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Uptake and Metabolism of Vitamins E and K by Pea Stem Sections[1]

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Summary. The uptake of α-tocopherol and vitamin K_1 by pea stem sections is described. Vitamin K_1 appears to be stable within the plant tissue and is found distributed in all particulate cell fractions following uptake. Only a small proportion of the tocopherol taken up is recoverable and the majority of the compound appears to undergo catabolism.

The oxidation of tocopherols by a cell-free system is described. This system requires oxygen and appears to involve enzyme activity but does not appear to be linked with the action of lipoxidase.

There have been a number of reports of the physiological effects of Vitamin E (tocopherols) and vitamin K_1 (K_1) when applied to plants (3, 10, 12). However, little is yet known of the natural occurrence of these compounds, and nothing at all of their distribution and fate following application. Among the effects of these 2 compounds is their synergism with auxins during the extension growth of pea stem sections (12). This paper describes some preliminary experiments which were performed to determine the fate of α-tocopherol (α-T) and K_1 following application to pea stem tissue.

Materials and Methods

Pea seeds (*Pisum sativum* L.) of the dwarf variety Progress No. 9 were grown in vermiculite at 23° under a weak red light source (0.1 ergs/cm^2 sec^{-1} between 600–800 mμ). For bioassay purposes 10 mm sections were cut from the third internode of 7 to 8 day shoots, approximately 2 mm below the apical bud. Sections were placed in petri dishes containing the following medium: 5 mM KH_2PO_4 buffer (pH 5.5), 0.05 mM $CoCl_2$, 1.5 % sucrose, 1.7 μM indole acetic acid, 0.3 μM gibberellic acid and ± 10 μM α-T or K_1. The lipid substrate was added as a stable emulsion, prepared as described by Stowe (11). Following incubation on a rotary shaker for 20 to 24 hours, sections were collected, washed, lyophilyzed and extracted in Soxhlet apparatus with chloroform for 3 hours. Analysis of the crude lipid extract for α-T and K_1 was performed as described previously (6). Residual lipid in the bioassay media was extracted into hexane and analyzed directly.

In order to investigate the distribution of K_1 following such an incubation, sub-cellular fractions were prepared from the washed pea stem sections. Pea stem tissue was ground with crystalline silicon carbide in a medium containing 0.6 M sucrose, 0.03 M KH_2PO_4 buffer (pH 6.0) at 0 to 4°. The homogenate was strained through 6 layers of cheese cloth and the crude cell-free extract centrifuged for 10 minutes at 500 × *g*, 10 minutes at 5000 × *g*, 1 hour at 20,000 × *g*, and for 3 hours at 100,000 × *g*. The lipid from each particulate fraction was extracted by boiling with 50 ml ethyl alcohol for 30 minutes in darkness. Each extract was evaporated to dryness under vacuum and analyzed for K_1. The supernatant fraction after centrifugation was extracted twice with hexane, and the extract analyzed as before.

The enzyme system that destroyed α-T was prepared by grinding pea shoots with crystalline silicon carbide in 0.22 M sucrose and 0.05 M KH_2PO_4 buffer, pH 6.0 (1 ml medium/1g fr wt) at 0 to 4°. The homogenate was squeezed through 6 layers of cheese cloth and centrifuged at 1000 × *g* for 15 minutes to give the crude cell-free extract used.

Substrates and other lipid additions were prepared as emulsions stabilized with Pluronic F-68 (Wyandotte Chemical Corp.) as described previously (11).

Enzyme Assay. Two ml substrate (1.2 mg α-T) were added to 28 ml enzyme preparation at 25° to start the reaction. A magnetic stirrer was used to mix and aerate the system. Samples of 2.0 ml were withdrawn at 0, 30 and 60 minutes. Each

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was added to 50 ml boiling ethyl alcohol and refluxed for 30 minutes in darkness. The mixture was then filtered and the extract evaporated to dryness under reduced pressure. The residue was taken up into hexane and water. After partition, the hexane was washed with water and concentrated to a small volume. This was added to a 5 g alumina column prepared as described previously (6). The column was eluted with 18 ml hexane, 25 ml 20 % (v/v) benzene in hexane and 25 ml 50 % (v/v) benzene in hexane. The last fraction was evaporated to dryness and the α-T present dissolved in ethyl alcohol for estimation by the Emmerie-Engel technique (1).

