The Zeanins: A Group of Growth-Promoting Compounds from Immature Maize Kernels

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Expressed maize-kernel juice was examined by Farrar, Bentley, Britton & Housley (1958) for activity in the *Avena* coleoptile straight-growth test of Bentley & Housley (1954). Activity of plant extracts in this test is often assumed to be due to the presence of indol-3-ylacetic acid, but it is not possible to account in this way for the activity of maize-kernel juice, most of which remains in the aqueous phase even after exhaustive ether extraction under conditions sufficiently acidic to remove indol-3-ylacetic acid completely.

The present paper describes further investigations of the growth-promoting properties of maize-kernel juice. An extraction procedure has been worked out, and the extract fractionated by countercurrent distribution. Paper chromatography of a series of fractions has shown that growth promotion is associated with the presence of a number of substances, all giving with the Salkowski reagent a purple colour quite distinct from the red given by indol-3-ylacetic acid. On paper chromatography in solutions containing ammonia these substances all decompose. One of the breakdown products appears, on the basis of R_F and a red Salkowski reaction, to be indol-3-ylacetic acid.

These reactions suggest a similarity sufficient to warrant a collective name, and since the plant source was *Zea mays* the term 'zeanin' was adopted. A zeanin is to be defined as a plant-growth substance occurring in *Zea mays*, giving a purple reaction with the Salkowski reagent, and breaking down on chromatography in ammoniacal solutions to give a Salkowski-reacting component similar to indol-3-ylacetic acid.

MATERIALS AND METHODS

Bio-assay. Ability to promote the growth of *Avena* coleoptiles was assayed by the straight-growth test of Bentley & Housley (1954).

Maize. The maize variety used for the extraction was Caldera 331, on trial in 1960 at Croston, Lancs., as a fodder crop. In spite of cool wet conditions this variety produced a large number of cobs. The cobs were harvested on 29 September, when most of them were at the 'milky' stage suitable for culinary use.

Solvents. For extraction and normally for chromatography a good commercial grade of butan-1-ol was used. When AnalaR butan-1-ol was used, the fact is stated. The 95% (v/v) ethanol conformed to the specification for 'ethyl alcohol', BS 50:1933.

Paper chromatography. The paper used was Whatman no. 1. Initially, chromatography was carried out at about 25° using butan-1-ol saturated with water as the solvent. However, since the R_F values of the zeanins were altered markedly by changes in the concentration of water in the butan-1-ol and by temperature fluctuations, later chromatograms were run at constant temperature (21°). The solvent used under these conditions was AnalaR butan-1-ol-water (6:1, v/v).

Spray reagents. Ehrlich's reagent, nitrous-nitric acid reagent and dilute and concentrated Salkowski reagents were as described by Bentley, Farrar, Housley, Smith & Taylor (1956). The *p*-dimethylaminocinnamaldehyde reagent was a 0.1% solution in ethanolic 1% (w/v) HCl.

Extraction procedure. Cobs (360), wt. 120 lb., were dehusked and the kernels removed. The remaining stalks were soaked for 1-2 hr. in water to be used for the extraction. The kernels were minced and mixed with water (1 l. to 20 cobs). The water extract was freed from pulp by centrifuging and the supernatant added to an equal volume of 95% (v/v) ethanol. The precipitate was removed by centrifuging. The aqueous-alcoholic supernatant was evaporated under reduced pressure below 50° to give a brownish gum; water was added to give 250 ml. of solution. This solution was extracted ten times with 300 ml. portions of butan-1-ol. As the extracts contained droplets of the aqueous phase which settled out only slowly, they were each washed in turn with two 50 ml. portions of water, the same two portions being retained and used in the same order throughout. The butan-1-ol extracts were combined and evaporated below 50° to 150 ml. During evaporation a solid began to separate. After standing overnight, the mother liquors were removed by decantation from the solid which was washed by decantation with butan-1-ol. The extremely hygroscopic solid (wt. about 30 g.) gave a strong purple colour reaction with the concentrated Salkowski reagent, whereas the mother liquors gave a redder, less intense colour. Paper-chromatographic studies of the mother liquors showed only traces of zeanins.

Fractionation procedure. The solid was dissolved in water (80 ml.). The solution was placed in the first two tubes of a 60-tube countercurrent apparatus with 40 ml. top and bottom phases. The solvent system was water-butan-1-ol.

After 60 transfers the total contents of the tubes were combined in sets of six to give ten fractions. Fraction 1 contained material which travelled right through the apparatus, i.e. the least-polar fraction, and fraction 10

contained the slow-moving polar material. Each fraction was evaporated under reduced pressure to 40 ml. and portions were chromatographed in butan-1-ol saturated with water. *p*-Dimethylaminocinnamaldehyde showed several spots whose R_F values are recorded in Table 1.

