Biosynthesis and Cytokinin Activity of 8-Hydroxy and 2,8-Dihydroxy Derivatives of Zeatin and N^6-(Δ^2-Isopentenyl)adenine†

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ABSTRACT: 8-Hydroxy and 2,8-dihydroxy derivatives of the cytokinins, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine and N^6-(Δ^2-isopentenyl)adenine, have been biosynthesized by xanthine oxidase oxidation. 8-Hydroxy derivatives have been shown to be the major intermediates. These compounds were tested for cytokinin activity in the tobacco bioassay. The results suggest that substitution of the 8 position with a hydroxyl group causes less decrease of cytokinin activity than substitution of both the 2 and 8 positions with hydroxyl groups.

Zeatin[1] (6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-purine) and N^6-(Δ^2-isopentenyl)adenine (i^6Ade or 2iP) are naturally occurring cytokinins which promote cell division and cell differentiation in plant tissue (Miller, 1961; Helgeson, 1968; Fox, 1969; Skoog and Armstrong, 1970; Kende, 1971; Hall, 1973). These compounds have been found free in higher plants and plant pathogens as well as in the tRNAs of many organisms (Skoog and Armstrong, 1970; Hall, 1970; Söll, 1971; Leonard, 1974; Chen et al., 1974a). An enzyme system which converts these compounds into various intermediates has been found both in mammalian (McLennan et al., 1968; Hall et al., 1971; Chheda and Mittelman, 1972; McLennan and Pater, 1973) and plant tissues (Chen et al., 1968; Paces et al., 1971; Chen et al., 1974b). One of these enzymes is xanthine oxidase. Cytokinins such as kinetin (Bergmann and Kwietny, 1958; Henderson et al., 1962) and i^6Ade (Chheda and Mittelman, 1972; McLennan and Pater, 1973) have been shown to be oxidized by xanthine oxidase with the formation first of the 8-hydroxy derivatives and then formation of the 2,8-dihydroxy derivatives. However, because of the versatility of xanthine oxidase which has capacity to catalyze the oxidation of many purine derivatives (Bray, 1963; Roussos, 1967), a more detailed kinetic analysis is needed to determine the nature of the oxidation reaction of the cytokinins, zeatin and i^6Ade.

There has been much interest in the structure–activity relationship of purine derivatives showing activity as cytokinins. Several authors (Skoog et al., 1967; Dyson et al., 1970; Hecht et al., 1970a,b; Schmitz et al., 1971; Letham, 1972; Fox et al., 1973; Hong et al., 1973; Dammann et al., 1974; Leonard, 1974) have studied the possibility of finding new cytokinins with the modification of the purine ring and of the N^6 side chain on adenine. However, there are no previous reports on the cytokinin activity of 8-hydroxy and 2,8-dihydroxy derivatives of i^6Ade and zeatin. This paper reports the oxidation of zeatin and i^6Ade to their 8-hydroxy and 2,8-dihydroxy derivatives by xanthine oxidase, confirmation of these oxidation products by ultraviolet absorption spectra and mass spectra, and the cytokinin activity of these products tested by relative promotion of growth in the tobacco tissue culture bioassay.

Materials and Methods

Chemicals and Enzymes. Xanthine, N^6-(Δ^2-isopentenyl)adenine, xanthine oxidase (milk, 40.6 mg of protein/ml), and catalase (beef liver) were obtained from Sigma Chemical Company, zeatin or 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine (a mixture of trans and cis isomers) was from Calbiochem, [8-^{14}C]adenosine (50 Ci/mol) was purchased from Schwarz/Mann, and Δ^2-isopentenyl bromide was from Columbia Organic Chemical Company, Inc.

Chromatography. Descending chromatography on Whatman No. 1 or No. 3MM paper was performed using the following solvent systems: A, ethyl acetate–1-propanol–water (4:1:2); B, 1-butanol–water–concentrated NH_4OH (86:14:5); C, 2-propanol–H_2O–concentrated NH_4OH (7:2:1); D, 95% ethanol–0.1 *M* $(NH_4)_3BO_3$ (pH 9.0) (1:9). Sephadex LH-20 resin (Pharmacia) was swollen in 35% aqueous ethanol and the column was eluted with this solvent.

Radioactivity. This was measured in a Nuclear Chicago Unilux II scintillation system. For liquid samples an aliquot, no more than 1.0 ml, was added to 10 ml of Bray's solution (Bray, 1960). For paper chromatograms, 1-cm or 2-cm sections were placed in vials containing scintillation fluid made up of 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 l. of toluene. Counting efficiency of liquid samples and chromatogram sections for ^{14}C were 86 and 74%, respectively.

