AN ABSTRACT OF THE THESIS OF

Ana Abdelnour-Esquivel for the degree of Master of Science in Plant Physiology presented on April 9, 1990.

Thesis: Interactions of Cytokinins and Phenethylamines in Cell-Free Enzyme Systems and Plant Callus Cultures.

Redacted for privacy

Abstract approved: _____

Donald J. Armstrong

1-Phenyl-3-(2-Thiazolyl)-2-Thiourea (PTTU), has been reported to inhibit dopamine-$\beta$-hydroxylase (a copper-containing enzyme involved in the pathway of biosynthesis of adrenaline in animal systems). In the present study, PTTU has been shown to have cytokinin activity in the tobacco bioassay. The cytokinin activity of PTTU suggested that cytokinin-active $N^6$-substituted adenine derivatives might be effective in inhibiting the activity of dopamine-$\beta$-hydroxylase. Tests of a number of cytokinin-active adenine derivatives have demonstrated that most of these compounds inhibit the activity of cell-free preparations of dopamine-b-hydroxylase. $N^6$-cyclohexylmethyladenine (the most effective of the compounds) was as active as PTTU in inhibiting the enzyme. The cytokinin $N^6$-($\Delta^2$-isopentenyl)adenine ($i^6$Ade) was one of compounds effective in inhibiting dopamine-$\beta$-hydroxylase. No inhibition of the enzyme was obtained with the corresponding $N^3$ substituted compound, $N^3$-($\Delta^2$-isopentenyl)adenine (triacanthine).

The ability of dopamine-$\beta$-hydroxylase to degrade labeled $i^6$Ade was tested using a variety of conditions, but no evidence was obtained of

any enzymatic attack of the enzyme on this compound. Tyramine (a substrate of dopamine-$\beta$-hydroxylase), was tested as a competitive substrate for the enzyme-catalyzed degradation of $i^6$Ade-2,8-$^3$H by cytokinin oxidase (an enzyme involved in the degradation of cytokinins in plant tissues). Some inhibition of $i^6$Ade degradation was observed at very high tyramine concentrations.

The biological activities of phenethylamines were tested in several plant tissue culture systems. Tyramine and hordenine (N,N-dimethyltyramine) did not show cytokinin activity in the tobacco callus bioassay. Octopamine, tyramine, hordenine, and phenylethylamine inhibited the growth of callus tissues of cytokinin-dependent Nicotiana tabacum and promoted the formation of a dark pigment by the callus tissues. Cytokinin-dependent callus tissues of Phaseolus genotypes, which require high levels of cytokinins, were less sensitive than the tobacco callus to the inhibitory effects of the phenethylamines.

The growth inhibitory effect of tyramine in the cytokinin-dependent callus tissues of tobacco was completely reversed by increased concentrations of kinetin. The possible mechanisms of interaction of cytokinins and phenethylamine derivatives in tobacco callus cultures are discussed.

# Interaction of Cytokinins and Phenethylamines in Cell-Free Enzyme Systems and Plant Callus Cultures

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of
Master of Science

Completed April 9, 1990

Commencement June 1990

APPROVED:

Redacted for privacy

Professor in charge of major

Redacted for privacy

Chairman of Plant Physiology Program

Redacted for privacy

Dean of Graduate School

Date thesis is presented: April 9, 1990

# ACKNOWLEDGEMENTS

I would like to express my most sincere appreciation to Dr. Donald J. Armstrong for his guidance, supervision, patience, and support during my graduate program.

I would also like to thank Dr. Thomas Moore for all his support during my graduate study and the other members of my committee, Drs. Derek Baisted and Alan Bakalinsky.

I am very grateful for the financial support that the Organization of American States awarded to me to pursue a Master's degree.

Help from the faculty and staff of the Department of Botany and Plant Pathology has been greatly appreciated.

Thanks to my husband and daughter for their patience and understanding.

I would like to express special thanks to my parents for their encouragement and support throughout my life.

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## I. INTRODUCTION

Cytokinin oxidase is widely distributed in plant tissues. The enzyme catalyzes the oxidative degradation of cytokinins bearing an unsaturated isoprenoid side chain (zeatin, $N^6$-($\Delta^2$-isopentenyl)adenine, and their ribonucleosides). Cytokinin-active urea derivatives, such as thidiazuron, inhibit the degradation of labeled $i^6$Ade by cell-free preparations of cytokinin oxidase (Chatfield and Armstrong, 1986). In tests of a number of urea derivatives, Kaminek and Armstrong (personal communication) found that 1-phenyl-3-(2-thiazolyl)-2 thiourea (PTTU) also inhibited the degradation of $i^6$Ade by cytokinin oxidase. In addition, PTTU promoted betacyanin formation in the Amaranthus bioassay for cytokinins and exhibited affinity for the cytokinin-binding protein from wheat germ (Brinegar et al, 1985). PTTU is known to be a potent inhibitor of dopamine-$\beta$-hydroxylase, the enzyme that catalyzes the conversion of dopamine to the neurotransmitter and hormone norepinephrine in animal systems (Johnson et al., 1969, 1970).

The biological activity of PTTU in the tobacco callus bioassay for cytokinins has been established in the present study. Interactions of cytokinins and phenethylamines have been examined in plant callus cultures and in cell-free enzyme systems. These investigations have established that cytokinin-active adenine derivatives can serve as inhibitors of dopamine-$\beta$-hydroxylase and have confirmed and extended the

observations of Christou and Barton (1989) concerning the interactions of phenethylamines and cytokinins in the regulation of growth and pigment formation in tobacco tissue culture.

# II. LITERATURE REVIEW

## A. Structure of Cytokinins

The isolation of kinetin (6-furfurylaminopurine) from an acid hydrolysate of DNA and its identification as a cell division factor in tobacco tissue cultures (Miller et al., 1955) initiated the studies of cytokinins and cytokinin structure-activity relationships. 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 structural requirements for cytokinin activity have been investigated using several types of biological responses in a number of different systems. Biological effects of cytokinins for which structure-activity data are available include: the promotion of cell division in tissue cultures of tobacco (Skoog and Leonard, 1968; Skoog and Armstrong, 1970; Skoog, 1971), soybean (Miller, 1963), and carrot (Letham, 1963); the promotion of germination in lettuce seeds (Skinner, 1957, and 1958; Bruce et al., 1965); the promotion of cell enlargement in radish leaf disks (Matsubara, 1980); the retardation of senescence in leaf disks of various species (Bruce et al, 1965); the promotion of lateral bud outgrowth in peas (Bruce et al., 1965); and the promotion of betacyanin synthesis in Amaranthus seedlings (Biddington, 1973; Elliot, 1979; Kaminek, 1987). The bioassays based upon in vitro growth of tobacco, soybean, and carrot callus tissues have been characterized as the most sensitive, specific, and reliable (Skoog and Armstrong, 1970; Matsubara, 1980).

Two major classes of cytokinins are known: $N^6$-substituted adenine derivatives and phenylurea derivatives. The cytokinins that have been identified unequivocally as naturally occurring are all adenine derivatives, and most of these have an $N^6$-isoprenoid side chain. Modifications of the purine ring and changes in the length, substitution, and degree of saturation of the $N^6$-side chain influence the activity of cytokinin-active adenine derivative (Skoog et al., 1967; Skoog and Armstrong, 1970). Diphenylurea was the first urea derivative demostrated to have cytokinin activity (Shantz and Steward, 1955; Bruce et al, 1965). Diphenylurea is rather weakly active as a cytokinin, but phenylurea derivatives have been synthesized with cytokinin activity as high or exceeding that of the most active adenine derivatives (Okamoto et al., 1974 and 1978; Isogai et al., 1976; Takahashi et al., 1978). The structural requirements for cytokinin activity of urea derivatives appear to be an intact -NH-CO-NH- bridge and a phenyl ring attached to one side of the bridge (Matsubara, 1980). At least two types of phenylurea derivatives, the pyridylureas and the thiadiazolylureas, show very high cytokinin activity (Mok et al., 1982).

Whether $N^6$-substituted adenine derivatives and phenylurea derivatives exibit cytokinin activity as the result of similar or different modes of action is still not certain. One hypothesis proposes that cytokinin-active urea derivatives act indirectly by promoting the synthesis of cytokinin-active adenine derivatives (Miller, 1961). However, it has also been argued that both classes of cytokinins must have a common action site (Kurosaki et al., 1981).

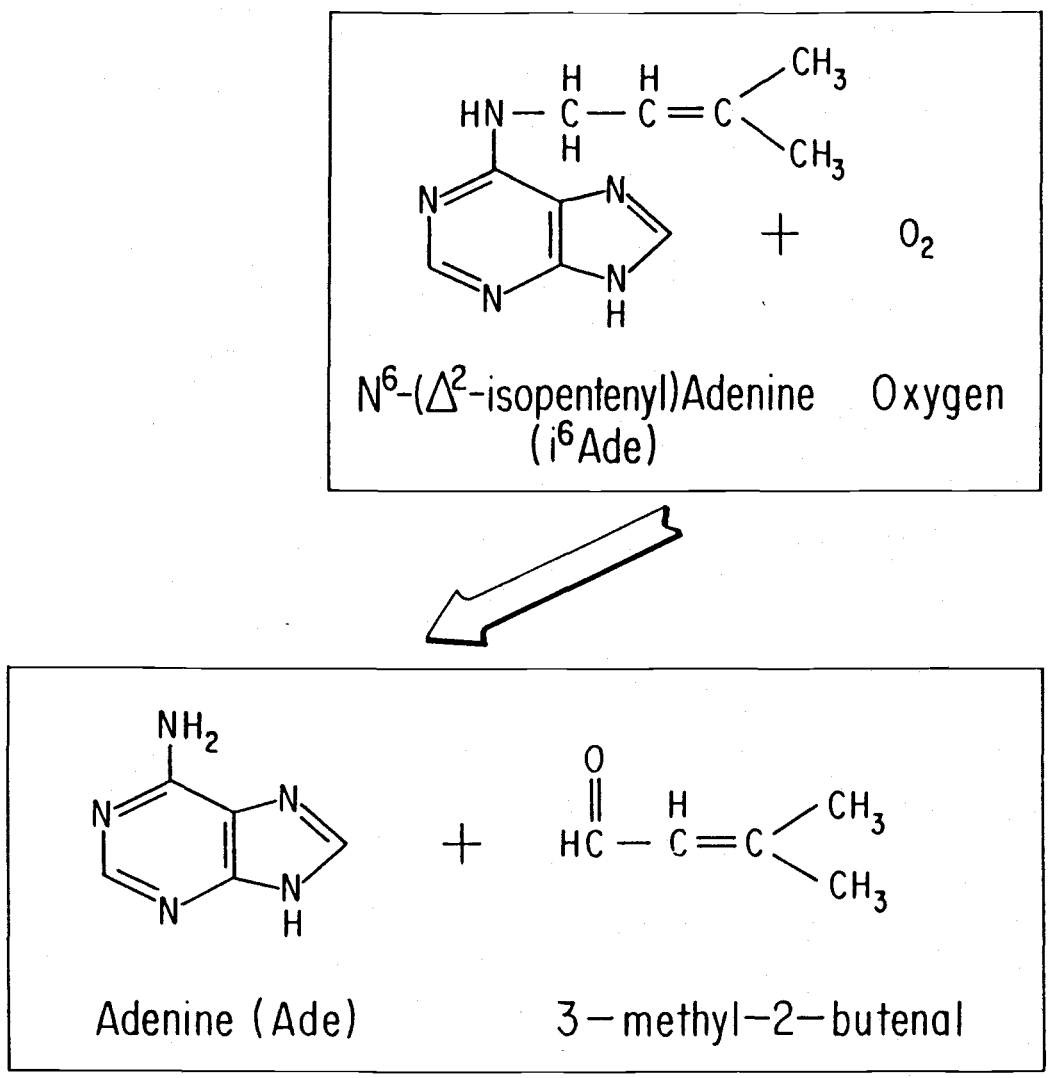
B. Cytokinin Oxidase

The degradation of cytokinins occurs as part of the regulatory control of cytokinin levels in plant tissues. The enzyme cytokinin oxidase catalyzes the degradation of cytokinins bearing unsaturated isoprenoid side chains. As shown in Figure 1, the products of the reaction are adenine or adenosine and an aldehyde derived from the $N^6$-side chain (Brownlee et al., 1973; Paces et al., 1971; Whitty and Hall, 1974; Paces and Kaminek, 1976; McGaw and Horgan, 1983). Molecular oxygen is required (Whitty and Hall, 1974; Chatfield and Armstrong, 1987), and an iminopurine intermediate has been isolated and identified from reaction mixtures containing 2-mercaptoethanol (Laloue and Fox, 1985).

Cytokinin oxidase activity was first detected in crude homogenates from callus tissues of Nicotiana tabacum (Paces et al., 1971). Similar enzyme activities have been demostrated in immature corn kernels (Whitty and Hall, 1974), Vinca rosea crown gall tissue (McGaw and Horgan, 1983), wheat germ (Laloue and Fox, 1989), Phaseolus callus tissues (Chatfield and Armstrong, 1986), and in the cellular slime mold Dictyostelium discoideum (Armstrong and Firtel, 1989). The latter is the only organism other than green plants from which a cytokinin oxidase activity has been obtained (Armstrong and Firtel, 1989).

The substrate specificity of cytokinin oxidase activities from several higher plant sources has been examined (Paces et al., 1971; Whitty and Hall, 1974; Paces and Kaminek, 1976; McGaw and Horgan, 1983; Chatfield and Armstrong, 1986; Laloue and Fox, 1989; Armstrong and Firtel, 1989; Kaminek and Armstrong, 1990). All enzymes from plant sources for which data are available appear to have very similar

Figure 1. Cytokinin oxidase reaction.



substrate specificities. A double bond in the $N^6$-side chain is essential for substrate activity, and the cytokinins $i^6$Ade, zeatin, and their ribonucleosides are substrates for cytokinin oxidase. The saturated analogs of zeatin and $i^6$Ade (dihydrozeatin and $N^6$-isopentyladenine, $hi^6$Ade), the cis-isomer of zeatin, and a number of synthetic cytokinins, including kinetin and $b^6$Ade, are resistant to degradation by cytokinin oxidase from higher plants. 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 has little effect on the ability of cytokinins to serve as substrates for cytokinin oxidase, but O-glucosylation confers resistance to attack by the enzyme (Paces et al., 1971; Paces and Kamenik, 1976; McGaw and Horgan, 1983; Chatfield and Armstrong, 1986).

The substrate specificity of cytokinin oxidase from the cellular slime mold, Distyostelium discoideum, has also been examined (Armstrong and Firtel, 1989). This enzyme resembles cytokinin oxidase from higher plants in its preference for $i^6$Ade as substrate. However, synthetic cytokinins ($b^6$Ade, kinetin and $hi^6$Ade) resistent to oxidation by cytokinin oxidase from higher plants sources were observed to inhibit the degradation of labeled $i^6$Ade in substrate competition tests with the Dictyostelium enzyme. Therefore, it appears likely that this enzyme has a somewhat broader specificity than the cytokinin oxidases from higher plants.

