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Conversion of 5-(1,2-Epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic Acid into Abscisic Acid in Plants

By B. V. MILBORROW and R. C. NODDLE*
Shell Research Limited, Milstead Laboratory of Chemical Enzymology, Broad Oak Road, Sittingbourne, Kent, U.K.

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(±)-5-(1,2-Epoxy-2,6,6-trimethylcyclohexyl)-3-methyl[2-^{14}C]penta-*cis*-2-*trans*-4-dienoic acid is converted into abscisic acid by tomato fruit in 1.8% yield (or 3.6% of one enantiomer if only one is utilized) and 15% of the abscisic acid is derived from the precursor. The 2-*trans*-isomer is not converted. The amounts of [2-^{3}H]mevalonate incorporated into abscisic acid have shown that the 40-times higher concentration of (+)-abscisic acid in wilted wheat leaves in comparison with unwilted ones reported by Wright & Hiron (1969) arises by synthesis. The conversion of (±)-5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methyl-[2-^{14}C]penta-*cis*-2-*trans*-4-dienoic acid into abscisic acid by wheat leaves is also affected in the same way by wilting and it is concluded from this that the epoxide or a closely related compound derived from it is on the biosynthetic pathway leading to abscisic acid. The oxygen of the epoxy group was shown, by ^{18}O-labelling, to become the oxygen of the tertiary hydroxyl group of abscisic acid.

The growth-inhibitory activity of some analogues of abscisic acid (I) has been reported (Anderson, 1969; Tamura & Nagao, 1969*a*,*b*) but there has been no indication whether these compounds are active *per se* or because they give rise to abscisic acid. The strong inhibitory activity of (±)-5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid(II)'epoxide'on plant growth could arise from the compound's molecular structure mimicking that of abscisic acid. Alternatively it could be converted into abscisic acid. (±)-5-(1,2-Epoxy-2,6,6-trimethylcyclohexyl)-3-methyl[2-^{14}C]penta-*cis*-2-*trans*-4-dienoic acid has been synthesized by Dr G. Ryback and Mr R. Mallaby in this laboratory and we have found that it is rapidly metabolized to abscisic acid by tomato fruit. The rapid rate of this conversion suggested that the epoxide itself or a closely related product formed from it could be an intermediate in the biosynthesis of abscisic acid.

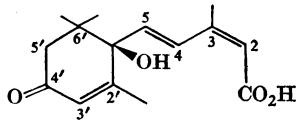
Wright & Hiron (1969) have reported that the abscisic acid concentration in wheat leaves increases 40-fold during the first 4h of wilting and we find that this increase is caused by synthesis of abscisic acid rather than by its release from a conjugate or an immediate precursor. This was shown by the increased incorporation of [2-^{3}H]mevalonic acid into abscisic acid by wilted wheat plants in comparison with turgid plants that had absorbed equal amounts of [2-^{3}H]mevalonic acid. When racemic 2-^{14}C-labelled epoxide (II) was supplied, larger amounts of labelled material were again incorporated into abscisic acid by wilted plants.

* Also: Department of Molecular Sciences, University of Warwick, Coventry, U.K.

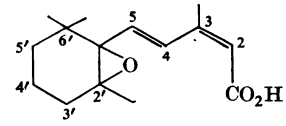
MATERIALS

Racemic 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (II) and 3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)-penta-*cis*-2-*trans*-4-dienoic acid (IV) were synthesized by Dr M. Anderson at Woodstock Agricultural Research Centre, Sittingbourne, Kent, U.K. (Anderson, 1969); the racemic epoxide (II) labelled with ^{14}C at C-2 was synthesized by Dr G. Ryback and Mr R. Mallaby in this laboratory and we thank them for providing the material.

Racemic 5-(1,2-[^{18}O]epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid was synthesized from ^{18}O-enriched oxygen and the methyl ester of compound (IV) as described by Tamura & Nagao (1969*b*). No reaction occurred in darkness but the ^{18}O-labelled epoxide was formed in 25% yield when the material was illuminated by diffuse sunlight for 5 days. The product was identified by co-chromatography with authentic material and with an authentic sample of the free acid after hydrolysis in 10% (w/v) KOH in aq. (1:1, v/v) ethanol for 24h at 20°C in the dark. Racemic abscisic acid was as used previously (Cornforth, Milborrow & Ryback, 1965). (±)-[2-^{3}H]Mevalonic acid (93mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. A.R.-grade chemicals were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K., Hopkin and



