AN ABSTRACT OF THE THESIS OF

Cynthia Ann Edwards   for the degree of Master of Arts

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Title: Cytokinin-active Ribonucleosides in Phaseolus vulgaris L.

Transfer Ribonucleic Acids

Abstract approved: Redacted for privacy
                          Donald J. Armstrong

The cytokinin-active ribonucleosides present in transfer
ribonucleic acid from etiolated Phaseolus vulgaris seedlings have
been isolated and identified as 6-(3-methyl-2-butenylamino)-9- -D-
ribofuranosylpurine, and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-
methylthio-9- -D-ribofuranosylpurine. The ribonucleosides were
extracted from an enzymatically hydrolyzed Phaseolus tRNA prepara-
tion and fractionated by Sephadex LH-20 chromatography. The struc-
tures of the compounds were established on the basis of their
chromatographic properties and the mass spectra of their permethy-
lated and perdeuteromethylated derivatives.

The distribution of cytokinin-active ribonucleosides in
Phaseolus tRNA isolated from etiolated seedlings has also been
examined. Phaseolus tRNA was fractionated by BD-cellulose and

RPC-5 chromatography, and the distribution of cytokinin activity
compared with the distribution of tRNA species expected to correspond
to codons beginning with U (ser, tyr, trp, phe, leu, and cys acceptor
activities). Cysteine, tyrosine and tryptophan tRNA species were
not associated with cytokinin activity. Cytokinin activity was
associated with tRNA fractions containing serine and leucine tRNA
species. Phenylalanine tRNA was not completely resolved from the
serine acceptor activity, but it presumably contains a base Y
derivative adjacent to the 3' end of the anticodon rather than a
cytokinin-active base. In addition to the cytokinin activity that
could be attributed to tRNA^{ser} and tRNA^{leu} species, several anomalous
peaks of cytokinin activity that did not correspond to any of the
U-group tRNA species were observed.

Cytokinin-active Ribonucleosides in <u>Phaseolus</u>
<u>vulgaris</u> L. Transfer Ribonucleic Acids

by

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LIST OF ABBREVIATIONS

A: adenosine

Ade: adenine

bzl^6A: N^6-benzyladenosine

bzl^6Ade: N^6-benzyladenine

C: cytosine

fr^6A: N^6-furfuryladenosine

fr^6A: N^6-furfuryladenine, kinetin

G: guanosine

i^6A: 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine,
N^6-(Δ^2-isopentenyl)adenosine

io^6A: 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofurano-
sylpurine, ribosylzeatin

m^1A: 1-methyladenosine

m^1G: 1-methylguanosine

ms^2i^6A: 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribo-
furanosylpurine

ms^2io^6A: 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-
β-D-ribofuranosylpurine

U: uridine

CYTOKININ-ACTIVE RIBONUCLEOSIDES IN <u>PHASEOLUS</u>

<u>VULGARIS</u> L. TRANSFER RIBONUCLEIC ACIDS

I. INTRODUCTION

Cytokinins are N^6-substituted adenine molecules that function

as hormones in the regulation of plant growth and development.

Cytokinins also occur as nucleoside constituents of transfer RNA

molecules in virtually all organisms examined to date (95). The

relationship between the cytokinin-active ribonucleosides in tRNA

molecules and the hormonal function of free cytokinins in plant tis-

sues is still uncertain.

The cytokinin, N^6-(Δ^2-isopentenyl)adenosine (i^6A) occurs in

all tRNA preparations that have been examined (40,64,95). All other

cytokinin-active ribonucleosides that have been isolated from tRNA

are structurally related to this compound. The cytokinins that have

been identified as constituents of tRNA molecules include i^6A, <u>cis</u>-

ribosylzeatin [io^6A, N^6-(4-hydroxy-3-methyl-2-butenyl)adenosine],

and the 2-methylthio derivatives of these two compounds (ms^2i^6A

and ms^2io^6A). The structures of cytokinin-active ribonucleosides are

shown in Figure 1. All four of these cytokinin-active ribonucleosides

have been identified in tRNA from plant sources, although ms^2i^6A is

typically present in only trace amounts (if at all) in plant tRNA

preparations.

Detailed studies of a number of microbial systems as well as
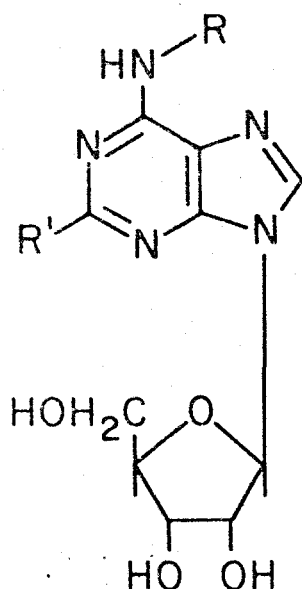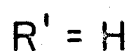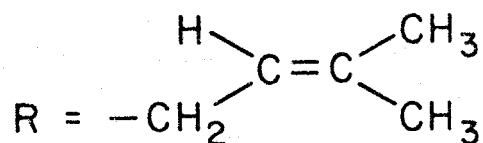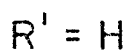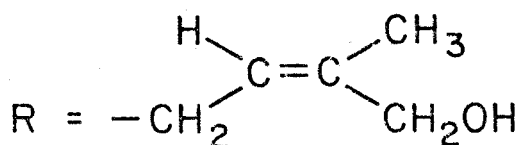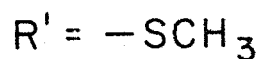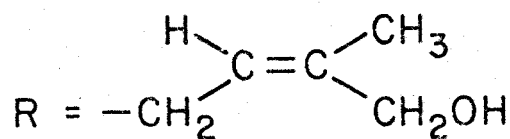
Figure 1. Structures of several cytokinin-active ribonucleosides.
A) N[6]-substituted adenosine, B) N[6]-(Δ^2-isopentenyl)adeno-
sine, C) N[6]-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-
ribofuranosylpurine, D) 6-(4-hydroxy-3-methyl-2-
butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine.

scattered information from other sources have shown that cytokinin-active ribonucleosides are found only in the 3' position adjacent to the anticodon in tRNA species which respond to codons beginning with uridine (40,64,95). (Serine, tyrosine, cysteine, phenylalanine, tryptophan, and tyrosine are represented by codons of this type.) Not all U-group tRNAs, however, have cytokinin moieties, and the distribution of cytokinin-active ribonucleosides among tRNA species is variable in different organisms.

In tRNA from microbial sources, cytokinin-active ribonucleosides appear to be present in most, if not all, of the tRNA species that respond to codons beginning with U (3,6,82). In higher organisms, the distribution appears to be more restricted. Wheat germ is the only plant system in which the distribution of cytokinin-active ribonucleosides with respect to individual tRNA species has been examined in detail (100). Cytokinin activity was restricted to wheat germ tRNA^{ser} species and a minor tRNA^{leu} species.

The isolation and identification of the cytokinin-active ribonucleosides in tRNA from etiolated seedlings of Phaseolus vulgaris are reported here. The distribution of cytokinins with respect to Phaseolus tRNA species has also been examined. The selection of this plant material was based, in part, on reports that dihydrozeatin (the saturated analog of zeatin) is a major free cytokinin in beans (97,109). Dihydrozeatin has not as yet been reported to occur as a constituent of tRNA molecules, and it was of interest to determine whether there were similarities in the metabolism of free cytokinins and the cytokinin constituents of tRNA molecules in this plant

system. In addition, it was of interest to determine whether the distribution of cytokinins in tRNA from a dicot plant source would be similar to that in wheat germ (a monocot source). The use of etiolated seedlings was intended to minimize the chloroplast tRNA content of the tRNA preparation and provide a more direct comparison with the wheat germ tRNA.

II. REVIEW OF LITERATURE

Isolation and Identification of Cytokinin-Active Ribonucleosides

The first nucleotide sequence of a transfer ribonucleic acid, brewer's yeast $tRNA^{ala}$, was published by Holley et al. in 1965 (48). Since that time, over 80 tRNAs have been sequenced, and a variety of modified nucleosides have been shown to occur as constituents of tRNA molecules. To date, more than 50 modified nucleosides have been isolated and identified from tRNA preparations (72). Most of these modified nucleosides are methylated or otherwise slightly modified derivatives of the four common nucleosides found in ribonucleic acids. However, some tRNA species also contain hypermodified bases, which bear rather complex side chains. Hypermodified bases are usually found in either the 5' "wobble" position of the anticodon (e.g. base Q and its derivatives) or the 3' position adjacent to the anticodon (e.g. base Y, N-(purin-6-ylcarbamoyl)threonine, i^6Ade, and their deri-vatives). Some of these hypermodified bases are shown in Figure 2.

The presence of cytokinin-active ribonucleosides in the 3' position adjacent to the anticodon was discovered in 1966 by Zachau et al. (11,112) in two yeast $tRNA^{ser}$ species. They identified the hypermodified nucleoside as $N^6-(\Delta^2-isopentenyl)adenosine$ (i^6A). At the same time, Hall and his coworkers (43,90) reported the isola-tion of i^6A from both yeast and calf liver tRNA hydrolysates. Shortly thereafter, Skoog et al. (96) demonstrated that _Escherichia_
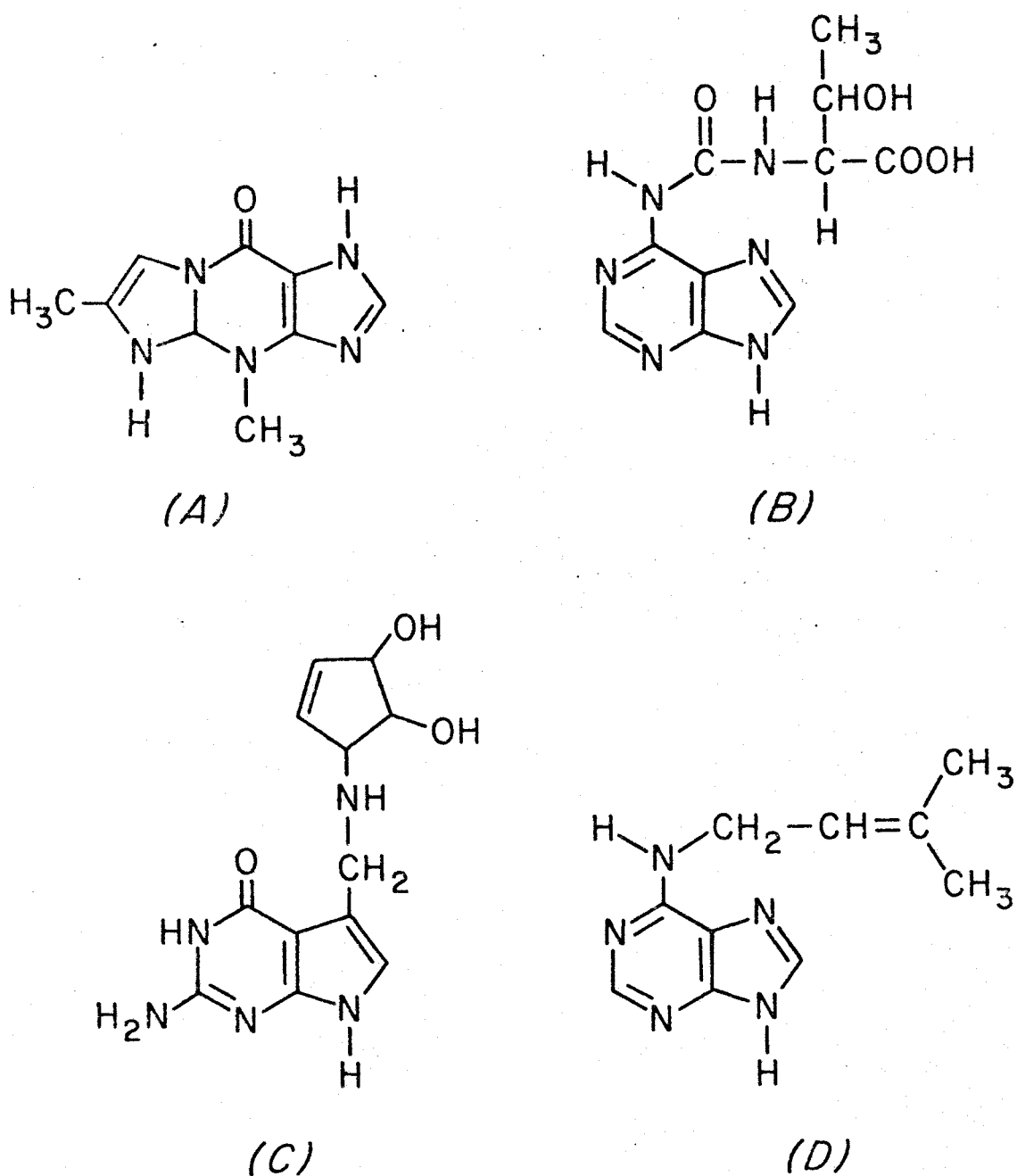
Figure 2. Structures of several hypermodified bases. A) Base Yt, B) N-[(N-9-β-D-ribofuranosylpurine-6-yl)N-methylcarbamoyl]-threonine, C) Base Q, D) N6-(Δ2-isopentenyl)adenosine.

coli tRNA hydrolysates were cytokinin-active, and by 1967, Hall and
his collaborators had isolated both i^6A and ribosylzeatin (io^6A)
from plant tRNA preparations (42). The side-chain hydroxyl group
of ribosylzeatin isolated from the plant tRNA preparations was found
to have the cis configuration.