Cytokinin-active urea derivatives have also been tested in substrate competition tests with cytokinin oxidase. Diphenylurea and N-(2-chloro-4-pyridyl)-N′-phenylurea strongly inhibited the degradation of $i^6$Ado by cytokinin oxidase from corn, wheat germ and Vinca rosea (McGaw and

Horgan, 1983; Laloue and Fox, 1989)). Thidiazuron, another cytokinin-active urea derivative, was reported to be as active as unlabeled $i^6$Ade in inhibiting the degradation of labeled $i^6$Ade by cytokinin oxidase from Phaseolus vulgaris callus tissues (Chatfield and Armstrong, 1986) and from Dictyostelium (Armstrong and Firtel, 1989). Whether thiadiazuron is a substrate or an inhibitor per se of cytokinin oxidase has not yet been established.

The cytokinin oxidase from Phaseolus vulgaris cv Great Northern has been characterized as a glycoprotein as evidenced by its strong affinity for the lectin concanavalin-A (Chatfield and Armstrong, 1988). Burch and Horgan (1989) have reported that the cytokinin oxidases from corn kernels and wheat germ also bind to concanavalin-A. However, the cytokinin oxidase from P. lunatus cv Kingston callus tissues does not bind to the lectin (Kaminek and Armstrong, 1990).

Enhanced in vitro activity of cytokinin oxidase from callus tissues of P. vulgaris has been observed in the presence of copper-imidazole complexes (Chatfield and Armstrong, 1987). The mechanism by which copper and imidazole enhance the activity of the enzyme has not been established. Based on the fact that the cytokinin oxidase reaction is not inhibited by anaerobic condition in the presence of copper-imidazole, it appears that these complexes are substituting for oxygen in the reaction mechanism by which cytokinin oxidase effects cleavage of the $N^6$-side chain of $i^6$Ade.

The degradation of $b^6$Ade and kinetin has been observed in some plant tissues. Therefore, cytokinin degradation in these tissues involves an enzyme system with properties distinct from the cytokinin oxidases described to date. A benzoic acid derivative appears to be the product

of the *in vivo* cleavage of $b^6$Ade (Fox *et al.*, 1972).

Degradation of cytokinins in mammalian and avian systems appears to be due to the activity of aminohydrolases (Hall *et al.*, 1971; Hall and Mintsioulis, 1973) or to xanthine oxidase (Chheda and Mittelman, 1972; Chen *et al.*, 1975).

C. Dopamine-$\beta$-Hydroxylase

Dopamine-$\beta$-hydroxylase is a mixed function oxidase that in mammalian systems is involved in the pathway of biosynthesis of adrenaline from tyrosine (Figure 2). As shown in Figure 3, dopamine-$\beta$-hydroxylase catalyzes the conversion of dopamine (3,4-dihydroxyphenylethylamine) to the neurotransmitter and hormone noradrenaline (norepinephrine) (Ljones and Skotland, 1984; Steward and Klinman, 1988). In addition to dopamine, the enzyme is capable of catalyzing the oxidation of phenylethylamine, epinine, tyramine, and other amines to the corresponding $\beta$-alcohol derivative (Pisano *et al.*, 1960; Friedman and Kaufman, 1965; Goldstein, 1966; Ljones and Skotland, 1984). Hydroxylations catalyzed by this enzyme have been observed in toad, cod, lobster, insects, fungi, and plants, as well as in mammalian systems (Steward and Wheaton, 1964; Sekeris and Karlson, 1966; Deacon and Marsh, 1971; Ljones and Skotland, 1984). However, most of the published work on this enzyme is related to its role in neurology and medicine.

The mammalian dopamine-$\beta$-hydroxylase has been characterized as a tetrameric, copper-containing glycoprotein that requires dioxygen and an exogenous electron donor such as ferricyanide (ascorbate *in vivo*) (Levin *et al.*, 1960; Wallace *et al.*, 1973). Copper is essential for catalytic activity and undergoes reduction and oxidation during the enzyme-

Figure 2. Biosynthesis of adrenaline.

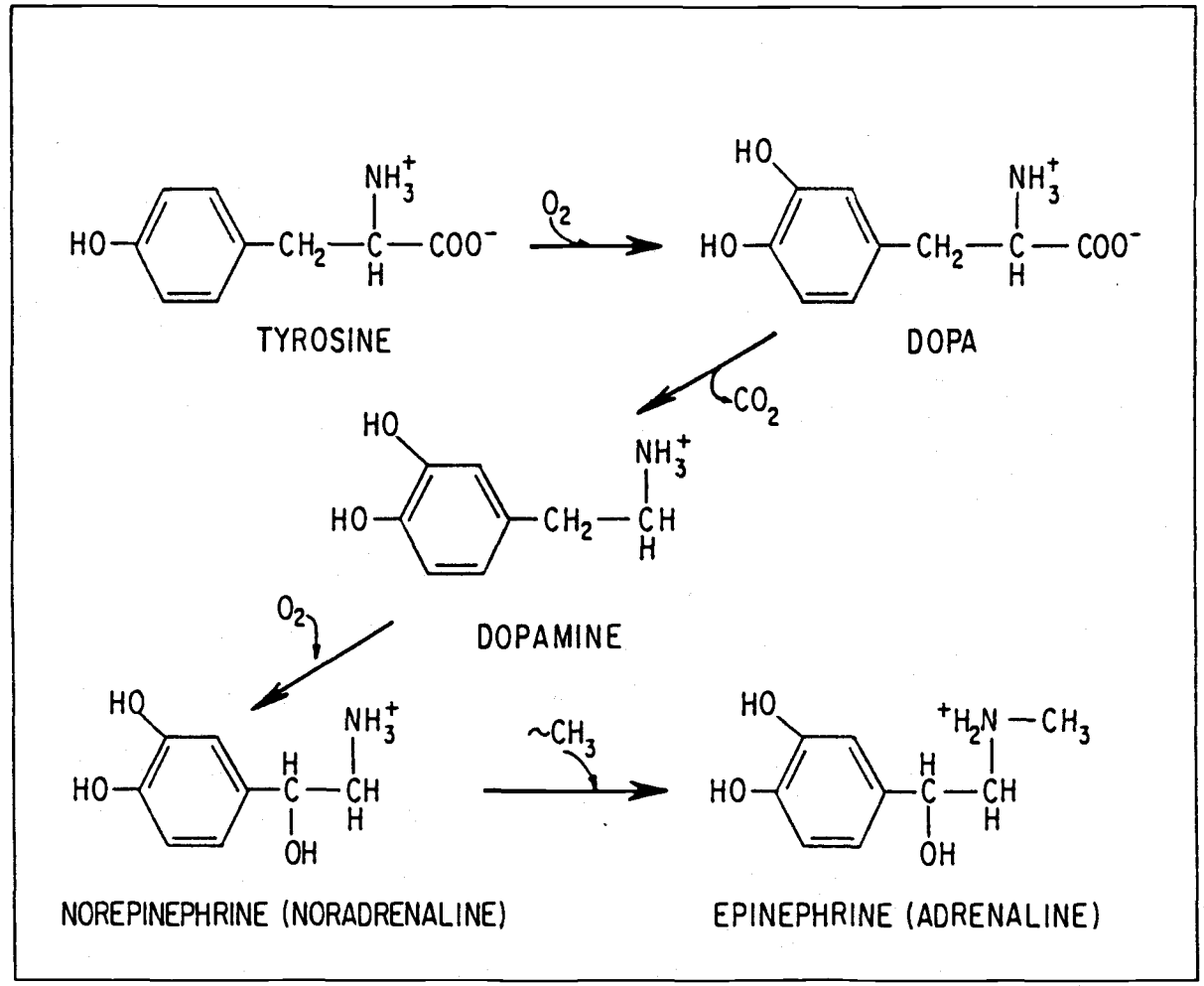
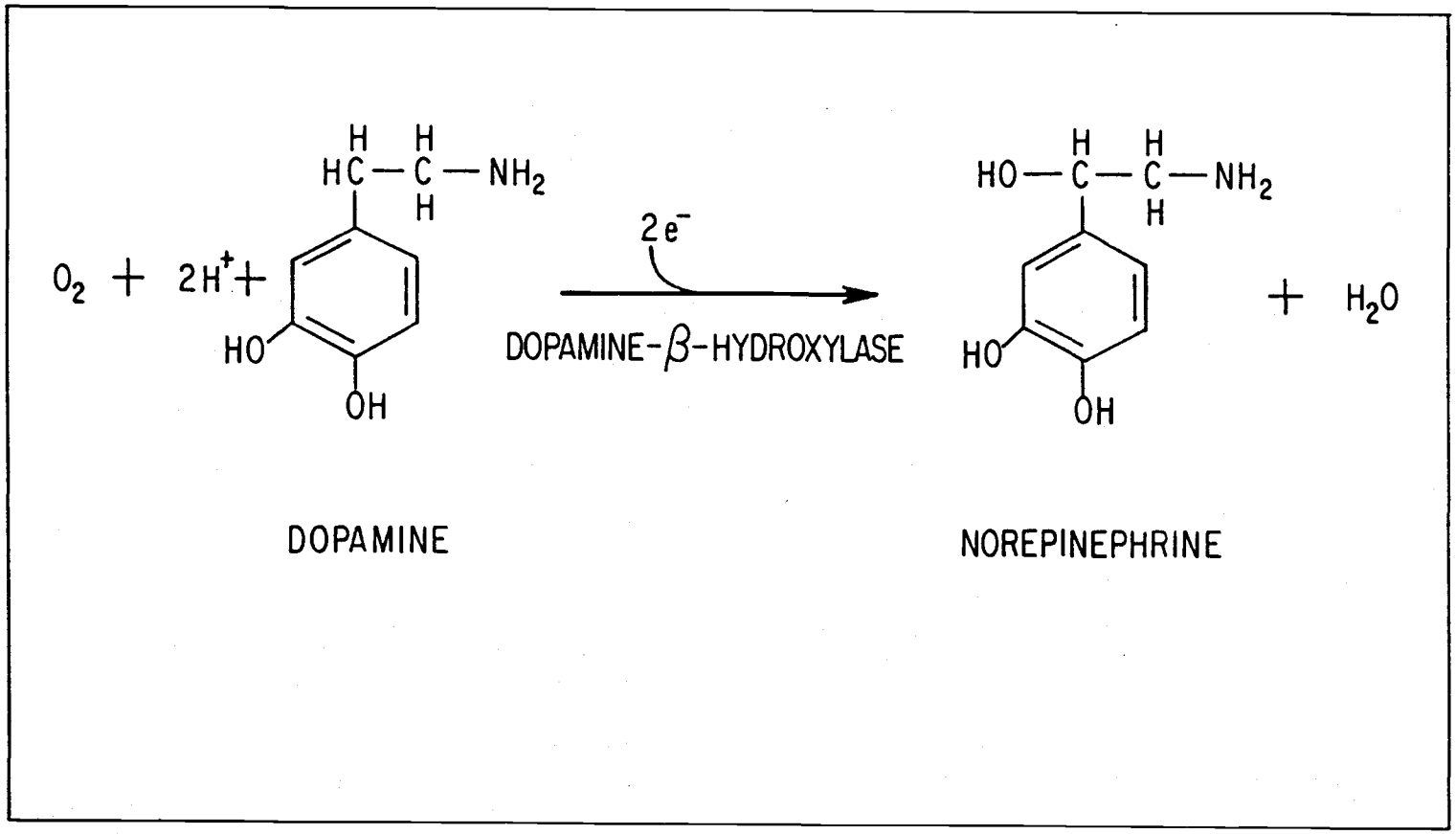


Figure 3. Dopamine-β-hydroxylase reaction.



catalyzed hydroxylation reaction (Kaufman and Friedman, 1965). In mammalian systems, dopamine-$\beta$-hydroxylase exists in both a soluble and a membrane-bound form. The soluble enzyme appears to be composed of four identical subunits. The membrane-bound form is also a tetramer, but it is composed of two subunits similar to those of the soluble enzyme and two subunits of distinctly larger size (Steward and Klinman, 1988).

The ability of dopamine-$\beta$-hydroxylase to catalyze the hydroxylation of tyramine to octopamine has often been used as an assay for the enzyme and to study the *in vitro* effects of inhibitors. Tyramine is more stable than dopamine, gives slightly higher activity, and yields data for which the kinetic interpretation is easier than with dopamine, which also functions as an electron donor (Pisano *et al.*, 1960; Creveling *et al.*, 1962; Wallace *et al.*, 1973).

Dopamine-$\beta$-hydroxylase is inhibited by compounds that block the formation of the catalytic copper-oxygen intermediates, either by forming an enzyme-Cu-chelator complex or by removing the copper from this metalloenzyme (*e.g.* sulfhydryl compounds, disulfiram and its reduced metabolite diethyldithiocarbamate, aromatic and alkylthioureas, phenylethylamine isomers (Johnson, 1969; Jonsson, 1967; Nagatsu *et al.*, 1967; Ljones and Skotland, 1984). Another class of dopamine-ß-hydroxylase inhibitors are aromatic compounds containing nitrogen at the benzylic carbon (*e.g.* N-phenylethylenediamines, benzylhydrazine). It has been postulated that the oxidation at the nitrogen leads to a homolytic cleavage producing benzyl radicals that interact with easily oxidized active-site residues and inactivate the enzyme (Fitzpatric and Villafranca, 1986; Steward and Klinman, 1988).

1-Phenyl-3-(2-thiazolyl)-2-thiourea (U-14,624, PTTU) has been reported to be a potent inhibitor of mammalian dopamine-$\beta$-hydroxylase, both *in vitro* and *in vivo*. When compared *in vitro* with many other aromatic and alkyl thioureas, PTTU was only surpassed in activity by disulfiram. However, when these two compounds were compared *in vivo* as inhibitors of dopamine hydroxylase, PTTU was as effective as disulfiram at half its concentration, and it was less toxic (Johnson *et al.*, 1969 and 1970; Von Voigtlander and Moore, 1970). Chelation of copper ion in dopamine hydroxylase has been proposed as the mechanism of inhibition by PTTU (Johnson *et al.*, 1969 and 1970). Like other inhibitors of dopamine-$\beta$-hydroxylase, PTTU has been used as tool in pharmacological and clinical studies. PTTU exhibits antihypertensive effects and acts as a central nervous system depressant (Johnson *et al.*, 1970; Von Voigtlander and Moore, 1970). In animal tests, PTTU also enhanced morphine analgesia and reduced the development of drug tolerance and dependence (Bhargava and Way, 1974).

$N^3$-substituted adenine derivatives have also been reported to inhibit the activity of dopamine-$\beta$-hydroxylase in cell-free systems (Fujii *et al.*, 1979). $N^3$-cyclohexylmethyladenine was among the most active analogues tested.

