#### AN ABSTRACT OF THE THESIS OF

 

 DONNER FRANKLIN BABCOCK (Name of Student)
 for the
 Ph. D. (Degree)

 in
 BIOCHEMISTRY (Major)
 presented on
 February 2, 1971 (Date)

 Title:
 INVESTIGATIONS OF THE ISOPRENOID NUCLEOSIDES

 OF PEA ROOT TRANSFER RIBONUCLEUC ACID Redacted for Privacy

 Abstract approved:
 R.O. Morris

Isoprenoid nucleosides are the most potent of the class of plant growth regulatory molecules known as cytokinins. Speculations concerning the molecular mode of action of cytokinins have centered around the fact that isoprenoid side chains occur specifically on the adenylic acid residue adjacent to the anticodon site of certain tRNAs and are vital for maximal activity of these tRNAs in protein synthesis.

To facilitate further investigations along these lines, an analytical method utilizing mass spectrometry in conjunction with partition and gas-liquid chromatography was developed. The method allowed the identification and quantitative measurement of the isoprenoid nucleosides present in very small amounts of tRNA. The occurrence of various isoprenoid nucleosides in hydrolysates of unfractionated but highly purified pea root tRNA and tRNA from several other species was determined. Pea root phenylalanine and tyrosine tRNA and a leucine tRNA subspecies (leu<sub>I</sub>, tRNA) were partially purified by reverse phase and benzoylated DEAE cellulose chromatography and their isoprenoid nucleoside contents examined.

Utilizing the above methods, evidence was found for the <u>in vitro</u> modification of tyrosyl, leucyl and possibly seryl tRNA. The modification of leucyl tRNA was examined in detail and found to be dependent on incubation with the ribonuclease-free aminoacyl synthetase preparation from pea roots. The product (leu<sub>L</sub> tRNA) migrated in a sharp band ahead of other tRNAs on gel electrophoresis, had unique mobility on reverse phase chromatography and could be deacylated but not reacylated, all of which suggested it arose as the result of a specific degradation of a leucyl tRNA. Analysis of the hydrolysate of leu<sub>L</sub> RNA indicated that its origin was the minor leucine tRNA(s) that contain an isoprenoid nucleoside. The relevance of these observations to the operation of mechanisms of control of gene expression is not totally understood but several alternatives are discussed.

## Investigations of the Isoprenoid Nucleosides of Pea Root Transfer Ribonucleic Acid

by

Donner Franklin Babcock

#### A THESIS

#### submitted to

#### Oregon State University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

June 1971

APPROVED:

# Redacted for Privacy

Associate Professor of Agricultural Chemistry in charge of major

# Redacted for Privacy

Chairman of Department of Biochemistry

# Redacted for Privacy

Dean of Graduate School

Date thesis is presented \_\_\_\_\_ February 2, 1971 \_\_\_\_\_

Typed by Opal Grossnicklaus for Donner Franklin Babcock

#### ACKNOWLEDGEMENTS

I wish to thank Dr. R. O. Morris for the encouragement and guidance given me during this project. Helpful discussion were also enjoyed with Dr. G. S. Beaudreau. I am also indebted to Dr. R. R. Claeys for his advice, to Mr. D. A. Griffin for operation of the Varian MATS spectrometer and to Dr. R. R. Haque for determination of the nuclear magnetic resonance spectrum of zeatin riboside and advice on its interpretation.

I am also most grateful for the gifts of isopentenyladenosine from Dr. R. H. Hall, <sup>14</sup>C-myeloblast RNA from Dr. A. O'C Deeney and pea seeds from the W. Brotherton Seed Co. The expert technical assistance of Mrs. Karen Zook was invaluable.

This research was conducted on a traineeship in the Center for Environmental Management supported by Public Health Service Grant P10-00210. Besides financial aid, numerous travel opportunities vital to this project were provided by the kind indulgence of Dr. I. J. Tinsley.

## DEDICATION

To J. B., L. D., to John and Carol, and to all the kids down at the Arctic Circle Drive-In

I	GENERAL IN TRODUCTION	1
	Regulation of Gene Expression: An Overview	1
	Isoprenoid Nucleosides as Cytokinins	15
	Statement of Purpose and Experimental Protocol	23
	Statement of I alpose and Enperimental I recees	
II	TRANSFER RNA AND AMINOACYL SYNTHETASES	
	FROM PEA ROOTS	25
	Introduction	<b>2</b> 5
	Materials and Methods	26
	$\frac{1}{2}$	26
	Polya crylamida Cel Electrophoresis	28
	Aminoacyl Synthetase and Aminoacyl	20
	tDNA Dronamation	30
	Decults and Discussion	22
	Characterization of Dec Boot (DNA	20
	Characterization of Pea Root IRNA	54
	Characterization of Pea Root Aminoacy	20
	Synthetases	39
	Determination of Conditions for Maximal	4.2
	Aminoacylation	43
III	GAS CHROMATOGRAPHIC ANALYSIS OF	
	ISOPRENOID NUC LEOSIDES	54
	Introduction	54
	Materials and Methods	58
	Procurement of Nucleoside Standards	58
	GLC Examination of tRNA Hydrolysates	59
	Mass and Nuclear Magnetic Resonance	5,
	Spectra of Isoprepoid Nucleosides	61
	Results and Discussion	63
		05
IV	TRANSFER RNA FRACTIONATION, PURIFICATION	
	OF SUBSPECIES AND ANALYSIS OF THEIR ISOPREN-	86
	OID NUCLEOSIDE CONTENT	00
	Introduction	86
	Materials and Methods	91
	Results and Discussion	93
	Purification and Fractionation of tRNA	
	Subspecies	93
	GLC Analysis of Hydrolysates of Purified	
	tRNAS	107

# TABLE OF CONTENTS

5

v	A DEGRADATORY ENZYME ACTIVITY SPECIFIC	
	FOR A LEUCINE tRNA	113
	Introduction	113
	Materials and Methods	114
	Results and Discussion	114
	Identification and Characterization of	
	leu <sub>t</sub> tRNA	114
	The Origin of leu $_{L}$ tRNA	120
	BIBLIOGRAPHY	1 31

## LIST OF FIGURES

Figure		Page
1.	Preparative gel electrophoresis of pea root tRNA.	34
2.	Analytical gel electrophoresis of tRNA.	36
3.	Gel electrophoresis of aminoacyl tRNA.	38
4.	Aminoacylation of leucine tRNA.	44
5.	Aminoacylation of serine tRNA.	45
6.	Aminoacylation of phenylalanine tRNA.	46
7.	Aminoacylation of tryptophan tRNA.	47
8.	Aminoacylation of tyrosine tRNA.	48
9.	Aminoacylation of cysteine tRNA.	49
10.	Structure of the isoprenoid nucleosides.	56
11.	Apparatus for collection of TMS-nucleosides.	62
12.	LH20 elution profile and absorbance spectrum of msIPA.	64
13.	Mass spectra of msIPA.	65
14.	Partition chromatography of a fortified tRNA hydrolysate.	69
15.	Partial mass spectra of $\underline{cis}$ and $\underline{trans}$ ZR enriched fractions.	71
16.	Partial nuclear magnetic resonance spectra of a <u>cis</u> and <u>trans</u> ZR mixture.	72
17.	GLC examination of tRNA hydrolysates.	75
18.	Additional GLC examinations of tRNA hydrolysates.	76

# Figure

19.	Mass spectra of $\underline{cis}$ ZR isolated from pea root tRNA.	79
20.	Reverse phase fractionation of aminoacyl tRNA.	95
21.	Benzoylated DEAE cellulose chromatography of bulk tRNA from pea roots	96
22.	Analytical gel electrophoresis of tRNA from BDC.	97
23.	Reverse phase chromatography of phenylalanyl tRNA.	100
24.	Chromatography of tyrosyl tRNA on BDC and reverse phase columns.	105
25.	GLC analysis of hydrolysates of purified tRNA.	110
26.	Reverse phase chromatography of leucyl tRNA from BDC salt and ethanol fractions.	115
27.	Gel electrophoresis of BDC ethanol fraction tRNA and reverse phase chromatography of a leucyl tRNA.	119
28.	Rechromatography of leucyl tRNA reverse phase column fractions on BDC.	122
29.	Production of leucyl tRNA leuL.	124
30.	Reverse phase chromatography of $^{32}$ P-tRNA.	126

#### LIST OF TABLES

Table		Page
1.	Purification of transfer RNA	33
2.	Purification of aminoacyl synthetases	· 40
3.	Ribonuclease activity in synthetase preparations	42
4.	Aminoacylation of pea root tRNA	51
5.	Source and occurrence of isoprenoid nucleosides	57
6.	Retention times of trimethylsilyl nucleosides	66
7.	Recovery of isoprenoid nucleosides from a tRNA hydrolysate	74
8.	Isoprenoid nucleoside content of unfractionated tRNAs	84
9.	Amino acid acceptance of BDC salt and ethanol fractions	98
10.	Purification of phenylalanine tRNA	101
11.	Purification of a leucine tRNA	103
12.	Purification of tyrosine tRNA subspecies	106
13.	Isop <b>r</b> enoid nucleoside analysis of purified tRNAS	108
14.	Discharging and reacylation of reverse phase fractions.	117

#### INVESTIGATIONS OF THE ISOPRENOID NUCLEOSIDES OF PEA ROOT TRANSFER RIBONUCLEIC ACID

#### I. GENERAL INTRODUCTION

#### Regulation of Gene Expression: An Overview

The regeneration of entire plants from single cells (Steward <u>et al.</u>, 1967) is a dramatic demonstration of the fact that mature, differentiated cells of a higher organism carry the complete genetic complement of the organism. Yet there is abundant evidence (Paul and Gilmour, 1966; Marushige and Bonner, 1966) that only a fraction of the DNA of a given cell is ever translated into protein and that this fraction differs from cell to cell. One of the major unanswered questions of developmental biochemistry is how differentiating cells control the expression of the appropriate portions of their genome.

Plant systems are unique in their dramatic physiological response to several classes of low molecular weight molecules implicated in control of their normal growth and development. The investigations described in this thesis were undertaken with the hope of elucidating the details of the mechanism of action of one such class of molecules, the cytokinins, in a higher plant. Before considering what is known of this mechanism in particular, it may be helpful to review some theoretical possibilities for gene control and the present state of knowledge as to their applicability, where possible, to higher plants.

Almost certainly some gene control is exerted at the transcriptional level. Negative transcriptional control by interaction of a protein repressor with the DNA of bacterial cells is well documented (Ptashne, 1967; Gilbert and Müller-Hill, 1967). Using a cell free protein synthesizing system and the DNA dependent RNA polymerase from <u>E</u>. <u>coli</u> with chromatin isolated from various organs of the pea plant, Bonner, Huang and Gilden (1963) found the following indications of a mechanism of transcriptional control operating on the DNA of this eukaryote. Pea globulin, a storage protein, is made only in developing pea cotyledons. Immunochemical examination of the protein made utilizing chromatin isolated from pea buds and developing cotyledons as template showed globulin to be synthesized only from the latter.

Various basic nuclear proteins, especially the histones, have been proposed to be rendering certain regions of the chromatin untranscribable (Bonner <u>et al.</u>, 1963; Bonner and Huang, 1963). In fact poly-L-arginine and poly-L-lysine have been shown (Shih and Bonner, 1970) to interact with DNA to form a complex that is not transcribed <u>in vitro</u>. Since the polymerase binds to the complex as well as to the free DNA and since the DNA-polypeptide complex has a quite high melting point, it is presumed that the complex is not able to undergo the local untwisting necessary for transcription. Koslov and Georgiev (1970) reported a similar study concerning the template activity of a DNA-histone complex in which they also concluded that the association prevents movement of the RNA polymerase along the DNA template.

Implicit in the histone theories of gene control first proposed is gene recognition by specific histone molecules and thus great histone heterogeneity. However, recent studies by Fambrough <u>et al</u>. (1968) have shown that the histones from different tissues are not qualitatively different, and indeed there appear to be only a small number of molecular species of histones. Other nuclear components, the acidic proteins (Paul and Gilmour, 1968) or chromosomal RNA (Huang and Huang, 1968; Bekhor, Kung, and Bonner, 1968) have been postulated as conferring specificity on the histone-DNA complex, but the evidence to confirm these hypotheses is still lacking.

More recent work from Bonner's laboratory (Matthysse and Phillips, 1969; Morgan and Bonner, 1970) has implicated hormonebinding proteins in a positive control mechanism operating on the DNA template. These protein fractions, in the presence of hormones, stimulate the <u>in vitro</u> template activity of chromatin presumably by making more of the genome accessible to the polymerase. Emmer <u>et al.</u> (1970) have shown that a hormone binding protein is involved in catabolite gene activation in <u>E. coli</u>. Noninducible mutants deficient in cell free lac operon DNA primed  $\beta$ -galactosidase synthesis were shown to have a defective cyclic AMP receptor protein. Although the system may be stimulated by partially purified wild type protein, it is unclear (Zubay, Schwartz and Beckwith, 1970) whether the protein associates with the promoter region of the template or with the polymerase.

Burgess et al. (1969) have isolated a protein factor, designated "sigma", dissociable from the E. coli polymerase, that does play a part in positive transcriptional controls operating on the RNA polymerase. The sigma factor apparently recognizes the regions of DNA appropriate for initiation of transcription (Travers and Burgess, 1969; Bautz and Bautz, 1970). Sigma-like factors with different specificities can thus control transcription of classes of mRNA initiated by diverse signals. Although attempts to discover more than one kind of sigma in  $\underline{E}$ . <u>coli</u> have failed, the production of new sigma-like factors plays an important role in controlling the development of the bacteriophage T4 (Travers, 1969; Schmidt et al., 1970; Travers, 1970). In addition, during sporulation of <u>B</u>. subtilis Losick, Shorenstein, and Sonenshein (1970) have shown that one of the subunits of the polymerase is altered so that it no longer recognizes the sigma of the vegetative cell, and presumably will no longer transcribe vegetative DNA. In a mammalian system, Lindell, Weinberg and Rutter (1970) have gathered preliminary evidence for a protein factor that differentially affects the nucleolar

and nucleoplasmic polymerases, but have not established its role in transcriptional regulation.

The discovery and characterization of another factor, which Travers, Kaman and Schleif (1970) have chosen to call "psi," was recently described. Psi and sigma appear to be simultaneously required for transcription of rRNA genes of E. coli. Psi and the cyclic AMP receptor protein have a number of strikingly similar properties. Each is involved in the specific initiation of synthesis of particular classes of RNA. They both require sigma and they both appear to be under the control of a nucleotide. The activity of the cyclic AMP receptor protein is stimulated by 3', 5'-AMP and that of psi is inhibited by ppGpp. Travers et al. suggested that psi and the cyclic AMP receptor protein are representative of a class of positive control elements, acting as secondary determinants for promoter recogni-They noted that the regulation of psi like factors by low molecution. lar weight effectors provides a readily reversible control system which would allow the cell to respond rapidly to environmental changes. Changes of a more irreversible nature, such as those associated with sporulation of B. subtilis or development of lytic phage, on the other hand, seem to be mediated by more permanent modifications of the RNA polymerase itself or of the sigma factor population.

The positive and negative controls operating on transcription may have analogs at the translational level that play a role in control

5

of gene expression. Again it is the bacterial and phage systems that have been most closely examined.

Although it is by no means certain that the RNA's of the RNA coliphages (f2, MS2, R17, etc.) represent typical messenger RNA (Rechler and Martin, 1970), they are the best characterized natural messages. R17 RNA is comprised of approximately 3300 bases (Strauss and Sinsheimer, 1963). Three proteins; coat protein, maturation protein, and RNA synthetase are encoded in approximately 2800 of these bases. The gene order has recently been shown to be 5'-maturation protein-coat protein-RNA synthetase-3' (Jeppesen <u>et</u> al., 1970).

These three genes are translated with vastly different efficiencies both <u>in vivo</u> and <u>in vitro</u>. In the f2 system Lodish (1968) has observed typical ratios of 6:20:1 for the maturation protein, coat protein and synthetase. At least two mechanisms have been postulated to be operating in this very simple system of control of gene expression. With the closely related phage MS2 Eggen and Nathans (1969) and Sugiyama and Nakada (1968) have demonstrated that a specific coat-protein-RNA complex prevents translation of the synthetase and maturation proteins <u>in vitro</u>. This complex may be a manifestation of negative gene control at the translational level.

Alternatively the differences in efficiency with which the three genes of R17 RNA are translated may be based on intrinsic properties of the RNA itself. Steitz (1969) has elucidated the sequence of the initiation regions in the R17 message. Each of the three regions is unique and it is possible that their initiation with various efficiencies is the basis of the observed ratios of translation. The functions of three dissociable ribosomal initiation factors,  $F_1$ ,  $F_2$ ,  $F_3$  of Iwasaki <u>et al.</u> (1968) corresponding to the A, C, B factors of Revel <u>et al.</u> (1968) have been elucidated by these workers.  $F_3$  (B) has been shown to be specifically required for formation of an initiation complex with natural messenger RNA. If different initiation sequences require different ribosomal  $F_3$  (B) factors for their recognition, these factors may act to control the translation of classes of genes in a manner analogous to the sigma factors of transcription.

Indeed, just such a positive translational control mechanism appears to be involved in the restriction of phage f2 RNA translation in cells infected with T4 (Hsu and Weiss, 1969; Schedl, Singer and Conway, 1970). Dube and Rudland (1970) have shown that several new proteins in the ribosomal "factor" fraction appear within two minutes after T4 infection and impart on these ribosomes the ability to discriminate against f2 and  $\underline{E}$ . <u>coli</u> mRNA while allowing efficient translation of T4 mRNA. More specifically Pollack <u>et al</u>. (1970) have shown that at least one modification in the B (F3) fraction is responsible for the alteration in template specificity resulting in the preferential translation of late T4 mRNA. Even more significant is the recent report from Revel <u>et al</u>. (1970) that the B (F3) fraction from uninfected <u>E</u>. <u>coli</u> contains at least three proteins which initiate the translation of various natural messages (and presumably different cistrons on the same message) with different efficiencies. This then implies that translational control of protein synthesis may be a general mechanism for regulation of gene expression.

The details of post-transcriptional control of protein synthesis in higher organisms has been examined in less detail partly because of the additional complexities imposed by the compartmentalization of the translational and transcriptional processes. In nucleated cells, only a small portion of the rapidly labeled nuclear RNA can be assigned as a precursor to cytoplasmic messenger RNA (Darnell, 1968; Dingman and Peacock, 1968). The transport of mRNA precursors across the nuclear membrane is thus obviously a possible site for operation of a gene control mechanism. Bell (1969), Spirin and Nemer (1965), and Henshaw (1968) have all presented evidence for the existence of cytoplasmic nucleic acid and protein containing particles. Although the DNA-protein particles seen by Bell may be artifacts (Fromson and Nemer, 1970; Müller, Zahn and Beyer, 1970), the existence of cytoplasmic RNA containing particles seems on firmer ground. Bell has postulated that these particles may be manifestations of a transport control mechanism. Spirin (1966) on

the other hand, cites studies on the development of non-nucleated fragments of sea urchin eggs and of fertilized eggs treated with actinomycin D (an inhibitor of RNA synthesis) which indicate that a form of "masked" messenger RNA is present in the unfertilized egg. He also describes numerous other studies indicating that the events of transcription and translation are often considerably separated in time during differentiation and development in a wide variety of eukaryotic organisms. The observed cytoplasmic nucleoprotein particles are thus proposed to be such "masked" or protected mRNA, whose translatability is presumed to be controlled by this association.

Tomkins <u>et al.</u> (1969) have studied the control of the enzyme tyrosine aminotransferase in synchronized populations of ETC (rat hepatoma) cells. This enzyme is inducible by glucocorticoid hormones only during  $G_1$  and S phases of the cell cycle. During the rest of the cell cycle the enzyme is synthesized constitutively, presumably from preexisting mRNA (Martin and Tomkins, 1970). Addition of insulin to induced cells results in a further two to three fold stimulation in tyrosine aminotransferase synthesis without concomitant RNA synthesis (Gelehater and Tomkins, 1970). To account for these observations, Tomkins has postulated a labile repressor of translation. The mammalian repressor may be an RNA, unlike the protein repressors of bacterial systems.

Until very recently the lack of fMet-tRNA in the cytoplasm of

9

nucleated cells (Marcker, Clark and Anderson, 1966; Mosteller, Culp and Hardesty, 1968) was taken by some investigators to imply that other (especially N-acetylated) tRNAs might serve as initiators of protein synthesis in eukaryotes. For example, Kim (1969) claimed the isolation of N-formylseryl tRNA from yeast and the labeling studies of Liew, Haslett and Allfrey (1970) indicated that f2a histones from regenerating liver were initiated with N-acetyl serine. Initiation by tRNAs other than fMet-tRNA would allow another possible site for translational control; by diverse specificities in multiple  $F_{l}$  (A) and  $F_2$  (C) factors which are involved in the binding of the initiator tRNA to the initiation complex. For example translation of a class of mRNAs initiated by N-blocked seryl tRNA could be controlled by the availability of such factors specific for this initiator. This possibility seems considerably more remote, however, in light of the recent work of Smith and Marcker (1970) and Brown and Smith (1970). The former have shown that two species of methionine tRNA can be isolated from the cytoplasm of mammalian tissue. One species is capable of being formylated by the bacterial transformylase (the transformylase is absent from mammalian tissue). Furthermore this species incorporates methionine specifically into the N-terminal position of peptides translated from encephalomyocarditus virus RNA in their cell-free system from Ascites tumor cells. Brown and Smith were able to initiate protein synthesis in this system with no synthetic

messages except those with an AUG sequence near their 3' terminus. These experiments imply that the initiation processes in nucleated and non-nucleated cells may differ only in the requirement for formylation of the initiating methionine tRNA. The idea that methionine is the universal initiator is also supported by the recent observations of Jackson and Hunter (1970), who showed that methionine is the N-terminal residue in short nascent hemoglibin chains bound to rabbit reticulocyte ribosomes. Similarly Wigle and Dixon (1970) demonstrated that methionine is transiently incorporated at the N-terminus of nascent trout testis protamine. Turning to plant systems, Leis and Keller (1970) have shown wheat leaves contain a eukaryotic type (non formylatable) met-tRNA in the cytoplasm and a procaryotic type (formylatable) met-tRNA in the chloroplasts.

