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Factors Affecting the Biosynthesis of Abscisic Acid

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ABSTRACT

Incorporation of labelled mevalonate into abscisic acid (ABA) has been demonstrated in the cotyledons of mature avocado seeds, embryos and endosperms of developing wheat seeds, and avocado stems. The increase in ABA concentration on wilting parallels the increased incorporation of [2-^{14}C]mevalonate into ABA in avocado leaves and stems, suggesting that the increase in ABA content occurs by synthesis rather than by release from a stored precursor. Incorporation of [2-^{14}C]mevalonate by avocado mesocarp segments is unaffected by an 18 per cent water loss. The ABA content of roots was hardly affected by a 30 per cent water loss, indicating that the wilt-activated mechanism is not fully operative in these tissues.

Submerged *Ceratophyllum* plants and submerged parts of *Callitriche* shoots show a twofold increase in ABA content on wilting whereas the aerial rosettes of the latter plant show a six-fold increase. This suggests that the occurrence of the wilt-induced mechanism is affected by previous growth conditions as well as by the morphology of the tissue.

INTRODUCTION

Wright (1969) and Wright and Hiron (1969, 1972) have found that when leaves wilt their content of abscisic acid (ABA) increases by about 40 times during the first half-hour of treatment. The high wilt-induced level is maintained until enough water has been supplied to the leaves to permit them to regain their turgor, whereupon the concentration of free abscisic acid falls to its original value during the next few days. The increase in ABA concentration has been attributed to synthesis from mevalonate (MVA) rather than to release from a pool of a precursor because nine times more tritiated MVA was incorporated into ABA by wilted wheat leaves than by turgid ones (Milborrow and Noddle, 1970). However, the increase in incorporation of radioactivity did not parallel the 40-fold increase in free ABA, although there were indications that this disproportion was attributable to slow penetration of the labelled precursor into the cells.

Abscisic acid has been isolated from a wide variety of different plant organs but until now synthesis from mevalonate has been demonstrated in fruit and leaf tissue only (Noddle and Robinson, 1969). The wilt-induced synthesis in leaves has been considered to be, at least in part, a device to close the stomata and so reduce water loss and thereby allow the leaves to regain their turgor. The fruits of avocado are the best system we have so far found for synthesizing ABA from added mevalonate but they are devoid of stomata and do not wilt. Consequently, it was of interest to

discover whether or not their biosynthetic system responded to water loss in the same way as that of leaves, or whether the control mechanism was specific to the leaf. The capacity of seeds to synthesize ABA from mevalonate was investigated at the same time. In addition the ability of leaves of plants growing in different environments, and of roots to respond to wilting was investigated so that the effect of environmental factors on the biosynthesis of ABA could be related to its presumed physiological role.

MATERIALS AND METHODS

Compounds

The sample of racemic abscisic acid used was synthesized by Cornforth, Milborrow, and Ryback (1965). (±)-[2-^{14}C]Abscisic acid (4·9 mCi/mM) was prepared by Cornforth, Mallaby, and Ryback (1968). (±)-[2-^{14}C]Mevalonolactone (7–10 mCi/mM) and (±)-[(*RS*)-2-$^{2}H_2$]-mevalonolactone (90 mCi/mM) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K.

A.R.-grade chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; A.R. solvents were from Hopkin and Williams, Chadwell Heath, Essex, U.K. The scintillator for liquid scintillation assay of radioactivity was 2, 5-bis(5-*t*-butylbenzoxazol-2-yl)thiophen (BBOT) obtained from CIBA (A.R.L.) Ltd., Duxford, Cambs., U.K.

Plant material

Avocado (*Persea gratissima* Gaertn.) fruit were bought from a local supplier and were imported from several countries at different times of the year. The fruits (200–300 g each) were used when they began to soften. Other plants were grown as specified below.

Extraction and purification

Methanol extracts of the various plant samples were fractionated to provide ether-soluble acids. All solvents used in extractions contained between 1 and 10 μg/ml 2, 6-di-*tert.*-butyl-4-methylphenol (BHT) and the TLC solvents contained 20 μg/ml of it. The ABA was isolated by chromatography of the ether-soluble acid fraction on precoated silica gel F_{254} thin-layer plates (obtained from E. Merck A.-G., Darmstadt, Germany) developed with toluene:ethyl acetate: acetic acid (25:15:2, v/v) (Milborrow, 1972). The area of the silica gel plates to which the sample, dissolved in ether+BHT, was applied was soaked beforehand with an ethereal solution of BHT (1 mg/ml); this prevents oxidation of ABA.