## D. Phenethylamine Metabolism in Plants

### D.1. Products of Phenethylamine Metabolism in Plants

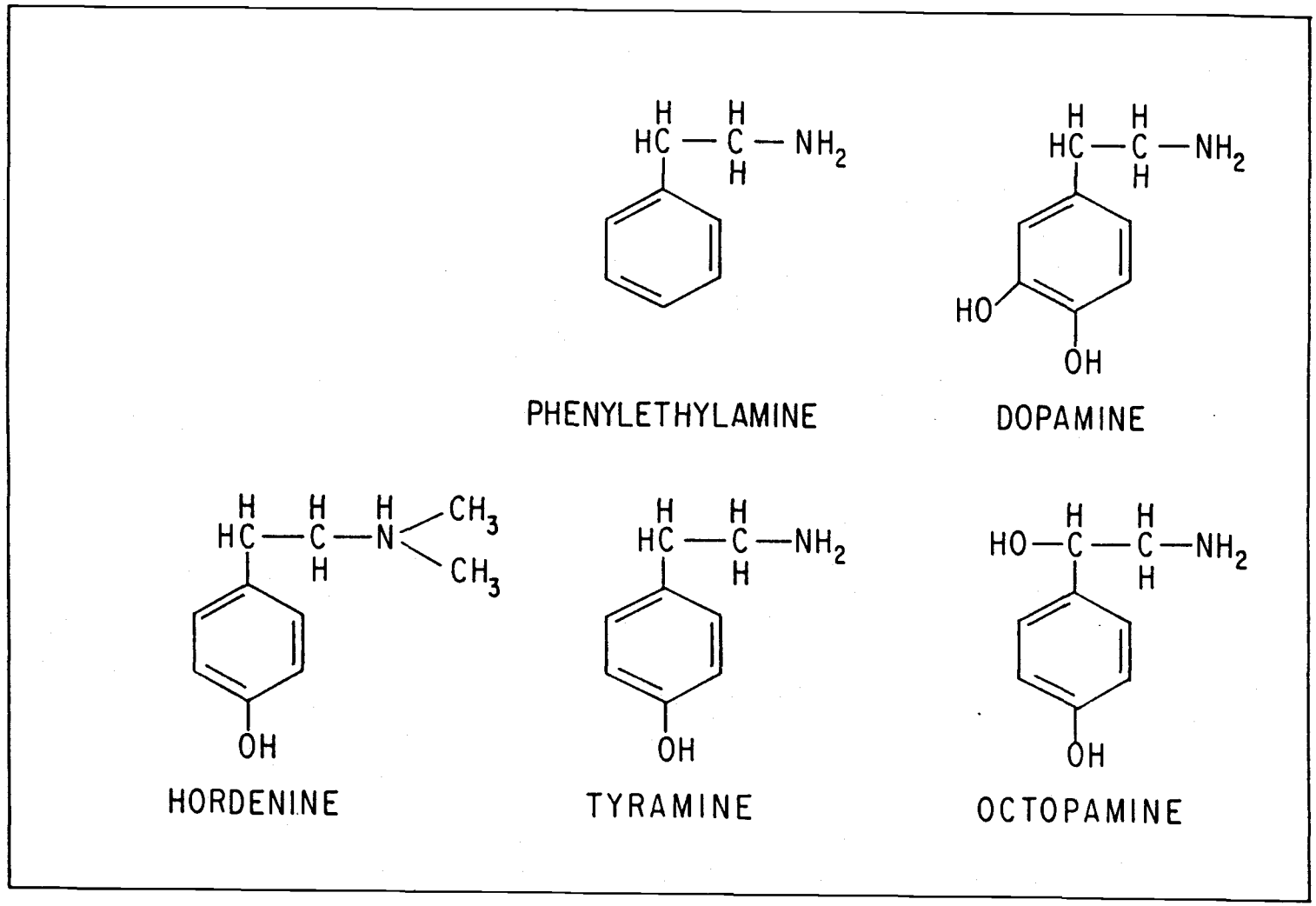
Phenethylamines are related to the central metabolism of plants through tyrosine and are associated with the synthesis of lignins,

alkaloids, and the pigments known as betalains (betacyanin and betaxanthin) and melanins. The structures of some phenethylamines are shown in Figure 4.

D.2. Distribution of Phenethylamines in Plants

A wide variety of phenethylamines have been identified in a number of plant species. Synephyne and octopamine have been identified in citrus (Steward and Wheaton, 1964) and in annual rye grass (Hardwich, 1969). Phenylethylamine, tyramine, dopamine, and noradrenaline have been isolated from banana fruit and peel (Udenfriend, et al., 1959; Palmer, 1963), and dopamine has been found in Papaver somniferum latex (Homeyer and Roberts, 1984). Hordenine (N,N-dimethyltyramine), N-methyltyramine, synephrine, and a number of phenethylamines derivatives have been identified in cactus species (McLaughlin and Paul, 1965; Neal et al., 1971; Rainieri and McLouughlin, 1977). In an extensive study of the phenolic amine content of plants, Wheaton and Steward (1970) examined 188 species from 97 families. Tyramine was found in 15% of the species under study. Members of the Anacardiaceae, Asteridae, Cyperaceae, Fabaceae, and Loranthaceae are among the species producing tyramine. N-methyltyramine and hordenine were less generally distributed. N-methyltyramine was identified in species of the Amaryllidaceae, and hordenine was found in the Berberidaceae family. Octopamine and sinephrine were identified in species of Rutaceae (citrus), Solanaceae (bell pepper), Lilliaceae, Cyperaceae, and Amarillidaceae.

Figure 4. Structure of phenethylamines.



D.3. Alkaloid formation from Phenethylamines

Dopa, dopamine, tyramine, N-methyltyramine, and hordenine (N,N-dimethyltyramine) are simple plant alkaloids derived from tyrosine. Some other alkaloids derived from phenethylamines are: anhalonidine in peyote cactus, salutadirine in opium poppy, and papaverine and sanguinarine in poppy. (Leete et al., 1952; Fodor, 1980; Cline and Coscio, 1987). In plant and mammalian systems, dopamine is the product of the decarboxylation of dopa (3,4-dihydroxyphenylalanine) which is formed from tyrosine by tyrosinase (a phenol oxidase) (Goodwin and Mercer, 1985; Stryer, 1988). In plants, dopamine can also be synthesized from tyramine. Tyramine is formed by decarboxylation of tyrosine. The hydroxylation of tyramine by a phenol oxidase gives rise to dopamine (Deacon and Marsh, 1970). N-methyltyramine and hordenine are formed in barley by methylation of tyramine (Leete et al., 1952).

The synthesis of N-methyltyramine and hordenine from tyramine in barley has been intensively examined (Mann et al., 1963; Steinhart et al., 1964; Mann and Mudd, 1963). Leete et al., (1952) administrated tyramine-$\alpha$-$C^{14}$ to sprouting barley and radioactive N-methyltyramine and hordenine were isolated from the roots. The radioactivity was found in the $\alpha$-carbon of these two species, but the activity of N-methyltyramine was 10 times that of hordenine. From these results, it was concluded that tyramine undergoes methylation in the barley roots to N-methyltyramine and hordenine. The enzyme responsible for these methylations, tyramine methylpherase, requires S-adenosylmethionine as a methyl donor (Mann and Mudd, 1963).

The synthesis of N-methyltyramine and hordenine are developmentally regulated in barley. These compounds were absent from barley seeds, but appeared in the roots of seedlings during the first day of germination (Mann et al., 1962). The concentration of these alkaloids reached a maximum in 4 to 5 days, and a month later, only traces could be detected. The decrease in the concentration of N-methyltyramine and hordenine was related to changes in metabolic functions associated with tissue maturation or differentiation. When the time courses of tyramine methylpherase activity was studied, it was demonstrated that the enzyme was absent from ungerminated seeds and upon germination rose to high levels of activity that were maintained until the end of the first week after germination, after which activity fell. The changes in tyramine methylpherase level correlated with the accumulation and disappearance of N-methyltyramine.

D.4. Pigment Formation from Phenethylamines

The biological pigments known as melanins and betalains are derived from phenolic amines.

Melanin is the common name given to dark brown and black pigments formed in biological systems. Melanins are the product of the oxidation of tyrosine or closely related compounds. In the first two steps in melanogenesis, these compounds are oxidized by the action of phenol oxidase to quinones, which by oxidative polymerization form the melanin. These melanin macromolecules are then conjugated with proteins (Thomson, 1976).

The melanins have been classified as eumelanins (which contain nitrogen) and catechol melanins (which lack nitrogen). Animal melanins are eumelanins. Plant melanins have been characterized as catechol

melanins based on the fact that they produce catechol as a product of degradation. However, due to the presence of phenylethylamines and phenol oxidases in cells that produce these pigments, it has been suggested that eumelanins also occur in plants (Goldwin and Mercer,1983).

Dark pigmentation is common in plants and may arise during normal development (e.g. black spots in Vicia faba) or as a result of injury, senescense, or death, but few of these pigments have been studied chemically (Thomson, 1976). The browning reactions in banana have been reported to result from oxidation of dopamine and tyramine by a monophenol oxidase (Palmer, 1963; Deacon and Marsh, 1970).

Betalains are water-soluble plant pigments found in a few, closely related, families of the order Centrospermae (e.g. Amaranthaceae, Cactaceae, Chenopodiaceae, and Portulacaceae among others) and in a few mushrooms (e.g. in the red cap of the agaric Amanita muscaria). Betacyanins are red-violet pigments, and betaxanthins are orange and yellow pigments (Mabry, 1980).

Betalains are formed from two molecules of dopa (Mabry, 1980). One dopa residue is oxidized to dopaquinone which spontaneously cyclises to cyclodopa. The other dopa molecule undergoes ring cleavage and recyclization to form betalamic acid. After glycosylation of cyclodopa, the two products condense to form betacyanins. If the betalamic acid condenses with some imino or amino acid, betaxanthins are formed (Mabry, 1980; Piatelli, 1981; Elliot, 1983). Experiments with radioactive precursors have shown the incorporation of tyrosine and dopa into indicaxanthin and betanin in Opuntia, amaranthin and betanin in Amaranthus seedlings, and into betanin in Beta vulgaris (Minale et al.,

1965; Garay and Towers, 1966; Liebisch et al., 1969; Bianco-Colomas, 1980; Elliot, 1983). Koehler (1972) demostrated the inhibition of betacyanin synthesis by the copper chelator phenylthiocarbamate.

The biosynthesis of betalains has been studied extensively in Amaranthus species. Bianco-Colomas (1980) studied qualitative and quantitative aspects of amaranthin biosynthesis in Amaranthus caudatus seedlings and found that addition of exogenous dopa led to diversified pigment formation. Besides amaranthin and betanin, another pigment was always present in the chomatography fractions. This substance showed an absorption maximum at 475 nm. When crude seedlings extracts were incubated with dopa for 30 min at 23 C, the same orange compound was detected. When the incubation lasted longer (120 min), the coloration became darker and finally black. The pigment was suggested to be dopachrome, an orange intermediate of melanogenesis with an absorption maximum at 475 nm. The same intermediate was recognized by Jimenez et al. (1984) as one of the products of dopamine oxidation by mushroom tyrosinase. They found that the steps for dopamine transformation to dopaminechrome were as follows: Dopamine to o-dopaminequinone-$H^+$ to o-dopaminequinone to leukodopaminechrome to dopaminechrome. No participation of oxygen was detected in the last step. Therefore, they proposed an Enzymatic-Chemical-Chemical mechanism for the reaction sequence.

The regulation of betalain synthesis in Amaranthus seedlings has been the subject of numerous investigations (Piatelli et al., 1970; Elliot, 1983; Stobart et al., 1979). Light is an absolute requirement for betalain formation in some species, and phytochrome appears to be involved in the regulation. It has been postulated that phytochrome

controls the synthesis of the enzymes involved in the process by regulating gene expresion (Piattelli, 1976). Testing the effect of Actinomycin D (a strong inhibitor of DNA-dependent RNA synthesis), and puromycin (an inhibitor of protein synthesis) on the synthesis of amaranthin in Amaranthus tricolor seedlings, Piattelli et al., (1970) found that inhibition of RNA and protein synthesis inhibited the synthesis of the pigment. This result supports the idea that light-stimulated synthesis of amaranthin involves the activation of genes controlling enzyme synthesis. The same results were obtained by Koehler (1972) and Guidici de Nicola et al. (1972) in A. caudatus seedlings.

## E. Cytokinin Effects on Phenethylamine Metabolism in Plants

### E.1. Effect of Cytokinins on Alkaloid Formation

Studies of the effect of cytokinins on alkaloid formation are limited, but effects of kinetin on the synthesis of N-methyltyramine and hordenine in barley seedling have been examined in some detail (Steinhart et al., 1964; Piattelli et al., 1970). Indirect evidence that cytokinins influence the synthesis of sanguinarine, an alkaloid from Papaver bracteateum, has also been reported (Cline and Cosio, 1987).

The formation of sanguinarine in cell suspension cultures of Papaver bracteatum growing in the presence of 2,4-D and $N^6$-benzyladenine was examined by Cline and Cosio (1987). The treatment of the cultures with a fungal elicitor preparation from a host-specific pathogen (Dendryphion penicillatum) resulted in a two-fold increase in sanguinarine levels compared to untreated control. The levels of dopamine (a precursor of sanguinarine) fluctuated but remained in the millimolar range. When the

elicitor from a general pathogen (Verticillium dahliae) was added to the cell suspension cultures, sanguinarine accumulation was dependent on elicitor dose, and the concentration of dopamine decreased in the treated cells. At the highest dose of the latter elicitor, sanguinarine and dopamine levels fell, and browning of the cells and cessation of growth was observed. When these tests were done in absence of the hormones, the cell suspensions treated with the elicitor from the general pathogen (Verticillium) produced sanguinarine in concentrations comparable to those produced in presence of the hormones, but the development of a browish-orange pigment in the medium and darkening of the cells occurred sooner than in the presence of hormones. Dopamine concentration decreased.

The synthesis of N-methyltyramine and hordenine in Hordeum vulgaris (barley) seedling has been studied intensively. Tyramine methylpherase is the enzyme that catalyzes the methylation of tyramine in this system. Mann et al. (1963), testing the effect of various compounds as stimulators of tyramine methylpherase activity in barley embryos, found that kinetin was the only compound that exerted a prolonged effect. Kinetin increased the activity of tyramine methylpherase even after the fifth day of germination. The effect of kinetin on the activity of tyramine methylpherase was examined by Steinhart et al.(1964) in barley embryos cultured on medium supplemented with a range of kinetin concentrations. All kinetin concentrations tested increased the levels of tyramine methylpherase in the embryos, and the maximal stimulation occurred at $4.6 \times 10^{-7}$ M kinetin. The levels of N-methyltyramine and hordenine were not affected at day 4, but at day 7, the concentrations of these alkaloids were twice as high as those in controls without

kinetin. This increase in alkaloids acompanied the increase in tyramine methylpherase resulted from the kinetin treatments. Based on the results of studies with inhibitors of RNA and protein synthesis, it was postulated that the kinetin-increased enzyme activity resulted from stimulation of enzyme synthesis rather than retardation of inactivation. $N^6$-benzyladenine and $N^6$-anilinopurine also stimulated the increase in tyramine methylpherase activity. The effects of other hormones were also tested. Gibberellic acid was ineffective. IAA and 2,4-D promoted some elevation of enzyme but less than half that given by kinetin. Kinetin caused additional stimulation in auxin-treated embryos, but this interaction was not investigated further.