(I)



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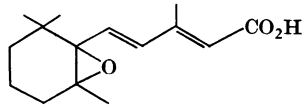

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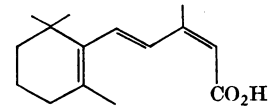

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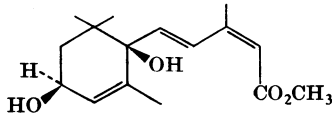

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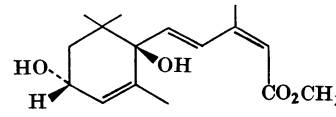
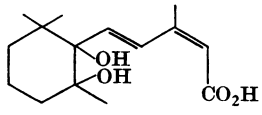
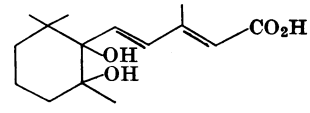


(VI)



(VII)



(VIII)

Williams, Chadwell Heath, Essex, U.K., or CIBA (A.R.L.) Ltd., Duxford, Cambs., U.K.

Plants were grown in a glasshouse at approximately 23°C and with mercury vapour lamps providing supplementary illumination when necessary. Green tomato fruit (*Lycopersicon esculentum*, cultivar. F_1 Supercross) were used for some experiments; for other experiments wheat seedlings (*Triticum vulgare*, cultivar. Capelle) were harvested 14 days after sowing and when the shoots, cut at ground level, weighed an average of 0.37g and measured 12cm in height.

Application of labelled compounds. An ethanolic solution of the epoxide (II) (1mg in 1ml) was supplied to green tomatoes (126g) by multiple injections; the fruits were then kept in darkness for 63h at 24°C. Wheat shoots were placed in solutions of the [2-^{14}C]epoxide or [2-^{3}H]-mevalonate in 0.01M-potassium phosphate buffer, pH7.3, (50ml) and ethanol (1ml). After 2h at room temperature in the first two experiments, or 18h at 7°C in the third, the plants were removed, the submerged parts rinsed in water, and blotted. Half of the batch from each solution was placed with the cut ends in water and kept in a damp atmosphere at 20°C whereas the other half was spread out on filter paper and exposed to a draught of air for 30min, by which time the plants had lost 25–30% of their weight. They were then covered with a sheet of transparent polythene for 3.5h; both sets were illuminated until harvested.

The experiments with 2-*cis*-(II) and 2-*trans*-isomers (III) of the epoxide were carried out in darkness except for a few essential manipulations; these were done at less than 300lx. Boiled tomatoes with intact cuticles were prepared in a steam bath for 5h, allowed to cool and treated in the same way as the living fruit.

Extractions. Ten tomatoes (125g total) were macerated in methanol (250ml) containing (±)-abscisic acid (156μg) and the slurry was filtered through cellulose powder and re-extracted with methanol (3×250ml). Saturated $NaHCO_3$ solution (100ml) was added to the combined extracts and the methanol was evaporated.

Wheat leaves (60g) were chopped and plunged into ice-cold methanol (2l) containing 0.05% of 2,6-di-*tert.*-butyl-4-methylphenol, 2ml of saturated aq. $NaHCO_3$ and unlabelled, racemic abscisic acid (20μg/60g of turgid leaves; 50μg/60g of wilted leaves). The solution was stirred at intervals for 5 days and then the leaves were extracted a second time with 1 litre of methanol.

The methanol from both extractions was removed at 30°C, 250ml of water was added, the pH was adjusted to 8.0 and neutral materials were removed with ether. The pH was then adjusted to 3.0 with dil. H_2SO_4 and the ether-soluble acid fraction (approx. 52μg/g of fruit) was extracted, dried and chromatographed. Any abscisic acid glucose ester (Koshimizu, Inui, Fukui & Mitsui, 1968; Milborrow, 1970) remaining in the aqueous phase was hydrolysed by heating at pH11 for 30min after a further sample of (±)-abscisic acid and 2,6-di-*tert.*-butyl-4-methylphenol had been added. Acids released by this treatment were extracted with ether at pH3.0.

