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XANTHOXIN AND ABSCISIC ACID

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Abstract—Abscisic acid and xanthoxin are endogenous plant growth inhibitors which differ from other known inhibitors in chemical nature and in the level of biological activity. A short review is presented of their discovery, physical and chemical properties, natural occurrence and biological rôles. The biosynthesis of ABA is discussed with particular reference to the possible involvement of xanthophylls and xanthoxin. ABA is rapidly metabolised by plants and the pathway by which this occurs is now known.

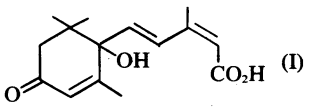
INTRODUCTION

Kefeli and Kadyrov refer[1] to plant growth inhibitors as regulating substances which retard such processes as root and stem elongation, seed germination and bud opening and depress the growth of isolated stem sections, acting as antagonists to the plant hormones. As many organic compounds will elicit this type of response if applied in sufficient quantity a more recent definition[2] sets a concentration limit, albeit an arbitrary one, of 10^{-5} M or less for activity. An additional stipulation could be that the compound must not produce symptoms of phytotoxicity. The above requirements would exclude most phenolic compounds which have previously been proposed as plant growth inhibitors but abscisic acid (ABA) and its naturally occurring analogue xanthoxin, the subjects of this short review, fully satisfy these conditions.

Since its isolation and structural determination in 1965 ABA has been the subject of many hundreds of papers. It has also been reviewed extensively[3–10] and the most recent article by Milborrow[10] is particularly comprehensive on all aspects of ABA research. The present review is largely confined to aspects of the chemistry and biochemistry of ABA and in particular its relationship with xanthoxin and the carotenoid pigments. Recent developments on the metabolism are also considered.

PROPERTIES OF ABA

Abscisic acid is the trivial name now ascribed to 3 - methyl - 5 - (1′ - hydroxy - 4′ - oxo - 2′,6′,6′ - trimethyl - 2′ - cyclohexen - 1′ - yl) - 2 - *cis*,4 - *trans* - pentadienoic acid (I). It is thus identical with the inhibitors "abscisin II" and "dormin" referred to in the earlier literature and is the major active compound of inhibitor β.[11]



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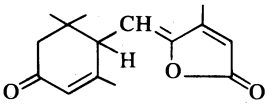
Natural ABA is dextrorotatory and has been obtained[12] as colourless crystals, m.p. 160–161°. The ultraviolet spectrum is pH dependent and is usually determined in acidified ethanol or methanol when the absorption maximum is at 262 nm with an ϵ of 21,400[13]. Infra-red,[12,14] nuclear magnetic resonance[8,12] and mass spectra[14] have all been reported and a detailed study[15] of the mass spectrum of the methyl ester has used metastable analysis, isotopic labelling and high resolution mass measurements to derive the cracking patterns. The interaction of the two chromophores in ABA with the single asymmetric centre produces an exceptionally intense and characteristic optical rotatory dispersion curve.[8,11] This ORD spectrum, measured in acidified alcohol, has [α]D (589) + 430, [α]269 and [α]225 nm 0°, [α]289 nm + 24000° and [α]246 – 69000°. The absolute configuration of ABA has been a matter of some dispute and is dealt with later when the relationship with certain carotenoid pigments is considered.

Only a limited amount of research has been carried out on the chemistry of ABA apart from the preparation of simple derivatives as aids to identification. Perhaps the most interesting reaction ABA is known to undergo is that with a mixture of formic and hydrochloric acids when the lactone (II) is formed as the major product.[16] This produces an intense violet-red colour with alkalis but as this fades with time its use as a quantitative method for ABA estimation is limited. However it appears to be an excellent qualitative test.



(II)

SYNTHESIS OF ABA

In view of the great difficulties involved in obtaining appreciable amounts of ABA from plant extracts, the synthesis has received considerable attention. Several syntheses are now available, most of which make use of the commercially available ionones as starting materials.

In the original method[17] (Fig. 1) 3 - methyl - 5 - (2′,6′,6′ - trimethylcyclohexa - 1′,3′ - dienyl) - 2 - *cis*,4 - *trans* - pentadienoic acid (III) was prepared by standard methods and converted to the epidioxide (IV) by irradiation with visible light in the presence of oxygen with eosin as photosensitizer. Treatment of the epidioxide with base gave racemic ABA with a m.p. of 188–190°, some 30° higher than the natural (+)-enantiomer.

