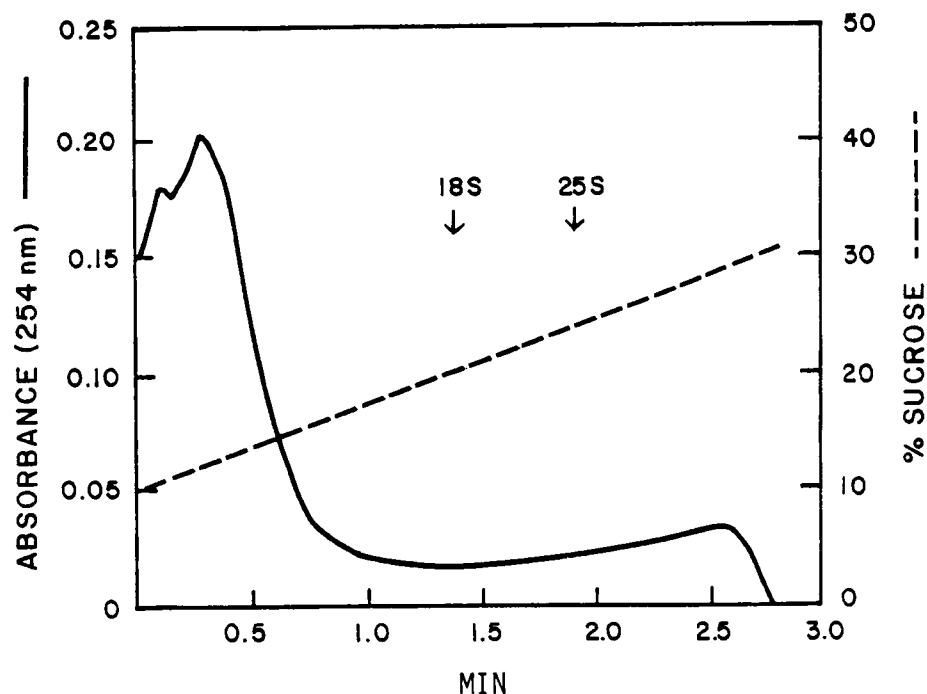


Figure IV. Sucrose density gradient centrifugation of RNA from 48 hour +GA treated half-seeds. Twenty μg of RNA was layered on top of a gradient consisting of 5 ml of 10 to 30% RNase-free sucrose in TnE plus 0.1% SDS. The sample was centrifuged for 5 hours at 43,000 rpm using a SW 50.1 rotor. The gradient was fractionated from the top at a rate of 0.5 ml/min using an Isco density gradient fractionator. Absorbance at 254 nm was monitored. The arrows indicate the position in the gradient of 18 and 25 S rRNA centrifuged under identical conditions in a separate tube.

by analyzing a gradient, containing no RNA, that was run under identical conditions. This profile clearly indicated that the tRNA preparations were not contaminated with a detectable amount of high molecular weight RNA.

Time Course of Aminoacylation

To determine the length of time needed to achieve maximum aminoacylation, the course of an aminoacylation reaction was followed over 60 minutes. These assays could also reveal the presence of RNase in a sample. A decrease in counts in 45 and 60 minute time points would indicate a breakdown of tRNA during the course of the reaction. The assay was scaled up to 200 μ l, with 40 μ l being drawn out at 15, 30, 45 and 60 minutes after starting the reaction. Figure V shows the amount of lysine tRNA aminoacylated over 60 minutes for a typical tRNA sample. As indicated by the curve, most of the tRNA was charged at 30 minutes and no RNase activity was detected. Only one sample, that extracted from embryo-containing half-seeds, showed evidence of RNase contamination.

The above experiment was used to determine charging efficiency for the tRNA samples. The charging efficiency value could be expressed either as counts per minute (cpm) per μ g of sample or as pmole per A_{260} unit. The cpm/ μ g number was used to calculate the amount of tRNA needed to prepare the sample for RPC-5 chromatography.

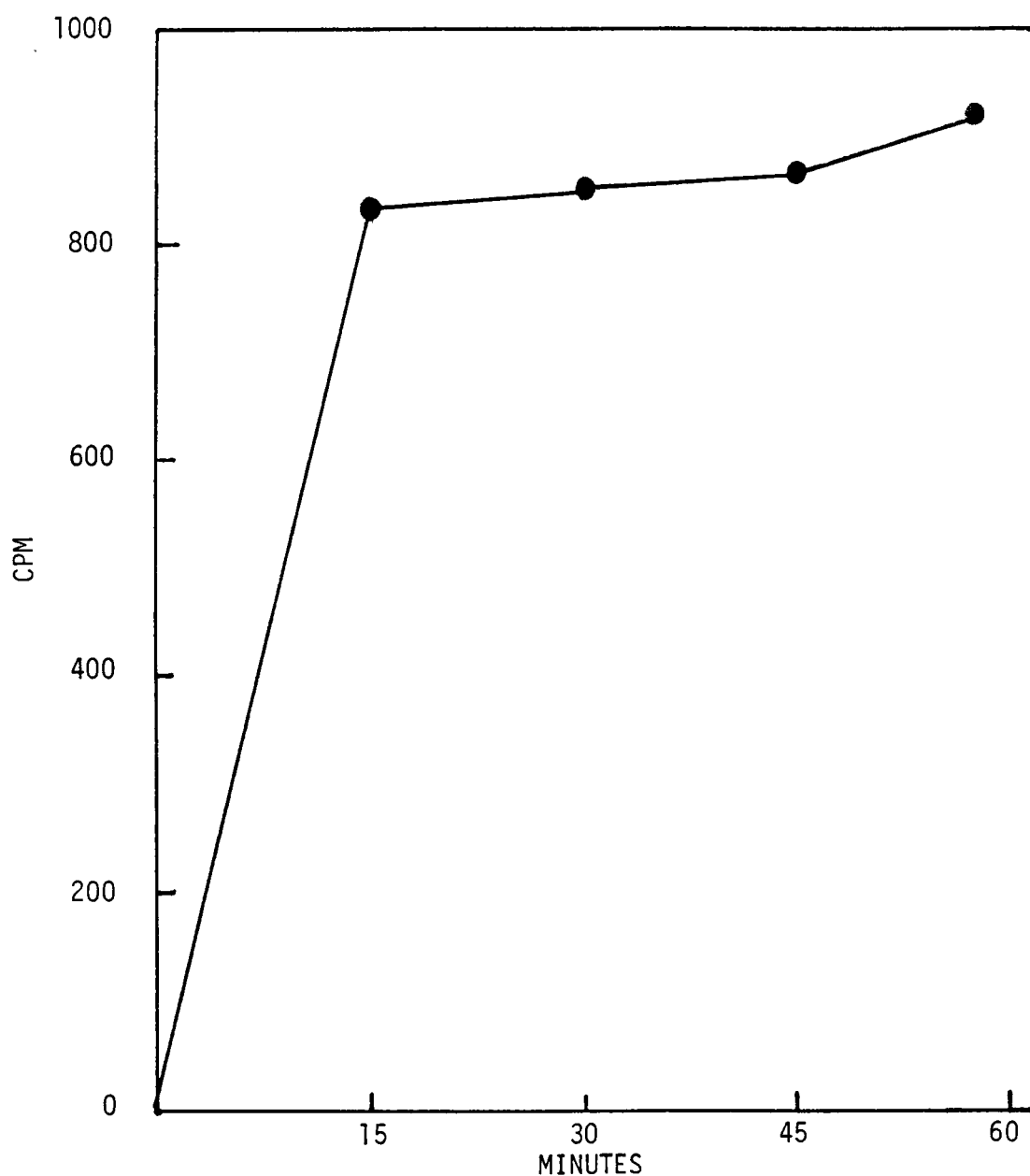


Figure V. Time course of aminoacylation where tRNA is limiting. Embryo-less half-seed tRNA aminoacylation was examined over 60 minutes. Duplicate assays were done, and the standard deviation for these measurements was 49 cpm. The assay contained 0.8 A₂₆₀ units of tRNA and 80 μ l of mixed synthetase in a solution of 0.01 M Tris-HCl pH 7.5, 0.008 M ATP, 0.02 M MgCl₂, 0.06 M BME and 20 μ M ³H-lysine (2Ci/mmol). At each time point, 40 μ l of a 200 μ l volume, was removed and pipetted onto a filter paper disk. Transfer RNA was precipitated and radioactivity was measured. Results are reported as cpm/40 μ l of a 50 μ l reaction mixture.

II. MIXED SYNTHETASE EXTRACTION

During the synthetase extraction, lysyl-tRNA synthetase was partially purified using Sephadex G-200 column chromatography. Figure VI shows the elution profile of total proteins eluting from the Sephadex column. The location of the lysyl-tRNA synthetase was determined by assaying the odd numbered fractions. Enzyme assays indicated the possibility of two isozymes of lysyl-tRNA synthetase. Fractions from the first peak, numbers 23 through 37, and the shoulder, number 38 through 50, were pooled. The first peak was used as the mixed synthetase preparation for all of the subsequent tRNA assays.

III. STANDARDIZATION OF THE ENZYME ASSAY

The assay for maximum aminoacylation of tRNA was standardized for temperature, concentration of reducing agent, concentration of enzyme and reaction time. Thirty-seven degrees and 0.06 M BME were shown to be the optimum temperature and concentration of reducing agent. The availability of ATP and/or phosphate inhibition did not limit the reaction. To determine the amount of the mixed synthetase preparation to use to achieve maximum aminoacylation of lysine tRNA, increasing amounts were added to the aminoacylation reaction. As demonstrated in Figure VII, 20 μ l of mixed synthetase gave the highest amount of lysyl-lysine tRNA formation.