Chromatographic separation and identification of abscisic acid. The acid fractions were chromatographed on Merck precoated, silica-gel F_{254} t.l.c. plates in benzene–ethyl acetate–acetic acid (15:3:1, by vol.) (Fig. 1) or in toluene–ethyl acetate–acetic acid (25:15:2, by vol.). The zones

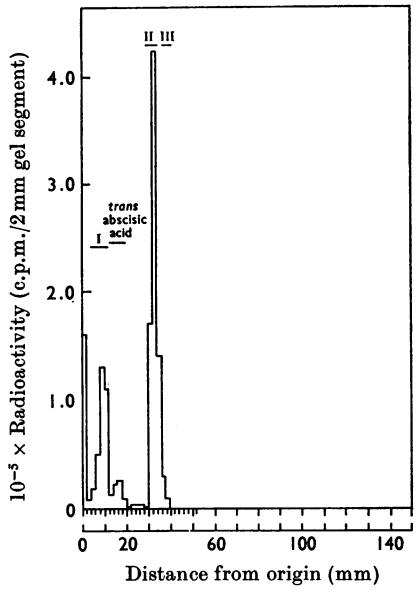


Fig. 1. Histogram of the distribution of ^{14}C from (±)-[2-^{14}C]epoxide (II) (1.3 mCi/mmol) in the ether-soluble acid fraction from tomato fruit. The extract was chromatographed in benzene–ethyl acetate–acetic acid (15:3:1, by vol.) on a precoated silica-gel F_{254} t.l.c. plate. The silica gel was put in the scintillation solution, which gave 50% counting efficiency.

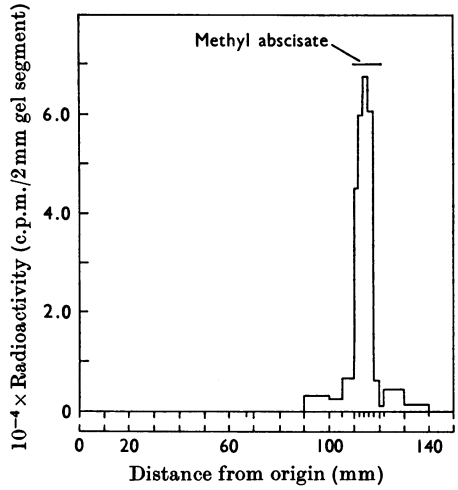


Fig. 2. Incorporation of ^{14}C from (±)-[2-^{14}C]epoxide into abscisic acid. The zone of the t.l.c. plate in Fig. 1 corresponding to abscisic acid was eluted with methanol and the acids were methylated and chromatographed in hexane–ethyl acetate (1:1, v/v).

adjacent to authentic markers were eluted with methanol, dried, and methylated with ethereal diazomethane. The methyl esters were chromatographed in hexane–ethyl acetate (1:1, v/v) (Fig. 2). Methyl abscisate was also converted into its two 1′,4′-diol esters (V, VI) in ice-cold water–methanol (1:2, v/v) when a crystal of $NaBH_4$ was added. They were separated by chromatography in the hexane–ethyl acetate solvent: *cis*-1′,4′-diol (V), R_F 0.38; *trans*-1′,4′-diol (VI), R_F 0.54 (Fig. 3).

Finally each 1′,4′-diol methyl ester was eluted with methanol, dried and dissolved in dry chloroform containing finely divided MnO_2. After being stirred at room temperature, in darkness, for 3 days both diols were oxidized to methyl abscisate, which was identified by chromatography and optical-rotatory-dispersion analysis. The optical-rotatory-dispersion spectra of all compounds were measured and compared with those of authentic materials. The circular-dichroism spectra (Milborrow, 1967) and mass spectra of some samples were also measured.

The [^{14}C]epoxide methyl ester was converted into 5-(1,2-dihydroxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (VII) methyl ester when treated with 2M-H_2SO_4 in aq. (1:4, v/v) methanol (Tamura & Nagao, 1969*a*). It was hydrolysed in ethanolic KOH and the free acids were purified by chromatography (Table 1).