Besides the negative translational controls operating on the message, postulated by Spirin (1966) and Tomkins (1969), there are two possible positive mechanisms operating on the translational machinery of eukaryotic cells. First, since the initiation factors from rabbit reticulocyte ribosomes have recently been shown to be similar in number and function to the bacterial factors (Pritchard et al., 1970) one might reasonably expect that the mammalian factor  $M_3$  (corresponding to the F3 (B) <u>E</u>. coli factor) may also prove heterogenous and show selectivity of recognition among its subfractions. Secondly, the investigations of Anderson and Gilbert (1969) indicate

that the concentration of an aminoacyl tRNA subspecies recognizing a minor code word will limit the <u>in vitro</u> of translation of a message such as that for the alpha chain of rabbit reticulocyte hemoglobin which contains such a minor code word.

Such a codon restriction mechanism of translational control, I believe, has been in the minds of the numerous investigators who have demonstrated alterations in the tRNA (Balinga <u>et al.</u>, 1969; Doi, Kaneko and Igaraski, 1968; Waters, 1969; Vold and Sypherd, 1968; Hung and Overby, 1968; Gallo and Pestka, 1970; Bick <u>et al.</u>, 1970) or the aminoacylating enzyme (Rennert, 1969; Anderson and Cherry; Neidhardt and Earhart, 1966) complement associated with changes in cell metabolism.

The operation of such a mechanism has been implicated in the restriction of host cell protein synthesis following infection by the T even phages. The intracellular concentration of the major leucine tRNA of <u>E</u>. <u>coli</u> ( $tRNA \ leu_I$ ) recognizing the code word CUG, (Dube, Marker and Yudelevich, 1970) was shown to decrease dramatically following T2 infection (Sueoka and Sueoka, 1968; Waters and Novelli, 1968). Moreover the proportion of this species found in the polysome fraction decreases even more markedly after T2 infection--implying that this tRNA species plays little part in T2 directed protein synthesis. This was further supported by measurement of the very low in vitro incorporation of radioactive leucine from leucyl tRNA leu<sub>I</sub>

using T2 mRNA as message (Kano-Sueoka and Sueoka, 1969).

It is not necessary that the intracellular concentration of a particular tRNA subspecies change to effect such a translational control. It has been shown that the biological activity of tRNA depends to a great extent on its complement of minor bases. For instance, Capra and Peterkofsky (1968) have reported an alteration in codon specificity in undermethylated leucine tRNA. Shugart, Novelli and Stulberg (1968) showed that unmethylated tRNA is aminoacylated at a fraction of the rate of its fully methylated counterpart. Hypermethylation has been shown to likewise impair aminoacylation (Pillinger, Hay and Borek, 1969) and ribosomal binding (Hay, Pillinger and Borek, 1970) of specific tRNAs. When the Y base (a hydrophobic residue of unknown structure) is specifically cleaved from yeast phenylalanine tRNA it can no longer be aminoacylated with the E. coli enzyme (Thiebe and Zachau, 1968). Recently Jacobson and Hedgcoth (1970) have proposed that the low dihydrouridine control of tRNA from chloramphenicol treated cells may make them inactive and be responsible for the slow recovery in growth rate after chloramphenicol treatment. However, the most significant example concerns the isoprenoid nucleosides (derivatives of  $N^{6}$ -( $\Delta^{2}$ -isopenteny) adenosine) found adjacent to the 3' end of the anticodon of certain tRNAs. These residues have been demonstrated to be vital for ribosomal binding competency of the tRNAs containing them. Preliminary evidence of this was

obtained by Fittler and Hall (1966) who showed yeast seryl-tRNA was no longer able to bind ribosomes after treatment with an aqueous iodine solution, a reagent which causes a 1 to 6 cyclization of N<sup>6</sup>isopentenyl adenosine, or after cleavage of the isopentenyl side chain from the N<sup>6</sup>-isopentenyl adenosine in intact serine tRNA by incubation with permanganate. Unfortunately both of these experiments left open the possibility that the chemical attack was nonspecific and that the impairment of ribosomal binding was due to loss of some other vital structural feature. Conclusive evidence was obtained from the elegant experiments of Gefter and Russell (1969). These investigators found normal <u>E</u>. <u>coli</u> tyrosyl-tRNA gave a single chromatographic peak in the RP-1 system of Kelmers, Novelli and Stulberg (1965). After infection with the defective transducing phage  $\phi$  80dsu<sup>+</sup> III, carrying the information for a tyrosine suppressor tRNA, two new peaks of tyrosine acceptor activity were found. In in vitro ribosomal binding assays peak I was inactive and peak II had only half the activity of peak III (which coincides with the tyrosyl-tRNA from uninfected cells). Examination of the oligonucleotide fragments obtained from partial digests of the three purified species allowed the conclusion that peaks I and II were modification deficient forms of the tyrosyl tRNA found in peak III, differing only in the residue adjacent to the 3' end of the anticodon. The inactive form, peak I, contained adenylic acid at this site. Peak II, the partially active form, contained

isopentenyl adenosine and the fully active peak III tRNA contained 2-methylthio-N<sup>6</sup>-isopentenyl adenosine.

Thus the presence of an isoprenoid nucleoside and even its proper degree of substitution are requisites for the full activity of certain tRNA molecules and we may imagine a translational control based on functional and non-functional forms of certain tRNAs differing in their minor base (especially isoprenoid nucleoside) content.

# Isoprenoid Nucleosides as Cytokinins

There is another reason to suppose that the isoprenoid nucleosides of tRNA may be involved in control of protein synthesis in plant systems: Isoprenoid nucleosides and their corresponding purines are the most potent of the class of plant growth regulators known as cytokinins.

The term cytokinin arose from the observation of Miller <u>et al</u>. (1955a) that a factor, commonly called kinetin and later characterized as 6-furfurylaminopurine (Miller <u>et al</u>., 1955b, 1956a), promoted cytokinesis (cell division) in tobacco callus tissue cultures. Numerous N<sup>6</sup>-substituted purines and their ribosides have been synthesized and cytokinin activity has been found associated with many having benzyl, pyridyl, alkyl or isoprenoid substituents (Skinner <u>et al</u>., 1956; Skoog <u>et al</u>., 1967). Cytokinin activity has also been found to be associated with N, N'-diphenylurea and its derivatives (Bruce and Zwar, 1966) and has been claimed for some purinone derivatives (Wood, 1970).

It soon became apparent that cytokinins were capable of promoting a wide variety of physiological responses in plant systems. This area has been reviewed by Miller (1961), von Overbeek (1966), Letham (1967) Hegelson (1968) and by Skoog and Armstrong (1970) so only a few examples are presented below.

Cytokinins are able to replace exposure to red light for the initiation of germination of lettuce seeds as shown by Miller (1956b). They have proved to be the most effective agents known for the retardation of senescence in excised plant tissues (Richmond and Lang, 1967). Stetler and Laetsch (1965) investigated the response of a tobacco tissue culture which did not require an exogenous cytokinin supply for growth. This system contained proplastid bodies which were capable of developing into mature chloroplasts when cytokinins were provided. Thus cytokinins were the first growth regulator shown to induce the development of a specific organelle.

Perhaps the observation with the most profound implications is the demonstration that different ratios of cytokinins to auxin (another class of plant growth regulators) in the growth medium greatly alters the morphology of tobacco callus cultures (Skoog and Miller, 1967). At low cytokinin to auxin ratios loose, friable tissue is obtained with roots forming in older cultures. At higher ratios flattened globules of tissue are obtained. At still higher ratios vigorous shoots are produced. Schaeffer and Smith (1963), also working with callus cultures, showed that tissue cultures of widely divergent parental species respond similarly and favorably to exogenous cytokinins whereas their tumor-forming hybrids give no response. This implied that the uncontrolled growth of tumorous plant tissue might be due to the ability of tumors to produce their own cytokinins. Indeed this appears to be the case for the tumor-like growths of fasciation disease (Klambt, Thies and Skoog, 1966; Thimann and Sachs, 1966). Moreover the work of Wood <u>et al</u>. (1969) demonstrated that crown gall tumors promote cytokinin synthesis in the affected tissue. More recent work by Wood (1970) and Lippincott and Lippincott (1970) indicated that this may be mediated through production of a growth factor of unknown nature.

Together the demonstration of experimental control of morphology of tissue cultures by auxin/cytokinin ratios in a completely defined growth medium and the alterations in morphology in abnormal tissue producing their own growth regulators has been taken as strong evidence that gradients of cytokinin and auxin concentration may be largely responsible for directing the growth and development of intact plants. It has not, however, been possible to directly test this hypothesis since the molecular basis of cytokinin action and thus the molecules whose concentrations would be critical, are not known. Many of the physiological and growth regulatory effects of cytokinins were discovered using kinetin in the nearly ten years (1955-1963) between its discovery and the discovery of the first naturally occurring cytokinin, zeatin. Letham (1963) and Letham, Shannon and McDonald (1964) first reported isolation and characterization of this hydroxyl substituted isoprenoid purine from immature corn seeds. Zeatin was later shown to be present as the nucleoside and nucleotide in the same tissue (Miller, 1965). The structure reported for zeatin is a <u>trans</u> configuration. The <u>cis</u> isomer of zeatin riboside as well as the unsubstituted N<sup>6</sup>-( $\Delta^2$ -isopenteny)adenosine have been found in other plant tissues (Hall <u>et al</u>., 1967). These compounds and the corresponding 2-methylthio analogs have recently been isolated from wheat germ (Hecht <u>et al</u>., 1969b).

Investigations of the molecular mode of action of cytokinins, however, date from the discovery of cytokinin activity associated with tRNA (Bellamy, 1966; Skoog <u>et al.</u>, 1966). Hall <u>et al</u>. (1966) soon showed that this activity in yeast tRNA was due to the presence of an isoprenoid nucleoside. What an astounding coincidence! Isoprenoid nucleosides, the most active cytokinins, molecules implicated in regulation of plant growth and development (presumably by control of protein synthesis) are also components of tRNA, macromolecules vital to the process of protein synthesis.

In the same year it was shown that isoprenoid nucleosides were

components of specific tRNA subspecies, where they occupy a position adjacent to the anticodon (Zachau, Dütting and Feldman, 1966) and that they are apparently necessary for full biological activity of these tRNAs (Fittler and Hall, 1966). At this point the way seemed clear to the elucidation of the mechanism of action of cytokinins. These molecules or their isopentenyl side chains, were postulated to be incorporated into certain minor tRNA species, conferring upon them competency for translation of classes of mRNAs containing the corresponding minor code words.

Fox (1966) and Fox and Chen (1967) conducted experiments that seemed to support this contention. Their isotope incorporation studies in soybean callus cultures were taken to imply that the synthetic cytokinin benzyladenine was incorporated, intact, into tRNA. It must be mentioned however, that the tRNA associated radioactivity found was very low and that association rather than incorporation could account for their observations. That their results were probably artifacts is indicated by the experiments described below.

Fittler, Kline and Hall (1968a) and Peterkofsky (1968) showed that isoprenoid side chains in the tRNA of microorganisms are derived from acetate via mevalonate. Fittler <u>et al.</u> (1968b) were later able to demonstrate the presence of an enzyme in yeast and rat liver extracts that attaches the isopentenyl side chain to specific adenosine residues in preformed tRNA. Chen and Hall (1969) have since found this same enzyme activity in tissue from a cytokinin requiring tobacco callus culture. This observation indicated that the isoprenoid nucleosides in the tRNA of a cytokinin requiring tissue are formed at the macromolecular level rather than at the mononucleoside level as proposed by Fox and Chen. Chen and Hall also demonstrated that in this tissue exogenously supplied cytokinin does not suppress the incorporation into tRNA of labeled  $a^2$ -isopentenyl side chains derived from mevalonate, although it does stimulate growth. Apparently exogenous cytokinins do not serve as isopentenyl donors to tRNA either. Rather clearly the cytokinin properties of isoprenoid nucleosides are not due to their incorporation, partial or complete, into tRNA as proposed by Fox and Chen.

Still it seems outside the realm of possibility that the presence of isoprenoid nucleosides in tRNA is not somehow related to their growth regulatory activity. For this reason Chen and Hall postulated that isoprenoid nucleoside containing tRNAs may actually serve as the source of the endogenous cytokinins. This hypothesis may be used to rationalize some otherwise disturbing observations on the effect of exogenous cytokinins on the leucine tRNAs of soybean cotyledons.

Anderson and Cherry (1969) found six leucine tRNA subspecies in soybean seedlings. Only four of these are charged by the aminoacyl synthetases derived from the cotyledon. Moreover the two subspecies ( $leu_5$  and  $leu_6$ ) not charged by the hypocotyl enzymes are present at much lower levels in the hypocotyl. Bick <u>et al.</u> (1970) showed that the relative proportion of  $leu_5$  and  $leu_6$  increases with age in the cotyledon. However application of exogenous cytokinins, while retarding the physiological changes associated with senescence of the cotyledon, produces the same molecular changes as ageing; increased levels of  $leu_5$  and  $leu_6$ . These last two observations may be reconciled if one postulates that:

- 1) Leu<sub>5</sub> and leu<sub>6</sub> tRNAs are a source of endogenous cytokinin
- On a cellular level loss of ability to produce cytokinins causes
   a loss of cytokinin induced capacities and results in senescence.
- On a molecular level this is manifested by increases in the levels of leu<sub>5</sub> and leu<sub>6</sub> with age of the cotyledon
- Endogenous cytokinin production is controlled so that exogenously supplied cytokinin prevents the degradation of leu<sub>5</sub> and leu<sub>4</sub> as well as producing the physiological response.

Such proposals lead to a hypothetical degradatory enzyme utilizing leu<sub>5</sub> and leu<sub>6</sub> tRNA as substrates. The proof of the existence of such an enzyme forms one portion of this thesis.

It should be noted that this hypothesis does not make any predictions about the mode of action of cytokinins subsequent to their release from tRNA. The key to this problem may lie in the recent report of Matthyssee (1969) concerning the effect of cytokinins on transcription in isolated nuclei, or the work of Berridge, Ralph and Letham (1970) indicating that cytokinins bind to plant ribosomes, possibly to the G factor known to be bound by cyclic AMP (Pastan and Perlman, 1970). There is however, another unrelated line of reasoning that must be considered.

Approaching the molecular role of isoprenoid nucleosides in regulation of plant growth and development from their apparent necessity to the tRNAs that contain them, it has been shown that various substitutions on the adenylic acid residue adjacent to the 3' end of the anticodon greatly alters the biological activity of <u>E</u>. <u>coli</u> tyrosine tRNA subspecies (Gefter and Russel, 1969). Recent work (Bautz <u>et al.</u>, 1970) indicated that the isoprenoid nucleoside content of <u>E</u>. <u>coli</u> tRNA from expotential and stationary phase cultures were quite different. This observation lends support to the hypothesis that modification variant forms of tRNA may be the basis of a translational restriction mechanism of control of protein synthesis.

The investigations of Vanderhoef and Key (1970) have shown that there are three chromatographically distinct tyrosine tRNAS in pea root tRNA. The relative proportions of these three species of tRNA from dividing tissue of the root tip were different than those of tRNA derived from nondividing root tissue, while identical profiles were obtained after fractionation of seven other isoaccepting species. A demonstration that these tyrosine tRNA subspecies from pea root

22

differ in their isoprenoid nucleoside content would be strong evidence for the operation of a translational restriction mechanism (possibly under control of the endogenous cytokinins) in regulation of gene expression in higher plants. Development of the means to examine the isoprenoid nucleoside content of the tyrosine tRNAs of pea roots constitutes the other work described in this thesis.

#### Statement of Purpose and Experimental Protocol

Based on the arguments presented above I have proposed a degradatory enzyme operating on the isoprenoid nucleoside containing leucine tRNAs of higher plants and have contracted to demonstrate its existence. Other equally valid arguments have led to an alternative proposition. I have also postulated a translational restriction mechanism for control of gene expression in plant systems. As a test of this hypothesis I have suggested a demonstration that the chromatographically separable tyrosine tRNAs of pea roots differ in their isoprenoid nucleoside content. To achieve these ends it was necessary to:

- Develop a technique for preparation of tRNA and aminoacyl synthetases from pea roots--as described in Part II.
- 2) Develop a method for identifying and measuring the isoprenoid nucleosides of pea root tRNA--as described in Part III.
- 3) Develop a means for purifying and fractionating the pea root

tyrosine (and other isoaccepting) tRNAs--as described in Part IV.

The acquisition of these techniques constitutes the bulk of the work presented in this thesis. Since each was developed rather independently of the others, each is described and discussed in a separate section. The actual application to the stated objectives and the relevance of the findings are covered in Parts IV and V.

#### II. TRANSFER RNA AND AMINOACYL SYNTHETASES FROM PEA ROOTS

#### Introduction

It has been known for several years that water-saturated phenol can be used for the extraction of relatively undegraded RNA from cell homogenates (Kirby, 1956). The addition of sodium dodecyl sulfate (SDS) to the homogenization medium assists in rupturing cell membranes and in liberating RNA (Fraenkel-Conrat, Singer and Williams, 1957). Low molecular weight material and the bulk of the phenol can be removed by precipitation of the RNA with ethanol. Transfer RNA is separated from most of the high molecular weight RNA by extraction with cold 1 M NaCl (Crestfield, Smith and Allen, 1955). Holley et al. (1961) showed further purification of tRNA may be achieved by chromatography on DEAE cellulose, for which single stranded RNA has a high affinity. However it is demonstrated below that pea root tRNA prepared by these procedures is still considerably contaminated by DNA, 5S RNA, oligonucleotide fragments and polysaccharides. Preparative polyacrylamide gel electrophoresis (Loening, 1967) was utilized to achieve final separation of tRNA from these contaminants.

A method for the partial purification of aminoacyl synthetases (amino acid; tRNA ligases E.C. 6.1.) from pea roots has been described by Scott and Morris (1969). It has proven necessary to
slightly mod ify their procedure to free the preparation of traces of contaminating ribonuclease activity. Aminoacyl synthetases obtained in this manner were used to determine optimal conditions for the aminoacylation of the pea root tRNA isoaccepting species presumed to contain isoprenoid nucleosides.

#### Materials and Methods

### Purification of tRNA

Alaska peas (<u>Pisum sativum</u> var. Alaska) were obtained from W. Brotherton Seed Company, Moses Lake, Washington. The dry peas were surface sterilized in 0.5% sodium hypochlorite for ten minutes then germinated in 3 mM CaCl<sub>2</sub> and lmM MgCl<sub>2</sub>. After imbibition for ten hours the peas were grown for an additional 38 hours between layers of cheesecloth under and overlayed with Vermiculite (W. R. Grace and Company, Portland, Oregon) saturated with 1 mM  $KH_2PO_4$ . For preparation of <sup>32</sup>P-labelled tRNA the dry peas (1 kg) were allowed to absorb a small volume of 20 mCi of carrier free <sup>32</sup>P-orthophosphate (International Chemical and Nuclear Corporation, Irvine, California) followed by periodic additions of the germination solution over the imbibition period.

The etiolated roots were harvested by freezing the seedlings with liquid nitrogen, shaking in a styrofoam ice bucket, then screening the fractured roots through 5 mm mesh stainless steel wire screen. Routinely 1 kg of roots were obtained from 10 kg of pea seeds. The frozen roots were powdered in a Waring Blender then stirred into three liters of 0.01 M trisCl buffer (pH 7.4) containing 0.5% SDS and 0.01 M MgCl<sub>2</sub> and homogenized with 1.5 liters of buffer-saturated phenol (reagent grade 88% phenol, redistilled before use). After shaking for 30 minutes the aqueous and phenol layers were separated by centrifugation at 5000 x g for ten minutes. The aqueous phase was removed and the phenol phase reextracted with 0.5 liters of fresh buffer. The combined aqueous layers were reextracted with 0.5 liters of buffer-saturated phenol. The aqueous phase from this extraction was made 0.1 M in potassium acetate (pH 4.5) and was added to four liters of 95% ethanol at -20°C. After precipitation overnight at -20°C the RNA was collected by centrifuga-The pelleted RNA was homogenized with two volumes of 1 M tion. NaCl, 0.01 M trisCl (pH 7.4), 0.1 mM dithiothreitiol, stirred for 30 minutes in the cold and centrifuged at 13,000 x g for ten minutes. The pellet was extracted twice more with equal volumes of the same solution and the combined extracts were again precipitated with ethanol. After collection by centrifugation the pellet was dissolved in 5-10 ml of 0.01 M tris Cl (pH 7.4), 0.01 M MgCl<sub>2</sub>, 0.1 mM dithiothreitol (TMD buffer). The solution obtained had a high viscosity indicating the presence of DNA. It was therefore treated with

27

electrophoretically purified deoxyribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) at a level of 50  $\mu$ g per ml for 15 minutes at 0°C. The solution was then diluted and applied to a 2.5 x 15 cm column of DEAE cellulose previously equilibrated against 0.1 M NaCl in TMD buffer. The column was washed with 500 ml of this solution and the tRNA was eluted with 1 M NaCl in the same buffer. A considerable amount of polysaccharides were removed by centrifugation at 20,000 x g for 30 minutes (Kirby, 1964) prior to storage at -70°C.

