Cytokinin oxidase activity in callus tissues of Phaseolus vulgaris L. cv. Great Northern has been examined using an assay based on the oxidation of radioactively labeled N\textsuperscript{6}(\Delta^2\text{-isopentenyl}) adenine (i\textsuperscript{6}Ade) to adenine (Ade). Conditions for the quantitative extraction and assay of the enzyme have been established. The substrate specificity of the enzyme appears similar to that reported for cytokinin oxidase preparations from other plant sources.

Solutions of exogenous cytokinins applied directly to the surface of Great Northern callus tissues induced relatively rapid (in less than an hour) increases in cytokinin oxidase activity. The cytokinin-induced increase in cytokinin oxidase activity appears to require RNA and protein synthesis. All cytokinin-active compounds tested, including substrates and non-substrates of cytokinin oxidase, were effective in inducing elevated levels of the
enzyme in Great Northern callus tissue. The cytokinin-active urea derivative, Thidiazuron, was as effective as any adenine derivative in inducing this response.

The addition of Cu^{+2} to cytokinin oxidase assay mixtures containing imidazole buffer enhanced the in vitro activity of the enzyme more than 20-fold. The effect was enzyme dependent and specific for copper and the cytokinin oxidase catalyzed reaction. In the presence of copper and imidazole, the degradation of i^{6}Ade to Ade catalyzed by cytokinin oxidase was observed to proceed under anaerobic conditions. This result suggests that the copper-imidazole complex is substituting for oxygen as an electron acceptor in the cytokinin oxidase reaction.

The chromatographic properties of the cytokinin oxidase activity have also been investigated. Most of the cytokinin oxidase activity extracted from callus tissues bound to concanavalin A-Sepharose 4B and was specifically eluted with methyl-mannose. This result suggests that the enzyme is a glycoprotein. DEAE-cellulose chromatography resolved the cytokinin oxidase activity into two peaks. The major fraction, which comprised 85% to 90% of the cytokinin oxidase activity extracted from the callus tissues, bound to concanavalin A-Sepharose 4B. The minor peak of cytokinin oxidase activity did not. These results provide evidence for the presence of multiple forms of cytokinin oxidase, but the possibility of artifacts
generated during enzyme preparation cannot yet be excluded.
Cytokinin Oxidase Activity from *Phaseolus vulgaris* L. cv. Great Northern Callus Tissues

by

J. Mark Chatfield

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Dean of Graduate School

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\( \text{i}^6 \text{AMP} \)  
\[ \text{N}^6-(\Delta^2\text{-isopenenyl)} \]  
adenosine-5'-monophosphate

\( \text{i}^6 \text{ADP} \)  
\[ \text{N}^6-(\Delta^2\text{-isopentenyl)} \]  
adenosine-5'-diphosphate

\( \text{i}^6 \text{ATP} \)  
\[ \text{N}^6-(\Delta^2\text{-isopentenyl)} \]  
adenosine-5'-triphosphate

\( \text{i}^6 \text{Ade} \)  
6-[4-hydroxy-3-methylbut-\text{trans}-2-enylamino]purine,  
(zeatin)

\( \text{i}^6 \text{Ado} \)  
zeatin riboside

\( \text{i}^6 \text{AMP} \)  
zeatin riboside-5'-monophosphate

\( \text{i}^6 \text{ADP} \)  
zeatin riboside-5'-diphosphate

\( \text{i}^6 \text{ATP} \)  
zeatin riboside-5'-triphosphate

\( \text{i}^6 \text{Ade-0-glucoside} \)  
0-\( \text{\AA}-\text{D}-\text{glucopyranosyl zeatin} \)

\( \text{i}^6 \text{Ado-0-glucoside} \)  
0-\( \text{\AA}-\text{D}-\text{glucopyranosyl zeatin riboside} \)

\( \text{i}^6 \text{AMP-0-glucoside} \)  
0-\( \text{\AA}-\text{D}-\text{glucopyranosyl zeatin riboside-5'-monophosphate} \)

\( \Delta^2\text{-IPP} \)  
\( \Delta^2\text{-isopentenylpyrophosphate, dimethylallylpyrophosphate} \)

\( \text{NADPH} \)  
nicotinamide-adenine  
dinucleotide phosphate

\( \text{tRNA} \)  
transfer ribonucleic acid
CHAPTER I.

GENERAL INTRODUCTION

The effective hormone levels in plant tissues are regulated by a variety of mechanisms that include specific pathways of degradation. In the case of cytokinins, a specific cytokinin oxidase has been identified and appears to be widely distributed in plant tissues (1,2,3,4,5,6,7,8). This enzyme catalyzes the oxidative cleavage of the side chains of cytokinins bearing unsaturated isoprenoid side chains (zeatin, N6-(Δ2-isopentenyl)adenine and their ribonucleosides). The specificity of the enzyme and its wide distribution in plant tissues suggest that it plays an important role in the regulation of cytokinin levels in plants tissues.

As part of a continuing effort to identify regulatory mechanisms controlling cytokinin metabolism in legumes, the physical and regulatory properties of a cytokinin oxidase identified in callus tissues derived from Phaseolus vulgaris L. cv. Great Northern have been examined. The work reported here summarizes these investigations. Cytokinin oxidase activity in these tissues has been demonstrated to be regulated by a mechanism that is sensitive to cytokinin supply and relatively rapid in response. The in vitro activity of the
enzyme has been shown to be greatly enhanced in the presence of copper and imidazole, and chromatographic studies have provided evidence that cytokinin oxidase activity in Great Northern callus tissues may be present in multiple forms. The major cytokinin oxidase activity extracted from the callus tissues has at least some of the properties of a glycoprotein. The possible significance of these observations is discussed.
LITERATURE CITED


CHAPTER II. LITERATURE REVIEW

A. The Biosynthesis and Metabolism of Cytokinins in Plants: An Overview

Discovery and Natural Occurrence.

Classes of Cytokinins.

An Overview of cytokinin metabolism.

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1. Nucleotide metabolism.
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4. Amino acid conjugation reactions.
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Phaseolus Callus tissues.

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Physical Properties of Cytokinin Oxidase.

In Vivo Studies.
A. The Biosynthesis and Metabolism of Cytokinin in Plants: An Overview.

**Discovery and Natural Occurrence.** A factor promoting cell division in tobacco pith tissue was isolated from an autoclaved sample of herring sperm DNA and characterized as N\(^6\)-furfuryladenine (kinetin) by Skoog and co-workers in 1955 (Miller et al., 1955). Subsequently, several synthetic N\(^6\)-substituted adenine derivatives were shown to exhibit biological activity similar to kinetin. The discovery of kinetin and early studies in this field have been described by Strong (1958).

The first isolation and purification of a naturally occurring compound with biological activity similar to that of kinetin was reported by Letham (1963). From Zea mays seeds, Letham and associates obtained a biologically active compound, which they identified as 6-(4-hydroxy-3-methylbut-trans-2-enylamino)purine (Letham et al., 1964). This compound was given the trivial name zeatin. Skoog et al. (1965) proposed the name "cytokinin" for all compounds that induced cell division in standard bioassay systems and exerted other plant growth regulatory functions in a manner similar to kinetin. The number of synthetic cytokinins now includes several hundred compounds (Kende, 1971), and more than thirty naturally occurring cytokinins have been identified as "free" compounds or as constituents of tRNA (Skoog and Armstrong, 1970; Kende,
Cytokinins are produced by all vascular plants examined, by bryophytes and perhaps other lower plant systems (Skoog and Armstrong, 1970), and by certain microbial symbionts and pathogens of plants (Green, 1980; Morris, 1986). In addition to occurring as free compounds, cytokinins also occur as component nucleosides in tRNA of plants, animals, and microorganisms (Skoog and Armstrong, 1970).

**Classes of Cytokinins.** Cytokinin activity as defined in standard bioassay systems is a property of at least two distinct classes of compounds: N⁶-substituted adenine derivatives and certain types of phenylurea derivatives (e.g. N,N'-diphenylurea). All of the naturally occurring cytokinins have been found to be structurally related to the cytokinin N⁶-(Δ²-isopentenyl)adenine (i⁶Ade) with the exception of two isolated reports of the natural occurrence of derivatives of N⁶-benzyladenine (Horgan et al., 1975; Ernst et al., 1983). Phenylurea and phenylthiourea derivatives have also been observed to exhibit weak cytokinin-like activity in various bioassay systems (Shantz and Steward, 1955; Bruce et al., 1965; Bruce and Zwar, 1966; Karanov et al., 1968; Kurosaki et al., 1981). Substituted urea derivatives with cytokinin activity in the tobacco callus bioassay approaching or exceeding that of the most active adenine derivatives have
been synthesized (Isogai et al., 1976; Okamoto et al., 1974; Okamoto et al., 1978; Okamoto et al., 1981; Takahashi et al., 1978).

An Overview of Cytokinin Metabolism. Cytokinin biosynthesis and metabolism have been the subject of several recent reviews (Letham and Palni, 1983; McGaw et al., 1984; Morris, 1986). A somewhat simplified representation of the biosynthesis and metabolism of natural plant cytokinins is shown in Figure II.1.

Plant tissues convert cytokinin bases into numerous metabolites. These include products of purine ring substitution (ribosides, nucleotides, N-glucosides and N-alanyl conjugates), isoprenoid side chain modification (zeatin and dihydrozeatin derivatives), isoprenoid side chain substitution (O-glucosides and O-xylosides), and side chain cleavage (adenine and derivatives). Some of these metabolites possess unique chemical structures. N-3 and -7 glucosyl derivatives of purines are quite uncommon in nature. In biological systems, only two cases of similar sugar-purine linkages have been described (Letham and Palni, 1983). Lupinic acid, an amino acid conjugate of zeatin in which an alanyl moiety is linked to the N9 position, is also an uncommon type of structure (Letham and Palni, 1983).

Biosynthesis of Cytokinins. Until 1978, information concerning the biosynthetic pathways involved in cytokinin production was limited to data obtained from in vivo
Figure II.1. A simplified representation of the pathways of cytokinin biosynthesis and metabolism in plants.
Figure II.1

\[ \Delta^2 \text{-IPP} + \text{AMP} \]

\( \text{i}^6\text{AMP} \rightarrow \text{i}^6\text{Ado} \rightarrow \text{i}^6\text{Ade} \)

\( \text{Ado} \)

\( \text{Ade} \)

\( \text{io}^6\text{AMP} \rightarrow \text{io}^6\text{Ado} \rightarrow \text{io}^6\text{Ade} \)

\( \text{hio}^6\text{AMP} \rightarrow \text{hio}^6\text{Ado} \rightarrow \text{hio}^6\text{Ade} \)

\text{NUCLEOSIDE DI and TRI PHOSPHATE DERIVATIVES}

\text{N-GLYCOSYLATION and AMINO ACID CONJUGATION of BASES and NUCLEOSIDES}

\text{O-GLYCOSYLATION}
experiments using radioactively labeled adenine and adenosine. Miller (1967) initially observed incorporation of labeled adenine into the zeatin, ribosylzeatin and zeatin ribonucleotide regions of chromatograms of corn kernel extracts. Later, Miura and Miller (1969) investigated the biosynthesis of cytokinins in *Rhizopogon roseolus*. The idea that *R. roseolus*, a mycorrhizal fungus, might produce cytokinins stemmed from the observation that the hypertrophy of root cortex cells that is common to ectotrophic mycorrhizal associations bears some resemblance to the hypertrophy that may result in cortex cells when roots are treated with kinetin. When labeled adenine, hypoxanthine or 4-amino-5-imidazole carboxamide (an intermediate in de novo synthesis of purines) were supplied to *Rhizopogon roseolus* cultures, radioactivity in ribosylzeatin was released into the culture medium. The same result was obtained regardless of the precursor used. Muira and Hall (1973) incubated *R. roseolus* with adenosine and found a pronounced incorporation of label into $i^6$Ado and $i^6$Ade. They also demonstrated that *R. roseolus* converts $i^6$Ado to ribosylzeatin. These data suggested the pathway for the synthesis of zeatin involved the attachment of the isopentenyl side chain to the amino group of adenine or adenosine followed by subsequent hydroxylation.

Other workers, using various plant materials, have observed similar incorporations of label from adenine and
adenosine into i^6\text{Ade}, i^6\text{Ado}, io^6\text{Ade}, and io^6\text{Ado}
(Beutelman, 1973; Einset and Skoog, 1973; Peterson and Miller, 1976; Chen and Petschow, 1978). More recent work (Stuchbury et al., 1979) has demonstrated that Vinca rosea tumor tissue is able to convert labeled adenine into io^6\text{Ado}, io^6\text{Ade}, and io^6\text{AMP} with maximum incorporation into these compounds occurring between 8 and 24 hours after the start of incubation. These products then decline with an associated increase in labeled cytokinin glucosides. These workers suggested AMP was directly converted into zeatin nucleotide with subsequent conversion into ribosylzeatin and zeatin.

The first cell-free system in which cytokinin biosynthesis could be demonstrated was obtained from the cellular slime mold, Dictyostelium discoideum, in 1978 (Taya et al., 1978). The Dictyostelium enzyme (cytokinin synthetase) catalyzed the formation of i^6\text{AMP} from AMP and \Delta^2-IPP. Chen and Melitz (1979) subsequently isolated cytokinin synthetase (\Delta^2-IPP : AMP- \Delta^2-isopentenyltransferase) from cytokinin-autonomous and cytokinin-dependent tobacco callus tissues. The tobacco enzyme catalyzed the same reaction as the slime mold enzyme. The tobacco enzyme has recently been further purified and characterized (Chen, 1982). The isopentenyl transferase reaction is specific for AMP and does not occur at the base or riboside level. Cytokinin synthetase activity has also been demonstrated in cell-free enzyme
preparations from microbial sources (Morris, 1986; Morris, 1982; Murai, 1981; Nishinari and Syono, 1980).

The cytokinin moieties in tRNA have been shown to be synthesized by a different enzyme using an analogous route involving the transfer of isopentenyl groups from $\Delta^2$-IPP to unmodified adenosine residues adjacent to the anticodon of appropriate tRNA species (Hall, 1973; Kline et al., 1969; Bartz and Soll, 1972; Holtz and Klambt, 1975, 1978). The suggestion by Chen and Hall (1969) that the degradation of tRNA molecules might serve as a pathway for the production of free cytokinins has been investigated (Klemen and Klambt, 1974; Leineweber and Klambt, 1974; Maab and Klambt, 1981; Short and Torrey, 1972), but the results have been inconclusive.

**Metabolism of Cytokinins.** The major features of cytokinin metabolism may be grouped into six classes of reactions: nucleotide metabolism, glycosylation reactions, side chain transformations, amino acid conjugation reactions, other types of purine ring substitutions, and degradation reactions.

1. **Nucleotide metabolism.** Interconversions of free base, nucleoside, nucleotide, and nucleoside triphosphate forms of cytokinins appear to be normal features of cytokinin metabolism in plant tissues (Fox et al., 1973; Doree and Guern, 1973; Parker and Letham, 1974; Sondheimer and Tzou, 1971; LaLoue et al., 1974). In wheat germ, enzymes that catalyze the interconversions of adenine, adenosine and
adenosine monophosphate will also utilize cytokinins as substrates (Chen, 1981; Chen and Kristopeit, 1981a, 1981b; Chen et al., 1982). Chen and co-workers in their studies on preparations from wheat germ have discovered an adenosine phosphorylase (Chen and Petschow, 1978) that strongly favors nucleoside formation and converts i$^6$Ade to i$^6$Ado, an adenosine kinase (Chen and Eckert, 1977) that converts i$^6$Ado to i$^6$AMP, an adenosine nucleosidase (Chen and Kristopeit, 1981a) that converts i$^6$Ado to i$^6$Ade, an adenine phosphoribosyltransferase (Chen and Eckert, 1977; Chen, Melitz and Clough, 1982) that converts i$^6$Ade to i$^6$AMP directly, and a 5'-ribonucleotide phosphohydrolase (Chen and Kristopeit, 1981b) that converts i$^6$AMP to i$^6$Ado. All of these enzymes exhibited lower affinities for the N$^6$-substituted substrates (i$^6$Ade, i$^6$Ado) than for adenine, adenosine, or AMP except for the 5'-ribonucleotide phosphohydrolase. In the case of the latter enzyme, N$^6$-substitution had little effect on the rate of phosphohydrolysis. Prior to these observations, Guern and co-workers (Pethe-Sadorge et al., 1972) had partially purified a purine phosphoribosyl transferase from Acer pseudoplatanus cells. Using cytokinin bases, the formation of cytokinin nucleotides was shown to occur in one step by the transfer of ribose-5'-phosphate from the donor phosphoribosyl-1-pyrophosphate.

2. Glycosylation reactions. A number of glucosyl derivatives of cytokinins are formed in plant tissues. The
glucosyl group may be attached to 3, 7, or 9 positions of the purine ring (N-glucosylation) (Parker et al., 1972; Yoshida and Orintani, 1972; Parker et al., 1973; Letham et al., 1975) or to the hydroxyl group present in the N6-side chains of the cytokinins zeatin and dihydrozeatin (O-glucosylation) (Parker et al., 1975). Letham and coworkers (Entsch and Letham, 1979) have described two enzymes from radish cotyledons that catalyze the formation of 7- and 9-glucosides of cytokinins using uridine diphosphoglucose as a glucose donor. Using N6-benzyladenine (b6Ade) as a substrate, the two enzymes were observed to produce the 7- and 9-glucosides in markedly different proportions. The ratio of 7-glucosyl-b6Ade/9-glucosyl-b6Ade was 1.5 for the major enzyme activity and 10.5 for the other enzyme. Zeatin was shown to be a substrate for the major enzyme and the cytokinin antagonist 3-methyl-7-(n-pentylamino)pyrazolo[4,3-d]pyrimidine was an effective competitive inhibitor of the enzyme. The occurrence of O-xylosyl derivatives of zeatin and ribosylzeatin in embryos (Lee et al., 1985) and seed coats of Phaseolus vulgaris (Turner et al., 1985) has been reported. A novel N9-disaccharide derivative of benzyladenine (b6Ade) has been recently isolated from Gerbera shoot cultures (Horgan, 1985). The chemical structure has not been elucidated but the disaccharide moiety appears to contain a pentose and a hexose.
3. Side chain transformations. The isopentenyl side chain of naturally occurring cytokinins may undergo a number of modifications including hydroxylation (to yield zeatin), hydroxylation and saturation (to yield dihydrozeatin), and multiple hydroxylations accompanied by saturation (Letham and Palni, 1983; Sondheimer and Tzou, 1971; Muira and Miller, 1969; Letham, 1973; Muira and Hall, 1973; Chen, 1982; Kimura et al., 1978; Watanabe et al., 1978). Chen and Leisner (1984) obtained a microsomal preparation from cauliflower that catalyzed the hydroxylation of i6Ade to zeatin. These workers suggested cytochrome P450 was involved in the hydroxylation reaction because NADPH was required and the reaction was completely inhibited by CO and metyrapone, which are potent inhibitors of cytochrome P450. Einset (1984) has reported differences in the conversion of i6Ade to zeatin in various tissue of plants belonging to the genus Actinidia. Studies on the biosynthesis of cytokinins in Pseudomonas syringae pv. savastanoi have resulted in the discovery of the novel cytokinin, 1"-methyl-trans-ribosylzeatin (Surico et al., 1985; Morris et al., 1985; Morris, 1986). Another 1"-methyl-zeatin derivative, 1"-hydroxymethyl-zeatin has been found in immature wheat seeds (Rademacher and Graebe, 1984). The enzymatic reactions responsible for these novel metabolites have not been described.

