THE ROLE OF tRNA IN DEVELOPMENT

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SUMMARY

Experiments that suggest a developmental regulatory role for tRNA in translation (such cytokinins in tRNA, suppressor mutations, tRNA methylases, excessive number of isoaccepting species, and others) are briefly presented. Reports that link changes in the tRNA population of cells with developmental changes are collated.

Our attempts to find changes in tRNA acceptor concentrations and in isoacceptor levels in the cotyledons of cotton seeds during their embryogenesis and germination are presented in detail. In this study we have found no pattern of change in the relative concentration of acceptors for each of the amino acids among the cytoplasmic tRNAs of the cotyledons during development. In fact the relative acceptor concentrations of the cotyledons are indistinguishable from those of cotton roots. We have observed a 7-fold increase per cell in the levels of chloroplastic tRNA that takes place during the first 5 days of germination. This increase does not require a light stimulation, and, further, chloroplastic tRNA is present in roots.

The assumptions and genetic implications of a system of developmental regulation based in part on tRNA levels and code word frequency in mRNA are discussed.

BACKGROUND

I would like to begin this presentation by briefly discussing the observations that were made during the 1960's that suggested to many people the possibility that tRNA molecules play a regulatory role in cellular processes including differentiation. As we all know, cell differentiation is ultimately a matter of getting different proteins and different amounts of the same protein into different cells, and consequently is a matter of specifying and regulating the synthesis of mRNA differentially in different cells. However, there are a number of reasons for thinking that perhaps the translation of mRNA into protein is a site of developmental regulation, since the final amount of the various proteins that a cell is equipped with could certainly be influenced by mechanisms that might treat different mRNA differently in the translation process. Quite naturally all the participants in the translation process became potential regulatory elements. Transfer RNA in particular seemed a likely candidate for a regulatory role for the following reasons.

1. LARGE NUMBER OF INDIVIDUAL tRNA SPECIES

As the genetic code was elucidated (1), it became apparent that 61 of the 64 possible nucleotide combinations are used to position amino acids in polypeptides, and a need for 61 tRNA species to translate the code could be assumed. But it soon was found that in many cases certain tRNA anticodons could "read" more than one code word. This phenomenon was shortly explained by CRICK (2), and his "wobble" hypothesis reduced the minimum number of tRNA species needed to translate the code to 31 (excluding specific initiation and termination requirements). However, as workers began to sum up the total number of tRNA species in cells and tissues, it became apparent that far more than 31 chemically different species of tRNA existed in all the cells examined (3,4,5). (Most values range between 55 and 65.) Some of these species were shown to read the same code words and others had overlapping code word recognitions. The unusual fact that these measurements emphasized is that there seem to be far more tRNA species than are required to translate the code.

2. INEQUALITY IN SPECIES CONCENTRATION

With the development of the Freon column (3), a method was found that would separate tRNA species that differed only slightly from one another, and with this tool it was possible to visualize the relative levels of the different species in cells. DAVID NOVELLI introduced the term "isoaccepting species" to denote those chemically different tRNA species that handled the same amino acid, and by means of Freon chromatography it was shown that there generally was a vast difference in the relative concentrations of isoaccepting species in cells (1,3,4,5). From this it could be inferred that the cellular concentration of tRNA for specific codewords is probably not equal in most cases.

Thus we find that there are more tRNA species than are required to read the code and that they are present in cells in unequal amounts.

3. CYTOKININS IN tRNA

The identification of the nucleotides in certain tRNA molecules uncovered the existence of numerous modified bases. Many of these modifications were simple methylations, but in some cases the modifications were found to have produced substituted purines that were identical to a class of compounds that were known to have dramatic effects on plant growth--the cytokinins (6,7). The presence of cytokinins were known to be required for normal plant tissue development in many instances. Thus with their discovery in the nucleotide chain of certain tRNA species (generally located on the 3' side of the anticodon), the idea that tRNA was involved in the regulation of development became quite irresistible.

4. METHYLASE ENZYMES

The modification of the nucleotides of tRNA by methylation was shown to be catalyzed by a group of methylases enzymes that individually placed methyl groups on specific positions of specific nucleotides. This phenomenon appeared to be without a function, until it was found in many developing systems that the levels and makeup of this group of enzymes changed during ontogeny, differentiation and maturation (8). It could now be asked, why should this enzyme population change with development unless their substrate-product tRNA was somehow involved in the developmental process?

5. SUPPRESSOR GENES

In microorganisms many mutants that suppressed other mutations were found to actually be mutations in the anticodon region of certain tRNA molecules (9). These species had changed so as to be able to bind with one of the three terminator nucleotide triplets. The

mutations they suppress apparently are cases in which the mRNA for the missing or non-functional protein contains, by mutation, the terminator triplet. The mutated tRNA is able to position an amino acid into the nascent protein in response to the triplet and functioning protein is produced.

The discovery that suppressor mutants could be explained in terms of tRNA and its role in translation demonstrated that the presence or absence of tRNA anticodons can determine what proteins get synthesized in cells, independent of the presence of the requisite mRNA. It was subsequently shown by FRENCH ANDERSON with an *in vitro* reaction that the rate of polypeptide synthesis from synthetic mRNA was controlled by the concentrations of the requisite tRNA species (10). He demonstrated that the rate of translation of an mRNA of known sequence was limited by the tRNA species present in the lowest amount.

Thus the synthesis of proteins was shown to depend on the presence of all the anticodons required by the mRNA, and the rate of synthesis of a specific polypeptide was shown to be affected by the relative amounts of the requisite anticodons. These demonstrations seemed to give meaning to the existence of so many tRNA species and to their unequal concentrations in cells.

With this background in mind many investigators explored the suggested involvement of tRNA species in the regulation of cell development. To date most studies of this sort have been aimed simply at detecting a changing level of tRNA species that appears to be coincident with a change in the pattern of cell growth and development. These searches have been successful in this respect as can be seen from Table I. In this table, which is a collation of the existing reports of changes in tRNA population, an attempt at organizing these reports into conceptually distinct categories has been made. As can be seen, environmentally induced changes in tRNA pools are as yet confined to bacteria, where alterations in the culture media or the atmosphere that determines the rate of growth have been reported to bring about specific changes in the levels of tRNA species. Whether or not these changes in growth rate constitute developmental situations depend upon the limits one places on what is meant by development.

Pathological conditions that result in changes in the tRNA pool of cells include the appearance of new tRNA species with bacteriophage infection of *E. coli*. Here, the new species apparently are coded for by the bacteriophage genome and are transcribed during bacteriophage proliferation inside the host cell. It is questionable that their existance is essential for bacteriophage replication, since bacteriophage genomes from which the tRNA cistrons have been deleted are replicated in the host cell. Numerous tumor cell lines that are maintained in culture have acquired tRNA populations that are different from the healthy tissue from which they are derived. Some investigators may consider these findings to have developmental overtones, since the pathological state here in many respects resembles a return to an embryonic, undifferentiated state.

In Table I the changes in tRNA pool composition that have been shown to accompany the more traditional type of developmental changes in tissues are seen to be both numerous and to cover a wide range of organisms. The sporulation of bacteria is perhaps the simplest developmental system, and both qualitative and quantitative changes in the tRNA population have been reported to accompany this process in *Bacillus subtilis*. Similar changes have been shown to occur in the classical systems in animal embryology (sea urchin and frog) and also to occur in a de-differentiating system (liver regeneration). Cells that specialize to the extent that, when mature, they synthesize large amounts of a few specific proteins have been found to have an acceptor population that reflects to some extent the amino acid composition of the specific proteins (rabbit reticulocytes and

Table I. Resume of Changes in tRNA Population.