# Polyacrylamide Gel Electrophoresis

The purification of the tRNA was monitored by electrophoresis on 10% polyacrylamide gels (Loening, 1967). N, N' methylenebisacrylamide, N, N, N', N' tetramethylethylenediamine (TEMED), and acrylamide were all purchased from Eastman-Kodak, Rochester, New York. The acrylamide was recrystallized from chloroform and the bis acrylamide from acetone before use. The buffer system employed was 0.02 M sodium acetate, 0.04M tris acetate, 0.02 M EDTA (pH 4.5). The polymerizing agents were 10% acrylamide, 0.25% bis acrylamide, 0.33% TEMED and 0.33% ammonium persulfate. All solutions were filtered through B6 membrane filters (Schleicher and Schuell, Keene, New Hampshire). The gel solution was degassed and pipetted into 0.5 x 7 cm glass tubes capped with

Parafilm (American Can Company, Neenah, Wisconsin). The gel solution was carefully overlayed with buffer and allowed to polymerize. Electrophoresis was carried out in the Canalco Model 6 apparatus (Canal Industrial Corporation, Rockville, Maryland). Gels were prerun for at least two hours at 6 ma per tube to remove excess ammonium persulfate. A sample of RNA (0.01-0.1 mg in 5-20  $\mu l$  of 20% sucrose) was then underlayed onto the gel surface with a capillary pipette and electrophoresis was continued for an additional three to four hours. At this time gels were removed from the tubes and either scanned at 260 nm on the Model 2000 Gilford recording spectrophotometer equipped with a Model 2410 linear transport cell (Gilford Instrument Laboratories Incorporated, Oberlin, Ohio) or were stained overnight with lanthanum acetate and acridine orange (Richards and Coll, 1965). Destaining was accomplished with 10% acetic acid and the gels were scanned at 440 nm. Nearly identical profiles were obtained with the ultraviolet and visible scans.

Preparative gel electrophoresis on the Canalco Prep Disc apparatus (Canal Industrial Corporation, Rockville, Maryland) was employed for some tRNA preparations. A 3 x 5 cm gel, capable of handling at least 25 mg of RNA, was prepared in the same manner as for analytical scale electrophoresis. Satisfactory resolution was obtained when the sample was applied in a volume of less than 1 ml. Electrophoresis was carried out at 30-40 ma and required six to eight hours for completion. The flow of eluant buffer (electrophoresis buffer made 0.1 mM in dithiothreitol) was adjusted to 0.2-0.5 ml per minute.

# Aminoacyl Synthetase and Aminoacyl-tRNA Preparation

Aminoacyl synthetase activity from pea roots was prepared by the method of Scott and Morris (1969) except that DEAE cellulose chromatography was performed in the presence of 5 mM MgCl<sub>2</sub> and dialysis against 10 mM potassium phosphate (pH 7.2), 5 mM MgCl<sub>2</sub> 0.1 mM dithiothreitol and 10% glycerol replaced the gel filtration step. Synthetase activities for most amino acids were stable for months at -70 °C although some, notably the seryl synthetase, were diminished or absent after a few weeks time.

Aminoacylation of tRNA was routinely performed in a volume of 0.2 ml although in some cases the reaction mixtures were scaled up to as much as 40 ml. Reaction mixtures were the same as described by Scott and Morris except that the trisCl buffer was replaced by N-2-hydroxyethylpiperazine-N<sup>1</sup>-2-ethane sulfonic acid (HEPES) purchased from Calbiochem, Los Angeles, California, and dithiothreitol was used rather than glutathione. Amino acids labelled with either  ${}^{14}C$  (specific activities of 50-500 mCi per mole) or  ${}^{3}H$ (specific activities of 500-1000 mCi per mmole) were procured from Amersham-Searle, Arlington Heights, Illinois. A 0.2 ml reaction mixture contained: 20  $\mu$ mole HEPES, 0.2-0.8  $\mu$ mole ATP, 0.5-2.5  $\mu$ mole MgCl<sub>2</sub>, 0.4-1.6  $\mu$ mole KCl, 0.08  $\mu$ mole dithithreitol, 0.004-0.01  $\mu$ mole amino acid, 20-50  $\mu$ g protein and 10-40  $\mu$ g tRNA.

Reaction mixtures were terminated by either the addition of 0.2 ml of 100% TCA (and subsequently plated on membrane filters) or were adjusted to pH 5.0 with 1 M sodium acetate, diluted 3-fold and chromatographed on miniature DEAE cellulose columns at pH 5.0 (Yang and Novelli, 1970). This procedure allows separation of the aminoacyl tRNA from protein and free amino acid. Aminoacyl tRNA prepared in this manner was found to be stable for weeks at -70 °C.

The gift of high specific activity <sup>14</sup>C-labelled high molecular weight myeloblast RNA from Dr. G. S. Beaudreau allowed the determination of ribonuclease activity associated with synthetase preparations. The <sup>14</sup>C-RNA was incubated with enzyme in the standard reaction mixture for zero minutes or 15 minutes and the radioactivity precipitated by TCA after the addition of 1 mg yeast carrier RNA was compared.

In some experiments aminoacyl tRNA was examined by gel electrophoresis. The gel was scanned at 260 nm, frozen in ethanoldry ice and sliced in 1 mm sections utilizing an automated apparatus designed in this laboratory (Morris and Jacobsen, 1970). The slices were shaken for one hour at room temperature with 10 ml of the Triton X-100 (a product of Hartman-Leddon Company, Philadelphia, Pennsylvania) scintillation system used for radioactivity determinations (Patterson and Green, 1965; Benson, 1966). All samples were counted in a Model 3214 Packard liquid scintillation spectrophotometer (Packard Instrument Company Incorporated, Downer's Grove, Illinois).

### **Results and Discussion**

#### Characterization of Pea Root tRNA

Table 1 summarizes the recovery of nucleic acid during a typical tRNA preparation starting with 10 kg dry peas. The overall yield of tRNA was 2-3 mg per kg dry peas or 20-30 mg per kg fresh weight of pea roots. This yield compares favorably with the 50 mg of RNA per kg of soybean hypocotyls reported for recovery of tRNA, similarly purified through the DEAE cellulose chromatography step (Cherry, 1970).

Figure 1 shows the preparative disc gel electrophoretic fractionation of the pea root RNA obtained after DEAE cellulose chromatography. In this experiment 520  $A_{260}$  units of impure RNA were applied in a volume of 1.0 ml, electrophoresis was carried out as described above and 1.0 ml fractions were collected. The first major peak consisted of 180  $A_{260}$  units of oligonucleotide fragments,

Fraction after	RNA (A <sub>260</sub> units)
Phenol extraction and ethanol precipitation	8000
NaCl extraction	3000
DEAE cellulose chromatography	2000
Preparative gel electrophoresis	450

# Table 1. Purification of transfer RNA.



Figure 1. Preparative gel electrophoresis of pea root tRNA.

the second peak contained 130  $A_{260}$  units of tRNA cleanly separated from the 80  $A_{260}$  units of (presumed) 55 RNA in the third peak. The last peak (40  $A_{260}$  units) could be followed as a yellow band migrating through the gel. The nature of this yellow chromaphore was not investigated. It was, however, found to disappear on treatment of the RNA solution with pronase, which is known to have broadly nonspecific esterase activity (Littauer <u>et al.</u>, 1969). The recovery of  $A_{260}$ absorbing material from gel electrophoresis was 85%. The recovered tRNA represented 25% of the applied material, although later tRNA preparations purified through the DEAE cellulose chromatography step contained up to 75% tRNA (see Figure 2B). High yields depend to a large extent on the immediate addition of phenol to the suspension of powdered roots in homogenization buffer.

Figure 2 shows scans of stained analytical scale gels of RNA after various stages of purification. The RNA recovered after salt extraction was applied to the gel whose scan is depicted in Figure 2A. The heavy band at the origin probably arose from DNA and high molecular weight RNA which did not enter the gel since Figure 2B shows that deoxyribonuclease treatment and DEAE cellulose chromatography removed these contaminants. An experiment kindly performed by Dr. G. S. Beaudreau (1970) indicated tRNA at this stage of purification contained less than 1% DNA as adjudged by its lack of primer activity for the DNA dependent RNA polymerase from avian



Figure 2. Analytical gel electrophoresis of tRNA.

myeloblastis virus. Still evident in the RNA after these treatments was a band also found in commercial preparations of baker's yeast and <u>E. coli</u> tRNA and tentatively identified as 5S RNA, a known contaminant of tRNA prepared by this procedure (Mirza and Cannon, 1967). Multiple, minor, slower moving bands and a considerable portion of material migrating faster than tRNA were also present. The mobility of the latter and the fact that it was stained by acridine orange suggested that it was oligonucleotide in nature. Vanderhoef <u>et al</u>. (1970b) have recently reported that tRNA preparations from plant tissue are somewhat more free of contaminants when extraction with 3 M sodium acetate rather than with 1 M NaCl is utilized. The applicability of this modification should be investigated.

The gel in Figure 2C was obtained using tRNA which had been finally purified by preparative gel electrophoresis. A single nearly symmetrical electrophoretic band was found even after prolonged storage of the tRNA at concentrations of 5-20 mg per ml in TMD buffer at -70°C. This indicated that formation of the dimeric forms of tRNA reported by Loehr and Keller (1968) and Adams and Zachau (1968) was not a problem under these conditions.

Figure 3 shows the results of an examination of aminoacyl tRNA by gel electrophoresis. RNA (5  $A_{260}$  units) which had been purified through the DEAE cellulose chromatography step was amino-acylated with either  ${}^{14}C$  amino acid mixture (Figure 3A) or with  ${}^{14}C$ 



Figure 3. Gel electrophoresis of aminoacyl tRNA.

leucine (Figure 3B). A total of 10,000 counts per minute of aminoacyl tRNA were applied to each gel which were then subjected to electrophoresis as described above, scanned at 260 nm, sliced and the radioactivity of each slice determined. It is evident from Figure 3A that nucleic acid associated radioactivity is restricted to the band we have identified as tRNA from its gel electrophoretic mobility, and confirms the supposition that there are no chargeable dimeric forms or chargeable tRNA fragments in our tRNA preparations. There is an indication of a slight shoulder on the leucyl tRNA profile of Figure 3B. The relevance of this observation will be discussed in Part V.

### Characterization of Pea Root Aminoacyl Synthetates

Table 2 shows the purification of the pea root aminoacyl synthetase activity, preparation B-1, used in the investigations described in this thesis. One unit represents the incorporation of 1  $\mu\mu$ mole of <sup>14</sup>C-leucine into tRNA in 15 minutes at 30 °C under enzyme limiting conditions. The specific activities shown are three to five times those reported by Scott and Morris, partially because aminoacylation was carried out at optimal Mg<sup>2+</sup>/ATP ratios and at saturating levels of amino acid. The total purification achieved was approximately eight-fold. It is probably unreasonable to expect high purification from a procedure designed to allow copurification of 20 or more

Fraction after	Volume (ml)	$\frac{A_{280}}{A_{260}}$	Total Protein (mg)	Specific Activity (units/mg)
Homogenization	400	0.54	2700	70
Protamine treatment	400	0.55	2700	75
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt.	50	0.80	1000	<b>2</b> 50
DEAE cellulose chromatography	100	0 <b>.86</b>	800	310
Dialysis	17	1.07	340	540

Table 2. Purification of aminoacyl synthetases.

enzymatic activities.

The data in Table 3 allow a comparison of the ribonuclease activity associated with the above (preparation B-1) and material prepared by the Scott and Morris procedure (preparation S-2). At enzyme levels and conditions standard to the aminoacylation reaction significant ribonuclease action was found in preparation S-2. While even with five times the normal level of protein, incubation with preparation B-1 produced only a barely perceptible loss of TCA precipitatable radioactivity from the <sup>14</sup>C-RNA. Under standard aminoacylation conditions it was judged that preparation B-1 was free of ribonuclease activity. Attempts to remove ribonuclease activity from preparation S-2 by hydroxyl apatite chromatography (Muench and Berg, 1966), adsorption on calcium phosphate gel (Vanderhoef <u>et al.</u>, 1970b), or IRC 50 chromatography (Littauer et al., 1969) were unsuccessful.

The major alteration of the Scott and Morris procedure employed in preparation B-1 was the inclusion of 0.005 M MgCl<sub>2</sub> in the buffer used for DEAE cellulose chromatography. The data in Table 2 demonstrate that this modification allows the separation of synthetase and ribonuclease activities as reported for the <u>E</u>. <u>coli</u> system by Muench and Berg (1966).

Added Enzyme Preparation	Incub <b>a</b> tion (min)	TCA ppt cpm	% Loss TCA ppt cpm
<b>S-2</b> (50 μg)	Q	5875	
		5814	
	15	4613	
		4682	34
(250 µg)	0	5766	
		5791	
	15	3107	46
		3049	
B-l (50μg)	0	5887	
		5819	
	15	5897	0
		5854	
(250 µg)	0	5776	
		5759	
		5705	2
		5647	

Table 3. Ribonuclease activity in synthetase preparations.

.

# Determination of Conditions for Maximal Aminoacylation

Optimal conditions were determined for the aminoacylation of those pea root tRNAs presumed to contain isoprenoid nucleosides, namely those specific for phenylalanine, cysteine, leucine, tyrosine, tryptophan and serine (Armstrong <u>et al.</u>, 1969a and b). Under enzyme saturating conditions the effects of varying  $Mg^{2+}/ATP$  ratios and ATP concentrations, were examined. Activity as a function of pH was also investigated and subsequent determinations of saturating levels of amino acid and tRNA were carried out at the pH, ATP concentration and  $Mg^{2+}/ATP$  ratio optima. Figures 4-9 represent the re sults of these experiments.

The pH optima for all activities were found to fall in the range 7.4-7.6 and in general acylation was quite insensitive to variation of the pH from 6.5-8.0. For all except tyrosine 55-80% of maximum activity was observed even at pH 6.0, and 80-90% at pH 8.0. At pH 6.0 only 30% and at pH 8.0 only 75% of maximal tyrosine acylation was obtained.

Amino acid concentrations of 20  $\mu$ M appeared to be saturating for all species except phenylalanine which required 60  $\mu$ M for maximal activity. Although 2 mM ATP gave the best acylation for all activities, the Mg<sup>2+</sup>/ATP ratios yielding maximal charging were quite characteristic; 0.5 for leucine, 2.0 for cysteine, 2.5 for



Figure 4. Aminoacylation of leucine tRNA.



Figure 5. Aminoacylation of serine tRNA.



Figure 6. Aminoacylation of phenylalanine tRNA.



Figure 7. Aminoacylation of tryptophan tRNA.

47



Figure 8. Aminoacylation of tyrosine tRNA.

48



Figure 9. Aminoacylation of cysteine tRNA.

tyrosine and tryptophan, 5 for serine and 6 for phenylalanine.

From data obtained for charging under tRNA limiting conditions (the linear portions of the curves in Figures 4C, 5C, 6C, 7C, 8C, and 9C) other pieces of information were obtained. First, since no acylation was found in reaction mixtures incubated without added tRNA, it was concluded that the synthetase preparation per se is devoid of contaminating tRNA. Secondly, the extent of aminoacylation for the six pea root tRNAs presumed to contain isoprenoid nucleosides were calculated. These results are presented in Table 4. The tRNA utilized in these experiments was examined by analytical scale gel electrophoresis. Integration of the peaks obtained from an ultraviolet scan of this gel allowed an estimate of 60% purity for this tRNA preparation. This factor was applied as a correction in the above calculations. Comparable or lesser acylation was found for tRNA which had been treated by the procedure of Lindahl, Adams and Fresco (1966), a method that converts some tRNAs to their active form.

The values shown in Table 4 are 5-50 fold greater than those reported by Scott and Morris for acylation of these pea root tRNA subspecies at suboptimal conditions. This fact assumes considerable importance in subsequent examination and purification of tRNA subspecies which usually involved the aminoacyl form of the tRNA. Unfortunately it was not possible to utilize tRNA purified by gel

Amino Acid	cpm	µµmole/A 260		
leu	24000	86		
ser	1600	15		
phe	18000	46		
trp	1400	31		
tyr	12000	12		
cys/2	5200	25		

# Table 4. Aminoacylation of pea root tRNA.

electrophoresis for the aminoacylation experiments, as this material showed greatly decreased amino acid acceptor activity.

It is interesting to compare the variability in optimal  $Mg^{2+}/ATP$  ratios found for pea root tRNA subspecies with the results reported for bean root tRNA by Burkard, Guillemaut and Weil (1970). These authors found a  $Mg^{2+}/ATP$  ratio of 1.5 gave maximal attachment for 18 amino acids. However, they did not examine the effect of ATP concentration on acylation and in fact conducted their experiments at 10 mM ATP, a concentration that is five times the optimum for the pea root system. The high ATP concentration utilized may have obscured the effects of variation of the  $Mg^{2+}/ATP$  ratio on aminoacylation since, in general, the  $Mg^{2+}/ATP$  ratios show a distinctive optimum characteristic of each amino acid (Novelli, 1967). Other investigators (Vanderhoef et al., 1970b) illustrated that the aminoacylation of soybean hypocotyl tRNA responds to varying  $Mg^{2+}/ATP$  ratios in a fashion very similar to the results reported here for pea root tRNA.

Burkhard <u>et al</u>. also claim that aminoacylation mixtures containing glutathione or in some cases no reducing agent at all, gave better acylation than those employing dithiothreitol. Clearly, similar experiments should be conducted with the pea root system.

In summary then, it was shown that preparative gel electrophoresis yields tRNA of high purity, suitable for quantitative analysis of its isoprenoid nucleoside content. Pea root tRNA purified through DEAE cellulose chromatography contained 25-75% tRNA. Its major contaminants were oligonucleotide fragments and a species presumed to be 5S RNA. It was demonstrated that aminoacyl synthetases free of ribonuclease activity may be prepared, and using these preparations the conditions necessary for maximal acylation of 6 tRNA subspecies were elucidated.

## III. GAS CHROMATOGRAPHIC ANALYSIS OF ISOPRENOID NUCLEOSIDES

### Introduction

Soon after tRNA hydrolysates were found to possess cytokinin activity (Bellamy, 1966; Skoog <u>et al.</u>, 1966) isoprenoid nucleosides were identified as tRNA constituents (Hall <u>et al.</u>, 1966). Utilizing such cytokinin induced responses as stimulation of growth, initiation of germination, or retardation of senescence, a large variety of bioassays allowing the detection of isoprenoid nucleosides were developed (Letham, 1967).

Since isoprenoid nucleosides are potent cytokinins, the bioassays are very sensitive--some are capable of detecting concentrations of less than 1  $\mu$ g per liter (Skoog, 1970). In general however, the measured response is a nonlinear function of cytokinin concentration. This property together with the natural variability of biological systems severely limits the usefulness of bioassays as quantitative tools. Bioassays are also time consuming, up to 30 days are required for maximum response (Letham, 1967).

Substances other than isoprenoid nucleosides also possess cytokinin activity (Giannattasio, Jeannin and Kavoor, 1969; Hecht <u>et al.</u>, 1970a; Wood <u>et al.</u>, 1969). Furthermore, since the bioassays yield no information about the chemical nature of the cytokinin, other methods must be used for identification.

Figure 10 shows the structures of the five isoprenoid nucleosides thought to be present in tRNA hydrolysates. Gram quantities of tRNA were hydrolyzed to obtain milligram quantities of these compounds, whose source and occurrence are shown in Table 5. Identification and measurement of the level of these substances in tRNA were based on combinations of chromatographic isolation, measurement of physical properties and bioassays. These techniques required milligram quantities of isoprenoid nucleosides for subjection to rather extensive purification prior to anlaysis. In addition to this limitation and the limitations inherent in the bioassays, the purification procedures employed had not been demonstrated to be quantitative and thus the reported estimates of the levels of isoprenoid nucleosides in tRNA

It is known that gas liquid chromatography (GLC) may be used for the identification of the plant hormones indole acetic acid and abscisic acid (Davis, Heinz and Addicott, 1968) and for the giberellins (Cavell <u>et al.</u>, 1967). Other workers (Hancock and Coleman, 1965; Hashizume and Sasaki, 1965) demonstrated that the trimethylsilyl (TMS) derivatives of purines and pyrimidines are volatile enough for GLC analysis. Recently a GLC method was shown to be capable of resolving a mixture of synthetic and natural cytokinins (Most, Williams and Parker, 1968).



Figure 10. Structure of the isoprenoid nucleosides.

Occurrence (w/w %)						
Source	IPA	<u>cis</u> ZR	<u>trans</u> ZR	msIPA	msZR	Reference
Baker's yeast tRNA	0.06					Robins <u>et al</u> . (1967)
Calf liver tRNA	0.05					
Chick embryo tRNA	0.03					
Human liver tRNA	0.05					
Immature pea tRNA	0.003	0.005				Hall <u>et</u> <u>al</u> . (1967a)
Spinach leaf tRNA	0.02	0.01				11
Immature corn kernels			present			Miller (1965)
Wheat germ tRNA	present	present	present	present	present	Hecht <u>et</u> <u>al</u> . (1969a)
<u>E. coli</u> B tRNA	0.05			0.003		Burrows <u>et al</u> . (1969)
L. acidophilus tRNA	present					Fittler <u>et al</u> . (1968)

Table 5.	Source	and	occurrence	of	isoprenoid	nucleosides.
----------	--------	-----	------------	----	------------	--------------

Besides possessing high resolving power, GLC is a sensitive, potentially quantitative technique. Moreover, when coupled to the mass spectrometer (McCloskey <u>et al.</u>, 1968) it becomes a powerful tool for the identification of purines, pyrimidines and their derivatives.