Fractions 1 and 2 appeared to contain indol-3-ylacetic acid. In a preliminary experiment in which butan-1-ol soluble material had not been removed, it was possible to isolate, from the corresponding fraction 1, indol-3-ylacetic acid, shown to be identical with synthetic indol-3-ylacetic acid by determinations of m.p., mixed m.p., and ultra-violet and infrared spectra.

Fractions 5-7 contained at least two different compounds giving spots between R_F 0.1 and 0.2. Fractions 7 and 8 gave a complex spot between R_F 0.0 and 0.1. The spot from fraction 9 was distorted in shape, and possibly in position, by the presence of impurities. This spot was also complex and was probably similar to the one in fraction 8.

Fractions 5-9 inclusive were again partitioned between butan-1-ol and water. Each fraction was made up to 120 ml. and placed in the countercurrent apparatus in the following order: fraction 9 in tubes 1, 2, 3 (initial tubes), fraction 8 in tubes 4, 5, 6, fraction 7 in tubes 7, 8, 9, fraction 6 in tubes 10, 11, 12 and fraction 5 in tubes 13, 14, 15. For this fractionation 120 tubes were used; other conditions were as before. After passing through the 120 tubes, the effluent butan-1-ol was collected in fractions. Individual fractions were combined in sets of ten, a bulked fraction comprising 400 ml.

The fractions were evaporated under reduced pressure to gums and then made up to 10 ml. with 95% ethanol. From this point onwards, the fractions were kept in a refrigerator. After 500 individual fractions (50 bulk fractions) had been collected, the countercurrent-distribution apparatus was emptied, and the total tube contents combined in sets of ten to give 12 bulk fractions of 800 ml. each. The procedure thus gave 62 fractions in all, each consisting of a solution in 10 ml. of 95% ethanol, or aqueous ethanol when this was necessary to achieve

solution. All the fractions from 10 to 54 inclusive showed Salkowski reactions. Fractions 1-9 and 55-62 gave no Salkowski reaction. The chromatographic examination of the fractions is discussed below.

RESULTS

Paper chromatography of alternate fraction nos. 10-56 from the second countercurrent-distribution experiment showed two distinct groups of zeatinins; one group, having R_F values of 0.1-0.2, was confined to fractions 10-22; the other group, R_F 0.0-0.1, was represented by some of its components in fractions 20-54.

Chromatography and bio-assay of fractions 13, 19 and 43

The zeatin of fraction nos. 10-22 was complex. With increasing fraction number the R_F decreased from 0.15 at fraction no. 12 to 0.12 at fraction no. 20. At first this was interpreted as being due to the presence of two components only. Fraction 13 appeared to be homogeneous with respect to the zeatin of R_F 0.15, while fraction 19 appeared to contain only the zeatin of R_F 0.12. Fractions 13 and 19 gave strong spots with spray reagents indicating a concentration high enough for bio-assay.

Accordingly, fractions 13 and 19 were each chromatographed in butan-1-ol-water, 1% of the total fraction being used in each case. After removal of a test strip of paper for spray reactions, the rest of the paper was sprayed repeatedly with water to remove butan-1-ol which interferes with the bio-assay. Both papers were cut into ten strips, each 0.1 R_F unit wide. Each strip was eluted with 15 ml. of water for bio-assay. Figs. 1 and 2 show the results, expressed as histograms, for fractions 13 and 19. In fraction 13, the coloured spot appeared at a slightly higher R_F than usual. However, both here and in fraction 19, activity in the straight-growth test was correlated with the position of the positively reacting spot.

Fraction 43 was complex, and appeared to contain most of the zeatinins with R_F values of 0.01-0.1. The procedure for this fraction differed slightly from that used for fractions 13 and 19. The fraction (3% of the total) was chromatographed in butan-1-ol-water for 5 days. The positions of fluorescent impurities showed that running had been fairly even. The edges of the paper were removed, and two suitable longitudinal test strips were cut and sprayed. The coloured bands of both strips were almost coincident. After repeated spraying with water, the rest of the paper was divided into ten horizontal strips, the width of each strip being one-tenth of the distance from the starting line to the bottom edge of the paper. The strips were eluted and assayed as before.

Table 1. R_F values of *p*-dimethylaminocinnamaldehyde-reactive substances from *Caldera* 331

Fractions are from a 60-tube butan-1-ol-water countercurrent distribution. Chromatography solvent system: butan-1-ol saturated with water at 25°.