E.2. Effect of Cytokinins on Betalain Formation

Effects of cytokinins on betalain synthesis have been demonstrated in Amaranthus. Koehler (1972) reported that kinetin markedly increased the light-stimulated synthesis of betacyanin in A. caudatus. Piattelli et al. (1970), working with A. tricolor seedlings, found that kinetin stimulated the synthesis of amaranthin in the dark and that the amount of pigment was proportional to kinetin concentration. Amaranthin synthesis showed a lag period of 10 hours from the time of application of the hormone and reached a maximum at 48 hours. After this period, a decrease in the pigment was observed. To test the interaction of kinetin and phytochrome, seedlings were treated with the hormone and irradiated with far-red light (converting $P_{FR}$ to $P_R$, the physiologically inactive form of phytochrome). In all conditions tested, far-red irradiation did not modify the kinetin-induced amaranthin synthesis. Therefore, it was concluded that the kinetin effect was not related to activation of phytocrome. Tests with actinomycin D and puromycin

demonstrated that the effect of kinetin was dependent on RNA and protein synthesis. When actinomycin was applied before kinetin, the synthesis of amaranthin was completely suppressed. When the antibiotic was given at increasing times after the application of kinetin, its effectiveness was progressively smaller, and when Actinomycin-D was given at the end of the lag period, the synthesis of amaranthin was not blocked at all. Based on these results it was suggested that mRNA synthesis required for pigment synthesis was induced shortly after kinetin application and that once induced the pigment forming system was stable. From these observations it was also concluded that kinetin substituted for the phytochrome effect in seedlings growing in the dark. Similar results were obtained by Piattelli et al. (1971) and Koehler (1972), and Stobart and Kinsman (1977).

Effects of metal ions on the synthesis of amaranthin have also been demostrated. Potassium, according to Stobart et al. (1979), enhanced the synthesis of amaranthin in seedlings of Amaranthus caudatus given a light treatment and in seedlings growing in the dark given a kinetin treatment. Based on experimentation with labeled tyrosine, they suggested that potassium stimulated the formation of amaranthin by increasing the active pool of tyrosine in the seedlings. Calcium ions also stimulated pigment formation (Stobart et al., 1979; Vallon et al., 1988). The synthesis of amaranthin induced by kinetin in Amaranthus was correlated with changes in intracellular calcium levels (Elliot et al., 1983, Vallon et al., 1988). The effect of calcium and calmodulin antagonists on the synthesis of betacyanin in Amaranthus cotyledons have

provided evidence that calcium and the calcium-dependent regulator protein calmodulin are involved in the regulation of pigment formation in Amaranthus (Elliot et al., 1983, Vallon et al., 1988).

F. Interaction of Cytokinins and Phenethylamines in Plant Callus Cultures

An interaction of phenethylamines and kinetin in cell cultures of tobacco, soybean, corn, and sunflower was reported by Christou and Barton (1989). They observed the formation of a black pigment and cessation of growth in cells treated with 10 mM octopamine, tyramine, synephrine, or epinephrine. When the callus tissues were transfered to media containing 1 mM kinetin, all callus types in the presence of octopamine survived and continued to grow. Synephine and epinephrine were lethal to the tissues. Callus cultures with cytokinin automomy (crown gall tumor tissue generated by inoculation of tobacco with Agrobacterium A208) survived the treatment with octopamine despite the accumulation of the compound in the cells. Christou and Barton (1989) suggested that the phenethylamines were acting as cytokinin antagonists in this system.

# III. MATERIALS AND METHODS

## A. Chemicals

1-Phenyl-3-(2-thiazolyl)-2-thiourea (PTTU), tyramine-HCl, octopamine-HCl, hordenine-hemisulfate, phenylethylamine-HCl, triacanthine, and commercial preparations of cytokinins were purchased from Sigma. Piçloram (4-amino-3,5,6-trichloropicolinic acid) was purchased from Aldrich Chemical Co.

$N^6$-Cyclohexanemethyladenine ($hb^6Ade$) and $N^6$-isopentyladenine ($hi^6Ade$) were synthesized as described by Kim and Armstrong (1986) and Leonard et al. (1968), respectively. $N^6$-($\Delta^2$-Isopentenyl)adenine-2,8-$^3H$ ($i^6Ade$-2,8-$^3H$) was synthesized as described by Chatfield and Armstrong (1987).

Dopamine-$\beta$-hydroxylase (from bovine adrenals, 2.7 units/mg protein) and catalase (from bovine liver, 14,000 units/mg protein) were purchased from Sigma. Polymin P (polyethyleneimine, 50% w/v) was obtained from Miles Laboratories, Inc. Polyvinylpolypyrrolidone (PVPP) was purchased from Sigma. Porapak Q (100-120 mesh) was purchased from Waters Associates.

## B. Tissue Culture

The cytokinin-dependent callus tissues of Nicotiana tabacum cv Wisconsin 38 used in this study were taken from 3- to 4-week old stocks maintained on medium containing the inorganic nutrients defined by Murashige and Skoog (1962) and the following organic substances (as

specified by Linsmaier and Skoog, 1965): thiamine-HCl (400 μg/l), myo-inositol (100 mg/L), sucrose (30 g/L), indole-3-acetic acid (IAA) (2 mg/L), kinetin (0.15 μM) and Difco Bacto-agar (10 g/L).

Cytokinin-dependent callus tissues of Phaseolus vulgaris cv Great Northern, P. vulgaris P.I. 200960, and P. lunatus cv Kingston were taken from 3-to 4-week old stocks mantained on media containing the inorganic nutrients defined by Murashige and Skoog (1962) and the following organic substances: thiamine-HCl (1 mg/L), nicotinic acid (5 mg/L), pyridoxine-HCl (0.5 mg/L), myo-inositol (100 mg/L), sucrose (30 g/L), picloram (2.5 μM), kinetin (5 μM), and Difco Bacto-agar (10 g/L).

The pH of all media was adjusted to 5.7 prior to the addition of agar. The agar was dissolved by heating (120 C for 10 min), and each medium was dispensed into 125 ml Erlenmeyer flasks (50 ml per flask) and sterilized by autoclaving at 120 C for 15 min.

For cytokinin activity tests, stock solutions of the test compounds were cold-sterilized and added to the culture flasks after autoclaving and prior to solidification of the medium. PTTU and kinetin were cold sterilized by dissolving these compounds in dimethylsulfoxide (DMSO) (Schmitz and Skoog, 1970). Aliquots (0.05 ml/50 ml medium) of the DMSO stock solutions were added to the autoclaved culture flasks. Aqueous stock solutions of phenylethylamine derivatives were cold sterilized by Millipore filtration. Aliquots (0.625 ml/50 ml medium) of the sterilized aqueous stock solutions were added to the autoclaved culture media.

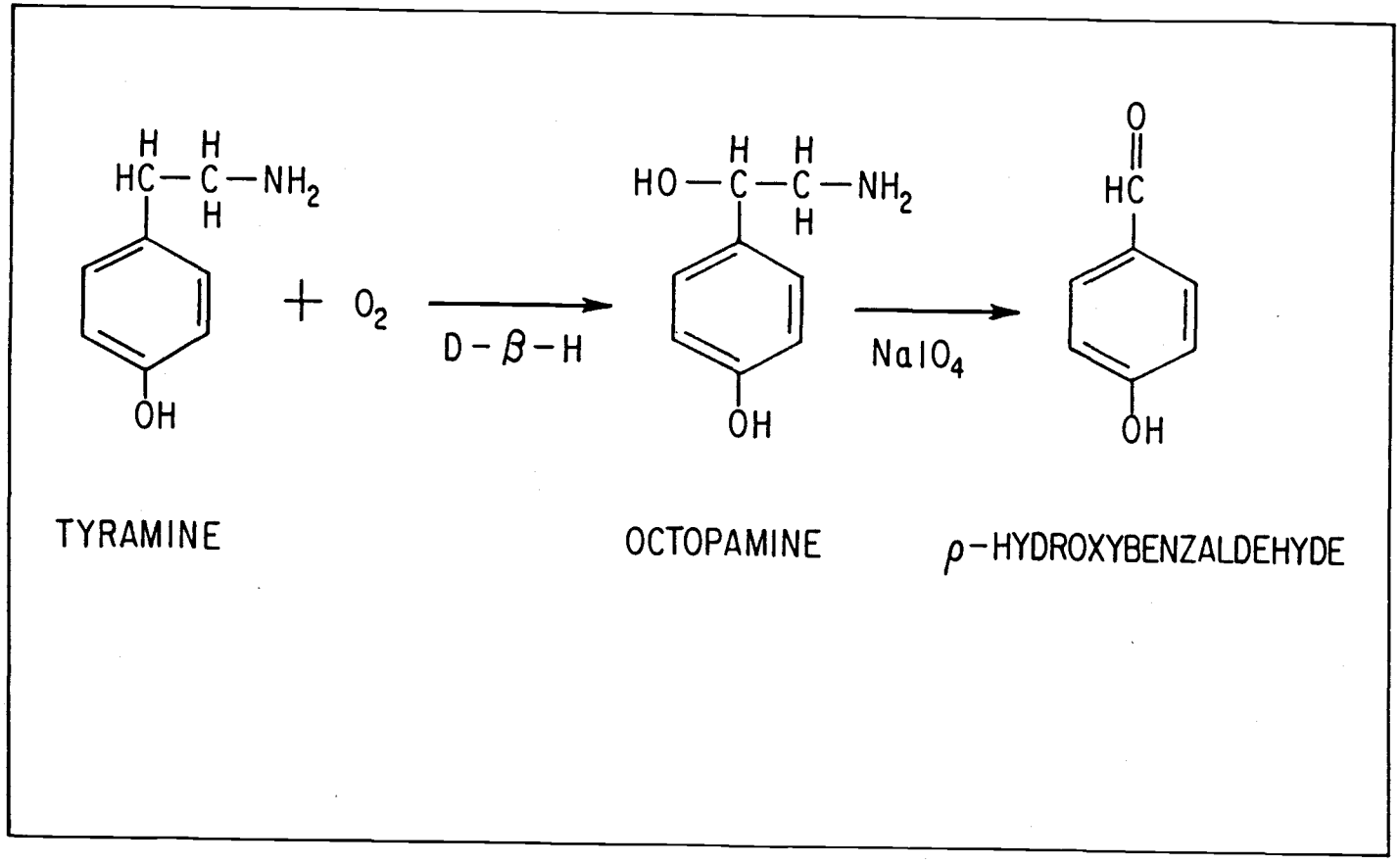
All callus tissues were grown at 28 C in the dark. Three pieces of stock callus tissue, weighing about 25 mg each, were planted per flask. Stock cultures were transferred at 3-week intervals. For growth

experiments, Phaseolus callus tissues were harvested and weighed after a 4-week growth period. Nicotiana callus tissues were harvested and weighed after a 5-week growth period. Unless otherwise indicated, fresh weight values are the average of two experiments using four replicate flasks for each treatment.

C. Assay of Dopamine-$\beta$-Hydroxylase Activity

The activity of dopamine-ß-hydroxylase was determined by the assay procedure described by Wallace et al. (1973), which is based upon the enzymatic oxidation of tyramine to octopamine (Figure 5). The octopamine formed is converted to p-hydroxybenzaldehyde by chemical oxidation with periodate, and this product is measured spectrophotometrically without separation from starting material. The standard incubation mixture contained 200 mM sodium acetate (pH 5), 4 mM tyramine-HCl, 10 mM sodium fumarate, 10 mM ascorbic acid, 8,000 units of catalase, and 0.1 units dopamine hydroxylase in a reaction volume of 2 ml. Compounds tested as inhibitors of dopamine-$\beta$-hydroxylase were dissolved in 50% dimethylformamide (DMF) and added to the reaction mixtures in 10 $\mu$l volumes. (The final DMF concentration in the reaction mixture was 0.5%.) The reaction mixtures were incubated for 20 min in a shaking-water bath at 37 C. The reactions were stopped by the addition of 0.4 ml of 4 N ammonium hydroxide followed by 0.8 ml of 2% (w/v) sodium periodate. After a 4 min incubation at room temperature, 0.8 ml of 10% (w/v) sodium bisulfite was added to reduce the excess periodate. Absorbance was measured at 330 nm against a blank containing no enzyme.

Figure 5. Dopamine-β-hydroxylase assay.



D. Preparation of Polyvinylpolypyrrolidone (PVPP), Porapak Q, and Polymin P

PVPP was prepared for use by boiling in 1 N HCl for 10 min. The adsorbent was washed with two bed volumes of 1 N HCl and then with double distilled water until the pH reached 5 or higher. The PVPP was dried at 60 to 80 C for 24 to 48 hours and stored until needed. Prior to use, the PVPP was hydrated in the appropriate buffer.

Porapak Q (100-120 mesh) 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.

E. Extraction and Assay of Cytokinin Oxidase Activity

Cytokinin oxidase activity was extracted from 17- to 18-day old callus tissues of Phaseolus vulgaris cv Great Northern as described by Chatfield and Armstrong (1986). The tissue was homogenized (1 min, Sorvall Omnimixer, setting 6) with an equal volume of cold 0.1 M bisTris-HCl (pH 6.5). All subsequent operations were performed at 4 C unless otherwise indicated. Homogenate aliquots equivalent to 5 g of tissue were each mixed with 1.5 g (dry weight) of polyvinylpolypyrrolidone (PVPP) hydrated with 50 mM bisTris-HCl (pH 6.5). The resulting suspensions were filtered under pressure (2 lb/in$^2$ $N_2$) through one layer of Miracloth. For each homogenate aliquot, the solids retained by Miracloth were washed with two 5 ml aliquots of the

50 mM 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 $\mu$l Polymin P per ml supernatant). After 10 min, the precipitated nucleic acids and associated proteins were removed by centrifugation (10,000 g, 10 min). Solid 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 an 0.5 ml Porapak Q column (0.8 x 1 cm) equilibrated with 0.05 M bisTris-HCl (pH 6.5) containing ammonium sulfate at 10% saturation. The column was rinsed with 2 ml volumes of the same buffer, and all eluates were combined. Solid ammonium sulfate was added to the combined elutes 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.

For cytokinin oxidase assays, the pellets from above were dissolved in 100 mM bisTris buffer (pH 6.5) (1 ml buffer per pellet equivalent to 5 g of tissue fresh weight), centrifuged (10,000 g, 10 min) to remove any particulate material, and assayed for cytokinin oxidase activity using $i^6$Ade-2,8-$^3$H as the substrate. The standard incubation mixture (50 $\mu$l) contained 100 mM bisTris (pH 6.5), 0.010 mM $i^6$Ade-2,8-$^3$H (0.05 $\mu$Ci, 100 $\mu$Ci/$\mu$mol) and enzyme extract equivalent to 0.2 g tissue fresh weight. Test substances were incorporated into the standard reaction mixtures as aqueous stock solutions to give the final concentration indicated for particular experiments. The assays were incubated for 30 min at 37 C. The reactions were stopped by adding 2 volumes of cold 95% (v/v) ethanol containing unlabeled Ade and $i^6$Ade (0.75 mM and 1 mM respectively) and stored at -20 C. For analysis, the precipitated

protein was removed by centrifugation (minifuge) and 100 $\mu$l volumes of the supernatant were streaked on 5 cm wide Si-$C_{18}$ TLC plates. The chromatograms were developed to a height of 5 or 15 cm in 38% (v/v) ethanol containing 100 m$\underline{M}$ $Na_4EDTA$. The locations of the Ade and $i^6$Ade standards were determined by inspection under UV light. The plates were divided into 5 cm wide bands, which were scraped and counted in 5 ml Ready Protein in a Beckman Model 1801 scintillation counter.