The major cytokinin-active ribonucleoside present in E. coli
tRNA was isolated and identified as 2-methylthio-N^6-(Δ^2-isopentenyl)-
adenosine (ms^2i^6A) by Skoog and his associates in 1968 (15). E.
coli tRNA was subsequently shown by the same workers to contain i^6A
as well as ms^2i^6A (16). The presence of methylthio substituted
cytokinins in plant tRNA preparations was established by Skoog and
co-workers in 1969 with the isolation of 2-methylthioribosylzeatin
(ms^2io^6A) from wheat germ tRNA (46). Wheat germ tRNA was later
shown to contain four cytokinin-active ribonucleosides: io^6A,
i^6A, ms^2io^6A, and ms^2i^6A (14).

The presence of small amounts of the trans-isomer of zeatin
and 2-methylthiozeatin in tRNA from plant sources was first reported
by Vreman et al. (106). However, in all plant tRNA preparations that
have been examined to date, the cis-isomer is the predominant form
of the zeatin side chain. In contrast, the trans-isomer has been
identified as the major free (hormonally-active) cytokinin present
in a number of plant tissues (95). The cis-isomer has not been
isolated in free form from plant tissues, although it is the major
cytokinin present in culture filtrates of the plant pathogen,
Corynebacterium fascians (92).

Since the initial discovery by Hall and Zachau, tRNA preparations

from a wide variety of organisms have been examined for cytokinin-active ribonucleosides (41,95). With the exception of one species of mycoplasma (45), all have been shown to contain cytokinin-active ribonucleosides. In every case examined to date, the cytokinin-active ribonucleoside is i^6A or a closely related derivative. At this time, i^6A is the only cytokinin that has been found in tRNA preparations from animal sources (41,110). Transfer RNA from plant sources has been shown to typically contain i^6A and io^6A, as well as their 2-methylthio derivatives (14,42,106). A number of bacterial tRNA preparations have been shown to contain only i^6A and/or ms^2i^6A (4,16,82) as cytokinin-active constituents. However, tRNA preparations from bacterial species that act as plant pathogens or symbionts (Corynebacterium fascians, Rhizobium sp., Pseudomonas sp., and Agrobacterium tumefascians) typically contain cytokinin-active ribonucleosides with hydroxylated side chains (20,69,84,104). Agrobacterium tumefasciens, the causative agent of crown gall disease, appears to be unique in that it is the only organism examined to date in which the trans-isomer of ribosylzeatin constitutes the major cytokinin-active ribonucleoside present in the tRNA (20).

Distribution of Cytokinins with Respect to tRNA Species

The early work by Hall (43,90) and Zachau (11,112) indicated that the concentration of cytokinin-active ribonucleosides in tRNA preparations was sufficiently low that this type of modification could not occur in all tRNA molecules. Based on independent studies of the distribution of cytokinins in Escherichia coli and yeast tRNA

species, Nishimura et al. (79) and Armstrong et al.(6) suggested that cytokinin-active ribonucleosides occurred in only those tRNA species that responded to codons beginning with U. Detailed information concerning the distribution of cytokinins with respect to tRNA species is now available for Lactobacillus (82), Agrobacterium (Armstrong, personal communication) and wheat germ (100) as well as yeast (6) and E. coli (79), and less extensive data is available for a number of other organisms (41,95,110). In every case, cytokinin moieties have been restricted to tRNA species in the U-group. Furthermore, in all organisms for which sequence information is available, the cytokinin-active ribonucleoside occurs at the 3' position adjacent to the anticodon, and it always occurs in the same sequence, 5' end ←—A (last nucleoside of anticodon)—modified A—A—ψ—C→3' end (95).

Transfer RNA species that correspond to codons beginning with U include tRNA^{ser}, tRNA^{phe}, tRNA^{cys}, tRNA^{trp}, tRNA^{leu}, and tRNA^{tyr}. The distribution of cytokinin-active ribonucleosides in isoaccepting species of tRNA^{ser} and tRNA^{leu} is of particular interest, because codons for serine and leucine begin with A and C respectively, as well as with U. The cytokinin-containing tRNA^{ser} species identified in yeast (112), rat liver (99), E. coli (6,106), S. edpidermidis (4), and Drosophila melanogaster (110) all respond to codons beginning with U; however, E. coli and D. melanogaster tRNA^{ser} species that have been identified as responding to codons beginning with A, all lack cytokinin-active ribonucleosides. Similar results have been obtained for the tRNA^{leu} species that respond to codons beginning

with U and C (3,78).

Although cytokinin-active ribonucleosides have not been found in tRNA species that respond to codons beginning with nucleosides other than U, they do not necessarily occur in all U-group tRNAs, nor are they found consistently in analogous tRNA species from different organisms. For example, most eukaryotic phenylalanine tRNA species contain base Y (or one of its derivatives) at the 3' position adjacent to the anticodon (24,95). Yeast tRNA^{trp} has an unmodified adenosine at this position (53), and wheat germ tRNA^{trp}, tRNA^{cys}, and tRNA^{tyr}, as well as tRNA^{phe}, are devoid of cytokinin-active ribonucleosides (100). In general, the available information indicates that the distribution of cytokinin-active ribonucleosides is much more restricted in eukaryotes than in prokaryotes.

The presence of organelle-specific tRNA populations in eukaryotes raises additional questions concerning the distribution of cytokinin moieties in tRNA from eukaryotic sources. On the basis of results obtained with tRNA isolated from different strains of _Euglena gracilis_, Swaminathan et al. (101) suggested that the methylthiolated derivatives of i^6A and io^6A were restricted to chloroplast tRNA species. Barnett and his associates have reported that _Euglena_ chloroplast tRNA^{phe} contains a modified adenosine (thought to be ms^2i^6A) at the 3' position adjacent to the anticodon, while the cytoplasmic tRNA^{phe} from _Euglena_ contains base Y at this position (18,47). Vreman et al. (107) compared the distribution of cytokinins in whole leaf and chloroplast tRNA isolated from spinach and found significant differences in the distribution of io^6A.

Biosynthesis of Cytokinin-Active Ribonucleosides in Transfer RNA

Cytokinin-active ribonucleosides in tRNA molecules appear to be synthesized via a route involving the transfer of an isopentenyl group from Δ^2-isopentenyl pyrophosphate (Δ^2-IPP) to the adenosine at the 3' position adjacent to the anticodon in a preformed tRNA molecule. The first evidence for this pathway came from studies involving the in vivo incorporation of radioactive mevalonic acid into Lactobacillus acidophilus tRNA (80,81).

The biosynthesis of cytokinin moieties in tRNA by cell-free systems requires a suitable tRNA substrate and was first demonstrated by Hall and his associates (30) using crude homogenates of yeast and rat liver as an enzyme source and tRNA that had been treated with permanganate under mild conditions to remove the isopentenyl side chains of i^6A residues. Undermodified tRNAs from natural sources (crude mycoplasma tRNA and undermodified isoaccepting tRNA species from E. coli) have also been used as substrates for cell-free biosynthetic systems (8,91). Radioactively labelled mevalonic acid is a suitable precursor of the isopentenyl side chain in studies with crude homogenates (22,30). In more purified enzyme systems, labeled Δ^3-IPP has been used as a precursor in reaction mixtures supplimented with an IPP isomerase from pig liver (8,59,91).

The partial purification of Δ^2-IPP:tRNA isopentenyl transferase activity from yeast (59), E. coli (8,91) and Lactobacillus (49) has been achieved. The enzyme was obtained in a 400-fold purification from E. coli (8,91). It shows a strict specificity for tRNA molecules, has a mass of 55,000 daltons, and has no requirement for

organic cofactors. The recognition site for the enzyme may be the oligonucleotide, 5' end←—A—i^6A—A—ψ—C—→3' end, in which the i^6A modifications have always been found.

Very little information is available on the biochemical mechanisms involved in methylthiolation of cytokinin-active ribonucleosides in transfer RNAs. In E. coli, the reaction appears to require S-adenosyl-methionine, cysteine, and Fe^{++} (35) and occurs after the addition of the isopentenyl moiety (36). In vivo experiments, using a $rel^-met^-cys^-$ mutant of E. coli, have shown that the biosynthesis probably proceeds via the donation of sulfur by cysteine, followed by the addition of a methyl group from S-adenosylmethionine (1). The data also indicated that these reactions occurred with both tRNA and precursor tRNA (1).

No direct information is available concerning the hydroxylation of cytokinin side chains in tRNA. Mevalonic acid can serve as a precursor for the in vivo biosynthesis of both i^6A and io^6A in tobacco callus tRNA (22,75). Presumably, hydroxylation occurs after the addition of the isopentenyl side chain to the appropriate adenosine residues in the tRNA molecules. In the case of free cytokinins, the in vivo conversion of radioactive i^6Ade to io^6Ade has been observed in both plant (73) and fungal systems (74).

Function of Cytokinins in tRNA Molecules

The function of the hypermodified bases occurring in the 3' position adjacent to the anticodon of tRNA molecules has been the subject of a number of investigations. Fittler and Hall (29)

selectively modified i^6A in yeast tRNA^{ser} by treating the RNA with aqueous iodine. The same procedure was used by Uziel and Faulkner (28) to alter ms^2i^6A in E. coli tRNA^{phe}. Both groups reported that the chemical modification of the cytokinin moiety had no effect on amino-acylation. However, ribosomal binding of yeast tRNA^{ser} was affected, and the ability of E. coli tRNA^{phe} to function in in vitro polypeptide synthesis was impaired. Treatment of E. coli tRNA^{tyr} with bisulfite (34) under conditions reported to specifically modify cytokinin moieties resulted in the same effect: unaltered aminoacylation capacity and decreased ability to bind to ribosome-mRNA complexes.

Chemical modification studies involve the introduction of an anomalous group into the tRNA molecule. The possibility exists that the modification may, in itself, interfere with protein synthesis. Thus, the studies described above do not provide un-equivocal evidence of the functional significance of cytokinin modifications in protein synthesis. More convincing evidence was provided by Gefter and Russell (35). They infected E. coli cells with a transducing phage carrying a suppressor tRNA^{tyr} gene and induced the lytic phase, thus producing a large number of tRNA^{tyr} molecules. Under these conditions, three tRNA^{tyr} species were pro-duced, differing only in the extent to which the adenosine adjacent to the 3' end of the anticodon was modified. One tRNA^{tyr} had an unmodified A, the second had i^6A, and the third, ms^2i^6A. The degree of modification had no effect on the aminoacylation process. However, the ability of the tRNA^{tyr} molecules to bind to ribosomes and act as suppressors was proportional to the extent of cytokinin

modification. The tRNA^{tyr} lacking any modification on the 3' end of the anticodon was completely ineffective in in vitro assays of suppression and had a greatly reduced capacity to bind to ribosomes.

The results of Gefter and Russell provide strong support for a critical role of cytokinin modifications in determining the capacity of particular tRNA molecules to bind to the ribosome and function in translation. However, the significance of these results has been questioned by some investigators. Litwack and Peterkofsky (66) were able to isolate both normal and i^{6}A-deficient tRNAs from Lactobacillus acidophilus by limiting the mevalonic acid uptake to that which was just adequate for maximum growth. Under these conditions, the i^{6}A content of the Lactobacillus tRNA was reduced to about one-half of the normal value. The undermodified tRNA was indistinguishable from normal Lactobacillus tRNA in in vitro protein synthesis systems, as well as in ability to accept amino acids.

