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Indoleacetaldehyde in Cucumber Seedlings[1]

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ABSTRACT

The presence of indoleacetaldehyde in cucumber (*Cucumis sativus* L.) cotyledons was demonstrated by thin layer chromatographic R_F values in three solvent systems, by the formation and hydrolysis of a bisulfite adduct, and by chemical reduction to indoleethanol and oxidation to indoleacetic acid. Bioassays indicated a minimum indoleacetaldehyde content in etiolated cotyledons of 0.7 μg per kg fresh weight. Tissue samples from all parts of both green and etiolated cucumber seedlings reduced exogenously supplied indoleacetaldehyde to indoleethanol.

In a continuing study of auxin biosynthesis and its regulation, we have demonstrated the occurrence of a number of enzymes and intermediates in the cucumber seedling. Some of these are directly involved in the metabolism of indole-3-acetaldehyde. They include three amine oxidases which can oxidize tryptamine to IAAld[4] (7), an indoleethanol oxidase which oxidizes indole-3-ethanol to IAAld (8, 13), IAAld reductases which reduce IAAld to IEt (1, 3), and an IAAld oxidase which oxidizes IAAld to IAA (2). These demonstrations could be considered irrelevant to the actual biosynthesis of auxin unless it is shown that IAAld occurs naturally in the cucumber seedling. Here it is demonstrated that IAAld is a normal constituent of both green and etiolated seedlings; in addition, it is shown that living cucumber tissue, from all organs of the seedling, catalyzes the reduction of exogenously supplied IAAld to IEt.

MATERIALS AND METHODS

Plants. Seeds of *Cucumis sativus* L. cv. National Pickling (Burpee Seed Co.) were soaked for 2 hr in distilled H_2O and sown in vermiculite saturated with tap water. Etiolated seedlings grew in darkness at 21 C for 5 or 6 days. Green seedlings grew on a 14-hr light, 10-hr dark cycle at 25 C for 5 or 6 days.

Growth Tests. The cucumber hypocotyl segment test (9) was used as a bioassay for IAAld. Segments 2 cm in length, taken immediately below the point of insertion of the cotyledons, were cut from 5-day-old green seedlings. Segments were incubated in groups of five in Stender dishes containing 2 ml of unbuffered medium and measured to the nearest 0.5 mm after 20 hr of growth in darkness. All treatments were run in duplicate, and the growth tests were repeated nine times in all.

Preparation of Free IAAld. IAAld was purchased as the bisulfite adduct from Sigma Chemical Co. and stored desiccated at −20 C. Each day, a freshly prepared 5 mM solution of IAAld bisulfite was adjusted to pH 10 with saturated Na_2CO_3 and allowed to stand briefly at room temperature. Free IAAld was extracted from the solution with anhydrous diethyl ether. Enough water was added to make the final concentration of IAAld 1 mM and the ether removed by flash evaporation. The free IAAld concentration was determined by the method of Brown and Purves (3).

Extraction and Partial Purification of Native IAAld. Cotyledons were harvested from etiolated or green seedlings and stored at −20 C. Samples of frozen cotyledons were briefly ground in methanol in a Waring Blendor and the homogenate filtered once through Miracloth (Calbiochem) and once through filter paper. The filtrate was mixed with activated charcoal at 35 g/100 g of etiolated tissue or 50 g/100 g of green tissue. The slurry was filtered and the filtrate discarded. The charcoal was then treated with acetone (250 ml/100 g of tissue). The acetone extract was combined with a small volume of water and the acetone evaporated at 30 C under vacuum in a rotary evaporator. Water was added to the aqueous residue to a final volume of 1 ml/g of tissue. The resulting solution was adjusted to pH 7.8 to 8.5 with KOH and extracted three times with equal volumes of anhydrous diethyl ether. The combined ether fractions were dried over Na_2SO_4 and evaporated to dryness. This residue is referred to as *fraction A*. For further purification, fraction A was subjected to TLC with solvent I (see below). The zone from R_F 0.5 to 0.6 was scraped from the sheet and eluted with ether, which was evaporated to dryness. This residue is referred to as *fraction B*. Fraction B was chromatographed with solvent II. Elution of the zone from R_F 0.05 to 0.15, followed by evaporation of the ether eluate, yielded *fraction C*.

Thin Layer Chromatography. Dried residues were dissolved in a minimal volume of acetone and streaked on 0.25-mm silica gel thin layer sheets (Eastman Chromogram Sheets, no. 6060). The following developing solvent systems were employed: *solvent I*: diethyl ether-heptane (7:1, v/v); *solvent II*: carbon tetrachloride-methanol (10:1, v/v); *solvent III*: acetone-methanol-chloroform (5:4:4, v/v); *solvent IV*: isopropyl alcohol-NH_4OH-H_2O (10:1:1, v/v). Some chromatograms were sprayed with Ehrlich's reagent (5) and dried under a stream of hot air to enhance color development. Others were sprayed with a modified Salkowski reagent (4) and kept in the dark during color development.