ENVIRONMENTAL

1) Changing growth media (bacteria) (11,12)
2) Aerobic vs anaerobic growth (bacteria) (13)

PATHOLOGICAL

1) Viral infection
   - Bacteriophage in *E. coli* (14,15,16)
   - Vaccinia in Hela cells (17)
2) Tumor induction, Neoplasia
   - Hepatic tumors (18)
   - Leukemic lymphoblasts (18)
   - Tumor cell lines in culture (18)

DEVELOPMENTAL

1) Bacterial Sporulation (19)
2) Tissue differentiation

   Animal

   - Sea urchin embryogenesis (20)
   - Tadpole metamorphosis (21)
   - Chick erythrocyte development (22)
   - Rabbit reticulocyte development (23)
   - Mammary gland development (24)
   - Rat liver regeneration (25)

   Plant

   - Seedling development (lupine,26; wheat,27;soybean,28)

3) Response to hormones
   - Estrogen in rooster liver (29)
   - Thyroxine in rat liver (30)
4) Mature tissues of organism

   Animal

   - Mammalian liver vs kidney vs brain (rats) (31)
   - Insect silkgland vs midgut (silk worm) (33)

   Plant

   - Hypocotyl vs cotyledon (soybean) (32)

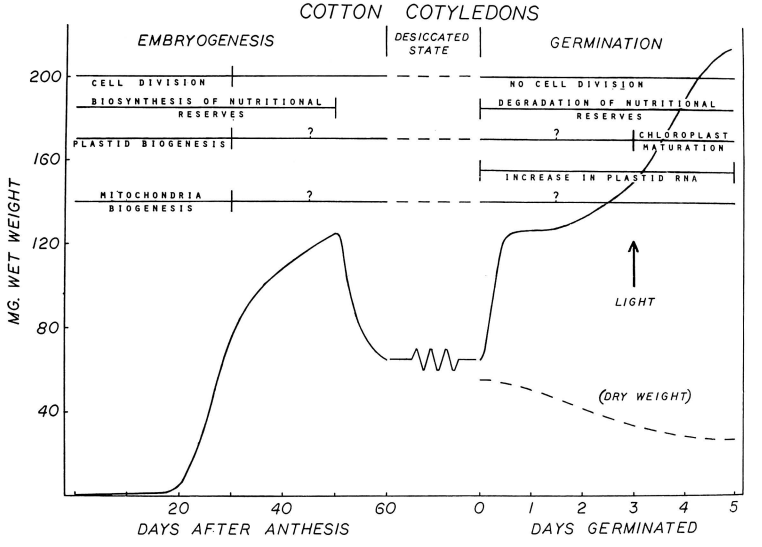
lactating mammary cells). In plants the seed germination process has been the developmental system used to look for changing populations of tRNA species.

Many hormones are known to regulate or to bring about profound changes in cell development, and, in two instances, hormone-mediated changes in cells have been shown to include a new distribution of isoaccepting species for certain amino acids. Curiously, if a rooster is given estrogen, the synthesis of phosvitin, an egg protein that is very rich in phosphoserine, is elicited in the rooster's liver. Simultaneously, a new $tRNA^{Ser}$ species has been shown to appear in this tissue.

Finally, there have been a number of comparisons of the levels of isoaccepting species for certain amino acids between the mature tissues of an organism. Differences have been found in each case, and the possibility that the unique population of isoacceptors that each tissue has is related to the highly specialized state of the tissue is an attractive idea to many people.

INTRODUCTION

With this background in mind I would like to present the results of our efforts to implicate tRNA in the regulation of developmental processes. The development of the cotyledon was followed during embryogenesis and germination of cotton seeds. Figure 1 gives a schematic presentation of some of the gross aspects of the development of this tissue during embryogenesis and germination along a temporal axis. Briefly, cotyledon development can be described as follows. During embryogenesis on the mother plant cotton embryos progress from the zygote to a mature embryo that weighs about 125 mg wet weight in about 50 days. The majority of the mature embryo is comprised of the two cotyledons which weigh about 120 mg at the end of embryogenesis. During the first 30 days after anthesis, which is a period of rapid cell proliferation, the cotyledons reach a wet weight of 85 mg. At this point there is a profound change in the development of this tissue. Cell division ceases and the transcription of mRNA begins for several enzymes that are to be used during germination; however, this mRNA is prevented from being translated



during the remainder of embryogenesis by the presence of the plant hormone, abscisic acid, and exists as "stored" mRNA until germination (34,35,36). The increase in cotyledon size during the remaining 20 days of embryogenesis is caused by the synthesis of large amounts of nutritional reserves in the form of protein granules and lipid globules. Storage protein synthesis during this period is so extensive that the storage protein itself comprises about 25% of the total protein of the mature seed (unpublished data). In this respect the cotyledons, after the 85 mg stage of development, resemble specialized cells that synthesize predominately one or two proteins. After 50 days the mature embryo dessicates and the dry seed is formed.

Upon germination the cotyledons do not resume cell division. However, the cells change the overall direction of their metabolism from one of synthesis of nutritional reserves to one of degradation of these reserves. The "stored" mRNA transcribed in embryogenesis is translated into enzymes that bring about this process, two of which have been identified as a carboxypeptidase C and isocitratase (36,37). During germination the cotyledons enlarge greatly, but lose dry weight in supplying nutrients to the rapidly growing root and shoot axis.

By the third day they have emerged from the soil, and are in position to receive the light stimulus that triggers the final maturation of their chloroplasts. By the fifth day the germination enzymes have reached their maximum level, and the cotyledons appear to be a fully matured tissue. Senescence sets in as the nutritional reserves are depleted, and within another two weeks the cotyledons are abscised from the young plantlet.

Against this developmental background, we have determined the percentage of the total tRNA present in cotton cotyledons at several stages in their development that will accept each of the 20 amino acids. We have also determined the number of isoaccepting species of tRNA for several amino acids and their concentrations relative to each other in this tissue during its development. The purpose of these measurements has been to determine if the developmental events that characterize the ontogeny and maturation of this tissue are accompanied by changes in its tRNA population. To represent the various developmental stages of this tissue, we prepared tRNA from a) young cotyledons in the rapid cell division stage, b) dry seed cotyledons which represent the post 85 mg stage that is characterized by the absence of cell division and the extensive synthesis of the storage protein, c) green cotyledons from 5-day germinated seedlings that are characterized by storage protein and lipid degradation, by cell and chloroplast maturation and also by the absence of cell division, d) cotyledons from 5-day germinated but etiolated seedlings which have developed the same as c) but without the final chloroplast maturation that is induced by light. In addition, we have utilized tRNA prepared from partially purified chloroplasts in order to identify chloroplast tRNA species, and, further, tRNA prepared from the roots of young seedlings to compare the tRNA population of cotyledons with that of a totally distinct tissue.

METHODS

Transfer RNA was extracted from the cotyledons, roots and partially purified cotton chloroplasts [isolated by the non-aqueous procedure of STOCKING (38)] by conventional phenol procedures and purified by DEAE chromatography. Preparations of crude aminoacyl-tRNA synthetases were made from cotyledons at the various stages of cotyledon development, and negligible differences between them were noted in the extent of charging tRNA with the different amino acids. However, the rates of charging were much greater in many cases with the synthetase preparations from 5-day germinated cotyledons, which presumably contain a higher concentration of chloroplast synthetases. Consequently, enzyme preparations from these cotyledons were used throughout. As part of another study the tRNA preparations were charged with each of the 20 amino acids by synthetases prepared from *E. coli* B, but, as will be seen, some of the data obtained with these synthetases were useful in this study also.

Since, in this study, we attempt to ascertain the relative amounts of the different tRNA species in a tissue as it develops and matures, we are obliged to establish that (a) all of the tRNA of the tissue is extracted and retained during purification and (b) that no loss of acceptor activity occurs during extraction and purification. As for the first obligation, we believe that the method of tRNA preparation that we have used yields all the tRNA in cotyledons and roots and in chloroplasts, since we have never obtained significantly greater amounts of tRNA per unit of tissue when the tissues were exhaustively extracted with solutions containing sodium dodecyl sulfate (SDS) and no attempt made to maintain acceptor activity. We have found that sodium deoxycholate is required in the initial homogenization medium; otherwise about 75% of the tRNA is sedimented at 17,000 x *g* from the initial extract. If the sodium deoxycholate is replaced by SDS, much of the tRNA acceptor activity is lost, presumably because of the extraction by SDS of nuclease enzymes that are carried along with the tRNA in the subsequent purification steps.

