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THE METABOLISM OF α-TOCOPHEROL BY PLANTS

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Key Word Index—*Pisum sativum*; Leguminosae; α-tocopherol oxidase; effect of ethanol.

Abstract—An enzyme system which metabolizes α-tocopherol has been identified in homogenates of etiolated pea shoots. Enzyme activity is considerably increased by the presence of 20% ethanol in the incubation mixture. The enzyme has an absolute requirement for phospholipid. The reaction utilizes molecular oxygen and it is proposed that the enzyme be called α-tocopherol oxidase.

INTRODUCTION

α-TOCOPHEROL has been reported to occur in all higher plants. It is the predominant tocopherol found in the vegetative parts of most plants.[1] On maceration of leaf tissue, oxidative destruction of the tocopherol has been reported with many species.[2–4] Tocopherol oxidation could be caused by two distinct processes. Lipid hydroperoxides could readily arise in plant homogenates following the action of lipoxygenase[5] on unsaturated lipids. Such compounds are known to oxidize α-tocopherol non-enzymically in the presence of haem proteins.[6] Alternatively a specific enzyme system could be responsible for direct metabolism of α-tocopherol. The latter possibility was suggested by Gaunt and Stowe[3] following studies on the breakdown of α-tocopherol in etiolated pea shoot homogenates.

The work reported here describes the partial characterization of an α-tocopherol degradation system in pea shoots which does not depend on the participation of lipid hydroperoxides.

RESULTS

Incubation of crude cell-free extracts of etiolated pea shoots with aqueous emulsions of α-tocopherol stabilized with Pluronic F68 resulted in slow and very variable rates of tocopherol breakdown in early attempts to study the activity of plant homogenates. In a search for a more satisfactory means of introducing α-tocopherol into the aqueous medium a number of organic solvents were investigated. The vitamin was dissolved in the solvent and then added to the crude cell-free extract to initiate reaction. The tocopherol either remained in solution or formed a stable emulsion, depending on the solvent and its concentration. Table 1 shows that in the presence of ethanol, tocopherol breakdown was rapid. The optimum level of ethanol was 20% and this was therefore used in all subsequent experiments. No other solvent was able to activate α-tocopherol breakdown. Although ethanol

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TABLE 1. THE EFFECT OF SOLVENTS ON THE ACTIVITY OF CRUDE CELL-FREE EXTRACTS

Solvent	Final concentration (%, v/v)	Activity (μg α-tocopherol degraded/min/g fr.wt)
Ethanol	5	1·1
	10	2·9
	20	11·7
	30	6·3
Diethyl ether	5	0
Butanol	5	0
Methanol	10	0
	20	0
Dimethyl sulphoxide	20	0

645 μg α-tocopherol were dissolved in the required amount of solvent and added to 3·5 ml of crude cell-free extract with buffer added to give a final vol. of 5 ml.

proved an effective means of introducing α-tocopherol into cell-free preparations, its use was not without problems. The rate of reaction decreased rapidly with time during incubations and after about 15 min little further breakdown of tocopherol occurred (Fig. 1). This was largely due to the slow inactivation of the system by ethanol. Preincubation of the cell-free extract for 15 min in 20% ethanol, before addition of α-tocopherol, resulted in almost complete loss of activity. In some experiments, the α-tocopherol level at zero time, as indicated by back extrapolation of the progress curve, was lower than the initial concentration actually present. This was commonly observed when following the activity of crude cell-free extracts. On purification of the enzyme system, the discrepancy became less obvious. The effect of this, combined with the rapid decline in activity during enzyme assays, was to make difficult the measurement of initial reaction velocity. This was always calculated from the extrapolated progress curve but was found to be reproducible and satisfactory for comparative experiments.

TABLE 2. EFFECT OF BOILING ETHANOL AND PROPAN-2-OL ON ENZYME ACTIVITY

Solvent	Amount of α-tocopherol broken down (expressed as % of starting concentration)
Ethanol, boiling	23
Propan-2-ol, boiling	1
Propan-2-ol, room temp.	1

52 μg α-tocopherol was dissolved in 4 ml solvent at the temperature shown and 0·8 ml of crude cell-free extract was added. The mixture was maintained at a uniform temperature for 10 min before residual α-tocopherol was estimated.