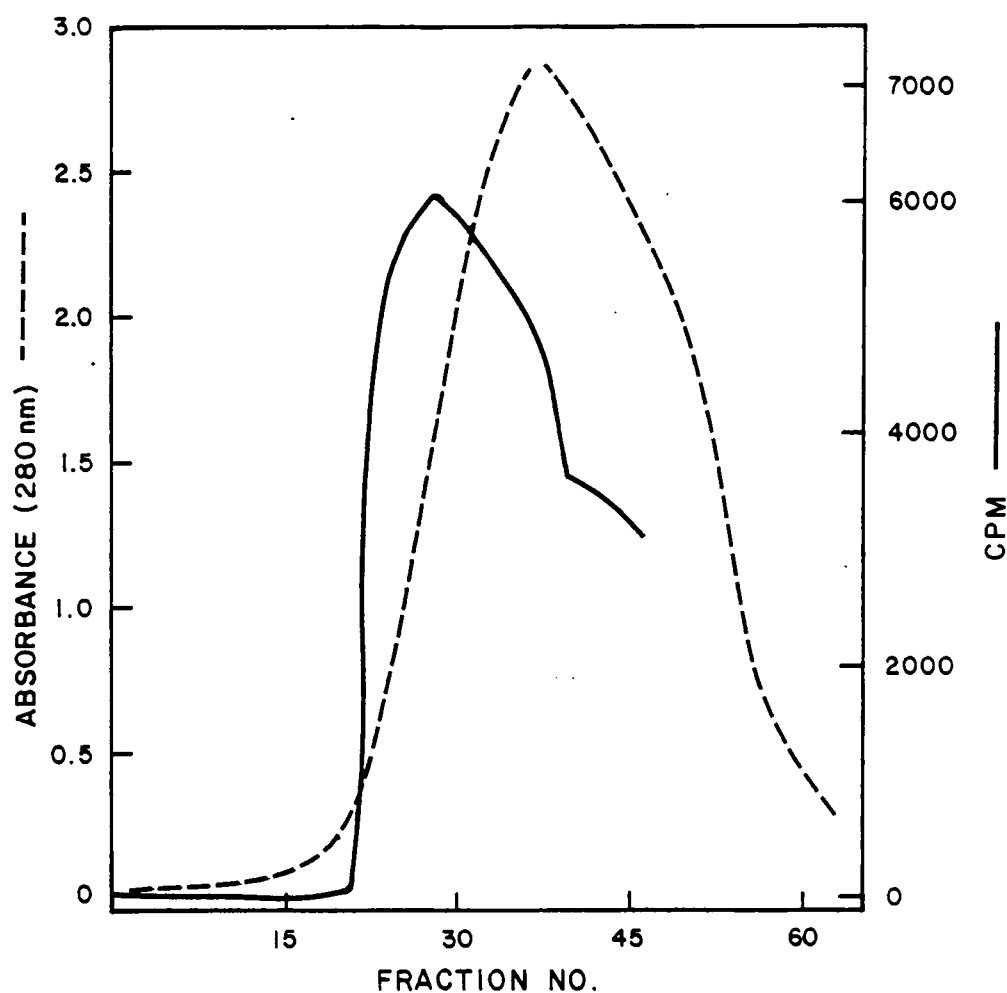


Figure VI. Partial purification of lysyl-tRNA synthetase using Sephadex G-200 column chromatography. The proteins which precipitated between 40 and 70% saturated ammonium sulfate were layered onto a 2.5 x 22 cm column. Samples were eluted in a buffer consisting of 15% glycerol, 0.01 M KPO_4 pH 7.4, 0.005 M KCl, 0.001 M MgCl_2 and 0.005 M BME. The column was run at 12°C, and 2 ml fractions were collected. Twenty-five μl of the odd numbered fractions were assayed for lysyl-tRNA synthetase activity. Fractions from the two peaks, 23-37 and 38-50, were pooled and stored at - 80°C. Results are reported as cpm/40 μl of a 50 μl reaction mix.

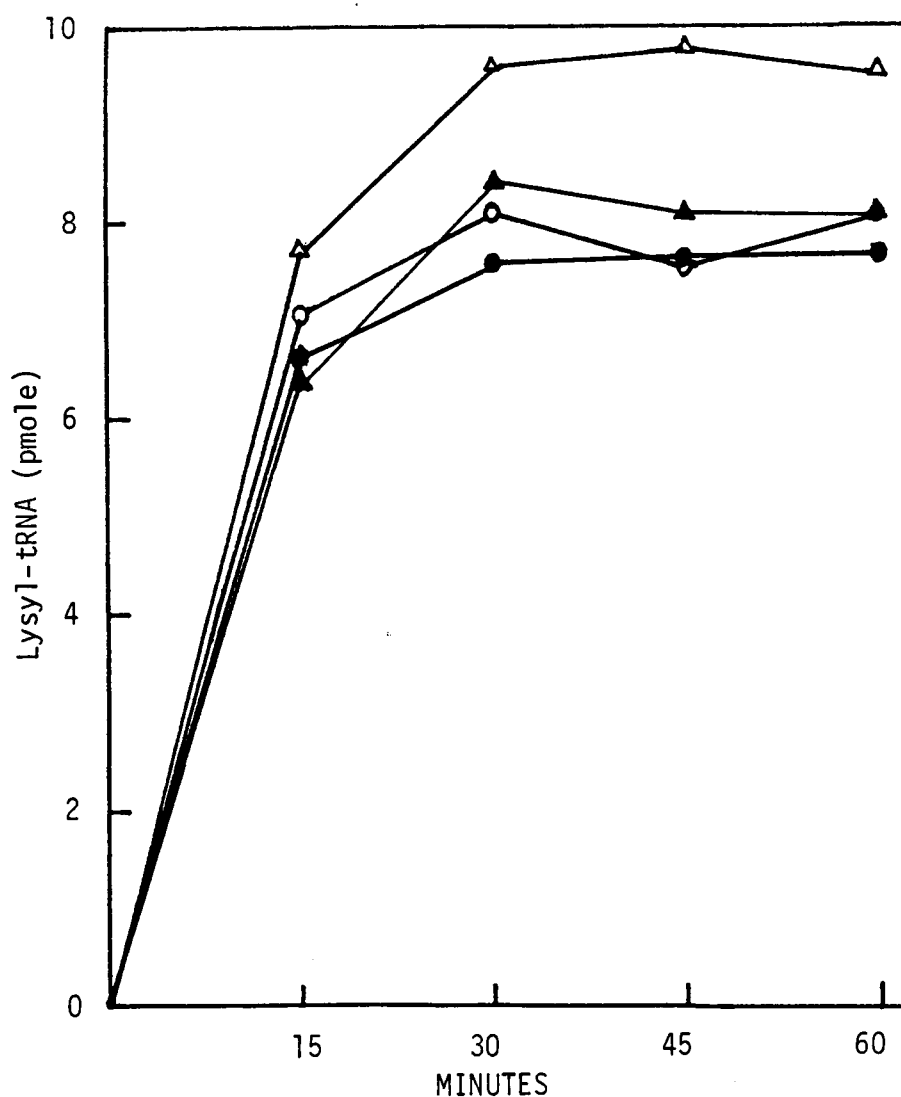


Figure VII. Effect of the concentration of lysyl-tRNA synthetase on lysyl-lysine tRNA formation. Duplicate assays were done, and the standard deviation for these measurements was 0.18 pmole. Assays contained 0.2 A₂₆₀ units of tRNA from embryo-containing half-seeds and one of the following: (●) 10 μ l, (○) 15 μ l, (Δ) 20 μ l or (▲) 22.5 μ l of mixed synthetase per 50 μ l total reaction mix containing 0.1 M Tris-HCl pH 7.5, 0.008 M ATP, 0.02 M MgCl₂, 0.06 M BME and 20 μ M ³H-lysine (1 Ci/mmol). In a reaction scaled up to 200 μ l total volume, 40 μ l was taken out at 15, 30, 45 and 60 minutes and was pipetted onto filter paper disks. Transfer RNAs were precipitated and radioactivity was measured. Results are reported as pmole/50 μ l of reaction mixture.

IV. COMPARISON OF LYSINE tRNA ISOACCEPTORS IN ALEURONE AND EMBRYO-LESS HALF-SEED TISSUE

Due to the difficulty in isolating dormant aleurone tissue, experiments were designed to determine whether profiles of lysine tRNA from germinating aleurone tissue were different from those of germinating embryo-less half-seed tissue. If the profiles were found to be the same, then embryo-less half-seed tissue could be used to observe changes in tRNA profiles over germination in place of isolated aleurone tissue.

Transfer RNAs were isolated from 300 (3.75 grams) embryo-less half-seeds that were sterilized, pre-incubated for 72 hours on sterile sand and incubated in media for 24 hours. Aleurone layers were then isolated from half of the seeds. Extractions from embryo-less half-seeds, aleurone layers and residual starchy endosperm were performed twice because yields obtained from the latter two tissues in the first extraction were not sufficient to obtain two lysine tRNA profiles. The tRNA extraction procedure was not standardized to achieve a quantitative yield of tRNA. Table II is a summary of yields and charging efficiencies from these two extractions. In general, extractions from embryo-less half-seeds gave the highest yield of tRNA (32.9 A_{260} units) per 150 half-seeds. These values compare to 4.1 A_{260} units for 1.875 grams of embryo-containing half-seeds from the preliminary extraction, to 0.013 A_{260} units for 1.875 grams of lupin seed and to an average of 0.00224 A_{260} units for

Table II. Yields and Charging Efficiencies of tRNA from Embryo-less Half-seeds, Starchy Endosperm and Aleurone Tissues.

Sample	Yield of tRNA ^a (A ₂₆₀)	Charging Efficiency (pmole/A ₂₆₀)	Yield of Lysine tRNA (pmole)
Embryo-less half-seed	32.9 ± 31.6	16.6 ± 10.3	545 ± 621
Starchy endosperm	12.2 ± 10.7	9.4 ± 1.3	114 ± 101
Aleurone layer	5.3 ± 0.9	49.4 ± 3.3	266 ± 53

^aFor each sample, values are based on an extraction of 150 embryo-less half-seeds.

^bValues were calculated by multiplying the yield by the charging efficiency.

the same amount of two day old, dark-grown bean seedlings (Weil, 1979). The tRNA preparation extracted from the aleurone tissue gave the highest charging efficiency (49.4 pmole/A₂₆₀). The charging efficiency for embryo-containing half-seed was in the range of 25 pmole/A₂₆₀.

Representative lysine tRNA profiles for these tissues are shown in Figures VIII, IX and X. Six lysine tRNA isoaccepting species were found to be present, with number two the dominant species. For each sample, areas under the curves were estimated and a percent of the total area was calculated for each peak. First, an ANOVA was calculated and an F test was used to compare the variation among extractions; little variation among extractions was found. However, because multiple reversed phase columns could not be run using all of the samples from the first extraction, and because larger than usual fractions (60 and 80 drops/fraction) had to be collected from columns of samples that were examined only once, the error associated with calculating the area under the peaks was increased. Thus, an ANOVA was calculated and an F test was used to compare the peaks among samples from the second extraction. Duplicate columns were run for each of these samples and 40 drop fractions were collected. The results of this test showed that, at the 95% level, there was no difference among the three samples for peaks 1, 2, 4 and 5. A Duncan's Multiple Range test was used to compare peaks 3 and 6. For peak 3, there was no difference between the aleurone and the embryo-less half-seed samples and the