Preparation of [^{14}C]i^6Ade. [8-^{14}C]i^6Ade was prepared by oxidation of [8-^{14}C]i^6Ado with $NaIO_4$ (Robins et al., 1967). The preparation of [8-^{14}C]i^6Ado (5 Ci/mol) was as described (Paces et al., 1971). A solution of 3.58×10^6 cpm (2.55×10^{-7} mol) of [8-^{14}C]i^6Ado in 2.0 ml of water was mixed with 3.0 ml of H_2O containing 2 μmol of $NaIO_4$. The oxidation reaction was allowed to proceed for 30 min at room temperature in the dark. At the end of the reaction, 4 μmol of glucose in 0.3 ml of H_2O was added to terminate the oxidation reaction. After 20 min the reaction mixture was made 1 *N* with respect to sodium hydroxide and boiled for 30 min. The sample was streaked on Whatman No. 3MM paper and developed in solvent D. Three ^{14}C radioac-

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[1] Abbreviations used are: i^6Ade, N^6-(Δ^2-isopentenyl)adenine; i^6Ado, N^6-(Δ^2-isopentenyl)adenosine; zeatin, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine; ZR, zeatin riboside or 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine.

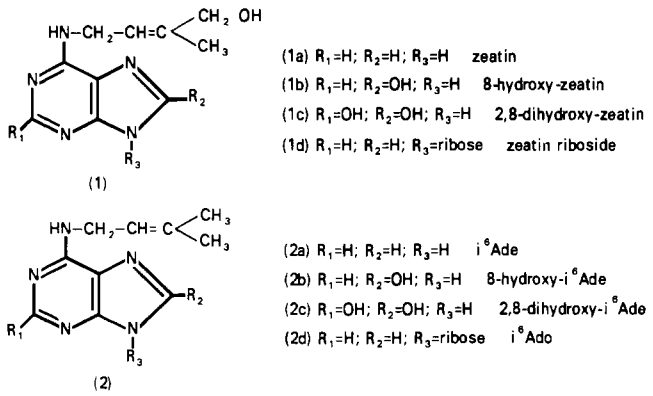

FIGURE 1: Structural formulas of the enzymatic oxidation products of zeatin and i^{6}Ade.

tive bands were obtained. (R_f values were: band 1, 0.11; band 2, 0.56, and band 3, 0.80.) Each band was further purified by chromatography on 20% ethanol washed Whatman No. 3MM paper in solvent C. The ^{14}C-labeled material from band 2 was characterized as [8-^{14}C]i^{6}Ade by cochromatography with the synthetic sample of i^{6}Ade (Figure 1; 2a) in solvent systems A–D (Table I). After final purification the percent recovery of the ^{14}C-labeled i^{6}Ade, based on cpm, was 52%.

Mass Spectral Determinations. Mass spectra were measured with a CH-5 single focusing mass spectrometer. The samples were admitted to the source via a direct insertion proble whose temperature could be precisely regulated between 10 and 500°. The electron impact source was adjusted to an electron beam energy of 70 eV and an accelerating voltage of 3000 V. Line spectra were drawn from the mass spectra; in each instance the most abundant ion was set to 100%.

Enzyme Assay. In the kinetic studies of the oxidation of zeatin, i^{6}Ade, and 8-hydroxy-i^{6}Ade (Figure 1) to their dihydroxy derivatives, the rates of increase in optical density at 306 nm were employed as measures of the rates of the reactions, whereas the oxidation of xanthine was followed at 290 nm (Kalckar, 1947). The incubation mixture of final volume 1.0 ml with 0.05 *M* phosphate buffer (pH 7.5) contained substrate (3–60 μM), xanthine oxidase (0.41 mg, or 0.069 unit; one unit catalyzes the conversion of 1.0 μmol of xanthine to uric acid per minute at pH 7.5 at 25°), and catalase (0.05 mg). The blank lacked substrate. Changes in absorbance were continuously recorded for 10 min on a Cary 14 spectrophotometer at room temperature (23–25°) in 1.0-cm light-path cells.

Cytokinin Bioassay. The cytokinin activities of xanthine oxidase oxidation products were determined in tobacco pith tissue culture bioassay as described by Fox (1963). The concentration of indole-3-acetic acid in the media was 11.4 μM. These compounds were treated with a small amount of dimethyl sulfoxide to facilitate solution (Schmitz and Skoog, 1970), sterilized by passage through a Nalgene filter (0.2-μ plain membrane), and added to the autoclaved media. The final concentration of dimethyl sulfoxide in the media did not exceed 0.05% (v/v), well below the concentration that affects tissue growth.