The key stage of the synthesis of Roberts *et al.*[18] involved the conversion of α-ionone (V) into 1 - hydroxy - 4 - keto - α - ionone (VI) by t-butyl chromate oxidation.

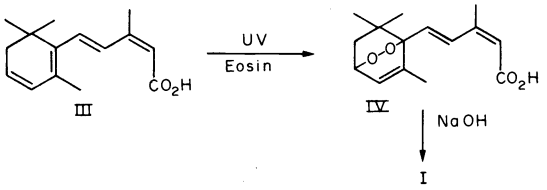


Fig. 1.

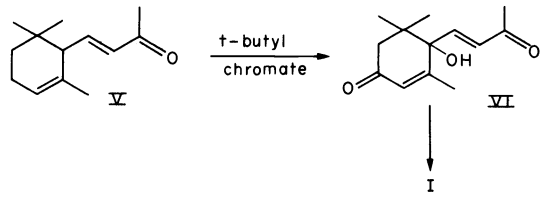


Fig. 2.

This was then converted into ABA by a Wittig reaction (Fig. 2).

In another variation,[19] 3,4-dehydro-β-ionone (VII) was prepared from β-ionone and oxidised with *m*-chloroperbenzoic acid to the epoxide (VIII). This on treatment with Jones reagent afforded the key intermediate (VI) which could be converted into ABA by standard methods.

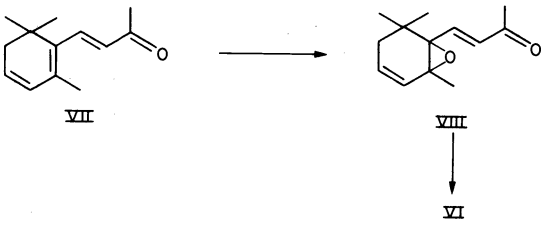


Fig. 3.

In the conversion of VI into ABA by reactions of the Wittig type, substantial quantities of the biologically inactive 2-*trans*, 4-*trans* isomer (t-ABA) are invariably formed. However irradiation of an acetone solution of this isomer with sunlight leads to the rapid formation of a 1:1 equilibrium mixture from which the 2-*cis*,4-*trans* isomer (ABA) can be recovered by chromatography.[20] Conversely ABA will also isomerise to t-ABA if exposed to sunlight hence reasonable care must be taken with its storage. A stereo selective synthesis of the 2-*cis*,4-*trans* racemate of ABA has been reported by Mayer, Schwieter and Weedon in the patent literature.[20a]

RADIO-ACTIVELY LABELLED ABA

Some of the above synthetic methods have been modified to provide labelled ABA for studies of transport and metabolism in plants. In the original synthesis[20] [2-^{14}C] labelled methyl dehydro-β-ionylideneacetate was prepared from dehydro-β-ionone and trimethyl [2-^{14}C] phosphonate using a Horner reaction. The ring functional groups were then altered photolytically to afford methyl abscisate from which ABA was obtained on hydrolysis.

An alternative synthesis[21] employed 1 - hydroxy - 4 - keto - α - ionone (VI) and a labelled Wittig reagent, [2-^{14}C]-methylene triphenylphosphorane. This method had the advantage of introducing the radioactive precursor at a later stage and has now been further improved[22] to provide ABA of very high specific activity. Other groups[23,24] have employed essentially the same procedures to obtain samples of [^{14}C]-ABA.

Tritiated ABA has been obtained by an exchange reaction in tritiated water and the stability of the fixed tritium is sufficient to permit utilization in biological work.[25]

RESOLUTION OF (±)-ABA

Synthetic (±)-ABA was first resolved with some difficulty by fractional crystallisation of the brucine salt.[26] A more recent variation[27] has involved resolution of t-ABA by crystallisation of the brucine salt followed by photoisomerisation of the resolved *trans*-enantiomers and isolation of (+)- and (−)-ABA by chromatography.