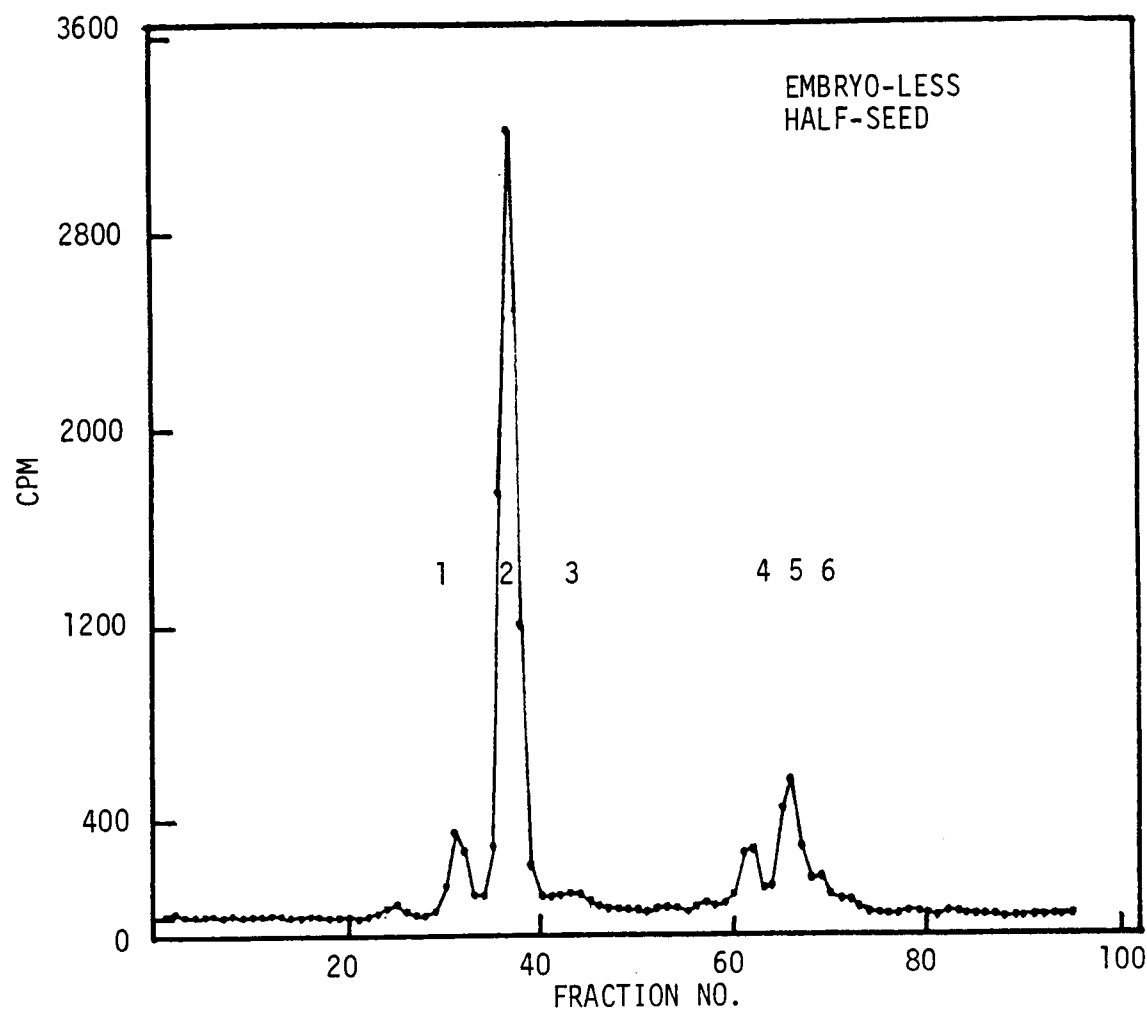


Figure VIII. RPC-5 chromatography of lysine tRNA from embryo-less half-seeds. The sample was eluted with a 200 ml gradient of 0.4 M to 0.6 M NaCl in 0.01 M sodium acetate pH 4.5, 0.01 M $MgCl_2$, 0.001 M EDTA and 0.005 M BME. Ninety-five 40 drop fractions were collected. Transfer RNA was precipitated and collected on glass fiber disks, and the radioactivity was determined as reported in the Materials and Methods section.

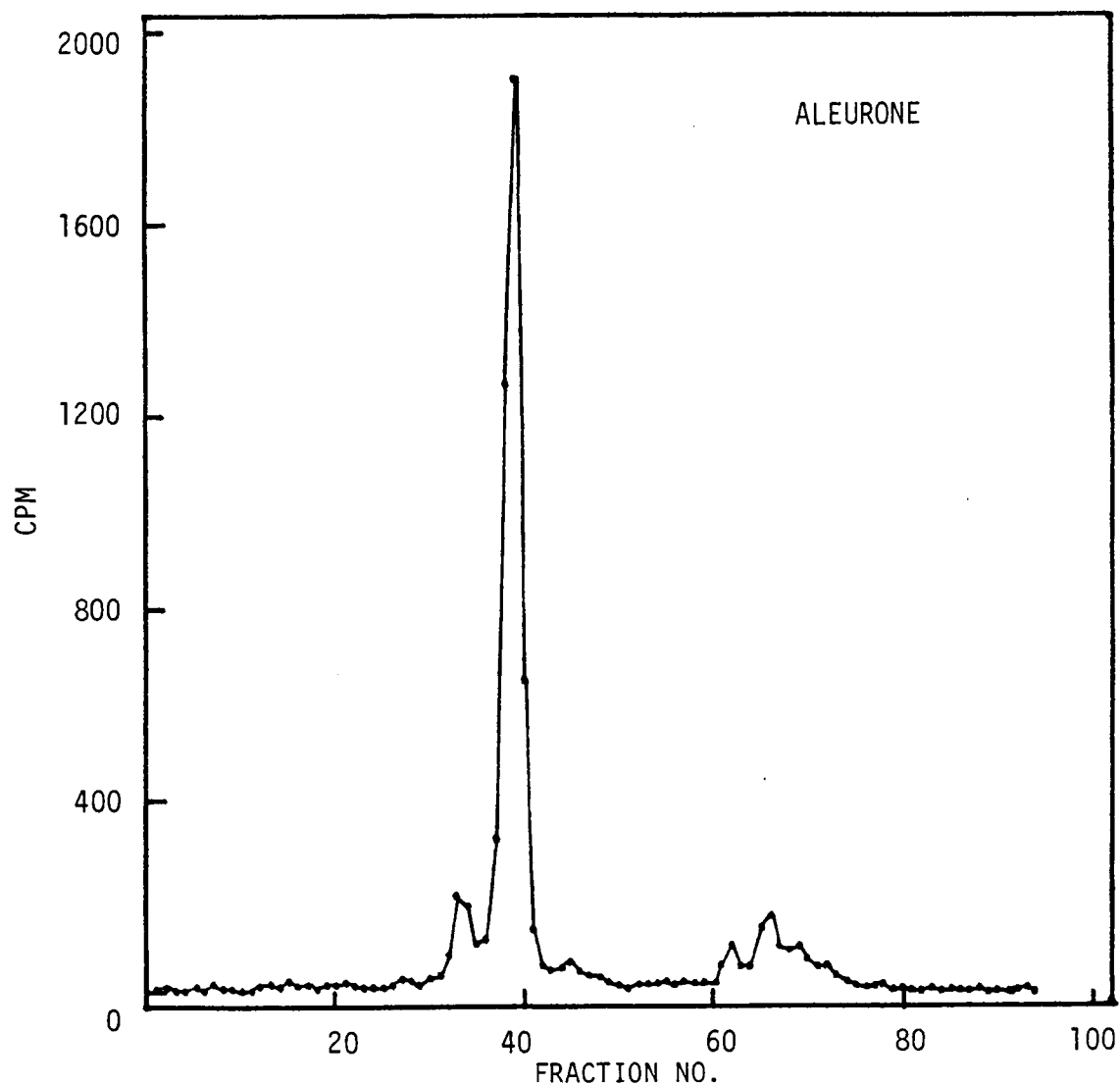


Figure IX. RPC-5 chromatography of lysine tRNA from aleurone. The sample was eluted with a 200 ml gradient of 0.4 to 0.6 M NaCl in 0.01 M sodium acetate pH 4.5, 0.01 M $MgCl_2$, 0.001 M EDTA and 0.005 M BME. Ninety-five 40 drop fractions were collected. Transfer RNA was precipitated and collected on glass fiber disks, and the radioactivity was determined as reported in the Materials and Methods section.

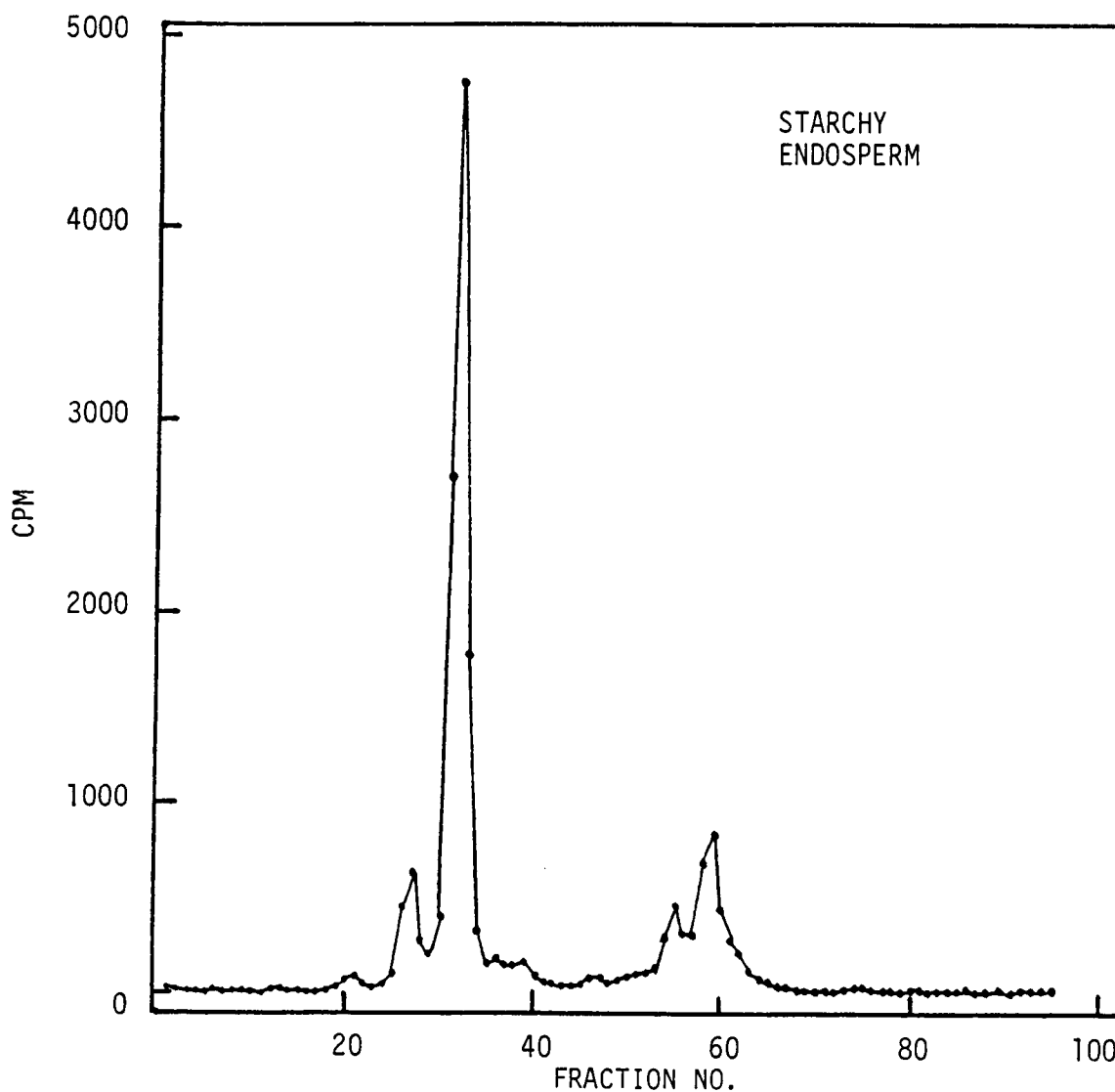


Figure X. RPC-5 chromatography of lysine tRNA from starchy endosperm. The sample was eluted with a 200 ml gradient of 0.4 to 0.6 M NaCl in 0.01 M sodium acetate pH 4.5, 0.01 M MgCl₂, 0.001 M EDTA and 0.005 BME. Ninety-five 40 drop fractions were collected. Transfer RNA was precipitated and collected on glass fiber disks, and the radioactivity was determined as reported in the Materials and Methods section.