Radioactively labelled 2-*cis*- and 2-*trans*-epoxides were freed of contamination with the unwanted radioactive

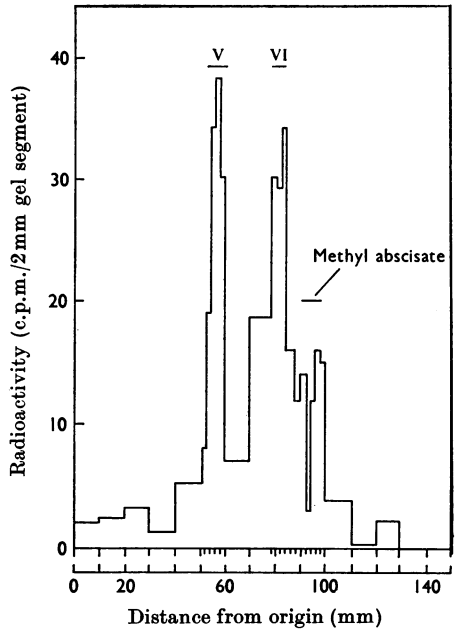


Fig. 3. Incorporation of ^{14}C from (±)-[2-^{14}C]epoxide into abscisic acid. The zone of the t.l.c. plate in Fig. 2 corresponding to methyl abscisate was eluted and reduced with $NaBH_4$. The ^{14}C incorporated into abscisic acid from the epoxide co-chromatographed with authentic 1′,4′-*cis*- (V) and 1′,4′-*trans*-diol (VI) markers in benzene–ethylacetate–acetic acid (15:3:1, by vol.).

Table 1. *R_F values of methyl esters of abscisic acid and related compounds*

Compounds were subjected to t.l.c. in hexane–ethyl acetate (1:1, v/v) on Merck precoated F_{254} plates.

Methyl ester	R_F
Abscisic acid (I)	0.41
2-*trans*-Abscisic acid	0.42
Epoxide (II)	0.65
2-*trans*- Epoxide (III)	0.66
1′,2′-Dihydroxy derivative (VII)	0.54
2-*trans*-1′,2′-Dihydroxy derivative (VIII)	0.57
2-*cis*-1′,4′-Diol of abscisic acid (V)	0.19
2-*trans*-1′,4′-Diol of abscisic acid (VI)	0.31

isomer by an addition of 10% of the unlabelled impurity; this was followed by a chromatographic separation.

The 2-*trans*-isomer of the epoxide (III) was converted into its 1,2-dihydroxy-2,6,6-trimethyl-1-cyclohexyl derivative (VIII) in boiled and in living tomato fruit. The [^{14}C]epoxide was converted into its 1,2-dihydroxy-derivative in boiled but not in living tomato fruit. These products and their methyl esters were identified by co-chromatography with authentic materials and by their u.v. spectra.

Measurement of abscisic acid. The quantity of (+)-abscisic acid in a purified extract was measured by its rotation of polarized light at 289nm (Cornforth, Milborrow & Ryback, 1966). The spectropolarimeter used, a modified Bellingham–Stanley (Bendix) Polarimatic 62 coupled to a Bryans *x–y* plotter (model 20180 S), could produce a deflexion of 2cm for 1μg of (+)-abscisic acid/ml per 1cm path length in acidic methanol. The (+)-abscisic acid methyl ester was dissolved in neutral methanol because its optical rotatory dispersion spectrum is unaffected by pH and is identical with that of the free acid in acidic methanol (2.5mM-H_2SO_4). Amounts as small as 0.3μg of (+)-abscisic acid could be determined.

The quantities of extractable (+)-abscisic acid present in the plant were determined by the 'Racemate Dilution' method (Milborrow, 1967). This technique depends on the optical activity of the natural abscisic acid and the absence of optical activity in the synthetic compound. A ratio is set up between an unknown quantity of (+)-abscisic acid in the plant tissue and a known amount of (±)-abscisic acid in the solution in which it is macerated. The total abscisic acid in a highly purified extract is measured from its u.v. absorption ($\epsilon_{260.5}$ 21400) in a Unicam SP. 800 or a Cary 14 spectrophotometer and the proportion of (+)-abscisic acid in the solution is measured by its optical rotatory dispersion. Provided no differential loss of one enantiomer occurs, e.g. by enzyme action or crystallization, then these data give the ratio, and hence the amount of free (+)-abscisic acid originally present in the tissue can be calculated. This value is unaffected by losses during purification.

Assay of radioactivity. Samples containing ^{14}C were dissolved in a scintillation solution of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen (4g/l) in redistilled methanol–toluene (1:1, v/v) and measured in a Packard Tri-Carb scintillation spectrometer (series 314E) that gave 50% efficiency of counting. Simultaneous measurements of ^{3}H and ^{14}C were carried out in a solution containing 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen (6g/l) and naphthalene (80g/l) in 1 litre of methoxyethanol–toluene (2:3, v/v) in a Packard Tri-Carb model 3375 that gave 56.6% counting efficiency for ^{14}C and 0.09% for ^{3}H in one channel and 18% ^{14}C, 24% ^{3}H in the other. Radioactive spots on precoated silica-gel t.l.c. plates were located either with a Packard radiochromatogram scanner (model 7200), rate-meter (model 385) and Honeywell Electronik 18 recorder or by covering the plates with Kodak Kodirex X-ray film and preparing the radioautograms with D II developer and Amfix fixative (May and Baker Ltd).