4. Amino acid conjugation reactions. Lupinic acid and dihydrolupinic acid, derivatives of zeatin and
dihydrozeatin in which the cytokinins are linked to L-alanine via a bond between the nitrogen at the 9-position of the purine ring and the 2-carbon of the amino acid, are produced in seeds of lupin and apple (Parker et al., 1975; Duke et al., 1978; Parker et al., 1978; Entsch et al., 1980). The enzyme responsible for this transformation, A-(9-cytokinin)alanine synthase (Murakoshi et al., 1977; Entsch et al., 1983) transfers the alanine moiety of O-acetylserine to zeatin to form lupinic acid. The b^6Ade analogue of lupinic acid has been isolated from derooted Phaseolus vulgaris seedlings fed with b^6Ade (Letham, Parker and MacLeod, 1979). Discadenine, a derivative of i^6Ade in which a 3-amino-3-carboxylpropyl group is covalently attached to the 3-position of the purine ring, has been isolated from the cellular slime mold Dictyostelium discoideum (Obata et al., 1973; Tanaka et al., 1975; Abe et al., 1976; Nomura et al., 1977). The enzyme discadenine synthase catalyzes transfer of the 3-amino-3-carboxypropyl moiety of S-adenosylmethionine to i^6Ade to form discadenine (Taya et al., 1978).

5. Other substitutions of the purine ring. The cytokinin-active constituents of tRNA include derivatives that bear a methylthio group at the 2-position of the purine ring (Skoog and Armstrong, 1970;). A derivative of zeatin in which the 2-position of the purine ring is hydroxylated has been reported to occur in corn (Letham, 1973).
Degradation of cytokinins. Degradation of cytokinins to inactive products that lack the N6-side chain is easily demonstrated in plant tissues (Skoog and Armstrong, 1970; Hall, 1973; Letham and Palni, 1983). A specific cytokinin oxidase, isolated from corn kernels (Whitty and Hall, 1974), tobacco callus (Paces et al., 1971), Vinca rosea tumor tissue (Scott et al., 1982; McGaw and Horgan, 1983a) and wheat germ (LaLoue and Fox, 1985) catalyzes the oxidative cleavage of the N6-side chain of i6Ade and zeatin to yield adenine. N6-benzyladenine, kinetin, and cytokinins bearing saturated isoprenoid side chains (dihydrozeatin and N6-isopentyladenine) are resistant to attack by the enzyme. In plant tissues, the cleavage of the side chains of io6Ade and i6Ade and their ribosides to form adenine or adenosine respectively is attributable to cytokinin oxidase. However, the degradation of b6Ade and f6Ade which is often observed in plant tissues cannot be thus explained. Therefore, another enzyme system distinct from cytokinin oxidase must be present in those plant tissues that cleaves benzyl and furfuryl groups from the N6 position of adenine and its derivatives. A benzoic acid derivative appears to be the product of the cleavage of b6Ade (Fox et al., 1972). Cytokinins have also been reported to serve as substrates for xanthine oxidase (Skoog and Armstrong, 1970; Chen et al., 1975) and an aminohydrolase (Terrine et al., 1969; Doree and Terrine, 1972; Hall et al., 1971) from animal tissues. The ability
of b6Ade, b6Ado, b6AMP, f6Ade, and various N6-substituted adenine derivatives to serve as substrates for adenosine and AMP aminohydrolases from rabbit muscle has been determined (Terrine et al., 1969; Doree and Terrine, 1972). All N6-substituted derivatives were degraded to inosine but at much slower rates than were observed for adenosine or AMP as substrates. An adenosine aminohydrolase prepared from chicken bone marrow (Hall et al., 1971) appeared to possess some characteristic that enabled effective degradation of N6-substituted 5-carbon derivatives to inosine whereas an adenosine aminohydrolase from calf intestinal mucosa very weakly catalyzed the oxidation of adenosine derivatives with N6-alkyl groups containing more than two carbon atoms. The chicken bone marrow enzyme exhibited considerable specificity as ribosylzeatin would not serve as a substrate. Van Staden and Forsyth (1985) have reported degradation of zeatin to adenine, N6-(2,3,4-trihydroxy-3-methylbutyl)adenine, and an unidentified compound following treatment with commercial peroxidase, but controls treated with hydrogen peroxide alone gave rather similar results.

Xanthine oxidase has been shown to oxidize zeatin and i6Ade to their 8- and 2,8- dihydroxy derivatives (Chen et al., 1975), and 2-hydroxyzeatin has been isolated as a minor component of immature Zea mays kernels (Letham, 1973). However, a role for these enzymes in the metabolism of cytokinins in plant tissues has yet to be demonstrated.
Although the analytical aspects of the purification and characterization of cytokinin metabolites have been developed, we still have little idea of the significance of this elaborate metabolism in plant growth and development. However, with this information as a basis, the enzymes responsible for cytokinin metabolism can now be specifically targeted for genetic, regulatory, and physiological studies that should give insight into the roles these cytokinin metabolites and enzymes play in plant development.

B. Cytokinin Metabolism in Phaseolus

The metabolism of cytokinins has been studied in many plant species and a number of different tissues. However, extensive metabolic data exist for only a few plant species. These include Zea mays, Vinca rosea crown gall tissue, Raphanus sativus, several Lupinus species, and Phaseolus vulgaris. In the case of Phaseolus, investigations of cytokinin metabolism have been done on most plant organs and on various tissues at different stages of plant development. Data concerning cytokinin metabolism in Phaseolus are available for seeds and isolated embryo axes, roots, shoots, leaves, flowers, and various callus tissues.

Phaseolus Callus Tissues. Investigations on the genetic regulation of hormone metabolism in food legumes have resulted in a series of reports on cytokinin activity
and metabolism in *Phaseolus* callus tissues. The activity of several cytokinins in promoting callus growth, were examined in *P. vulgaris* cv. Great Northern and *P. lunatus* cv. Kingston (Mok et al., 1978). The structural feature which contributed to the major genotypic difference in cytokinin structure-activity relationships was the presence of a double bond at the 2,3-position of the N⁶ side chain. In Great Northern callus tissues, io⁶Ade and i⁶Ade were 30- to 100 fold less active than their saturated analogs (hio⁶Ade and hi⁶Ade) in promoting the growth of the cytokinin dependent callus tissues. In Kingston callus tissues, on the other hand, the cytokinins with unsaturated side chains were either more active (io⁶Ade) or equally as active (i⁶Ade) as their saturated counterparts. The activities of cytokinins with saturated side chains were comparable in the two genotypes. It was suggested that these differences in cytokinin structure-activity relationships between Kingston and Great Northern callus tissues might be related to differences in cytokinin destruction in the two tissues.

In a more recent study, the activities of i⁶Ade, hi⁶Ade, io⁶Ade, and hio⁶Ade were compared, and the metabolism of i⁶Ado-8-¹⁴C was examined in callus tissues derived from *P. vulgaris* cv. Great Northern, *P. lunatus* cv. Kingston, and the interspecific hybrid Great Northern x Kingston (Mok et al., 1982). The differences in cytokinin structure-activity relationships for Kingston
and Great Northern described above were correlated with
differences in the metabolism of i6Ado-8-14C. In Great
Northern callus tissues, i6Ado-8-14C was rapidly degraded
to adenosine, in Kingston callus tissues, the major
metabolite was i6AMP. The growth responses of callus
tissues of the interspecific hybrid were intermediate
between the parental tissues, and the metabolism of i6Ado-
8-14C by the hybrid callus tissue exhibited
characteristics of both parents. The patterns for i6Ado-8-
14C metabolism in Great Northern and Kingston callus
tissues provided support for the hypothesis that
differences in cytokinin destruction in these two
genotypes might explain the differences in cytokinin
structure-activity relationships. Thus, the low activity
of i6Ade and io6Ade in promoting growth of callus tissue
in Great Northern, could be explained if these cytokinins
were converted to inactive metabolites.

Genotypic differences in responses of Phaseolus
callus tissues to N,N'-diphenylurea (DPU) have been
reported (Mok et al., 1979) and the observed ability of
DPU to induce cytokinin autonomy in P. lunatus genotypes.
It led these investigators to suggest that the cytokinin
activity of DPU might be due to the promotion of
endogenous cytokinin biosynthesis in the systems where it
was active. The effects of N-phenyl-N'-1,2,3-thidiazol-5-
ylurea (Thidiazuron) and other substituted urea compounds,
on the growth of callus tissues of P. lunatus cv. Kingston
have been described (Mok et al., 1982). The effects of Thidiazuron and zeatin on the metabolism of i$_6$Ado-8-$_{14}$C were compared in callus tissues derived from P. lunatus cv. Jackson Wonder and P.I. 260415 (Capelle et al., 1983). These two lines of callus tissue differ in their biological response to Thidiazuron. After exposure to culture medium containing Thidiazuron, callus tissues of Jackson Wonder could be subcultured onto medium without cytokinins and continue to grow (i.e. exhibit cytokinin autonomy). However, callus tissues of P.I. 260415 exposed to Thidiazuron, would not exhibit autonomy and continued to require exogenous cytokinin for growth. Under the same culture conditions, the metabolism of i$_6$Ado-8-$_{14}$C was similar for callus tissues of the two genotypes, but the pattern of metabolism varied with the cytokinin used in to culture the tissues. Thus, i$_6$Ado was converted to i$_6$AMP by callus tissues grown on zeatin-containing medium, whereas only traces of the nucleotide were formed in tissues grown on Thidiazuron. Callus tissues grown in the presence of Thidiazuron, were shown to rapidly metabolize i$_6$AMP to i$_6$Ado, but i$_6$AMP persisted in tissues grown on zeatin. It was suggested that Thidiazuron either inhibits the conversion of i$_6$Ado to i$_6$AMP (nucleoside kinase activity) or enhances the conversion of i$_6$AMP to i$_6$Ado (5’-nucleotidase activity).

The metabolism of $_{14}$C-Thidiazuron was examined in callus tissues of P. lunatus cv. Kingston (Mok and Mok,
1985). No degradation of $^{14}$C-Thidiazuron was observed in callus tissues incubated for up to 33 days in medium containing labeled thidiazuron. However two glucosyl derivatives and one hydroxyl derivative of Thidiazuron were identified as metabolic products. All three metabolites had lower cytokinin activity in callus tissue bioassays than Thidiazuron. It was suggested that Thidiazuron is the biologically active form, and that the metabolism of Thidiazuron in *Phaseolus* callus tissues involves modification of the intact structure to conjugates that have lower cytokinin activity than Thidiazuron itself.

In a genetic analysis of cytokinin autonomy in callus tissues, the inheritance of the cytokinin requirement (autonomy) in *P. vulgaris* callus tissues appeared to be regulated by one set of nuclear alleles (Mok et al., 1979).

**Phaseolus Seeds and Embryos.** The cytokinins present in developing seeds of *P. coccineus* have been isolated and identified as io$^6$Ade, io$^6$AMP, hio$^6$Ade, hio$^6$Ado, and hio$^6$AMP (Sodi and Lorenzi, 1982). It was estimated that the dihydrozeatin derivatives predominated. In a study of zeatin metabolism in isolated embryonic axes of *P. vulgaris*, the nucleosides of zeatin and dihydrozeatin were the major metabolites recovered after feeding radioactive zeatin (Sondheimer and Tzou, 1971). Surprisingly, little ureide metabolism was observed. The conversion of zeatin
to dihydrozeatin in the embryonic axes could be inhibited by exogenous ABA treatment. When labeled zeatin was applied to the tips of the radicles of germinating Phaseolus vulgaris seeds, Hutton and van Staden (1982) observed metabolism to Ade, Ado, and io^6Ado. This is contrary to Sondheimer and Tzou's result in which conversion to dihydrozeatin comprised 60% of zeatin metabolism. With regard to cytokinin transport, Hutton and van Staden observed rapid movement of cytokinins from P. vulgaris embryonic axis to the cotyledons. In a companion study on the endogenous cytokinins in P. vulgaris seeds, it was suggested that cytokinins are not synthesized in cotyledons, but are provided by the embryonic axis (Hutton and van Staden, 1982).

The metabolism of zeatin-8-\(^{14}\)C has been compared in developing embryos of P. vulgaris and P. lunatus cv. Kingston (Lee et al., 1985). Ribosylzeatin (io^6Ado), io^6AMP, io^6Ado-O-glucoside and two novel metabolites (tentatively identified as O-ribosyl derivatives of io^6Ade and io^6Ado) were isolated from Great Northern embryo extracts. In Kingston, io^6Ado, io^6AMP, and the O-glucosides were isolated but the two novel metabolites could not be detected. The difference in zeatin metabolism in Great Northern and Kingston embryos appeared to be tissue specific. These novel metabolites were not found in tests with other plant organs and tissues of Great Northern. The novel O-pentose derivatives of zeatin and
ribosylzeatin have been conclusively identified as the 0-xylosides of io₆Ade and io₆Ado (Mok, MC, personal communication). Zeatin-8-¹⁴C metabolism in seed coats and pod tissues of P. lunatus cv. Kingston and P. vulgaris cv. Great Northern has been described by Turner et al. (1985). Zeatin was metabolized to 0-glucosyl derivatives in seed coats of Kingston and 0-xylosyl derivatives in seed coats of Great Northern. This pattern of zeatin metabolism was similar to that observed in embryos of these two genotypes. However, the degradation of zeatin to adenine derivatives was much higher in seed coats than in embryos. The major metabolite recovered from the pod tissues of both genotypes was io₆AMP.

Phaseolus Roots. The major cytokinin extracted from P. vulgaris roots was zeatin-0-glucoside (Scott and Horgan, 1984). Minor cytokinins present in root tissue were tentatively identified as io₆Ade, io₆Ado, and io₆Ado-0-Glucoside. This result contrasted with an earlier study (Wareing et al, 1977) in which io₆Ade-8-¹⁴C was fed to disbudded seedlings of P. vulgaris, and io₆AMP was found to be the major cytokinin in roots. Scott and Horgan (1984) have suggested that uptake and transport processes may explain the contrast between their analysis of endogenous root cytokinins and the earlier study on zeatin metabolism in rooted cuttings. Uptake of exogenous cytokinins in plant tissues often yield high levels of nucleotides (Scott et al., 1982; Letham et al., 1982), and
this may not correspond to the normal situation. Nucleotide formation may be associated with the uptake and transport of cytokinins across membranes. The rate of uptake of purine bases, in cultured mammalian cells is strictly a function of the rate of their phosphoribosylation (Marz et al, 1979). A similar situation may exist in plants, where uptake of cytokinin bases and their phosphoribosylation may be coupled phenomena.

**Phaseolus Shoots and Flowers.** Palmer et al. (1981) determined the major endogenous cytokinins in stems of decapitated, disbudded *P. vulgaris* plants to be io^6^AMP, hio^6^AMP, io^6^Ado, and hio^6^Ado. Minor cytokinins found were hio^6^Ade-O-glucoside and io^6^AMP-O-glucoside. The major metabolite of dihydrozeatin (hio^6^Ade) in this system was hio^6^AMP. The identification of ribonucleosides and ribonucleotides as the major cytokinins in stems of decapitated, disbudded, *P. vulgaris* plants, in conjunction with the observation that these are the primary cytokinin metabolites observed in bean stem tissues when cytokinins are applied to whole plants or isolated stem segments, suggests that cytokinins may be delivered to the leaves as ribonucleosides or ribonucleotides where they accumulate as glucosides. It will be necessary to identify cytokinins in root and stem exudates to confirm this hypothesis. Degradation of labeled cytokinins to adenine and ureides was not evident in this study.
The transport of labeled zeatin in *P. vulgaris* was examined by Hutton and van Staden (1984). Sites of cytokinin accumulation changed as the plants progressed from a vegetative to a reproductive phase of growth. In this study, labeled zeatin was applied to the xylem and phloem of exposed stems, and cytokinins were extracted from all tissues after incubation. In the phloem applications, flowering and fruiting plants exhibited increased transport of cytokinin glucosides and/or adenosine (identification was by polar nature only) to the reproductive organs. In vegetative plants, there was little transport of cytokinins to the apices, and the majority of the label was recovered in the stem and roots, suggesting basipetal transport. In xylem applications of labeled zeatin, most of the label was found in the apices of vegetative plants, in stems and flowers of flowering plants, and in the leaves, stems and fruits of fruiting plants.

**Phaseolus Leaves.** The metabolism of $^{14}C\text{Ade}$, $^{32}P\text{Ade}$, and their O-glucosides has been studied in detached leaves of *P. vulgaris* (Palmer et al., 1981a). The radioactively labeled cytokinins were taken up by the petioles, and the leaves were incubated for three days. The O-glucosides were more stable than the free bases and the dihydrozeatin derivatives were more stable than the zeatin derivatives. The observed order of stability of the cytokinins was dihydrozeatin-O-glucoside > zeatin-O-glucoside >
dihydrozeatin > zeatin. Zeatin was extensively metabolized to adenine derivatives suggesting that cytokinin oxidase activity was significant in this tissue. Major metabolites of all four compounds were ureides and urea. This is not surprising since ureides are well known products of purine degradation (Guranowski, 1982) and are also a major transport form of nitrogen in Phaseolus (Thomas and Schrader, 1981). With all four cytokinins very little ribonucleotide (<5%) was recovered. It was suggested that O-glucosylation may be the major form of cytokinin metabolism in leaves.

Further studies of detached leaves, leaf discs, and petioles of P. vulgaris, suggest that the site of glucosylation is the lamina (Palmer, 1980). In the petiole, bio6Ade is typically metabolized to cytokinin ribonucleosides and ureides. Therefore, leaf metabolism is quite different from that of intact or detached stems (Palmer, 1980). Dihydrozeatin-O-glucoside is unstable in detached leaves and in attached leaves of plants with outgrowing buds (Palmer et al, 1981a) but accumulates in attached leaves of decapitated, debudded plants. The authors suggested that O-glucosides are storage forms and N7- and N9-glucosides (which appear resistant to enzymatic degradation) may be irreversible inactivation forms. Other workers have observed zeatin-O-glucoside formation (Hutton and van Staden, 1984) or extracted high levels of zeatin-
and dihydrozeatin-0-glucosides (Wang et al., 1977) from leaves of P. vulgaris.

In Phaseolus leaves, side chain cleavage is the predominant pathway of cytokinin metabolism. The io6Ade-0-glucoside and N9-alanyl conjugates have not been found (Wareing et al., 1977). This contrasts with Lupinus, where N9-alanyl conjugates are major cytokinin metabolites (Parker et al., 1978). However the N9-alanyl conjugate of b6Ade was formed in P. vulgaris leaves fed b6Ade, suggesting the enzymatic capacity to form such conjugates is present in Phaseolus (Letham et al., 1979).

