

AN ABSTRACT OF THE THESIS OF

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TUMEFACIENS

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Abstract approved: _____

Roy Owen Morris

Certain plant-associated prokaryotes such as Corynebacterium fascians and Agrobacterium tumefaciens are known to produce substantial levels of cytokinins. In view of the fragmentary and presumptive evidence available on the quantitative and qualitative levels of cytokinins produced by A. tumefaciens, it was decided to conduct a study of the cytokinins produced by A. tumefaciens.

In order to accomplish this objective, a sequence of methods was developed which allowed the isolation, purification and rigorous chemical identification of cytokinins at the sub-microgram level. The first step of purification utilized the adsorption of cytokinins onto C18 silica by a process known as trace enrichment. Further purification was effected by high performance liquid chromatography and cytokinin-active fractions were located by bioassay. Material in

biologically active fractions was permethylated and analyzed by combined capillary gas-liquid chromatography and mass spectrometry.

A. tumefaciens tRNA was found to contain i^6 Ado, $ms^2 i^6$ Ado, $ms^2 io^6$ Ado and trans- io^6 Ado. A. tumefaciens culture filtrates were found to contain i^6 Ade, i^6 Ado, $ms^2 i^6$ Ado, $ms^2 io^6$ Ado and the trans isomers of io^6 Ade and io^6 Ado. Possible mechanisms for the production of these cytokinins were discussed.

Preliminary evidence on three strains of A. tumefaciens showed that neither the production of $N^6-\Delta^2$ -isopentenyladenine or zeatin correlated with the presence of the tumor-inducing plasmid.

Cytokinin Production by
Agrobacterium tumefaciens

by

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DEDICATION

To my children:

Aaron Dylan Chapman
Melissa Dyan Chapman

"See what everyone has seen,
but think what no one else has thought."

Szent-Györgyi

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CYTOKININ PRODUCTION BY AGROBACTERIUM TUMEFACIENS

I. INTRODUCTION

Since the original discovery of the plant cell division factor termed kinetin (Miller et al., 1955) and the subsequent delineation of its structure as 6-furfurylaminopurine (f^6 Ade) (Miller et al., 1955b), numerous naturally occurring kinetin analogues have been found. The generic term, cytokinin, is now used for compounds which elicit a kinetin-like response in higher plants (Skoog et al., 1965). Miller (1961) isolated and partially characterized a naturally occurring cytokinin from corn kernels which was later identified as 6-(4-hydroxy-3-methyl-but-2-enyl)-aminopurine (io^6 Ade) by Letham et al. (1964). Since these basic discoveries, the number of known synthetic cytokinins has increased to greater than one hundred (Kende, 1971) and more than twenty naturally occurring cytokinin-active compounds are known. Aspects of cytokinin physiology have been the subject of frequent reviews (Burrows, 1975; Leonard, 1974; Skoog, 1973; Kende, 1971; Skoog and Armstrong, 1970) and earlier reviews provide additional material of historical interest (Strong, 1958; Miller, 1961).

The most active group of cytokinins are the N^6 -substituted aminopurines. Some of these compounds promote cell division in

the tobacco callus bioassay (Linsmaier and Skoog, 1965) at concentrations as low as 5×10^{-5} μ M. The structures of several naturally occurring or synthetic cytokinins are shown in Figure 1. Studies have shown that in order for a molecule to have maximum cytokinin activity, it must meet certain structural criteria. The linkage between the purine nucleus and isopentenyl or benzyl moiety must be a nitrogen (Henderson et al., 1975). The trans isomer of zeatin is more active in the callus bioassay than the cis isomer (Schmitz et al., 1972). The cytokinin activity of free bases is greater than that of nucleosides. The cytokinin activity of nucleotides is greater than that of cyclic nucleotides (Schmitz et al., 1975) and the optimum side chain length is five carbons with unsaturation at the two position in the side chain (Skoog and Armstrong, 1970). In general, the isopentenyl cytokinins are more active than the benzyl cytokinins in the soybean callus bioassay (Horgan, 1975). Skoog (1973) and Leonard (1974) have reported the synthesis of a series of cytokinin antagonists, the most potent of which is 3-methyl-7-(3-methylbutylamino)-pyrazolo-[4,3-d]-pyrimidine. This compound appears to compete specifically with cytokinins with respect to cell division in the tobacco callus bioassay. However, as Kende (1971) has noted, studies on structure-activity relationships using unlabeled cytokinins cannot always be considered conclusive. Low cytokinin activity may be a reflection of slow uptake of the compound or

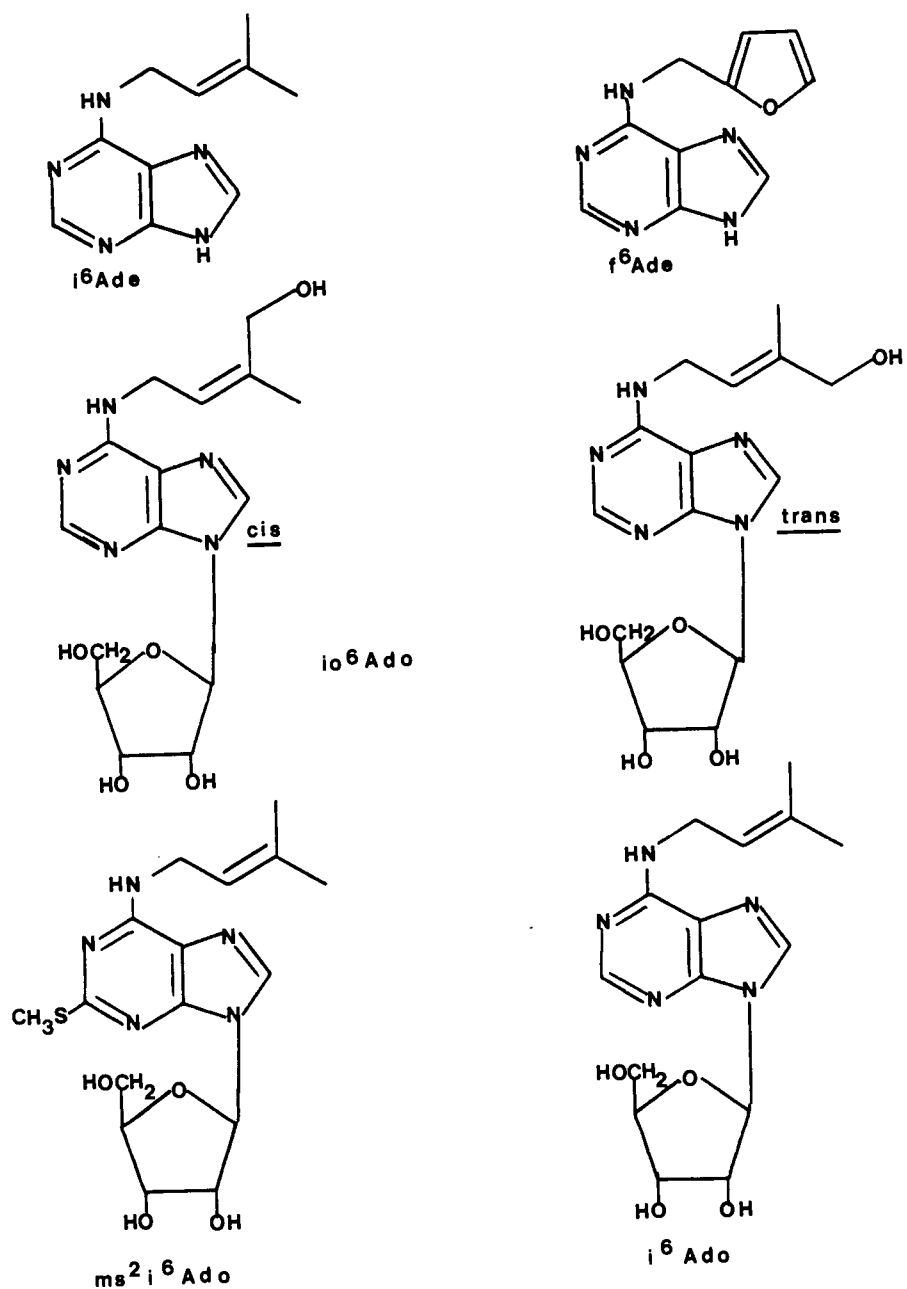


Figure 1. Chemical structures of several naturally occurring or synthetic cytokinins with currently used abbreviations.

rapid metabolism to an inactive form in the test tissue.

In addition to free cytokinins, Zachau et al. (1966) and Hall et al. (1966) isolated and characterized a cytokinin-active nucleoside (i^6 Ado) from a transfer ribonucleic acid (tRNA) hydrolysate of yeast. Since that time, cytokinin-active nucleosides have been found in tRNA isolated from many sources, including bacteria, plants, fungi and animals (Hall, 1970). Table 1 shows the distribution of some tRNA-bound and free cytokinins. When total tRNA preparations were fractionated into amino acid-isoaccepting species using reverse-phase chromatography, the cytokinin-containing tRNA species were found to be those which recognize condons starting with uridine (Skoog and Armstrong, 1970). In addition, in all cases studied thus far, the cytokinin-active nucleoside is located adjacent to the 3' side of the anticodon loop of the specific tRNA (Zachau et al., 1966; Skoog, 1973). The function of the cytokinin moieties in tRNA has been the subject of considerable speculation. Fittler and Hall (1966) found the 6-(3-methyl-2-butenyl)-amino-purine (i^6 Ado) moiety to be required for ribosome binding; however, modification of the moiety did not affect aminoacylation of the tRNA. Geftter and Russell (1969) resolved amber supressor SU_3^+ tyrosine tRNAs into three species differing in the degree of modification at the adenosine moiety adjacent to the 3' side of the anticodon. All of these species were equally acylated with tyrosine but there were

distinct differences in the ability of the tRNA to bind ribosomes. In vitro, the unmodified tRNA containing an adenylate residue adjacent to the anticodon was ineffective in binding to ribosomes. The highly modified ($ms^{2,6}i^6$ Ado) tRNA bound most efficiently to the ribosomes and i^6 Ado-containing tRNA bound at an intermediate level. In more recent work, Fox and Erion (1975) report that ribosomes have two binding sites for cytokinins, one high-affinity site and one low-affinity site. It is now generally accepted that the cytokinin moiety is required for proper ribosome binding but has no effect on the aminoacylation process.

Table 1. Occurrence of cytokinins in natural sources. (Hall, 1970).

Organism	tRNA	Free	Cytokinin		
			i^6 Ado	io^6 Ado	$ms^{2,6}i^6$ Ado
Human liver	+		+		
Yeast	+		+		
Peas	+		+	+	
Corn	+		+		
<u>E. coli</u>	+				+
<u>Lactobacillus</u>	+		+		
<u>Agrobacterium</u>	+	+	+	+	+
<u>Corynebacterium</u>		+	+	+	+

The physiological effects of endogenous cytokinins are many. The stimulation of plant cell divisions is the most frequently cited response to cytokinins. In addition, cytokinins are known inducers of specific enzymes such as nitrate reductase (E.C. 1.6.6.3) (Kende et al., 1971) and cytokinins act to retard senescence in excised plant tissue (Richmond and Lang, 1967). Under certain conditions, cytokinins may substitute for light exposure in phytochrome-mediated germination of lettuce seeds (Speer et al., 1974). Cytokinins have long been known to cause accumulation of metabolites against concentration gradients (Mothes et al., 1959) and this has led to speculations on the mechanism of retardation of senescence (Kende, 1971). Some of the most interesting effects of cytokinins are those which elicit morphogenic responses. The ratio, auxin/cytokinin, determines the growth pattern of tobacco callus tissue. At a high auxin/cytokinin ratio (2 mg l^{-1} IAA: 0.02 mg l^{-1} kinetin) root formation is favored while a low auxin/cytokinin ratio (2 mg l^{-1} IAA: 0.5 mg l^{-1} kinetin) favors the production of buds and shoots (Skoog and Miller, 1957). Spiess (1975, 1976) has noted the stimulatory effect of certain cytokinins on bud production in the moss, Funaria. The loss of apical dominance following infection of woody dicots by Corynebacterium fascians has been ascribed to alteration of hormone levels in vivo. This organism is known to produce significant levels of cytokinins (Armstrong et al.,

1976). The causative agent of the plant tumor, crown gall, has been shown to produce high levels of free cytokinins (Kaiss-Chapman and Morris, 1977) and tRNA-bound cytokinins (Chapman et al., 1976). In both cases, it appears that cytokinin-producing prokaryotes elicit complex morphogenic changes in higher plants.

The biosynthesis of cytokinins has been the subject of intensive scientific investigation. This problem may be divided into two parts: the biosynthesis of tRNA-bound cytokinins and the biosynthesis of free cytokinins. The primary transcripts from genes coding for tRNA molecules exhibit few of the distinctive features found in the mature, functional tRNA. Some of the common features of mature tRNA molecules are a size of 75-90 nucleotides in length, the presence of 10-16% modified bases and a common 3' sequence, -C-C-A-OH (Altman, 1975). Transfer RNA precursor molecules were found to have a low content of modified nucleotides and this modified nucleotide content could be increased by incubation with cell extracts in vitro (Burdon, 1971). The extent to which a precursor RNA is found modified depends upon the half-life of the precursor (Altman, 1975). In examining the biosynthesis of the cytokinin-active modified base, $i^6\text{Ado}$, the tRNA of Lactobacillus, Peterkofsky (1968) observed that the radioactive label from $[2-^{14}\text{C}]$ -mevalonic acid (MVA) was found in the $i^6\text{Ado}$ residues of tRNA. The incorporation of label from MVA into $i^6\text{Ado}$ was not observed

with yeast (Fittler et al., 1968). The lack of incorporation in yeast was attributed to the fact that yeast cells only take up small amounts of MVA from the medium. Chen and Hall (1969) noted that the radioactive label of MVA was incorporated specifically into the i^6 Ado in the tRNA of tobacco callus tissue. Kline et al., (1969) reported the partial characterization of an enzyme from rat liver and from yeast which would transfer in vitro Δ^2 -isopentenylpyrophosphate to an adenosine residue in tRNA. Rosenbaum and Geftter (1972) isolated unmodified tRNA^{tyr} from E. coli cells infected with bacteriophage ϕ 80. They utilized this tRNA^{tyr} as a test substrate for biosynthesis of cytokinins. They found (and purified 550 fold) an enzyme which transferred the Δ^2 -isopentenyl group derived from MVA to the correct adenosine residue in the modification-minus tRNA^{tyr}. This was unequivocal proof of the biosynthesis of a tRNA-bound cytokinin. In addition, the radioactive label from MVA has subsequently been shown to be incorporated into cis- i^6 Ado of tobacco tissue tRNA (Murai et al., 1975). The in vitro donor of the methyl group in tRNA-bound ms^2, i^6 Ado has been shown by Geftter (1969) to be S-adenosylmethionine. The enzyme system responsible for this transfer was not further characterized.

The incorporation of the intact cytokinin 6-benzylaminopurine (BAP) into tobacco callus tRNA has also been documented. By the use of double-labeled BAP, it was determined that about one BAP

molecule was incorporated per 10^4 tRNA molecules (Walker et al., 1974). Armstrong et al. (1976) also report the incorporation of labeled BAP into the ribosomal ribonucleic acid (rRNA) of tobacco callus tissue. The low levels of incorporation of BAP into rRNA may be viewed as a transcriptional error or as specific incorporation into a minor polynucleotide component of the rRNA. It thus appears that the major route of biosynthesis for tRNA-bound cytokinins is the enzymatic transfer of the isopentenyl group to a specific adenosine residue in a pre-formed tRNA molecule, although intact incorporation of cytokinins into tRNA does occur by an unknown mechanism. A possible explanation of this phenomenon in eukaryotic cells could be different mechanisms of synthesis in different cell organelles.

The possibility that catabolic processing of tRNA could regulate production of cytokinins has been frequently proposed. Klemen and Klämbt (1974) examined tRNA turnover rates in root tip cells of Zea mays using pulse-chase experiments. The pulse medium contained ^{14}C -orotic acid and the pulse period was 12 hours. The estimate of tRNA half-life obtained was 2.5 days. Using these data, cytokinin production due to tRNA turnover in the root system of an 18-day old corn plant was estimated to be 2×10^{-10} mol/day. The actual production of cytokinins by the root system was not determined. During the course of this experiment, the roots grew

several millimeters and any quantitative determination was clearly impossible. The tRNA turnover rate in the prokaryote, Lactobacillus acidophilus was determined by Leineweber and Klämbt (1974). The cells were maintained in logarithmic phase by dilutions to a specific cell density. The pulse conditions utilized ^{14}C -orotic acid for six hours followed by a chase period. The generation time for the cells was found to be two hours during logarithmic phase and the tRNA half-life was found to be three to four hours. During stationary phase, the tRNA half-life was found to be 10-12 hours. Based on these data, they estimated that one gram of bacteria could produce 4×10^{-9} mol of cytokinins in 3.5 hours. The authors compare this value to the production of cytokinins by Corynebacterium fascians which has been found to produce 2×10^{-9} mol of cytokinin/gram of bacteria (Klämbt et al., 1966). However, a critical evaluation of these data must be held in abeyance until Lactobacillus has been shown to produce culture filtrate cytokinins. Using Agrobacterium tumefaciens, strain B6, Hahn et al. (1976) observed a generation time of about 19 hours and a tRNA half-life of 27.5 hours. They measure a free cytokinin production (presumed $i^6\text{Ade}$) of 0.3 $\mu\text{g/l}$ of culture. They estimate cytokinin production due to tRNA turnover to be more than sufficient to account for the actual cytokinin levels found. In more recent work using A. tumefaciens strain C58, $i^6\text{Ade}$ was found to account for only 30% of the cytokinin

content of the culture filtrate (Kaiss-Chapman and Morris, 1977) and hence the report by Hahn et al. may represent an inaccurate estimate of cytokinin production. Measurements of tRNA turnover rates are subject to errors due to pool size changes resulting from the growth of the cells. In addition to quantitative estimates, qualitative examination of tRNA-bound cytokinins in various A. tumefaciens strains (Chapman and Morris, 1975; Chapman et al., 1976; Chapman and Morris, 1976) indicate that a variety of cytokinins are present. The unequivocal proof that free cytokinins arise from tRNA turnover remains to be demonstrated.

If wholesale tRNA turnover does not account for free cytokinin production, several other hypotheses merit consideration. The possibility that specific cytokinin-containing tRNA molecules are degraded selectively has been examined by Babcock and Morris (1973). They found that a specific leucyl-tRNA was partially degraded by some unknown factor. The cleavage of the tRNA^{leu} probably occurred in the anticodon region and did not of itself release a cytokinin. The possibility of further degradation of the tRNA^{leu} to yield a cytokinin-active nucleotide remains. An alternate hypothesis is a site-specific endoribonuclease which would excise the cytokinin-active nucleotide from the intact tRNA molecule. While the existence of site-specific endodeoxyribonucleases is well documented (Nathans and Smith, 1975), no

site-specific endoribonuclease has been reported.