F. Test of $i^6$Ade as a Substrate of the Dopamine-$\beta$-Hydroxylase Reaction

The ability of $i^6$Ade to serve as a substrate for dopamine-$\beta$-hydroxylase was tested by a modification of the standard dopamine-$\beta$-hydroxylase assay. The following modifications were made: the final volume of the incubation mixture was 0.1 ml and 10 or 30 $\mu\underline{M}$ $i^6$Ade-2,8-$^3$H (specific activity 100 $\mu$Ci/$\mu$mole) was used as a substrate in the presence or absence of 4 m$\underline{M}$ tyramine. The reaction was stopped by adding 2 volumes of cold 95% (v/v) ethanol containing unlabeled Ade and $i^6$Ade (0.75 and 1 m$\underline{M}$ respectively). Aliquots of 0.1 ml were treated as described above for cytokinin oxidase activity analysis.

G. Test of Dopamine-$\beta$-Hydroxylase for Cytokinin Oxidase Activity

Dopamine-$\beta$-hydroxylase preparations were tested for cytokinin oxidase activity using $i^6$Ade as substrate and reaction conditions similar to those employed in the assay of cytokinin oxidase. The standard incubation mixture (50 $\mu$l) contained 100 m$\underline{M}$ buffer (sodium acetate, pH 5; bisTris, pH 6.5; or Taps, pH 8.4). The copper imidazole enhanced reaction contained 100 m$\underline{M}$ imidazole, 20 m$\underline{M}$ $CuCl_2$, and 25 m$\underline{M}$

sodium acetate (pH 5.5). All assay mixtures contained 10 $\mu$M $i^6$-Ade-2,8-$^3$H (0.05 $\mu$Ci, 100 $\mu$Ci/$\mu$mol) as substrate. The samples were analyzed as described for cytokinin oxidase assay.

H. Cell-Free Enzyme Preparations from Tobacco Callus Tissues

Cell-free enzyme preparations were obtained from 22-day old cytokinin-dependent N. tabacum cv Wisconsin 38 callus tissues. The tissues was homogenized (1 min, Sorvall Omnimixer, setting 6) with an equal volume of 0.01 M $KH_2PO_4$ (pH 7). All subsequent operations were performed at 4 C unless otherwise indicated. Homogenate aliquots equivalent to 5 g of tissue were each mixed with 1.5 g (dry weight) of polyvinylpolypyrrolidone (PVPP) hydrated with 0.05 M $KH_2PO_4$ buffer (pH 7). The resulting suspensions were filtered under pressure (2 lb/in$^2$ $N_2$) through one layer of Miracloth. For each homogenate aliquot, the solids retained by Miracloth were washed with two ml aliquots of 0.05 M buffer, and the filtrates were combined and centrifuge (10,000 g, 10 min). The resulting suspensions were used as the enzyme preparations.

For pigment formation assays, the reactions mixtures (5 ml) contained: 0.05 M $KH_2PO_4$ buffer (pH 7), tyramine and kinetin as specified, and tobacco cell-free enzyme preparation equivalent to 0.5 g of tissue fresh weight. The reaction mixtures were incubated in a water bath at 37 C until the pigment developed.

# IV. RESULTS

## A. Biological Activity of 1-Phenyl-3-(2-Thiazolyl)-2-Thiourea (PTTU)

The biological activity of PTTU in promoting the growth of cytokinin-dependent callus tissues of Nicotiana tabacum cv Wisconsin 38, Phaseolus vulgaris cv Great Northern, and P. lunatus cv Kingston was tested and compared with the activity of kinetin.

The effect of PTTU on the growth of callus tissues of N. tabacum is shown in Figure 6. PTTU promoted the growth of the tobacco callus at concentrations from 1 to 32 $\mu$M. The optimum PTTU concentration for maximum growth of the callus tissue was 10 $\mu$M. This concentration was much higher than the optimum kinetin concentration (0.1 $\mu$M). Thus, PTTU exhibited cytokinin activity in the tobacco callus bioassay, but the compound was considerably less active than kinetin.

The effects of PTTU on the growth of cytokinin-dependent Phaseolus callus tissues are shown in Figures 7 and 8. No growth promotion was observed. However, as shown in earlier work by Mok et al. (1978), these Phaseolus callus tissues require a much higher concentration of cytokinin to promote growth than are required by Wisconsin 38 tobacco callus tissue. The difference in sensitivity of these tissues to kinetin is evident from the kinetin response curves obtained here. Therefore, the failure to obtain a growth promotive effect in these Phaseolus cultures is presumed to be due to the relatively weak cytokinin activity of PTTU.

Figure 6. Cytokinin activity of 1-phenyl-3-(2-thiazolyl)-2-thiourea (PTTU) in the tobacco callus bioassay.

Cytokinin-dependent Nicotiana tabacum cv Wisconsin 38 callus tissue was grown as described in "Materials and Methods". PTTU and kinetin were cold-sterilized in dimethylsulfoxide (DMSO) and added to the autoclaved tissue culture medium. The final DMSO concentration was 0.1 % (0.05 ml DMSO per 50 ml medium per 125 ml Erlenmeyer flask). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 35 days of growth at 28 C in the dark. The average data from two replicate experiments are plotted.

Figure 6.

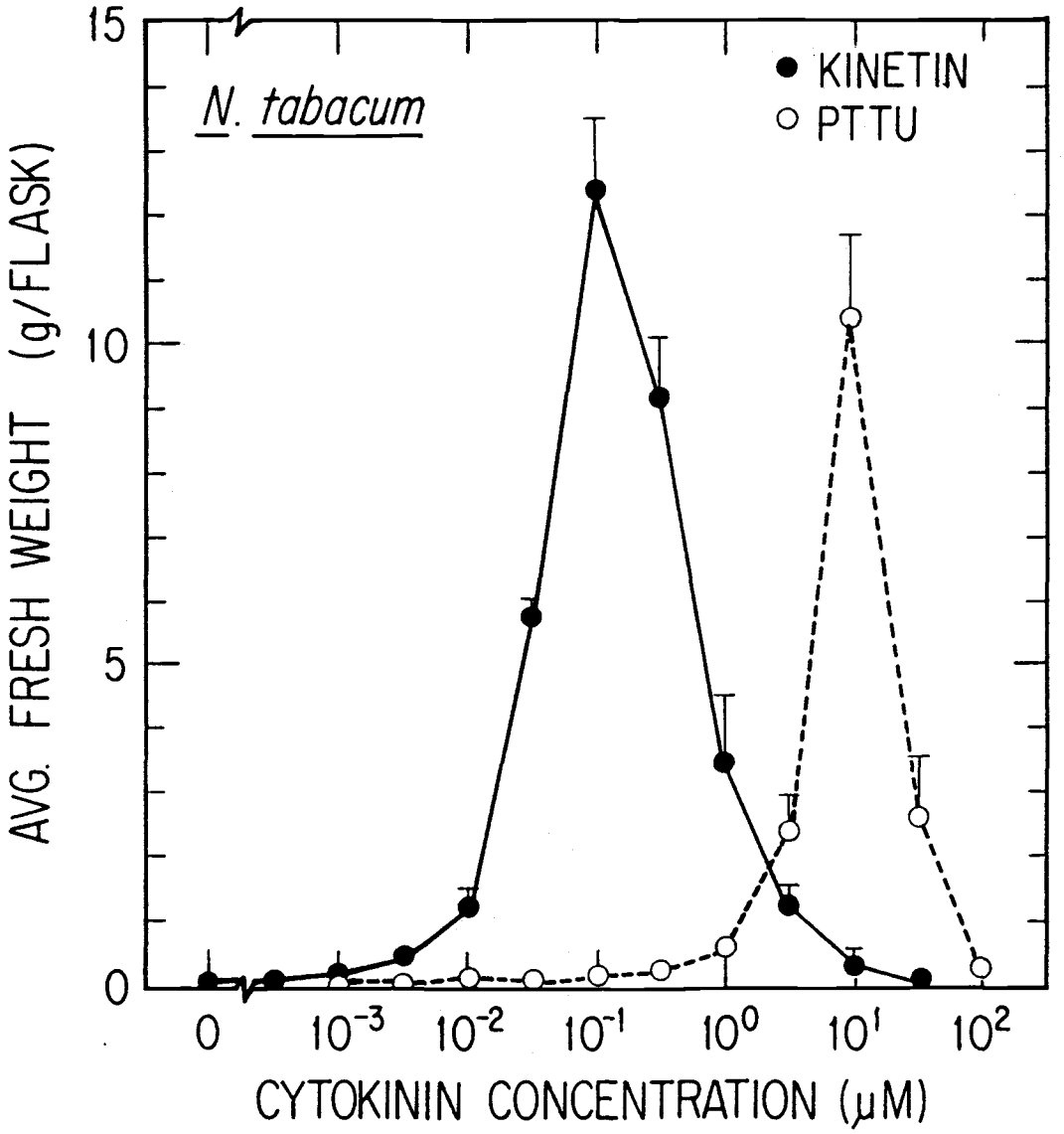

15
10
5
0
AVG. FRESH WEIGHT (g/FLASK)
N. tabacum
KINETIN
PTTU
0
$10^{-3}$
$10^{-2}$
$10^{-1}$
$10^{0}$
$10^{1}$
$10^{2}$
CYTOKININ CONCENTRATION (μM)

Figure 7. Cytokinin activity of 1-phenyl-3-(2-thiazolyl)-2-thiourea PTTU in cytokinin-dependent Phaseolus vulgaris cv Great Northern callus culture.

Cytokinin-dependent P. vulgaris cv Great Northern callus tissue was grown as described in "Materials and Methods". PTTU and kinetin were cold-sterilized in dimethylsulfoxide (DMSO) and added to the autoclaved tissue culture medium. The final DMSO concentration was 0.1% (0.05 ml DMSO per 50 ml medium per 125 ml Erlenmeyer flask). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28 C in the dark. The average data from two replicate experiments are plotted.

Figure 7.

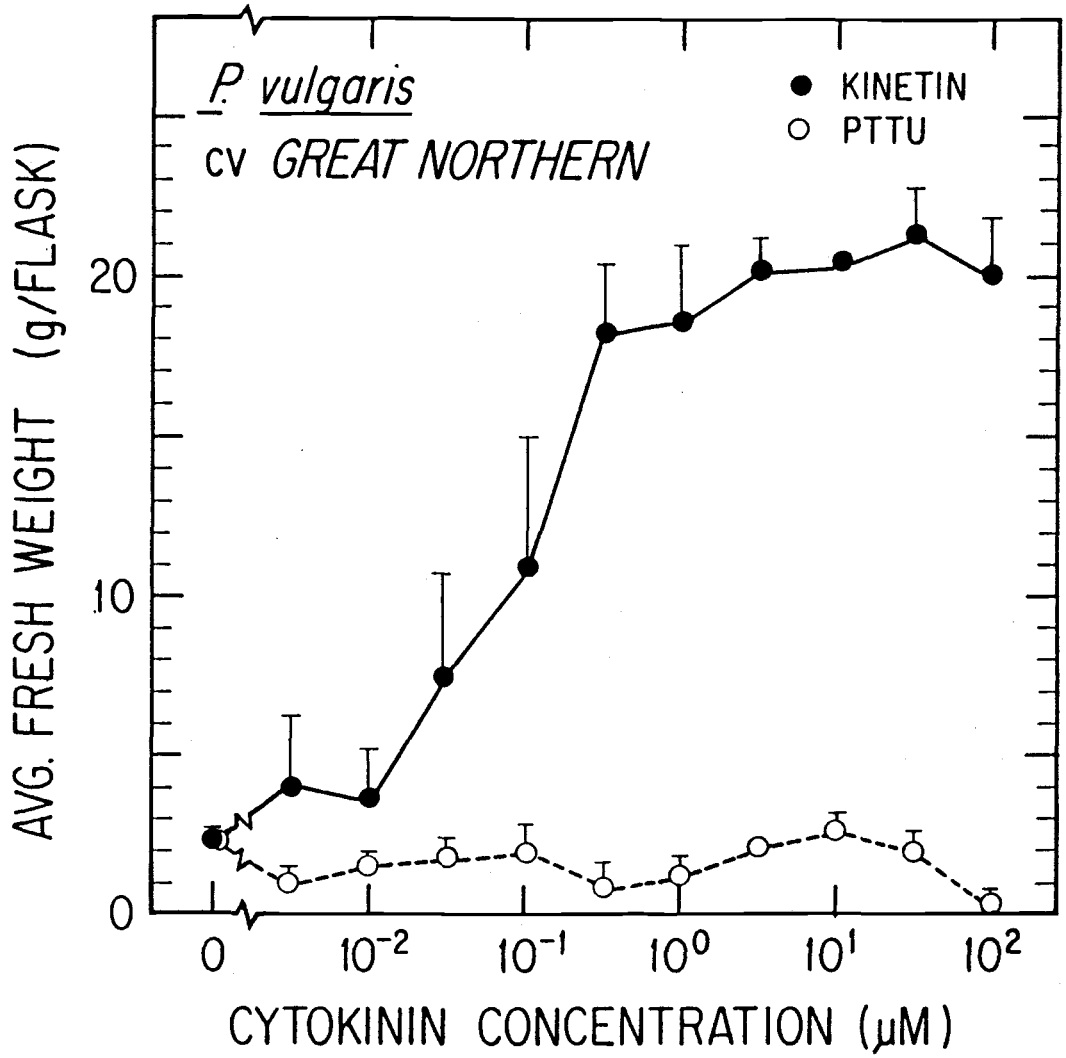
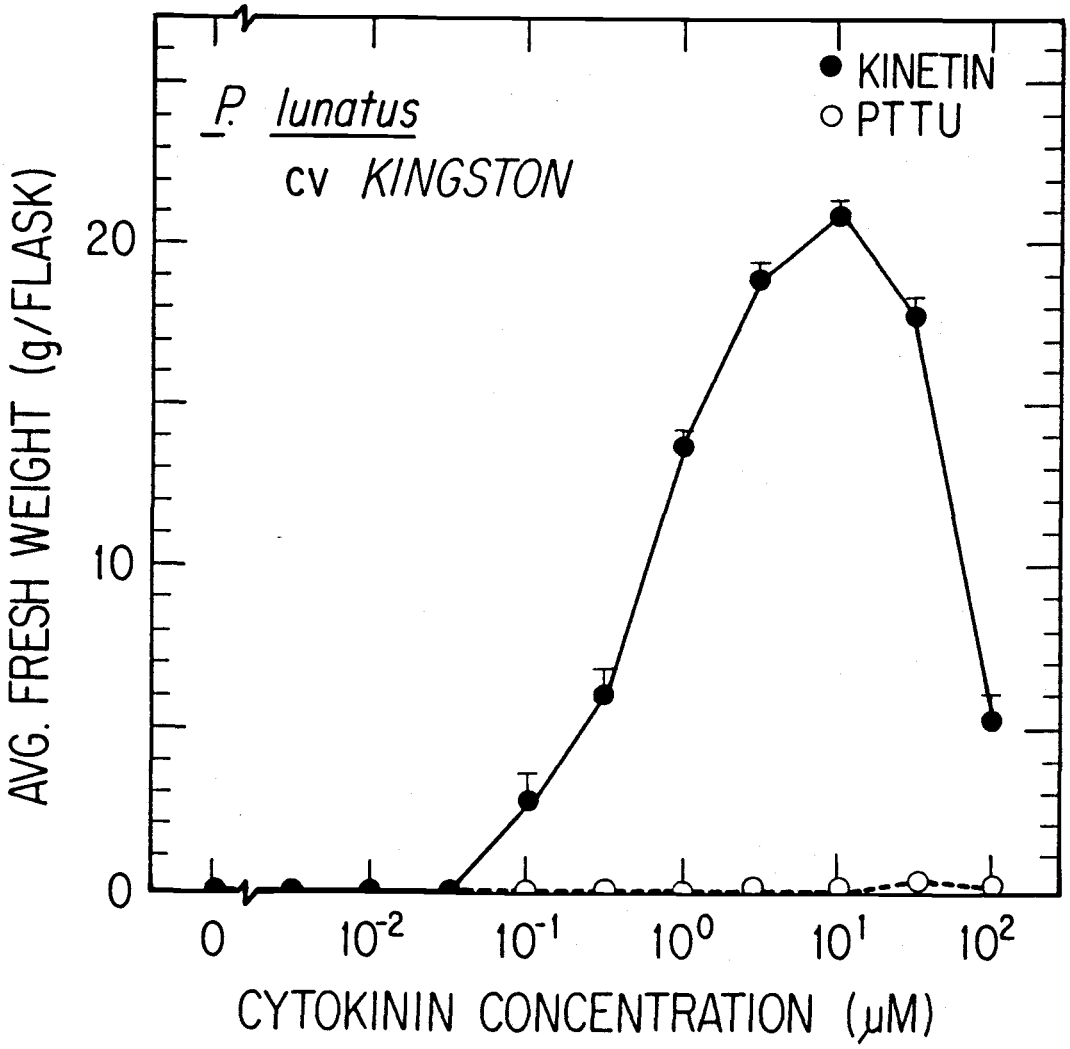