Further, we observed that some tRNA is precipitated when tRNA is separated from rRNA by the high salt precipitation of rRNA, or by ultracentrifugation. Consequently, the procedure we have used separates tRNA from the bulk of the other nucleic acids by DEAE-cellulose chromatography.

The amount of tRNA present in each of the preparations was determined prior to the DEAE-cellulose column step and in the final preparations by SDS polyacrylamide electrophoresis as described by LOENING (39). The amount of tRNA per unit tissue and the concentration of tRNA present in the final preparations were calculated from these gel profiles. The tRNA in the final preparations was often contaminated with small amounts of DNA and rRNA. These contaminants did not affect the acceptor activity of the tRNA, and therefore no effort was made to remove them. The amount of 5S RNA contaminating the tRNA in the final preparations was determined by SDS gel electrophoresis using less porous gels, and the tRNA concentrations were corrected for this contamination.

The tRNA purified in the above manner was not appreciably damaged as shown by the following measurements. (a) The total amount of tRNA in each of the preparations that could be charged with the 20 amino acids was at least 90% of the total tRNA present, as will be brought out in RESULTS. This percentage was determined by a summation of the *p*moles of amino acid accepted per 100 *p*moles of tRNA, assuming an average tRNA molecular weight of 25,000 and an absorbancy at 260 mμ of 25 for 1 mg of tRNA in 1 ml of 0.01 M Tris-HCl buffer, pH 7.2, at 25°C. (b) The tRNA preparations showed no loss of acceptance for any of the twenty amino acids after they had been heated to 80°C for 5 minutes and quickly cooled. This indicated that there were no breaks in the polynucleotide chains. However, a considerable loss of acceptor activity for several amino acids was observed when tRNA that had been dialyzed at any stage of purification was heated and quickly cooled. Consequently, dialysis was avoided during tRNA purification.

The formation of aminoacyl-tRNA was measured in a 0.25 ml reaction mixture containing 0.5 to 2 A_{260} units of tRNA, crude aminoacyl-tRNA synthetase, ATP, a ^{3}H or a ^{14}C amino acid, and 0.01 M NH_4Cl, all in a Tris buffer containing 0.01 M $MgCl_2$. The reaction mixture was incubated at 30°C, aliquot samples of 0.05 ml were removed at intervals and the acid precipitable radioactivity formed was determined by the cellulose disk method of BOLLUM (40). The incubation times varied with different amino acids, and were long enough to give the total extent of tRNA charged with each amino acid (plateau value). Only in the case of proline did a rapid discharge complicate the determination of plateau values. The assay system was optimized for each amino acid with respect to the concentration of ATP, amino acid and synthetase preparation required to give complete charging within 20 to 40 min. The presence of cotton tyrosyl-tRNA synthetase activity could not be demonstrated in the cotton synthetase preparation, and the level of $tRNA^{Tyr}$ species was estimated with the *E. coli* synthetase preparation.

To visualize the number and relative levels of isoaccepting tRNA species for an individual amino acid we employed two techniques. The first of these involved the chromatographic separation of the various isoaccepting species acylated with a radioactive amino acid on the RPC-2 (Freon) column developed by WEISS and KELMERS (3). This column separates whole molecules of acylated tRNA on the basis of small differences in charge and in reactivity of the tRNA with a non-mobile organic phase on the column matrix. Transfer RNAs differing only trivially from one another can be separated on this column. However, spurious peaks (that is, elution peaks of radioactive amino-acyl tRNA that do not represent a unique oligonucleotide composition) were encountered with this column from a variety of causes. Furthermore, the recovery of the radioactivity applied to the column was often below 70%. These two facts limited the usefulness of this

column for a developmental study, which prompted us in most instances to resort to a second technique for resolving different isoaccepting tRNA species and determining their relative amounts.

This technique involved the digestion of tRNA acylated with a specific radioactive amino acid with ribonuclease T_1. This enzyme, which specifically cleaves polynucleotide chains at guanine residues leaving the G $3'PO_4$, produces only one radioactive oligonucleotide fragment from each aminoacyl tRNA--the one carrying the radioactive amino acid. The nucleotide length and composition of this fragment depends upon the position of the guanine residues nearest the CpCpA-amino acid terminus of the molecule. Isoaccepting tRNA species that produce different aminoacyl-oligonucleotides in the digestion can be distinguished and their relative concentrations measured if the ^{14}C aminoacyl-oligonucleotides are chromatographed on DEAE or CM-cellulose at pH 4.5 without urea. This pH is used to produce about 1/2 a net positive charge on cytosine residues and a slight net positive charge on adenine residues. Urea is omitted in this procedure to allow a maximum interaction between the nucleoside residues and the column matrix, which promotes the separation of aminoacyl-oligonucleotides that differ in nucleotide composition but not in nucleotide number. This procedure is diagrammatically presented in Figure 2. There is obviously a serious limitation to this technique

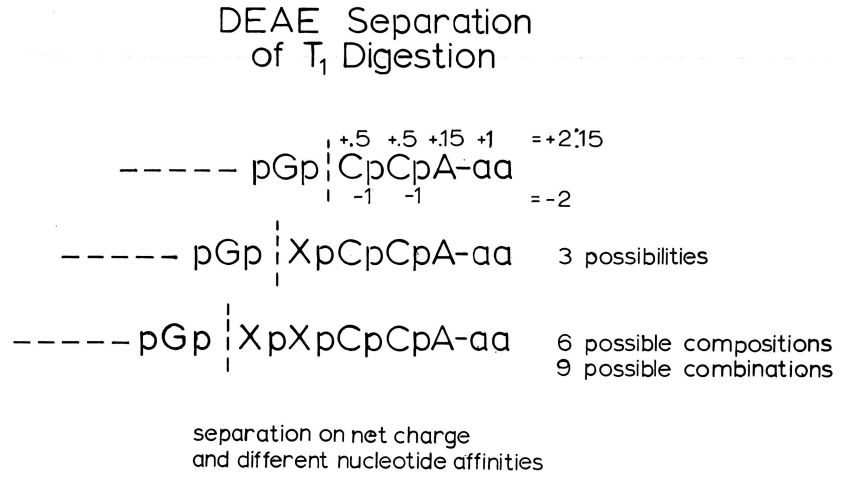


FIGURE 2. Diagram of the action of RNAse T_1 on ^{14}C aminoacyl-tRNA. Vertical dashed line indicates position of cleavage. Approximate electrostatic charge on the ionized groups of the ^{14}C aminoacyl-oligonucleotides at pH 4.5 are shown.

in ascertaining the number and relative levels of isoaccepting tRNA species. Isoaccepting species that differ in nucleotide composition only in other parts of the polynucleotide chain, including the anticodon region, will generate the same aminoacyl-oligonucleotide fragment upon digestion with RNAse T_1, and thus not be distinguished by this technique.

Utilizing these methods we determined the distribution of isoaccepting tRNA species for a number of amino acids in cotyledon tRNA prepared from the developmental stages listed above and from chloroplasts and roots. A detailed description of these procedures has been published (41,42).

RESULTS

1. DETERMINATION OF tRNA ACCEPTOR LEVELS

In Table II the percentage of the total tRNA of each preparation that can be charged with each of the 20 amino acids is given. These percentages are based on the plateau values of amino acid incorporated into tRNA and calculated from the assumptions stated earlier. The values given for tyrosine however, are estimated from the percentage of the tRNA in each preparation that was acylated with tyrosine by an *E. coli* B synthetase preparation. Table II shows that over 90% of the tRNA in each preparation was charged with the 20 amino acids (when the $tRNA^{Tyr}$ estimates are included). However, the values for glutamine are very low and may reflect unstable glutaminyl-tRNA synthetase(s). Unfortunately, no glutaminyl-tRNA whatsoever was formed with the *E. coli* enzyme preparation. In contrast, very high amounts of tRNA accepting glycine, leucine, arginine and valine were found in all the preparations.

Table II. Percent of tRNA Charged with Each Amino Acid.