Before use in the bioassays, the stock tissue was subcultured for 8 days on a basic medium with only 11.4 μM auxin. Four replicate cultures, with three pieces of callus, each weighing 25–40 mg, planted on 50 ml of agar medium, were used for each treatment. The cultures were kept at room temperature (23–25°) in the dark. After a 35-day growth period, the total fresh weights were determined.

Table I: R_f Values of the Enzymatic Oxidation Products of Zeatin and i^{6}Ade.

Compound	Solvent System A	B	C	D
Zeatin	0.62	0.70	0.77	0.63
i^{6}Ade	0.88	0.87	0.84	0.54
2,8-Dihydroxyzeatin	0.06	0.05	0.42	0.66
2,8-Dihydroxy-i^{6}Ade	0.31	0.16	0.56	0.83
8-Hydroxyzeatin	0.53	0.51	0.68	0.60
8-Hydroxy-i^{6}Ade	0.85	0.77	0.80	0.51
Z-X		0.41	0.77	
i-X		0.38	0.83	

Results

Oxidation of Zeatin and i^{6}Ade by Xanthine Oxidase. When zeatin (38.2 nmol) was incubated with a mixture of xanthine oxidase (0.41 mg of protein) and catalase (0.05 mg of protein) in 1.0 ml of 0.05 *M* phosphate buffer (pH 7.5) the absorption spectrum changed in a characteristic way (Figure 2). During the 95-min incubation at room temperature (23–25°), the original absorption maximum at 269 nm was converted to 306 nm with a transient appearance of an intermediate absorption peak at 275 nm. A constant rate of the conversion of the absorption spectrum from 269 to 306 nm can be detected during the initial 50 min of this incubation. A similar absorption spectrum change was observed when i^{6}Ade was used as a substrate.

In order to investigate the numbers of intermediates formed from i^{6}Ade or zeatin by xanthine oxidase, [8-^{14}C]i^{6}Ade (7500 cpm) was incubated with xanthine oxidase and catalase in 1.0 ml of 0.05 *M* phosphate buffer (pH 7.5) as previously described. After 10 min at 37° the reaction was stopped by addition of an equal volume of 95% ethanol. The reaction mixture was reduced to less than 0.1 ml in a flash evaporator at 38°, mixed with 3.0 A_{269} units of unlabeled i^{6}Ade, and chromatographed on Whatman No. 1MM paper in solvent B system; 2-cm sections of the chromatogram were counted. The radiochromatographic results indicate that there are two major products and possibly a minor one formed from [8-^{14}C]i^{6}Ade. The amounts of ^{14}C radioactivity, normalized to 100% (7.2×10^3 cpm), were peak 1, 50.41 (2,8-dihydroxy-i^{6}Ade); peak 2, 3.47; peak 3, 26.67 (8-hydroxy-i^{6}Ade); and peak 4, 19.45%. The authentic marker, i^{6}Ade, moved coincidently with the radioactive peak 4. The compound in peak 2 was not identified.

When i^{6}Ado, ZR, and a ribose modified i^{6}Ado, 2-*O*-[1(*R*)-[9-*N*^{6}-(Δ^2-isopentenyl)adenyl]-2-hydroxyethyl]glycerol (Chen et al., 1974b), were incubated with xanthine oxidase no spectrophotometric change was detected even after 3 hr of incubation. Thus they are not substrates for the enzyme.

Characterization of the Oxidation Products. In order to identify the metabolites formed from zeatin and i^{6}Ade several large-scale incubations of zeatin or i^{6}Ade with the enzymes were executed. A typical incubation mixture contained xanthine oxidase (101.5 mg of protein) and zeatin (20 mg) or i^{6}Ade (20 mg) in 200 ml of 0.05 *M* phosphate buffer (pH 7.5). Catalase (25 mg of protein) was included only for the preparation of 2,8-dihydroxy derivatives. After 1–3 hr at 37°, the reaction was stopped by addition of an