Cytokinin-active bases are not the only hypermodified bases that occur adjacent to the anticodon in tRNA molecules. N-(purin-6-ylcarbamoyl)threonine and its derivatives have typically been found at this position in tRNA species that respond to codons beginning with A (41). [This base has also been reported to occur in tRNA^{tyr} isolated from mammalian sources and silk worm (12)]. Base Y is a hypermodified nucleoside found adjacent to the 3' end of the anticodon in most eukaryotic phenylalanine tRNAs. It is a flourescent molecule and, therefore, easily monitored. A number of studies concerning the function of this nucleoside have been published. It has been observed that excision of base Y causes

inability of the tRNA^{phe} to participate in polyU-induced ribosome binding (103), a decrease in the rate of tRNA-aminoacyl synthetase complex formation (61), and a differential preference for the codon UUC instead of UUU (37,86). Ghosh and Ghosh (37) suggested that the occurrence of the latter phenomenon was perhaps due to disruption of the stacking arrangement of anticodon nucleosides in the absence of Y. This suggestion was supported by proton magnetic resonance studies showing the importance of the Y base in stabilizing the stacking of nucleosides in a hexanucleotide containing Y (52). It appears likely that the function of base Y in tRNA^{phe} is analogous to that of the cytokinin-active ribonucleosides in other tRNA species that respond to codons beginning with U.

The possibility that the cytokinin modifications in tRNA may have regulatory significance is suggested by a number of reports of variations in the relative abundance of cytokinin-containing tRNA species and types of cytokinin modifications that occur in tRNA (9,42,57,67,85,93). An enzyme that cleaves the isopentenyl side chain from i^{6}A in tRNA molecules has been reported to occur in Lactobacillus (71), and it is conceivable that the production of cytokinin modifications in tRNA may be reversible. Regulatory roles have been established for other modified bases in tRNA (94), but there is as yet no definitive evidence that cytokinin modifications in tRNA are involved in regulatory mechanisms.

Relationship of Cytokinins in tRNA to the Hormonal Function of

Cytokinins in Plants

The discovery of cytokinin-active ribonucleosides in tRNA mole-

cules provoked a great deal of speculation on the nature of the

relationship between cytokinins in tRNA and the hormonal function

of free cytokinins in plant tissues. As early as 1962, McCalla et

al. (70) noted the incorporation of small quantities of the cytokinin

N^6-benzyladenine (bzl^{6}Ade) into RNA when [^{14}C]-bzl^{6}Ade was applied

to cocklebur leaves. Others obtained similar results with kinetin

and tobacco leaves (111). In 1966, Fox (31) reported the specific

incorporation of a small amount of [^{14}C]-bzl^{6}Ade into the soluble

RNA fraction isolated from cytokinin-dependent tobacco and soybean

callus tissue. Fox and Chen later confirmed this report (32).

Srivastava (98), however, observed uniform distribution of cytokinin

incorporation into sRNA and rRNA. Others did not observe any in-

corporation of cytokinins into RNA (89), and Key (54) raised the

possibility that the apparent cytokinin incorporation reported by

Fox might be due to noncovalent association of cytokinins with the

tRNA molecules.

Kende and Tavares (55) attempted to devise a test to separate

the hormonal action of cytokinins from their incorporation into

RNA. A bzl^{6}Ade derivative, [^{14}C]-6-benzylamino-9-methylpurine, was

synthesized. The radioactivity incorporated into the RNA of cyto-

kinin-dependent soybean callus tissue grown on [^{14}C]-bzl^{6}Ade and

[^{14}C]-6-benzylamino-9-methylpurine was compared. The results indi-

cated that the presence of a methyl group in the nine position

prevented detectable incorporation into RNA, although the cytokinin activities of the two compounds were identical. The design of this experiment was later criticized on several counts (95,33), and it is not clear whether bzl^{6}Ade incorporation was, or could have been, detected in either tissue.

The possibility that the apparent incorporation of cytokinins into RNA molecules was due to contamination with free cytokinins was given support by Bezemer-Sybrandy and Veldstra (10), who examined the metabolism of [^{14}C]-bzl^{6}Ade in Lemna. They observed that the 5'-monophosphate ester of bzl^{6}A, rather than the 3'-ester of bzl^{6}A, was obtained after alkaline hydrolysis of Lemna tRNA. They concluded that it was unlikely that bzl^{6}A was actually incorporated into the tRNA, but rather that the tRNA hydrolysate was contaminated with 5'-bzl^{6}AMP. Non-covalent absorption of cytokinins to RNA preparations was also observed by Elliott and Murray (26) in studies with the soybean callus system.

The demonstration of the Δ^2-IPP:tRNA isopentenyl transferase pathway in microbial systems raised additional doubts concerning the possible role of free cytokinins as precursors of the cytokinin ribonucleosides in tRNA. Chen and Hall (22) reported that tRNA from cytokinin-dependent tobacco callus cultures could be labeled with mevalonic acid, both in vivo and in crude cell-free systems, and concluded that in this tissue the pathway for the formation of cytokinin-active ribonucleosides in tRNA was similar to that in microbial systems.

In an attempt to clarify some of the questions concerning

incorporation of cytokinins into plant tRNA molecules, Burrows et al.
(17) isolated and unequivocally identified the cytokinin-active
ribonucleosides from tRNA of cytokinin-dependent tobacco callus
grown on bzl^{6}Ade. The cytokinins typically found in plant tissue
tRNA, ms^{2}io^{6}A, i^{6}A, and io^{6}A, were identified as the major cytokinin-
active constituents of tobacco callus tRNA, but the tRNA also con-
tained a small amount of bzl^{6}A. Later studies (108) using double-
labeled bzl^{6}Ade demonstrated that the intact base was incorporated
into RNA.

The problem of cytokinin incorporation into plant RNA prepara-
tions was placed in a new perspective by Martin (68) and Dyson (25)
working in Fox's laboratory. In contrast to the earlier results of
Fox (31) and Fox and Chen (32), they found a preferential incorpora-
tion of labeled bzl^{6}Ade into high molecular weight RNA (presumably
ribosomal RNA) isolated from tobacco and soybean tissue cultures.
In fact, in short term labeling experiments, little if any label
could be detected in the tRNA fraction. These results were confirmed
by Armstrong et al.(5), who presented extensive evidence that the
observed incorporation actually involved covalent attachment to
polynucleotides. Murai et al. (77) found that kinetin (N^6-furfuryl-
adenine, fr^{6}Ade), a synthetic cytokinin, was also incorporated into
tobacco callus RNA and at concentrations four times greater than
that of bzl^{6}A. As in the case of bzl^{6}Ade, kinetin was preferentially
incorporated into the ribosomal RNA fraction. However, it was not
specifically associated with either of the two major cytoplasmic
ribosomal RNA components (both rRNA species being equally labeled) (76).

In summary, it appears clear that free cytokinins are not the normal precursor molecules for cytokinin-active ribonucleosides in plant tRNA molecules. Although the possibility still exists that the hormonal function of free cytokinins may involve incorporation into some type of RNA molecule, the low levels of the observed incorporation and the lack of any clear specificity are consistent with incorporation as the result of transcriptional errors.

An alternate precursor-product relationship between the cytokinin ribonucleosides of tRNA and free cytokinins has been proposed by Chen and Hall (22) who suggested that degredation of cytokinin-containing tRNA molecules might serve as a route for free cytokinin biosynthesis. The biosynthetic pathway for free cytokinins has not been conclusively established, but the synthesis of other free modified bases and their corresponding nucleosides in tRNA appear to involve separate pathways (62,105). Chen et al. (21) have used studies with adenine analogs to provide evidence for a separate biosynthetic pathway for free cytokinins, and a cell-free system that synthesizes cytokinins from Δ^2-IPP and AMP has recently been obtained from Dictyostelium (102). In addition, the turnover rate for the rather stable tRNA molecules would have to be fairly high to account for the amount of free cytokinins in plant tissues. Attempts have been made to calculate the half-life of Lactobacillus and Zea tRNAs with the ambiguous conclusion that the turnover rate might be sufficient to account for free cytokinin formation (58, 63).

Cherry has suggested that free cytokinins might inhibit ribonucleases that specifically cleave cytokinin-containing tRNA molecules

(23). Babcock and Morris (7) have reported the occurrence of an en-
zyme in peas that specifically cleaves a cytokinin-containing tRNA^{leu}
species. Enzymes which specifically excise other hypermodified
bases, such as base Q, are also known (27). However, cytokinins
were without effect on the enzyme activity detected by Babcock and
Morris. Thus, the relationship (if any) between the hormonal func-
tion of cytokinins in plant systems and the presence of cytokinin-
active modified bases in tRNA is still uncertain.

III. MATERIALS AND METHODS

Materials. Seeds of Phaseolus vulgaris (var. Bush Blue Lake 274) were kindly supplied by Asgrow Seed Company.

The following enzyme preparations were purchased from Sigma Chemical Company: lyophilized snake venom (Crotalus adamenteus), alkaline phosphatase (from calf intestinal mucosa, 1025 units per mg protein), and ribonuclease T_1 (Grade III, about 340,000 units per mg protein).

Whatman DE-23 cellulose was used for diethylaminoethyl-cellulose (DEAE-cellulose) chromatography. Sephadex LH-20 and Sephadex G-25 are products of Pharmacia. The RPC-5 column packing material was purchased from Astro Enterprises, Inc., Powell, Tennessee. Benzoylated DEAE-cellulose was prepared as described by Gillam et al. (38).

The $\left[^{14}C\right]$-amino acids were purchased from New England Nuclear Corporation. Cetyltrimethylammonium bromide was purchased from Sigma.

Silanized glassware was prepared by treatment with 5% (v/v) dichlorodimethylsilane in benzene.

Growth of Etiolated Bean Seedlings. Phaseolus vulgaris seeds (var. Bush Blue Lake) were planted in vermiculite and grown in the dark at 25° C for seven days. The plants were watered under a dim green safe-light. The length of the hypocotyls varied between four and eight cm at the time of harvest. Approximately one kg of

seedling tissue was obtained from 250 g of seeds.

Isolation of Phaseolus tRNA. All operations prior to homo-
genization were performed under a dim green safe-light. The intact,
etiolated seedlings were divided into 225 g lots. Each lot was
homogenized in a Waring blender (30 sec low speed, 30 sec high speed)
in 75 ml 0.1 M Tris-HCL buffer (pH 7.5) containing 2% (w/v) naph-
thalene-1,5-disulfonate (disodium salt) and 200 ml of buffer-
saturated phenol containing 0.1% (w/v) 8-hydroxyquinoline. The
homogenate was stirred for no less than 30 minutes at room tempera-
ture and then centrifuged (10,400 g, 30 min). The aqueous phase
was combined with one-third volume of a buffer-saturated phenol/m-
cresol mixture (10/1,v/v), stirred, and centrifuged as described
above. RNA was precipitated from the aqueous phase of the second
phenol treatment by addition of one-tenth volume of 20% (w/v)
potassium acetate (pH 6.0, glacial acetic acid) and 2 volumes of
cold ethanol. All subsequent steps were performed in the cold
except as indicated. All centrifugations were at 9000 g for 30
min except as indicated.

The RNA precipitate was stored overnight at -20 C, collected by
centrifugation, drained as dry as possible, and extracted twice
with 2.5 M potassium acetate (pH 6.0, glacial acetic acid) containing
0.2% (w/v) tetraethyl ammonium bromide (TEAB). Each extraction was
performed by suspending the precipitate in 125 ml of the solution
per kg of original tissue, homogenizing (Sorvall Omnimixer; medium
speed; 30 sec on, 30 sec off, for 5 min), stirring for 25 min, and
then centrifuging. The supernatant liquids from the two extractions

were combined, and the RNA precipitated from the extract by addition of two volumes of cold ethanol.

The RNA recovered from the potassium acetate extract was washed twice with cold ethanol containing 0.1 $\underline{M}$ NaCl and then further purified using the methoxyethanol partition method of Kirby (56) as modified by Ralph and Bellamy (87). The washed pellets were homogenized (as above) in 0.025 $\underline{M}$ Tris-HCl (pH 8.0) containing 0.025 $\underline{M}$ NaCl and 0.2% (w/v) TEAB (100 ml per kg of original tissue). Equal volumes (100 ml per kg of original tissue) of 2.5 $\underline{M}$ K_2HPO_4 (pH 8.0, phosphoric acid) and methoxyethanol (ethylene glycol monomethyl ether) were added to the homogenate. The mixture was stirred for 15 min and centrifuged (5900 $\underline{g}$, 15 min). The upper phase (approximately 5/6 of the total liquid volume) was removed and stirred until it reached room temperature. An equal volume of 0.2 $\underline{M}$ sodium acetate (pH 8.0, glacial acetic acid), followed by one-half volume of 1.0% (w/v) cetyltrimethyl ammonium bromide (CTAB) were added (at room temperature). The precipitated CTA-RNA complex was recovered by centrifugation and the RNA converted to the sodium form by repeated suspension in cold 1.0 $\underline{M}$ sodium acetate (pH 8.0, glacial acetic acid) followed by precipitation with two volumes of cold ethanol. The conversion was judged to be complete when the precipitate dissolved almost completely in the sodium acetate solution and foam was no longer evident.