In order to assay tocopherol levels during enzyme assays, 1 ml samples of incubation mixture were transferred into 4 ml propan-2-ol at room temperature. This was found to

completely inhibit further breakdown of tocopherol. It was noted that transfer into boiling ethanol was not immediately effective in this respect (Table 2).

All activity of the cell-free system was lost under anaerobic conditions or following treatment at 100° for 5 min. Activity was examined over a pH range from 3·0 to 8·5. There was no breakdown of tocopherol at pH 4 or below and the optimum pH was 5·5.

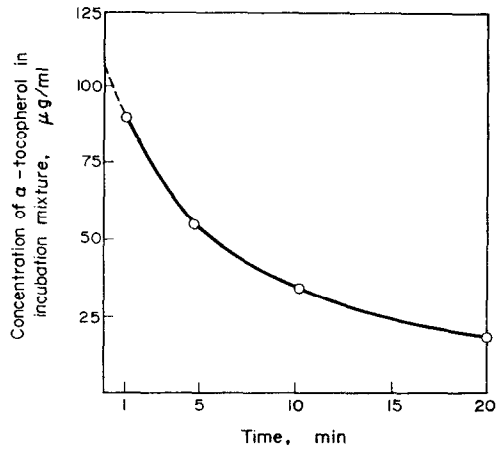

FIG. 1. THE BREAKDOWN OF α-TOCOPHEROL BY A CRUDE CELL-FREE EXTRACT PREPARED FROM ETIOLATED PEA SHOOTS.
The crude cell-free preparation (8 ml) was mixed with 2 ml of α-tocopherol dissolved in ethanol (540 μg/ml).

The crude cell-free extract was fractionated by centrifugation at 7800 and 100 000 *g*. Neither of the two particulate fractions nor the 100 000 *g* supernatant had any activity against α-tocopherol. However, if either of the particulate fractions was suspended in the 100 000 *g* supernatant, activity was restored (Table 3). Thus at least two factors appeared necessary for α-tocopherol breakdown, one associated with the membrane-containing fractions of the cell and the other a soluble component.

TABLE 3. ACTIVITY OF SUB-CELLULAR FRACTIONS

Fraction	Activity (μg α-tocopherol degraded/min/g fr. wt)
7800 *g* pellet	0
100 000 *g* pellet	0·1
100 000 *g* supernatant	0
7800 *g* pellet suspended in supernatant	8·0
100 000 *g* pellet suspended in supernatant	15·6

ca. 500 μg α-tocopherol dissolved in 2 ml EtOH were added to various sub-cellular fractions. 8 ml of the 100 000 *g* supernatant was used. Each particulate fraction was suspended either in 8 ml buffer or in 8 ml supernatant.

Properties of the Membrane-associated Component

The total particulate fraction from a crude cell-free extract was divided into two equal parts. One fraction was suspended in buffer. The lipids were extracted from the second and emulsified in an equal quantity of buffer. The activities of each after mixing with the 100 000 *g* supernatant were found to be identical. It is evident that the active component of the particulate fraction was a lipid and that total replacement of the membrane fraction was achieved by the lipid extract.

The lipids extracted from the sub-cellular particulate fractions were separated by column chromatography, partially characterized by TLC, and the activity of each fraction examined. Table 4 shows that only those fractions containing phospholipids were effective in restoring tocopherol-oxidizing activity to the supernatant. Neutral lipids were quite ineffective.

