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INDOLE-3-ACETIC ACID AND INDOLE-3-ETHANOL IN LIGHT-GROWN *PISUM SATIVUM* SEEDLINGS AND THEIR LOCALIZATION IN CHLOROPLAST FRACTIONS

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Key Word Index—*Pisum sativum*; Leguminosae; indole-3-acetic acid; indole-3-ethanol; chloroplasts; HPLC; GC-MS.

Abstract—Studies have been carried out on the compartmentation of indole-3-acetic acid (IAA) and related indoles in *Pisum sativum* cv. Meteor. By the use of HPLC, GC and combined GC-MS, data were obtained demonstrating the presence of IAA and indole-3-ethanol (IEt) in light-grown pea seedlings. HPLC, GC and GC-MS analyses also confirmed IAA as an endogenous constituent in pea chloroplast fractions while HPLC and GC provided strong evidence for the presence of IEt in chloroplast preparations.

INTRODUCTION

4-Chloroindole-3-acetic acid (**1**), methyl 4-chloroindole-3-acetic acid (**2**), monomethyl 4-chloroindole-3-acetyl-L-aspartate, (**3**), α-N-carbomethoxyacetyl-D-4-chlorotryptophan (**4**) and α-N-carboethoxyacetyl-D-4-chlorotryptophan (**5**) have been identified in extracts from immature seeds of *Pisum sativum* [1–4]. In contrast, there are few characterizations of endogenous indoles from pea seedlings based on either mass spectrometric evidence or high resolution chromatographic data [see 5, 6]. Evidence for the occurrence of indole-3-acetic acid (IAA) (**6**) and indole-3-acetyl-L-aspartate (**7**) in semi-purified extracts from dwarf seedlings has been obtained using HPLC with a selective electrochemical detector [7]. A glucoside of indole-3-ethanol (IEt) (**8**) has been tentatively identified as a putative metabolite of tryptamine (**9**) in etiolated pea stem sections [8]. It has also been reported that etiolated pea stem sections decarboxylate IAA to produce indole-3-aldehyde (**10**), indole-3-methanol (IM) (**11**) and indole-3-carboxylic acid (ICA) (**12**) [9]. Unfortunately there are certain limitations with this study as (i) none of the substrates was isotopically-labelled, (ii) they were fed in very large amounts (3–4 mg per 10 g of tissue) and (iii) the putative products were identified only on the basis of thin layer chromatography data and colour reactions.

The current study was initiated in order to investigate the occurrence of IAA and selected indoles in shoot tissue and chloroplast preparations from *Pisum sativum* cv Meteor. Compounds of special interest were IEt (**8**), IM (**11**) and ICA (**12**). *Pinus sylvestris* needles contain IM and ICA as well as IEt which is synthesized from tryptophan (**13**) and acts as an intermediate in the production of IAA [10–12]. IEt is also an established endogenous constituent of cucumber seedlings [13].

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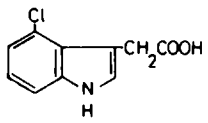
RESULTS AND DISCUSSION

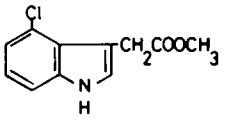
As a preliminary to analyses of intracellular preparations, an attempt was made to identify several indoles in extracts from seedlings of *Pisum sativum* cv Meteor. About 70 g fr. wt of 14-day-old pea shoot tissue were harvested and added to 150 ml 0.1 M pH 8 Pi buffer containing 0.02 M sodium diethyldithiocarbamate as an anti-oxidant. After homogenization in ice, the extract was filtered before being passed through Amberlite XAD-7 concentrator columns at pH 8 and pH 2.7. The resultant neutral and acidic fractions were analysed by HPLC.

The pH 8 fraction was first subjected to normal phase HPLC using a Nucleosil CN column eluted isocratically with ethyl acetate–hexane (1:3). A substantial fluorescent peak corresponding to the R_t of IEt was observed. This peak co-chromatographed with authentic IEt. A minor peak, which co-chromatographed with IM was also detected. The IEt and IM zones were collected and further analysed by gradient elution from a reverse phase HPLC column. While the IEt-like fluorescent peak again showed identical retention properties to authentic IEt, the putative IM did not co-chromatograph with authentic IM and therefore no further analysis was carried out on this fraction. Since IM is notoriously unstable [10] the sample may well have broken down. The remainder of the putative IEt sample was subsequently divided in two and one aliquot was silylated to produce di-trimethylsilyl (TMSi)-IEt. Both underivatized and silylated samples produced capillary GC profiles which closely matched those of authentic standards. GC-MS of both these samples confirmed the presence of IEt in pea seedlings. To date, this is only the third occasion on which IEt has been identified on the basis of acceptable analytical data as an endogenous constituent of higher plant tissues [see 5, 6].

