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REGULATORS OF CELL DIVISION IN PLANT TISSUES—III[1]

THE IDENTITY OF ZEATIN

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Abstract—Zeatin, a cytokinin isolated from *Zea mays* kernels, has been shown to be 6-(4-hydroxy-3-methylbut-*trans*-2-enyl)aminopurine.

SUBSTANCES which induce cell division in certain excised plant tissues in the presence of exogenously supplied auxin are now termed cytokinins[2] and not kinin as originally proposed.[3] A number of synthetic 6-substituted purines, 1-substituted adenines and urea derivatives belong to this group of growth stimulants. Plant extracts are known to contain highly active cytokinins. The first of these compounds to be isolated in a state of purity has been termed zeatin,[4] 0·2–1 mg being obtained crystalline from about 60 kg of sweet corn (*Zea mays*) kernels.[1] As a stimulant of cell division in plant tissue cultures, zeatin is markedly more effective than any of the known synthetic cytokinins.[1,5] About 3 mg of zeatin were available for determination of structure. Evidence to establish the identity of zeatin is now presented. A brief preliminary account of this work has been published.[6]

On paper chromatograms, zeatin gave colour reactions (reagents of Reguera and Asimov,[7] red spot; silver nitrate–formic acid,[8] yellow spot) consistent with it being a purine. Pyrimidines do not give these reactions. An attempt was first made to degrade zeatin to some known purine derivative. Hydrolysis with 1N HCl at 100° yielded two products separable by paper chromatography but neither could be identified with the amounts available. Treatment with 0·2N KOH at 100° did not appear to degrade zeatin. Oxidation with nitric acid, permanganate or manganese dioxide, however, yielded adenine which was identified by UV spectra, chromatographic behaviour and conversion to hypoxanthine. The mass spectrum of zeatin[9] showed a peak (*m/e* 135) attributable to the adenine ion and one of *m/e* 108 which is prominent in the mass spectra of adenine and certain adenine derivatives.[9] A mol. wt of 219 (cf adenine 135) was indicated by the mass spectrum while that calculated from the absorption

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[4] D. S. Letham, *Life Sciences* **2**, 569 (1963).
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[6] D. S. Letham, J. S. Shannon and I. R. McDonald, *Proc. Chem. Soc.* 230 (1964).
[7] R. M. Reguera and I. Asimov, *J. Amer. Chem. Soc.* **72**, 5781 (1950).
[8] D. S. Letham, *J. Chromatog.* **20**, 184 (1965).
[9] J. S. Shannon and D. S. Letham, *New Zealand J. Sci.*, in press.

spectrum of zeatin picrate (assumed to be a monopicrate) was 239. The discrepancy was probably due to hydration of the picrate which was recrystallized from water. The above observations established that zeatin was a substituted adenine, the substituent group or groups being eliminated by oxidation.

The location of the substitution was determined by consideration of the pK_a values, the PMR spectrum and the bathochromic shift accompanying oxidation of zeatin with xanthine oxidase. A study of the absorption spectrum of zeatin over the pH range 2–12 detected two ionizations, the pK_a values being 4·4 and 9·8 (in water, 20°, ionic strength 0·1). Since these values differ markedly from those of 1- and 3-substituted adenines,[10,11] the adenine moiety of zeatin was probably not substituted at positions 1 or 3. The pK_a of 9·8, attributable to dissociation of the imidazole —NH— group, indicated that positions 7 and 9 were unsubstituted. The pK_a of 4·4, attributable to protonation of the exocyclic nitrogen, could be given by 6-(mono- and di-substituted amino) purines and by 2- or 8-substituted adenines. In the aromatic region, the PMR spectrum of zeatin picrate (Table 1) was closely similar to the spectra of 6-hexyl- and 6-(2-hydroxyethyl)-aminopurine suggesting that the zeatin picrate spectrum was consistent with an adenine structure unsubstituted at C_2 and C_8. The assignment of the high field peak τ 2·12 to the C_8 proton and the low field peak τ 2·04 to the C_2 proton follows from the reported PMR spectrum of purine.[12] Oxidation of zeatin with xanthine oxidase, an enzyme which hydroxylates adenine at positions 2 and 8, displaced the absorption maximum to longer wavelengths by 37·5 mμ (Fig. 1). The displacements to longer wavelengths observed under identical conditions with known adenine derivatives were as follows: 6-methylaminopurine, 38 mμ; 2-methyladenine, 8 mμ; 8-methyladenine, 0 mμ; 6-(2-hydroxyethyl)aminopurine, 38 mμ. Under similar conditions, displacements recorded for adenine,[13] 6-furfurylaminopurine[14] and 2,6-diaminopurine[15] are 45 mμ, 36 mμ and 11·5 mμ respectively. A bathochromic shift of the magnitude observed with zeatin appears to be given only when positions 2 and 8 are unsubstituted. The absence of a substituent at these positions in zeatin was thus established and zeatin was therefore substituted in the 6-amino group only. Additional evidence for this mode of substitution was provided by the mass spectrum of zeatin.[9] This showed ions at *m/e* 148, 135 and 119 which are characteristic of 6-alkylaminopurines.