TABLE 4. EFFECT OF LIPID FRACTIONS ON ACTIVITY OF THE 100 000 *g* SUPERNATANT

Sample	Solvent which eluted from silica gel column	Activity (μg α-tocopherol degraded/min/g fr. wt tissue)
Neutral lipid	Chloroform	0
Glycolipid	Chloroform: methanol, 4:1	0·3
Phospholipid (peak 1)	Chloroform: methanol, 1:4	7·8
Phospholipid (peak 2)	Chloroform: methanol, 1:4	3·3
Phospholipid	Methanol	5·3

The total lipid extract from sub-cellular particulate fractions was added to a silica gel/celite column and eluted with the solvents shown above. Lipids eluted from the column were collected separately and partially characterized. An emulsion of 1 mg of each fraction in 1 ml buffer was added to 3 ml of 100 000 *g* supernatant and incubated with 580 μg of α-tocopherol dissolved in 1 ml EtOH.

Crude phospholipid was subjected to methanolysis and the water-soluble and light petroleum-soluble reaction products were examined for activity. None was observed showing that neither the methyl esters of constituent fatty acids nor the water-soluble hydrolysis products could replace the intact phospholipid molecule.

A study was made of the effect of phospholipid concentration on the activity of the 100 000 *g* supernatant. There was a nearly linear increase in activity with increasing phospholipid concentration up to a level of 5 mg phospholipid per ml supernatant. Increasing the concentration above this point did not further increase the rate of tocopherol breakdown.

Commercial soybean lecithin (Sigma) was subsequently found to be active in the tocopherol oxidation system and could replace crude pea phospholipid.

Properties of the Soluble Factor

A 12-hr dialysis of the 100 000 *g* supernatant led to a 40% loss of activity which did not increase further even on prolonged (2 day) dialysis against EDTA. Original activity could not be restored to the dialysed supernatant by addition of a concentrate of the dialysate. The active factor in the supernatant was also found to be the heat and ethanol-labile component of the crude cell-free system. It thus seemed likely that it was a protein. Treat-

ment of the supernatant with trypsin or with a mixture of α-chymotrypsin and carboxypeptidise *A* resulted in almost complete loss of activity. Finally, the active factor was quite stable to ammonium sulphate precipitation. Ammonium sulphate was added to the 100 000 *g* supernatant to bring it to 0·91 saturation. The precipitated protein was taken up in buffer and dialysed. Its activity was identical to that of supernatant fraction that had been dialysed.

Storage of the 100 000 *g* supernatant for 5 days at 4° led to no loss of activity. This contrasts with a rapid decline in the activity of crude cell-free extracts on storage for even a few hours at 4°.

Observations on the Involvement of Lipid Hydroperoxides

From the data so far presented, the possibility that α-tocopherol breakdown resulted from haem protein catalysed oxidation by lipid hydroperoxides could not be excluded. Thus a series of experiments were designed to test this hypothesis.

The ability of the crude cell-free extract to catalyse the production of lipid hydroperoxides from added linoleate was determined in the presence and absence of ethanol. Table 5 shows that hydroperoxides are formed but that ethanol inhibits their production. In the presence of 20% ethanol very little hydroperoxide formation would be expected.

TABLE 5. EFFECT OF ETHANOL ON LIPID PEROXIDATION ACTIVITY OF THE CELL-FREE EXTRACT

Final ethanol concentration (%, v/v)	Activity (units/g fr. wt tissue)	% Inhibition
0	1·92	0
5	0·28	85
10	0·16	92
25	0·13	93

10 ml of crude cell-free extract were mixed with the required volume of ethanol and the volume made up to 15 ml with phosphate buffer, pH 7·2. Reaction was initiated by addition of 5 ml of an emulsion of linoleic acid.

However, hydroperoxides could be formed in crude cell-free extracts between the time of tissue homogenization and enzyme assay. Tocopherol breakdown could be due to residual peroxides. To test this possibility, the rate of tocopherol metabolism was followed in two incubations with phospholipids of different hydroperoxide contents. Table 6 shows that the activity of each was the same although peroxide levels differed by a factor of 36. When the concentration of phospholipid in the incubation medium was doubled, the breakdown of tocopherol was also doubled. It is apparent, therefore, that the activity of the phospholipid does not depend on the hydroperoxide level, but rather on the phospholipid concentration as such.