It is difficult either to prove or disprove (from the evidence currently available) the contention that cytokinin production, in planta, occurs via one of the postulated mechanisms of tRNA degradation. The alternative is an entirely novel biosynthetic mechanism for cytokinin production. It is known that cytokinin-dependent tobacco callus tissue when grown in culture does not convert appreciable amounts of ^{14}C -adenine into cytokinins. However, a strain of cytokinin-autotrophic tobacco callus has been derived from the cytokinin-dependent strain. When the cytokinin-autotrophic strain was supplied with ^{14}C -adenine, cytokinins having properties similar to io^6Ade and i^6Ade were produced. Both the cytokinin-dependent and the cytokinin-autotrophic strains of tobacco callus tissue contains cytokinins in the tRNA molecules (Burrows et al., 1971; Dyson and Hall, 1972). While the presence of tRNA-bound cytokinins in a cytokinin-dependent tobacco callus strain does not exclude the possibility of a specific tRNA-degradation mechanism, it makes the production of free cytokinins by wholesale tRNA turnover unlikely since these tissue lines have not been shown to lack ribonuclease activity. Direct evidence for biosynthesis of free cytokinins has been published by Chen et al., (1976). They supplied ^{14}C -labeled adenine and a double-labeled adenosine analogue, 2-0-[1-(R)-(9-adenyl)-7-(hydroxy)-ethyl] glycerol to cytokinin-

autotrophic tobacco tissue cultures. The chemical structure of this analogue precluded its incorporation into tRNA. After a 12-day incubation period, three radioactive components were isolated. One of these components had properties identical with the authentic N⁶-isopentenyl derivative of the analogue. The authors concluded that while the data do not preclude the turnover of cytokinin-containing tRNA as the source of cytokinins, they do show that an alternate pathway for cytokinin biosynthesis may exist.

Thus it appears that at least two routes of synthesis may exist in higher plants, while there is no conclusive evidence to support two routes of synthesis in prokaryotic bacteria.

The use of prokaryotic organisms in biochemical research offers numerous advantages. The rapid growth of large homogenous cultures allows isolation of even trace biochemical components. Large varieties of organisms are available and construction of desired mutants is greatly facilitated in comparison to diploid eukaryotes. A disadvantage in using a prokaryote as a test system is that it is possible that different metabolic processes may occur in eukaryotes. The choice of Agrobacterium tumefaciens as the model system for cytokinin biosynthesis was based on the large amount of background genetic data available on this organism. This bacterium has been of interest since 1907 when Smith and Townsend showed A. tumefaciens (then called Bacterium tumefaciens) to be

the agent responsible for crown gall, a tumorous disease of dicotyledonous plants. Braun (1947) postulated the production of a metabolic product of A. tumefaciens which converted the susceptible plant into a tumorous state. Not only does presumptive evidence exist that A. tumefaciens produces cytokinins (Upper et al., 1970) but A. tumefaciens responds to kinetin (10^{-7} M) by a 158% increase in cell numbers over control cultures (Maruzzella and Garner, 1963). In addition to producing a cytokinin, A. tumefaciens has long been known to produce the auxin, indole-3-acetic acid (Berthelot and Amoureux, 1938). The similarities between the phytohormone production of A. tumefaciens and eukaryotic plants are remarkable, thus making this an ideal system for phytohormone research.

Agrobacterium tumefaciens (Smith and Townsend) Conn. is included in the family Rhizobiaceae in Bergey's Manual (Buchanan and Gibbons, 1974). The agrobacteria are mesophilic soil inhabitants consisting of gram-negative rods averaging $1.5 \times 0.8 \mu\text{m}$ in size. Figure 2 shows a transmission electron micrograph of a single cell of A. tumefaciens, strain A178. They are motile and characteristically have one to six peritrichous flagella. Many strains produce large amounts of 3-ketoglucosides and they are frequently auxotrophic for biotin, nicotinic acid, or pantothenate (Lippincott and Lippincott, 1975). Virulent strains of A. tumefaciens rarely produce plant tumors unless the bacterium is introduced into

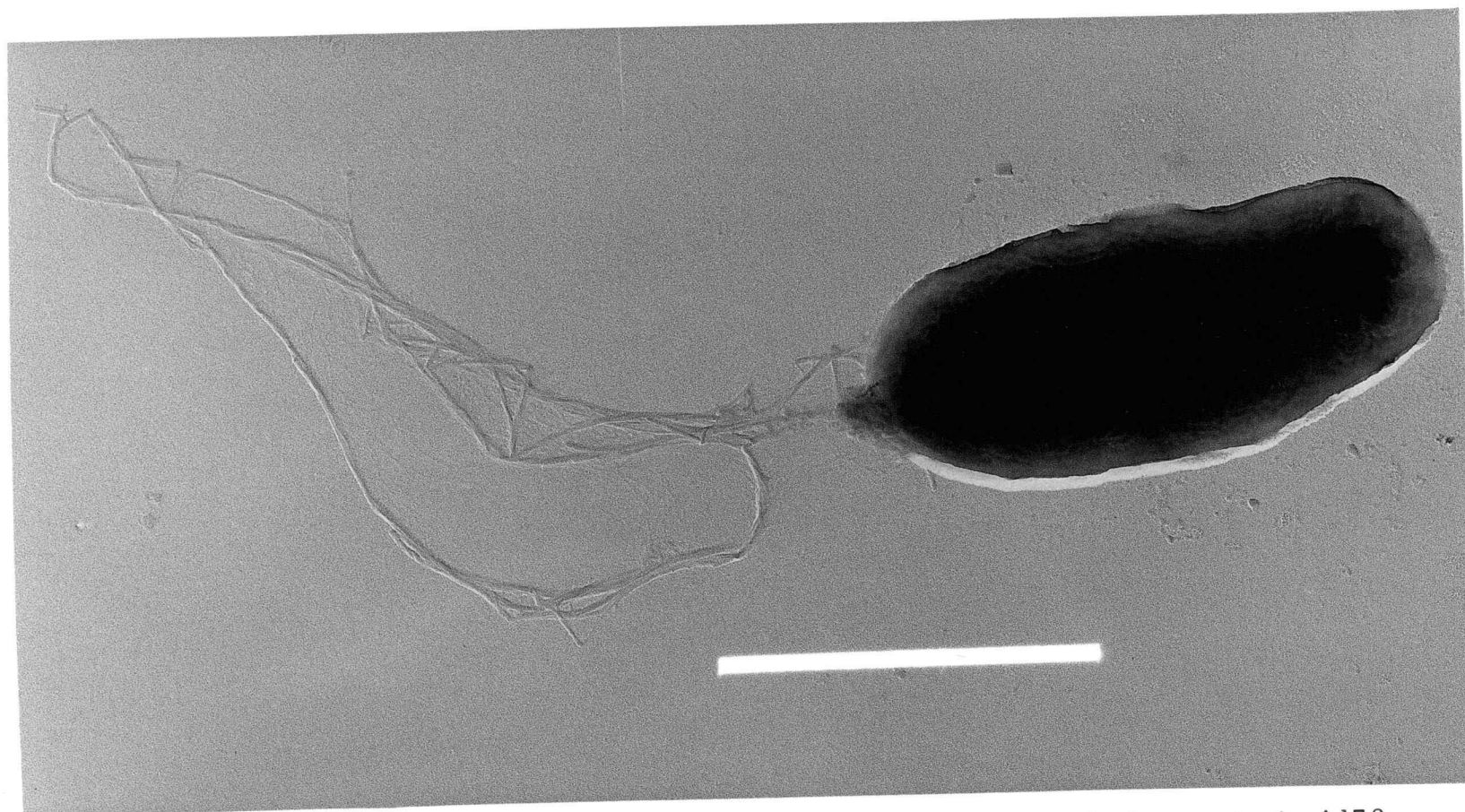


Figure 2. Transmission electron micrograph of a single cell of A. tumefaciens, strain A178.
The black bar indicates one μm .

the host by wounding. Tumor (gall) tissue consists of either disorganized masses of hyperplastic tissue or partially organized teratomas. Secondary tumors arise at sites remote from the primary tumor site and these secondary tumors are frequently bacteria-free. Another interesting biochemical relationship exists between the bacterium and the tumor. Crown gall tumor tissue may produce any of several unusual amino acids. These amino acids are N^2 -(D-carboxyethyl)-L-arginine (octopine), N^2 -(1,3-dicarboxypropyl)-L-arginine (nopaline) and N^2 -(D-carboxyethyl)-L-lysine (lysopine). Whether octopine or nopaline is present in the neoplastic tumor tissue depends on the bacterial strain which induced the tumor. Strains which induce tumors containing octopine are able to utilize octopine as sole nitrogen source, whereas strains which induce nopaline-containing tumors are able to utilize nopaline as a nitrogen source (Klapwijk et al., 1976). It appears that the genes determining the specificity of degradation of these amino acids (Bomhoff et al., 1976) are located on a large plasmid (Ti) that is present in virulent strains of Agrobacterium (Zaenen et al., 1974).

Other aspects of nitrogen metabolism seem to link cytokinin production with crown gall. Peterson and Miller (1976) found that addition of glycine, aspartate, asparagine, glutamate, proline, arginine, ammonium chloride or ammonium nitrate increased the

cytokinin level in Vinca crown gall tissue cultures. The major cytokinin produced by this tumor tissue was identified as trans-ribosylzeatin (Miller, 1974).

The transformation of normal plant tissue, which is known to require a cytokinin for growth in tissue culture, to a neoplastic crown gall tumor which is cytokinin-independent involves a true genetic transformation. The identity of the tumor-inducing principle (TIP) (Braun, 1947) has been the subject of much speculation in plant physiology literature. Various workers have considered bacterial DNA, plasmid DNA or bacteriophage DNA as the TIP. Since virus particles demonstrate the ability to induce tumors in animal systems, much work has been done on the bacteriophages of Agrobacterium. Thus far, there have been no reproducible reports showing that a bacteriophage is involved as the TIP (Lippincott and Lippincott, 1975). There are conflicting reports in the literature concerning the presence of bacterial DNA in the tumor tissue. Some of these studies (Merlo and Kemp, 1976) report the presence of low levels of bacterial DNA in tumor tissue using DNA:DNA hybridization techniques. The amount of hybridization in these experiments is at the lower threshold of detection and these results should be viewed with skepticism until corroborating evidence is available. The lower limit in these experiments using intact bacterial DNA would be about 5% of the bacterial genome.

Integration of smaller amounts of bacterial DNA into tumor tissue would be undetectable (Chilton et al., 1974). Recent experiments tend to indicate that the Ti plasmid DNA as being present in the tumor tissue (Matthysse and Stump, 1976; Chilton et al., 1977). Matthysse and Stump estimate the amount of plasmid DNA in bacteria-free tumor cells to be about 0.1% of the tumor cell DNA. The data presented show great variability and may be used only as an upper limit on the amount of plasmid DNA present in the tumor cells. Instead of attempting marginal hybridization experiments using intact Ti plasmid DNA (1.2×10^7 daltons), Chilton et al. (1977) cleaved the plasmid into nineteen fragments using the restriction deoxyribonuclease Sma I which cleaves at specific sites in the plasmid. Each of the nineteen restriction fragments was then isolated using gel electrophoresis and used in hybridization studies. Only one fragment, band 3, was found to hybridize significantly with tumor cell DNA. This fragment did not hybridize with normal cell DNA. Further electrophoresis of band 3 resolved it into two components, band 3a and 3b. From renaturation kinetic analysis, it was found that 40% of band 3b hybridized with tumor DNA. This means that 3.7×10^6 daltons of foreign (Ti plasmid) DNA is present in tumor tissue DNA but not in normal tissue DNA. Whether foreign DNA in the tumor cell is in the form of a plasmid or integrated into the tumor cell chromosomes is unknown. It thus appears that in this

case the presumed barrier to natural genetic interaction between prokaryotic and eukaryotic DNA may be absent.

Further confirmation of the oncogenic nature of the Ti plasmid has been derived in an independent manner. Kerr et al. (1977) have reported an in planta and ex planta transfer of virulence from a pathogenic strain to a non-pathogenic strain of Agrobacterium using the plasmid-carried trait of octopine utilization as a marker. The in planta transfer supports Lippincott and Lippincott's (1975) previous hypothesis that genetic exchange between Agrobacterium strains may occur in crown gall tumors. The work of Genetello et al. (1977) indicates that ex planta transfer of virulence is due to the Ti plasmid acting in a conjugative manner.

The agrobacteria are useful organisms for studying the biochemistry and molecular genetics of phytohormone production and the processes involved in neoplastic growth. There are a number of virulent and avirulent forms available and inter-conversion between these forms is possible. If cytokinin production is correlated with the tumorigenic process, it may be apparent from examination of cytokinin production of various pathogenic and non-pathogenic forms. Examination of the tRNA-bound and culture filtrate cytokinins produced by these bacteria may offer an insight into the biosynthetic pathway involved in cytokinin production.

II. STATEMENT OF RESEARCH OBJECTIVES

The major research objective was the rigorous chemical identification of the cytokinins present in the tRNA and culture filtrate of Agrobacterium tumefaciens strains. However, this objective required the development of extraction, purification and identification methods as preliminary objectives.

Rapid Quantitative Isolation of Cytokinins From Biological Sources

Most of the methods for cytokinin isolation in use at the initiation of this study involved extractions of large amounts of aqueous biological material with large volumes of organic solvents such as n-butanol or ethyl acetate. Such extractions are time consuming and hazardous. In addition, the yield of cytokinins is variable (Kaiss-Chapman and Morris, unpublished results; Hemberg and Westlin, 1973; Hemberg, 1974). The first research objective was development of an isolation procedure which was both rapid and quantitative, and which bypassed the use of large quantities of organic solvent.

Development of HPLC and GLC Separations of Cytokinins

Conventional procedures for purification of cytokinins utilize

large columns which require long periods of time to run (Scarborough et al., 1973). In addition, paper and thin-layer chromatography in various solvent systems have been frequently used (Webb et al., 1973; Thomas et al., 1975). Gas-liquid chromatography (GLC), when used, employed thermally labile stationary phases such as QF1 (Upper et al., 1970) and DC11 (Babcock and Morris, 1970). With few exceptions, the basic criterion for cytokinin identification involved comparison of R_f values of biologically active fractions with those of known cytokinins. When mass spectra were obtained, it was on pre-purified samples via the direct inlet system. The second research objective was the development of high performance liquid chromatographic (HPLC) techniques for cytokinin separation. Further, volatile cytokinin derivatives were sought for use on high resolution ($\geq 10,000$ theoretical plates), thermostable, support coated open glass capillary GLC columns used in conjunction with the mass spectrometer.

Unequivocal Identification of Cytokinins from *Agrobacterium tumefaciens*

The third and major research objective was the rigorous chemical identification of the tRNA-bound and culture filtrate cytokinins from several virulent and avirulent strains of *Agrobacterium tumefaciens*. At the point this work was started,

the only free cytokinin thought to be produced by A. tumefaciens was i⁶Ade (Upper et al., 1970; Hahn, 1976).

Determination of the Relationship Between Plasmid
Status and Cytokinin Production in
Agrobacterium tumefaciens

The fourth research objective was to determine the relationship (if any) between the Ti plasmid status and cytokinin production of a variety of A. tumefaciens strains. At the beginning of this study, there was no data in existence correlating cytokinin production with Ti plasmid status. If the genes coding for the enzymes which produce cytokinins were carried on the Ti plasmid, it could have direct bearing on the tumorigenic transformation process and provide a possible explanation for the production of eukaryotic phytohormones by this bacterium.

III. MATERIALS AND METHODS

Organism and Culture Conditions

The various strains of Agrobacterium tumefaciens used in this study and their Ti plasmid and virulence status are listed in Table 2. The general maintenance medium used throughout the study was medium 523 (Kado and Heskett, 1970). The composition of this medium is shown in Table 3. This is also the medium used for the growth of cells for tRNA extraction. The defined medium used in the isolation and identification of culture filtrate cytokinins was highly modified from that of Valera and Alexander (1965). The composition of this medium is shown in Table 4. Growth of cells in defined medium was accomplished in Fernbach flasks which were agitated on a rotary shaker at 150 cycles/minute at a temperature of 26°C. The growth of cells for tRNA preparation was done in a high density fermenter (Lab Line, New Brunswick, NJ). Routinely, 5 l of culture were grown at a bath temperature of 27°C, an O₂ flow rate of 8 l/min and a rotation speed of 360 rpm. Growth was monitored by using a Gilford spectrophotometer (Gilford, Inc., Oberlin Ohio) set at a wavelength of 660 nm.

Table 2. Plasmid and virulence status of organisms used in this study.

Organism	Strain	Ti Plasmid	Virulence
<u>A. tumefaciens</u>	C58	+	+
<u>A. tumefaciens</u>	NT1	-	-
<u>A. tumefaciens</u>	II BV7	+	+
<u>A. tumefaciens</u>	II BNV6	-	-
<u>A. tumefaciens</u>	A 174	+	+

Table 3. Components of Medium 523 (Kado and Heskett, 1970), the general maintenance medium used in this study.

Component	grams/liter
Casein hydrolysate	8
Yeast extract	4
K_2HPO_4	2
$MgSO_4 \cdot 7 H_2O$	0.3
Sucrose	10

Table 4. Components of defined medium. (highly modified from Valera and Alexander, 1965).

Component	g/l	mg/l
Sucrose	15	
$(\text{NH}_4)_2\text{SO}_4$	2	
$\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$	0.3	
NaCl	0.4	
FeCl_3	0.1	
CaNO_3	0.25	
KH_2PO_4	0.2	
K_2HPO_4	0.8	
Na_2MoO_4		2
Zn acetate		2
H_3BO_3		2
MnCl_2		2
CuCl_2		0.1
CoCl_2		0.15
m-inositol		1.0
Thiamine•HCl		1.0
Ca•pantoate		1.0
Riboflavin		1.0
Pyridoxamine•HCl		1.0
Biotin		1.0
p-aminobenzoate		0.2

Partition Behavior of Cytokinins

In order to determine the effect of pH and organic solvent on the partition behavior of cytokinins, known amounts of i^6 Ade, io^6 Ade, i^6 Ado and io^6 Ado were added to a buffer at pH 2.50 (0.5 M, glycine-HCl) or pH 8.0 (0.5 M, Tris-HCl). An equal volume of the organic solvent to be tested was added and the mixture sealed in a silanized glass ampoule. Duplicate samples were prepared. The sealed ampoules were agitated until equilibrium was established (two hours). The two phases were allowed to separate and the amount of each cytokinin was determined for both the organic and aqueous phase using gas-liquid chromatography with computer-controlled peak area integration.