Multiple development of the chromatograms was frequently used and the zones containing ABA or derivatives were located by the presence of marker compounds on either side of the extract. The silica gel carrying a compound of interest was scraped into a glass sinter thimble and eluted with methanol. ABA-containing fractions were rechromatographed in the same solvent system if particularly impure. Material from the ABA zone was methylated with an ethereal solution of diazomethane and the product was chromatographed in the same way in hexane:ethyl acetate (2:1, v/v). The abscisic acid methyl ester was eluted and was found to be the only u.v.-absorbing organic material from the plant and also the only product in the solution to contain label derived from mevalonate. This was shown, by subsequent reduction of the methyl ABA with sodium borohydride in ice-cold methanol:water (2:1, v/v) for up to 3 h, to give labelled 1′, 4′-*cis*- and 1′, 4′-*trans*-diol esters in approximately equal amounts. No evidence was obtained to suggest that these compounds were not radiochemically pure. Retention of radioactivity from mevalonate in equal amounts in the methyl diols of ABA is considered to be adequate evidence for its having been incorporated into ABA.

Determination of radioactivity

Aliquots of the solutions to be assayed were dried in glass vials to which were added 10 ml of a solution of 2-methoxyethanol:toluene (2:3, v/v) containing 80 mg naphthalene and 6 mg BBOT. The samples were counted to a standard deviation of less than 1 per cent in a Packard Tri-Carb model 3375 liquid scintillation spectrometer which gave 80 per cent counting efficiency for [^{14}C] and 30 per cent for [^{3}H].

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Spectropolarimetry

Optical rotatory dispersion curves were recorded on a Bellingham and Stanley (Thorn Bendix) Polarmatic 62 spectropolarimeter coupled to a Bryans $x-y$ recorder. Provided that the sample to be measured exhibited the appropriate Cotton effect parameters (a reflection of the degree of purity), quantitative estimations were made from the rotations at one or two specified wavelengths. In most cases such measurements were made on the methyl ester of abscisic acid (dissolved in MeOH) where specific rotations at the extrema are $-65\,500°$ (246 nm) and $+22\,800°$ (286 nm). The sensitivity of the original instrument has been improved (Ryback and Robinson, 1970) such that deflections of 6·7 mm and 10 mm per millidegree may be obtained at these respective wavelengths at a noise level of better than 1 millidegree. Samples containing as little as 0·5 μg/ml in a 10-mm light path could thus be accurately measured.

ORD measurements were supplemented by u.v. absorption spectrometry: for methyl abscisate in MeOH, $\epsilon\lambda_{max}$ (265 nm) = 20 900.

Biosynthesis of ABA *in avocado fruit*

Two radial cylinders (20 g) were cut from the equator of a single fruit. The skin was removed and each cylinder was further cut equatorially into three slices. To the newly exposed surfaces of each group of slices was applied a solution of ($\pm$)-[2-^{14}C]mevalonolactone ($5{\cdot}4 \times 10^6$ d/min; 0·34 μmol) in 0·2 ml Tween-20: acetone: water (1:1:8, v/v). The slices were replaced in their original position and each stack was cut radially into 35 segments which were then just separated from one another. One set (21·7 g) was placed in a water-saturated atmosphere on the bench top for 24 h; the other was exposed to a gentle stream of air for 3 h after which the weight had dropped from 21·4 to 17·5 g (loss of 18 per cent). This set was then covered with a Petri-dish lid and left for the remainder of the 24-h period. The tissue was then homogenized and thoroughly extracted with methanol containing BHT and ($\pm$)-ABA. ABA was isolated and assayed.

