AN ABSTRACT OF THE THESIS OF

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Title: Cytokinin Oxidase Activity from Phaseolus vulgaris
L. cv. Great Northern Callus Tissues

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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 $N^6-(\Delta^2-isopentenyl)$adenine ($i^6$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^6Ade 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 accepter 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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ABBREVIATIONS

ABA	abscisic acid
Ade	adenine
Ado	adenosine
AMP	adenosine-5'-monophosphate
b^6Ade	N^6-benzyladenine
b^6Ado	N^6-benzyladenosine
f^6Ade	N^6-furfuryladenine, (kinetin)
7-glucosyl-b^6Ade	N^6-benzyladenine-9-Ã-D-glucopyranose
9-glucosyl-b^6Ade	N^6-beznyladenine-9-Ã-D-glucopyranose
hi^6Ade	N^6-isopentyladenine
hi^6Ado	N^6-isopentyladenosine
hio^6Ade	6-[4-hydroxy-3-methylbutylamino]purine, (dihydrozeatin)
hio^6Ado	dihydrozeatin riboside
hio^6AMP	dihydrozeatin riboside-5'-monophosphate
hio^6ADP	dihydrozeatin riboside-5'-diphosphate
hio^6ATP	dihydrozeatin riboside-5'-triphosphate
hio^6Ade-O-glucoside	O-Ã-D-glucopyranosyl dihydrozeatin
hio^6Ado-O-glucoside	O-Ã-D-glucopyranosyl dihydrozeatin riboside
i^6Ade	N^6-($\triangle^2$-isopenenyl)adenine
i^6Ado	N^6-($\triangle^2$-isopentenyl) adenosine

i^{6}AMP	N^6-($\triangle^2$-isopenenyl) adenosine-5'-monophosphate
i^{6}ADP	N^6-($\triangle^2$-isopentenyl) adenosine-5'-diphosphate
i^{6}ATP	N^6-($\triangle^2$-isopentenyl) adenosine-5'-triphosphate
io^{6}Ade	6-[4-hydroxy-3-methylbut-_trans_-2-enylamino]purine, (zeatin)
io^{6}Ado	zeatin riboside
io^{6}AMP	zeatin riboside-5'-monophosphate
io^{6}ADP	zeatin riboside-5'-diphosphate
io^{6}ATP	zeatin riboside-5'-triphosphate
io^{6}Ade-O-glucoside	O-Ä-D-glucopyranosyl zeatin
io^{6}Ado-O-glucoside	O-Ä-D-glucopyranosyl zeatin riboside
io^{6}AMP-O-glucoside	O-Ä-D-glucopyranosyl zeatin riboside-5'-monophosphate
$\triangle^2$-IPP	$\triangle^2$-isopentenylpyrophosphate, dimethylallylpyrophosphate
NADPH	nicotinamide-adenine dinucleotide phosphate
tRNA	transfer ribonucleic acid

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

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, N^6-(Δ^2-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.

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CHAPTER II. LITERATURE REVIEW

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A. The Biosynthesis and Metabolism of Cytokinins 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,

1971; Hall, 1973; Letham and Palni, 1983; McGaw et al.,
1984).

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: N^6-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 N^6-(Δ^2-isopentenyl)adenine (i^6Ade) with the
exception of two isolated reports of the natural
occurrence of derivatives of N^6-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
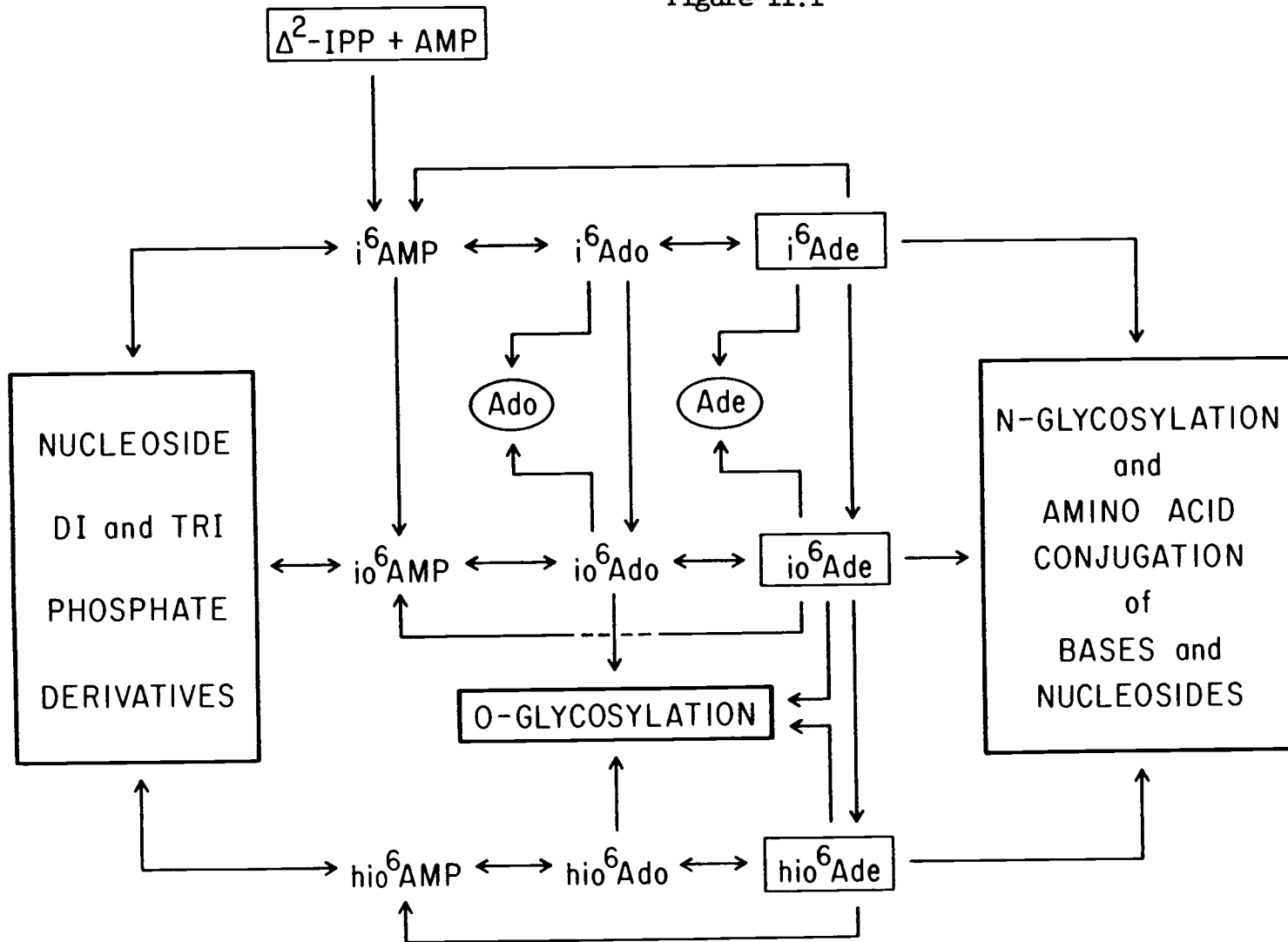
experiments using radioactively labeled adenine and adenosine. Miller (1967) initially observed incorporation of labeled adenine into the zeatin, ribosylzeatin and zeatin ribonucleotide regions of chromatograms of corn kernel extracts. Later, Miura and Miller (1969) investigated the biosynthesis of cytokinins 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^6Ado and i^6Ade. They also demonstrated that R. roseolus converts i^6Ado 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^6Ade, i^6Ado, io^6Ade, and io^6Ado
(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^6Ado, io^6Ade, and io^6AMP 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^6AMP from AMP and
Δ^2-IPP. Chen and Melitz (1979) subsequently isolated
cytokinin synthetase (Δ^2-IPP : AMP- Δ^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 moities in tRNA have been shown to be synthesized by a different enzyme using an analogous route involving the transfer of isopentenyl groups from Δ^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^6Ade to i^6Ado, an adenosine kinase (Chen and Eckert, 1977) that converts i^6Ado to i^6AMP, an adenosine nucleosidase (Chen and Kristopeit, 1981a) that converts i^6Ado to i^6Ade, an adenine phosphoribosyltransferase (Chen and Eckert, 1977; Chen, Melitz and Clough, 1982) that converts i^6Ade to i^6AMP directly, and a 5'-ribonucleotide phosphohydrolase (Chen and Kristopeit, 1981b) that converts i^6AMP to i^6Ado. All of these enzymes exhibited lower affinities for the N^6-substituted substrates (i^6Ade, i^6Ado) 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 N^6-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 N^6-benzyladenine (b^6Ade) as a substrate, the two enzymes were observed to produce the 7- and 9-glucosides in markedly different proportions. The ratio of 7-glucosyl-b^6Ade/9-glucosyl-b^6Ade 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 (b^6Ade) has been recently isolated from Gerbera shoot cultures (Horgan, 1985). The chemical structure has not been elucidated but the disccharide moity 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 i^6Ade to
zeatin. These workers suggested cytochrome P_{450} 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
P_{450}. Einset (1984) has reported differences in the
conversion of i^6Ade 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, β-(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 N^6-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 N^6-side chain of i^6Ade and
zeatin to yield adenine. N^6-benzyladenine, kinetin, and
cytokinins bearing saturated isoprenoid side chains
(dihydrozeatin and N^6-isopentyladenine) are resistant to
attack by the enzyme. In plant tissues, the cleavage of
the side chains of io^6Ade and i^6Ade and their ribosides to
form adenine or adenosine respectively is attributable to
cytokinin oxidase. However, the degradation of b^6Ade and
f^6Ade 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
N^6 position of adenine and its derivatives. A benzoic acid
derivative appears to be the product of the cleavage of
b^6Ade (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 b^6Ade, b^6Ado, b^6AMP, f^6Ade, and various N^6-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 N^6-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 N^6-substituted 5-carbon derivatives to inosine whereas an adenosine aminohydrolase from calf intestinal mucosa very weakly catalyzed the oxidation of adenosine derivatives with N^6-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, N^6-(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 i^6Ade 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 <u>Phaseolus</u>

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 <u>Zea mays</u>, <u>Vinca rosea</u> crown gall
tissue, <u>Raphanus sativus</u>, several <u>Lupinus</u> species, and
<u>Phaseolus vulgaris</u>. In the case of <u>Phaseolus</u>,
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 <u>Phaseolus</u> are available for seeds and
isolated embryo axes, roots, shoots, leaves, flowers, and
various callus tissues.

<u>Phaseolus Callus Tissues.</u> 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 N^6
side chain. In Great Northern callus tissues, io^6Ade and
i^6Ade were 30- to 100 fold less active than their
saturated analogs (hio^6Ade and hi^6Ade) 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
(io^6Ade) or equally as active (i^6Ade) 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 i^6Ade,
hi^6Ade, io^6Ade, and hio^6Ade were compared, and the
metabolism of $i^6Ado-8-^{14}C$ 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 $i^6Ado-8-^{14}C$. In Great Northern callus tissues, $i^6Ado-8-^{14}C$ was rapidly degraded to adenosine, in Kingston callus tissues, the major metabolite was i^6AMP. The growth responses of callus tissues of the interspecific hybrid were intermediate between the parental tissues, and the metabolism of $i^6Ado-8-^{14}C$ by the hybrid callus tissue exhibited characteristics of both parents. The patterns for $i^6Ado-8-^{14}C$ 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 i^6Ade and io^6Ade 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[6]Ado. 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[6]Ado), io[6]AMP, io[6]Ado-O-glucoside and two novel metabolites (tentatively identified as O-ribosyl derivatives of io[6]Ade and io[6]Ado) were isolated from Great Northern embryo extracts. In Kingston, io[6]Ado, io[6]AMP, 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 O-
xylosides of io[6]Ade and io[6]Ado (Mok, MC, personal
communication). Zeatin-8-^{14}C 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 O-glucosyl derivatives in seed
coats of Kingston and O-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 io[6]AMP.

