Stereochemical Aspects of the Formation of Double Bonds in Absciscic Acid

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The stereochemistry of the hydrogen elimination that occurs during the formation of the Δ^4 - and Δ^2 '-double bonds of absciscic acid has been determined from the $^{14}\text{C}/^3\text{H}$ ratios in absciscic acid biosynthesized by avocado fruit from $[2\text{-}^{14}\text{C}, (2R)\text{-}2\text{-}^3\text{H}_1]\text{-}$, $[2\text{-}^{14}\text{C}, (2S)\text{-}2\text{-}^3\text{H}_1]\text{-}$ and $[2\text{-}^{14}\text{C}, (5S)\text{-}5\text{-}^3\text{H}_1]\text{-}$ mevalonate. Setting the $^{14}\text{C}/^3\text{H}$ ratio at 3:3 for $[2\text{-}^{14}\text{C}, (2R)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate, the corresponding ratio in derived methyl abscisate was 3:2.28; the analogous ratio for methyl abscisate from $[2\text{-}^{14}\text{C}, (2S)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate was 3:1.63. Removal of the 3'-hydrogen atom of absciscic acid by base-catalysed exchange altered the ratios to 3:1.55 and 3:1.44 respectively. It was concluded that this 3'-hydrogen atom is derived from the *pro*-2R-hydrogen atom of mevalonate. Removal of the 4-hydrogen atom from methyl abscisate by formation of a derivative, a lactone, lacking this hydrogen atom changed the ratio to 3:1.04 for material derived from $[2\text{-}^{14}\text{C}, (2R)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate and to 3:1.05 for $[2\text{-}^{14}\text{C}, (2S)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate, showing that this hydrogen atom also is derived from the *pro*-2R-hydrogen atom of mevalonate. These ratios of the lactones are consistent with their retaining one ^3H atom at the 6'-methyl position of absciscic acid from the $[(2R)\text{-}2\text{-}^3\text{H}_1]\text{-}$ and $[(2S)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate. The presence of some label at positions 3' and 4 when $[(2S)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate was the precursor is attributed to the action of isopentenyl pyrophosphate isomerase. The hydrogen atom at C-5 of absciscic acid is derived from the *pro*-5S-hydrogen atom of mevalonate.

Much of the elegant work of Cornforth and Popják and their collaborators on the stereochemistry of the formation of polyisoprenoids from mevalonic acid was made possible by the synthesis (Cornforth *et al.*, 1966) of the stereospecifically tritiated pairs of mevalonic acids labelled at the 2- and 4-positions. The $[(5R)\text{-}5\text{-}^3\text{H}_1]\text{-}$ mevalonic acid has been made by Donniger & Popják (1964) and recently the $[(5S)\text{-}^3\text{H}_1]\text{-}$ mevalonic acid, the final member of the sextet, has been synthesized (Cornforth & Ross, 1970; Scott *et al.*, 1970; Seiler *et al.*, 1970).

In the enzymic formation of the *trans,trans*-farnesyl pyrophosphate all three *pro*-4S-hydrogen atoms of the three mevalonate units participating are eliminated (Popják & Cornforth, 1966) when the three double bonds are formed. This is also true of carotenoids (Goodwin, 1971), but in the rubber hydrocarbon (which has *cis* double bonds) the *pro*-4S-hydrogen atoms survive and the *pro*-4R-hydrogen atoms are eliminated. Robinson & Ryback (1969) therefore looked for a possible distinction between the pathways of biosynthesis of carotenoids and of absciscic acid (which has a 3-*cis* double bond), but it was found that the *pro*-4R-hydrogen atom of mevalonate was retained at C-2 (and also C-5') of absciscic acid, suggesting that the Δ^2 -double bond is formed *trans* and hence the labelling pattern could have arisen from either of the proposed routes.

The retention of ^3H from $[(2R)\text{-}2\text{-}^3\text{H}_1]\text{-}$ and $[(2S)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate at C-4 and C-3' of absciscic acid and from $[(5S)\text{-}5\text{-}^3\text{H}_1]\text{-}$ mevalonate at C-5 of absciscic acid has now been studied and the stereochemistry of the elimination of the hydrogen atom deduced. The nominal $[(2R)\text{-}2\text{-}^3\text{H}_1]\text{-}$ and $[(2S)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonic acids used are present as a racemic mixture in which both enantiomers carry ^3H at C-2. The nominal $[(2R)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate has the naturally occurring 3R configuration whereas its antipode, which carried a 2S- ^3H atom, is 3S. Similarly, the nominal $[(2S)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate is the natural 3R enantiomer and is present with 3S- $[(2R)\text{-}2\text{-}^3\text{H}_1]\text{-}$ mevalonate. Many workers have found, both in plants and animals, that mevalonate kinase utilizes the 3R enantiomer of mevalonate only and the absence of incorporation of ^3H from nominally $[(4S)\text{-}4\text{-}^3\text{H}_1]\text{-}$ mevalonate of analogous constitution by avocado fruit pulp (Robinson & Ryback, 1969) demonstrated that the discrimination operates in this tissue also.

The probable derivation of the hydrogen atoms from C-2 and C-5 of mevalonate in the hypothetical saturated sesquiterpenoid precursor of absciscic acid can be deduced from previous work (Popják & Cornforth, 1966). Fusion of the C₅ units proceeds with inversion of the arrangement of the hydrogen atoms on both carbon atoms involved in the new bond (III and IV). During the formation of absciscic acid,

(I) Absciscic acid showing numbering used

(II) Postulated origin of the carbon atoms of abscisic acid showing their numbering in mevalonic acid

(III) Arrangement of hydrogen atoms of C-2 and C-5 portrayed as in mevalonate

(IV) Stereochemistry of hydrogen atoms of C-2 and C-5 of mevalonate after junction of the isoprene residues

four of the C-5-hydrogen atoms of mevalonate are lost when the oxo and carboxyl groups are formed, so that unless hydrogen migration occurs the ^3H atom from C-5 of mevalonate can be retained at C-5 of abscisic acid only. Formation of the Δ^4 -double bond can be expected to be stereospecific, and therefore one only of the six C-5-hydrogen atoms of mevalonate should be retained per molecule of abscisic acid.