Amino Acid	Source of tRNA					
	Young Embryo Cotyledons	Dry Seed Cotyledons	Roots	Green Cotyledons	Etiolated Cotyledons	Chloroplasts
Ala	5.1	5.1	5.0	4.8	4.7	4.7
Arg	9.0	8.7	8.7	9.3	9.3	9.5
Asn	1.4	2.5	2.5	1.3	1.4	2.1
Asp	6.8	6.5	6.6	6.1	6.0	5.0
Cys	0.8	0.7	0.8	0.7	0.8	0.9
Gln	0.2	0.2	0.2	0.2	0.2	0.2
Glu	2.0	2.1	2.3	2.2	2.3	3.0
Gly	10.0	10.1	10.3	9.7	9.6	8.6
His	3.4	3.3	3.3	3.7	3.7	3.6
Ile	3.2	3.2	3.3	3.3	3.4	4.4
Leu	10.0	9.8	10.2	11.2	11.0	11.1
Lys	5.2	5.3	5.5	3.6	3.7	4.0
Met	3.5	3.3	3.5	4.7	4.7	5.8
Phe	4.6	4.6	4.7	5.0	5.1	6.7
Pro	4.6	4.6	4.4	4.1	4.0	3.2
Ser	3.4	3.2	1.3	3.8	3.7	4.1
Thr	5.6	5.6	5.7	5.4	5.5	4.7
Trp	2.1	1.9	1.9	2.3	2.3	1.9
Tyr*	2.7	2.7	2.7	2.7	2.7	3.0
Val	9.0	8.9	9.0	8.8	8.6	6.6
TOTAL	92.6	92.3	91.9	92.9	92.7	93.1

*Estimated from charging with *E. coli* synthetases as explained in METHODS.

The striking feature of the data in Table II is the near identity of the amount of tRNA accepting each amino acid in the preparations from young embryo and dry seed cotyledons and from roots. The fundamental developmental differences between cotyledons from these two stages have been pointed out, and their developmental and functional distinctness from roots is obvious.

There are two instances where this apparent constancy in the tRNA population of these three preparations is not borne out by the data in Table II. These are the low value for $tRNA^{Asn}$ in the cotyledons from young embryos, and the low value for $tRNA^{Ser}$ in roots (underlined in Table II). These low values were reproducible and were obtained in the several tRNA preparations made from these tissues.

Unfortunately, we were unable to determine if these two low values were the result of a lower level or absence of particular isoaccepting species because only one asparaginyl-oligonucleotide and one seryl-oligonucleotide was produced in any of the tRNA preparations by the RNAse T_1 digestion (see Figure 8).

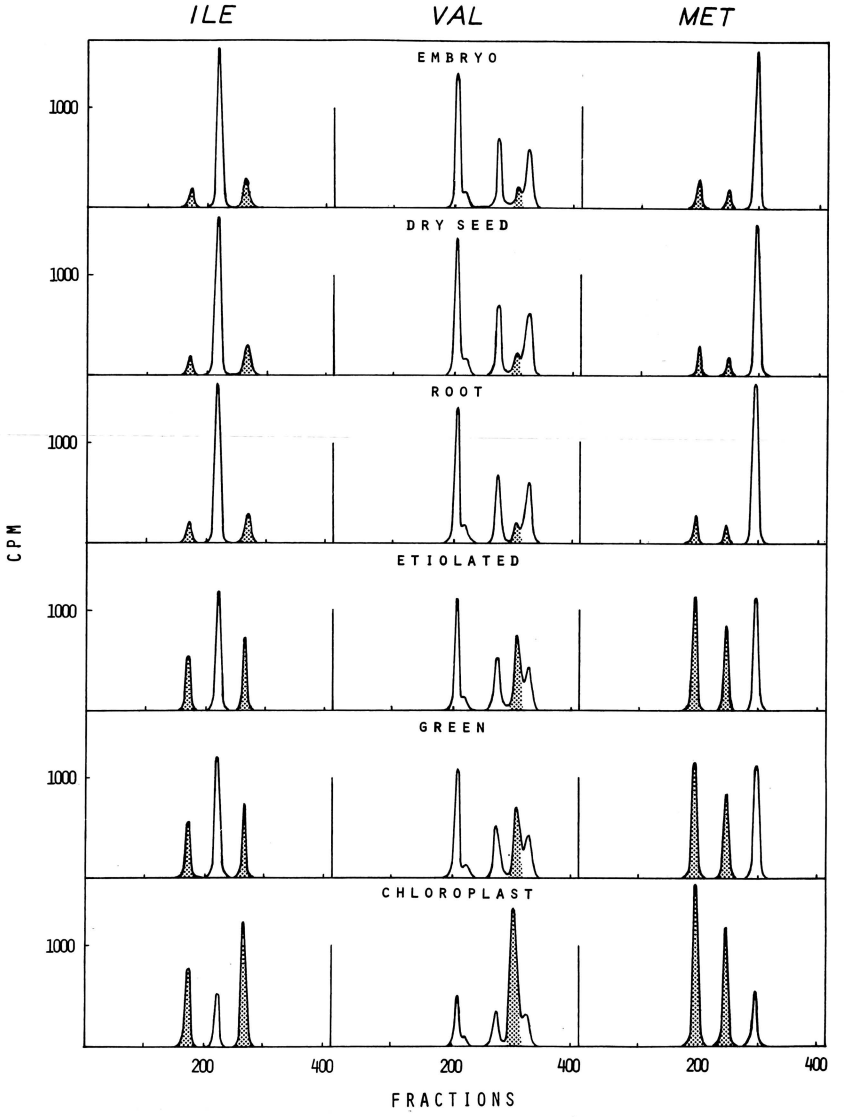


FIGURE 3. Radioactivity elution profiles of [^{14}C]isoleucyl-, [^{14}C]valyl- and [^{14}C]methionyl-oligonucleotides from DEAE-32 columns. Transfer RNA from the tissues indicated in the figure were charged with the indicated ^{14}C amino acid by a cotton cotyledon enzyme preparation and ^{14}C aminoacyl-oligonucleotides were prepared from the charged tRNA and chromatographed as described in METHODS. Shaded elution peaks represent aminoacyl-oligonucleotides from chloroplastic aminoacyl-tRNA.

The values for the relative tRNA acceptor concentrations in germinated cotyledons on the other hand differ somewhat from the values obtained for the young embryo and dry seed cotyledons and for roots. However, there is again a striking constancy in the values for green cotyledon tRNA and etiolated cotyledon tRNA. Chloroplast tRNA shows a distribution of amino acid acceptors distinct from all the other tRNA preparations. The differences here may be even greater than those given in Table II, because the chloroplast tRNA is contaminated with between 20 and 25% cytoplasmic tRNA, as will be shown below.

From the data in Table II no shift in the relative amounts of tRNA acceptors for each amino acid is observed to accompany the developmental changes in cotyledons during embryogenesis, and the small changes that are observed during cotyledon germination apparently result from an increase in chloroplast tRNA (as will be seen below) and not from changes in the levels of cytoplasmic species.

2. DETERMINATION OF NUMBER AND LEVELS OF ISOACCEPTING tRNA SPECIES

To more closely examine the levels of tRNA acceptors in the developing cotyledon tissue, the number and levels of isoaccepting tRNA species were determined by RPC-2 chromatography of ^{14}C aminoacyl-tRNA or by the DEAE or CM-cellulose chromatography of RNAse T_1 digestion products of ^{14}C aminoacyl-tRNA.