P. vulgaris
cv GREAT NORTHERN
KINETIN
PTTU
AVG. FRESH WEIGHT (g/FLASK)
0
10
20
0
$10^{-2}$
$10^{-1}$
$10^{0}$
$10^{1}$
$10^{2}$
CYTOKININ CONCENTRATION (μM)

Figure 8. Cytokinin activity of 1-phenyl-3-(2-thiazolyl)-2-thiourea PTTU in cytokinin-dependent Phaseolus lunatus cv Kingston callus culture.

Cytokinin-dependent P. lunatus cv Kingston callus tissue was grown as described in "Materials and Methods". PTTU and kinetin were cold-sterilized in dimethylsulfoxide (DMSO) and added to the autoclaved tissue culture medium. The final DMSO concentration was 0.1% (0.05 ml DMSO per 50 ml medium per 125 ml Erlenmeyer flask). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28 C in the dark. The data from one experiment is plotted.

Figure 8.


P. lunatus
cv KINGSTON
KINETIN
PTTU
AVG. FRESH WEIGHT (g/FLASK)
20
10
0
0
$10^{-2}$
$10^{-1}$
$10^{0}$
$10^{1}$
$10^{2}$
CYTOKININ CONCENTRATION (μM)

B. Effect of Cytokinins on the Activity of Cell-Free Preparations of Dopamine-$\beta$-Hydroxylase

The cytokinin activity of PTTU in the tobacco callus bioassay suggested that cytokinins might interact with dopamine-$\beta$-hydroxylase in a manner similar to PTTU. To test this hypothesis, the effects of a number of cytokinin-active adenine derivatives on the activity of dopamine-ß-hydroxylase were determined and compared with the effect of PTTU on cell-free preparations of dopamine-$\beta$-hydroxylase. Dopamine-$\beta$-hydroxylase activity was assayed using tyramine as a substrate as described in "Materials and Methods". As reported in the literature (Johnson et al., 1969, 1970; Von Voigtlander and Moore, 1970), PTTU strongly inhibited the activity of dopamine-$\beta$-hydroxylase in these tests (Table 1). Most of the cytokinin-active adenine derivatives tested also inhibited the activity of dopamine-$\beta$-hydroxylase. Of these compounds, $N^6$-cyclohexylmethyladenine was the most active in inhibiting dopamine-ß-hydroxylase. Zeatin and the cytokinin ribonucleosides, $b^6$Ado and $i^6$Ado, were the only cytokinin-active compounds that failed to inhibit the enzyme.

The inhibitory effects of $N^6$-cyclohexylmethyladenine and PTTU on the activity of dopamine-$\beta$-hydroxylase were further examined and compared (Table 2). The two compounds were approximately equally active in inhibiting the activity of the enzyme.

The specificity of the inhibitory effect of cytokinins on dopamine-$\beta$-hydroxylase activity was tested by comparing the inhibitory effects of the cytokinin $i^6$Ade with the corresponding $N^3$-substituted compound, $N^3$-

Table 1. Effect of Cytokinins on the Activity of a Purified Cell-Free Preparation of Dopamine-$\beta$-Hydroxylase.[a]

| Cytokinin | Cytokinin Concentration ($\mu$M) | |
|---|---|---|
| | 32 | 100 |
| | Enzyme Activity (% Control)[b] | |
| Adenine | 102 | 94 |
| 1-Phenyl-3-(2-thiazolyl)-2-thiourea | 19 | - |
| $N^6$-Benzyladenine | 42 | 22 |
| $N^6$-Cyclohexylmethyladenine | 24 | 9 |
| $N^6$-Furfuryladenine (Kinetin) | 85 | 65 |
| $N^6$-Hexyladenine | 43 | 29 |
| $N^6$-(4-Hydroxy-3-methyl-2-butenyl)adenine (Zeatin) | 96 | 103 |
| $N^6$-($\Delta^2$-Isopentenyl)adenine | 54 | 35 |
| $N^6$-Isopentyladenine | 52 | 30 |
| $N^6$-Benzyladenosine | 98 | 102 |
| $N^6$-($\Delta^2$-Isopentenyl)adenosine | 107 | 100 |

[a]Dopamine-$\beta$-hydroxylase (a purified commercial preparation from bovine adrenals) was assayed using 4 m$\underline{M}$ tyramine as a substrate as described in "Materials and Methods".

[b]The activity of controls incubated in the absence of any inhibitor was equal to 131.3 nmoles of octopamine formed per hour per 2 ml assay volume. All values are the average of 3 experiments.

Table 2. Comparison of the Inhibitory Effects of 1-Phenyl-3-(2-Thiazolyl)-2-Thiourea and $N^6$-Cyclohexylmethyladenine on the Activity of Cell-Free Preparations of Dopamine-$\beta$-Hydroxylase.[a]

| Cytokinin | Cytokinin Concentration ($\mu$M) | | |
|---|---|---|---|
| | 3.2 | 10 | 32 |
| | Enzyme Activity (% Control)[b] | | |
| 1-Phenyl-3-(2-thiazolyl)-2-thiourea | 85.4 | 53.6 | 26.1 |
| $N^6$-Cyclohexylmethyladenine | 86.4 | 51.7 | 27.0 |

[a]Dopamine-$\beta$-hydroxylase (a purified commercial preparation from bovine adrenals) was assayed using 4 m$\underline{M}$ tyramine as a substrate as described in "Materials and Methods".

[b]The activity of the controls incubated in the absence of any inhibitor was equal to 97.5 nmoles of octopamine formed per hour per 2 ml assay volume. All values are the average of 3 experiments.

($\Delta^2$-isopentenyl)adenine (triacanthine). The results are shown in Table 3. Triacanthine did not exhibit any inhibitory effect on the activity of the enzyme.

C. Test of $i^6$Ade as a Substrate for Dopamine-$\beta$-hydroxylase

The ability of dopamine-$\beta$-hydroxylase to utilize labeled $N^6$-($\Delta^2$-isopentenyl)adenine ($i^6$-Ade-2,8-$^3$H) as a substrate was tested using both the reaction conditions employed in the dopamine-$\beta$-hydroxylase assay and the reaction conditions used to assay for cytokinin oxidase activity. TLC analysis of reaction mixtures in which $i^6$Ade-2,8-$^3$H was substituted for tyramine did not provide any evidence that $i^6$Ade was converted to other products in the standard dopamine-$\beta$-hydroxylase assay (Tables 4, 5). Similarly, no evidence of conversion of $i^6$Ade to Ade (or other oxidation products) was observed when $i^6$Ade was incubated with dopamine-$\beta$-hydroxylase under the conditions for either the standard cytokinin oxidase assay or the copper-imidazole enhanced assay for this enzyme (Table 6).

D. Test of Tyramine Interaction with Cytokinin Oxidase

The possibility that tyramine might serve as a substrate or inhibitor of cytokinin oxidase was examined by substrate competition tests using a crude enzyme preparation from callus tissues of Phaseolus vulgaris cv Great Northern. Tyramine was tested for its ability to inhibit the enzyme-catalyzed degradation of labeled $i^6$Ade under the cytokinin oxidase standard assay conditions as described in "Materials and Methods". As shown in Table 7, no inhibitory effect on the degradation of $i^6$Ade was observed at low concentrations of tyramine.

Table 3. Comparison of the Inhibitory Effects of $N^6$- and $N^3$-Substituted Adenine Derivatives on the Activity of Cell-Free Preparations of Dopamine-$\beta$-Hydroxylase.[a]

| Test Compound | Test Compounds ($\mu$M) | | |
|---|---|---|---|
| | 10 | 32 | 100 |
| | Enzyme Activity (% of Control)[b] | | |
| $N^6$-($\Delta^2$-Isopentenyl)adenine | 79.7 | 56.5 | 32.5 |
| $N^3$-($\Delta^2$-Isopentenyl)adenine (Triacanthine) | 101.5 | 98.1 | 99.1 |

[a]Dopamine-$\beta$-hydroxylase (a purified commercial preparation from bovine adrenals) was assayed using tyramine as a substrate as described in "Materials and Methods".

[b]The activity of the controls incubated in the absence of inhibitor was equal to 67.5 nmoles of octopamine formed per hour per 2 ml assay volume. All values are the average of 3 experiments.

Table 4. Test of Dopamine-$\beta$-Hydroxylase for Cytokinin Oxidase Activity using Reaction Conditions similar to those Employed to Assay for Dopamine-$\beta$-Hydroxylase Activity.[a]

| Assay Conditions[b] | Radioactivity Recovered as Adenine | |
|---|---|---|
| | cpm | % Total Counts |
| 10 $\mu$M $i^6$Ade-2,8-$^3$H | | |
| - Enzyme Control | 247 | 0.95 |
| + Enzyme | 229 | 0.94 |
| + Enzyme + 4 mM tyramine | 240 | 0.91 |
| 30 $\mu$M $i^6$-Ade-2,8-$^3$H | | |
| - Enzyme Control | 688 | 0.88 |
| + Enzyme | 676 | 0.85 |
| + Enzyme + 4 mM tyramine | 708 | 0.89 |

[a]Dopamine-ß-hydroxylase used in the assays was a purified commercial preparation from bovine adrenals.

[b]The assay conditions for Dopamine-ß-hydroxylase (as described in "Materials and Methods") were used except that $i^6$Ade-2,8-$^3$H (specific activity 100 $\mu$Ci/$\mu$mol) was included in the reaction volumes, tyramine was omitted from the reaction volumes except as indicated, and assay volumes of 100 $\mu$l were employed. The reactions were stopped by adding 2 volumes of cold 95% (v/v) ethanol containing unlabeled Ade and $i^6$Ade (0.75 and 1 mM respectively). The samples were analyzed as described for cytokinin oxidase assay. 100 $\mu$l volumes were streaked on 5 cm wide $SiC_{18}$ TLC plates and the chromatograms were developed to a height of 5 cm.

Table 5. Test of $N^6$-($\Delta^6$-isopentenyl)adenine as a substrate for Dopamine-$\beta$-Hydroxylase[a]: TLC analysis of reaction products.

| Distance from Origin (cm) | Radioactivity Recovered from TLC Plate cpm | |
|---|---|---|
| | - Enzyme (Control) | + Enzyme |
| 0 to 1 | 1374 | 1677 |
| 1 to 2 | 1874 | 1655 |
| 2 to 3 | 46545 | 35146 |
| 3 to 4 | 11469 | 23137 |
| 4 to 5 | 968 | 926 |
| 5 to 6 | 206 | 230 |
| 6 to 7 | 937 | 894 |
| 7 to 8 | 199 | 151 |
| 8 to 9 | 73 | 98 |
| 9 to 10 | 104 | 116 |
| 10 to 11 | 291 | 285 |
| 11 to 12 | 111 | 93 |
| 12 to 13 | 82 | 91 |
| 13 to 14 | 53 | 62 |
| 14 to 15 | 32 | 33 |

[a]Dopamine-$\beta$-hydroxylase used in the assays was a purified commercial preparation from bovine adrenals. Assay conditions for dopamine-$\beta$-hydroxylase as described in "Materials and Methods" using label $i^6$Ade as substrate. Assay volume was 100 $\mu$l. The reactions were stopped by the adding 2 volumes of 95% (v/v) ethanol. The samples were analyzed by TLC chromatography methods similar to those used in assays of cytokinin oxidase. 100 $\mu$l aliquots were streaked on 5 cm wide $SiC_{18}$ TLC plates and the chromatograms were developed to a height of 15 cm.

Table 6. Test of Dopamine-$\beta$-Hydroxylase for Cytokinin Oxidase Activity using Reaction Conditions Similar to those Employed to Assay of Cytokinin Oxidase Activity in Plant Tissues.

| Assay Conditions[a] | Radioactivity Recovered as Adenine | |
|---|---|---|
| | cpm | (% total counts) |
| Standard Assay[b] | | |
| pH 5 | | |
| - Enzyme Control | 380 | 1.73 |
| + Enzyme | 261 | 1.20 |
| pH 6.5 | | |
| - Enzyme Control | 228 | 1.01 |
| + Enzyme | 235 | 1.10 |
| pH 8.4 | | |
| - Enzyme Control | 236 | 1.07 |
| + Enzyme | 247 | 1.10 |
| Copper-Imidazole enhanced assay[c] | | |
| pH 5 | | |
| - Enzyme Control | 342 | 1.49 |
| + Enzyme | 283 | 1.30 |

[a]Experiment was conducted and analyzed by cytokinin oxidase assay procedures as described in "Materials and Methods".

[b]Standard assays contained 100 mM of appropriate buffer: sodium acetate (pH 5), bisTris-HCl (pH 6.5),or taps (pH 8.4).

[c]Copper-imidazole enhanced assays contained 100 mM imidazole, 20 mM $CuCl_2$, and 25 mM NaOAc (pH 5).
All assays contained 10 $\mu$M $i^6$-Ade-2,8-$^3$H and dopamine-$\beta$-hydroxylase (purified commercial preparation from bovine adrenals) in an assay volume of 50 $\mu$l.