Results and Discussion

Uptake by Pea Stem Sections. During incubation of α-T or K_1 with pea stem sections under the bioassay conditions described by Stowe and Obreiter (12) about 25 to 30 % of the lipid was found to enter the plant tissue (table I). In the case of K_1 over 90 % of the lipid taken up was found unchanged in extracts of the sections. However, only 20 % of α-T was recoverable. This suggests considerable breakdown of this compound by pea stem tissue. Such a conclusion was supported by the finding of an abnormal chromatographic spot during examination of section extracts. This spot was always observed in extracts of plant tissues that had been treated with α-T but was never detected in normal tissues. Presumably it is a metabolite of α-T. On column chromatography it appeared in fraction II (see ref 6) and on paper chromatography it was detected as a pinkish, UV absorbing, Emmerie-Engel positive streak. Rf Values are shown in table II. On elution into ethyl alcohol, the UV spectrum of the substance showed maximum absorption at 301 mμ.

It should be noted that in these incubation experiments, each pea stem section had 2 cut surfaces exposed to the medium. Thus it is possible that breakdown of α-T occurred in these regions and not within the undamaged cells of the section. However, if this were so, some of the metabolite would possibly leak to the incubation medium. In fact, none was found outside the sections. Furthermore, this metabolite should surely be found during in vitro experiments (discussed below) if it was produced by damaged tissue. Again, none was found during these experiments, suggesting that quite different reactions are involved.

Table II. *Chromatographic Properties of Metabolites of α-T*

Extracts were run on zinc-ammonium carbonate and fluorescein impregnated Whatman No. 1 chromatography paper as described in (6). The solvent for the first dimension was 30 % (v/v) benzene in cyclohexane, and for the second dimension (reversed phase) 95 % ethyl alcohol. Spots were detected by viewing the developed chromatogram under UV light at 254 mμ and at 320 mμ.

Sample	Rf 1st dimension	Rf 2nd dimension	Emmerie-Engel reaction
α-Tocopherol	0.7	0.8	+
in vivo Metabolite from pea stem sections	0.1–0.6	0.1	+
in vitro Metabolite 1	0.55	0.7	...
in vitro Metabolite 2	0.4	0.8	...
in vitro Metabolite 3	0.05	0.85	...

The metabolism of α-T in pea tissues makes it impossible to determine the actual compound acting as an auxin synergist in this bioassay system.

The path of entry of the applied lipids into the sections is uncertain. Since both K_1 and α-T are highly hydrophobic compounds, it is likely that the majority entered via the cuticle by diffusion, but this was not investigated.

Intracellular Location of Absorbed K_1. Since K_1 was stable within pea tissue, the eventual intracellular distribution of this lipid was determined in pea stem sections after incubation under normal bioassay conditions. The contents of K_1 in each subcellular fraction are shown in table III. The compound was found in all membrane-containing fractions of the cell in amounts several times greater than endogenous levels. Penny and Stowe (9) found the same result after incubation of ^{14}C-methyl oleate with pea stem sections. While these findings fail to indicate a definite site of action of lipids during auxin-lipid synergism, they do show that under normal bioassay conditions, the applied lipid penetrates to all parts of cells within the tissue.

Metabolism of α-T. In a previous paper (6) it was shown that endogenous α-T was rapidly broken

Table I. *Uptake of α-Tocopherol and Vitamin K_1 by Pea Stem Sections*

200 10-mm Third internode sections (8 g fr wt) were incubated for 18 hours in 120 ml medium containing 1.2 μ moles of either α-tocopherol or vitamin K_1. Disappearance from the medium during incubation and section contents were then estimated. Allowance is made for losses during analysis.

Compound	Amount lost from medium	Found in tissue	Normal content of 200 sections	Breakdown in tissue
	mμ moles	mμ moles	mμ moles	%
α-Tocopherol	420	88	12	82
Vitamin K_1	315	290	2.5	8.5

Table III. *Intracellular Distribution of Adsorbed Vitamin K_1*

600 10 mm Third internode pea stem sections were incubated for 24 hours with 360 ml 0.05 M sucrose, 0.005 M phosphate buffer pH 5.5 containing 7 μmoles vitamin K_1. Sections were then harvested, washed, homogenized and the homogenate fractionated prior to extraction and analysis.