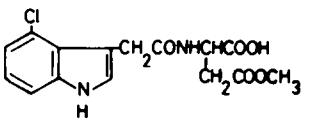
The acid fraction from the XAD-7 column was analysed by similar procedures. Aliquots of sample were eluted isocratically from a normal phase HPLC column using a mobile phase of ethyl acetate–hexane (1:3) in



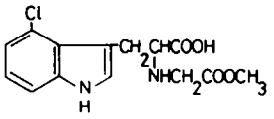
4-CHLOROINDOLE-3-ACETIC ACID (1)



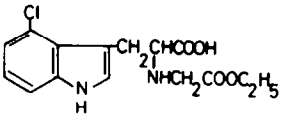
METHYL 4-CHLOROINDOLE-3-ACETIC ACID (2)



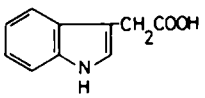
MONOMETHYL 4-CHLOROINDOLE-3-ACETYL-L-ASPARTATE (3)



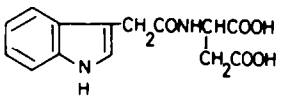
α-N-CARBOMETHOXYACETYL-D-4-CHLOROTRYPTOPHAN (4)



α-N-CARBOETHOXYACETYL-D-4-CHLOROTRYPTOPHAN (5)



INDOLE-3-ACETIC ACID (6)



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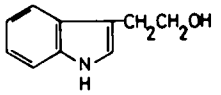
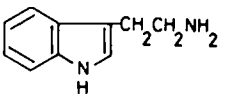
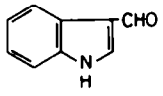


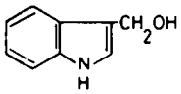
INDOLE-3-ETHANOL (8)



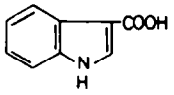
TRYPTAMINE (9)



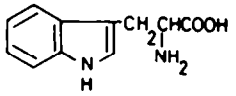
INDOLE-3-ALDEHYDE (10)



INDOLE-3-METHANOL (11)



INDOLE-3-CARBOXYLIC ACID (12)



TRYPTOPHAN (13)

1% acetic acid. A fluorescent component which co-chromatographed with IAA was collected and further analysed by reversed phase HPLC. This yielded a single fluorescent peak which again co-chromatographed with IAA. The putative IAA peak was collected, reduced to dryness and methylated with ethereal diazomethane. The extract was then divided into two portions, one of which was silylated and analysed by capillary GC and GC-MS. GC of the putative IAA methyl ester (IAA-Me) indicated the presence of a single peak with the same R_t as authentic IAA-Me. Similarly GC of the methylated and silylated sample yielded a trace containing a clearly-resolved peak at the same R_t as authentic TMS-IAA-Me. GC-MS analysis of both samples further confirmed the presence of IAA in the *Pisum* shoot extract. No evidence was obtained for the possible presence of ICA.

Prior to analysis of chloroplast preparations, enzyme marker assays were routinely carried out to determine the mitochondrial, microbody and cytoplasmic presence in the supernatant and pellet fractions. The occurrence of mitochondria was assessed by assaying cytochrome *c* oxidase activity while catalase activity signified the presence of microbodies in the fraction. General cytoplasmic contamination was assayed by acid phosphatase activity. Typical results are given in Table 1. These results show that, although detectable, the degree of contamination by microbodies and cytoplasm was low. While mitochondrial contamination was predominant, this was to be expected when using differential centrifugation techniques since mitochondria have a density approaching that of broken chloroplasts and hence would sediment in the same band.

Pellets from six separate chloroplast isolations were combined to give a suspension containing a total of 115 mg chlorophyll from *ca* 560 g fr. wt pea leaf tissue. After the addition of 140 ml 0.1 M pH 8 Pi buffer containing anti-oxidant, the preparation was ultrasonicated for 20 min in ice and the buffer extract treated as described above to yield neutral and acidic XAD-7 fractions. These samples were analysed in a similar manner to the equivalent fractions derived from shoot tissue. Normal phase HPLC was carried out and the IEt zone was collected and analysed by reversed phase HPLC which indicated that the sample contained a single fluorescent peak which co-chromatographed with authentic IEt. It was estimated that the purified sample contained *ca* 7 ng IEt. GC of the putative IEt, following silylation, indicated the presence of a component with an identical R_t to di-TMSi-IEt. Although lack of sample prevented GC-MS analysis, the HPLC and GC data that were obtained, summarized in Table 2, represent strong chromatographic evidence for the presence of IEt in pea chloroplast fractions.