The RNA precipitate from above was dissolved in 0.1 $\underline{M}$ Tris-HCL (pH 7.5) containing 0.2 $\underline{M}$ NaCl, and applied to a DEAE-cellulose column (20 ml bed volume per kg original tissue) equilibrated with

the same buffer. The column was then washed with about 30 bed volumes of 0.1 $\underline{M}$ Tris-HCl (pH 7.5) containing 0.2 $\underline{M}$ NaCl. The tRNA was eluted from the column with 0.1 M Tris-HCL containing 1.0 $\underline{M}$ NaCl ($\underline{ca}$. 8 bed volumes). The tRNA was recovered from the column eluate by ethanol precipitation. The final yield was about 1500 A_{260} units tRNA per kg of original tissue.

Isolation of Cytokinin-Active Ribonucleosides from Phaseolus tRNA. The Phaseolus tRNA preparation was hydrolyzed to nucleosides by treatment with ribonuclease T_1 (75), followed by snake venom phosphodiesterase and alkaline phosphatase (39). Phaseolus tRNA (6070 A_{260} units) was dissolved in 110 ml distilled water, and the solution adjusted to pH 7.5 with 0.1 $\underline{N}$ NaOH. Ribonuclease T_1 (5 units per A_{260} unit tRNA) was added to the tRNA solution. The solution was incubated at 35 C for four hours. Toluene was added (1 or 2 drops) periodically to prevent bacterial growth. The pH was checked every 30 min and maintained at pH 7.5 with 0.1 $\underline{N}$ NaOH throughout the RNase T_1 digestion. At the end of the four hour incubation period, the pH of the solution was adjusted to 8.6. Magnesium sulfate (0.01 ml 0.1 $\underline{M}$ MgSo$_4$ per ml incubation volume), lyophilized snake venom (0.01 mg per A_{260} unit tRNA), and alkaline phosphatase (0.01 unit per A_{260} unit tRNA) were then added to the solution. The solution was incubated at 35 C for eight hours. The pH was checked periodically and adjusted to pH 8.6 as needed. A few drops of toluene were also added as needed. Additional MgSO$_4$ (0.01 ml 0.1 $\underline{M}$ MgSO$_4$ per ml incubation volume), snake venom (0.006

mg per A_{260} unit), and alkaline phosphatase (0.01 units per A_{260} unit) were added after eight hours incubation. The incubation was then continued for sixteen hours. The hydrolysate was adjusted to pH 7.5 with 0.1 $\underline{N}$ HCl and two volumes of ethanol were added. Any precipitate that formed was removed by centrifugation at 3000 $\underline{g}$ for 10 min. The supernatant liquid was evaporated to dryness under reduced pressure at 37 C in a silanized evaporating flask. Silanized glassware was used in all subsequent procedures with the exception of the column used for Sephadex LH-20 chromatography.

The dry solids recovered from the hydrolysate were fractionated according to the procedure of Armstrong et al.(2). The solids were extracted six times (15 min each) with water-saturated ethyl acetate (30 ml per extraction). The extracts were combined and evaporated to dryness as described above. The ethyl acetate soluble nucleosides thus obtained were dissolved in 6 ml of 33% (v/v) ethanol and chromatographed on a Sephadex LH-20 column (2 x 58.5 cm) in the same solvent. The elution positions of i^6A and io^6A were determined by chromatographing authentic samples of these compounds on the same column after completion of the fractionation of the Phaseolus tRNA nucleosides. Fractions from the Sephadex LH-20 column eluate were pooled on the basis of the elution position of the standards and taken to dryness under reduced pressure at 37 C. The solids recovered from the pooled fractions corresponding to the elution position of io^6A were redissolved in 5 ml of distilled water and rechromatographed on a Sephadex LH-20 column (2 x 60 cm) with distilled water as the eluent. The pooled cytokinin-active fractions

recovered from the Sephadex LH-20 colums were used for the identification of cytokinin ribonucleosides as described below.

Identification of Cytokinin-Active Ribonucleosides. The identification of the cytokinin ribonucleosides isolated from Phaseolus tRNA was performed by Dr. Roy O. Morris and Dr. Richard W. Kaiss-Chapman (Dept. of Agricultural Chemistry, Oregon State University). The composition of the cytokinin-active fractions from Sephadex LH-20 columns was examined by high performance liquid chromatography (HPLC) using a Waters ALC 202 HPLC System equipped with two Model 600 pumps and a Model 660 Solvent Programmer (Waters Associates, Milford, MA). Gas-liquid chromatography-mass spectrometry (GLC-MS) was carried out with a Varian 1200 Gas-Liquid Chromatograph interfaced with a Varian MAT CH7 Mass Spectrometer via a single-stage glass jet separator. Data acquisition from this system was by means of an on-line System 150/PDP-8 computer (System Industries, Sunnyvale, CA).

For HPLC analysis, samples consisting of 1% of the cytokinin-active materials recovered from Sephadex LH-20 columns were applied to a 2.1 x 250 mm μBondapak C_{18} column (Waters Associates) equilibrated with 0.02 M ammonium acetate (pH 3.5). The column was eluted with a linear ethanol gradient (0 to 70%, v/v, in the ammonium acetate buffer) over 18 min at a flow rate of 1.5 ml per min. The HPLC chromatograms of the samples were compared to similar chromatograms of authentic cytokinins and their ribosides.

For GLC-MS analysis, samples consisting of 70% of the cytokinin-active materials recovered from the Sephadex LH-20 columns were

permethylated by a modification of the method of Hakomori (49) as described by Kaiss-Chapman (19). The remainder of the cytokinin-active material was perdeuteromethylated in the same manner. The derivatized samples were introduced into the GLC-MS system by injection into a capillary glass column (0.5 mm x 20 m) coated with a solution containing 8 mg per ml Dexsil 300 (Analabs, Inc., North Haven, CT) and 1 mg per ml Silanox 101 (Cabot Corp., Boston, MA) in chloroform. The temperature was held at 200 C for 3 min following sample injection and then raised to 325 C at a rate of 6 C per min. Helium was used as the carrier gas at a linear flow rate of 30 cm per sec. Approximately one-sixth of the column effluent was diverted to the flame ionization detector of the gas chromatograph. The mass spectra of suspected cytokinin ribonucleosides were compared with mass spectra of authentic cytokinin ribonucleosides obtained in the same manner.

Chromatographic Fractionation of Phaseolus tRNA. Crude Phaseolus tRNA was fractionated by BD-cellulose chromatography (38). The crude tRNA preparation (7300 A_{260} units dissolved in 75 ml of 0.4 M NaCl) was applied to an unbuffered BD-cellulose column equilibrated with the same solvent. The tRNA was eluted with a 3000 ml linear salt gradient (0.4 M to 1.0 M NaCl). Fractions (20 ml) were collected at a flow rate of ca. 1.7 ml per min. The salt gradient was discontinued at ca. 0.86 M, and the column purged with 1.0 M NaCl in 15% (v/v) ethanol.

Cytokinin-active tRNA fractions recovered from the BD-cellulose column eluate were further fractionated by RPC-5 column chromatography

(83). The tRNA samples were chromatographed on an RPC-5 column (1.27 x 47 cm, packed at 150 p.s.i.) equilibrated with 0.01 $\underline{M}$ Tris-HCl (pH 7.5) containing 0.01 $\underline{M}$ MgCl$_2$, 0.001 $\underline{M}$ β-mercaptoethanol and 0.45 $\underline{M}$ NaCl. The tRNA samples were applied to the column in 5 ml of the equilibrating buffer solution and eluted with a 1680 ml linear salt gradient (0.45 $\underline{M}$ or 0.50 $\underline{M}$ NaCl to 0.70 $\underline{M}$, 0.75 $\underline{M}$, or 0.85 $\underline{M}$ NaCl, depending on the tRNA sample) in the same buffer solution. After most of the tRNA sample appeared to have been eluted from the column, the initial gradient was discontinued, and the column purged with buffer solution containing 1.2 $\underline{M}$ NaCl. The column was maintained at 37 C and 120 p.s.i. during chromatography. Fractions (12 ml) were collected at a flow rate of $\underline{ca}$. 2 ml per min.

Preparation of Aminoacyl-tRNA Synthetases. All precedures were performed at 4 C. Etiolated bean seedlings (50 g) were macerated with a mortar and pestle in 50 ml 0.1 $\underline{M}$ Tris-HCl buffer containing 0.01 $\underline{M}$ MgCl$_2$, 0.04 $\underline{M}$ β-mercaptoethanol, and 20% (v/v) glycerol. The macerate was strained through cheesecloth, sonicated (Bronwill Biosonic IV, 100% at low setting for 2 min), and then centrifuged (12,000 $\underline{g}$, 15 min). The supernatant liquid was adjusted to pH 7.5 with 0.2 $\underline{M}$ ammonium hydroxide and recentrifuged at 27,000 $\underline{g}$ for one hour. The pellet was discarded and the supernatant liquid readjusted to pH 7.5 as described above. Streptomycin sulfate was added to a final concentration of 2.0% (w/v) by adding an appropriate volume of a 10% (w/v) streptomycin sulfate solution dropwise with stirring. The suspension was allowed to stand in the cold for 15 min and then centrifuged (27,000 $\underline{g}$, 30 min). The supernatant liquid was

recovered, and solid ammonium sulfate was added to 75% saturation.
The precipitated protein was recovered in one tube by repeated
centrifugation (27,000 $\underline{g}$, 25 min). The pellet was dissolved in
3.5 ml 0.01 $\underline{M}$ K_2HPO_4 (pH 7.5, phosphoric acid) containing 0.001 $\underline{M}$
β-mercaptoethanol and 0.002 $\underline{M}$ EDTA (ethylenediamine-tetraacetic acid,
disodium salt) and centrifuged (27,000 $\underline{g}$, 25 min) to remove any un-
dissolved solids. The resulting supernatant liquid was applied to
a Sephadex G-25 column (1.5 x 60 cm) equilibrated with the same
buffer. The column eluate was collected in 4.0 ml fractions. The
five fractions containing the highest concentration of protein were
pooled and mixed with an equal volume of glycerol. The crude enzyme
preparation was stored at -20 C. (Some of the aminoacyl-tRNA syn-
thetases were stable under these conditions, but the activity of
others declined rapidly within a few days after the preparation was
made.) The crude synthetase preparation was diluted 1:1 (v/v) with
8 m$\underline{M}$ dithiothreitol immediately before use in the amino acid acceptor
assays. For serine acceptor assays, the enzyme-dithiothreitol
mixture was preincubated at 30 C for 30 min prior to use.

Amino Acid Acceptor Assays. Aminoacylation reactions were
carried out in 0.1 ml reaction volumes containing the components
listed in Table I. (Optimum conditions for each of the aminoacyla-
tion reactions were determined in preliminary experiments.) Each
reaction volume was prepared from 50 μl of a concentrated assay
mix (containing buffer, $MgCl_2$, ATP, [^{14}C]-amino acid, and KCl at
twice the concentrations specified in Table I), 25 μl of the ap-
propriate tRNA fraction, and 25 μl of the aminoacyl-tRNA synthetase

Table I. CONCENTRATION OF ACCEPTOR ASSAY COMPONENTS IN FINAL
          REACTION VOLUMES

[^{14}C]-Amino Acid (0.1 μCi/0.1 ml)	HEPES (pH 8) (m$\underline{M}$)	MgCl$_2$ (m$\underline{M}$)	ATP (m$\underline{M}$)	KCl (m$\underline{M}$)	Dithio-threitol[1] (m$\underline{M}$)
Cys	50	25	5	0	3
Leu	100	10	10	0	1
Phe	100	10	5	50	1
Ser	50	10	5	0	1
Try	100	10	5	0	1
Tyr	50	10	5	0	1

[1] These values include the dithiothreitol contributed to the reaction
volumes by the synthetase preparation after dilution with dithio-
threitol solution as described in Materials and Methods.

preparation diluted with dithiothreitol as described above. (The

cysteine assay mix also contained 4 m$\underline{M}$ dithiothreitol. ATP was

neutralized (pH 7.5) before addition to the assay mixes. An aliquot

part (0.5 ml) of each tRNA sample had been precipitated with 1/10

volume 0.6 $\underline{M}$ MgCl$_2$, stored at -20 C for 24 hours, and redissolved in

an equal amount (0.5 ml) of distilled water. This sample was used

for accepter assays because preliminary tests indicated that the

aminoacyl-tRNA synthetases were inhibited by NaCl.) The reaction

volumes were incubated at 30 C for 60 min except in the case of the

serine acceptor assays which were incubated for 120 min. At the

end of the incubation period, 50 μl aliquots of the reaction volumes

were removed and applied to 2.3 cm diameter paper of 2.4 cm diameter

glass fiber discs. (Whatman 3MM filter paper discs were used for

the serine, leucine, and phenylalanine assays; Whatman GF/C glass

fiber discs were used for the tryptophan tyrosine, and cysteine

assays.) The discs were immediately placed in cold 10% (w/v) tri-

choloroacetic acid (10 ml per disc), and washed for 10 min. This

wash was repeated, followed by three washes in 5% (w/v) trichloroacetic

acid (5 ml per disc, 5 min), and two washes in Hokin's reagent

(10 ml per disc, 10 min, then 5 ml per disc, 5 min) (51). The discs

were dried, placed in scintillation vials with 5 ml of a toleune-

based scintillation fluid (Omnifluor, New England Nuclear), and

counted in a Packard Model 2405 scintillation counter. Counting

efficiencies were determined by applying a known amount of [^{14}C]-

amino acid mix to a filter disc.