Phaseolus Roots. The major cytokinin extracted from
P. vulgaris roots was zeatin-O-glucoside (Scott and
Horgan, 1984). Minor cytokinins present in root tissue
were tentatively identified as io[6]Ade, io[6]Ado, and io[6]Ado-
O-Glucoside. This result contrasted with an earlier study
(Wareing et al, 1977) in which io[6]Ade-8-^{14}C was fed to
disbudded seedlings of P. vulgaris, and io[6]AMP 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 io^{6}Ade, hio^{6}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, hio^{6}Ade 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 io^{6}Ade-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
b^{6}Ade was formed in P. vulgaris leaves fed b^{6}Ade,
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 rinucleosides 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^{6}AMP in the roots, to hio^{6}Ado in stems and petioles, and to io^{6}Ado-0-glucoside and hio^{6}Ade-0-glucoside in laminae (Wareing et al., 1977).

6. When hio^{6}Ade or hio^{6}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
(io^{6}Ade) and N^6-($\triangle^2$-isopentenyl)adenine (i^{6}Ade) 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]Ade and i[6]Ado 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]Ade 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]Ade 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

cochomatographs 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 N^6 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 i^6Ado 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-(Δ^2-isopentenylimino)purine. This characterization was based on the reduction of the intermediate to i^6Ado 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-(Δ^2-isopentenyl)adenine $\xrightarrow{\text{CKO}}$ Adenine + 3-methyl-2-butenal

Oxygen N^6-(Δ^2-isopentenyl)adenine   Cytokinin   Adenine   3-methyl-2-butenal
           (i^6Ade)   oxidase
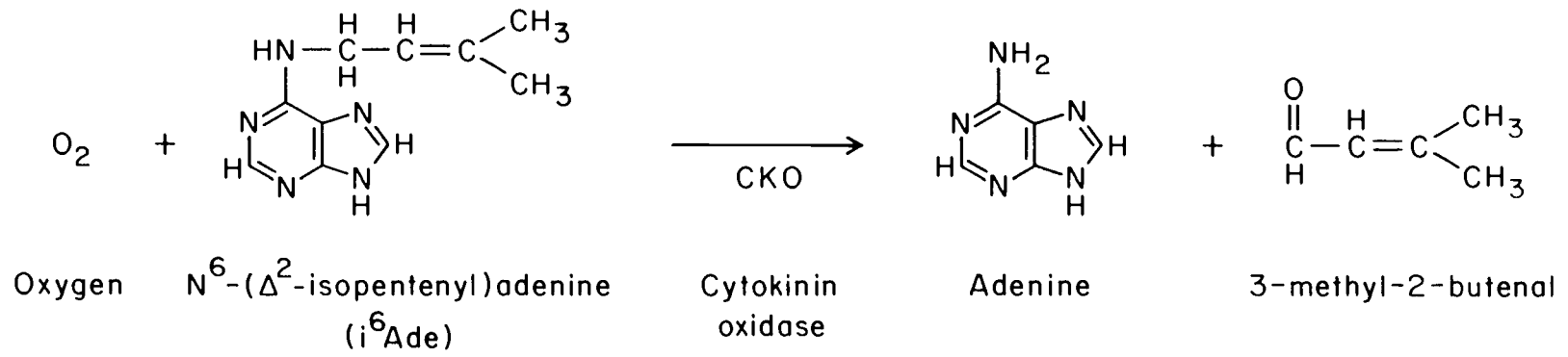
i^6Ado-8-^{14}C as a substrate, observed the conversion of i^6Ado to Ado and Ade by a crude homogenate from tobacco cells. An adenosine hydrolase activity present in the extracts was shown to convert Ado to Ade, and this activity confounded the interpretation. Adding saturating amounts of Ado to assays (to negate the effect of the hydrolase) elevated the recovery of Ado-8-^{14}C. It was concluded that i^6Ado metabolism may proceed via either i^6Ado to Ado or i^6Ade to Ade. The specificity of cytokinin oxidase from corn was examined by Whitty and Hall (1974). Using i^6Ade-8-^{14}C as a substrate, unlabeled i^6Ado 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^6Ado 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 Ado during chromatography. The substrate specificity of the reaction as determined by this spectrophotometric assay was shown

to be in the following order: $i^6Ado = i^6Ade > io^6Ade > io^6Ado$. Side chain saturation, shifting the double bond from the Δ^2 to the Δ^3 position, or substitution of other functional groups on the side chain resulted in less active substrates. The synthetic cytokinins b^6Ade, f^6Ade, and hi^6Ade 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 Km 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^6Ado 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 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 Δ^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 <u>Vinca</u> enzymes. Interestingly, diphenylurea and N-(2-chloro-4-pyridyl)-N'-phenyl-urea (both cytokinin-active urea derivatives) strongly inhibited the degradation of i^6Ado.

<u>Physical Properties of Cytokinin Oxidase</u>. 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_2O_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^6Ado 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^6Ade, i^6Ade or io^6Ade. These data suggest a cytokinin oxidase-like activity may be providing adenine that is necessary for growth. Interestingly, f^6Ade 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[1][2]

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 N^6-(Δ^2-isopentenyl)adenine-8-^{14}C (i^{6}Ade-8-^{14}C) 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

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 i^6Ade-8-^{14}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 kernals by Whitty and Hall (24) and, more recently, from <u>Vinca</u> <u>rosea</u> 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^6-side chains of N^6-(Δ^2-isopentenyl)adenine (i^6Ade)[3] and zeatin (io^6Ade) and their ribonucleosides (i^6Ado and io^6Ado). 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^6Ade (2). The mechanism of the reaction is uncertain, but an iminopurine intermediate has been hypothesized (2). Cytokinins bearing saturated isoprenoid side chains (N^6-isopentyladenosine, hi^6Ado, and dihydrozeatin, hio^6Ade) and some synthetic cytokinins (N^6-benzyladenine, b^6Ade, and kinetin, f^6Ade) 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 <u>Phaseolus</u>, we are investigating the regulation of cytokinin oxidase activity

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[3]Abbreviations: i^6Ade, N^6-(Δ^2-isopentenyl)adenine; Ade, adenine; Ado, adenosine; b^6Ade, N^6-benzyladenine; c-io^6Ade, <u>cis</u>-zeatin; f^6Ade, N^6-furfuryladenine (kinetin); Hex^6Ade, N^6-hexyladenine; hi^6Ade, N^6-isopentyladenine; hi^6Ado, N^6-isopentyladenosine; hio^6Ade, dihydrozeatin; i^6Ado, N^6-(Δ^2-isopentenyl)adenosine; t-io^6Ade, <u>trans</u>-zeatin; PVPP, polyvinylpolypyrrolidone.

in callus tissues derived from selected <u>Phaseolus</u> genotypes. The effects of short-term perturbations in cytokinin supply on the levels of cytokinin oxidase activity in callus tissues of <u>P</u>. <u>vulgaris</u> 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

<u>Chemicals</u>. 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. N^6-Isopentyladenine was synthesized as described by Leonard <u>et al</u> (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-^{14}C (57 mCi/mmol) was obtained

from Amersham Co. Lipofluor and the $Si-C_{18}$ 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 u$\underline{M}$) and kinetin
(5 u$\underline{M}$). 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 $\underline{N}$ HCl
for 10 min. The adsorbent was then washed with 5 bed
volumes each of 1 $\underline{N}$ 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 i[6]Ade-8-[14]C. Ado-8-^{14}C was adjusted to a specific activity of 25 mCi/mmol by the addition of unlabeled Ado and used in the synthesis of i[6]Ado-8-^{14}C according to Paces et al. (20) as modified by Mok et al. (17). The i[6]Ado-8-^{14}C was purified by chromatography on a Sephadex LH-20 column in 33% (v/v) ethanol (1) and deribosylated to i[6]Ade as described by LaLoue et al. (10). The i[6]Ade-8-^{14}C 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-^{14}C 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.
N_2). 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^6Ade-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^6Ade-8-14C (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^6Ade (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^6Ade 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 othrwise 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 $(NH_4)SO_4$ 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.
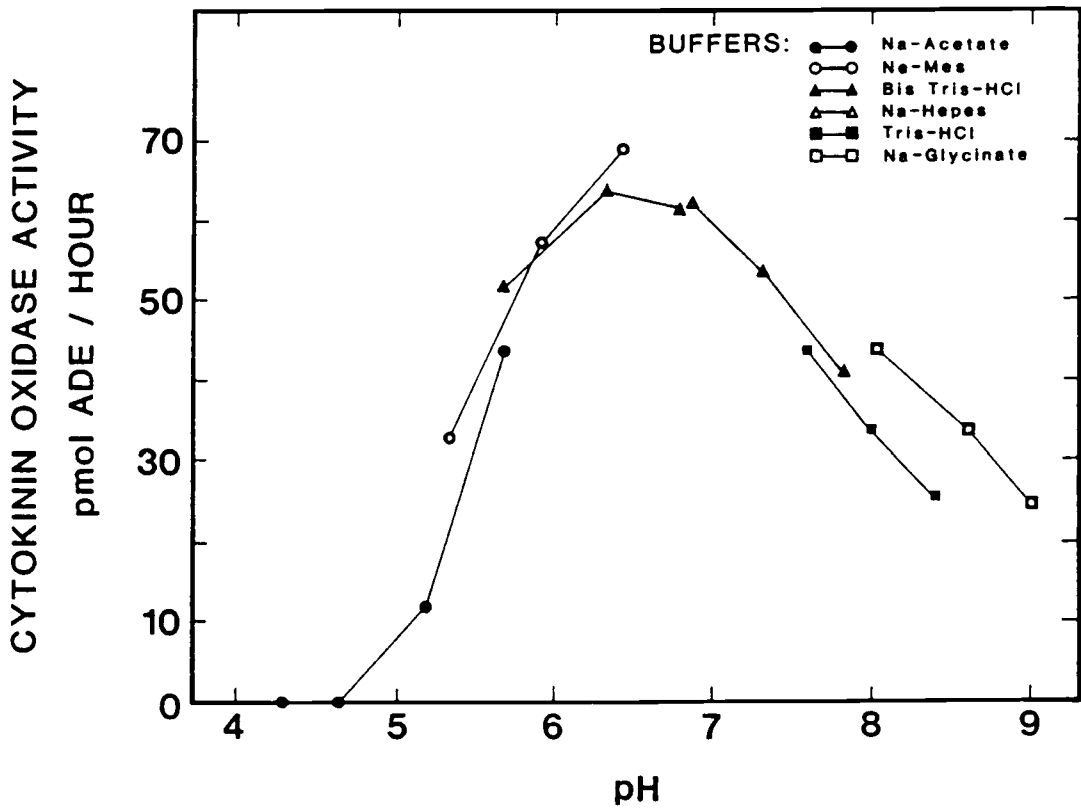
their effect on the activity of the <u>Phaseolus</u> enzyme (Table III.1). The use of imidazole resulted in an approximately two-fold increase in cytokinin oxidase activity, and this buffer has been used in all our standard assays of cytokinin oxidase activity. As shown in Table III.1, histidine would 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 <u>P</u>. <u>vulgaris</u> 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 i^6Ade to tissue homogenates (data not shown).

Table III.1. Effect of Buffer Composition on Cytokinin Oxidase Activity.

Buffer (45 mM, pH 6.6)	Cytokinin Oxidase Activity[a]
	$nmol \cdot mg^{-1}$ protein$\cdot h^{-1}$
Experiment 1	
Bis tris	0.95
Ada	0.95
Imidazole	1.93
KH_2PO_4	0.93
NaH_2PO_4	1.05
Malonic acid	1.02
Mes	0.98
Experiment 2	
Bis tris	0.90
Imidazole	1.45
Histidine	0.78

[a]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 $\pm 4\%$ of the means.

Table III.2. Recovery of Cytokinin Oxidase Activity from
P. _vulgaris_ cv. Great Northern Callus Tissue.