Preparation of tRNA-bound Cytokinins

The methods used have been described in detail in earlier publications (Chapman et al., 1976; Kaiss-Chapman and Morris, 1977) and will only be outlined here. The cell paste recovered from centrifugation was freeze-thawed and extracted in the presence of a phenol, m-cresol mixture. This solution was partitioned against a pH 8.0 buffer. The pooled aqueous phases from three extractions were back washed once with additional phenol-cresol. Two volumes of -20°C ethanol were added and the nucleic acids were allowed to

precipitate overnight at -20°C . The precipitated nucleic acids were then extracted three times with 1M NaCl. This resulted in the precipitation of the rRNA and the extraction of tRNA and DNA into the supernatant. The pooled supernatants were again precipitated overnight with ethanol. The overnight precipitate was dissolved in buffer (0.5% disodium naphthalene disulfonate, 0.01% 8-hydroxy-quinoline, 3% sodium-p-toluene sulfonate and 4% sodium chloride: pH 7.95 at 22°C). Sodium benzoate (20% w/v) was added followed by addition of 15% (v/v) m-cresol. The temperature was maintained at 22°C throughout this procedure. DNA precipitated during this procedure and the supernatant was then precipitated with ethanol overnight as before. The final precipitate consisting mainly of 4s and 5s RNA was examined by electrophoresis on 10% polyacrylamide gels (Loening, 1967). The purity of the 4s tRNA was normally found to be 85-95%. The tRNA was hydrolyzed in 0.3 N KOH at 37°C for 24 hours. The pH was then adjusted to 8.8 with Tris-HCl and 10 mM Mg^{++} was added along with bacterial-alkaline phosphatase. The mixture was incubated at 37°C for 18 hours with a periodic readjustment of the pH to 8.8. After 18 hours, additional enzyme and Mg^{++} were added and the incubation continued for an additional 18 hours at 37°C . This procedure resulted in the production of nucleosides from the tRNA.

Preparation of Culture Filtrate Cytokinins

In general, either 50 l or 20 l of defined medium was prepared (1500 ml/Fernbach flask), autoclaved (121°C , 17 psig), cooled, and inoculated with five ml of the desired starter culture per Fernbach flask. The starter culture was grown from a clonal isolate and checked microscopically for contamination after being stained with a Gram stain. All glassware in these experiments was treated with a silane solution (10% dichlorodimethylsilane in benzene) to prevent cytokinin adsorption onto glass surfaces. The cells were harvested from the medium using the Szent-Görygi, Blum continuous-flow centrifuge at 14,500 g and a flow rate of 50 ml/min. The cell-free effluent was collected in silane-treated glassware and used as the filtrate for further purification.

Trace Enrichment of Cytokinins

To avoid solvent extraction of cytokinins, the following procedure was developed. The source of cytokinins, either culture filtrate or tRNA-hydrolysate, was adjusted to pH 3.50 using 10 N acetic acid. The solution was then passed through an octadecylsilica (C18/Porasil B, 37-75 μm ; Waters Assoc., Milford, MA) reverse phase column using a modified pressure bomb (PMS Instrument Co., Corvallis, OR) at 50 bar pressure, the adsorbed cytokinins were

eluted with 50 ml of redistilled 95% ethanol. The column was then re-equilibrated by flushing 300 ml of buffer (ammonium acetate, pH 3.50, 0.02 M) through the column (Morris et al., 1976). This procedure was repeated until the desired amount of cytokinin source solution had been processed. In some experiments, [8-¹⁴C]-6-furfurylaminopurine (20 mCi/mM, Amersham/Searle, Arlington Heights, IL) was added to the cytokinin source prior to trace enrichment and aliquot samples of the column eluate were counted in a Packard liquid scintillation counter (Packard Inst., Downers Grove, IL) in order to estimate cytokinin recoveries. After trace enrichment, the pooled ethanol eluates containing the cytokinins were taken to dryness, in vacuo, at 35°C and stored at -20°C under a nitrogen atmosphere until used.

Analytical High Performance Liquid Chromatography (HPLC)

The dried ethanol eluates were reconstituted in HPLC buffer (0.02 M, pH 3.50, ammonium acetate) and injected into a Waters, ALC 202, HPLC equipped with two model 600 pumps and a model 660 solvent programmer (Waters Assoc., Milford, MA). The column used was an analytical μ C18/Porasil reverse-phase column (2.1 mm x 25 cm). The column conditions used were a flow rate of two ml/min, a two minute isocratic hold pumping HPLC buffer

followed by a linear increasing ethanol gradient to 60% ethanol in a time of 18 minutes. Two ml fractions were collected in silane-treated tubes. Elution was monitored by recording the absorbance at 260 nm. Ten percent of each fraction was reserved for bioassay and the remainder of each fraction was lyophilized for 18 hours using a Virtis lyophilizer (Virtis, Inc., Gardiner, NY) with a vacuum of 10^{-3} torr. at a temperature of -55°C . The dry samples were stored over P_2O_5 at -20°C until bioassays were completed.

Cytokinin Bioassay

The cucumber cotyledon bioassay as modified from that of Fletcher and McCullagh (1971) was used to assay the HPLC fractions for cytokinin activity. Each of the dried aliquot samples reserved for bioassay was dissolved in 500 μl of bioassay buffer (pH 5.0, 10 mM phosphate with 3 mM CaCl_2) and five 100 μl replicates were dispensed for testing. Three day old cucumber cotyledons were excised at the junction of the cotyledons and placed into the replicate solutions to be tested. All these operations were performed in a darkroom under a weak green safelight. The cotyledons were allowed to imbibe the test solution for 18 hours in the dark. The cotyledons were then exposed to cool-white fluorescent light for a two-hour period. After light exposure, the cotyledons were rapidly frozen and the chlorophyll extracted by

homogenization in tetrahydrofuran:ethyl cellosolve (1:1). Cytokinin levels were determined by measuring the absorbance difference $|A_{665\text{nm}} - A_{698\text{nm}}|$. The absorbance difference produced by known amounts of cytokinins was correlated with the absorbance difference of the sample to be tested. The bioassay results were typically plotted as histogram overlays on the $A_{260\text{nm}}$ HPLC elution profile.

Preparation of Samples for Gas-Liquid Chromatography

The lyophilized contents of HPLC fractions which exhibited bioassay activity were transferred to Microflex v-vials (Kontes, Inc., Vineland, NJ). Samples to be trimethylsilated (TMS) were placed in 300 μl vials and those which were to be permethylated were placed in 1000 μl vials. The TMS derivatives were prepared in essentially the same manner as used by Babcock and Morris (1970). The dry samples were reconstituted with 10 μl of dry pyridine and 40 μl of Regisyl RC-1 (Regis Chemical Co., Morton Grove, IL) were added. The reaction mixture was heated for two hours at 60°C. When cool, the samples were ready for chromatography. These samples were very sensitive to hydrolysis and were only stable for 3 days. A more satisfactory method, a modification of Hakomori's (1964) method, was used in most of the experiments. We have previously published the details of the method (Kaiss-Chapman and Morris, 1977). The dry sample was dissolved in 10 μl dry

dimethylsulfoxide (DMSO) and an excess of dimethylsulfinyl anion was added. After standing for 30 min at 25°C, the methyl or deuteromethyl ester was prepared by adding five µl of methyl iodide (Baker Chemical Co., Phillipsburg, NJ) or deuteromethyl iodide (Wilmad Glass Co., Buena, NJ) and allowing the reaction mixture to stand at 25°C for one hour. The reaction was terminated by the addition of a five-fold excess of distilled water and the derivatives were extracted into 300 µl of distilled chloroform. The chloroform was backwashed three times with distilled water to remove traces of the DMSO. These samples appeared to be stable about two weeks when stored dry at -20°C.

Gas-Liquid Chromatography (GLC)

During the initial phases of the study, GLC was accomplished on packed columns. The columns utilized Dexsil 300 coated as a three percent load on Anakrom A, 110-120 mesh (both from Analabs, Inc., North Haven, CT). This material was packed in 2 mm x 100 cm glass columns. Typical plate values for these columns were 800-1100 theoretical plates. During the latter phase of the study, open capillary glass columns were prepared by drawing 100 cm x 0.6 mm glass tubing into capillary columns (0.5 mm x 100 m) using a Hewlett-Packard (Avondale, PA) capillary drawing instrument (Jennings, 1974). The capillary columns were coated, using the

static method, with a solution containing 8 mg/ml (Dexsil 300: chloroform) and 1 mg/ml Silanox 101 (Cabot Corp., Boston, MA). These columns exhibited in excess of 10,000 theoretical plates for a 10 m column. As much as 1 μ l of the permethylated derivatives could be injected on these columns with no apparent damage to the column.

The carrier gas utilized for these columns was helium and a linear flow rate (μ) of 30 cm/sec was found to permit baseline resolution of the cis and trans isomers of zeatin. The typical conditions used for temperature programming were a three minute isothermal hold at 200°C followed by a 2°C/min increase in temperature to 325°C.

Mass Spectrometry

Throughout this study, a Varian MAT CH7 (Varian Assoc., Palo Alto, CA) single-focusing, magnetic sector mass spectrometer was used. Data acquisition was accomplished with an on-line System 150/PDP-8 computer (System Industries, Sunnyvale, CA). Sample introduction was via the direct inlet at ambient to 200°C or through the GLC inlet. A Varian 1200 GLC was interfaced to the mass spectrometer via a single-stage glass jet separator. The mass spectrometer had been modified to add differential pumping capability. Spectra were recorded continuously under the direction

of the computer system at an ionizing voltage of 70 eV (300 μ Amp) and the photomultiplier voltage was typically set at 18.5 kVolts. During a typical run, 1/6 of the total GLC column effluent was routed through an all-glass system to the flame ionization detector (FID) of the gas chromatograph. The FID was typically operated at an amplifier setting of 10^{-11} amps/mVolt. In addition to acquiring spectra, the System 150 acquires total ion current (TIC) data for reconstructing the gas chromatogram (RGC).

IV. RESULTS

Extraction, Purification and Identification of Cytokinins

Extraction of Cytokinins by Partitioning with Organic Solvents

At the initiation of this study, typical methods for the extraction of cytokinins utilized partitioning of acidic (pH 2.5) biological material against an organic solvent such as ether or ethyl acetate as a wash procedure. The organic phase was then discarded and the aqueous phase was adjusted to pH 8.0. The aqueous phase was then extracted several times with ethyl acetate or n-butanol (Webb et al., 1973). Since quantitative data on the recovery of cytokinins from these procedures were sparse, a model experiment was designed to determine the partition behavior of $i^6\text{Ade}$, $io^6\text{Ade}$, $i^6\text{Ado}$, and $io^6\text{Ado}$ against various organic solvents at pH 2.5 or 8.0. The solvents tested were hexane, ether, n-butanol, ethyl acetate and a t-butanol:ethyl acetate (2:1) mixture. After partitioning to equilibrium in sealed, silane-treated ampoules, the distribution of cytokinin in the organic and aqueous phase was determined by GLC.

The sum of the cytokinins in the organic and aqueous phases were found to be identical to the initial amount of cytokinins present.

The results of three extractions at either pH 2.5 or pH 8.0 are shown in Tables 5 and 6 respectively. It is clear from the data in Table 5 that washing with any solvent except hexane results in the substantial loss of cytokinins. The data in Table 6 indicate that both n-butanol and the t-butanol: ethyl acetate mixture gave reasonable yields at pH 8.0 in this model system. Extractions with n-butanol resulted in an average removal of 99% of the cytokinins tested and extractions with the t-butanol:ethyl acetate mixture resulted in the removal of 93% of these cytokinins. Since the t-butanol:ethyl acetate mixture was much easier to remove, in vacuo, it was used as the system of choice when solvent extraction was necessary.

Although the use of hexane as a wash solvent and the use of t-butanol:ethyl acetate as an extraction solvent gave reasonable results, this method was difficult to use on large volumes of material.

Trace Enrichment as a Method for Isolation of Cytokinins

Solvent extraction of a large volume of aqueous biological material was a time-consuming procedure. For example, extracting 50 l of bacterial medium three times with half-volumes of organic solvent left 75 l of organic solvent to be reduced to dryness in vacuo. As an alternate procedure, it was decided to attempt the

Table 5. Percent of cytokinins extracted into organic phase following three partitions; pH 2.50.

Cytokinin	pH 2.50				
	Hexane	Ether	Ethyl Acetate	t-butanol:ethyl acetate	n-butanol
i ⁶ Ade	0	50.3	71.9	89.8	99.0
i ⁶ Ado	0	46.9	84.8	66.9	99.4
io ⁶ Ade	0	26.4	28.5	39.0	71.0
io ⁶ Ado	0	0	trace	62.7	70.6

Table 6. Percent of cytokinins extracted into organic phase following three partitions; pH 8.0

Cytokinin	pH 8.0				
	Hexane	Ether	Ethyl Acetate	t-butanol:ethyl acetate	n-butanol
i ⁶ Ade	0	98.0	99.9	99.5	99.9
i ⁶ Ado	0	68.6	98.6	99.9	100.0
io ⁶ Ade	0	25.9	54.7	93.2	99.4
io ⁶ Ado	0	0	trace	80.4	97.7

direct adsorption of cytokinins from aqueous solutions onto octadecylsilane (C18/Porasil B) columns. After adsorption onto the column, the cytokinins were eluted by pumping 50 ml of ethanol through the column. Model experiments using $[8-^{14}\text{C}]$ -kinetin or a mixture of standard cytokinins showed that trace enrichment of cytokinins from pH 3.5 buffer (0.02 M, ammonium acetate) was essentially quantitative (Morris et al., 1976). Up to five liters of solution could be processed through the trace enrichment column for each ethanol elution of cytokinins. In order to determine the actual recovery of cytokinins from biological material, a known amount of $[8-^{14}\text{C}]$ -kinetin was added to the first and tenth liter of Agrobacterium tumefaciens culture filtrate. The total amount of culture filtrate processed was 48.5 l processed in five separate experiments. As shown in Table 7, the overall average recovery was 94.6%. By use of this procedure, the cytokinins in 48.5 l of culture filtrate were recovered in 600 ml of ethanol and the bulk of the polar contaminants were removed. The amount of ethanol used in this procedure represents less than one percent of the volume of solvent generated in a solvent extraction of a similar volume of bacterial medium with an excellent recovery of cytokinins.

The trace enrichment method was also used for cytokinin recovery from Douglas fir xylem exudate with essentially quantitative recovery (Morris et al., 1976). Further, the trace enrichment

Table 7. Trace enrichment recovery of [8-¹⁴C]-kinetin, added to A. tumefaciens culture filtrates (1.62 x 10⁵ dpm were added to the first and last liter processed each day).

Day	Initial Liter		% Recovery	Final Liter		% Recovery
	dpm in	dpm out		dpm in	dpm out	
1	1.62 x 10 ⁵	1.64 x 10 ⁵	101	1.62 x 10 ⁵	1.44 x 10 ⁵	89
2	1.62 x 10 ⁵	1.61 x 10 ⁵	99	1.62 x 10 ⁵	1.45 x 10 ⁵	89.5
3	1.62 x 10 ⁵	1.60 x 10 ⁵	99	1.62 x 10 ⁵	1.45 x 10 ⁵	89.5
4	1.62 x 10 ⁵	1.61 x 10 ⁵	99	1.62 x 10 ⁵	1.46 x 10 ⁵	90
5	1.62 x 10 ⁵	1.62 x 10 ⁵	100	1.62 x 10 ⁵	1.46 x 10 ⁵	90

technique was also used to recover cytokinin nucleosides from tRNA hydrolysates (Chapman et al., 1976; Kaiss-Chapman and Morris, 1977).

Analytical High Performance Liquid Chromatography and Bioassay

The pooled ethanol eluates from the trace-enrichment column were sufficiently impure to require additional purification prior to GLC. A chromatographic analytical fractionation procedure was therefore developed which utilized chromatography on a micro-particulate, (10 μ m) reverse phase, octadecylsilica HPLC column.

Cytokinin standards elute from this column in the order of decreasing polarity as shown in Figure 3. Thus, io⁶Ado and io⁶Ade elute before i⁶Ado and i⁶Ade with the least polar of these cytokinins, ms^{2,6}i⁶Ado eluting last. In order to evaluate this technique on biological material, 900 ml of Douglas-fir xylem exudate was obtained from Dr. J. B. Zaerr (O.S.U., Forest Sciences Dept.) and trace enriched. The enriched ethanol eluate was reduced to dryness in vacuo and fractionated on the analytical HPLC column. Fractions were collected and assayed for biological activity by means of the chlorophyll greening assay of Fletcher and McCullagh (1971). Figure 4 shows the results. The absorbance at 260 nm is shown overlayed with a histogram of biological activity. The elution positions of

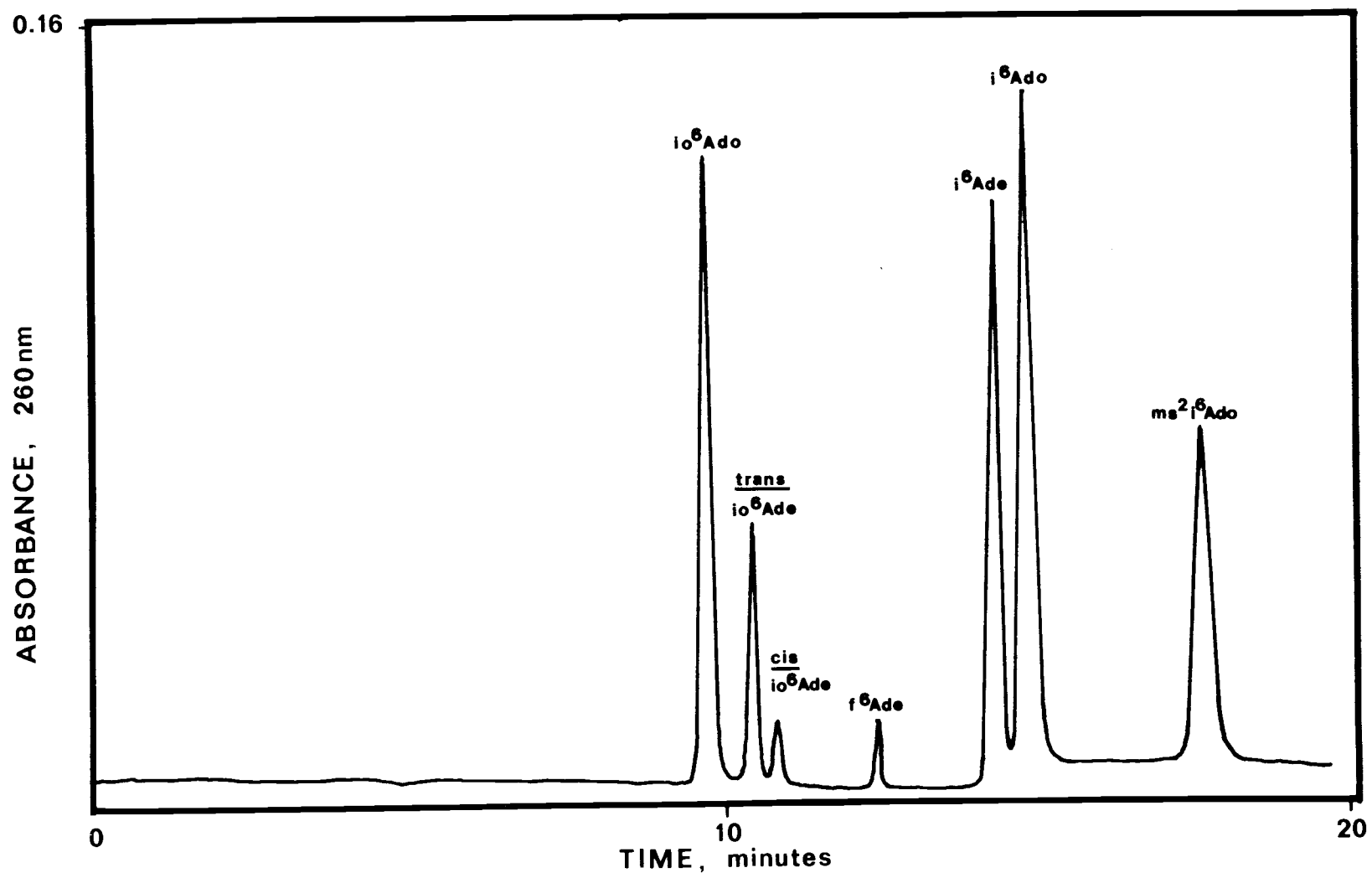


Figure 3. Analytical HPLC of standard cytokinins.