Experiments performed in this laboratory and described below demonstrate how GLC in conjunction with mass spectrometry may be used to identify the isoprenoid nucleosides of pea root (or other) tRNA. It is also shown that GLC may be applied to the quantitative estimation of submicrogram quantities of isoprenoid nucleosides in hydrolysates of tRNA from pea roots and other sources. The extremely low level of isoprenoid nucleosides present in bulk tRNA necessitates a partial purification prior to formation of the TMS derivatives. Partition chromatography (Hall, 1967c) was found to be ideally suited for this purpose.

#### Materials and Methods

#### Procurement of Nucleoside Standards

Adenosine, cytidine, guanosine and uridine were purchased from Sigma Chemical Company, St. Louis, Missouri. Authentic  $N^{6}-\Delta^{2}$ -isopentenyl adenosine (IPA) was the generous gift of Dr. Ross H. Hall, McMaster University, Hamilton, Ontario, Canada. Calbiochem, Los Angeles, California provided  $N^{6} - \Delta^{2}$ -isopentenyladenine and N<sup>6</sup>-(4-hydroxy-3-methyl-2-enyl) adenosine, commonly known as zeatin riboside (ZR). The ZR from this source was later shown to be a mixture of the cis and trans isomers. 2-methylthio- $N^{6}-\Delta^{2}$ -isopentenyl adenosine (msIPA) was isolated from <u>E</u>. <u>coli</u> B tRNA, supplied by Schwarz Bio-Research Incorporated, Orangeburg, New York. The technique employed, described by Burrows et al. (1969), follows. The tRNA (210 mg) was hydrolyzed as below, lyophylized, and extracted six times with 2 ml portions of watersaturated ethyl acetate. The extracts were taken to dryness, dissolved in 0.5 ml of 35% ethanol and applied to a 1.2 x 45 cm column of Sephadex LH 20 (Pharmacia Fine Chemicals Incorporated, Piscatataway, New Jersey) equilibrated with 35% ethanol. Elution was with the same solvent at 0.1 ml per minute, and 4 ml fractions were collected. Examination of the appropriate fractions showed them to contain a component which had ultraviolet and mass spectra identical to those reported by Burrows et al.

#### GLC Examination of tRNA Hydrolysates

Baker's yeast, <u>E. coli</u> B and <u>E. coli</u> W tRNAs were purchased from Schwarz Bio-Research Incorporated, Orangeburg, New York. Rat liver tRNA was donated by Dr. R. J. Dejmal. The tRNAs were degraded to their constituent nucleosides by incubation in 0.3 M KOH at 37°C for 24 hours, followed by digestion with bacterial alkaline phosphatase (Sigma Chemical Company, St. Louis, Missouri) as reported by Fittler <u>et al</u>. (1968). Following the procedure of Robins <u>et al</u>. (1967) the hydrolysate was lyophilized, dissolved in a small volume (0.3-1.5 ml) of the aqueous phase of their solvent system E (ethyl acetate:1-propanol:water, 4:1:2) and mixed with Celite 545 (Johns-Mannvile Company, New York, N. Y.) in the ratio of 0.5 ml per gram. The sample was applied to a column (0.6 x 60 cm or 0.3 x 60 cm) containing Celite 545 equilibrated against the aqueous phase of solvent system E in the same ratio. The column was eluted with the organic phase of system E at 0.5 ml per minute.

Early fractions (1.0 ml each) containing the isoprenoid nucleosides were evaporated under a stream of dry nitrogen and the TMS derivatives were prepared by addition of 40  $\mu$ l of N, O-bis(trimethylsilyl)-trifloroacetamide (Regis Chemical Company, Chicago, Illinois) and 10  $\mu$ l dry pyridine. The reaction was found to be complete in five minutes at 60 °C and the TMS derivatives were found to be stable for months at -70 °C.

Retention times and all quantitative data were obtained on silanized Pyrex gas chromatography columns (0.2 x 12 cm) prepared in this laboratory. The columns were coiled and packed with 10% DC-11 (Dow Chemical Company, Midland, Michigan) on 60-80 mesh Gas Chrom Q (Johns-Mannville Company, New York, N. Y.) Analysis was performed in the Varian 1520 instrument (Varian-Aerograph, Walnut Creek, California) using a hydrogen flame detector. Column operating parameters were: nitrogen carrier gas flow, 20 cc per minute; hydrogen flow, 30-40 cc per minute; air flow, 200 cc per minute. Injector port and detector oven temperatures were maintained at 20-50 °C above column operating temperature. Columns were preconditioned at 270 °C for 48 hours before use. Retention times were measured from the time of injection (of 2-5  $\mu$ l of the reaction mixture).

### Mass and Nuclear Magnetic Resonance Spectra of Isoprenoid Nucleosides

Mass spectra of isoprenoid nucleosides were determined on a Varian MATS CH7 spectrometer (Varian Aerograph, Walnut Creek, California) using the direct inlet at 250-275°C and ionization at 70 eV. Before measurement samples from the partition column were dried and extracted with cold ether to remove impurities, those from the LH 20 and GLC columns were dried and applied directly. To obtain the mass spectrum of the free nucleoside from a GLC peak, a specially constructed glass trap was butted to the outlet of a 0.2 x 160 cm helical Teflon-lined aluminum column (Chemical Research Service Incorporated, Addison, Illinois) packed with 10% DC-11 on Gas Chrom Q. The apparatus is shown in Figure 11. Carbon dioxide carrier gas and immersion of the trap in liquid nitrogen were utilized to minimize aerosol formation. The trapped TMS derivative was hydrolyzed with


Figure 11. Apparatus for collection of TMS-nucleosides.

water (15 minutes at 25°C) and mass spectra were obtained as above.

The nuclear magnetic resonance spectrum of commercial zeatin riboside was obtained on the Varian HA-100 instrument (Varian Aerograph, Walnut Creek, California). The low solubility (2 mg/ml) of zeatin riboside in D<sub>2</sub>O and contamination with H<sub>2</sub>O limited examination to the methyl protons of the sample.

## Results and Discussion

Figure 12A shows the profile obtained when the ethyl acetate extract of the hydrolysate of <u>E</u>. <u>coli</u> tRNA was subjected to LH 20 chromatography. The two early peaks probably represent adenosine and methylated adenosines which are somewhat soluble in the extraction solution (Grimm and Leonard, 1967). GLC examination of the TMS derivative of an aliquot of Fraction 21 indicated this material was IPA, known to be present in small amounts in <u>E</u>. <u>coli</u> tRNA (Burrows <u>et al.</u>, 1969). The ultraviolet absorbance spectrum (Figure 12B) and the mass spectrum (Figure 13) of the material in Fraction 36 are identical to those reported by Burrows <u>et al</u>. for msIPA isolated in a similar manner. The extinction coefficient reported by these authors ( $E_{280} = 18,000$ ) allowed calculation of the concentration of msIPA in the pooled fractions from this peak. This material was used as the msIPA standard in the experiments which followed.

Table 6 summarizes data collected on retention times as a



Figure 12. LH20 elution profile and absorbance spectrum of msIPA.



Figure 13. Mass spectra of msIPA.

			Retention Time (min.)		
Compound Tem	rature (°C)	235	245	255	270
$\frac{1}{1}$ -( $^2$ -isopentenyl) adenosine	······································	14.0	9.8	6.4	4.0
-methythio-N <sup>6</sup> -( $^2$ -isopentyl)	nosine	30.5		13.8	7.8
Leatin riboside					
$ZR_{\Delta}$		29.5	,19.7	12.5	7.2
ZR <sub>B</sub>		34.5	22.7	14.2	8.1
Adenosine		6.2	4.4	2.4	1.8
Cytidine		8.9	6.1	4.1	2.7
Guanosine		8.9	6.0	4.0	
		9.9	6.6	4.3	2.6
Uridine		3.8	2.6	1.8	1.2
$\sqrt[6]{-(^2-isopentenyl)}$ adenine		1.3	1.1	0.75	0.5 <b>5</b>
Uridine N <sup>6</sup> -( <sup>2</sup> -isopentenyl) adenine		9.9 3.8 1.3	6.6 2.6 1.1	4.3 1.8 0.75	2 1 0

Table 6. Retention times of trimethylsilyl nucleosides.

function of temperature for the TMS derivatives of authentic samples of the major nucleosides and the isoprenoid nucleosides known to occur in tRNA at the time of these investigations. In all cases, the isoprenoid nucleoside derivatives were separated from each other, from those of the major nucleosides and from those of the free bases. Presumably the greater retention time of the TMS isoprenoid nucleosides are due to interaction of the isoprenoid side chain with the hydrophobic stationary phase. Although retention times were found to be very sensitive to column temperature and carrier gas flow, careful regulation of these parameters enabled the tabulated values to be reproduced to within  $\pm 5\%$ . The nucleosides, with two exceptions, gave single peaks when examined by this technique. Guanosine contained, beside the major peak, a minor slow-moving component previously reported by Jacobson, O'Brien and Hedgcoth (1968). Commercial zeatin riboside gave two major peaks whose relative abundance was independent of the conditions and duration of derivatization. The nature of these peaks will be discussed below.

The sensitivity of the technique to authentic samples of isoprenoid nucleosides was high. Calibration curves were obtained by injecting aliquots of reaction mixtures containing known amounts of IPA, msIPA, and ZR and were linear in the range  $0.01-2.0 \mu g$ . Relative detector response to each compound at 255 °C was IPA 100, msIPA 75. ZR 60. Neither peak height nor retention time were sensitive to the injection volume within the range 2-5  $\mu$ l.

Figure 14 illustrates how partition chromatography separates the isoprenoid nucleosides from the bulk of the nucleosides present in a tRNA hydrolysate. Yeast tRNA (15 mg) was hydrolyzed, lyophilized and mixed with 20  $\mu$ g of authentic IPA and 20  $\mu$ g of commercial zeatin riboside. The mixture was fractionated on a 0.9 x 60 cm partition column and the early fractions were examined by GLC. In Figure 14 one unit of nucleoside represents 1  $\mu$ g of adenosine, 2  $\mu$ g of IPA or 0.1  $\mu$ g ZR. The IPA (and in experiments with <u>E</u>. <u>coli</u> tRNA, the msIPA) eluted with the void volume of the column (fraction 1-5). The two peaks of zeatin riboside eluted between fractions 5 and 20. Adenosine eluted beyond fraction 8. Fractions eluting before the maximum of the adenosine peak (as indicated by the optical absorbance) were routinely pooled for examination by GLC.

As shown in Figure 14,  $ZR_A$  and  $ZR_B$  (designated on the basis of retention times; see Table 5) were differentially distributed across fractions 5-20, indicating that the commercial ZR contains two components. Partial separation of the two compounds occurred when 500 µg of commercial ZR was fractionated on a partition column. An early fraction from the column (corresponding to fraction 7, Figure 14) was shown to contain 56%  $ZR_A$  and 44%  $ZR_B$  by GLC analysis. A late fraction (corresponding to fraction 14, Figure 14) contained 15%  $ZR_A$  and 85%  $ZR_B$ . Portions of the nearly identical



Figure 14. Partition chromatography of a fortified tRNA hydrolysate.

mass spectra obtained from these two fractions are shown in Figure 15A and B respectively. Hall <u>et al.</u> (1967) reported the mass spectra of <u>cis</u> and <u>trans</u> ZR to differ most notably in the relative intensities of peaks at m/e 192, 228 and 331. The major difference we observed was at m/e 228. Comparison of our findings with the published spectra and mobilities in solvent system E identified ZR<sub>A</sub> as <u>cis</u> ZR and ZR<sub>B</sub> as <u>trans</u> ZR. Additional support for this interpretation was obtained from a partial nuclear magnetic resonance spectrum of the commercial material in D<sub>2</sub>O. As seen in Figure 16 the methyl peak was split by approximately 4 ppm at 4°C indicating the presence of <u>cis</u> and <u>trans</u> isomers within the mixture. It was therefore concluded that commercial ZR is a mixture of 30% <u>cis</u> ZR and 70% <u>trans</u> ZR.

In order to investigate the stability of the isoprenoid nucleosides during hydrolysis and subsequent conversion to the TMS derivatives, 30 mg of salt-extracted commercial yeast RNA was mixed with 20  $\mu$ g each of IPA and ZR, hydrolyzed, lyophilized and subjected to partition chromatography. The appropriate fractions were derivatized and examined by GLC. An unfortified 0.001 M KH<sub>2</sub>PO<sub>4</sub> blank was treated in a like manner. No extra peaks indicative of degradation products were obtained from the fortified hydrolysates, and recoveries were near quantitative.

Actual recovery data were obtained by fortifying <u>E</u>. <u>coli</u> B tRNA hydrolysates with  $1-20\mu g$  of isoprenoid nucleoside. Samples



Figure 15. Partial mass spectra of cis and trans ZR enriched fractions.



Figure 16. Partial nuclear magnetic resonance spectra of a <u>cis</u> and <u>trans</u> ZR mixture.

were lyophilized, fractionated on  $0.3 \times 75$  cm partition columns, derivatized and analyzed by GLC. Table 6 summarizes the data collected which indicated that recovery was quantitative to at least the l µg level. The msIPA as well as the IPA content of the unfortified samples were determined as discussed below. These values were used for calculation of the levels of isoprenoid nucleosides in <u>E</u>. <u>coli</u> B tRNA included in Table 7.

Figures 17 and 18 illustrate GLC profiles obtained when the method was applied to analysis of tRNA from various sources: Figure 17A, phosphate buffer carried through the analytical procedure; Figure 17B, 11.2 mg of electrophoretically purified pea root tRNA (first partition column fraction, containing 100% of the IPA and 50% of the <u>cis</u> ZR present in the hydrolysate); Figure 17C, 30 mg of baker's yeast tRNA; Figure 18A, 30 mg of <u>E. coli</u> W tRNA; Figure 18B, 15 mg of rat liver tRNA. Analysis was performed isothermally at 255 °C. Attenuation factors are noted on the figure.

Early peaks (with retention times less than five minutes) were due to solvent, solvent impurities, adenosine, and possibly methylated adenosines. An unidentified peak at ten minutes was seen in all samples including the phosphate buffer blank. The nature of the peak with a retention time of 16.6 minutes present in the yeast tRNA (and sometimes pea root tRNA) hydrolysates is obscure. It may be the second isoprenoid nucleoside reported to occur in yeast tRNA by

tRNA (mg)	Added IPA (µg)	Content by IPA (µg)	GLC Analysis msIPA (µg)	% Recovery of added IPA
2		0.08	1.48	
2		0.08	1.36	
5		0.37	4.80	
5		0.39	5.30	
10		0.85	10.2	
30		2.64	29.8	
5	1.00	1.22	3.90	85
5	5.00	5.55	4.70	103
5	10.00	10.5	4.80	101
5	20.00	19.6	4.75	96

Table 7. Recovery of isoprenoid nucleosides from a tRNA hydrolysate.



Figure 17. GLC examination of tRNA hydrolysates.



Figure 18. Additional GLC examinations of tRNA hydrolysates.

Hall <u>et al</u>. (1967b) since its mobility on partition chromatography and the extended retention time of its TMS derivative are both indicative of a nonpolar molecule. Hecht <u>et al</u>. (1969b) reported that wheat germ tRNA contains 2-methylthiozeatin riboside which would be expected to have such properties. Attempts to isolate msZR from wheat germ tRNA by their procedure for use as a standard, were, however, unsuccessful.

Coinjection with standards allowed us to conclude that each of the tRNA hydrolysates shown in Figures 17 and 18 contained IPA. <u>E. coli</u> tRNA also contained msIPA, and pea root tRNA contained <u>cis</u> ZR. Because the presence of IPA in tRNA from many sources is well established it was felt that appropriate mobility in both gaseous and liquid systems was sufficient to establish its identity. In the case of <u>cis</u> ZR however, further confirmation was sought by subjecting the appropriate GLC peak to analysis by mass spectrometry.

Direct coupling of GLC to the mass spectrometer has the advantages of elegance and ease of obtaining experimental data. However preliminary experiments demonstrated that spectra were not obtainable from the available instruments with injection of  $\mu$ g quantities of TMS nucleosides. Other experiments were therefore designed to evaluate the feasibility of trapping GLC peaks. An appropriate interval after injection of a TMS nucleoside, the trap (see Figure 11)

was connected to the GLC column outlet, and the sample was collected. After allowing the trap to come to room temperature the TMS nucleosides were washed into the trap reservoir with hexane and an aliquot was reinjected. The GLC peak produced indicated that less than 10% of the TMS nucleoside had survived the trapping procedure intact, although after removal of the hexane and derivitization of the residue, reanalysis showed 40-70% of the TMS nucleoside had been trapped (and subsequently undergone hydrolysis). While it is possible to utilize trapped TMS nucleosides for mass spectral analysis (Alam and Hall, 1970), the ease with which the TMS ethers are hydrolyzed would require the use of special precautions. Furthermore, in subsequent experiments mass spectra identical to the parent compound were obtained after trapping and hydrolyzing GLC peaks produced from injection of standard TMS isoprenoid nucleosides.

This method was then applied to the GLC peak identified as TMS <u>cis</u> ZR from a hydrolysate of 20 mg of pea root tRNA. After trapping and hydrolysis the resulting free nucleoside (10  $\mu$ g by GLC analysis) was examined in the mass spectrometer. The resulting spectrum shown in Figure 19 proved to be nearly identical to that for authentic <u>cis</u> ZR. Major peak assignments were m/e: 351, molecular ion; 334, less of OH; 320, less of CH<sub>2</sub>OH; 262 and 248, less of sugar fragments; 219, free base; 202, free base less OH; 188, free base less CH<sub>3</sub> and OH; 135, adenine. The peak at m/e



Figure 19. Mass spectra of <u>cis</u> ZR isolated from pea root tRNA.

281 is found in background scans at 50 °C. Peaks at m/e 73 and 147 are characteristic of polysilated compounds (McCloskey <u>et al.</u>, 1968).

Hall <u>et al</u>. (1967a) hydrolyzed 5 g of pea tRNA to report that it contained components which had physical and chemical properties indistinguishable from <u>cis</u> ZR and IPA. The data above, obtained from 20 mg of pea root tRNA, confirm these findings and indicate the general usefulness of the described technique for the identification of the isoprenoid nucleosides of systems which yield small amounts of tRNA.

Figure 17 also illustrates the sensitivity of GLC as a quantitative tool. The peaks of Figure 17B correspond to 0.15  $\mu$ g of IPA and 0.19  $\mu$ g of <u>cis</u> ZR respectively, based upon calibration curves determined with authentic samples.

The isoprenoid nucleoside content of each tRNA (Table 8) was calculated from such data. The IPA and msIPA contents of <u>E</u>. <u>coli</u> W and <u>E</u>. <u>coli</u> B tRNA agree quite closely with those obtained on analysis of gram quantities of <u>E</u>. <u>coli</u> B tRNA (0.003 and 0.05%, respectively) by Burrows <u>et al</u>. (1969).

The isoprenoid nucleoside content of <u>E</u>. <u>coli</u> tRNA has been shown to change with age of the culture (Bartz <u>et al.</u>, 1970) and to be dependent on the nutritional state of the organism (Rosenberg and Gefter, 1969). These factors may be the basis for the differences observed in isoprenoid nucleoside contents of <u>E</u>. <u>coli</u> B and <u>E</u>. <u>coli</u> W tRNAs.

The IPA content of base digested yeast tRNA in Table 8 (0.17%)does not agree with the value of 0.065% reported by Robins <u>et al.</u> (1967) for an examination of enzymatic hydrolysates of yeast tRNA. Fittler <u>et al.</u> (1968) however, observed incomplete release of IPA from tRNA hydrolysates using enzymatic hydrolysis, possibly due to decreased activity of diesterases to N- and O-methyl substitution of the adjacent nucleotide. Because of the possibility of incomplete hydrolysis and the possible difficulty of quantitative elution of isoprenoid nucleosides from the paper chromatograms utilized in their procedure, the value reported by Robins <u>et al.</u> should be considered a minimal value.

Using a similar procedure, Hall <u>et al</u>. (1967a) reported yields corresponding to 0.0025% and 0.0051% respectively for IPA and <u>cis</u> ZR from pea tRNA. While these values can not be directly compared to the values obtained for pea root tRNA, they may be indicative that the procedure employed by Robins <u>et al</u>. and by Hall <u>et al</u>. was not quantitative. It is interesting to note that a 2:1 ratio of <u>cis</u> ZR: IPA was found in the tRNA from both pea roots and whole peas.

In the realm of speculation it should be noted that partition chromatography has been applied by Hall (1967c) to purification of a large number of minor tRNA constituents. Thus the use of the GLC method described here may prove advantageous for identification and

measurement of other unusual tRNA components. The catalog of mass spectra of numerous minor nucleosides compiled by Hecht, Gupta and Leonard (1969b, 1970b) might serve as a valuable source of information for such investigations.