Enzyme Preparation	Recovery per Gram Callus Tissue		
	Total protein	Total cytokinin oxidase activity	
	mg	nmol/h	%Recovery[a]
Crude Homogenate[b]	3.60	1.57	100
Std. Assay Preparation[c]	1.27	1.52	97

[a]All values are the average of four assays. The SE were less than ±2% 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.
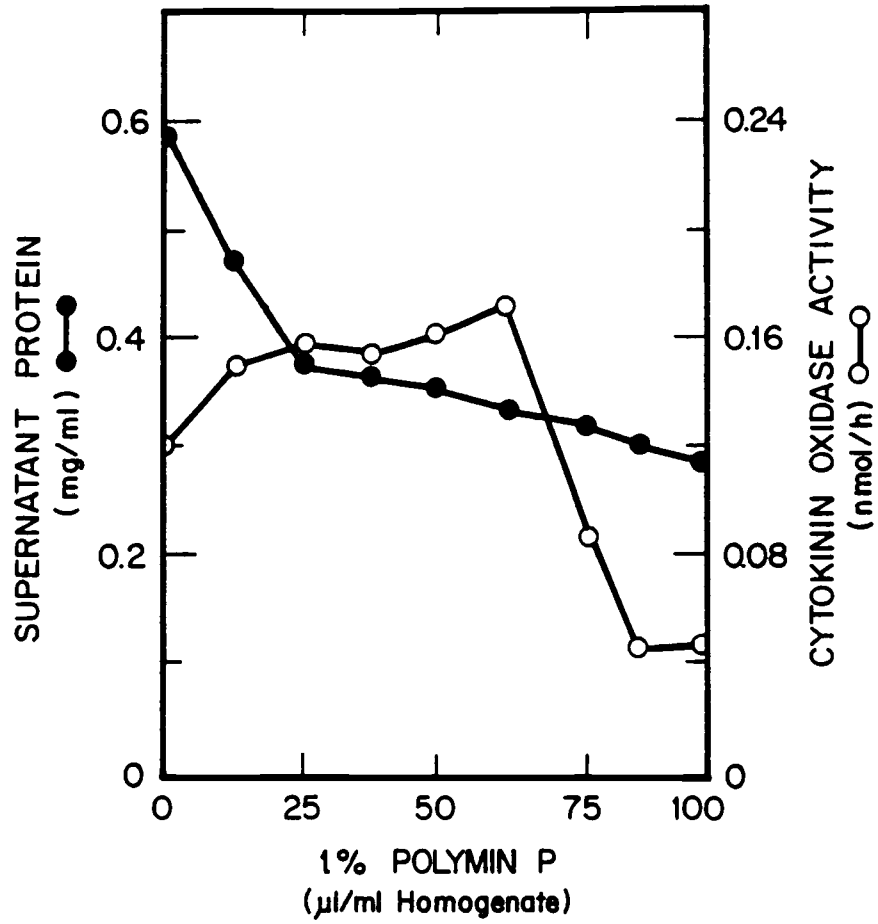
The effects of varying enzyme concentration and time of incubation on the reaction rates obtained with cytokinin oxidase preparations prepared as described above are shown in Figure III.3. A 30 min incubation time was selected for the standard assay procedure because enzyme concentrations could be varied 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[6]Ade-8-^{14}C 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[6]Ade-8-^{14}C 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[6]Ade substrate (10 u$\underline{M}$) and in ten-fold excess (100 u$\underline{M}$). As expected, Ade was completely ineffective in inhibiting the degradation of i[6]Ade-8-^{14}C, whereas the addition of unlabeled i[6]Ade to the reaction mixture markedly inhibited oxidation of the labeled substrate. Suprisingly, the cytokinin-active urea derivative Thidiazuron was as effective as i[6]Ade in inhibiting label degradation. The nucleoside form of i[6]Ade (i[6]Ado) was somewhat less effective than the free base. Zeatin ($\underline{t}$-io[6]Ade) rather weakly inhibited the reaction, and $\underline{cis}$-zeatin was even less effective than the $\underline{trans}$ 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.
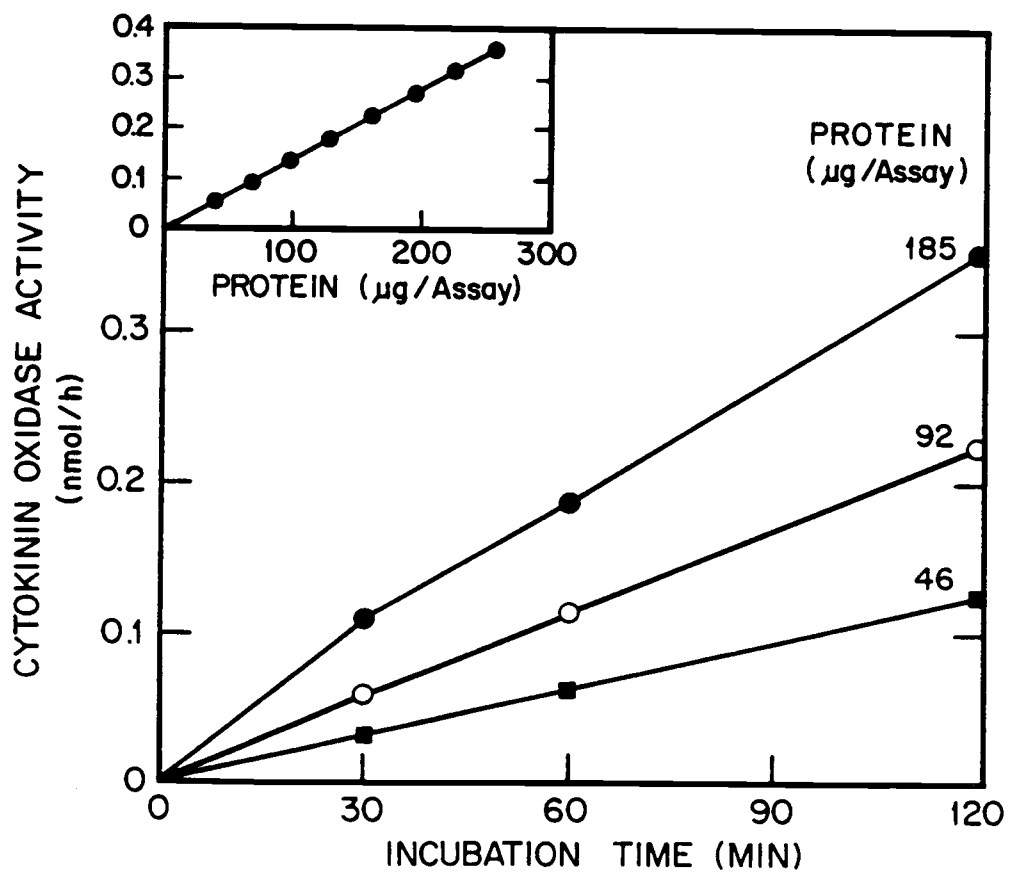
Table III.3. Effects of Unlabeled Cytokinins on the Degradation of i^6Ade-8-^{14}C by Cytokinin Oxidase

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

Unlabeled	Concentration of unlabeled cytokinin	
Cytokinin	10 u$\underline{M}$	100u$\underline{M}$
	Cytokinin Oxidase Activity[a] % control	
Ade	100	101
i^6Ade	54	14
i^6Ado	74	46
hi^6Ade	98	89
hio^{6}Ade	98	92
t-io^{6}Ade	83	63
c-io^{6}Ade	99	82
f^{6}Ade	96	98
b^{6}Ade	95	94
Thidiazuron	50	14

[a]The cytokinin oxidase activity of controls incubated without the addition of unlabeled cytokinins was equal to 1.27 nmol/h·mg protein. This value was taken as 100% activity. All values are the average of four assays. SE averaged ±2% of the means and did not exceed ±6% of the means.

isomer preparations used here contained 7-10% of the other isomer). Cytokinins bearing saturated isoprenoid side chains (hi^{6}Ade, hio^{6}Ade) or side chains with ring structures (b^{6}Ade and f^{6}Ade) did not significantly inhibit the degradation of i^{6}Ade-8-^{14}C.

Cytokinin Oxidase Activity in Callus Tissues Treated with Exogenous Cytokinins. 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 u$\underline{M}$ f^{6}Ade. Cytokinin oxidase activity was monitored over a 4 hour incubation period following the cytokinin application. The results of treatment with zeatin (io^{6}Ade) and dihydrozeatin (hio^{6}Ade) 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 i^{6}Ade and hi^{6}Ade also induced increases in cytokinin oxidase activity in Great Northern callus tissues (Figure III.4B). Again, the cytokinin with the saturated N^6-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 at as described in "Materials and Methods". Cytokinin solutions (100 u$\underline{M}$) 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 u$\underline{M}$. Following cytokinin application, the tissues were incubated for the times indicated at 27^6 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.
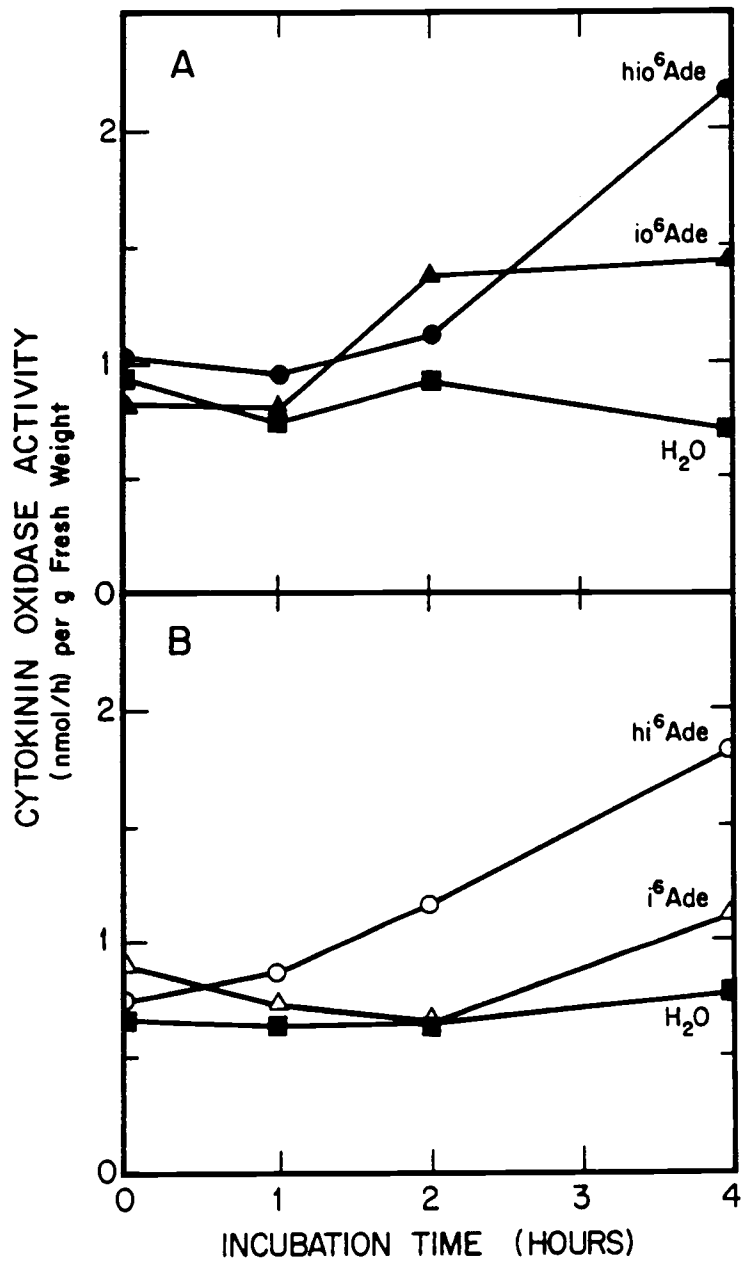
(hi^{6}Ade) was more effective than the analog bearing the corresponding unsaturated side chain (i^{6}Ade) in inducing elevated levels of cytokinin oxidase in the callus tissue.

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 u$\underline{M}$) 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 u$\underline{M}$. 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".

Cytokinin	Cytokinin Oxidase Activity[a]	
	Time 0	Time 4 h
	nmol·g^{-1} tissue·h^{-1}	
Ade	0.92	0.95
b^{6}Ade	0.82	2.31
Hex^{6}Ade	0.53	1.74
hi^{6}Ade	0.82	2.31
f^{6}Ade	0.76	2.02
Thidiazuron	0.78	2.56

[a]All 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 $\underline{P}$. $\underline{vulgaris}$ cv. Great Northern
callus tissue following treatment with N^6-
isopentyladenine (hi^{6}Ade).

Callus tissues (5 g/flask) were grown and selected at
as described in "Materials and Methods". Following a
single application of hi^{6}Ade (100 u$\underline{M}$) 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
$\pm$5% of the means and did not exceed $\pm$8%.