Figure 3 shows the elution profile of isoleucyl, valyl and methionyl-oligonucleotides produced by the RNAse T_1 digestion. This figure shows that with every tRNA preparation 3 different isoleucyl-oligonucleotides are produced from isoleucyl-tRNA. These isoleucyl-oligonucleotides indicate that at least 3 $tRNA^{Ile}$ species exist in these tissues and that their relative levels are the same in the tRNA from the young embryo and dry seed cotyledons and from roots. Further, two of these species have increased relative to the third during germination, in etiolated cotyledons as well as in green cotyledons, to the extent that they comprise about 50% of the $tRNA^{Ile}$, after 5 days of germination. The same two species are seen to be the predominant species in chloroplastic tRNA. Our interpretation of these changes in isoleucine isoacceptor levels is that the species which increase in cotyledons during germination and which predominate in the tRNA from partially purified chloroplasts are chloroplastic tRNA species. The alleged chloroplast species are shaded in this and other figures. It may be argued that the isoleucyl-oligonucleotides that are seen here to increase in relative amount are not the result of an increase in the levels of existing species, but represent the *de novo* appearance of two new $tRNA^{Ile}$ species that happen to yield the same isoleucyl-oligonucleotide on RNAse T_1 digestion as existing species. However, the chromatography of isoleucyl-tRNA on the RPC-2 column (Figure 4) shows that the two minor species existing in the dry seed cotyledons are the species that increase in amount during germination and that are concentrated in chloroplasts. The elution profiles of isoleucyl-tRNA from this column also show that two cytoplasmic $tRNA^{Ile}$ rather than one exist in these tRNA preparations. These two cytoplasmic species apparently produce identical isoleucyl-oligonucleotides on RNAse T_1 digestion, and thus appear as one species on the DEAE-cellulose column elution profile.

The information from the two columns taken together shows that two chloroplastic and two cytoplasmic $tRNA^{Ile}$ species exist in all the tissues examined (including the young embryo cotyledons and roots) and that the chloroplastic species increase markedly during germination. We have found that the amount of tRNA per cell increases approximately 80% in cotyledons during germination (unpublished data), which supports our contention that the level of chloroplast species increases per cell during this period, as distinct from there being reduction in the level of cytoplasmic species per cell. However, there appear to be no difference in the amounts of the two cytoplasmic species relative to each other nor in the quantitative relation-

ship between the two chloroplastic species in any of the tRNA preparations. Further, the increase in the chloroplastic species is shown not to be light dependent. The quantitative identity of the DEAE column profiles obtained with young embryo and dry seed cotyledon tRNA and root tRNA shows that not only do these tissues have the same levels of total $tRNA^{Ile}$ relative to the total tRNA (Table II), but that they have the same levels of isoaccepting $tRNA^{Ile}$ species.

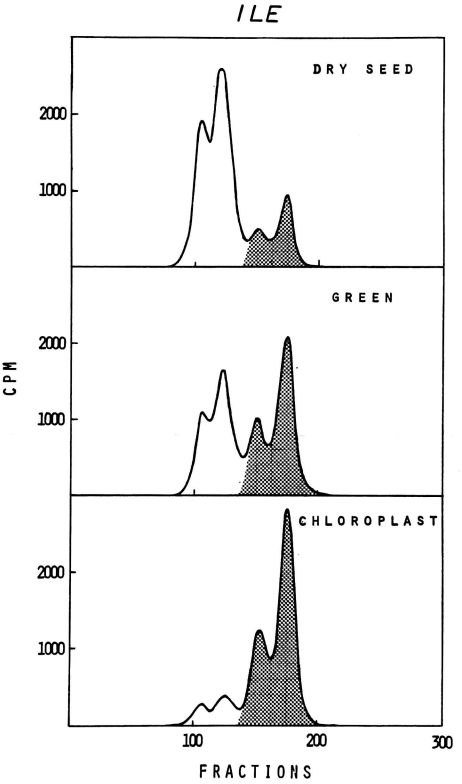


FIGURE 4. Radioactivity elution profiles of [^{14}C]isoleucyl-tRNA from RPC-2 columns. Transfer RNA from the tissues indicated were charged with [^{14}C]isoleucine by a cotton cotyledon enzyme preparation, separated from the reaction mixture and chromatographed as described in METHODS. Shaded elution peaks represent chloroplastic isoleucyl-tRNA species.

The same pattern observed for $tRNA^{Ile}$ species is reiterated in the case of $tRNA^{Val}$ and $tRNA^{Met}$ in Figure 3. There are 5 valyl-oligonucleotides evident in this figure, one of which appears to be derived from chloroplastic tRNA by the criteria given above. Four code words exist for valine (1) and, if chloroplastic and cytoplasmic protein synthesis are mutually exclusive in their use of tRNA, and if chloroplast protein synthesis uses the entire genetic code, there should exist at least one other chloroplastic $tRNA^{Val}$. Unfortunately, the RPC-2 column did not resolve valyl-tRNA well enough to determine if the chloroplastic valyl-oligonucleotide seen in the DEAE cellulose column elution profile was produced by two chloroplastic $tRNA^{Val}$ species. Here again the levels of the cytoplasmic $tRNA^{Val}$ species are found not to change relative to one another during cotyledon development nor to differ from that found in root tRNA.

The DEAE column chromatography of methionyl-oligonucleotides shows two chloroplastic species and one cytoplasmic species. We have found that the methionyl-tRNA that produces the middle methionyl-oligonucleotide in the elution profile can be formylated to form N-formylmethionyl-tRNA by an endogenous cotton enzyme and by *E. coli* transformylase (41). Figure 5 shows how the formylation of this species by an endogenous cotton transformylase was demonstrated. Here the DEAE elution profile of methionyl-oligonucleotides from green cotyledon tRNA is compared with the elution profile of the same preparation that was incubated with N-10-formyltetrahydrofolate before being digested with the RNAse T_1. One of the chloroplastic methionyl-oligonucleotide fragments is seen to have acquired greater net negative charge causing it to elute at a higher salt concentration. This is what would be expected of an N-formylmethionyl-oligonucleotide. The radioactive amino acid recovered from this elution peak (peak #3) was shown to be N-formylmethionine, which demonstrated that one of the putative chloroplastic $tRNA^{Met}$ species is a $tRNA_f^{Met}$. This fact further substantiates our assumption that species that increase during germination and that are concentrated in the chloroplast tRNA preparation are chloroplastic species, since formylmethionyl-tRNA has been shown to initiate chloroplastic but not cytoplasmic protein synthesis in plants (43). There should be at least 2 cytoplasmic $tRNA^{Met}$ species also; one for polypeptide chain initiation and another for the internal positioning of methionine in translation, but we were not able to demonstrate more than one by RPC-2 chromatography.

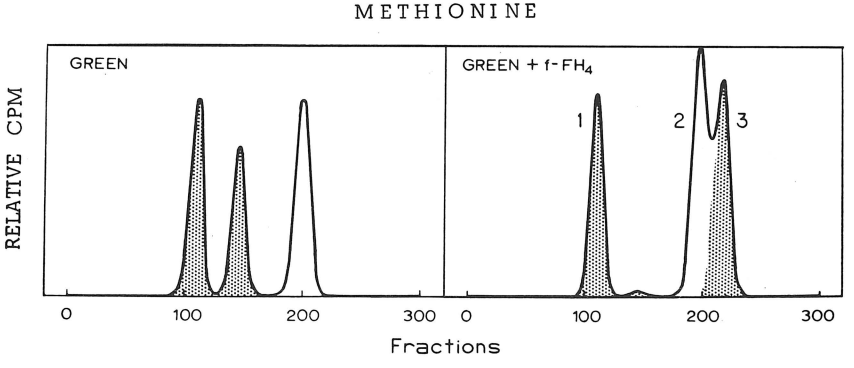


FIGURE 5. Radioactivity elution profiles of [^{14}C]methionyl and [^{14}C]N-formylmethionyl-oligonucleotides from DEAE columns. Transfer RNA from green cotyledons was charged with [^{14}C]methionine and digested with RNAse T_1 and chromatographed (left) or incubated with N-10-formyl-tetrahydrofolate (f-FH_4) before digestion and chromatography (right). Peaks of radioactivity (1-3) were collected, the ^{14}C amino acid recovered and electrophoresed for identification. Peaks 1 and 2 yielded [^{14}C]methionine and Peak 3 yielded N-formylmethionine.