Cytokinin Metabolism in the Whole Plant: A Synthesis.
The identification of the major cytokinins in many Phaseolus tissues have been described. The metabolism of exogenously applied cytokinins, in conjunction with information on cytokinin transport, can be used to design models of the metabolism of cytokinins in the whole plant. Letham has suggested cytokinin ribonucleosides and ribonucleotides may have a particular role in cytokinin transport (Letham, 1978). The identification of cytokinin ribonucleosides and ribonucleotides as the major endogenous forms in stems tissues, and the metabolism of exogenous cytokinins to these conjugates in plant stems, have led to the suggestion that cytokinins may be delivered to the leaves as ribonucleosides or ribonucleotides where glucosylation allows them to accumulate (Palmer, Horgan and Wareing, 1981). Palmer et
al. (1981) have suggested that the relative activities of cytokinin oxidase and dihydrozeatin-zeatin transformations maintain a decreasing gradient of zeatin derivatives, and an increasing gradient of dihydrozeatin derivatives from the roots to the laminae. Evidence for this hypothesis is as follows:

1. In stems of decapitated, debudded bean plants, the levels of cytokinin ribonucleotides and ribonucleosides are comparable to the levels of dihydrozeatin-0-glucoside which accumulate in the leaves (Palmer, 1980).

2. The major cytokinins in leaves are the ribonucleoside and O-glucoside of dihydrozeatin (Wang, 1977; Wang and Horgan, 1978).

3. The major cytokinin in bean roots has been identified as zeatin-0-glucoside (Scott and Horgan, 1984).

4. In leaves, dihydrozeatin-0-glucoside and dihydrozeatin are more stable than zeatin-0-glucoside and zeatin (Palmer et al., 1981).

5. In rooted bean plants, exogenously applied zeatin is predominantly metabolized to io°AMP in the roots, to hio°Ado in stems and petioles, and to io°Ado-0-glucoside and hio°Ade-0-glucoside in laminae (Wareing et al., 1977).

6. When hio°Ade or hio°Ade-0-glucoside are applied to a petiole of a decapitated bean plant, there is a higher recovery of zeatin derivatives in the stem than in the leaves (Palmer, 1980).

7. A major site of cytokinin biosynthesis in beans appears to be in the roots (Skene, 1975; Letham, 1978).

These facts may suggest that the metabolism of cytokinins in shoots serves to control the distribution and activity of cytokinins produced in the roots, additional controls
presumably are exerted at the level of cytokinin biosynthesis.

These studies on *P. vulgaris* illustrate the complexity of cytokinin metabolism in whole plants. The different organs of the plant exhibit very distinct patterns of cytokinin metabolism, and the various types of cytokinins have different metabolic characteristics in the plant. The significance of these complexities to the regulation of plant growth and development has yet to be determined.

C. Cytokinin Oxidase Activity in Plant Tissues.

The application of cytokinins to plant tissues has, in almost every case, led to the production of adenine (Ade), adenosine (Ado) or adenine nucleotides (Entsch et al., 1979; Summons et al., 1979; Horgan et al., 1981; LaLoue et al., 1977; Skoog and Armstrong, 1970; Hall, 1973; Letham and Palni, 1983). This destruction of cytokinins has been suggested as a mechanism for control of total endogenous cytokinin activity (McGaw and Horgan, 1983). The presence of an inactivation mechanism for zeatin (i6Ade) and N6-(Δ2-isopentenyl)adenine (i6Ade) had been suggested previously by Nitsch (1968) and later by Hall (1970), who initiated a series of studies on the enzymatic degradation of cytokinins in plant and animal tissues.

**Characteristics of the Reaction Catalyzed by Cytokinin Oxidase.** An enzyme that catalyzes the cleavage
of isopentenyl side chains from i$_6^{\text{A}}$de and i$_6^{\text{A}}$do to yield Ade and Ado was originally detected by Paces et al. (1971) in crude homogenates of cultured cells derived from *Nicotiana tabacum* cv. Wisconsin 38 pith tissue. A similar enzyme activity was subsequently isolated and partially purified from immature corn kernels by Whitty and Hall (1974). The reaction catalyzed by the corn enzyme was indirectly shown to require oxygen, and the enzyme was therefore named cytokinin oxidase. The reaction products generated by cytokinin oxidase were further characterized by Brownlee et al. (1975). Using i$_6^{\text{A}}$de as a substrate and cytokinin oxidase from corn, the major side chain product was shown to be 3-methyl-2-butenal. This product, tested in the form of a semicarbazone, was identical with authentic standard by several criteria, including chromatographic behavior, mass spectrum, and ultraviolet spectrum. The other expected product of the catalysis of i$_6^{\text{A}}$de by cytokinin oxidase was identified as adenine on the basis of chromatography on paper (Pace et al., 1971; Whitty and Hall, 1974; Pace and Kaminek, 1976). Van Staden (1982) has suggested the oxidation products were misidentified. By analogy with permanginate oxidation products, Van Staden (1982) proposed that 6-(2,3,4-trihydroxy-3-methylbutylamino)purine was the product of cytokinin oxidase action on zeatin. This compound cochromatographs with adenine on paper and Sephadex LH-20 (Van Staden, 1982). However, McGaw and Horgan (1983) were
able to positively identify adenine as the sole purine product of cytokinin oxidase from corn. Their identification of adenine was based on the mass spectrum, ultraviolet absorbance spectrum and HPLC chromatography.

The cytokinin oxidase reaction deduced from these studies is shown in Figure II.2. The mechanism of the enzyme reaction has not been described, but early studies suggested an unstable intermediate was involved in the reaction (Whitty and Hall, 1974). Brownlee et al. (1975) suggested that the reaction mechanism involved oxidation of the N⁶ to C-1 bond to give an iminopurine which then hydrolyzed to yield the C-5 aldehyde and a 6-aminopurine moiety. Recently, LaLoue and Fox (1985) reported the isolation and characterization of this reaction intermediate. Using i⁶Ado as a substrate and a cytokinin oxidase preparation from wheat, a stable intermediate could be isolated from reaction mixtures containing 2-mercaptoethanol. The intermediate was identified as 6-(Δ²-isopentenylimino)purine. This characterization was based on the reduction of the intermediate to i⁶Ado by cyanoborohydride, rapid hydrolysis to Ado in acetic acid, its formation of two characteristic adducts in the presence of cyanide, and the ultraviolet and mass spectra.

**Substrate Specificity of Cytokinin Oxidase.** Interest in defining the biochemical function of cytokinin oxidase has resulted in several studies on the substrate specificity of the enzyme. Paces et al. (1971), using
Figure II.2. The cytokinin oxidase reaction.
Figure II.2

$O_2 + N^6-(\Delta^2\text{-isopentenyl})\text{adenine (i}^6\text{Ade)} \xrightarrow{\text{CKO}} \text{Adenine} + 3\text{-methyl-2-butenal}$
\[ i^6\text{Ado-8-}^{14}\text{C} \] as a substrate, observed the conversion of \[ i^6\text{Ado} \] to \[ \text{Ado} \] and \[ \text{Ade} \] by a crude homogenate from tobacco cells. An adenosine hydrolase activity present in the extracts was shown to convert \[ \text{Ado} \] to \[ \text{Ade} \], and this activity confounded the interpretation. Adding saturating amounts of \[ \text{Ado} \] to assays (to negate the effect of the hydrolase) elevated the recovery of \[ \text{Ado-8-}^{14}\text{C} \]. It was concluded that \[ i^6\text{Ado} \] metabolism may proceed via either \[ i^6\text{Ado} \] to \[ \text{Ado} \] or \[ i^6\text{Ade} \] to \[ \text{Ade} \].

The specificity of cytokinin oxidase from corn was examined by Whitty and Hall (1974). Using \[ i^6\text{Ade-8-}^{14}\text{C} \] as a substrate, unlabeled \[ i^6\text{Ado} \] and (to a lesser degree) zeatin inhibited the enzyme reaction. Kinetin and benzyladenine were much less effective, suggesting that these compounds were not substrates. A spectrophotometric assay, using change in absorbance at 250 nm to measure the rate of reaction, was devised to further characterize the substrate specificity of the reaction. This value was selected because the absorbance maximum for \[ i^6\text{Ado} \] is 269 nm, and an increase in absorbance at 248 nm with a concomitant loss of absorbance at 269 nm was observed upon reaction. This particular shift in absorbance was unexpected (because the absorbance maximum for adenosine is 260 nm) and was interpreted as suggesting that an intermediate product was formed which was stable during the reaction and converted to \[ \text{Ado} \] during chromatography. The substrate specificity of the reaction as determined by this spectrophotometric assay was shown
to be in the following order: \(i^6\text{Ado} = i^6\text{Ade} > io^6\text{Ade} > io^6\text{Ado}\). Side chain saturation, shifting the double bond from the \(\Delta^2\) to the \(\Delta^3\) position, or substitution of other functional groups on the side chain resulted in less active substrates. The synthetic cytokinins \(b^6\text{Ade}, f^6\text{Ade},\) and \(hi^6\text{Ade}\) were resistant to side chain cleavage. The spectrophotometric assay was zero order with respect to substrate but was protein concentration dependent. This suggests other factors may be required to facilitate a first order reaction. A kinetic analysis performed under these conditions would be meaningless, which probably explains why no \(K_m\) data were reported for these substrates.

The substrate specificity of cytokinin oxidase was further defined by Paces and Kaminek (1976) using an enzyme preparation from cultured tobacco cells. Trans-ribosylzeatin was shown to be a more effective inhibitor of \(i^6\text{Ado}\) degradation catalyzed by the enzyme than cis-ribosylzeatin.

Recently, McGaw and Horgan (1983) compared the substrate specificities of the corn enzyme and cytokinin oxidase preparation from \textit{Vinca rosea} crown gall tissue. Substrate specificity studies were performed on a number of synthetic and naturally occurring cytokinins. Both enzymes were found to have similar specificities. A \(\Delta^2\) double bond in the \(N^6\)-side chain was essential for substrate activity. The presence of glucosyl or ribosyl
groups in the 7 or 9 position of the purine ring or an alanyl group in the 9 position had little effect on the ability of cytokinins to serve as substrates, but the O-glucosyl derivatives were resistant to oxidation. LaLoue and Fox (1985) have described the substrate specificity of a cytokinin oxidase from wheat germ. The specificity for purine cytokinins was similar to that of the corn and Vinca enzymes. Interestingly, diphenylurea and N-(2-chloro-4-pyridyl)-N'-phenyl-urea (both cytokinin-active urea derivatives) strongly inhibited the degradation of \( i^6 \text{Ado} \).

**Physical Properties of Cytokinin Oxidase.** Initial characterization of the physical properties of a cytokinin oxidase was by Whitty and Hall (1974). Using corn kernels as a source material, the enzyme was purified 9000 fold by 40-60% acetone fractionation, Sephadex G-100 gel filtration, and hydroxyapatite chromatography. Surprisingly, 150% of the total activity loaded on the column was recovered after passage through hydroxyapatite. This suggests some inhibitory factor was being removed or some stimulatory factor was acquired during chromatography. A step gradient with increasing phosphate was used to elute cytokinin oxidase from hydroxyapatite, and a cytokinin oxidase active fraction was obtained after each increase in phosphate. A gel electrophoretic analysis was performed on the two most active fractions. Both cytokinin oxidase preparations comigrated in the same area.
of the gel. Staining replicate samples for mixed function oxidase (peroxidase) activity with 3-3'-dimethoxybenzidine and H$_2$O$_2$ revealed peroxidase positive staining in the cytokinin oxidase regions of the gel. It was concluded that cytokinin oxidase had the properties of a mixed function oxidase, but it was not equivalent to horse radish peroxidase (HRP) because HRP would not degrade i$^6$Ado to Ade. The cytokinin oxidase from corn was shown to have a pH optimum between 5.5 and 6.0. The enzyme did not require Mg, and was partially inhibited by potassium cyanide. Concentrated protein solutions of cytokinin oxidase could be stored for several months at -20 C.

Cytokinin oxidase has recently been isolated from *Vinca rosea* crown gall tissue and compared with the corn enzyme (McGaw and Horgan, 1983). The two enzymes exhibited different pH optima and different molecular weights. The cytokinin oxidase from corn had a pH optimum of 6.0 compared to a pH optimum of 7.0 for the cytokinin oxidase from *Vinca* crown gall tissue. The molecular weights of the two enzymes were determined on a Sephadex G-150 gel filtration column. The molecular weight for the cytokinin oxidase from corn was ca. 90,000 daltons compared to ca. 25,000 daltons for the *Vinca* enzyme. The estimation of the apparent molecular weight for the corn enzyme was in agreement with data obtained by Whitty and Hall (1974) using the same technique. LaLoue and Fox (1985) have detected a cytokinin oxidase in wheat germ and partially
purified it by conventional techniques and chromatofocusing. The molecular weight of the wheat enzyme has been estimated at 40,000 daltons by Spherogel TSK-3000 SW and they have suggested this enzyme is different from previously described cytokinin oxidases.

In Vivo Studies of Cytokinin Oxidase. Cytokinin oxidase levels have been measured in several tissues under various conditions (Whitty and Hall, 1974; Scott et al., 1982). Whitty and Hall (1974) measured cytokinin oxidase activity levels in developing corn kernels at one, two and three weeks post-anthesis. At three weeks post anthesis, the developing corn kernel had a higher specific enzyme activity (on a protein basis), and also more total activity on a fresh weight basis, than one and two week post-anthesis samples. These data suggest a trend of increasing cytokinin oxidase activity with corn kernel maturation. Scott et al. (1982) compared cytokinin oxidase activity in Vinca rosea crown gall and normal callus tissues at different times after subculture. On a fresh weight basis, there was little difference between the two tissues in the maximal enzyme levels observed. This comparison was made using data obtained at times when both tissues were vigorously growing. If the levels of cytokinin oxidase activity were compared on a time in culture basis, several fold differences were observed between the two tissues. However, these differences in
enzyme levels probably reflect the large difference in growth rate and water content in the two tissues.

Evidence for a cytokinin oxidase activity in *Saccharomyces cerevisiae* has been presented by Laten (1985). Using an adenine auxotrophic strain that would not utilize hypoxanthine, growth could be obtained in cultures provided \( f^6 \text{Ade}, i^6 \text{Ade} \) or \( io^6 \text{Ade} \). These data suggest a cytokinin oxidase-like activity may be providing adenine that is necessary for growth. Interestingly, \( f^6 \text{Ade} \) appears to be the most effective substrate which suggests that the specificity of this putative cytokinin oxidase is different from the plant enzyme.

The significance of cytokinin oxidase in plant growth and development is still obscure. Evidence for an interaction between cytokinins and oxidative enzymes has been reported by Lee (1971, 1974). Treatment of a cytokinin requiring tobacco tissue culture with exogenous cytokinins changed the distribution of indoleacetic acid oxidase isoenzymes. It has been suggested (Lee, 1971, Whitty and Hall, 1974) that oxidases and peroxidases may play a role in the known physiological interactions of auxins and cytokinins. The substrate specificity of cytokinin oxidase suggests that it may play a role in the regulation of cytokinin levels in plant tissues. Although it is attractive to speculate that cytokinin oxidase plays a role in maintaining specific levels of cytokinins or in modulating cytokinin pool levels during developmental
sequences, the actual physiological significance of the enzyme has yet to be established.
CHAPTER III.

REGULATION OF CYTOKININ OXIDASE ACTIVITY IN CALLUS TISSUES OF PHASEOLUS VULGARIS L. CV. GREAT NORTHERN

ABSTRACT

The regulation of cytokinin oxidase activity in callus tissues of *Phaseolus vulgaris* L. cv. Great Northern has been examined using an assay based on the oxidation of N6-(Δ2-isopentenyl)adenine-8-14C (i6Ade-8-14C) to adenine. Solutions of exogenous cytokinins applied directly to the surface of the callus tissues induced relatively rapid increases in cytokinin oxidase activity. The increase in activity was detectable after one hour and continued for about eight hours, reaching values two- to three-fold higher than the controls. The cytokinin-induced increase in cytokinin oxidase activity was inhibited in tissues pretreated with cordycepin or cycloheximide, suggesting that RNA and protein synthesis were required for the response. Rifampicin and chloramphenicol, at concentrations that inhibited the growth of Great Northern callus tissues, were ineffective in inhibiting the increase in activity. All cytokinin-active compounds tested, including substrates and non-substrates of

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cytokinin oxidase, were effective in inducing elevated levels of the enzyme in Great Northern callus tissue. The cytokinin-active urea derivative, Thidiazuron, was as effective as any adenine derivative in inducing this response. The addition of Thidiazuron to the reaction mixtures used to assay cytokinin oxidase activity resulted in a marked inhibition of the degradation of the labeled $\text{i}^6\text{Ade-8-}^{14}\text{C}$ substrate. It is possible that Thidiazuron may serve as a substrate for cytokinin oxidase, but other mechanisms of inhibition have not been excluded.

INTRODUCTION

The catabolism of cytokinins in plant tissues appears to be due, in a large measure, to the activity of a specific enzyme, cytokinin oxidase. The metabolism of cytokinins to inactive products that lack the $N^6$-side chain has been observed in many plant tissues (5,12,22). Although cytokinins have been reported to serve as substrates for xanthine oxidase (4,22) and adenosine aminohydrolase (6) prepared from animal sources, a role for these enzymes in the metabolism of cytokinins in plant tissues has yet to be demonstrated.

The properties of cytokinin oxidase have been the subject of several investigations (2,13,14,20,24). The enzyme was originally detected by Paces et al. (20) in crude homogenates of cultured tobacco cells. It was subsequently isolated and partially purified from immature
corn kernels by Whitty and Hall (24) and, more recently, from *Vinca rosea* tumor tissue by McGaw and Horgan (14). As isolated from either monocot (24) or dicot (14,22) plant sources, the enzyme requires molecular oxygen and catalyzes the oxidative cleavage of the N\textsuperscript{6}-side chains of N\textsuperscript{6}-(\Delta\textsuperscript{2}-isopentenyl)adenine (i\textsuperscript{6}Ade)\textsuperscript{3} and zeatin (io\textsuperscript{6}Ade) and their ribonucleosides (i\textsuperscript{6}Ado and io\textsuperscript{6}Ado). The products of the reaction are adenine (or adenosine) and a side chain fragment, identified as 3-methyl-2-butenal in the case of the oxidation of i\textsuperscript{6}Ade (2). The mechanism of the reaction is uncertain, but an iminopurine intermediate has been hypothesized (2). Cytokinins bearing saturated isoprenoid side chains (N\textsuperscript{6}-isopentyladenosine, hi\textsuperscript{6}Ado, and dihydrozeatin, hio\textsuperscript{6}Ade) and some synthetic cytokinins (N\textsuperscript{6}-benzyladenine, b\textsuperscript{6}Ade, and kinetin, f\textsuperscript{6}Ade) are reported to be resistant to attack by the enzyme (14,24).

The specificity of cytokinin oxidase suggests that it plays an important role in the regulation of cytokinin levels in plant tissues, but the actual physiological and developmental significance of the enzyme has yet to be established. As part of a continuing study of the genetic regulation of cytokinin metabolism in *Phaseolus*, we are investigating the regulation of cytokinin oxidase activity

\textsuperscript{3}Abbreviations: \textit{i}\textsuperscript{6}Ade, N\textsuperscript{6}-(\Delta\textsuperscript{2}-isopentenyl)adenine; \textit{Ade}, adenine; \textit{Ado}, adenosine; \textit{b}\textsuperscript{6}Ade, N\textsuperscript{6}-benzyladenine; \textit{c}-io\textsuperscript{6}Ade, cis-zeatin; \textit{f}\textsuperscript{6}Ade, N\textsuperscript{6}-furfuryladenine (kinetin); \textit{Hex}\textsuperscript{6}Ade, N\textsuperscript{6}-hexyladenine; \textit{hi}\textsuperscript{6}Ade, N\textsuperscript{6}-isopentyladenidine; \textit{hi}\textsuperscript{6}Ado, N\textsuperscript{6}-isopentyladenosine; hio\textsuperscript{6}Ade, dihydrozeatin; \textit{t-\textit{i}}\textsuperscript{6}Ado, N\textsuperscript{6}-(\Delta\textsuperscript{2}-isopentenyl)adenosine; \textit{t-\textit{i}}\textsuperscript{6}Ado, trans-zeatin; PVPP, polyvinylpolypyrrolidone.
in callus tissues derived from selected *Phaseolus* genotypes. The effects of short-term perturbations in cytokinin supply on the levels of cytokinin oxidase activity in callus tissues of *P. vulgaris* L. cv. Great Northern are reported here. We have found that transient increases in the supply of exogenous cytokinins result in elevated levels of cytokinin oxidase activity in Great Northern callus tissues. The increase in enzyme activity occurs relatively rapidly (within a few hours following cytokinin treatment) and appears to require RNA and protein synthesis.