Sondheimer *et al.*[28] achieved the resolution of (±)-ABA by using a chromatographic column of optically active acetylcellulose. Under the conditions employed (+)-ABA was eluted initially and after enantiomorphic enrichment from several runs the enriched fractions were treated with 2.5% *n*-butanol in isooctane. This solvent selectively solublized the optically active components and eventually (+)- and (−)-ABA were obtained with acceptable optical purity. In some bioassays the unnatural (−)-ABA was found to be as active as the natural enantiomer but in others ABA was appreciably more active.[28]

DETECTION AND ESTIMATION OF ABA IN PLANTS

In view of the importance of ABA as a plant hormone inhibitor it is not surprising that there is a considerable literature on its detection and estimation in plants. Both physical and biological methods have been used with success.

Extraction of plant material is usually carried out with an organic solvent such as ether, ethyl acetate or methanol. This is then followed by partitioning between solvents, frequently aqueous bicarbonate and ether, when the ABA can be recovered from the "strong acid fraction". This procedure has the advantage of removing neutral, alkaline and weakly acid substances (e.g. certain phenols) at an early stage of the purification.

Further purification is usually by chromatography, the variations used including silica gel thin layer,[11,29] silica gel dry-column,[30] polyvinylpyrrolidine column[31] and Sephadex chromatography.[32] The techniques most frequently employed for ABA estimation in purified fractions are spectropolarimetry and gas liquid chromatography. The former makes use of the highly characteristic ORD curve of ABA and is a sensitive and specific method.[11,33,34] For gas chromatography volatile derivatives are essential and the most convenient are the trimethylsilyl ether[35,36] and the methyl ester.[37] Gas chromatography has been coupled with mass spectrometry to provide an unambiguous identification procedure[38,39] and in a recent modification[40] ABA is recovered from a TLC plate and methylated for GLC simultaneously. ABA has also been estimated by spectrofluorimetry.[41]

All of the above procedures involve losses of ABA in the various purification steps and for an exact estimation of the inhibitor in the original tissue an assessment of these losses is essential. An ingenious "racemic dilution" method has been devised whereby a known quantity of (±)-ABA is added to the tissue homogenate. After purification the total ABA can be calculated from the UV spectrum, the (+)-ABA content from the ORD spectrum and from a knowledge of the percentage recovery of the racemate an exact estimate of the (+)-ABA in the tissue can be obtained. Other methods employed for calculating losses include the addition of labelled ABA[42] and the addition of a known amount of the *trans*-isomer.[43] In the last method light catalysed isomerisation of the two isomers must be carefully avoided.

Other methods developed for the estimation of ABA depend upon its biological activity. Bioassays involving such processes as inhibition of seed germination and coleoptile extension have been used extensively but suffer from the disadvantage that other compounds possessing

similar chromatographic properties to those of ABA can affect the result. It is certainly desirable that such non-specific assays be used in conjunction with a sound physical method if the compound is to be positively identified as ABA.

Potentially the most sensitive and useful bioassay procedure for ABA involves its antitranspirant activity. When isolated epidermal strips of *Commelina communis* L. were incubated in ABA solutions there was found to be[44] a linear correlation between the size of stomatal openings and the ABA concentration over the range 10^{-8}–10^{-4} M. This bioassay was improved by the use of buffers[45] so that as little as 26 pg could be detected. The assay possesses the great merit of being unaffected by other growth regulators such as auxins, gibberellins and cytokinins.

By the use of physical and bioassay methods ABA has now been detected in very many species of higher plants.[3,10] It appears to be widely distributed among the various plant parts having been found in leaves, stems, buds, roots, tubers, rhizomes, seeds and fruits. ABA has also been reported in a fern,[11] a moss[46] and in algae[47] but confirmation of these sources is required if ABA is to be considered a ubiquitous constituent of lower plants.

PHYSIOLOGICAL RÔLE OF ABA

The *in vivo* rôle of ABA is an area of intensive research[10] of which only a brief outline can be given here. In retrospect the name given to the inhibitor may prove to be misleading in that abscission is probably not the principal biological function. Certainly there is little evidence that ABA is responsible for leaf abscission but largely as a result of the work of Davis and Addicott[48] an involvement of ABA in controlling certain types of fruit abscission is more certain.

An early suggestion[49] was that ABA was involved in the regulation of dormancy. This has been the subject of conflicting reports in the literature and it is certainly desirable that much of the earlier work be repeated. Indeed contrary to the first indications Lenton *et al.*[43] were able to show that the ABA contents of beech, maple and sycamore plants did not vary with photoperiod although short photoperiods induced dormancy in these species. However Wright[50] has recently found that the ABA content of blackcurrant and beech buds is highest in autumn at the time of the onset of winter dormancy. A conjugate of ABA is apparently very important in the dormancy cycle and builds up during the winter months. A similar phenomenon has been observed with birch buds.[51].