Determination of Cytokinin Activity. The cytokinin activity

of nucleoside fractions and tRNA fractions was determined in the tobacco callus bioassay (65,6).

Transfer RNA samples were recovered from the column eluates by addition of one/tenth volume of 0.6 $\underline{M}$ $MgCl_2$, followed by two and one-half volumes of cold 95% (v/v) ethanol (88). The precipitated RNA was allowed to stand at -20 C for at least 24 hours and then recovered by centrifugation (27,000 $\underline{g}$, 15 min). Nucleoside fractions from Sephadex LH-20 fractionations were evaporated to dryness under reduced pressure at 37 C prior to testing for cytokinin activity.

The sample sizes used in bioassays of tRNA fractions varied from 20% of the RNA recovered from appropriately pooled column fractions (BD-cellulose fractionation) to 50% of the RNA recovered (RPC-5 fractionations). Bioassays of nucleoside fractions from Sephadex LH-20 colums were performed using 20% aliquot parts of the pooled column fractions. All bioassay samples, including nucleoside fractions, were acid hydrolyzed in 5 ml of 0.1 N HCl (100 C, 45 min) prior to bioassay. The neutralized hydrolysates were incorporated into 100 ml of RM-1964 medium containing 2 mg per liter indole-3-acetic acid and tested in five-fold serial dilutions in the tobacco bioassay as described by Armstrong et al. (6).

Cytokinin activity is expressed as Kinetin Equivalents (μg KE), defined as the μg of kinetin, 6-furfurylaminopurine, required to give the same growth response as the test sample under the specified bioassay conditions.

IV. RESULTS

Isolation of Cytokinin-Active Ribonucleosides from Phaseolus

tRNA. Crude Phaseolus tRNA was hydrolyzed to nucleosides with snake

venom phosphodiesterase and alkaline phosphatase and the lyophilized

hydrolysate extracted with water-saturated ethyl acetate as des-

cribed in Materials and Methods. The ethyl acetate soluble nucleo-

sides thus obtained were fractionated by chromatography on a

Sephadex LH-20 column in 33% ethanol (Fig. 3). The elution profile

was similar to that obtained for other plant tRNA preparations (14,

106). Cytokinin activity eluted as two peaks (fractions III and V),

which corresponded to the elution positions of the io^6A and i^6A

standards. (Under these conditions, ms^2io^6A elutes slightly later

and overlapping on i^6A.) No cytokinin activity was detected in the

latter portion of the elution profile where ms^2i^6A would be expected

to elute.

The solids recovered from fraction V were subjected to HPLC

and GLC-MS analysis as described below. The solids recovered from

fraction III were rechromatographed on a Sephadex LH-20 column in

water (Fig. 4). Most of the cytokinin activity eluted in fraction

III-4, corresponding to the elution position of io^6A. The solids

recovered from this fraction were subjected to HPLC and GLC-MS

analysis. The small amount of cytokinin activity eluting prior to

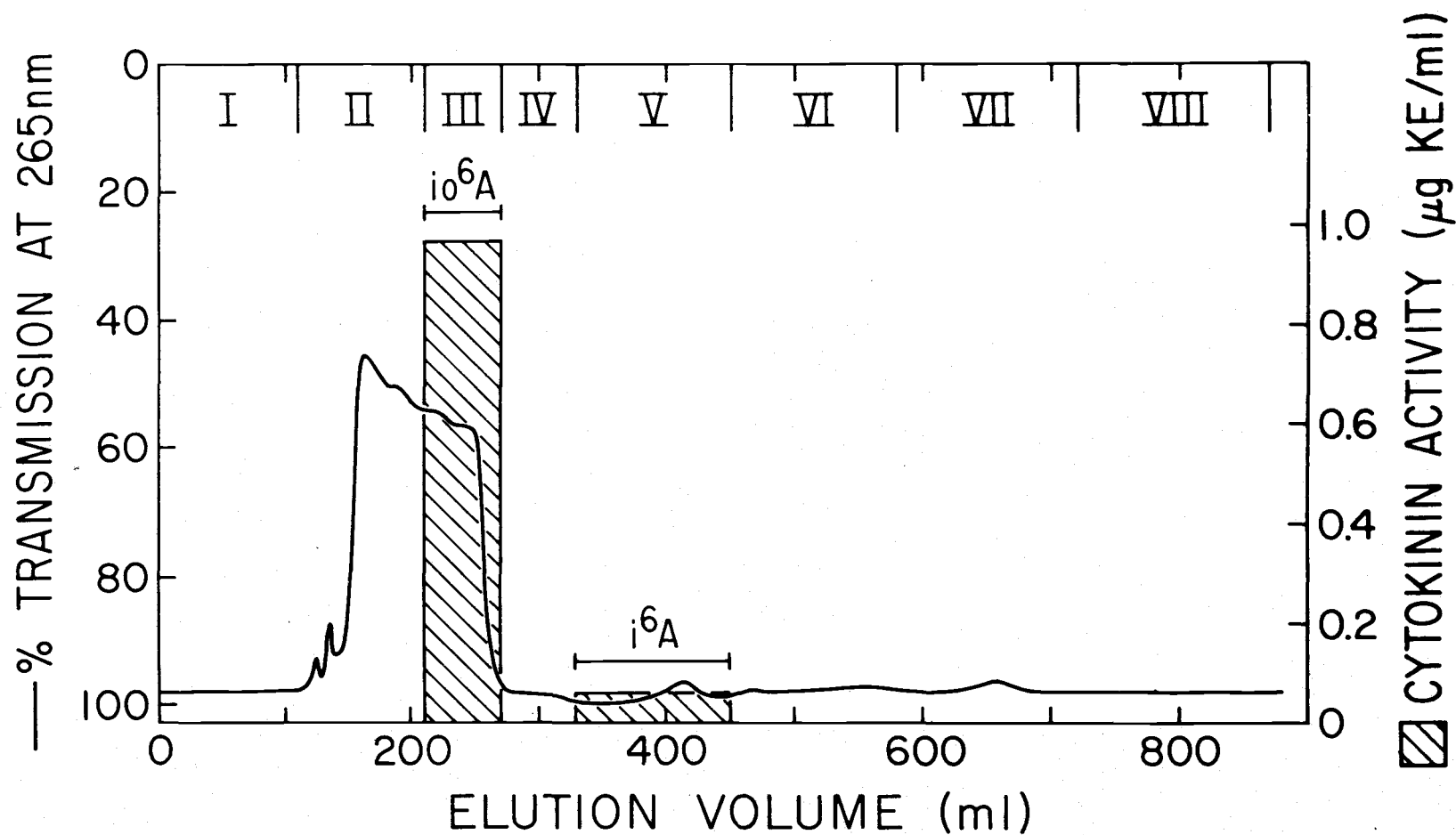the elution position of the io^6A standard can probably be attributed

Figure 3. Sephadex LH-20 chromatography of ethyl acetate-soluble nucleosides from _Phaseolus vulgaris_ tRNA. Nucleosides were chromatographed in 33% ethanol as described in Materials and Methods.
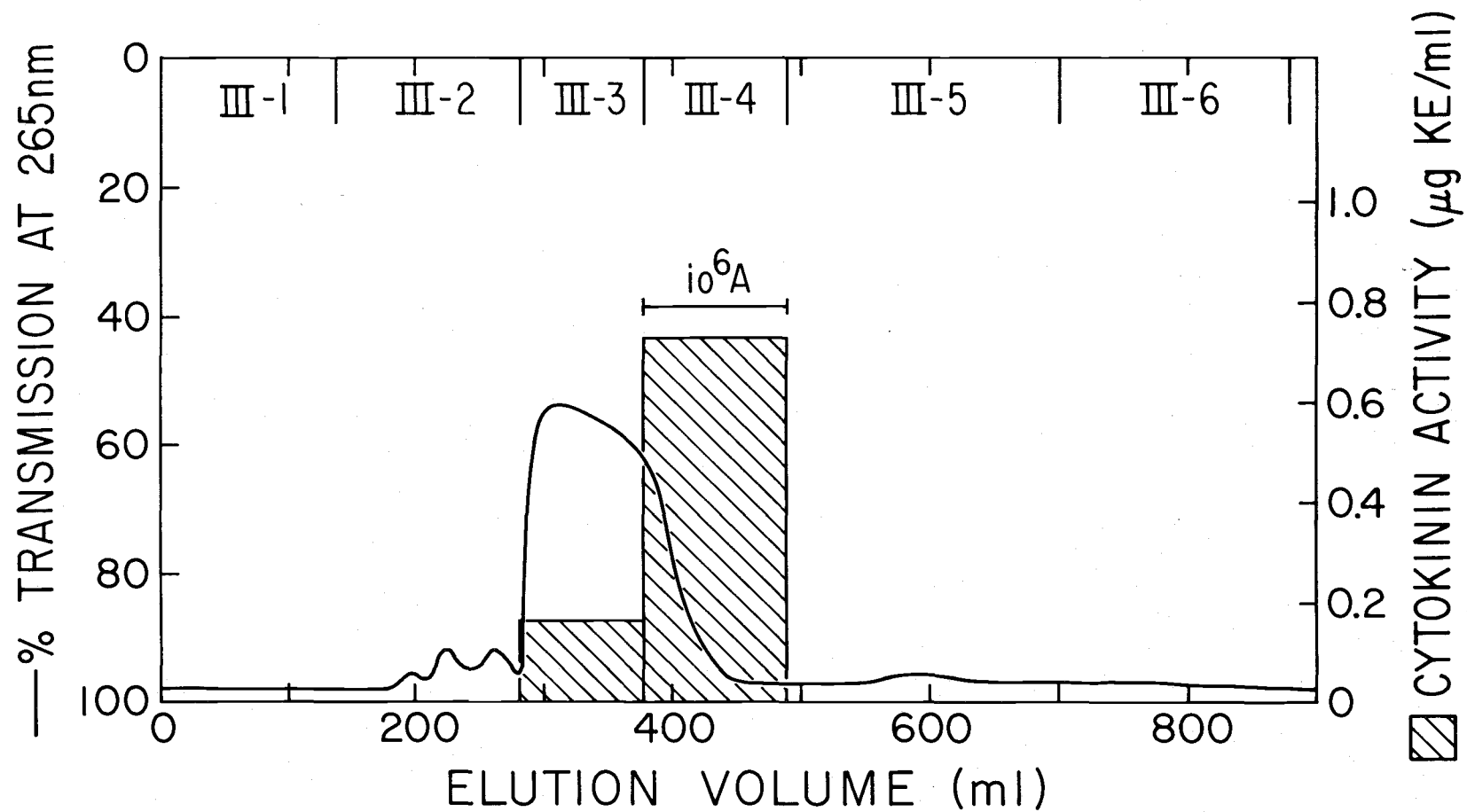
Figure 4. Rechromatography of fraction III nucleosides on Sephadex LH-20 in distilled water. Details of the chromatographic procedures are given in Materials and Methods.

to spreading of the _Phaseolus_ io^{6}A peak but analysis of this fraction in not complete at this time.

Identification of Cytokinin-Active Ribonucleosides. The identification of the cytokinin ribonucleosides recovered from the Sephadex LH-20 columns was performed by Dr. Roy O. Morris and Dr. Richard Kaiss-Chapman (Dept. of Agricultural Chemistry, Oregon State University). The results are summarized here.

The HPLC fractionation of small aliquots of the cytokinin-active fractions (V and III-4) recovered from the Sephadex LH-20 column gave the results shown in Figure 5. (The elution positions of authentic cytokinins chromatographed under the same conditions are shown in Figure 5A.) Fraction V (from the Sephadex LH-20 column in 33% ethanol) was separated into two UV-absorbing peaks by HPLC chromatography. One peak exhibited a mobility identical to that of an authentic sample of i^{6}A; the second peak exhibited a mobility intermediate between 6-furfurylaminopurine (fr^{6}A) and i^{6}A. Fraction III-4 (from the Sephadex LH-20 column in distilled water) also exhibited two peaks when fractionated on the HPLC column. One peak eluted at approximately the same position as a _cis_-io^{6}Ade standard. The large, early peak of UV-absorbing material did not correspond in mobility to any of the standards and was later identified as deoxyadenosine. (Presumably this compound originated from DNA contaminants of the tRNA preparation.)