In Figure 6 the elution profiles of leucyl-oligonucleotides from the DEAE-cellulose column and lysyl-oligonucleotides from the DEAE- and CM-cellulose columns are presented. Five leucyl-oligonucleotides were resolved indicating the existence of at least 5 $tRNA^{Leu}$ species. However, when these preparations of tRNA were charged with the *E. coli* synthetase preparation and then digested with RNAse T_1, only a portion of the leucyl-oligonucleotide eluting

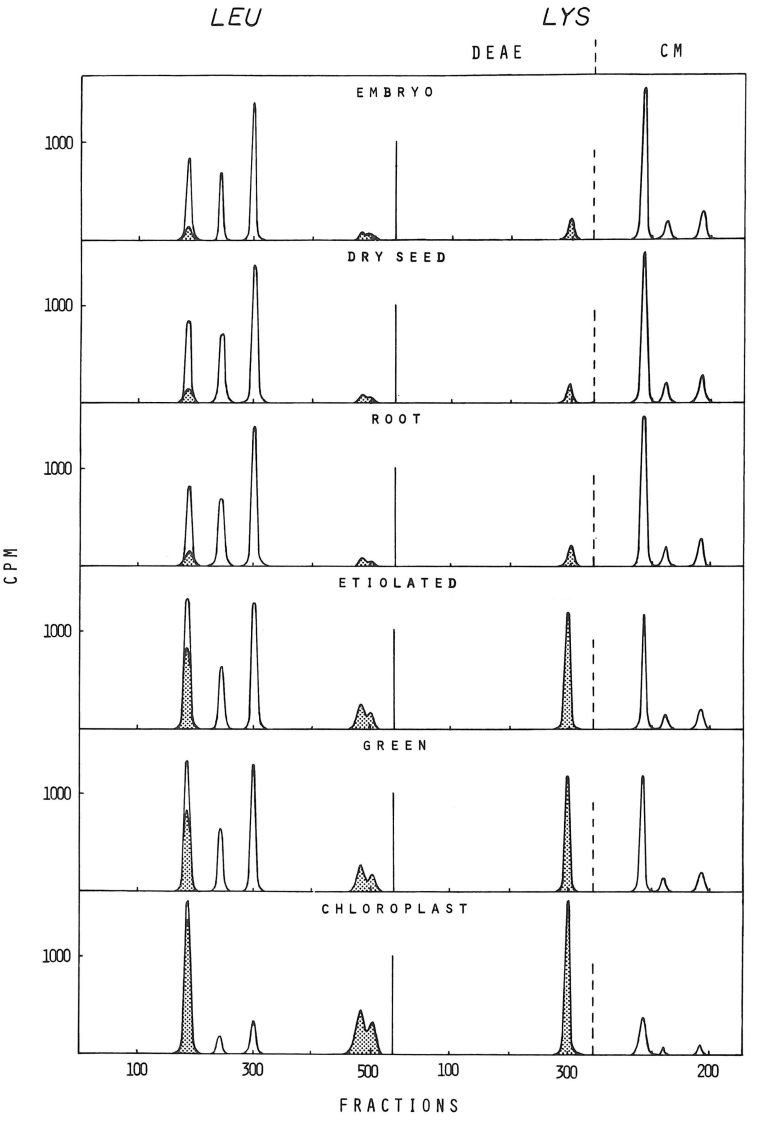


FIGURE 6. Radioactivity elution profiles of [^{14}C]leucyl- and [^{14}C]lysyl-oligonucleotides from DEAE-32 and CM-32 columns. Experimental details are presented in the legend to Figure 3 and in METHODS.

first from the DEAE column was formed. This portion was larger in tRNA from germinated cotyledons than from younger cotyledons and roots and was almost equivalent to that formed with the cotton synthetase preparation in the chloroplast tRNA. This we interpret as indicating that the first eluting leucyl-oligonucleotide is produced from two $tRNA^{Leu}$ species--one cytoplasmic and one chloroplastic --and that the *E. coli* synthetase preparation charges only the chloroplastic species. Thus the amount that the chloroplastic $tRNA^{Leu}$

species contributes to the first eluting leucyl-oligonucleotide (shaded portion of the first peak in Figure 6) was obtained with the *E. coli* synthetase preparation. From this there appear to be 6 $tRNA^{Leu}$ species in cotton--three cytoplasmic and three chloroplastic. Assuming that these species contain a "wobble" capability in their anticodons (2), three species are sufficient to recognize the 6 leucine code words. Notice again that, although the chloroplast species increase in amount per cell during germination, there is no change in the levels of the cytoplasmic species relative to each other nor in the levels of the chloroplastic species relative to each other during cotyledon development nor between young cotyledon and root tRNA.

Only one of the lysyl-oligonucleotides was retained by the DEAE-cellulose column, whereas three others were retained by the CM-cellulose column, indicating that they bear a net positive charge at pH 4.5. Only a limited number of lysyl-oligonucleotides would be positively charged at this pH, and consequently it is possible to assume the nucleotide composition of these three lysyl-oligonucleotides as being, in order of elution, ApCpCpA-lysine, CpCpCpA-lysine and CpCpA-lysine, with positive charges of about 0.4, 0.7 and 1.2 ESU respectively. (Free lysine is also retained by the CM-cellulose column at pH 4.5, but it elutes from this column earlier in the salt gradient than any of these three lysyl-oligonucleotides.)

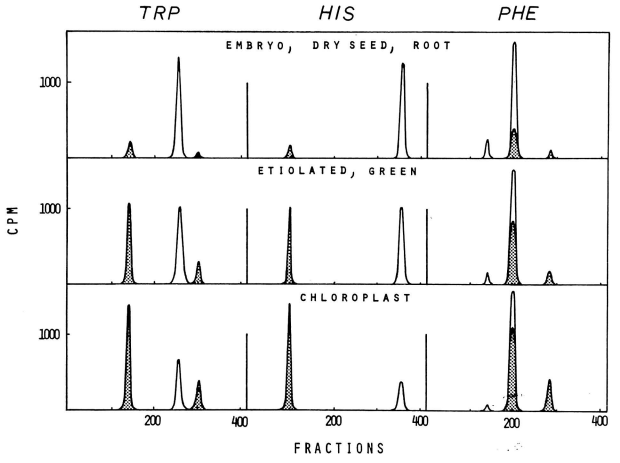


FIGURE 7. Radioactivity elution profiles of [^{14}C]tryptophanyl-, [^{14}C]histidyl- and [^{14}C]phenylalanyl-oligonucleotides from DEAE-32 columns. Experimental details are presented in the legend to Figure 3 and in METHODS.

In Figure 7 the elution profiles of tryptophanyl-, histidyl- and phenylalanyl-oligonucleotides obtained from the tRNA preparations are presented. Since the profiles obtained from young embryo and dry seed cotyledon tRNA and from root tRNA were once again identical, only one profile is presented for these three tRNA preparations. Likewise, the profiles obtained from both etiolated and green germinated cotyledons were again identical. In the case of all three amino acids, charged oligonucleotides can be identified that appear to represent chloroplastic tRNA species. In the case of phenylalanine, as with leucine, charging with the *E. coli* synthetase preparation indicated that the phenylalanyl-oligonucleotide eluting second from the DEAE-cellulose column was produced from the two $tRNA^{Phe}$ species--one cytoplasmic and one chloroplastic, which was acylated by the

E. coli synthetase. It is interesting that there are two chloroplastic $tRNA^{Trp}$ species in cotton although only a single code word exists for tryptophan (1). Again the quantitative relationship between the chloroplastic species of tryptophan and phenylalanine tRNA and between the cytoplasmic species of phenylalanine tRNA are unchanged in all the tRNA preparations.

In Figure 8 the position of elution from the DEAE- and CM-cellulose columns of the single aminoacyl-oligonucleotide produced in each case from all the tRNA preparations charged with alanine, asparagine, aspartic acid, glycine, serine, or threonine is presented. Obviously all the isoaccepting tRNA species for each of these amino acids, both cytoplasmic and chloroplastic, have the same nucleotide composition from the 3'terminal adenine in to the first guanine residue. In the case of asparagine this sequence must be simply CpCpA, since the single asparaginyl-oligonucleotide is retained by the CM-cellulose column but not the DEAE-cellulose column, and only CpCpA-asparagine has a net positive charge at pH 4.5. The relative amounts of each of these aminoacyl-oligonucleotides given in Figure 8 reflect the relative concentrations of $tRNA^{Ala,Asn,Asp,Gly,Ser}$ and Thr in the tRNA from green and etiolated cotyledons. (The differences in specific radioactivity of the amino acids have been corrected for.)