Perhaps the most convincing rôle yet demonstrated for ABA involves the response by plants to stress conditions. Wright[52] found that when cut wheat leaves were wilted there was a rapid increase in the level of inhibitor β. The compound responsible was further identified[34] as ABA and a water loss of 10% of the total fresh weight was found to be sufficient to produce a forty-fold increase. Furthermore exogenously applied ABA was shown[53,54] to induce stomatal closure and reduce transpiration and hence the increase in the level of endogeneous ABA on wilting could be seen as a regulating mechanism used by the plant to close stomata and reduce water loss. A remarkable experimental demonstration of this was provided by Hiron and Wright [55] who showed that when a continuous stream of hot air was directed onto leaves of dwarf bean seedlings, wilting at first occurred but the plants then regained turgor even though the warm air treatment was continuing. The leaf water deficit caused by the warm air treatment was found to be accompanied by a large increase in ABA which then closed the stomata enabling the plants to recover. The stress accompanying waterlogging is also sufficient to cause a large increase in the ABA content of many species[55] but predictably in rice, a species well adapted to a flooded environment, there was no ABA increase on waterlogging.

Very recent research suggests that ABA may also be involved in the light induced inhibition of root growth and also in the geotropic response. The root cap is now believed to be the zone sensitive to light and gravitational stimuli and there are reports[56,57] of the presence of ABA in the dissected caps. Movement of ABA from the cap to the growing zone is essential for it to be involved in growth responses and Pilet was now demonstrated[58] the basipetal and lateral movement of exogenously applied ABA in maize root segments. Exogenous ABA applied to intact maize roots in darkness can also bring about growth inhibition and bending.[59]

BIOSYNTHESIS OF ABA

ABA is a C15 compound whose structure is formally that of a sesquiterpene. However it is also very similar to the end portion of certain carotenoids and hence it could arise in nature either directly from mevalonic acid (MVA) or by the degradation of a carotenoid such as violaxanthin. These possibilities are outlined in Fig. 4.

The first experimental work on ABA biosynthesis demonstrated the incorporation of [^{14}C]MVA into ABA in avocado and tomato fruit.[60] With the use of [4R–$^{3}H_1$]MVA it was further shown[61] that the ABA arose from an all *trans* precursor, probably farnesyl pyrophosphate, and that the Δ^2-*cis*-double bond must have been formed from a Δ^2-*trans* bond at a later stage in the process. The stereochemistry of the hydrogen elimination that occurs during the formation of the Δ^4- and $\Delta^{2'}$-double bonds has also been determined[29] using the ^{14}C/^{3}H ratio in the ABA biosynthesised from appropriately labelled mevalonate. The results of all these experiments demonstrated no important differences between ABA and carotenoid biosynthesis.

At this time the evidence most in favour of a direct pathway for ABA biosynthesis was the assignment of absolute configurations. Violaxanthin, the most likely pigment precursor of ABA, had been allotted[62] the configuration shown in Fig. 4, an assignment which has been recently confirmed.[63] However by application of Mill's rule to the diols formed by reduction of (+)- and (−)-ABA, the tertiary hydroxyl in ABA was thought[26] to have the opposite configuration to that of the epoxide in violaxanthin. A reversal of configuration during the biosynthesis of ABA from violoxanthin would be extremely unlikely.

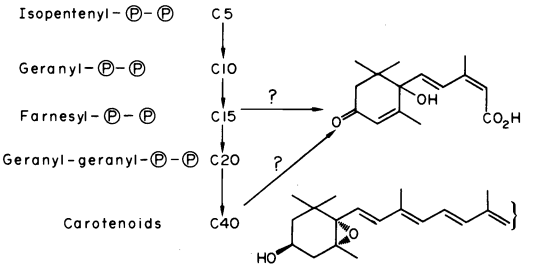
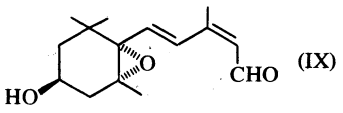


Fig. 4.