**MATERIALS AND METHODS**

**Chemicals.** Picloram was purchased from Aldrich. Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea, DROPP, SN 49537) was a gift from NOR-AM Chemical Co. N6-Isopentyladenine was synthesized as described by Leonard *et al* (11). The other cytokinin-active purine derivatives used in this study were purchased from Sigma.

Porapak Q (100-120 mesh) was purchased from Waters Assoc. Polymin P (polyethyleneimine, 50% w/v) was obtained from Miles Laboratories, Inc. Polyvinylpolypyrrolidone, Sephadex LH-20, bis tris, imidazole, cycloheximide, rifampicin and cordycepin were purchased from Sigma. Ammonium sulfate was purchased from Schwarz/Mann. Adenosine-8-14C (57 mCi/mmol) was obtained
from Amersham Co. Lipofluor and the Si-C<sub>18</sub> TLC plates used in this study are products of J. T. Baker Co.

**Plant Materials.** Seeds of *Phaseolus vulgaris* L. cv. Great Northern were obtained from Atlee Burpee Co. The seeds were surface sterilized for 5 min in 50% Clorox containing 0.1% (v/v) Tween 20, rinsed three times in sterile water, and germinated under aseptic conditions. Tissue cultures of *P. vulgaris* cv. Great Northern were established from the hypocotyl tissue of 5-day-old seedlings as described previously (15).

**Tissue Culture.** The medium used to culture *Phaseolus* callus tissues consisted of the mineral nutrients defined by Murashige and Skoog (18) with the following organic substances added: sucrose (30 g/l), myo-inositol (100 mg/l), thiamine·HCl (1 mg/l), nicotinic acid (5 mg/l), pyridoxine·HCl (0.5 mg/l), picloram (2.5 uM) and kinetin (5 uM). The pH of the medium was adjusted to 5.7 and Difco Bacto-agar (10 g/l) was added. The medium was dispensed into 125 ml Erlenmeyer flasks (50 ml/flask) and autoclaved at 120° C for 15 min.

**Preparation of Polyvinylpolypyrrolidone and Porapak Q and Polymin P.** PVPP was prepared for use by boiling in 1 N HCl for 10 min. The adsorbent was then washed with 5 bed volumes each of 1 N HCl and glass distilled water and resuspended in water. The suspension was neutralized with KOH, and the PVPP was washed with water until free of chloride. The acid-cleaned PVPP was washed with glass-
distilled 95% (v/v) ethanol until no UV-absorbing (260 nm) material was detectable in the eluate. The ethanol was removed by washing with water, and the adsorbent was dried at 50° C. The PVPP was hydrated in excess buffer for several hours just prior to use.

Porapak Q was suspended in 95% (v/v) ethanol and washed in this solvent until absorbance at 260 nm could no longer be detected in the wash solutions. The washed Porapak Q was stored in 70% ethanol at 4° C. The ethanol was removed by water washes, and the resin was equilibrated with the appropriate buffer prior to use.

Solutions of Polymin P were adjusted to pH 6.5 with HCl prior to use.

**Preparation of i6Ade-8-14C.** Ado-8-14C was adjusted to a specific activity of 25 mCi/mmol by the addition of unlabeled Ado and used in the synthesis of i6Ado-8-14C according to Paces et al. (20) as modified by Mok et al. (17). The i6Ado-8-14C was purified by chromatography on a Sephadex LH-20 column in 33% (v/v) ethanol (1) and deribosylated to i6Ade as described by LaLoue et al. (10). The i6Ade-8-14C was isolated from the reaction mixture by chromatography on a Sephadex LH-20 column in 20% (v/v) ethanol and stored at -20° C in 50% (v/v) ethanol. The yield from Ado-8-14C was about 24%.

**Extraction and assay of cytokinin oxidase.** Callus tissue of P. vulgaris cv. Great Northern was homogenized (1 min, Sorvall Omnimixer, full speed) with an equal volume of
cold 0.1 M bis tris-HCl (pH 6.5). All subsequent operations were performed at 4° C unless otherwise indicated. An aliquot of the homogenate equivalent to 5 g of tissue was mixed with 1.5 g (dry weight) of PVPP hydrated with 0.05 M bis tris-HCl (pH 6.5) prepared as described above. The resulting suspension was filtered through two layers of Miracloth under pressure (2 p.s.i. N₂). The filtrate and two 5 ml rinses with the same buffer were combined and centrifuged (10,000 g, 10 min). Polymin P (1% v/v, pH 6.5) was added dropwise with stirring to the supernatant (40 ul Polymin P /ml supernatant). After 10 min, the precipitated nucleic acids and associated proteins (3) were removed by centrifugation (10,000 g, 10 min). Ammonium sulfate was added to the supernatant from the Polymin P step to give 10% saturation. Endogenous cytokinins were removed from the resulting solution by passage through a 1 ml Porapak Q column (0.8 x 2 cm) (8) equilibrated with 0.05 M bis tris-HCl (pH 6.5) containing ammonium sulfate at 10% saturation. The column was rinsed with two 2 ml volumes of the same buffer, and all eluates were combined. Solid ammonium sulfate was added to the combined eluates to give 80% saturation. The resulting suspension was allowed to stand 30 min prior to centrifugation (20,000 g, 20 min). The pellets were stored at -20° C overnight. If sealed under nitrogen, these preparations could be stored for at least 10 weeks with no detectable loss in cytokinin oxidase activity.
The protein pellets from above were dissolved in 0.1 M imidazole buffer (pH 6.5) and assayed for cytokinin oxidase activity using i^6 Ade-8-^{14}C as a substrate. The assay volumes contained imidazole buffer (pH 6.5) at a final concentration of 90 mM, 0.01 mM i^6 Ade-8-^{14}C (0.0125 uCi), and either 95 or 190 ug protein (equivalent to 75 or 150 mg of callus tissue) in a total volume of 50 ul. The assays were incubated for 30 min at 37° C. The reactions were terminated by the addition of 2 volumes of cold 95% (v/v) ethanol containing unlabeled Ade and i^6 Ade (0.75 mM each). After 10 min on ice, precipitated protein was removed by centrifugation. Aliquots (100 ul) of the supernatants were streaked on 5 cm wide Si-C_{18}F TLC plates. The chromatograms were developed to a height of 5 cm in 47% (v/v) ethanol containing 0.15 M NaCl. The locations of the Ade and i^6 Ade standards were determined by inspection under UV light, and bands (1.5 cm wide) centered on the standards were removed and counted in 5 ml Lipofluor in a Packard Model 2405 scintillation counter. To obtain accurate count values in the presence of the fluorescent indicator from the TLC plates, it was necessary to leave the samples in contact with Lipofluor in the dark for 12 to 24 hours prior to counting.

Unless otherwise indicated, all values for cytokinin oxidase activity are the average of four assays (two replicate assays performed on each of two homogenates).
Protein assays. Protein solutions were mixed with equal volumes of cold 20% (w/v) trichloroacetic acid and allowed to stand on ice for 30 min. The precipitated protein was collected by centrifugation (20,000 g, 10 min) and dissolved in 0.1 N NaOH for protein determination using the Folin phenol method of Peterson (21). Bovine serum albumin was used as a standard.

Cytokinin and antibiotic treatments. Callus tissues of P. vulgaris cv. Great Northern were selected for use at an average fresh weight per flask of 5 g (± 0.5 g). At this time, the tissues were rapidly growing and had reached approximately one-fourth of their final weight at 28 days. Culture age at the 5g stage averaged 20 days. Cytokinins and antibiotics were applied as aqueous solutions (0.1 ml/g tissue) directly to the surface of these callus pieces. Following treatment, the tissues were incubated at 27° C in the dark for the time intervals indicated.

RESULTS

Assay of Cytokinin Oxidase Activity in P. Vulgaris L. cv. Great Northern Callus Tissue. The cytokinin oxidase activity extracted from callus tissues of P. vulgaris cv. Great Northern exhibited a pH optimum at about 6.5 (Figure III.1), which is similar to values reported for cytokinin oxidases from other plant tissues (14,24). The activity of the enzyme was reduced 50% at pH values of 5.5 and 8.0. Various buffers with pKa values near 6.5 were tested for
Figure III.1. Effect of pH on cytokinin oxidase activity from homogenates of *P. vulgaris* cv. Great Northern callus tissues.

Callus tissues (12 g/flask) were homogenized, centrifuged to remove debris, treated with Polymin P (0.1 ml/ml homogenate) and (NH$_4$)$_2$SO$_4$ as described in "Materials and Methods". Protein was dissolved in 1 mM bis tris, pH 6.5 and dialyzed against this buffer overnight. The enzyme preparation was mixed with buffer stocks to give final concentrations of 50 mM at the pH values indicated and cytokinin oxidase activity was determined as described in "Materials and Methods". All values are the average of two replicate assays.
Figure III.1.
their effect on the activity of the Phaseolus enzyme (Table III.1). The use of imidazole resulted in an approximately two-fold increase in cytokinin oxidase activity, and this buffer has been used in all our standard assays of cytokinin oxidase activity. As shown in Table III.1, histidine would not substitute for imidazole in stimulating the activity of cytokinin oxidase in crude enzyme preparations from the callus tissue.

The recovery of cytokinin oxidase activity from callus tissues of P. vulgaris cv. Great Northern is illustrated in Table III.2. The final enzyme preparation contained 97% of the activity present in a crude homogenate and exhibited a 2.7-fold increase in specific activity. The use of Polymin P to remove nucleic acids and associated proteins from the crude homogenates facilitated subsequent concentration of cytokinin oxidase activity by ammonium sulfate precipitation. Conditions for the use of Polymin P were determined from titration curves of the type illustrated in Figure III.2. The Polymin P concentration (40 ul 1% per ml homogenate) used for cytokinin oxidase preparations, precipitated about 40% of the protein in the homogenate and left all of the cytokinin oxidase activity in the supernatant. The effectiveness of the Porapak Q step in the removal of endogenous cytokinins was demonstrated by direct addition of i6Ade to tissue homogenates (data not shown).
Table III.1. Effect of Buffer Composition on Cytokinin Oxidase Activity.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Cytokinin Oxidase Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol·mg&lt;sup&gt;-1&lt;/sup&gt;·protein·h&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Bis tris</td>
<td>0.95</td>
</tr>
<tr>
<td>Ada</td>
<td>0.95</td>
</tr>
<tr>
<td>Imidazole</td>
<td>1.93</td>
</tr>
<tr>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.93</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1.05</td>
</tr>
<tr>
<td>Malonic acid</td>
<td>1.02</td>
</tr>
<tr>
<td>Mes</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>Bis tris</td>
<td>0.90</td>
</tr>
<tr>
<td>Imidazole</td>
<td>1.45</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.78</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cytokinin oxidase activity was assayed as described in "Materials and Methods except for the indicated buffer substitutions in the final reaction volumes. Each value is the average of four assays. The SE were less than ±4% of the means.
Table III.2. Recovery of Cytokinlin Oxidase Activity from \textit{P. vulgaris} cv. Great Northern Callus Tissue.

<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>Total protein</th>
<th>Total cytokinin oxidase activity</th>
<th>%Recovery$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Homogenate$^b$</td>
<td>3.60</td>
<td>1.57</td>
<td>100</td>
</tr>
<tr>
<td>Std. Assay Preparation$^c$</td>
<td>1.27</td>
<td>1.52</td>
<td>97</td>
</tr>
</tbody>
</table>

$^a$All values are the average of four assays. The SE were less than $\pm2\%$ of the means.

$^b$Prior to assay, cellular debris was removed from the crude homogenate by centrifugation at 10,000 g for 10 min.

$^c$The standard assay preparation was obtained by treating the homogenate with PVPP, Polymin P, and Porapak Q as described in the "Materials and Methods".
Figure III.2. Effect of Polymin P concentration on the recovery of protein and cytokinin oxidase activity from homogenates of *P. vulgaris* cv. Great Northern callus tissues.

Callus tissues (7 g/flask) were homogenized and treated with PVPP as described in "Materials and Methods". The indicated volumes of 1 % Polymin P were added to 10 ml aliquots of PVPP-treated crude homogenate while vortexing. After 10 min, the samples were centrifuged to remove precipitated material. Cytokinin oxidase activity and protein were measured on samples of the supernatant as described in the "Materials and Methods". SE (n=4) averaged ±3% of the means and did not exceed ±6%.
Figure III.2.
The effects of varying enzyme concentration and time of incubation on the reaction rates obtained with cytokinin oxidase preparations prepared as described above are shown in Figure III.3. A 30 min incubation time was selected for the standard assay procedure because enzyme concentrations could be varied more than an order of magnitude (from 30 to 300 ug protein/assay) with no deviation in the linearity of response.

**Effects of unlabeled cytokinins on the degradation of i^6Ade-8-14C by cytokinin oxidase.** The substrate specificity of the cytokinin oxidase from Great Northern callus tissue was examined by testing the effects of unlabeled cytokinins on the degradation of i^6Ade-8-14C in the standard cytokinin oxidase assay (Table III.3). Unlabeled cytokinins were added to the assay mixture at concentrations equal to that of the labeled i^6Ade substrate (10 uM) and in ten-fold excess (100 uM). As expected, Ade was completely ineffective in inhibiting the degradation of i^6Ade-8-14C, whereas the addition of unlabeled i^6Ade to the reaction mixture markedly inhibited oxidation of the labeled substrate. Suprisingly, the cytokinin-active urea derivative Thidiazuron was as effective as i^6Ade in inhibiting label degradation. The nucleoside form of i^6Ade (i^6Ado) was somewhat less effective than the free base. Zeatin (t-io^6Ade) rather weakly inhibited the reaction, and cis-zeatin was even less effective than the trans isomer. (Both of the zeatin
Figure III.3. Effect of enzyme concentration and time of incubation on the rate of cytokinin oxidase activity.

Assays were performed as described in "Materials and Methods" except for the indicated modifications in protein concentration and incubation time. The data plotted in the insert were obtained using the standard incubation time of 30 min. All values are the average of two replicate assays.
Figure III.3.
Table III.3. Effects of Unlabeled Cytokinins on the Degradation of \( \text{i}^6\text{Ade}-8^{-14}\text{C} \) by Cytokinin Oxidase

Unlabeled cytokinins were added to standard assay mixtures containing 95 \( \mu \text{g} \) protein and \( \text{i}^6\text{Ade}-8^{-14}\text{C} \) at a concentration of 10 \( \mu \text{M} \). All other conditions were as described in "Materials and Methods".

<table>
<thead>
<tr>
<th>Unlabeled Cytokinin</th>
<th>Concentration of unlabeled cytokinin</th>
<th>Cytokinin Oxidase Activity(^a) % control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ( \mu \text{M} )</td>
<td>100</td>
</tr>
<tr>
<td>Ade</td>
<td></td>
<td>101</td>
</tr>
<tr>
<td>( \text{i}^6\text{Ade} )</td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>( \text{i}^6\text{Ado} )</td>
<td></td>
<td>74</td>
</tr>
<tr>
<td>hi( ^6\text{Ade} )</td>
<td></td>
<td>98</td>
</tr>
<tr>
<td>hi( ^6\text{Ado} )</td>
<td></td>
<td>98</td>
</tr>
<tr>
<td>t-( ^6\text{Ade} )</td>
<td></td>
<td>83</td>
</tr>
<tr>
<td>c-( ^6\text{Ade} )</td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>f( ^6\text{Ade} )</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>b( ^6\text{Ade} )</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>Thidiazuron</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

\(^a\) The cytokinin oxidase activity of controls incubated without the addition of unlabeled cytokinins was equal to 1.27 nmol/h/\( \mu \text{g} \) protein. This value was taken as 100% activity. All values are the average of four assays. SE averaged \( \pm 2\% \) of the means and did not exceed \( \pm 6\% \) of the means.
isomer preparations used here contained 7-10% of the other isomer). Cytokinins bearing saturated isoprenoid side chains (hi6Ade, hic6Ade) or side chains with ring structures (b6Ade and f6Ade) did not significantly inhibit the degradation of i6Ade-8-14C.

Cytokinin Oxidase Activity in Callus Tissues Treated with Exogenous Cytokinin. The effect of increases in cytokinin concentration on in vivo levels of cytokinin oxidase activity in callus tissues of P. vulgaris cv. Great Northern was tested by direct application of exogenous solutions of cytokinins to the surface of callus tissues. At the time of treatment, the callus tissues had reached 5 g/flask on a medium containing 5 uM f6Ade. Cytokinin oxidase activity was monitored over a 4 hour incubation period following the cytokinin application. The results of treatment with zeatin (io6Ade) and dihydrozeatin (hic6Ade) are shown in Figure III.4A. Tissues treated with zeatin exhibited elevated levels of cytokinin oxidase activity by the end of the 4 hour incubation period. Tissues receiving dihydrozeatin exhibited even larger increases in cytokinin oxidase activity than those observed in tissues treated with zeatin. During the same time period, little change in cytokinin oxidase activity was observed in control tissues treated with water. Treatments with the cytokinins i6Ade and hi6Ade also induced increases in cytokinin oxidase activity in Great Northern callus tissues (Figure III.4B). Again, the cytokinin with the saturated N6-side chain
Figure III.4. Effects of exogenously applied cytokinins on cytokinin oxidase activity in P. vulgaris cv. Great Northern callus tissue.

Callus tissues (5 g/flask) were grown and selected as described in "Materials and Methods". Cytokinin solutions (100 uM) were applied to the callus tissues at a rate of 0.1 ml/g tissue to give an estimated concentration in the tissues of 10 uM. Following cytokinin application, the tissues were incubated for the times indicated at 27°C in the dark. Cytokinin oxidase activity was assayed as described in "Materials and Methods". SE (n=4) averaged ±9% of the means and did not exceed ±11%.
Figure III.4.

Cytokinin oxidase activity (nmol/h per g fresh weight) vs. incubation time (hours).

---

A

- hio^6Ade
- io^6Ade
- H_2O

B

- h^6Ade
- i^6Ade
- H_2O
(hi$^6$Ade) was more effective than the analog bearing the corresponding unsaturated side chain (i$^6$Ade) in inducing elevated levels of cytokinin oxidase in the callus tissue.

A number of other cytokinins have been tested for their ability to induce increases in cytokinin oxidase activity in Great Northern callus tissues (Table III.4). The cytokinins b$^6$Ade, Hex$^6$Ade, hi$^6$Ade, and f$^6$Ade all induced 2 to 3 fold increases in cytokinin oxidase activity over the 4 hour incubation period. Adenine had no effect on the levels of cytokinin oxidase activity in the callus tissue. Interestingly, the cytokinin-active urea derivative, Thidiazuron was just as effective as the purine derivatives in inducing elevated levels of cytokinin oxidase activity.