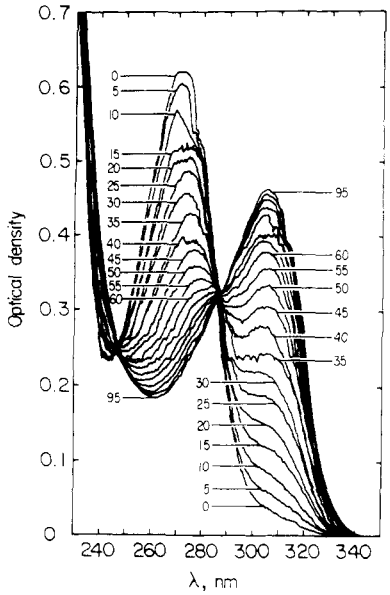

FIGURE 2: Change in the absorption spectrum of zeatin (3.82×10^{-5} *M*) when incubated with xanthine oxidase (0.4 mg of protein) and catalase (0.05 mg of protein) in 1.0 ml of 0.05 *M* phosphate buffer (pH 7.5) at room temperature (23–25°) for 95 min. The numbers on the curves represent time of incubation in minutes.

equal volume of 95% ethanol to the incubation mixture or by boiling the incubation mixture. The solution was reduced to less than 3 ml in a lyophilizer. The solution was passed onto a Sephadex LH-20 column (1.5 × 24 cm) and eluted with 35% ethanol (Table II); 2-ml fractions were collected and the presence of ultraviolet absorbing materials in the elute was located by using a Cary 14 spectrophotometer and by reading the absorbance of each tube at 269, 275, 295, and 306 nm. Purification of the constituent of each peak was achieved by descending Whatman No. 3MM paper chromatography with solvents C and A. Identification of the constituents of each purified sample was made partially on the basis of absorption spectra at pH 1, 7, and 12. As shown in Table III, the metabolites of zeatin or i^{6}Ade have characteristic ultraviolet absorption spectra of 8-hydroxy-, and 2,8-dihydroxypurine derivatives. The R_f values of the substrates and their purified oxidation products, in a variety of solvent systems, are given in Table I. On the basis of these data it is concluded that the behavior of both zeatin and i^{6}Ade toward xanthine oxidase is similar to that of adenine (Wyngaarden and Dunn, 1957) and kinetin (Bergmann and Kwietny, 1958) which are oxidized to their respective 2,8-dihydroxy derivatives with the corresponding 8-hydroxy derivatives formed as intermediates. One of the metabolites (Z-X from zeatin or i-X from i^{6}Ade) having λ_{max} (pH 7.0) 295 (Table III) was repeatedly isolated (about 3.0–3.5% of total products) from the reaction products. However, when the purified Z-X or i-X was treated with xanthine oxidase, no formation of oxidation products was detected. It is clear that 2,8-dihydroxyzeatin could not have arisen from its substrate, zeatin, via Z-X as an intermediate. This is also true for i-X which is not a substrate for xanthine oxidase. In order to see if the products, Z-X or i-X, were derived from an impurity of the substrates, both zeatin and i^{6}Ade were purified by chromatography on 20% ethanol-washed Whatman No. 1MM paper in solvent C. In the presence of xanthine oxidase, the purified substrates yielded about 1.0–1.5% of either Z-X or i-X, less than the amount yielded when using a nonpurified substrate. Thus,

Table II: Relative Mobilities of the Enzymic Oxidation Products on Sephadex LH-20 Columns.[a]

Compound	Relative Mobility
Zeatin	1.16
i^{6}Ade	1.82
2,8-Dihydroxyzeatin	0.89
2,8-Dihydroxy-i^{6}Ade	1.10
8-Hydroxyzeatin	1.26
8-Hydroxy-i^{6}Ade	1.97
Z-X	0.79
i-X	0.72

[a] The value 1.0 represents an elution volume equivalent to one column volume. The columns (1.5 × 24.0 cm) were eluted with 35% ethanol. The values given are compiled from five different columns. Total recovery of optical density from the columns ranged from 97 to 100%.

Table III: Ultraviolet Absorption Spectra (nm).

	pH 1.0		pH 7.0		pH 12.1	
	λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}	λ_{min}
Zeatin	275	235	269	233	276	242
i^{6}Ade	273	235	269	225	275	240
8-Hydroxyzeatin	282	237	275	235	284	247
2,8-Dihydroxyzeatin	309	264	306	266	302	265
8-Hydroxy-i^{6}Ade	282	251	275	246	284	251
2,8-Dihydroxy-i^{6}Ade	309	264	306	266	303	264
Z-X	300	267	295	263	293	265
i-X	300	272	295	268	295	272

these results do not rigorously exclude the possibility that either Z-X or i-X is a derivative of a chemical degradation product of the substrate.

In order to confirm the structures assigned on the basis of the enzymatic and ultraviolet absorption data, the oxidation products of zeatin and i^{6}Ade were analyzed by mass spectrometry.