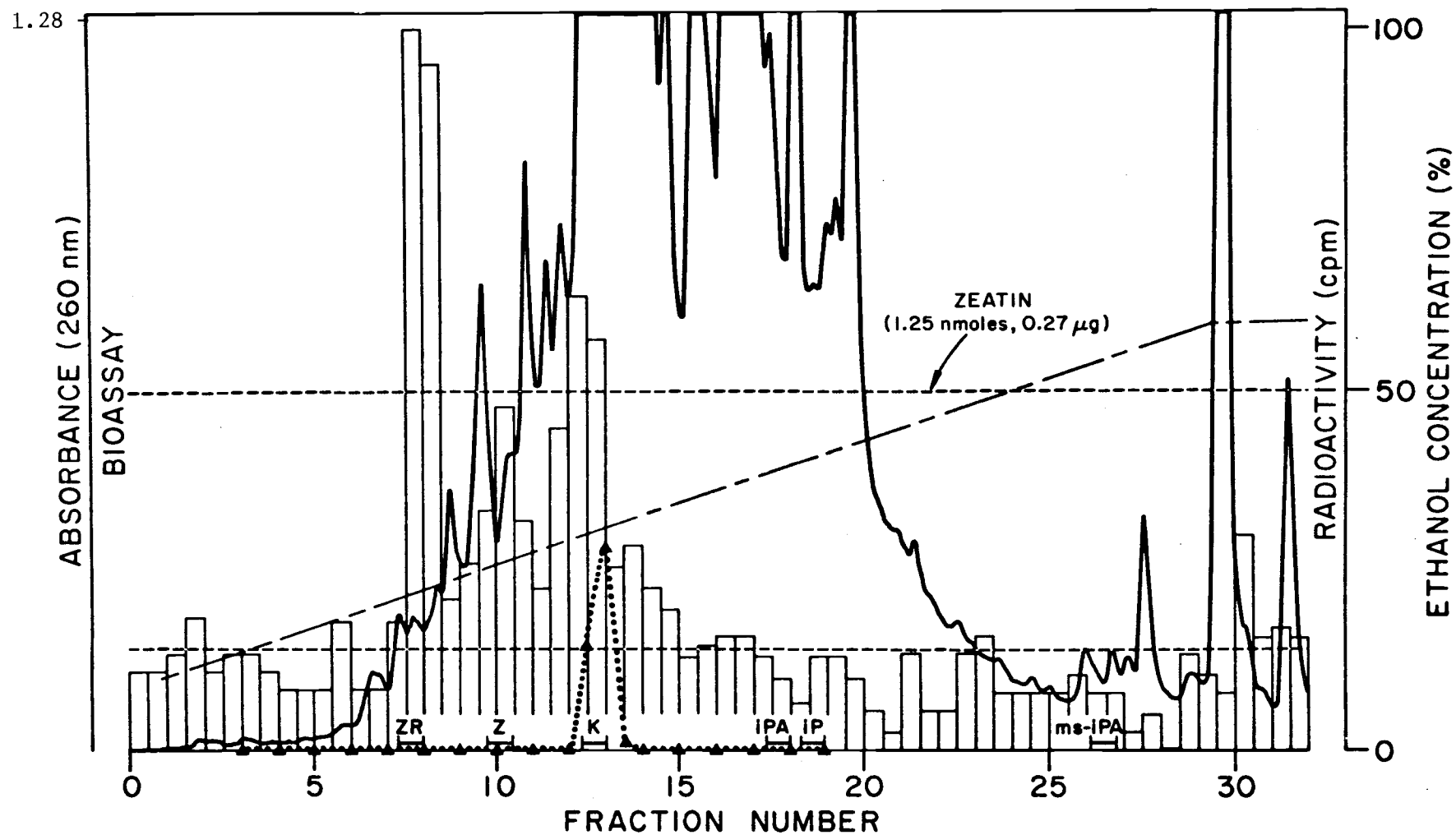


Figure 4. Analytical HPLC of Douglas fir xylem exudate showing the absorbance profile, bioassay activity and recovery of $[8-^{14}\text{C}]$ -kinetin (Morris *et al.*, 1976).

known cytokinins are shown on the abscissa. The elution of $[8-^{14}\text{C}]$ -kinetin, as determined by liquid scintillation counting, is shown as a dotted peak. At least four fractions were found to have biological activity. Fractions 16-17 had a relative mobility (R_f) similar to that of io^6Ado . The R_f of fractions 20-22 was similar to that of trans- io^6Ade . Further, fractions 24-30 and 60-62 had biological activity. Positive identification of the cytokinin-active components was not attempted due to the amount of xylem sap available at that time (Morris et al., 1976).

In order to evaluate these techniques on another system, A. tumefaciens culture filtrate was also processed by trace enrichment, analytical HPLC and bioassay with the results shown in Figure 5. Two distinct peaks of biological activity were present. The ability of these techniques to resolve complex mixtures into discrete components is apparent.

These experiments were primarily to test analytical methods we developed to ensure they would work with diverse biological materials. The chlorophyll bioassay of Fletcher and McCullagh (1971) was considerably less sensitive than the callus bioassay of Linsmaier and Skoog (1965) but was used because of its rapidity (3 days vs. 28 days) and the need to screen large numbers of fractions resulting from analytical HPLC.

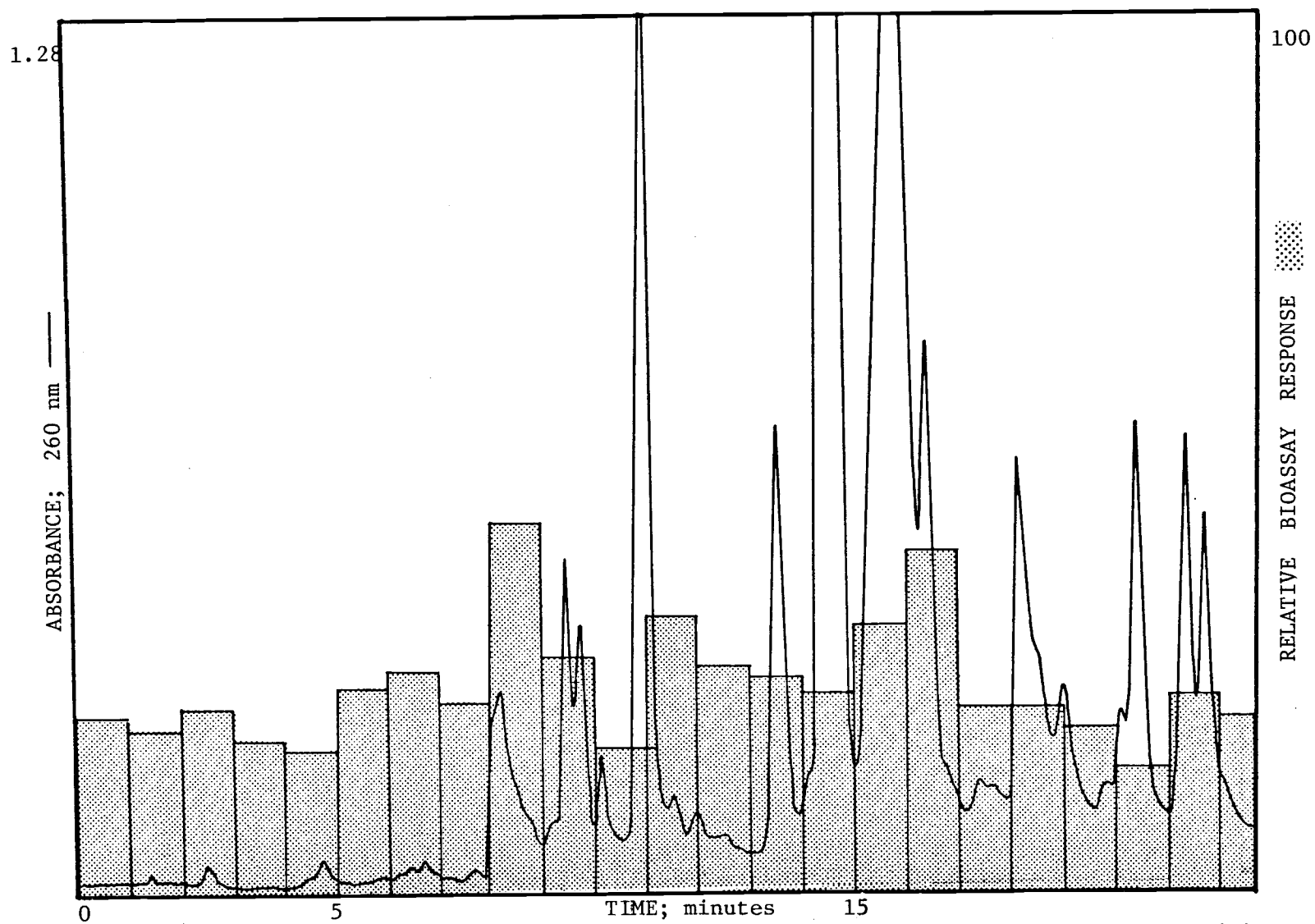


Figure 5. Analytical HPLC of A. tumefaciens, C58 culture filtrate, showing bioassay activity.

Volatile Cytokinin Derivatives
for GLC-MS

Even though analytical HPLC techniques effect excellent separations of cytokinins, the fractions are still not sufficiently pure for direct introduction into the mass spectrometer. It was therefore necessary to make cytokinin derivatives of sufficient volatility to permit GLC separation of the components prior to introduction to the mass spectrometer. The derivatives in use at the initiation of this study were the trimethylsilyl ethers (TMS), prepared by heating anhydrous cytokinin samples with commercially available silyl donors such as bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Babcock and Morris, 1970; Upper et al., 1970). Using this procedure, active (i.e., exchangeable) protons are replaced with trimethylsilyl groups. These derivatives are simple and rapid to prepare but have two disadvantages; they are sensitive to water and decompose rapidly in the presence of trace moisture and each TMS group increases the apparent molecular weight of the compound by 72 atomic mass units (amu). For example, the molecular weight of underivatized io⁶Ado is 351 while the TMS derivative of io⁶Ado has a molecular weight of 639, an increase of 182%. High molecular weight compounds are difficult to chromatograph except at high temperatures and are unstable in the mass spectrometer. To avoid

these problems, it was decided to prepare cytokinin permethyl derivatives using a modification of Hakomori's (1964) procedure.

The Hakomori procedure, which is mediated by dimethylsulfinyl anion in non-aqueous solution, results in the substitution of methyl groups for all active protons as shown in Figure 6. This should be contrasted to TMS which misses the N⁶ nitrogen. These derivatives are not moisture sensitive but are highly volatile and of moderate molecular weight (e.g. methyl io⁶Ado has a molecular weight of 421, an increase of only 122%). The permethyl derivatives proved to have an additional advantage since they exhibited mass spectra which generally had strong molecular ions (M^+) together with a simple fragmentation pathway. In contrast, the TMS derivatives of cytokinins gave complex mass spectra and, in general, have weak M^+ ions.

Separation of Cytokinin Derivatives by GLC

At the inception of this study, separations of TMS-cytokinins by GLC were performed on a packed column using thermally labile stationary phases such as QF1 (Upper et al., 1970) and DC11 (Babcock and Morris, 1970). During this study, it was found that packed columns using Dexsil 300 as a stationary phase were both thermally stable and afforded reasonable resolution of most cytokinin peaks. However, since cytokinins with hydroxylated side

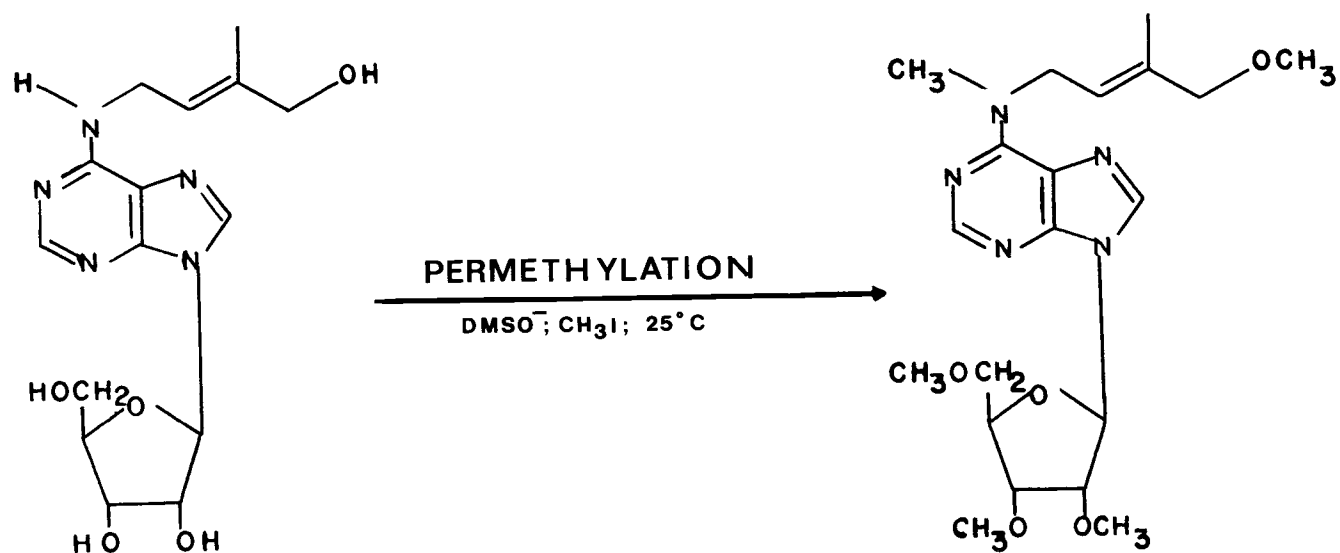


Figure 6. Permethylation of io⁶Ado.

chains can exist in either of two isomeric forms (cis or trans) and the forms are known to differ in biological activity, it was desirable to be able to achieve baseline resolution of these isomers. This resolution proved to be impossible using classical packed-column GLC. Therefore, it was decided to construct and coat support-coated open capillary columns (SCOT) (Jennings et al., 1974). These columns, drawn on a capillary drawing instrument and coated with a Dexsil-Silanox mixture, had greater than 1,000 theoretical plates per meter. A 20 meter column permitted baseline resolution of the cis and trans isomers of io^6Ade . Figure 7 is a typical separation of the permethyl derivatives of cytokinin standards on one of these columns, showing the excellent resolution achieved in a 60 minute separation.

Mass Spectra of Permethyl Cytokinins

By coupling the GLC to the mass spectrometer via an all glass system and removing excess carrier gas through a single-stage glass jet separator, it was possible to obtain mass spectra of cytokinin peaks as they eluted from the GLC column. When operated in this mode, the MS was under the direction of a system 150, PDP-8 computer. Up to 600 mass spectra were acquired and catalogued from a single GLC run. This catalogue was then examined for the

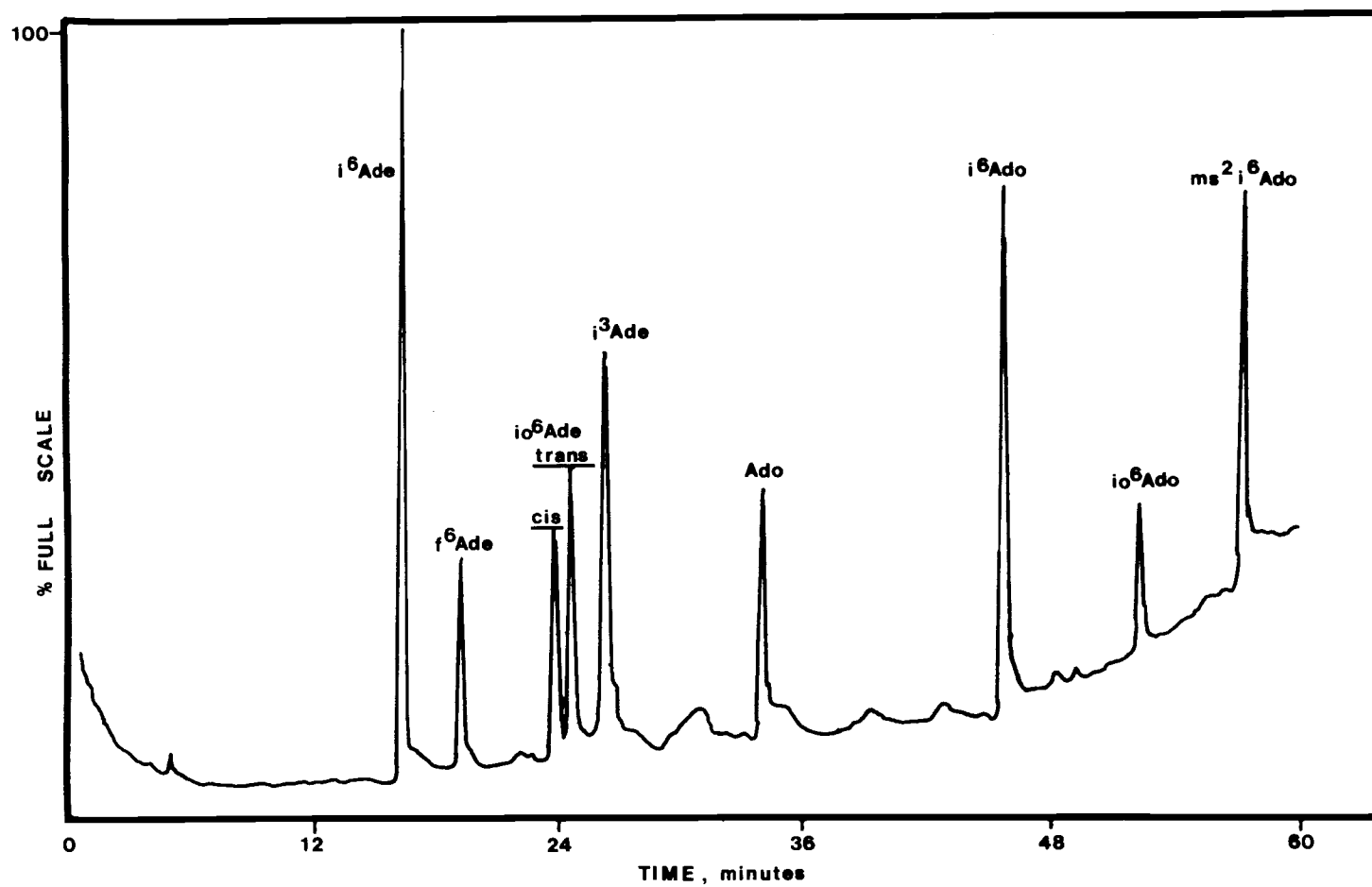


Figure 7. Separation of permethyl derivatives of cytokinins on Dexsil SCOT GLC column. Zero time starts with the first scan of the mass spectrometer.

presence of spectra typical of methyl cytokinins and, if found, "hard" copies of the spectrum were prepared.