embryo-less half-seed and endosperm sample; both were significantly different from the aleurone sample. However, values used in calculating the ANOVA were subject to several sources of error. One source was the counting error associated with the scintillation counter. Minor peaks were not counted long enough to achieve a high degree of confidence in the counts. Other sources of error include error in calculating the area under the curve as well as error in calculating the area under each peak. These three sources of error are compounded when percent area under the curve was used as the value to be compared. However, the number of counts in the total sample layered on the column varied, making percentages the only possible way to compare the samples. Empirically none of the profiles appeared to differ appreciably. Since four of the peaks, including the major peak, were the same for all samples, there was no difference in peak 3 for embryo-less half-seed and aleurone samples, and peak 6 was a minor peak, it was concluded that the three profiles were essentially the same and that the embryo-less half-seed tissue could be used in place of isolated aleurone layers for the time course experiments.

V. COMPARISON OF LYSINE tRNA ISOACCEPTORS FROM EMBRYO-LESS HALF-SEEDS GERMINATED IN THE PRESENCE AND ABSENCE OF GA

Since tRNAs have been shown to regulate protein synthesis in systems similar to the barley aleurone system, experiments were designed to observe lysine tRNA profiles from dormant embryo-less

half-seeds and from embryo-less half-seeds incubated for 24 and 48 hours with and without GA. Table III shows the yields and charging efficiencies from the five treatments outlined above. When tRNA was extracted from the dormant and 24 hour +GA tissue, DNA was spooled out after the first addition of ethanol. This severely decreased yields. Dormant tissue was re-extracted and the second sample (Zero-2), yielding 12.3 A_{260} units was used for assays and for RPC-5 chromatography. The 24 hour +GA sample yielded 4 A_{260} units of tRNA, which was sufficient to obtain two RPC-5 profiles. It was decided not to remove DNA from samples during the extraction procedure. As mentioned above, yields of tRNA cannot be considered as absolute values of tRNA present in the tissue.

Figures XI through XV show representative RPC-5 profiles of lysine tRNA from dormant embryo-less half-seeds and those incubated for 24 and 48 hours in the presence and absence of GA. Duplicate columns were run for each sample. In all cases, six lysine tRNA isoacceptors were found. As described above, an ANOVA was used to determine if differences existed among the peaks of the five samples. There were no differences among samples for peaks 1, 3, 5 and 6. Peaks 2 and 4 were further analyzed using a Duncan's Multiple Range test. Peak 4, ranging from 3.0 to 6.5% of the total lysine tRNA, was different for each sample. For peak 2, the 24 hour -GA sample was slightly different from the rest. As mentioned previously, much error could be associated with counts in the lower peaks and also with the method used to approximate areas under all

Table III. Yields and Charging Efficiencies of tRNA from Germinating Embryo-less Half-seeds.

Sample	Yield of tRNA ^a (A ₂₆₀)	Charging Efficiency (pmole/A ₂₆₀)	Yield of Lysine tRNA ^b (pmole)
Zero	1.5	N.D. ^d	N.D.
Zero-2 ^c	12.3	28	340
24 hour + GA	4.0	18	72
48 hour + GA	24.2	7.6	180
24 hour - GA	11.0	2.5	28
48 hour - GA	16.0	23	370

^aFor each sample, values are based on an extraction of 200 embryo-less half-seeds.

^bValues were calculated by multiplying the yield by the charging efficiency.

^cThe first time tRNA was extracted from dormant embryo-less half-seeds, the yield was insufficient for RPC-5 chromatography, therefore a second extraction was performed (Zero-2).

^dNot determined.

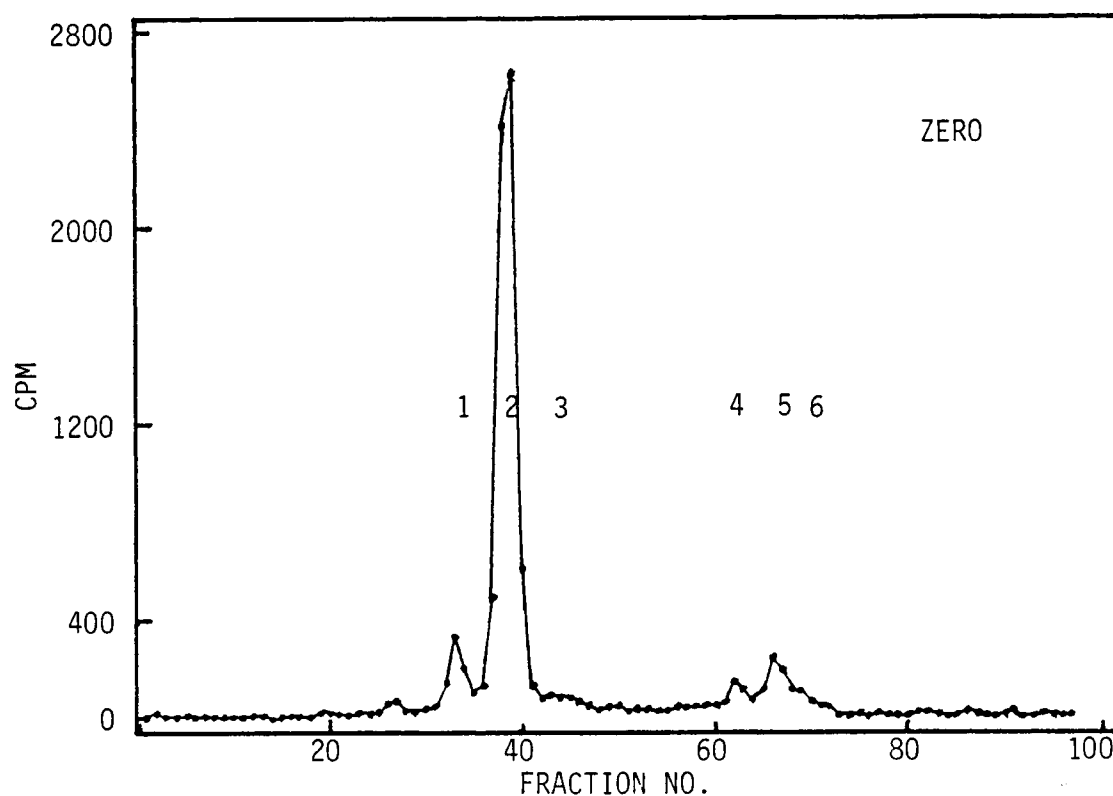


Figure XI. RPC-5 chromatography of lysine tRNA from zero-2 half-seeds. The sample was eluted with a 200 ml gradient of 0.4 to 0.6 M NaCl in 0.01 M sodium acetate pH 4.5, 0.01 M $MgCl_2$, 0.001 M EDTA and 0.005 M BME. Ninety-seven 40 drop fractions were collected. Transfer RNA was precipitated and collected on glass fiber disks, and the radioactivity was determined as reported in the Materials and Methods section.

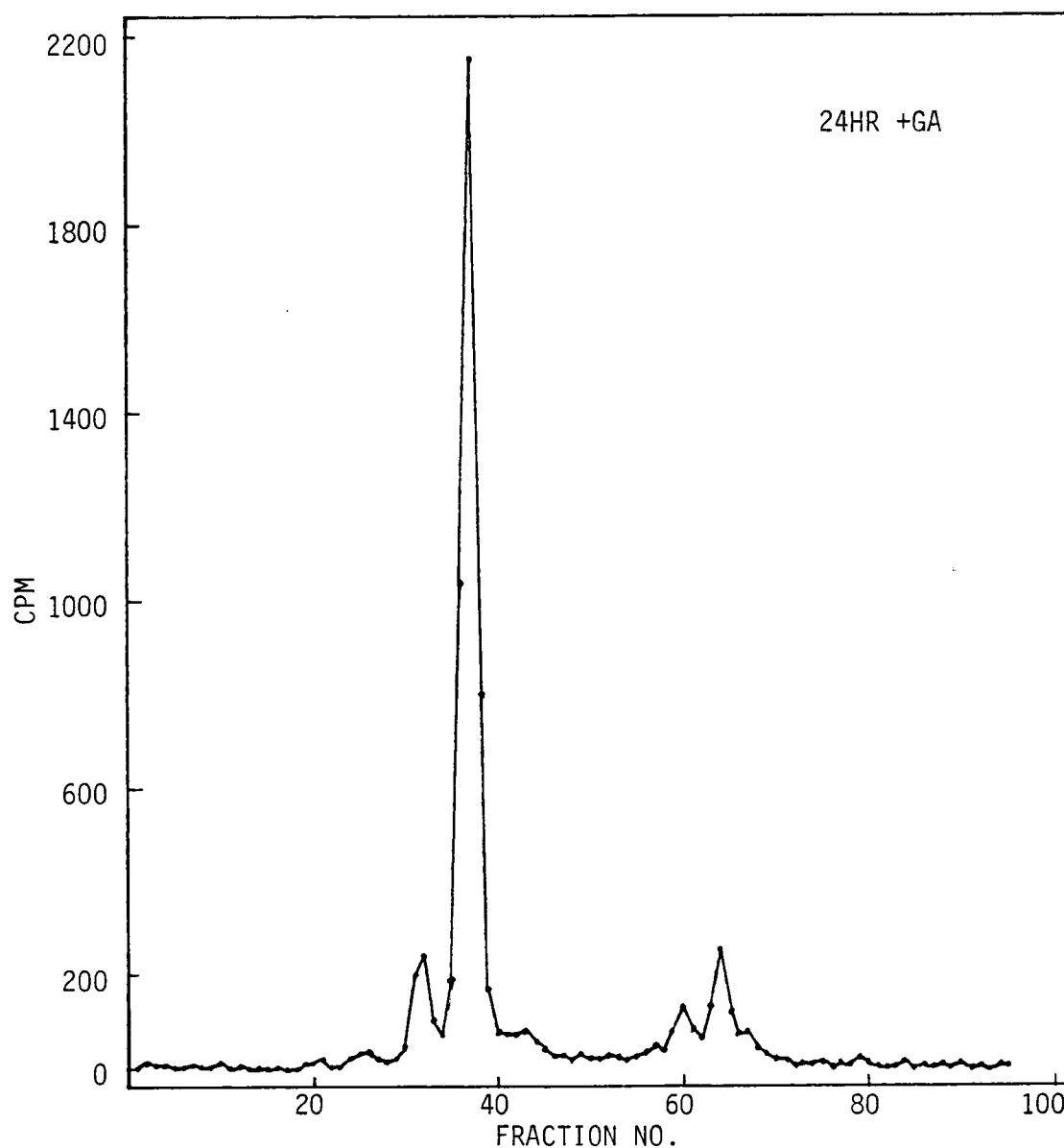


Figure XII. RPC-5 chromatography of lysine tRNA from 24 hour +GA half-seeds. The sample was eluted with a 200 ml gradient of 0.4 to 0.6 M NaCl in 0.01 M sodium acetate pH 4.5, 0.01 M MgCl₂, 0.001 M EDTA and 0.005 M BME. Ninety-five 40 drop fractions were collected. Transfer RNA was precipitated and collected on glass fiber disks, and the radioactivity was determined as reported in the Materials and Methods section.