***In Vivo* Assay for IAAld Reductase Activity.** Tissue samples from green or etiolated seedlings were prepared as follows: cotyledons: five cotyledons, each cut into five strips; hypocotyls: five apical 1-cm segments, each cut into 2-mm pieces; roots: five 1-cm segments, taken at random and cut into 2-mm pieces; epicotyls (green only): 10, intact. The samples were placed in 1 ml of distilled H_2O, and the experiment was initiated by adding 2 ml of 0.8 mM IAAld to each sample. After 3 hr at room temperature, under normal laboratory illumination, 0.2 ml of

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[4] Abbreviations: IAAld: indole-3-acetaldehyde; IEt: indole-3-ethanol.

each reaction mixture was combined with 0.2 ml of 0.8 mM IAAld to insure that enough IAAld was present to interact in the Salkowski test with the IEt produced during incubation. (Vickery and Purves [13] have shown that a mixture of IAAld and IEt gives a strong pink color [λ_{max} = 529 nm] with the Salkowski reagent. IAAld or IEt alone gives little color in this test.) The Salkowski reagent (0.5 ml) was added and A_{529} measured after 20 min, with a Beckman Acta V spectrophotometer. This *in vivo* assay is of limited value in comparing samples of differing tissue types, since it depends closely upon the area of cut surface. It may prove to be useful in comparing the effects of various treatments upon identical tissue samples.

RESULTS AND DISCUSSION

Isolation and Identification of IAAld. Aliquots of fraction A obtained from either green or etiolated cotyledons were subjected to TLC in solvent I. Ehrlich's reagent revealed a pinkish blue spot at the R_F of IAAld (both the test samples and authentic IAAld gave R_F values of 0.54). Chromatography of fraction B from green or etiolated tissue in solvent II, followed by Ehrlich's spray, produced a violet spot at R_F 0.10 (R_F of authentic IAAld = 0.10). Another aliquot of fraction B was chromatographed with solvent III; Ehrlich's spray gave a violet spot at R_F 0.86 (R_F of IAAld = 0.85). The data from these three chromatographic systems are consistent with the presence of IAAld in both green and etiolated cucumber cotyledons.

Fraction B from 90 g of etiolated cotyledons was dissolved in diethyl ether and shaken with aqueous $NaHSO_3$ (35%, w/v) for 5 min. The aqueous phase was isolated, adjusted to pH 10 with NaOH, and extracted three times with equal volumes of diethyl ether. The combined ether wash fractions were evaporated, taken up in acetone, and chromatographed with solvent II, yielding an Ehrlich-positive spot at R_F 0.10, the R_F of authentic IAAld. These results suggest that IAAld present in fraction B formed a bisulfite adduct which partitioned into the aqueous phase at neutral pH. Alkaline conditions hydrolyzed the adduct, so that the free aldehyde was extractable with diethyl ether.

Fraction C from another sample of 90 g of etiolated cotyledons was dissolved in diethyl ether and evaporated into 2 ml of water, to which was added 7 mg of $NaBH_4$. After 2 hr of reaction at room temperature, the solution was acidified to pH 2.5 with HCl and extracted with 25 ml of diethyl ether. The ether phase was dried over Na_2SO_4 and evaporated to dryness. The residue was chromatographed with solvent I, yielding an Ehrlich-positive spot at R_F 0.35 (R_F of IEt = 0.36, of IAAld = 0.54). These results are consistent with the reduction of IAAld to IEt by borohydride. There was no IEt present in the original sample of fraction C.

Fraction B from 300 g of etiolated cotyledons was resuspended in 1.5 ml of 0.02 M Ag_2SO_4, and 0.5 ml of 0.12 M NaOH was added in the dark. After 1.5 hr of reaction, the solution was adjusted to pH 2.5 with HCl and extracted with 30 ml of diethyl ether, which was dried with Na_2SO_4 and evaporated to dryness. The residue was chromatographed with solvent IV, yielding a Salkowski-positive spot at R_F 0.45 (R_F of authentic IAA = 0.45, of IAAld = 0.90). This indicates the oxidation of IAAld to IAA by Ag^+. No IAA was present in untreated fraction B.

The presence of IAAld in cucumber cotyledons was clearly established by these several means. IAA1d has previously been demonstrated to be a normal constituent of peak (6, 10, 12) and sunflower seedlings (10).