Figure III.5.
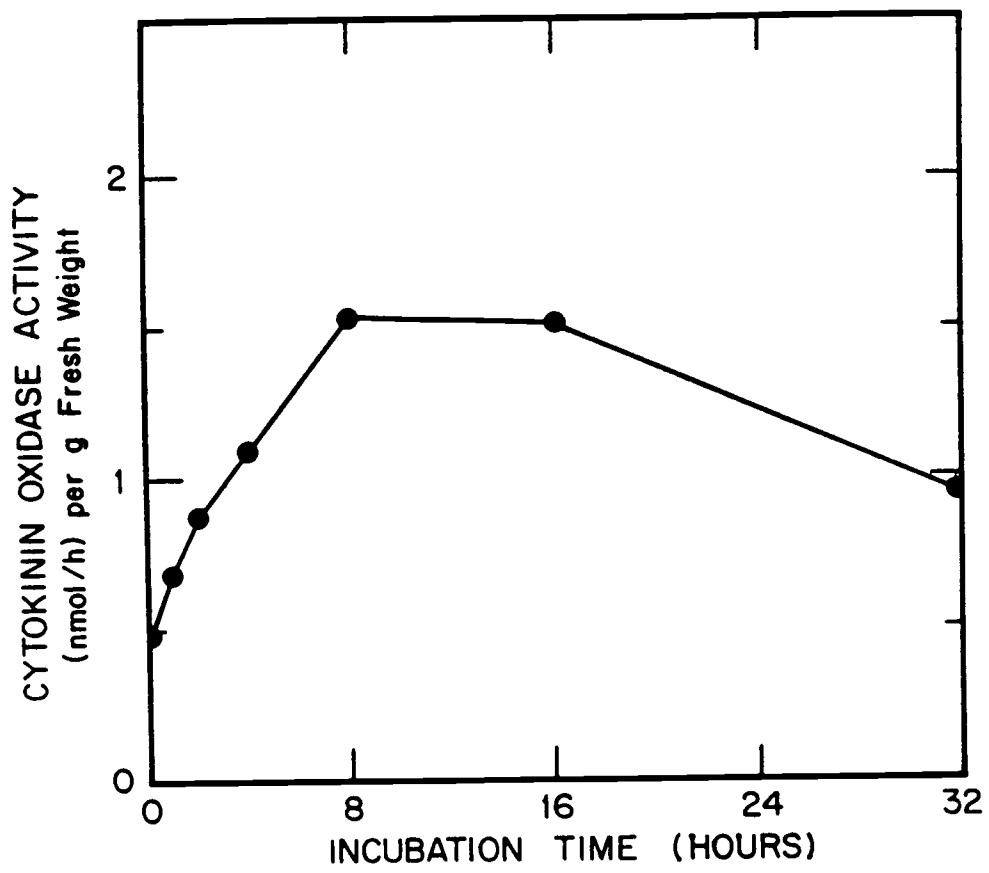
in Figure III.6. As measured 4 hours following treatment,
a slight increase in cytokinin oxidase activity could be
detected after treatment with hi⁶Ade concentrations
equivalent to as little as 0.1 nmoles per gram tissue.
Cytokinin oxidase activity increased with increasing
concentrations of hi⁶Ade up to a plateau value
representing better than a three-fold increase in activity
at an estimated $\underline{in}$ $\underline{vivo}$ hi⁶Ade concentration of about 20
u$\underline{M}$.

**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 m$\underline{M}$ 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^6-isopentyadenine
concentration on the induction of cytokinin
oxidase activity in P. vulgaris cv. Great
Northern callus tissues.

Callus tissues (5 g/flask) were grown and selected as
described in "Materials and Methods". N6-isopenteyladenine
solutions (0.1 uM to 320 uM) were applied to the surfaces
of the callus tissues at a rate of 0.1 ml/g tissue to give
estimated hi^{6}Ade concentrations in the tissue as
indicated. The tissues were incubated for 4 hours at 27° C
in the dark. Cytokinin oxidase activity was assayed as
described in "Materials and Methods". SE (n=4) averaged
±3% of the means and did not exceed ±5%.

Figure III.6.
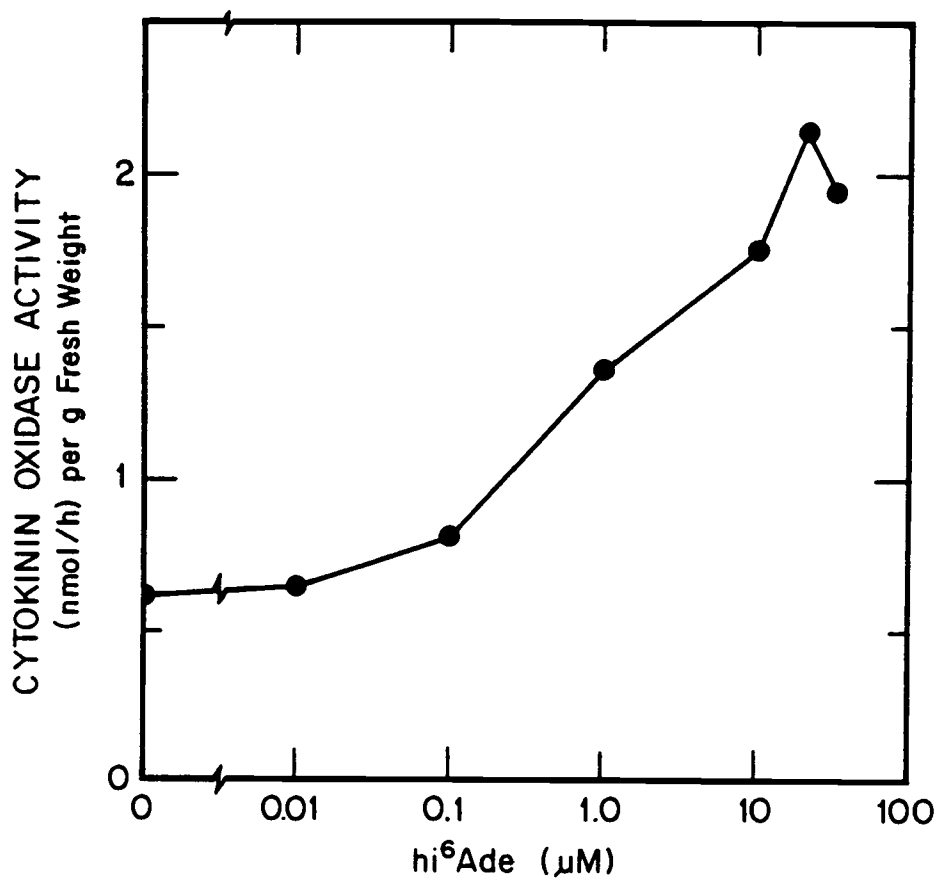
Figure III.7. Effects of antibiotic inhibitors of
            transcription and translation on the growth
            of P. vulgaris cv. Great Northern callus
            tissues.

    The antibiotics were dissolved in DMSO and added to
autoclaved tissue culture medium at a rate of 50 ul/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.
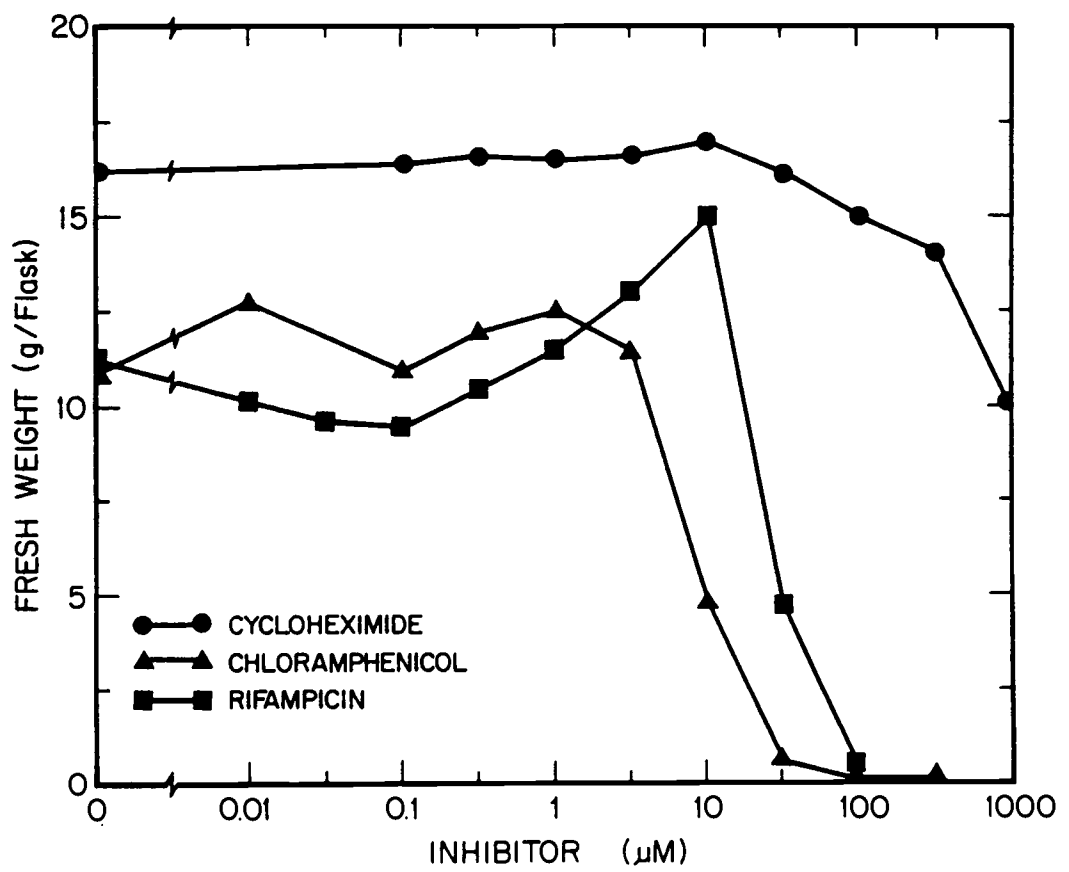
Table III.5. Effects of Inhibitors on Cytokinin-induced Increases in Cytokinin Oxidase Activity in P. vulgaris cv Great Northern Callus Tissue

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 hi⁶Ade (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".

Inhibitor	Inhibitor Concentration in vivo[a]	Cytokinin Oxidase Activity[b]		Increase in CKO Activity Induced by hi⁶Ade Treatment[c]	
		Expt 1	Expt 2	Expt 1	Expt 2
	u$\underline{M}$	nmol·g^{-1} tissue·h^{-1}		%	
None		1.60	1.42	146	92
Cordycepin	320	0.84		29	
Rifampicin	32	1.50		131	
Cycloheximide	3200		0.94		27
Chloramphenicol	320		1.68		127

