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Inhibition of *Ent*-Kaurene Oxidation by Cytokinins*,**

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Abstract. Cytokinins, which have some structural similarities to ancymidol, a plant growth retardant, were tested for their effects on the cell-free oxidation of *ent*-kaurene. Results indicate that several cytokinins inhibit this reaction in microsomal extracts of liquid endosperm from immature wild cucumber seeds. N^6-cyclohexanemethyladenine was the most active (inhibiting 50% of the control *ent*-kaurene oxidation at 2×10^{-6} M). N^6-isoamyladenine, N^6-benzyladenine, N^6-(Δ^2-isopentenyl)adenine and dihydrozeatin were active at successively higher concentrations. Zeatin, kinetin, adenine, N^6-benzyladenosine, and N^6-(isopentenyl)adenosine were inactive in this system.

The basis for the inhibition of *ent*-kaurene oxidation by cytokinins may be similar to that of ancymidol: interaction with cytochrome P-450. A binding spectrum similar to that of ancymidol with cytochrome P-450 from wild cucumber endosperm microsomes was obtained with four active cytokinins. The cytokinin binding properties of this protein are currently under investigation.

No metabolism of N^6-benzyladenine could be detected under conditions in which the cytokinin inhibited the oxidation of *ent*-kaurene to *ent*-kaurenol.

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Abbreviations. Ancymidol, α-cyclopropyl-α-(*p*-methoxyphenyl)-5-pyrimidine methyl alcohol; EL-509, the α-isopropyl derivative of ancymidol (see Coolbaugh et al. 1982); *ent*-Kaurene, *ent*-kaur-16-ene; *ent*-kaurenol, *ent*-kaur-16-en-19-ol; isopentenyladenine, N^6-(Δ^2-isopentenyl)adenine; benzyladenine, N^6-benzylaminopurine; kinetin, 6-(furfurylamino)purine; zeatin, 6-(4-hydroxy-3-methyl-trans-2-butenylamino) purine.

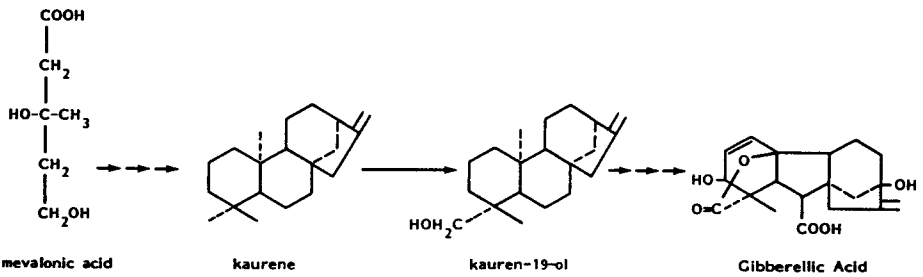


Fig. 1. Biosynthetic pathway for gibberellins.

Ancymidol, a plant growth regulator that inhibits natural internode elongation (Tschabold et al. 1970) and gibberellin-induced growth (Leopold 1971), will commonly elicit other effects such as increased stem diameter (Montague 1975), increased greening, and increased root initiation in shoot cuttings (Kefford 1973). Although ancymidol may have more than one mode of action, we have been most interested in its inhibition of gibberellin biosynthesis (see Fig. 1). In cell-free preparations from liquid endosperm of immature wild cucumber seeds, ancymidol has been shown to block the oxidation of kaurene to kaurenol (Coolbaugh and Hamilton 1976). Because of some superficial similarities in the actions and structures (see Fig. 2) of ancymidol and cytokinins, the effects of several cytokinins on the cell-free oxidation of kaurene were examined. Ancymidol was also tested for possible effects in standard cytokinin bioassays.