The time course of the cytokinin-induced elevation in cytokinin oxidase activity was investigated in more detail using hi$^6$Ade. Figure III.5 shows the changes in cytokinin oxidase activity over a 32 hour time course following a single application of hi$^6$Ade to the Great Northern callus tissue. An increase in cytokinin oxidase activity was detectable by 1 hour after the cytokinin treatment. The activity continued to increase for about 8 hours, reaching a plateau value corresponding to an approximately three-fold increase in activity. The enzyme activity had begun to decline by 32 hours of incubation.

The effect of varying the hi$^6$Ade concentration used in the treatment of the Great Northern callus tissue is shown
Table III.4. Effects of Exogenously Applied Cytokinins on Cytokinin Oxidase Activity in *P. vulgaris* cv. Great Northern Callus Tissue.

Callus tissues (5 g/flask) were grown and selected as described in "Materials and Methods". At time zero, cytokinin solutions (100 μM) were applied to the surface of the callus tissues at a rate of 0.1 ml/g tissue to give a final estimated concentration in the tissue of 10 μM. Following cytokinin application, the tissues were incubated for 4 hours at 27°C in the dark. Cytokinin oxidase activity was assayed as described in "Materials and Methods".

<table>
<thead>
<tr>
<th>Cytokinin</th>
<th>Cytokinin Oxidase Activitya</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time 0</td>
<td>Time 4 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nmol·g⁻¹ tissue·h⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ade</td>
<td>0.92</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>b⁶Ade</td>
<td>0.82</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td>Hex⁶Ade</td>
<td>0.53</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td>hi⁶Ade</td>
<td>0.82</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td>f⁶Ade</td>
<td>0.76</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>Thidiazuron</td>
<td>0.78</td>
<td>2.56</td>
<td></td>
</tr>
</tbody>
</table>

aAll values are the average of four assays. SE averaged ±7% of the means and did not exceed ±13% of the means.
Figure III.5. Time course of changes in cytokinin oxidase activity in P. vulgaris cv. Great Northern callus tissue following treatment with N°-isopentyladenine (hi°Ade).

Callus tissues (5 g/flask) were grown and selected as described in "Materials and Methods". Following a single application of hi°Ade (100 uM) to the surface of the callus tissue at a rate of 0.1 ml/g tissue, the tissues were incubated in the dark at 27°C at the indicated times. Cytokinin oxidase activity was assayed as described in "Materials and Methods". SE (n=4) averaged ±5% of the means and did not exceed ±8%. 
Figure III.5.

![Graph showing cytokinin oxidase activity (nmol/h) per g fresh weight over time (hours). The activity increases initially, reaches a peak, and then decreases.]
in Figure III.6. As measured 4 hours following treatment, a slight increase in cytokinin oxidase activity could be detected after treatment with hi⁶Ade concentrations equivalent to as little as 0.1 nmoles per gram tissue. Cytokinin oxidase activity increased with increasing concentrations of hi⁶Ade up to a plateau value representing better than a three-fold increase in activity at an estimated in vivo hi⁶Ade concentration of about 20 uM.

**Effects of Inhibitors on Cytokinin-Induced Increases in Cytokinin Oxidase Activity.** The effects of antibiotic inhibitors of transcription and translation on the induction of elevated levels of cytokinin oxidase activity in Great Northern callus tissue have also been examined. Where feasible, appropriate concentrations of the inhibitors were selected by determining the concentrations at which they affected the growth of Great Northern callus tissue (Figure III.7). The effects of four such inhibitors on the induction of increases in cytokinin oxidase activity by hi⁶Ade are shown in Table III.5. Cordycepin, at a concentration corresponding to 320 mM in the tissue, inhibited the hi⁶Ade-induced increase in cytokinin oxidase activity by 71%.

The effect of cordycepin on hi⁶Ade-induced increases in cytokinin oxidase activity was investigated in more detail (Figure III.8). The inhibitory effects of cordycepin on induction were evident at concentrations
Figure III.6. Effect of variations in N\textsuperscript{6}-isopentyadenine concentration on the induction of cytokinin oxidase activity in \textit{P. vulgaris} cv. Great Northern callus tissues.

Callus tissues (5 g/flask) were grown and selected as described in "Materials and Methods". N\textsuperscript{6}-isopenteyladenine solutions (0.1 \textmu M to 320 \textmu M) were applied to the surfaces of the callus tissues at a rate of 0.1 ml/g tissue to give estimated \texttt{hi}^\text{6}\text{Ade} concentrations in the tissue as indicated. The tissues were incubated for 4 hours at 27\textdegree C in the dark. Cytokinin oxidase activity was assayed as described in "Materials and Methods". SE (n=4) averaged \pm 3\% of the means and did not exceed \pm 5\%. 

Figure III.6.

[Graph showing the relationship between Cytokinin Oxidase Activity (nmol/h) per g Fresh Weight and hi^6Ade (μM).]
Figure III.7. Effects of antibiotic inhibitors of transcription and translation on the growth of *P. vulgaris* cv. Great Northern callus tissues.

The antibiotics were dissolved in DMSO and added to autoclaved tissue culture medium at a rate of 50 μl/50 ml medium. Three callus pieces weighing ca. 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days growth at 28° C in the dark. The *Phaseolus* tissue culture medium is described in "Materials and Methods".
Figure III.7.
### Table III.5. Effects of Inhibitors on Cytokinin-induced Increases in Cytokinin Oxidase Activity in *P. vulgaris* cv Great Northern Callus Tissue

Callus tissues (5 g/flask) were grown and selected as described in "Materials and Methods". Inhibitor solutions were applied to the surface of the callus tissues at a rate of 0.1 ml/g tissue. The tissues were incubated for 2 hours at 27°C in the dark and then treated with 200 uM hi°Ade (0.1 ml/g tissue) to give a final estimated concentration of 20 uM in tissue. After an additional 4 hour incubation period, cytokinin oxidase activity (CKO) was determined as described in "Materials and Methods".

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Concentration in vivo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cytokinin Oxidase Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Increase in CKO Activity Induced by hi°Ade Treatment&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>Expt 1 nmol·g⁻¹ tissue·h⁻¹</td>
<td>Expt 2</td>
</tr>
<tr>
<td>None</td>
<td>1.60</td>
<td>1.42</td>
<td>146</td>
</tr>
<tr>
<td>Cordycepin</td>
<td>320</td>
<td>0.84</td>
<td>29</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>32</td>
<td>1.50</td>
<td>131</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>3200</td>
<td>0.94</td>
<td>27</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>320</td>
<td>1.68</td>
<td>127</td>
</tr>
</tbody>
</table>

<sup>a</sup>Concentrations were calculated assuming 1 g callus equals 1 ml volume.

<sup>b</sup>All values are the average of four assays. SE averaged ±5% of the means and did not exceed ±6% of the means.

<sup>c</sup>The cytokinin oxidase activity in untreated control tissues which had not been exposed to either inhibitors or hi°Ade was equal to 0.65 and to 0.74 nmol/h·g tissue in experiments 1 and 2, respectively.
Figure III.8. Effect of cordycepin on the induction of cytokinin oxidase activity by N6-isopentyladenine (hi6Ade) in P. vulgaris cv. Great Northern callus tissue.

Callus tissues were grown and selected as described in "Materials and Methods". Cordycepin solutions (0.01 to 10 mM) were applied to the surface of callus tissues at a rate of 0.1 ml/g tissue to give estimated hi6Ade concentrations in the tissue as indicated. The tissues were incubated for 2 hours at 27°C in the dark and then treated with 200 uM hi6Ade (0.1 ml/g tissue) to give a final estimated concentration of 20 uM in the tissue. After an additional 4 hour incubation period, cytokinin oxidase activity was assayed as described in "Materials and Methods". Cytokinin oxidase activity is expressed as percent of the activity of control tissues that had not been exposed to either cordycepin or hi6Ade. The activity of the control tissues was equal to 1.27 nmol/h per gram fresh weight, and this value was taken as 100% activity.
Figure III.8.

![Graph showing cytokinin oxidase activity (as % of control) vs. cordycepin concentration (μM). The graph shows an increase in cytokinin oxidase activity from 0 to 10 μM followed by a sharp decrease at concentrations above 10 μM.](image-url)
equivalent to 100 \text{ uM} in the tissue, and a cordycepin concentration of 1000 \text{ uM} was sufficient to completely inhibit the increase in cytokinin oxidase activity caused by hi$^6$Ade treatment. On the basis of the results shown in Figure III.7, cycloheximide was tested at a concentration equivalent to 3200 \text{ IM} in the tissue. At this concentration, the hi$^6$Ade-induced increase in cytokinin oxidase activity was inhibited 73\%. Neither cordycepin nor cycloheximide has any significant effect on cytokinin oxidase activity when these compounds were added directly to the enzyme assays. Thus, the effects of these compounds do not appear to result from direct inhibition of enzyme activity per se. Rifampicin and chloramphenicol, which would be expected to be relatively specific inhibitors of RNA and protein synthesis in organelles, had little inhibitory effect on the induction of elevated cytokinin oxidase levels, although these compounds did inhibit the growth of the callus tissue (Figure III.7). In fact, chloramphenicol appeared to enhance the response of the callus tissues to hi$^6$Ade treatment.

**DISCUSSION**

The regulation of cytokinin oxidase activity in callus tissues of *P. vulgaris* cv. Great Northern has been examined using an assay based on the oxidation of i$^6$Ade-8-\textsuperscript{14}C to Ade. The use of imidazole as a reaction buffer was important in obtaining reproducible results in the assay.
of cytokinin oxidase activity in extracts of Phaseolus callus tissue. Reaction rates were nonlinear and unpredictable with crude enzyme preparations in the absence of imidazole, and the observed cytokinin oxidase activity was approximately doubled in the presence of imidazole. The biochemical basis for the stimulation of cytokinin oxidase activity by imidazole is uncertain, but it is possible that the compound may inhibit other reactions that compete with cytokinin oxidase for activated oxygen when crude enzyme preparations are used in the assay. To obtain accurate and reproducible assays of cytokinin oxidase activity in Phaseolus callus tissues, it was also necessary to concentrate the enzyme activity and to remove endogenous cytokinins present in the homogenates. Concentration of the enzyme was achieved by ammonium sulfate precipitation after removal of nucleic acids and associated proteins with Polymin P. In practice, it was efficient to remove cytokinins by passage of the extracts over small Porapak Q columns prior to performing the ammonium sulfate precipitation.

The substrate specificity of the cytokinin oxidase from P. vulgaris cv. Great Northern callus tissues appears rather similar to that observed in previous studies with cytokinin oxidase preparations from other plant sources (14, 24). On the basis of substrate competition experiments, both the Phaseolus and Zea mays enzymes (24) appear to utilize i^6Ade and io^6Ade as substrates and to be
ineffective in degrading hi^6Ade, hio^6Ade, b^6Ade, or f^6Ade. Where data are available, similar specificities have been observed using the corresponding ribonucleosides. The trans isomer of io^6Ado has been reported to be more effective than the cis isomer in competitively inhibiting the degradation of labeled io^6Ado by a cytokinin oxidase from tobacco (19). Similar results were obtained in the present study using the Phaseolus enzyme and comparing the cis and trans forms of io^6Ade. However, the trans isomer itself was somewhat less effective as a substrate for the oxidase than had been anticipated on the basis of published results.

The observed ability of Thidiazuron to inhibit the in vitro degradation of io^6Ade-8-14C by cytokinin oxidase was unexpected and raises the possibility that cytokinin-active urea derivatives may also serve as substrates for the enzyme. However, the possibility that Thidiazuron is simply a potent inhibitor of the enzyme cannot be excluded.

As shown in the present study, the levels of cytokinin oxidase activity in callus tissues of P. vulgaris cv. Great Northern are regulated, at least in part, by the supply of exogenous cytokinins. Treatment of the callus tissue with solutions of cytokinins induced relatively rapid increases in cytokinin oxidase activity. Under the conditions used here, the increase in activity was detectable one hour following the cytokinin treatment.
and continued for about 8 hours, reaching values two- to three-fold higher than the controls. Although cytokinin oxidase activity was always present in the control tissues, these basal levels of enzyme activity may be the inevitable consequence of the necessity of growing the tissues on cytokinin-containing media.

The structural requirements for the induction of cytokinin oxidase activity in Great Northern callus tissues appear to differ from those determining the ability of compounds to function as substrates for cytokinin oxidase. Thus, all cytokinin-active compounds tested were effective in inducing increased levels of cytokinin oxidase activity in the callus tissue. Derivatives with saturated N⁶-side chains (hi⁶Ade and hio⁶Ade), which were resistant to attack by the enzyme, were actually more effective than i⁶Ade and io⁶Ade in promoting increases in cytokinin oxidase activity. The effectiveness of hi⁶Ade and hio⁶Ade as inducers may be related to their persistence in the tissue. Thidiazuron was also effective in inducing increases in cytokinin oxidase activity, indicating that the regulatory mechanisms controlling the induction of cytokinin oxidase activity in Great Northern callus tissues are sensitive to cytokinin-active urea derivatives as well as to N⁶-substituted adenine derivatives. The ability of Thidiazuron to induce cytokinin oxidase activity and to inhibit the in vitro assay for the enzyme is consistent
with the hypothesis that cytokinin-active adenine and urea derivatives share at least some common sites of action (7,9).

Cytokinin-induced increases in cytokinin oxidase activity were inhibited in Great Northern callus tissues pretreated with cordycepin or cycloheximide. Inhibitor studies of this type must be interpreted with caution, but these studies suggest that the increase in enzyme activity is dependent on RNA and protein synthesis. Rifampicin and chloramphenicol, at concentrations that inhibited the growth of Great Northern callus tissues, were ineffective in inhibiting the increase in cytokinin oxidase activity. Therefore, if increased synthesis of the enzyme occurs in response to cytokinin treatment, the inhibitor data suggest that this synthesis does not occur in organelles. Additional work will be needed to establish these points beyond question.

We have not yet determined whether elevated levels of cytokinin oxidase are induced in other plant tissues treated with exogenous cytokinins. However, Terrine and LaLoue (23) have remarked that the degradation of i^6Ado appeared to be stimulated in tobacco cells that had been previously exposed to cytokinins. This observation is compatible with an induction of cytokinin oxidase activity similar to what we have observed with Great Northern callus tissues.
The results presented here indicate that cytokinin oxidase activity in *Phaseolus* callus tissues is regulated by a mechanism that is sensitive to cytokinin supply and capable of making relatively rapid adjustments in the levels of the enzyme. Enzymes involved in hormonal metabolism are likely to be subject to multiple controls, and it will be of interest to determine whether additional types of regulatory mechanisms are operative in the control of cytokinin oxidase activity in these callus tissues.
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6. Hall RH, SN Alan, BD McLennan, C Terrine, J Guern 1971 N\textsuperscript{6}-(\Delta\textsuperscript{2}-isopentenyl)adenosine: Its conversion to inosine, catalyzed by adenosine aminohydrolases from chicken bone marrow and calf intestinal mucosa. Can J Biochem 49: 623-630


CHAPTER IV.

CYTOKININ OXIDASE FROM CALLUS TISSUES OF PHASEOLUS VULGARIS L. CV. GREAT NORTHERN: II. ENHANCEMENT OF THE IN VITRO ACTIVITY OF THE ENZYME BY COPPER-IMIDAZOLE COMPLEXES

ABSTRACT

The effects of metal ions on cytokinin oxidase activity from Phaseolus vulgaris L. cv. Great Northern have been examined using an assay based on the oxidation of i$^6$Ade-2,8-3$^3$H to adenine. The addition of Cu$^{+2}$ to enzyme reaction mixtures containing imidazole buffer enhanced cytokinin oxidase activity more than 20-fold. The effect was enzyme dependent and specific for copper. The addition of EDTA to reaction mixtures eliminated the copper enhancement, but EDTA did not inhibit the basal (unenhanced) levels of cytokinin oxidase activity. The substrate specificity of the copper-enhanced reaction, as judged by substrate competition tests, was the same as that observed in the absence of copper. The elution positions of cytokinin oxidase activity fractionated by DEAE-cellulose chromatography were identical using either the copper-imidazole enhanced assay or an assay without the addition of copper. In the absence of copper, reducing the oxygen concentration in cytokinin oxidase reaction mixtures inhibited the oxidation of i$^6$Ade to adenine. However, in the presence of copper-imidazole, no inhibition of cytokinin oxidase activity was observed.
under anaerobic conditions. This result suggests that the copper-imidazole complex is substituting for oxygen as an electron accepter in the cytokinin oxidase reaction.

INTRODUCTION

Reactions catalyzed by cytokinin oxidase appear to constitute a major pathway of cytokinin degradation in plant tissues \((2,4,6,10,11,16,17,19,22)\). The enzyme catalyzes the oxidative cleavage of the \(N^6\)-side chains of zeatin and \(N^6-(\Delta^2\text{-isopentenyl})\text{adenine}\) and their ribonucleosides to form adenine or adenosine and the corresponding side chain fragment \((2,10)\). Although details of the reaction mechanism are uncertain, molecular oxygen is required, and an iminopurine intermediate has been isolated from reaction mixtures containing 2-mercaptoethanol \((6)\). The nature of the prosthetic group of the enzyme has yet to be established, but the observation by Whitty and Hall \((22)\) that cytokinin oxidase activity from corn is inhibited by cyanide suggests that the enzyme may be a metalloprotein.

The properties of cytokinin oxidase activity isolated from callus tissues of \textit{Phaseolus vulgaris} cv. Great Northern have been examined in the present investigation. The substrate specificity and regulatory properties of the enzyme from this source have been described previously \((4)\). We report here that copper-imidazole complexes greatly enhance the \textit{in vitro} activity of cytokinin oxidase
and present evidence that such complexes function as alternative electron accepters in the enzyme-dependent degradation of cytokinins.

MATERIALS AND METHODS

**Chemicals.** Picloram (4-amino-3,5,6-trichloropicolinic acid) was purchased from Aldrich. Thidiazuron (N-phenyl-N'1,2,3-thidiazol-5-ylurea, DROPP, SN 49537) was a gift from NOR-AM Chemical Co. N6-Isopentyladenine was synthesized as described by Leonard et al (8). All other cytokinin-active compounds used in this study were purchased from Sigma.

Polymin P (polyethylenimine, 50% w/v) was obtained from Miles Laboratories, Inc. Ammonium sulfate (enzyme grade) was purchased from Schwarz/Mann. Polyvinylpolypyrrolidone (PVPP) was purchased from Sigma and prepared for use as previously described (4). DE52-cellulose was obtained from Whatman. Sephadex LH-20 was purchased from Sigma. The Si-C18 TLC plates (without fluorescent indicator) used in this study are products of J. T. Baker Co.

Adenosine-2,8-3H (40 Ci/mmol) was obtained from ICN Radiochemicals. Ready-Solv HP/b is a Beckman product.

**Plant Materials.** Seeds of Phaseolus vulgaris L. cv. Great Northern were obtained from Atlee Burpee Co. The seeds were surface sterilized for 5 min in 50% Chlorox containing 0.1% (v/v) Tween 20, rinsed three times in
sterile water, and germinated under aseptic conditions. Tissue cultures of *P. vulgaris* cv. Great Northern were established from the hypocotyl tissue of 5-day-old seedlings as described previously (13). The callus tissues were subcultured on fresh medium at approximately 24 day intervals.