Formation of the Δ^4 - and Δ^3 -double bonds of abscisic acid occurs with the loss of one or other of the ^3H atoms of C-2 of mevalonate at these positions. The third isoprene residue should retain both the $2R$ - and $2S$ - ^3H atoms of mevalonate in one of the geminal methyl groups of abscisic acid.

Thus four possible labelling patterns of abscisic acid can originate from $[(2R)\text{-}2\text{-}^3\text{H}_1]$ - and $[(2S)\text{-}2\text{-}^3\text{H}_1]$ -mevalonate, i.e. $[6',3',4\text{-}^3\text{H}_3]$, $[6',3'\text{-}^3\text{H}_2]$, $[6',4\text{-}^3\text{H}_2]$ and $[6'\text{-}^3\text{H}_1]$. The location and amount of ^3H in abscisic acid biosynthesized by ripening avocado fruit that had been supplied with either $[2\text{-}^{14}\text{C},(2R)\text{-}2\text{-}^3\text{H}_1]$ - or $[2\text{-}^{14}\text{C},(2S)\text{-}2\text{-}^3\text{H}_1]$ -mevalonate was determined from the $^{14}\text{C}/^3\text{H}$ ratio of purified abscisic acid methyl ester. The hydrogen atom removed from C-3' by exchange and then the loss of the hydrogen atom from C-4 by lactone formation were also measured.

Materials and Methods

Materials

The sample of racemic abscisic acid used was prepared by Cornforth *et al.* (1965). The $1',4'\text{-cis}$ - and $1',4'\text{-trans}$ -diols of abscisic acid were prepared by reduction of this material with methanolic NaBH_4 in ice-cold methanol-water (4:1, v/v). The methyl esters of these compounds were made by treatment

with ethereal diazomethane. A.R.-grade chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; A.R. solvents used were from Hopkin and Williams, Chadwell Heath, Essex, U.K.

$\text{RS-[2-}^{14}\text{C]Mevalonolactone}$ (10.3 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The $[(2R)\text{-}2\text{-}^3\text{H}_1]$ - and $[(2S)\text{-}2\text{-}^3\text{H}_1]$ -mevalonolactones (both $60\mu\text{Ci}/\mu\text{mol}$) were synthesized by Dr. R. H. Cornforth by the method described by Cornforth *et al.* (1966). The $[(5S)\text{-}5\text{-}^3\text{H}_1]$ -mevalonolactone ($0.2\mu\text{Ci}/\mu\text{mol}$) was synthesized by F. P. Ross (Cornforth & Ross, 1970). Mevalonolactones of defined $^{14}\text{C}/^3\text{H}$ ratios were prepared by mixing benzene solutions of the above materials to give the desired values. The mixtures were assayed for ^{14}C and ^3H after chromatography in toluene-ethyl acetate-acetic acid (25:12:2, by vol.) on silica-gel t.l.c. plates.

Fruit

The plant material used was ripening fruit of avocado (*Persea gratissima* Gaertn., c. var. Evenrond) obtained commercially in June 1971. The fruits (250–300 g each) were used when they began to soften; they were cut in half and the stones were removed. Longitudinal and transverse cuts, 5–10 mm apart, were made in the mesocarp and then the aqueous mevalonic acid solutions, which contained 0.2% Triton X-100 as wetting agent (5 ml/fruit), were pipetted into the cavities and spread along the cuts.

Extraction

After incubation for 16 h, the fruits were homogenized in 1 litre of methanol containing 10 mg of 2,6-di-*t*-butyl-4-methylphenol and 2.0 mg of racemic

abscisic acid. The solid material was precipitated by centrifugation at 1000rev./min at 0°C and re-extracted a further three times with methanol alone. The methanol was removed in a Buchi rotary evaporator at 40°C until the solution was concentrated to approx. 400ml. A 10%-satd. solution of NaHCO₃ (200ml) was then added and the volume was decreased to 100ml. The ether-soluble neutral compounds were removed by extraction with diethyl ether (4×50ml). The pH was adjusted to 3.0 with 0.5M-H₂SO₄ and the ether-soluble acid fraction was extracted into ether (4×50ml).

When [3'-³H]abscisic acid was expected to be present, the whole extraction was carried out under acidic conditions to prevent loss of this hydrogen atom. Acetic acid (20ml) was added to the methanol, and water was added during the final stages of its evaporation. The ether-soluble acid and neutral compounds were then extracted together in 6×50ml of ether. The acid fraction was removed from the ether by shaking the ether with small volumes of 0.2M-sodium phosphate buffer, pH 6.0, until the pH of the buffer remained constant at 6.0. Two further extracts were then made and combined with the preceding ones (Table 1). H₂SO₄ (1M) was then added to lower the pH of the buffer to 3.0, and then the acid fraction was extracted with ether (6×50ml).

Chromatography

Chromatography was carried out on pre-coated silica-gel G₂₅₄ thin-layer plates (200mm×200mm) obtained from E. Merck A.-G., Darmstadt, Germany. The ether-soluble acid fractions were applied to

2.5mm-thick plates, which required approx. 4h for the solvent front to run 120mm. The smaller quantities of materials eluted from a specific zone of such plates were chromatographed on 0.25mm-thick plates which developed to the same distance in 2h. The solvent system for free acids was toluene-ethyl acetate-acetic acid (25:15:2, by vol.) containing 200μg of 2,6-di-*t*-butyl-4-methylphenol/ml, and for methyl esters and compound (VI), hexane-ethyl acetate mixtures (from 1:1 to 3:1, v/v) containing 200μg of 2,6-di-*t*-butyl-4-methylphenol/ml were used. Multiple development of the chromatograms was frequently done, particularly with the whole ether-soluble acid fractions. Authentic marker compounds were invariably applied as 20mm bands towards the edges of the plates so that the appropriate zone of the plate containing a given compound could be located exactly. Most compounds were detected by their quenching of the fluorescence of the phosphor in silica gel when the plates were viewed under weak u.v. irradiation (254nm). Mevalonic acid was detected by exposing the edge of the plate to iodine vapour; this region was then discarded.