Materials and Methods

Kaurene Oxidation

Marah oreganus fruits were harvested in western Oregon in July 1978 and 1981. Seeds were removed and frozen at −20°C until used. Liquid endosperm from immature seeds was removed, homogenized in a Thomas teflon-to-glass homogenizer, squeezed through four layers of cheesecloth and centrifuged for 15 min at 10,000 × *g*. The supernatant was then centrifuged 60 min at 100,000 × *g*. The resulting pellet (P_{100}) was resuspended in one-third to one-fifth volume of 100 mM Tris-HCl buffer (containing 25% glycerol) at pH 7.5 and stored in liquid nitrogen until used as the enzyme source in assay reactions. Reaction mixtures consisted of 50 µl P_{100} enzyme (~100 µg protein), 5×10^{-5} M FAD, 5×10^{-4} M NADPH, 75 mM Tris buffer, 0.17 µM [^{14}C]-kaurene (54.6 µCi/µmole) and 100 µl potential inhibitor (in 50% MeOH) or 50% MeOH in a total volume of 1.0 ml. Reaction mixtures were incubated for 10 min (linear activity through 20 min) at 30°C in open 20-ml vials in a shaking waterbath. Extractions and product determinations were as described previously (Coolbaugh et al. 1982). Each potential inhibitor was tested over the range of concentrations ($0-10^{-3}$ M) in independent experiments, and each experiment was repeated two to four times with essentially the same results. The percent kaurene oxidation was calculated by dividing the cpm oxidized products (at origin

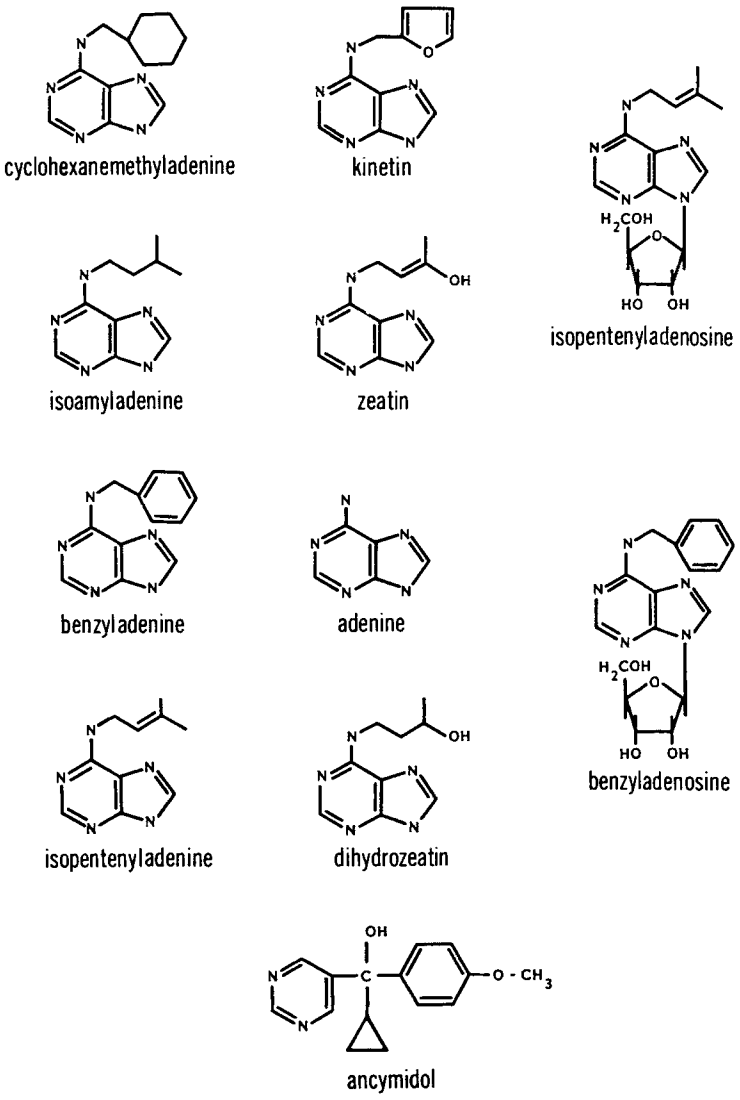


Fig. 2. Structures of cytokinins and ancymidol.

of TLC developed in hexane) by the total cpm recovered from TLC (oxidized products plus kaurene). Small amounts of nonenzymic oxidation as determined in zero-time controls were subtracted from other percent oxidation values. For comparative purposes the data from each experiment were normalized against the percent oxidation in reactions run in the absence of inhibitor.

A series of confirming experiments was conducted using seeds of *Marah macrocarpus* collected in the Santa Monica Mountains in the spring of 1983 and provided by Charles A. West. These reactions were as above, except that

the microsomes were pelleted at 150,000 × *g* for 90 min and ^{14}C-kaurene concentration was 0.13 μM (40 μCi/μmole).