GLC-MS analysis of the permethylated and perdeuteromethylated derivatives of the cytokinin-active samples from the Sephadex LH-20 columns revealed that fraction V contained two cytokinins, i^{6}A and
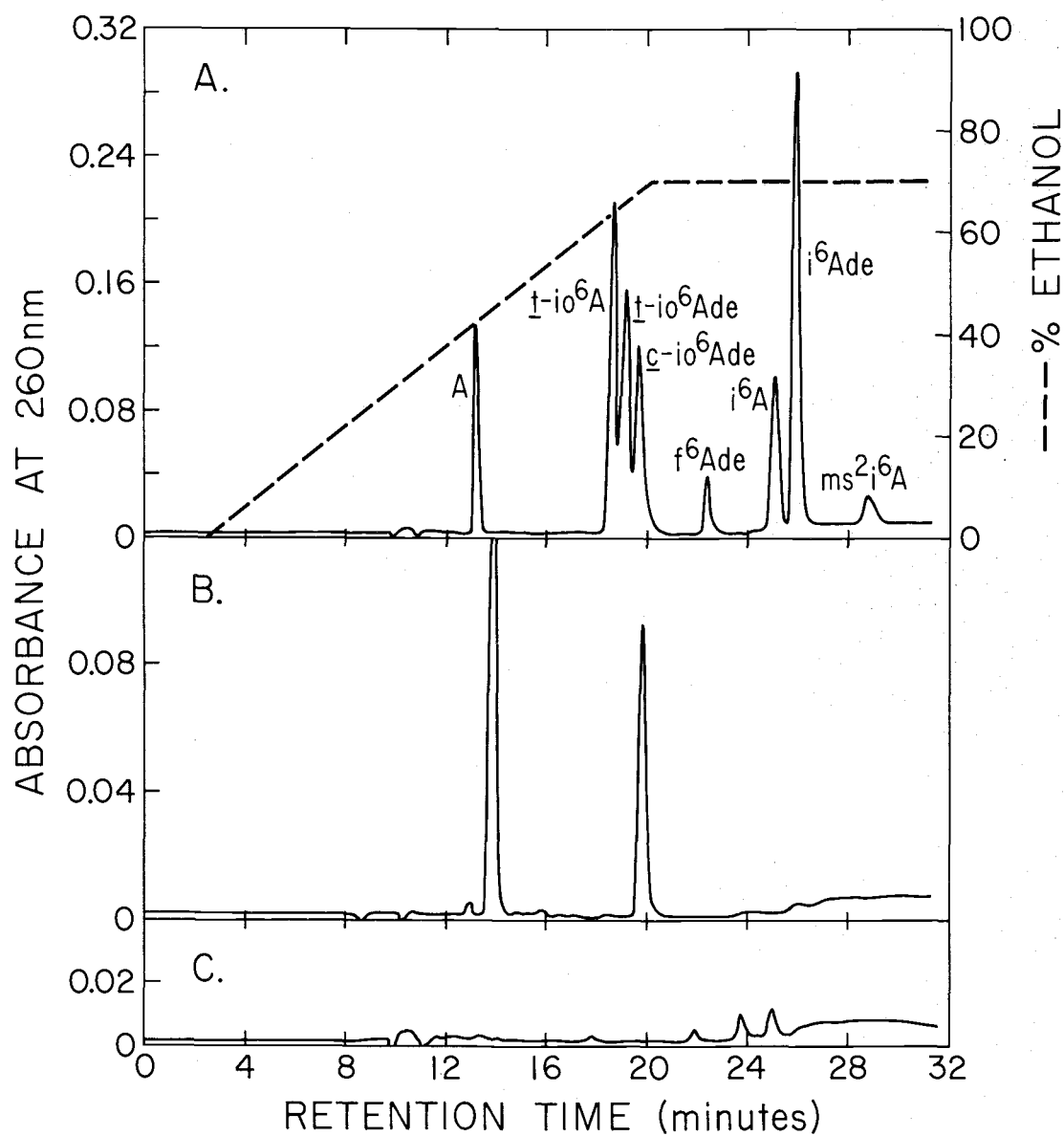
Figure 5. HPLC analysis of cytokinin-active nucleosides from <u>Phaseo-</u>
<u>lus</u> <u>vulgaris</u> tRNA. A) Cytokinin standards, B) Fraction
III-4 nucleosides, and C) Fraction V nucleosides. Details
of chromatographic procedures are described in Materials
and Methods.

ms^2io^6A. (the isomeric configuration of the side-chain hydroxyl group in ms^2io^6A has not yet been determined.) Fraction III-4 was shown to contain c-io^{6}A and deoxyadenosine. The permethyl derivatives of these compounds showed mass spectra and GLC retention times identical to those previously published (20,19). The permethyl derivative of i^{6}A showed a molecular ion at m/e = 391, with major fragment ions at m/e values of 348, 217, and 174. The permethyl derivative of ms^2io^6A had a molecular ion at m/e = 467, with major fragment ions at m/e values of 452, 439, 394, 278, 262, 220, and 193. The permethyl derivative of the third cytokinin-active compound, c-io^{6}A, had a molecular ion of m/e = 421 with the major fragment ions at m/e values of 390, 348, and 216. The perdeuteromethylated derivatives of the cytokinin-active compounds showed the expected increases in mass units. Computer integration of peak areas from the gas-liquid chromatogram indicated that 21.4 μg of c-io^{6}A was recovered from a sample equivalent to 3885 A_{260} units of tRNA. The corresponding values for i^{6}A and ms^2io^6A were 4.06 μg and 4.55 μg, respectively, obtained from a sample equivalent to 4855 A_{260} units of tRNA.

BD-Cellulose Chromatography of Phaseolus tRNA. Crude Phaseolus tRNA was fractionated by chromotography on BD-cellulose (Fig. 6). The distribution of tRNA species expected to correspond to codons beginning with U (tRNA^{cys}, tRNA^{leu}, tRNA^{phe}, tRNA^{ser}, tRNA^{trp}, tRNA^{tyr}) was compared with the elution profile of cytokinin activity. The cytokinin activity eluted in the latter half of the salt gradient (Pooled Fractions V, VI, VII, and VIII) and in the ethanol purge
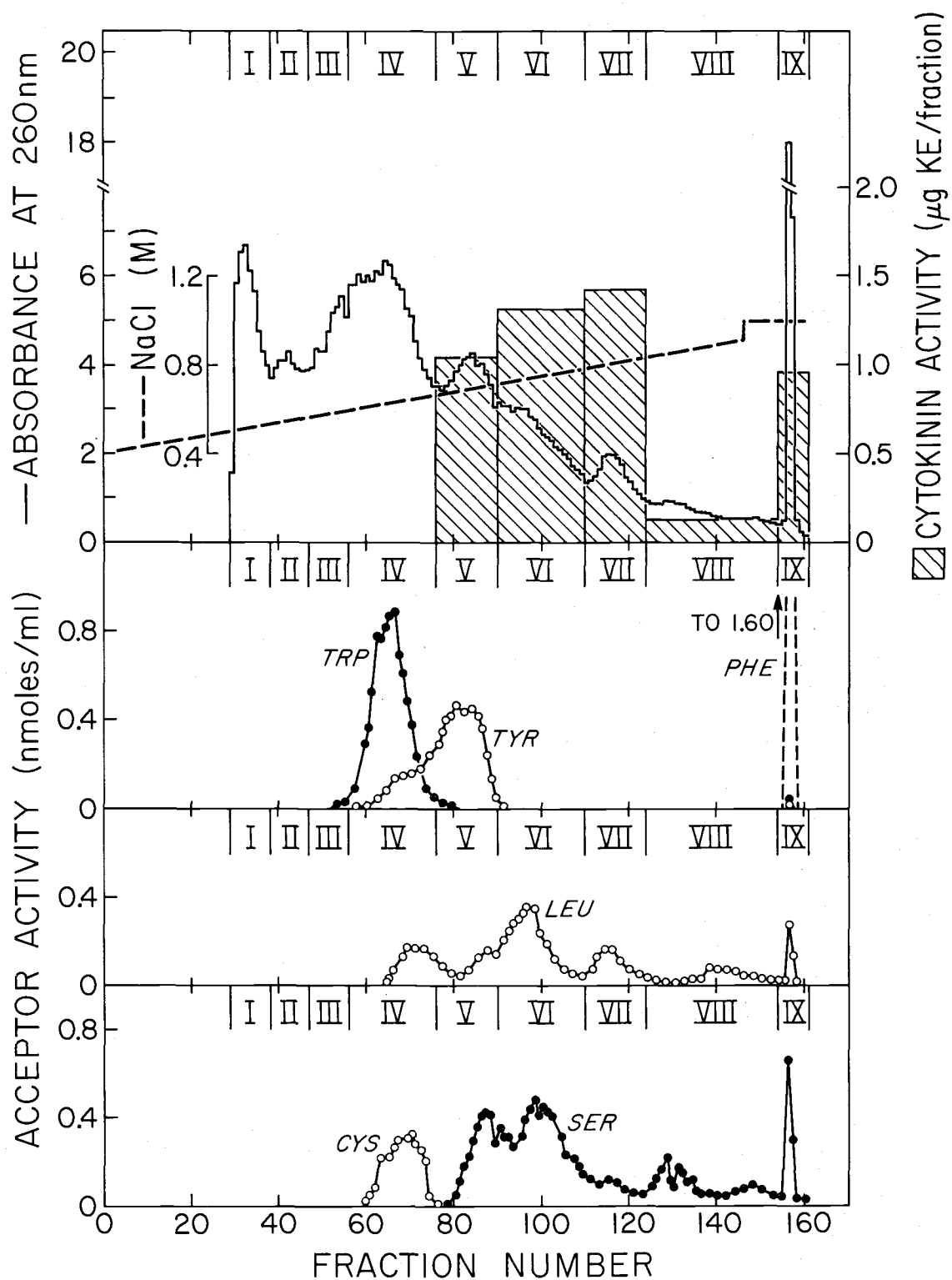
Figure 6. BD-cellulose chromatography of crude Phaseolus vulgaris
tRNA. Details of chromatographic procedures are described
in Materials and Methods.

(Pooled Fraction IX). All of the cysteine and tryptophan acceptor activity eluted early in the salt gradient, in fractions that were inactive in the tobacco bioassay. A portion of the leucine and tyrosine acceptor activities eluted in fractions devoid of cytokinin activity. The cytokinin activity eluting in the salt gradient was associated with peaks of tyrosine (fraction V), serine (fractions V to VIII), and leucine (fractions V to VIII) acceptor activity. Virtually all of the phenylalanine acceptor activity and minor peaks of serine and leucine acceptor activity were associated with the cytokinin activity eluting in the ethanol purge region of the BD-cellulose profile.

RPC-5 Chromatography of Phaseolus tRNA Fractions. The cytokinin-active tRNA fractions (V, VI, VII, VIII, and IX) recovered from the BD-cellulose column were rechromatographed on an RPC-5 column. The elution profile for cytokinin activity was compared with the distribution of the appropriate amino acid acceptor activities.

RPC-5 chromatography of BD-cellulose fraction V (containing tyr, leu and ser acceptor activity) gave the elution profiles shown in Figure 7. All of the cytokinin activity was associated with a complex peak of tRNA^{ser} and was completely separated from the peaks of tRNA^{tyr} and tRNA^{leu}.

The RPC-5 fractionation of BD-cellulose fraction VI (containing ser and leu acceptor activity) is shown in Figure 8. Single peaks of serine and leucine acceptor activity eluted early in the profile and were almost completely separated from each other. The cytokinin activity was coincident with the tRNA^{ser} peak.

The RPC-5 fractionation of BD-cellulose fraction VII (containing ser and leu acceptor activity) is shown in Figure 9. The single peak of leucine acceptor activity eluted prior to the cytokinin activity. Cytokinin activity was associated with a complex tRNA^{ser} peak, but there was also considerable cytokinin activity which eluted prior to the tRNA^{ser} peak.

RPC-5 chromatography of BD-cellulose fraction VIII (containing ser and leu acceptor activity) is shown in Figure 10. Cytokinin activity was distributed throughout the elution profile and was present in all fractions containing leucine and serine acceptor activities. However, a significant amount of cytokinin activity eluted late in the salt gradient and in the high salt purge region of the elution profile where U-group tRNAs were not detected.