Semiquantitative Determination of IAAld. We processed several 50-g samples of etiolated cotyledons to the fraction C stage. Each of these residues was dissolved in 2 ml of water and used as a test sample for hypocotyl segment growth tests. In some instances, these gave maximal growth responses (equivalent to 0.1–1 mM IAAld). In no case did the test solution give less growth than that obtained with 0.1 μM IAAld. A sample experiment is illustrated in Figure 1. From such data, we calculate that etiolated cucumber cotyledons contain no less than 0.7 μg of IAAld/kg fresh wt. This presumably represents an underestimation of the true IAAld content since extraction may not have been complete and, more importantly, free IAAld is relatively unstable in aqueous solution.

Reduction of IAAld *in Vivo*. We have shown previously that cucumber extracts are capable of reducing IAAld to IEt, and we have isolated and partially characterized the indoleacetaldehyde reductases responsible for this activity (1, 3). Having now demonstrated the presence of IAAld in the cucumber seedling, we tested the ability of living cucumber tissue to reduce exogenously supplied IAAld. In these experiments, segments of various seedling organs were incubated in aqueous solutions of free IAAld without added reduced pyridine nucleotide (required in *in vitro* assays of the enzymes [3]); and IEt accumulation in the medium was assayed by Salkowski color reaction (13) and confirmed by TLC. Under these conditions, no oxidation of exogenous IAAld to IAA was observed, perhaps because of rapid destruction or sequestration of IAA. Results of a typical *in vivo* experiment are presented in Table I. It can be seen that exogenously supplied IAAld was metabolized to IEt by all organs of both green and etiolated plants. It is inappropriate to compare the relative rates of IEt production among the various organs since variation in tissue handling (in

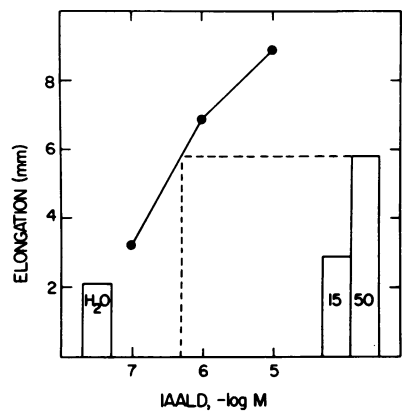

FIG. 1. Semiquantitative determination of IAAld by bioassay. IAAld extracted from etiolated cucumber cotyledons was purified to the fraction C stage as under "Materials and Methods." This fraction was dissolved in water and assayed for growth-promoting activity in the cucumber hypocotyl test. The curve is a dosage response reference for known IAAld in this experiment. The bar to the left represents the water control; the bars to the right represent growth elicited by fraction C samples corresponding to 15 and 50 g of plant material. In this experiment, as indicated by the dotted line interpolation, the 50-g sample gave growth corresponding to 0.5 μM IAAld. This yields an estimate of IAAld concentration in this tissue sample of 3 μg/kg of tissue.

TABLE I. *Reduction of indoleacetaldehyde to IEt by living cucumber tissue*

Samples of green and etiolated seedling organs (35 to 350 mg) were sliced and added to IAAld solutions without added pyridine nucleotide. IEt production was measured by the Salkowski method of Vickery and Purves (13). Specific activities are presented as increase in absorbance at 529 nm per gram fresh weight per hour. Data from two replicate experiments are shown. Note that values for different organs or light treatments may not be compared, since uncontrolled variables (such as number of cut surfaces) are present.

Plant	Organ	IEt Production, $\Delta A_{529} \cdot g^{-1} \cdot hr^{-1}$ Sample I	Sample II
Green	Cotyledon	0.281	0.316
	Hypocotyl	0.763	0.767
	Epicotyl	0.485	0.535
	Root	0.110	0.623
Etiolated	Cotyledon	0.464	0.430
	Hypocotyl	0.610	0.591
	Root	0.844	1.11

particular, the number of cut surfaces) can markedly affect this activity. No detectable IEt diffused from tissue samples not treated with IAAld. It should be recognized that the production of IEt from IAAld in this system represents the resultant of IAAld reductase (3), IEt oxidase (13), and IAAld oxidase (2) activities. It is difficult to determine the relative contributions of each of these metabolic activities. In any case, the metabolism of exogenous IAAld to IEt seems common to all parts of the seedling. Rajagopal (11) has shown that the ability of living plant tissues to reduce IAAld to IEt is of widespread, and possibly ubiquitous, occurrence. Cucumber was not included in Rajagopal's survey of 27 species in 14 angiosperm families.

The results of this investigation indicate that IAAld is a normal constituent of the cucumber seedling and that the IAAld reductases we have examined *in vitro* (1, 3) function *in vivo* as well.

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