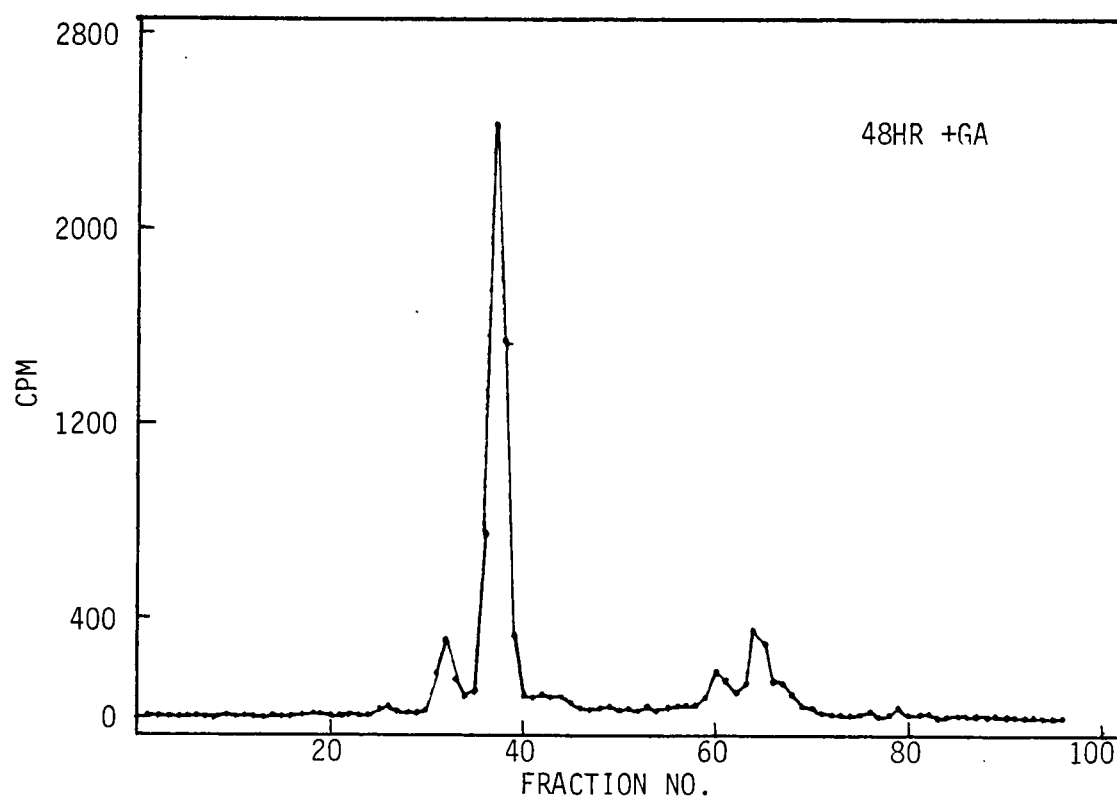


Figure XIII. RPC-5 chromatography of lysine tRNA from 48 hour +GA half-seeds. The sample was eluted with a 200 ml gradient of 0.4 to 0.6 M NaCl in 0.01 M sodium acetate pH 4.5, 0.01 M MgCl₂, 0.001 M EDTA and 0.005 M BME. Ninety-six 40 drop fractions were collected. Transfer RNA was precipitated and collected on glass fiber disks, and the radioactivity was determined as reported in the Materials and Methods section.

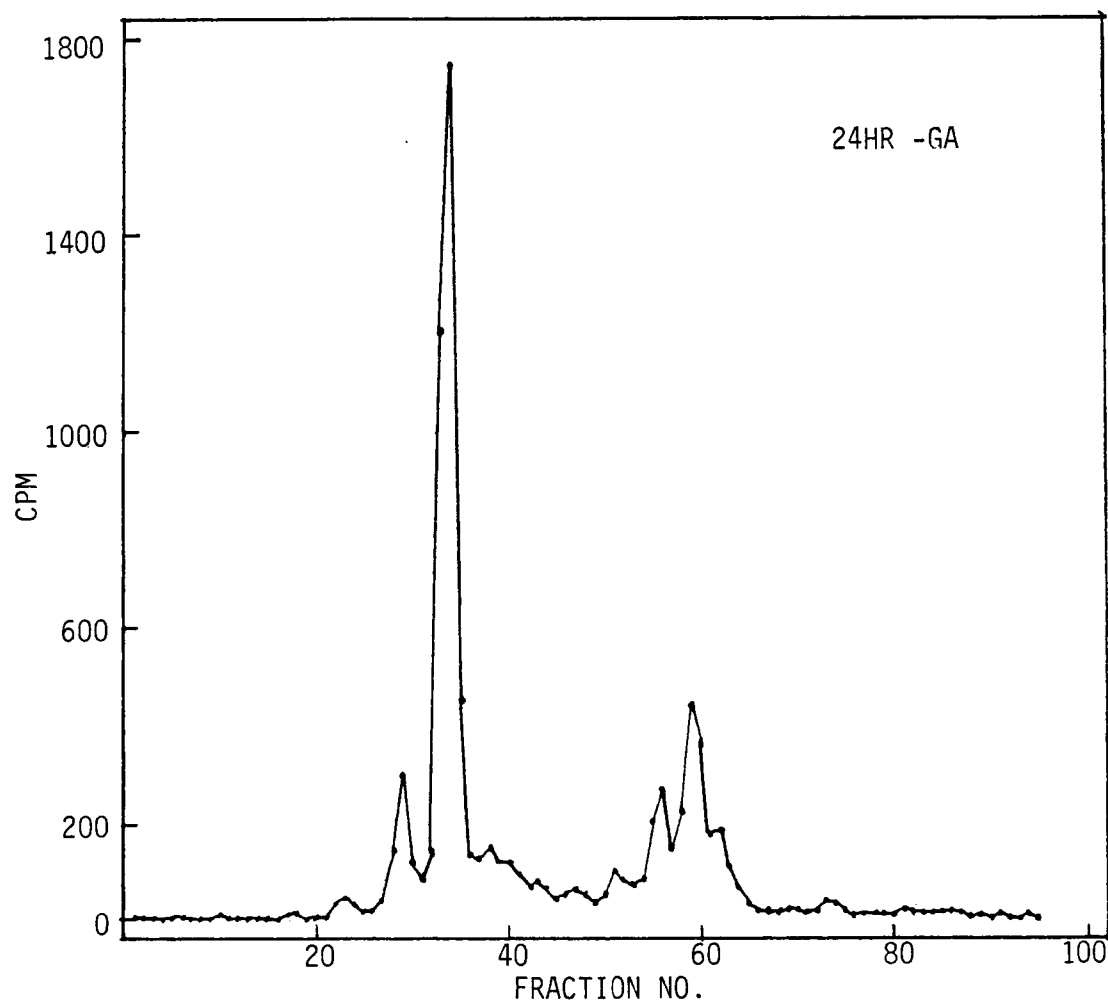


Figure XIV. RPC-5 chromatography of lysine tRNA from 24 hour -GA half-seeds. The sample was eluted with a 200 ml gradient of 0.4 to 0.6 M NaCl in 0.01 M sodium acetate pH 4.5, 0.01 M MgCl₂, 0.001 M EDTA and 0.005 M BME. Ninety-five 40 drop fractions were collected. Transfer RNA was precipitated and collected on glass fiber disks, and the radioactivity was determined as reported in the Materials and Methods section.

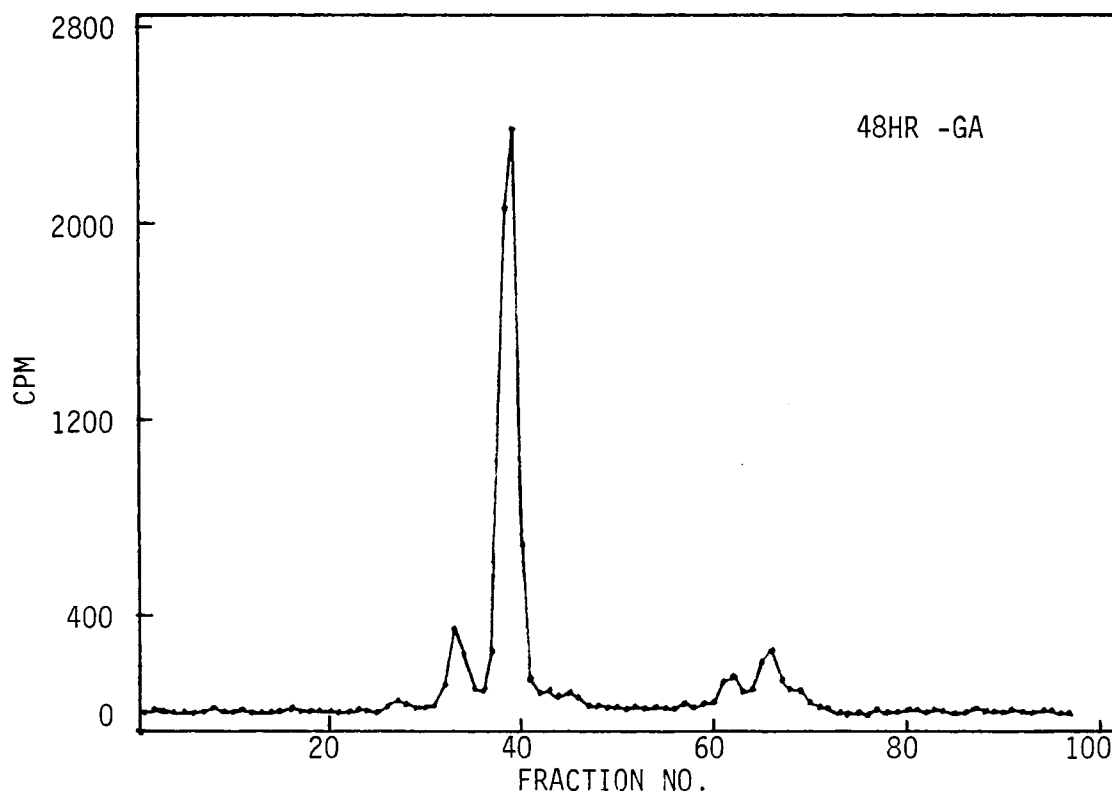


Figure XV. RPC-5 chromatography of lysine tRNA from 48 hour -GA half-seeds. The sample was eluted with a 200 ml gradient of 0.4 to 0.6 M NaCl in 0.01 M sodium acetate pH 4.5, 0.02 M MgCl₂, 0.001 M EDTA and 0.005 M BME. Ninety-seven 40 drop fractions were collected. Transfer RNA was precipitated and collected on glass fiber disks, and the radioactivity was determined as reported in the Materials and Methods section.

peaks. All of the peaks were the same for the zero, 24 and 48 hour +GA samples, except for peak 4, a minor peak. Considering the error associated with the calculations for that peak, it was concluded that the lysine tRNA profiles did not change appreciably over germination. The 24 hour -GA sample differed slightly from the rest for peak 2; however, because endosperm is normally exposed to GA, this sample was not physiologically significant. Again, peak 4 was the only peak found to be different among the dormant, 24 and 48 hour +GA samples and the 48 hour -GA sample. It was thus concluded that GA had little effect on the tRNA profile. A profile generated from dormant embryo-containing half-seeds (data not shown) was the same as those shown from embryo-less half-seeds.