**Tissue Culture Medium.** The medium used to culture *Phaseolus* callus tissues consisted of the mineral nutrients defined by Murashige and Skoog (15) with the following organic substances added: sucrose (30 g/l), myo-inositol (100 mg/l), thiamine•HCl (1 mg/l), pyridoxine•HCl (0.5 mg/l), picloram (2.5 uM) and kinetin (5 uM). The pH of the medium was adjusted to 5.7 and Difco Bacto-agar (10 g/l) was added. The medium was dispensed into 125 ml Erlenmeyer flasks (50 ml/flask) and autoclaved at 120° C for 15 min.

**Preparation of i<sup>6</sup>Ade-2,8-3H.** Ado-2,8-3H was adjusted to a specific activity of 250 mCi/mmol by the addition of unlabeled Ado and used in the synthesis of i<sup>6</sup>Ado-2,8-3H according to methods previously described for the synthesis of i<sup>6</sup>Ado-8-14C (14). The i<sup>6</sup>Ado-2,8-3H was purified by chromatography on a Sephadex LH-20 column in 33% (v/v) ethanol (1) and deribosylated to i<sup>6</sup>Ade as described by LaLoue et al. (7). The i<sup>6</sup>Ade-2,8-3H was isolated from the reaction mixture by chromatography on a Sephadex LH-20 column in 20% (v/v) ethanol and stored at -20° C in 50% (v/v) ethanol. The yield from Ado-2,8-3H was
ca. 20%. Prior to use in enzyme assays, the i^6^Ade-2,8-^3^H was adjusted to a specific activity of 100 uCi/umol with unlabeled i^6^Ade, and the ethanol was removed by evaporation.

**Standard Extraction and Assay of Cytokinin Oxidase Activity.** Callus tissues of *P. vulgaris* cv. Great Northern (5 to 7.5 g/flask, 19 to 22 days old) were homogenized (1 min, Sorvall Omnimixer, full speed) with an equal volume of cold 0.1 M bis tris-HCl (pH 6.5). All subsequent operations were performed at 4° C unless otherwise indicated. An aliquot of the homogenate equivalent to 5 g of tissue was mixed with 1.5 g (dry weight) of PVPP hydrated with 0.05 M bis tris-HCl (pH 6.5). The resulting suspension was filtered under pressure (2 lbs/in^2^ N_2_) through two layers of Miracloth. The solids retained by the Miracloth were washed with two 5 ml aliquots of the same buffer, and the filtrates were combined and centrifuged (10,000 g, 10 min). Polymin P (1% v/v, pH 6.5) was added dropwise with stirring to the supernatant (40 ul Polymin P /ml supernatant). After 10 min, the precipitated nucleic acids and associated proteins (2) were removed by centrifugation (10,000 g, 10 min). Solid ammonium sulfate was added to the supernatant from the Polymin P step to give 80% saturation. The resulting suspension was allowed to stand without stirring for 30 min prior to centrifugation (20,000 g, 20 min). If necessary, the pellets were stored at -20° C. At this
temperature, pellets sealed under nitrogen could be stored for at least 10 weeks with no detectable loss in cytokinin oxidase activity.

The protein pellets from above were dissolved in bis tris or imidazole buffer (pH 6.5) and assayed for cytokinin oxidase activity using i6Ade-2,8-3H as a substrate. The assay mixtures contained bis tris-HCl or imidazole-HCl buffer (both at pH 6.5) at the concentrations indicated (0.05, 0.1 or 0.2M), 0.01 mM i6Ade-2,8-3H (0.05 uCi, specific activity 100 uCi/ummol), and enzyme in a total volume of 50 ul. The assays were incubated for 30 min at 37° C. The reactions were terminated by the addition of 2 volumes of cold 95% (v/v) ethanol containing unlabeled Ade and i6Ade (0.75 mM each) and 20 mM Na2EDTA. After 10 min on ice, precipitated protein was removed by centrifugation. Aliquots (100 ul) of the supernatants were streaked on 5 cm wide Si-C18 TLC plates. The chromatograms were developed to a height of 5 cm in 37.5% (v/v) ethanol containing 0.1 M Na4EDTA. (The addition of Na4EDTA to the developing solvent was necessary to prevent copper and other metals added to the reaction mixtures from interfering with the chromatography.) The locations of the Ade and i6Ade standards were determined by inspection under UV light, and bands (1.5 cm wide) centered on the standards were removed and counted in 5 ml Ready-Solv HP/b in a Beckman Model 1801 scintillation counter.
Large Scale Extraction of Cytokinin Oxidase. The extraction procedure used to obtain the cytokinin oxidase preparation for DEAE-cellulose chromatography was modified from that described above. The PVPP treatment was omitted, and the homogenates of Great Northern callus tissue were filtered directly through 4 layers of cheesecloth and 2 layers of Miracloth without rinsing. Polymin P (1% v/v, pH 6.5) was added to the centrifuged filtrate at a rate of 75 ul Polymin P/ml supernatant. All other steps were essentially as described for the standard extraction procedure.

Preparation of the DEAE-Cellulose Column. DE52-cellulose was precycled in HCl and NaOH (each at 0.5 N), degassed, and fines removed as recommended by Whatman (21). The exchanger was titrated to pH 7.5 with tris base, washed with 10 bed volumes of 20 mM tris-HCl (pH 7.5) containing 1 M KCl and packed into a 2 x 32 cm column (100 ml bed volume) in the same buffer-salt solution at a flow rate of 300 ml/h. The packed column was washed with 20 mM tris-HCl (pH 7.5) until the pH and conductivity of the eluate were the same as the wash buffer.

Assay of Cytokinin Oxidase in Column Fractions. The cytokinin oxidase activity in fractions resulting from chromatography on DEAE-cellulose was determined using a modified assay procedure in which undegraded i6Ade was removed from the reaction mixtures by solvent partitioning. For the copper-enhanced assay, the assay
mixtures contained 100 mM imidazole-HCl (pH 6.5), 0.01 mM i6Ade-2,8-3H (0.05 uCi, specific activity 100 uCi/uM), 10 mM CuCl2, and 40 ul aliquots of the column fractions in a final volume of 50 ul. For the unenhanced enzyme assays, 100 mM bis tris-HCl (pH 6.5) was substituted for imidazole and CuCl2 was omitted from the reaction mixtures. All assays were incubated at 37 C for 30 min and terminated by the addition of 50 ul of 20 mM Na2EDTA containing i6Ado and Ade (each at 1 mM) followed by the addition of 300 ul of butyl acetate saturated with a 1 mM Ade solution. The assay tubes were vortexed for 15 sec, and the two phases separated by centrifugation. The organic phase, containing undegraded i6Ade, was removed, and the extraction with butyl acetate (saturated with 1 mM Ade) was repeated two times. Aliquots (80 ul) of the aqueous phase, containing the adenine produced in the enzyme reaction, were mixed with 5 ml of Ready-Solv HP/b and counted in a Beckman LS 1801 scintillation counter.

Protein assays. Protein solutions were mixed with equal volumes of cold 20% (w/v) trichloroacetic acid and allowed to stand on ice for 30 min. The precipitated protein was collected by centrifugation (20,000 g, 10 min) and dissolved in 0.1 N NaOH for protein determination using the Folin phenol method of Peterson (18). Bovine serum albumin was used as a standard.
RESULTS

To test for possible stimulating effects of metal ions on cytokinin oxidase activity, salts of various metals known to incorporate into the active sites of enzymes were added to reactions mixtures containing 0.05 M bis tris (pH 6.5) as the assay buffer. The results are shown in Table IV.1. Copper was the only metal that had a significant effect on cytokinin oxidase activity. When imidazole was used as the assay buffer, copper stimulated cytokinin oxidase activity approximately 4 fold.

The effects of increasing concentrations of CuCl₂ on cytokinin oxidase activity measured in reaction mixtures containing imidazole buffer are illustrated in Figure IV.1. At the imidazole concentration used here (200 mM), cytokinin oxidase activity increased with increasing concentrations of CuCl₂ to values representing an approximately 30-fold enhancement of activity at 30 mM CuCl₂. As shown in Figure IV.1, the rate of the copper enhanced reaction at any given copper concentration was dependent on enzyme concentration. The stimulation of cytokinin oxidase activity by copper appeared to plateau only at the limits of copper solubility in the system (Figure IV.2). This limit appears to be determined by the imidazole concentration.

The enzyme dependence of the copper enhanced reaction is further illustrated by the data shown in Table IV.2. If
Table IV.1. Effects of Metals on Cytokinin Oxidase Activity

<table>
<thead>
<tr>
<th>Metal</th>
<th>Bis Tris</th>
<th>Imidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuCl₂</td>
<td>113</td>
<td>410</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>102</td>
<td>99</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>91</td>
<td>105</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>96</td>
<td>104</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>102</td>
<td>106</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>98</td>
<td>91</td>
</tr>
</tbody>
</table>

Cytokinin Oxidase Activity \% of Control

\( a \) Cytokinin oxidase activity was determined in reaction mixtures containing 100 mM bis tris-HCl or imidazole-HCl (pH 6.5), 0.01 mM \( ^{15} \text{Adenosine-2,8-}^3 \text{H} \) (0.05 uCi, Spec. Act. 100 uCi/umol) and protein equivalent to 250 mg (fresh wt.) of P. vulgaris cv. Great Northern callus tissue, and the indicated concentration of metal salts.

\( b \) The cytokinin oxidase activity of controls incubated without the addition of metals was equal to 0.97 and 1.51 nmol per hour per mg protein for the bis tris and imidazole assays respectively. These values were taken as 100% activity. All values are the average of three assays, SE averaged ±1% of the means and did not exceed ±2% of the means.
Figure IV.1. The effect of CuCl$_2$ on cytokinin oxidase activity from *P. vulgaris* cv. Great Northern callus tissues.

Callus tissues (7 g/flask) were homogenized, centrifuged to remove debris, and treated with Polymin P and ammonium sulfate as described in the "Materials and Methods". Protein was dissolved in 100 mM imidazole-HCl (pH 6.5) and centrifuged. Reaction mixtures contained 7.5 yg protein (○) or 15 ug protein (●), 0.01 mM i$^6$Ade-2,8-$^3$H, 200 mM imidazole-HCl (pH 6.5) and the CuCl$_2$ concentrations indicated. Cytokinin oxidase activity was assayed as described in "Materials and Methods". Each value is the average of 3 assays. SE averaged ±2% of the means and did not exceed ±4% of the means.
Figure IV.1

[Graph showing the relationship between CuCl₂ concentration and cytokinin oxidase activity]
Figure IV.2. Effects of CuCl$_2$ on cytokinin oxidase activity in reaction mixtures containing 100 mM or 200 mM imidazole as the assay buffer.

Cytokinin oxidase activity was determined in reaction mixtures containing 100 mM (●) or 200 mM (○) imidazole-HCl (pH 6.5), 0.01 mM $[^6]$Ade-2,8-$^3$H (0.05 uCi, Spec. Act. 100 uCi/umol) and protein equivalent to 21 or 125 mg (fresh wt.) of *P. vulgaris* cv. Great Northern callus tissue, and the indicated concentrations of CuCl$_2$. All values are the average of three assays, SE averaged ±1% of the means and did not exceed ±2% of the means.
Figure IV.2

[Graph showing the effect of CuCl₂ concentration on cytokinin oxidase activity per mg protein]
Table IV.2. Characteristics of the Copper-Imidazole Stimulation of Cytokinin Oxidase Activity from *P. vulgaris* cv. Great Northern callus tissues

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Cytokinin Oxidase Activity&lt;sup&gt;a&lt;/sup&gt; (pmol/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus Enzyme Control</td>
<td>0</td>
</tr>
<tr>
<td>Standard Assay&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Standard Assay +CuCl&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>124 ± 1</td>
</tr>
<tr>
<td>Standard Assay +CuCl&lt;sub&gt;2&lt;/sub&gt; +EDTA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Boiled Enzyme Control&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cytokinin oxidase activity was assayed as described in "Materials and Methods". Each value is the average of three assays ±SE.

<sup>b</sup>Standard assay mixtures contained 8 ug protein, 0.01 mM i<sup>6</sup>Ade-2,8-<sup>3</sup>H, 200 mM imidazole-HCl (pH 6.5) and 8 ug of protein extracted from the callus tissues by the standard extraction procedure described in the "Materials and Methods".

<sup>c</sup>Prior to assay, CuCl<sub>2</sub> was added at a concentration of 10 mM.

<sup>d</sup>Prior to assay, Na<sub>2</sub>EDTA was added to a final concentration of 20 mM.

<sup>e</sup>Prior to assay, the enzyme solution was incubated in boiling water for 5 min.
the enzyme preparation was denatured or omitted from the reaction mixture, the degradation of \( i^6 \text{Ade} \) to Ade did not occur in either the presence or absence of copper. As shown in the same table, the addition of EDTA to the reaction mixtures inhibited the reaction and reduced the activity to that of the controls without copper. It should be noted that EDTA did not inhibit the basal (unenhanced) levels of enzyme activity.

The results described above indicated that the copper-enhanced degradation of \( i^6 \text{Ade} \) observed in assays for cytokinin oxidase activity was enzyme dependent, but the nature of the reaction stimulated by copper was not precisely defined. To determine whether cytokinin oxidase activity per se was enhanced by the addition of copper, the substrate specificity of the copper-enhanced assay was examined and compared with the substrate specificity of cytokinin oxidase activity measured in assays without copper. Substrate competition tests, in which unlabeled cytokinins were added to reaction mixtures containing \( i^6 \text{Ade-2,8-}^3\text{H} \) as the labeled substrate, were used to test the specificity of the assay. Unlabeled cytokinins were added to the assay mixture at concentrations equal to that of the labeled \( i^6 \text{Ade} \) substrate (10 \( \mu\text{M} \)) and in 10-fold excess (100 \( \mu\text{M} \)). The results obtained in comparisons of the copper-imidazole enhanced assay with unenhanced assays performed using either imidazole or bis tris as the assay buffer are shown in Table IV.3. The substrate specificity
Table IV.3. Effect of Unlabeled Cytokinins on the Degradation of \(^{15}\text{Ade}-2,8-^3\text{H}\) by Cytokinin Oxidase in Bis Tris, Imidazole and Copper-Imidazole Buffer

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>Unlabeled cytokinin(^a)</th>
<th>(\text{Cytokinin Oxidase Activity(^c)}) % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uM</td>
<td>i(^6)Ade</td>
<td>hi(^6)Ade</td>
</tr>
<tr>
<td>Bis Tris</td>
<td>10</td>
<td>61</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>13</td>
<td>91</td>
</tr>
<tr>
<td>Imidazole</td>
<td>10</td>
<td>62</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15</td>
<td>92</td>
</tr>
<tr>
<td>Imidazole +CuCl(_2)</td>
<td>100</td>
<td>60</td>
<td>92</td>
</tr>
</tbody>
</table>

\(^a\)Unlabeled cytokinins were added to assay mixtures containing \(^{15}\text{Ade}-2,8-^3\text{H}\) at a concentration of 0.01 mM, 120 ug protein in the cases of the bis tris and the imidazole assays, and 5 ug protein in the copper-imidazole assays. Buffer concentrations were 0.1 M and CuCl\(_2\) was 0.01 M in the copper-imidazole assays. All other conditions were as described in the "Materials and Methods".

\(^b\)Thidiazuron, (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea)

\(^c\)The cytokinin oxidase activity of bis tris, imidazole and copper-imidazole controls incubated without the addition of unlabeled cytokinins was equal to 1.02, 1.24 and 29.8 nmol per hour per mg protein respectively (nmol·h\(^{-1}\)·mg\(^{-1}\)). These values were taken as 100% activity. All values are the average of three assays. SE averaged ±1% of the means and did not exceed ±2% of the means.
of cytokinin oxidase in imidazole buffer has been
described previously (4). The substrate specificity
observed here was essentially the same for all three assay
systems tested. Thidiazuron and i6Ade were equally
effective in inhibiting the oxidation of the labeled
substrate. Zeatin rather weakly inhibited the reaction and
the addition of benzyl adenine (b6Ade) and N6-
-isopentyladenine (hi6Ade) to the reaction mixtures had no
significant effect on the rate of the reaction.

The specificity and reliability of the copper-
enhanced assay for cytokinin oxidase activity were further
tested in studies involving chromatographic fractionation
of the activity extracted from Great Northern callus
tissue. To accommodate the large number of samples
generated by column chromatography procedures, the
standard assay procedure for cytokinin oxidase activity
was modified and simplified. Extractions with butyl
acetate were used to remove undegraded i6Ade from the
reaction mixtures and thus eliminate the necessity of
separating the reaction products by TLC. The validity of
the assay procedure was established by comparing the
results obtained using solvent partitioning with those
obtained in the standard assay using TLC and by
determining the actual solvent partitioning of i6Ade and
Ade generated in the enzyme reaction. The results obtained
using ethyl acetate and butyl acetate as extraction
solvents are compared in Table IV.4. The i6Ade in the
Table IV.4. Partitioning of $i^6$Ado and Ade into Ethyl Acetate and Butyl Acetate Extractions of Cytokinin Oxidase Reaction Mixtures

The assays were incubated at 37 C for 30 min, then terminated by the addition of 50 ul of 20 mM EDTA·Na$_2$ containing $i^6$Ado and Ade (each at 1 mM) and 300 ul of 1 mM Ade saturated ethyl acetate or butyl acetate saturated with 1 mM Ade. These mixtures were extracted by vortexing for 15 sec, and centrifuged to separate the two phases. The organic phase was removed and the extraction repeated two times. The solutions from both phases were chromatographed on SiC$_{18}$ TLC plates. The developing solvent was 37.5% (v/v) ethanol containing 0.1 M EDTA·Na$_4$. The bands centered on Ade and $i^6$Ade standards were mixed with 5 ml Ready-Solv HP/b, and counted in a Beckman LS 1801 scintillation counter. For a comparison, replicate assays were processed by the SiC$_{18}$ TLC method.

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Ethyl Acetate Extraction</th>
<th>Butyl Acetate Extraction</th>
<th>Std. Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled Compound</td>
<td>Aqueous Phase</td>
<td>Organic Phase</td>
<td>Aqueous Phase</td>
</tr>
<tr>
<td>Buffe$^b$</td>
<td>Ade</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control$^b$</td>
<td>$i^6$Ade</td>
<td>1</td>
<td>97</td>
</tr>
<tr>
<td>Enzyme Assay$^c$</td>
<td>Ade</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>$i^6$Ade</td>
<td>1</td>
<td>65</td>
</tr>
</tbody>
</table>

$^a$Each value is the average of three assays. SE averaged less than ±1% of the means.

$^b$Reaction mixtures contained at a final concentration, 100 mM imidazole·HCl (pH 6.5), 0.01 mM $i^6$Ado-2,8-3H (0.05 uCi, 25 uCi/umol) and 10 mM CuCl$_2$.

$^c$In addition to the components described for buffer controls, the standard enzyme assays contained 8 ug protein.
cyclokinin oxidase reaction mixtures was quantitatively extracted into both ethyl acetate and butyl acetate. However, adenine recovery in the aqueous phase from the ethyl acetate extraction was 52% compared to 77% when butyl acetate was used. Therefore, butyl acetate was selected as the extraction solvent in subsequent assays of the cytokinin oxidase activity present in fractions generated by column chromatography.