Retention of the 3'-hydrogen atom

The acidic mixture of compounds believed to contain [3'-³H]abscisic acid was applied to the origin of the chromatograms, which had been sprayed with hexane-acetic acid (10:1, v/v) before use, and was run in ether-acetic acid (10:1, v/v) containing 5mg of 2,6-di-*t*-butyl-4-methylphenol/ml. The abscisic acid zone of the chromatogram was eluted through a fine glass-sinter with methanol-acetic acid (49:1,

Table 1. Distribution of ¹⁴C and ³H in avocado fractions

For experimental details see the text. Radioactivity supplied to the fruit was as follows: 3R-[2-¹⁴C,(2R)-2-³H₁]-mevalonolactone and 3S-[2-¹⁴C,(2S)-2-³H₁]-mevalonolactone, 2712000 d.p.m. of ¹⁴C, 61396000 d.p.m. of ³H; 3R-[2-¹⁴C,(2S)-2-³H₁]-mevalonolactone and 3S-[2-¹⁴C,(2R)-2-³H₁]-mevalonolactone, 2656000 d.p.m. of ¹⁴C, 39342000 d.p.m. of ³H; 3R-[2-¹⁴C,(5S)-5-³H₁]-mevalonolactone and 3S-[2-¹⁴C,(5S)-5-³H₁]-mevalonolactone, 157000 d.p.m. of ¹⁴C, 1250000 d.p.m. of ³H. These values were obtained from the experiments reported in Tables 2 and 4. All values of radioactivity are corrected for quenching.

Precursor ...	10 ⁻³ × Radioactivity (d.p.m.)					
	[2- ¹⁴ C,(2R)-2- ³ H ₁]- Mevalonolactone		[2- ¹⁴ C,(2S)-2- ³ H ₁]- Mevalonolactone		[2- ¹⁴ C,(5S)-5- ³ H ₁]- Mevalonolactone	
	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C	³ H
Fraction						
Methanol extract	—	—	1205	25200	120	922
Residue of fruit	352	6500	644	6700	55	303
Methanol distillate	6	968	—	—	10	90
Neutral fraction	596	11810	489	7260	43	277
Aqueous residue	413	15220	397	8440	24	238
Acid fraction	415	5020	331	5270	9	51

v/v). After evaporation of the eluting solvent to a convenient volume, the acid was methylated with excess of ethereal diazomethane for 2 min, and then excess of acetic acid was added to destroy the remaining diazomethane and to maintain the acidity of the solution. Chromatography of the methyl ester was carried out on similarly acid-sprayed plates in hexane-ethyl acetate-acetic acid (15:5:1, by vol.).

Determination of ^3H and ^{14}C

Samples containing ^{14}C and ^3H were placed in a scintillation solution of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen (BBOT) [CIBA (A.R.L.) Ltd., Duxford, Cambs., U.K.] dissolved (6 g/l) in a solution of naphthalene (80 g) in 1 litre of methoxyethanol-toluene (2:3, v/v). The samples were counted for radioactivity in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer which gave, at the settings used, 50.9% counting efficiency for ^{14}C and 0.5% for ^3H in one channel and 16.72% for ^{14}C and 16.67% for ^3H in the other channel. Coloured solutions were counted for radioactivity before and after additions of internal standards of [^3H]toluene (22539 d.p.m./10 μl) and [^{14}C]toluene (4933 d.p.m./10 μl) (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.). All samples were counted for radioactivity until the standard deviation fell to 1% or less of the total c.p.m.

Identification and measurement of abscisic acid

The criteria used to demonstrate the incorporation of a labelled compound into abscisic acid have been stated previously (Milborrow & Noddle, 1970). Briefly, these are that the label should co-chromatograph with abscisic acid and then with the methyl abscisate formed from it. The label should also co-chromatograph with the 1',4'-*cis*- and 1',4'-*trans*-diol of methyl abscisate when these are formed from the methyl abscisate. These requirements were fulfilled in these experiments, and no labelled contaminant was observed in the methyl abscisate eluted on rechromatography. Any radioactivity, therefore, that co-chromatographed with methyl abscisate was deemed to have been incorporated into abscisic acid. In the experiments reported here, the 3'- ^3H atom was lost on the formation of the 1',4'-*cis*- and 1',4'-*trans*-diols because of the alkaline conditions produced in the aqueous methanolic NaBH_4 . However, ^{14}C and the remaining ^3H appeared at the R_F values corresponding to the *cis* and *trans* diols. Optical-rotatory-dispersion spectra of zones of the chromatograms opposite authentic markers were obtained as before (Milborrow & Noddle, 1970), and were identical with those of natural (+)-abscisic acid or the products derived from it. Further, labelled material formed from methyl abscisate co-chromatographed

with authentic compound (VI) (see below) prepared by Mallaby & Ryback (1972).

Position of ^3H

The ^3H on the 3'-double bond in the methyl ester of abscisic acid was removed by incubating the material with 0.2 ml of 1 M-NaOH for 1 h at 20°C. In a preliminary experiment, this treatment hydrolysed most of the methyl ester, so that the abscisic acid was not then extracted into ether. To overcome this, oxalic acid dihydrate (20 mg) was added to each tube. The abscisic acid and any unhydrolysed ester were then taken up together by 4 $\times$ 1 ml extractions with ether. Diazomethane was added to re-esterify any abscisic acid. The aqueous solution remaining was mixed with scintillation solution and counted for radioactivity directly. Internal standards of first [^3H]toluene and then [^{14}C]toluene showed that there was little quenching (7.2% for ^3H ; 0% for ^{14}C) of the radioactivity in this solution.

The amount of ^3H derived from C-2 of mevalonate that was present on C-4 of abscisic acid was determined by conversion into the lactone (VI). The reactions leading to this compound have been elucidated by Mallaby & Ryback (1972). Methyl abscisate was dissolved in 0.1 ml of formic acid and was heated at 90°C for 1 h. The formic acid was then volatilized in a stream of N_2 and the lactone was separated from other substances, which were formed in low yield, by chromatography in hexane-ethyl acetate (3:1, v/v). The remaining ^3H in the molecule is assigned to a 6'-methyl group, but this is at present inaccessible to chemical degradation.

Results and Discussion

One factor that could affect the changes in $^{14}\text{C}/^3\text{H}$ ratios reported here is whether the three isoprene residues comprising abscisic acid are contributed with equal efficiency from the labelled mevalonate. The results of Robinson & Ryback (1969), with [$2\text{-}^{14}\text{C}$, (4*R*)-4- ^3H]₁mevalonate, show that the two terminal isoprene residues carry almost exactly equal amounts of ^3H . Further, the overall $^{14}\text{C}/^3\text{H}$ ratio of the abscisic acid formed was consistent with the loss of an equivalent amount of ^3H from the central isoprene residue during biosynthesis. Thus the added mevalonate contributes equally to the three isoprene residues of abscisic acid synthesized by avocado fruit and the validity of the method has been established.