As mentioned in the literature review (p. 18), Janowicz et al. (1979) compared the major isoacceptors of phenylalanine tRNA from wheat and barley germ and found their nucleotide sequence to be the same. The profile of barley seed lysine tRNA is similar to that of wheat germ (Hatfield and Rice, 1978). The major isoacceptor for wheat germ lysine tRNA recognizes the codon AAG. This is the same codon which is used most frequently to code for lysine in the barley α -amylase mRNA (Muthukrishnan, personal communication). This suggests that the major lysine tRNA isoacceptor in barley, peak 2, might also preferentially bind to the AAG codon. Its availability in aleurone cells could promote the synthesis of α -amylase.

VI. KINETICS OF THE AMINOACYLATION REACTION

The lysine analog AEC is known to inhibit the expression of α -amylase in the aleurone system (Henke et al., 1981). This inhibition can be counteracted by the addition of lysine. The biochemical mechanism for AEC inhibition is unknown. One possibility is for the AEC to interfere in the aminoacylation of lysine tRNA. The AEC could compete with lysine and either block the aminoacylation of the lysine so that less protein can be made, or AEC can itself be aminoacylated to lysine tRNA and thus be incorporated into proteins possibly rendering them inactive. Experiments were designed to observe the effects of AEC on the formation of lysyl-lysine tRNA. Figure XVI shows that AEC does inhibit lysyl-lysine tRNA formation, using conditions for maximum aminoacylation. To determine an apparent K_m value for lysine in the aminoacylation reaction, it was necessary to establish conditions where lysine was limiting. As shown in Figure XVII, lysine became limiting at concentrations below 20 μ M. Once the effect of varying the lysine concentrations was determined, it was necessary to alter the assay conditions to insure zero-order kinetics. Figure XVIII shows that, over four lysine concentrations, this was accomplished by decreasing the concentration of enzyme to one fifth its original level and by decreasing the reaction time to less than 10 minutes.

Since the enzyme was not pure and since the reaction was not completely characterized for all its components, absolute kinetic

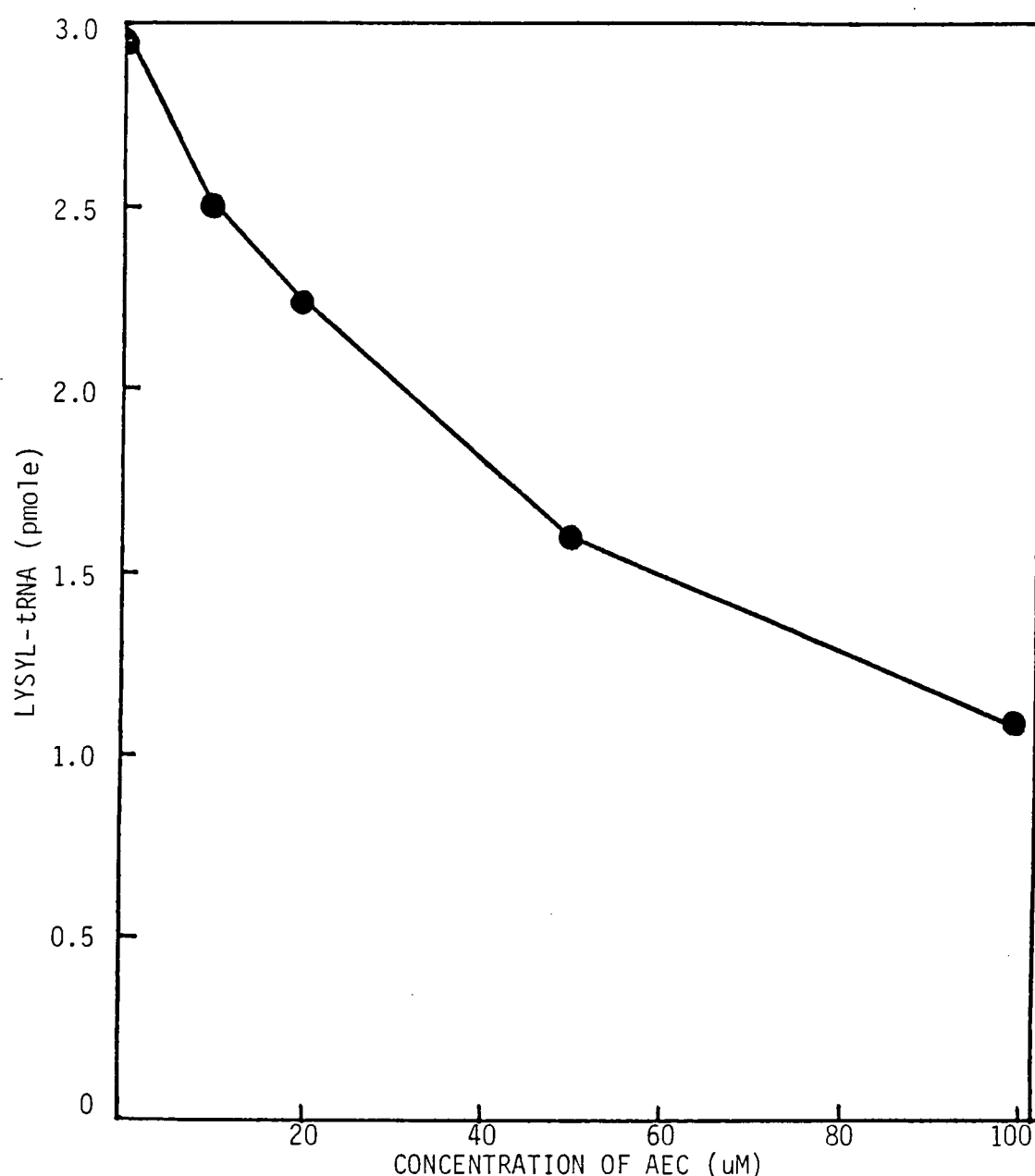


Figure XVI. Inhibition of lysyl-tRNA formation caused by AEC. Duplicate assays were done, and the standard deviation for these measurements was 0.15 pmole. The assays contained 0.2 A₂₆₀ of tRNA (48 hour -GA) and 20 μ l of mixed synthetase preparation in a 50 μ l reaction mixture containing 0.1 M Tris-HCl pH 7.5, 0.008 M ATP, 0.02 M MgCl₂, 0.06 M BME, 20 M ³H-lysine (2 Ci/mmol) and varying amounts of AEC. At 30 minutes, a 40 μ l sample was pipetted onto filter paper disks. Transfer RNA was precipitated, and radioactivity was measured. Results are reported as pmole/50 μ l reaction mixture.

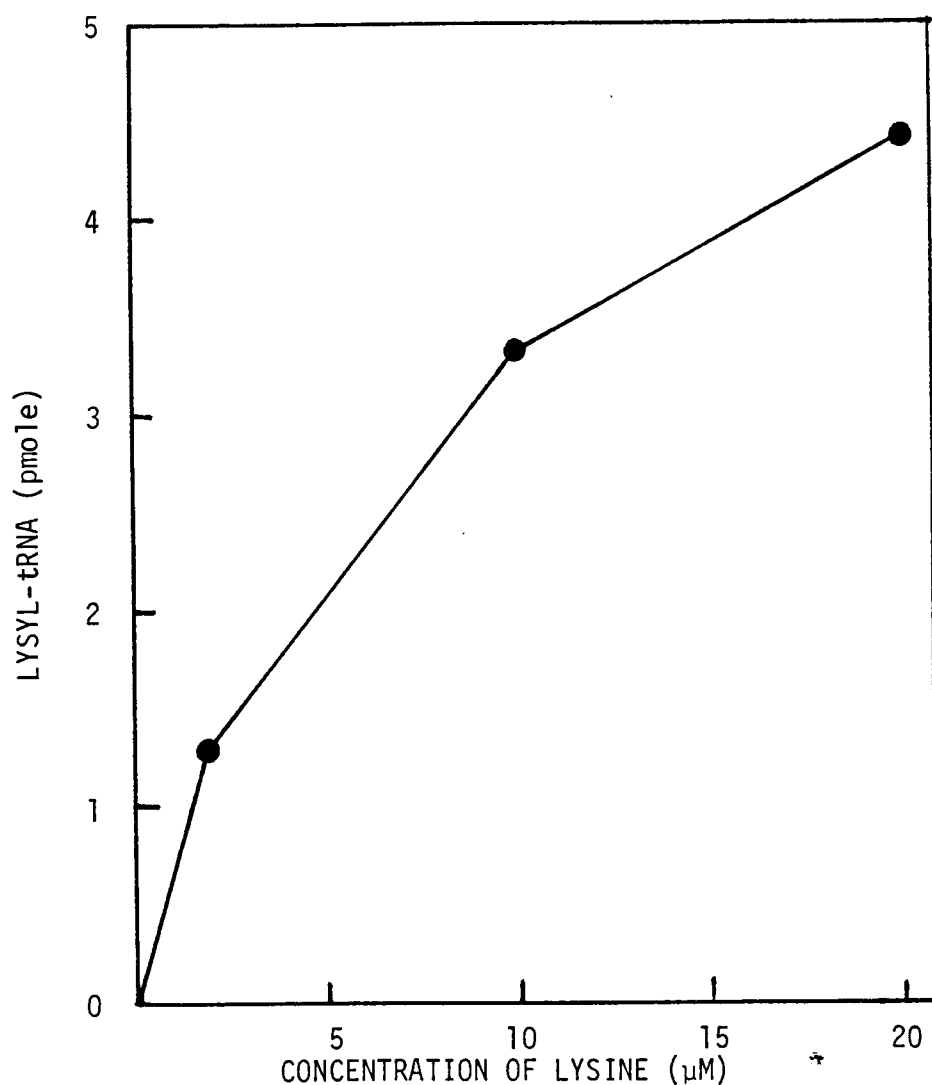


Figure XVII. Effect of lysine concentration on lysyl-tRNA formation. Assays were done in duplicate, and these measurements had a standard deviation of 0.07 pmole. Assays contained 0.2 A₂₆₀ of tRNA (48 hour -GA) and 20 μl of mixed synthetase preparation in a 50 μl reaction mixture containing 0.1 M Tris-HCl pH 7.5, 0.008 M ATP, 0.02 M MgCl₂, 0.06 M BME and varying amounts of ³H-lysine (2 Ci/mmol). At 30 minutes, a 40 μl sample was pipetted onto filter paper disks. Transfer RNA was precipitated and radioactivity was measured. Results are reported as pmole/50 μl reaction mixture.