Table 7. Test of Tyramine as Substrate for Cytokinin Oxidase using the Standard Cytokinin Oxidase assay.[a]

| Tyramine Concentration (mM) | Cytokinin Oxidase Activity | |
|---|---|---|
| | nmoles adenine/assay | % Control |
| 0 (Control) | 0.094 | 100 |
| 0.01 | 0.094 | 100 |
| 0.1 | 0.088 | 94 |
| 1 | 0.088 | 94 |
| 10 | 0.072 | 77 |

[a]Substrate activity of tyramine for cytokinin oxidase was assayed as described in "Materials and Methods". Pellets of enzyme preparation from P. vulgaris cv Great Northern were redissolved in 0.1 M bisTris buffer. The assay volume was 50 $\mu$l. Aqueous stock solutions of tyramine were added to the reaction mixture Standard assay contained 100 mM bisTris buffer (pH 6.5), 10 $\mu$M i$^{6}$Ade-2,8-$^{3}$H (specific activity 100 $\mu$Ci/$\mu$mol) and bean enzyme preparation equivalent to 200 mg of tissue.

However, at the highest concentration of tyramine tested (10 mM), the conversion of $i^6$Ade to Ade was inhibited approximately 20% relative to controls without tyramine. The cause of this inhibition was not established, but it is possible that tyramine at high levels may interact with some site on cytokinin oxidase.

## E. Effects of Phenethylamines on Callus Cultures

### E.1. Biological Activity of Tyramine and Hordenine

The biological activities of tyramine and hordenine in promoting the growth of cytokinin-dependent N. tabacum cv Wisconsin 38 callus tissues were tested and compared to the activity of kinetin. As shown in Figure 9, neither tyramine nor hordenine exhibited cytokinin activity in the tobacco callus bioassay. The tissues treated with tyramine, even at the lowest concentration tested, showed a dark brown coloration in a few hours after planting the tissues on the culture medium. In 2 or 3 days, the tissues were completely black. However, at the end of the 5-week growth period the tissues on 0.032 mM tyramine were almost as white as the ones on medium without tyramine. The tissues growing in the highest concentration of hordenine exhibited a red-violet coloration by the end of the growth period.

### E.2. Effect of Phenethylamines on Cytokinin-Dependent N. tabacum Callus Cultures

The effects of octopamine, tyramine, hordenine and phenylethylamine on the growth of cytokinin-dependent N. tabacum cv Wisconsin 38 callus tissue growing on medium containing 0.1 $\mu$M kinetin (the concentration near optimal for growth) were examined. As shown in Figure 10, all of the phenethylamines compounds were inhibitory to callus growth at

Figure 9. Cytokinin activities of tyramine and hordenine in the tabacco callus bioassay.

Cytokinin-dependent Nicotiana tabacum cv Wisconsin 38 callus tissue was grown as described in "Materials and Methods". Kinetin stocks were dissolved in dimethylsulfoxide (DMSO) and added to the tissue culture medium before its sterilization. The final DMSO concentration was 0.1% (0.05 ml DMSO per 50 ml medium per 125 ml Erlenmeyer fask). Aqueous stock solutions of tyramine and hordenine were cold sterilized by Millipore filtration and added to the autoclaved tissue culture medium (0.625 ml per 50 ml medium per 125 ml Erlenmeyer flask). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 35 days of growth at 28 C in the dark. The average data of one experiment is plotted.

Figure 9.

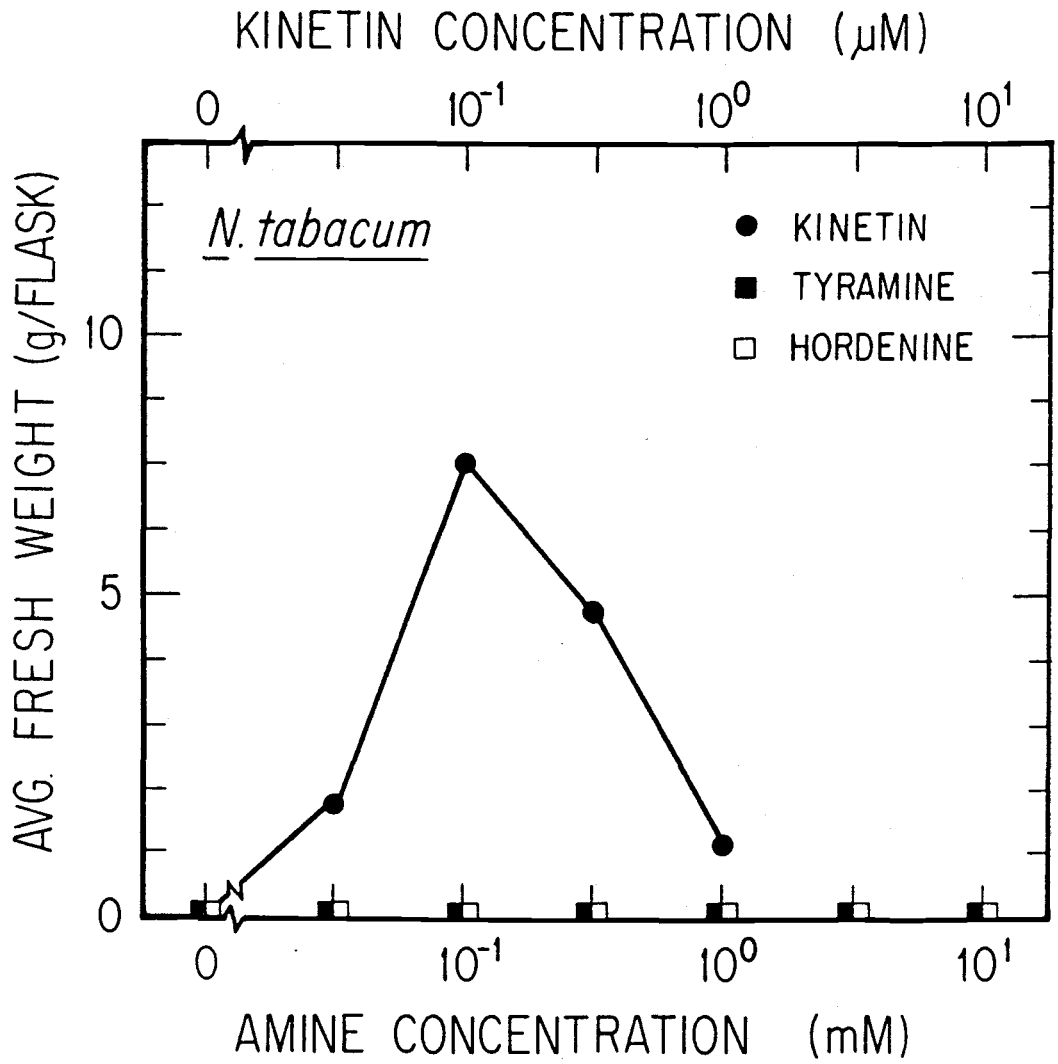
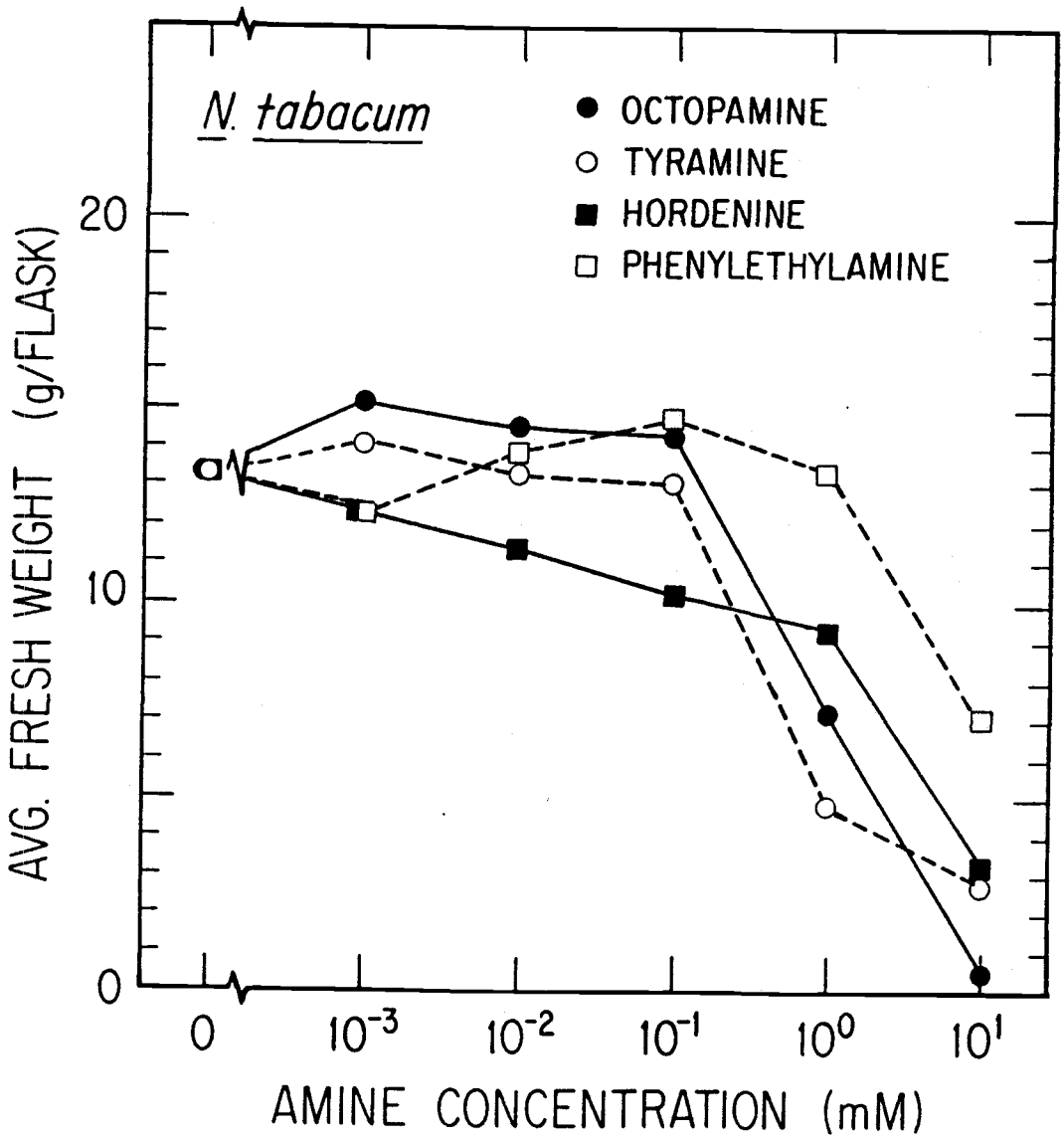

KINETIN CONCENTRATION (μM)
0
10⁻¹
10⁰
10¹
N. tabacum
KINETIN
TYRAMINE
HORDENINE
AVG. FRESH WEIGHT (g/FLASK)
10
5
0
0
10⁻¹
10⁰
10¹
AMINE CONCENTRATION (mM)

Figure 10. Effects of phenylethylamines derivatives on the growth of cytokinin dependent Nicotiana tabacum cv Wisconsin 38 callus cultures.

Cytokinin-dependent N. tabacum cv Wisconsin 38 callus tissues was grown as described in "Materials and Methods". Aqueous stock solutions of phenylethylamine derivatives were cold-sterilized by Millipore filtration and added to the autoclaved tissue culture medium (0.625 ml stock solution per 50 ml medium per 125 ml Erlenmeyer flask). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 35 days of growth at 28 C in the dark. The average data from two replicate experiments are plotted. The maximum standard errors for octopamine, tyramine, hordenine and phenylethylamine treatments were 25%, 21%, 22%, and 21% respectively.

Figure 10.


N. tabacum
OCTOPAMINE
TYRAMINE
HORDENINE
PHENYLETHYLAMINE
AVG. FRESH WEIGHT (g/FLASK)
20
10
0
0
$10^{-3}$
$10^{-2}$
$10^{-1}$
$10^{0}$
$10^{1}$
AMINE CONCENTRATION (mM)

concentrations of 1 mM and higher. However, the inhibitory activity of phenylethylamine was less than that of the other compounds. Tissues growing at high concentration (1 and 10 mM) of octopamine and tyramine showed the same dark pigmentation observed in the callus tissues inoculated on medium without kinetin. Tissues growing in 10 mM hordenine showed the formation of a red-violet pigment during the fourth week in culture, and at the end of the 5-week growth period, the tissues were completely colored.

E.3. Effect of Octopamine on Cytokinin-Dependent Phaseolus callus tissue

The effect of octopamine on cytokinin-dependent P. vulgaris P.I. 200960, P. vulgaris cv Great Northern, and P. lunatus cv Kingston callus tissues growing on medium containing 5 μM kinetin was examined (Figure 11). Callus tissues of P. vulgaris cv Great Northern were inhibited by octopamine at the highest concentration tested (10 mM). However, the callus tissues of P. vulgaris P.I. 200960 and P. lunatus cv Kingston were not significantly affected by octopamine at the concentrations tested. No pigment formation was exhibited by callus tissues of any of the genotypes.

E.4. Cytokinin Reversal of the Inhibitory Effects of Tyramine in Tobacco Callus Tissues

The ability of increasing concentrations of kinetin to reverse the inhibitory effects of phenethylamines on the growth of cytokinin-dependent N. tabacum cv Wisconsin 38 was tested using tyramine. The results are shown in Figure 12. The inhibitory effect of tyramine on

Figure 11. Effect of octopamine in cytokinin-dependent Phaseolus callus cultures.

Cytokinin-dependent Phaseolus callus tissues were grown as described in "Materials and Methods". Aqueous stock solutions of octopamine were cold-sterilized by Millipore filtration and added to the autoclaved tissue culture medium (0.625 ml of stock solution per 50 ml medium per 125 ml Erlenmeyer flask). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flask were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28 C in the dark. The average data from two experiments are plotted.

Figure 11.