Biosynthesis of ABA *in avocado leaves*

Three experiments were undertaken with detached leaves, 10–15 cm long, cut from greenhouse-grown plants. (1) Immediately on excision from the plants half of the leaves were extracted with methanol and half were wilted (10 per cent weight loss in 1 h) and maintained in this state for a further 7 h before extraction. (2) Excised leaves (20 g) were allowed to take up a solution of ($\pm$)-[2-^{14}C]mevalonolactone in 2 per cent ethanol over a 6-h period (total absorbed: $5{\cdot}2 \times 10^6$ d/min; 0·33 μmol) after which one-half of the batch was wilted (15 per cent weight loss in 1 h) and maintained in this state for a further 6 h; the others were kept wet for a further 7 h. Both batches were then extracted. (3) Excised leaves (50 g) were allowed to take up mevalonolactone solution as in experiment 2 but in this case over a 30-h period at room temperature followed by 18 h at 4 °C (total absorbed: $24{\cdot}0 \times 10^6$ d/min; 1·5 μmol). Half of the sample was then wilted (15 per cent weight loss in 2 h) and maintained in this state for a further 5 h; the other half was kept wet for a further 7 h. Both batches were then extracted. In all experiments wilting was induced by a gentle air stream and maintained by placing the leaves in plastic bags. During periods of wet incubation the leaves were placed in previously wetted plastic bags. All manipulations were undertaken in normal laboratory light. ABA was extracted and assayed (Table 2).

Biosynthesis of ABA *in avocado stems*

Ten stems, each up to 30 cm long, were cut from greenhouse-grown plants and, with all of the leaves removed, were stood in 2 ml of an aqueous solution of ($\pm$)-[2-^{14}C]mevalonolactone containing 2 per cent ethanol. After 1·5 h a 2-ml water wash was supplied and taken up over 2·5 h. The total label uptake was $19{\cdot}8 \times 10^6$ d/min; 1·2 μmole. The stems were then stood in excess water at 4 °C for 19 h after which five stems (61 g) were covered with a plastic bag and allowed to remain in water at room temperature for a further 20 h. The remaining five stems (59 g) were placed on the bench top for 20 h after which a 30 per cent weight loss was recorded. The stems were chopped and extracted thoroughly with methanol containing BHT and ($\pm$)-ABA. ABA was isolated and assayed.

Biosynthesis of ABA *in avocado seed (cotyledon)*

Cubes for cotyledonary tissue (and mesocarp from the same fruit for comparison), 2·5 g each, were sliced into six sections. To the cut surfaces was applied a solution of

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(±)-[2-^{14}C]-mevalonolactone (6·4 × 10^{6} d/min; 0·4 μmol) in 0·1 ml Tween-20:acetone:water (1:1:8, v/v).The cubes were then reassembled from the slices and incubated at room temperature for 24 h. The tissues were homogenized and extracted with methanol containing BHT and (±)-ABA. ABA was isolated and assayed (Table 5).

Content of (+)-ABA *in avocado roots*

Root systems were cut from 32 greenhouse-grown avocado plants each several moths old, washed, drained, and divided into two 400 g batches (equivalent to 344 g fresh weight when allowance is made for approximately 14 per cent surface water). One batch was immersed in aerated tap water for 2 h. The other batch was exposed to a gentle stream of air over a 2-h period after which the weight was 240 g (loss of 30 per cent water). Both batches were then placed in closed flasks and allowed to remain in the dark at room temperature for a further 4 h. The roots were then extracted by soaking over a period of days in several changes of methanol containing BHT and (±)-[2-^{14}C]ABA. ABA was recovered and assayed (Table 4).

Biosynthesis of ABA *in wheat seeds*

Developing wheat (*Tricum sativum*, c.v. Kloker) seeds were harvested (21 July 1972) and 50 embryos were separated from the endosperms by a razor cut close to and parallel with the scutellum (which remained with the embryo). The endosperm was milky and its skin was readily removable, leaving the aleurone layer bright green. The embryos were also green and approximately half their final size. The excised embryos and endosperms were placed in 40-mm Petri-dishes with their cut surfaces against the glass which had been moistened with 1·0 ml water containing 7·6 × 10^{6} d/min (±)-[(*RS*)-2-$^{3}H_2$]MVA (90 mCi/mM). The water was allowed to dry during the next 20 h when the lids of the dishes were put on and incubation continued in dull light for a further 28 h. The samples were dried in a desiccator for 16 h, then frozen at −20 °C and homogenized in methanol (20 ml) to which (±)-ABA (0·25 mg) and 2, 6-di-*tert.*-butyl phenol has been added.