Since 6-dialkylaminopurines appear resistant to attack by xanthine oxidase,[16,17] the 6-amino group of zeatin was probably monosubstituted. The UV absorption spectra of zeatin supported this conclusion. The spectra of zeatin in three solvents were almost indistinguishable from those of certain 6-(monosubstituted amino)purines but were divergent from those of 6-(disubstituted amino)purines (Table 2). The numbers of exchangeable hydrogens in zeatin and its fragment ions[9] were also consistent with zeatin being a 6-(monosubstituted amino)purine.

[10] P. Brookes and P. D. Lawley, *J. Chem. Soc.* 539 (1960).
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[12] M. P. Schweizer, S. I. Chan, G. K. Helmkamp and P. O. P. Ts'o, *J. Amer. Chem. Soc.* **86**, 696 (1964).
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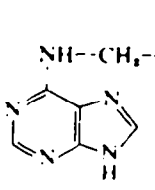
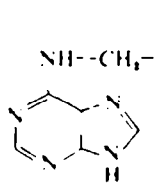
[16] T. R. Henderson, C. G. Skinner and R. E. Eakin, *Plant Physiol.* **37**, 552 (1962).
[17] F. Bergmann, H. Kwietny, G. Levin and H. Engelberg, *Biochim. Biophys. Acta* **37**, 433 (1960).

The structure of the substituent was determined mainly from the mass and PMR spectra. Deuterium exchange in the mass spectrometer[9] indicated that the zeatin molecule contained three active hydrogens. Two must be assigned to the aminopurinyl moiety[9] and one to the substituent. The presence in the mass spectrum of zeatin[9] of both an M-31 peak (*m/e* 188 (190*)) and a metastable peak at *m/e* 188²/219 established the presence of a $-CH_2OH$ group which accounted for the exchangeable hydrogen of the substituent. The intense M-17 peak (*m/e* 202 (204)), the base peak of the spectrum, strongly suggested that the hydroxymethyl group was attached to an unsaturated carbon in the substituent which being of mass 85 possessed the empirical formula C_5H_9O. The PMR spectrum of zeatin picrate (Table 1) indicated an allylic methyl group not coupled with adjacent protons, two methylene groups revealed as an asymmetric doublet and a single olefinic proton revealed as a multiplet. The chemical shifts of the methylene protons indicated that the methylene groups were attached to O or N atoms and were probably allylic. The above findings are consistent with the *cis* and *trans* isomers of only two structures (I and II).

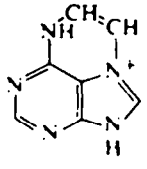


I



II

The mass spectrum of zeatin showed a strong peak at *m/e* 160 (162) which was also present in the spectrum of 6-(3-methylbut-2-enyl)aminopurine and which was attributed to structure III.[9]



III

Of structures I and II, I is much more likely to yield this ion. Mass spectral evidence, therefore, favoured structure I. Oxidation of zeatin with permanganate yielded three UV-absorbing products—N-(purin-6-yl)glycine, adenine and an unidentified compound (probably a 1,2-glycol, but not a ketone). I on oxidation would readily yield N-(purin-6-yl)glycine but II would be expected to give instead a methyl ketone. Oxidative degradation, therefore, also favoured structure I which was assigned to zeatin.

The *trans* form of I was synthesized[10] by an unambiguous route. Chromatography, spectroscopy, mixed m.ps, biological activity and chemical degradation established

* *m/e* values in brackets derived from the spectrum of (NNO-D_9) zeatin.