The 100 000 *g* supernatant of a cell-free preparation was replaced by a mixture of lipoxygenase and haemoglobin which was pre-incubated with the particulate cell fractions for several minutes. Following addition of α-tocopherol in ethanol no breakdown of the vitamin was observed. Thus the supernatant could not be replaced by proteins catalysing the production of lipid hydroperoxides.

TABLE 6. EFFECT OF HYDROPEROXIDE CONTENT ON ACTIVITY OF THE PHOSPHOLIPID FRACTION

Sample	Peroxide content (nmol I_2 formed/1·65 mg phospholipid)	Activity (μg α-tocopherol degraded/min/g fr. wt tissue)
1·65 mg untreated phospholipid	27·4	3·1
1·65 mg lipoxygenase treated phospholipid	985	3·1
3·3 mg untreated phospholipid	27·4	6·6

100 000 *g* supernatant (3 ml) was mixed with phospholipid emulsion (1 ml) and reaction initiated by addition of 645 μg α-tocopherol in 1 ml EtOH.

The phospholipid requirement was substituted in a normal assay by linoleate or trilinolein, both of which readily form hydroperoxides. No breakdown of tocopherol was observed.

Effect of Substrate Concentration

The effect of α-tocopherol concentration on enzyme activity was followed by using the oxygen electrode to assay reaction velocity. Maximum activity was observed at a tocopherol concentration of 1·3 mM and the K_m was approximately 0·25 mM.

Products of Metabolism

^{3}H-α-Tocopherol was incubated with supernatant and phospholipid for 20 min. All metabolites were found to be extracted into light petroleum. Chromatography showed three products (Table 7), none of which was phenolic. The major metabolite (*B*) had a UV spec-

TABLE 7. R_f VALUES OF α-TOCOPHEROL METABOLITES

Metabolite	R_f in 30% benzene in cyclohexane	R_f in reversed phase 95% ethanol	Relative proportions present (%)
A	0·05	0·00	25
B	0·85	0·00	58
C	0·35	0·70	17
α-Tocopherol	0·80	0·60	

trum with λ_{max} 288 nm. IR analysis showed peaks at 2920, 2850, 1740, 1685 and 1365 cm^{-1} and confirmed the absence of a free hydroxyl group. It was also eluted from an alumina chromatography column with diethyl ether in light petroleum (4%, v/v). Further characterization of the metabolites was not undertaken.

Distribution of the Enzyme System in Plant Tissues

Table 8 summarizes the activity of the α-tocopherol degradation system in various plant tissues. Of the few plants examined, the shoots of etiolated peas were most active. Light-grown plant tissue showed considerably less activity. The enzyme was found in all parts of plants, leaves, buds and roots. It appears not to occur in all species, however, and cell-free preparations from the two etiolated monocotyledonous plants investigated were inactive.

TABLE 8. α-TOCOPHEROL OXIDATION BY CELL-FREE EXTRACTS OF VARIOUS PLANT TISSUES

Plant tissue	Activity (μg α-tocopherol degraded/min/g fr. wt tissue)
Etiolated pea stems	17·5
Apical buds of etiolated pea shoots	15·3
Etiolated pea roots	12·3
Light-grown pea shoots	4·5
Light-grown clover leaves	7·3
Light-grown nettle leaves	3·4
Etiolated wheat shoots	0
Etiolated oat shoots	0

600 μg α-tocopherol dissolved in 1 ml EtOH was added to 4 ml crude cell-free extract of each plant tissue.

DISCUSSION

It is by no means uncommon to find plant enzymes which are active in the presence of organic solvents.[7,8] However, the observed stimulation by ethanol of α-tocopherol breakdown in pea shoot homogenates was most unexpected. It could be explained on the basis of an increase in the solubility and thus the surface area of the substrate.[9] Alternatively the ethanol may have a direct activating effect on the enzyme. Such an effect is not unknown and two phenolases have been reported to be directly activated by treatment with organic solvents.[10,11]

A secondary consequence of the use of ethanol in enzyme incubations was the inhibition of hydroperoxide production during enzyme assay. This effect is in agreement with reports of Mitsuda *et al.*[12] of the inhibition of lipoxygenase by low concentrations of ethanol.