It has been shown (Milborrow, 1972) that a total of half of the tritium from (±)-[(*RS*)-2-$^{3}H_2$]-mevalonolactone is lost during its incorporation into ABA and during the isolation procedure. Although the amount of mevalonate incorporated by the embryos is small it is of the same order as that of avocado mesocarp on a weight basis.

Content of (+)-ABA *in sunflower roots*

Field-grown sunflower plants (20) were pulled from the ground after heavy rain (September 1972) and the brickearth soil was washed from the roots with a strong jet of water. The secondary roots were then cut off the taproot and divided into two equal batches, blotted, and weighed (481 g). One-half was immediately re-wetted while the other was spread out on dry filter-paper in a draught of warm air. After approximately half an hour the roots had lost 28 per cent of their weight; they were then covered with a transparent polythene sheet for 2 h when they were plunged into methanol (2 l) containing 2,6-di-*tert.*-butyl phenol (20 mg) and (±)-ABA (143 μg, [2-^{14}C] 0·046 μCi/μM) and cut into short lengths (*c.* 10 mm). The unwilted sample was blotted dry and treated similarly at the same time; both were then steeped for 5 d in methanol (2 l) and re-extracted twice (2 l + 1 l).

Content of (+)-ABA *in hornwort* (Ceratophyllum demersum)

The hornwort (*Ceratophyllum demersum*) plants were collected locally and grown entirely submerged in 2·7 l natural pondwater held in 3-l glass jars. They were grown for 10 weeks in a glasshouse whose mean daily temperature was 22 °C, and supplementary illumination was provided by mercury-vapour lamps during the later afternoon and evening.

The plant material (2 kg blotted fresh weight) was divided into two equal batches. One batch was dampened with water and spread out in a polythene bag while the other batch was spread out on a dry filter-paper and dried out in a draught of air. After 30 min the wilted plants were placed in a similar polythene bag and kept for 3 h. Both batches were then rapidly frozen at −20 °C, and crushed to powder; each was poured into ice-cold methanol (2 l) which contained BHT (20 mg) and (±)-[2-^{14}C]ABA (60 μg, 20000 d/min).

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Content of (+)-ABA *in water starwort* (Callitriche stagnalis)

The water starwort (*Callitriche stagnalis*) plants used were collected from flowing, clear water in the Stour at Chilham, a trout stream near Canterbury. The rosettes, some in flower, were growing on the surface and were picked between 15.00 and 17.00 hours on 13 July 1972. They were kept in polythene bags until 18.00 hours. All surface leaves were cut off the submerged parts which were stored similarly. Both batches were blotted dry, halved and weighed. One half of each sample was immediately re-wetted and stored for 3·5 h in a polythene bag while the other half was spread out on dry filter-paper at 22 °C under tungsten illumination (1800 lx) so that the surface parts lost 47 per cent of their weight and submerged parts 42 per cent during 50 min. The wilted samples were then stored in polythene bags for 3 h during which time they remained wilted but did not lose any more water. The wet samples were shaken free of adhering water and all were rapidly frozen at −20 °C at the same time as the wilted ones. The plants were crushed while frozen and then the powder was poured into 2 l of ice-cold methanol containing BHT (10 mg) and (±)-[2-^{14}C]ABA (14·3 μg, 0·046 μCi/μmol).

Stomata were present on the surface leaves of *Callitriche* and also on the totally submerged ones in similar numbers. No stomata were found on any leaves or stems of *Ceratophyllum*. Avocado leaves bear stomata on their lower surfaces only. Avocado fruit have no stomata in their epidermis although lenticel-like structures are present in some varieties.

Ceratophyllum grows entirely submerged; *Callitriche*, on the other hand, forms terminal, aerial rosettes which bear the flowers and both the aerial and submerged leaves carry large numbers of stomata.

RESULTS AND DISCUSSION

Biosynthesis of ABA *by avocado fruit*

The synthesis of abscisic acid by wilted fruit slices was measured and compared with that in the unwilted sample on two bases: (i) the incorporation of labelled MVA into ABA; (ii) the concentration of abscisic in the tissues. It was considered that both methods were necessary because permeability barriers to the penetration of the precursor, rate of turnover of ABA, and release of an unlabelled precursor could all affect the result.