Several of the results with [$2\text{-}^{14}\text{C}$, (2*R*)-2- ^3H]₁- and [$2\text{-}^{14}\text{C}$, (2*S*)-2- ^3H]₁-mevalonate (Tables 2, 3, 4 and 5) show variations from the expected $^{14}\text{C}/^3\text{H}$ ratios, but these can be explained by the action of isopentenyl pyrophosphate isomerase and the differences observed are in good agreement with this explanation.

The conversion of isopentenyl pyrophosphate into

Scheme 1. Stereochemistry of the C-2-hydrogen atoms of mevalonate in isopentenyl pyrophosphate after the action of isomerase

dimethylallyl pyrophosphate, mediated by isopentenyl pyrophosphate isomerase, is reversible (Scheme 1). The activity of this enzyme relative to that catalysing the next step in polyisoprenoid biosynthesis (isopentenyl pyrophosphate transferase) is much greater in plants than in animals; in plants, therefore, the dimethylallyl pyrophosphate is more likely to be reconverted into isopentenyl pyrophosphate and back again before being used for synthesis (Goad, 1970). Although the removal and replacement, on isopentenyl pyrophosphate isomerase, of the *pro*-4*S*-hydrogen of mevalonate is stereospecific, three hydrogen atoms have become equivalent in the methyl group of dimethylallyl pyrophosphate, the two originally present and one supplied from the medium. A second isomerization back to isopentenyl pyrophosphate is accompanied by a loss of one of these three hydrogen atoms of the dimethylallyl pyrophosphate methyl group. Thus one cycle of the isomerase will randomize all the ^3H of isopentenyl pyrophosphate derived from stereospecifically 2-tritiated mevalonate and further cycles will continue the loss of the ^3H to the medium. In the absence of isotope effects, one-third of the product will be the original isopentenyl pyrophosphate with the two hydrogen atoms occupying their former positions. In the other two-thirds of the isopentenyl pyrophosphate re-formed from dimethylallyl pyrophosphate, one or other of the two original hydrogen atoms has

been eliminated, and in each case the stereochemical location of the other has been reversed. In isopentenyl pyrophosphate formed from a stereospecifically 2-tritiated mevalonate, one-third of the label would be lost in regenerated isopentenyl pyrophosphate and the remaining label would be equally distributed between the two available positions on the methylene group (Scheme 1). This equal sharing of the label between two positions will persist even if an isotope effect resists the elimination of ^3H (Cornforth *et al.*, 1970).

Thus dimethylallyl pyrophosphate derived from $[2\text{-}^3\text{H}_1]\text{mevalonate}$ may have suffered some loss of ^3H from the methyl group before being utilized for biosynthesis, and the label in isopentenyl pyrophosphate similarly formed may be more-or-less randomized between the two hydrogen atoms of the terminal methylene group before being utilized in turn. Partially random labelling was observed at both C-4 and C-3' of abscisic acid synthesized from stereospecifically 2-tritiated mevalonates. The presumed 6'-methyl label of abscisic acid, originating from a methyl group of dimethylallyl pyrophosphate, showed no attenuation of its ^3H label, an indication either that a large isotope effect operated against removal of ^3H in the isomerization of dimethylallyl pyrophosphate to isopentenyl pyrophosphate, or that no dimethylallyl pyrophosphate that had been through the double sequence

was available for biosynthesis of abscisic acid.

Formation of the $\Delta^{2'}$ -double bond

The hydrogen atom on C-3' of abscisic acid is stable in acid conditions and exchanges with water, together with the two hydrogen atoms on C-5' and the three hydrogen atoms of the C-2'-methyl group, at pH 9 and above (Milborrow, 1969). The abscisic acid formed from $[2-^{14}\text{C}, (2R)-2-^3\text{H}_1]\text{mevalonate}$, and isolated under acidic conditions throughout, not only showed a $^{14}\text{C}/^3\text{H}$ ratio (3:2.28) consistent with the retention of ^3H at this position, but also lost close to the expected proportion of the ^3H (the ratio changed to 3:1.55) when treated with 1M-NaOH; the ^3H lost (equivalent to a ratio of 3:0.584) was assayed by

counting the radioactivity in the aqueous exchange medium (Table 2). The methyl abscisate formed from $[2-^{14}\text{C}, (2S)-2-^3\text{H}_1]\text{mevalonate}$ (Tables 4 and 5) contained more ^3H than expected for the retention of 1 ^3H atom per molecule of abscisic acid, as determined from the $^{14}\text{C}/^3\text{H}$ ratio (3:1.63). A smaller proportion of ^3H was lost on exchange of the hydrogen atom on C-3' of abscisic acid (the $^{14}\text{C}/^3\text{H}$ ratio of 3:1.63 was changed to 3:1.44, i.e. a difference of 3:0.19, and ^3H equivalent to 3:0.23 was found in the aqueous exchange medium). The amount exchanged tallies with a small retention of ^3H from $[(2S)-2-^3\text{H}_1]\text{-mevalonate}$ at this position because of randomiza-

Table 2. Incorporation of $3R\text{-}[2-^{14}\text{C}, (2R)-2-^3\text{H}_1]\text{mevalonate}$ into abscisic acid by avocado fruit and the position of the ^3H

The ^3H at C-3' of abscisic acid is accounted for by the loss of ^3H on base-catalysed exchange. The ^3H at C-4 is accounted for by the loss of ^3H during the formation of compound (VI). The remaining ^3H is assigned to one of the geminal methyl groups. For experimental details see the text. Calculated radioactivity of $3RS\text{-}[2-^{14}\text{C}, (2R)-2-^3\text{H}_1]\text{-mevalonolactone}$ was 2712000 d.p.m. of ^{14}C and 61396000 d.p.m. of ^3H . The radioactivity present in a subsample after t.l.c. was 14590 d.p.m. of ^{14}C and 308297 d.p.m. of ^3H , i.e. $^{14}\text{C}/^3\text{H}$ ratio of 1:21.0. This is normalized to 3:3. Total ^{14}C radioactivity in the methyl ester of (+)-abscisic acid was 4446 d.p.m. [8.9 d.p.m./ μg of (+)-abscisic acid]. The total calculated weight of (+)-abscisic acid in the two half-fruits, which weighed a total of 207 g, was 499 μg [2.41 mg of (+)-abscisic acid/kg of mesocarp]. This experiment corresponds to that in Table 4 in which the other two halves of the fruits were used.