Spectral Measurements of Cytochrome P-450

Hemoprotein contents were measured in dense suspensions of microsomes containing 2 mg protein per ml. Resuspended microsomes as described above (*Marah macrocarpus*) were clarified by centrifugation at 10,000 × *g* for 15 min. Cytochrome P-450 was measured by the methods of Omura and Sato (1967) by determining the ΔA between 450 and 490 nm in a carbon monoxide difference spectrum with reduction by dithionite. An extinction coefficient of 91 cm^{-1} mM^{-1} was used.

Cytochrome P-450 binding spectra were obtained by determining a baseline with two oxidized samples (792 μl). Then 8 μl of 10^{-2} M to 10^{-4} M cytokinin (in MeOH) was added to the sample cuvette and 8 μl MeOH was added to the reference cuvette. The difference spectra were recorded on a Varian Cary model 210 spectrophotometer coupled to an Apple II Plus microcomputer using the Master Scan Storage program. The ΔA between 410 nm and the absorption peak (425–440 nm) was used as an estimate of binding.

Potential Metabolism of Benzyl Adenine and EL-509

To test for the potential metabolism of benzyladenine in the cell-free enzyme system, ^{14}C-benzyladenine (10^{-4} M; 13.4 μCi/μmole) was used in routine reaction mixtures with unlabeled kaurene. After incubation, the products were extracted and chromatographed on TLC in 50% EtOH. Similar experiments were conducted with [^{14}C]-EL-509. Labeled ancymidol was unavailable, but EL-509 is a very close analog of ancymidol with a similar K_i value (~2×10^{-9} M) (Coolbaugh et al. 1982). After incubation and extraction, the products were chromatographed on TLC plates developed 15 cm in chloroform/ethyl acetate/acetic acid (60:40:5).

Cytokinin Bioassays

Benzyladenine and ancymidol were comparatively tested for activity in the cucumber cotyledon chlorophyll induction assay (Fletcher and McCullagh 1971), the tobacco callus assay (Lindsmaier and Skoog 1965), and the apical dominance assay (Sachs and Thimann 1964, 1967).

Reagents

R-[2-^{14}C]-MVA lactone (13.65 μCi/μmole and 53 μCi/μmole) was purchased from Amersham and hydrolyzed with 100% excess NaOH. High sp. act. MVA

was diluted with unlabeled MVA (Sigma) to 10 μCi/μmole. [^{14}C]-kaurene was prepared biosynthetically as described previously (Coolbaugh et al. 1982). Ancymidol, α-cyclopropyl-α-(*p*-methoxyphenyl)-5-pyrimidine methyl alcohol (technical), and [^{14}C]-EL-509, α-isopropyl α-(*p*-methoxyphenyl)-5-pyrimidine methyl alcohol (2.53 μCi/μmole), were gifts from Eli Lilly and Co. N^6-cyclohexanemethyladenine and N^6-isoamyladenine were provided by Donald J. Armstrong, Oregon State University. Other cytokinins (structures illustrated in Fig. 2) and other potential inhibitors of kaurene oxidation were purchased from Sigma. [^{14}C]-Benzyladenine (13.4 μCi/μmole) was purchased from Amersham.

Protein Determinations

The protein contents of enzyme extracts were determined by using the Bio Rad Protein Assay. Ten-μl samples of enzyme extracts were routinely measured with bovine albumin as the standard.