The results in Table 1 show that there was no rise in (+)-ABA content when the fruit tissue lost 18 per cent of its fresh weight, nor was the amount of labelled mevalonate incorporated into ABA significantly increased.

TABLE 1. *Biosynthesis of* ABA *in wet and dried avocado fruit segments*

Each sample was incubated with (±)-[2-^{14}C]mevalonolactone ($5·4 \times 10^6$ d/min; 0·34 μmol). Each was extracted in the presence of (±)-ABA (140 μg)

	% recovery of ABA by racemate dilution	(+)-ABA/original fresh weight (corrected for recovery) (μg/kg)	d/min incorporated into ABA/sample (corrected for recovery)
Wet mesocarp	69·0	4650	3020
Dried mesocarp (18% weight loss)	79·5	4050	3650

No role has been suggested for the ABA which is present in fruit nor has an explanation been advanced for the large amounts that are synthesized during ripening. Sondheimer, Tzou, and Galson suggested (1968) that the ABA in the pericarp of Ash (*Fraxinus americana* and *F. ornus*) has no influence on the seed; we find

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that the seed of the avocado has usually broken its connection with the fruit at the onset of softening when the rate of synthesis of ABA is greatest. Although this does not preclude a role for the ABA in the fruit relating to seed germination, it would seem that transfer to the seed would be haphazard and depend on the factors affecting decay or survival of the fruit pulp. We suggest that the increase in ABA which has been reported to occur in ripening fruit (Rudnicki and Pieniązek, 1971) and which is associated with rapid biosynthesis from added mevalonate in avocado mesocarp is involved with the induction of the climacteric of the fruit. [Since this paper was written we have discussed the role of ABA in ripening fruit with Dr. B. G. Coombe of the Waite Institute and thank him for allowing us to cite his unpublished work on grapes. He finds that ABA is intimately concerned with fruit expansion, the development of colour in the skin, and other reactions of ripening.] Abscisic acid hastens leaf senescence when applied as a solution to detached leaves but the effects of sprays on attached fruit (e.g. olives, Barnsley, Gabbott, and Milborrow 1968, and Hartman, Heslop, and Whisler 1968) have been equivocal.

TABLE 2. *Biosynthesis of* ABA *in wet and wilted avocado leaves*

Leaves in Experiments 2 and 3 were supplied with (±)-[2-^{14}C] mevalonolactone (see Experimental Section) and (±)-ABA was included in the extraction medium to measure recoveries

	Hours elapsed between excision and extraction	% recovery of ABA by racemate dilution	(+)-ABA/original fresh weight (corrected for recovery) (μg/kg)	d/min incorporated into ABA/sample (corrected for recovery)
Experiment 1				
Wet leaves	0	..	20	..
Wilted leaves (10% weight loss)	8	..	825	..
Experiment 2				
Wet leaves	13	76·0	210	0
Wilted leaves (15% weight loss)	13	48·0	550	42
Experiment 3				
Wet leaves	55	65·0	540	544
Wilted leaves	55	69·5	2670	1370

Biosynthesis of ABA *in avocado leaves*

Avocado leaves are somewhat resistant to wilting and tend to retain their turgor after being picked until ventilated with a draught of warm air. The ABA content of the wilted sample was then found to be 40 times higher than that of the turgid sample (Table 2). Avocado leaves differ from fruit tissues by increasing their ABA content when they lose water and also in the resistance of their tissue to penetration by mevalonate. In the first experiment with labelled substrate the leaves took up the mevalonate solution but failed to incorporate label into ABA when wilted

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immediately. In the second and third experiments the precautions taken to ensure penetration of the mevalonate before any wilting was induced (a long period of uptake (30 h) and storage at 4 °C for 18 h) caused the concentration of ABA in the nominally turgid leaves to rise out of proportion to the increase in the wilted leaves. The concentration increase was only fourfold. The incorporation of mevalonate showed a similar but even less pronounced difference. Whether the high initial concentration was caused by incipient wilting or by the physiological stress of the unnaturally low temperature is unknown.

Similar difficulty of penetration of MVA was encountered by Goodwin (1958*a*, *b*) in early experiments on carotenoid synthesis. It was later found that damaged chloroplasts synthesized carotenoids from labelled mevalonate, suggesting that the chloroplast membrane was a major barrier to penetration.