DISCOVERY OF XANTHOXIN

Possible evidence for the production of ABA from a carotenoid was first provided by Taylor and Smith who showed[64] that *in vitro* illumination of violaxanthin and neoxanthin from nettle leaves produced a growth inhibitor. However the inhibitory activity was found to be present in the neutral fraction of the photoproducts and hence the compound responsible was not ABA. The large scale isolation of violaxanthin from orange peel and the development of a zinc permanganate oxidation method for cleaving the polyene chain has now enabled the inhibitor to be isolated and characterised.[65–67] It is 2 - *cis*,4 - *trans* - 5 - (1',2' - epoxy - 4' - hydroxy - 2',6',6' - trimethyl - 1' - cyclohexyl) - 3 - methyl - pentadienal (IX) and has been given the name "xanthoxin".



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Xanthoxin as produced by the oxidation of violaxanthin is accompanied by other products and the purification presents difficulties. The biologically inactive *trans,trans*-isomer is particularly difficult to remove and the best method[67] involves reduction of xanthoxin to the diol with sodium borohydride, separation of the *cis,trans* from the *trans,trans*- isomer by TLC and reoxidation with manganese dioxide. Interconversion of the separated xanthoxins in illuminated solutions is particularly facile.

PROPERTIES OF XANTHOXIN

Xanthoxin is normally obtained as a colourless syrup. It has a u.v. absorption maximum of 281.5 nm and details of the mass spectrum of the acetate have been published.[67] Xanthoxin on thin layer plates is particularly sensitive to 2,4-dinitrophenylhydrazine and as little as 0.1 μg of the inhibitor can be detected as an orange spot after warming.

Undoubtedly the most interesting feature of the chemistry of xanthoxin is the reaction with chromium trioxide in pyridine. This reagent not only oxidises the secondary alcohol to a ketone but also effects a subsequent rearrangement to form abscisic aldehyde (Fig. 5). This can be further oxidised to abscisic acid.[67] The chemical conversion of xanthoxin to ABA is important for it relates the stereochemistry of violaxanthin to that of ABA. Contrary to expectation, the ABA formed from xanthoxin was found to be the natural dextrorotatory enantiomer and this could only mean that the configuration of either violaxanthin or ABA had been incorrectly assigned. Several papers subsequently demonstrated that the configuration of ABA required revision. The methods employed were degradation of ABA and correlation with malic acid,[68] a partial stereospecific synthesis of ethyl (−)-abscisate from (−)-α-ionone,[69] correlation with the grasshopper ketone of known stereochemistry[70] and calculations from ORD and CD data using the exciton chirality method.[71,72] According to the Cahn, Ingold and Prelog notation of 1966 the configuration of (+)-ABA is now defined as (S). This revised absolute configuration of ABA has an important bearing on the biosynthesis for a carotenoid precursor cannot now be excluded on the grounds of stereochemistry.

SYNTHESIS OF XANTHOXIN

Although ABA has been prepared from the readily available α- and β-ionones, a synthesis of xanthoxin based on these sources has not yet been achieved. However (±)-O-methylxanthoxin has been synthesised from β-ionone, the key reaction being an addition of methanol to dehydro-β-ionone.[73] The only total synthesis of (±)-xanthoxin so far reported[74] is outlined in Fig. 6.

A most valuable partial synthesis (Fig. 7) employs

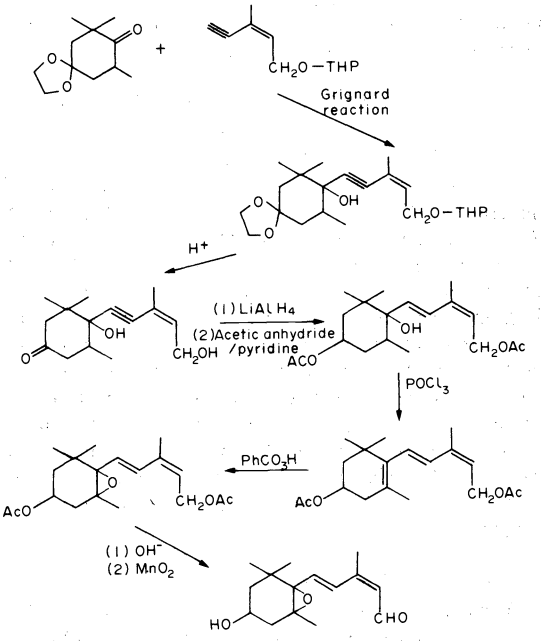


Fig. 6.