	^{14}C (d.p.m.)	^3H (d.p.m.)	$^{14}\text{C}/^3\text{H}$ ratio (normalized)
Methyl abscisate	818	12750	3:2.28
3'-Exchanged methyl abscisate after t.l.c.	855	9260	3:1.55
Aqueous exchange medium (this subsample contained 422 d.p.m. of $[^{14}\text{C}]\text{methyl abscisate}$ and the ^3H d.p.m. are corrected for the 1% of methyl abscisate which remained in the exchange medium)	0	1730	3:0.584
Compound (VI)	322	2433	3:1.04

Table 3. Repeat of experiment reported in Table 2 with $3RS\text{-}[2-^{14}\text{C}, (2R)-2-^3\text{H}_1]\text{mevalonolactone}$

For experimental details see the text. Calculated radioactivity of $3RS\text{-}[2-^{14}\text{C}, (2R)-2-^3\text{H}_1]\text{mevalonolactone}$ was 3334000 d.p.m. of ^{14}C and 79746000 d.p.m. of ^3H . The radioactivity present in a sub-sample after t.l.c. was 2998 d.p.m. of ^{14}C and 62213 d.p.m. of ^3H , i.e. $^{14}\text{C}/^3\text{H}$ ratio of 1:20.75. This is normalized to 3:3. Total ^{14}C radioactivity in the methyl ester of (+)-abscisic acid was 1238 d.p.m. (8.5 d.p.m./ μg of (+)-abscisic acid). The total calculated weight of (+)-abscisic acid in one half-fruit was 146 μg /144 g [1.01 mg of (+)-abscisic acid/kg of mesocarp]. This experiment corresponds to that in Table 5 in which the other half of the same fruit was used.

	^{14}C (d.p.m.)	^3H (d.p.m.)	$^{14}\text{C}/^3\text{H}$ ratio (normalized)
Methyl abscisate	192	2389	3:1.80
3'-Exchanged methyl abscisate after t.l.c.	371	3652	3:1.42
Aqueous exchange medium (this subsample contained 305 d.p.m. of $[^{14}\text{C}]\text{methyl abscisate}$ and the ^3H d.p.m. are corrected for the 1.4% of methyl abscisate which remained in the exchange medium)	0	565	3:0.21
Compound (VI)	96.9	393	3:0.59

Table 4. *Incorporation of 3R-[2-¹⁴C,(2S)-2-³H₁]mevalonate into abscisic acid by avocado fruit and the position of the ³H*

The ³H at C-3' of abscisic acid is accounted for by the loss of ³H on base-catalysed exchange. The ³H at C-4 is accounted for by the loss of ³H during the formation of compound (VI). The remaining ³H is assigned to one of the geminal methyl groups. For experimental details see the text. Calculated radioactivity of 3RS-[2-¹⁴C,(2-S)-2-³H₁]mevalonolactone was 2656000 d.p.m. of ¹⁴C and 39342000 d.p.m. of ³H. The radioactivity present in a subsample after t.l.c. was 1311 d.p.m. of ¹⁴C and 19418 d.p.m. of ³H, i.e. ¹⁴C/³H ratio of 1:14.8. This is normalized to 3:3. Total ¹⁴C radioactivity in the methyl ester of (+)-abscisic acid was 4312 d.p.m. [6.3 d.p.m./μg of (+)-abscisic acid]. The total calculated weight of abscisic acid in the two half-fruits, which weighed a total of 207 g, was 684 μg [3.3 mg of (+)-abscisic acid/kg of mesocarp]. This experiment corresponds to that in Table 2 in which the other two halves of the fruits were used.

	¹⁴ C (d.p.m.)	³ H (d.p.m.)	¹⁴ C/ ³ H ratio (normalized)
Methyl abscisate	2856	22870	3:1.63
3'-Exchanged methyl abscisate after t.l.c.	824	5590	3:1.44
Aqueous exchange medium (this subsample contained 844 d.p.m. of [¹⁴ C]methyl abscisate and the ³ H d.p.m. are corrected for the 1.5% of methyl abscisate which remained in the exchange medium)	0	958	3:0.23
Compound (VI)	399	2054	3:1.05

Table 5. *Repeat of experiment reported in Table 4 with 3R-[2-¹⁴C,(2S)-2-³H₁]mevalonolactone*

For experimental details see the text. Calculated radioactivity of 3RS-[2-¹⁴C,(2S)-2-³H₁]mevalonolactone was 2705000 d.p.m. of ¹⁴C and 46009000 d.p.m. of ³H. The radioactivity present in a subsample after t.l.c. was 690 d.p.m. of ¹⁴C and 11462 d.p.m. of ³H, i.e. a ¹⁴C/³H ratio of 1:16.61. This is normalized to 3:3. Total ¹⁴C radioactivity in the methyl ester of (+)-abscisic acid was 1330 d.p.m. [8.2 d.p.m./μg of (+)-abscisic acid]. The total calculated weight of (+)-abscisic acid in one half-fruit was 163 μg/144 g [1.13 mg of (+)-abscisic acid/kg of mesocarp]. This experiment corresponds to that in Table 3 in which the other half of the same fruit was used.

	¹⁴ C (d.p.m.)	³ H (d.p.m.)	¹⁴ C/ ³ H ratio (normalized)
Methyl abscisate	267	1890	3:1.28
3'-Exchanged methyl abscisate after t.l.c.	740	4415	3:1.08
Aqueous exchange medium (this subsample contained 768 d.p.m. of [¹⁴ C]methyl abscisate and the ³ H d.p.m. are corrected for the 1.3% of methyl abscisate which remained in the exchange medium)	0	567	3:0.13
Compound (VI)	171	556.5	3:0.60

tion, together with a slight loss of some of this ³H by exchange during isolation (Table 6). Complete randomization of ³H labels in isopentenyl pyrophosphate, even when combined with complete retention of ³H in dimethylallyl pyrophosphate, could not give, in abscisic acid, a ratio higher than 3:2.0 for both stereospecifically labelled mevalonic acids. The ratio of 3:2.28 found when [(2R)-2-³H₁]mevalonate was the precursor is thus an index that both the 3'- and the 4-hydrogen atoms in abscisic acid originate preferentially from the *pro*-2R-hydrogen atom of mevalonate. The label introduced at these positions from [(2S)-2-³H₁]mevalonate, and the less-than-quantitative incorporation of label from [(2R)-2-³H₁]mevalonate,

can be attributed to the partial, but not total, randomization at the stage mediated by isopentenyl pyrophosphate isomerase.