Three typical spectra, permethyl i^6 Ade, permethyl i^6 Ado and permethyl i^3 Ade (triacanthine) are shown as they are output from the computer in Figures 8, 10 and 12. Peak assignments of major fragment ions (as confirmed by deuteropermethylation) are illustrated in Figures 9, 11 and 13 respectively.

The mass spectra of the permethyl derivatives were analogous to those of native cytokinins (Shannon and Letham, 1966; Morris, 1977). Thus, the mass spectrum of permethyl i^6 Ade (Figure 8) showed a prominent M^+ at m/e 231. The peak at m/e 216 arose by characteristic loss of one of the side chain methyl groups. Loss of 43 amu ($C_3H_7\cdot$) from the side chain and ring closure yields the stabilized tricyclic ring structure m/e 188 (Figure 9). The purine nucleus with N^9 methyl group yields a fragment ion at m/e 135. Elimination of HCN from this nucleus gives the peak at m/e 107. The peaks at m/e 98 and m/e 69 are side chain fragments.

The mass spectrum of the corresponding riboside, permethyl i^6 Ado (Figure 10), shows a large M^+ at m/e 391. Loss of 43 amu from the side chain and formation of the tricyclic structure, m/e 348 is similar to that of i^6 Ade. Cleavage of the ribose moiety and transfer of a proton to the base gives the very characteristic m/e 217 peak. Loss of the N^6 methyl group gives a peak at m/e

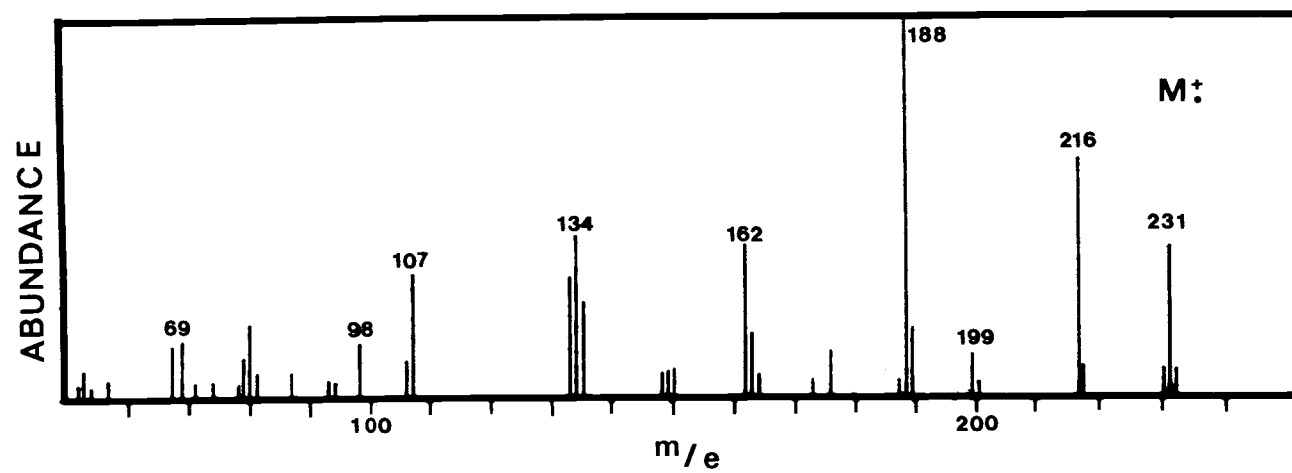


Figure 8. Mass spectrum of permethyl i^6 Ade showing the M^+ at 231 and the base peak, 188.

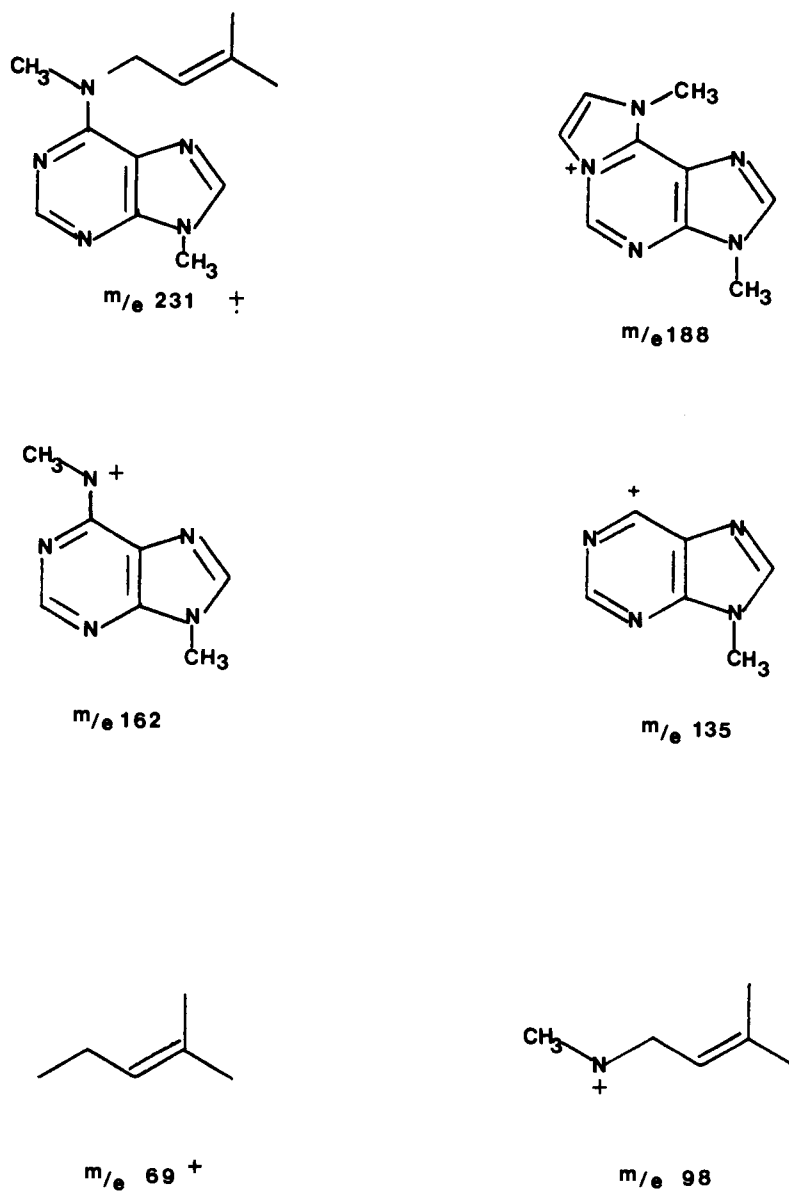


Figure 9. Structure of the major fragment ions in the mass spectrum of permethyl *i*⁶Ade.

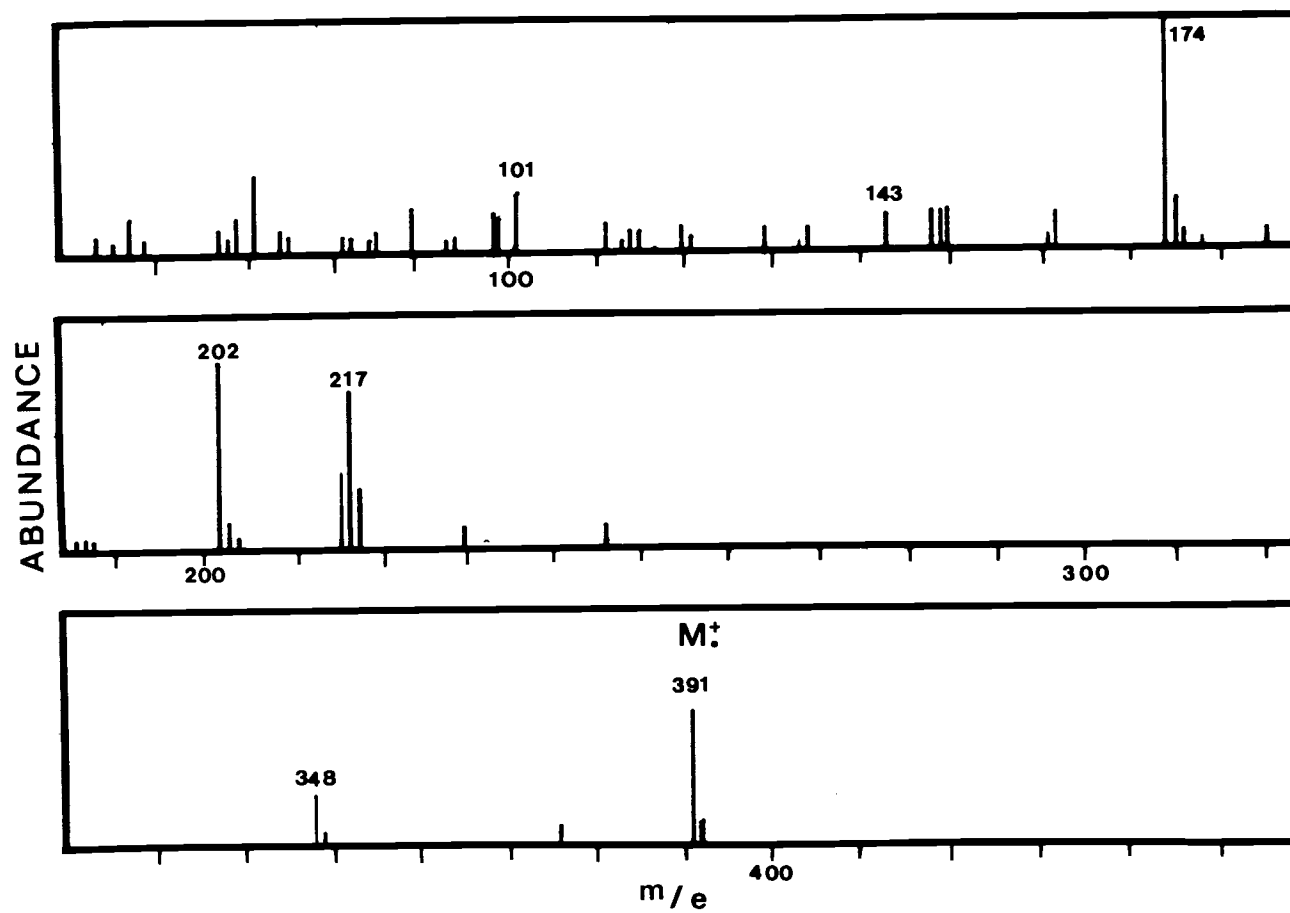


Figure 10. Mass spectrum of permethyl *i*⁶Ado showing the *M*⁺ at 391 and the base peak at 174.

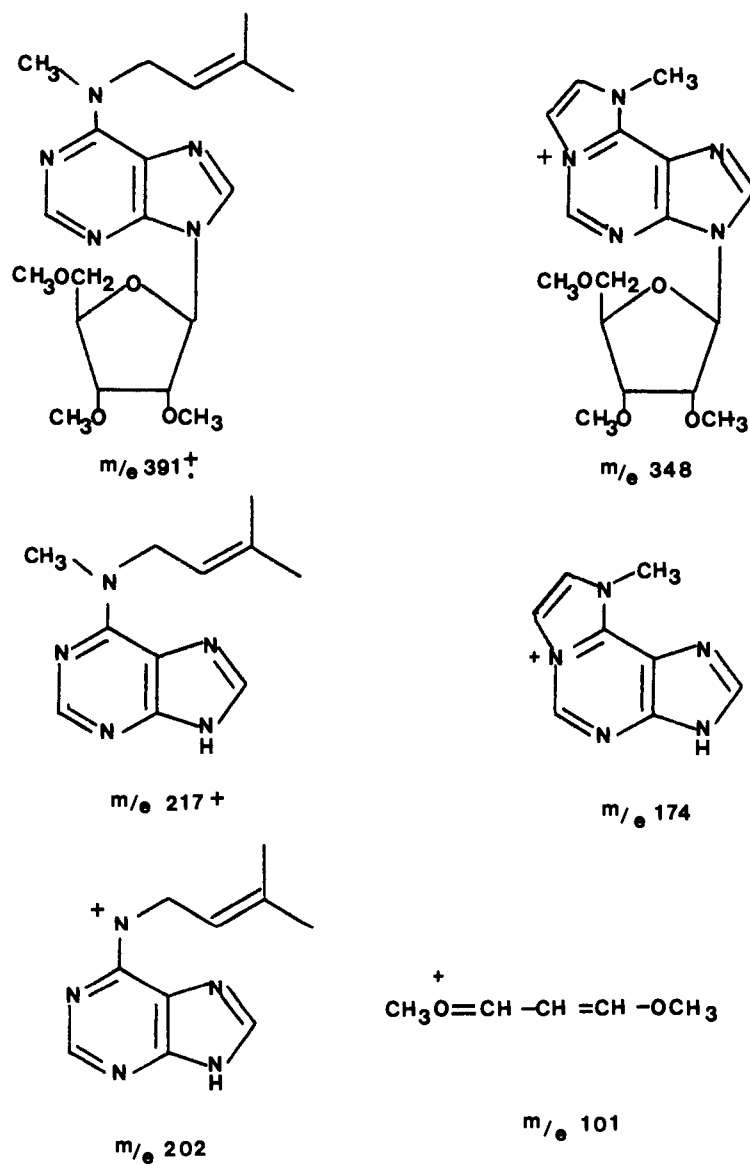


Figure 11. Structure of the major fragment ions in the mass spectrum of permethyl i^6 Ado.

202. Loss of 43 amu from the side chain of the base with ring closure yields the base peak of the spectrum at m/e 174. The peak at m/e 143 is that of trimethyl ribose and a ribose fragment peak is seen at m/e 101. This spectrum is strictly analogous to that of permethyl i^6 Ade.

The mass spectrum of permethyl i^3 Ade (triacanthine) is shown in Figure 12. It is interesting to note that although i^3 Ade and i^6 Ade have the same empirical chemical formulae ($C_{12}H_{17}N_5$), they have quite different mass spectra. This is an excellent illustration of the power of the mass spectrometer in discriminating between isomeric structures. The M^+ peak was at m/e 231 as was the M^+ peak for i^6 Ade. Similarly, there was loss of a methyl group to yield the m/e 216 peak. Loss of the isopentenyl side chain yields a peak at m/e 162 followed by the loss of one N^6 methyl group and the transfer of a proton to the base to yield a peak at m/e 148. Transfer of the methyl group to the base plus the expulsion of CH_2NH yields the base peak of the spectrum, m/e 134, with the usual structure shown in Figure 13. Loss of H_2CN from this structure yields a peak at m/e 120 and further loss of HCN gives a peak at m/e 93 (McCloskey, 1974). The side chain fragment ion is seen at m/e 69. Note the absence of any m/e 188 for fragment ion since i^3 Ade cannot form this cyclic structure.

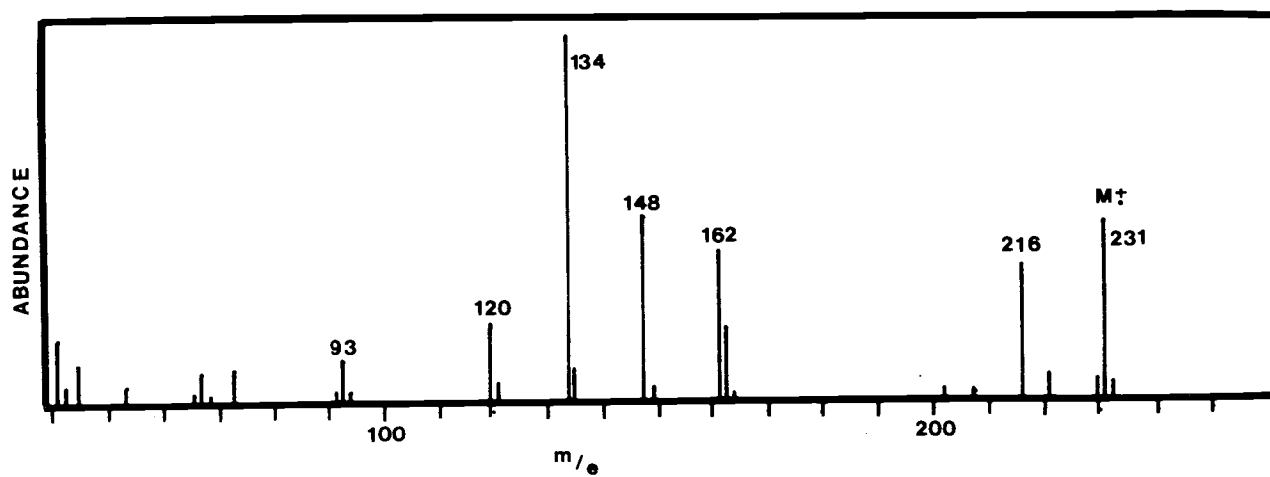


Figure 12. Mass spectrum of permethyl triacanthine (i^3 Ade).