Assay of ^{18}O. The ^{18}O/^{16}O ratio in the epoxide, and in abscisic acid formed from it, was determined by mass spectrometry of their methyl esters. This was carried out with a Varian G.L.C. (series 1200) coupled to an A.E.I. MS9 via a Llewellen–Type separator (Hawes, Mallaby & Williams, 1969). The mean intensity of the parent ion of the ^{18}O-labelled epoxide at *m/e* 266 was 66% of the height of the parent ion at *m/e* 264 (40 atoms % enrichment with ^{18}O). ^{18}O was absent from the side chains of (I) and (II) because there was no satellite peak two mass units greater than the *m/e* 125 peak which is attributed to the methyl dienoate side-chain-fragment ion in both compounds. The mass spectrum of the epoxide methyl ester contained peaks characteristic of authentic material at *m/e* 232, 189, 179 (25%), 174, 147 (23%), 138, 133, 125 (10%), 123 (100%), 95, 93, 79 (38%), 69 (17%) and 52 (20%).

RESULTS AND DISCUSSION

Green tomato fruit have already been shown to synthesize abscisic acid from mevalonic acid (Noddle & Robinson, 1969) and were, therefore, used for the first experiments to find whether 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methyl-[2-^{14}C]penta-*cis*-2-*trans*-4-dienoic acid (II) can be converted into abscisic acid (I).

The criteria we use to demonstrate incorporation of a labelled compound into abscisic acid require that the radioactivity should not only co-chromatograph with abscisic acid but also with methyl abscisate after methylation, should be divided between the methyl esters of the 1′,4′-*cis*- (V) and 1′,4′-*trans*- (VI) diols formed from methyl abscisate by treatment with methanolic borohydride, and should reappear in methyl abscisate after oxidation of both 1′,4′-diols (Figs. 1, 2 and 3; Table 2). Optical-rotatory-dispersion spectra of the zones of the chromatograms opposite authentic markers were also obtained and when they contained natural (+)-abscisic acid, or the products derived from it, they exhibited the characteristic optical-rotatory-dispersion and u.v. spectra (Cornforth, Draber, Milborrow & Ryback, 1967).

The 2-*trans*- isomer, 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (III), was not converted into abscisic acid; there was a progressive loss of radioactivity as the various derivatives were formed and purified. The

Table 2. *Incorporation of* (±)-[2-^{14}C]*epoxide* (II) *and its* 2-trans-*isomer* (III) *into abscisic acid*

The compounds (1.3mCi/mmol) were injected in ethanolic solution into green or boiled green tomato fruits (125g)

	Dose injected (d.p.m.)	Radioactivity in abscisic acid (methyl ester) after racemate dilution correction (d.p.m.)	Incorporation (%)	(+)-Abscisic acid isolated (μg/125g, assayed by racemate dilution method)	Radioactivity in 1′,2′-dihydroxy derivative (d.p.m.)
Epoxide	11687000	206300	1.8	129	—
2-*trans*-Epoxide	12191000	5124	0.042	97	—
Epoxide into boiled tomatoes	5747000	2196	0.038	24.6	200000
2-*trans*-Epoxide into boiled tomatoes	6411000	920	0.014	27.3	1392000

2-*cis*-epoxide and 2-*trans*-epoxide were also injected into boiled tomatoes and both compounds were converted into the 1,2-dihydroxy-2,6,6-trimethylcyclohexyl derivatives (VII, VIII) (Table 2).

As these products were also formed from the epoxides by treatment with dilute acids, the large amounts found in boiled fruit were probably formed by non-enzymic reactions. The 1′,2′-dihydroxy derivative (VII) is not inhibitory to coleoptile extension growth (Tamura & Nagao, 1969*c*), and is unlikely, therefore, to be an intermediate in the series of reactions between the epoxide and abscisic acid. (±)-5-(1,2-Dihydroxy-2,6,6-trimethylcyclohexyl)-3-methyl[2-^{14}C]penta-*cis*-2-*trans*-4-dienoic acid was not converted into abscisic acid by tomatoes (Table 3).