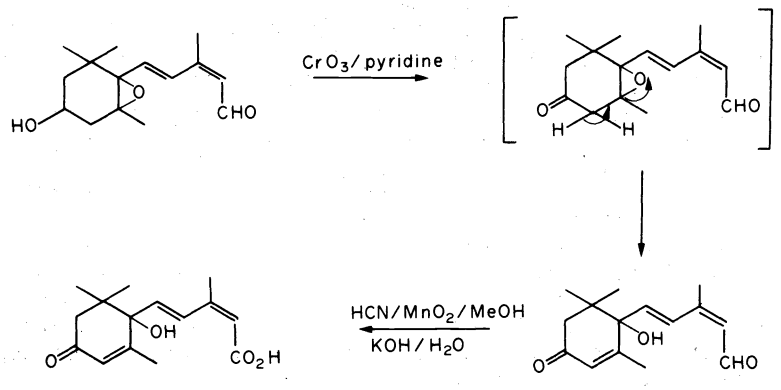


Fig. 5.

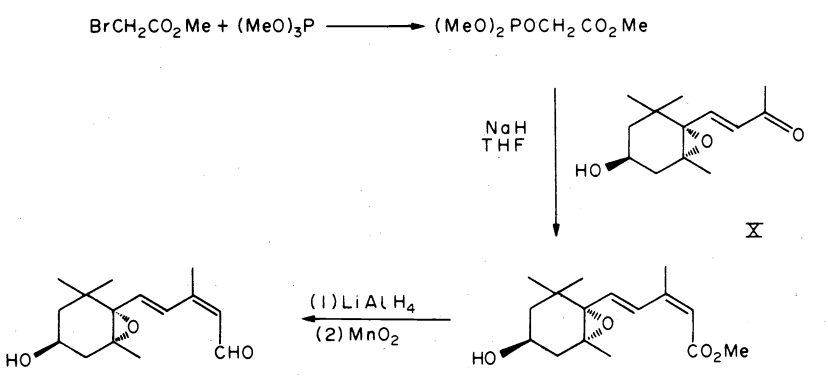


Fig. 7.

another degradation product of violaxanthin, the butenone (X) and this method has been used[75] to synthesise [2-^{14}C]-labelled xanthoxin. A great advantage of the method is that it produces the naturally occurring enantiomers exclusively and this is very useful in studies of xanthoxin metabolism.

A recent attempt[76] to use microorganisms to introduce an hydroxyl group into the required position of the β-ionone ring has not been successful. However allylic hydroxylation of β-ionone was found to occur in good yield with several fungal species.

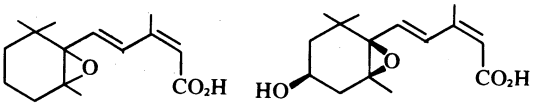
DETECTION AND ESTIMATION OF XANTHOXIN IN PLANTS

Xanthoxin is a potent plant growth inhibitor having activity comparable to ABA in many bioassays and superior to ABA in a seed germination test.[67] It is the only ABA analogue which has been found to occur naturally and it has been isolated from several species of higher plants and also from ferns.[77,78] The recommended isolation procedure employs solvent partitioning, thin layer chromatography and finally glc analysis of the acetate derivative. The *cis,trans*- and *trans,trans*-isomers can be separated and estimated in this manner. The origin of endogenous xanthoxin is uncertain; it may be formed from violoxanthin photolytically or alternatively by enzymes. A soybean lipoxygenase capable of cleaving violoxanthin to xanthoxin and other products has been isolated.[79]

FURTHER ASPECTS OF ABA BIOSYNTHESIS

When [2-^{14}C] xanthoxin was fed to cut shoots of bean and tomato it was converted to (+)-ABA in high yield.[75] Together with the natural occurrence of xanthoxin this established a viable biogenetic route to ABA from carotenoids. However the importance of this route in normal plant metabolism has yet to be established. Experiments quoted by Milborrow[9] show that when [^{14}C] phytoene and [2-^{3}H]MVA are fed to avocado pears only the tritium label is found in ABA although the carotenoids become [^{14}C] labelled. This is considered good evidence that a direct biosynthetic route operates under these circumstances.