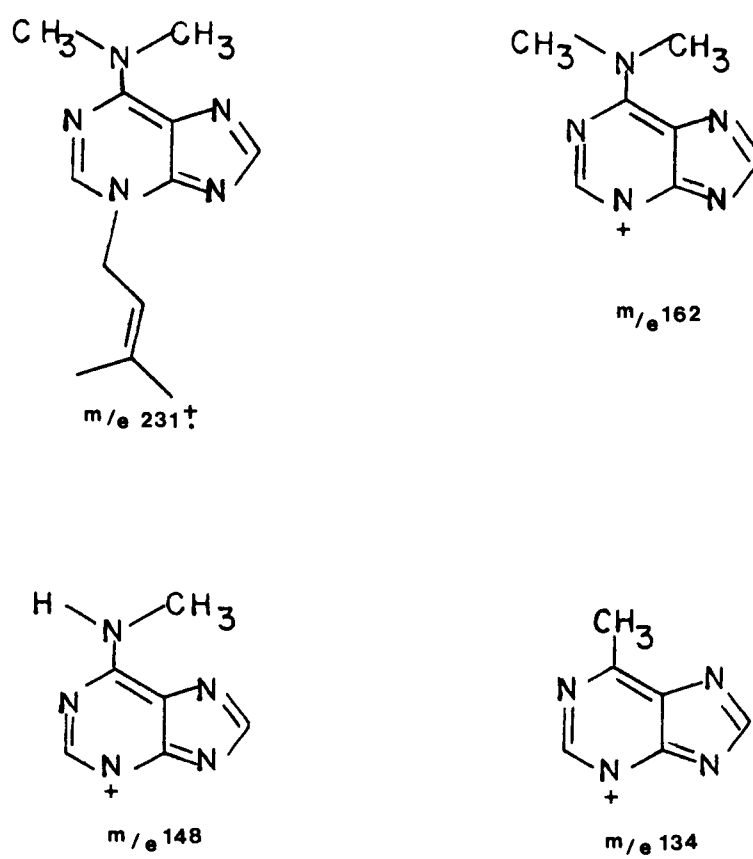


Figure 13. Major fragment ions in the mass spectrum of penthyl i³Ade (triacanthine).

Mass spectrometry thus offers a method for rigorous chemical identification of cytokinins. Interpretation of mass spectra of hydroxylated side chain cytokinins will be covered later in the results section.

Quantitative Estimation of Cytokinin Levels Using GLC-MS

Using the data acquired by the computer system during the GC-MS run, it is possible to integrate the peak intensities acquired during a run and, by comparing these integrations to the base peak areas of standard cytokinins, determine the amount of each cytokinin present. The lower limit of definitive identification of cytokinins was about 0.5 μg . This is a direct function of the hardware available and with improvements currently in progress, it should be possible to identify 0.1 μg of any cytokinin.

The methods developed in this study permit the rapid, quantitative extraction of cytokinins by trace enrichment, the analytical HPLC and determination of biological activity and not only the rigorous chemical identification of the cytokinins present, but also their side-chain geometry and the quantitative levels present. Using these techniques, I then examined the cytokinins of Agrobacterium tumefaciens tRNAs and culture filtrates.

Cytokinin Production by Agrobacterium

Growth of Agrobacterium

Figure 2 shows a transmission electron micrograph (15,000 magnification) of a single cell of A. tumefaciens, strain A178. The external morphology ($0.8 \times 1.5 \mu\text{m}$) and the flagella are visible. When grown in shake-cultures in the chemically defined medium of Table 4, A. tumefaciens strains exhibited typical bacterial growth curves, as monitored by the change in absorbance at 660 nm ($A_{660\text{nm}}$), approaching a maximum absorbance of about one in a period of 24 hours. A representative growth curve for A. tumefaciens strain C58, is shown in Figure 14. The viable cell count, obtained by serial dilution and plating, showed a maximum at 24 hours followed by a steady decline without visible cell lysis. Figure 15 shows the relationship between the viable cell number and the $A_{660\text{nm}}$. When A. tumefaciens strains were grown in a rich medium (Table 3) in the high-density fermenter, the $A_{660\text{nm}}$ typically reached five. The relationship between the $A_{660\text{nm}}$ and the grams (wet weight) of cells produced per liter of medium 523 is shown in Figure 16. A typical yield of cells from one cycle in the high density fermenter was 30-50 g. The normal yield of cells grown in shake culture in defined medium was 1 g/l. Prior to

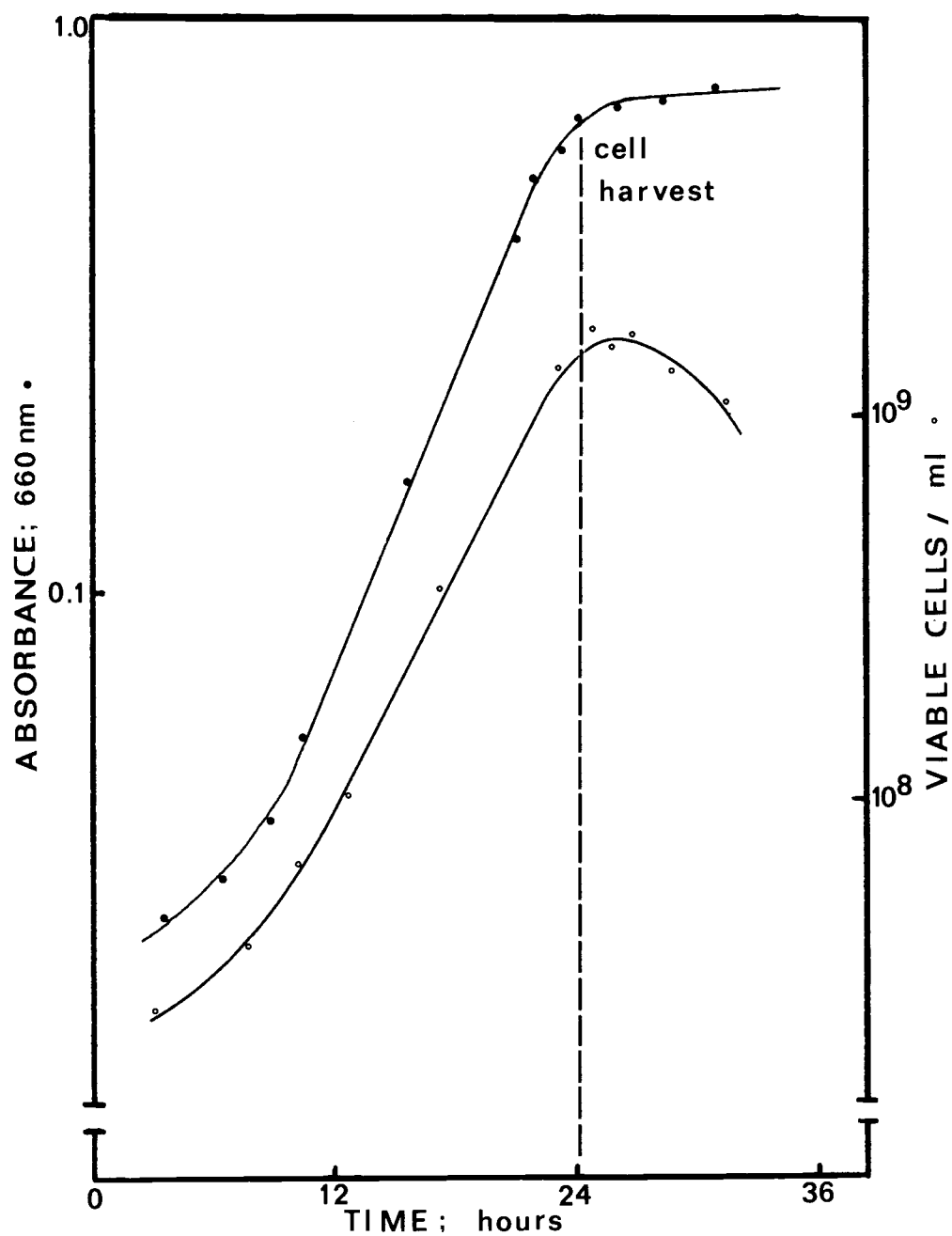


Figure 14. Typical growth curve of *A. tumefaciens*, strain C58, grown in defined medium. The viable cell count is indicated by the closed circles.

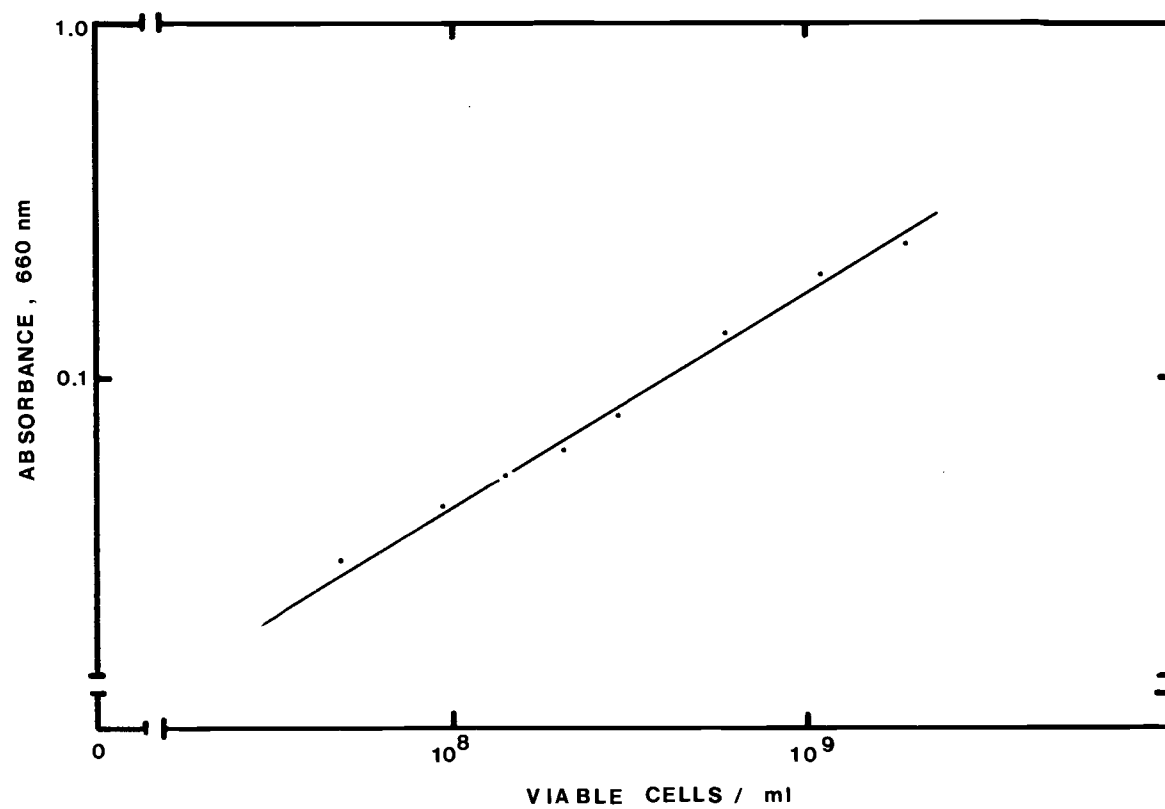


Figure 15. Relationship between $A_{660\text{nm}}$ and the number of viable cells/ml.

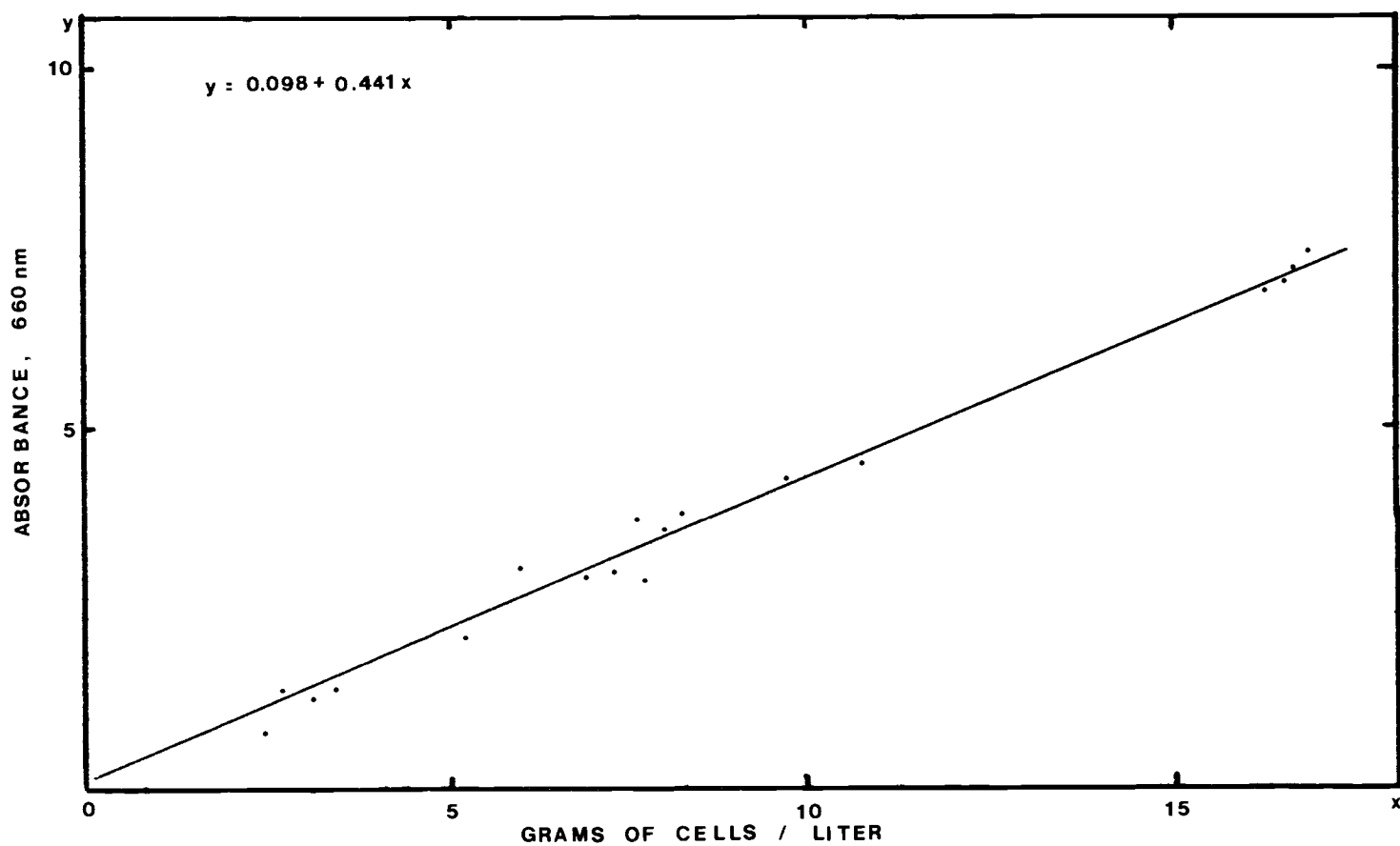


Figure 16. Relationship between $A_{660\text{nm}}$ and the yield of cells (wet weight) per liter of bacterial medium.

harvesting any experimental cultures, aliquot samples were gram-stained and examined microscopically for contaminating organisms. Any culture found to be contaminated was rejected. Bacterial strains were periodically checked for virulence on tomato plants.

Cytokinins in Agrobacterium tRNA

The procedure used for the isolation of tRNA began with the growth of 50-100 g of cells. When done in batches, the harvested cells were stored in liquid nitrogen (-196°C) until used. The amounts of nucleic acids present at various steps in the purification were monitored by measuring the absorbance at 260 nm. Table 8 shows the yields, of nucleic acids from 88 g of A. tumefaciens, strain C58.

Cytokinins were extracted from tRNA by hydrolysis after the purity of the tRNA was estimated by polyacrylamide gel electrophoresis. The amounts of various nucleic acids were determined by scanning the gel at 260 nm and calculating the area of each peak. A typical gel-scan is shown in Figure 17. The purity of 4s tRNA was normally about 90% and the only significant contaminant was 5s RNA, which is known not to contain cytokinin-active nucleosides. Yields of 4s RNA were adjusted to compensate for this minor contaminant.

Table 8. Yield of nucleic acids from 88 g of A. tumefaciens cells.

Nucleic Acid	A _{260nm} Units	Yield, mg
DNA	6.0×10^3	250
rRNA	1.43×10^4	595
tRNA	3.72×10^3	155

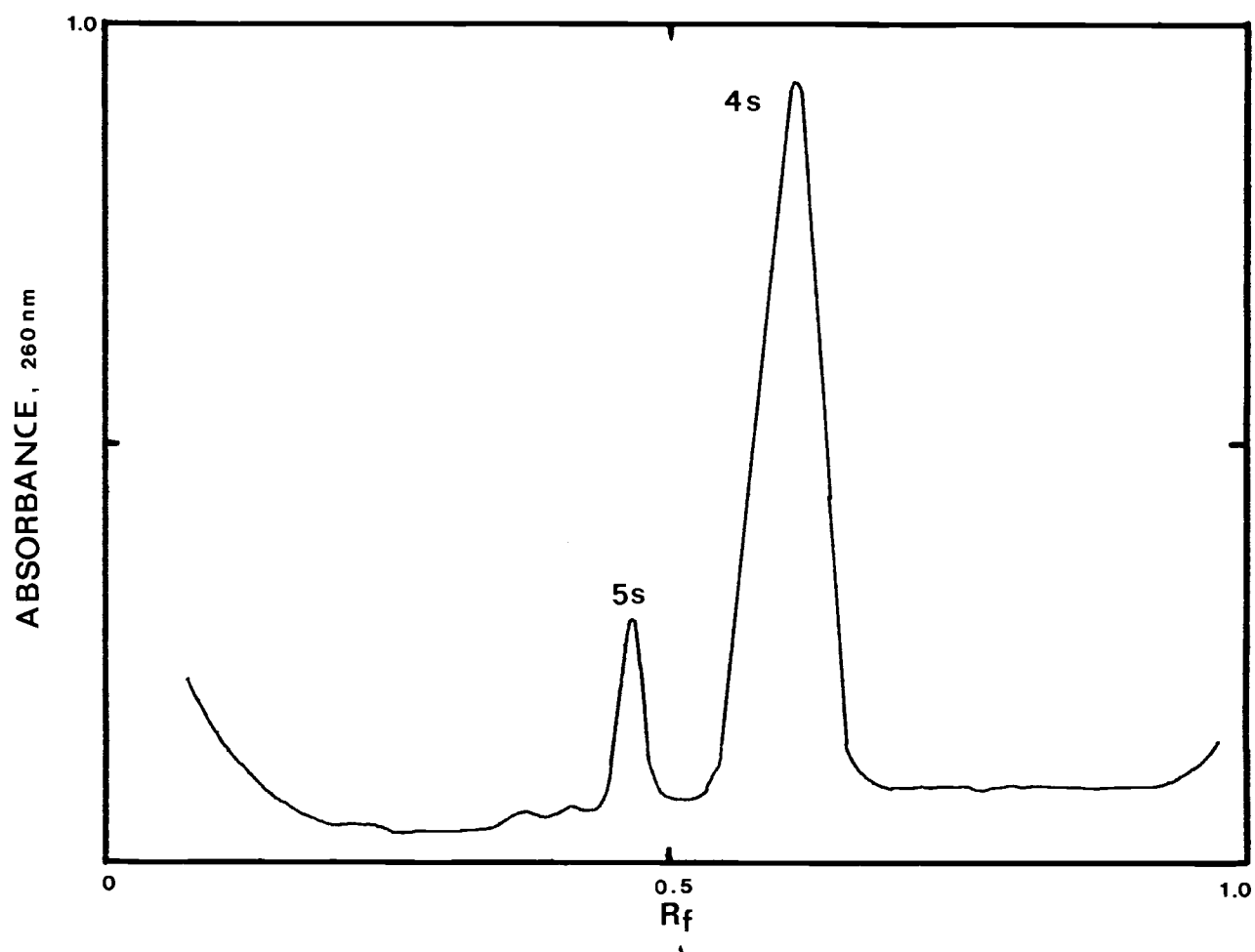


Figure 17. Scan of 10% polyacrylamide gel at 260nm. The gel was electrophoresed 4 hours at 4 milliamps/tube.