All isolates of naturally-occurring abscisic acid have given almost identical, positive, specific rotations so it appears that only (+)-abscisic acid is synthesized by plants. Racemic 5-(1,3-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (II) was injected into the tomatoes but it is most unlikely that both enantiomers of the epoxide are converted into abscisic acid. Measurements of radioactivity found in abscisic acid indicate that 1.8% of the total epoxide (or 3.6% if one enantiomer only is metabolized to abscisic acid) is recovered as abscisic acid after 48h. This amount represents 15% of the abscisic acid in the plant. The unchanged epoxide recovered from the tomatoes does indeed show slight optical activity and this could result from the conversion of one enantiomer only into abscisic acid, leaving an excess of the other; the rotatory power of the pure epoxide enantiomers would in any case be expected to be far less than that of abscisic acid. On the other hand the optical activity of the epoxide remaining in the plant could also arise from a stereospecific metabolism of either enantiomer to some other product. The amount of ^{14}C-labelled 2-*trans*-epoxide (III) incorporated into abscisic acid by tomato fruit was so small in comparison with the amount of the 2-*cis*-isomer (0.0016% compared to 0.44%) that it may be attributed to photolytic isomerization during isolation or residual contamination of the starting material and it is concluded that the 2-*trans*-isomer (III) cannot act as a precursor of abscisic acid (Table 2). The 2-*cis* double bond of abscisic acid is probably first formed in the *trans* configuration (Robinson & Ryback, 1969) and no enzymic isomerization of 2-*trans*-abscisic acid has been detected (Milborrow, 1970). The non-incorporation of the 2-*trans*-epoxide puts the stage at which this bond is isomerized to its final *cis* configuration to an earlier phase of the biosynthesis.

Wright & Hiron (1969) have found that wheat seedlings normally contain about 20μg of extractable (+)-abscisic acid/kg fresh weight but when

Table 3. *Incorporation of* (±)-(1,2-*dihydroxy*-2,6,6-*trimethylcyclohexyl*)-3-*methyl*-[2-^{14}C]*penta*-cis-2-trans-4-*dienoic acid* (1.3mCi/mmol) *and its* 2-trans-*isomer* (1.3mCi/mmol) *into abscisic acid by green tomato fruit* (83 *and* 99g) *during* 48*h at* 20°C *in the dark*

	Radioactivity (d.p.m.)		(+)-Abscisic acid isolated, assayed by racemate dilution method (μg)
	Injected	In abscisic acid methyl ester	
2-*cis*-1′,2′-Dihydroxy derivative (VII)	199700	34	36
2-*trans*-1′,2′-Dihydroxy derivative (VIII)	223800	0	27

caused to wilt for 4h the concentration increases to 500μg/kg. This rise could be caused by the release of bound abscisic acid or by its formation from a large pool of a precursor but we have found that wilted wheat shoots, previously supplied with (±)-[^{3}H]mevalonic acid solution for 2h, convert nine times as much mevalonate into abscisic acid as do unwilted plants. Parallel determinations of the amount of (+)-abscisic acid in the extracts by means of its optical rotatory dispersion showed that the concentration rose from less than 7μg to 120μg/kg original fresh wt. (Table 4). The discrepancy between the ratios of incorporation of labelled mevalonate in turgid and wilted plants in comparison with the amounts of (+)-abscisic acid determined by optical rotatory dispersion is considered to result from slow penetration of the labelled mevalonate because in a second experiment approximately twice the amount of mevalonate was incorporated into abscisic acid in wilted seedlings although the concentrations of abscisic acid determined by optical rotatory dispersion showed an approximately 25-fold difference. A subsample of these wilted plants was maintained in a wilted condition for a further 18h and incorporated 8 times as much label from mevalonate into abscisic acid as during the first 4h (Table 5).

These results suggested a novel way of investigating whether the conversion of the epoxide (II) into abscisic acid was regulated in the same way as the pathway by which abscisic acid is made *in vivo*. In a second wheat experiment with [^{3}H]mevalonate the ^{14}C-labelled epoxide was included, alone and mixed with [^{3}H]mevalonate. The samples of plants that had absorbed these solutions were divided and exposed to wet and dry conditions and analysed as before. The greater incorporation of [2-^{14}C]epoxide into abscisic acid by wilting leaves suggests that the epoxide, or a close derivative of it, is a natural intermediate because the amount incorporated is regulated in the same way as the endogenous biosynthesis of abscisic acid. If the conversion were brought about by an adventitious series of reactions this parallelism would not be expected to occur. The response to an environmental factor of the endogenous synthesis of abscisic acid and of the incorporation of the epoxide indicates that a site of regulation of biosynthesis exists between the epoxide and abscisic acid.