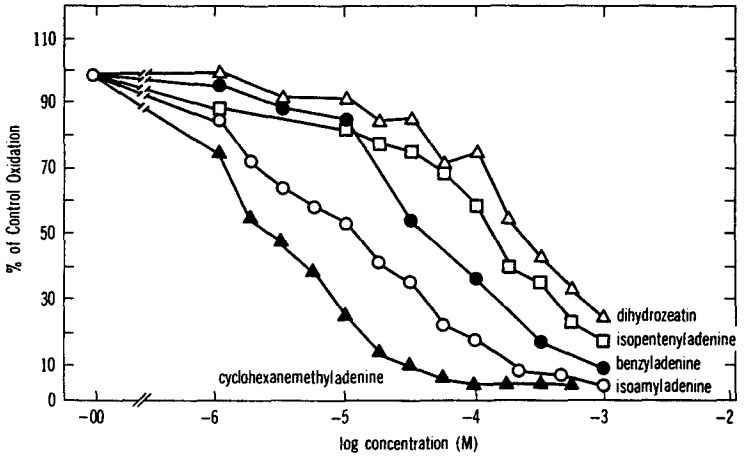
Results

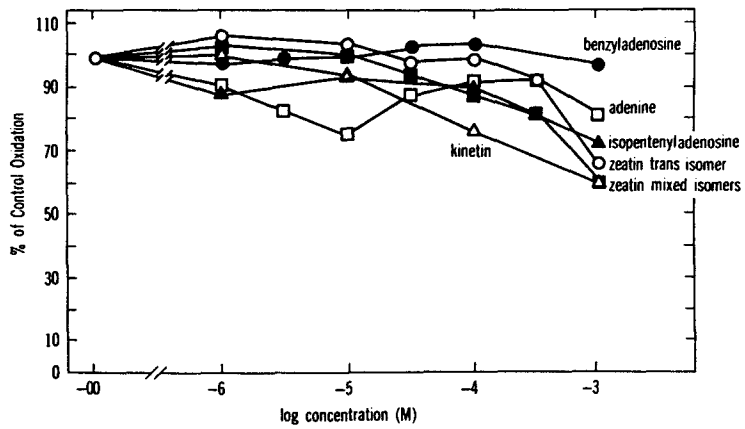
Effects of Cytokinins on Kaurene Oxidation

The effects of five cytokinins tested over a range of concentrations on cell-free kaurene oxidation are shown in Fig. 3. N^6-cyclohexanemethyladenine and N^6-isoamyladenine were the most active among the compounds tested. They inhibited the oxidative reaction by approximately 50% at a concentration of $2-5 \times 10^{-6}$ M. Benzyladenine, isopentenyladenine, and dihydrozeatin gave similar effects only at about 10-fold or greater concentrations. Kinetin and zeatin showed little or no activity at 10^{-4} M and 30–40% inhibition at 10^{-3} M (Fig. 4). Identical assays with benzyladenosine and isopentenyladenosine, the ribosides of two active cytokinins, as well as adenine itself, showed these compounds to have little effect (Fig. 4).

Spectral Interactions

Cytochrome P-450 was detected in the cell-free extracts by its CO-difference spectrum. Its concentration was approximately 0.033 nmole/ml (0.017 nmole/mg protein). As ancymidol has been shown previously to cause a spectral change when added to microsomes containing cyt P-450, it was of interest to test for such an interaction with cytokinins. Fig. 5 illustrates the difference spectra obtained when cytokinins or ancymidol were added to the sample cuvette (final concentration of 10^{-4} M to 10^{-6} M). The cytokinins and ancymidol gave similar difference spectra with absorption peaks between 425 and 440 nm.





Figs. 3 (top) and 4 (bottom). Effects of cytokinins on cell-free kaurene oxidation. Cytokinins were incubated with microsomal preparations (50 μl) from liquid endosperm of immature *Marah oreganus* seeds, FAD, NADPH, and ^{14}C-kaurene in 75 mM Tris buffer, pH 7.5. After 10-min incubations at 30°C ^{14}C-products were determined. The effects of potential inhibitors over the full range of concentrations were determined in separate experiments. In these experiments, 15–35% of the added substrate was oxidized in the absence of inhibitors (controls). These values were normalized to 100% for comparison. Each point represents a mean value from two to four independent experiments.

Potential Metabolism of Benzyladenine

The possibility that the cytokinins were metabolized in the cell-free enzyme system was tested by incubating [^{14}C]-benzyladenine (10^{-4} M) in reaction mixtures identical to those above. After extraction and TLC in 50% EtOH, only a single peak of radioactivity was detected which coincided with the position of authentic unlabeled benzyladenine at Rf 0.80 (data not shown). [^{14}C]-EL-509 was similarly tested with no evidence of metabolism. When a solvent

system consisting of chloroform/ethyl acetate/acetic acid (60:40:5) was used, a single peak of radioactivity was detected at Rf 0.65.