[18] D. B. Dunn and J. D. Smith, *Biochem. J.* **68**, 627 (1958).

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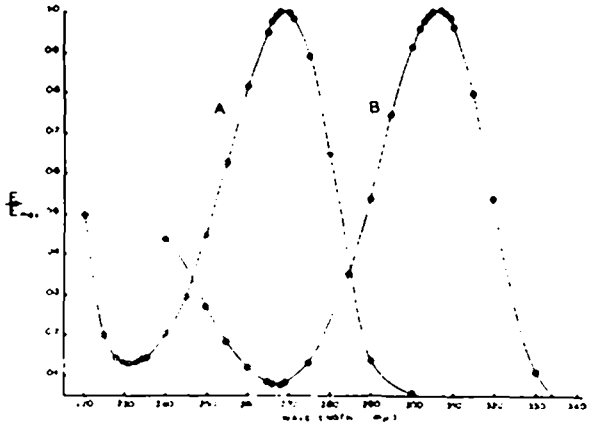

FIG. 1. UV spectra of zeatin (A) and the product formed by oxidation of zeatin with xanthine oxidase (B). Solvent: 0·05M phosphate buffer (pH 8·0).

TABLE 1. PMR SPECTRA OF PICRATES OF ZEATIN AND OF 6-(4-HYDROXY-3-METHYLBUT-*trans*-2-ENYL)AMINOPURINE

Zeatin picrate[a]				6-(4-Hydroxy-3-methylbut-*trans*-2-enyl)-aminopurine picrate[a]		
Chemical shifts (τ values)	Splitting pattern	No. of protons	Assignments	Chemical shifts[b] (τ values)	Splitting pattern	No. of protons
8·47	Singlet	3	$=C(CH_3)-$	8·50	Singlet	3
6·17, 6·08	Asymmetrical doublet	4	$-C(CH_2-O/N)-$	6·26[c], 6·20[c]	Asymmetrical doublet	4
4·58	Multiplet	1	$-CH_2-CH=C-$	4·65[d]	Multiplet	1
2·12	Singlet	1	Purine C_8	2·24	Singlet	1
2·04	Singlet	1	Purine C_2	2·16	Singlet	1
1·67	Singlet	2	Picrate H	1·90	Singlet	2
2·76, 2·42, 1·30	—	—	Pyridine	2·94, 2·48	—	—

[a] Solvent: D_2O–C_5D_5N (ca. 20:1 v/v). In this solvent hydroxyl and amino protons would exchange and were thus not detected.

[b] The chemical shift differences between these spectra is attributed to difficulties in establishing an accurate calibration under the extreme amplifications necessary for the zeatin picrate spectrum. Additional preparations of the synthetic product gave chemical shifts identical to these values.

[c] This asymmetrical doublet was simplified by a spin decoupling technique (−93 c/s) to a single peak with a chemical shift of τ 6·24. It is suggested that the original doublet was a methylene singlet $C(Me)=C-CH_2OH$ (τ 6·26), and a methylene doublet $N-CH_2-CH=C$ (τ 6·23, J ca. 4 c/s). The complementary decoupling signal (+93 c/s) simplified the τ 4·65 multiplet to a clear singlet with minor methyl coupling.

TABLE 2. SPECTRAL CHARACTERISTICS OF ZEATIN AND 6-(MONO- AND DI-SUBSTITUTED AMINO)PURINES[a]

Compound	0·1N HCl		0·1N NaOH		95% EtOH	
	λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}	λ_{min}
Zeatin	273·5	234	218 275	241	269	230
6-Methylaminopurine	267	232	273	239		
6-Hexylaminopurine	270·5	233	218 275	240	269	229
6-(3-Methylbut-2-enyl)aminopurine	273	233·5	218 275	241	269	230
6-(2-Hydroxyethyl)aminopurine	272·5	234	217 274	240	268	229
6-Dimethylaminopurine	277	236	281	245		
6-(N-methyl-N-isopentylamino)purine[b]	278	237	220 283	246	277	234
6-Dipropylaminopurine[c]	277·5	235·5	222 284	246·5	278·5	234
6-Bis(2-hydroxyethyl)aminopurine	283	238	221 282	245	277·5	234

[a] The values for 6-methylaminopurine and 6-dimethylaminopurine are those given by Dunn and Smith.[18] The remainder of the data has not previously been recorded.