After this work was completed, it was learned that Upper <u>et al</u>. (1970) had also developed a GLC method for examination of cytokinins from natural sources. While these workers did report retention times of the TMS derivatives of IPA, msIPA and ZR and the corresponding purines, they failed to examine the TMS derivatives of methylthio zeatin and its riboside. This is somewhat surprising for this compound should have been readily available to them as its discovery was reported by other investigators (Hecht <u>et al</u>., 1969) at the same institution. Even with the use of temperature programming the method described by Upper <u>et al</u>. was unable to achieve resolution of the TMS derivatives of <u>trans</u> ZR and msIPA. In addition, the extraction procedure employed was not demonstrated to be quantitative, which also limits the usefulness of their method.

The isoprenoid nucleoside contents determined by the GLC method do, of course, depend on the purity of the tRNA. Commercial <u>E. coli</u> and yeast tRNA were found to give only  $15-17 \text{ A}_{260}$  units per mg in 0.005 M MgCl<sub>2</sub>, 0.01 M trisCl (pH 7.4). In addition, analytical gel electrophoresis showed these materials to be contaminated with at least 10% 5S RNA. For these reasons the actual isoprenoid

nucleoside contents of these species are probably 20-30% higher than the values of Table 8. The rat liver tRNA subjected to hydrolysis and GLC examination was of doubtful purity, thus its IPA content (Table 8) must be considered a minimum value. Hall (1970) has noted that IPA seems to be the only isoprenoid nucleoside found in tRNA from mammalian tissue. The data from rat liver tRNA agrees with this observation. The pea root tRNA utilized for GLC analysis had been prepared by preparative gel electrophoresis and was of high purity. The IPA and <u>cis</u> ZR contents of pea root tRNA reported therefore reflect the best currently attainable values.

The procedure discussed here enables quantitative determination of the isoprenoid nucleoside content of very small samples of RNA. The least amount of IPA giving a reproducible response using the hydrogen flame detector was 0.01  $\mu$ g, comparable to the detection of 0.005  $\mu$ g reported by Most <u>et al.</u> (1969). Injecting one-tenth aliquots, one could detect IPA in a hydrolysate containing 0.1  $\mu$ g of IPA. Data in Table 7 demonstrate that 5 mg of unfractionated tRNA is therefore sufficient for analysis when the isoprenoid nucleosides are present at a minimal level of 0.01%. Based on similar calculations, 0.05 mg of a purified isoprenoid nucleoside containing subspecies could be analyzed. The usefulness of GLC in conjunction with mass spectrometry was also illustrated. A mass spectrum of the component identified as <u>cis</u> ZR was obtained from a hydrolysate of 20 mg of pea root

tRNA Source	Nucleoside Content (w/w%)			
	IPA	msIPA	<u>cis</u> ZR	
<u>E. coli</u> W.	0.010	0.048		
<u>E. coli</u> B	0.009	0.085		
Pea root	0.031		0.075	
Yeast	0.17			
Rat liver	0.04			

Table 8. Isoprenoid nucleoside content of unfractionated tRNAs.

tRNA.

The resolution of the GLC method is high and is equal to that achieved by earlier workers. Hancock (1969) was able to separate the alpha and beta anomers of psuedouridine. In our hands the method proved capable of the comparable resolution of <u>cis</u> and <u>trans</u> zeatin ribosides. The separation and retention times observed for the TMS derivatives of IPA and ZR are similar to those obtained by Most <u>et al</u>. (1968).

## IV. TRANSFER RNA FRACTIONATION, PURIFICATION OF SUBSPECIES AND ANALYSIS OF THEIR ISOPRENOID NUCLEOSIDE CONTENT

#### Introduction

A large number of fractionation procedures has been devised in attempts to achieve purified tRNA subspecies. Only a few of the methods more commonly employed are considered here. More detailed discussions can be found in reviews by Brown (1963) and Tanaka (1966).

These procedures fall into two groups, based on physical or chemical principles. The physical methods utilize differences in such properties as electrophoretic mobility, partition coefficients, or the strength of binding to ion-exchange materials. Column methods involving the use of DEAE cellulose and DEAE Sephadex (Kawade, Okamoto and Yamamoto, 1963; Beck and Cherayil, 1967) or hydroxyl apatite (Hartmann and Coy, 1961; Muench and Berg, 1966b) have been employed, but in general offer poor resolution. Columns of methylated serum albumin adsorbed to keiselguhr (MAK columns) have been utilized for tRNA fractionation (Sueoka and Yamane, 1964; Doi, Kaneko and Igarashi, 1968) but the low capacity of MAK limits its usefulness as a preparative method.

Perhaps the most well known method of tRNA fractionation is countercurrent distribution (Holley <u>et al.</u>, 1961) the method employed

for the purification and subsequent elucidation of the structure of yeast alanine tRNA (Holley <u>et al.</u>, 1965). This procedure although of undeniable use for large scale preparative work, requires rather elaborate apparatus, has limited resolution and has fallen into relative disuse as more powerful techniques have been developed.

Khym (1965) showed that tRNAs were quantitatively extracted by a solution of tricaprylammonium chloride in trichlorotrifluoroethane (Freon TF) from a 0.25 M NaCl solution and were completely reextracted into 0.4 M NaCl from the Freon layer. Between 0.25 M and 0.4 M NaCl the tRNAs were distributed in the two phases according to their partition coefficients. By immobilizing the solution of quaternary ammonium compound on hydrophobic diatomaceous earth, Kelmers, Novelli and Stulberg (1965) were able to combine the advantages of column chromatography with both ion exchange and differential solubility phenomena in a technique offering very high resolution. Four such reversed phase chromatographic systems have been described (Kelmers et al., 1965; Weiss and Kelmers, 1967; Weiss, Pearson and Kelmers, 1968) each offering unique advantages. These techniques have been widely employed for tRNA fractionation (Epler, 1969; Bick et al., 1970; Anderson and Cherry, 1969; Vanderhoef and Key, 1970; Waters and Novelli, 1968; Kan, Kano-Sueoka and Sueoka, 1968). Their use for the purification of two E. coli formylmethione tRNAs and a valine tRNA have been reported

(Weiss, Pearson and Kelmers, 1968).

Chemical modification of tRNAs for purposes of fractionation have generally employed the high specificity of the aminoacyl synthetases to esterify the cognate amino acid to the desired tRNAs in a mixture. The most recent (and elegant) methods have utilized the synthetase-tRNA complex itself for isolation of tRNA. Preddio (1969) caused gel filtration to separate the tryptophanyl synthetasetRNA complex from a mixture of tRNAs. Denburg and De Luca (1970) coupled the isoleucine synthetase to Sepharose with CNBr. The coupled synthetase retained enzymatic activity and interacted specifically with isoleucine tRNA, allowing its isolation from bulk tRNA. The application of this technique to the purification of other tRNAs remains to be demonstrated.

The differences in chemical reactivity of the aminoacylated and uncharged tRNAs have also been exploited to separate them. One approach is to chemically link a large molecule to the attachment site of uncharged tRNAs and then remove the complex by a physical method. Procedures employed include oxidation of the unprotected tRNAs by periodate and subsequent reaction with hydrazine (Zamecnik <u>et al.</u>, 1960), or binding of the terminally oxidized tRNA to aminoethyl cellulose columns (Zubay, 1962). Alternatively the aminoacyl group attached to tRNA was reacted with a reagent giving an insoluble derivative which was separable from the unesterified tRNA (Peterkofsky and Jesensky, 1969; Littauer <u>et al.</u>, 1969; Mehler and Banks, 1963).

Gillam <u>et al.</u> (1968) have described a technique in which the soluble phenoxyacetyl derivative of aminoacyl tRNA was made. This procedure subsequently utilized the affinity of benzolyated DEAE cellulose (BDC) for aromatic residues to achieve 8-20 fold purification of several isoaccepting tRNAs. The method has found widespread use, and has been employed for purification of the following <u>E. coli</u> tRNAs: alanine, Schulman and Chambers (1968); valine, Yaniv and Barrel (1969); isoleucine, Yarus and Berg (1969); methionine and formylmethionine, Henes, Krauskopf and Ofengaard (1969); tyrosine, Gefter and Russel (1969); leucine, Lurquin, Metzger and Buchet-Mahieu (1969).

The phenoxyacetylation technique of Gillam <u>et al</u>. was considered the most promising method for purification of the isoprenoid nucleoside containing tRNAs from pea roots (originally an objective of the research reported here). However, while the other techniques necessary for achieving this goal were being developed, Armstrong <u>et al</u>. (1969a) identified the isoprenoid nucleoside containing <u>E</u>. <u>coli</u> tRNAs as phenylalanine, leucine, serine, tyrosine, tryptophan and cysteine. Examination of yeast (Armstrong <u>et al</u>., 1969b; Hecht et al., 1969c) and <u>Lactobacillus acidophilus</u> (Peterkofsky and Jesensky, 1969) showed the same distribution except for phenylalanine tRNA. Yeast (Raj Bhandary <u>et al.</u>, 1967), wheat germ (Dudock <u>et al.</u>, 1969) and beef liver (Yoshikami <u>et al.</u>, 1968) phenylalanine tRNA all contain hydrophobic, fluorescent residues of unknown structure, rather than any of the known isoprenoid nucleosides.

It seemed reasonably certain that pea root tRNA would also have isoprenoid nucleosides in those tRNAs which recognize codons starting with the letter U. Attention was therefore focused on the isoprenoid nucleoside content of tyrosine and leucine tRNA subspecies from pea roots--the species most strongly implicated in a regulatory role (by the reasons discussed in Part I).

For purification of these species the affinity of BD cellulose for hydrophobic residues provides an additional advantage. Highly purified phenylalanine (Wimmer, Maxwell and Tener, 1968) and tyrosyl (Maxwell, Wimmer and Tener, 1968) tRNAs may be obtained without chemical modification. Described below is the use of BDC and reverse phase chromatography for the purification and fractionation of tyrosine tRNA, phenylalanine tRNA and a leucine tRNA subspecies from pea roots, and the analysis of their hydrolysates for isoprenoid nucleoside content by the GLC method developed

### Materials and Methods

Aminoacyl tRNA was prepared as described in Part II. For preparative work 10% of the tRNA was charged with labelled amino acid and 90% with unlabelled amino acid under identical conditions. The pH was adjusted to 4.5 and the samples were mixed and chromatographed on DEAE cellulose.

BD cellulose was either prepared by the method of Gillam et al. (1968) or purchased from Regis Chemical Company, Chicago, Illinois. Pea root tRNA was applied at a concentration of 5 mg per ml in starting buffer to a 1.2 x 30 cm column of BD cellulose. An 800 ml linear gradient of 0.25 M to 1.0 M NaCl in 0.02 M sodium acetate (pH 5.0), 0.01 M MgCl<sub>2</sub> and 0.1 mM dithiothreitol was followed by 300 ml of 1 M NaCl then 100 ml of 2.5 M NaCl and 20% ethanol in the same buffer. Elution was at 1 ml per minute and 4 ml fractions were collected. It was necessary to dilute the ethanol-containing fractions with one volume of water before the addition of more ethanol to prevent precipitation of NaCl with the tRNA. Precipitated samples were dissolved in a small volume of TMD buffer and examined for amino acid acceptor activity or gel electrophoretic mobility as previously described. The fluorescence of a sample of the BDC ethanol fraction tRNA was compared to a quinine sulfate standard solution as described by Yoshikami <u>et al</u>. (1968) using the Aminco-Bowman spectrofluorometer (American Instrument Company Incorporated, Silver Springs, Maryland).

Reverse phase packing material was prepared by the procedure of Waters and Novelli (1970). 1,1,1,3 tetrachlorotetrafluoropropane and tricaprylmethyl ammonium chloride was purchased from E. I. Dupont de Nemours, Wilmington, Delaware and General Mills, Chemical Division, Kankakee, Illinois. Equivalent results were obtained with material purchased from Penninsular Chemical Research Incorporated, Gainsville, Florida. Columns of 0.6 x 120 cm or 0.9 x 120 cm were prepared from a slurry of packing material which had been briefly degassed on the house vacuum. The material was compacted with a mechanical vibrator as it settled. Columns were eluted with 800 ml linear gradients of 0.35 M to 0.7 M NaCl in 0.02 M sodium acetate (pH 5.0), 0.01 M MgCl<sub>2</sub>, 0.001 M EDTA, 0.1 mM dithiothreitol applied at 18-30 ml per hour. Fractions of 2.0 ml were collected and aliquots were either counted directly or were precipitated with 0.5 ml of 100% TCA after the addition of 1 mg of salmon sperm DNA as carrier. The precipitated activity was then collected on membrane filters and counted.

Samples taken for GLC analysis were precipitated with ethanol, taken up in a small volume of 0.3 M trisCl (pH 8.8) and incubated at 37°C for 30 minutes to discharge the amino acids (Yang and Novelli, 1970). This treatment was followed by dialysis and the alkaline hydrolysis, lyophilization and partition chromatography steps described in Part III.

#### Results and Discussion

# Purification and Fractionation of tRNA Subspecies

Figure 20 shows the fractionation of five isoaccepting pea root aminoacyl-tRNAs presumed to contain isoprenoid nucleosides. The data were obtained by cochromatography of aliquots of bulk tRNA separately aminoacylated with <sup>3</sup>H-leucine and the <sup>14</sup>C-amino acid. Gradients of 0.35-0.7 M NaCl produced six leucine species, four of tyrosine and phenylalanine, two of serine and a single tryptophan species, in addition to a small peak of non-TCA-precipitable radioactivity for each species, appearing at the void volume of the column. This material was presumed to be mainly free amino acid. These data may be compared with that of Vanderhoef and Key (1970) who obtained five leucine, three tyrosine, four phenylalanine and three serine peaks for chromatography of pea root aminoacyl tRNA under similar conditions. The differences in these data may be explained by differences in the tRNA and/or synthetase preparations, or by the different ages (two days and four days) of the pea roots utilized by the two laboratories. It should be noted that Cherry and Osbourne

(1970) reported a reverse phase chromatographic profile for pea leucyl tRNA that appears identical to that shown in Figure 10.

It is obvious from Figure 20 that the resolution obtained by reverse phase chromatography alone is not sufficient to allow direct examination of the isoprenoid nucleoside containing tRNAs. For this reason the application of BDC chromatography to tRNA fractionation was investigated.

The fractionation of 2700 A<sub>260</sub> units of bulk pea root tRNA on BDC is depicted in Figure 21. Approximately 85% of the tRNA was eluted with a salt gradient and wash, while 12% was eluted only with buffer containing ethanol (cf. 5% of yeast tRNA reported by Gillam et al., 1968). Aliquots of fraction 33 and of pooled salt and pooled ethanol fractions were examined by analytical scale gel electrophoresis. Figures 22A, B and C respectively depict the scans obtained from these gels. A comparison of the scan of fraction 33 with that for the pooled salt fractions show fraction 33 to be enriched in 5S RNA. This agrees well with the observation of Sedat, Lyon and Sinsheimer (1969) that BDC retains RNA with higher degrees of secondary and tertiary structure less strongly than RNA with more single stranded regions. This property might be used for a simple separation of 4S and 5S RNAs.

The capability of the tRNA in the BDC eluate to accept amino acids was also examined. As shown in Table 9, the ethanol fraction



Fraction number

Figure 20. Reverse phase fractionation of aminoacyl tRNA.



Figure 21. Benzoylated DEAE cellulose chromatography of bulk tRNA from pea roots.



Figure 22. Analytical gel electrophoresis of tRNA from BDC.
	BDC Salt Fraction		BDC Ethanol Fraction		
amino acid	cpm per 0.5 A 260	μμ mole per A 260	cpm per 0.14 A 260	μμmole per A 260	% tRNA in BDC E.F.
Phe	2760	7	16500	150	70
Leu	24000	89	4900	60	6
Ser	1460	15	1000	36	1 <b>2</b>
Trp	1400	32	350	27	6
Cys/2	3100	30	2400	75	17
Tyr	12000	33	900	10	2
Val	7000	33	400	8	2

Table 9. Amino acid acceptance of BDC salt and ethanol fractions.

contains 70% of the phenyla lanine activity. It accepts 150  $\mu\mu$ moles of phenylalanine per A<sub>260</sub> unit representing a four to five fold purification. It is also enriched in serine and cysteine activity and accepts considerable amounts of leucine and tryptophan. Other activities such as valine and tyrosine are nearly absent from the ethanol fraction.

Reexamination of Figure 20 indicates that, of the other activities present in the BDC ethanol fraction, leucine is the most likely to coelute with phenylalanine from reverse phase chromatography. Efforts were therefore concentrated on separation of leucine and phenylalanine tRNA, Dube, Marcker and Yudelevich (1970) have described the use of preparative gel electrophoresis to purify E. coli leucine tRNA. Besides removing the low molecular weight material present in the BDC ethanol fraction RNA, this method might separate leucyl and phenylalanyl pea root tRNAs. BDC ethanol fraction tRNA (150 A<sub>260</sub> units) charged with <sup>3</sup>H-leucine and <sup>14</sup>C-phenylalanine was therefore subjected to preparative gel electrophoresis. The elution profile is shown in Figure 27A, Partial separation of leucyl and phenylalanyl tRNAs was indeed obtained. The peak tubes of the phenylalanine activity were pooled then chromatographed and rechromatographed on reverse phase columns. The results are shown in Figures 23A and B respectively, and the purification is summarized in Table 10.



Figure 23. Reverse chromatography of phenylalanyl tRNA.

tRNA	A260	µµmole Phe	μμmole A 260	μμmole leu	μμmole A 260
Unfractionated	1000	21000	21	90000	90
BDEF	150	145000	100	5500	35
After Gel Electrophoresis	5 <b>2</b>	7700	150	1280	25
After reverse phase	8.2	2150	260	340	7
Reverse phase rechromatography	3.3	1030	340		

Table 10. Purification of phenylalanine tRNA.

Although the specific activity of the purified tRNA indicated approximately 25% of theoretical phenylalanine acceptance (Tanaka, 1966), this should be considered a minimal value because the purified material may contain substantial quantities of unacylated phenylalanine tRNA as well as other non-tRNA material absorbing at 260 nm. This contention is supported by an experiment which indicated that BDC ethanol fraction tRNA accepting 100  $\mu\mu$ moles of phenylalanine per A<sub>260</sub> unit had an activity of more than 180  $\mu\mu$ moles per A<sub>260</sub> unit as judged by the fluorescence assay of Yoshikami <u>et al.</u> (1968). The complete separation of leucyl and phenylalanyl activities and the overall purification of 10-12 fold, rather than its acceptor activity, are probably more indicative of the suitability of this material for analysis of the isoprenoid nucleoside content of phenylalanine tRNA.

A leucyl tRNA subspecies was also purified from the BDC ethanol fraction. Peak tubes of the fore-running leucine activity from the preparative gel electrophoresis were collected and chromatographed on a reverse phase column. The elution profile is shown in Figure 27B and the purification is characterized in Table 11. Total purification was at least 25 fold and the final material was more than 50% pure.

Purification of tyrosyl tRNA from the BDC salt fraction involved aminoacylation, chromatography on BDC and fractionation of aliquots of the tyrosyl tRNA containing BDC ethanol fraction on

102

A260	µµmole leu	uµmole/A 260
150	5500	35
15.5	1650	110
0.62	540	830
	A <sub>260</sub> 150 15.5 0.62	A μμmole leu   150 5500   15.5 1650   0.62 540

## Table 11. Purification of a leucine tRNA.

reverse phase columns. Figures  $^{24}$  A and B show the results of these last two steps. Approximately 85% of the tyrosyl tRNA was retained in the BDC ethanol fraction. When an aliquot of this was fractionated on a reverse phase column, a profile vastly different than that of tyrosyl tRNA from unfractionated tRNA (Figure 20) was observed. In the latter case of the four peaks found the last predominated. For the tyrosyl tRNA found in the BDC ethanol fraction, however, the second peak predominated. An aliquot of the late salt fraction from the BDC column was also examined on a reverse phase column. This experiment, the results of which are shown in Figure 24C, indicated that the fourth peak was not selectively lost into the salt fraction. It must be concluded that some steps associated with aminoacylation and/or BDC chromatography produced the redistribution of tyrosyl tRNA subspecies evidenced by reverse phase chromatography of unfractionated and BDC ethanol fraction tRNA. The nature and significance of this alteration will be discussed below.

The purification of tyrosyl tRNA is summarized in Table 12. Overall purification of the subspecies was 20-25 fold. Again it is difficult to explain the low specific acceptance of this material, but it does seem to correlate with the observations of other workers. Kelmers (1970) stated that recovery of activity for isoprenoid nucleoside containing <u>E</u>. <u>coli</u> tRNAs from reverse phase columns is lower than the recovery of activities of other tRNAs under the same

104



Figure 24. Chromatography of tyrosyl tRNA on BDC and reverse phase columns.

tRNA	A260	µµmole tyr	μμmole/A 260
BDSF	960	10500	11
BDSF (late)	23	1400	60
BDEF	134	9200	70
After reverse phase			
Tyrl	0.96	190	200
<sup>Tyr</sup> 2	1.77	530	300
Tyr <sub>3</sub>	0.45	92	210
Tyr <sub>4</sub>	1.07	240	220

Table 12. Purification of tyrosine tRNA subspecies.

chromatographic conditions.