The incorporation of ^{14}C-labelled epoxide into abscisic acid shows that the carbon skeleton of the epoxide becomes the carbon skeleton of abscisic acid. The fate of the epoxy oxygen was investigated with wilting wheat because any ^{18}O-containing abscisic acid formed during the experiment would not be diluted by a large pool of endogenous ^{16}O material. The plants took up a solution of (±)-[2-^{14}C,1′,2′-^{18}O]epoxide for 18h at 7°C before they were wilted at 20°C. The abscisic acid formed during the 4h of wilting was isolated and the proportion of ^{18}O determined from the ratio of the parent ions, at m/e^{+} 278 and m/e^{+} 280, of the methyl ester. There is no indication of an m/e 127 peak in the mass spectrum of either the epoxide methyl ester or the abscisic acid methyl ester, therefore there was no ^{18}O in the dienoic acid side chain of either compound. Unpublished results in our laboratory show that the oxygen of the tertiary hydroxyl group is retained in the fragment ion of m/e 190 (base peak) in abscisic acid and its methyl ester and, as expected, the mass spectrum of ^{18}O-labelled abscisic acid showed a new peak at m/e 192. The proportion of ^{18}O to ^{16}O in the methyl abscisic acid showed that 97% had been derived from the ^{18}O-labelled precursor. The amount of ^{14}C-labelled abscisic acid derived from the epoxide compared with the total optically active abscisic acid determined by optical-rotatory-dispersion analysis is in close agreement with the result obtained by mass spectrometry (Table 6). The oxygen of the 1,2-epoxy group, therefore, becomes the tertiary hydroxyl of abscisic acid and the conversion is quantitative. Further, the weight of abscisic acid calculated to be derived from the epoxide of known specific radioactivity, together with the weight of abscisic acid determined by optical rotatory dispersion, and in agreement with the proportion of ^{18}O present, excludes the presence of any but trace amounts of racemic abscisic acid. For the ^{18}O to be retained the C-1′–oxygen bond

Table 4. *Incorporation of* (±)-[2-^{3}H]*mevalonate* (93mCi/mmol) *into* (+)-*abscisic acid by cut wheat leaves*

The wheat plants absorbed 19 μg of mevalonate in 20ml of 0.01M-potassium phosphate buffer, pH 7.3, 3% (v/v) in ethanol during 2h. The batch was then divided and one half was allowed to wilt so that it lost 20% of its weight; the other half was placed with the cut ends in water for 5h.

Treatment	Original wt. (g)	(+)-Abscisic acid (μg/kg)	(+)-Abscisic acid (assayed by racemate dilution method) (μg/kg)	Radioactivity in abscisic acid (assayed by racemate dilution method) (d.p.m.)	S.E. (%)	% (±)-mevalonate incorporated	% abscisic acid formed from mevalonate
Water	75	<4	<6.6	286	1	0.0013	—
Wilt	70	60	120	2600	1	0.012	0.012

Table 5. *Incorporation of* (±)-[2-^{3}H]*mevalonate and* (±)-[2-^{14}C]*epoxide into abscisic acid by cut wheat shoots*

The plants were treated as before except that the wilted batches lost 29% of their original weight during 6h of treatment and ethanol was omitted. Small subsamples of two (*) were kept for a further 18h before extraction (24h). (±)-[2-^{3}H]Mevalonate (10.8 μmol) (93mCi/mmol) and (±)-[2-^{14}C]epoxide (1.3mCi/mmol) were supplied as the potassium salt. Incorporation results are calculated from the amount of racemate taken up by the plants and assuming that four ^{3}H atoms from mevalonate are incorporated into one molecule of abscisic acid. The relatively small amounts of the precursors incorporated in 6h are attributed to their slow rates of penetration into the cells. R.D., racemate dilution.