[a]Concentrations were calculated assuming 1 g callus equals 1 ml volume.
[b]All values are the average of four assays. SE averaged ±5% of the means and did not exceed ±6% of the means.
[c]The cytokinin oxidase activity in untreated control tissues which had not been exposed to either inhibitors or hi⁶Ade 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 N^6-
isopentyladenine (hi^{6}Ade) 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 hi^{6}Ade
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 hi^{6}Ade (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 hi^{6}Ade. 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.
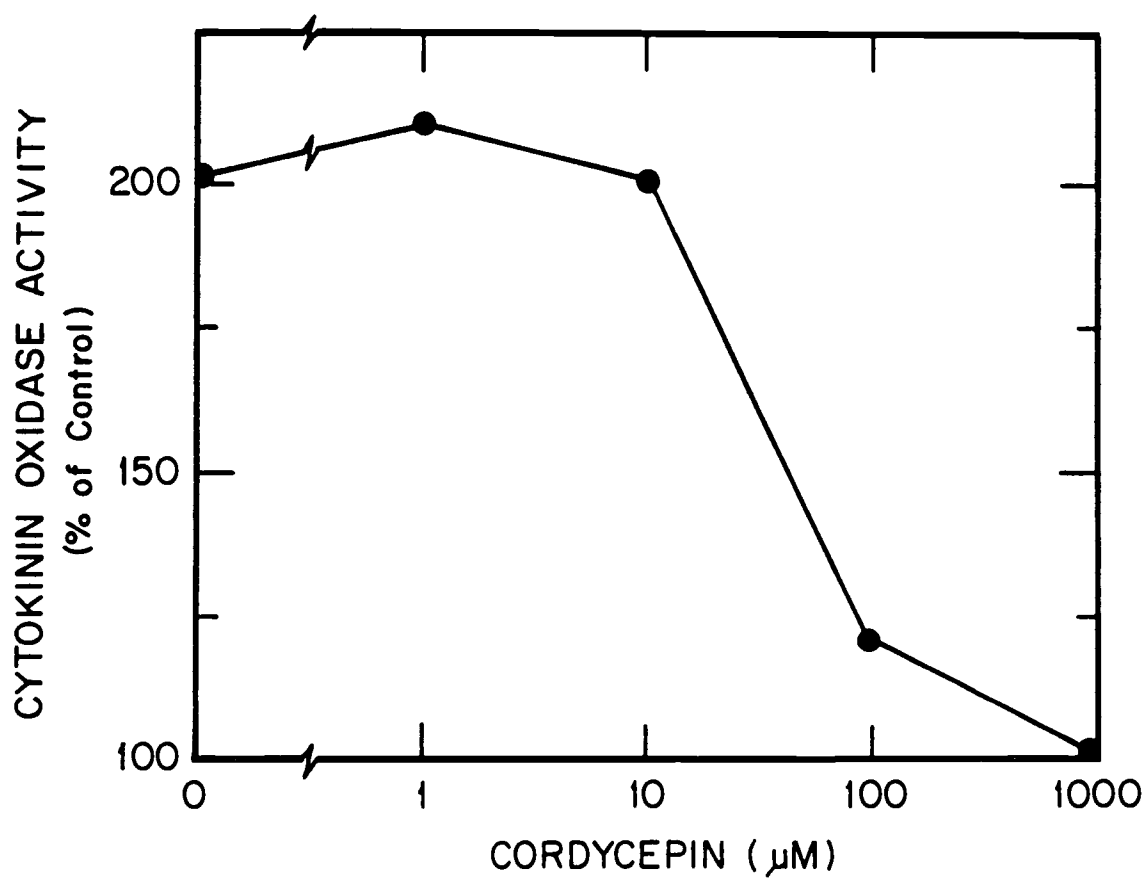
equivalent to 100 u$\underline{M}$ in the tissue, and a cordycepin concentration of 1000 u$\underline{M}$ was sufficient to completely inhibit the increase in cytokinin oxidase activity caused by hi[6]Ade treatment. On the basis of the results shown in Figure III.7, cycloheximide was tested at a concentration equivalent to 3200 $\dot{\imath}\underline{M}$ 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 $\underline{P}$. vulgaris cv. Great Northern has been examined using an assay based on the oxidation of i[6]Ade-8-^{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 $\underline{P}$.
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^{6}Ade, hio^{6}Ade, b^{6}Ade, or f^{6}Ade.
Where data are available, similar specificities have been
observed using the corresponding ribonucleosides. The
trans isomer of io^{6}Ado has been reported to be more
effective than the cis isomer in competitively inhibiting
the degradation of labeled i^{6}Ado 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^{6}Ade. 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 i^{6}Ade-8-^{14}C 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 N^6-side chains (hi^6Ade and hio^6Ade), which were resistant to attack by the enzyme, were actually more effective than i^6Ade and io^6Ade in promoting increases in cytokinin oxidase activity. The effectiveness of hi^6Ade and hio^6Ade 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 N^6-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[6]Ado 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 is _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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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^6Ade-2,8-^{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^6Ade 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-(Δ^2-isopentenyl)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 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 in vitro activity of cytokinin oxidase

and present evidence that such complexes function as alterative 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. N^6-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 (polyethyleneimine, 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 $Si-C_{18}$ TLC plates (without fluorescent indicator) used in this study are products of J. T. Baker Co.

Adenosine-2,8-^{3}H (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^6Ade-2,8-3H. Ado-2,8-^{3}H was adjusted to a
specific activity of 250 mCi/mmol by the addition of
unlabeled Ado and used in the synthesis of i^6Ado-2,8-^{3}H
according to methods previously described for the
synthesis of i^6Ado-8-^{14}C (14). The i^6Ado-2,8-^{3}H was
purified by chromatography on a Sephadex LH-20 column in
33% (v/v) ethanol (1) and deribosylated to i^6Ade as
described by LaLoue et al. (7). The i^6Ade-2,8-^{3}H 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-^{3}H was

<u>ca</u>. 20%. Prior to use in enzyme assays, the i[6]Ade-2,8-^{3}H was adjusted to s 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 <u>P</u>. <u>vulgaris</u> 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 <u>M</u> 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 <u>M</u> 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 i^6Ade-2,8-^{3}H 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 0.2$\underline{M}$), 0.01 m$\underline{M}$
i^6Ade-2,8-^{3}H (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 i^6Ade (0.75 m$\underline{M}$ each)
and 20 m$\underline{M}$ Na_2EDTA. 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} TLC
plates. The chromatograms were developed to a height of 5
cm in 37.5% (v/v) ethanol containing 0.1 $\underline{M}$ Na_4EDTA.(The
addition of Na_4EDTA 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 i^6Ade
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 $\underline{N}$), 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 m$\underline{M}$ tris-HCl (pH 7.5) containing
1 $\underline{M}$ 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 m$\underline{M}$ 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 i[6]Ade was
removed from the reaction mixtures by solvent
partitioning. For the copper-enhanced assay, the assay

mixtures contained 100 m$\underline{M}$ imidazole-HCl (pH 6.5), 0.01 m$\underline{M}$

i^{6}Ade-2,8-3H (0.05 uCi,specific activity 100 uCi/uM)), 10

m$\underline{M}$ CuCl$_2$, and 40 ul aliquots of the column fractions in a

final volume of 50 ul. For the unenhanced enzyme assays,

100 m$\underline{M}$ bis tris-HCl (pH 6.5) was substituted for imidazole

and CuCl$_2$ 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 m$\underline{M}$ Na$_2$EDTA containing i^{6}Ado

and Ade (each at 1 m$\underline{M}$) followed by the addition of 300 ul

of butyl acetate saturated with a 1 m$\underline{M}$ Ade solution. The

assay tubes were vortexed for 15 sec, and the two phases

separated by centrifugation. The organic phase, containing

undegraded i^{6}Ade, was removed, and the extraction with

butyl acetate (saturated with 1 m$\underline{M}$ 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 $\underline{N}$ 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 $\underline{M}$ 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 $CuCl_2$ on cytokinin oxidase activity measured in reaction mixtures containing imidazole buffer are illustrated in Figure IV.1. At the imidazole concentration used here (200 $m\underline{M}$), cytokinin oxidase activity increased with increasing concentrations of $CuCl_2$ to values representing an approximately 30-fold enhancement of activity at 30 $m\underline{M}$ $CuCl_2$. 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

Metal	Buffer	
	Bis Tris	Imidazole
(1 m$\underline{M}$)		
	Cytokinin Oxidase Activity[a] % of Control[b]	
CuCl$_2$	113	410
CaCl$_2$	102	99
NiCl$_2$	98	95
CoCl$_2$	100	97
ZnCl$_2$	91	105
MgSO$_4$	96	104
MnSO$_4$	102	106
FeSO$_4$	98	91

[a]Cytokinin oxidase activity was determined in reaction mixtures containing 100 m$\underline{M}$ bis tris-HCl or imidazole-HCl (pH 6.5), 0.01 m$\underline{M}$ i^{6}Ade-2,8-^{3}H (0.05 uCi, Spec. Act. 100 uCi/umol) and protein equivalent to 250 mg (fresh wt.) of $\underline{P}$. $\underline{vulgaris}$ 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
µg protein (o) or 15 ug protein (●), 0.01 mM i^6Ade-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
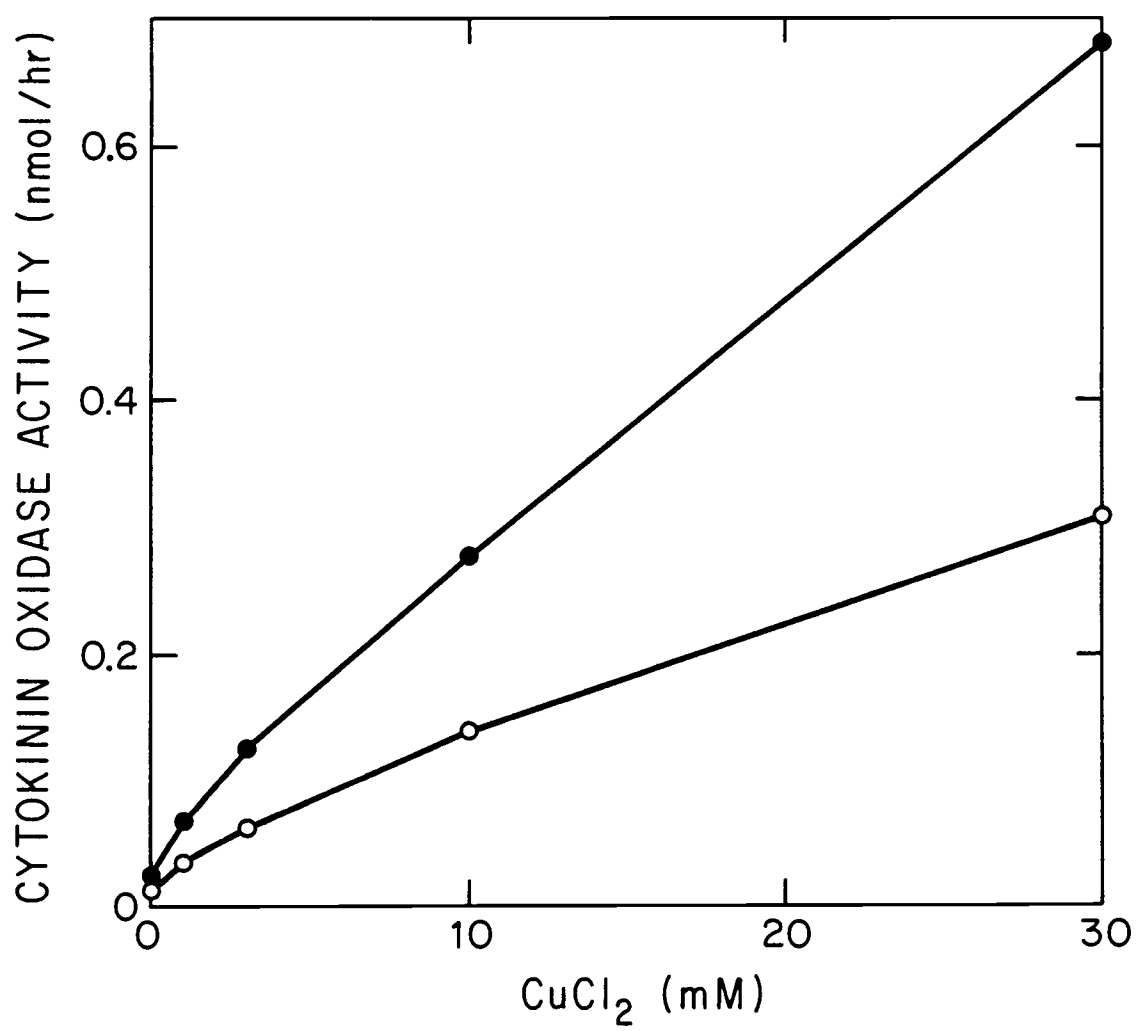
Figure IV.2. Effects of CuCl$_2$ on cytokinin oxidase
            activity in reaction mixtures containing 100
            m$\underline{M}$ or 200 m$\underline{M}$ imidazole as the assay buffer

    Cytokinin oxidase activity was determined in reaction
mixtures containing 100 m$\underline{M}$ (●) or 200 m$\underline{M}$ (○) imidazole-
HCl (pH 6.5), 0.01 m$\underline{M}$ i^{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 $\underline{P}$. $\underline{vulgaris}$ cv. Great Northern callus
tissue, and the indicated concentrations of CuCl$_2$. All
values are the average of three assays, SE averaged $\pm$1% of
the means and did not exceed $\pm$2% of the means.