[b] 6-(N-methyl-N-butylamino)purine exhibited identical characteristics.

[c] The characteristics for 6-dipropylaminopurine, 6-diisopentylaminopurine and 6-dihexylaminopurine were almost identical (greatest difference 1·5 mμ).

TABLE 3. SPECTRAL CHARACTERISTICS OF DEGRADATION PRODUCTS OF ZEATIN

Compounds	0·1N HCl		0·1N NaOH		95% EtOH	
	λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}	λ_{min}
Oxidation products:						
OX 1	262·5	229	269	237	261	—[a]
OX 2	262·5	229	269	237	260·5	—
OX 3[b]	273·5	234	274	240	—	—
OX 4	273	233	274	241	267	228
OX 5	262·5	229	269	237	261	225
Acid hydrolysis products:						
R_f 0·59 (solvent 1)	269	235	274	242	270·5	243
R_f 0·84 (solvent 1)	277·5	237	279	246	—	—
Adenine	262·5	229	269	237	261	225
N-(purin-6-yl)glycine[c]	273·5	234	274	240	—	—

[a] A dash indicates value not determined.

[b] $\lambda_{max}^{pH\,9\cdot0?}$ = 272·5 mμ.

[c] $\lambda_{max}^{pH\,9\cdot0?}$ = 273 mμ.

that the synthetic product and zeatin were identical. Zeatin was therefore 6-(4-hydroxy-3-methylbut-*trans*-2-enyl)aminopurine. Zeatin has since been synthesized by a different method[20] which confirms the structure assigned above.

EXPERIMENTAL

Methods. All m.ps are corrected. When only small samples were available, a Kofler apparatus was used and the values observed are termed "micro" m.ps. The hot stage was heated to about 15° below the m.p. before the sample was inserted. pK_a values were determined spectrophotometrically.[21] An ultramicro adaption of the method of Cunningham *et al.*[22] was used to determine spectrophotometrically the mol. wt of zeatin; ε for the picrate ion was determined with a pure preparation of adenine picrate using the same spectrophotometer. A Mettler UM 7 balance was used for all ultramicro weighings. For paper chromatography, Whatman No. 1 paper used.

UV spectra: a Beckman model DU instrument fitted with a photomultiplier attachment; PMR spectra: in D_2O–C_5D_5N sols (ca. 20:1 v/v) with a Varian D. P. 60 instrument. Because of the small sample of zeatin picrate (ca. 2 mg) it was necessary to modify the usual techniques. Commercial microcells were not successful, but solvent volumes were considerably reduced when a 15-mm close fitting glass slug was placed in a normal sample tube. Total sample height was restricted to 22 mm. Spectra were calibrated by a ±284 c/s side band from the central HOD peak and by external TMS. Chemical shifts were calculated in tau values. The determination of mass spectra is described in the following paper.[9]

6-(Substituted amino)purines used for spectral comparison with zeatin

New compounds prepared by condensing 6-chloropurine with the appropriate amine in boiling butan-1-ol were:

6-(*N-methyl-N-isopentylamino*)*purine*, m.p. 193° (Found*: C, 60·27; H, 7·78; N, 32·14. $C_{11}H_{17}N_5$ required: C, 60·25; H, 7·81; N, 31·94%.)

6-(*N-methyl-N-butylamino*)*purine*, m.p. 175–175·5° (Found: C, 58·82; H, 7·41; N, 34·03. $C_{10}H_{15}N_5$ required: C, 58·51; H, 7·37; N, 34·12%.) Both compounds were recrystallized from aqueous EtOH.

6-Dipropylaminopurine (Found: C, 60·31; H, 7·87; N, 31·75. Calc. for $C_{11}H_{17}N_5$: C, 60·25; H, 7·81; N, 31·94%) was also prepared in the above way, but the m.p. (163·5–164·5°) differed from the lit.[23] value (143–144°). 6-Dihexylaminopurine was the gift of Dr C. G. Skinner, University of Texas. All other compounds were prepared by published methods (observed m.ps in agreement with lit. values) or were purchased and recrystallized if impure.