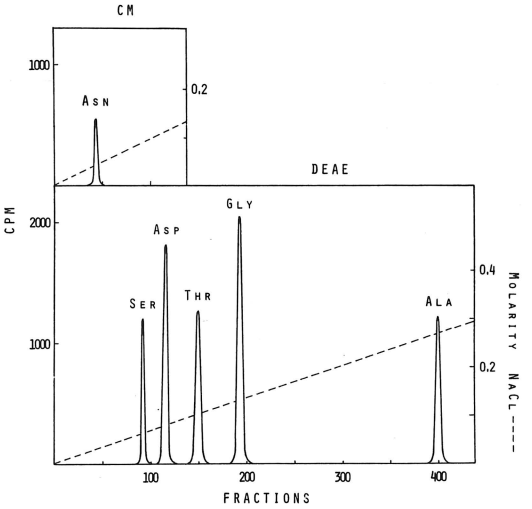


FIGURE 8. Radioactivity elution profile of the [^{14}C]seryl-, [^{14}C]asparty1, [^{14}C]threonyl, [^{14}C]glycyl, [^{14}C]alanyl- and [^{14}C]asparaginyl-oligonucleotide from DEAE-32 and CM-32 columns. The elution profile of each [^{14}C]aminoacyl-oligonucleotide was obtained separately but they are plotted together with respect to their position of elution on the salt gradient. Experimental details are presented in the legend to Figure 3 and in METHODS.

Table III presents a summary of our data on the number of isoaccepting tRNA species that we have observed from both DEAE- and CM-cellulose and RPC-2 chromatography for 15 amino acids, and gives their putative assignment as cytoplasmic and chloroplastic species where possible. Of the 5 amino acids that were not examined, prolyl-tRNA discharged too rapidly for chromatography, tyrosyl-tRNA was not formed by the cotton enzyme preparation, and the levels of cysteinyl, glutamyl and glutaminyl-tRNA were considered too low for meaningful chromatography. The RPC-2 elution profiles that were obtained with

alanyl, arginyl, aspartyl, glycyl, leucyl, lysyl, methionyl, phenylalanyl and valyl tRNA are not shown because they resolved the same number of species (Met) or fewer species (Lys,Phe,Val) than did the DEAE column profiles, or because the overlapping of the eluting species prohibited their division into cytoplasmic or chloroplastic species (Ala,Arg,Asp,Gly,Leu).

Table III. Number of Isoaccepting tRNA Species

Transfer RNA prepared from cotton cotyledons at several stages of development was enzymatically aminoacylated and chromatographed on an RPC-2 column or digested with RNAse T_1 as described in METHODS. The aminoacyl-oligonucleotides produced by the digestion were chromatographed on DEAE- or CM-cellulose columns as described in METHODS and, based on the criteria given in RESULTS, were designated as cytoplasmic or chloroplastic species.

	Number of Species					
	DEAE, CM Chromatography			RPC-2 Chromatography		
	Total	Cytoplasmic	Chloroplastic	Total	Cytoplasmic	Chloroplastic
Ala	1[a]			4[b]		
Arg	7[b]			4[b]		
Asn	1[a]			-[c]		
Asp	1[a]			4[b]		
Cys	-[c]			-[c]		
Gln	-[c]			-[c]		
Glu	-[c]			-[c]		
Gly	1[a]			3[b]		
His	2[d]	1	1	-[c]		
Ile	3[e]	1	2	4[h]	2	2
Leu	6[f,g]	3	3	6[b]		
Lys	4[f]	3	1	2[b]		
Met	3[e]	1	2	3[b]	1	2
Phe	4[d,g]	2	2	3[b]		
Pro	-[c]			-[c]		
Ser	1[a]			-[c]		
Thr	1[a]			-[c]		
Trp	3[d]	1	2	-[c]		
Try	-[c]			-[c]		
Val	5[e]	4	1	4[b]		

[a] See Figure 8
[b] Column elution profiles not shown
[c] Not determined
[d] See Figure 7
[e] See Figure 3
[f] See Figure 6
[g] Additional species inferred from charging with *E. coli* synthetases
[h] See Figure 4

The single aminoacyl-oligonucleotide produced by the digestion of alanyl, asparaginyl, aspartyl, glycyl, seryl or threonyl-tRNA reflects the limitation of this technique for examining isoaccepting species, since, in the case of alanine, aspartic acid and glycine, the existence of several isoaccepting species was indicated by RPC-2 chromatography. The recovery of radioactivity from the RPC-2 column was below 70% with asparaginyl-, seryl- and threonyl-tRNA which made meaningful chromatography of these species impossible. The DEAE column profiles of arginyl-oligonucleotides which gave 7 radioactive peaks is also not shown. There was a large amount of free arginine released during the digestion of arginyl-tRNA with RNAse T_1, and hence it was not possible to determine if these arginyl-oligonucleotides were derived from cytoplasmic or chloroplastic tRNA.

DISCUSSION

In this study we have attempted to examine the tRNA population of a developing tissue with a great deal of effort directed towards quantitative results. Care was taken to demonstrate a quantitative extraction of tRNA and to demonstrate the integrity of the purified tRNA polynucleotide chains. The frequent occurrence of spurious tRNA species in the elution profiles and the incomplete recovery of radioactivity from RPC-2 columns led to the use of DEAE- and CM-cellulose column chromatography of RNAse T_1 digests to examine the number of isoaccepting tRNA species. We have obtained values for the amount of tRNA in the several tRNA preparations that will accept each of the amino acids, except possibly for tyrosine and glutamic acid and possibly for asparagine and serine in two specific preparations (Table II). We have determined the relative levels of individual isoaccepting tRNA species for 8 amino acids and, based on our criteria, assigned a cytoplasmic or chloroplastic origin to these species (Figures 3,4,6 and 7). We attempted with less success to visualize the isoaccepting species for 6 other amino acids (Figure 8, Table III), and did not attempt these experiments with the remaining 5 amino acids.

The salient feature of our results taken as a whole is that there is no pattern of change in the tRNA population as the cotyledon tissue develops and matures, except for the increase in the amount of chloroplast tRNA in the tissue that takes place during germination. The amount of tRNA that accepts each amino acid does not appear to change in cotyledons in their development prior to germination, and the changes that are shown in Table II to occur during germination can be attributed to the increased contribution of chloroplast species. There is no cell division in cotyledons during this period and the increase in tRNA per cotyledon that occurs during germination reflects the increase in chloroplast tRNA per cell. This increase in chloroplast tRNA is in step with the increase in chloroplast rRNA (and presumably in chloroplast ribosomes) that occurs during germination. There is about a 7-fold increase in chloroplast rRNA during this period (unpublished data), and a commensurate increase in the amount of chloroplast tRNA species is shown in Figures 3,4,6 and 7. We have found that the number of tRNA molecules per ribosome, which is between 14 and 15, remains constant in both the cytoplasm and in chloroplasts during cotyledon development (unpublished data).

The apparent constancy in the cytoplasmic tRNA population is also reflected in the unchanging relationship in the amounts of cytoplasmic isoaccepting species that was found for isoleucine, valine, leucine, lysine and phenylalanine. Further, no variations in the levels of chloroplastic isoaccepting tRNA species relative to one another were found in those instances where more than one chloroplastic isoaccepting species was distinguished by the chromatography (isoleucine, leucine, tryptophan and phenylalanine).

These results contrast markedly with the numerous reports of changes in tRNA population with tissue development and differentiation presented in Table I. A marked change in the cotyledon tRNA population might have been anticipated to accompany the cessation of cell division and the formation of the germination mRNA that takes place at about the 85 mg stage of embryogenesis. Furthermore, after this point in embryogenesis, cotyledons are engaged in synthesizing principally a single storage protein which appears to be comprised of but two polypeptide chains (unpublished data). In several instances tissues that synthesize predominately a few specific proteins have been found to have tRNA pools that reflect the amino acid composition of these proteins as was pointed out in the BACKGROUND. Yet when the tRNA populations of young embryo and dry seed cotyledons are compared in either acceptor concentrations or in isoacceptor levels, no change is found to accompany these developmental events.