Mass spectra of the i^{6}Ade oxidation product assigned the 8-hydroxy-i^{6}Ade (Figure 1, 2b) structure supported this assignment. The expected molecular ion at *m/e* of 219 was present [i^{6}Ade (203) + 16] and the spectrum was essentially identical with that recorded by McLennan and Pater (1973). Attempted mass spectral analysis of 2,8-dihydroxy-i^{6}Ade (Figure 1, 2c), however, was unsuccessful. This confirms the observations of McLennan and Pater (1973). We have no explanation for the apparent instability of the 2,8-dihydroxy-i^{6}Ade.

The mass spectral characterization of the zeatin derivatives was more successful (Figures 3 and 4). The observed molecular ion of 8-hydroxyzeatin (Figure 3) was 235; this is 16 more than the molecular ion of zeatin (219). Careful comparison of the mass spectra of zeatin and the zeatin oxidation product clearly locates the additional oxygen atom in the purine ring portion of the substrate. Fragmentation of the hydroxyisopentenyl side chain leads to ions of *m/e* 218, 204, 176, 164, and 151. The corresponding peaks in zeatin are at *m/e* 202, 188, 160, 148, and 135. The absence of a peak at *m/e* 220 as reported for 2-hydroxyzeatin (Hecht et al., 1970a) as well as the ultraviolet spectral characteristics (Table III) clearly establish the material as 8-hydroxyzeatin.

The observed molecular ion of 2,8-dihydroxyzeatin (Figure 4) was at *m/e* 151; this is 32 more than zeatin, molecular weight 219. Again comparison of the fragmentation pattern with that of zeatin and 8-hydroxyzeatin (Figure 3)

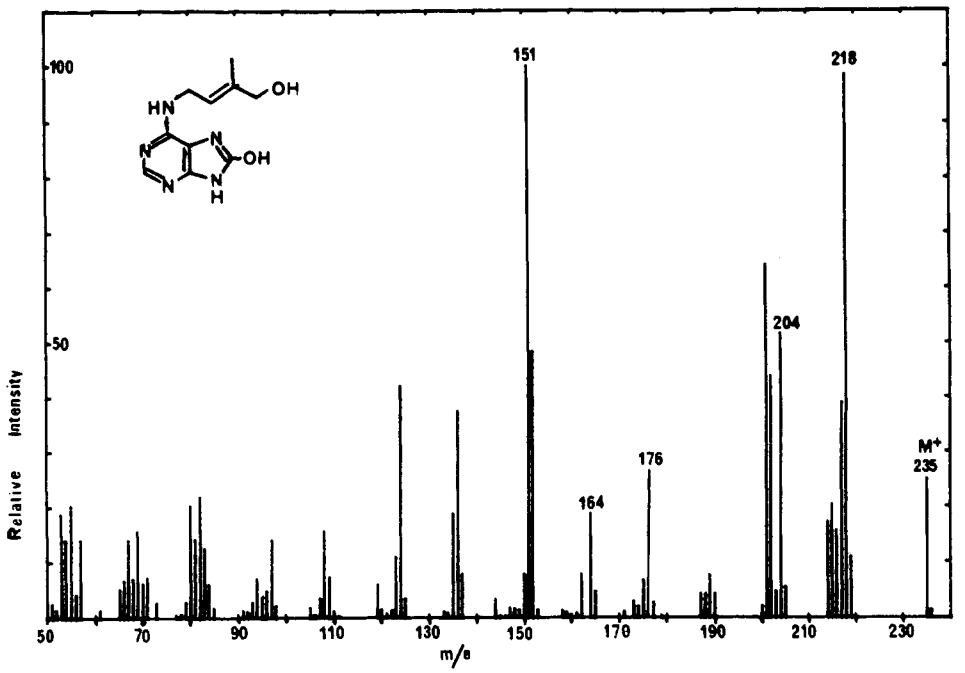

FIGURE 3: Mass spectrum of 8-hydroxyzeatin. Major peak assignments *m/e:* 235, molecular ion; 218(−17), loss of OH; 204(−31), loss of CH_2OH; 176(−59), loss of $C(CH_3)CH_2OH$ and H; 164(−71), loss of $CHC(CH_3)CH_2OH$; and 151(−84), 8-hydroxyadenine.