### GLC Analysis of Hydrolysates of Purified tRNAS

Purified phenylalanine and tyrosine tRNA and leucyl and tyrosyl tRNA subspecies as well as BDC salt and ethanol fractions tRNA were hydrolyzed and their isoprenoid nucleoside content were examined by GLC as shown in Table 13. It is interesting to note that 95% of the cis ZR but only 40% of the IPA containing tRNAs are found in the BDC salt fraction. Calculation of the levels of IPA and cis ZR on a  $\mu\mu$ mole per A<sub>260</sub> unit basis indicated that the sum of the IPA and cis ZR contents of the ethanol fraction (240  $\mu\mu$ mole/A<sub>260</sub>) is nearly identical to the sum of the acceptor activities of the presumed isoprenoid nucleoside containing tRNAs (220  $\mu\mu$ mole/A<sub>260</sub>) in the ethanol fraction, excluding phenylalanine. The sum of the cis ZR and IPA contents of the salt fraction (130  $\mu\mu$ mole/A<sub>260</sub>) also agrees well with the sum of the acceptor activities of the presumed isoprenoid nucleoside containing tRNAs (115  $\mu\mu$ mole/A<sub>260</sub>) in the salt fraction, excluding phenylalanine and leucine. If one assumes that phenylalanine tRNA contains no isoprenoid nucleosides and that the leucine species which do are retained in the ethanol fraction, as Roy and Soll (1968) have shown for  $\underline{E}$ . <u>coli</u> tRNA, then the isoprenoid nucleosides in pea root tRNA, distributed one per tRNA, may be accounted for by the observed serine, tryptophan, cysteine, and ethanol fraction

tRNA	Amount I		oprenoid Nucleos GLC	de Content Theoretical	
	$(A_{260} units)$	$\frac{\text{cis}}{\text{ZR}}$	(μg)	(µg)	
BDC salt fraction	640	3.7	24		
BDC ethanol fraction	80	5.2	1.3		
Phe	3.3			0.35	
Tyr (unfractionated)	17	0.28	0.16	0.78	
Tyrl	2.3			0.16	
Tyr <sub>2</sub>	2.1			0.38	
Tyr <sub>3</sub>	4.0			0.13	
Tyr <sub>4</sub>	2.5			0.21	
Leu	0.6		1.04	0.20	

Table 13. Isoprenoid nucleoside analysis of purified tRNAS.

leucine acceptor activities. In other words, their distribution would be consistent with their known distribution among the isoaccepting tRNAs of other organisms (Armstrong <u>et al.</u>, 1969a and 1969b; Peterkofsky and Jesensky, 1969; Hecht et al., 1969c).

The theoretical isoprenoid nucleoside content for the purified tRNAs is also included in Table 13. These calculations are based on one  $\mu\mu$ mole of isoprenoid nucleoside per  $\mu\mu$ mole of amino acid acceptance. For instance, if all phenylalanine tRNAs possessed an isoprenoid nucleoside their hydrolysate should have contained 0.38  $\mu$ g of these substances. This is five times the estimated limit of detection. Since no isoprenoid nucleosides were detected, less than 20% of the pea root phenylalanine tRNAs contain IPA or <u>cis</u> ZR, in agreement with their absence in yeast, wheat germ and beef liver phenylalanine tRNA (Yoshikani et al., 1968).

During purification two minor phenylalanine tRNAs were lost. It is interesting to speculate that these minor species may be of mitochondrial origin (Epler, 1969), in which case they might be expected to resemble the bacterial phenylalanine tRNAs some of which are known to contain isoprenoid nucleosides (Armstrong <u>et al.</u>, 1969a).

The GLC response to the derivatized hydrolysate of the purified tRNAs that were found to contain isoprenoid nucleosides are shown in Figure 25. The hydrolysate of tyrosyl tRNA purified through the



Figure 25. GLC analysis of hydrolysates of purified tRNA.

BDC chromatography step was found to contain both <u>cis</u> ZR and IPA. No isoprenoid nucleosides were found in the purified tyrosine tRNA subspecies. However, of those examined, only tyr<sub>2</sub> was calculated to have a theoretical content sufficiently above the limits of detection to allow reasonable certainty of the absence of <u>cis</u> ZR and IPA. A pea root tyrosine tRNA subspecies that contains neither <u>cis</u> ZR nor IPA probably has adenylic acid at its codon adjacent site (Gefter and Russel, 1969; Hall, 1970).

The rationale for the attempts made here to confirm the existence of chromatographically separable, modification variant forms of tyrosine tRNA were based on experiments (Vanderhoef and Key, 1970) which showed that the pea root tyrosine tRNA species with the strongest affinity for reverse phase columns  $(tyr_3/tyr_4)$  were much less prominent in tRNA from mature pea root tissue than in the tRNA from dividing tissue, while the species with the least affinity  $(tyr_1)$ were present at higher levels. If a translational control mechanism to restrict protein synthesis in mature tissue operates as was postulated, then tyr<sub>1</sub>, the species increased in mature tissue, should be modification deficient (contain no isoprenoid nucleosides) and presumably be least active in protein synthesis (Gefter and Russel, 1969). Those species found to be diminished  $(tyr_3/tyr_4)$  should be fully modified (contain cis ZR) and be the most active in protein synthesis. Tyr<sub>2</sub> would be predicted to be an intermediate (IPA containing)

species.

The data obtained from GLC analysis of partially purified but unfractionated tyrosyl tRNA (Table 13) confirm that some tyrosyl tRNA subspecies probably do contain <u>cis</u> ZR and/or IPA but it was not possible to decide which ones. The preliminary finding is that  $tyr_2$  is a modification deficient species, in contradiction to the predictions made from the findings of Vanderhoef and Key. Furthermore, the apparent ease with which the distribution of tyrosyl tRNA subspecies may be altered <u>in vitro</u> (Figures 20 and 24) raises grave doubts about the original observation of these authors that this distribution is different for dividing and nondividing tissues of the pea root. Clearly the basis of the <u>in vitro</u> alteration in distribution needs to be investigated. Only then should larger quantities of tyrosine tRNA subspecies be purified and their hydrolysates examined by GLC.

It should be noted that Cherry and Osbourne (1970) have mentioned unpublished data that indicate that they too may have encountered <u>in vitro</u> alteration of the tyrosine tRNA subspecies. Whether or not these alterations are related to the findings in Part V is still an open question. Another possibility is that the altered distributions have their basis in the observation of Kelmers (1970) that the isoprenoid nucleoside containing tRNAs seem to be less stable under various chromatographic conditions than do other tRNAs.

## V. A DEGRADATORY ENZYME ACTIVITY SPECIFIC FOR A LEUCINE tRNA

### Introduction

The effects of exogenous cytokinin application on soybean cotyledons were discussed in Part I. It was argued that the seeming inconsistency of the gross physiological effects of cytokinin application with the effects observed on the molecular level (changes in the population of leucine tRNAs) might be reconciled by the existence of a specific degradatory enzyme utilizing the isoprenoid nucleoside containing leucine tRNAs as substrates.

Other enzymes associated with tRNA metabolism, such as the aminoacyl synthetases and the isopentenyl transfer enzyme (Hall, 1970), exhibit specific recognition of tRNA subspecies. Although the isolation of a nuclease with this high degree of specificity has not been demonstrated the existence of such an activity has been postulated by other workers. Cherry and Osborne (1970), for example, mentioned unpublished data that led them to believe that a tyrosine tRNA specific nuclease is found in the aminoacyl synthetase preparations from soybean hypocotyls. In addition, the concept of a nuclease specific for certain leucine tRNA molecules was invoked by Sueoka and Kano-Sueoka (1970) to explain some of the very early events following infection of E. coli by the bacteriophage T2. During the course of the other investigations described in this thesis a new leucine containing RNA was discovered. This molecule has properties that suggest that it arises as a result of the degradation of a pea root leucyl tRNA. Its characteristics, possible mode of production and significance are discussed below.

### Materials and Methods

The materials utilized and the techniques employed in these investigations have been described in previous sections.

### **Results and Discussion**

# Identification and Characterization of leu tRNA

It was shown in Table 9 that after BDC chromatography of pea root tRNA (see Figure 21), portions of the leucine acceptor activity are found in tRNA from both the salt and ethanol fractions. Figure 26A shows the results of cochromatography of <sup>3</sup>H-leucyl tRNA prepared from tRNA of the BDC salt fraction (2.5  $A_{260}$  units) with <sup>14</sup>C-leucyl tRNA from the BDC ethanol fraction (1.5  $A_{260}$  units) on a reverse phase column using the standard gradient of 0.35-0.7 M NaCl. It is apparent that the ethanol fraction tRNA contains very little of the major leucine tRNA species, leu<sub>1</sub> and leu<sub>2</sub>. It is however enriched in leu<sub>3</sub>, leu<sub>4</sub>, leu<sub>5</sub> and leu<sub>6</sub> and also gives rise to an



Figure 26. Reverse phase chromatography of leucyl tRNA from BDC salt and ethanol fractions.

increased amount of radioactivity appearing at the void volume of the column.

Figure 26B presents the profile obtained when tRNA from the BDC salt (20 A<sub>260</sub> units) and ethanol (10 A<sub>260</sub> units) fractions were separately charged with <sup>14</sup>C-leucine and cochromatographed on a reverse phase column eluted with a linear 800 ml 0.3-0.7 M NaCl gradient. The lower initial salt concentration allowed separation of the radioactivity at the void volume into two distinct peaks.

Aliquots of selected fractions (identified in Figure 26B) from this column were precipitated with TCA before and after deacylation. Attempts were then made to recharge the deacylated material with <sup>14</sup>C-leucine. The results of this experiment are found in Table 14. The radioactivity in fraction 32 was not precipitated by TCA and was assumed to be mainly free amino acid. The label associated with the new peak characteristic of BDC ethanol fraction tRNA (and the label in fractions from other leucyl tRNA peaks) were precipitated when TCA was added. Furthermore, the labelled leucine in this and all other fractions known to discharge amino acids from tRNA (Yang and Novelli, 1970). However, while deacylated tRNA from the other leucine peaks will reaccept leucine, this material is not capable of being recharged.

This new peak of leucine activity was further characterized by

Fraction	TCA p	opt. cpm		
	Before deacylation	After deacylation	Reacylation TCA ppt. cpm	%
32	22	7	1	
51	492	14	6	1
68	344	18	293	77
			233	
94	561	11	557	95
			516	
102	386	7	291	85
			367	
180	243	7	125	45
			98	

Table 14. Discharging and reacylation of reverse phase fractions.<sup>1</sup>

A 0.2 ml aliquot of each fraction was TCA precipitated by the addition of 1 mg salmon sperm DNA and 0.1 ml 100% TCA. To another 0.3 ml aliquot, 0.15 ml 1 M TrisCl (pH 7.6) was added and the sample was incubated at 37°C for 30 minutes. A 0.2 ml aliquot of the deacylated sample was removed and precipitated with TCA as before. Duplicate 0.1 ml aliquots of the deacylated sample were acylated with <sup>14</sup>C-leucine in the standard 0.2 ml reaction mixture without added buffer. After 15 minutes these samples were also TCA precipitated. All precipitated samples were collected on nitrocellulose filters and counted. its mobility on polyacrylamide gels. Figure 27A presents the profile obtained when preparative gel electrophoresis was carried out on 150 A<sub>260</sub> units of BDC ethanol fraction tRNA charged with <sup>3</sup>H-leucine and <sup>14</sup>C-phenylalanine. The single peak of phenylalanine activity coincides with the optical absorbance peak, but the leucyl-tRNA was resolved into two components, one of which migrates faster than tRNA. Figure 27B shows that when this species, hereafter called leucyl-tRNA<sub>leuL</sub>, was subjected to chromatography on a reverse phase column it had a mobility identical to the fore-running leucine peak seen on reverse phase chromatography of the leucyl-tRNA from the BDC ethanol fraction. The mobility of leucyl-tRNA<sub>leuL</sub> on gel electrophoresis and reverse phase chromatography are both indicative of a degraded tRNA (Waters, 1971), possibly as small as onehalf a tRNA molecule in size.

After gel electrophoresis and reverse phase chromatography the pooled  ${}^{14}$ C-leucyl-tRNA<sub>leuL</sub> had an A<sub>260</sub>/A<sub>280</sub> ratio of 1.95 (indicating that it contained little or no protein) and a specific activity of 830 µµmole of leucine per A<sub>260</sub> unit. Its purity was considered high enough to allow examination of its hydrolysate by GLC. The analysis (Figure 25A and Table 8) indicated that the 0.62 A<sub>260</sub> units of tRNA<sub>leuL</sub> contained 1.04 µg of <u>cis</u> ZR or 5 µµmoles <u>cis</u> ZR per µµmole of attached leucine. Thus as much as 80% of the leu<sub>L</sub> tRNA may be uncharged. If this is the case then fully charged



Figure 27. Gel electrophoresis of BDC ethanol fraction tRNA and reverse phase chromatography of a leucyl tRNA.

119

leucyl-tRNA<sub>leu</sub> would have  $4150 \ \mu\mu$ mole leucine per A<sub>260</sub> unit-twice the acceptance of a purified tRNA (Tanaka, 1966), but not unreasonable for a molecule that may be half the size of a normal tRNA.

The experiments described above have yielded considerable information about the nature of leucyl-tRNA<sub>leu<sub>L</sub></sub>. Precipitation by TCA indicated that the leucine is chemically linked to the RNA. It was shown that although the attached leucine may be discharged with mild alkaline digestion, the deacylated leu<sub>L</sub> tRNA can no longer be recognized by the leucyl synthetase. The unique mobility of leucyltRNA<sub>leu<sub>L</sub></sub> on reverse phase chromatography and gel electrophoresis imply that it is considerably smaller than normal tRNA. Finally, GLC analysis of the hydrolysate of leu<sub>L</sub> tRNA demonstrated that it contains an isoprenoid nucleoside. But these are precisely the properties that would be expected of the product of the hypothetical degradatory enzyme! What then can be said about the origin of leucyltRNA<sub>leu<sub>I</sub></sub>?

# The Origin of leu<sub>L</sub> tRNA

It was previously shown (Table 3) that the synthetase preparation used here is devoid of detectable ribonuclease activity under the conditions standard to the aminoacylation reaction. It was also shown (Figure 27) that tRNA species with gel electrophoretic properties resembling those of leucyl-tRNA<sub>leuL</sub> are not generated from phenylalanyl tRNA, nor are molecules with mobilities similar to leucyl $tRNA_{leu_L}$  on reverse phase columns produced by aminoacylation of phenylalanine, or four other tRNAs (with the possible exception of serine), present in unfractionated pea root tRNA (Figure 20). Neither is leucyl-tRNA<sub>leu\_L</sub> produced from the major leucine tRNAs (leu<sub>1</sub> and leu<sub>2</sub>, see Figure 26A) found in the BDC salt fraction. Together these data demonstrate that production of leucyl-tRNA<sub>leu\_L</sub> is unlikely to be the result of the relatively nonspecific action of the known ribonucleases.

Figures 28A and B respectively show the rechromatography on BDC of pooled fractions 48-55 (containing  ${}^{14}$ C-leucyl-tRNA<sub>leu<sub>L</sub></sub>) and pooled fractions 177-88 (containing  ${}^{14}$ C-leucyl-tRNA<sub>leu<sub>6</sub></sub>) from the reverse phase column whose elution profile is depicted in Figure 26B. It is evident that leucyl-tRNA<sub>leu<sub>L</sub></sub> now appears in the salt fraction while leucyl-tRNA<sub>leu<sub>6</sub></sub> is still retained in the ethanol fraction. Apparently leucyl-tRNA<sub>leu<sub>L</sub></sub> has an affinity for BDC that is vastly different than other aminoacyl tRNAs produced from tRNA of the BDC ethanol fraction. Leucyl-tRNA<sub>leu<sub>L</sub></sub> is found only on aminoacylation of BDC ethanol fraction tRNA, yet it is not itself retained in the ethanol fraction upon subsequent rechromatography on BDC. Presumably then, leucyl-tRNA<sub>leu<sub>L</sub></sub> arises from modification of BDC ethanol fraction tRNA<sub>leu</sub> as a result of the aminoacylation procedure.



Figure 28. Rechromatrography of leucyl tRNA reverse phase column fractions on BDC.

This supposition is confirmed by the results of the next experiments, which are illustrated in Figure 29. Figure 29A shows cochromatography of <sup>3</sup>H-leucyl-tRNA from the BDC ethanol fraction  $(4 A_{260})$  units, incubated for 15 minutes in the standard reaction mixture) with <sup>14</sup>C-leucyl-tRNA also from the BDC ethanol fraction  $(2 A_{260})$  units, incubated for 45 minutes in the standard reaction mixture). In this and the following experiment the two reaction mixtures were adjusted to pH 4.5, immediately mixed and passed through a miniature DEAE cellulose column. Free amino acids and protein were washed from the column and the aminoacyl tRNAs eluted as previously described. The diluted eluant was applied directly to a 0.6 x 120 cm reverse phase column equilibrated against 0.2 M NaCl in the standard buffer. A linear 800 ml gradient from 0.2-0.7 M NaCl was applied at 20 ml per hour. One ml of each fraction was counted directly.

A comparison of the  ${}^{3}$ H- and  ${}^{14}$ C-leucyl-tRNA<sub>leuL</sub> peaks in Figure 29A indicated that the production of leucyl-tRNA<sub>leuL</sub> is time dependent. If one assumes that the apparent normalization around peaks of leu<sub>3</sub>, leu<sub>4</sub> and leu<sub>5</sub> tRNA is real, then a decrease in leucyltRNA<sub>leu<sub>6</sub></sub> is also evident. The same pattern was observed when the labels were reversed. In a similar experiment,  ${}^{14}$ C-leucyl-tRNA from the BDC ethanol fraction (2 A<sub>260</sub> units, incubated for 15 minutes in the standard reaction mixture with 0.5 the normal amount of



synthetase preparation) was cochromatographed with  ${}^{3}$ H-leucyltRNA also from the BDC ethanol fraction (4 A<sub>260</sub> units, incubated for the same period in the standard reaction mixture with 2.5 times the normal amount of synthetase preparation). An examination of the results (Figure 29B) indicates that the production of leucyltRNA<sub>leu</sub> from its precursor (possibly tRNA<sub>leu6</sub>) is also dependent on concentration of the synthetase preparation. That this precursor is not present in the synthetase preparation itself is evidenced by the lack of detectable leucyl-tRNA formation in reaction mixtures incubated in the absence of added tRNA (Figure 4C).

The obligatory coupling of leucyl-tRNA<sub>leu</sub> formation to aminoacylation in the above experiments, however, leaves open the possibility that the production of tRNA<sub>leu</sub> is catalyzed by a non-protein factor (for instance a heavy metal ion) present in the synthetase preparation. To allow independent detection of tRNA<sub>leu</sub>, <sup>32</sup>P-labelled tRNA from pea roots was prepared. This material (specific activity 10,000 cpm per A<sub>260</sub> unit) was fractionated on BDC and the <sup>32</sup>P-tRNA from the BDC ethanol fraction was utilized in the following experiment. The elution profile presented in Figure 30A was produced by reverse phase chromatography of <sup>3</sup>H-leucyl-<sup>32</sup>PtRNA from the BDC ethanol fraction (2. A<sub>260</sub> units, incubated with <sup>3</sup>H-leucine in the standard reaction mixture) and unlabelled leucyl-<sup>32</sup>P-tRNA from the BDC ethanol fraction (20 A<sub>260</sub> units, incubated



with <sup>1</sup>H-leucine in the standard reaction mixture). Figure <sup>30</sup>B shows the control experiment in which <sup>3</sup>H-leucyl-(unlabelled)-tRNA from another BDC ethanol fraction (0.5 A<sub>2.60</sub> units, aminoacylated with  $^{3}$ H-leucine to serve as a marker) was cochromatographed with  $^{32}$ PtRNA from the BDC ethanol fraction (50  $A_{260}$  units, incubated in a reaction mixture containing unlabelled leucine and synthetase preparation whose synthetase activity had been destroyed by boiling). The two columns were developed with the same gradient as in the preceding experiments. Some loss of resolution is apparent and is probably due to the increased load applied (Weiss et al., 1968). However, the <sup>32</sup>P profiles in Figures 30A and B are quite similar, implying that the aminoacylation procedure has no gross effects on the mobility of the tRNA. The difference to note though, is in the region of the <sup>3</sup>H-leucyl-tRNA<sub>leu</sub> marker. In the control experiment depicted in Figure 30B there is no  ${}^{32}P-tRNA_{leu_{\tau}}$  to be seen in this region. Apparently a heat-labile factor (presumably a protein) present in the synthetase preparation is necessary for the specific production of the small, isoprenoid nucleoside containing  $tRNA_{leu_T}$  species. In other words, the appearance of tRNA seems to be the result of leu the action of a specific degradatory enzyme as postulated in Part I.

The evidence that  $tRNA_{leu_L}$  arises from  $leu_5$  and/or  $leu_6$  is more presumptive. Leu<sub>6</sub> tRNA is probably one of the tRNAs which contain an isoprenoid nucleoside since it is retained in the BDC ethanol fraction (Roy and Söll, 1968) and since its chromatographic mobility on reverse phase columns coincides with the <u>E</u>. <u>coli</u> leucine tRNA identified as containing an isoprenoid nucleoside (Kan, Nirenberg and Sueoka, 1970; Armstrong <u>et al.</u>, 1969b). More directly, leucyl-tRNA<sub>leu6</sub> seems to decrease as leucyl-tRNA<sub>leu</sub> increases (Figure 29).

Conclusive evidence as to the origin of  $leucyl-tRNA_{leu_L}$  might be obtained from hybridization of  ${}^{32}P$ -tRNA with  ${}^{3}H$ -leucyl-tRNA\_{leu\_L}. The efficacy of hybridization of aminoacyl-tRNA with DNA has been demonstrated by several workers (Weiss <u>et al.</u>, 1968; Nass and Buck, 1969 and 1970). The separation of  ${}^{32}P$ -tRNA<sub>leux</sub>:  ${}^{3}H$ -leucyl-tRNA<sub>leu</sub><sub>L</sub> hybrids from tRNA dimers,  ${}^{32}P$ -tRNA and  ${}^{3}H$ -leucyl-tRNA<sub>leu</sub><sub>L</sub> should be possible using the gel filtration technique of Loehr and Keller (1968).