Oxidation of zeatin

(a) *With nitric acid.* Zeatin (about 60 μg) was dissolved in HNO_3 (d = 1·42; 0·5 ml) and kept at 40° for 30 hr. Evaporation of the acid at 40° yielded a residue which was chromatographed on paper using as solvent butan-1-ol–water–AcOH (12:5:3; solvent 1). The UV printing technique[24] detected four components with R_f values (listed in order of decreasing spot intensity) of 0·16, 0·41, 0·67 and 0·84. The component of R_f 0·41 (termed OX 1) was eluted with 95% EtOH and rechromatographed in solvent 1 to free it from small amounts of an impurity. The spectral characteristics (Table 3) of purified OX 1 were in agreement with those of adenine. It was indistinguishable from adenine when chromatographed on paper using four solvents.

(b) *With manganese dioxide.* Manganese dioxide (B.D.H. technical grade) was thoroughly washed with boiling water and dried at 75°. Dried oxide (10 mg) was added to an aqueous soln of zeatin (about 150 μg in 0·5 ml). The suspension was heated under reflux for 6 hr, acidified with AcOH and centrifuged. The clarified soln was evaporated and the residue chromatographed on

* Analyses by A. Bernhardt, Mulheim, Germany.

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[21] D. Shugar and J. J. Fox, *Biochim. Biophys. Acta* 9, 199 (1952).

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[23] C. G. Skinner, W. Shive, R. G. Ham, D. C. Fitzgerald and R. E. Eakin, *J. Amer. Chem. Soc.* 78, 5097 (1956).

paper using solvent 1. UV printing[84] revealed two components (R_f values 0·54 and 0·85) which gave spots of approximately equal intensity and a third component (R_f 0·75) which appeared as a much fainter spot. UV spectra (Table 3) and paper chromatography using several solvents indicated that the component of R_f 0·54 (termed OX 2) was adenine. The conversion of OX 2 into hypoxanthine by the procedure next described confirmed this identification.

To a soln of OX 2 in 2N AcOH (about 20μg in 0·1 ml), 0·25M $NaNO_2$ (0·04 ml) was added. After the resulting soln had been kept at 55° for 15 min, a further 0·04 ml of $NaNO_2$ soln was added and the mixture was then kept at 55° for 1 hr and at 40° overnight. To free the reaction product from AcONa, the resulting soln was evaporated on a 5 × 15 mm area at the bottom of a sheet of Whatman cellulose phosphate paper (P20, previously converted to acid form) which was then irrigated with 0·1N AcOH. The resulting chromatogram possessed two UV-absorbing zones—one (faint) near the origin due to a little unreacted OX 2 and one of higher R_f which was eluted with 1N NH_4OH at room temp. Paper chromatography using several solvents indicated that this compound was hypoxanthine.

(c) *With potassium permanganate.* Zeatin (100 μg) was stirred with 0·3 ml $KMnO_4$ soln (2 g/l; pH 7) at room temp for 8 min. Excess permanganate was then destroyed by adding a slight excess of a dilute soln of crotyl alcohol. The mixture was evaporated onto a 5 × 15 mm area on a sheet of chromatography paper and subjected to two-dimensional chromatography using butan-2-one saturated with water (solvent 2) followed by solvent 1. Three UV-absorbing oxidation products which gave spots of approximately equal intensity were detected on the resulting chromatogram. These were designated OX 3, OX 4 and OX 5 and gave R_f values of 0·01, 0·13 and 0·28 respectively in solvent 2 and 0·53, 0·46 and 0·60 respectively in solvent 1.

UV spectra (Table 3) and paper chromatography using several solvents indicated that OX 5 was adenine. Paper electrophoresis showed that at pH 7 OX 3 was negatively charged. Because of this and since other evidence established that zeatin was a 6-(hydroxyalkenyl)aminopurine, OX 3 was probably an N-(purin-6-yl)monoamino acid. OX 3 could be distinguished from 2-(purin-6-ylamino)propionic acid and from 3-(purin-6-ylamino)propionic acid (both prepared according to Ward *et al.*[85]) by paper chromatography with solvent 1. N-(purin-6-yl)glycine and OX 3, however, were indistinguishable when compared chromatographically on paper using several solvents. Electrophoresis at pH 7 and UV spectroscopy (Table 3) confirmed that these two compounds were identical.