The cytokinin oxidase activity extracted from callus tissues of P. vulgaris cv. Great Great Northern was fractionated by DEAE-cellulose chromatography, and the column fractions were assayed for cytokinin oxidase activity using both the copper-imidazole enhanced assay and an unenhanced assay in which copper was omitted from the reaction mixtures and bis tris was used as the assay buffer. The elution profiles for cytokinin oxidase activity determined with the two types of reaction mixtures are compared in Figure V.3. The elution positions of the activity were identical using either the copper-enhanced assay or the bis tris assay. However, as expected, the cytokinin oxidase activity observed in the copper-imidazole assays was markedly higher than the activity determined in bis tris.

The results obtained in tests of substrate specificity and in the chromatographic comparison described above indicated the copper-enhanced assay was specific for cytokinin oxidase. However, the high copper
Figure IV.3. DEAE-cellulose chromatography of cytokinin oxidase.

An homogenate of Great Northern callus tissue was treated with Polymin P and ammonium sulfate as described in the "Materials and Methods", dissolved in 20 mM tris-HCl (pH 7.5) and desalted on a Sephadex G-25 column. A 140 ml sample (300 mg protein) of the desalted fraction was applied to a 2 x 32 cm DE52-cellulose column (100 ml bed volume) equilibrated in 20 mM tris-HCl (pH 7.5). The column was rinsed with 100 ml of the same buffer. The cytokinin oxidase activity was eluted with a linear gradient from 0 to 0.3 M KCl over a volume of 2 liters. Fraction of 20 ml were collected at a flow rate of 50 ml/h. Cytokinin oxidase activity was measured in bis tris and imidazole-copper buffers as described in the "Materials and Methods".
Figure IV.3
concentration required for enhancement suggested that copper was acting in some manner other than as a cofactor for the enzyme. The possibility that copper or copper-imidazole complexes might be functioning as an alternative electron accepter (replacing oxygen) in the assay was tested by examining the effect of copper addition on enzyme activity measured under anaerobic conditions (Table IV.5.). Reducing oxygen concentration in mixtures from which copper was omitted and bis tris was used as the assay buffer resulted in a reduction in cytokinin oxidase activity to 16% of that observed in the same reaction mixtures in the presence of oxygen. In contrast to this result, the removal of oxygen from reaction mixtures containing copper and imidazole as a buffer, had little effect on the rate of the cytokinin oxidase catalyzed reaction.

**DISCUSSION**

The *in vitro* activity of cytokinin oxidase extracted from callus tissues of *P. vulgaris* cv. Great Northern has been shown here to be markedly enhanced in reaction mixtures containing copper and imidazole. The enhanced degradation of i6Ade observed under these conditions is enzyme dependent and appears to be specifically catalyzed by cytokinin oxidase. The evidence for the latter assertion is provided by the substrate specificity of the reaction and the results obtained in assays of
Table IV.5. The Effect of Anaerobic Conditions on the Enhanced Cytokinin Oxidase Activity by Copper

Bis tris reaction mixtures contained 160 ug protein and 0.1 M bis tris-HCl (pH 6.5). Copper-imidazole assays contained 16 ug protein, 0.1 M imidazole-HCl (pH 6.5) and 0.01 M CuCl₂. The reaction mixtures were placed in 0.5 ml centrifuge tubes and sealed with serum stoppers. All tubes were connected to a manifold and evacuated to 0.05 atm, then flushed with purified N₂. This process was repeated 9 times. The reactions were initiated by adding the substrate to the enzyme mixtures with a Hamilton syringe. All other assay conditions were described in the "Materials and Methods".

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Cytokinin Oxidase Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>+O₂</th>
<th>-O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol/hour</td>
<td></td>
</tr>
<tr>
<td>Bis Tris</td>
<td></td>
<td>65 ± 1</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Copper-Imidazole</td>
<td></td>
<td>186 ± 6</td>
<td>188 ± 5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cytokinin oxidase activity was assayed as described in "Materials and Methods". Each value is the average of 3 assays ± SE.
chromatographic fractionations of cytokinin oxidase activity. The substrate specificity of cytokinin oxidase activity in the copper-enhanced assay was identical to that observed in assays using either imidazole or bis tris as the assay buffer without added copper, and the elution positions of cytokinin oxidase in preparations fractionated by chromatography on DEAE-cellulose were identical using either bis tris or copper-imidazole as the assay buffers.

The observed ability of cytokinin oxidase preparations to catalyze the degradation of $i^6$Ade to Ade under anaerobic conditions if copper and imidazole are included in the reaction mixture suggests that copper-imidazole complexes may be acting as alternative electron accepters in the reaction. If oxygen-dependent cleavage of an iminopurine intermediate normally limits the rate of the cytokinin oxidase reaction, copper or copper-imidazole complexes may enhance the reaction rate by increasing the effective concentration and/or reactivity of the electron accepter for the reaction, thus facilitating the breakdown of the intermediate. It is possible that the formation of copper-cytokinin complexes may also be involved in the enhancement of the cytokinin oxidase reaction. Adenine is known to combine strongly with Cu$^{2+}$ ions (5,9,20), and Miller (12) has described an interaction of cupric ions with cytokinin bases that stimulated the oxidation of NADH by horseradish peroxidase. Whatever the mechanism of
enhancement, the magnitude of the response raises the interesting possibility that manipulation of the reaction mechanism for cytokinin oxidase could be used in the \textit{in vivo} regulation of the activity of the enzyme.

The extent to which the results obtained with cytokinin oxidase may be extended to other enzyme catalyzed reactions in which oxygen serves as an electron accepter is not certain. In preliminary experiments with galactose oxidase, we have found that the addition of copper and imidazole to reaction mixtures for this enzyme did enhance the oxidation of 3-methoxybenzyl alcohol to the corresponding aldehyde. However, the mechanism of enhancement in this reaction may not be the same as that observed with cytokinin oxidase.

Significant questions remain concerning the effects of copper and imidazole on the reaction mechanism for cytokinin oxidase. Nevertheless, the evidence presented here indicates that the copper-imidazole enhanced reaction provides a reliable and specific assay of cytokinin oxidase activity. The increase in assay sensitivity observed in the presence of copper and imidazole should facilitate work with the enzyme, particularly in studies involving chromatographic or electrophoretic separations.
LITERATURE CITED


17. Paces V, E Werstiuk, RH Hall 1971 Conversion of N^6-(Δ^2-isopentenyl)adenosine to adenosine by enzyme activity in tobacco tissue. Plant Physiol 48: 775-778


CHAPTER V.

CYTOKININ OXIDASE ACTIVITY FROM CALLUS TISSUES OF PHASEOLUS VULGARIS L. CV. GREAT NORTHERN

III. AFFINITY FOR CONCANAVALIN A-SEPHAROSE 4B AND OTHER CHROMATOGRAPHIC PROPERTIES OF THE ENZYME

ABSTRACT

The chromatographic properties of cytokinin oxidase activity from callus tissues of *P. vulgaris* L. cv. Great Northern have been investigated using an assay based on the oxidation of $N^6-(\Delta^2$-isopentenyl)adenine-$[2,8-^3H]$ to adenine. Most of the cytokinin oxidase activity extracted from Great Northern callus tissues bound to concanavalin A-Sepharose 4B and was specifically eluted with methylmannose. This result suggests the enzyme is a glycoprotein. Ion exchange chromatography on DEAE-cellulose resolved the cytokinin oxidase activity from Great Northern callus tissues into two peaks. The major fraction, which comprised 85 to 90% of the cytokinin oxidase activity extracted from the callus tissues, bound to concavavalin A-Sepharose 4B. The minor fraction did not. The apparent pI of the major peak of cytokinin oxidase activity was estimated by chromatofocusing to be approximately 5.0. Chromatofocusing and polyacrylamide gel electrophoresis separated cytokinin oxidase activity from the major peroxidase activities present in the callus tissues.
INTRODUCTION

Cytokinin oxidase activity appears to be widely distributed in plant tissues (2,4,8,10,11,15,16,17,19). The enzyme catalyzes the oxidative cleavage of the side chains of cytokinins bearing unsaturated isoprenoid side chains (zeatin, $N^6$-(\(\Delta^2\)-isopentenyladenine, and their ribonucleosides). The products of the reaction are adenine or adenosine and the corresponding side chain fragment (2,10). The degradation of cytokinins to inactive products by the action of cytokinin oxidase may be important in the regulation of cytokinin levels in plant tissues.

The substrate specificity of cytokinin oxidase appears to be similar in enzyme preparations from different plant sources (4,8,11,15,17,19), but other properties of the enzyme vary with the source of the activity. The molecular weight of a cytokinin oxidase from immature corn kernels was estimated to be 88,000 daltons (19). The molecular weights of the corresponding enzymes isolated from Vinca rosea crown gall tissue (11,17) and from wheat germ (8) were estimated as 25,000 and 40,000 daltons respectively. Differences in pH optima and stability have also been reported for cytokinin oxidase preparations from different plant sources (11,17). These variations in properties suggest that more than one form of the enzyme may be present in plant tissues.
Cytokinin oxidase activity is readily extracted from callus tissues of Phaseolus vulgaris cv. Great Northern. The substrate specificity and regulatory properties of the enzyme from this source have been described in an earlier publication (4). The chromatographic properties of cytokinin oxidase activity from Great Northern callus tissues have been examined in the present study. This investigation was undertaken to determine whether multiple forms of the enzyme might exist in the callus tissue and to identify properties of the enzyme that might be useful in its purification. The results of attempts to fractionate the enzyme activity by affinity chromatography on concanavalin A-Sepharose 4B, chromatofocusing, DEAE-cellulose chromatography, and polyacrylamide gel electrophoresis are reported here.

MATERIALS AND METHODS

Chemicals. Picloram (4-amino-3,5,6-trichloropicolinic acid) was purchased from Aldrich. Polymin P (polyethyleneimine, 50% w/v) was obtained from Miles Laboratories, Inc. Ammonium sulfate (enzyme grade) was purchased from Schwarz/Mann. Adenosine-2,8-3H (40 Ci/mmol) was obtained from ICN Radiochemicals. Ready-solv HP/b is a Beckman product. Polyexchanger PBE94 and Polybuffer 74 were obtained from Pharmacia. DE52-cellulose anion exchanger was purchased from Whatman. Concanavalin A-
Sepharose 4B, Sephadex LH-20, and all other organic chemicals used in this study were purchased from Sigma.

**Preparation of i6Ade-2,8-3H.** Ado-2,8-3H, adjusted to a specific activity of 250 mCi/mmol by the addition of unlabeled Ado, was used in the synthesis of i6Ado-2,8-3H by methods described previously for the preparation of i6Ado-8-14C (13). The i6Ado-2,8-3H was purified by chromatography on a Sephadex LH-20 column in 33% (v/v) ethanol (1) and deribosylated to i6Ade as described by LaLoue et al. (9). The i6Ade-2,8-3H was isolated from the reaction mixture by chromatography on a Sephadex LH-20 column in 20% (v/v) ethanol and stored at -20 C in 50% (v/v) ethanol. The yield from Ado-2,8-3H was about 20%.

Prior to use in enzyme assays the i6Ade-2,8-3H was adjusted to a specific activity of 100 mCi/mmol with cold i6Ade, and the ethanol was removed by evaporation.

**Tissue Culture.** The medium used to culture Phaseolus vulgaris cv. Great Northern callus tissues consisted of the mineral nutrients defined by Murashige and Skoog (14) with the following organic substances added: sucrose (30g/L), myo-inositol (100 mg/L), thiamine-HCl (1 mg/L), nicotinic acid (5 mg/L), pyridoxine-HCl (0.5 mg/L), picloram (2.5 uM), and kinetin (5 uM). The pH of the medium was adjusted to 5.7 and Difco Bacto-agar (10 g/L) was added. The medium was dispensed into 125 ml Erlenmeyer flasks (50 ml/flask) and autoclaved at 120 C for 15 min.
Cultures were transferred to fresh medium at approximately 24 day intervals.

**Preparation of DEAE-Cellulose, Concanavalin A-Sepharose 4B, and Polyexchanger PBE94 Chromatography Columns.** DE52-cellulose was precycled in HCl and NaOH (each at 0.5 N), degassed, and fines removed as recommended by Whatman (Information Leaflet IL 2). The exchanger was titrated to pH 7.5 with tris base, washed with 10 bed volumes of 20 mM tris-HCl (pH 7.5) containing 1 M KCl, and packed into a 1 x 20 cm column in this buffer at a flow rate of 80 ml/h. The packed column was washed with 20 mM tris-HCl (pH 7.5) until the pH and conductivity of the eluate were the same as the wash buffer.

Concanavalin A-Sepharose 4B (10-14 mg concanavalin A per ml settled gel) was packed into small columns (0.9 x 3 cm or 1 x 6 cm) and washed with 50 bed volumes of 0.05 M bis tris-HCl (pH 6.5) containing 0.25 M (NH₄)₂SO₄, 0.1 M methyl-mannose, 0.001 M CaCl₂, and 0.001 M MnCl₂. The initial wash was followed by 50 bed volumes of the same buffer without methyl-mannose, CaCl₂, or MnCl₂.

Polyexchanger PBE94 was washed with 25 bed volumes of degassed 25 mM imidazole-HCl (pH 7.4) and packed into a 0.9 x 32 cm column under gravity. The packed column was equilibrated in 25 bed volumes of the same buffer. The Polybuffer 74 used to elute the Polyexchanger PBE94 column was titrated to pH 4.0 with HCl, diluted 8-fold from the original concentration, and degassed. The chromatofocusing
exchanger and buffers were degassed with the vacuum obtained on a sink aspirator for 5 min, and maintained under a 75 ml column of Ascarite (which served as a CO$_2$ trap) throughout the entire equilibration and elution process.

**Extraction of Cytokinin Oxidase Activity from Phaseolus callus tissues.** Callus tissues of *P. vulgaris* cv. Great Northern were harvested at 19 to 22 days of age (5 to 7.5 g/flask). The callus tissue was homogenized (1 min, Sorvall Omnimixer, full speed) with an equal volume of cold 0.1 M bis tris-HCl (pH 6.5). All subsequent operations were performed at 4 C unless otherwise indicated. The homogenate was filtered through 4 layers of cheesecloth and 2 layers of Miracloth. The filtrate was centrifuged (15,000 g for 10 min). Polymin P (1% v/v, pH 6.5) was added dropwise with stirring to the supernatant (75 μl Polymin P/ml supernatant). After 10 min, the precipitated nucleic acids and associated proteins (3) were removed by centrifugation (15,000 g for 10 min). Solid ammonium sulfate was added to the supernatant from the Polymin P step to give 80% saturation. The resulting suspension was allowed to stand 1 h prior to centrifugation (20,000 g for 20 min). The protein pellets were stored at -20 C.

**Assay of Cytokinin Oxidase Column Fractions for Activity.** Cytokinin oxidase activity in fractions resulting from chromatography on DEAE-cellulose, concanavalin A-Sepharose
4B, and Polyexchanger PBE 94 was assayed by procedures established previously (see Chapter IV). The assay mixtures contained 100 mM imidazole-HCl (pH 6.5), 0.01 mM i^6 Ade-2,8-3H (0.05 uCi, specific activity 100 uCi/umol), 1 or 10 mM CuCl₂, and 10 ul of the column fraction to be assayed in a final volume of 50 ul. The assays were incubated at 37 °C for 30 min. The reactions were terminated by the addition of 50 ul of 20 mM Na₂EDTA containing i^6 Ado and Ade (each at 1 mM) followed by 300 ul of either ethyl acetate or butyl acetate, each saturated with 1 mM Ade. The assay tubes were vortexed (15 sec), and the phases were separated by centrifugation. The organic phase, containing undegraded i^6 Ade, was removed and the extraction was repeated two times. Aliquots of the aqueous phase (80 ul), containing the adenine produced in the enzyme reaction, were mixed with 5 ml of Ready-Solv HP/b and counted in a Beckman LS 1801 scintillation counter. This procedure underestimates the adenine produced in the reaction (see Chapter IV). Adenine recovery was 52% or 80% of the total using ethyl acetate or butyl acetate extraction respectively.

Assay of Column Fractions for Peroxidase Activity. A modification of the assay procedure described in the Worthington Enzyme Manual 1977 (20) was used to determine peroxidase activity in fractions obtained from the chromatofocusing column. Aliquots of column fractions were assayed in reaction mixtures containing 100 mM potassium
phosphate buffer (pH 7), 1 mM 4-aminoantipyrene, 80 mM phenol, and 1 mM H₂O₂ at 25 C. Peroxidase activity was determined by the increase in absorbance at 510 nm over time relative to the change in absorbance in reaction mixtures without enzyme.

**Polyacrylamide Gel Electrophoresis.** Nondissociating discontinuous gels were cast with a 7.5% acrylamide concentration in the resolving gel as described by Hames (7) and using the high pH buffer system of Davis (5) with two modifications. The stacking gel contained 2% (w/v) methyl-mannose, and the sample wells were filled with 125 mM tris-HCl (pH 6.8) containing 2% methyl-mannose. The methyl-mannose was added to the system to reduce the binding of any lectins present in the samples. The stacking buffer was used in the sample wells to reduce the effects of an extreme pH change of 6.8 to 8.3 (the pH of the reservoir buffer). This pH change was observed to precipitate some of the sample protein in test tubes and also in sample wells.

Cytokinin oxidase activity was localized in polyacrylamide gels by sectioning the entire resolving gel lanes (0.15 x 0.8 x 12 cm) into 0.25 cm long sections. Gel sections were added to the reaction mixtures specified in the assay procedure described above. To accommodate gel sections, all volumes were doubled. Other details of the assays were as described.
To detect peroxidase activity, the gels were incubated in 100 mM bis tris-HCl (pH 6.5) containing 0.5 mM 4-chloro-1-naphthol, 10 mM H$_2$O$_2$, and 0.95% (v/v) ethanol. (The 4-chloro-1-naphthol was dissolved in 95% ethanol and added to the buffer just prior to assaying the gel.) The oxidation of 4-chloro-1-naphthol by peroxidase results in the formation of an intense purple-blue color (6).

**RESULTS**

**Concanavalin A-Sepharose 4B Chromatography of Cytokinin Oxidase from *P. vulgaris* Callus Tissues.** To determine whether cytokinin oxidase extracted from callus tissues of *P. vulgaris* cv. Great Northern might have the properties of a glycoprotein, the affinity of the enzyme for concanavalin A-Sepharose 4B was examined. The results are shown in Figure V.1. Over 80% of the total cytokinin oxidase activity extracted from the callus tissues bound to the concanavalin A column and was recovered only after the addition of methyl-mannose to the eluting buffer. The remaining 20% of the cytokinin oxidase activity did not bind to the affinity column and eluted with the bulk of the protein in the sample flow through. On the basis of these results, it appears that most (but not all) of the cytokinin oxidase activity present in Great Northern callus tissue may be associated with a glycoprotein.
Figure V.1. Concanavalin A-Sepharose 4B chromatography of cytokinin oxidase.