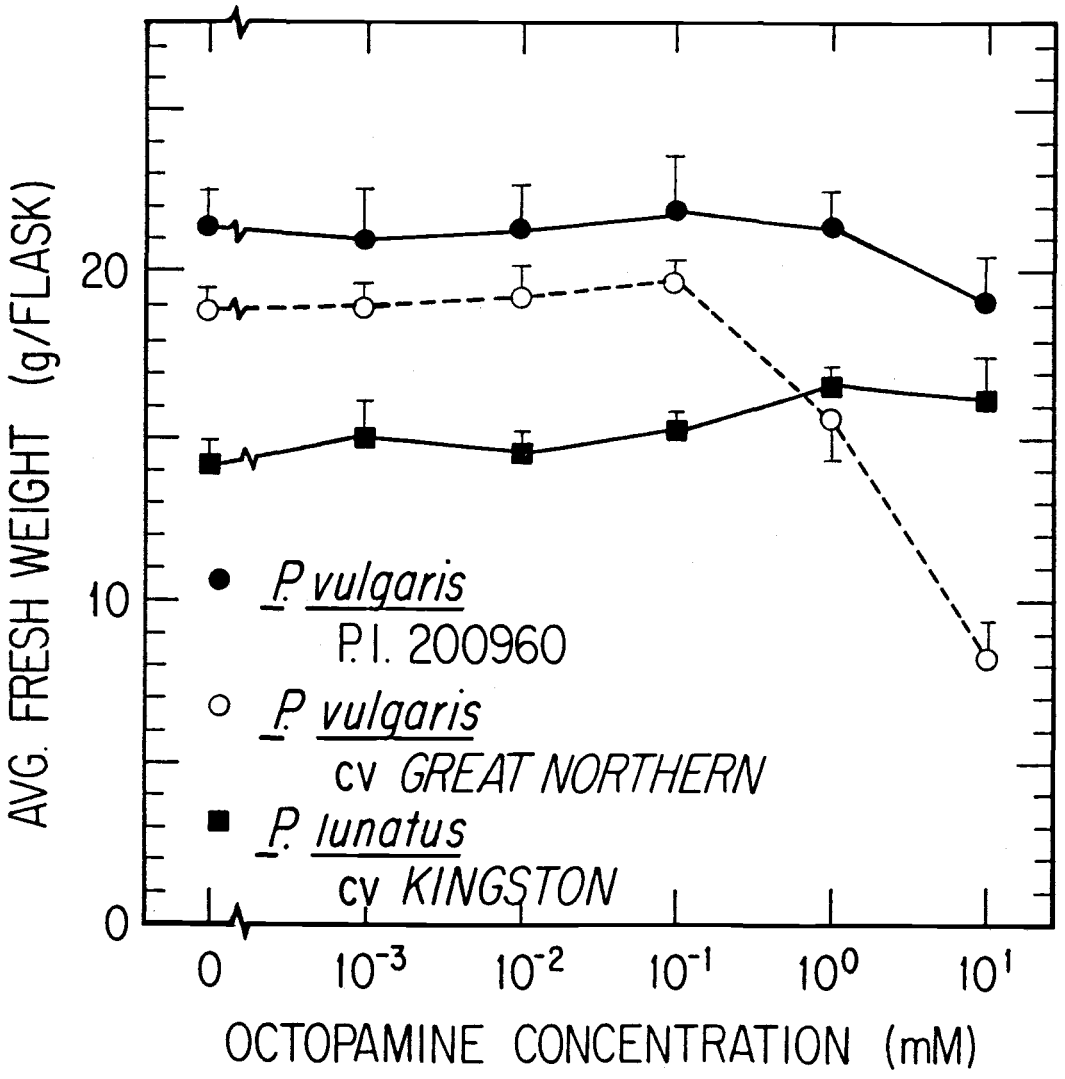
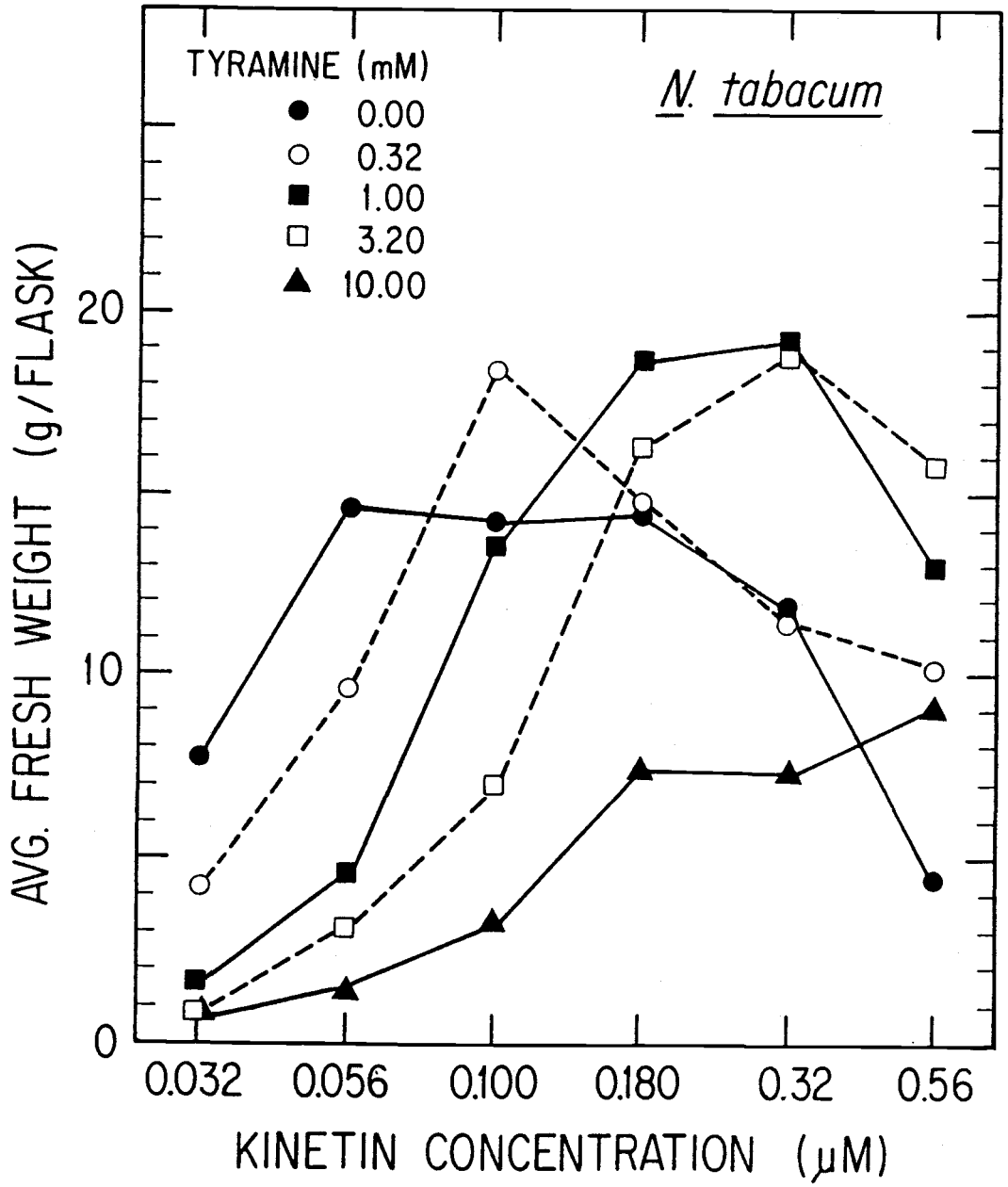

AVG. FRESH WEIGHT (g/FLASK)
20
10
0
P. vulgaris
P.I. 200960
P. vulgaris
cv GREAT NORTHERN
P. lunatus
cv KINGSTON
0
10-3
10-2
10-1
100
101
OCTOPAMINE CONCENTRATION (mM)

Figure 12. Interaction of kinetin and tyramine in cytokinin-dependent Nicotiana tabacum cv Wisconsin 38 callus cultures.

Cytokinin-dependent N. tabacum cv Wisconsin 38 was grown as described in "Materials and Methods". Kinetin was dissolved in dimethylsulfoxide (DMSO) and added to the culture media (0.05 ml DMSO per 50 ml medium) prior to sterilization of the media. Aqueous stocks solutions of tyramine were cold-sterilized by Millipore filtration and added to the autoclaved tissue culture medium (0.625 ml stock solution per 50 ml medium per 125 ml Erlenmeyer flask). Three callus pieces weighing about 25 mg were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 35 days of growth at 28 C in the dark. The average data from two replicate experiments are plotted. Note that kinetin concentration is plotted on a log scale. The maximum standard errors for each tyramine treatment (0, 0.32, 1, 3.2, and 10 mM) were 21%, 15%, 20%, 22%, and 25% respectively.

Figure 12.


TYRAMINE (mM)
0.00
0.32
1.00
3.20
10.00
N. tabacum
AVG. FRESH WEIGHT (g/FLASK)
0
10
20
0.032
0.056
0.100
0.180
0.32
0.56
KINETIN CONCENTRATION (μM)

the growth of callus tissues of cytokinin-dependent tobacco was completely, or almost completely, reversed by increasing the concentration of kinetin. The reversal of the inhibitory effect of tyramine at doses of 1 and 3.2 mM was complete, and no dark pigmentation was observed. The inhibitory effect of tyramine at 10 mM was only partially reversed by the highest concentration of kinetin tested, and the same result was obtained with respect to pigmentation. The lowest concentration of tyramine tested (0.32 mM) did not have a significant effect on the growth of the callus tissues at concentrations of kinetin equal to or greater than 0.1 $\mu$M. Dark pigmentation was observed at suboptimal concentrations of kinetin (0.032 and 0.056 $\mu$M). However, as the kinetin concentration increased, the pigmentation present in the tissue decreased to the point that tissues growing at 0.1 $\mu$M kinetin did not show pigmentation. All tyramine concentrations tested at suboptimal kinetin concentrations were inhibitory to the growth of the cytokinin-dependent *N. tabacum* callus tissues.

## F. Preliminary Results Concerning Pigment Formation in a Cell-Free Enzyme Preparation from Cytokinin-Dependent Tobacco Callus

Crude cell-free enzyme preparations from cytokinin-dependent *N. tabacum* cv Wisconsin 38 were prepared as described in "Materials and Methods". Incubation of these preparations with tyramine resulted in the enzyme dependent formation of a dark pigment within a few hours. Using 1 mM tyramine in the reaction mixture, the development of pigmentation was observed to be inhibited in the presence of 10 $\mu$M kinetin. Quantitative data for this effect have not yet been obtained.

# V. DISCUSSION

PTTU, a potent inhibitor of the enzyme dopamine-ß-hydroxylase, has been shown in the present study to have cytokinin activity in the tobacco callus bioassay. Cytokinin-dependent tobacco callus cultures achieved the same maximum fresh weight on medium containing either kinetin or PTTU. However, the PTTU concentration required for maximum growth of the callus tissues was 100-fold greater than the equivalent kinetin concentration. The failure of PTTU to promote the growth of cytokinin-dependent callus tissues of Phaseolus genotypes may be explained by the high cytokinin concentration (5 $\mu$M) required to support the growth of these callus tissues and the relatively weak cytokinin activity of PTTU.

The cytokinin activity of PTTU suggested that other cytokinin-active compounds might be effective in inhibiting the activity of dopamine-$\beta$-hydroxylase. The effects of cytokinin-active $N^6$-substituted adenine derivatives on the enzyme were of particular interest. $N^3$-substituted adenine derivatives have been reported to inhibit dopamine-$\beta$-hydroxylase (Fujii, et al., 1979), but these compounds are not active as cytokinins (Skoog, et al., 1967). In the present study, most of the cytokinins tested inhibited the activity of the enzyme. $N^6$-(cyclohexylmethyl)adenine (the most effective of these compounds) was as active as PTTU in inhibiting the enzyme. Interestingly, the $N^3$-substituted compound that was reported by Fujii et al. (1979) to be the most effective inhibitor of dopamine-$\beta$-hydroxylase was $N^3$-

cyclohexylmethyladenine. The relative inhibitory activities of $N^6$- and $N^3$-substituted adenine derivatives were tested in the present study by comparing $i^6$Ade and and $i^3$Ade [$N^3$-($\Delta^2$-isopentenyl)adenine; triacanthine). No inhibition of dopamine-ß-hydroxylase activity was obtained in tests with triacanthine. This result was unexpected and inconsistent with the results of Fujii et al. (1979). Triacanthine was not among the $N^3$-substituted compounds tested by these investigators, but it appears unlikely that the properties of this compound are significantly different from the tested compounds. $N^3$-substituted adenines exposed to high temperatures are known to be susceptible to ring opening and rearrangement that results in migration of the side chain substituent to the $N^6$-position (Skoog, et al., 1967). No details of solution handling were given by Fujii et al. (1979), but $N^3$ to $N^6$ rearrangement might explain the discrepancy between their results and the results reported here.

The interaction of PTTU with the copper residues in dopamine-ß-hydroxylase has been suggested as a possible mechanism for the inhibition caused by this compound (Johnson, 1970). Dopamine-$\beta$-hydroxylase contains a hydrophobic region near the active-site (Ljones and Skotland, 1984) and that site may serve to bind PTTU. Adenine and cytokinins have also been reported to form complexes and interact with copper ions (Sletten, 1967; Eichhorn, 1973; Marzilli, 1981; Miller, 1985), and it is possible that cytokinins may have the same mechanism of inhibition as PTTU. The substrate activity of labeled $i^6$-Ade for dopamine-$\beta$-hydroxylase was tested in the present study, using a variety of conditions, but no evidence was obtained of any enzymatic attack of the enzyme on these compounds. Tyramine, when tested as a competitive

substrate for the enzyme-catalyzed degradation of $i^6$Ade-2,8-$^3$H by cytokinin oxidase, inhibited degradation of the labeled substrate by about 20%. This result suggests that an interaction between cytokinin oxidase and this phenethylamine might exist.

The ability of cytokinins to inhibit dopamine-$\beta$-hydroxylase in cell-free systems raises the possibility that similar interactions of cytokinins with enzymes involved in phenethylamine metabolism may occur in vivo. In tests of the biological activity of phenethylamine derivatives, tyramine and hordenine did not show cytokinin activity in the tobacco callus bioassay, and the callus tissues treated with tyramine produced a dark pigment. The formation of a dark pigment was also observed by Bianco-Colomas (1980) and Jimenez et al. (1984) in Amaranthus fed with dopa. In this case, the pigment was identified by its absorption spectrum as dopachrome, an intermediate in melanogenesis and a product of the oxidation of dopamine by mushroon tyrosinase. All phenethylamines tested (octopamine, tyramine, hordenine, and phenylethylamine) inhibited the growth of the cytokinin-dependent tobacco callus grown in the presence of 0.1 $\mu$M kinetin. Tissues treated with octopamine and tyramine, showed the greater inhibitory effect and also the formation of a dark pigment at higher concentrations (1 and 10 mM) of these amine derivatives.

The inhibitory effects of tyramine on the growth of cytokinin dependent tobacco callus tissues were reversed by increasing kinetin concentration. Similar results were obtained by Christou and Barton (1989) when octopamine was added to cytokinin-dependent tobacco callus tissues. These investigators found that in cytokinin-autonomous tobacco tumor tissues (a crown gall tissue generated by inoculation of tobacco

with Agrobacterium A208) an inhibitory effect of octopamine on the growth of the tissue was not observed. Presumably, the lack of effect of octopamine on this tissue is related to the greatly elevated levels of cytokinins in the tumor tissues. Epinephrine proved to be lethal for the cytokinin-dependent tissues and even affected the tobacco tumor tissue.

Christou and Barton (1989) suggested that phenethylamines were acting as cytokinin antagonists in the tobacco callus system. However, an alternative hypothesis is that kinetin reversal of the negative effect of these phenethylamines on the tobacco callus tissues could be due to effects on phenethylamine metabolism. For example, cytokinins may also stimulate the synthesis of enzymes that metabolize phenethylamines. The action of cytokinins in promoting the synthesis of tyramine methylpherase in barley (Stainhart et al., 1964) provides a precedent for such an effect.

The ability of cytokinins to inhibit dopamine hydroxylase activity, as shown in the present study, suggests an additional possibility. Cytokinin reversal of the inhibitory effects of phenethylamine could be due to the inhibition of an oxidase that converts phenethylamines to toxic products. Copper-containing phenol oxidases have been reported in plant tissues (Gregory and Bendal, 1966). A preliminary experiment with crude enzyme preparations from cytokinin-dependent tobacco callus indicated that reactions leading to pigment formation from 1 mM tyramine were inhibited by the addition of kinetin to the cell-free system. This result is consistent with such a hypothesis. Further work will be needed to determine whether phenethylamine metabolism is altered by cytokinin treatment of these tissues, whether cytokinins compete with

phenethylamines for the same molecular sites, and whether these results have significance for the normal regulation of the metabolism and growth of plant tissues.

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