Table 1. Balance sheet of components measured in first supernatant, supernatant after washing crude pellet and final chloroplast pellet

Fraction	% component in fraction			
	Chlorophyll	Cyt. *c* oxidase	Catalase	Acid phosphatase
1st supernatant	54.9	87.6	99.2	97.1
2nd supernatant	20.4	8.1	0.6	1.7
Chloroplast pellet	24.7	4.3	0.2	1.2

Data is expressed as a percentage of total crude filtered homogenate.

Table 2. Chromatographic properties of endogenous IEt-like compound from pea chloroplast fractions

Chromatographic system	R_t			
	Authentic IEt	Putative IEt	Authentic di-TMSi-IEt	Putative di-TMSi-IEt
Normal phase HPLC	18.7 min	18.7 min	—	—
Reversed phase HPLC	16.4 min	16.4 min	—	—
GC	6.41 min	6.42 min	11.30 min	11.29 min

Normal phase HPLC, reverse phase HPLC, GC and combined GC-MS analyses were carried out on the acidic fraction from the chloroplast preparation. At each chromatographic step a clearly-resolved IAA-like peak was present which co-chromatographed with authentic IAA. The methylated sample also produced a peak which co-eluted with the IAA-Me standard. The presence of IAA as an endogenous constituent of pea chloroplast fractions was confirmed by GC-MS of the methylated and methylated-TMSi-derivatives.

Although the limitations of the chloroplast isolation procedure with regard to purity are well appreciated, the demonstration of the presence of IAA and its intermediate IEt in chloroplast preparations provides encouragement for future research into the compartmentation of the production of IAA and related indoles in peas. It should be noted, however, that in the current study chloroplast preparations were the only sub-cellular fraction to be investigated. The distribution of IAA and IEt is thus not necessarily restricted to this organelle.

EXPERIMENTAL

Plant material. Seeds of *P. sativum* L. cv Meteor (Sinclair McGill, Boston, Lincolnshire, U.K.) were soaked in running H_2O overnight and then sown in moist vermiculite. The seedlings were grown in a controlled growth room at a temp. of 20° under a bank of 'Atlas' warm white (80 W) fluorescent lights giving an intensity of *ca* 80 W/m^2 at plant level. The illumination was set for a 12 hr photoperiod and the seedlings were grown for 12–14 days.

Chloroplast isolation. Pea seedlings were harvested within 1 hr of the onset of the 12 hr light period in order to minimize the production of starch grains by the chloroplasts [14]. The pea leaf tissue (*ca* 100 g) was cut finely with scissors and then homogenized in 200 ml ice-cold grinding medium in a Waring blender at high speed for 3 sec. The grinding medium consisted of 0.33 M sorbitol; 50 mM Na_2HPO_4; 50 mM KH_2PO_4; 5 mM $MgCl_2$; 0.1% NaCl and 0.2% sodium isoascorbate [15]. This was adjusted to pH 6.5 with HCl and stored at −20° until needed. Prior to homogenization, 200 μg/ml of freshly-prepared penicillin-G and streptomycin sulphate were added to the grinding medium. The crude homogenate was filtered through 2 + 8 layers of muslin, (with non-absorbent cotton wool spliced between the 8 layers), by squeezing through the two uppermost layers but allowing the homogenate to drip through the remaining layers of muslin and cotton wool. The filtrate was then centrifuged in clean, scratch-free 100 ml-centrifuge tubes at 4000 *g* for 50 sec in a swing-out head. The supernatants were poured off and retained for enzyme marker assays. The pellets were then resuspended in 2–3 ml wash medium using a soft paintbrush in order to cause as little mechanical damage to the chloroplasts as possible. The medium consisted of 0.33 M sorbitol, adjusted to pH 7.6 with Tris base [16]. After a second centrifugation at 4000 *g* for 35 sec the tubes were gently shaken to resuspend the top layer of the pellets only. This supernatant contained a larger proportion of broken chloroplasts and mitochondria and was carefully pipetted off. The pellets were subsequently resuspended in a known vol. of assay medium using the soft paintbrush. The assay medium consisted of 0.33 M sorbitol; 2 mM EDTA; 1 mM $MgCl_2$; 1 mM $MnCl_2 \cdot H_2O$ and 50 mM HEPES. This was prepared double-strength, adjusted to pH 7.6 with KOH and stored at −20° until needed. It was diluted by half before use. The combined chloroplast suspension was covered with foil and stored on ice until needed. Using the procedure as described, 100 g of pea leaf tissue yielded a chloroplast fraction corresponding to 12–15 mg chlorophyll. Chlorophyll was determined spectrophotometrically by the method of Arnon [17].