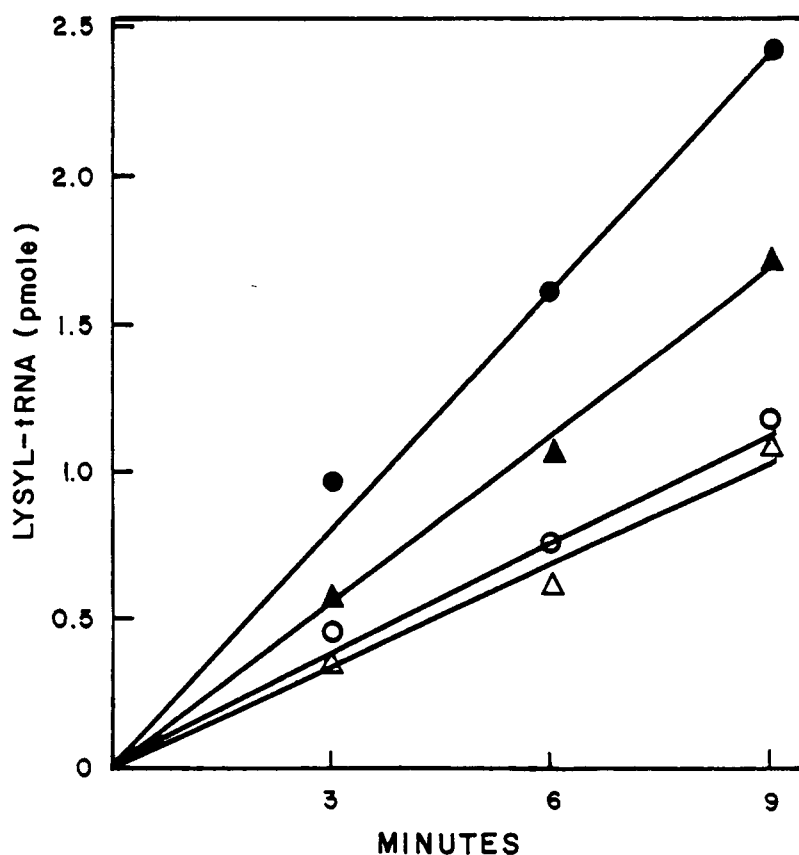


Figure XVIII. Kinetics of aminoacylation. Assays were done in triplicate, and these measurements had a standard deviation of 0.10 pmole. One hundred μ l assays contained 0.4 A₂₆₀ of whole seed tRNA and 8 μ l of mixed synthetase in 0.1 M Tris-HCl pH 7.5, 0.008 M ATP, 0.02 M MgCl₂, 0.06 M BME and one of the following: (Δ) 2 μ M, (○) 3 μ M, (▲) 5 μ M or (●) 20 μ M ³H-lysine (2 Ci/mmol). At 3, 6 and 9 minutes, 30 μ l was pipetted onto glass fiber disks. Transfer RNA was precipitated and radioactivity was measured. Results are reported as pmole/30 μ l of reaction mixture. Mean values for each time point were used to calculate the regression lines shown.

constants could not be measured. An apparent K_m for lysine and an apparent K_i for AEC were obtained. The apparent K_m for lysine was determined to be $2.72 \pm 0.39 \mu\text{M}$. Figure XIX is one of five Lineweaver-Burke plots used to determine this value. As indicated in Chapter II (page 19), most K_m values reported for aminoacyl-tRNA synthetases and their cognate amino acids were in the range of 10^{-5} to 10^{-6} M. The relative affinity of the enzyme for AEC was determined by measuring an apparent K_i . This was accomplished by running the reaction, using the same lysine concentrations, in the presence of 6 and 12 μM unlabeled AEC. Figure XIX shows a double reciprocal plot of these values. Each line represents one experiment. Since the V_{max} was relatively constant (the y-intercept equals $1/V_{\text{max}}$), it is shown that AEC is a competitive inhibitor in this reaction. The K_i value was calculated from each line. The average value determined over five experiments was $9.2 \pm 7.3 \mu\text{M}$. It was concluded that the lysyl-tRNA synthetase has affinities for lysine and AEC in the micromolar range.

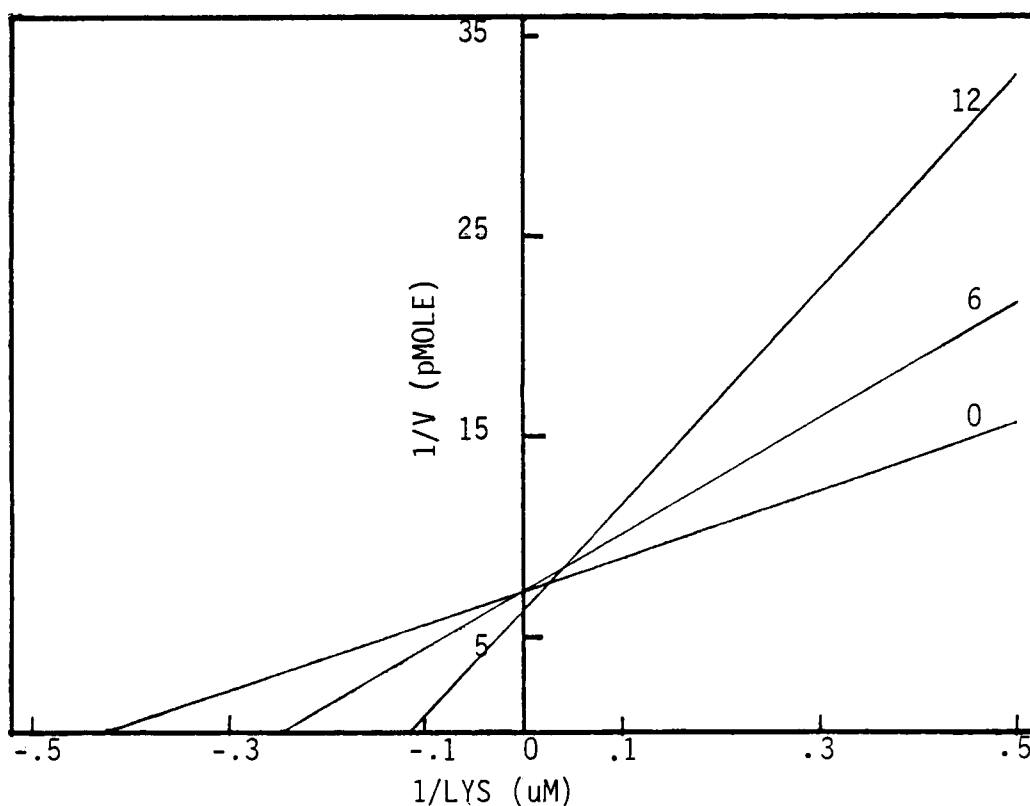


Figure XIX. Lineweaver-Burke double reciprocal plots of lysyl-tRNA formation with varying concentrations of lysine and AEC. Concentrations of lysine used were 2, 3, 5 and 20 μM . All assays were done in triplicate. The plot shows an apparent K_m for lysine of 2.3 μM (line designated 0). An apparent K_i for the lysine analog AEC was determined with 6 and 12 μM AEC present in the assay (lines 6 and 12). The plot shows the apparent K_i value for AEC to be 6.5 μM .

CHAPTER V

CONCLUSION

Lysine tRNA and lysyl-tRNA synthetase from barley (Hordeum vulgare L. cv. Atlas 57) endosperm were characterized. Lysine tRNA isoaccepting species were examined for changes during germination in the presence and absence of GA. An apparent K_m ($2.3 \mu M$) for lysine and an apparent K_i ($9.2 \mu M$) for AEC were determined for lysyl-tRNA synthetase.

Six lysine tRNA isoacceptors were found in all tissues examined. The chromatographic profiles of the lysine tRNA do not change during germination. This is in contrast to several similar systems where a tissue mainly produces one protein product, and the availability or amount of isoacceptor(s) are known to be correlated with the amount of the major protein product. Also, GA has no dramatic effect on the barley lysine tRNA profile. There is evidence suggesting that the most dominant isoacceptor, number two, could bind specifically to the AAG codon (Hatfield and Rice, 1978 and Janowicz et al., 1979), the codon most commonly used for lysine in the α -amylase mRNA (Muthukrishnan, personal communication). Thus, there is a possibility that the availability of this lysine tRNA in aleurone tissue could play a part in enhancing the synthesis of α -amylase.

Other tRNAs could be examined for their role in the regulation of α -amylase synthesis. Himalaya barley α -amylase has 22 lysine

residues, but it also contains 33 alanine, 46 glycine, 22 valine, 49 asparagine plus aspartate and 26 glutamine plus glutamate residues (Rodaway, 1978). Profiles of these aminoacyl-tRNAs could change in response to an influx of GA thus promoting the synthesis of α -amylase.

The lysine analog AEC was shown to be a competitive inhibitor of the lysyl-tRNA synthetase reaction. Henke et al. (1981) showed AEC to inhibit the expression of α -amylase in barley endosperm. This inhibition was reversed by the addition of lysine. Also, α -amylase from endosperm incubated in the presence of AEC has an altered electrophoretic mobility on nondenaturing gels (Mullin and Thompson, unpublished). Together, these results are evidence suggesting that AEC is being incorporated into newly synthesized aleurone proteins.

Further experimentation needs to be done in order to prove conclusively that AEC is incorporated into α -amylase. Aminoacylation of labeled AEC could not be done due to the commercial cost of the compound and due to the inability to synthesize the radiolabeled compound with a sufficient specific activity and quantity for assays. Measuring for activation of AEC using the ATP/PPi exchange assay could support AEC incorporation. A periodate oxidation experiment could demonstrate aminoacylation of AEC onto lysine tRNAs. Finally, α -amylase from endosperm incubated in the presence of AEC could be isolated, purified and hydrolysed. An amino acid analysis of the products could determine whether or not AEC is incorporated. Care

must be taken to make certain that the preparation is free of soluble amino acids and that the AEC is not destroyed in the hydrolysis reaction.