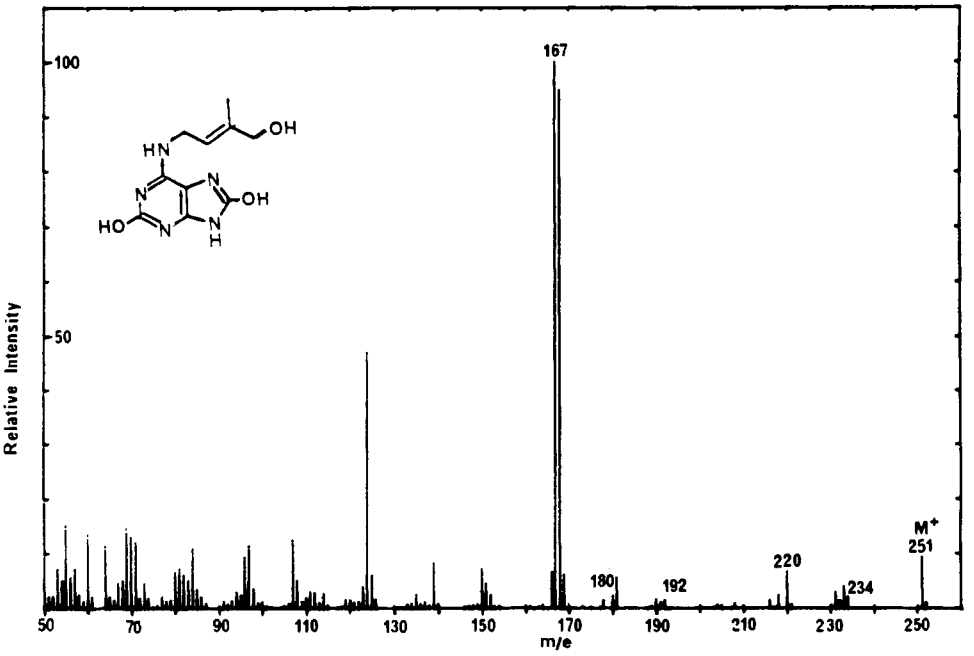

FIGURE 4: Mass spectrum of 2,8-dihydroxyzeatin. Major peak assignments *m/e:* 251, molecular ion; 234(−17), loss of OH; 220(−31), loss of CH_2OH; 192(−59), loss of $C(CH_3)CH_2OH$ and H; 180(−71), loss of $CHC(CH_3)CH_2OH$; and 167(−84), 2,8-dihydroxyadenine.

confirms the presence of the oxygen atoms on the purine ring portion of the substrate: side chain fragmentation giving ions at *m/e* 234, 220, 192, 180, and 167. The presence of the additional substituent has some effect upon fragmentation since loss of water from the side chain is greater in 2,8-dihydroxyzeatin (resulting in fragment ion *m/e* 233) and the major fragment ion (*m/e* 167) represents a substantial increase in relative intensity. This ion comprises the 2,8-dihydroxypurine ring with retention of the 6-amino group. These data clearly establish the structure of the oxidation product to be 2,8-dihydroxyzeatin.

Affinity of Purine Derivatives for Xanthine Oxidase and Rate of Reaction. A kinetic study of the oxidation of zeatin and i^6Ade was carried out as described under Materials and Methods, and comparisons with xanthine were made. Comparison of initial oxidation rates was made at an arbitrary substrate concentration of 1.10×10^{-5} *M* in 1.0 ml of 0.05 *M* phosphate buffer (pH 7.5) at room temperature (23–25°). The Michaelis constants and rates of reaction were calculated by the method of least squares, using a modification of the computer program of Barton and Fisher (1971). It is apparent from Table IV that the affinity of zeatin, i^6Ade, and its hydroxy derivative for the enzyme approximates that of xanthine. Nevertheless, the initial oxidation rate of these compounds is only about 31.5–42% that of xanthine. Some cautions should be exercised in interpreting

Table IV: Michaelis Constants and Initial Velocities for the Oxidation of Purine Derivatives by Xanthine Oxidase.[a]

Compound	ϵ_{max} ($\times 10^{-4}$)[b]	K_m ($M \times 10^{-5}$)	Initial Oxidation Rate (μmol per ml per min)	Relative Rate
Xanthine	1.02	1.16	1.08	100
Zeatin	1.62	2.02	0.38	35.2
i^{6}Ade	1.94	2.21	0.34	31.5
8-Hydroxyzeatin	1.63	1.81	0.45	41.7
8-Hydroxy-i^{6}Ade	1.95	1.89	0.41	38.0

[a] Initial oxidation rates were determined with 1.10×10^{-5} M of purine derivatives in 1.0 ml of 0.05 M phosphate buffer (pH 7.5), in air at room temperature. Each incubation mixture contained 0.41 mg of xanthine oxidase and 0.05 mg of catalase. K_m values were determined under similar conditions with data on at least five different substrate concentrations of each purine derivative. [b] The molar extinction coefficients (at pH 7.0) used in calculation of kinetic data for xanthine, zeatin and i^{6}Ade were taken from Dunn and Hall (1968), for 8-hydroxyzeatin, 8-hydroxy-i^{6}Ade, 2,8-dihydroxyzeatin (1.74×10^{-4}), and 2,8-dihydroxy-i^{6}Ade (1.98×10^{-4}) were determined by authors.