Bioassay of Ancymidol

Ancymidol was tested for activity in three bioassay systems that are known to be affected by several cytokinins over a wide range of concentrations. Under conditions in which benzyladenine was quite active, ancymidol did not stimulate greening in cucumber cotyledons, or growth in tobacco callus tissue, and did not break the apical dominance in pea seedlings (data not shown). It was therefore concluded that ancymidol does not act as a cytokinin in these systems.

Discussion

The initial impetus for the studies reported here was the observation that ancymidol, a plant growth inhibitor that blocks gibberellin biosynthesis, elicits increased greening, increased stem diameter, and increased rooting of shoot cuttings. Because of these observations and superficial structural similarities between cytokinins and ancymidol, it was thought that ancymidol might have some cytokinin activity. No such activity was observed in three standard cytokinin bioassays. Experiments testing the opposite relationship, however, revealed that some cytokinins have structural features that make them analogs of ancymidol.

The inhibitory effects of cytokinins on kaurene oxidation in cell-free microsomal preparations from wild cucumber endosperm are quite dramatic and reproducible. Cyclohexanemethyladenine, the most active cytokinin tested, inhibited kaurene oxidation by 50% at approximately micromolar concentrations. Other cytokinins were less active, but several affected this reaction in a similar range of concentrations. It is noteworthy that dihydrozeatin is more active than zeatin, N^6-isoamyladenine more active than N^6-Δ^2-isopentenyl)adenine, and N^6-cyclohexanemethyladenine more active than N^6-benzyladenine in inhibiting kaurene oxidation. In all of these cases, the compound with the saturated side chain is more active than the corresponding compound with an unsaturated side chain. The absence of activity in this system with ribosides of active cytokinins, and with adenine itself, also suggests a degree of specificity involved in the observed effect.

In some ways this specificity is in contrast to that observed in standard bioassays. For example, kinetin and zeatin are normally very active, ribosides frequently have similar activity to the corresponding free bases, and saturation of side chains often reduces activity (see Skoog and Schmitz 1979). Furthermore, most bioassays of cytokinins are sensitive to much lower levels than those shown here to be active in inhibiting kaurene oxidation (Skoog et al. 1967, Schmitz et al. 1975, Mok et al. 1978). However, it is not uncommon for investigators to use micromolar and higher concentrations in bioassays and experiments involving cytokinin applications. Indeed, 10 μM kinetin was re-

ported to be optimum for bud induction in tobacco callus (Linsmaier and Skoog 1965).

Numerous effects of cytokinins on enzyme activities have been reported previously and were reviewed recently by Skoog and Schmitz (1979). Most of the effects appear to be of stimulatory nature, including the stimulation of IAA oxidase and peroxidase, but not catalase, activities by concentrations of cytokinins that inhibit growth of *Lens culinaris* roots (Gaspar and Xhaufflaire, 1967). Lee (1971, 1972, 1974) has also reported stimulation by kinetin (0.2 μM optimal) of the formation of isoperoxidases and two isozymes of IAA oxidase.

Recently, Norman et al. (1982) reported that a number of cytokinins inhibit abscisic acid biosynthesis in the fungus *Cercospora rosicola*. Nine cytokinins were tested at 500 μM and 100 μM. Kinetin riboside and benzyladenine, the two most active compounds investigated, inhibited ABA production by 76% and 74%, respectively, at 100 μM. By using ^{14}C-farnesyl pyrophosphate (FPP) as a substrate, these investigators demonstrated that the inhibited step is beyond FPP in the biosynthetic pathway. It is noteworthy that the ABA biosynthetic pathway and the gibberellin biosynthetic pathway are common up to FPP, and both include subsequent cyclization and oxidation steps. It will be interesting to see if the oxidative steps in ABA synthesis are cytochrome P-450 catalyzed and if this is the site of action of the cytokinins.

The spectral interactions between cytokinins and cytochrome P-450 in microsomal preparations are quite similar to those obtained with ancymidol (Fig. 5). The latter has been described previously (Coolbaugh et al. 1978). The appearance of an absorption peak between 430 nm and 441 nm and a trough at about 410 nm is reminiscent of a type II interaction in liver and bacterial cytochrome P-450 preparations that have interacted with amines and certain other oxidase substrates and inhibitors (Orrenius et al. 1972, Yoshida and Kumaoka 1975). The absorption maxima and minima are slightly different for the binding spectra from animal systems, which often give intense peaks at 430 nm and absorption minima at 395 nm. Orrenius et al. (1972) and Hodgson (1974) have summarized evidence that such spectral changes reflect the interaction of the supplied substance with cytochrome P-450 and that maxima and minima may vary depending upon the source of cytochrome P-450 and the chemical nature of the interacting substance. It is concluded that the difference spectra seen in the present instance are a result of the interaction of cytokinins with cytochrome P-450.