Ammonium sulphate precipitation, dialysis experiments and the use of proteolytic enzymes show that a protein is involved in tocopherol oxidation. This enzyme has an absolute requirement for phospholipid as a co-factor. No other lipid was found to replace phospholipid, not even the methyl esters of the constituent fatty acids of the phospholipids. The partial loss of activity following dialysis suggested that an additional small molecular weight co-factor could be involved. However, no direct evidence for such a co-factor was observed.

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Although none of the products of metabolism was identified, the properties of the major metabolite resemble those of a tocopherol dimer, a spirodienone ether described by Draper *et al.*[13–16] The presence of more than one metabolite suggests either a complex enzyme reaction or possibly an unstable primary reaction product that undergoes non-enzymically catalysed secondary reactions.

As the metabolism of α-tocopherol utilizes molecular oxygen, the enzyme must be an oxidase and it is suggested that it be named α-tocopherol oxidase. Certain other oxidases and particularly electron transfer oxidases are known to require phospholipids as co-factors.[17,18] Green and Fleischer[19] and Jones and Wakil[20] consider that activity of the phospholipid to be the result of a surface effect. It may well be that a compound such as α-tocopherol with a single polar hydroxyl group at one end of a large lipophilic molecule can interact in a very specific way with phospholipids, for example as suggested by Stowe.[21] Only when in such a combination is it available for enzyme attack.

It is clear from the data presented that in the system investigated lipid hydroperoxides play no part in the oxidation of α-tocopherol. This does not, however, exclude the possibility that such compounds may contribute to tocopherol oxidation in entirely aqueous plant homogenates. Etiolated pea shoot homogenates were shown to be capable of forming lipid hydroperoxides and these substances are known to cause destruction of α-tocopherol.[6] At this stage it is not possible to determine how much of the destruction of endogenous tocopherol that occurs on tissue disruption is due to direct enzyme action.

EXPERIMENTAL

Growth of plants. Pea seeds (*Pisum sativum* L. var. Progress No. 9) were germinated and grown in vermiculite in a dark room maintained at 24° and 60% relative humidity. A green safe-light was used for inspection. The etiolated pea shoots were harvested when about 6 in. long, 8–10 days after planting. Wheat and oat seeds were germinated and grown under similar conditions and the etiolated shoots were harvested after 10 days. The light-grown stems and leaves of the pea plant were obtained from peas which had germinated and grown in vermiculite in a greenhouse for 3 weeks. Light-grown clover and nettle leaves were obtained from the field.

Purification of solvents. All solvents were redistilled before use.

Preparation of crude cell-free extract. All operations were performed at 0–4°. The fresh tissue (2 g/ml) was ground in 0·05 M phosphate–citrate buffer, pH 6·7 (referred to as 'buffer' except where otherwise specified) with acid washed silicon carbide (Carborundum Co. Ltd., Trafford Park, Manchester 17). The brei was squeezed through cheese-cloth, made up to a volume equal to the original weight of tissue with buffer, and centrifuged for 10 min at 1000 *g*. The supernatant was termed the crude cell-free extract.

Sub-cellular fractionation. A crude cell-free extract, prepared with buffer containing 0·5 M sucrose, was centrifuged at 7800 *g* for 10 min. The pellet was suspended in a vol. of buffer or 100000 *g* supernatant equal to the vol. of the cell-free extract from which the pellet was precipitated. The remaining supernatant was then centrifuged at 100 000 *g* for 60 min. The pellet was suspended as above.

Ammonium sulphate precipitation. The amount of $(NH_4)_2\,SO_4$ necessary was calculated[22] and the precipitated protein was removed by centrifugation at 15 000 *g* for 10 min, dissolved in buffer and dialysed against the same buffer.

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Treatment with proteolytic enzymes. 100 mg Trypsin (B.D.H) were added to 3 ml of 100 000 *g* supernatant and the solution incubated at room temp. for 10 hr. After this treatment the pH of the solution was adjusted to 6·7. 3 mg Chymotrypsin (Sigma Chemicals) and 1 mg carboxypeptidase A (Sigma Chemicals) were added to 3 ml of the supernatant prepared in phosphate buffer pH 6·7. The solution was incubated at 30° for 4 hr, after which the pH was adjusted to 6·7.