The similarity of the carbon skeleton of ABA to that of carotenoids suggests that both may be synthesized by evolutionarily related enzymes; this, together with the slow penetration of MVA to the site of synthesis of ABA in leaves, suggests the possibility that ABA is synthesized in the chloroplast.

Biosynthesis of ABA *in avocado stems*

A single experiment was performed with green, supple stems of avocado (Table 3). Conditions similar to those applied to leaves were used in anticipation of problems

TABLE 3. *Biosynthesis of* ABA *in wet and wilted avocado stems*

Each sample had taken up (±)-[2-^{14}C]mevalonolactone ($9{\cdot}9 \times 10^6$ d/min; 0·6 μmol). Each was extracted in the presence of (±)-ABA (50 μg)

	% recovery of ABA by racemate dilution	(+)-ABA/original fresh weight (corrected for recovery) (μg/kg)	d/min incorporated into ABA/sample (corrected for recovery)
Wet stems	70·0	500	40
Wilted stems (30% weight loss)	42·0	930	3640

with penetration of equilibration of the supplied mevalonate. Eight to ninefold differences were observed in both total extracted (+)-ABA and in biosynthesis. The latter measurement in fact exhibited the slightly higher ratio, adding support to the notion that wilt-induced increases in ABA are due solely to synthesis.

Content of ABA *in roots*

The ability of avocado roots to synthesize ABA in response to drying was examined by measuring the net increase in ABA by spectropolarimetry. The percentage recovery of the natural (+)-ABA was monitored by assaying the simultaneous recovery of (±)-[2-^{14}C]ABA added to the methonal extraction medium. This monitoring was necessary as it had been found that wilted tissues often gave cleaner extracts than did unwilted ones, and the extra manipulations necessary to isolate the ABA from the latter would affect the percentage recovery.

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The separated avocado roots did not show a marked increase in ABA content on wilting (Table 4); the twofold rise could have been brought about by synthesis but since labelled mevalonate was not used the possibility of release from a precursor has not been excluded. The low concentrations of ABA in the roots of avocado in relation to the amount of u.v.-absorbing material made the measurement somewhat imprecise, and the experiment was repeated with sunflower roots (Table 5). These gave extracts that were well resolved by chromatography; consequently the measurements of ABA concentrations are more accurate. Sunflower roots show a negligible increase in (+)-ABA content on wilting.

TABLE 4. *Content of* ABA *in wet and wilted avocado roots*

Each sample was extracted in the presence of (±)-[2-^{14}C]ABA (6960 d/min, 86 μg)

	% recovery of labelled ABA	(+)-ABA/original fresh weight (corrected for recovery) (μg/kg)
Wet roots	24·5	27
Wilted roots (30% weight loss)	52·5	85

TABLE 5. *Concentration of* (+)-ABA *in wilted and wet sunflower lateral roots (original, blotted fresh weight of each sample* 392 *g; final weight of wilted sample* 284 *g)*

(±)-[2-^{14}C]ABA (64 μg containing 5520 d/min) was added to each methanol extract

	Original fresh wt. (g.)	Final fresh wt. (g)	(+)-ABA isolated (μg)	d/min in methyl ABA	% recovery	Extracted dry wt. (g)	(+)-ABA/original fresh weight (corrected for recovery) (μg/kg)
Wet roots	392	392	2·7	2950	53·4	43·5	12·9
Wilted roots 27·5% weight loss	392	284	4·2	2940	53·3	35·0	19·4

Thus roots fail to respond to wilting by increasing their ABA content in the same manner as leaves and stems; although ABA is present in roots its synthesis there has not been demonstrated conclusively.

Biosynthesis of abscisic acid in seeds

The seed of the avocado contains a large and well-formed axis at the time of fruit softening. The seeds germinate sporadically and most show evidence of dormancy. The concentrations of ABA within the massive cotyledons are lower than in mesocarp and our initial attempts to demonstrate incorporation of labelled mevalonate into ABA in the seed failed. A subsequent experiment showed that incorporation of label can occur in the seed but to a much lesser extent than in the mesocarp under

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the same conditions (Table 6). It is possible that ABA is formed in the seed principally or solely during its development, and the biosynthesis found may have reflected residual activity from the final stages of maturation. Since it is difficult to obtain immature avocado fruit in England we have been unable to demonstrate this possibility experimentally nor yet to determine to what extent, if any, transport from fruit to seed occurs.