Assuming the absorbance difference between the peak and the trough at 410 nm is a meaningful measure of the degree of binding, there is a clear dependence on concentration for four of the five added cytokinins. A correlation may also be drawn between the activity of a cytokinin as an inhibitor of kaurene oxidation and its ability to elicit a binding spectrum. This is illustrated in Table 1 in which binding (Fig. 5) and inhibition (Figs. 3 and 4) are summarized and compared. The two cytokinins having the greatest activity in inhibiting kaurene oxidation (isoamyladenine and cyclohexanemethyladenine) also have the most intense binding spectra. Benzyladenine and isopentenyladenine are less active inhibitors and bind less readily. Kinetin, which has essentially no inhibitory activity, also has almost no binding spectrum. It seems quite likely,

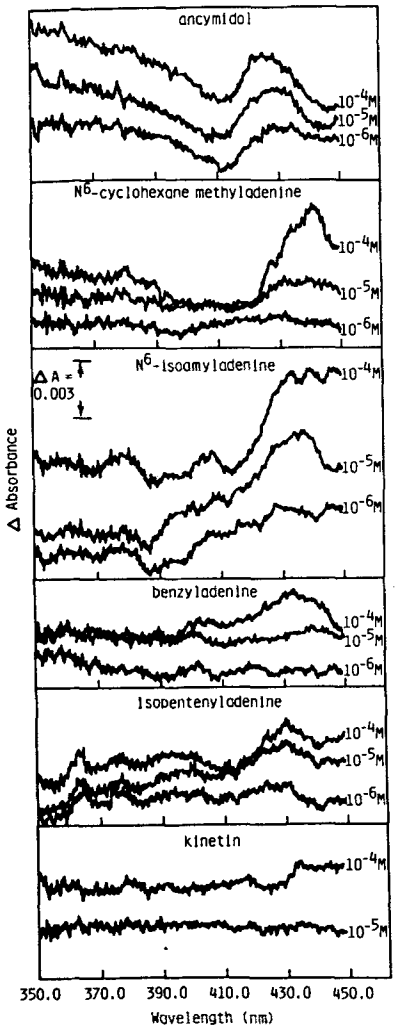


Fig. 5. Interaction of cytokinins and ancymidol with cytochrome P-450 in microsomal extracts from *Marah macrocarpus*. A baseline was established with oxidized enzymes (792 μl) in both sample and reference cuvettes. Indicated cytokinins or ancymidol was added in MeOH (8 μl) to the sample cuvette, 8 μl MeOH was added to the reference cuvette, and the difference spectrum was recorded. Protein content was 2 mg/ml; cytochrome P-450 was 0.017 nmol/mg protein.

based upon these results, that the binding of P-450 by cytokinins is the basis for inhibition of kaurene oxidation.

As most of the cell-free inhibition studies were done with microsomes from *M. oreganus* and most of the binding spectra were done with microsomes from *M. macrocarpus,* the inhibition of kaurene oxidation by microsomes of *M. macrocarpus* and the induction of binding spectra in microsomes of *M. oreganus* were confirmed in separate experiments (data not shown).

Cytokinins are known to be metabolized in plant tissue and tissue culture (see, for example, Deleuze et al. 1972, McGaw and Horgan 1983, and references therein). Particularly pertinent to the present investigations are the reports of hydroxylation of isopentenyladenine and isopentenyladenosine to form zeatin and ribosylzeatin, respectively, by the fungus *Rhizopogon roseolus* (Miura

Table 1. Comparison of inhibitory potential (Figs. 3 and 4) and binding potential (Fig. 5) of cytokinins and ancymidol.