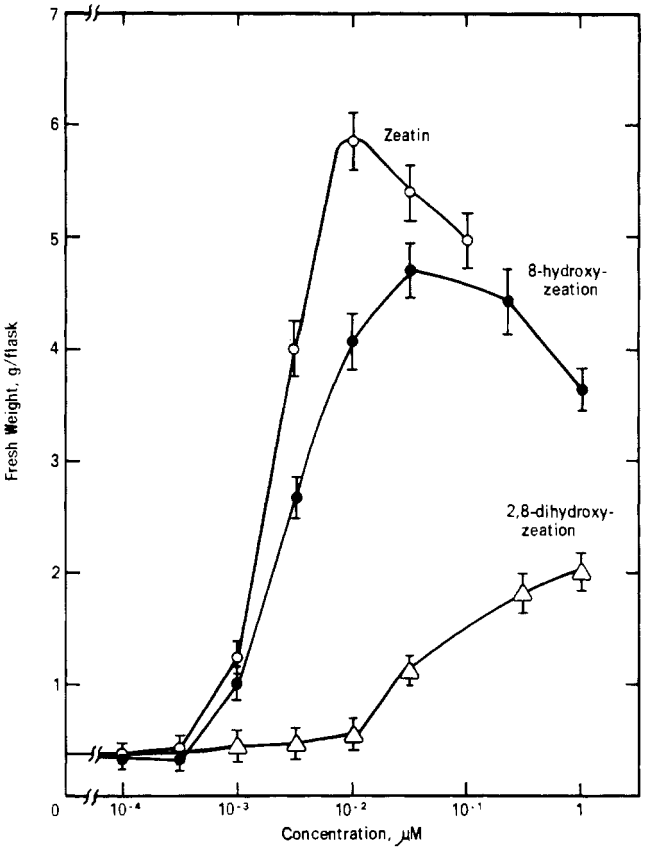

FIGURE 5: Cytokinin activity of 8-hydroxy- and 2,8-dihydroxyzeatin compared with zeatin in the tobacco bioassay.

the obtained kinetic parameters. The oxidation of zeatin or i^{6}Ade by xanthine oxidase can be complex as in the case of hypoxanthine oxidation described by Jezewska (1973). She demonstrated that hypoxanthine oxidation is not a two-step reaction, since the intermediate, xanthine, is detached from the enzyme; during hypoxathine oxidation two subsequent reactions occur in parallel in the time usually taken for experiments.

Cytokinin Activity. The relative cytokinin activities of the four xanthine oxidase products, 8-hydroxyzeatin, 2,8-dihydroxyzeatin, 8-hydroxy-i^{6}Ade, and 2,8-dihydroxy-i^{6}Ade, have been determined and compared with the activities of the parent compounds, zeatin and i^{6}Ade, in the tobacco bioassay (Figures 5 and 6). To avoid breakdown from autoclaving, the test samples were filter-sterilized and added to the medium when it was close to the gelation point. From the results presented in Figures 5 and 6, it is apparent that the 8-hydroxyzeatin and 8-hydroxy-i^{6}Ade are at least ten times less active than the parent compounds, zeatin and i^{6}Ade. Substitution of hydroxyl groups at both the 2 and 8 positions on the adenine nucleus of zeatin and i^{6}Ade further decreases the cytokinin activity. These results are in general agreement with the finding of Dammann et al. (1974). They showed that substitution of i^{6}Ade in the 2 or 8 position, or both the 2 and 8 positions, with MeS, Me, $MeSO_2$,$C_6H_5CH_2S$, HS, and/or Cl lowers the cytokinin activity with the exception of 8-methyl and the 2,8-dimethyl derivatives.