The preliminary results of J. R. Vose, G. Britton & T. W. Goodwin cited by Goodwin (1971) indicate that the 2R-³H of mevalonate is retained at the analogous position of α-carotene (Scheme 2).

However, the Δ^{2'}-double bond of abscisic acid may not arise by the same mechanism that produces the analogous double bond in α-carotene. Work on the incorporation of racemic compound (V) (Milborrow & Noddle, 1970) has shown that the 1',2'-epoxide oxygen atom is retained in the tertiary hydroxyl group of (+)-abscisic acid, and that tomatoes contain an

Table 6. Loss of ^3H from C-3' and C-4 of abscisic acids formed from $2R\text{-}[^3\text{H}]$ - and $2S\text{-}[^3\text{H}]$ -mevalonate

The values are from Tables 2, 3, 4, 5 and 7. Expected $^{14}\text{C}/^3\text{H}$ ratios are calculated by assuming that the hydrogen atom at C-3' and C-4 of abscisic acid is derived from the *pro*-2*R*-hydrogen atom of mevalonate without racemization. For experimental details see the text.

	Precursor ($^{14}\text{C}/^3\text{H}$ ratio normalized to 3:3)	$^{14}\text{C}/^3\text{H}$ ratio of abscisic acid	$^{14}\text{C}/^3\text{H}$ ratio after exchange of ^3H at C-3		$^{14}\text{C}/^3\text{H}$ ratio of ^3H from C-3' of abscisic acid		$^{14}\text{C}/^3\text{H}$ ratio of compound VI		$^{14}\text{C}/^3\text{H}$ ratio of ^3H from C-4 of abscisic acid	
			Found	Expected	Found	Expected	Found	Expected	Found	Expected
Expt. 1 (Tables 2 and 4)	$[2\text{-}^{14}\text{C}_1(2R)\text{-}2\text{-}^3\text{H}_1]\text{Mevalonolactone}$	3:2.28	3:1.55	3:1.0	3:0.73	3:1.0	3:1.04	3:1.0	3:0.51	3:1.0
Expt. 2 (Tables 3 and 5)	$[2\text{-}^{14}\text{C}_1(2S)\text{-}2\text{-}^3\text{H}_1]\text{Mevalonolactone}$	3:1.63	3:1.44	3:0.0	3:0.19	3:0.0	3:1.05	3:0.0	3:0.39	3:1.0
	$[2\text{-}^{14}\text{C}_1(2R)\text{-}2\text{-}^3\text{H}_1]\text{Mevalonolactone}$	3:1.80	3:1.42	3:0.38	3:0.38	3:1.0	3:0.59	3:1.0	3:0.83	3:1.0
Expt. 3 (Table 7) + iodoacetamide	$[2\text{-}^{14}\text{C}_1(2S)\text{-}2\text{-}^3\text{H}_1]\text{Mevalonolactone}$	3:1.28	3:1.08	3:0.20	3:0.20	3:0.0	3:0.60	3:0.0	3:0.48	3:0.0
	$[2\text{-}^{14}\text{C}_1(2R)\text{-}2\text{-}^3\text{H}_1]\text{Mevalonolactone}$	3:2.55	3:1.99	3:0.56	3:0.56	3:1.0	3:1.19	3:1.0	3:0.80	3:1.0
	$[2\text{-}^{14}\text{C}_1(2S)\text{-}2\text{-}^3\text{H}_1]\text{Mevalonolactone}$	3:1.12	3:0.92	3:0.20	3:0.20	3:0.0	3:0.90	3:0.0	3:0.02	3:0.0

enzyme system that can incorporate the oxygen atom of an epoxide into a tertiary hydroxyl group. The $\Delta^{2'}$ -double bond may be formed concurrently.

The elimination of a hydrogen atom from C-3' of a precursor of abscisic acid may occur by a reaction of this kind. The analogous double bond of α -carotene is formed in position, not by isomerization of a β -double bond to the α -position (Goodwin & Williams, 1965; Williams *et al.*, 1967) (Scheme 2).

As the precursors of abscisic acid are still unknown, there is no evidence for a mechanism of formation of the $\Delta^{2'}$ -bond or for the stereochemistry at C-2'. Indeed, even the absolute configuration of the tertiary hydroxyl group of abscisic acid is uncertain (Scheme 3).

Derivation of the hydrogen atom at C-4

The hydrogen atom on C-4 of abscisic acid cannot be lost by exchange during isolation, but the C-2-hydrogen atoms of mevalonate in the third isoprene unit can be expected to have changed places and to have suffered slight loss under the action of isopentenyl pyrophosphate isomerase to the same extent as the C-3'-hydrogen atom. The C-4-hydrogen atom of abscisic acid is removed by formation of compound (VI) (Mallaby & Ryback, 1972). The $^{14}\text{C}/^3\text{H}$ ratios of the samples of abscisic acid and their derivatives (Table 6) indicate that the hydrogen atom at C-4 of abscisic acid is derived from the 2*R*-hydrogen atom of mevalonate. The occurrence of 39% of 2*S*- ^3H , and the value for the 2*R*- ^3H being 49% less than the theoretical, are attributed to partial randomization with a concurrent loss of a small proportion $[100 - (51 + 39)\% = 10\%]$ of the ^3H from this position. The $^{14}\text{C}/^3\text{H}$ ratios of compound (VI) (^3H in the 6'-methyl group), compared with those of the original mevalonic acids, give a measure of the loss of ^3H caused by the action of isopentenyl pyrophosphate isomerase. Both measurements show slightly more than one equivalent of ^3H remaining in the molecule and attributed to this position; this may indicate, as suggested above, that little dimethylallyl pyrophosphate was used in the biosynthesis that had been 'formed twice' (i.e. isopentenyl pyrophosphate $\rightarrow$ dimethylallyl pyrophosphate $\rightarrow$ isopentenyl pyrophosphate $\rightarrow$ dimethylallyl pyrophosphate) and that tritiated molecules of dimethylallyl pyrophosphate may revert to isopentenyl pyrophosphate somewhat less rapidly than non-tritiated molecules (including those containing ^{14}C). It is also possible that a slight degree of 'scrambling' of ^3H occurred into other positions of abscisic acid where it is unavailable for exchange.