Figure IV.2
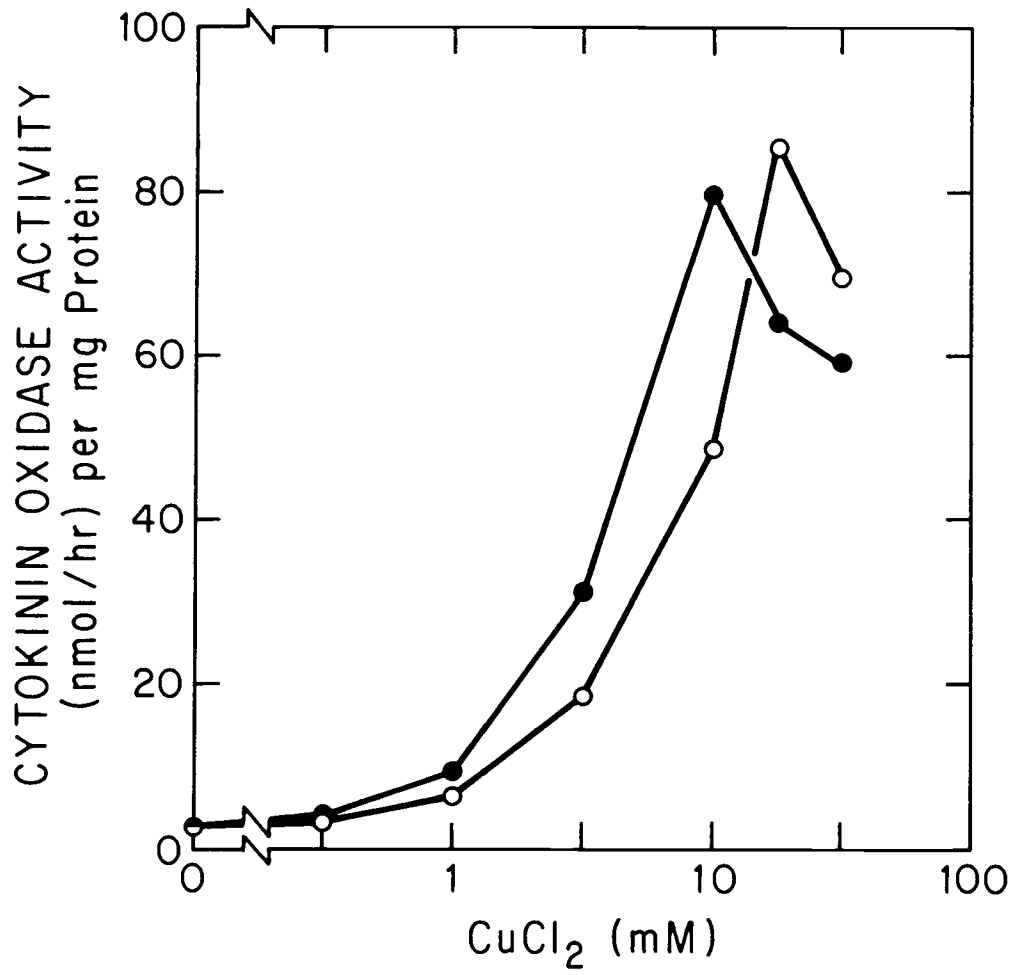
Table IV.2. Characteristics of the Copper-Imidazole
Stimulation of Cytokinin Oxidase Activity from P. vulgaris
cv. Great Northern callus tissues

Assay Conditions	Cytokinin Oxidase Activity[a] (pmol/hour)
Minus Enzyme Control	0
Standard Assay[b]	6 ± 1
Standard Assay +CuCl$_2$[c]	124 ± 1
Standard Assay +CuCl2 +EDTA[d]	6 ± 1
Boiled Enzyme Control[e]	0

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

[b]Standard assay mixtures contained 8 ug protein, 0.01
mM i^{6}Ade-2,8-^{3}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".

[c]Prior to assay, CuCl$_2$ was added at a concentration
of 10 mM.

[d]Prior to assay, Na$_2$EDTA was added to a final
concentration of 20 mM.

[e]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^6Ade 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^6Ade 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^6Ade-2,8-^{3}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^6Ade substrate (10 u$\underline{M}$) and in 10-fold excess (100 u$\underline{M}$). 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 i[6]Ade-2,8-^{3}H by Cytokinin Oxidase in Bis Tris, Imidazole and Copper-Imidazole Buffer

Buffer	Concentration Unlabeled Cytokinin	Unlabeled cytokinin[a]				
		i[6]Ade	hi[6]Ade	Thi[b]	io[6]Ade	b[6]Ade
	uM	Cytokinin Oxidase Activity[c] % of Control				
Bis Tris	10	61	98	54	82	98
	100	13	91	17	65	90
Imidazole	10	62	98	56	81	98
	100	15	92	18	65	95
Imidazole +CuCl$_2$	10	60	92	52	82	95
	100	16	87	17	45	92

[a]Unlabeled cytokinins were added to assay mixtures containing i[6]Ade-2,8-^{3}H at a concentration of 0.01 m$\underline{M}$, 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 $\underline{M}$ and CuCl$_2$ was 0.01 $\underline{M}$ 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 $\pm$1% of the means and did not exceed $\pm$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 i^6Ade were equally effective in inhibiting the oxidation of the labeled substrate. Zeatin rather weakly inhibited the reaction and the addition of benzyl adenine (b^6Ade) and N^6-isopentyladenine (hi^6Ade) 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 i^6Ade 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 i^6Ade 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 i^6Ade in the

Table IV.4. Partitioning of i^{6}Ade 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 m$\underline{M}$ EDTA·Na$_2$ containing i^{6}Ado and Ade (each at 1 m$\underline{M}$) and 300 ul of 1 m$\underline{M}$ Ade saturated ethyl acetate or butyl acetate saturated with 1 m$\underline{M}$ 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 $\underline{M}$ 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.

Assay Conditions		ASSAY METHOD				
		Ethyl Acetate Extraction		Butyl Acetate Extraction		Std. Assay
	Labeled Compound	Aqueous Phase	Organic Phase	Aqueous Phase	Organic Phase	Single Phase
		% of Total Radioactivity Recovered Per Assay[a]				
Buffer Control[b]	Ade	1	1	1	1	2
	i^{6}Ade	1	97	1	97	98
Enzyme Assay[c]	Ade	18	16	29	9	37
	i^{6}Ade	1	65	1	61	63

[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 m$\underline{M}$ imidazole-HCl (pH 6.5), 0.01 m$\underline{M}$ i^{6}Ade-2,8-^{3}H (0.05 uCi, 25 uCi/umol) and 10 m$\underline{M}$ CuCl$_2$.

[c]In addition to the components described for buffer controls, the standard enzyme assays contained 8 ug protein.

cytokinin 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
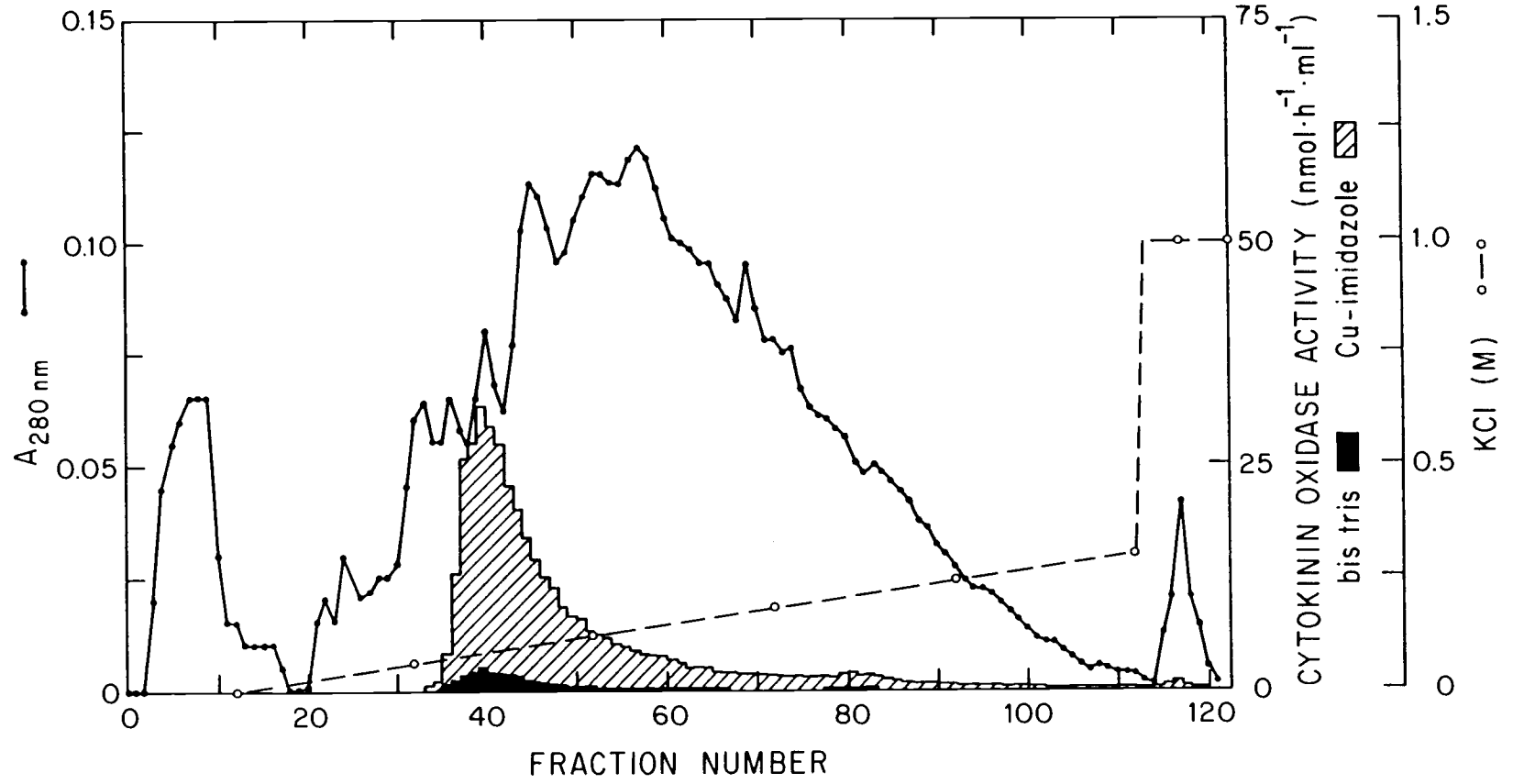
concentration required for enhancement suggested that copper was acting in some manner other than as a cofactor for the enzyme. The possibility that copper or copper-imidazole complexes might be functioning as an alternative electron accepter (replacing oxygen) in the assay was tested by examining the 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 i[6]Ade 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 $\underline{M}$ bis tris-HCl (pH 6.5). Copper-imidazole assays
contained 16 ug protein, 0.1 $\underline{M}$ imidazole-HCl (pH 6.5) and
0.01 $\underline{M}$ CuCl$_2$. 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 N$_2$. 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".

Buffer	Cytokinin Oxidase Activity[a]	
	+O$_2$	-O$_2$
	pmol/hour	
Bis Tris	65 $\pm$ 1	10 $\pm$ 1
Copper-Imidazole	186 $\pm$ 6	188 $\pm$ 5

[a]Cytokinin oxidase activity was assayed as described
in "Materials and Methods". Each value is the average of 3
assays $\pm$ 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^6Ade 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 _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-methyoxybenzyl 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.