RPC-5 chromatography of BD-cellulose fraction IX (containing ser, leu and all of the phe acceptor activity) is shown in Figure 11. Most of the serine acceptor activity eluted near the front of the elution profile and overlapped major and minor peaks of phenylalanine acceptor activity. Minor peaks of leucine acceptor activity were scattered throughout the elution profile. Cytokinin activity was distributed throughout the elution profile. Two major peaks of cytokinin activity eluting late in the salt gradient and in the high salt purge region of the profile were not associated with acceptor activity corresponding to any of the U-group tRNA species.
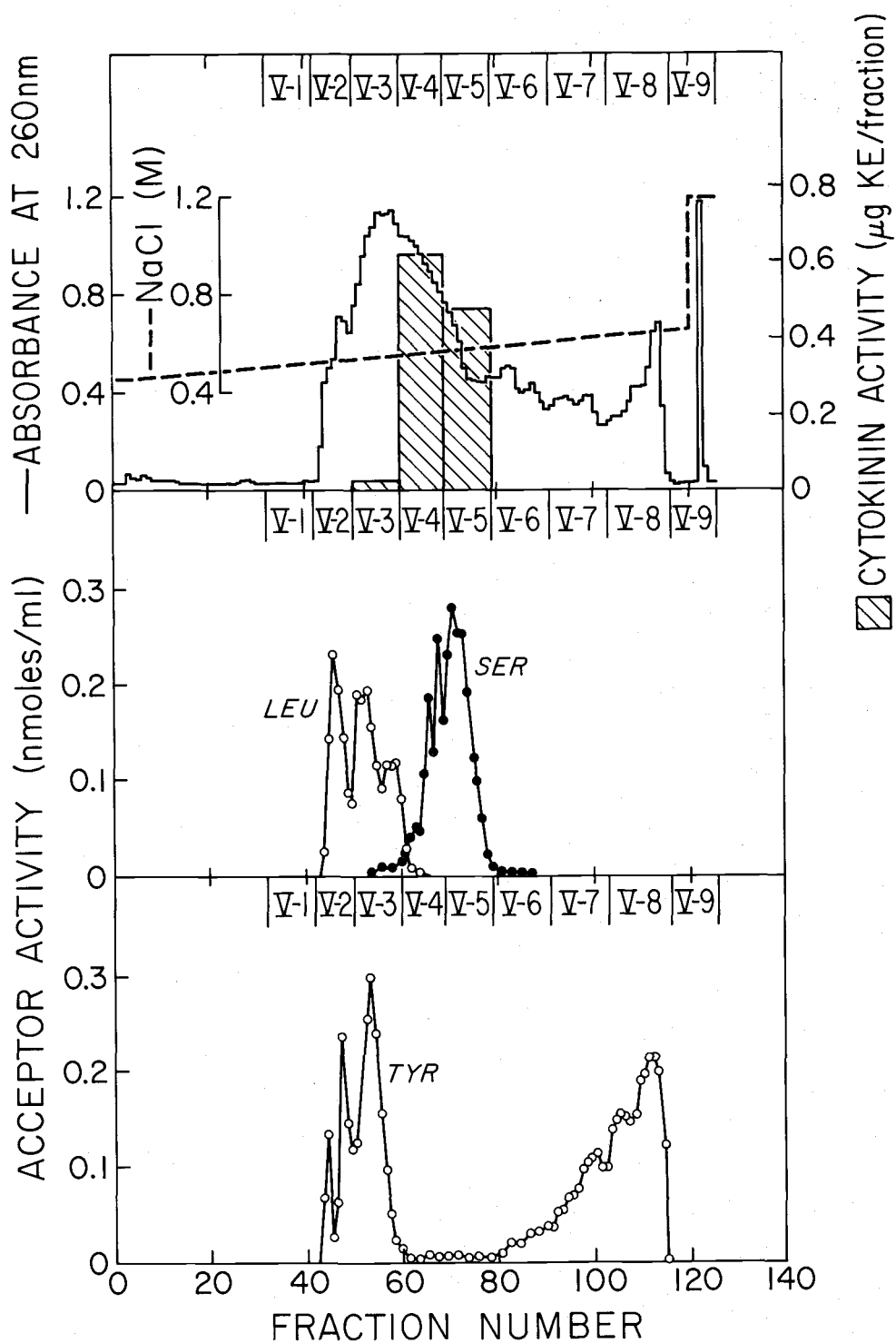
Figure 7. RPC-5 chromatography of BD-cullulose fraction V tRNA.
Details of chromatographic procedures are described in
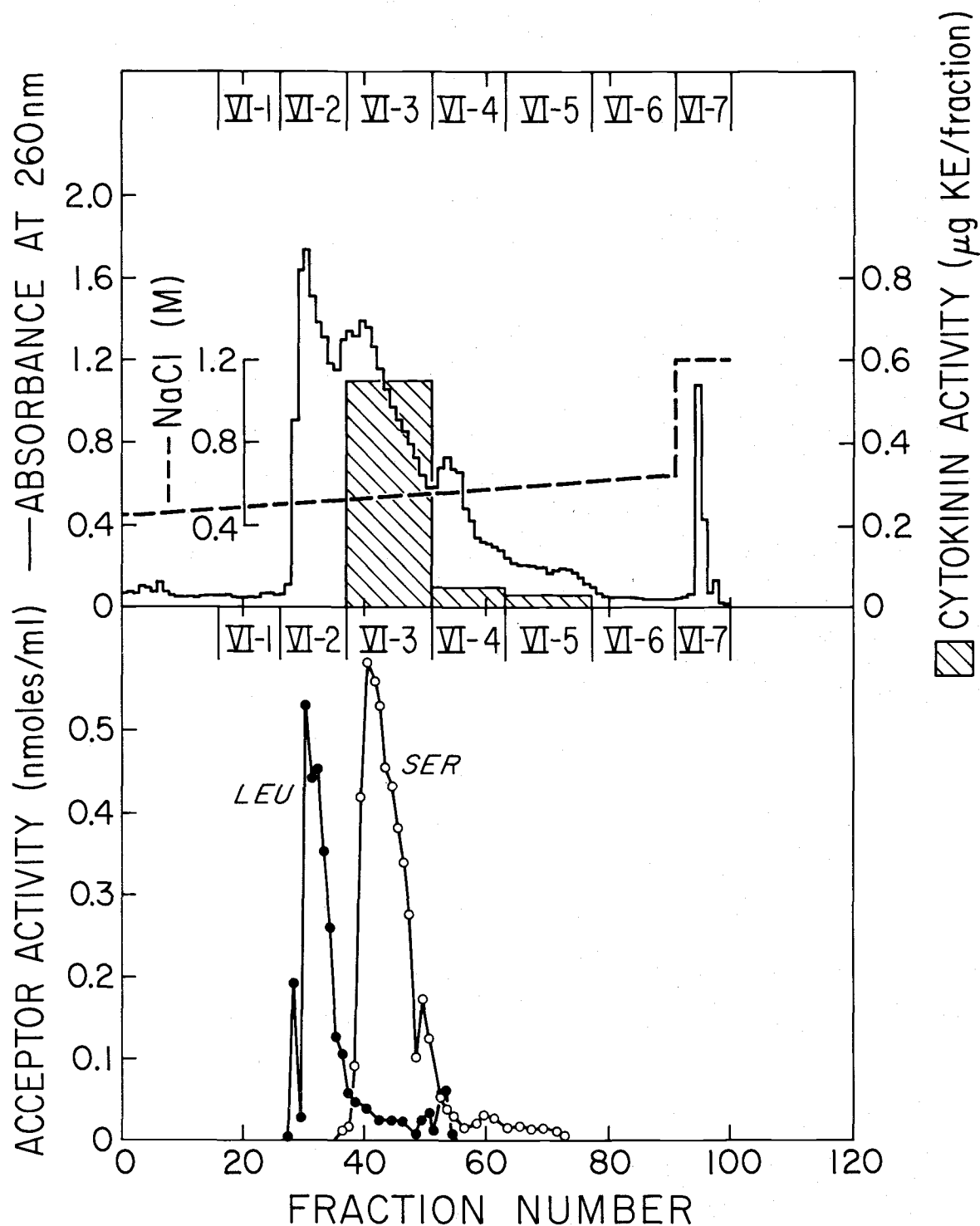Materials and Methods.

Figure 8. RPC-5 chromatography of BD-cellulose fraction VI tRNA.
Details of chromatographic procedures are described in
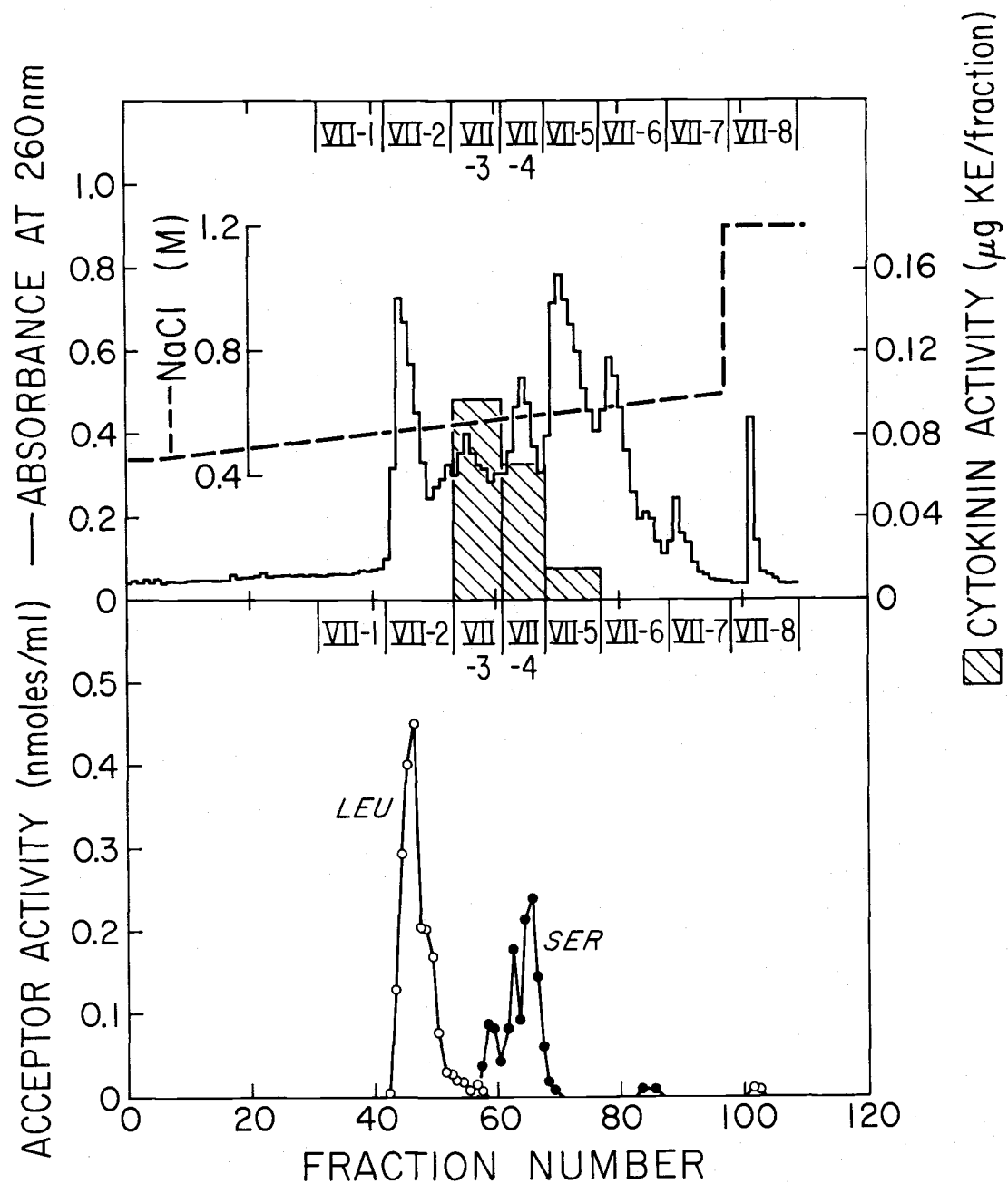Materials and Methods.