Even more in contrast to the prevailing literature collated in Table I is our evidence that the tRNA populations of the two distinct tissues, cotyledons and roots, are almost identical before the amplification of chloroplastic species takes place in germination.

However, it must be emphasized that our procedure for distinguishing isoaccepting species by the chromatography of RNAse T_1 digests does not allow modifications of tRNA species by methylation, alkylation or other processes to be manifested, since these modifications are not found on the C-C-A containing stem. It is conceivable that certain tRNA species become more or less modified during cotyledon development, without influencing the DEAE or CM-cellulose column elution profiles of the aminoacyl-oligonucleotides. However, these modifications are not thought to influence directly the cell's capacity for translating specific code words. In fact it should be pointed out that in no instance has a developmentally related change in tRNA population been shown to result in an increased or decreased capacity for translating individual code words. It is true that "codon restriction" has been suggested as a causal factor in tissue aging (44), but whether or not tissue senescence constitutes regulated development and differentiation remains to be seen. It is also true that changes in the tRNA pool often accompany the transition of tissue to a pathological state (see series in ref. 18). However, these changes may represent a conceptually different situation from regulated development and may be related to the pathological state itself.

In fact, the idea that a change in tRNA population may play a developmental regulatory role directly by altering the rate of translation of specific code words, thereby altering the levels of specific proteins, appears to be a poor one. Implicit in this idea is the assumption that synonym code words do not occur randomly in mRNA, but are used with a frequency that is "in phase" with the change in tRNA population so that the proper change in translation rates of certain mRNAs takes place. Such a restriction imposed on code word usage would appear incompatible with the idea that the genetic code is highly evolved towards the prevention of lethal mutations. That is, a single base change in DNA could conceivably alter greatly the levels of specific proteins, if their levels were regulated by code word frequency and tRNA population.

In Table IV the theoretical drawbacks to a system of developmental regulation based on tRNA population and code word frequency are diagrammatically presented. Here a hypothetical transition from one developmental stage to another is visualized as being in part mediated by an increase in a $tRNA^{Arg}$ species that reads AGA and a decrease in a $tRNA^{Arg}$ that reads CGA. For such a change to have an effect on the cell's complement of proteins, it is a requirement that these two code words not be utilized indiscriminately in mRNA but occur so as to insure that those proteins that characterize developmental stage I are synthesized from mRNA that position arginine with the code word CGA, and those that characterize stage II are synthesized from mRNA that utilize AGA. This requirement for selective code word usage in mRNA that codes for developmentally significant proteins we consider an unlikely result of evolutionary processes. In our opinion these processes would more likely have effected a randomization of code word frequency.

The other undesirable feature of this regulatory mechanism is the frequent lethal effect of single base changes in DNA. In the hypothetical case given in Table IV were base changes to occur in the mRNA for the developmentally important proteins (such as AGA to CGA), the mRNA would change categories. That is, the rate of its translation would change from one extreme to the other. This would result in a reversal in the amount of protein produced by the mRNA in the two developmental stages. It would seem that such an event could hardly be tolerated.

Table IV. Developmental Regulation by tRNA Population and Codeword Frequency.

	Developmental Stage I		Developmental Stage II	
	Change in concentration of tRNA Arg isoaccepting species			
$tRNA^{Arg}$ with anticodon	UCU *(low)*	GCU *(high)*	UCU *(high)*	GCU *(low)*
	↓	↓	↓	↓
mRNA with codeword	AGA	CGA	AGA	CGA
	↓	↓	↓	↓
Number of protein molecules	*Few*	*Many*	*Many*	*Few*
(Mutation to give codeword)	(CGA)		↓	
	↓			
Number of protein molecules	*Many*		*Few*	
Features of System:	1) Evolution of specific codeword usage in specific mRNA 2) Deleterious effects of single base change			

From this, it would seem that the changes in tRNA pools that have been reported to accompany tissue differentiation do not establish that tRNA participates in the differentiation process by influencing the rate of translation of specific proteins. A change in the rate of total or non-specific protein synthesis resulting from a change in the tRNA pool remains, of course, a feasible possibility.

Perhaps a more likely regulatory role for tRNA molecules would be in the allosteric regulation of steady state processes that serve to control the internal concentration of cell constituents. In this context, the modification of individual tRNA species by methylases and other enzymes could be considered to regulate the molecule's allosteric effectiveness. The genetic repression of several amino acid biosynthetic pathways in microorganisms are thought to involve the cognate aminoacyl-tRNAs in some fashion (see Review in Ref. 45) and, more recently, a species of $tRNA^{Tyr}$ has been shown to allosterically inactivate a mutant form of the enzyme, tryptophan pyrrolase, in *Drosophila melanogaster* (46).

Another interesting feature of our results concerns the chloroplastic tRNA species. The chromatography of isoaccepting species shows that chloroplast species exist in very young embryo cotyledons and in roots in about the same amounts. It is not surprising that chloroplastic tRNA, and presumably the entire chloroplast protein synthesizing system, exists in the young embryo. Conceptually, it would seem that this system must perpetuate itself during the reproductive cycle, otherwise its reestablishment would have to be carried out in part by the cytoplasmic system. We have no evidence that the tRNA species that we consider chloroplastic are transcribed from chloroplast DNA. However, it has been found that 35% of the leucyl-tRNA of bean leaves hybridizes specifically with bean leaf chloroplastic DNA (47), which is approximately the amount of chloroplastic $tRNA^{Leu}$ that we have found in germinated cotton cotyledons (Figure 6). The observation that chloroplast tRNA exists in roots strengthens the long held contention that root amyloplasts are

derived from the same proplastid progenitor as are chloroplasts (48). Finally the data show that the large increase in chloroplastic tRNA that occurs during germination does not require an induction by light. In this respect cotton cotyledons differ from chloroplast-bearing unicellular organisms such as Euglena in which the induction of chloroplast tRNA synthesis is in response to a light stimulus (49).

No mention has been made of a possible contribution to the tRNA population of the cotyledon or root tissue by mitochondria. To date we have been unable to demonstrate quantities of tRNA in cotton mitochondria that would affect the data we have presented.

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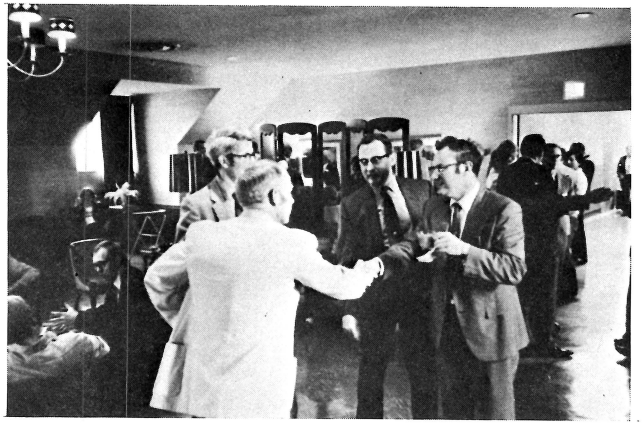
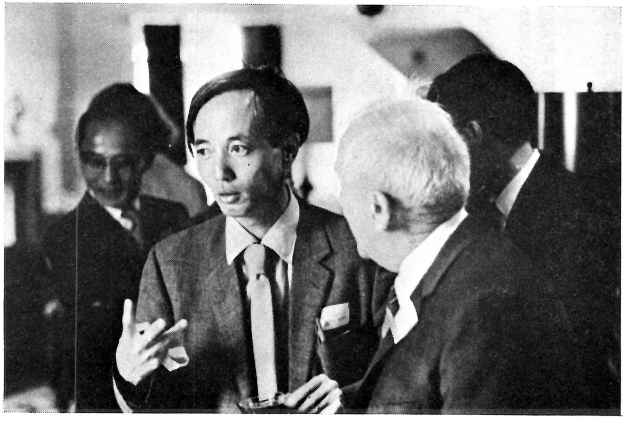
Dr. Leon Dure at the Symposium.





The reception in the Alumni Lounge on April 21, 1972.