Even with the origin of leucyl-tRNA<sub>leu</sub> uncertain, the enzymatic production of this isoprenoid nucleoside containing species has profound implications for future investigations concerning the relationship of the isoprenoid nucleoside containing tRNAs and the role of isoprenoid nucleosides in regulation of plant growth and development.

For instance, the experiments described here have demonstrated the presence of a degradatory activity seemingly specific for a leucine tRNA that contains these minor nucleosides. It was predicted from the experiments of Bick <u>et al.</u> (1970) that such an activity would be involved in the release of isoprenoid nucleosides from tRNA and would be under feedback control. If this is really the function of the degradatory activity, then there must be additional activities involved with release, but not present in the synthetase preparation, since  $tRNA_{leu}_{L}$  still contains <u>cis</u> ZR. Attempts to show <u>in vitro</u> inhibition of the degradatory enzyme by free IPA (at a concentration of 1 mM in the aminoacylation reaction mixture) were unsuccessful. However, other forms such as <u>cis</u> ZR, the purine or the nucleotide, or possibly  $tRNA_{leu}_{L}$  itself might be the effective agent.

The specificity of the degradatory activity also needs to be further investigated. Preliminary experiments indicate that a seryl tRNA species similar to tRNA<sub>leu</sub> may also exist. Serine and leucine have a common feature, unique to themselves (and arginine). They are the only amino acids possessing two sets of unrelated code words; UUPu and CUX for leucine, UCPy and AGPy for serine, CGX and AGPu for arginine. This property makes them ideally suited for involvement in a translational restriction control mechanism. It would be very exciting if a tRNA<sub>ser</sub> species is also found.

The work presented here assumes an added dimension when compared to that of Kano-Sueoka and Sueoka (1968). These authors found that the transient production of a leucine tRNA species they called  $tRNA_{leu_F}$  is one of the earliest events following T2 infection of <u>E</u>. <u>coli</u>, and is apparently intimately connected with the restriction of host cell protein synthesis in phage infected cells. Kano-Sueoka and Sueoka also demonstrated that  $tRNA_{leu_F}$  is a small molecule that can be discharged but not reacylated and is presumably produced by degradation of one of the <u>E</u>. <u>coli</u> leucine tRNAs. Although they proposed that it originated from leu<sub>1</sub> tRNA as the result of the action of a hypothetical nuclease,  $tRNA_{leu_F}$  had codon recognition properties that suggested that it arose instead from the isoprenoid nucleoside containing leu<sub>5</sub> tRNA. Aside from a possible discrepancy as to their origins,  $tRNA_{leu_F}$  from phage infected <u>E</u>. <u>coli</u> and  $tRNA_{leu_L}$ from pea roots have strikingly similar if not identical properties.

It may be worthwhile to consider an analogy between restriction of host cell protein synthesis following phage infection with the restriction of protein synthesis that presumably follows commitment of the differentiating cells near the apical meristem of a growing pea root to a particular developmental pathway. The evidence presented here indicates that a common mechanism of a translational control may be involved in both cases.

#### BIBLIOGRAPHY

- Adams, A. and H. G. Zachau. 1968. Serine specific ribonucleic acids 15. Some properties of the aggregates from serine specific ribonucleic acids. European Journal of Biochemistry 5: 556-8.
- Alam, S. A. and R. H. Hall. 1970. Separation of the  $^2$  and  $^3$  isomers of N<sup>6</sup>-( $^2$ -isopentenyl) adenosine by gas chromatography and characterization by mass spectrometry. Submitted for publication in Biochemistry.
- Anderson, M. B. and J. H. Cherry. 1969. Differences in leucyltransfer RNA's and synthetase in soybean seedlings. Proceedings of the National Academy of Science 62:202-9.
- Anderson, W. F. and J. M. Gilbert. 1969. Translational control of <u>in vitro</u> hemoglobin synthesis. Cold Spring Harbor Symposium on Quantitative Biology 34:585-588.
- Armstrong, D. J., <u>et al.</u> 1969. Cytokinins: Distribution in species of yeast transfer RNA. Proceedings of the National Academy of Science 63:504-511.
- Armstrong, D. J., <u>et al.</u> 1969. Cytokinins: Distribution in transfer RNA species of <u>Escherichia coli</u>. Proceedings of the National Academy of Science 63:834-41.
- Baliga, B. S., et al. 1969. Differences in the transfer RNAs of normal liver and Novikoff hepatoma. Proceedings of the National Academy of Science 62:899-905.
- Bartz, J., <u>et al.</u> 1970. Identification of the cytokinin active ribonucleosides in pure <u>Escherichia coli</u> transfer RNA species. Proceedings of the National Academy of Science 67:1448-53.
- Bautz, K. F. and A. Bautz. 1970. Initiation of RNA synthesis: the functions of  $\sigma$  in the binding of RNA polymerase to promoter sites. Nature 226:1219-22.
- Beaudreau, G. S. Associate Professor, Oregon State University, Department of Agricultural Chemistry. Personal Communications. Corvallis, Oregon. November, 1970.

- Bekhor, I., G. M. Kung and J. Bonner. 1968. Sequence specific interaction of DNA and chromosomal protein. Journal of Molecular Biology 39:351-64.
- Bell, E. 1969. I-DNA: Its packaging into I-somes and its relation to protein synthesis during differentiation. Nature 224:326-8.
- Bellamy, A. R. 1966. Cytokinins in RNAs: cytokinin activity in animal transfer RNA. Nature 211:1093-5.
- Benson, R. H. 1966. Limitations of tritium measurements by liquid scintillation counting of emulsions. Analytical Chemistry 38: 1353-1356.
- Berridge, M. V. and R. K. Ralph. 1970. The binding of kinetin to plant ribosomes. Biochemical Journal 119:75-84.
- Bick, D., <u>et al.</u> 1970. Changes in leucyl- and tyrosyl-RNA of soybean cotyledons during plant growth. Biochimica et Biophysica Acta 204:175-82.
- Bock, R. M. and J. D. Cherayil. 1967. Chromatography of sRNA on DEAE cellulose and DEAE sephadex. In: Methods in Enzymology. Vol. 12, part A. Ed. by L. Grossman and K. Moldove. New York, Academic. p. 638-44.
- Bonner, J., R-C. C. Huang and R. V. Gilden. 1963. Chromosomally directed protein synthesis. Proceedings of the National Academy of Science 50:893-900.
- Bonner, J., and R-C. C. Huang. 1963. Properties of chromosomal nucleohistone. Journal of Molecular Biology 6:169-74.
- Brown, G. L. 1963. Preparation, fractionation and properties of sRNA. In: Progress in Nucleic Acid Research. Vol. 2. Ed. by J. N. Davidson and W. E. Cohn. New York, Academic. p. 260-310.
- Brown, J. C. and A. E. Smith. 1970. Initiation codons in eukaryotes. Nature 226:610-12.
- Bruce, M. I. and J. A. Zwar. 1966. Cytokinin activity of some substituted ureas and thioureas. Proceedings of the Royal Society, series B, 165:245-65.

- Burgess, R. R., et al. 1969. Factor stimulating transcription by RNA polymerases. Nature 221:43-6.
- Burkard, G., P. Guillemaut and J. H. Weil. 1970. Comparative studies of the tRNAs and the amino-tRNA synthetases from the cytoplasm and the chloroplasts of <u>Phaseolus</u> <u>vulgaris</u>. Biochimica et Biophysica Acta 224:184-98.
- Burrows, W. J., et al. 1968. Cytokinin from soluble RNA of Escherichia coli: 6-(3-methyl-2 butenylamino)-2-methylthio-9-β-Dribofuranosylpurine. Science 161:691-3.
- Burrows, W. J., <u>et al</u>. 1969. The isolation and identification of two cytokinins from Escherichia coli tRNAs. Biochemistry 8:3071-6.
- Burrows, W. J., et al. 1970. Isolation and identification of four cytokinins from wheat germ tRNA. Biochemistry 9:1867-72.
- Capra, J. D. and A. Peterkofsky. 1968. Effect of <u>in vitro</u> methylation on the chromatographic coding properties of methyl deficient leucine tRNA. Journal of Molecular Biology 33:591-607.
- Cavell, B. D., <u>et al.</u> 1967. Plant hormones V. Thin-layer and gasliquid chromatography of the giberellins; direct identification of the gibberellins in a crude plant extract by gas-liquid chromatography. Phytochemistry 6:867-74.
- Chen, C. and R. H. Hall. 1969. Biosynthesis of N<sup>6</sup>-(<sup>2</sup>-isopentenyl) adenosine in the tRNA of cultured tobacco pith tissue. Phytochemistry 8:1687-95.
- Cherry, J. H. Associate Professor, Department of Horticulture, Purdue University. Personal communication. Bloomington, Indiana. August, 1970.
- Cherry, J. H. and D. J. Osborne. 1970. Specificity of leucyl-tRNA and synthetase in plants. Biochemical and Biophysical Research Communications 40:763-69.
- Crestfield, A. M., K. C. Smith and F. W. Allen. 1955. The preparation and characterization of ribonucleic acids from yeast. Journal of Biological Chemistry 261:185-193.
- Darnell, J. E. 1968. Ribonucleic acids from animal cells. Bacteriological Revues 32:262-90.
- Davis, L. A., D. E. Heinz and F. T. Addicott. 1968. Gas liquid chromatography of trimethylsilyl derivatives of abscisic acid and other plant hormones. Plant Physiology 43:1389-1394.
- Denburg, J. and M. De Luca. 1970. Purification of a specific tRNA by Sepharose-bound enzyme. 67:1057-1062.
- Dingman, C. W. and A. C. Peacock. 1968. Analytical studies on nuclear ribonucleic acid using polyacrylamide gel electrophoresis. Biochemistry 7:659-668.
- Doi, R. H., I. Kaneko and R. T. Igarashi. 1968. Pattern of valine transfer ribonucleic acid of <u>Bacillus subtilis</u> under different growth conditions. Journal of Biological Chemistry 243:945-951.
- Dube, S. K. and P. S. Rudland. 1970. Control of translation by T4 phage: altered binding of disfavoured messengers. Nature 226: 820-3.
- Dube, S. K., K. A. Marcker and A. Yudelevich. 1970. The nucleotide sequence of a leucine transfer RNA from <u>E. coli</u>. Federation of European Biochemical Societies, Letters 9:168-170.
- Dudock, B. S., <u>et al.</u> 1969. Primary structure of wheat germ phenylalanine transfer RNA. Proceedings of the National Academy of Science 62:941-945.
- Eggen, K. and D. Nathans. 1969. Regulation of protein synthesis directed by coliphage MS2 RNA II. <u>in vitro</u> repression by phage coat protein. Journal of Molecular Biology 39:293-305.
- Emmer, M., <u>et al.</u> 1970. Cyclic AMP receptor protein of <u>E. coli</u>: its role in the synthesis of inducible enzymes. Proceedings of the National Academy of Science 66:480-487.
- Epler, J. L. 1969. The mitochondrial and cytoplasmic transfer ribonucleic acids of <u>Nuerospora</u> <u>crassa</u>. Biochemistry 8: 2285-2290.
- Fambrough, D. M., F. Fujimura and J. Bonner. 1968. Quantitative distribution of histone components in the pea plant. Biochemistry 7:575-585.

- Fittler, F. and R. H. Hall. 1966. Selective modification of yeast seryl-tRNA and its effect on the acceptance and binding functions. Biochemical and Biophysical Research Communications 25:441-446.
- Fittler, F., L. K. Kline and R. H. Hall. 1968. N<sup>6</sup>-(<sup>2</sup>-isopentenyl) adenosine: Biosynthesis <u>in vitro</u> by an enzyme extract from yeast and rat liver. Biochemical and Biophysical Research Communications 31:571-576.
- Fittler, F., L. K. Kline and R. H. Hall. 1968. The precursor relationship of acetate and mevalonate to the △<sup>2</sup>-isopentenyl groups of the transfer ribonucleic acid of microorganisms. Biochemistry 7:940-944.
- Fox, J. E. 1966. Incorporation of a kinin, N, 6-benzyladenine into soluble RNA. Plant Physiology 41:75-82.
- Fox, J. and C-M. Chen. 1967. Properties of RNA from tissue grown on cytokinins. Journal of Biological Chemistry 242:4490-4.
- Fraenkel-Conrat, H., D. Singer and R. C. Williams. 1957. Infectivity of viral nucleic acid. Biochimica et Biophysica Acta 25:87-96.
- Fromson, D. and M. Nemer. 1970. Cytoplasmic extraction: polyribosomes and heterogenous ribonucleoproteins without associated DNA. Science 168:266-7.
- Gallo, R. C. and S. Pestka. 1970. Transfer RNA species in normal and leukemic human lymphoblasts. Journal of Molecular Biology 52:195-219.
- Gefter, M. L. and R. L. Russell. 1969. Role of modifications in tyrosine transfer RNA: A modified base affecting ribosome binding. Journal of Molecular Biology 39:149-157.
- Gelehater, T. D. and G. M. Tomkins. 1970. Post-transcriptional control of tyrosine aminotransferase synthesis by insulin. Proceedings of the National Academy of Science 66:390-397.
- Giannattasio, M., G. Jeannin and A. Kovoor. 1969. Cytokinins and transfer ribonucleic acids II. Presence of cytokinins in different amino-acid specific transfer ribonucleic acids of Brewer's yeast as revealed by the growth response of tobacco pith callus tissue and <u>Scorzonera</u> crown-gall tissue. Physiologia Plantarum 22:458-61.

- Gilbert, W. and B. Müller-Hill. 1966. Isolation of the lac represser. Proceedings of the National Academy of Science 56:1891-1898.
- Gillam, I., <u>et al.</u> 1968. A general procedure for the isolation of specific transfer ribonucleic acids. Biochemistry 7:3459-3468.
- Grimm, W. A. H. and N. J. Leonard. 1967. Synthesis of the "minor nucleotide"  $N^{6}$ -( $\gamma$ ,  $\gamma$ -dimethylallyl)adenosine-5'-phosphate and relative rates of rearrangements of  $1-N^{6}$ -dimethylallyl compounds for base, nucleoside and nucleotide. Biochemistry 6: 3625-3631.
- Hall, R. H., et al. 1966. Isolation of N<sup>6</sup>-(γ, γ-dimethylallyl)adenosine from soluble ribonucleic acid. Journal of the American Chemical Society 88:2614-2615.
- Hall, R. H., <u>et al</u>. 1967a. Cytokinins in the soluble RNA of plant tissue. Science 156:69-71.
- Hall, R. H., B. Hacker, and L. Kline. 1967b. Biosynthesis of  $N^{6}$ - $(\Delta^{2}$ -isopentenyl) adenosine: precursor relationship of mevalonate to the isopentenyl group of sRNA. Federation Proceedings: Federation of the American Society for Experimental Biology 26:733.
- Hall, R. H. 1967c. Partition chromatography of nucleic acid components (isolation of minor nucleosides) In: Methods in Enzymology. Vol. 12, part A. Ed. by L. Grossman and K. Moldave. New York, Academic. p. 305-15.
- Hall, R. H. 1970. N<sup>6</sup>-(<sup>2</sup>-isopentenyl)adenosine: Chemical reactions, biosynthesis, metabolism and significance to the structure and function of tRNA. In: Progress in Nucleic Acid Research. Vol. 10. Ed. by J. N. Davidson and W. E. Cohn. New York, Academic. p. 57-86.
- Hancock, R. L. and D. L. Coleman. 1965. Gas chromatographic studies on nucleosides. Analytical Biochemistry 10:365-367.
- Hartmann, G. and U. Coy. 1961. Fraktionierung der Aminosäure spezifischen lösishen Ribonukleinsäuren. Biochimica et Biophysica Acta 47:612-613.

- Hashizume, T. and Y. Sasaki. 1966. Gas chromatographic separation of ribonucleotides by means of trimethylsilyl (TMS) derivatives. Analytical Biochemistry 15:199-203.
- Hay, J., D. J. Pillinger and E. Borek. 1970. The effect of hypermethylation on the functional properties of transfer ribonucleic acid: Ribosome binding and polypeptide synthesis. Biochemical Journal 114:429-435.
- Hecht, S. M., A. S. Gupta and N. J. Leonard. 1969a. Mass spectra of ribonucleoside components of tRNA. Analytical Biochemistry 30:249-270.
- Hecht, S. M., <u>et al.</u> 1969b. Cytokinin of wheat germ transfer RNA: 6-(4-hydroxy-3-methyl-2-butenylamine)-2-methylthio-9-3-Dribofuranosylpurine. Science 166:1272-1274.
- Hecht, S. M., et al. 1969c. Cytokinins: isolation and identification of 6-(3-methyl-2-butenyl)-9-β-D-ribofuranosylpurine (2iPA) from yeast. Biochemical and Biophysical Research Communications 35:205-9.
- Hecht, S. M., A. S. Gupta and N. J. Leonard. 1970. Mass spectra of ribonucleoside components of tRNA II. Analytical Biochemistry 38:230-251.
- Hegelson, J. P. 1968. The cytokinins. Science 161:974-981.
- Henes, C., M. Krauskopf and J. Ofengaard. 1969. A simple method for the preparation of highly purified formylmethionine transfer ribonucleic acid and methionine transfer ribonucleic acid from E. coli. Biochemistry 8:3024-3036.
- Henshaw, E. C. 1968. Messenger RNA in rat liver polyribosomes: evidence that it exists as ribonucleoprotein particles. Journal of Molecular Biology 36:401-411.
- Holley, R. W., <u>et al</u>. 1961. A simplified procedure for the preparation of tyrosine-and valine-acceptor fractions of yeast "soluble ribonucleic acid". Journal of Biological Chemistry 236:200-202.
- Holley, R. W., <u>et al.</u>, 1965. Nucleotide sequence in the yeast alanine transfer ribonucleic acid. Journal of Biological Chemistry 240:2122-2128.
- Hsu, W-T. and S. B. Weiss. 1969. Selective translation of T4template RNA by ribosomes from T4 infected E. coli. Proceedings of the National Academy of Science 64:345-351.

- Huang, R-C. C. and P. C. Huang. 1968. Effect of protein bound RNA associated with chick embryo chromatin on template activity of the chromatin. Journal of Molecular Biology 39:365-378.
- Hung, P. P. and L. R. Overby. 1968. Alteration in function of transfer ribonucleic acid of <u>Escherischia coli</u> after infection with phase Qβ. Journal of Biological Chemistry 243:5525-5531.
- Iwasaki, K., et al. 1968. Translation of the genetic message VII. Role of initiation factors in formation of the chain initiation complex with <u>E. coli</u> ribosomes. Archives of Biochemistry and Biophysics 125:542-547.
- Jackson, R. and T. Hunter. 1970. Role of methionine in the initiation of hemoglobin synthesis. Nature 227:672-676.
- Jacobson, M. and C. Hedgcoth. 1970. Levels of 5,6-dihydrouridine in relaxed and chloramphenicol transfer ribonucleic acid. Biochemistry 9:2513-2519.
- Jacobson, M., J. F. O'Brien and C. Hedgcoth. 1968. Determination of nucleoside composition of ribonucleic acid by gas-liquid chromatography. Analytical Biochemistry 25:363-369.
- Jeppesen, P. G. N., <u>et al.</u> 1970. Gene order in the bacteriophage R17: 5'-A protein-coat protein-synthetase-3'. Nature 226: 230-237.
- Kan, J., M. W. Nirenberg, and N. Sueoka. 1970. Coding specificity of <u>Escherichia coli</u> leucine transfer ribonucleic acids and effect of bacteriophage T2 infection. Journal of Molecular Biology 52: 179-193.
- Kano-Sueoka, T. and N. Sueoka. 1969. Leucine tRNA and cessation of <u>Escherichia coli</u> protein synthesis upon phage T2 infection. Proceedings of the National Academy of Science 62:1229-1236.
- Kano-Sueoka, T. and N. Sueoka. 1968. Characterization of a modified leucyl tRNA of <u>Escherichia coli</u> after bacteriophage T2 infection. Journal of Molecular Biology 37:475-491.
- Kawade, Y., T. Okamoto and Y. Yamamoto. 1963. Fractionation of soluble RNA by chromatography on DEAE ion exchangers. Biochemical and Biophysical Research Communications 10:200-203.

- Kelmers, A. D. 1970. Effects of divalent metal ions on the reverse phase chromatographic separation of transfer ribonucleic acid. Biochemistry 9:4401-4404.
- Kelmers, A. D., G. D. Novelli and M. P. Stulberg. 1965. Separation of transfer ribonucleic acid by reverse phase chromatography. Journal of Biological Chemistry 240:3974-3983.
- Khym, J. X. 1965. Separation of transfer ribonucleic acids by a simple extraction process. Journal of Biological Chemistry 240:PC1488-9.
- Kim, W. S. 1965. N-formylseryl-transfer RNA. Science 163:947-949.
- Kirby, K. S. 1956. A new method for the isolation of ribonucleic acids from mammalian tissues. Biochemical Journal 64:405-408.
- Kirby, K. S. 1964. Isolation and fractionation of nucleic acids. In: Progress in Nucleic Acid Research. Vol. 3. Ed. by J. N. Davidson and W. E. Cohn. New York, Academic. p. 1-31.
- Klambt, D., G. Thies and F. Skoog. 1966. Isolation of cytokinins from <u>Cornebacterium fascians</u>. Proceedings of the National Academy of Science 56:52-59.
- Koslov, Y. V. and G. P. Georgiev. 1970. Mechanism of inhibitory action of histones on DNA template activity <u>in vitro</u>. Nature 228:245-247.
- Leis, J. P. and E. B. Keller. 1970. Protein chain-initiating methionine tRNAs in chloroplasts and cytoplasm of wheat leaves. Proceedings of the National Academy of Science 67:1593-1599.
- Letham, D. S. 1963. Kinetin, a factor inducing cell division, isolated from Zea mays. Life Sciences 8:569-573.
- Letham, D. S. 1967. Chemistry and physiology of kinetin-like compounds. In: Annual Revues of Plant Physiology. Vol. 18. Ed. by L. Machlis. Palo Alto, Annual Revues Inc. p. 349-364.
- Letham, D. S., J. S. Shannon and I. R. McDonald. The structure of zeatin, a factor inducing cell division. Proceedings of the Chemical Society, 1964, p. 230-231.