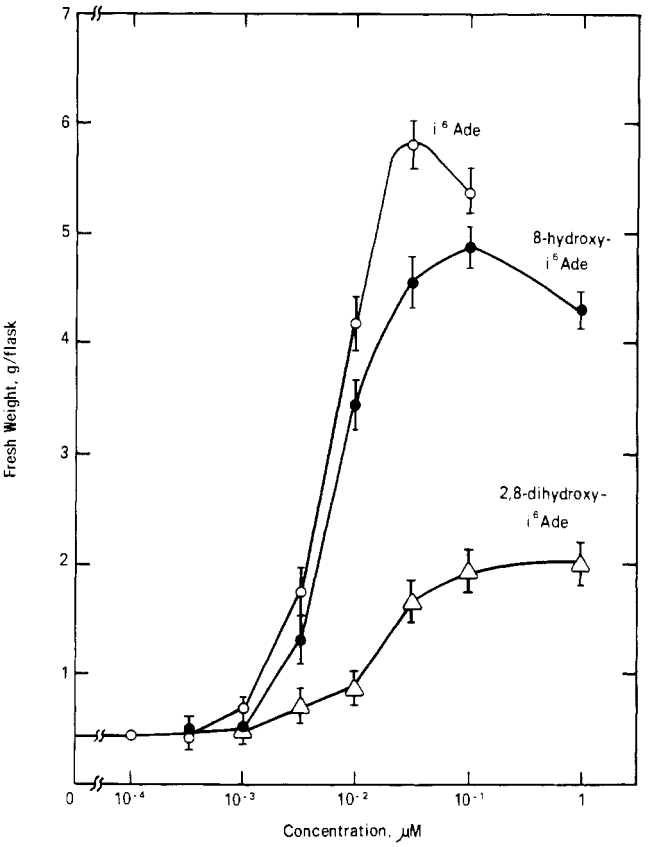

FIGURE 6: Cytokinin activity of i^{6}Ade, 8-hydroxy-, and 2,8-dihydroxy-i^{6}Ade.

Discussion

Several workers (Klenow, 1952, Wyngaarden and Dunn, 1957; Bergmann and Kwietny, 1958; Chheda and Mittelman, 1972; McLennan and Pater, 1973) report that xanthine oxidase catalyzes conversion of adenine and its N^6 derivatives to the corresponding 8-hydroxy and 2,8-dihydroxy derivatives. Zeatin, like other N^6 derivatives of adenine, is attacked first at position 8 and thereafter at position 2 of the purine moiety. This is demonstrated in Figure 2, where a peak at 275 nm appears in the intermediary stages of the enzymatic reaction and vanishes again toward the end of the oxidation. If the corresponding 2-hydroxy derivative would have been formed, its presence would have been noted by a temporary peak at about 284 nm (Hecht et al.,

1970a). Furthermore, the spectral change in time revealed that this oxidation is not a one-step reaction. There is only an isosbestic point near the end of the reaction (285 nm), and the time course curve (306 nm) is not linear. The accumulation of an intermediate is obvious. The formation of an intermediate has also been demonstrated by radiochromatography of this reaction mixture. These results, however, do not exclude the possibility that the alternative pathway may be used to a very small extent.

The fact that i^6Ado, ZR, and i^6Ado analog, 2-*O*-[(*R*)-[9-N^6-(Δ^2-isopentenyl)adenyl]-2-hydroxyethyl]glycerol, are resistant to the enzyme attack is in agreement with the specific structural requirements of adenine. Adenine is oxidized by xanthine oxidase only if (a) the imidazole ring contains a free NH group and (b) at least one hydrogen atom is present in the 6-amino group (Bergmann et al., 1960). The various published Michaelis constants for the oxidation of xanthine by milk xanthine oxidase are in the range from 5.0×10^{-5} *M* (Mackler, 1954) to 2.65×10^{-6} *M* (Fridovich and Handler, 1958). Our results (Table IV) are in the range of these reported values. While inclusion of the bulky N^6-isopentenyl side chain lowers the relative oxidation rate to about one-third of that of xanthine, there seem to be no significant differences between zeatin and i^6Ade in the affinity for the enzyme (Table IV). Although zeatin and i^6Ade are converted to 8-hydroxy and 2,8-dihydroxy derivatives in vitro, it is still unknown whether this same reaction occurs in vivo in the plant systems in a manner analogous to the oxidation of adenine to 2,8-dihydroxyadenine when the former is administered in larger doses to rats (Bendich et al., 1950) and man (Stone and Spies, 1948).

From the results presented in Figures 5 and 6, it is obvious that the 8-hydroxy and 2,8-dihydroxy derivatives of either zeatin or i^6Ade have lower cytokinin activity than zeatin or i^6Ade itself. This result is in accord with the findings of Dammann et al. (1974). While the question how or why hydroxylation of zeatin and i^6Ade lower their cytokinin activity remains to be determined, these hydroxy derivatives may change the rate of uptake and transport of the cytokinins in the tissues. Alternatively, these more polar derivatives may be less easily transformed into an active form or interact less effectively at a cytokinin receptor site.

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