Figure 9. RPC-5 chromatography of BD-cellulose fraction VII tRNA. Details of chromatographic procedures are described in Materials and Methods.
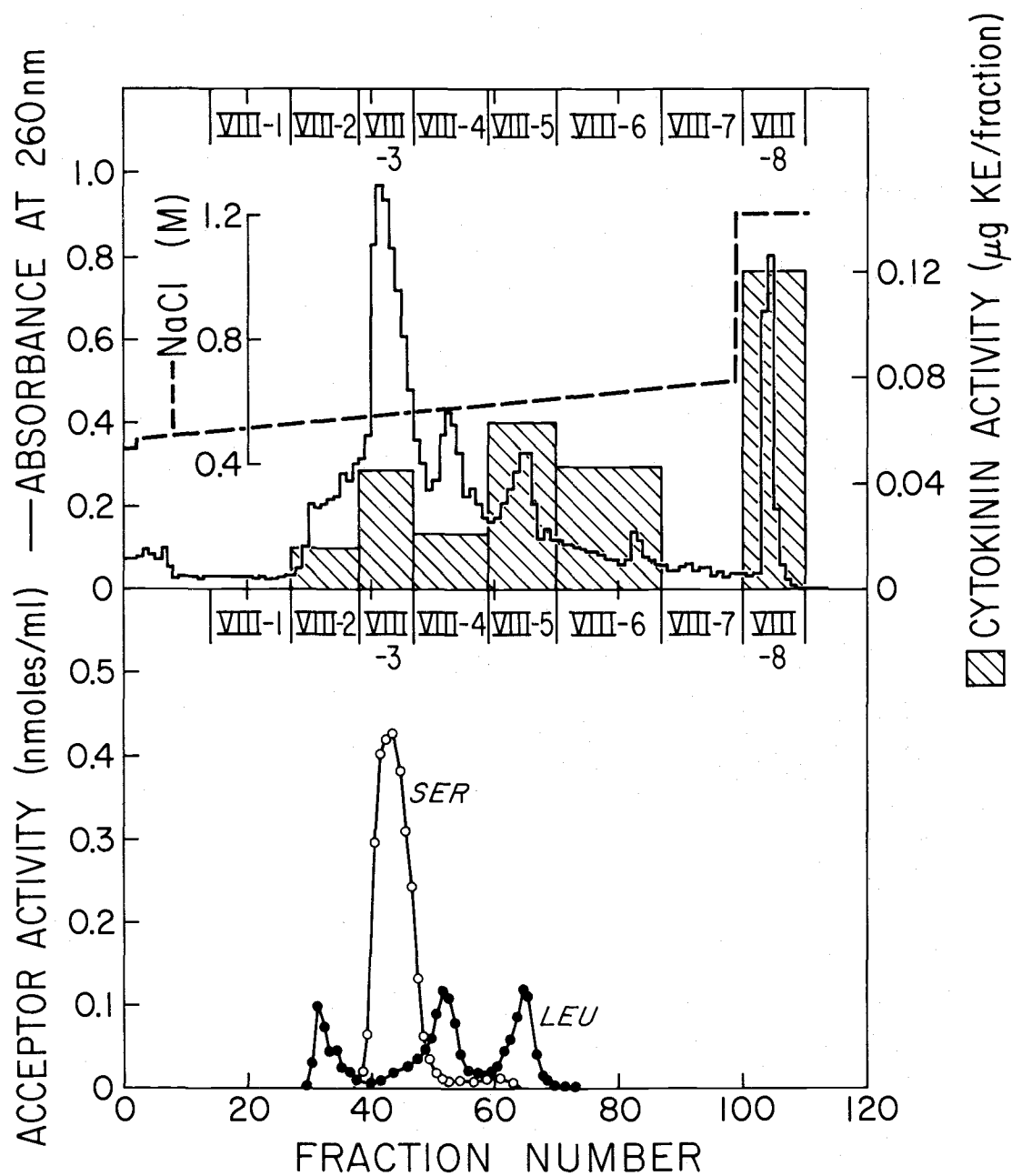
Figure 10. RPC-5 chromatography of BD-cellulose fraction VIII tRNA.
Details of chromatographic procedures are described in
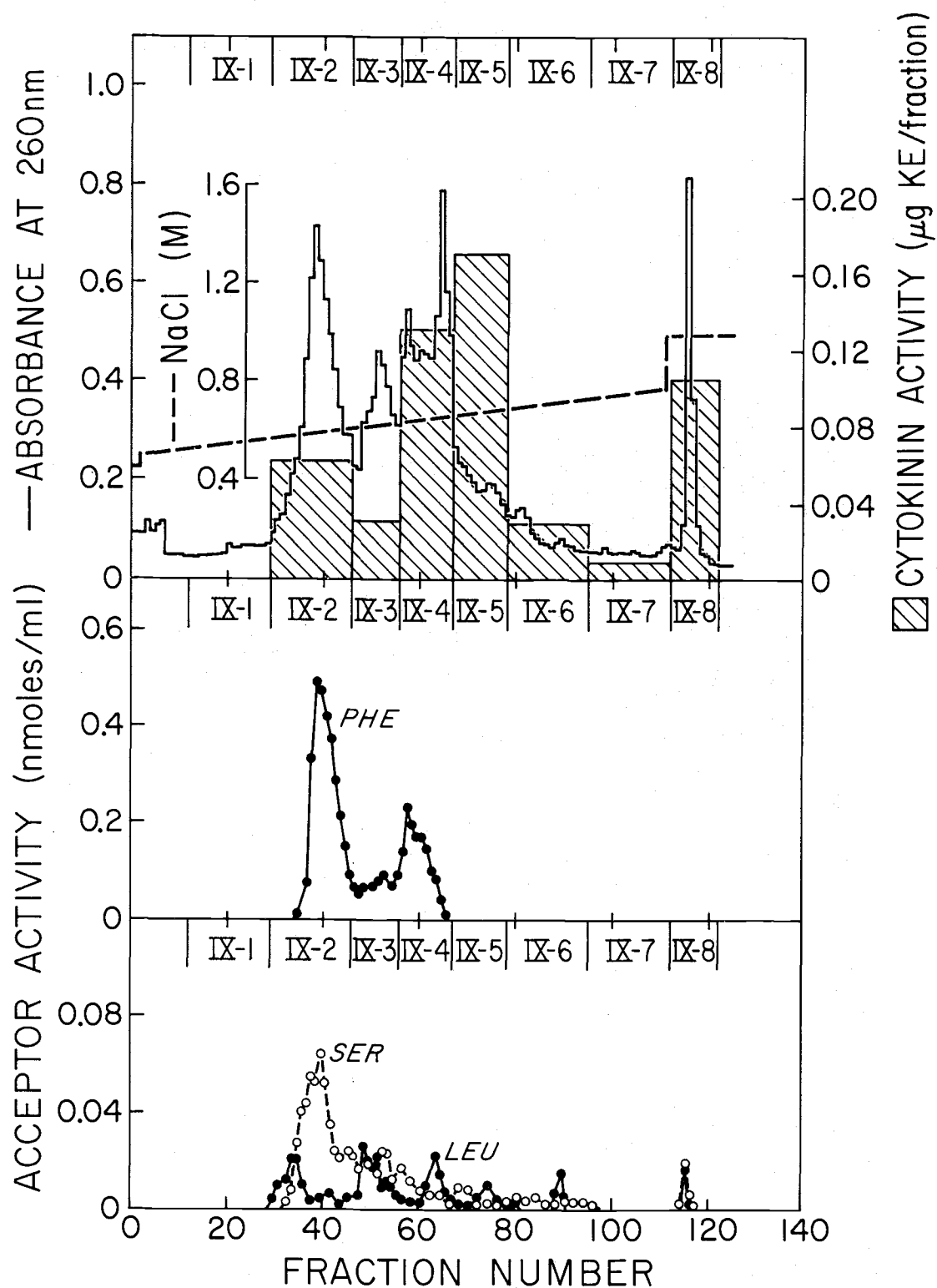Materials and Methods.

Figure 11. RPC-5 chromatography of BD-cellulose fraction IX tRNA. Details of chromatographic procedures are described in Materials and Methods.

V. DISCUSSION

The cytokinin composition of _Phaseolus_ _vulgaris_ tRNA is similar
to that reported for other plant tRNA preparations (14,17,42,106).
The predominant cytokinin-active ribonucleoside in _Phaseolus_ tRNA
was found to be _cis_-ribosylzeatin. This cytokinin accounted for
76% of the total cytokinin-active ribonucleosides present in
Phaseolus tRNA. Cis-ribosylzeatin has also been reported to be
the major cytokinin component in tRNA preparations from tobacco
callus tissue and wheat germ (14,17). Green plant tissues appear
to have somewhat higher proportions of i^6A and ms^2io^6A, probably
due to the contribution of chloroplast tRNAs (42,101,106,107).

The riboside of dihydrozeatin was not detected in _Phaseolus_
tRNA. This result concurs with the recent report by Burrows (13)
that dihydrozeatin does not occur in tRNA isolated from lupine seeds.
[Dihydrozeatin is the major free cytokinin isolated from lupine
seeds. (60)] Burrows was also unable to detect N^6-(2-hydroxybenzyl)
adenosine in tRNA from _Populus_ _robusta_, although this cytokinin
has been reported to occur in the free form in _Populus_ leaves (50).

The distribution of cytokinin-active ribonucleosides in
Phaseolus tRNA species that correspond to codons beginning with U
is restricted to a limited number of tRNA species. As in the case
of wheat germ tRNA (100), the only other plant system in which the
distribution of cytokinin moieties has been examined in detail,
Phaseolus tRNA^{tyr}, tRNA^{cys}, and tRNA^{trp} species do not appear

to have cytokinin-active ribonucleoside constituents. On the basis

of the results obtained here, it was not possible to determine whether

Phaseolus tRNA^{phe} contains a cytokinin moiety. However, it is pro-

bable that the Phaseolus tRNA^{phe} species are similar to wheat germ

tRNA^{phe} and contain a base-Y hypermodification in the 3'-position

adjacent to the anticodon (24). Thus, in both wheat germ tRNA and

tRNA from etiolated Phaseolus seedlings, the distribution of cyto-

kinins in U-group tRNA species appears to be restricted to tRNA^{ser}

and tRNA^{leu} species. It should be noted, however, that the elution

profiles of Phaseolus tRNA^{ser} and tRNA^{leu} species are considerably

more complex than those of the corresponding tRNA species from wheat

germ. It is not clear whether this is due to a taxonomic difference

(dicots vs. monocots) or to a developmental difference in the two

plant materials.

The structures of the nucleosides adjacent to the 3'-end of

the anticodon in tRNA^{cys}, tRNA^{tyr}, and tRNA^{trp} species from Phaseolus

and wheat germ are not known. However, tRNA^{trp} from yeast has been

shown to contain an unmodified adenosine at this position (53),

and tRNA^{tyr} from rat liver and silk worm has been reported to

contain N-[N-(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]-threonine (12).

The most interesting, and potentially the most important, re-

sult of this study is the detection of cytokinin activity that does

not chromatograph with U-group tRNA species. Possible explanations

for this observation may be summarized as follows:

1) In Phaseolus (and perhaps other eukaryotes), cytokinin-

active ribonucleosides may not be restricted to U-group tRNA species.

This apparent breach of the general rules governing the types of hypermodifications found in the 3'-position adjacent to the anticodon is not unprecedented. As mentioned above, the carbamoylthreonine derivatives which were previously thought to occur in only those tRNA species that respond to codons beginning with A have recently been reported to occur in tRNA^{tyr} isolated from eukaryotic sources (53). If cytokinins are indeed associated with tRNA species other than the U-group, it would appear likely that they will be found in tRNA species that respond to codons beginning with A, as this is the only other group of tRNA species in which hypermodified bases have been found adjacent to the anticodon.

2) The anomalous cytokinin-active tRNA fractions may contain isoaccepting species of U-group tRNAs that were not aminoacylated in the acceptor activity assays. This phenomenon could occur if specific aminoacyl-tRNA synthetases were a) not extracted from the plant tissue, b) not present at that particular developmental stage, or c) rendered inactive by the extraction or storage procedures.

3) A more remote possibility is that the anomalous cytokinin activity is due to the presence of cytokinin-containing RNA species other than tRNA. The activity in question was detected by rechromatography of tRNA samples recovered from the latter part of the BD-cellulose elution profile. These fractions are usually enriched in the small amounts of high molecular weight RNA fragments (or species) that contaminate most crude tRNA preparations (Armstrong, unpublished).

In summary, the distribution of cytokinin-active ribonucleosides

in _Phaseolus_ tRNA species that respond to codons beginning with U is very similar to that in wheat germ tRNA with the exception of the additional complexity of the _Phaseolus_ tRNA^{ser} and tRNA^{leu} profiles. Although the distribution of cytokinins in tRNA species from green tissues is likely to be more complex, the results reported here suggest that the distribution of cytokinins in cytoplasmic U-group tRNAs will be similar in different plant systems and restricted to tRNA^{ser} and tRNA^{leu} species. (It is of interest that the only cytokinin-containing tRNA species identified to date in tRNA from animal sources have been tRNA^{ser} species.) Further work will be needed before the significance of the anomalous cytokinin activity detected in fractionations of _Phaseolus_ tRNA can be adequately assessed.

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