An 18 hour alkaline hydrolysis of tRNA at 37°C was sufficient to liberate all cytokinin-active nucleotides. To check that cytokinins were unaffected by alkaline hydrolysis, authentic standard cytokinins were subjected to 0.3M KOH treatment for 24 hours at 37°C. When extracted, derivitized (TMS), and examined by GLC, no decrease in peak areas (compared to untreated samples) was seen, nor were there any spurious peaks.

In order to determine if the qualitative presence of tRNA-bound cytokinins was related to virulence, four strains of A. tumefaciens were examined. The strains used were C58, NT1, II BV7 and II BNV6. Strain NT1 is an avirulent, plasmid-negative strain derived from the virulent strain strain C58. They differ only in the presence of the Ti plasmid. Strain II BV7 and II BNV6 (arivulent) were both derived from the parent strain II B. These paired strains are thus closely related except for virulence and the presence of the Ti plasmid. The nature of the tRNA-bound cytokinins in each of these strains is shown in Table 9. In addition to the cytokinins expected in prokaryotes ($i^6\text{Ado}$, $ms^2i^6\text{Ado}$), all strains had trans $io^6\text{Ado}$. Strains C58 and NT1 has $ms^2io^6\text{Ado}$. This was the first reported instance of trans $io^6\text{Ado}$ from the tRNA of a prokaryote. The only other confirmed report of $io^6\text{Ado}$ production by prokaryotes was that of cis $io^6\text{Ado}$ in the culture filtrate of Corynebacterium (Scarborough et al., 1973). It should be noted

Table 9. Occurrence of cytokinins in tRNA from various strains of A. tumefaciens. (Chapman et al., 1976).

Strain	Cytokinin Present				Virulence
	i ⁶ Ado	ms ^{2,i} ⁶ Ado	<u>trans</u> -io ⁶ Ado	ms ² io ⁶ Ado	
C58	+	+	+	+	+
NT1	+	+	+	+	-
II BV7	+	+	+	-	+
II BNV6	+	+	+	-	-

that there was no apparent correlation of tRNA-bound cytokinins with virulence in these strains.

Quantitative Determination of Culture Filtrate and tRNA-bound Cytokinins

Using the techniques already discussed, the levels of cytokinins present in the culture filtrate and tRNA of various strains of A. tumefaciens were determined. The bacteria were grown in defined medium (Table 4). To preclude the possibility that production of cytokinin-active material could result from autoclaving the medium, one liter of medium was prepared and autoclaved. The medium was not inoculated but was trace-enriched and fractionated on the analytical HPLC. Bioassay of the fractions showed that no cytokinin-active material was produced. Hence, 48.5 l of culture filtrate from strain C58 were examined for cytokinins and the tRNA-bound cytokinins from the 45.9 g of cells were isolated. Figure 18 shows the analytical HPLC separation and bioassay data for the culture filtrate (panel A), tRNA hydrolysate (panel B) and a standard mixture of cytokinins fractionated under identical conditions (Kaiss-Chapman and Morris, 1977). Bioassay-active fractions were pooled as indicated on Figure 17, lyophilized and derivatized for GLC-MS. Fractions designated A1, A2, A3, B1, B2, and B3 were permethylated for GLC-MS. Fraction B4, because

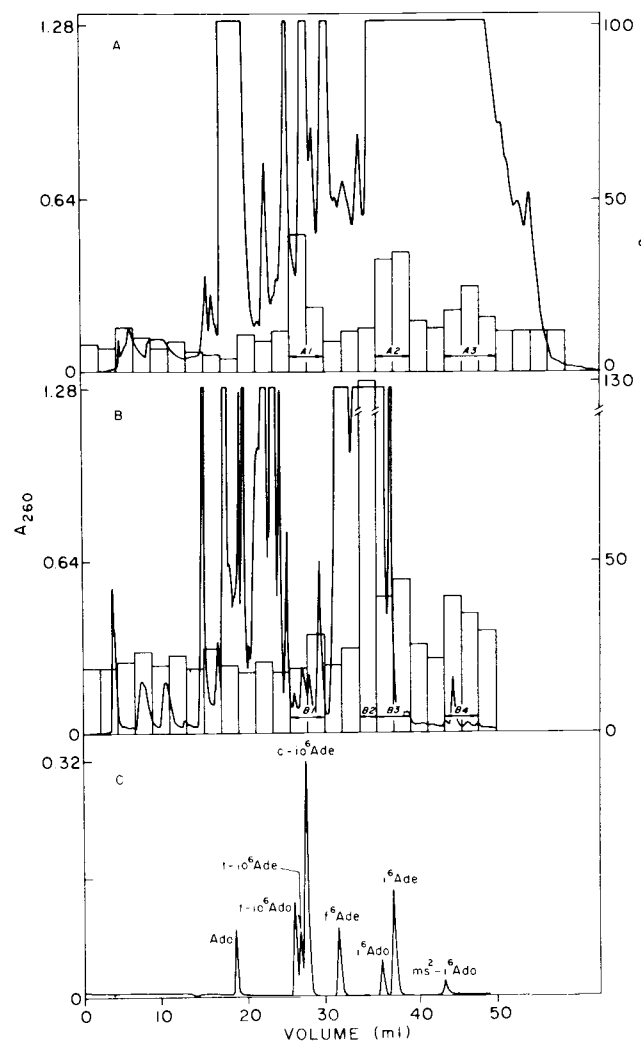


Figure 18. Analytical HPLC of *A. tumefaciens*, C58, culture filtrate (panel A), tRNA hydrolysate (panel B) and a cytokinin standard (panel C) under identical conditions of elution.

of its apparent purity by analytical HPLC, was converted to the trimethylsilyl derivative for direct-inlet MS. The apparent R_f values for the bioassay-active HPLC fractions indicated the possible presence in the culture filtrate of io^6 Ado, io^6 Ade (A1), ms^2io^6 Ado, i^6 Ado, i^6 Ade (A2) and ms^2i^6 Ado (A3). In the tRNA hydrolysate the R_f of bioassay-active HPLC fractions indicated the possible presence of io^6 Ado (B1), ms^2io^6 Ado (B2), i^6 Ado (B3) and ms^2i^6 Ado (B4).

Figure 19 (panel A) shows the mass spectrum obtained from culture filtrate fraction A1. It is identical to that of authentic io^6 Ade and exhibits a M^+ at m/e 261. The molecular weight of permethyl io^6 Ade. The base peak of the spectrum, m/e 230, represents the facile loss of OCH_3 from the hydroxylated side chain. The peaks at m/e 216, 188, 162 and 134 are similar to those in the spectrum of i^6 Ade.

The mass spectrum from fraction B2, shown in Figure 19, panel C, exhibits a M^+ at m/e 467, the molecular weight of permethyl ms^2io^6 Ado. There is a peak at m/e 452 from the loss of a methyl group. The base peak, m/e 436, represents the loss of OCH_3 from the hydroxylated side chain. Additional side chain loss and formation of a tricyclic structure yields a peak at m/e 394. Loss of ribose gives a peak at 292 followed by a loss of a methyl group to give a peak at m/e 262. Ring closure of the base gives a

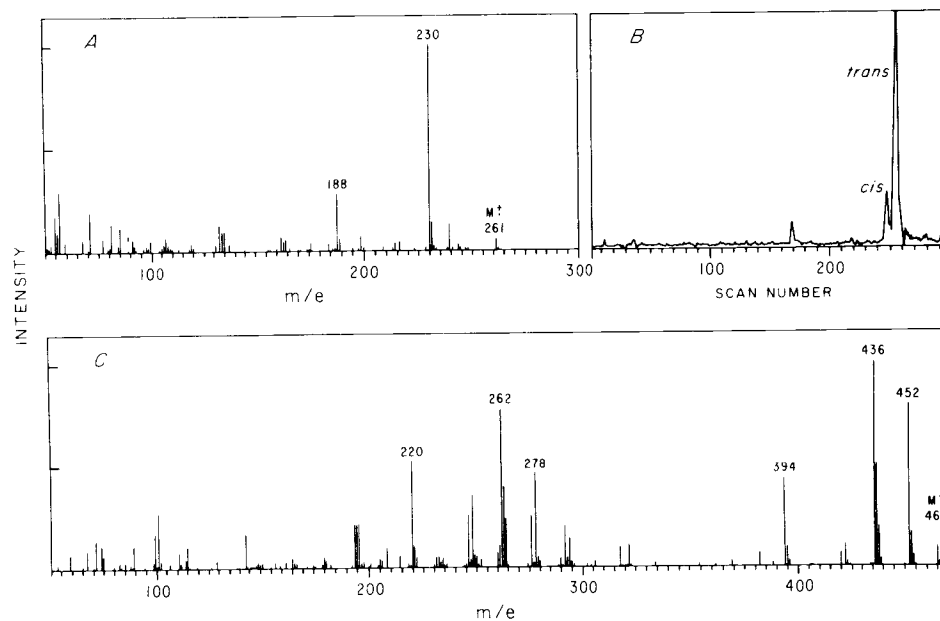


Figure 19. Mass spectrum of $io^6 Ade$ (panel A) obtained from culture filtrate (fraction A1) of A. tumefaciens, C58. Panel B shows the relative amounts of the *cis* and *trans* isomers of $io^6 Ade$. Panel C shows the mass spectrum of $ms^2io^6 Adeo$ obtained from tRNA hydrolysate fraction B2.

peak at m/e 220. The fragmentation of this compound is similar to that of io^6 Ado (Figure 20) from fraction A1 except that its peaks are shifted up by 46 amu, the mass of the methylthio group.

Fraction A3 had a component which gave the mass spectrum shown in Figure 21. This spectrum is similar to that of i^6 Ado (Figures 10 and 11) except that peaks are again shifted up by 46 amu. The spectrum is identical to that of authentic permethyl $ms^2 i^6$ Ado.

Figure 22 shows the mass spectrum of TMS $ms^2 i^6$ Ado from fraction B4. The M^+ ion is rather weak and all ions above m/e 100 had to be multiplied by a factor of two for clarity. The base peak in the spectrum m/e 73 is due to the facile loss of the OTMS⁺ group. The peak at m/e 147 is a frequent rearrangement ion, TMSOTMS⁺. This spectrum is identical to that of authentic TMS $ms^2 i^6$ Ado.

The quantitative levels of cytokinins present in the tRNA and culture filtrate of strain C58 are shown in Table 10. These levels were determined by integration of the base peak intensity in each of the mass spectra. The three major cytokinins found in the culture filtrate were i^6 Ade, trans- io^6 Ade and $ms^2 io^6$ Ado. The major cytokinins present in tRNA were i^6 Ado and $ms^2 io^6$ Ado. In addition to measuring the levels of cytokinins present, the ratio of the cis and trans isomers of io^6 Ade was determined by integrating the base peak ($m/e=230$) of the separated isomers (panel B, Figure 19). The trans isomer predominated over the cis isomer in a ratio of 85:15.

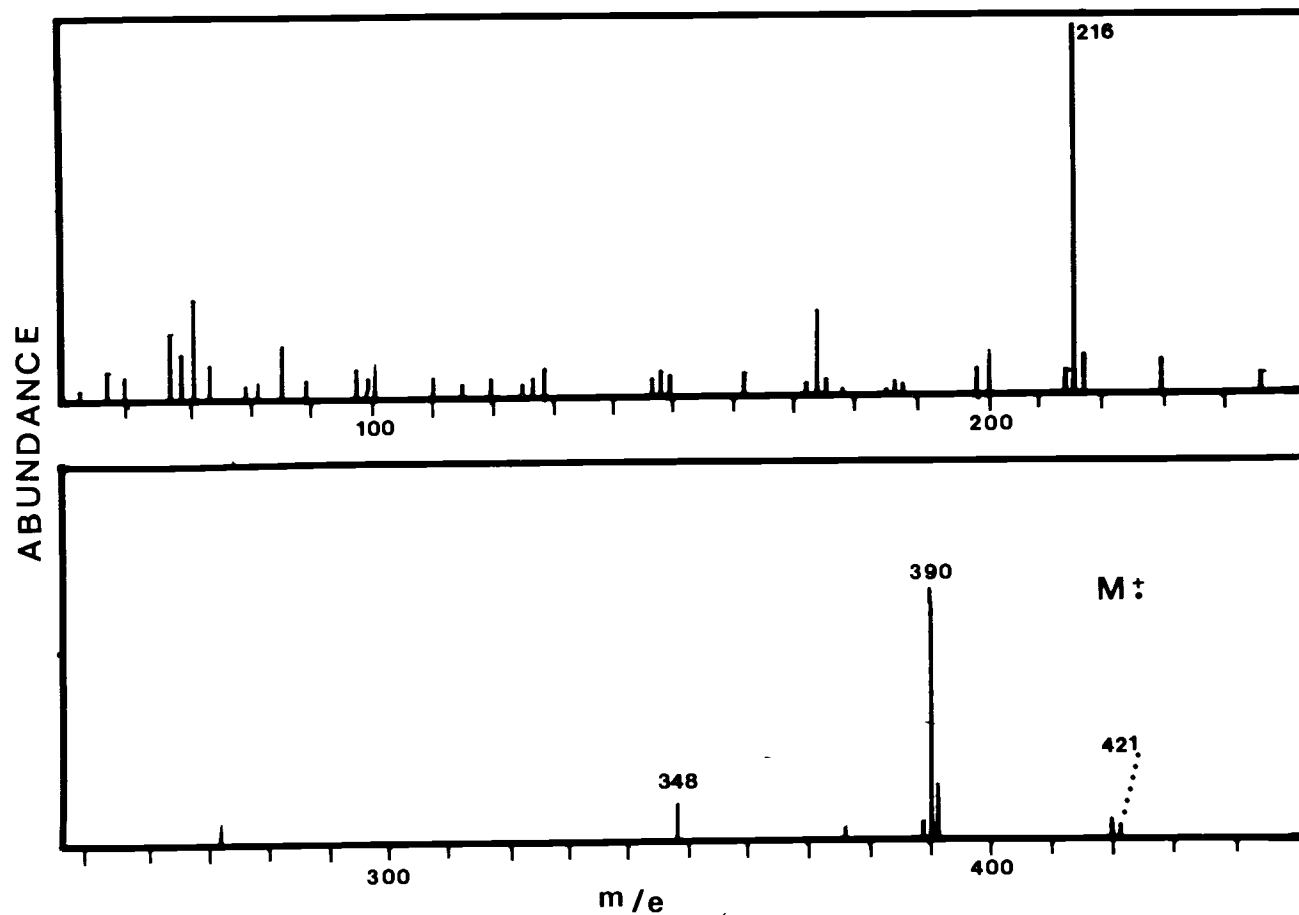


Figure 20. Mass spectrum of permethyl io⁶Ado obtained from A. tumefaciens C58, culture filtrate fraction A1.

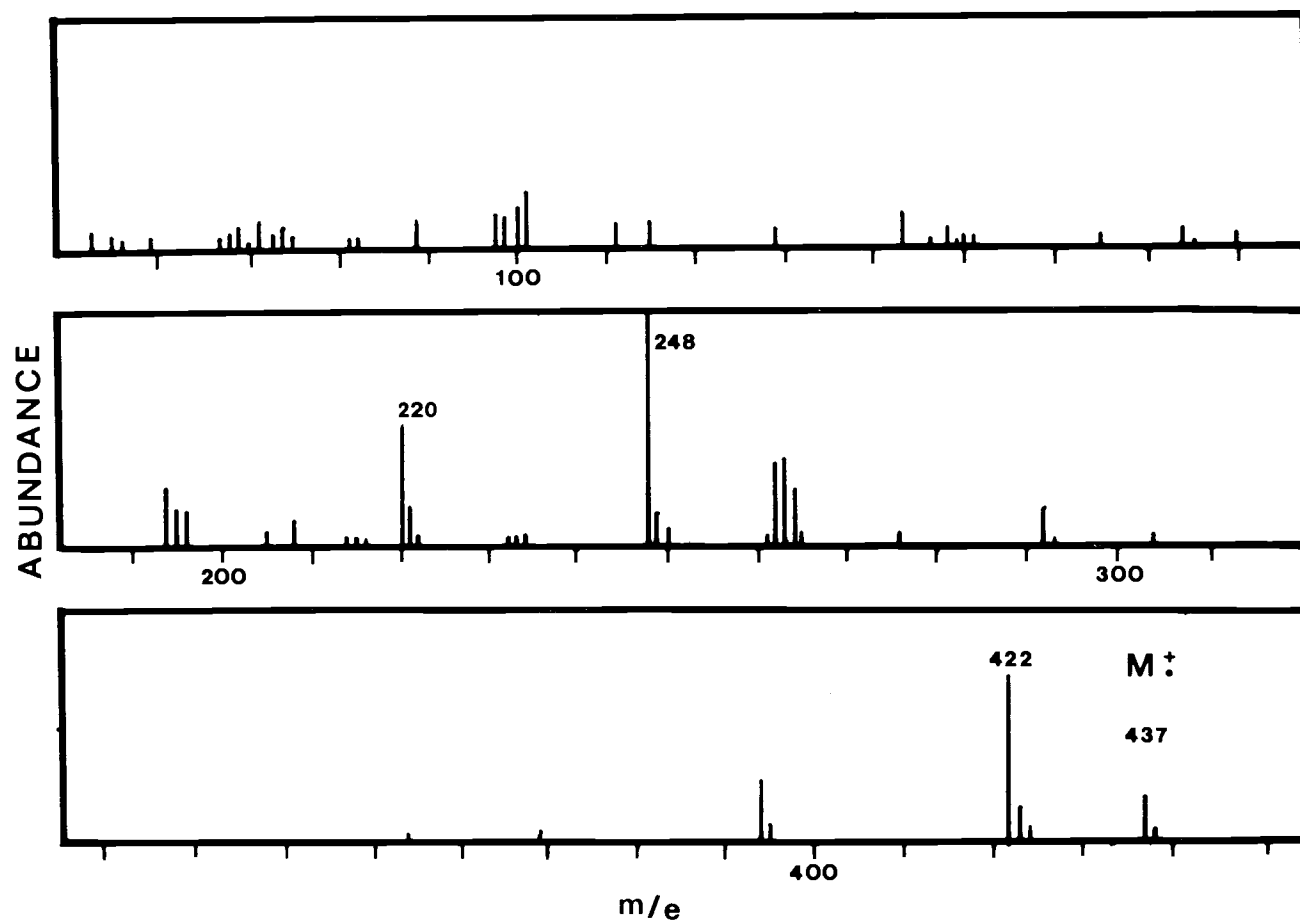


Figure 21. Mass spectrum of permethyl $ms^{2,6}$ Ado obtained from A. tumefaciens C58, culture filtrate fraction A3.