Enzyme assay. All enzyme assays were performed at 30°. DL-α-Tocopherol (Roche Products Ltd.) was added either: (1) as a stable emulsion prepared with Pluronic F68 (Kodak Ltd.) according to the method of Stowe,[23] or (2) as an EtOH solution, whose concentration was determined by measuring the absorptivity at 292 nm ($E^{1\%}_{1cm}$ = 75·8). Samples were withdrawn from the incubation mixture mixed with 4 ml of isoPrOH, the level of α-tocopherol being determined by the Emmerie–Engel technique.[24] 10 ml of light petroleum (b.p. 40–60°) were added followed by 10 ml H_2O. 8 ml of the upper organic phase were evaporated to dryness under N_2 and the residue dissolved in 3·5 ml of α,α′-dipridyl in EtOH (0·07%, w/v). 0·5 ml $FeCl_3 . 6H_2O$ in EtOH (0·9%, w/v) was added and the absorptivity read at 520 nm after 2 min. The α-tocopherol content was calculated from $E^{1\%}_{1cm}$ = 102.

When crude cell-free preparations from green plant tissue were used in enzyme assays, light petroleum extracts of the incubation medium contained carotenoid and chlorophyll pigments which interfered with the assay of α-tocopherol. Such extracts were applied in light petroleum to a column containing 5 g of neutral alumina (Grade 1) hydrated with 4·25% (w/w) H_2O. The column was eluted with 10 ml of light petroleum to remove β-carotene. α-Tocopherol was then eluted with 20 ml Et_2O: light petroleum (20%, v/v).

Examination of the oxygen requirement of the enzyme. The experiment to determine if the system required O_2 was performed in Thunberg tubes. All the tubes for the anaerobic experiment and aerobic control were repeatedly evacuated and the vacuum released under N_2 or air respectively.

Enzyme assay using an oxygen electrode. Buffer (1·1 ml) was mixed with 0·4 ml of an emulsion of commercial soybean lecithin (20 mg/ml buffer) and 0·4 ml EtOH or an EtOH solution of α-tocopherol (100–10 000 μg/ml). The mixture was equilibrated for 10 min in the O_2 electrode (Rank Bros., Bottisham, Cambridge). 100 000 *g* supernatant (0·1 ml) was then added and the O_2 uptake recorded. The enzyme activity is expressed in terms of chart units per min.

Extraction of lipids. The lipids were extracted from the particulate cell fractions with a mixture of $CHCl_3$–MeOH according to the procedure of Folch *et al.*[25] The organic phase was washed with 0·58% (w/v) NaCl and reduced to dryness under N_2.

Chromatography of lipids. Crude lipid extracts were fractionated by column chromatography on silica gel H (Merck, Dormstadt)/hyflo-super-cel (Koch–Light Ltd.) in the ratio of 2:1.[26] 1·5 g of this mixture was used to separate 10 mg of lipid. The column was first washed with $CHCl_3$ (20 ml/g silica gel). The lipid extract in $CHCl_3$ was added to the column which was eluted in a step-wise manner with mixtures of $CHCl_3$–MeOH as described. The Liquid Chromatograph System 1 (W. G. Pye Ltd., Cambridge) was used to detect organic compounds eluted from the column. Lipid fractions obtained from the column were further examined by TLC on silica gel G plates developed with $CHCl_3$–MeOH–H_2O (80:25:3, by vol.). Organic compounds were detected by charring with H_2SO_4[27] Phospholipids were detected by the method of Dittmer and Lester[28] using molybdenum dissolved in sulphuric acid.