Inhibitor (Concentration)	% Inhibition	ΔA × 100
	M. oreganus	*M. macrocarpus*
None		
Ancymidol (10^{-5}M)	100	0.24
Ancymidol (10^{-6}M)	100	0.20
N^6-cyclohexanemethyladenine (10^{-4}M)	95	0.53
N^6-cyclohexanemethyladenine (10^{-5}M)	75	0.17
N^6-cyclohexanemethyladenine (10^{-6}M)	25	0.07
N^6-isoamyladenine (10^{-4}M)	82	0.49
N^6-isoamyladenine (10^{-5}M)	47	0.36
N^6-isoamyladenine (10^{-6}M)	16	0.17
Benzyladenine (10^{-4}M)	63	0.20
Benzyladenine (10^{-5}M)	16	0.07
Benzyladenine (10^{-6}M)	5	0.03
Isopentenyladenine (10^{-4}M)	41	0.25
Isopentenyladenine (10^{-5}M)	18	0.17
Isopentenyladenine (10^{-6}M)	12	0.08
Kinetin (10^{-4}M)	24	0.10
Kinetin (10^{-5}M)	8	0.00

and Miller 1969) and corn endosperm (Miura and Hall 1973). More recently Chen (1982) reported the same reactions in cell-free microsomal extracts prepared from tobacco tissue cultures. On the basis of these results, Chen suggested the involvement of microsomal mixed function oxidases in cytokinin metabolism. Type II binding spectra, however, are thought to result from interaction of the inhibitor with the protein at other than the active site (Orrenius et al. 1972). It is therefore not expected that the cytokinins and the plant growth regulators that elicit Type II binding spectra would be metabolized in this system. This is consistent with the noted absence of detectable metabolites of ^{14}C-benzyladenine and ^{14}C-EL-509 in the present study under conditions in which the inhibitors prevent the oxidation of kaurene. Further studies with labeled isopentenyl adenine will be necessary to determine whether it is metabolized by wild cucumber microsomes.

Cytokinin binding proteins have been isolated and characterized in a number of studies using a variety of techniques (Berridge et al. 1970, Fox and Erion 1975, Takegami and Yoshida 1977, Gardner et al. 1978, Sussman and Kende 1978, Moore 1979, Erion and Fox 1981). These proteins are of great interest in elucidating the molecular site and mechanism of action of cytokinins. Since the results presented in this paper suggest that cytokinins inhibit kaurene oxidation by binding to kaurene oxidase, a cytochrome P-450, at other than the active site, it is appealing to consider whether cytochrome P-450 is a natural cytokinin binding protein. Although most of the quantitative aspects of this binding and its physiological significance remain to be established, some com-

parisons may be made. Kobayashi et al. (1981) reported that a particulate fraction from carrot suspension cells contains high affinity binding sites with a dissociation constant of 33 ± 6 nM and a frequency of 1133 ± 120 fmol g^{-1} f.w. (994 ± 106 fmol mg^{-1} protein). Low affinity binding sites had a k_d of 6400 ± 50 nM and a frequency of 44 ± 8 pmol g^{-1} f.w. (39 ± 7 pmol mg^{-1} protein). In the present study, in which routine reaction mixtures contained approximately 100 μg microsomal protein per ml, the most active cytokinins inhibited kaurene oxidation up to 50% at a concentration of 2×10^{-6} M. Dense suspensions of microsomes containing 2 mg/ml protein and approximately 0.033 nmole cytochrome P-450 were required to observe spectral interactions between the cytochromes and cytokinins. Furthermore, these spectral changes required 10^{-5} to 10^{-4} M cytokinins. The concentration of cytochrome P-450 in these dense suspensions was approximately 33 pmol ml^{-1} (17 pmol mg^{-1} protein). Thus, the concentration of cytochrome P-450 in microsomal preparations of *M. macrocarpus* appears to be in the same concentration range as the low affinity cytokinin binding protein of carrot suspension cells. Purification of the cytochrome P-450, further kinetic studies, and specific cytokinin binding assays need to be done before any conclusions can be drawn regarding the binding affinity and its significance.

In summary, several cytokinins have been shown to inhibit kaurene oxidation in cell-free microsomal preparations of liquid endosperm from immature wild cucumber seeds. The inhibition is accompanied by an observed difference spectrum in the microsomes with added active cytokinins. The inhibition and the apparent binding are similar to those observed with ancymidol and its analogs with growth regulatory activity. The physiological significance of these interactions remains open to speculation, but these results suggest that some cytokinins may affect gibberellin biosynthesis under experimental conditions.

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