OX 4 gave a negative 2,4-dinitrophenylhydrazine test[86] for aldehyde and ketone groups, but reacted positively to a periodate–benzidine spray[87] which detects compounds oxidised by periodate.

Oxidation of adenine derivatives with xanthine oxidase

The catalase used to prevent inactivation of the xanthine oxidase by H_2O_2 was purchased from Sigma Chemical Co. (St. Louis) as a dry powder (3,000 μM units per mg). Xanthine oxidase, also obtained from this source, was supplied as a suspension in 60% $(NH_4)_2SO_4$ (8 μM units per ml). One volume was diluted with 9 volumes of distilled water to give the xanthine oxidase soln used below.

The adenine derivative (about 30 μg) was dissolved in phosphate buffer (3·2 ml; 0·05 M; pH 8·0) in a silica absorption cell and the UV absorption spectrum determined with phosphate buffer (3·2 ml) in the reference cell. Catalase soln (1 mg/ml; 0·2 ml) and xanthine oxidase soln (0·2 ml) were then added to both cells which were kept at 23° for 12–16 hr. The absorption spectrum of the oxidation product was then determined.

Acid hydrolysis of zeatin

A soln of 50 μg of zeatin in 1N HCl (0·3 ml) was heated under reflux for 3·5 hr and then evaporated to dryness in a vacuum desiccator containing NaOH pellets and conc. H_2SO_4. Paper chromatography of the residue using solvent 1 revealed three UV-absorbing components with R_f values (listed in order of decreasing spot intensity) of 0·59, 0·84 and 0·76. The latter component was not present in sufficient amount for exact spectral characterization but spectral data for the other two are given in Table 3.

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[85] D. N. Ward, J. Wade, E. F. Walborg and T. S. Osdene, *J. Org. Chem.* **26**, 5000 (1961).
[86] E. Stahl, *Thin-layer Chromatography* p. 490, Academic Press, New York (1965).
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Comparison of zeatin and 6-(4-hydroxy-3-methylbut-trans-2-enyl)aminopurine

Synthetic 6-(4-hydroxy-3-methylbut-*trans*-2-enyl)aminopurine (*trans*-I) crystallized from water[19] was recrystallized from EtOH to remove impurities revealed by mass spectrometry. This yielded clusters of thick needles with micro m.p. 208–209°, c.f. zeatin 209–210°; mixed m.p. 208–209°. Micro m.ps for zeatin picrate, *trans*-I picrate and a mixture of the two were 188–190°, 189–191° and 188–190° respectively. Addition of a saturated EtOH soln of 3-iodo-2,4,6-trinitrophenol (synthesized according to Hodgson and Moore[20]) to aqueous solns (pH 2) of zeatin and *trans*-I followed by recrystallization of the washed ppts from water yielded the 3-*iodo*-2,4,6-*trinitrophenolates* of zeatin and *trans*-I as needles. The two derivatives had the same micro m.p. (205–206°) unaltered by admixture. (Found for derivative of *trans*-I: I, 22·40. $C_{10}H_{13}ON_5 \cdot C_6H_3O_7N_3I$ required: I, 22·10%.)

Zeatin picrate and *trans*-I picrate gave identical PMR spectra in deuteropyridine—D_5O and identical UV spectra in 95% EtOH. The UV spectra of zeatin and *trans*-I (solvents 0·1N HCl, 0·1N NaOH and 95% EtOH) were superimposable and the mass spectra were in agreement. Zeatin and *trans*-I could not be distinguished by paper chromatography in six solvents or by TLC on Merck aluminium oxide G using 5 solvents. Both yielded the same products when degraded with 1N HCl at 100°, HNO_2 at room temp and dilute permanganate at room temp.

Zeatin picrate and *trans*-I picrate showed identical activity in the carrot phloem assay for cytokinins.[1] At concentrations of 20, 2, 0·2 and 0·02 μg/l, zeatin picrate induced increments in explant weight of 31·8, 25·5, 17·2 and 7·6 mg respectively, while *trans*-I picrate caused increments of 28·0, 26·7, 16·6 and 6·4 mg respectively. Differences between the two sets of values are not significant.

[20] H. H. Hodgson and F. H. Moore, *J. Chem. Soc.* 630 (1927).