Treatment	Original fresh wt. (g)	Weight absorbed (μg)		(+)-Abscisic acid extracted (μg/kg)	(+)-Abscisic acid assayed by R.D. method (μg/kg)	Radioactivity in abscisic acid after R.D. correction (d.p.m./sample)		% incorporation		% of abscisic acid formed from precursor	
		^{3}H	^{14}C			^{3}H	^{14}C	Mevalonate	Epoxide	Mevalonate	Epoxide
Wet control	56.6	—	—	1.1	19.4	—	—	—	—	—	—
Wilted control	43.8	—	—	29.2	670	—	—	—	—	—	—
Wet+ mevalonate	33.1	7.72	—	0.37	15.1	280	—	0.000262	—	0.049	—
Wilted+ mevalonate*	57.0	6.84	—	29.6	520	6771	—	0.0072	—	0.0015	—
* 24h	1.4	0.168	—	—	—	138	(not R.D.)	0.054	—	—	—
Wet+mevalonate +epoxide	53.5	11.18	9.5	0.62	15.0	310	1339	0.000192	1.28	0.033	15
Wilted+ mevalonate+ epoxide	37.2	9.99	10.6	22.1	590	1302	2068	0.0094	1.77	0.0038	0.90
Wet+epoxide	57.0	—	8.6	1.7	31.6	—	1054	—	1.12	—	5.6
Wilted+epoxide*	57.0	—	8.0	23.7	415	—	3018	—	3.42	—	1.2
* 24h	2.0	—	0.028	—	—	(not R.D.)	111	—	36	—	—

Table 6. *Formation of the tertiary hydroxyl group of abscisic acid from a 1′,2′-epoxy oxygen*

(±)-[2-^{14}C,1′,2′-^{18}O]Epoxide (II) (1.13mg) (24.4μCi of ^{14}C/mmol; 33.2 atoms % ^{18}O) was taken up by 103g of wheat leaves in 35ml of 0.01M-potassium phosphate buffer, pH7.3, during 18h at 7°C. The plants were then wilted so that they lost 48% of their weight during the first 20min of a 4h treatment at 20°C. Abbreviation: o.r.d., optical rotatory dispersion.

(+)-Abscisic acid by o.r.d. (μg)	Mean atoms % ^{18}O in methyl abscisate $m/e\left(\frac{280}{278+280}\right)$	S.E. (%)	% of abscisic acid formed from epoxide by ^{18}O	Wt. of abscisic acid from [^{14}C]epoxide (μg)	% of abscisic acid formed from epoxide by ^{14}C and o.r.d.
20.0	32.2	2	97	19.2	96

must remain intact and epimerization at C-1′ is not possible. It follows that only one enantiomer of the epoxide is converted into abscisic acid: that one in which the epoxide is on the same side of the six-membered ring as the hydroxy group in abscisic acid.

The formation of abscisic acid from the epoxide requires the net addition of keto oxygen on C-4′ of the cyclohexyl ring and could be achieved by a hydroxylation followed by oxidation. If an early intermediate in the conversion contained a C-4′ keto group then this structure could facilitate the rearrangement of the 1′,2′-epoxide group to give the tertiary hydroxyl of abscisic acid and the allylic double bond. A cyclohexyl ring with the same carbon skeleton and with an epoxy- and a hydroxy-group in the same relative positions is known in violaxanthin.

The conversion of the epoxide into abscisic acid is unlikely to proceed by hydrolytic cleavage of the 1′,2′-epoxide because 5-(1,2-dihydroxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (VII) is inactive as a growth inhibitor and is not converted into abscisic acid. However, the compound tested was formed by acid hydrolysis and under these conditions epoxy groups give 1′,2′-*trans*-dihydroxy products. The possibility, although unlikely, that a 1′,2′-*cis*-dihydroxy intermediate is formed enzymically cannot be excluded.

Phillips & Wareing (1959) and Robinson & Wareing (1964) observed increases in the concentration of growth-inhibitory material, later identified as abscisic acid, in sycamore leaves over several days, in response to changes in day-length treatments. Wright & Hiron (1969) have shown a 40-fold increase in concentrations of abscisic acid in wheat leaves but until now all reports of the synthesis of abscisic acid from labelled mevalonate (Noddle & Robinson, 1969) have been for fruits. The experiments reported here show that abscisic acid is synthesized in both leaves and fruit.

The rapid conversion of the epoxide into abscisic acid by tomatoes and wheat provides an explanation for the reported growth-inhibitory activity of the former compound. It also indicates that the growth-inhibitory activity of other related analogues should be re-examined to discover whether the effects they produce could also occur as a result of conversion into abscisic acid.

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