The 2*R*- and 2*S*- ^3H lost from C-4 of abscisic acid gives a measure of the amount of randomization undergone by the ^3H before the isopentenyl pyrophosphate residues are joined. As expected from work with plants (Goad, 1970), the degree of randomization

Scheme 2. Postulated stereochemistry of the hydrogen atoms derived from C-2 of mevalonate in precursors of α -carotene and abscisic acid

The retention of the *pro-2R*-hydrogen atom of mevalonate in both products is shown. [Note added in proof: the absolute configuration of (+)-abscisic acid has recently been found to be as shown (B. V. Milborrow, R. S. Burden & H. F. Taylor, unpublished work).]

is quite considerable, but there is a clear preferential retention of the $2R\text{-}^3\text{H}$ at both C-3' and C-4. The $^{14}\text{C}/^3\text{H}$ ratios of methyl abscisic acid, 3'-exchanged methyl abscisic acid, 3'- ^3H in the exchange medium and compound VI derived from methyl abscisic acid were calculated for different degrees of randomization. The smallest mean difference between these values and the $^{14}\text{C}/^3\text{H}$ ratios measured with $[2\text{-}^{14}\text{C}, (2R)\text{-}2\text{-}^3\text{H}_1]$ - and $[2\text{-}^{14}\text{C}, (2S)\text{-}2\text{-}^3\text{H}_1]$ -mevalonate were obtained when it was assumed that 36% of the $2R\text{-}^3\text{H}$ had come to occupy the position preferred by the $2S$ -hydrogen and vice versa.

The experiment with $2(R)$ - and $2(S)$ -tritiated mevalonolactones was repeated, but the avocado fruit used incorporated a smaller percentage of the labelled mevalonate and abscisic acid. Consequently these $^{14}\text{C}/^3\text{H}$ ratios (Tables 3 and 5) were less precise than those obtained in the first experiment. The $^{14}\text{C}/^3\text{H}$

ratios in the second experiment confirm those obtained in the first experiment and clearly show that there is greater incorporation of the $2(R)\text{-}^3\text{H}$ of mevalonate and abscisic acid at C-4 and C-3' (Tables 2, 3, 4, 5 and 6). In the second experiment less ^3H from C-2 of mevalonate was retained in all three positions of abscisic acid, possibly because the activity of the isomerase was greater, compared with that of the polymerase, than in the previous experiment.

Inhibition of the isopentenyl pyrophosphate isomerase

Isopentenyl pyrophosphate is particularly sensitive to inhibition by iodoacetamide; in a cell-free system isolated from liver, for instance, Cornforth *et al.* (1966) reported that the isomerase was completely blocked by 5mM-iodoacetamide. This suggested that the enzyme could be inhibited *in vivo* by the application of a suitable concentration of the inhibitor: accordingly, in the third experiment the 5ml of aqueous mevalonic acid solution contained 2mM-iodoacetamide in an attempt to lower the isomerase activity so that it became the rate-limiting step in the biosynthesis, but was not completely blocked. It would not, therefore, completely prevent incorporation of labelled mevalonate into abscisic acid because of a lack of dimethylallyl pyrophosphate.

The percentage incorporation of the labelled mevalonic acid into abscisic acid per unit weight of avocado mesocarp in the presence of 2mM-iodoacetamide was similar to that in the other experiments (Table 7), so the overall rate of abscisic acid synthesis did not appear to have been seriously affected. However, the

Scheme 3. Stereochemistry of (+)-abscisic acid suggested by two methods

[Note added in proof: the absolute configuration of violaxanthin and of (+)-abscisic acid according to Burden & Taylor (1970) has recently been confirmed (B. V. Milborrow, R. S. Burden & H. F. Taylor, unpublished work).]

Table 7. Incorporation of 3*R*-[2-¹⁴C,(2*R*)-2-³H₁]mevalonate and 3*R*-[2-¹⁴C,(2*S*)-2-³H₁]mevalonate into abscisic acid in the presence of 2.0 mM-iodoacetamide

The same procedures were used for this experiment as were described in Tables 2, 3, 4 and 5, except that the solutions applied to the fruits contained 2.0 mM-iodoacetamide. The two parts of the experiment were carried out on the two halves of one avocado fruit (216 g total). Calculated radioactivity: for 3*RS*-[2-¹⁴C,(2*R*)-2-³H₁]mevalonolactone was 2020000 d.p.m. of ¹⁴C and 35778000 d.p.m. of ³H; for 3*RS*-[2-¹⁴C,(2*S*)-2-³H₁]mevalonolactone was 2020000 d.p.m. of ¹⁴C and 65373000 d.p.m. of ³H. The radioactivity present in subsamples after t.l.c. was 980 d.p.m. of ¹⁴C and 17370 d.p.m. of 2*R*-³H, i.e. a ¹⁴C/2*R*-³H ratio of 1:17.72, and 1630 d.p.m. of ¹⁴C and 52741 d.p.m. of 2*S*-³H, i.e. a ¹⁴C/2*S*-³H ratio of 1:32.36.

	¹⁴ C (d.p.m.)	³ H (d.p.m.)	¹⁴ C/ ³ H ratio (normalized)
Methyl abscisate (2 <i>R</i>)- ³ H	823	12370	3:2.55
Methyl abscisate (2 <i>S</i>)- ³ H	875	10610	3:1.12
3'-Exchanged methyl abscisate (2 <i>R</i>)- ³ H ₁	485	5685	3:1.99
3'-Exchanged methyl abscisate (2 <i>S</i>)- ³ H ₁	923	9117	3:0.92
Compound VI (2 <i>R</i>)- ³ H ₁	90.6	635	3:1.19
Compound VI (2 <i>S</i>)- ³ H ₁	248	2415	3:0.90

amount of 2(*S*)-³H incorporated at positions 2' and 4 of abscisic acid was much less than in the two previous experiments. Also considerably more 2(*R*)-³H was retained at these positions than in the previous experiments. Thus it appears that less randomization of the 2(*R*)- and 2(*S*)-³H of mevalonate had taken place by recycling of the labelled precursors through the isopentenyl pyrophosphate → dimethylallyl pyrophosphate → isopentenyl pyrophosphate cycle under the action of isopentenyl pyrophosphate isomerase.