Part of the rationale for conducting this research was to learn more about the basic biochemistry of the Atlas 57 seed in order to facilitate the characterization of mutagenized, AEC-resistant Atlas 57 seeds. The endosperm from these mutants was shown to produce active α -amylase in the presence of AEC. Five possible categories of mutations could account for this AEC resistance. They are: 1) exclusion of AEC from the cells, 2) detoxification, by enzymatic breakdown of AEC, once it enters the cells, 3) overabundance of lysine that would out-compete AEC for aminoacylation, 4) changes in the lysine tRNA and/or lysyl-tRNA synthetase which would lead to an inability of AEC to be aminoacylated to lysine tRNA thereby excluding it from protein and 5) overproduction of α -amylase. The focus of this project was on the fourth category, the tRNA/synthetase mutants. As mentioned above, the profile of lysine tRNAs in aleurone of normal seeds was characterized. Kinetic parameters for the lysyl-tRNA synthetase were determined. The apparent K_m for lysine and the apparent K_i for AEC were found to be 2.7 and 9.2 μM , respectively.

In summary, two conclusions can be made from this research. Methods have been established for the extraction of tRNAs from barley endosperm. These methods could be applied to the extraction of tRNAs from any grain, mutant or non-mutant. Until this study

was done, tRNAs from germinating barley endosperm had not been examined. The isoaccepting species of lysine tRNA from this tissue have now been examined. Although no changes in profiles were seen, and no hormonal effects were demonstrated, our knowledge of germination physiology has increased. The characterization of the lysyl-tRNA synthetase from barley aleurone and its interaction with lysine analog AEC and lysine tRNA is only a beginning.

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APPENDIX

APPENDIX

PREPARATION FOR COLUMN CHROMATOGRAPHY

DEAE Cellulose Column Chromatography

A stock of DEAE cellulose was prepared for column chromatography by taking the dry resin through a series of washes. The procedure is as follows. Thirty grams of DEAE cellulose (Bio-Rad Celle D) was suspended in 1 l of ddH₂O, stirred with a stirring rod for 10 minutes and allowed to stand for 30 minutes. Fines were removed by decantation. The remaining water was removed by filtering over a buchner funnel fitted with filter paper. Resin was suspended in 300 ml of 1 N NaOH, stirred for 10 minutes, filtered and washed with 1 l of ddH₂O. The procedure was repeated using 300 ml of 1 N HCl, a water wash, and then 300 ml of 1 N NaOH. The NaOH was washed out using three l of ddH₂O followed by 300 ml of 0.025 M NaCl. Resin was then suspended in 300 ml of 0.025 M NaCl and placed in a buchner funnel fitted with a closable outlet. The DEAE-cellulose was washed with ddH₂O until it was free of chloride ions. This was tested by adding a single drop of eluate to 0.5 ml of 0.1% AgNO₃ solution. A white precipitate (AgCl) indicated the presence of chloride ions. The resin was stored refrigerated in 400 ml of ddH₂O containing 0.02% sodium azide.

RPC-5 Column Chromatography

RPC-5 beads were prepared by Sheghemi Simms of the Biology Division (ORNL) by coating plaskon beads with Adogen 464 (Pearson et al., 1971). The beads were vigorously stirred overnight in a high salt buffer (2 M NaCl). The column was prepared by pipetting a few ml of resin into a Glenco 0.6 x 30 cm jacketed column, held at 37°C and by packing the beads under 300 psi pressure. Only a high salt buffer was used during the packing procedure. After those beads were packed, a few more ml of resin were added and pressure was applied. This procedure was repeated until a final packed column height of 17 to 18 cm was achieved. Pressure was lowered to about 180 psi and the column was further packed by taking it through a series of washes. These began with a 200 ml gradient of 0.4 to 0.6 M NaCl RPC-5 buffer (0.01 M sodium acetate pH 4.5, 0.01 M MgCl₂ and 0.001 M EDTA) followed by 30 ml of 2 M NaCl RPC-5 buffer. The upper column plunger was adjusted to a position right above the column matrix. The column was equilibrated with about 100 ml of 0.4 M NaCl RPC-5 buffer and was rinsed with an additional 200 ml 0.4 to 0.6 M gradient. The plunger height was re-adjusted and the column was stored in 2 M NaCl RPC-5 buffer until further use.

Sephadex G-200 Column Chromatography

Sephadex beads were swollen by autoclaving them in Sephadex column buffer (15% glycerol, 0.01 M KPO₄ pH 7.5, 0.005 M KCl and 0.001 MgCl₂). Sephadex G-200 was poured into a 2.5 x 22 cm

refrigerated column. Approximately 2 cm of Sephadex G-25, prepared as above, was layered on top to give a firmer surface on which to layer the sample. The column was equilibrated with 250 ml of column buffer, using a 30 cm pressure head.

PREPARATION OF LYSINE STOCK

One mCi L-[4,5 $^3\text{H}(\text{N})$] lysine (New England Nuclear, NET-376) was purchased with a specific activity of approximately 60 Ci/mmol. The lysine was in one ml of 0.01 N HCl. The specific activity of the lysine stock varied according to the experiment in which it was to be used. Routine assays to achieve maximal tRNA aminoacylation used lysine with a specific activity of one or two Ci/mmol, depending on the concentration of the tRNA. For enzyme kinetic experiments a stock of five Ci/mmol was used. All lysine stocks were made to 100 μM .

The procedure for diluting the purchased lysine began with determining the molarity of the lysine by dividing the specific activity into one mCi. This gave the total nmoles of lysine in the purchased vial. A dilution factor was calculated by dividing one mCi over the molarity calculated above by the specific activity of the desired stock. The molarity was then multiplied by one minus the dilution factor. This determined the nmol of cold lysine needed. Summing the nmol of hot lysine available with the amount of cold lysine needed gave the total number of nmol for the desired 100 μM stock, and thus determined the total volume of stock to be

made. The ^3H -lysine was neutralized with an equal number of equivalents of NaOH. The proper amount of cold lysine was added from a stock solution, and the final volume was made using ddH₂O. The lysine stocks were stored frozen in 500 μl aliquots.

DETERMINATION OF COUNTING EFFICIENCY

A quench curve was constructed to determine the conditions for counting in aqueous fluor. One hundred μl of sample in 1 M NaCl was counted in 5 ml of Scintaverse plus 0, 100, 300, 700, 1100 or 1400 μl of ddH₂O. Samples were counted for 10 minutes each. Figure XX shows that 300 μl of ddH₂O was optimum for achieving maximum counting efficiency.

Counting efficiency was determined by placing a known amount of lysine (dpms) onto paper and glass fiber disks and in the liquid system described above. Disks were counted in 5 ml of Scintanalysed (Fisher) toluene containing 0.4 g/l PPO and 0.05 g/l POPOP. Counting efficiency was defined as the ratio of cpms to dpms.

For the conversion of cpms to dpms for the filter paper system, 100 μl of aminoacylated tRNA was spotted onto paper and glass disks. The same amount was also prepared in the liquid system. Radioactivity was measured and the ratio of cpm in the liquid system to cpm on the disks was determined. This ratio, in conjunction with the counting efficiency in liquid, determined above, could be used to calculate the dpms in any sample, thus allowing for the conversion of cpms to pmol of aminoacylated tRNA.

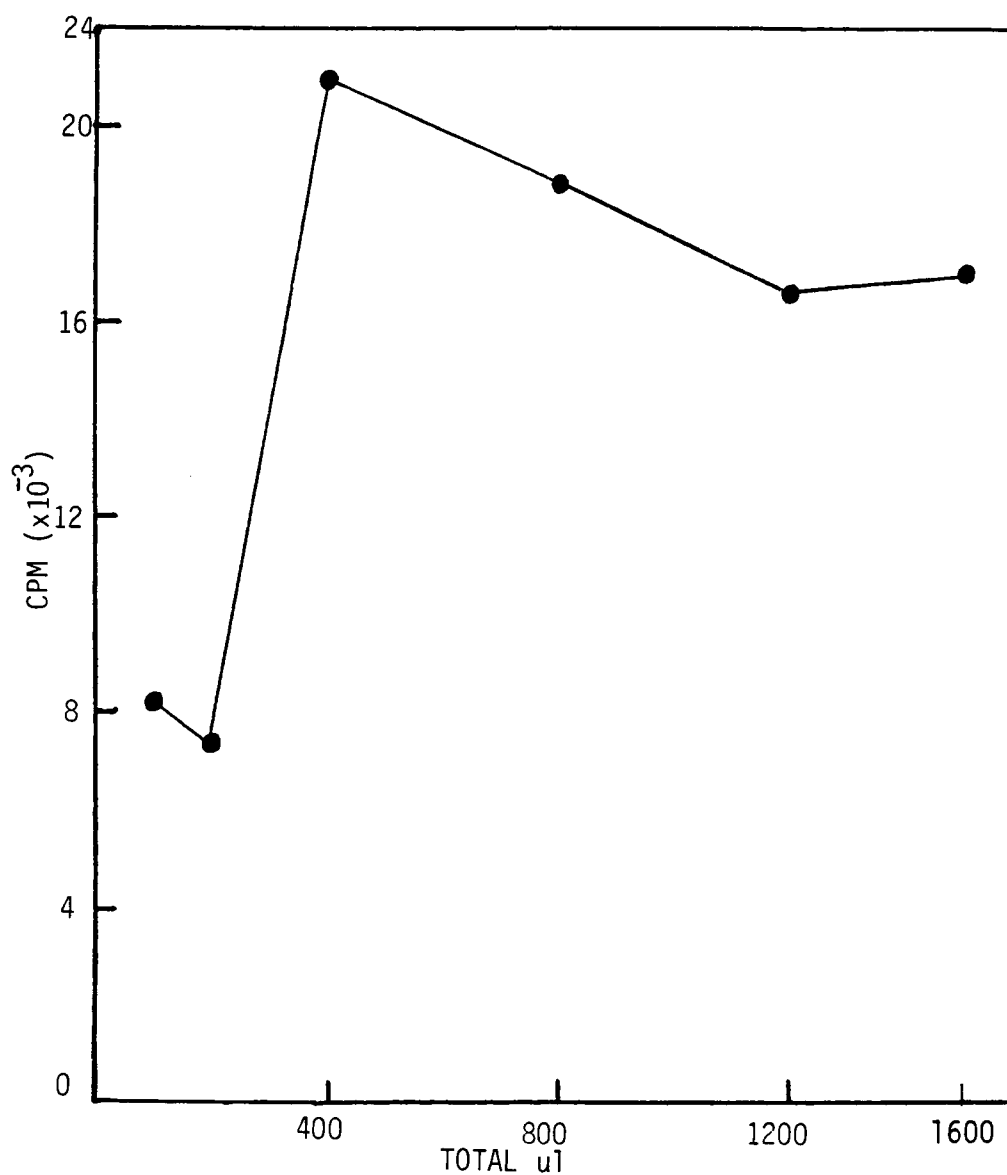


Figure XX. Quench curve for ^3H counting using a Beckman LS-200B liquid scintillation system. One hundred μl of ^3H -dTTP in 1M NaCl plus one of the following: 0, 100, 300, 700, 1100, or 1400 μl of ddH₂O was counted in 5 ml of Scintaverse.

VITA

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