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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-(Δ^2-isopentenyl)adenine-[2,8-^{3}H] 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 methyl-
mannose. 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-(Δ^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 i[6]Ade-2,8-3H**. Ado-2,8-^{3}H, adjusted to a specific activity of 250 mCi/mmol by the addition of unlabeled Ado, was used in the synthesis of i^{6}Ado-2,8-^{3}H by methods described previously for the preparation of i^{6}Ado-8-^{14}C (13). The i^{6}Ado-2,8-^{3}H was purified by chromatography on a Sephadex LH-20 column in 33% (v/v) ethanol (1) and deribosylated to i^{6}Ade as described by LaLoue et al. (9). The i^{6}Ade-2,8-^{3}H 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-^{3}H was about 20%. Prior to use in enzyme assays the i^{6}Ade-2,8-^{3}H was adjusted to a specific activity of 100 mCi/mmol with cold i^{6}Ade, 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 m$\underline{M}$ tris-HCl (pH 7.5) containing 1 $\underline{M}$ 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 m$\underline{M}$ 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 $\underline{M}$ bis tris-HCl (pH 6.5) containing 0.25 $\underline{M}$ $(NH_4)_2SO_4$, 0.1 $\underline{M}$ methyl-mannose, 0.001 $\underline{M}$ $CaCl_2$, and 0.001 $\underline{M}$ $MnCl_2$. The initial wash was followed by 50 bed volumes of the same buffer without methyl-mannose, $CaCl_2$, or $MnCl_2$.

Polyexchanger PBE94 was washed with 25 bed volumes of degassed 25 m$\underline{M}$ 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 ul 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 m$\underline{M}$ imidazole-HCl (pH 6.5), 0.01 m$\underline{M}$ i[6]Ade-2,8-[3]H (0.05 uCi, specific activity 100 uCi/umol), 1 or 10 m$\underline{M}$ CuCl$_2$, 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 m$\underline{M}$ Na$_2$EDTA containing i[6]Ado and Ade (each at 1 m$\underline{M}$) followed by 300 ul of either ethyl acetate or butyl acetate, each saturated with 1 m$\underline{M}$ 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 m$\underline{M}$ potassium

phosphate buffer (pH 7), 1 m$\underline{M}$ 4-aminoantipyrene, 80 m$\underline{M}$
phenol, and 1 m$\underline{M}$ H_2O_2 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 m$\underline{M}$ bis tris-HCl (pH 6.5) containing 0.5 m$\underline{M}$ 4-chloro-1-naphthol, 10 m$\underline{M}$ H_2O_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.

Figure V.1
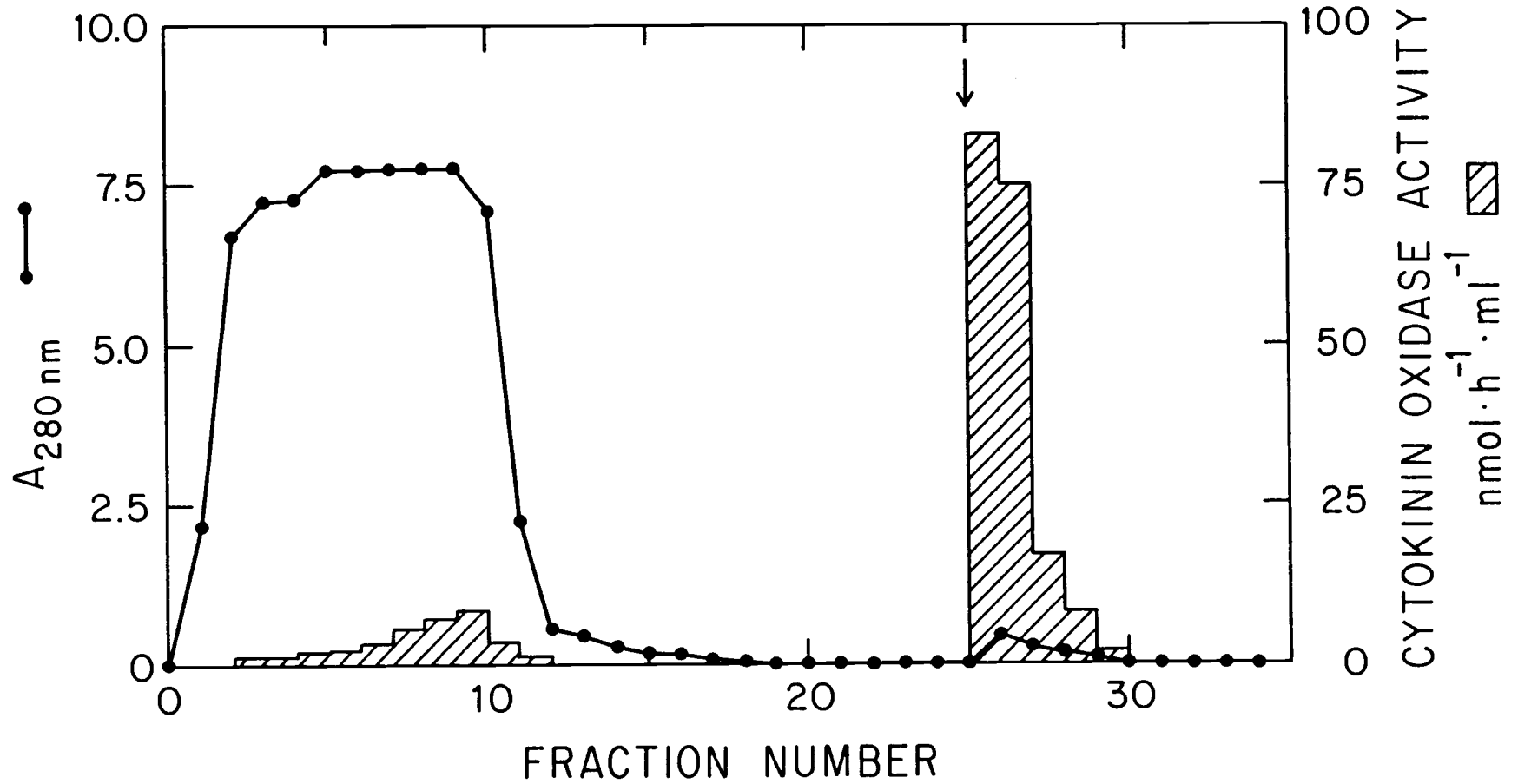
<u>Chromatofocusing of Cytokinin Oxidase from P. vulgaris</u>

<u>callus tissues.</u> 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 methyl
mannose 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. <u>vulgaris</u>
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 $\underline{M}$ imidazole buffer and
10 m$\underline{M}$ CuCl$_2$). 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).

Figure V.2. Chromatofocusing of a cytokinin oxidase
preparation from Great Northern callus tissue.

The cytokinin oxidase fractions (# 26-29) obtained
from the concanavalin A Sepharose 4B fractionaion (Figure
V.1) were pooled and this sample was exchanged into 25 m$\underline{M}$
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 N_2, followed by dilution to 10 ml with 25 m$\underline{M}$
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
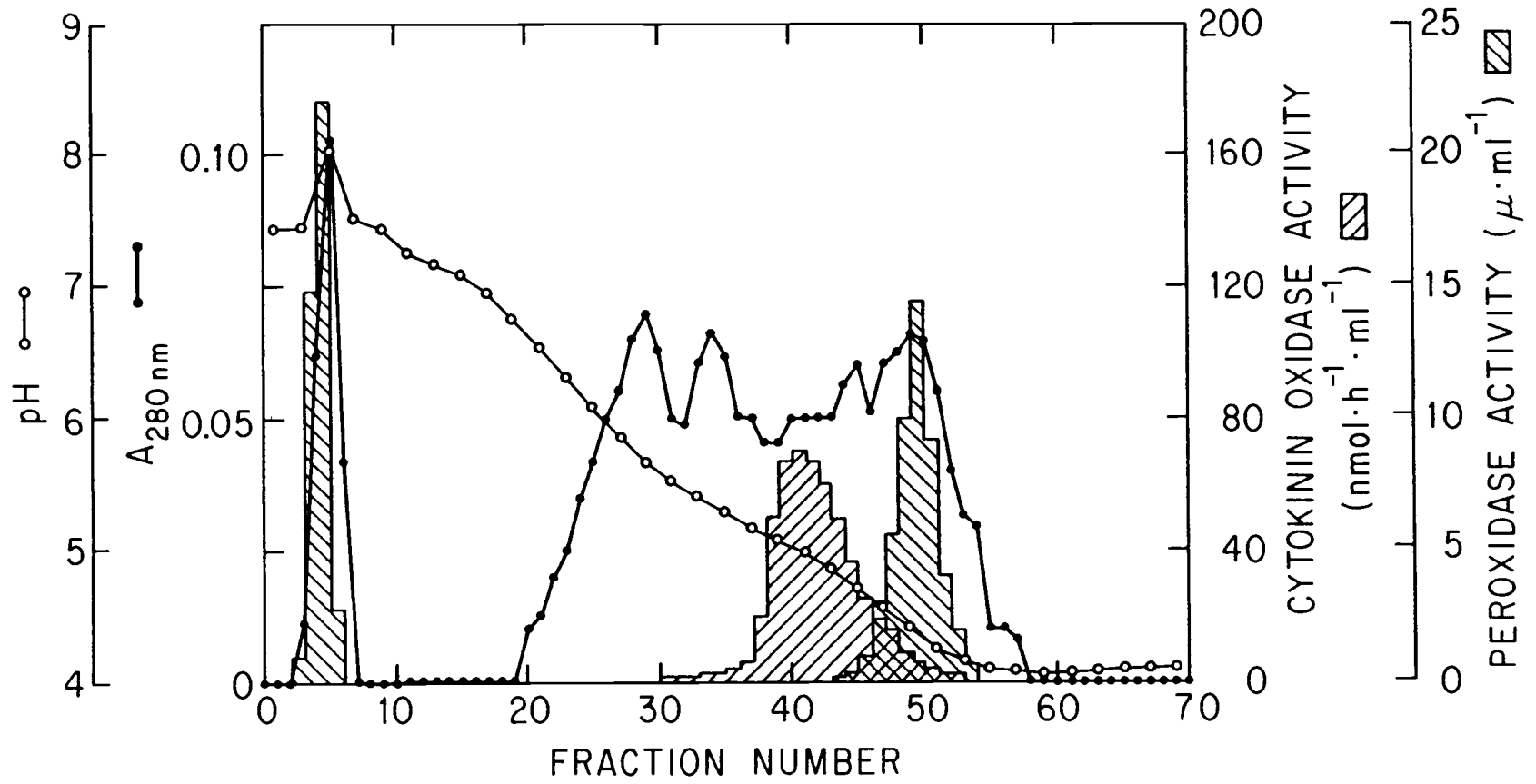
Table V.1. Summary of the Purification of Cytokinin Oxidase from Callus Tissue of P. vulgaris cv. Great Northern [a]

Fraction	Total Protein	Total Activity	Specific Activity	Fold Purification	Yield
	mg	$umol \cdot h\text{-}1$	$umol \cdot h^{-1} \cdot mg$		%
Crude Homogenate	1180	33.7	0.028	1	100
Polymin P·$(NH_4)SO_4$ Treated Fraction	755	28.6	0.038	1.3	85
Concanavalin A-Sepharose 4B (fractions 26-29)	28.7	22.8	0.796	28	68
Chromatofocusing (fractions 39-43)	1.4	18.3	13.1	455	54

[a]From 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".