The ¹⁴C/³H ratios in this experiment may not be exactly comparable with those of the other experiments, because one of the basic assumptions may no longer hold, namely that all three isoprene residues of abscisic acid are derived in the same proportion from the labelled mevalonate supplied. The decrease in the supply of dimethylallyl pyrophosphate could have altered the proportion of the *gem*-dimethyl isoprene residue of abscisic acid derived from the labelled mevalonate. The proportions of the central and carb-

oxyl-terminal isoprene residues would not be expected to differ from each other.

The results show, more clearly than before, that the hydrogen atoms on C-3' and C-4' of abscisic acid are both derived from the *pro*-(*R*)-hydrogen of C-2 of mevalonate. The exaggeration of the differences in $^{14}\text{C}/^3\text{H}$ ratios by iodoacetamide, a potent inhibitor of isopentenyl pyrophosphate isomerase, supports the suggestion that this enzyme is responsible for the partial randomization of ^3H from C-2 of mevalonate. The amount of labelled mevalonate incorporated into abscisic acid in the presence of 2mM-iodoacetamide shows that the other stages of abscisic acid formation are not so affected by iodoacetamide that biosynthesis is prevented.

Origin of the hydrogen atom at C-5

The nominal $[(5S)\text{-}5\text{-}^3\text{H}_1]\text{mevalonate}$ used in this experiment was, like the $[(2R)\text{-}2\text{-}^3\text{H}_1]$ - and $[(2S)\text{-}2\text{-}^3\text{H}_1]$ -mevalonate, a mixture in which the biologically active 3*R* enantiomer carried ^3H at the 5*S*-position only. The $^{14}\text{C}/^3\text{H}$ ratios of methyl abscisic acid (also the 1',4'-*cis*-, 1',4'-*trans*-diols of methyl abscisic acid

and compound VI) formed from $[2\text{-}^{14}\text{C},(5S)\text{-}5\text{-}^3\text{H}_1]\text{mevalonate}$ show that one 5*S*- ^3H atom was retained per three mevalonate residues in a molecule of abscisic acid (Table 8). Although only 88% of the expected amount of ^3H was retained in abscisic acid the $[(5S)\text{-}5\text{-}^3\text{H}_1]\text{mevalonate}$ was later found to contain 12.6% of a labelled impurity (F. P. Ross, personal communication); thus the percentage incorporation of the 5*S*- ^3H into abscisic acid was close to the expected value. The hydrogen atoms of the other two isoprene units would be lost on formation of the carboxyl and 4'-oxo groups.

According to a scheme of reactions elucidated by Mallaby & Ryback (1972), the C-5-hydrogen atom of abscisic acid should be removed by enolization of a ketone formed at C-4 on treatment of the compound (VI) with base. When this was done, by using the same conditions as for exchange of the C-3'-hydrogen, most of the 5*S*- ^3H was lost from the molecule (Table 8), so the attribution of the ^3H label to C-5 of abscisic acid is compatible with the proposed reactions.

The stereochemistry of the formation of the analogous *trans*-double bond of carotenoids has been investigated by Goodwin and his collaborators

Table 8. Incorporation of 3*R*- $[2\text{-}^{14}\text{C},(5S)\text{-}5\text{-}^3\text{H}_1]\text{mevalonate}$ into abscisic acid by avocado fruit

The $^{14}\text{C}/^3\text{H}$ ratio of the purified methyl abscisate, and compound (VI) formed from it, indicate that the C-5-hydrogen atom is derived from the (5*S*)-hydrogen atom of mevalonate. As expected, the ^3H can be removed from C-5 by base-catalysed rearrangement. Calculated radioactivity of 3*RS*- $[2\text{-}^{14}\text{C},(5S)\text{-}5\text{-}^3\text{H}_1]\text{mevalonolactone}$ was 156500 d.p.m. of ^{14}C and 1250000 d.p.m. of ^3H , i.e. a $^{14}\text{C}/^3\text{H}$ ratio of 1:7.99. This is normalized to 3:3. Total ^{14}C radioactivity in the methyl ester of (+)-abscisic acid was 544 d.p.m. [0.94 d.p.m./ μg of (+)-abscisic acid]. The total abscisic acid in the fruit, which weighed 266 g, was 564 μg [2.12 mg of (+)-abscisic acid/kg of mesocarp]. For experimental details see the text.

	^{14}C (d.p.m.)	^3H (d.p.m.)	$^{14}\text{C}/^3\text{H}$ ratio
Methyl abscisate	544	1275	3:0.88
Compound (VI)	137	318	3:0.87
Repeat of experiment with the $^{14}\text{C}/^3\text{H}$ ratio in methyl abscisate normalized to 3:1	190	1680	3:1
Compound (VI)	187.4	1683	3:1.02
Compound (VI) after base-catalysed rearrangement	48	68	3:0.16

(VII) Stereochemistry of the hydrogen atoms at C-4 and C-5 of a hypothetical precursor of abscisic acid before the formation of the Δ^4 -double bond

(VIII) Origin of the hydrogen atoms of the Δ^4 -double bond of abscisic acid

(Williams *et al.*, 1967; Walton *et al.*, 1969) by using similar methods, except that they compared the incorporation of ^3H from [(5*R*)-5- $^3\text{H}_1$]mevalonate with that of [(5*RS*)-5- $^3\text{H}_2$]mevalonate. The Δ^4 -*trans*-double bond of abscisic acid is formed, as it is in carotenoids, by the elimination of the 2*S*-hydrogen and 5*R*-hydrogen of mevalonate (formula VII). Thus the positions of the hydrogen atoms that are lost during the formation of the Δ^4 -double bond (formula VIII) are such that they could undergo a *trans*-elimination. In the formation of this double bond, as with the Δ^2 - and Δ^2' -bonds, there is no difference between abscisic acid and carotenoids in the stereochemistry of the hydrogen atom eliminated.

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