Purity of chloroplast fraction. In order to assess the degree of contamination by other organelles in the chloroplast preparation, enzyme marker assays were routinely carried out. The chloroplast isolations were carried out as described above. After the first centrifugation the supernatants were combined and stored in darkness at 4°. The pellets were subsequently

resuspended in wash medium and re-centrifuged, after which the supernatants were again combined and retained. The pellets were then resuspended and the vol. of each fraction determined. Two aliquots from each fraction were removed and ultrasonicated in ice for 5 min to disintegrate all intracellular constituents. On each fraction the following determinations were performed: catalase activity denoting the presence of microbodies [18], cytochrome *c* oxidase for mitochondrial contamination [19] and acid phosphatase activity representing general cytoplasmic activity [20, 21]. The chloroplast content of each fraction was determined by measuring chlorophyll [17].

Extraction and purification. Pi buffer (0.1 M, pH 8) containing 0.02 M sodium diethyl dithiocarbamate as anti-oxidant was added to the chloroplast preparations to 3–4 times the initial vol. After ultrasonication and filtration at 4°, the residue was washed and $(NH_4)_2SO_4$ added to the combined filtrates to satn level. After 40 min centrifugation at 10000 *g* the supernatant fraction was re-adjusted to pH 8 and eluted onto a 300 cm × 5 mm i.d. column packed with 0.3 g Amberlite XAD-7 (300–1000 μm) which had been pre-eluted with 0.1 M pH 8 Pi buffer. The eluent was retained, adjusted to pH 2.7 and run through a second XAD-7 column which had previously been equilibrated to pH 2.7. After washing the first column with *ca* 10 ml buffer soln, it was eluted with 10 ml EtOAc–hexane (7:3) which removed the neutral indoles from the column. The aq. phase of the eluent was pipetted off and the organic phase reduced to dryness under a stream of N_2. To remove acidic indoles, the second XAD-7 column was eluted with 10 ml EtOAc–hexane (2:3) [7]. Both pH 8 and pH 2.7 fractions were stored in darkness at −20° until HPLC analysis.

HPLC. Aliquots of extract were injected off-column via a Rheodyne injector fitted with a 200 μl sample loop. Column eluates were monitored with a spectrophotofluorimeter fitted with a 16 μl flow-cell. The detector was set to an excitation at 280 nm and an emission at 350 nm for optimum sensitivity for IAA. Normal phase chromatography was carried out using a 240 cm × 4.6 mm i.d. 5 μm Nucleosil CN column eluted at a flow rate of 1 ml/min. The mobile phase consisted of EtOAc–hexane (1:3) and 1% HOAc was added to the solvents when acidic indoles were analysed. Reversed phase chromatography was on a 250 cm × 5 mm i.d. ODS Hypersil column eluted with MeOH in 20 mM NH_4OAc buffer. Both isocratic and gradient elution were carried out at a constant flow rate of 1 ml/min. When analysing acidic indoles, the NH_4OAc was adjusted to pH 3.5. This was adjusted to pH 6.5 when analysing samples containing indoles of neutral pH. All solvents used for chlomatography were HPLC-grade and the aq. buffer was purified by elution through a 10 cm × 100 mm glass column containing 10 μm ODS silica gel.

GC was carried out on several methylated and silylated samples. The equipment comprised a chromatograph equipped with a flameless NP detector. The column was a 15 m × 0.25 mm i.d. fused silica column and the crosslinked stationary phase was DB-1, with a film thickness of 0.25 μm. The splitless inj. technique was used, with the inj. temp. at 250°. The He carrier gas flow rate was 1 ml/min. The oven temp. was held at 170° and the detector temp. was 200°. To avoid column memory effects from standards a blank injection was carried out immediately prior to the analysis of extracts.

GC-MS analysis of endogenous extracts was carried out using a GC linked via a jet separator to a magnetic sector MS equipped with a computer system. Silylated and methylated aliquots from extracts were introduced via an injector at 240° onto a glass column (2 m × 2 mm i.d.) packed with 1% SP2100. The column temp. was held at 120° for 4 min after inj. and then programmed from 120 to 200° at 16°/min. The He carrier gas flow rate was 30 ml/min. The interface temp. was 240°. Positive ion EIMS were recorded at 70 eV with a 1 sec/decade scan rate and a 0.5 sec interscan delay. Column memory effects were avoided as described in the previous section.

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