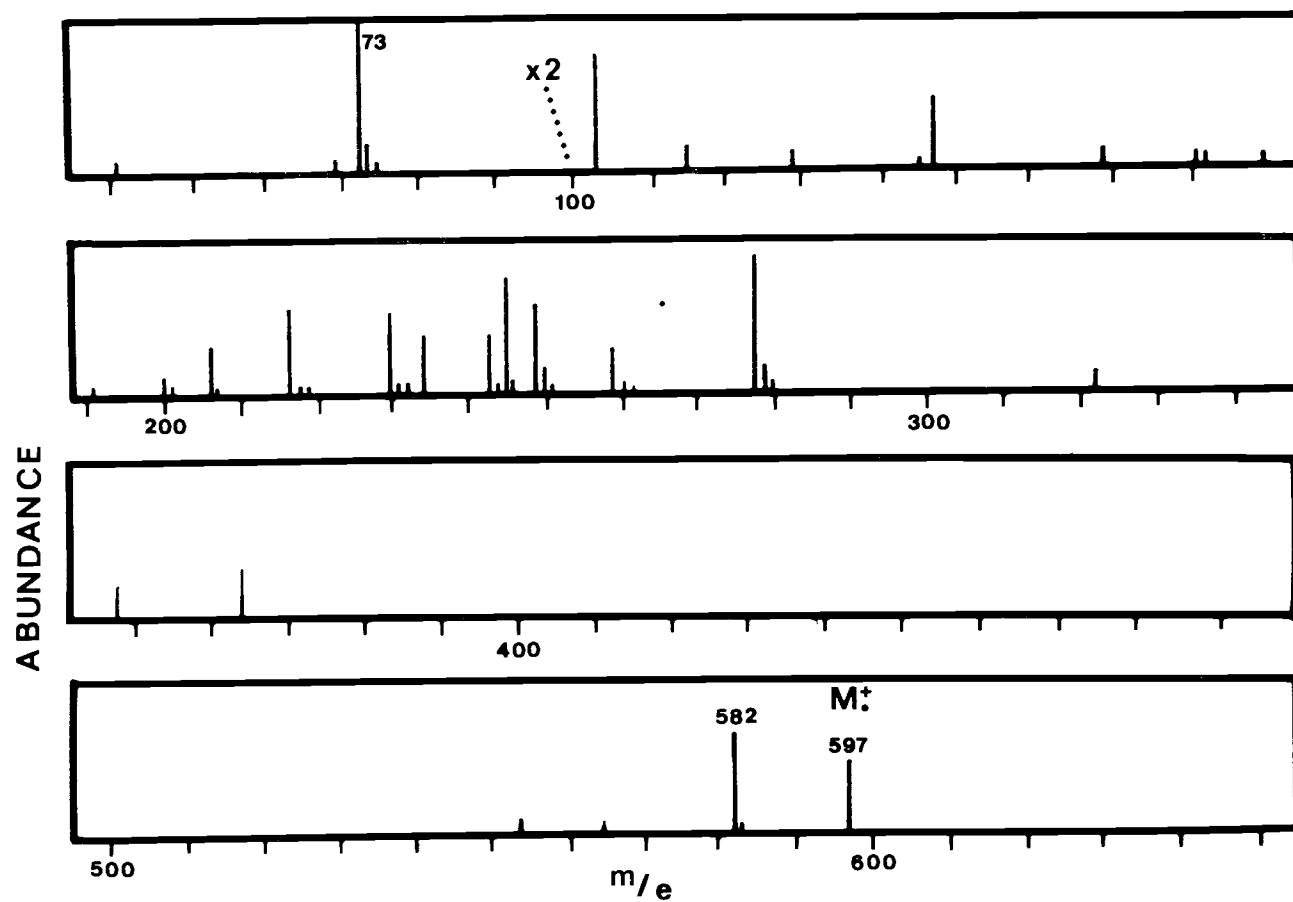


Figure 22. Mass spectrum of trimethylsilyl $ms^{2,6}$ Ado obtained from *A. tumefaciens*, C58, tRNA hydrolysate fraction B4. All peaks starting at m/e 100 are multiplied by two.

Table 10. Occurrence of cytokinins in tRNA and culture filtrates of A. tumefaciens, strain C58 (Kaiss-Chapman and Morris, 1977).

Cytokinin	Source	
	tRNA ^a Mole %	Culture Filtrate ^b μg/g cells
i ⁶ Ade	--	0.28
i ⁶ Ado	0.11	0.09
<u>c</u> -io ⁶ Ade	--	0.036
<u>t</u> -io ⁶ Ade	--	0.204
<u>t</u> -io ⁶ Ado	0.011	0.08
ms ² i ⁶ Ado	0.015	0.02
ms ² io ⁶ Ado	0.048	0.21

^a Yield of tRNA from 45.87 g cells = 80.3 mg.

^b One liter of medium gave 1.01 g of cells.

This is the first reported instance of the trans isomer of io⁶Ade as the major hydroxylated-side chain cytokinin produced in the culture filtrate of a prokaryote. In conjunction with the occurrence of the trans isomer of io⁶Ado in the tRNA (Table 9) of A. tumefaciens, this evidence makes A. tumefaciens unusual among prokaryotes.

Relative Levels of Cytokinins Present in
Various Strains of *A. tumefaciens*

The relative amounts of i^6 Ade and io^6 Ade present in several strains of *A. tumefaciens* were determined. Strain C58 is a virulent, Ti plasmid-positive strain, and strain NT1 is an avirulent, Ti plasmid-negative strain. Strain NT1 was derived from strain C58 by heat treatment. The two strains are presumably isogenic except for the Ti plasmid. Strain A174 is a genetic construct consisting of the Ti plasmid from strain C58 in an avirulent NT1 derivative. If the genetic determinants of cytokinin production were plasmid-borne, this should be evident in the relative levels of cytokinins produced. Table 11 shows preliminary results obtained from this experiment. The level of i^6 Ade produced by all three strains is remarkably similar, differing only by a factor of two. However, we were unable to detect io^6 Ade in either NT1 or A174 culture filtrates. The lower level of detection is estimated to be 0.2-0.5 μ g of cytokinin in these experiments.

Table 11. Amount of i^6 Ade and io^6 Ade present in the culture filtrate of various A. tumefaciens strains.

Strain	Virulence	Relative Amount ⁺	
		i^6 Ade	io^6 Ade
C58	+	1.0	1.0
NT1	-	0.6	N.D.*
A174 [§]	+	0.5	N.D.

⁺ A relative amount of 1.0 represents 1.4×10^5 digital integrator counts for the base peak of the mass spectrum. For i^6 Ade, $2.5 \mu\text{g} = 1.4 \times 10^5$ counts; for io^6 Ade, $1.7 \mu\text{g} = 1.4 \times 10^5$ counts.

* N.D. indicates not detected at a limit of 10% of the C58 values.

[§] This strain was derived by placing the C58 Ti plasmid into the NT1 background.

V. DISCUSSION

Definitive identification and measurement of the low levels of cytokinins present in the tRNA and culture filtrate of A. tumefaciens strains required the development of methods for isolation and purification which were sensitive, rapid and quantitative.

The technique of trace enrichment (Morris et al., 1976; Chapman et al., 1976; Kaiss-Chapman and Morris, 1977), previously described, was found to be superior to solvent extraction methods which have been in general use (Webb et al., 1973; Hemberg and Westlin, 1973; Hemberg, 1974). Using trace enrichment, excellent recovery of cytokinins from such diverse biological material as Douglas-fir xylem exudate and bacterial culture filtrates was obtained.

Although the trace enrichment procedure provides substantial purification of cytokinins, a further purification was required prior to capillary column GLC-MS. Fractionation on micro-particulate C18-silica provided such a step and when coupled with the use of the rapid bioassay of Fletcher and McCullagh (1971) allowed the purification and location of cytokinin-active fractions. We have found (and Carnes et al. (1975) have confirmed) that the resolution of analytical HPLC was sufficient to separate all standard cytokinins which were available including the cis and trans isomers of io⁶ Ade.

Rigorous chemical identification was provided by GLC-MS using permethyl derivatives (Hakamori, 1964). These derivatives possessed several advantages over the trimethylsilyl derivatives previously used (Upper et al., 1970). They are highly volatile, stable, of moderate molecular weight and yield simple mass spectra with strong fragment ions.

The use of Dexsil-coated glass capillary GLC columns provided the final purification step necessary before introduction to the mass spectrometer. The sequence of methods developed in this study, trace enrichment, analytical HPLC, permethylation and capillary GLC-MS have potential for use in the identification of cytokinins from a variety of biological sources. Further, these techniques are sufficiently sensitive to allow recovery, purification and definitive identification of as little as 0.5 μ g of an individual cytokinin. This allows the use of smaller amounts of biological materials.

The results of this study have shown that the nature of the cytokinins produced by A. tumefaciens is distinct from that of other prokaryotes such as E. coli. The culture filtrate of A. tumefaciens contains i^6 Ade, trans- i^6 Ade and $ms^2 i^6$ Ado along with other cytokinins. The tRNA of A. tumefaciens contains i^6 Ado and $ms^2 i^6$ Ado as well as trans- i^6 Ado. The production of the trans isomers of hydroxylated cytokinins implies that A. tumefaciens is unusual even

among other plant-associated prokaryotes. Table 12 contains a comparison of cytokinins produced by the plant pathogen, C. fascians, with those produced by A. tumefaciens. In contrast to A. tumefaciens, C. fascians produces primarily the cis isomers of io^6Ade and io^6Ado . C. fascians infection results in the loss of apical dominance and the formation of a "witches broom" by the susceptible host. A. tumefaciens results in neoplastic tumorous growths on the host plant. Since trans- io^6Ade is more active than cis zeatin in the tobacco callus bioassay (Skoog and Armstrong, 1971), one may speculate that the production of trans- io^6Ade by A. tumefaciens may be related to the etiology of crown gall disease.

Estimates of the levels of cytokinins produced by A. tumefaciens strains range from a high of 100 μg of io^6Ade per liter of bacterial medium (Upper et al., 1970) to a low value of 0.24 μg of io^6Ade per liter of bacterial medium (Hahn et al., 1976). It must be emphasized that in both of these reports, identification of the cytokinin was presumptive. In this study, 0.28 μg of io^6Ade was found per liter of medium. However, this amount represented only 30% of total cytokinin actually present (Table 10). The total amount of cytokinin found in one liter of medium was 0.92 μg . Because of the death of cells in stationary phase (Figure 14), the choice of harvest time for these experiments was critical. Upper et al. (1970) harvested cells at 60 hours whereas in this study cells were

Table 12. Production of cytokinins by plant pathogenic prokaryotes.

Organism		Cytokinins Produced							
		i^6 Ade	i^6 Ado	ms^2i^6 Ado	ms^2i^6 Ado	io^6 Ade		io^6 Ado	
						<u>cis</u>	<u>trans</u>	<u>cis</u>	<u>trans</u>
<u>C. fascians</u> *	C.f. ^s	+	+			+	trace	+	trace
<u>A. tumefaciens</u> [#]	tRNA	-	+	+	+	-	-	trace	+
	C.f.	+	+	+	+	trace	+	trace	+

* Armstrong et al., 1976; Scarborough et al., 1973.

[#] Chapman et al., 1976; Kaiss-Chapman and Morris, 1977.

^s C.f. = culture filtrate

harvested at the peak of the viable cell curve (24 hours). Lysis of moribund cells and catabolic processing of tRNA could account for the high value obtained by Upper et al. The amount of cytokinin produced by A. tumefaciens is small, however, similarly low levels of cytokinins produced by Rhizobium were assumed to be sufficient to influence the nodulation process (Phillips and Torrey, 1972). In addition, application of cytokinins to susceptible plants can mimic symptoms of C. fascians infection (Armstrong et al., 1976).

Mechanisms of cytokinin biosynthesis by prokaryotic organisms have been a source of much speculation. By inspection of Tables 9 and 10, it can be seen that the cytokinin profiles of tRNA and culture filtrate are similar, so that metabolic turnover of tRNA and the presence of a nucleosidase could provide a mechanism for the production of the culture-filtrate cytokinins actually found. Figure 23 is a schematic diagram illustrating this process. Since no free methylthiolated cytokinin bases were observed, it may be possible that methylthiolation at the two position of the purine ring confers an immunity to nucleosidase action. However, the data of Table 11 may support an independent biosynthetic route. If simple tRNA turnover coupled with nucleosidase activity generates free cytokinins, why is io^6Ade not found in the culture filtrate of strain NT1 when this strain is known to contain trans io^6Ado (Table 9)? One possible alternate explanation for this would be a selective

degradation of specific tRNA molecules to release only certain cytokinins. In support of this point, Babcock and Morris (1973) observed a factor in peas which degraded the anticodon loop of tRNA^{leu}₆. Further degradation of the tRNA^{leu}₆ could yield cytokinin-active nucleotides. The data of this study do not exclude the possibility of an independent route of cytokinin biosynthesis nor do they prove that biosynthesis occurs via degradation of tRNA.

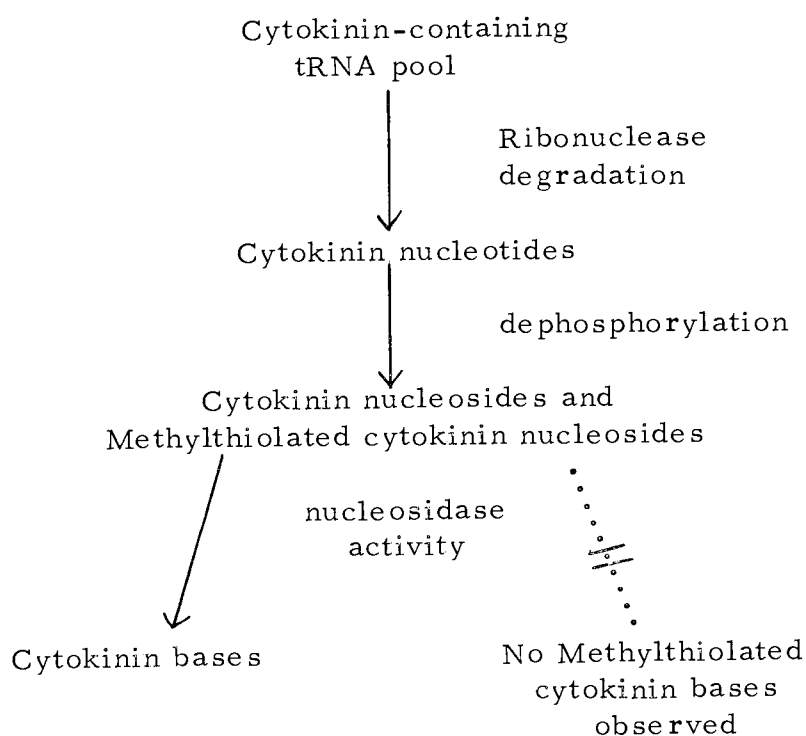


Figure 23. Schematic diagram of catabolic processing of tRNA to yield cytokinin bases.

Finally, the preliminary data of Table 11, presented because of its interest, shows that the production of i^6 Ade does not correlate with the presence of the Ti plasmid nor does the production of io^6 Ade. Strain C58 (virulent, Ti plasmid positive), strain NT1 (avirulent, Ti plasmid negative) and strain A174 (the C58 Ti plasmid in the NT1 background) produce levels of i^6 Ade differing only by a factor of two. Neither strain NT1 nor strain A174 produced detectable levels of io^6 Ade. The lack of io^6 Ade in the culture filtrate of strain A174 may be real or reflect a partial deletion in the reinserted Ti plasmid. It is known that strain K27 carries genes for both octopine and nopaline on the Ti plasmid but when this Ti plasmid is placed in the NT1 background to give strain A178, strain A178 acquires virulence and the ability to utilize octopine but cannot utilize nopaline (Dr. Eugene Nester, personal communication). Further research, using a large number of virulent, avirulent and genetically constructed strains, is necessary before any conclusions may be made relating the Ti plasmid to cytokinin production.

These experiments have resulted in a definitive profile, both quantitative and qualitative, of the cytokinins produced by A. tumefaciens. Further, they have shown that neither catabolic tRNA processing or an independent mechanism of cytokinin biosynthesis can be excluded as the source of culture filtrate cytokinins.

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APPENDIX

Appendix. Useful data for cytokinins and related compounds.

Compound	Molecular Weights			ϵ Max $\times 10^{-3}$		λ Max, nm		MP °C
	MW	TMS	METHYL	acid	base	acid	base	
Ade	135.055	279.13		13.1	12.3	263	269	220
Ado	267.097	555.25		14.6	14.9	257	260	234
i ⁶ Ade	203.117	275.16	231.15	18.6	18.1	273	275	213
i ⁶ Ado	335.159	551.28	391.22	20.4	20.0	265	269	146
io ⁶ Ade	219.112	363.19	261.16	14.5	15.9	207	220	275
io ⁶ Ado	351.154	639.31	421.23			264	268	181
ms ^{2,6} _i Ado	381.147	597.27	437.21	18.6	24.9	246	243	194