Figure V.3
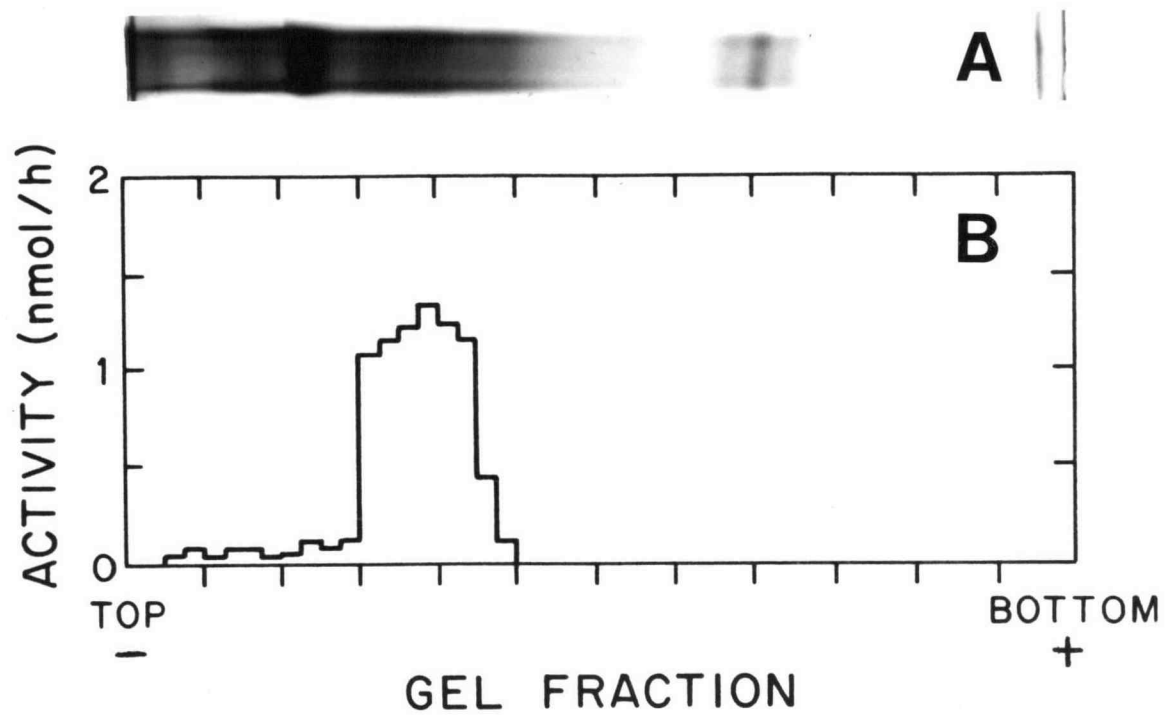
DEAE-Cellulose Fractionation of Cytokinin Oxidase from P. vulgaris Callus Tissues. The apparent separation of cytokinin oxidase activity into two fractions by concanavalin A-Sepharose 4B chromatography suggested that multiple forms of the enzyme might be present in the P. vulgaris cv. Great Northern 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 m$\underline{M}$ 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 m$\underline{M}$ 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 $\underline{M}$ over a total volume of 0.24 liters.
Fraction of 4 ml were collected at a flow rate of 16 ml/h
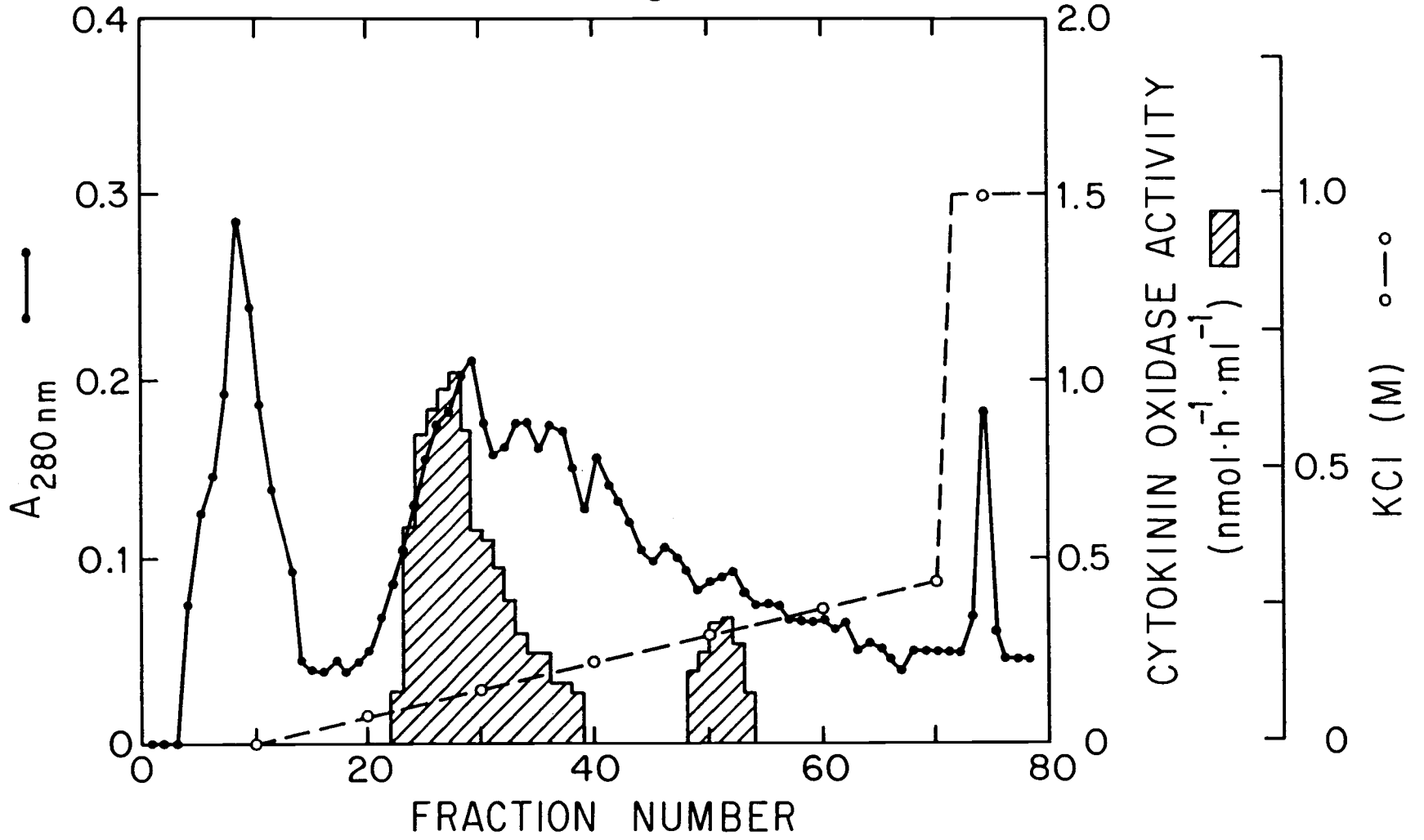(12 cm·h^{-1}).

Figure V.4

Figure V.5. Concanavalin A-Sepharose 4B chromatography of
            DEAE-cellulose fractionated cytokinin oxidase.

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
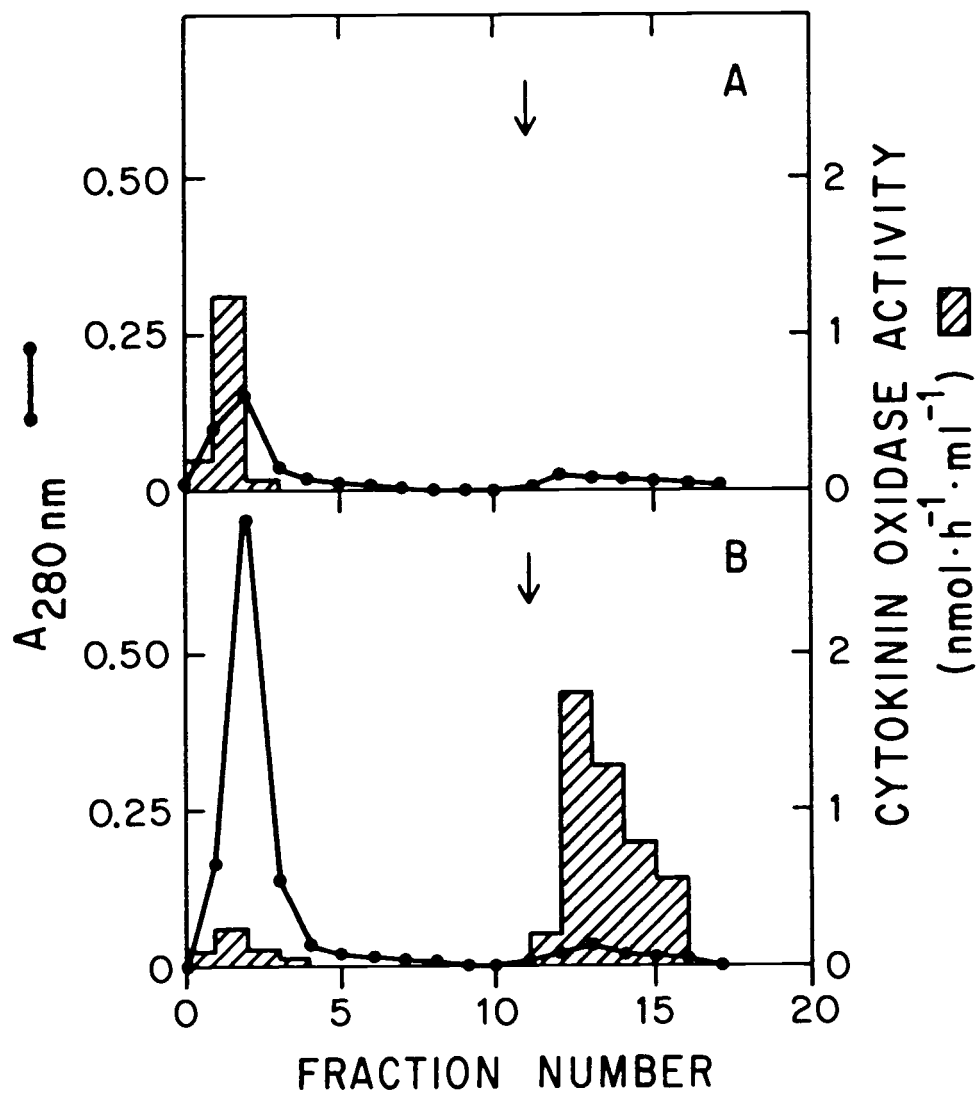
affinity columns, the two cytokinin oxidase fractions were examined by native polyacrylamide gel electrophoresis. The results are shown in Figure V.6. The cytokinin oxidase activities in the two fractions were electrophoretically distinct from each other. Peroxidase activity was detected in the cytokinin 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 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
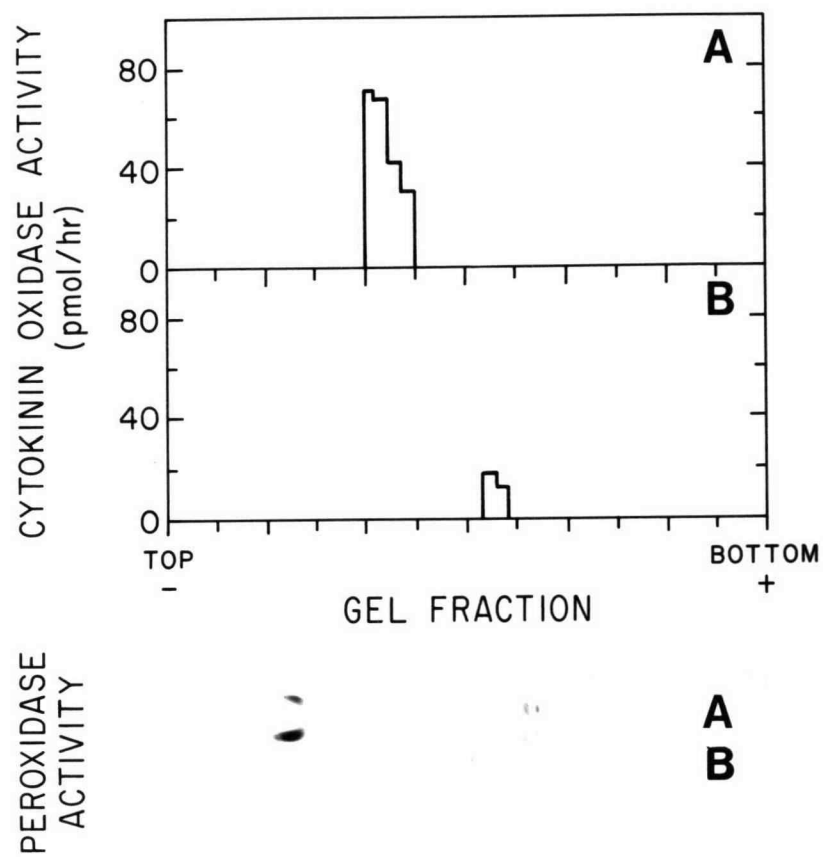
chromatography and chromatofocusing. The pI of the enzyme was estimated by chromatofocusing to be 5.0. Because the cytokinin oxidase activity eluted in the last third of the pH gradient, where the increasing ion strength of the Polybuffer could also become a factor in the elution, this pI estimate is 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 <u>Phaseolus</u> <u>vulgaris</u> L. cv. Great Northern have
been examined in the work reported here. An assay based on
the oxidation of radioactively labeled $N^6-(\triangle^2-$
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 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 i^6Ade 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 N-(2-chloro-4-pyridyl)-N'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 chromatigraphically 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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