By the use of [^{14}C] and [^{18}O] labelling techniques it has been demonstrated[80] that the epoxide (XI) is converted into ABA by plants and the oxygen of the epoxide group becomes the tertiary hydroxyl group of ABA. However although the racemic epoxide was supplied, only pure (+)-ABA was biosynthesised. Subsequent experiments[81] led to the isolation of (−)-1′,2′-epi-2-*cis*-xanthoxin acid (XII) as a metabolic product and it was hence suggested



XI XII

that although hydroxylation occurs at C-4′ with both enantiomers of the epoxide, further metabolism to ABA occurs only with the isomer having the hydroxyl and epoxide *trans* to each other. This of course is the configuration found in xanthoxin and there is thus a possibility that xanthoxin or a related compound may be an intermediate on a direct biosynthetic route to ABA from MVA.

Recent work by Milborrow has shown that ABA is probably biosynthesised in the plastids[82] and that the cyclisation of a precursor follows the same stereochemistry as that observed for carotenoids.[83] He concludes that the enzymes of ABA biosynthesis have probably evolved from those of carotenoid biosynthesis.

From all this it may concluded that despite the considerable amount of effort in this area of research the precise *in vivo* relationship of carotenoids, xanthoxin and ABA has yet to be unravelled. Xanthoxin is certainly a widespread naturally occurring growth inhibitor derived from carotenoids and probably possesses a high level of biological activity in *in vitro* assays as a result of conversion to ABA. However there does appear to be a considerable weight of evidence that most ABA in plants is produced directly from MVA, albeit by enzymes very similar in characteristics and in cellular location to those involved in carotenoid biogenesis. It is probably significant that when the ABA content on leaves undergoes a sudden and massive increase of wilting[34] the level of xanthoxin remains unchanged.[84,85] It is hard to reconcile this with the indirect route of ABA biosynthesis but such a route may be operated in other circumstances.

It is now known that the xanthoxin content of red light grown peas is some 5–7 times higher than that the dark grown plants.[86] This finding which has been recently confirmed[87] may help to explain why short periods of red light have a dwarfing effect on etiolated seedlings.

METABOLISM OF ABA

The metabolism of ABA was first studied by Milborrow who found that when (±)-[2-^{14}C] ABA was fed to tomato shoots it was metabolised to a water soluble conjugate, the glucose ester[88] and the 6′-hydroxymethyl derivative, metabolite C.[89] Metabolite C rapidly rearranged to phaseic acid, a compound isolated from bean and for which an

incorrect structure has been previously assigned.[90] Several subsequent attempts to reisolate metabolite C failed, phaseic acid being isolated each time.[9]

When Walton and Sondheimer investigated the metabolism of (±)-[2-^{14}C] ABA by embrionic bean axes[91] they found that it gave rise to two metabolites with very low growth inhibitory activity. They designated these as metabolites M-1 and M-2 and it was subsequently found[92] that M-1 was phaseic acid and M-2 was the dihydroderivative in which the 4'-keto group of ABA had been reduced. Metabolism studies with resolved (+)-[2-^{14}C] ABA and (−)-[2-^{14}C] ABA have shown[28] that only the natural (+)-enantiomer is hydroxylated to give phaseic acid. Phaseic acid and dihydrophaseic acid are naturally occurring metabolites for both have been isolated[93] from bean seeds where dihydrophasic acid accumulates in relatively large quantities. The absolute configurations of these metabolites have now been assigned[94] and are as given in Fig. 8. This pathway appears to be the major way

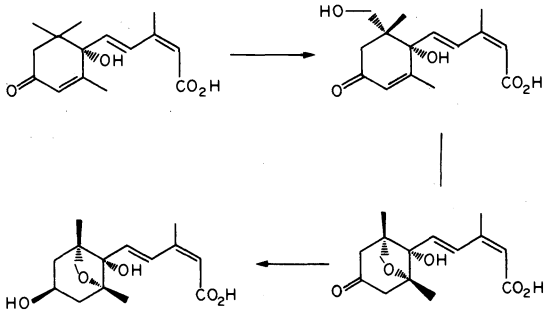


Fig. 8.

by which ABA is deactivated in the plant but the rôle of the glucose ester is still uncertain. It is possible that as suggested by Hiron and Wright[55] it may act as a metabolic backstop, leading to a continuing high ABA level after a period of water stress.

An unidentified metabolite of [2-^{14}C] ABA has been isolated from lettuce seeds[95] while [1-^{14}C] ABA appears to be decarboxylated by apple seeds during stratification.[96]

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