An homogenate of Great Northern callus tissue (500 g fresh wt) was treated with Polymin P and ammonium sulfate as described in the "Materials and Methods", and dissolved in 50 mM bis tris-HCl (pH 6.5) and the conductivity adjusted to that of 50 mM bis tris-HCl (pH 6.5) containing 0.25 M ammonium sulfate. The sample (400 mg) was loaded on a 1 x 6 cm (5ml bed volume) column of concanavalin A-Sepharose 4B equilibrated in 50 mM bis tris pH 6.5 containing 0.25 M ammonium sulfate. The column was rinsed with 150 ml of buffer to remove unbound protein, followed by 100 ml of buffer containing 0.1 M methy-mannose. Fractions of 10 ml were collected at a flow rate of 25 ml/h. The arrow denotes the point at which methyl mannose elution was initiated.
Chromatofocusing of Cytokinin Oxidase from P. vulgaris callus tissues. The cytokinin oxidase activity that bound to concanavalin A-Sepharose 4B was further characterized by chromatofocusing (Figure V.2). The protein preparation recovered from the affinity column by elution with methylmannose was fractioned on a Polyexchanger PBE94 column with a pH gradient from pH 7 to pH 4. The cytokinin oxidase activity eluted from the column in the pH range of 4.8 to 5.1. The activity peaked at pH 5.0. Fractions from the chromatofocusing column were also assayed for peroxidase activity. The peak of cytokinin oxidase activity was clearly resolved from the peroxidase activity, which eluted as two peaks early and late in the elution profile.

The partial purification of cytokinin oxidase activity that resulted from affinity chromatography and chromatofocusing of the enzyme extracted from P. vulgaris callus tissues is summarized in Table V.1. Cytokinin oxidase activity was purified 455-fold to a specific activity of 13.1 umol per hour per mg protein (measured in reaction mixtures containing 0.1 M imidazole buffer and 10 mM CuCl₂). Native polyacrylamide gel electrophoresis of this enzyme preparation is shown in Figure V.3. The cytokinin oxidase activity was distributed over a 1.5 cm region of the gel (Figure V.3B) that corresponded to a diffuse protein band observed in replicate gel lanes silver-stained for protein (Figure V.3A).
The cytokinin oxidase fractions (# 26-29) obtained from the concanavalin A Sepharose 4B fractionation (Figure V.1) were pooled and this sample was exchanged into 25 mM imidazole-HCl, pH 7.4 with an Amicon ultrafiltration cell using a YM10 membrane. The buffer exchange was accomplished by ultrafiltration of the sample to 0.5 ml at 50 psi N₂, followed by dilution to 10 ml with 25 mM imidazole-HCl pH 7.5. The ultrafiltration and buffer dilution steps were repeated two more times. After the third dilution to 10 ml, the preparation was recovered from the cell. An 8 ml sample (8 mg protein) was loaded on a 0.9 x 32 cm (20 ml bed volume) Polyexchanger PBE94 column, equilibrated in the sample buffer. The cytokinin oxidase activity was eluted with 0.32 l of Polybuffer-HCl, pH 4.0 (diluted 1:8). Fractions of 4 ml were collected at a flow rate of 20 ml/h (12 cm/h).
Figure V.2

Graph showing fraction number on the x-axis and various activities on the y-axis, including pH, A_{280}, cytokinin oxidase activity, and peroxidase activity.
Table V.1. Summary of the Purification of Cytokinin Oxidase from Callus Tissue of *P. vulgaris* cv. Great Northern

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Fold Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Homogenate</td>
<td>1180</td>
<td>33.7</td>
<td>0.028</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Polymin P*(NH₄)SO₄ Treated Fraction</td>
<td>755</td>
<td>28.6</td>
<td>0.038</td>
<td>1.3</td>
<td>85</td>
</tr>
<tr>
<td>Concanavalin A-Sepharose 4B (fractions 26-29)</td>
<td>28.7</td>
<td>22.8</td>
<td>0.796</td>
<td>28</td>
<td>68</td>
</tr>
<tr>
<td>Chromatofocusing (fractions 39-43)</td>
<td>1.4</td>
<td>18.3</td>
<td>13.1</td>
<td>455</td>
<td>54</td>
</tr>
</tbody>
</table>

*aFrom 1 kg of Callus Tissue*
Figure V.3. Native polyacrylamide gel electrophoresis of cytokinin oxidase.

The cytokinin oxidase fractions (39-43) obtained from chromatofocusing were concentrated and the buffer exchanged into 60 mM tris-HCl (pH 6.8) by ultrafiltration in an Amicon concentrating cell (using a YM10 membrane). A 7.5% discontinuous acrylamide gel was prepared as described in the "Materials and Methods". Lanes were loaded with 10 ug protein. Following electrophoresis, the gel lanes were silver-stained for protein according to BioRad (A), or cut into 0.25 cm sections and assayed for cytokinin oxidase activity (B) as described in the "Materials and Methods".
DEAE-Cellulose Fractionation of Cytokinin Oxidase from *P. vulgaris* Callus Tissues. The apparent separation of cytokinin oxidase activity into two fractions by concanavalin A-Sepharose 4B chromatography suggested that multiple forms of the enzyme might be present in the *P. vulgaris* cv. Great Northern callus tissues. To further test this hypothesis, cytokinin oxidase activity extracted from the callus tissue was chromatographed on DEAE-cellulose. DEAE-cellulose chromatography resolved the cytokinin oxidase activity into two peaks of activity separated by more than two bed volumes (Figure V.4). The early eluting peak comprised approximately 85% of the total cytokinin oxidase activity recovered from the columns. Both peaks of cytokinin oxidase activity remained chromatographically distinct when rechromatographed on DEAE-cellulose (data not shown).

The two peaks of cytokinin oxidase activity resolved on DEAE-cellulose were rechromatographed on concanavalin A-Sepharose 4B columns (Figure V.5). The cytokinin oxidase activity that eluted as an early peak from the DEAE-cellulose column bound to concanavalin A and was recovered from the column only after the addition of the competing ligand, methyl-mannose (Figure V.5A). The small, late eluting peak of cytokinin oxidase activity recovered from the DEAE-cellulose column had no apparent affinity for concanavalin A and eluted in the void volume of the affinity column (Figure V.5B). After passage through the
Figure V.4. DEAE-cellulose chromatography of cytokinin oxidase.

An homogenate of Great Northern callus tissue was treated with polymin P and ammonium sulfate as described in the "Materials and Methods", dissolved in 20 mM Tris-HCl, pH 7.5 and desalted on a Sephadex G-25 column. A 20 ml sample of the desalted fraction (33 mg) protein was applied to a 1 x 20 cm DEAE-cellulose column (16 ml bed volume) equilibrated in 20 mM Tris-HCl, pH 7.5. The column was washed with 20 ml of the same buffer. The cytokinin oxidase activity was then eluted with a linear gradient from 0 to 0.3 M over a total volume of 0.24 liters. Fraction of 4 ml were collected at a flow rate of 16 ml/h (12 cm·h⁻¹).
Figure V.4

FRACTION NUMBER

A$_{280}$ nm

Cytokinin oxidase activity (nmol·h$^{-1}$·ml$^{-1}$)

KCl (M)

0.0 0.5 1.0 1.5

0 20 40 60 80

0.0 0.1 0.2 0.3 0.4

0 0.1 0.2 0.3
The DEAE-cellulose column fraction which contained cytokinin oxidase activity were pooled as follows: tubes 22 to 38 (designated as early eluting fraction) and tubes 49 to 54 (designated as the late eluting fraction). The pooled fractions were brought to 80% saturation ammonium sulfate and the precipitated protein was collected by centrifugation. These preparations were dissolved in 2 ml of 20 mM Tris-HCl, pH 7.5 and the conductivity adjusted to that of 20 mM Tris-HCl, pH 7.5, containing 0.25 M ammonium sulfate, then loaded on 1 x 3 cm concanavalin A-Sepharose 4b columns by gravity. The columns were rinsed with 20 ml of buffer to remove unbound protein, followed by 20 ml of buffer containing 0.1 M methyl-mannose. Fractions of 2 ml were collected at a flow rate of 20 ml/h. Arrows denote the point at which methyl-mannose elution was initiated.
Figure V.5

A

B

A_280 \text{ nm}

Cytokinin oxidase activity (nmol h^{-1} ml^{-1})

Fraction Number

0 5 10 15 20
affinity columns, the two cytokinin oxidase fractions were examined by native polyacrylamide gel electrophoresis. The results are shown in Figure V.6. The cytokinin oxidase activities in the two fractions were electrophoretically distinct from each other. Peroxidase activity was detected in the cytokinin oxidase fraction with affinity for concanavalin A (Figure V.6A) but was well separated from the cytokinin oxidase activity.

DISCUSSION

The chromatographic properties of cytokinin oxidase activity extracted from callus tissues of P. vulgaris cv. Great Northern have been investigated in the work reported here. Most of the cytokinin oxidase activity recovered from the callus tissue bound to concanavalin A-Sepharose 4B and was specifically eluted from the affinity column in the presence of methyl-mannose. The affinity of the enzyme for concanavalin A provides a convenient and rapid method for initial purification of the activity and indicates that the major fraction of cytokinin oxidase activity extracted from the callus tissue may be a glycoprotein. Definitive proof of glycosylation awaits more extensive biochemical characterization of the enzyme.

The cytokinin oxidase fraction that bound to concanavalin A was further fractionated by chromatofocusing. This cytokinin oxidase activity was purified 450-fold by the combination of affinity
Figure V.6. Native polyacrylamide gel electrophoresis of cytokinin oxidase and peroxidases from concanavalin A-Sepharose 4B purified fractions.

The cytokinin oxidase fractions resulting from concanavalin A-Sepharose 4B chromatography were brought to 80% saturation with ammonium sulfate, and the precipitated protein was collected by centrifugation. These preparations were dissolved in 60 mM tris-HCl, pH 6.8 and desalted on Sephadex G-25 columns in the same buffer. Gels containing a linear gradient of 5% to 15% acrylamide were prepared as described in the "Materials and Methods". Lanes were loaded with 15 ug protein from the early eluting DEAE-cellulose fraction (A) and 30 ug protein from the late eluting DEAE-cellulose fraction (B). Following electrophoresis, the gel lanes were stained for peroxidase activity or cut into 0.25 cm sections and assayed for cytokinin oxidase activity as described in the "Materials and Methods".
Figure V.6
chromatography and chromatofocusing. The pI of the enzyme was estimated by chromatofocusing to be 5.0. Because the cytokinin oxidase activity eluted in the last third of the pH gradient, where the increasing ion strength of the Polybuffer could also become a factor in the elution, this pI estimate is probably on the high side of the actual pI of the cytokinin oxidase.

Whitty and Hall (19) reported electrophoretic comigration of cytokinin oxidase activity with peroxidase activity in enzyme preparations extracted from corn kernels. More recently, Van Staden (18) reported that a commercial preparation of horse radish peroxidase could bring about the oxidation of zeatin, although the rate of degradation was very low. The data presented here indicate that the major peroxidase activities present in Great Northern callus tissues are distinct from cytokinin oxidase. Both cytokinin oxidase activities extracted from the P. vulgaris callus tissue were separated from the major peroxidase activities by chromatofocusing (Figure V.2.) and/or by polyacrylamide gel electrophoresis (Figure V.6).

The cytokinin oxidase activity from callus tissues of P. vulgaris cv. Great Northern was resolved into two fractions by either DEAE-cellulose chromatography or by affinity chromatography on concanavalin A-Sepharose 4B. The fraction eluting early from DEAE-cellulose was bound by concanavalin A columns. This fraction represented the
majority (more than 80%) of the total cytokinin oxidase activity recovered from the callus tissue. The minor fraction of cytokinin oxidase activity, eluting late from DEAE-cellulose did not bind to concanavalin A. The two cytokinin oxidase fractions were electrophoretically distinct and remained chromatographically distinct when rechromatographed on DEAE-cellulose.

The results described above raise the possibility that more than one form of cytokinin oxidase activity may be present in callus tissues of P. vulgaris cv. Great Northern. However, we can not be certain that the two peaks of activity observed in these studies represent distinct enzymes. The minor peak of cytokinin oxidase activity is somewhat variable in amount in cytokinin oxidase preparations from different batches of Great Northern callus tissues, and it is possible that this fraction results from the action of proteases or glycosidases during isolation and fractionation of the enzyme activity. Differences in post-translational modifications of cytokinin oxidase could also account for the occurrence of two activities. It should be noted that some heterogeneity in cytokinin oxidase activity might be expected if the enzyme is a glycoprotein. The occurrence of multiple forms of cytokinin oxidase activity could have functional and regulatory significance, but further work will be needed to establish this point beyond question.
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CHAPTER VI.

GENERAL CONCLUSIONS AND DISCUSSION

The catalytic, regulatory, and chromatographic properties of cytokinin oxidase activity from callus tissues of *Phaseolus vulgaris* L. cv. Great Northern have been examined in the work reported here. An assay based on the oxidation of radioactively labeled N\(^6\)-\(\Delta^2\)-isopentenyl)adenine to adenine was used to characterize the enzyme. Conditions for the quantitative extraction and assay of the enzyme activity from Great Northern callus tissues have been established.

Cytokinin oxidase activity in Great Northern callus tissues appears to be regulated by cytokinin supply. Transient increases in the supply of exogenous cytokinins resulted in elevated levels of cytokinin oxidase activity in the callus tissues. The increase in activity occurred rapidly (within a few hours following cytokinin treatment) and appeared to require RNA and protein synthesis. All cytokinin-active compounds tested, including substrates and non-substrates of the enzyme, were effective in inducing elevated levels of the enzyme in this callus tissue. Therefore the structural requirements for induction are distinct from the structural requirements for a compound to serve as a substrate. The cytokinin-active urea derivative Thidiazuron was as effective as any adenine derivative in inducing the
response. It is not clear whether this compound is also a substrate for the enzyme.

The results described above indicate that the site controlling induction of cytokinin oxidase activity is distinct from the active site of the enzyme. It would be useful to identify cytokinins that do not induce increases in cytokinin oxidase activity and to have available compounds that lack cytokinin activity but are capable of inducing increases in cytokinin oxidase activity. Compounds with these properties would be useful in manipulating the levels of cytokinin oxidase activity in plant tissues and in investigating the underlying regulatory process controlling the induction phenomenon.

The effects of plant hormones other than cytokinins on the induction of cytokinin oxidase activity in Great Northern callus tissues has been examined in preliminary experiments (data not shown). Ethylene, indoleacetic acid, abscisic acid and gibberellic acid (GA$_3$) have been tested for the ability to induce cytokinin oxidase activity. The hormones were applied to the callus tissue, and cytokinin oxidase levels were determined after four hour incubations. No effects on the endogenous levels of cytokinin oxidase activity in Great Northern callus tissues were observed in these experiments. However, applications hormones simultaneously or prior to cytokinin treatment of the callus tissue have not yet been attempted and may reveal evidence of interactions in hormonal
metabolism. Abscisic acid is of particular interest in this regard, because it has been reported to inhibit certain aspects of cytokinin metabolism and to have a "cytokinin sparing" effect in some plant tissues.

The substrate specificity of the cytokinin oxidase from Great Northern callus tissues has been examined by substrate competition tests and appears similar to that reported for cytokinin oxidase from other plant sources. Somewhat surprisingly, the addition of the cytokinin-active phenylurea derivative, Thidiazuron, to the reaction volumes used to assay cytokinin oxidase activity resulted in a marked inhibition of the degradation of the labeled \( \text{i}^6 \text{Ade} \) substrate. On the basis of this result, it is possible that Thidiazuron may serve as a substrate for cytokinin oxidase, but other mechanisms of inhibition have not been excluded. The hydroxylation of labeled Thidiazuron has been shown to occur in Phaseolus callus tissues and this oxidation may be catalyzed by cytokinin oxidase. The fact that diphenylurea and \( \text{N-(2-chloro-4-pyridyl)-N'} \text{phenylurea} \) exhibit a similar inhibition of cytokinin oxidase activity in substrate competition tests indicates that phenylurea derivatives in general interact with cytokinin oxidase in some manner, either as substrates or as potent inhibitors of the enzyme.

An enhanced assay for cytokinin oxidase activity has been developed, which is at least 30 times more sensitive than previously described assays. In this procedure, the
addition of copper to reaction mixtures containing imidazole buffer stimulated cytokinin oxidase activity approximately 30 fold. The stimulation of cytokinin oxidase activity appeared to plateau only at the limits of copper solubility in the buffer. This effect was dependent on cytokinin oxidase and specific for copper. No inhibition of cytokinin oxidase activity was observed under anaerobic conditions in the presence of copper and imidazole. This result suggests that copper-imidazole complexes may be substituting for oxygen as an electron accepter in cytokinin oxidase catalysis. In any event, the increase in sensitivity obtained with the enhanced assay should facilitate work with cytokinin oxidase, particularly in studies involving chromatographic and electrophoretic separations.

The chromatographic properties of cytokinin oxidase from callus tissues of *P. vulgaris* cv. Great Northern callus tissue were investigated on concanavalin A-Sepharose 4B, DEAE-cellulose, and chromatofocusing columns. Most of the cytokinin oxidase activity extracted from Great Northern callus tissues bound to concanavalin A-Sepharose 4B and was specifically eluted with methyl-mannose, suggesting the enzyme is a glycoprotein. However definitive proof of glycosylation, awaits further biochemical characterization of the enzyme. The apparent pI of the cytokinin oxidase fraction that bound to concanavalin A was estimated to be approximately 5.0.
Cytokinin oxidase activity was purified 450 fold by the combination of affinity chromatography and chromatofocusing.

The cytokinin oxidase activity from callus tissues of *P. vulgaris* cv. Great Northern was resolved into two fractions by either DEAE-cellulose or by affinity chromatography on concanavalin A-Sepharose 4B. The fraction eluting early from DEAE-cellulose was bound by concanavalin A columns. This fraction represented the majority of (more than 80%) of the total cytokinin oxidase activity recovered from the callus tissue. The minor fraction eluting late from DEAE-cellulose did not bind to concanavalin A. This fraction is somewhat variable in amount in different cytokinin oxidase preparations from Great Northern callus tissues. The two fractions of cytokinin oxidase activity were electrophoretically distinct and remained chromatographically distinct when rechromatographed on DEAE-cellulose.

The results described above raise the possibility that more than one form of cytokinin oxidase activity may be present in callus tissues of *P. vulgaris* cv. Great Northern. However, we can not be certain that the two peaks of activity represent distinct enzymes. The minor peak of cytokinin oxidase activity may be the result of the action of proteases or glycosidases during the isolation of the enzyme activity. Differences in post translational modifications of cytokinin oxidase could
also account for the occurrence of two activities. It should be noted that heterogeneity in cytokinin oxidase activity might be expected if the enzyme is a glycoprotein. The occurrence of multiple forms of cytokinin oxidase activity could have functional and regulatory significance, but further work will be needed to establish this point beyond question.

The use of lectin columns provides a rapid and convenient method for initial purification of glycoproteins, and the affinity for concanavalin A of the major cytokinin oxidase activity is of practical significance in purifying the enzyme. It will be of interest to determine whether this methodology can be applied to cytokinin oxidases from other plant sources.

Protein glycosylation appears, in part, to function in the packaging and delivery of enzymes and structural proteins to specific locations within cells and tissues. If cytokinin oxidase is a glycoprotein, it may be packaged to a specific organelle, cell wall, or membrane site. The subcellular location of cytokinin oxidase is of theoretical interest in evaluating the function of the enzyme and its role in cytokinin metabolism. The indication that cytokinin oxidase is a glycoprotein provides a number of new opportunities for investigating the regulation of the enzyme.
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