Fraction no.	R_F
1 (least polar fraction)	0.79
2	0.76
3	No detectable spot
4	No detectable spot
5	0.17 (weak reaction)
6	0.17, 0.12 (overlapping)
7	0.06, 0.12, 0.18
8	0.06
9	0.12*
10 (most polar fraction)	No clear spot

* This spot was heart-shaped. Fluorescence in u.v. light showed that fraction 9 contained other substances that possibly increased the R_F value.

Fig. 3 shows the results for fraction 43. Two distinct coloured bands seen on the test strips again corresponded in position with areas active in the straight-growth test. The more slowly moving component gave a coloured spot extending from 6.3–8.7 cm. from the origin and not obviously multiple. This component was named zeatin Z. The more rapidly moving spot lay between 10.5

and 16.2 cm. from the origin and consisted of at least three different substances. These were named collectively zeatin complex Y.

Further examination of the zeatins with R_F 0.1–0.2

More concentrated solutions of the two components having R_F values between 0.1 and 0.2 were obtained by heavily loading a chromatogram with a mixture of 10% of fractions 14 and 17; development in butan-1-ol–water proceeded for 64 hr. Although running was somewhat uneven, the position of fluorescent contaminants made it possible to extrapolate the positions of the zeatins, as shown by spray reactions, across the paper. Instead of the two spots expected, three appeared, completely separated from each other. The absence of tailing between the spots made it appear to be unlikely that any of the spots were breakdown products formed during chromatography.

The distances travelled by the spots were in the proportions 1:0.80:0.66. They were named zeatin A, B and C respectively. The leading edge of spot A had travelled 41.7 cm. from the starting line. The strongest colour reaction (with *p*-dimethylaminocinnamaldehyde) was given by zeatin A; zeatin B gave the weakest reaction.

The three zeatins were eluted from appropriate sections of the chromatogram to give three 30 ml. extracts. Results of bio-assay in comparison with indol-3-ylacetic acid show (Fig. 4) that the extract of zeatin A induced more growth than a solution of indol-3-ylacetic acid (20 mg./l., a concentration

Fig. 1. Histogram of butan-1-ol-water chromatogram of fraction 13 assayed on *Avena* coleoptiles. Tenfold dilutions are indicated by broken lines. Arrows indicate growth in control solutions. 1, Water; 2, 3 and 4, 0.02, 0.2 and 2.0 mg. of indol-3-ylacetic acid/l. respectively.

Fig. 2. Histogram of butan-1-ol-water chromatogram of fraction 19 assayed on *Avena* coleoptiles. Tenfold dilutions are indicated by broken lines. Arrows indicate growth in control solutions. 1, Water; 2, 3 and 4, 0.02, 0.2 and 2.0 mg. of indol-3-ylacetic acid/l. respectively.

Fig. 3. Histogram of the first 30 cm. of a chromatogram of fraction 43, run for 5 days in butan-1-ol-water, assayed on *Avena* coleoptiles. Tenfold dilutions are indicated by broken lines. Arrows indicate growth in control solutions. 1, Water; 2, 3 and 4, 0.02, 0.2 and 2.0 mg. of indol-3-ylacetic acid/l. respectively.

approaching the optimum for this test). The actual concentration of zeatin in the zeatin A extract was unknown, but ultraviolet spectroscopy indicated that its concentration could not be more than 30 mg./l. if the compound were assumed to be a 3-substituted indole.

Reactions of the zeatinins with spray reagents

In Table 2, reactions of the zeatinins with spray reagents are compared with those of indol-3-ylacetic acid. The different zeatinins behaved similarly, although the rates of fading of the spots given with the concentrated Salkowski reagent varied somewhat.

Decomposition of the zeatinins during chromatography in ammoniacal solvents

When the zeatinins were chromatographed in solvent systems containing ammonia, a red Salkowski reaction always appeared in the indol-3-ylacetic acid region of the chromatogram. However, if solutions of any of the zeatinins were kept overnight at room temperature in test tubes with a solvent which would cause decomposition on a paper chromatogram (e.g. propan-2-ol-water-ammonia (sp.gr. 0.88) (16:3:1, by vol.), and then chromatographed in a non-ammoniacal solvent (e.g. butan-1-ol saturated with water), no red Salkowski-reacting component could be detected. This suggests that the decomposition of zeatinins in the presence of ammonia may be oxidative.

DISCUSSION

Fawcett (1961) listed the unidentified indole auxins present in plants that have been detected by paper chromatography. Only the auxins of maize-kernel juice (Farrar *et al.* 1958) were stated to give a purple Salkowski reaction. In much of the work discussed by Fawcett, ethereal extracts only were examined. When aqueous extracts have been studied, chromatography has usually been carried out in ammoniacal solutions. However, the importance of using ammonia-free solvents in this work cannot be too strongly stressed.