Methanolysis of phospholipids. The crude phospholipid fraction was dissolved in 14% (w/v) BF_3 in MeOH using 1 ml reagent per mg phospholipid. The solution was refluxed for 2 hr using a $CaCl_2$ tube at the top of the condenser to ensure anhydrous conditions, and then allowed to cool. This was sufficient time to cause complete dissociation of the fatty acid groups from the phospholipids, even from the sphingomyelins, the amide bond of which is relatively difficult to split.[29] Light petroleum was used to extract the MeOH solution, which was washed with several vol. H_2O. Both the H_2O and the light petroleum extract were taken to dryness, and the residue stored at −20° until required.

Preparation of lipid emulsions. Both the neutral lipids and the phospholipids were prepared as aqueous emulsions by the use of sonic waves for 2 min. This gave very stable emulsions. Linoleic acid was prepared as an approx. $7·5 \times 10^{-3}$ M emulsion containing 0·25% (v/v) Tween 20 according to the method of Surrey.[30] Commercial maize oil was used as a source of trilinolein.[31] 0·5 ml of oil and 0·5 ml of Tween 20 were added to 200 ml buffer. The mixture was emulsified using a Silverson homogenizer.

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Measurement of the lipid peroxidation. The activity was determined using the method described by Surrey,[30] measuring the production of linoleate hydroperoxides at 234 nm. A cell-free extract was prepared by homogenizing 50 *g* of pea shoots with 100 ml phosphate buffer pH 7·2. The homogenate was centrifuged at 2000 *g* for 15 min and the supernatant used as the cell-free extract. 10 ml of this were mixed with 5 ml of buffer and/or EtOH and 5 ml of linoleic acid emulsion. 0·2 ml samples were withdrawn every 2 min and pipetted into 0·4 ml of EtOH. Dilutions of both test and blank samples were made with 60% EtOH and the absorptivity measured. A unit of activity is defined as an increase in absorptivity of 1·0 at 234 nm in 1 min in a total vol. of 10 ml of 60% EtOH solution.

Incubation of phospholipids with lipoxygenase. 2·5 mg phospholipid in an emulsion were incubated at 25° for 6·5 hr with 2 mg soybean lipoxygenase in 0·75 ml buffer.

Estimation of lipid hydroperoxides. The method is based on that of Kokatnur and Jelling[32] by measuring the amount of I_2 produced from KI.[33] However, the peroxide concentration within the phospholipid fraction was extremely low: therefore, the amount of I_2 released was difficult to estimate by titration. Iodine has a UV spectrum with maximum extinction at 290 nm and 355 nm; this extinction is enhanced by I' producing I_3 ions. Into 4 ml of isoPrOH and 0·2 ml HOAc was added 0·1 ml of the sample and N_2 was passed for 3 min after which air was excluded. The contents were refluxed at 80–85° for 3 min. IsoPrOH (1 ml) saturated with NaI was then added, the contents refluxed for a further 15 min, cooled, made to 12 ml with isoPrOH and centrifuged. The absorptivity was measured at 290 nm.

Chromatography of α-tocopherol metabolites. 560 μg DL-(5-Methyl-^{3}H)-α-tocopherol (Radio-chemical Centre, Amersham, diluted to a specific activity of 1 μCi/mg) were incubated with 3 ml 100 000 *g* supernatant, 1 ml phospholipid emulsion and 1 ml EtOH for 20 min. All lipids were then extracted into light petroleum and chromatographed on $ZnCO_3$ impregnated Whatman No. 1 paper.[24] After development with 30% (v/v) benzene in cyclohexane for the first dimension and 95% (v/v) EtOH for the reversed phase dimension,[34] the chromatogram was cut into strips. Radioactive spots were detected with a radio-chromatogram scanner (Packard Model 7201). Phenolic compounds were detected by spraying with 0·5% (w/v) α,α′-dipyridyl in EtOH mixed with 0·2% (w/v) $FeCl_3.6H_2O$ in EtOH (1:1, v/v).[24] Extraction of the metabolites from the paper was performed in a soxhlet with a mixture of light petroleum–EtOH (1:1, v/v), refluxing for 24 hr. Column chromatography was performed using grade 1 neutral alumina (5 g) hydrated H_2O (0·3 ml). The lipids were added to the column in light petroleum and the column developed with Et_2O in light petroleum using gradient elution.

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