The early failure with avocado seeds led us to examine developing wheat grains to find whether or not mevalonate was incorporated into ABA by seeds. The results in Table 7 show that incorporation did occur; the immature seeds were cut parallel to the scutellum and although the embryo portion comprised one-sixth of the fresh weight of the seed it incorporated more than twice as much mevalonate as the endosperm portion.

TABLE 6. *Biosynthesis of* ABA *in avocado seed*

Each sample was extracted in the presence of (±)-ABA (40 μg)

	% recovery of ABA by racemate dilution	(+)-ABA/original fresh weight (corrected for recovery) (μg/kg)	d/min incorporated into ABA/sample (corrected for recovery)
Cotyledonary tissue segments	70·0	500	40
Mesocarp tissue segments from the same fruit	64·5	5950	2300

TABLE 7. *Biosynthesis of* ABA *in wheat embryos and endosperm*

(±)-[2-(*RS*)—$^{3}H_{2}$]mevalonolactone (3·42 μg; 90 μCi/μM) were supplied to 50 excised embryos and 50 endosperms of developing wheat seeds. The d/min shown are those present in the methyl diols formed

	Weight, g	d/min in methyl ester of 1′, 4′-*trans* diol	dpm in methyl ester of 1′, 4′-*cis* diol	% recovery of ABA by u.v. absorption of diols
Endosperms	2·67	27·5	33·3	34
Embryos	0·54	69·8	67·2	21

The effect of wilting on water plants

The results of the experiments reported above show that the ability of a tissue to synthesize large amounts of ABA on wilting is associated with the tissue's regulation of water loss by stomata. Stomata are closed rapidly by applications of ABA and it has been suggested that the prime function of the extra ABA, formed on wilting, is to close the stomata. If this were so then one would expect that an aquatic plant, which lacks stomata and has such a thin cuticle that it cannot regulate water loss in drying conditions by adjustment of stomatal aperture, would obtain no advantage from an ABA-induced closure of stomata on wilting. Aquatic plants would not, therefore, be expected to form large amounts of ABA on wilting. In the first

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experiment to test this prediction hornwort (*Ceratophyllum demersum*) was used; in the second experiment water starwort (*Callitriche stagnalis*) was used. *Ceratophyllum* appears to be more thoroughly adapted to an aquatic environment than *Callitriche* because it grows entirely submerged and has no stomata on its stems and leaves. *Callitriche*, on the other hand, forms terminal aerial rosettes which bear the flowers, and both aerial and submerged leaves carry large numbers of stomata. The measurements of (+)-ABA extracted from *Ceratophyllum* (Table 8) showed that the concentration of ABA was lower than the normal values for unwilted leaves of terrestrial plants. Furthermore, there was only a 1·7-fold increase on wilting.

TABLE 8. (+)-ABA *isolated from wet and wilted hornwort* (Ceratophyllum demersum)

(±)-[2-^{14}C]ABA (60 μg containing 20 000 d/min) was added to the extraction medium

	Original fresh wt. (g)	Final fresh wt. (g)	(+)-ABA isolated (μg)	d/min in methyl ABA	% recovery	(+)-ABA/ original fresh weight (corrected for recovery) (μg/kg)
Wet sample	1000	1000	3·0	9190	45·9	6·5
Dry sample	1000	382	8·5	9545	47·7	17·7

TABLE 9. (+)-ABA *isolated from wet and wilted parts of water starwort* (Callitriche stagnalis)

(±)-[2-^{14}C]ABA (14 μg containing 5520 d/min) was added to the extraction medium

	Original fresh wt. (g)	Final fresh wt. (g)	(+)-ABA isolated (μg)	d/min in methyl ABA	% recovery	(+)-ABA/ original fresh weight (corrected for recovery) (μg/kg)
Wet sample aerial rosettes	269	269	0·33	506	9·2	13·4
Dry sample aerial rosettes	269	142	7·25	1720	31	86·6
Wet sample submerged parts	368	368	0·48	1432	25·9	5·0
Dry sample submerged parts	368	212	0·39	388	7·0	14·9