Zenk (1961) has described the isolation of a new auxin, 1-(indol-3-ylacetyl)- β -D-glucose, from *Colchicum neapolitanum*. This compound was readily split by ammonia to give, by a combination of ammonolysis and hydrolysis, indol-3-ylacetic acid and indol-3-ylacetamide. Bentley *et al.* (1956) found that indol-3-ylpyruvic acid, claimed by Stowe & Thimann (1954) as a growth-promoting constituent of maize kernels, decomposed very rapidly on chromatography in ammoniacal solutions; ascorbigen, obtained from cabbage and in-

Fig. 4. Variation of growth-promoting activity of zeatinins A, B, and C with concentration, compared with that of indol-3-ylacetic acid. Assayed on *Avena* coleoptiles. ●, Zeatin A; ▲, zeatin B; ○, zeatin C; ■, indol-3-ylacetic acid.

Table 2. Reactions with some spray reagents of the zeatinins compared where applicable with the reactions of indol-3-ylacetic acid

Spray reagent	Reaction with zeatinins	Reaction with indol-3-ylacetic acid
Conc. Salkowski reagent	*Purple, fading to brown	Red, fading slowly to brown
Dil. Salkowski reagent	*Purple, fading slowly to brown	Red, fading only on long standing
Ehrlich's reagent	Blue	Blue
Nitrous-nitric acid reagent	Yellow (faint)	Red
Ninhydrin	Negative	—
Ninhydrin after attempted acid hydrolysis	Negative	—
Aniline phthalate	Negative	—
5% (w/v) NaOH followed after 1 hr. by the conc. Salkowski reagent	Red, like indol-3-ylacetic acid	—

* Zeatinins require stronger heating than indol-3-ylacetic acid for development of colour with the Salkowski reagents.

vestigated by Procházka (1954, 1959), Procházka, Šanda & Šorm (1957) and Procházka & Šanda (1958), was found by Kutáček, Icha, Valenta, Tupy & Holkova (1959) to give indol-3-ylacetic acid under similar conditions.

It seems possible that there might be a relationship between the zeatinins and these last two readily oxidized compounds. Fawcett has suggested that in plants there is an 'indole cycle' involving 1,2-dihydroxy-3-(indol-3-yl)prop-1-ene as a hypothetical intermediate capable of combining with ascorbic acid to give ascorbigen. 1,2-Dihydroxy-3-(indol-3-yl)prop-1-ene could give rise to the zeatinins by combining with moieties other than ascorbic acid.

The biological role of the zeatinins is unknown, but it is possible that they may be the factors of corn milk which are effective in the culture of excised embryos of corn, studied by Haagen-Smit, Siu & Wilson (1945). Any process in which the zeatinins act decisively must be completed before the kernels are mature, since at maturity all the auxin potential is present as either 'free auxin' which is ether-extractable, or 'bound auxin', both of these forms differing from the zeatinins. Avery & Shalucha (1942) and Avery, Berger & Shalucha (1942) studied the auxins of mature maize kernels. The 'free auxin' observed in these studies and in the present investigation appears to be indol-3-ylacetic acid. 'Bound auxin', sometimes called 'indol-3-ylacetic acid precursor', liberates indol-3-ylacetic acid (characterized chemically) on autoclaving at alkaline pH. If 'bound auxin' contains residues derived from the zeatinins, this treatment could liberate indol-3-ylacetic acid, so that an alternative role of the zeatinins would be as intermediates in the formation of 'bound auxin'.

The complexity of the zeatin complement of maize is rather surprising. There is evidence that other zeatinins exist apart from those already mentioned. In one extraction of frozen corn-off-the-cob, supplied by Birds-Eye Products Ltd., a further zeatin-like component was detected. It appeared in fraction 4 (Table 1), and travelled to about the same position on paper chromatograms as indol-3-ylacetic acid. This zeatin was not detected on paper chromatograms of any of the other zeatinins under any of the conditions employed, nor

did it appear in the Caldera 331 extract, worked up under similar conditions, so that it seems unlikely to have been a breakdown product. The Caldera 331 extract itself contained at least seven zeatinins.

It may be that each component has a specialized function in development and maturation.

SUMMARY

1. An extract of immature kernels of *Zea mays* was fractionated by a countercurrent-distribution procedure.

2. Several indole compounds, named zeatinins, capable of promoting the growth of oat coleoptiles, were shown to be present.

3. Possible relationships of the zeatinins to other indole auxins are discussed.

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