- Liew, C. C., G. W. Haslett and V. G. Allfrey. 1970. N-acetylseryl-tRNA and polypeptide chain initiation during histone biosynthesis. Nature 226:414-417.
- Lindahl, T., A. Adams and J. R. Fresco. 1966. Renaturation of transfer ribonucleic acids through site binding of magnesium. Proceedings of the National Academy of Science 55:941-948.
- Lindell, T., F. Weinberg and W. J. Rutter. 1970. Functionally distinct nuclear RNA polymerases. Federation Proceedings: Federation of the American Society for Experimental Biology 26:669.
- Lippincott, B. B. and J. A. Lippincott. 1970. Bioassay and attributes of a growth factor associated with crown gall tumors. Plant Physiology 46:708-714.
- Littauer, U. Z., et al. 1969. Synthesis of peptidyl aminoacyl transfer RNA: a chemical method for the purification of transfer RNAs. Biochemica et Biophysica Acta 195:29-49.
- Lodish, H. A. 1968. Bacteriophage f2 RNA: control of translation and gene order. Nature 220:345-350.
- Loehr, J. S. P. and E. B. Keller. 1968. Dimers of alanine transfer RNA with acceptor activity. Proceedings of the National Academy of Science 61:1115-1122.
- Loening, U. E. 1967. The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochemical Journal 102:251-257.
- Losick, R. P. Metzger and J. Buchet-Mahieu. 1969. Some properties of the leucine specific tRNAs from <u>Bacillus stearothermoph-</u> <u>ilus</u>. Federation of European Biochemical Societies, Letters 5: 265-269.
- Marcker, K. A., B. F. C. Clark and J. S. Anderson. 1966. N-formyl-methionyl-sRNA and its relationship to protein biosynthesis. Cold Spring Harbor Symposium on Quantitative Biology 31:279-285.
- Martin, D. W. and G. M. Tomkins. 1970. The appearance and disappearance of the post-transcriptional repressor of tyrosine aminotransferase synthesis during the HTC cell cycle. Proceedings of the National Academy of Science 65:1064-1068.

- Marushige, K. and J. Bonner. 1966. Template properties of liver chromatin. Journal of Molecular Biology 15:160-174.
- Matthysse, A. G. Interaction of effector substances with chromosomal effectors. Abstracts of the International Botanical Congress, 11th. Seattle, 1969, p. 143.
- Matthysse, A. G. and C. Phillips. 1969. A protein intermediary in the interaction of a hormone with the genome. Proceedings of the National Academy of Science 63:897-903.
- Maxwell, I. H., E. Wimmer and G. M. Tener. 1968. The isolation of yeast tyrosine and tryptophan transfer ribonucleic acids. Biochemistry 7:2629-33.
- McCloskey, J. A., <u>et al.</u> 1968. Mass spectrometry of nucleic acid components. Trimethylsilyl derivatives of nucleotides, nucleosides and bases. Journal of the American Chemical Society 90:4182-4184.
- Mehler, A. H. and A. Banks. 1963. Isolation of specific transfer ribonucleic acids by a general method using N-carboxy anhydrides. Journal of Biological Chemistry 238:PC2888-2889.
- Miller, C. O. 1956. Similarity of some kinetin and red light effects. Plant Physiology 31:318-319.
- Miller, C. O. 1961. Kinetin and related compounds in plant growth. In: Annual Reviews of Plant Physiology. V. 12. Ed. by L. Machlis. Palo Alto, Annual Reviews Inc. p. 395-408.
- Miller, C. O. 1965. Evidence for the natural occurrence of zeatin and derivatives; compounds from maize which promote cell division. Proceedings of the National Academy of Science 54: 1052-1058.
- Miller, C. O. <u>et al</u>. 1955. Kinetin, a cell division factor from deoxyribonucleic acid. Journal of the American Chemical Society 77:1392.
- Miller, C. O., <u>et al.</u> 1956. Isolation, structure and synthesis of kinetin, a substance promoting cell division. Journal of the American Chemical Society 78:1375-1380.

- Mirza, M. A. A. and M. Cannon. 1967. A comparison of different methods for the preparation of soluble ribonucleic acid from Escherichią coli. Biochemica et Biophysica Acta 149:596-598.
- Morgan, C. R. and J. Bonner. 1970. Template activity of liver chromatin increased by <u>in vitro</u> administration of insulin. Proceedings of the National Academy of Science 65:1077-1080.
- Morris, R. O., Associate Professor and G. B. Jacobsen, Research Associate, Dept. of Agricultural Chemistry, Oregon State University, unpublished results. August 1970.
- Most, B. H., J. C. Williams and K. J. Parker. 1968. Gas chromatography of cytokinins. Journal of Chromatography 38: 136-138.
- Mosteller, R. D., W. J. Culp and B. Hardesty. 1968. Deacylated transfer ribonucleic acid as a factor for peptide chain initiation in rabbit reticulocyte systems. Journal of Biological Chemistry 243:6343-52.
- Muench, K. H. and P. Berg. 1966a. Preparation of aminoacyl transfer ribonucleic acid synthetases from <u>Escherichia coli</u>. In: Procedures in Nucleic Acid Research. Ed. by G. L. Cantoni and D. R. Davies. New York, Harper and Row. p. 375-383.
- Muench, K. H. and P. Berg. 1966b. Resolution of aminoacyl transfer ribonucleic acid by hydroxyl-apatite chromatography. Biochemistry 5:982-987.
- Müller, W. E. G., R. K. Zahn and R. Beyer. 1970. Is "I-DNA" derived from nuclear DNA? Nature 227:1211-1212.
- Nass, M. M. K. and C. A. Buck. 1969. Comparative hybridization of mitochondrial and cytoplasmic aminoacyl transfer RNA with mitochondrial DNA from rat liver. Proceedings of the National Academy of Science 62:506-513.
- Nass, M. M. K. and C. A. Buck. 1970. Studies on mitochondrial tRNA from animal cells II. hybridization of aminoacyl-tRNA from rat liver mitochondria with heavy and light complementary strands of mitochondrial DNA. Journal of Molecular Biology 54:187-198.

- Needhardt, F. C. and C. F. Earhart. 1966. Phage-induced appearance of a valyl sRNA synthetase activity in <u>Escherichia coli</u>. Cold Spring Harbor Symposium on Quantitative Biology 31: 557-563.
- Novelli, G. D. 1967. Amino acid activation for protein synthesis. In: Annual Reviews of Biochemistry. Vol. 36. Ed. by P. D. Boyer. Palo Alto, Annual Reviews Inc. p. 449-484.
- Pastan, I. and R. Perlman. 1970. Cyclic adenosine monophosphate in bacteria. Science 169:339-344.
- Patterson, M. S. and R. C. Green. 1965. Measurements of low energy beta-emitters in an aqueous solution by liquid scintillation counting of emulsions. Analytical Chemistry 37:854-857.
- Paul, J. and S. Gilmour. 1966. Restriction of deoxyribonucleic acid template activity in chromatin is organ specific. Nature 210: 305-316.
- Paul, J. and S. Gilmour. 1968. Organ specific restriction of transcription in mammalian chromatin. Journal of Molecular Biology 34:305-316.
- Peterkofsky, A. 1968. The incorporation of mevalonic acid into the N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenosine of transfer ribonucleic acid in Lactobacillus acidophilus. Biochemistry 7:472-482.
- Peterkofsky, A. and C. Jesensky. 1969. The localization of N<sup>6</sup>-(<sup>2</sup>isopentenyl)adenosine among the acceptor species of transfer ribonucleic acid of <u>Lactobacillus acidophilus</u>. Biochemistry 8: 3798-3809.
- Pillinger, D. J., J. Hay and E. Borek. 1969. The effect of hypermethylation on the functional properties of transfer ribonucleic acid. Formation of aminoacyl-transfer-ribonucleic acid. Biochemical Journal 114:429-435.
- Pollack, Y. et al. 1970. Role of initiation factor B (F3) in the preferential translation of T4 late messenger RNA in T4 infected <u>E. coli</u>. Federation of European Biochemical Societies, Letters 9:218-221.

- Preddie, E. C. 1969. Tryptophanyl transfer ribonucleic acid synthetase from Bovine Pancreas III. A complex of tryptophanyl transfer ribonucleic acid synthetase and transfer ribonucleic acid accepts tryptophan: the purification of <sup>32</sup>P-tryptophan transfer ribonucleic acid. Journal of Biological Chemistry 244:3969-3972.
- Prichard, P. M. et al. 1970. Factors for the initiation of haemoglobin synthesis by rabbit reticulocyte ribosomes. Nature 226: 511-514.
- Ptashne, M. 1967. Specific binding of the  $\lambda$  phage repressors to  $\lambda$  DNA. Nature 214:232-234.
- Raj Bhandary, U. L. <u>et al</u>. 1967. Studies on polynucleotides LXVIII. The primary structure of yeast phenylalanine transfer RNA. Proceedings of the National Academy of Science 57:751-758.
- Rechler, M. M. and R. G. Martin. 1970. The intercistronic divide: translation of an intercistronic region of the histidine operon of <u>Salmonella typhimurium</u>. Nature 226:908-911.
- Rennert, O. M. 1969. Aminoacyl-tRNA synthetase reaction unique to early embryonic tissue of the mouse. Proceedings of the National Academy of Science 63:878-884.
- Revel, M. <u>et al.</u> 1968. Function of three protein factors and of ribosomal subunits in the initiation of protein synthesis in <u>E. coli</u>. Nature 219:1016-1021.
- Revel, M. et al. 1970. Fractionation of translation initiation factor B (F3) into cistron-specific species. Federation of European Biochemical Societies, Letters 9:213-217.
- Richards, E. G. and J. A. Coll. 1965. Disc electrophoresis of ribonucleic acid in polyacrylamide gels. Analytical Biochemistry 12:452-471.
- Richmond, A. E. and A. Lang. 1967. Effect of kinetin on protein content and survival of detached <u>Xanthium</u> leaves. Science 125:650-651.

- Robins, M. J., R. H. Hall and R. Thedford. 1967. N<sup>6</sup>; (<sup>2</sup>-isopentenyl) adenosine. A component of the transfer ribonucleic acid of yeast and of mammalian tissue; methods of isolation and characterization. Biochemistry 6:1837.
- Rosenberg, A. H. and M. L. Gefter. 1969. An ion-dependent modification of several transfer RNA species in <u>Escherischia coli</u>. Journal of Molecular Biology 46:581-584.
- Roy, K. L. and D. Söll. 1968. Fractionation of <u>Escherichia coli</u> transfer RNA on benzoylated DEAE-cellulose. Biochemical et Biophysica Acta 161:572-574.
- Schaeffer, G. W. and H. H. Smith. 1963. Auxin-kinetin interaction in tissue cultures of <u>Nicotiana</u> species and tumor conditioned hybrids. Plant Physiology 38:291-297.
- Schedl, P. D., R. E. Singer and T. W. Conway. 1970. A factor required for the translation of bacteriophage f2 RNA in extracts of T4 infected cells. Biochemical and Biophysical Research Communications 38:631-637.
- Schmidt, D. A. <u>et al</u>. 1970. Involvement of a phage T4 factor and an anti-terminator protein in the transcription of early T4 genes in vivo. Nature 225:1012-1016.
- Schulman, L. H. and R. W. Chambers. 1968. Transfer RNA, II. A structural basis for alanine acceptor activity. Proceedings of the National Academy of Science 61:308-315.
- Scott, P. C. and R. O. Morris. 1969. Preparation of aminoacyl synthetases from higher plants. Biochemica et Biophysica Acta 185:474-477.
- Sedat, J., A. Lyon and R. L. Sinsheimer. 1969. Purification of <u>Escherichia coli</u> pulse-labeled RNA by benzoylated DEAEcellulose chromatography. Journal of Molecular Biology 44: 415-434.
- Shih, T. Y. and J. Bonner. 1970. Template properties of DNApolypeptide complexes. Journal of Molecular Biology 50:333-344.

- Shugart, L., G. D. Novelli and M. P. Stulberg. 1968. Isolation and properties of undermethylated phenylalanine transfer ribonucleic acids from a relaxed mutant of <u>Escherichia coli</u>. Biochemica et Biophysica Acta 157:83-90.
- Skinner, C. G. et al. 1956. Effects of some 6-(substituted) purines on regeneration of <u>Hydra</u>. Journal of the American Chemical Society 78:5997-5100.
- Skoog, F., Professor, Department of Botany, University of Wisconsin, personal communication, letter. September 1970.
- Skoog, F. and C. O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissue cultures in vitro. Symposium: Society of Experimental Biology 11:118-131.
- Skoog, F. <u>et al.</u> 1966. Cytokinin activity: localization in transfer RNA preparations. Science 154:1354-1356.
- Skoog, F. <u>et al.</u> 1967. Cytokinins: structure/activity relationships. Phytochemistry 6:1169-1192.
- Skoog, F. and D. J. Armstrong. 1970. Cytokinins. In: Annual reviews of Plant Physiology. Vol. 21. Ed. by L. Machlis. Palo Alto, Annual Reviews Inc. p. 359-384.
- Smith, A. E. and Marcker, K. A. 1970. Cytoplasmic methionyl transfer RNAs from eukaryotes. Nature 226:607-610.
- Spirin, A. S. 1966. On "masked" forms of messenger RNA in early embryogenesis and in other differentiating systems. In: Current Topics in Developmental Biology. Vol. 1. Ed. by A. A. Moscona and A. Monroy. New York, Academic Press. p. 1-38.
- Spirin, A. S. and N. Nemer. 1965. Messenger RNA in early sea urchin embryos: cytoplasmic particles. Science 150:214-217.
- Strauss, J. H. and R. L. Sinsheimer. 1963. Purification and properties of bacteriophage MS2 and its ribonucleic acid. Journal of Molecular Biology 7:43-54.
- Steitz, J. A. 1969. Peptide chain initation: nucleotide sequences of the three ribosomal binding sites in bacteriophage R17 RNA. Nature 224:957-964.

- Stetler, D. A. and W. M. Laetsch. 1965. Kinetin-induced chloroplast maturation in cultures of tobacco tissue. Science 149: 1387-1388.
- Steward, F. C. et al. 1963. Growth and organization in free cell cultures. Brookhaven Symposia in Biology 16:73-86.
- Sueoka, N. and T. Kano-Sueoka. 1970. Transfer RNA and cell differentiation. In: Progress in Nucleic Acid Research. Vol. 10. Ed. by G. L. Cantoni and D. R. Davies. New York, Academic, p. 23-55.
- Sueoka, N. and T. Yamane. 1964. Leucine tRNA and cessation of <u>E. coli</u> protein synthesis upon phage T2 infection. Proceedings of the National Academy of Science 52:1535-1543.
- Sugiyama, T. and D. Nakada. 1968. Translational control of bacteriophage MS2 RNA cistrons by MS2 coat protein: polyacrylamide gel electrophoretic analysis of proteins synthesized in vitro. Journal of Molecular Biology 31:431-440.
- Tanaka, K. 1966. Methods of isolation of amino acid specific RNA. In: Procedures in Nucleic Acid Research. Ed. by G. L. Cantoni and D. R. Davies. New York, Harper and Row. p. 466-479.
- Thiebe, R. and H. G. Zachau. 1968. The role of the anticodon region in homologous and heterologous charging of tRNA. Biochemical and Biophysical Research Communications 33:260-5.
- Thimann, K. V. and T. Sachs. 1966. The role of cytokinins in the "fasciation" disease caused by <u>Cornebacterium fascians</u>. American Journal of Botany 53:731-739.
- Tomkins, G. M., <u>et al</u>. 1969. Control of specific gene expression in higher organisms. Science 166:1474-1480.
- Travers, A. A. 1969. Bacteriophage sigma factor for RNA polymerase. Nature 223:1107-1110.
- Travers, A. A. 1970. Positive control of transcription by a bacteriophage sigma factor. Nature 225:1009-1012.
- Travers, A. A. and R. R. Burgess. 1969. Cyclic reuse of the RNA polymerase sigma factor. Nature 222:537-540.

- Travers, A. A., R. I. Kamen and R. F. Schleif. 1970. Factor necessary for ribosomal RNA synthesis. Nature 228:748-751.
- Upper, C. D., <u>et al</u>. 1970. Gas-liquid chromatographic isolation of cytokinins from natural sources. Plant Physiology 45:543-547.
- Vanderhoef, L. N. and J. L. Key. 1970a. The fractionation of transfer ribonucleic acid from roots of pea seedlings. Plant Physiology 46:284-288.
- Vanderhoef, L. N., R. F. Bohannon and J. L. Key. 1970b. A study of transfer RNA, aminoacyl-tRNA synthetases, and transfer RNA aminoacylation in higher plants. Phytochemistry 9:2291-2304.
- von Overbeek, J. 1966. Plant hormones and regulators. Science 152:721-731.
- Vold, B. S. and P. S. Sypherd. 1968. Modification in transfer RNA during the differentiation of wheat seedlings. Proceedings of the National Academy of Science 59:454-458.
- Waters, L. C. 1969. Altered chromatographic properties of tRNA from chloramphenicol-treated <u>Escherichia</u> <u>coli</u>. Biochemical and and Biophysical Research Communications 37:297-304.
- Waters, L. C., Research Scientist, Oak Ridge National Laboratories, Oak Ridge, Tennessee, personal communication, telephone conversation, January, 1971.
- Waters, L. C. and G. D. Novelli, 1968. The early change in <u>E</u>. <u>coli</u> leucine tRNA after infection with bacteriophage T2. Biochemical and Biophysical Research Communications 32:971-976.
- Waters, L. C. and G. D. Novelli. Analytical reversed phase chromatography of <u>E</u>. <u>coli</u> aminoacyl-tRNAs. In: Methods in Enzymology. Vol. 12, part C. Ed. by L. Grossman and K. Moldave. New York, Academic, in the press.
- Weiss, J. F. and A. D. Kelmers. 1967. A new chromatographic system for increased resolution of transfer ribonucleic acids. Biochemistry 6:2507-2513.

- Weiss, J. F., R. L. Pearson and A. D. Kelmers. 1968. Two additional reversed phase chromatographic systems for the separation of transfer ribonucleic acid and their application to the preparation of two formylmethionine and a valine transfer ribonucleic acid from <u>Escherichia coli</u> B. Biochemistry 7: 3479-3487.
- Weiss, S. B., W. T. Hsu and J. W. Fort. 1968. Transfer RNA coded by the T4 bacteriophage genome. Proceedings of the National Academy of Science 59:114-121.
- Wigle, D. T. and G. H. Dixon. 1970. Transient incorporation of methionine at the N-terminus of protamine newly synthesized in trout testis cells. Nature 226:676-680.
- Wimmer, E., I. H. Maxwell and G. M. Tener. 1968. A simple method for isolation of highly purified yeast phenylalanine transfer ribonucleic acid. Biochemistry 7:2623-2628.
- Wood, H. N. 1970. Revised identification of the chromaphore of a cell division factor from crown gall tumor cells of <u>Vinca rosea</u>
  L. Proceedings of the National Academy of Science 67:1283-1287.
- Wood, H. N., <u>et al.</u> 1969. Studies on the distribution and properties of a new class of cell division-promoting substances from higher plants. Proceedings of the National Academy of Science 62: 349-356.
- Yang, W. K. and G. D. Novelli. Analysis of isoaccepting tRNAs in mammalian tissues and cells. In: Methods in Enzymology. Vol. 12, part C. Ed. by L. Grossman and K. Moldave, New York, Academic, in the press.
- Yaniv, M. and B. G. Barrell. 1969. Nucleotide sequence of <u>E</u>. <u>coli</u> tRNA, <sup>val</sup>. Nature 222:278-279.
- Yarus, M. and P. Berg. 1969. Recognition of tRNA by isoleucyltRNA synthetase. Journal of Molecular Biology 42:171-189.
- Yoshikami, D., <u>et al</u>. 1968. A fluorescence assay for phenylalanine transfer RNA. Biochemica et Biophysica Acta 166:714-717.

- Zachau, H. G., D. Dütting and H. Feldman. 1966. Nucleotidsequenzen zweir serinspezifischer transfer-ribonukleinsäuren. Angewantische Chemie 78:392-393.
- Zamecnik, P. C., M. L. Stephenson and J. F. Scott. 1960. Partial purification of soluble RNA. Proceedings of the National Academy of Science 46:811-822.
- Zubay, G. 1962. The isolation and fractionation of soluble ribonucleic acids. Journal of Molecular Biology 4:347-356.
- Zubay, G., D. Schwartz and J. Beckwith. 1970. Mechanism of activation of catabolite sensitive genes: a positive control system. Proceedings of the National Academy of Science 66: 104-110.