A similar experiment was then carried out with *Callitriche* except that the aerial rosettes were separated from the submerged shoots. Both sets were divided and halves were wilted as before (Table 9). The submerged parts behaved like the *Ceratophyllum* shoots and the initial and final concentrations of both were almost identical with the *Ceratophyllum* values. When the rosettes were wilted the concentration of ABA increased to 86·6 μg/kg, i.e. 6·5 times its original value. Although this value is

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still low for a wilted land plant it does indicate a considerable difference in the physiological mechanism controlling ABA concentration from that operating in the submerged parts. The leaves of the terminal, aerial rosettes of *Callitriche* have thicker cuticles than have the submerged leaves and their ABA content when wet (13·4 μg/kg) is close to the range of values for unwilted leaves of land plants; nevertheless they are in very close proximity to water and the difference in both their normal concentration of ABA and the magnitude of the wilt-induced rise indicates that the control mechanism is highly sensitive to its environment. While the proximity and ready availability of water to the floating rosettes might be expected to minimize the differences between them and submerged parts of the plant, the constitution of the latter samples would tend to exaggerate the difference. The submerged samples contained a higher proportion of stem tissue than did the rosettes; consequently, if the increase in ABA induced by wilting were confined to the leaves, then smaller differences between the samples would be distorted by expressing the results in terms of total fresh weight.

The increase in ABA content which occurs in shoots of land plants on wilting was first described by Wright (1969) and he has suggested that the high concentrations formed in wilting plants tend to close the stomata and thereby decrease further water loss. Leaves of avocado show a considerable increase in ABA content on wilting while the net amount of ABA in the stems also increases slightly although the incoroporation of labelled MVA into ABA was considerably higher in wilted stems then in unwilted ones. An exact proportionality between the amount of labelled MVA incorporated into ABA, and the increase in free ABA, cannot be expected because the rate of turnover in normal, turgid material is unknown. In all previous experiments relatively more ABA was made in wilted than in turgid tissues in comparison with the amount of MVA incorporated. The result does show that a plant organ other than a leaf responds to wilting by synthesizing extra ABA.

The small increase in ABA content that occurs in the submerged parts of water plants suggests that the mechanism regulating ABA biosynthesis is affected by the environmental conditions under which the plants have grown. Thus ABA biosynthesis appears to be regulated in at least three different ways:

(i) In maturing avocado fruit the synthesis of ABA is rapid and ABA contents are the highest found so far. The rate of synthesis and ABA content are unaffected by wilting.

(ii) The endogenous levels of ABA in leaves are much lower than in fruit but increase up to 40-fold when the leaves wilt.

(iii) In roots and submerged water plants the ABA content is low. On wilting it increases by a very small or negligible amount.

Other publications (e.g. Corgan and Peyton, 1970) have suggested that the ABA contents of buds, seeds, and tubers increase during development in response to changing daylength or the degree of maturity of the organs. Consequently, there may be a fourth way in which ABA contents are adjusted; the rate of biosynthesis and the equilibrium concentration may depend on the state of development of the tissue.

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CONCLUSION

Abscisic acid can be synthesized from added mevalonate by the fruit, stems, and leaves of avocado and the developing seeds of wheat. The data for roots of avocado and sunflower are inconclusive.

The only known latent source of ABA, the glucose ester, is usually present at concentrations of between one-third and one-tenth of that of the free ABA and these amounts alone are quite insufficient to account for a significant rise in the ABA concentration. No leaf extracts we have examined have been found to contain appreciable amounts of an inhibitor (other than ABA) which would, if it were a stored precursor of ABA be expected to show marked inhibitory activity. The failure to find such a precursor is weak and indirect evidence against the occurrence of such a compound but taken together with the avocado leaf experiment in Table 2 and the earlier results with wheat (Milborrow and Noddle, 1970) it appears that the majority, if not all, the 'extra' ABA present in wilted leaves arises by synthesis rather than by release from 'latent' ABA.

Roots separated from the plant hardly increase their ABA content on wilting. The approximately twofold rise may be brought about by synthesis but the possibility that this 200 per cent 'extra' ABA is released from a precursor has not been excluded, However, the small size of the increase induced by wilting of roots, in comparison with the 4000 per cent increase which occurs in leaves when they wilt, shows that the biosynthesis is controlled differently in the two organs.

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