Fraction	Vitamin K_1 content	Endogenous level of vitamin K_1 expected
	mμ moles	mμ moles
Residue in cheese cloth	220	
500 × *g* pellet	54	1
5000 × *g* pellet	54	1
20,000 × *g* pellet	107	2
100,000 × *g* pellet	22	1.5
Supernatant	0	0

down during homogenization of pea tissue. It also appeared from observations presented above that applied α-T was metabolized by intact pea stem sections. Booth (2) had earlier suggested that an enzyme system occurred in several plant species that metabolized α-T. Following these observations, the system responsible for the breakdown α-T in etiolated pea plants was further investigated.

Crude cell-free extracts of pea shoots were active in metabolizing added α-T and γ-T but had no effect upon the related substances plastoquinone and ubiquinone. All activity was lost on boiling and in the presence of $AgNO_3$. Table IV shows some of the properties of the system. Molecular oxygen was required and presumably a metal co-factor, as indicated by the inhibitory effects of metal chelating agents such as cyanide, ethylene diamine tetracetic acid and diethyldithiocarbamate.

Table IV. *Properties of the System Metabolizing α-Tocopherol*

Incubation mixtures contained 3 μmoles α-tocopherol in 30 ml crude cell-free extract. Samples were withdrawn for analysis at 0, 30 and 60 minutes. The loss of α-tocopherol in the first 30 minutes was used to calculate metabolism figures shown below.

Treatment	Rate of metabolism	% Of control
	mμmoles g^{-1} fr wt tissue min^{-1}	
Control	1.9	100
Boiled enzyme	0.0	0
No oxygen	0.0	0
5×10^{-3} M $AgNO_3$	0.0	0
5×10^{-3} M KCN	0.4	21
5×10^{-3} M Diethyl dithiocarbamate	0.6	32
3×10^{-3} M EDTA	1.0	53
10^{-2} M Linoleate	1.6	84
5×10^{-3} M Diphenylamine	1.9	100
5×10^{-3} M Ascorbate	1.9	100

The breakdown of α-T in pea shoot homogenates could result from either direct enzyme oxidation or from purely chemical oxidation. This substance is very sensitive to oxidation and functions as an antioxidant in many systems. Indeed there is some belief that the biological function of α-T is due to its antioxidant properties (13). Perhaps the most likely chemical breakdown of α-T is attack by hydroperoxides produced by the action of lipoxidase with linoleate, as has been suggested by Kunkel (7). In fact O'Brien and Titmus have very recently confirmed this reaction in vitro (8) and linoleate hydroperoxide was shown to oxidize α-T to a number of products, the reaction being catalyzed by haematin. Lipoxidase is a widely distributed enzyme and was found to be present in the pea homogenates used in these experiments. If tocopherol breakdown was mediated either by a direct acting oxidase or via lipoxidase activity, then molecular oxygen would be required and the reaction would be inhibited by boiling and $AgNO_3$. However, if lipoxidase were involved, the addition of linoleate to the system would be expected to stimulate α-T breakdown. In fact linoleate was found to slightly inhibit the reaction. Again the addition of antioxidants to the system would be expected to inhibit breakdown, and indeed O'Brien and Titmus (8) showed that ascorbate completely stopped α-T oxidation by hydroperoxides. In the pea shoot homogenate both ascorbate and diphenylamine were without effect. It thus seems unlikely that lipoxidase is responsible for α-T metabolism in pea shoot homogenates. This situation resembles that in the field of carotenoid biochemistry. Oxidation of carotenoids by plant homogenates had long been assumed to be a by-product of lipoxidase action until careful investigation showed quite separate enzyme systems to be involved (4, 5). It is still possible that some other non-specific oxidizing system could be acting in tocopherol breakdown. These preliminary experiments merely report some properties of the system in peas, properties which suggest the participation of at least 1 enzyme.

The products of this in vitro metabolism of α-T were only briefly investigated. Oxidation in the cell-free system gave 3 distinct non-phenolic products which were observed on paper chromatograms run as in (6). Chromatography details are given in table II. None of the products resembles the single metabolite found during in vivo metabolism in stem sections and described above. It is thus uncertain how many enzyme systems are involved in the breakdown of α-T in the pea plant, and their significance remains to be explained.

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